ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

CONSTRUCTING PEPTIDE (GEPI)-PROTEIN MOLECULAR HYBRIDS BY USING GENETIC ENGINEERING METHODS FOR MATERIALS AND MEDICAL APPLICATIONS

Ph.D. Thesis by

Deniz ŞAHİN

Advanced Technologies Department

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DECEMBER 2011

İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

MALZEME VE MEDİKAL UYGULAMALAR İÇİN GEN MÜHENDİSLİĞİ YOLUYLA PEPTİD (GEPI)-PROTEİN HİBRİTLERİN OLUŞTURULMASI

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ARALIK 2011

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Date of Submission : 02 October 2010
Date of Defense : 21 December 2011

FOREWORD

First, I would like to thank to my supervisors, Prof. Candan Tamerler and Prof. Dr. Mehmet Sarıkaya. From a fresh MSc student to an experienced PhD candidate, they were all the time there for me, guiding and believing.

I think it is a big deal to have a PhD degree, but i am sure it is more important to purify your mind from the effects of endless rainstorm of nonscientific beliefs and habits, and developing a true scientific mind as much as possible. These were the years for me not just full of studying on my thesis but imroving myself as a true candidate for being a scientist and that was possible with the friends who had the similar view of life. Thank you my friends; Ece, Pınar, Anıl, Ceren, Urartu, Abdullah, Hasan, Volkan, Hilal, Esra, Koray, Turgay, Orkun, Günseli, Sakip and Öykü and many others for deepest friendship and endless support.

My niece Doğa who is now about 2.5 years old, was always the light in the darkness, and the cheer in the sadness.

There are so much to say for my family in Manisa, but i guess they know what i feel about them. Thank you very much for everything.

I would like to send special thanks to Oren family. Emre, Çiğdem and Doğa were always my second family:)

And finally, I would also like to acknowledge the funding agencies. This study was supported by GEMSEC (Genetically Engineered Materials and Engineering Center, PI: Prof. Dr. Mehmet Sarıkaya) through NSF-MRSEC at the UW, ITU Institute of Science and Technology through Turkish State Planning Organization and TUBITAK/NSF-IRES joint project 107T250.

JANUARY 2012 Deniz ŞAHİN

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ABBREVIATIONS

ABTS : 2,20-azino-bis(3- ethylbenzothiazoline-6-sulfonic acid)
Amp : Ampicillin
AP : Alkaline Phosphatase
AuBP : Gold Binding Protein
Bp : Base pair
Cam : Chloramphenicol
CSD : Cell Surface Display
dH2O : Distilled water
dsDNA : Double stranded DNA
DNA : Deoxyribonucleic acid
EB : Elution Buffer
EDTA : Ethylenediaminetetraacetic acid
EtBr : Ethidium bromide
GBP : Gold Binding Protein
GEPI : Genetically Engineered Peptides for Inorganics
GFPuv : Recombinant Green Fluorescent Protein
GST : Glutathione S-transferase
His-TAG : Hexahistidine TAG
IMAC : Immobilized Metal Affinity Chromatography
IPTG : Isopropyl-b D- thiogalactopyranoside
Kb : Kilobase
LacZ : α-galactosidase
LB- broth : Luria Bertani broth
MBP : Maltose Binding Protein
MCO : Multi Copper Oxidase
mRNA : Messenger ribonucleic acid.
Na-Ac : Sodium acetate
NSL : Non Specific Linker
OD : Optical density
PCR : Polymerase Chain Reaction
PD : Phage Display
QBP : Quartz Binding Peptide
RBS : Ribosome Binding Site
SA-QD : Streptaavidin coated Quantum Dots
SDS : Sodium dodecyl sulfate
SDS-PAGE : Sodium Dodecyl Sulphate
ssDNA : Single stranded DNA
TAE : Tris acetate EDTA
TBE : Tris-borat-EDTA
X-Gal : 5-Bromo-4-chloro-3-indolyl-D-galactoside

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CONSTRUCTING PEPTIDE (GEPI)-PROTEIN MOLECULAR HYBRIDS BY USING GENETIC ENGINEERING METHODS FOR MATERIALS AND MEDICAL APPLICATIONS

SUMMARY

For nearly two decades, molecular biomimetics makes the solid-binding short peptides available for nano-biotechnological applications. The new trend for selecting the GEPIs (Genetically Engineered Peptides for Inorganics) is the combination of experimental knowledge with bioinformatics tools to design GEPIs computationally. Combinatorially selected and then computationally enhanced silica binding peptide, QBP1, can be used as a TAG peptide for protein purification process. Affinity tags are highly effcient tools for purifying proteins from crude extracts. The selected peptide's ability to bind strongly and specifically on silica material makes it a strong candidate to form a novel tag system for protein purification purposes.

In nature, as a result of evolutionary selection processes, biological systems present a high degree of sophistication by having the advantage of using key biomacromolecules such as lipids, proteins and polysaccharides. Among these macromolecules, proteins with unique and specific interactions with other macromolecules and inorganics control the structures and the functions of all hard and soft tissues in organisms. Bones, teeth, shells, skeletal units and spicules are examples of hard tissues produced as biocomposites in many multicellular organisms and they are composed of not only minerals of different kinds, including hydroxyapatite, calcium and silica but also structural macromolecules, especially proteins**.** They are the key molecules to form the inorganics-organics hybrids to acquire different functionalities. The examples include; magnetic nanoparticles formed by magnetotactic bacterium (*Aquaspirillum magnetotacticum*) which helps the bacteria to use the gravity to move, nanostructurally ordered thin film calcite on the outer layer of an S layer bacterium, *Synechococcus* strain GL24, that serves as a protective coating, mouse tooth enamel which is a hard, wear-resistant material with a highly ordered micro/ nano architecture consisting of hydroxyapatite crystallites that assemble into a woven rod structure.

In these examples, biomaterials are highly organized from the molecular to the nano-, micro- and macroscales with complex architectures that each hierarchical manner has its own different function. Their organization and formation are self-directed; their interaction with their surroundings is dynamic; their structures, functions, self-healing in damage control are complex and their physical and chemical properties are multifunctional so their characteristics are difficult to achieve in purely synthetic systems. On the other hand, forming the similar nanotechnological systems in technology, have limitations in the synthesis and assembly into useful functional structures and devices. If harnessed well, novel engineering systems at nano-meter scale dimensions could have unique mechanical, electronic, magnetic and solution properties of nanostructured composites, low-dimensional semiconductors, single-

domained particles and colloidal suspensions, respectively. Therefore mimicking nature has an enormous potential to achieve novel technological products and systems.

Molecular biomimetics, mimicking the processes of biology at the molecular scale in materials formation for addressable structures and genetically tailorable properties, is a new approach for future bio-inspired materials. Such materials could be based on proteins binding to inorganics specifically and controlling their synthesis, formation and assembly so that these proteins can take place as integral parts of the final hybrid composite system.

Molecular biomimetic approach has three solutions to the development of heterofunctional nanostructures: DNA-based technology, molecular and nanoscale recognition and self-assembly. Therefore, for nano- and nanobiotechnology applications, in the control of assembly and formation of functional inorganic and hybrid materials and systems, inorganic binding polypeptides are used as molecular building blocks..

For obtaining inorganic binding peptides, there is a consensus on using combinatorial biological techniques. A large library of different peptides having same number of amino acids is created and screened if there is a specific sequence that binds an inorganic material of practical interest strongly. The final aim is to produce a molecular erector set promoting the assembly of complex, hybrid structures composed of inorganics, proteins and even functional polymers. The proteins and their binding characteristics can be manipulated by using DNA technologies. These short polypeptides are referred as genetically engineered proteins for inorganics (GEPI). Post selection engineering followed the experimental knowledge by using bioinformatics to design GEPIs computationally. Sequence similarity scores are assigned by using genetic algorithm procedures so that *de novo* structure predictions can be made and tuned-up GEPIs can be developed for the desired affinity and specificity. The engineered peptides are used as molecular manipulators for a wide range of applications. From the molecular tool box, a protein data bank is available with containing fully characterized GEPIs. Furthermore, GEPIs can be fused to functional proteins that lead to multifunctional protein based constructs to be used for many nanobiotechnological applications.

In this part of the study, we used one of the second generation GEPIs (Quartz Binding Peptide, (QBP-1)), which was developed using computational tools based on the knowledge generated through phage display technology (first generation binder peptides). Genetically engineered vector systems with the required enzyme cleavage sites can make possible *in vivo* production of any desired peptide-protein construct. A peptide with specificity and affinity to a desired material, e.g., QBP-1, quartz binding peptide, can be linked to a desired functional biomacromolecule, such as laccases to form the molecular hybrid. The vectors can be used as hosts for the production of any other constructs just by changing either the peptide (ligand) or the protein (functional moiety). The selected peptide's ability to bind strongly and specifically on silica material will be used to form a Tag system. And these fusion proteins will be tried to produce in large quantities in novel protein expression systems such as the newly called SilTag. This system will be optimized to be a conventional method for the new coming GEPI and designer protein fusions.

Laccases (EC 1.10.3.2) are lignolytic enzymes which carry two or four copper atoms per molekül.They are extracellular phenoloxidases with the ability to oxidize

phenolic substances by reducing molecular oxygen into water. Laccases are key enzymes for many important industrial applications such as; detoxification of waste material from paper and textile industries, cleaning of herbicides and pesticides, water purification and production of cosmetic materials.

In our research group, the expression of laccase gene from *Pycnoporus sanguineus* strain in *Pichia pastoris* was achieved. In this part of the project, QBP1 peptide (PPPWLPYMPPWS) is placed at the N terminus of the laccase enzyme (lcc1) in *Pichia pastoris* yeast cells to form a hybrid fusion molecule. Firstly, fusion protein was formed at DNA level and transformed into yeast cells, then the hybrid fusion was expressed at protein level. The activities of wild type enzyme (here, lcc1 laccase enzyme) and fusion protein were compared showing no significant effect of the tag peptide on laccase enzyme. For the next part of the study, QBP1 peptide is used as a "TAG" molecule for purification of fusion molecule through silica/ quartz packed columns.

Overall, what we are showing here is that Molecular Biomimetics and bioinformatic tools, collectively, offer a good way of designing our own tag peptide for purification purposes with increased properties such as using cheaper material and single step easy purification protocols.

MALZEME ve MEDİKAL UYGULAMALAR İÇİN GEN MÜHENDİSLİĞİ YOLUYLA PEPTİD (GEPI)- PROTEİN HİBRİTLERİN OLUŞTURULMASI

ÖZET

Doğada, milyonlarca yıl süren evrimsel süreçler sonucunda, lipidler, polisakkaritler ya da proteinler gibi biyomakromoleküller yüksek seviyede sofistike olmuş sistemlerin oluşmasını sağlamıştır. Oluşan bu sistemlerle canlılar, zorlu çevre şartlarına daha iyi uyum sağlamanın yollarını keşfetmişlerdir. Bu biyomakromoleküller arasında proteinler özel bir öneme sahiptir. Proteinler diğer makromoleküller ve inorganikler ile tepkimeye girerek tüm sert ve yumuşak dokuların yapısında bulunurlar ve tüm fonksiyonların çalışmasında etkili olurlar. Kemikler, dişler, deniz kabukları ve iskeletler, proteinlerin diğer organik ve inorganik moleküllerle birlikte çalıştıkları örneklerdir. Bu yapılarda yer alan proteinler sadece organik moleküllerle değil, aynı zamanda inorganik moleküllerle de bağ yaparak değişik fonksiyonel özelliklerin kazanılmasını sağlarlar. Örneğin, magnetotaktik bakteride (*Aquaspirillum magnetotacticum*) hücre içerisinde inorganik nanoyapılar bakterinin yerçekimi yardımı ile hareketini sağlar. Diğer bir örnek olan S-layer bakterisi (*Synechococcus* strain GL24) yüzeyinde oluşan kalsit tabaka koruyucu bir yapıdadır. Benzer şekilde fare dişindeki enamel, hidroksiapatit kristallerinden oluşan ve dişe hem esneklik hem dayanıklık sağlayan bir yapıdır.

Bu örneklerde görülen, nano boyuttan makroya, yüksek derecede organize olmuş yapılar hiyerarşik bir şekilde oluşmuşlar ve inorganik moleküller ile organik moleküller birlikte rol almışlardır. Evrimsel süreçler sonucunda elde edilen bu fonksiyonlar ile canlılar çevresel şartlar karşısında avantaj elde etmişlerdir.

Diğer yandan, doğada gözlemlenen bu tür fonksiyonel yapıların endüstride ve teknolojide yeniden yapılması son derece zorlu bir süreçtir. Bu yapılar detaylı incelendiğinde, proteinlerin düzenleyici, kontrol eden, bir araya getiren özellikleriyle bu yapıların oluşmasının temelinde olduğunu görüyoruz. O halde bu tür fonksiyonel özellikleri elde edebilmemiz için, inorganiklere seçici olarak bağlanabilen proteinleri kullanabiliriz.

Moleküler Biyomimetik doğada moleküler seviyede gerçekleşen süreçleri izleyerek, benzer sistemleri oluşturmayı ve fonksiyonel sistemler kurmayı amaçlar. Canlıların milyonlarca yıl süren evrimsel süreçlerle elde ettikleri yararlı fonksiyonların gözlenmesi ile benzer fonksiyonel sistemler laboratuvar ortamında elde edilebilir. Moleküler Biyomimetik'in temelini de inorganiklere spesifik olarak bağlanabilen peptidlerin bulunması ve uygulamalarda kullanılması yatar.

İnorganiklere spesifik olarak bağlanabilen peptidlerin bulunması için aşamalı bir yol izlenmesi genel kabul görmektedir. Öncelikle, kombinatoryel biyolojik teknikler (faj gösterim, hücre yüzey gösterim metodları vb.) yoluyla istenilen inorganik

malzemeye spesifik peptidler bulunur ve bağlanma dereceleri sınıflandırılarak optimize edilir. Devamında ise, deneysel verilerden elde edilen bilgiler ışığında, biyoinformatik yöntemler ve benzerlik analizleri yoluyla daha iyi bağlanabilen peptidler elde edilir ve bu peptidler de test edilerek bağlanabilirliği kontrol edilir**.**

Bu çalışmada kullanılan QBP1 peptidi 12 amino asit uzunluğunda ve kuartza bağlanabildiği deneysel çalışmayla gösterilmiş dizilerin biyoinformatik işlemden geçirilmesi sonucunda ortaya çıkarılmıştır. QBP (Quartz binding peptid), kuartza seçici olarak ve yüksek afinitede bağlanabilen 12 amino asit uzunluğunda bir peptidtir. Kuartz yüzeyine bağlanabilme özelliği sayesinde, istenilen bir protein QBP1 yoluyla kuartz yüzeyinde sabitlenebilir. Ancak bunun için ilk yapılması gereken, DNA seviyesinde QBP1 peptidinin dizisini iatenilen proteinin DNA dizisine eklemektir. Burada QBP1 peptidini AP (alkalin fosfataz) enziminin N-terminal ucuna eklendi. Protein üretilmesi aşamasında ise, bakteri hücresi kullanıldı ve sonuçlar optimize edilmeye çalışıldı. Burada amaçlanan, QBP1 yoluyla istenilen bir proteinin kuartz yüzeyine bağlanabileceğini ve uygulamalar açısından kullanışlı olacağını göstermektir. Ancak dikkat edilmesi gereken en önemli konuların başında, bakteri hücresinde protein üretimi sırasında meydana gelebilecek sıkıntılardır.

Projemizde 2. jenerasyon GEPI'ler (Kuartza bağlanabilen peptidler, QBP-1) kullanıldı. Bu peptidler grubumuzda daha önceden faj gösterim metodu ile elde edilen 1. jenerasyon QBP'lerin biyoinformatik yöntemlerle işlenmesi sonucu elde edildi (1). Kuartz yüzey üzerine seçici olarak bağlanmada diğer QBP'ler arasından öne çıkan QBP-1 bu çalışma için seçildi. QBP-1 ile herhangi bir fonksiyonel molekül DNA seviyesinde biraraya getirilerek hibrid moleküller oluşturulabilir ve QBP-1'in kuartz yüzeyine seçici olarak bağlanabilme özelliğinden yararlanılarak istenilen molekülün fonksiyonel özellikleri kullanılabilir.

Lakkazlar (EC 1.10.3.2) ligninolitik enzimler olarak molekül başına iki ya da dört bakır atomu tasıyan ve fenolik maddeleri moleküler oksijeni suya indirgeyerek okside edebilme özelligi olan ekstraselüler fenoloksidazlardır. Kagıt ve tekstil sanayiinden gelen endüstriyel atıkların detoksifikasyonu gibi çesitli biyoteknolojik süreçlerde çok önemli uygulamaları bulunan enzimler olup, herbisit ve pestisitlerin temizlenmesi, belli su saflastırma sistemleri için temizleme ajanları ve kozmetik malzemesi olarak da kullanılabilmektedir (2,3,4). Grubumuzda *Pycnoporus sanguineus* suşundan elde edilen lakkaz geninin *Pichia pastoris*'te ekspresyonu sağlanmıştır. Projenin bu kısmında ise, QBP1 peptidi (PPPWLPYMPPWS), *Pichia pastoris* maya hücrelerinden elde edilen lakkaz (lcc1) enziminin N-terminal ucuna yerleştirilerek füzyon protein oluşturuldu. Öncelikle DNA seviyesinde oluşturulan füzyon protein *Pichia pastoris* hücrelerinde üretildi. Oluşturulan füzyon protein ile lcc1 enziminin aktiviteleri karşılaştırılarak, QBP-1 peptidinin bağlanmış olduğu enzim aktivitesi üzerine etkisi ortaya konuldu. QBP1 peptidi, N-terminale bağlı olduğu halde, enzim aktivitesinde değişikliğe neden olmamaktadır. Bu ise bir "tag" molekülü olarak QBP1 için oldukça avantajlı bir özelliktir. Yapılacak uygulamaya göre, saflaştırma sonrasında tag molekülü, enzim ile bağlı olarak bırakılabilir. Çalışmanın devamında QBP-1 peptidi bir "tag" peptid olarak görev yaptırılarak hücre ortamından füzyon proteinin saflaştırılmasında kullanılmıştır. Hazırlanan kuartz kolonlar ve batch sistem yollarıyla, QBP-1'in kuartz yüzeyine bağlanabilme özelliği kullanılarak füzyon proteini hücre ortamından saflaştırmak mümkün olmuştur.

Sonuç olarak burada, Moleküler Biyobenzetim ve biyoinformatik tekniklerinin birlikte kullanılmasıyla, daha ucuz ve basit saflaştırma yöntemleri geliştirme amaçlı yeni TAG moleküllerinin elde edilebileceğini göstermiş oluyoruz. Kuartza seçici olarak bağlanabilen QBP1 peptidi, sahip olduğu özellikler sayesinde, mevcut ticari "tag" (afinite etiketi) sistemleri ile rekabet edebilecek bir tag molekülüdür.

1. INTRODUCTION AND BACKGROUND

1.1 Inorganic-binding Proteins and Their Importance in Organismal Biomineralization

As the main building material of the cell, proteins do most of a cell's work. Through their unique and specific interactions with other macromolecules and inorganics, they control the formation of cellular structures and functional activities in the biological tissues. Soft (Kaplan *et al.*, 1994) and hard (Lowenstam and Weiner, 1989) tissues, as the main biological tissues in which the proteins do most of the work, have very organized structure from molecular to the nanoscale. Soft tissues are the biomaterials containing only organic part as in the cases of muscle, membranes, skin, tendon, spiders' silks, and cuticles and they are of great interest in soft tissue engineering (Kaplan *et al.*, 1994, Sakiyama and Hubbell, 2001).

On the other hand, hard tissues contain organic-inorganic hybrid systems (Lowenstam and Weiner, 1989, Mann, 1996) such as bones, dental tissues (i.e., dentine and enamel), spicules, spines, shells, skeletal units of single-celled organisms (e.g., radiolarian) or plants, bacterial thin film, and nanoparticles (Lowenstam and Weiner, 1989, Mann, 1996). For more than 3 decades, they have been an inspiration for materials design and engineering due to their key properties. They have an architectural organization from molecular to nano-, micro-, and macro-scales which have often been formed in a hierarchical manner, with very complex nano-architectures and infinite number of functional diversity. They are ''smart,'' dynamic, complex, self-healing, and multifunctional. At the center of all these hard tissues, there is a common proteinaceous phase working as the main organizer of the system.

After hundred thousands years of evolutionary processes, nature has built abundant examples of materials and systems where inorganics and organics have met for improved and refined properties. Figure 1.1 shows three examples of naturally occuring organic-inorganic hybrid material formations in different species. The first

one is a single-celled bacteria called *Aquaspirillum magnetotacticum* that incorporates a magnetic nanoparticle system (Figure 1.1A). The bacteria forms magnetic nanoparticles which are magnetite (Fe3O4) formed as perfect cubo-octahedral single crystals, about 50 nms. in diameter. About 25 nanoparticles align within the cell to to create a magnetic moment large enough to sense the Earth's magnetic field.

The second one is mother-of pearl of seashells, the natural armor of mollusks (Figure 1.1B), which has a layered shell structure in the form of $CaCO_3$ and biopolymer mixture (proteins and polysaccharides). The soft and hard component phases in the shell structure gives the necessary toughness and fracture strength for the organism's protection.

The third one is a mammalian tooth (Figure 1.1C). One of its tissues is enamel which is the hardest material in the body, protecting the tooth. Under the enamel, there is dentin layer which is softer and absorbs the stress during chewing. A group of proteins control the formation of ordered fibers of 3 microm-diameter enamel rods, each consisting of thousands of 30 nm-diameter, mm-long, and crystallographically-aligned elongated hydroxyapatite (HA) crystallites. This organics-inorganics hybrid structure prevents premature fracture or failure.

All these examples of hybrid systems are coming from the organisms which have naturally found the ways to use some of their proteins to produce and bind the inorganic materials *in vivo*, and then use these excellent systems for their benefit. This systems are formed through years of evolutionary processes in aqueous environments under mild physiological conditions to perform excellent functions such as forming protective layers, supportive tissues, transferring charge and ion, developing some optical and mechanical properties. The key molecule to form such functional systems is proteins with indispensable properties in every process within the cells such as working for mineralization, serving as enzymatic catalysts, being a usefull transport and storage molecules, mediating cell responses, being active in immune protection and cell differentiation. By observing the functions of the proteins in nature, we can genetically engineer polypeptides to specifically bind to selected inorganic compounds to form hybrid materials to get excellent functions for applications in nano- and biotechnology. (Sarıkaya and Aksay, 1995, Mann, 1996, Lowenstam and Weiner, 1989).

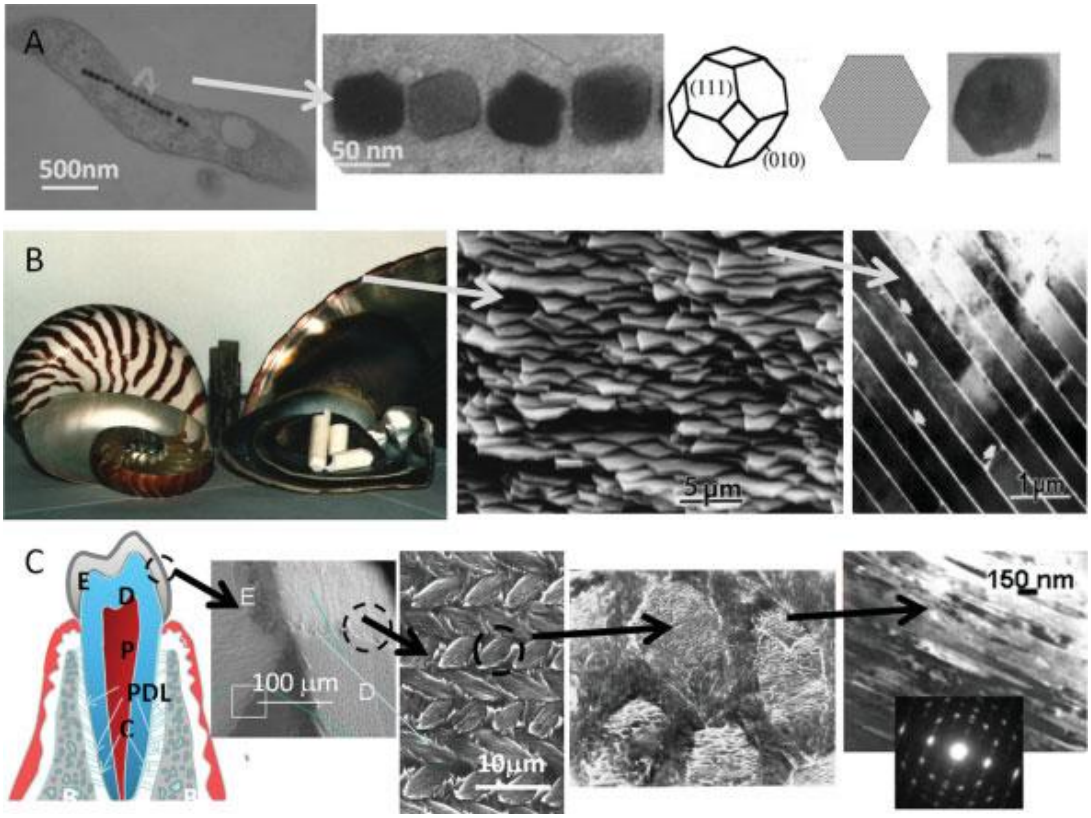


Figure 1.1: Examples of biologically synthesized organic-inorganic hybrid materials with a variety of physical properties: (A) Single-crystalline, single-domained magnetic magnetite nanoparticles (Fe_3O_4) formed by a magnetotactic bacterium (*Aquaspirillum magnetotacticum*) (inset: higher magnification image of the magnetite nanoparticles revealing cubo-octahedral particle shape). (B) Nacre, mother-of-pearl is a segmented laminated composite of calcium carbonate and biomolecules having mechanical properties that provide an armor to the mollusks such as red abalone, pearl oyster, and nautilus. (C) Mammalian tooth is a hierarchical multimaterial system composed of enamel (e), dentin (d), pulp (p), cementum (c) and periodontal ligaments (PDL). Enamel, such as the one shown here from mouse, is ~100% hydroxyapatite crystals that have a hierarchically ordered woven structure providing stability against complex mastication mechanical stresses. (Sarıkaya *et al.*, 2010).

1.2 Applications of Inorganic-binding Proteins in Nano and BioTechnology

For decades, traditional approaches try to overcome the limitations of nano-technological systems. The difficulty in the synthesis of nano-technological systems and their assembly into useful functional structures and devices prevents us to benefit from the full potential of nanotechnology in sciences and technology. Synthesis of nanoscale materials by traditional approaches such as melting and solidification processes, followed by thermo-mechanical treatments, or solution/vacuum deposition and growth processes (DeGarmo *et al.*, 1988), is inefficient, requiring stringent

conditions such as high heat and pressure, and often producing toxic byproducts (Niemeyer, 2001, Sarıkaya *et al.*, 2003). Even most advanced micro and nanotechnology processes have the limitations of large scale synthesis of complex nano-scale architectures. The problem of scaling-up in nanotechnology is still standing against traditional approaches. The product is so small in quantity and not reproducible because of nonspecific interactions and uncontrolled agglomeration. Even today's one of the most successful nano-technological systems, carbon nano-tubes, have the same kind of limitations such as uncontrolled surface chemistry, and difficulties in multidimensional assembly for the widespread use (Harris, 1999).

For the control and production of large scale nano-structures, practical strategies are needed (Schmid, 1994). Observing and analyzing the examples from the nature can show the way to find those strategies. As exampled in Figure 1.1, the nature itself produces ordered and complex nanostructures where inorganic materials are synthesized in aqueous environment under mild conditions such as ambient temperature, pressure and neutral pH. Magnetite (Fe_3O_4) particles in magnetotactic bacteria or teeth of chiton (Frankel and Blakemore, 1991); silica (SiO_2) as skeletons of radiolarian (Lowenstam and Weiner, 1989) or tiny light-gathering lenses and optical wave guides in sponges (Fong *et al.*, 2000); hydroxyapatite ($Ca_2C(OH)_3$) in bones (Glimcher and Nimni, 1992) and dental tissues of mammals (Paine *et al.*, 2000); calcium carbonate ($CaCO_3$) in the shells of mollusks as armor (Mayer and Sarıkaya, 2002) or as thin protective films in some species of S-layer bacteria (Schultze *et al.*, 1992); and spines and tests of sea-urchins (Lowenstam and Weiner, 1989) are all examples of natural occurring nanostructures and hard tissues where a proteinaceous phase is active in the synthesis (Sarıkaya, 1999). In all these examples, proteins are the workhorses that control the fabrication of biological structures by orchestrating the assembly of materials in two and three dimensions.

1.3 Obtaining Inorganic-binding Proteins

To get the similar functional properties as shown in Figure 1.1, we need to somehow produce or get inorganic binding polypeptides. There are several possible ways to obtain the proteins or polypeptides, specific to inorganic substances. Some of them are naturally occurring inorganics-binding proteins such as ice-binding (antifreeze) proteins which are synthesized in many fish species, plants, and insects (Liou *et al.*,

2000). They bind to ice in the internal fluids of the organisms and control particle size, morphology, or disruption. The examples in Figure 1.1 are systems in which naturally occuring proteins are used to form inorganic material *in vivo* and then the organism uses these inorganics for its benefit. First way of having inorganic-binding polypeptides such as the examples given before, is to extract biomineralizing proteins from hard tissues, followed by purification and the cloning of their genes. So far, several proteins isolated in this fashion. They have been used as nucleators, growth modifiers, or enzymes in the synthesis of certain inorganics (Cariolou and Morse, 1988, Paine and Snead, 1996, Berman *et al.*, 1988, Weizbicki, 1994, Kroger *et al.*, 1999, Cha *et al.*, 1999). Amelogenins in mammalian enamel synthesis (Paine and Snead, 1996); silicatein, in sponge spicular formation (Cha *et al.*, 1999); and calcite- and aragonite-forming polypeptides in mollusk shells (Cariolou and Morse, 1988, Berman *et al.*, 1988, Weizbicki *et al.*, 1994) are the examples for that case. The problem with this way is the limitations with the extraction process and further analysis steps. It is difficult and time consuming. Limited number of known naturally occuring proteins is another drawbacks for this way of getting inorganics-binding polypeptides.

Another way of obtaining inorganic-binding peptides is designing them using a theoretical molecular approach similar to those employed for the design and development of pharmaceutical drugs (Schneider and Wrede, 1998, Schonbrun *et al.*, 2002). But that is currently impractical and too expensive for current materials research.

Finally, there is a consensus on using another way of obtaining such inorganic binding peptides which are referred as genetically engineered proteins for inorganics (GEPI) (Sarıkaya *et al.*, 2004). This way is using combinatorial biological techniques (Sarıkaya *et al.*, 2003, Naik *et al.*, 2002) which is basically using a large library of different peptides having same number of amino acids to screen if there is a specific sequence that binds an inorganic material of practical interest strongly. This technology have been used for more than two decades for obtaining organics-binding peptides and been adapted for selection of inorganics-binding peptides.

1.4 Molecular Biomimetics

Combination of traditional physical and biological fields brings us a new field called molecular biomimetics. It is an emerging field where assembly of hybrid materials can be achieved at the molecular scale using the specific recognition properties of proteins. Its essence is basically mimicking the processes of biology at the molecular scale. The ability of recognition and specifically binding to inorganics makes the proteins to form inorganic-organic hybrid materials which form the base for this new field (Sarıkaya *et al.,* 2003, Ball, 2001).

Three basic solutions are offered by molecular biomimetics for the problem of control and fabrication of large-scale nanostructures and ordered assemblies of materials in two and three dimensions as schematically. The new field offers three unique advantages derived from Nature.

The first, complex nano-, and possibly hierarchical structures, similar to those found in nature (self-assembly) can be formed by using the ability of biological molecules to self- and co-assemble into ordered nanostructures. The ultimate goal of molecular biomimetics is to generate a molecular erector set in which different proteins, each engineered to bind to a specific surface, size, or morphology of an inorganic compound, promote the assembly of intricate, hybrid structures composed of inorganics, proteins, and even functional polymers (Sarıkaya *et al.*, 2003). Some potential acting ways of inorganic-binding proteins are exampled in Figure 1.2.

Secondly, synthetic entities, including nanoparticles, functional polymers, or other nanostructures can be bind onto molecular templates where the molecular and nanoscale recognition by the use of surface specific proteins are effective. In this step, the protein template is the linker or in other words “molecular erector sets” to join synthetic entities and the inorganic surface which the protein template is specific.

Final one is designing the protein templates through genetics at the molecular level. This is DNA-based technology and inorganic binding peptides and proteins are selected and designed. The designed protein template is the result of the DNA which codes for that protein.

Current progress in the field points to a possible beginning of a new era where biological principles are not only used in designing materials which mimic the

properties of biological counterparts, but also to take advantage of the functions and underlying principles of biomolecular processes to produce novel materials, systems and fabrication techniques that exhibit exquisite sensitivity, and accuracy, self healing, adaptability, control and, even, self replication.

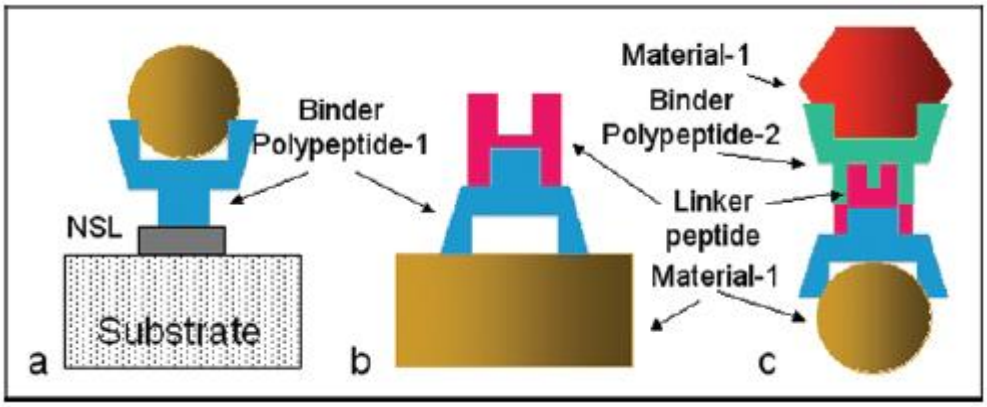


Figure 1.2: Potential uses of inorganic-binding polypeptides. (*a*) linkers for nanoparticle immobilization, (*b*) functional molecules that assemble on specific substrates, and (*c*) heterofunctional linkers involving two (or more) binding proteins adjoining several nanoinorganic units. NSL, nonspecific linker (Sarıkaya *et al.*, 2004).

1.5 Peptide Display Technologies

Development of combinatorial selection techniques more than two decades ago led to enormous progress in the characterization of antibody-receptor, protein- or peptideligand interaction for a myriad of biotechnological and biological applications. Among them, phage (Smith *et al.*, 1997) and cell surface displays (Boder *et al.*, 1997, Lu *et al.*, 1997, Brown *et al.*, 2000) have been widely used. Both of the techniques rely on the link between phenotype and genotype of the organisms. Random peptide sequences that are encoded in either phage genome or plasmid bacterial DNA are displayed within the context of proteins localized on the surface of the phage particle or the cell, respectively. Outer membrane proteins, lipoproteins, fimbriae and flagellar proteins have been used to display the randomized peptide library on surface of bacteria,5 while phage display utilizes the major or minor coat proteins of bacteriophage M13 to display the random peptides on the virus surface.

Phage display (PD) (Smith, 1985, Hoess, 2001) and cell-surface display (CSD) (Wittrup, 2001) are well-established in vivo display techniques for the isolation of polypeptides capable of binding inorganic materials with high affinity. They have been used to screen and identify various biological activities, such as catalytic properties or altered affinity and specificity to target molecules in many applications including the design of new drugs, enzymes, antibodies, DNA-binding proteins and diagnostic agents (Rosander *et al.*, 2002, Petrouna *et al.*, 2000, Smith and Petrenko, 1997, Benhar, 2001). Ribosomal and messenger RNA display technologies are the main *in vitro* methods (Amstatz *et al.*, 2001) which have been developed for increased library size (10^{15}) compared to those of *in vivo* systems (10^{7}–10^{10}).

1.6 Adaptation of Combinatorial Techniques for the Selection of Inorganic-binding Proteins

To use display techniques for selecting inorganic-binding polypeptides, combinatorial selection procedures were adapted and optimized for the organic-inorganic interaction. Among biocombinatorial *in vivo* and *in vitro* methods, phage and cell surface displays became the predominant tools for material-specific peptide selection.

In the standard biopanning round, the library of phage or cell clones, displaying a vast population of randomized peptides on their surfaces, is exposed to inorganic targets. Spectroscopic and imaging techniques were used to characterize inorganic surfaces before and after biopanning rounds to detect any change on the surface (Dai *et al.*, 2004). If evidence of surface modification is obtained, buffer conditions should be optimized to guarantee compatibility with the target inorganic. The effect of the conditions in the rounds on the biological materials, phages or bacteria, need also be optimized. In general, a large, random library of peptides with the same number of amino acids, but of different sequence compositions, is screened to identify specific sequences that strongly bind to a chosen inorganic material surface. There is no need for a priori knowledge of the desired specific polypeptide or single amino acid. The specific polypeptide sequence is simply selected and enriched.

In Figure 1.3, the adaptation of *in vivo* display techniques, phage display (PD) and cell surface display (CSD), is simply presented. In these techniques, libraries are generated by inserting randomized oligonucleotides within certain genes encoded on

phage genomes (Whaley *et al.*, 2000, Naik *et al.*, 2002) or on bacterial plasmids (Brown, 1997, Schembri *et al.*, 1999, Brown *et al.*, 2000) (step 1 in Figure 1.3). When the libraries are created, a random polypeptide sequence is incorporated within a protein on the surface of the selected organism. This protein is a coat protein of a phage for phage display selection procedure or an outer membrane or flagellar protein of a bacterial cell for cell surface display selection methodology; (step 2). The peptide is displayed randomly on each phage or cell, and different than others (step 3). The next step is introduction of the random peptide library which contains a heterogeneous mixture of recombinant cells or phages, and the selected inorganic substrate (step 4). After several washing cyles, non-binder cells or phages are eliminated. The weak interactions with the substrate and the cells or phages are disrupted via washing buffers (step 5). Better binders stay on the surface and can be eluted by using strong buffers (step 6). The eluted phages are amplified by reinfecting the host in phage display (step 7); on the other hand, in cell surface display, cells are allowed to grow (steps 7, 8). This is the end of a round of biopanning which is generally repeated 3-5 times to to enrich the population with clones having high-binding affinity to inorganic solid targets.

In the final step, individual clones from the selected bound pool are isolated and binding peptide sequences are identified (step 9) by DNA analysis of the phage genome (Figure 1.3). The advantage of the bacterial cell surface display system over phage display library is its greater efficiency in generating peptide sequences, simply because of the fact that the former does not need a second host organism to amplify and replicate genomic and plasmid sequences.

Since the adaptation of combinatorial techniques for obtaining inorganic-binding polypeptides, many short peptide sequences have been identified for inorganic targets including noble metals; Au (Lu *et al.*, 1995, Huang *et al.*, 2005), Ag (Naik *et al.*, 2002, Nam *et al.*, 2008), Pt (Seker *et al.*, 2007), oxides; SiO2 (Oren *et al.*, 2007), ZnO (Thai *et al.*, 2004), Cu2O (Thai *et al.*, 2004), TiO2 (Dickerson *et al.*, 2008, Sano *et al.*, 2005, Sano *et al.*, 2003), minerals; hydroxyapatite (Gungormus *et al.*, 2008), calcite (Gaskin *et al.*, 2000), graphite, sapphire (Krauland *et al.*, 2007) and semiconductors; GaN (Whaley *et al.*, 2000), ZnS and CdS (Lee *et al.*, 2002).

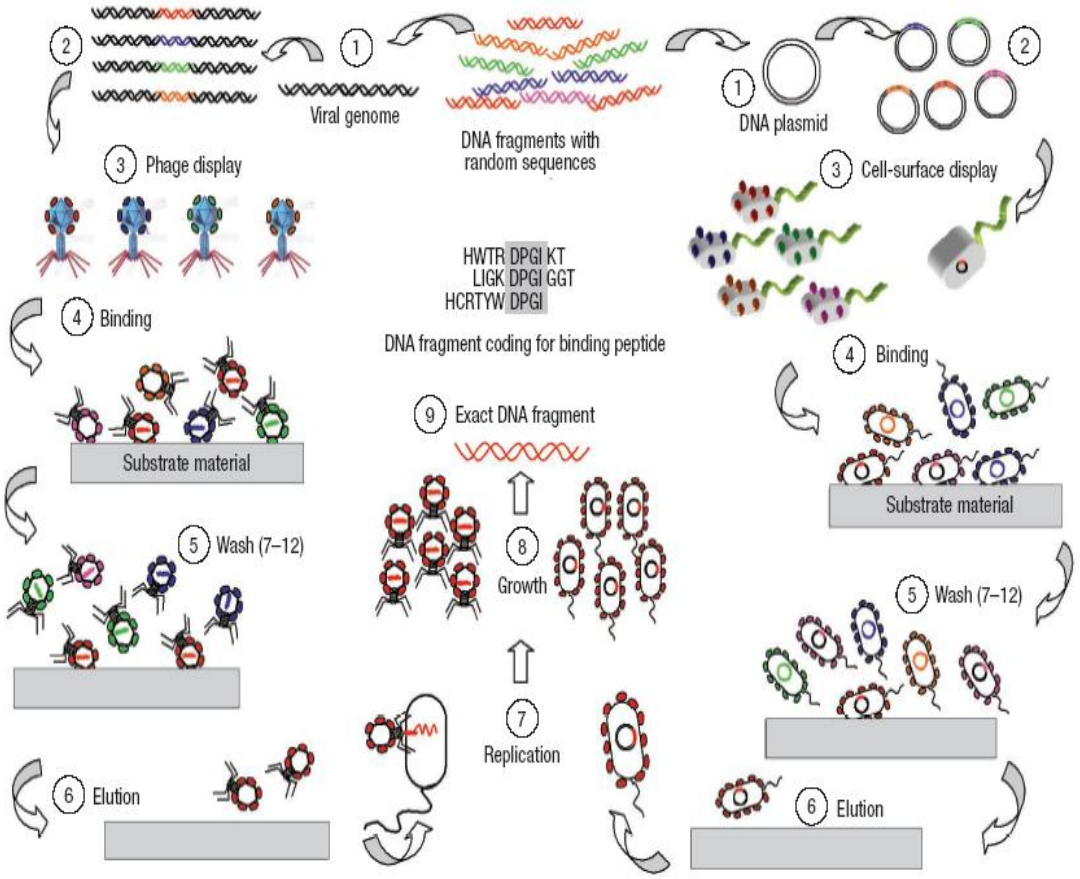


Figure 1.3: Phage display and cell-surface display. Principles of the protocols used for selecting inorganic binding polypeptide sequences (Sarıkaya *et al.*, 2004).

All these binders have been used in the proof-of-principle synthesis, morphogenesis and assembly of inorganics, finding practical applications in a wide ranging and diverse areas of nanotechnology and medicine (Sarikaya *et al.*, 2004, Naik *et al.*, 2002).

1.7 Computational Selection Methodology: Combining Experimental Knowledge with Bioinformatics

The new trend for selecting the GEPIs is the combination of experimental knowledge with bioinformatics tools to design GEPIs computationally. The inspiration for this trend is coming from evolutionary biology. As we know from the examples of nature, there is a correlation between the sequence and the function. Having similar sequences usually means performing similar functions due to biochemical, biophysical and evolutionary constraints.43. Combining Experimental Knowledge

with Bioinformatics is a general one and can be used to design new sequences that bind to a given inorganic solid with predictable and enhanced affinity (Oren *et al.*, 2007).

To do that, as shown in Figure 1.4 (Tamerler *et al.*, 2010), GEPIs are selected experimentally and characterized so that they are grouped into three sequence clusters, i.e., strong, moderate and weak, according to their material-binding affinities. Then, this experimental knowledge is combined with sequence alignment methods (Needlema *et al.*, 1970, Smith *et al.*, 1981) and the standard scoring matrices (Henikoff *et al.*, 1992, Dayhoff *et al.*, 1978), to generate a novel material specific sequence scoring matrix (Oren *et al.*, 2007) that would account for the specific sequence patterns responsible for binding. So that, *de novo* structure predictions can be made and finally, tuned-up GEPIs can be developed for the desired affinity and specificity. To design high affinity GEPIs, random sequences can be generated and by using scoring matrixes their sequence similarities can be compared to the experimentally known strong binding sequences.

Different material specific matrices can be combined for the selection of GEPIs with multiple inorganic material recognition and binding functionalities (Figure 1.4) (Tamerler *et al.*, 2009).

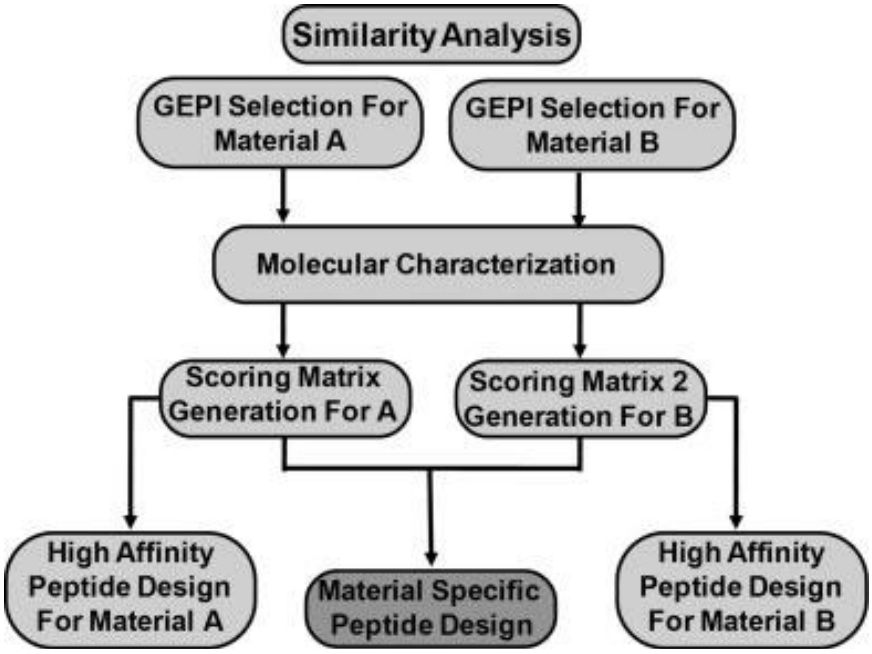


Figure 1.4: Knowledge based approach for generating next generation of peptides with better binding properties. (Tamerler *et al.*, 2010).

1.8 Current and Potential Applications of Inorganic Binding Peptides (GEPIs)

Among the others, two review papers (Tamerler et al 2008, 2010) which were published by our research group summarize the current and potential applications of inorganic binding polypeptides (GEPIs). They can be usefull for a wide range of applications at the core of nano-technology (Drexler *et al.* 1994), bio-nanotechnology (Niemeyer *et al.* 2001), as well as biological materials science and engineering.

Firstly, GEPIs are assemblers and linkers. Functional nano particles, proteins as well as organic dyes can be immobilized onto specific inorganic surfaces by using inorganic binding peptides. (Krauland *et al.* 2010, Zin *et al.* 2007, Kacar *et al.* 2009, Zin *et al.* 2005, Park *et al.* 2006).

GEPIs provide the self assembly of the nano-components on the surface for various applications, e.g., protein immobilization, biosensing, microarray fabrication. Two basic properties of GEPIs make them suitable for this type applications: which are their high affinity and material specificity. (Sarikaya *et al.* 2003, Brown *et al.* 1997, Tamerler *et al.* 2006).

Figure 1.5 shows an example of using GEPIs to directly assemble a nanoparticle on a micropatterned substrate composed of multimaterial regions, e.g. gold, platinum, silica (Tamerler *et al.* 2010). Gold binding peptide (AuBP), three repeats of a platinum binding peptide (3R-PtBP) and a quartz binding peptide (QBP) were used in immobilization of streptavidin-coated quantum dots (SAQD) that emit red light under the fluorescence microscope (FM).

They can also be used as as ink and printed onto the solid surfaces specifically using different lithography techniques, e.g., soft lithography (Zin *et al.* 2007, Kacar *et al.* 2009) and dip-pen lithography (Zhao *et al.* 2006). GEPIs can be chemically linked to other linker molecules too, e.g., biotin (Figure 1.5) and organic molecules, e.g. FITC.

Secondly, GEPIs are used as molekuler linkers in biomaterial applications. They bind quite strongly to their respective materials. Several of them have already been shown to form densely packed monolayers in entirely aqueous solutions under ambient conditions (e.g., pH *7.0). So that, they are uniquely suited for modification of

biomaterial surfaces. Bioactive and bioinert surfaces can be created via GEPI linkers on a number of materials.

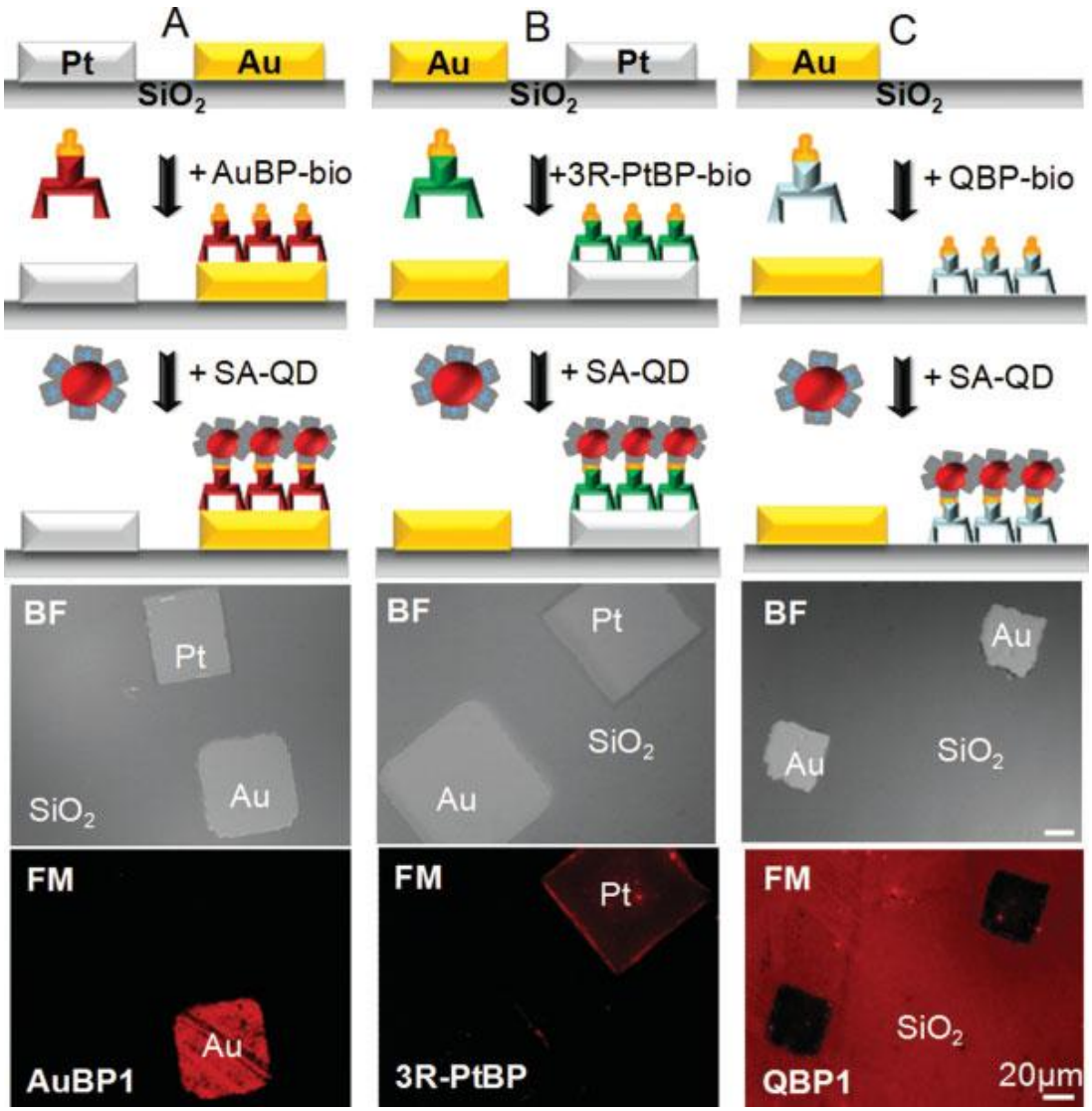


Figure 1.5: Use of GEPI as linker for preferential assembly of SA-QD on different inorganic surfaces. The substrates composed of either Pt and Au or only micropads on silica were incubated with either AuBP-bio (A), or 3R PtBP-bio (B) or QBP-bio (C). After the procedure schematically shown for each peptide was carried out the substrates were examined under FM. (Tamerler *et al.* 2010).

Figure 1.6 shows immobilization of RGD integrin-binding sequence on silica glass using QBP1 peptide for bone grafts and other tissue-integrated applications.

Thirdly, using GEPIs is a practical approach in biomineralization purposes for restorative and regenerative tissue engineering. GEPIs have been a usefull agent in fabrication of inorganic substrates, templated conjugates, production of protein-

encapsulated particles and controlled synthesis of nano-particles and minerals (Gungormus *et al.* 2008, Gaskin *et al.* 2000). Besides these peptides can be utilized to control the formation of biologically important hard tissues, i.e., bone and teeth.

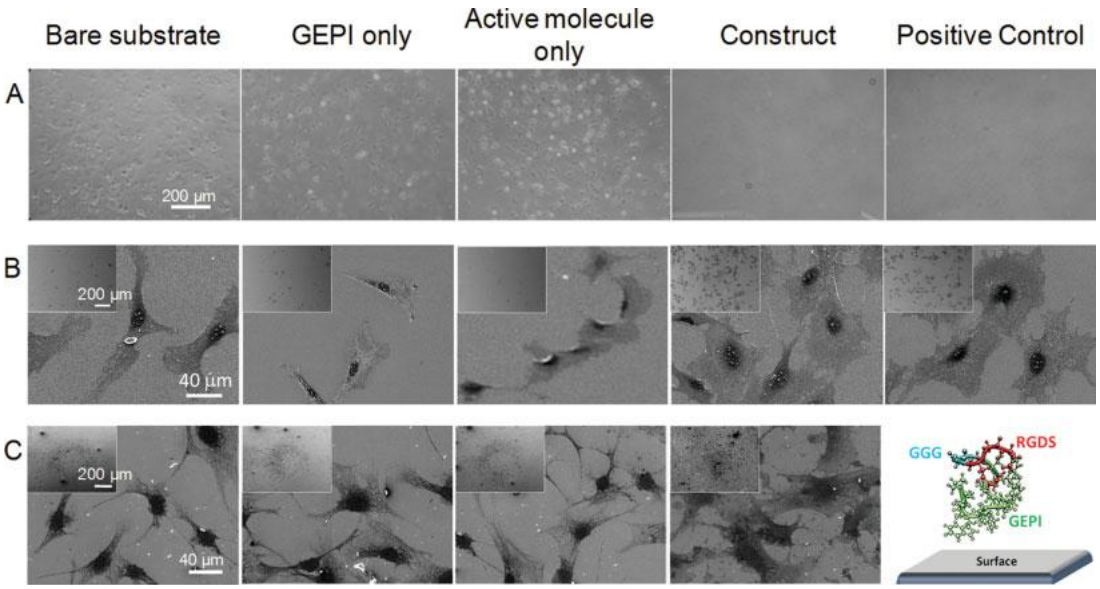


Figure 1.6:(A) Light optical microscopy images of NIH3T3 fibroblast cells (3 3 105 cells, 1.5 hrs, 2003) on gold substrate modified with GEPI-PEG conjugate; (B) Scanning electron microscopy (SEM) images of NIH3T3 (5 3 103 cells, 24 hrs) on glass substrate modified with GEPI-RGD conjugate; (C) SEM images of NIH3T3 (83 104 cells, 24 hrs) on titanium surface modified with GEPI-RGD conjugate.

Additionally, GEPIs can also be used as as ink and printed onto the solid surfaces specifically using different lithography techniques, e.g., soft lithography (Kacar *et al.* 2009, Zin *et al.* 2007) and dip-pen lithography (Zhao *et al.* 2006). They can be chemically linked to other linker molecules too, e.g., biotin and organic molecules, e.g. FITC.

1.9 TAG Technology-Affinity Tags

1.9.1 Significance of affinity tags in purification of recombinant proteins

Affinity tags are highly efficient and mostly used tools for structural and functional proteomics research. They have been developed to facilitate the detection and purification of recombinant proteins from cells and in recent years, it has become

clear that affinity tags have become indispensable tools for protein studies for excellent reasons (Terpe *et al.* 2003).

They allow the detection and purification of nearly any protein with hundred or even thousand fold purification from crude extracts. There is no need for prior steps to remove nucleic acid or other cellular material. Simple experimental procedure and mild elution conditions, and for some tag systems, positive impact on the yield, solubility and even the folding of their fusion partners, are the strong qualities about the tag systems which make them prefferrable over conventional chromatography in which highly customized procedures are associated with (Lichty *et al.* 2005).

They can be categorized mainly into two classes depending on the nature of the affnity tag and its target; First class is the peptide or protein tags which recognize small ligands linked to a solid support. The hexahistidine tag and glutathione *S*-transferase protein fusions are such tags which bind to immobilized metal and glutathione attached to chromatography resin, respectively.

In the second class, there are peptide tags which bind on a protein binding partner immobilized on chromatography resin as the calmodulin-binding peptide tag binds specifically to calmodulin allowing proteins fused to the peptide to be purified over calmodulin resin. The protein binding partner can also be an antibody, recognizing a specific peptide epitope as in the FLAG peptide tag, which can be used with one of several anti-FLAG antibody resins.

1.9.2 Comparison of commercially available peptide/protein tags

In the last 25 years, a variety of proteins, domains, or peptides have been used as affinity tags (reviewed in Nilsson *et al.* 1997, Heam *et al.* 2001, Terpe *et al.* 2003, Arnau *et al.* 2006). For example, a hexahistidine tag (His-tag) (Hochuli *et al.* 1987, Hochuli *et al.* 1988), glutathione S-transferase and maltose-binding protein (Smith *et al.* 1988, di Guan *et al.* 1988) and peptide epitopes like the FLAG-tag (Einhauer *et al.* 2001), the calmodulin-binding peptide (Vaillancourt *et al.* 2000), the Strep-tag or Streptag II (Schmidt *et al.* 1993, Voss *et al.* 1997) and the biotin acceptor peptide (Schatz *et al.* 1993) were all used as tag system for the purification of proteins form cell extract.

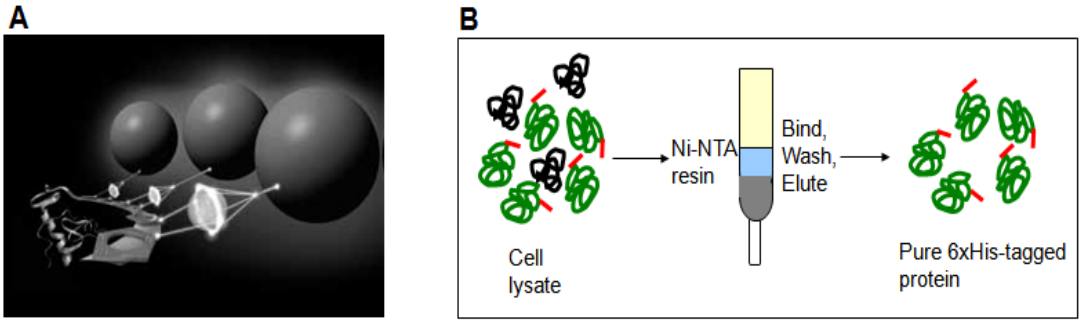


Figure 1.7: A. Interaction between Ni-NTA and a 6xHis-tagged protein B. Purification of 6xHis-tagged proteins (The QIAexpressionist Handbook, 2003).

No single affinity tag is optimal, each one has its weakness and advantages which must be considered beforehand. The properties of most of the available affinity tags is compared in reviews in which the tags were classified according to cost, yield, easiness of experimental procedure (Nilsson *et al.* 1997, Hearn *et al.* 2001, Terpe *et al.* 2003, Arnau *et al.* 2006, Lichty *et al.* 2005, Waugh *et al.* 2005). Although they have originally been developed to facilitate the detection and purification of recombinant proteins, they can also have a positive impact on the yield, solubility and even the folding of their fusion partners which are the reasons for preference of the tag system over others.

Table 1.1 is from a review paper (Lichty *et al.* 2005) comparing the basic tag systems in terms of ability to increase the yield, enhance the solubility, and facilitate the purification of their fusion partners.

Peptide epitopes like the FLAG-tag (Nilsson *et al.* 1997), the calmodulin-binding peptide (Hearn *et al.* 2001), the Strep-tag or Streptag II (Terpe *et al.* 2003, Arnau *et al.* 2006) and the biotin acceptor peptide (Hochuli *et al.* 1987) all exhibit a high degree of specificity for their cognate binding partners.

However, the resins (immobilized proteins) that they interact with tend to be expensive, are easily fouled and have relatively low binding capacities, making them less than ideal for high-throughput applications.

By contrast, large protein tags usually recognize small ligands that make for less expensive and more robust chromatography matrices.

Table 1.1 Advantages and disadvantages of some commonly used fusion partners [Lichty *et al.* 2005].

Tag[a]	Advantages	Disadvantages
GST	Efficient translation initiation	High metabolic burden
	Inexpensive affinity resin	Homodimeric protein
	Mild elution conditions	Does not enhance solubility
MBP	Efficient translation initiation	High metabolic burden
	Inexpensive affinity resin	
	Enhances solubility	
	Mild elution conditions	
NusA	Efficient translation initiation	High metabolic burden
	Enhances solubility	
	Not an affinity tag	
Thioredoxin	Efficient translation initiation	Not an affinity tag[b]
	Enhances solubility	
Ubiquitin	Efficient translation initiation	Not an affinity tag
	Might enhance solubility	
FLAG	Low metabolic burden	Expensive affinity resin
	High specificity	Harsh elution conditions
BAP	Low metabolic burden	Expensive affinity resin
	Mild elution conditions	Variable efficiency of enzymatic biotinylation
	Provides convenient means of immobilizing proteins in a directed orientation	Co-purification of *E. coli* biotin carboxyl carrier protein on affinity resin
		Does not enhance solubility
His_6	Low metabolic burden	Specificity of IMAC is not as high as other affinity methods
	Inexpensive affinity resin	
	Mild elution conditions	
	Tag works under both native and denaturing conditions	Does not enhance solubility
STREP	Low metabolic burden	Expensive affinity resin
	High specificity	Does not enhance solubility
	Mild elution conditions	
SET	Enhances solubility	Not an affinity tag
CBP	Low metabolic burden	Expensive affinity resin
	High specificity	Does not enhance solubility
	Mild elution conditions	
S-tag	Low metabolic burden	Expensive affinity resin
	High specificity	Harsh elution conditions (or on-column cleavage)
		Does not enhance solubility

aGST, glutathione S-transferase; MBP, maltose-binding protein; NusA, N-utilization substance A; FLAG, FLAG-tag peptide; BAP, biotin acceptor peptide; His6, hexahistidine tag; STREP, streptavidin-binding peptide; SET, solubility-enhancing tag; CBP, calmodulin-binding peptide. bDerivatives of thioredoxin have been engineered to have affinity for immobilized metal ions (His-patch thioredoxin) or avidin/streptavidin.

The most popular examples are glutathione S-transferase (GST) (Hochuli *et al.* 1988) and MBP (Smith *et al.* 1988). One disadvantage of GST is that it is a homodimer (di Guan *et al.* 1988), which can complicate purification of fusion proteins and renders this affinity tag unsuitable for the isolation of oligomeric proteins. Moreover, whereas MBP contains no cysteine residues, the four solvent exposed cysteines in each subunit of the GST dimer can lead to a significant degree of oxidative aggregation (di Guan *et al.* 1988).

A disadvantage of large protein affinity tags in general is that they devour more metabolic energy during overproduction than small tags.

The hexahistidine tag (His-tag), which binds to immobilized transition metals, is by far the most commonly used affinity tag for high-throughput protein purification.

Virtually all large structural genomics centers use immobilized metal affinity chromatography (IMAC) as their principal affinity strategy. Ni(II)-nitrilotriacetic acid (Ni-NTA), which exhibits a high affinity for adjacent histidine residues, is the most commonly used matrix for IMAC (Einhauer *et al.* 2001, Vaillancourt *et al.* 2000).

The His-tag combines the advantages of small size with the added benefit of interacting with a chromatographic matrix (e.g. Ni-NTA resin) that is relatively inexpensive, able to withstand multiple regeneration cycles under stringent sanitizing conditions, and exhibits a high binding capacity.

Moreover, elution conditions are mild and flexible (100–250 mM imidazole, pH!5.0, or 10 mM EDTA).

The His-tag also works well under denaturing conditions, adding yet another dimension to its versatility. If a His-tagged recombinant protein is insoluble in E. coli, then it can still be purified by IMAC under denaturing conditions and refolded.

Additionally, because affinity tags have the potential to interfere with structural or functional studies, provisions must also be made for removing them.

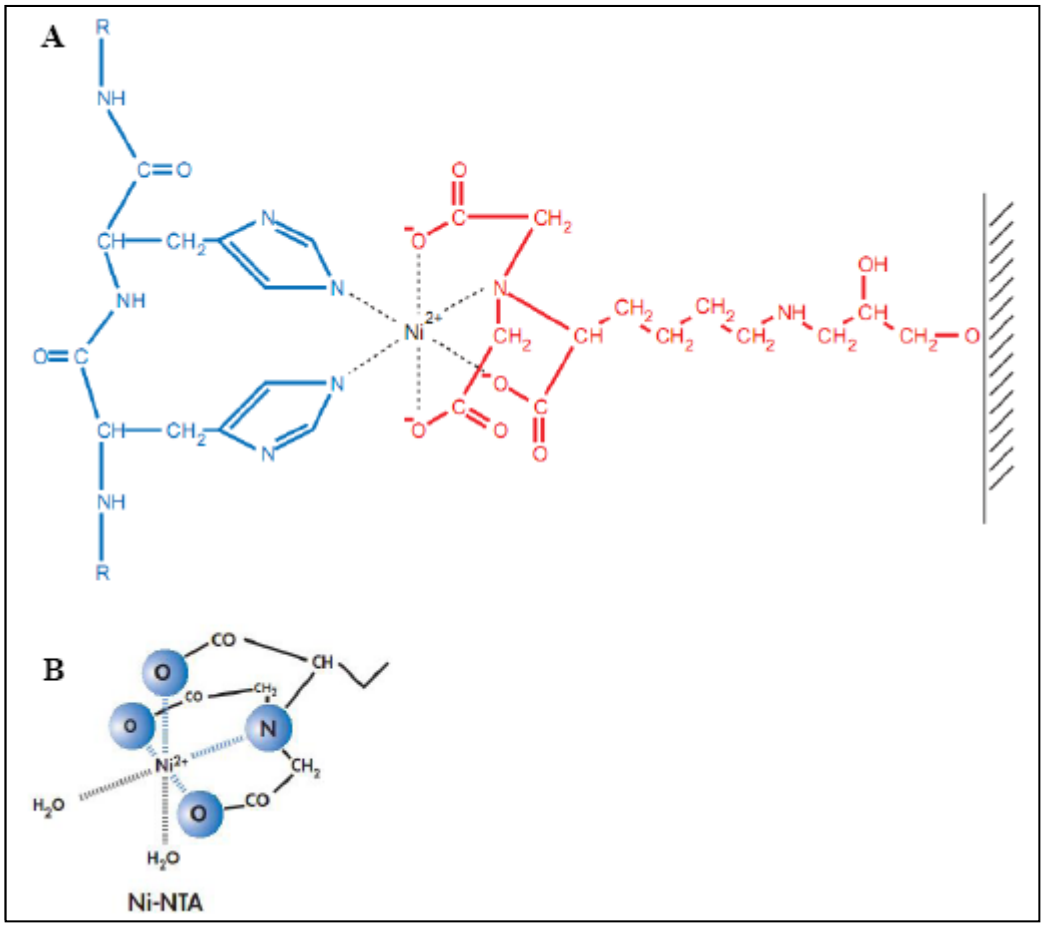


Figure 1.8: A. Interaction between neighboring residues in the 6xHis tag and Ni-NTA matrix. B. Interactions of metal chelate matrices with nickel ions (The QIAexpressionist Handbook, 2003).

1.10 Expression Systems

Expression systems can be basically defined into two groups. Cell-based expression systems and cell-free expression systems. Cell based systems are the most widely used expression systems and they are a combination of an expression vector, its cloned DNA, host cell enabling high level production of foreign protein (Baneyx, 1999).

There are many different host cells which may be used for expression. Each one has distinct advantages and liabilities. They are normally referred to by the host and the DNA source or the delivery mechanism for the genetic material. For example, common expression hosts are bacteria (such as *E.coli*, *B.subtilis*), yeast (such as *S.cerevisiae*) or eukaryotic cell lines. Common DNA sources and delivery mechanisms are viruses (baculovirus, retrovirus, adenovirus etc.), plasmids, artificial

chromosomes and bacteriophage (such as lambda phage) (Baneyx, 1999, Cregg *et al.*, 2000, Kost *et al.*, 2005).

Choosing the best expression system depends on the gene involved. For example, yeast cells are preferred for proteins that require significant post-translational modification, insect or mammal cell lines are used when human-like splicing of the mRNA is required. On the other hand, bacterial expression has the advantage of easily producing large amounts of protein.

Cell-free protein expression has been used for several decades for the analysis of proteins. The biggest drawback of cell-free systems was usually the low yield of target proteins. The widely used *E. coli* and wheat germ or reticulocyte lysates were commercialized by various companies (e.g. Roche, Invitrogen, Qiagen, Novagen) indicating that cell-free expression is a valuable method for the analysis of proteins (Rosser *et al.*, 2005, Lackner *et al.*, 2008).

1.10.1 Prokaryotic expression systems

Prokaryotic recombinant protein expression systems have several advantages including ease of culture, and very rapid cell growth. IPTG can be used to easily induce bacterial protein expression. Purification is quite simple and there are many commercial kits available for recombinant protein expression.

On the other hand, most proteins become insoluble in inclusion bodies and are very difficult to recover as functional proteins (Baneyx, 1999).

Additionally, most post-translational modifications are not added by bacteria and therefore your protein of interest may not be functional.

1.10.1.1 *Eschericia coli*

E. coli is one of the most widely used expression hosts. The overexpression techniques in *E. coli* are well developed and DNA is normally introduced in a plasmid expression vector. DNA sequence for a protein of interest could be cloned or subcloned into a high copy-number plasmid containing the *lac* promoter, which is then transformed into the bacterium *Escherichia coli*. Addition of IPTG (a lactose analog) activates the lac promoter and causes the bacteria to express the protein of interest (Fux *et al.*, 2005, Blattner *et al.*, 1997).

1.10.2 Eukaryotic expression systems

Eukaryotic genes are not really present in prokaryotic cells. *E. coli* cells frequently recognize the protein products of cloned eukaryotic genes as outsiders and destroy them. Also, prokaryotes do not carry out the same kinds of posttranslational modification as eukaryotes do. Another serious problem is that the interior of a bacterial cell is not proper for folding of eukaryotic proteins as the interior of a eukaryotic cell resulting improperly folded, inactive products of cloned genes.

Yeast cells, mammalian cells and insects are available eukaryotic expression systems. Advantages of eukaryotic protein expression systems include the fact that you can get very high levels of expression. The proteins are easy to purify using special tags which are included into the vectors including His, Myc and other tags.

There will be no inclusion bodies to worry about and proteins will have intact post-translational modifications. These are vital parameters if the function of the protein and/or protein-protein interactions are studied.

As the disadvantage of using eukaryotic protein expression systems, eukaryotic cells grow slower than prokaryotic cells (Cregg *et al.*, 2000, Kost *et al.*, 2005).

1.10.2.1 *Pichia pastoris*

Pichia pastoris is frequently used as an expression system for the production of proteins. It has a high growth rate and is able to grow on a simple, inexpensive medium. It can grow in either shake flasks or a fermenter, making it suitable for both small and large scale production.

It has two alcohol oxidase genes, AOX1 and AOX2, which have a strongly inducible promoter. These genes allow *Pichia* to use methanol as a carbon and energy source. The AOX promoters are induced by methanol and are repressed by e.g. glucose.

The gene for the desired protein is introduced under the control of the AOX1 promoter so that protein production can be induced by the addition of methanol (Cregg *et al.*, 2000).

1.11 Functional Proteins

1.11.1 Recombinant green fluorescent protein (GFPuv)

1.11.1.1 Green fluorescent protein (GFP)

Green fluorescent protein (GFP) is a 27-29 kDa protein composed of 238 amino acids. It forms an environment for three residues (Ser65–Tyr66–Gly67) to act as a fluorophore (Chalfie and Kain, 1998). It has a typical beta barrel structure, consisting of one β-sheet with alpha helix(s) containing the chromophore running through the center as shown in Figure 1.9.

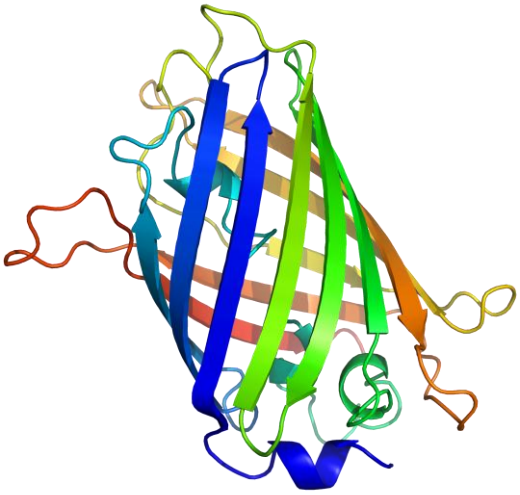


Figure 1.9: GFP ribbon structure. (Ormö *et al.*, 1996).

Many marine organisms have green fluorescent proteins but GFP traditionally refers to the protein first isolated from the jellyfish *Aequorea victoria*. The GFP from *A. victoria* has a major excitation peak at 395 nm and a minor one at 475 nm. The emission peak is at 509 nm which is in the lower green portion of the visible spectrum.

1.11.1.2 Application areas and GFP derivatives

GFP gene is frequently used as a reporter of expression (Phillips, 2001). It has been introduced and expressed in many bacteria, yeast and other fungi, fish (such as zebrafish), plant, fly, and mammalian cells, including human. It has been expressed as a proof-of-concept that a gene can be expressed throughout a given organism.

Many different mutants of GFP have been engineered (Shaner *et al.*, 2005). The spectral characteristics of GFP have been improved resulting in increased fluorescence, photostability or a shift of the major excitation peak.

The availability of GFP and its derivatives has thoroughly redefined fluorescence microscopy and the way it is used in cell biology and other biological disciplines (Yuste, 2005). Most small fluorescent molecules such as FITC (fluorescein isothiocyanate) are strongly phototoxic when used in living cells but fluorescent proteins such as GFP are usually much less harmful when illuminated in living cells. In cells where the gene is expressed, and the tagged proteins are produced, GFP is produced at the same time. Thus, only those cells in which the tagged gene is expressed, or the target proteins are produced, will fluoresce when observed under fluorescence microscopy. Through its ability to form internal chromophore without requiring accessory cofactors, enzymes or substrates other than molecular oxygen, GFP makes for an excellent tool in all forms of biology (Stepanenko *et al.*, 2008).

1.11.1.3 Recombinant green fluorescent protein-GFPuv

GFPuv was developed by introducing point mutations that replaced three amino acid codons in the native GFP DNA sequence (Chalfie *et al.*, 1994, Crameri *et al.*, 1996). It has been shown to be resistant to heat ($T > 70°C$) and alkaline pH (between 5.5 and 12.0; optimum = 8.0).

GFPuv is expressed two to three times faster in *E. coli* and exhibits eighteen-fold greater fluorescence intensity than native GFP. With the excitation/emission maxima of 395/509 nm (Chalfie and Kain, 1998, Chalfie *et al.*, 1994), the fluorescence characteristics of GFPuv can be quantified for *in vivo* and *in vitro* studies by a variety of techniques using fluorescence microscopy, flow cytometry and spectrofluorometry.

1.11.2 Alkaline phosphatase

1.11.2.1 Characteristics

Alkaline phosphatase (AP, EC 3.1.3.1) is a dimer hydrolase enzyme. It is a dimer (MW = 140.000) of identical or nearly identical sub-units (MW = 69,000).

Each momoner contains 2 molecules of Zn2+, one tightly bound and necessary for structural stability, the other loosely bound and required for catalytic activity. There is a reactive serine in active site.

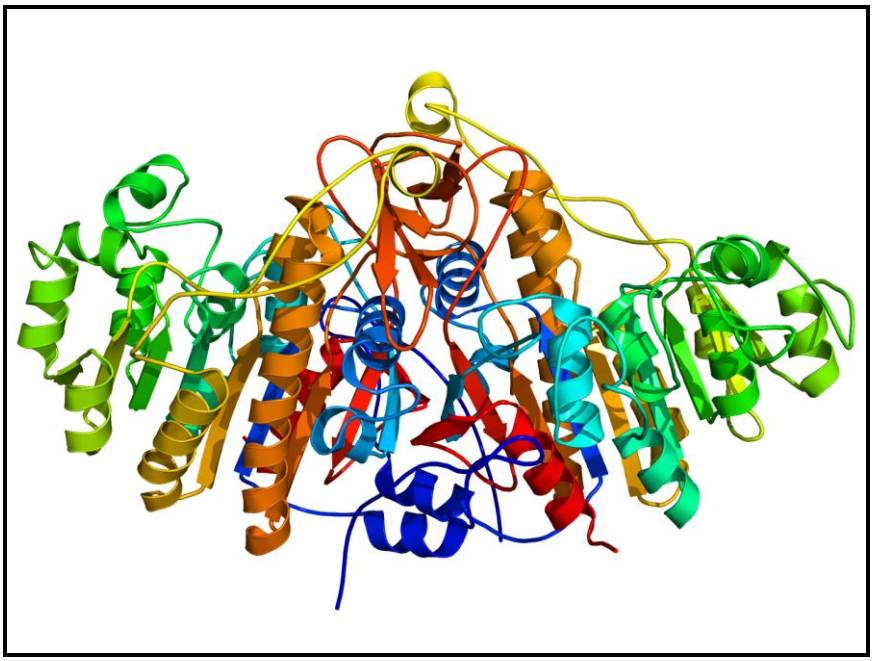


Figure 1.10: Cartoon diagram (rainblow colored, N-terminus = blue, C-terminus = red]) of the dimeric structure of bacterial alkaline phosphatase based on the PDB 1ALK coordinates (Kim *et al.*, 1991).

Alkaline phosphatase (AP) hydrolyzes phosphate esters of primary and secondary alcohols, cyclic aliphatic alcohols, sugar alcohols, phenols, and amines. AP also hydrolyzes inorganic pyrophosphate (at a pH optimum of approx. 8) and 5'-terminal phosphates of single and double-stranded DNA or RNA (Kim *et al.*, 1991).

1.11.2.2 Application areas

It has become a useful tool in molecular biology laboratories. It has been widely used in immunoassays for removing inorganic phosphate groups from various types of molecules such as proteins and DNA (Anderson *et al.*, 1975).

DNA possesses phosphate groups on the 5' end. Removing these phosphates prevents the DNA from ligating, thereby keeping DNA molecules linear until the next step of the process for which they are being prepared; also, removal of the phosphate groups allows radiolabeling (replacement by radioactive phosphate groups) in order to measure the presence of the labeled DNA through further steps in the process or experiment.

Another important use of alkaline phosphatase is as a label for enzyme immunoassays.

The wide utility of AP in biochemical, immunological, and medical assays resulted in numerous studies investigating the immobilization of AP via fusion peptides on solid supports (Brennann *et al.*, 1995, Tominaga *et al.*, 2005, Zhang *et al.*, 2001).

1.11.3 Laccases

1.11.3.1 Characteristics

Laccases (benzenediol:oxygen oxidoreductases, EC 1.10. 3.2) are the largest subgroup of blue multicopper oxidases (MCO). They contain copper atoms in the catalytic centre which gives their redox ability to catalyze the oxidation of a wide range of aromatic substrates simultaneously with the reduction of molecular oxygen to water (Solomon *et al.*, 1996).

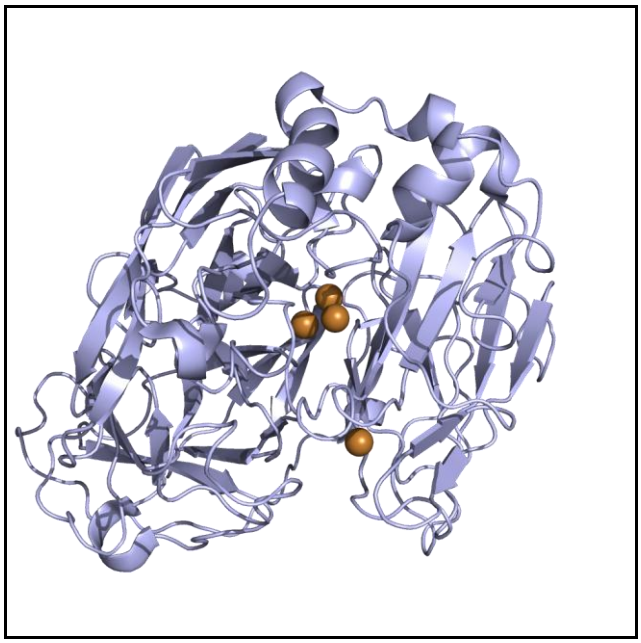


Figure 1.11: Laccase from *T. Versicolor.*

Laccases have been found in nearly all woodrotting fungi (Heinzkill *et al.*, 1997) and isolated from plants, from some kinds of bacteria, and from insects too (Bao *et al.*, 1993, Sato *et al.*, 2001, Sharma *et al.*, 2007, Roberts *et al.*, 2002). In fungi, laccases carry out a variety of physiological roles including morphogenesis, fungal plant pathogen/host interaction, stress defense, and lignin degradation (Thurston *et al.*, 1994, Ginfreda *et al.*, 1999). In plants, laccases have been found in the wood and

cellular walls of herbaceous species (Bao *et al.*, 1993), and participate in the radical-based mechanisms of lignin polymer formation (Sterjiades *et al.*, 1992; Liu *et al.*, 1994; Boudet, 2000; Ranocha *et al.*, 2002; Hoopes & Dean, 2004). In bacteria, laccases have a role in morphogenesis, in the biosynthesis of the brown spore pigment and in the protection afforded by the spore coat against UV light and hydrogen peroxide, and in copper homeostasis. In insects, laccase-type proteins is believed to affect the sclerotization of the cuticle in the epidermis.

The physiological function of laccase in the organism usually determines the localization of the enzyme in the cell. Because of lignin degradation process, most laccases are extracellular enzymes. On the other hand, the laccases of wood-rotting fungi are also found intracellularly. Periplasmic laccases in bacterial cells could play role in the transformation of low molecular weight phenolic compounds in the cell. (Eggert *et al.*, 1995; Galhaup & Haltrich, 2001).

The majority of the fungal laccases are monomeric globular proteins of approximately 60–70 kDa.They are generally glycosylated and acidic isoelectric point (pI) is around pH 4.0. Laccases contain three types of copper. One of these coppers gives lacccase its characteristic blue colour.

Laccases catalyze the reduction of oxygen to water accompanied by the oxidation of a substrate (as hydrogen donors) such as ortho and para-diphenols, aminophenols, polyphenols, polyamines, and aryl diamines as well as some inorganic ions. Commonly used phenolic substrates for laccases are syringaldazine, DMP and guaiacol. On the other hand, they are also able to oxidize electron donor substrates such as ABTS [2,20-azino-bis(3- ethylbenzothiazoline-6-sulfonic acid)].

1.11.3.2 Application areas

Because of their high nonspecific oxidation capacity, and because they use readily available molecular oxygen as an electron acceptor, laccases are useful biocatalysts for a wide range of biotechnological applications. They do not need the addition or synthesis of a low molecular weight cofactor like hydrogen peroxide, as its cosubstrate – oxygen – is usually present in its environment. Most laccases are extracellular enzymes, making the purification procedures very easy. They generally exhibit a considerable level of stability in the extracellular environment.

There are many potential application areas for lacceases such as in pulp delignification and biobleaching (Bajpai *et al.*, 1999), treatment of industrial plant wastewater (Dura´n *et al.*, 2000) , enzymatic modification of fibers and dye-bleaching in the textile and dye industries (Abadulla *et al.*, 2000), enzymatic removal of phenolic compounds in beverages, fruit juice processing (Minussi *et al.*, 2002), and biosensor and biofuel cell construction (Amir *et al.*, 2009).

1.12. Aim of the Study

We used one of the Quartz Binding Peptides, QBP-1 (PPPWLPYMPPWS), which was developed using computational tools based on the knowledge generated through phage display technology. The selected peptide's ability to bind strongly and specifically on silica material makes it a strong candidate to form a novel tag system for protein purification purposes.

Firstly, prokaryotic expression systems were used for the formation of QBP1-functional protein fusion proteins. Alkaline phosphatase and recombinant GFP proteins were tagged with QBP1 peptide at the DNA level and optimization of protein expressin was performed.

Secondly, eukaryotic expression systems were used for protein expression and then purification purpose. QBP1 peptide was placed at the N terminus of the laccase enzyme (lcc1) in *Pichia pastoris* yeast cells to form a hybrid fusion molecule. After the optimization of protein expression, QBP1 peptide was used as tag molecule to purify the tagged protein form cell extract using silica/quartz columns.

Here, we show that combinatorially selected and then bioinformatically enhanced quartz binding peptides can be used as a tag molecule for prokaryotic and eukaryotic protein expression purposes.

The tag molecule does not have a significant effect on the activity of the main protein and also, it does not affect the esecretion of the tagged protein out of the cell. Finally, QBP1 peptide is used as a "TAG" molecule for purification of fusion molecule through silica/ quartz packed columns.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Bacterial and yeast strains

2.1.1.1. *E. coli* DH5αTM-T1R host strain

F^- φ80lacZΔM15 Δ(lacZYA$^-$argF) U169 deoR recA1 endA1 hsdR17 (rk^-, mk^+) phoA supE44 λ^- thi$^-$1 gyrA96 relA1 tonA, chemically competent cells were supplied with Gene-TailorTM Site-Directed Mutagenesis System (Catalog#12297-016, Invitrogen).

2.1.1.2. *E. coli* TunerTM(DE3)pLacI host strain

F^- ompT hsdSB (rB^- mB^-) gal dcm lacY1 (DE3) pLacI (CmR), chemically competent cells compatible with expression from pETBlueTM vectors, were supplied with pETBlue™ System (Catalog#70674-3, Novagen).

2.1.1.3 *E. coli* One Shot® TOP10 host strain

F^- mcrA Δ(mrr$^-$hsdRMS$^-$mcrBC) φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara$^-$ leu)7697 galU galK rpsL (StrR) endA1 nupG, electrocompetent and chemically competent cells were supplied with One Shot® TOP10 Competent Cells (Catalog# C4040-52, Invitrogen)

2.1.1.4 *E. coli* BL21(DE3) host strain

B F^-dcm ompT hsdS(rB^- mB^-) gal λ(DE3)BL21(DE3) strain electrocompetent and chemically competent cells were supplied with (Catalog #C6000-03 Invitrogen)

2.1.1.5 *Pichia pastoris* host strain

Yeast host strain was a commonly used strain of *Pichia pastoris* which was supplied in "EasySelect™ *Pichia* Expression Kit, For Expression of Recombinant Proteins Using pPICZ and pPICZα in *Pichia pastoris"* kit from Invitrogen. (Cat. no. K1740-0)

2.1.2. Inorganic targets: quartz and silica beads

Quartz and silica powders were obtained from Merck Company (ABD). Silica gel was 0,063-0,200 mm in diameter (Lot no: 1.07734.1000), quartz was 0,2-0,8 mm in diameter (1.07536.1000).

2.1.3. Bacterial culture media

2.1.3.1. Luria Bertani (LB) medium

10 g tryptone (Acumedia), 5 g yeast extract (Acumedia), 5 g NaCl (Riedel-de-Haen) were dissolved in distilled water and completed up to 1 lt and the pH was adjusted to 7.0-7.5 with 10 M NaOH and sterilized for 15 min. under 1.5 atm at 121 ˚C. The medium was stored at room temperature.

2.1.3.2. LB agar medium

10 g tryptone (Acumedia), 5 g yeast extract (Acumedia), 5g NaCl (Riedel-de-Haen), 15 g bactoagar (Acumedia) were dissolved in distilled water and completed up to 1lt and the pH was adjusted to 7.0-7.5 with 10 M NaOH and sterilized for 15 minutes under 1.5 atm at 121˚C. Following autoclaving, tetracycline solution (Sigma) (final concentration of 10 μg/ml) and X-gal/IPTG solution (final concentration of 40 μg/ml) (Fermentas/Sigma) were added when the temperature of the medium was cooled down to 45-50 ºC. The medium was shaken properly and poured into the plates by avoiding any bubble formation (3.5 ml for small plates and 15 ml for big plates). After the medium was solidified in the plates, they were turned upside down and stored at 4 ˚C for later use.

2.1.3.3. 2xYT medium

16 g tryptone (Acumedia), 10 g yeast extract (Acumedia), 5g NaCl (Riedel-de-Haen) were dissolved in distilled water and completed up to 1lt and the pH was adjusted to 7.0 with 10 M NaOH and sterilized for 15 minutes under 1.5 atm at 121˚C. per liter. The medium was stored at room temperature.

2.1.3.4. MagicMedia *E.coli* expression medium

MagicMedia™ *E. coli* Expression Medium is specifically designed to dramatically increase the yield of recombinant proteins in T7-regulated *E. coli* expression systems

without monitoring culture O.D. (optical densities) or adding inducer. The proprietary MagicMedia™ formulation enables *E. coli* growth to reach culture densities 3-10-fold higher than those achieved with traditional LB + IPTG methods. The medium was supplied in "MagicMedia™ *E. coli* Expression Medium SoluPouch™" kit from Invitrogen. (Cat. no. K6802)

2.1.3.5. SOC medium

20 g tryptone, 5 g yeast extract, 0.5 g NaCl was dissolved in 950 mL deionized water. 10 mL of 250 mM KCl was added and pH was adjusted to 7.0 with NaOH. Water was added up to 1lt and sterilized by autoclaving. Just before use, 10mM of $MgCl_2$ and 20mM of glucose were added.

2.1.3.6. *E. coli* overnight culture

5 ml LB solution containing 1 mM $MgCl_2$ and tetracycline, was inoculated with *E. coli* ER-2738 stock (from -80°C). The culture was left in the shaker overnight at 37°C, 200 rpm.

2.1.4. Yeast culture media

2.1.4.1. Minimal methanol medium

13.4 g of yeast nitrogen base with ammonium sulfate and without amino acids was dissolved in 993 ml of water, 15 g of agar was added and sterilized for 15 minutes on liquid cycle. 5 ml of methanol and 2 ml of filter-sterilized 500x biotin were added. (1.34 % YNB, 4 x 10^{-5} % Biotin and 0.5 % Methanol). Medium was cooled to ~60°C and poured.

2.1.4.2. Buffered minimal glycerol and buffered minimal methanol media, BMG and BMM

13.4 g of yeast nitrogen base with ammonium sulfate and without amino acids was dissolved in 890 ml of distilled water and sterilized for 15 minutes on liquid cycle. Following to cooling the medium to ~60°C, 100 ml of pre-sterilized 1 M potassium phosphate buffer, pH 6.0, 2 ml of filter-sterilized 500x biotin and 10 ml of glycerol was added. 5 ml of methanol was added for BMM instead of glycerol. (100 mM potassium phosphate buffer, pH 6.0, 1.34 % YNB, 4 x 10^{-5} % Biotin and 0.5 % Methanol or 1 % Glycerol)

2.1.4.3. Buffered glycerol-complex medium, BMGY and BMMY

10 g of yeast extract, 20 g of mycological peptone and 13.4 g of yeast nitrogen base with ammonium sulfate and without amino acids was dissolved in 890 ml of distilled water and sterilized for 15 minutes on liquid cycle. Following to cooling the medium to ~60°C, 100 ml of pre-sterilized 1 M potassium phosphate buffer, pH 6.0, 2 ml of filter-sterilized 500x biotin and 10 ml of glycerol was added. 5 ml of methanol was added for BMMY instead of glycerol. (100 mM potassium phosphate buffer, pH 6.0, 1.34 % YNB, 1 % Yeast Extract, 4 x 10^{-5} % Biotin, 2 % Mycological peptone and 0.5 % Methanol or 1 % Glycerol)

2.1.5. Stock solutions

2.1.5.1. Tetracyline-HCl stock solution

20 mg/ml tetracycline-HCl (Sigma) was dissolved in distilled water. It was then stored at -20°C at dark to protect from the light. Tetracycline is light-sensitive.

2.1.5.2. Ampicillin stock solution

1g ampicillin sodium salt (Fisher BP1760-25) was dissolved in 10 ml distilled water (100 mg/ml) and filter sterilized using 0.22µm syringe filter. It was then stored at -20°C.

2.1.5.3. Kanamycin stock solution

1 g kanamycin sulfate was dissolve in 35 ml of distilled water and the volume was made up to 40 ml with distilled water (25 mg/ml) and filter sterilized using 0.22µm syringe filter. It was then stored at -20°C.

2.1.5.4. Chloramphenicol stock

34 mg/mL chloramphenicol was dissolved in 100% ethanol, filter-sterilized and stored at -20°C.

2.1.5.5. Zeocin stock solution

1g zeocin was dissolved in 10 ml distilled water (100 mg/ml) and filter sterilized using 0.22µm syringe filter. It was then stored at -20°C.

2.1.5.6. Xgal/ IPTG stock

1.25 g IPTG (isopropyl β-D-thiogalactoside) (Sigma) and 1 g Xgal (5-Bromo-4-chloro-3 indolyl-β-D-galactoside) (Fermentas) were dissolved in 25 ml Dimethyl formamide (Riedel-de-Haen). Solution was stored at –20°C at dark to protect from the light.

2.1.5.7. Detergent stock

20 % (w/v) Tween 20 (Riedel-de-Haen) and 20 % (w/v) Tween 80 (Merck) were mixed and distilled water was added up to 20 ml.

2.1.5.8. Glycerol stock solution

50 ml of 100% glycerol (Sigma) was mixed with distilled water up to 100 ml total volume to have 50 % glycerol solution. It was sterilized for 15 minutes under 1.5 atm at 121˚C and then stored at room temperature.

2.1.5.9. $MgCl_2$ stock solution

1M $MgCl_2.6H_2O$ (Fisher) was dissolved in distilled water up to 100 ml. and sterilized with 0.2 μm single use syringe filter.

2.1.6 Buffer solutions

2.1.6.1. Phage display buffers

- **PC (Potassium Phosphate-Sodium Carbonate Buffer) (no detergent):** 55 mM KH_2PO_4 (Fisher), 45 mM Na_2CO_3 (Fisher), 200 mM NaCl (Sigma) were dissolved in distilled water up to 500 ml and the solution was sterilized by using 0.2 μm single use syringe filter. The pH value was adjusted to 7.2-7.5.

- **PC (containing 0.02% detergent):** 55 mM KH_2PO_4 (Fisher), 45 mM Na_2CO_3 (Fisher), 200 mM NaCl (Sigma), 0.5 ml detergent stock solution were dissolved in distilled water up to 500 ml and the solution was sterilized by using 0.2 μm single use syringe filter. The pH value was adjusted to 7.2-7.5.

- **PC (containing 0.1% detergent):** 55 mM KH_2PO_4 (Fisher), 45 mM Na_2CO_3 (Fisher), 200 mM NaCl (Sigma), 2.5 ml detergent stock solution were dissolved in distilled water up to 500 ml. and the solution was sterilized by using 0.2 μm single use syringe filter. The pH value was adjusted to 7.2-7.5.

- **PC (containing 0.5% detergent):** 55 mM KH_2PO_4 (Fisher), 45 mM Na_2CO_3 (Fisher), 200 mM NaCl (Sigma), 12.5 ml detergent stock solution were dissolved in distilled water up to 500 ml. and the solution was sterilized by using 0.2 μm single use syringe filter. The pH value was adjusted to 7.2-7.5.

Note: PC buffer can not be sterilized because carbonate ions convert to $C0_2$ due to high pressure in the autoclave. This causes an increasing of pH up to 10.

- **Elution buffer:** 0.2 M glycine (Merck) and 1mg /ml BSA (Sigma) were dissolved in distilled water up to 50 ml. and pH was adjusted to 2.2 with 10 M HCl and 0,1M HCl. The solution was sterilized by using 0.2 μm single use syringe filter.

- **Tris Buffer:** 5% casein (Sigma), 10 mM Tris-base (Merck), 150 mM NaCl, 1% tween 20 (Riedel-de-Haen) was dissolved in 0.1 M NaOH. pH was adjusted to 8.2 and distilled water was added up to 50 ml.

2.1.6.2. Electrophoresis buffers

Agarose gel electrophoresis, SDS-PAGE and native-PAGE buffers are given in Section 2.2

2.1.6.3. Sil-TAG application buffers

- **Binding Buffer:** PC buffer (pH.7,5) containing 0% detergent was used as binding buffer.

- **Washing Buffer:** Different concentrations and pH values of PC buffer (pH.6,5-7,5 + 0-0.5%Tween20) were used as washing buffers.

- **Elution Buffers:** Different concentrations and pH values of PC buffer (pH.3-7,5 ± 0-5%Tween20 ± 2M $MgCl_2$ ± 1M Imidazole) were used as elution buffers.

2.1.7. DNA and protein molecular weight markers

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

2.1.8. Oligonucleotides

Oligonucleotides given in Appendix B were synthesised by Alpha DNA on automated computer-controlled synthesizers using standard phosphoramidite chemistry. The oligonucleotides were purchased as lyophilized form.

2.1.9. Cloning and expression vectors

All the cloning and expression vectors were given in Appendix B.

2.1.9.1. pCR®2.1-TOPO® cloning vector

pCR®2.1-TOPO® cloning vector (See Appendix-A) was purchased from Invitrogen™ (Catalog#K4560-40) to be used for cloning of PCR products containing the single A (Adenine) overhang at each end. Adenine nucleotide was added at 3' ends of blunt-ended PCR products using QIAGEN A-Addition Kit (Catalog# 231994, Qiagen).

2.1.9.2. pGEM®-T and pGEM®-T easy vector systems

The pGEM®-T and pGEM®-T Easy Vector Systems are convenient systems for the cloning of PCR products which were purchased from Promega and supplied in a kit including a 2X Rapid Ligation Buffer for ligation of PCR products. (Catalog# A1360, Promega)

2.1.9.3. pPICZ-B expression vector

pPICZ-B expression vector (See Appendix-A) was purchased from Invitrogen™ (Catalog#V190-20). It is 3.3 kb expression vector used to express recombinant proteins in *Pichia pastoris*. The vector allows high-level, methanol inducible expression of the gene of interest in *Pichia*, and can be used in any *Pichia* strain including X33, GS115, SMD1168H, and KM71H.

2.1.9.4. pETBlue-2 expression vector

The pETBlue™ vectors are designed to identify recombinants by traditional blue/white screening while also allowing T7lac promoter based expression of target genes. pETBlue-2 vector (See Appendix A) was purchased from Novagen (Catalog#70674-3, EMD Biosciences, Inc.).

2.1.9.5. pQE-60 expression vector

pQE-60 Expression Vector is designed for high-level expression of 6xHis-tagged proteins in *E. coli,* based on the T5 promoter transcription–translation system. (See Appendix A) It was purchased from Qiagen (Catalog#32903).

2.1.10. Enzymes

2.1.10.1. Restriction enzymes

***EcoR*I** (G^AATTC, Fermentas), ***Pst*I** (CTGCA^G, Fermentas), ***Bgl*II** (A^GATCT, Fermentas) and ***Nco*I** (C^CATGG, Fermentas) restriction endonucleases and their reaction buffers were obtained from Fermentas (Catalog #ER0611, ER0081).

2.1.10.2. *Taq* DNA polymerase

The *Taq* DNA Polymerase is a highly thermostable DNA polymerase that catalyzes template-dependent polymerization of nucleotides into duplex DNA in the 5'=>3' direction. The *Taq* DNA Polymerase possesses low 5'=>3' exonuclease activity and has no detectable 3'=>5' exonuclease (proofreading) activity. Like other DNA polymerases without 3'=>5' exonuclease activity, *Taq* DNA Polymerase exhibits deoxynucleotidyl transferase activity, which frequently results in the addition of extra adenines at the 3'-end of PCR products (Catalog # EP0402, Fermentas).

2.1.10.3. *Pfu* DNA polymerase

The *Pfu* DNA Polymerase is a highly thermostable DNA polymerase that catalyzes the template-dependent polymerization of nucleotides into duplex DNA in the 5'=>3' direction. The *Pfu* DNA Polymerase also exhibits 3'=>5' exonuclease (proofreading) activity, that enables the polymerase to correct nucleotide incorporation errors. The enzyme has no 5'=>3' exonuclease activity (Catalog#EP0502, Fermentas).

2.1.10.4. Quick T4 DNA ligase

Quick T4 DNA ligase was supplied with Quick Ligation™ Kit purchased from New England Biolabs, Inc (Catalog # M2200S).

2.1.11. Lab equipment

Autoclave **:** Yamato sterilizer SE 510.

Balances **:** Denver Toledo AB 54

Centrifuge Products	**:**Sorvall RC 5B Plus Kendro Laboratory Eppendorf Centrifuge 5415D
Centrifuge rotors	**:** SA-600, SLA-1500, SH-3000, PN-11779.
Deep freezes and refrigerators Nordic	**:** Heto Polar Bear 4410 ultra freezer, JOUAN A/S, catalog# 003431.
Deionized water	**:** Millipore Milli Q Synthesis A 10
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.
Glassware	**:** Technische Glaswerke Ilmenau GmbH.
Ice Machine	**:** Cornelius Ice Systems
Incubators VWR 1310	**:** Quincy Lab. Inc. Model 10-14 Incubator
Laminar Flow Cabinet Bioexpress	**:** Airclean 600 PCR Workstation ISC
Orbital shaker Brunswick	**:** Innova 3100 Water Bath Shaker New Scientific
Magnetic stirrer 10.0162,	**:** AGE 10.0164, VELP Scientifica srl.**:** ARE VELP Scientifica srl.
Pipettes	**:** Pipetteman 1-20-200-1000-5000 ml
pH meter Inolab pH Technische	**:** MP 220, Mettler Toledo International Inc.**:** level 1, order# 1A10-1113,Wissenschaftlich-Werkstätten GmbH & Co KG.
PCR cycler	**:** Eppendorf Master Cycler Gradient

Power supply **:** EC 250-90, E-C Apparatus

Sequencer **:** Applied Biosystems 3730XL

Spectrophotometer **:** Tecan Safire

: DU530 Life Science UV/ Vis, Beckman.

: UV-1601, Shimadzu Corporation.

Sterilizer **:** FN 500, Nuve.

Ultrasonic bath **:** Branson 1200

Vortexing machine **:** Reax Top, product# 541-10000, Heidolph2.2.

2.2 Methods

2.2.1 Identification of silica-binding peptides

The quartz-binding peptide-QBP-1 (PPPWLPYMPPWS) has been obtained by previous experiments in our group as followed; firstly, quartz binding peptides have been selected by Phage Display Methodology and the selected peptides were characterized and grouped using Fluorescence Microscopy, Similarity Analysis and Biomineralization Experiments. Peptides were produced using phage-display qtechniques as described in Naik et al. (2002), Sarikaya et al. (2003), Smith (1985) and Whaley et al. (2001).

2.2.2 Enhancement of binders by bioinformatic techniques

The properties of the experimentally selected binders have been used to generate *De novo* binders using bioinformatics tools, naming the best *De novo* binder as QBP-1.To accomplish this, we first generated random sequences based on the observed amino acid frequencies in the phage library used for the combinatorial selection. We then calculated the TSS between each of these sequences and the experimentally determined strong binder group. Sequences with the highest and lowest similarity scores were considered to represent the strongest and weakest binders, respectively. QBP1 sequence is formed as the best silica binder according to the details of enhancement procedure, which are given elsewhere (Oren et al. 2007).

2.2.3. DNA based studies

2.2.3.1 QBP1 oligo construction and cloning

The designed 143 bp long ssDNA (Figure 3.1) (QBP1+14 aa linker+enzyme cutting sites) was synthesized commercially (Alpha DNA/Quebec/Canada). The designed single strand QBP-1 oligo was PCR amplified and cloned into pGEM-T vector for practical uses.

Synthesis of double stranded QBP-1 oligonucleotide: Optimized PCR conditions to make dsDNA oligo was as followed: Initial denaturation at 95^{o}C for 4 min, denaturation at 95 ^{o}C for 1 min, annealing at 60 ^{o}C for 1 min, extension at 72 ^{o}C for 1 min and final elongation at 72 ^{o}C for 5 min. 30 cycles of reaction was performed using Taq and Pfu polymerases (Fermentas).

Polymerase chain reaction (PCR) was obtained with forward and reverse primers in order to obtain double-stranded DNA from the synthesized oligonucleotide. Reaction conditions are described below:

Table 2.1: Polymerase Chain Reaction (PCR) ingredients and reaction conditions.

Ingredients	Volume/Concentration	Reaction Steps	Temp.	Time
Template DNA	0.5 µl (10 pmol)	Initial Denaturation	95°C	4 min
Forward Primer	1 µl (10 pmol)	Denaturation	94°C	1 min
Reverse Primer	1 µl (10 pmol)	Annealing	60°C	1 min
dNTP	2 µl (10mM each)	Extension	72°C	1 min
Taq Buffer	5 µl (10X)	No. of Cycles	30	
Taq/Pfu polymerase	0.5 µl	Final Elongation	72°C	5 min
dH_2O	40 µl			
Total Volume	50 µl			

PCR product was run on 2.5% agarose gel and desired gene product was extracted from the gel.

Agarose gel electrophoresis of PCR products: After the amplification of the oligo, PCR products were analyzed on agarose gel electrophoresis. Firstly, for %1 gel, 0.5 gr agarose was added in 50 ml 0.5X TAE buffer and then gel solution was boiled in a microwave until the agarose was completely dissolved. After cooling the solution to about 60°C, ethidium bromide was added to the gel solution to a final concentration of 0.5 ug/ml.

Then, gel solution was poured into gel casting tray and a proper comb was placed in gel tray. After the gel had been solidified, the comb was removed and gel was placed

in electrophoresis chamber containing the appropriate amount of 0.5X TBE buffer. An appropriate molecular weight marker (Marker 9, Fermentas) and PCR product samples that were mixed with the 6X loading dye depending on volume were pipeted into the gel wells. Gel was run at 80 V for 20 min. EtBr-stained DNA bands were visualized by UV trans-illuminator.

TBE (Tris / Borate / EDTA) solution: 10X TBE buffer was prepared by dissolving 108 g tris-base (Merck), 55 g boric acid (Riedel-de-Haen.) and 4 % (v/v) 0.5M EDTA (Merck) pH 8.0. Distilled water was added to complete up to 1 l.

Gel extraction procedure: Gel extraction reactions in this study are performed by MinElute Gel Extraction Kit (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^{o}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 dissolved, 1 gel volume of room temperature 100% isoproponal was added and mixed by inverting the tube several times.
6. Sample was applied to the MinElute 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.
10. In order to wash, 750 µl PE buffer was applied to the column and centrifuged for 1 min. at 13000 rpm.

11. Flow-through was discarded from collection tube and centrifuged for an additional 1 min.

12. Spin column was placed in a microfuge and 10 µl EB buffer was applied to elute the DNA. After 1 min. incubation the tube was centrifuged for 1 min.

    Eluted DNA is stored at –4^{o}C prior to usage

Cloning of QBP-1 oligo into a TA vector: QBP-1 oligo was then ligated into pGEM-T cloning vector by using Promega pGEM-T vector system and Qiagen Quick Ligation™ Kit. Ligation reactions was set up as following; 5µl of 2X Rapid Ligation Buffer, 1µl of pGEM®-T (50ng), 2µl of PCR product (8ng), 1µl of T4 DNA Ligase (3 Weiss units/µl) and deionized water to a final volume of 10µl. The reactions was mized by pipetting and incubated for 1 hour at room temperature.

Table 2.2: Ligation reaction, performed according to Promega pGEM-T vector system and Qiagen Quick Ligation™ Kit.

Reaction Component	Standard Reaction	Positive Control	Background Control
2X Rapid Ligation Buffer, T4 DNA Ligase	5µl	5µl	5µl
pGEM®-T or pGEM®-T Easy Vector (50ng)	1µl	1µl	1µl
PCR product	Xµl*	–	–
Control Insert DNA	–	2µl	–
T4 DNA Ligase (3 Weiss units/µl)	1µl	1µl	1µl
nuclease-free water to a final volume of	**10µl**	**10µl**	**10µl**
*Molar ratio of PCR product:vector may require optimization.			

Ligation reaction was also performed according to TOPO TA Cloning® Kit (Catalog # K 4500-01, Invitrogen) into pCR2.1-TOPO TA Cloning Vector.

Table 2.3: Ligation reaction, performed according to TOPO TA Cloning® Kit.

Reagent*	Chemically Competent *E. coli*	Electrocompetent *E. coli*
Fresh PCR product	0.5 to 4 µl	0.5 to 4 µl
Salt Solution	1 µl	--
Dilute Salt Solution	--	1 µl
Water	add to a total volume of 5 µl	add to a total volume of 5 µl
TOPO® vector	1 µl	1 µl
Final Volume	6 µl	6 µl

The reaction was mixed gently and incubated for 15 minutes at room temperature (22-23°C). The reaction was placed on ice and proceeded to transformation.

Chemical transformation procedure: 2 µl of the cloning reaction was added into a vial of One Shot DH5α-T1 chemically competent *E. coli* and/or a sterile 1.5ml microcentrifuge tube on ice containing 50µl of thawed JM109 High Efficiency Competent Cells.

The samples were incubated on ice for 15 minutes. Heat-shock was applied to the cells for 30 seconds at 42°C without shaking. The tubes were transferred immediately to ice. 250 µl of room temperature S.O.C. medium was added. The tube was capped tightly and shaken horizontally (200 rpm) at 37°C for 1 hour.

Plating and colony selection: 100 µl of transformation culture is plated onto the LB Agar plates (100µg/ml Amp + Xgal-IPTG) for overnight incubation at 37 ºC. White colonies are picked from the plate with a pipette tip 5 ml LB Medium with the pipette tip was inoculated Incubation for 4.5 hours at 37 ºC (OD600 : 1.5-5) After incubation, 2 ml of culture is mixed with glycerol (15%) and vortexed to keep at -80 ºC.

Plasmid purification: White colonies obtained subsequent to transformation were selected and incubated overnight in 5 mL LB containing 100 µg/mL ampicillin. 700 µl of this bacterial culture was mixed with 300 µl 50% glycerol and stored at –80°C. Plasmid DNA was purified from 4 mL bacterial culture for sequencing and further applications by High Pure Plasmid Isolation Kit (Catalog # 11 754 785 001, Roche). Procedure is described in detail below.

1. 4 mL bacterial culture was divided into two 2 mL microfuge tubes and centrifuged in a standard bench-top microcentrifuge at 6 000 x g for 1 minute. The supernatant was discarded.
2. 250 µl Suspension Buffer containing RNase was added to each microfuge tube containing the bacterial pellet. The bacterial pellet was resuspended and mixed well.
3. 250 µl Lysis Buffer was added to the resuspended bacterial pellet and mixed gently by inverting the tube 3 to 6 times. The tube was incubated for 5 min at room temperature (25°C).
4. 350 µl chilled Binding Buffer was added to the lysed solution and mixed gently by inverting the tube 3 to 6 times. Incubated on ice for 5 min.
5. Centrifuged for 10 min at approximately 13 000 x g (full speed) in a standard tabletop microcentrifuge.
6. After centrifugation one High Pure Filter Tube was inserted into one Collection Tube. The entire entire supernatant from Step 5 was transferred into upper buffer reservoir of the Filter Tube. The entire High Pure Tube assembly was inserted into a standard tabletop microcentrifuge and centrifuged for 1 min at 13 000 x g.
7. After centrifugation the Filter Tube was removed from the Collection Tube, the flowthrough liquid was discarded, and the Filter Tube was reinserted in the same Collection Tube.
8. To wash the cells 700 µl Wash Buffer II was added to the upper reservoir of the Filter Tube and centrifuged for 1 min at 13 000 x g in a standard tabletop microcentrifuge.
9. After discarding the flowthrough liquid the entire High Pure Tube assembly was centrifuged for an additional 1 min to remove residual Wash Buffer. The collection tube was discarded.
10. To elute the DNA the Filter Tube was inserted into a clean, sterile 1.5 ml microcentrifuge tube. 100 µl Elution Buffer or double distilled water (pH adjusted to 8.0 – 8.5) was added to the upper reservoir of the Filter Tube. The

tube assembly was centrifuged for 1 min at 13 000 x g in a standard tabletop microcentrifuge.

11. The microcentrifuge tube containing the eluted plasmid DNA was either used directly in applications such as cloning or sequencing or stored at –20°C for later analysis.

Plasmid DNA sequencing:

The sequencing reaction conditions were given below at Table 2.4.

Table 2.4: Sequencing reaction conditions.

Ingredients	Volume/Concentration	Reaction conditions	Temp.	Time
Ready Reaction Mix*	2 µl	Initial Denaturation	95°C	4 min
BigDye Terminator Sequencing Buffer (5X)*	1 µl	Denaturation	95°C	1 min.
Template DNA	1 µl (80ng)	Annealing	50°C	30 sec.
M13 –20 Forward Primer	1 µl (3.2 pmole)	Extension	60°C	4 min
dH_2O	5 µl	No. of Cycles	35	
Total volume	10 µl	Final Elongation	72°C	5 min

*BigDye® Terminator v3.1 Cycle Sequencing Kit from Applied Biosystems.

Sodium acetate-Ethanol precipitation was applied to the PCR products before sequence analysis.

1. 1 µl 3M pH 4.6 sodium acetate and 25 µl 95 % ethanol 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 14000 rpm.
4. Supernatant was discarded and DNA pellet was washed with 250 µl cold 70% ethanol.
5. Samples were centrifuged for 15 min at 14000 rpm.
6. Ethanol was discarded and samples were incubated for 5 min at 95^{o}C for the evaporation of all residual ethanol.

7. DNA was dissolved in 20 µl Hi-Di Formamide.

8. Samples were denatured by first putting at 95°C for 5 min and then at -20°C.

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

2.2.3.2 QBP1-pETBlue2 construction

pETBlue-2 (Novagen) vector has been chosen as the host vector for construct formation. QBP-1 oligo was enzyme digested from the pGEM-T cloning vector and ligated into pETBlue-2 expression vector. QBP-1 vector is ligated into the host vector in a way that any functional protein can be added downstream of the QBP-1 oligo. When expressed, the fusion protein has the oligo part at N terminal and the desired protein at the C terminal, fused to each other.

Restriction enzyme digestion of pGEM-T-QBP-1/ pCR2.1-TOPO-QBP1 and pETBLUE-2 vectors: Both vectors are digested with the same restriction digestion enzymes, *BglII* and *EcoRI* (Fermentas). Next, they are loaded into agarose gel and following the isolation of the DNA fragments from gel, we have QBP-1 oligo and pETBlue-2 ready to ligate with sticky ends.

Table 2.5 : Restriction Enzyme Digestion Conditions.

Ingredients	Volume/Concentration	Ingredients	Volume/Concentration
pCR2.1-TOPO-QBP1	10 µl (1.2 ug)	pETBlue-2	1.5 µl (750ng)
Buffer Orange	2 µl	Buffer Orange	1 µl
Bgl*II* (5U)	0,5 µl	Bgl*II* (5U)	0.4 µl
EcoR*I* (5U)	0,5 µl	EcoR*I* (5U)	0.4 µl
dH_2O	7 µl	dH_2O	6.7 µl
Total volume	20 µl	Total volume	10µl

Reaction conditions: The reactions were incubated at 37°C for 4 hours and 15 minutes at 65C for enzyme inactivation. Digestion reactions were run on 1-2.5% agarose gel electrophoresis and the desired QBP1 and pETBlue-2 sequences were extracted from the gel according to previously described procedure.

Ligation of QBP-1 oligo into pETBlue-2: pCR2.1-TOPO TA cloning vector was digested with restriction enzymes and desired DNA fragment was gel extracted.

Insert DNA which was cleaned and concentrated was ligated into pETBlue-2 expression vector. Procedure is described in detail below.

Ligation reaction was performed with Quick Ligation™ Kit (New Enland Biolabs, Inc., Catalog # M2200S)

1. 1 µl (60 ng) pETBlue-2 vector was combined with 1 µl (5 ng) of insert (3-fold molar excess). Volume was adjusted to 10 µl with dH_2O.
2. 10 µl of 2X Quick Ligation Buffer was added and mixed.
3. 1 µl of Quick T4 DNA Ligase was added and mixed thoroughly.
4. Centrifuged briefly and incubated at room temperature (25°C) for 15 minutes.
5. Chilled on ice and transformed or stored at –20°C for longer storage.
6. It was not heat inactivated bacause heat inactivation dramatically reduces transformation efficiency.

Transformation of pETBlue2-QBP1 into host cells: The newly formed pETBlue2-QBP1 vector was transformed into JM109 and TOP10 competent cells as described before (in section 2.1.1.4.) and colony selection was performed.

3 µl of the ligation reaction was transformed into a vial of One Shot TOP10 chemically competent *E. coli* cells as previously described. Plates were incubated overnight at 37°C.

White colonies obtained subsequent to transformation were selected and incubated overnight in 5 mL LB containing 100 µg/mL ampicillin. 700 µl of this bacterial culture was mixed with 300 µl 50% glycerol and stored at $-80^{o}C$. Plasmid DNA was purified from 4 mL bacterial culture for sequencing and further applications by High Pure Plasmid Isolation Kit as previously described.

The purified plasmids were sequenced using BigDye® Terminator v3.1 Cycle Sequencing Kit from Applied Biosystems according to the protocol given before and the sequence was verified.

Sodium acetate-Ethanol precipitation was applied to the PCR products before sequence analysis as previously described. ABI 3100 Avant (PE, Applied Biosystem, CA) automated sequencer was used for DNA sequencing.

2.2.3.3 Construction of pETBlue2-QBP1-GFPuv and pETBlue2-QBP1-AP

pETBLUE-2 vector serves as the host where the QBP-1 oligo and a functional protein meets to form a fusion protein. DNA sequences of GFPuv and alkaline phosphatase (AP) proteins were amplified from pGFPuv and pSB2991 plasmids, respectively, with the addition of required enzyme cutting sites. The sequences were first ligated into pGEM-T cloning vectors and then enzyme digested for ligating into previously formed pSilTAG vector to form the fusion proteins. The pSilTAG vector containing fusion of QBP-1 and functional protein was transformed into Tuner pLacI and Bl21 (DE3) Star competent cells for the protein expression studies.

2.2.3.4 Construction of QBP1-lcc1

For the formation of QBP1-lcc1 fusion molecule at the DNA level, firstly, 2 primers were designed and commercially synthesized (Alpha DNA/Quebec/Canada). Forward primer was a long, 75 base containing primer which contained EcoRI enzyme restriction site and QBP1 sequence. The primer binds on 5' end of lcc1 gene. Reverse primer binds on the 3' end of lcc1 gene and contains NotI enzyme restriction site. The template DNA was pPICZ-B plasmid vector containing lcc1 gene. The details of the primers are given in the Appendix section.

PCR (Polymerase Chain Reaction) amplification: Optimized PCR conditions to amplify lcc1 gene with QBP1 at 5' end was as followed: Initial denaturation at 95^{o}C for 1 min, denaturation at 95 ^{o}C for 1 min, annealing at 60 ^{o}C for 1 min, extension at 72 ^{o}C for 1 min and final elongation at 72 ^{o}C for 5 min. 30 cycles of reaction was performed using Pfu polymerase enzyme (Fermentas).

Amplified DNA was run on 1% agarose containing jel (in TBE Buffer:Tris/Borate/EDTA) and extracted from gel by using QIAquick Gel Extraction Kit.

Ligation of QBP1-lcc1 oligo into cloning vector (pDrive): QBP1-lcc1 oligo was then ligated into pDrive cloning vector system (Qiagen PCR Cloning Kit). Ligation reactions was set up as following; 5µl of 2X Rapid Ligation Buffer, 1µl of pGEM®-T (50ng), 2µl of PCR product (8ng), 1µl of T4 DNA Ligase (3 Weiss units/µl) and deionized water to a final volume of 10µl. The reactions was mized by pipetting and incubated for 1 hour at room temperature.

Transformation into host cells: 2µl of ligation reaction was added into a sterile 1.5ml microcentrifuge tube on ice containing 50µl of thawed JM109 High Efficiency Competent Cells. The cells were mixed by gently flicking the tube and placed ice for 20 minutes. The cells was Heat-shocked for 45-50 seconds in a water bath at exactly 42°C and returned to ice for 2 minutes. 950µl room temperature SOC medium was added to the tubes and incubated for 1.5 hours at 37°C with shaking (~150rpm).

Plating and colony selection: 100 µl of transformation culture is plated onto the LB Agar plates (100µg/ml Amp + Xgal-IPTG) for overnight incubation at 37 ºC. White colonies are picked from the plate with a pipette tip 5 ml LB Medium with the pipette tip was inoculated Incubation for 4.5 hours at 37 ºC (OD600 : 1.5-5) After incubation, 2 ml of culture is mixed with glycerol (15%) and vortexed to keep at -80 ºC.

Restriction enzyme digestion of pDrive-QBP1-lcc1 and pPICZ-B vectors: Both vectors were digested with the same restriction digestion enzymes, EcoRI and NotI (Fermentas). Next, they are loaded into agarose gel and following the isolation of the DNA fragments from gel, we have QBP1-lcc1 oligo and p-PICZ-B ready to ligate.

Ligation of QBP1-lcc1 oligo into pPICZ-B vector: Enzyme digested QBP1-lcc1 oligo and pPICZ-B sequences were ligated by using Quick Ligation Kit (New England Biolabs/ABD) as followed; 50 ng of digested vector was combined with a 3-fold molar excess of digested insert. The volume was adjusted to 10 µl with dH_2O and 10 µl of 2X Quick Ligation Buffer was added and mixed. 1 µl of Quick T4 DNA Ligase was added, mixed and incubated at room temperature (25°C) for 5 minutes.

Transformation of pPICZ-B (QBP1-lcc1) into bacterial host cells: The newly formed vector was transformed into TOP10 competent cells as described before (in section 2.1.1.4.) and colony selection was performed. The sequence of newly formed fusion vector was checked before transforming into *Pichia pastoris* yeast cells.

The purified plasmids were sequenced using BigDye® Terminator v3.1 Cycle Sequencing Kit from Applied Biosystems according to the protocol given before and the sequence was verified.

Sodium acetate-Ethanol precipitation was applied to the PCR products before sequence analysis as previously described. ABI 3100 Avant (PE, Applied Biosystem, CA) automated sequencer was used for DNA sequencing.

Transformation of pPICZ-B (QBP1-lcc1) into yeast host cells: pPICZ-B (QBP1-lcc1) vector was transformed into Pichia pastoris yeast cells as written in *Pichia* Expression Kit (Invitrogen) and colonies were selected on Zeocin containing medias.

2.2.4 Protein based studies

2.2.4.1 QBP1-GFPuv and QBP1-AP expression in *Eschericia coli*

pSilTAG vectors (pETBlue2-QBP1) containing GFPuv and AP proteins were transformed into Tuner (DE3)pLacI and BL21 (DE3)Star (Novagen) host cells. Different expression procedures were tried;

Firstly, 3 ml of LB media plus 1% glucose plus antibiotics (ampicillin: 50ug/ml and/or chloramphenicol: 34 ug/ml) in a culture tube was inoculated with a sterile loop of cells taken from glycerol stocks of Tuner (DE3)pLacI and BL21 (DE3) cells (2 sets of experiment). The culture was incubated at 37°C with shaking at 250 rpm to an OD600 of approximately 0.6–1.0 and stored overnight at 4°C. The following morning, the cells was collected by centrifugation and resuspended in 3 ml fresh medium plus 1% glucose plus antibiotic(s) and this culture was used to inoculate 100 ml medium plus 1% glucose plus antibiotic(s) (in another sets of experiments, this step was repeated without addition of 1% glucose). The 100-ml culture was shaked at 37°C until the OD600 is approximately 0.5–1.0. Just prior to induction, the 100-ml culture was splitted into 10-ml cultures. For full induction, IPTG was added to 0, 0.05, 0.1, 0.5, 1 and 3 mM. The cultures were incubated with shaking at 37°C for 10 more hours. After 3rd, 6th and 10th hours, samples were taken from each cultures for SDS-PAGE analysis.

As a second way, the transformed cells were grown in LB media plus 0-1% glucose plus antibiotics (ampicillin: 50ug/ml and/or chloramphenicol: 34 ug/ml) for 16 h by shaking vigorously at 37°C. Then the bacterial cells were collected by centrifugation at 2,000 x g for 20 min at 4°C. The bacteria were resuspended in LB medium

with/without glucose. IPTG was added to the final concentration of 0, 0.1, 0.5 and 1 mM. The samples were analyzed by SDS-PAGE.

As a third way, MagicMedia™ E. coli Expression Medium was also used as an alternative. 2-10 ml LB + antibiotic was inoculated with an expression colony and incubated overnight at 37°C, 300 rpm shaking. Pre-warmed complete MagicMedia™ + antibiotic was inoculated with overnight culture at a 1:40 dilution. The culture was incubated at 37°C (for AP), and 30°C (for GFPuv) 300 rpm 18-24 hours.

Protein expression analysis: It is important to determine whether the protein is soluble in the cytoplasm or located in cytoplasmic inclusion bodies to set up the best purification strategy. Many proteins, when they are expressed at high levels in bacteria, form inclusion bodies while others are tolerated well by the cell and remain in the cytoplasm in their native configuration.

Determination of target protein solubility:

1. A single colony from a freshly streaked plates from glycerol stocks of Tuner (DE3) PlacI harboring pETBlue-2-GFPuv or pETBlue-2 were picked up and inoculated into 5 ml LB medium containing 50µg/ml ampicillin and 34µg/ml chloramphenicol in 50 ml Falcon tube to analyze the target protein production and its solubility. Culture was grown overnight at 37°C or 30°C with shaking at 250 rpm.
2. 50 ml pre-warmed LB containing 50 µg/ml ampicillin and 34µg/ml chloramphenicol was inoculated with 0.5 ml of the overnight culture and grown at 37°C or 30°C with vigorous shaking (250 rpm) until the OD_{600} was 0.5-0.8.
3. 1 ml sample was taken immediately before induction (noninduced control). The cells were centrifuged at 14.000 rpm and the pellet was resuspended in 50 µl 1xSDS-PAGE sample buffer. The sample was frozen at –20°C until needed for SDS-PAGE.
4. Cells were induced with 0.1-1.0 mM IPTG and incubated for 3 to 12 hours at 37°C or 30°C.

5. 1 ml sample (induced control) was taken each hour and centrifuged. Pellets were resuspended in 100 µl 1x SDS-PAGE sample buffer and frozen at –20°C until needed for SDS-PAGE.
6. Cells were harvested by centrifugation at 4 000 x *g* for 20 min. Cell pellet was resuspended in 50 µl of lysis buffer and 3 µl of lysozyme (25 mg/ml) was added to the sample.
7. Cell lysate was sonicated 6 x 10 s with 10 s pauses at 400 W on ice. Lysate was centrifuged at 10 000 x *g* at 4°C for 25 min. The supernatant was decanted (soluble protein) and saved on ice.
8. The pellet was resuspended in 50 µl of lysis buffer which constitutes the suspension of insoluble matter (insoluble protein).
9. 50 µl of 2xSDS-PAGE sample buffer was added to each of the soluble protein and insoluble protein extract.
10. Samples were heated with the frozen noninduced and induced cell samples at 95°C for 5 min.

Induced, non-induced and 2.5 ml of soluble protein mixtures were centrifuged at 15.000 x g for 1 min. All the samples were stored at –20°C until SDS-PAGE analysis.

SDS-PAGE analysis of fusion expression: Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), was carried on a 12.5 % separating gel and a 5 % stacking gel. Electrophoresis was run two hours with a voltage of 120 mV. Protein bands were stained with Coomassie brilliant blue R250. Gels were then destained with a mixture of acetic acid and ethanol (5%; 10%). The Protein Molecular Weight Marker mix (SM #0431) included; β-galactosidase (116 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), lactate dehydrogenase (35 kDa), endonuclease Bsp 981 (25 kDa), β-lactoglobin (18.4 kDa) and lysosym (14.4 kDa). The gel components are given in Tables below.

Table 2.6 : Components of Lower Resolving Gel (12% Acrylamide/Bis-acrylamide).

Components	*Volume*
H_2O	3.3 ml
1.5 M Tris-HCI (pH:8.8)	2.5 ml
SDS (10%, w/v)	0.1 ml
Acrylamide:bis-acrylamide (29:1) (30%, w/v)	4.0 ml
Ammonium Persulfate (APS) (10%, w/v)	0.1 ml
TEMED	10 ul
Total	10 ml

Prepared solution was poured into the gel casting forms provided that leaving 2 cm below the bottom of the comb for the upper stacking gels. Top of the gels were layered with 200 ul isopropanol to remove the bubbles. After the polymerization of the lower resolving gels, isopropanol was washed out completely.

Table 2.7 : Components of Upper Stacking Gel (5 % Acrylamide/Bis-acrylamide).

Components	**Volume**
H_2O	1.4 ml
1 M Tris-HCI (pH:6.8)	0.25 ml
SDS (10%, w/v)	0.02 ml
Acrylamide:bis-acrylamide (29:1) (30%, w/v)	0.33 ml
Ammonium Persulfate (APS) (10%, w/v)	0.02 ml
TEMED	6 ul
Total	2 ml

Prepared upper stacking gel solution was poured on the top of the lower resolving gels and combs were inserted. After this, gels were allowed for complete polymerization. 12 ul of each sample obtained in purification steps were mixed with 6 ul of 3X SDS loading dye and boiled for 5 min at 95 °C for protein denaturation. After loading all the samples and protein marker (Fermentas, Appendix D) to the gel lanes, gels were run at 80 volt for upper stacking gels and 140 volt for lower resolving gels until the loading dyes reached the bottom of the gels.

Visualization of the protein bands were accomplished by staining the gels with 100 ml of gel stain solution (Appendix B) with shaking at 40 rpm for 1 hour at 37 °C. Finally, gels were destained by 100 ml of gel destain solution (Appendix B) with gentle shaking at 30 rpm for 2 hours at 37 °C.

2.2.4.2 QBP1-lcc1 expression in *Pichia pastoris*

Pichia pastoris colonies containing pPICZ-B (QBP1-lcc1) were selected. For the expression of fusion proteins, *Pichia* Expression Kit (Invitrogen) has been followed.

25 ml of BMGY medium was inoculated using a single colony in a 250 ml flask and grown at 30°C in a shaking incubator (250 rpm) until culture reached an OD600 = 2–6. The cells were harvested by centrifuging at 2000 g for 5 minutes at room temperature. Supernatant was decanted and cell pellet was resuspended to an OD600 of 1.0 in BMMY medium to induce expression (approximately 100–200 ml). 100% methanol was added to a final concentration of 0.5% every 24 hours to maintain induction. 1 ml of the expression culture was transferred to a 1.5 ml microcentrifuge tube to analyze expression levels.

2.2.5. Assays

2.2.5.1 Laccase enzyme activity assay

ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)) is the substrate used to observe the reaction kinetics of laccase. Laccase enzyme facilitates the reaction of ABTS with hydrogen peroxide, turning it into a green and soluble end product which gives absorbance maximum of 420 nm light. 3 selected colonies and control group were induced for 5 days, and samples of every 24 hours were collected and mixed with ABTS (2.5 mM) in NaCitrate (pH.3.0, 30mM) buffer. Enzyme kinetics experiment was performed at 421 nm of light.

2.2.5.2 BCA assay

Microplate procedure (sample to working reagent-WR ratio = 1:8)

The procedure for BCA assay is as follows:

1. Pipette 25 µl of each standard or unknown sample replicate into a microplate well (working range = 20-2,000 µg/ml).

However, the working range of the assay in this case will be limited to 125-2,000 µg/ml.

2. Add 200 µl of the WR to each well and mix plate thoroughly on a plate shaker for 30 seconds.

3. Cover plate and incubate at 37°C for 30 minutes.

4. Cool plate to RT.

5. Measure the absorbance at or near 562 nm on a plate reader.

6. Subtract the average 562 nm absorbance measurement of the Blank standard replicates from the 562 nm measurements of all other individual standard and unknown sample replicates.

7. Prepare a standard curve by plotting the average Blank-corrected 562 nm measurement for each BSA standard vs. its concentration in µg/ml. Use the standard curve to determine the protein concentration of each unknown sample.

2.2.6. Preparation of silica and quartz resin

- Around 100 mg (for batch), 1-2 g (for column) of silica/quartz powder was weighed.
- Serial washing steps were applied by using 50 % isopropanol and distilled water.
- Powder samples were vortexed for 5-10 min and then sonicated for 20 min in the ultrasonic bath.
- The powder was vortexed quickly to resuspend and spinned down at 200 g for 3 min.
- After washing steps, 0-0.5 % PC buffer was added to equilibrate the resin surface.

2.2.7 Column packing / batch procedure

For the purification steps, both column and batch procedures were applied. After the silica/quartz resin was cleaned, it was poured into empty purification columns and waited to settle down. In column case, the samples and buffers were run through the

column using peristaltic micro-pump to arrange the liquid flow speed. The system is given in Figure 3.26. For the batch application, the samples can be introduced with resin material in a micro centrifuge tube. Washing and elution buffers were applied onto the resin material in the micro centrifuge tube, and after incubation period, the resin was precipitated using centrifugation.

3. RESULTS AND DISCUSSION

3.1. Bacterial Cloning and Expression Systems (*E.coli*)

3.1.1 Cloning optimization

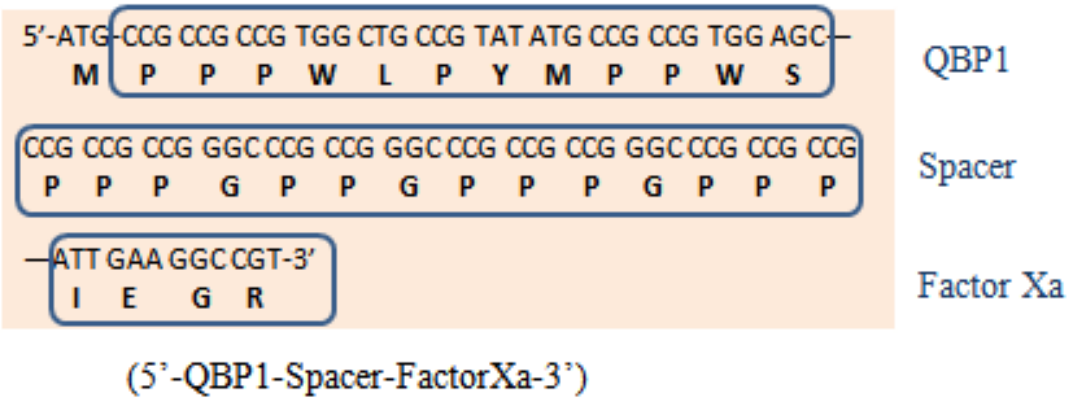
3.1.1.1 QBP1 oligo construction

To use QBP-1 peptide as N-terminal fusion molecule, bacterial cloning and expression systems have been selected as host systems. The idea is forming a QBP1-functional protein fusion molecule at the DNA level and transform it into a bacterial host system (e.g. *Eschericia coli*) and express the fusion molecule for later use. At the end, QBP1 peptide can be used as a "tag" molecule for the purification of functional protein from cell extract using silica/quartz resin.

For the formation of the vector systems at the DNA level, 3 parts were needed to be brought together. The first one was a host vector, pETBLUE-2 (or pQE60), which serves as a place where QBP-1 oligo and a desired functional protein can form a fusional protein**.** So the experimental work starts with the design of QBP-1 oligo which includes previously selected QBP-1 peptide sequence, a designed 14 aa linker (proline and glycine rich) to hold the QBP-1 peptide out of the functional protein, Factor Xa and restriction enzyme cutting sites.

The QBP1 oligo complex contains the basic parts for different purposes. The restriction enzyme cutting sites form the necessary positions for relocation of the oligo complex in other expression systems. QBP1 oligo is followed by a 14 amino acid long linker sequence. The idea of using a linker sequence is basically; when the protein fusion molecule is formed, with the help of the linker molecule, QBP1 sequence can easily be hold outside of the protein. So QBP1 peptide can freely bind on the resin material. The composition of linker (spacer) sequence is formed just theoretically, including proline and glycine residues. Prolines help to form a rigid structure and glycine is needed for some flexibility. The last part is Factor Xa cutting site. When the fusion molecule is expressed and purified by using silica/quartz resin,

QBP1-functional protein fusion molecule is obtained. If necessary, QBP1 oligo can be separated from functional protein by using Factor Xa enzyme which recognizes IEGR sequence and cuts the protein. This is an optional choice. If the tag molecule does not affect the activity of the functional protein, it can stay on N-terminal of the protein and there is no need for Factor Xa usage. If there is an effect of QBP1 molecule on the functionality of the protein, Factor Xa can be used to separate them and the parts can be run through silica resin for a final step.



(5'-QBP1-Spacer-FactorXa-3')

Figure 3.1: QBP1 oligo includes QBP-1peptide, 14 amino acid spacer and Factor Xa Protease Cleavage Site.

When expressed, QBP-1 peptide will have just a methionine residue at the N terminal end. Here, Figure 3.1 shows the designed QBP-1 oligo sequence which will be placed at the N-terminal end of the functional protein. The oligo basically includes a start codon for methionine residue, 12 amino acid long QBP1 sequence, 14 amino acid long spacer which is rich in proline and glycine, and finally Factor Xa cutting site for later protease digestion of fusion molecule. The final construct is given schematically in Figure 3.2.

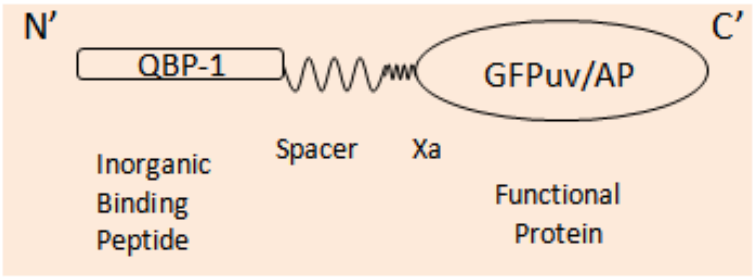


Figure 3.2: Schematic presentation of QBP1-Functional protein fusion molecule which includes QBP-1peptide at N-terminal, functional protein (GFPuv/AP) at C-terminal and 14 amino acid spacer and Factor Xa Protease Cleavage Site in between.

So, the experimental procedure starts with the design of QBP1 oligo complex. Because the designed oligo was synthesized commercially as ssDNA, it was needed to be turned into ds form before ligating into the host vector. It is a short one that is why the conditions for the amplification need to be carefully optimized.

PCR optimization for QBP1 oligo: Figure 3.3 shows the amplified ds oligo QBP-1 around 150 base long marker DNA. Dealing with a short DNA sequence can be problematic. In our case, the main problem is the very high GC content of QBP1 oligo with spacer sequence. About 80% GC content of the oligo makes the PCR protocol extremely problematic. The annealing temperature of the primers must be carefully optimized for getting a sharp DNA band on the gel for later use. Because of the high GC content, the oligo has a very high melting temperature which overlaps with the working temperature of the polymerase enzyme. Best ways to decrease the melting temperature is using agents such as betain, GC-melt or DMSO which will inhibit the formation of secondary structure in DNA template and primers, and proceed the PCR reaction.

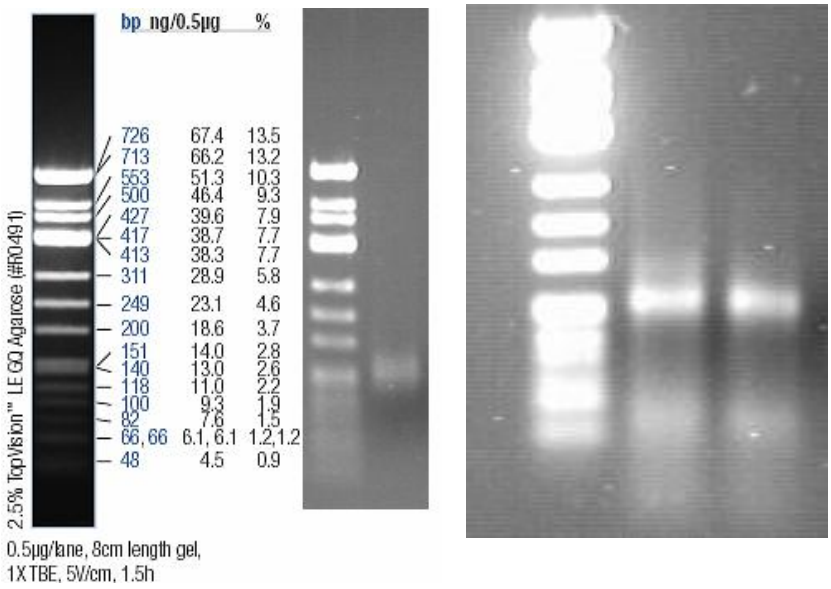


Figure 3.3: Agarose gel electrophoresis image: ssDNA oligo was PCR amplified and turned into dsDNA before the ligation step. On the left; Marker 10 and amplified oligo was seen around 140 bp. On the right; there is Marker 10 and amplified oligo with 10% DMSO (lane 2-3 with different template amounts) usage in PCR reaction. (PCR with Taq enzyme and 61°C annealing temperature).

We have also tried Klenow reaction to synthesize dsDNA from single stranded oligo but a sharp bant could not be achieved. The best result was obtained by using DMSO in the PCR reaction. The later part is the colony screening for the sequence without mutation.

Ligation into cloning vector: Amplified ds QBP-1 oligo was ligated into a cloning vector, pGEM-T, and transformed into appropriate host cells. These vectors including QBP-1 oligo, (Figure 3.4) can now be used as a kind of library for the formation of any desired fusion protein. QBP-1 and/or the linker part can just be enzyme digested or PCR amplified according to the needs of another system to form the fusion constructs. This kind of libraries was prepared for each component which was necessary in our systems, such as QBP-1 oligo, a differentiated QBP-1 oligo with linker sequence or functional proteins (GFPuv, AP and lcc1).

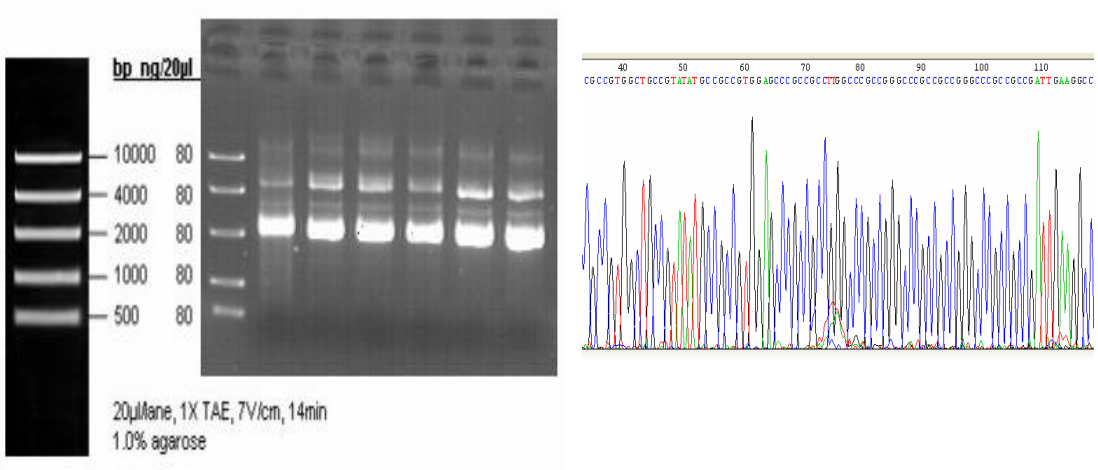


Figure 3.4: (On the left) Agarose gel electrophoresis image for pGEM-T-QBP-1 vectors acting as library for QBP1 sequence for any later ligation process for different functional proteins. (On the right) After all the ligation procedures, the sequences were verified by sequence analysis to check if there is any mutation in the sequence.

The next step was the ligation of QBP-1 oligo into pETBLUE-2 (or pQE60) host vector where any functional protein can be added to C terminal of the linker peptide to form the construct. Figure 3.4 shows the single digestion (EcoR*I*) of the ligation product around 3600 bp DNA marker. This vector is now the main expression vector for addition of any desired protein, so at the end the protein will have an N terminal

QBP-1 tag with 14 aa spacer molecule. In the ligation step, the main problem is the low amount of QBP1 oligo DNA before ligating into host vector. Theoretical 3:1 molar ratio rule is not always in use for the ligation protocol. The ratio starts from 1:1 to 9:1 which makes obtaining the necessary amount of oligo DNA difficult.

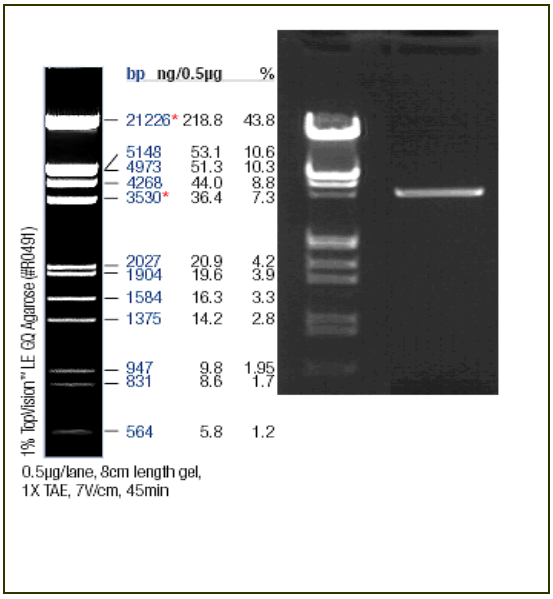


Figure 3.5: Agarose gel electrophoresis image for single eznyme digested (*Eco*RI) pETBlue-2 vector including QBP-1 oligo.

3.1.1.2 Addition of functional proteins at the dna level: recombinant green fluorescent protein (GFPuv) and alkaline phosphatase (AP)

The DNA sequences of GFPuv and AP was obtained from pGFPuv and pSB2991 vectors respectively, by PCR amplification with the addition of necessary enzyme cutting sites. The sequences were first ligated into pGEM-T cloning vector to form the library for each of them. The sequences were then enzyme digested and ligated into host vectors such as pETBLUE-2 and pQE60, host vector containing QBP1 oligo complex for the protein expression studies.

Working with GFPuv and AP is much easier than working with QBP1 oligo at the DNA level. GFPuv is 717 bp long and AP is 1434 bp long which are long enough at the DNA level for PCR, enzyme restriction or ligation. If necessary, AP or GFPuv proteins can be replaced by any desired protein for expression process.

Figure 3.6 Agarose gel electrophoresis image for pETBlue–2 (QBP1-GFPuv). On the left; double digested pETBlue2(+QBP1) and GFPuv sequences in which QBP1 first ligated to host vector and then GFPuv was ligated to pETBlue2(+QBP1), in the middle; QBP1 and GFPuv were ligated first and then were placed into host vector, on the right; single and double digested full vector pETBlue2(+QBP1+GFPuv).

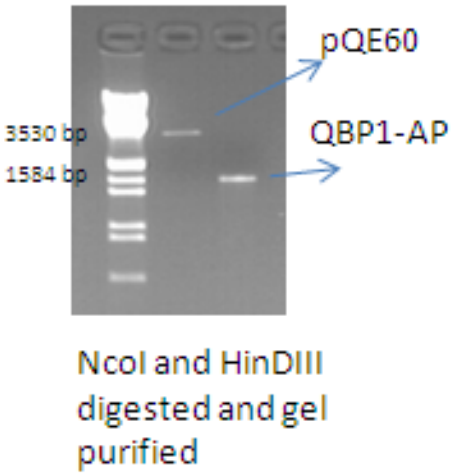


Figure 3.7 Agarose gel electrophoresis image for double digested (NcoI and HinDIII) pQE60 and QBP1-AP sequences.

The final construct structure can be shown in Figure 3.8. pETBlue-2 vector (or pQE60) acts as host vector at the DNA level for QBP1-GFPuv and QBP1-AP fusion molecules, or any desired fusion structures.

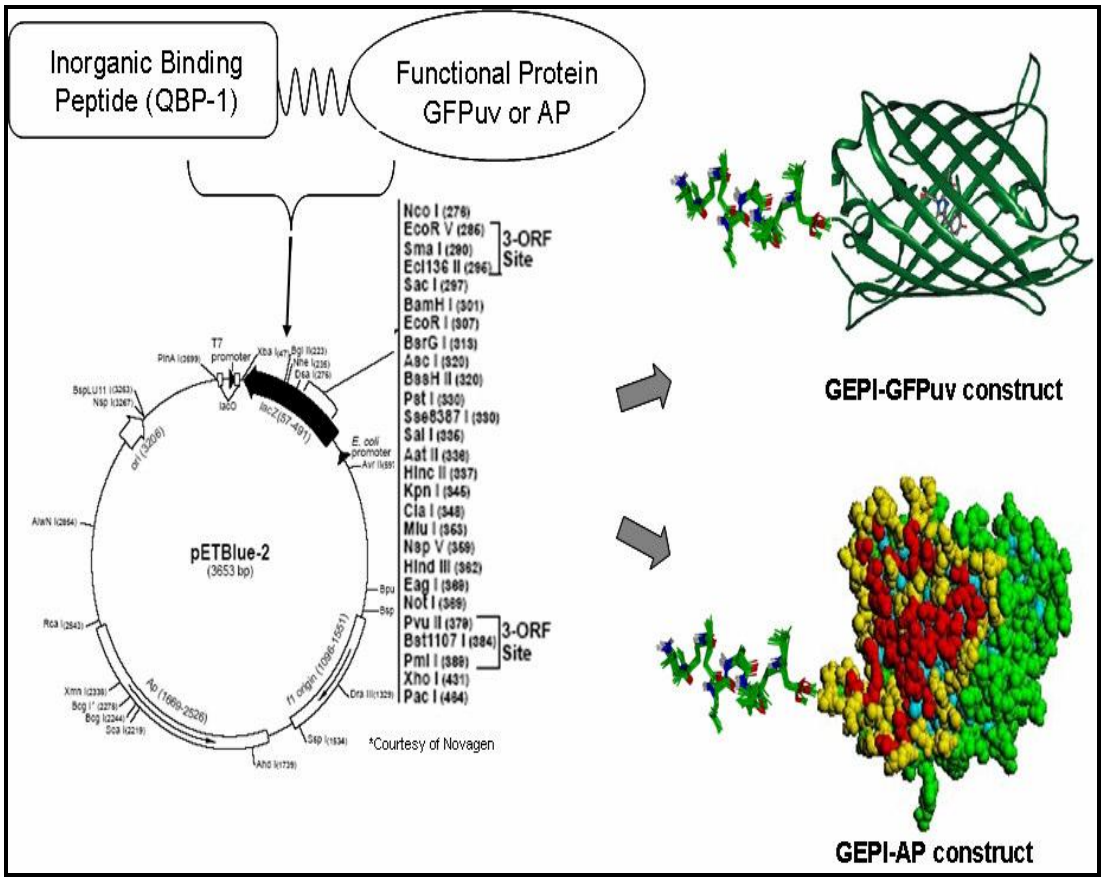


Figure 3.8. pETBlue2 (or pQE60) acts as the host vector to form GEPI-Functional protein fusion molecules.

3.1.2 Expression optimization

Although working at the DNA level can be tricky or problematic, the critical point is the expression of the QBP1 tagged protein. The tag molecule is located at the N terminal of the protein. The reason why it is located at that way, when quartz binding peptides were selected, they are located at the N terminal of the phage proteins. Change of direction of tag molecule might have an effect on the level of binding on the silica resin, that is why, the tag molecule is located at the N terminal position in the vector systems.

3.1.2.1 QBP1-GFPuv

There can be many parameters affecting the expression of the protein in the cell. The type of the cell, the media used for the incubation, the temperature and time ranges and the composition of the DNA sequence are the main affecting factors. What we are doing is, checking as much as possible parameters and limiting the possible effective steps on the expression.

The formed QBP1+GFPuv vector system was transformed into 2 different host cells, Tuner (DE3) pLacI and BL21(DE3)Star, for the protein expression studies. Each protein and each vector system needs its own optimized expression conditions. Different media, temperatures, IPTG concentrations etc. were tried. These are the parameters affecting the level of protein expression which must be optimized. pETBLUE-2 system generally needs a host cell with an extra pLacI plasmid for the tight control of protein expression.

The protein is supposed to be in soluble portion of the extract, and also maximum expression is expected around 30°C and 300 rpm shaking. Temperature was decreased from 37°C to 30°C to slow the increase in cell number but to have a high level of expressed protein.

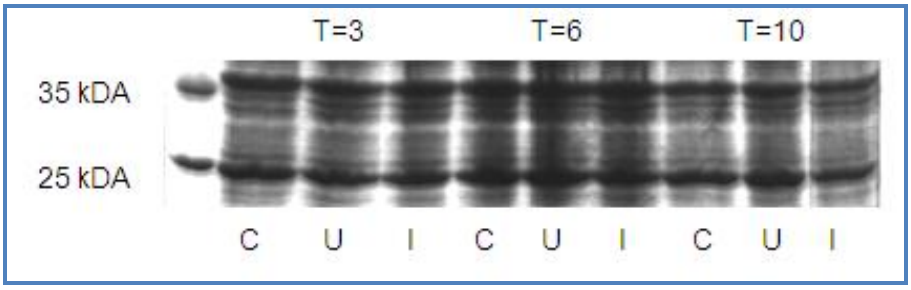


Figure 3.9. SDS-PAGE image for pETBlue2-QBP1-GFPuv expression in Tuner (DE3) pLacI cells at 30°C.

Addition of alanine as the second amino acid: Due to the failure in protein expression after trying several cell lines, temperature and IPTG ranges, DNA sequence has been modified. The new vector system was formed by similar experimental procedures as in the first vector system. In the first vector system, QBP1 oligo comes after the first methionine residue. It is a differentiated form of pETBLUE-2 vector in a way that the expressed QBP-1 peptide has just an extra methionine residue at the N terminal, aiming not to affect the binding of QBP-1 on the silica surface.

But in the modified vector system, QBP-1 oligo was PCR amplified in a way that an alanine residue was added as the second amino acid after the first methionine residue, as recommended in pETBLUE-2 system. It produces QBP-1 peptide with extra methionine and alanine residues at the N terminal. The reason for that is to

optimize the vector efficiency during the protein expression, but on the negative side, this extra alanine can affect the binding of the QBP-1 on the silica surface.

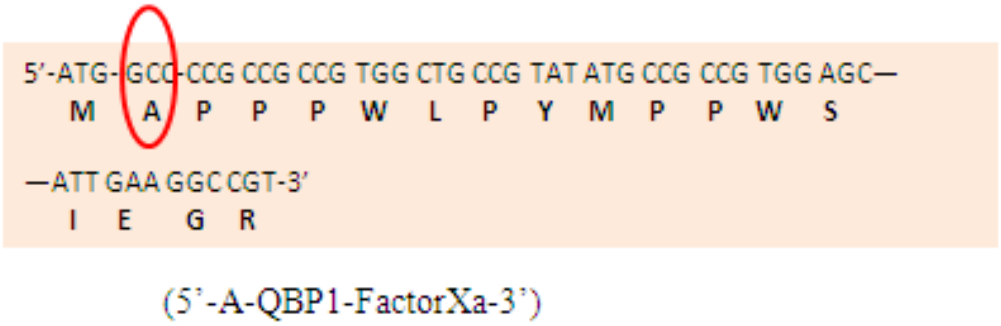


Figure 3.10: Alanine residue has been added as the second amino acid in front of QBP1 sequence.

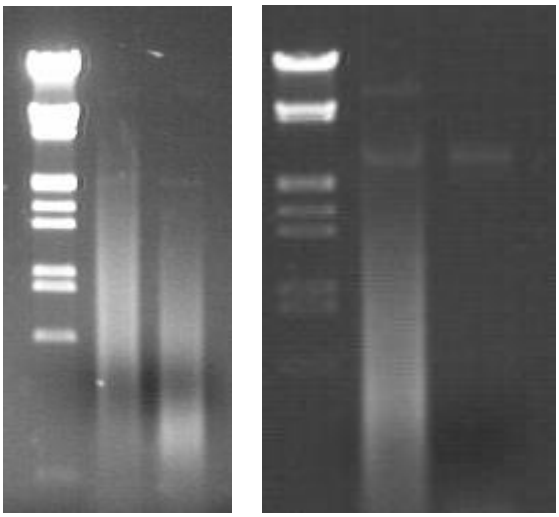


Figure 3.11: (On the left) Agarose gel electrophoresis images: Direct PCR and use of GC-Melt for PCR Reaction, (On the right); 5% and 10% DMSO for Site Directed Mutagenesis for Addition of Alanine Residue, (annealing 80ºC, Pt HiFi enzyme).

Effect of linker sequence: QBP1-GFPuv (without linker): As a second modification to the vector system, to increase the efficiency of the expression of the fused proteins, linker sequence has been removed from the system.

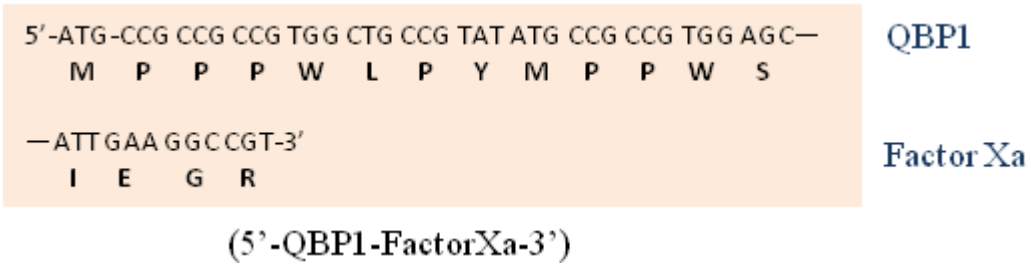


Figure 3.12: QBP1-Functional Protein Sequence Structure without 14 aa spacer sequence.

The new system has the pETBlue-2 vector as the host vector as in previous vector systems but 14 aa long linker peptide between QBP1 peptide and the functional proteins were deleted. QBP1 peptide directly binds with the functional proteins. Two versions of the vector system were created, one containing AP (alkaline phosphatase) and the other one containing GFPuv as the functional protein. Molecular biology procedures were followed as given in Section 2 to form the constructs. After the formation of the vector products, they were transformed into host cells such as Tuner (DE3) pLacI, BL21 (DE3) Star for the protein expression experiments. Different media such as 2YT, LB and commercial Magic Media (Invitrogen) were tried to optimize the protein expression conditions.

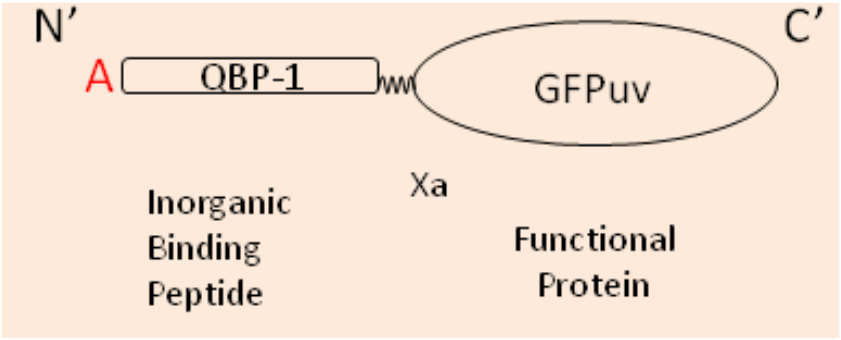


Figure 3.13 Schematic represantation of QBP1-GFPuv construct protein after removal of 14 amino acid spacer sequence and addition of Alanine (A) residue as second amino acid.

The schematic represantation of construct protein after removal of 14 amino acid spacer sequence and addition of Alanine (A) residue as second amino acid were given in Figure 3.13.

The best result obtained was the QBP1+GFPuv fusion protein, with alanine added as the second amino acid, in the BL21 (DE3) Star cells, after 21 hours of induction in MagicMedia solution as given in Figure 3.14.

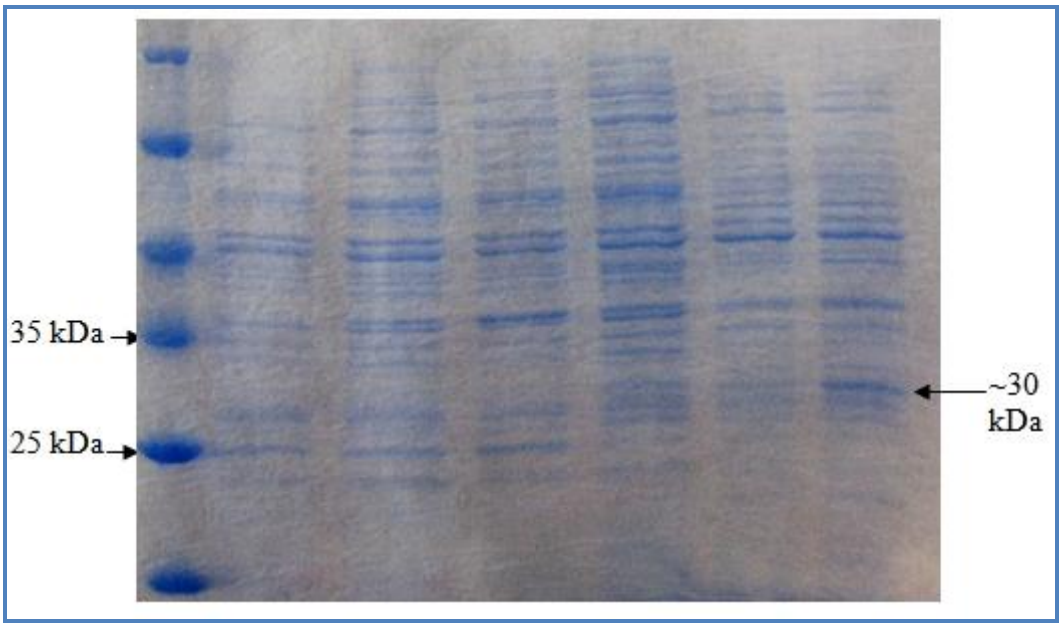


Figure 3.14 Unmodified/Modified Vector systems , pETBlue2-QBP1-GFPuv in the BL21(DE3) Star cells, after 21 hours of induction in MagicMedia solution.Lane1: Protein marker, Lane 2-4: t=10 hrs of induction, C, VS (+linker) and VS (-linker), Lanes 5-7: t=21 hrs of induction, C, VS (+linker) and VS (-linker). (C: Tuner (DE3) pLacI with a control pETBLUE-2 vector, VS1: Vector System with linker and VS2: Vector System without linker.

When the vectors including GFPuv functional protein with/without linker sequences were expressed in different host cells and culture media, there was still problem of expressing the protein. The best results obtained were with using commercial MagicMedia solution and after 21 hours of incubation. But the problem is that, the results were not repeatable. After applying the same experimental conditions, similar results were not obtained. GFPuv is a small protein in a hollow-cylindirical structure. One possible explanation of low expression level might be the affect of QBP1 peptide during the translation process.

3.1.2.2 QBP1-AP

Similar conditions were applied for pETBlue2-QBP1-AP construct. Modifications have been applied on DNA sequence. Alanine residue was placed as the second

amino acid and 14 aa. linker sequence was removed as shown in Figure 3.15. The type of the cell, the media used for the incubation, the temperature and time ranges have been tried for an optimized protein expression.

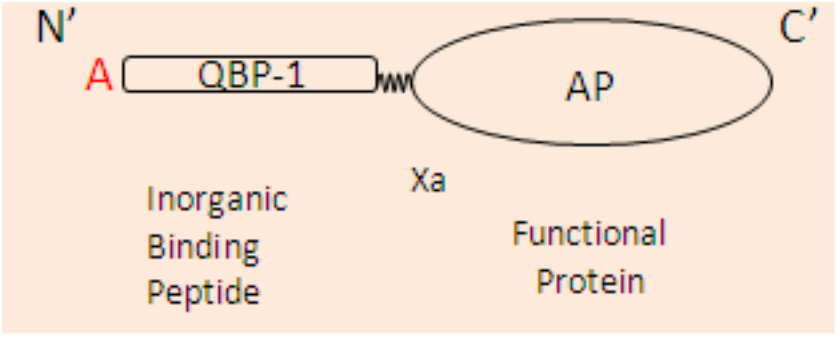


Figure 3.15: Schematic represantation of QBP1-AP construct protein after removal of 14 amino acid spacer sequence and addition of Alanine (A) residue as second amino acid.

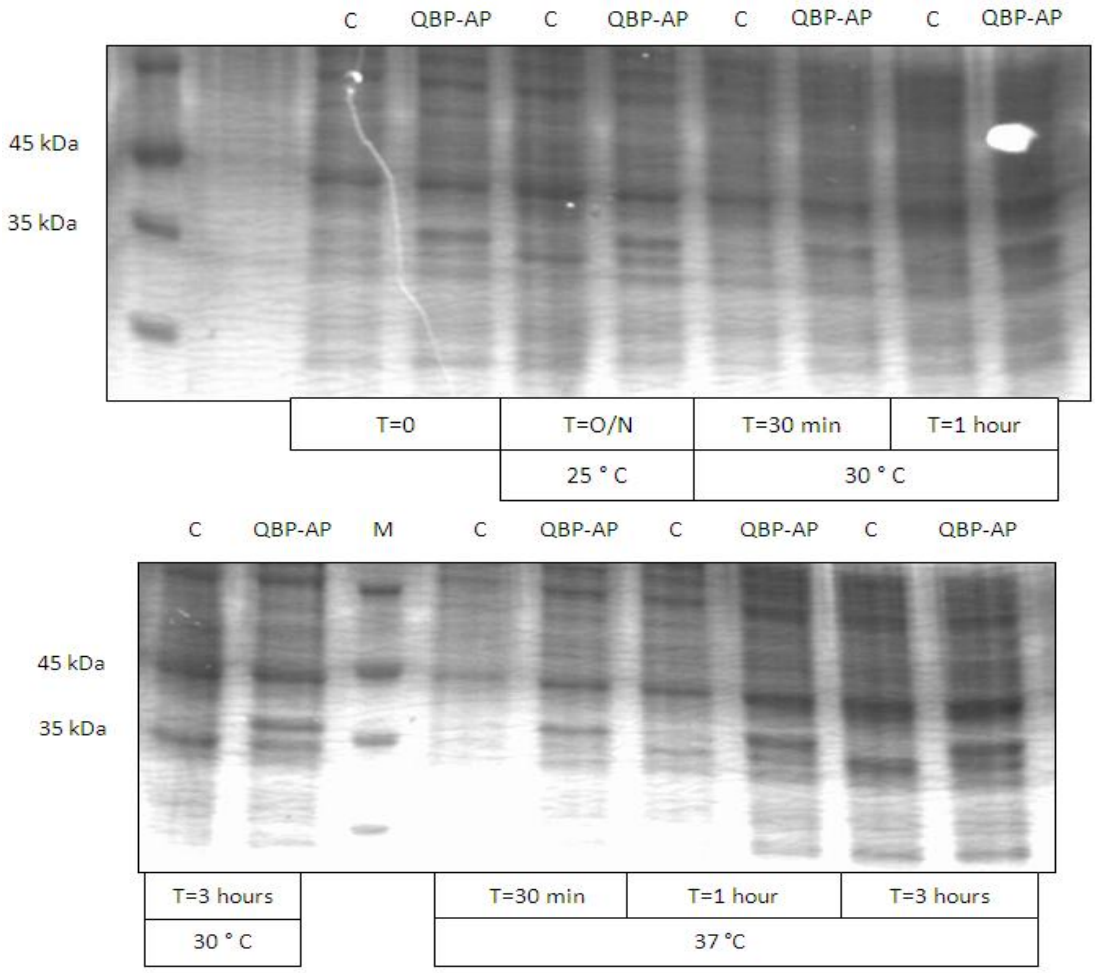


Figure 3.16: SDS-PAGE gel results for QBP1-AP fusion proteins (no linker) in pETBLUE-2, in TUNER cells.

For AP case, the protein has normally a leading peptide sequnce at the N terminal which sends the protein to the periplasmic space. In here, the leading peptide is replaced with QBP-1 peptide, that's why the localization of the protein needs to be determined.

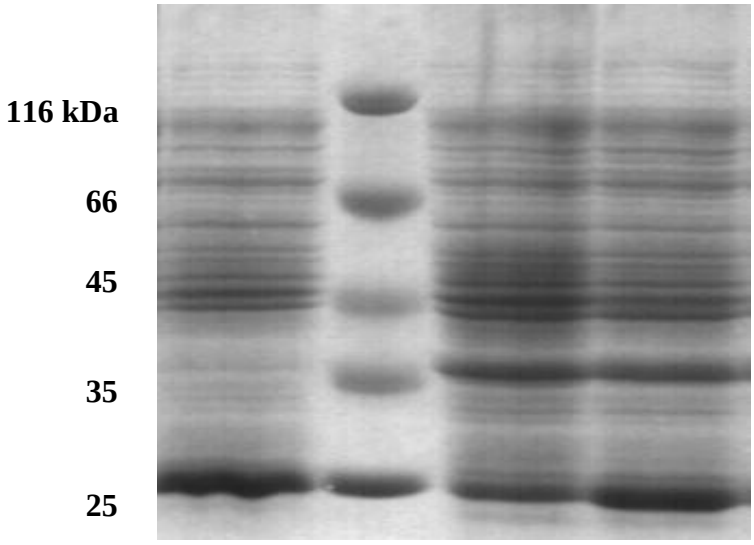


Figure 3.17: SDS Gel Eectrophoresis Analysis for QBP1-AP (no linker) construct: Lane 1: 3 hours of 1mM IPTG induction, pETBlue-2 from TUNER (DE3) pLacI cells, Lane 2: Protein Marker, Lane3: 3 hours of 0,5 mM IPTG induction pETBlue-2 (QBP1+AP) from TUNER (DE3) pLacI cells, Lane 4: 1mM IPTG induction of construct containing TUNER cells.

As it is seen in Figure 3.16 and Figure 3.17, the QBP1-AP fusion protein was produced around 40 kDa length. After 3 hours of induction, the cells were disrupted by using lysozyme and the cell extract were run on SDS-PAGE gel. In the 3rd and 4th lanes, the expressed proteins are shown at about 37–40 kDa range. AP protein is normally about 50 kDa and with the addition of QBP1 complex, the fusion molecule must be in 51–52 kDa range. But the gel results show a problem in protein expression. The translation process might be stopped before producing all protein so instead of 51–52 kDa protein, only 37–40 kDa protein is observed. One of the possible explanation for this; QBP1 tag molecule could bend and interfere with the mRNA molecule during translation process. This might be because of rigid proline residues in QBP1 peptid.

Purification experiments using silica columns, QBP1 acting as tag peptide

For purification of the QBP1-tagged proteins, silica columns (60–80μm) were prepared and cell extract was applied onto the column. The columns were washed

with buffer (pH: 6,4) and the eluted sample was boiled in loading dye before applying onto SDS-PAGE gel.

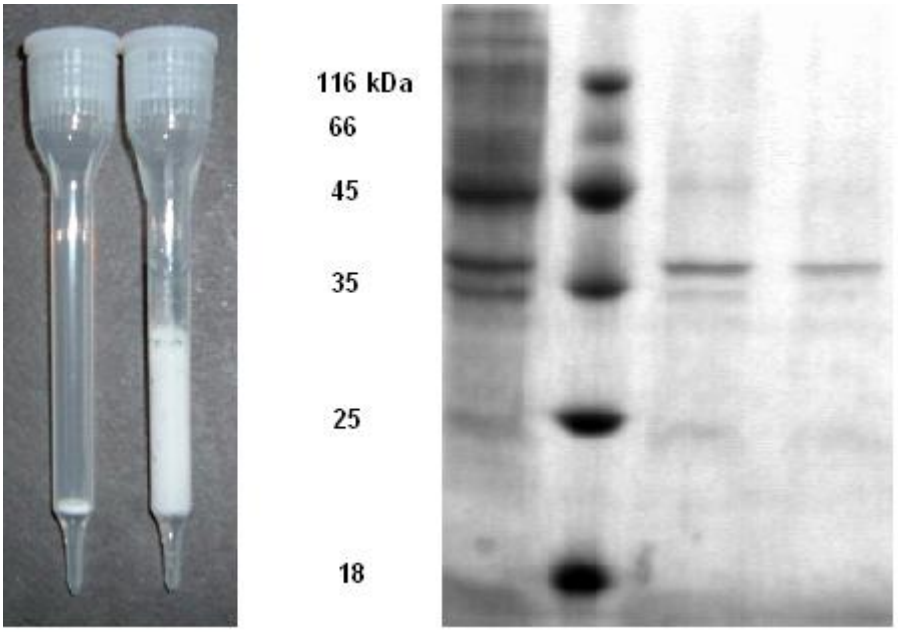


Figure 3.18: (On the left): Silica columns containing 60-80 μm Silica particles. (On the right): SDS Gel Electrophoresis Results: Lane 1: After 1 hours of incubation of cell extract on silica resin, the non-binding sample. Lane 2: Protein marker, Lane 3-4: The samples after boiling the resin.

The experiments previously carried out showed us that QBP-1 peptide is a strong silica-binder.

The results show that a protein which is about 37-40 kDa stayed on the silica resin after the washing steps. This protein is most possibly half produced QBP1-AP protein which must have a right composition of QBP1 sequence to bind on silica resin. When bringing both data together, QBP1-AP fusion molecule is supposed to be about in 51-52 kDa range but there is protein expression only in 37-40 kDa range which is obtained after purification on silica resin. QBP1 peptide must be formed right but the remaining part of the protein is not produced. The amino acid composition of QBP1 peptide is most possible reason for that due to high ratio of rigid proline residues.

3.2. Eukaryotic Cloning and Expression Systems (*Pichia pastoris*)

3.2.1. QBP1-lcc1 construct formation

Laccase gene, *lcc1,* has been obtained from *Pycnoporus sanguineus* by our research group in a former study. What we are doing here is taking lcc1 gene from *Pycnoporus sanguineus* and fuse it with QBP1 oligo and transform it into *Pichia pastoris* for the expression of QBP1-lcc1 fusion protein. QBP1 peptide (PPPWLPYMPPWS) is placed at the N terminus of the laccase enzyme (lcc1) in *Pichia pastoris* yeast cells to form a hybrid fusion molecule. Firstly, fusion protein was formed at DNA level and transformed into yeast cells, then the hybrid fusion was expressed at protein level.

For the formation of QBP1-lcc1 fusion molecule at the DNA level, firstly, 2 primers were designed and commercially synthesized (Alpha DNA/Quebec/Canada). Forward primer was a long, 75 base containing primer which contained *EcoRI* enzyme restriction site and QBP1 sequence. The primer binds on 5' end of *lcc1* gene. Reverse primer binds on the 3' end of *lcc1* gene and contains *NotI* enzyme restriction site. The template DNA was pPICZ-B plasmid vector containing *lcc1* gene (Figure 3.19).

PCR amplified QBP1-lcc1 DNA sequence has been ligated into pDrive cloning vector and transformed into JM109 High Efficiency Competent Cells. QBP1-lcc1 sequence was verified by DNA sequencing and restriction enzyme digested for ligation process.

QBP1-lcc1 oligo was placed into pPICZ-B vector and translated into *Pichia pastoris* yeast cell for expressing the fused QBP1-lcc1 fusion protein. The selected colonies were incubated on MM (Minimal Media) plates containing Zeocin as shown in Figure 3.20.

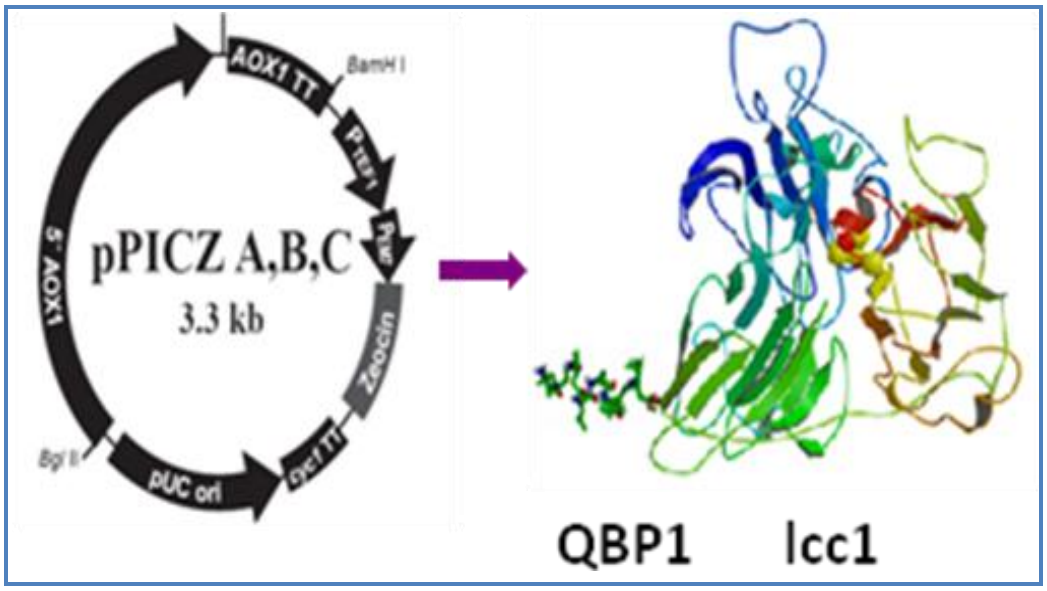


Figure 3.19: pPICZ-B vector acts as a host for fusion of QBP1 and lcc1 sequences.

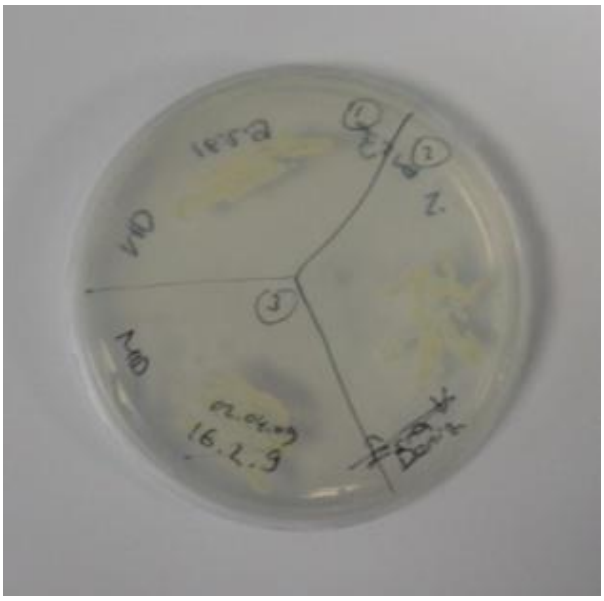


Figure 3.20: Three colonies were selected on Zeocin (100 mg/ul) containing Minimal media plates (MM).

The selected colonies will have lcc1 protein with N-terminal QBP1 sequence fused, but yet the enzyme activities of each selected colonies may differ from each other. All selected colonies need to be checked for enzyme activity levels.

Enzyme activities can be checked first on MM plates with the addition of ABTS chemical substrate. The green color in Figure 3.21 shows that; the three selected colonies are expressing laccase protein and releasing outside the cells where the enzyme reacts with ABTS and forms a green color which can be easily seen visually.

3.2.2 Production of "QBP1 Tagged lcc1" protein and its secretion out of the host cell

The first question when the QBP1-lcc1 construct is formed and transformed into the host cell, is whether the tagged enzyme would be produced and secreted outside of the cell or not. What we see here is a simple visual presentation of the produced tagged enzyme activity outside of the cell. When control cells (*Pichia pastoris* without lcc1), lcc1 containing cells (*Pichia pastoris*+lcc1) and QBP1-lcc1 cells (*Pichia pastoris*+QBP1lcc1) were grown on YPD media containing ABTS substrate of lcc1 enzyme, the blue colour on the plate shows the secreted active enzyme outside of the cell.

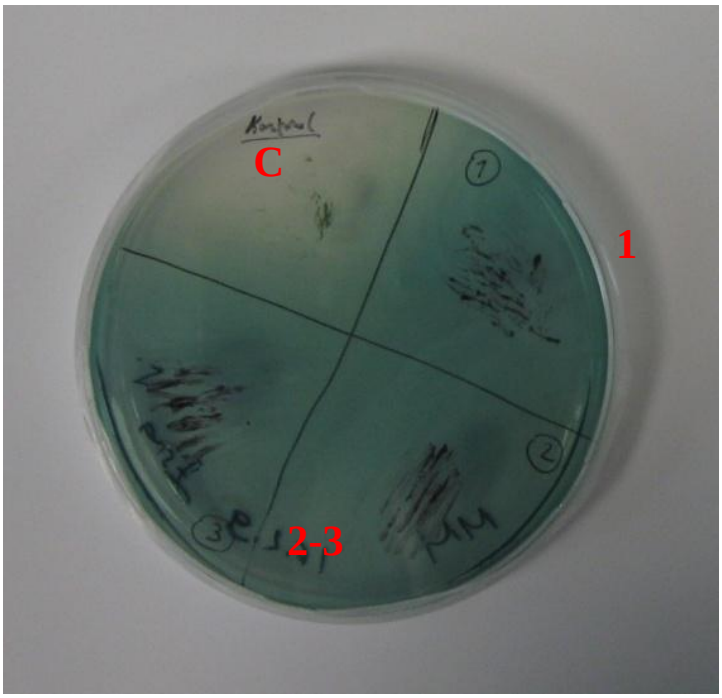


Figure 3.21: Secretion of QBP1 tagged lcc1 protein outside of the cell. C: *Pichia pastoris*, 1: *Pichia pastoris* (+lcc1), 2–3: *Pichia pastoris* (QBP1-lcc1)

QBP1 tag molecule does not affect the production of the enzyme and its secretion out of the cell even when it has been located at the N-terminal of the enzyme.

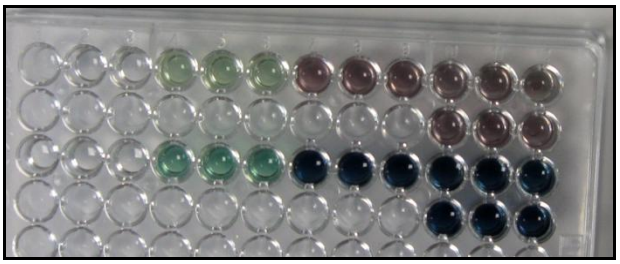


Figure 3.22: As the extracellular samples are reacted with 2,5 mM ABTS, the colour turns into green according to the level of active enzyme. (Samples are in triplets, first two lanes are waited measurings, 3rd and 4th lanes are fresh measurings).

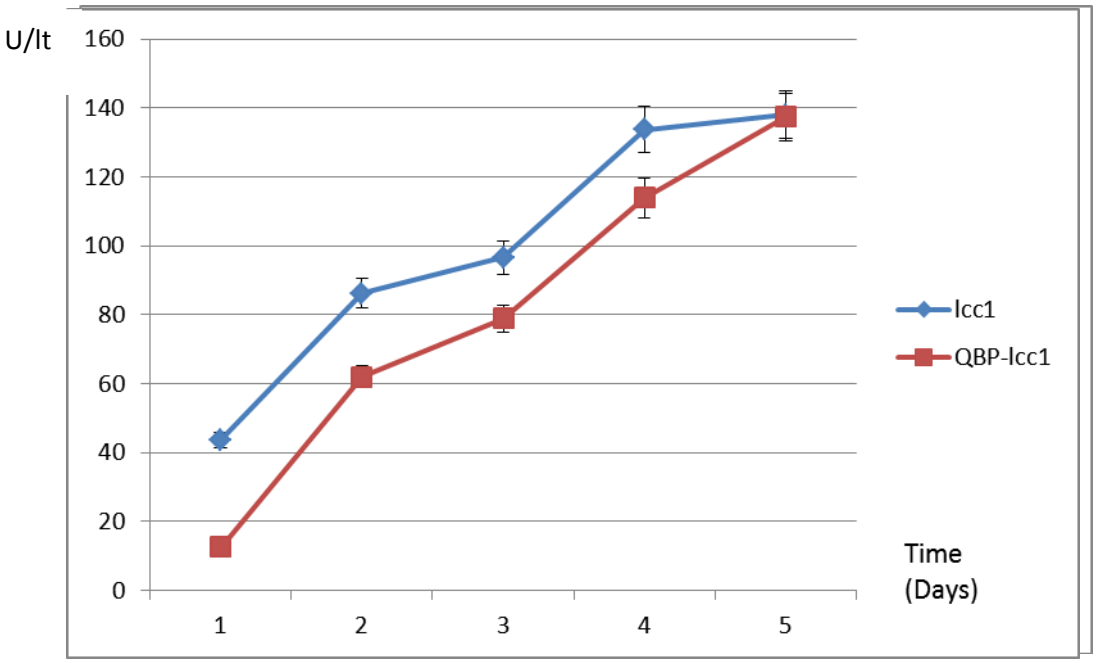
3.2.3 Effect of the "Tag" molecule on the enzyme activity

After 5 days incubation of control (*Pichia pastoris* (+lcc1)) and construct (*Pichia pastoris* (QBP1-lcc1)) cells, the enzyme activity levels were compared.

Methanol was used to induce the protein expression and samples were collected every 24 hours for enzyme activity assay. Samples were centrifuged to get the extracellular supernatant where the expressed proteins localize.

Figure 3.23 shows the enzyme activity levels for tagged enzyme (QBP1-lcc1) and control sample (lcc1) for five days.

The results means that tag does not affect the activity of the enzyme. First, the activity of the tagged enzyme was a little different from the control sample but after 5 days of incubation tagged enzyme shows the same level of expression with the laccase from the control sample. This shows that QBP1 tag on the N-terminal does not have a significant effect on the activity of lcc1 enzyme.



	Day: 1	2	3	4	5
Icc1 (U/lt)	43,7	86,18	96,59	133,64	137,9
QBP-Icc1 (U/lt)	12,49	62,04	78,82	113,85	137,46

Figure 3.23 : Laccase activity levels from selected *Pichia pastoris* colonies and a control sample were compared for five days by reacting laccase containing extracellular medium with ABTS and measuring the absorbance level spectrophotometrically.

3.3 Vector Set for QBP1 tagged functional protein systems

Before starting the details of SilTAG application, all the vector systems have been summarized here. For eukaryotic and bacterial cell lines, totally 4 cell types were used as host cells to express the vectors containing several versions of QBP1-Functional protein fusion constructs. DNA sequences for QBP1, AP, GFPuv and lcc1 alone, or fused, with or without spacer, or Alanine residue, have been prepared in different vectors and in different host cells, both bacterial and eukaryotic. In case of need, any one of them can easily be amplified, or removed and a new sequence can be added. The list of host vectors, cells with construct details is given in Figure 3.24.

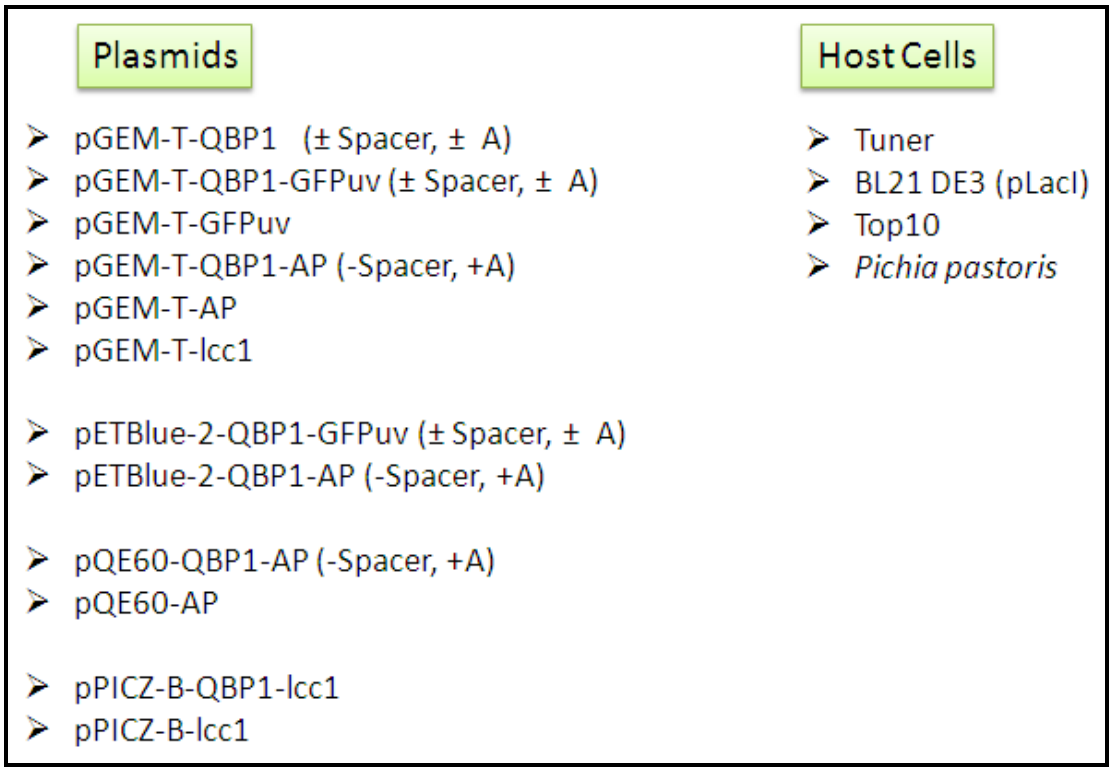


Figure 3.24 : List of constructed QBP1-Functional protein versions in different host cell for eukaryotic and bacterial sources.

3.4 SIL-TAG application

3.4.1 Selection of resin material

Although quartz binding peptides were initially selected by using quartz crystals (SiO_2), for the purification purpose, different kinds of resin material were applied. Crushed glass particles, silica and quartz beads were used to bind the fusion proteins on the surface. Silica resin was 60 to 200 µm in diameter and quartz resin was 200-800 µm in diameter. All resin materials were cleaned with isopropanol, ethanol and distilled water in sonic bath before introducing the proteins.

After expressed in *Pichia pastoris* cells, QBP1-lcc1 fusion protein is secreted outside of the cell. The extracellular solution, including QBP1-lcc1 fusion molecule, was applied on to cleaned resin materials, crushed glass, silica and quartz beads. The materials were washed several times with PC buffer (pH: 7) solution and beads were taken into fresh PC solution for remaining total protein and enzyme activity analysis. BCA analysis was applied for measuring the remaining total protein amount on the surface and Na-citrate analysis was used for measuring the enzyme activity levels.

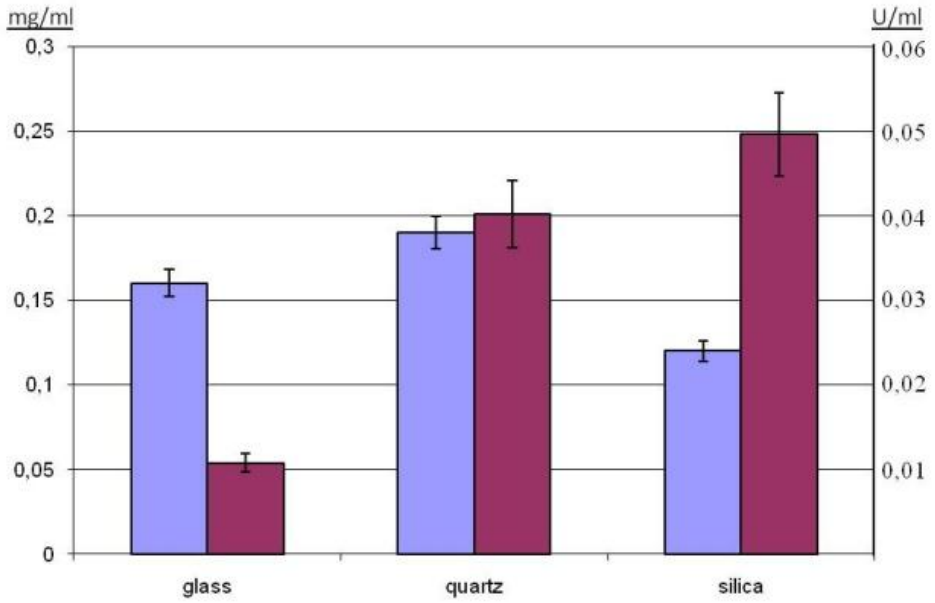


Figure 3.25: Relative total protein and enzyme activity levels after several wash steps of glass, quartz and silica beads which have been incubated with QBP1-lcc1 extracellular solution.

The basic binding experiments show that glass and quartz hold more protein on the surface but active anzyme level is more for silica and quartz. For the batch and column purification experiments, silica and quartz resins were selected.

3.4.2 Batch and/or column purification

Both methodologies can be used for the purification process. The samples can be introduced with resin material in an eppendorph tube or the sample can be sent through resin packed columns. Here three different forms of purification were tried. Simple eppendorph tubes were for the batch application. Samples were run through packed columns simply by gravitational force or mircopump system.

3.4.3 Denatured / Native form of the purified protein

One of the main advantegous sides of a tag system is its availability to use both in purification of desired protein in native or denatured forms. For different biotechnological applications, protein can be preferred in denatured or native forms. So, a better purification system must be able to purify the proteins in both forms. If after the purification process the enzyme is needed in active form, the samples should run through the column in native conditions without any denaturing detergent

or heat application. There might not be need for preserving the native form of the protein, so the enzyme can be first denatured using heat and detergents and then run through the column.

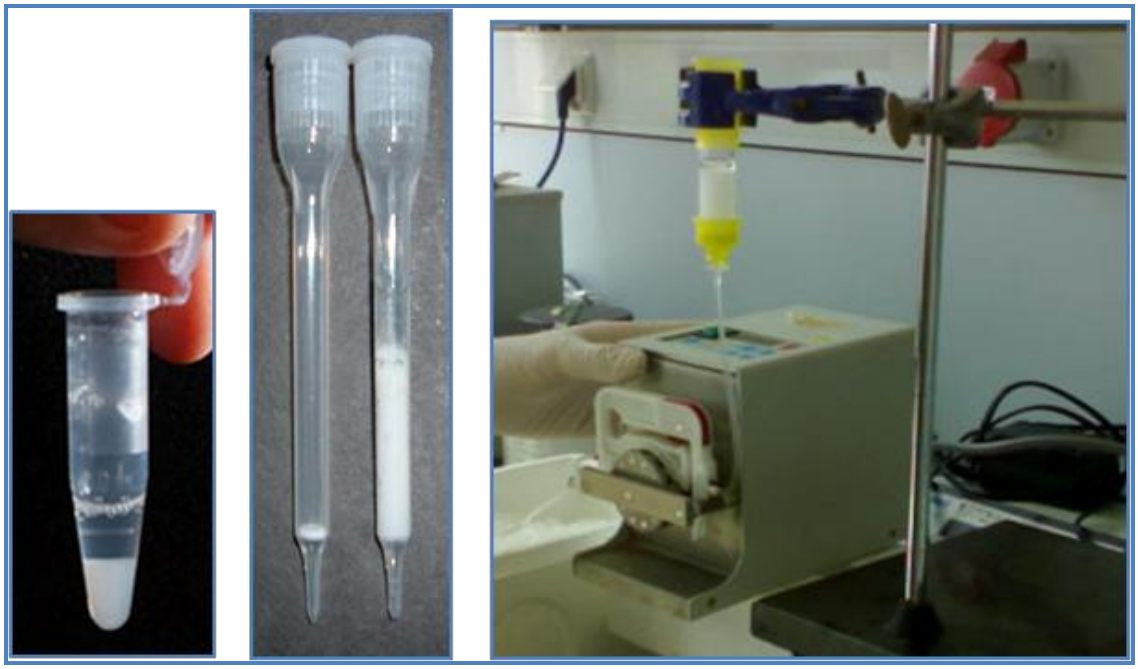


Figure 3.26: Mini centrifuge tubes can be used for batch purification (on the left). Resin packed columns can be run via gravitational force (in the middle), or peristaltic pump (on the right).

3.4.4. Optimization for binding buffer

Silica powder was used as a resin material for purification assay. The bead size of powder was from 60 to 200 um. The powder was cleaned with isopropanol, ethanol and distilled water in sonic bath and packed in a column. Extracellular sample containing the expressed conjugated protein was taken into PC buffer (4 different pH values). After running the extracellular sample through the column, flowthrough samples were collected and enzyme activity levels were measured in sodium citrate buffer (laccase activity assay). Below is the tables giving the activity levels and protein amounts for each collected flowthrough samples for different pH values.

Table 3.1 shows the enzyme activity levels for extracellular sample and the flowthrough. There is not much loss in enzyme activity levels when pH 6,5-7,5 buffers were used as binding buffers. As shown in Table 3.2, total protein loss is much less with pH 7,5 buffer. Totally, the binding buffer was selected as pH 7.5

buffer in which there is not much protein loss to flowthrough and enzyme avtivity level is high.

Table 3.1: Enzyme Activity Assay (BCA Assay)-Binding levels of QBP1-lcc1 conjugate protein on silica resin for 4 different pH values.

	Input (U)	Flowthrough (U)	Binding Percentage %
pH:5.5	0,04329	0,01369	68
pH:6.5	0,02525	0,0006	98
pH:7.5	0,01975	0,0004	97
pH:8.5	0,01537	0,0036	76

Table 3.2: Total Protein Amount -Binding levels of QBP1-lcc1 conjugate protein on silica resin for 4 different pH values.

	Control (mg)	Flowthrough (mg)	Binding Percentage %
pH:5,5	0,76	0,09	87,38
pH:6,5	0,76	0,15	79,81
pH:7,5	0,68	0,07	90,04
pH:8,5	0,62	0,13	78,74

Table 3.3 is showing specific activity levels meaning low level of QBP1-lcc1 fusion molecule in flowthrough sample for pH 6,5-7,5 buffers.

The graph given below shows the binding percentage levels for each different pH values. Acoording to the results, PC buffer (pH: 6,5 and pH:7.5) are suitable as binding buffer. The extracellular sample which includes the conjugate protein must first transferred into the optimum binding buffer.

Table 3.3: Specific Activity Levels-Binding levels of QBP1-lcc1 conjugate protein on silica resin for 4 different pH values.

	Control (U/mg)	Flowthrough (U/mg)
pH:5,5	0,057	0,15279
pH:6,5	0,03322	0,004
pH:7,5	0,02892	0,0058
pH:8,5	0,02479	0,02753

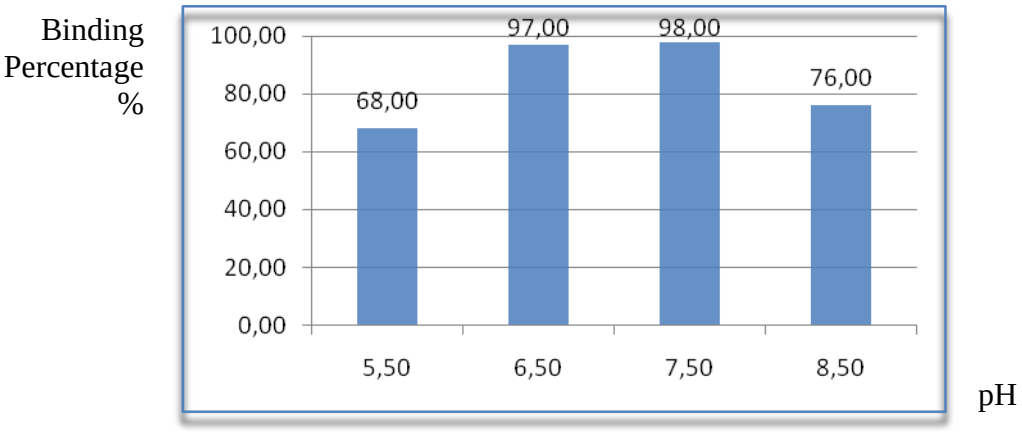


Figure 3.27: Binding levels of QBP1-lcc1 conjugate for different pH values.

3.4.5. Optimization for washing buffer

To find an optimum wash buffer, extracellular sample was first transferred into optimum binding buffer (PC buffer, pH: 7.5) which was given in the previous section.

After binding is complete, the column is washed with PC buffer (pH: 6.5 or pH: 7.5). As given in the next table, PC buffer (pH: 6.5) acts well as a wash buffer.

PC buffer (pH.7.5) was used as binding buffer and PC buffer (pH.7,5 + 0.5%Tween20) was used as washing buffer. The extracellular protein sample was in PC buffer (pH.7,5) to have the protein in native form, or in PC buffer (pH.7,5 + 8M

Urea) to have the protein in denatured form, to see the effect of protein folding on binding level.

Table 3.4: Total Protein Amounts after Wash Steps - QBP1-lcc1 conjugate protein on silica resin for 2 different pH values.

	Control (mg/ml)	FT+Wash (mg/ml)	Binding Percentage %
pH:6.5	0,73	0,32	55,75
pH:7.5	0,51	0,11	79,21

Table 3.5: Protein Activity Level- wash buffers on binding levels.

	Control Sample (U/lt)	Flowthrough (U/lt)	Binding Percentage %
pH:6.5	21,77	2,54	88
pH:7.5	16,68	3,89	76

Table 3.6: Specific Activity-Effects of wash buffers on binding levels.

	Control Sample (U/g)	Flowthrough (U/g)
pH:6.5	29,82	7,94
pH:7.5	32,7	35,36

Binding and washing conditions were optimized showing pH 7.5 as the best binding pH and 6.5 as better washing pH condition.

The graph given below shows the binding percentage levels for each different pH values. Acoording to the results, PC buffer (pH: 6,5 and pH:7.5) are suitable as binding buffer. The extracellular sample which includes the conjugate protein must first transferred into the optimum binding buffer.

To find an optimum wash buffer, extracellular sample was first transferred into

optimum binding buffer (PC buffer, pH: 7.5) which was given in the previous section. After binding is complete, the column is washed with PC buffer (pH: 6.5 or pH: 7.5).

As given in the next table, PC buffer (pH: 6.5) looks better as a wash buffer.

Effect of pH on enzyme activity: Another result from here is the effect of pH on the enzyme activity. As the pH increases from 5.5 to 8.5 the activity decreases as shown in the next graph. When optimizing for the purification steps, buffers with different pH values are tried. For measuring the activity levels at different pH values, control samples at that pH values must be prepared for each step.

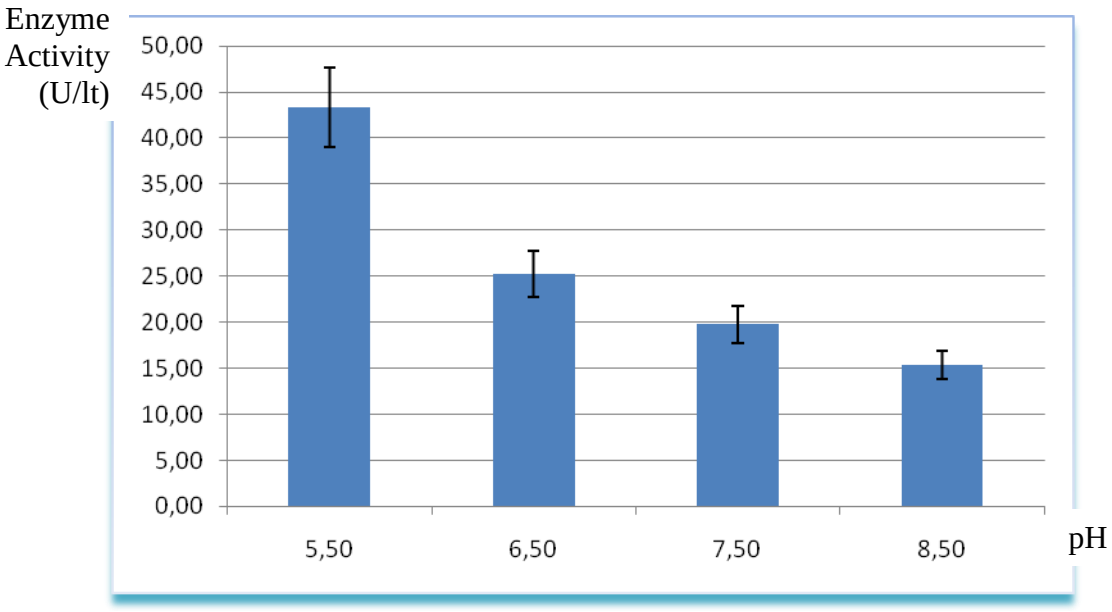


Figure 3.28: Effect of pH on the QBP1-lcc1 enzyme activity.

3.4.6. Optimization for elution buffer

Different buffers were tried as elution buffers with different detergent ratios and pH values. For all samples, PC buffer (pH 7,5) was used as binding buffer and PC buffer (pH 6,5) was used as washing buffer. Each elution steps were proceeded in 4 steps collecting samples in different tubes for increasing enzyme purity and yield levels. The table given below shows Protein Activity, Total Protein Amount, Specific Activity, percent yield and fold levels for each input and eluted samples. Each elution buffer is formed basically from PC buffer with different pH levels, detergent or chemicals. After using PC buffer (pH.3) for elution step, ~7 fold purification has

been obtained but yield was low as 12%. The case looks like similar with using PC buffer (pH.4.5), as the fold increases the yield decreases dramatically. The reason for using low pH levels for the elution part mainly aims the breaking of the ionic interactions.

In the case of using PC buffer (pH.4.5+ 0,5%Tween 20), fold was lower than the previous cases. Overall, increasing protein activity levels might results in better fold and yield levels.

Although strong acid solutions and detergent containing solutions were somewhat able to release QBP1 tag from the particles, these conditions could be destructive for proteins.

High concentrations of MgCl2 will also release Sil-tag, even at neutral pH. Divalent cations with high ionic potential play an important role in dissociating Sil-tag from silica. They are electrostatically attracted to negatively charged silica surfaces.Sil-tagged proteins on silica surfaces are dissociated by an ion exchange effect, wherein Mg 2+ act as competing ions for silanol groups. Moreover, after the dissociation of Sil-tag, the divalent cations are expected to bind to the surface silanol group, with each alkaline earth metal ion linked to approximately one silanol group as follows:

-SiOH+M2+ ---SiOM+ + H+

This positively charged surface site should neutralize the total surface charge, repel the Sil-tag polypeptide with a net positive charge, and therefore prevent re-adsorption of the released Siltag, resulting in the efficient elution of Sil-tag. So, better results were obtained with PC buffer containing MgCl2 (2 M, pH.8). ~60-70% yield has been obtained with 6-7 fold purification.

As in HisTag purification system, imidazole has been tried as an elution agent to compete with QBP1 tag. Using imidazole as an elution agent aims the mimic the cyclic structure of proline amino acid, which is the main part of QBP1 peptide. The binding mechanism of QBP1 might be due to higher percentage of proline residues so a similar structure such as imidazol would break the interaction between silica and QBP1 peptide.

Table 3.7: Eluted protein properties for different elution buffers.

	Total Protein (mg)	Activity (U)	Specific Activity (U/mg)	Yield %	FOLD
pH:3					
Input	0,538	0,1194	0,221	100	1
E	0,181/0,060	0,0250	0,138/0,416	21	1,88
E1	0,026/0,009	0,014	0,538/1,55	12	7,01
pH:4,5					
Input	0,301	0,004	0,0130	100	1
E	0,087	0,003	0,0340	75	2,6
E1	0,028	0,002	0,0710	50	5,5
E2	0,013	0,001	0,0760	25	5,8
pH:4,5+Tw20					
Input	0,350	0,004	0,0110	100	1
Elution	0,089	0,002	0,0250	50	2,3
Elution Step 4	0,023	0,001	0,0430	25	3,9
pH:7.5+MgCl2					
Input	0,532	0,121	0,2270	100	1
E1	0,063	0,091	1,4200	75	6,3
E2	0,045	0,076	1,6800	62	7,4
pH:7.5+imidazole					
Input	0,510	0,036	0,0700		1
Elution	0,080	0,0110	0,125	31	1,8
E2	0,020	0,008	0,4000	22	5,7

Figure 3.29 shows together the different conditions for QBP1-lcc1 protein purification process. Silica (60 to 200 um in diameter) and quartz (200-800 um in diameter), were used as resin material. Both materials are cheap and commercially available requiring no initial process before the purification steps. They were cleaned with isopropanol, ethanol and distilled water in sonic bath and packed in separate columns or used as batch system. PC buffer (pH.7,5) was used as binding buffer and PC buffer (pH.6,5 + 0.5%Tween20) was used as washing buffer. The extracellular protein sample was in PC buffer (pH.7,5) to have the protein in native form, or in PC buffer (pH.7,5 + 8M Urea) to have the protein in denatured form, to see the effect of protein folding on binding level. The elution buffer was PC buffer (pH4.5, 0,5% Tween20).

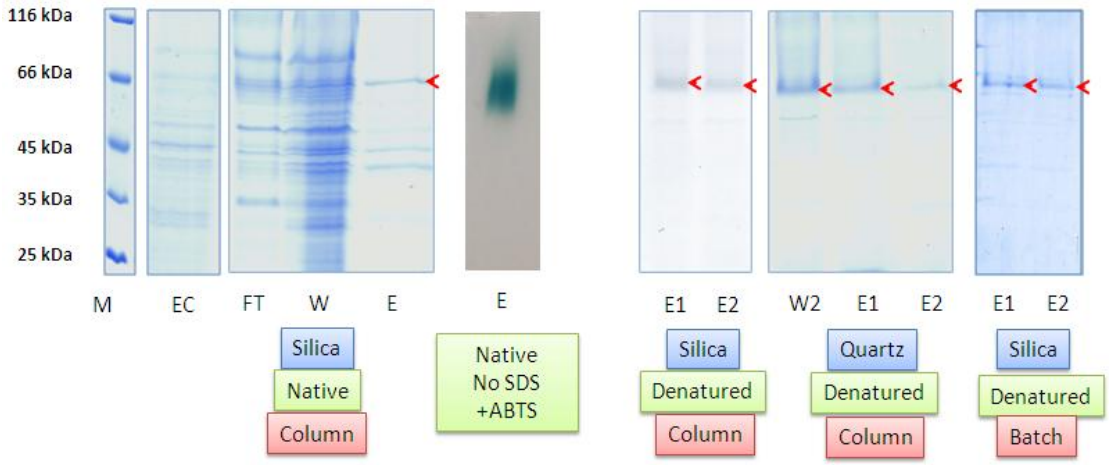


Figure 3.29: SDS-PAGE and native-PAGE results showing the purification steps of native or denatured QBP1-lcc1 protein through silica or quartz resins with column/ batch procedure. (M:Marker, EC: Extracellular protein sample, FT: Flowthrough, W: Wash, E: Elution).The red arrows show the QBP1-lcc1 bands. Native gel was applied into ABTS containing buffer to observe the enzyme activity.

Purification conditions given in Figure 3.29 shows that ”QBP1” peptide is very promising as a tag molecule for purification purpose. Each experiment needs some more optimization steps but as overall, tag works in native and denatured states of the protein and also through silica and quartz resins. Extra bands in Elution sample (E) can easily be removed with one extra ultrafiltration step or tuning the pH of elution or washing buffers. It is same for denatured cases. Increasing the washing steps can increase the purity of eluted protein.

3.4.7. QBP1 as a TAG peptide

Paramaters to select a Tag system: During the last 25 years many alternative affinity tag systems have been described, all having advantages and disadvantages depending on the application and the requirement for specificity, solubility, and the binding and elution conditions. No single affinity tag is optimal. Depending on the structure and the procedure, each has weak and strong sides (Nilsson *et al.* 1997, Hearn *et al.* 2001, Terpe *et al.* 2003, Arnau *et al.* 2006, Lichty *et al.* 2005, Waugh *et al.* 2005).

To select a suitable tag system for the purification of a desired biomolecule, there are some parameters which must be considered beforehand.

The first parameter is the the translation efficiency. The tag molecule can be placed at N or C terminal of the protein or inside the protein where a permissive site occurs. A suitable tag molecule must not decrease the efficiency of translation. In general, bigger tag molecules (protein tags) such as MBP or GST tags are known as having less effect on translation efficiency. These tags are already naturally translated proteins and they do not interfere with the translation process of the fusion protein. In contrast, depending on the amino acid sequence, short peptides can decrease the efficiency of the translation process. In this case, tag peptide must be located at C terminal of the protein or somewhere permissive on the protein, if the interaction between tag peptide and the resin material is not been blocked (Lichty *et al.* 2005).

The second parameter is the metabolic burdendue to using bigger protein tags. Using short peptides does not cause any extra metabolic burden for the host cell but protein tags can be as large as the functional protein which is intented to be purified. So, the cell needs to translate a whole tag protein too which causes a metabolic burden for the host cell.

The third parameter is the specificity of the tag molecule to the resin material. Peptide tags such as the FLAG-tag (Nilsson *et al.* 1997), the calmodulin-binding peptide (Hearn *et al.* 2001), the Strep-tag or Streptag II (Terpe *et al.* 2003, Arnau *et al.* 2006) and the biotin acceptor peptide (Hochuli *et al.* 1987) have a high degree of specificity for their binding resin partners. On the other hand, the resins for these short peptide tags are generally expensive and have low binding capacities.

The fourth parameter is whether tag molecule enhances solubility of the fusion molecule or not. For most of the applications, tagged protein is better to be in soluble form instead of forming insoluble inclusion bodies which needs extra steps to be solubilized. In general, protein tags are known better enhancers for a soluble tagged protein. For short peptide tags, the peptide sequence of the tag and the location of the tag on the protein must be considered beforehand which will be effective on the solubility of the fusion protein.

Resin price, mild elution conditions and easiness of the protocol are some other parameters to be considered. These are the parameters which are necessary to be considered before the experimental procedure. A peptide or protein tag may have high specificity and less metabolic burden for the cell or other advantageous sides but relatively expensive resins may make the system useless for most applications.

Main objective of the purification is obtaining the tagged protein unharmed and still active at the end of the process. That is why, using mild elution conditions as HisTag system uses (100–250 mM imidazole, pH: 5.0, or 10 mM EDTA) would be critical step. And finally, easiness of the purification protocol especially one step purification must be aimed.

Additionally, because affinity tags have the potential to interfere with structural or functional studies, removing them may be necessary at the end of the purification process. If the tag molecule does not interfere with the activity or structure of the purified protein, depending on the application, it may stay fused to the purified protein but for most of the tag systems, especially for protein tags, extra steps must be added to the purification protocol to remove the the tag.

QBP1 as a "tag" peptide: Here, we are presenting a methodology of designing your own TAG peptide for any desired resin material. QBP1 is a silica/quartz binding peptide which is 12 amino acid long (PPPWLPYMPPWS), and it has been developed

by combining the combinatorial and bioinformatic techniques. So, the first advantage is the flexibility of designing a new peptide system. QBP1 works good as a tag peptide, but if necessary, the flexibility of methodology enables you using other quertz binders (QBP2, QBP3 etc.) or selecting new tag peptides for any other resin material.

The purification protocol for QBP1-tag system is relatively easy and simple. There is no need for extra purification steps, single step elution is possible. The resin material can be quartz, silica or even crushed glass which do not need for any prior activation steps. No peptide, metal ion or any ligand must bind on the resin. The ligand for the tag is already the resin material itself. So, after cleaning the resin, it is ready to be used for the binding.

Purification protocols are also flexible. The tagged protein can be purified in native or denatured form depending on the later application in which the purified protein is needed. Also, batch or column protocols are available for the process. Different elution buffers were tried and in general mild elution conditions were available for the purification protocol such as neutral pH, low salt conditions or addition of MgCl2 (2 M).

Using SilTAG is much less expensive than conventional affinity purification methods, which require a ligand to be immobilized on a resin, because the silica itself serves as both a resin and ligand for SilTAG. On the basis of costs reported by Lichty et al. (2005), our method is one of the least expensive of several commonly used methods for isolating tagged proteins; the cost was approximately $3 per 10 mg of total protein using silica particles (calculated from the retail price of $1.2 per gram dry weight of the particles used), whereas the conventional methods cost $12 to $5000 to purify the same amount of tagged polypeptide.

QBP1 has no effect on the enzyme activity which makes extra purification steps unnecessary. Simply, the tag can be left on the enzyme. If necessary, it can be cleaved off via Factor Xa cutting site. This is one of the generally used the strategies of removing tag molecule from the purified protein.

Because it is short peptid, it causes no metabolic burden for the host cell but depending on the cell type, protocol must be formed carefully. Results show that in eukaryotic host cell, there is no problem about the translation efficiency but in

bacterial host cells, the tag peptide may be interfering with the translation process. This is most possibly due to the amino acid sequence of the peptide. the provisions to overcome the problem might be locating the tag molecule at C terminal of the protein, using eukaryotic host cell or changing to another quartz binding peptide.

8 fold purification with the level of 60% yield is obtained using MgCl2 as the elution material.

Overall, what we are showing here is that Molecular Biomimetics and bioinformatic tools, collectively, offer a good way of designing our own tag peptide for purification purposes with increased properties and QBP1 can be used as a tag molecule successfully.

3. CONCLUSION AND RECOMMENDATIONS

The experiments previously carried out showed us that QBP-1 peptide is a strong silica-binder which makes it suitable for medical and nanobiotechnological applications via making available the formation of fusion proteins. One of the applications is using the QBP-1 peptide as a tag peptide for the isolation of any desired protein.

The selection procedure of Quartz Binding Peptides was simply as followed; firstly, Phage Display Methodology was used for the selection of quartz binding peptides. Library of M13 phages (~10^9 colonies/ml) each containing random 12 amino acid peptide sequences at N-terminal of one of the coat proteins, P3. After 5 biopanning cycles, 50 first generation quartz binding peptides were isolated which then, grouped into 3 groups as strong, moderate and weak binders according to their binding strengths by using semi-quantitative fluorescence microscopy experiments. Next step was taking the strong binders' properties and designing theoretical binders by using bioinformatics tools. These were the second generation quartz binding peptides which then top six of the strong binders were experimentally produced and their binding strengths were verified. QBP1 peptide sequence was the top one among the second generation theoretically designed binders.

Using QBP1as a tag peptide for biomolecule purification purpose is composed of two main parts. The first one is the formation of QBP1 tagged functional proteins at the DNA level and then expression of these molecules in prokaryotic or eukaryotic cell lines.

QBP1 peptide was designed and placed at the N terminal of functional proteins which were recombinant green fluorescent protein (GFPuv) and alkaline phosphatase (AP) protein. DNA construct included necessary restriction enzyme cutting sites, spacer sequence, Factor Xa cutting site for later protease cutting purpose. Different versions of cloning and expression vectors were formed for each functional proteins. The constructs were formed suitable for later replacement of any desired protein or tag molecule. All the sequences were verified by sequencing analysis to prevent any

mutation occurence which would affect the expression steps severely. QBP1-GFPuv construct was transformed into different prokaryotic cells for expression analysis. The construct was loosely expressed which was generally unrepeateble. Different media conditions, vector choices, time and IPTG ranges had not affect the level of expression. QBP1-AP construct case had the similar problems. But at the end, the construct was formed incompletely, about 42 kDa instead of 51 kDa fusion protein. For both QBP1-GFPuv and QBP1-AP cases, the problems affecting the expression levels might be most possibly about the amino acid composition of QBP1 sequence. QBP1 is located at the N-terminal of the functional proteins. During the translation process, it is produced first, then the functional produced as a fusion molecule. There are many prolin residues in QBP1 sequence, especially the first three residues, which might be the reason of a loop formation and incomplete ending of protein translation process in QBP1-AP or complete loss of QBP1-GFPuv expression. Expressed QBP1-AP construct protein was purified using silica columns and a clear 42 kDa band has been obtained. That shows us QBP1 is produced properly and binds on silica surface through the column but the remaining AP protein is not properly expressed. These results show the importance of selection process for QBP sequence for prokaryotic protein purification process. QBP1 sequence can be located at the C-terminal end of the fusion protein or another quartz binding peptide which have been selected computationally and enhanced via bioinformatic techniques can be easily replaced with QBP1 sequence.

For eukaryotic expression of QBP1 tagged functional protein, we constructed a QBP1-lcc1 fusion molecule, first on the DNA level, and expressed it to show the enzyme activity. DNA sequences were verified before protein expression steps. The problems which occured with prokaryotic expression systems did not occur and QBP1-lcc1 functional protein was produced properly. The first result here is that, QBP1 does not affect the expression of the construct protein as it happens in prokaryotic cells. Secondly, the construct was successfully sent to the extracellular space whic means that QBP1 tag molecule does not affect the secretion of the construct molecule extracellularly. Finally, the tagged protein has the same level of enzyme activity with the control bare lcc1 protein. That means QBP1 tag does not affect functioning of the protein and it can be left on lcc1 protein after purification process which eliminates the following purification steps.

At the end of the first part, a set of GEPI-Functional Protein Constructs have been formed for bacterial and eukaryotic cells. QBP1-GFPuv, QBP1-AP and QBP1-lcc1 constructs with/out linker sequences for 4 different cell types and in 12 different vector form for them were formed. QBP1-lcc1 fusion protein was expressed successfully. The fusion protein was secreted extracellularly and activity of the enzyme was ensured.

The second part of the study was using the QBP1 peptide as the "TAG" molecule for purification of lcc1from extracellular cell medium through silica/quartz packed columns. The results show that QBP1 is a very promising tag molecule which have the main advantageous sides. The purification protocol for QBP1-tag system is relatively easy and simple. Single step elution is possible making the purification protocol time saving and simple. The resin materials, quartz, silica and glass, do not need any prior steps for surface activation, ready to use after cleaning and relatively much more cheaper than other resins for commercially available tag systems.

QBP1 has no effect on the secretion of enzyme extracellularly or enzyme activity which makes extra purification steps unnecessary. Simply, the tag can be left on the enzyme according to the application in which the purified protein is needed.

Batch or column protocols are available for the purification of tagged protein both in native or denatured forms. By tuning the pH levels of binding, washing and elution buffers, or using extra chemicals to compete with QBP1 peptide on binding, a better purification level was obtained. Among the different elution buffer selections, $MgCl_2$ (2 M, pH: 7.5) containing buffer gave a better result with 8 fold purification with 60% yield level.

Overall, Molecular Biomimetics gives us a way to select our own tag peptide for any desired inorganic material which can be used in prokaryotic or eukaryotic cell types, in different purification protocols and protein folds. It is very important to get the protocol strategy as a principle for any new desired application. And finally, QBP1 can be used as a tag molecule successfully.

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APPENDICES

Appendix A. : Cloning and Expression Vectors

Appendix B. : Gene DNA Sequences

Appendix C. : Oligonucleotides and Primer Sequences

Appendix D. : Molecular Weight Markers

APPENDIX A.1 :

Sequence Landmarks of pETBlue-2 Vector

Element	**Position (bp)**
Lac operator	3606–3625
T7 promoter	1–17
Lac operator	22–42
T7 transcription start	18
Multiple cloning region (Nco I–Pac I)	276–467
His•Tag® coding sequence	437–454
HSV•Tag® coding sequence	395–430
LacZ start codon	491
LacZ α-peptide ORF	57–491
E. coli promoter	541–569
F1 origin	1096–1551
Bla coding sequence	1669–2526
pUC origin	3206

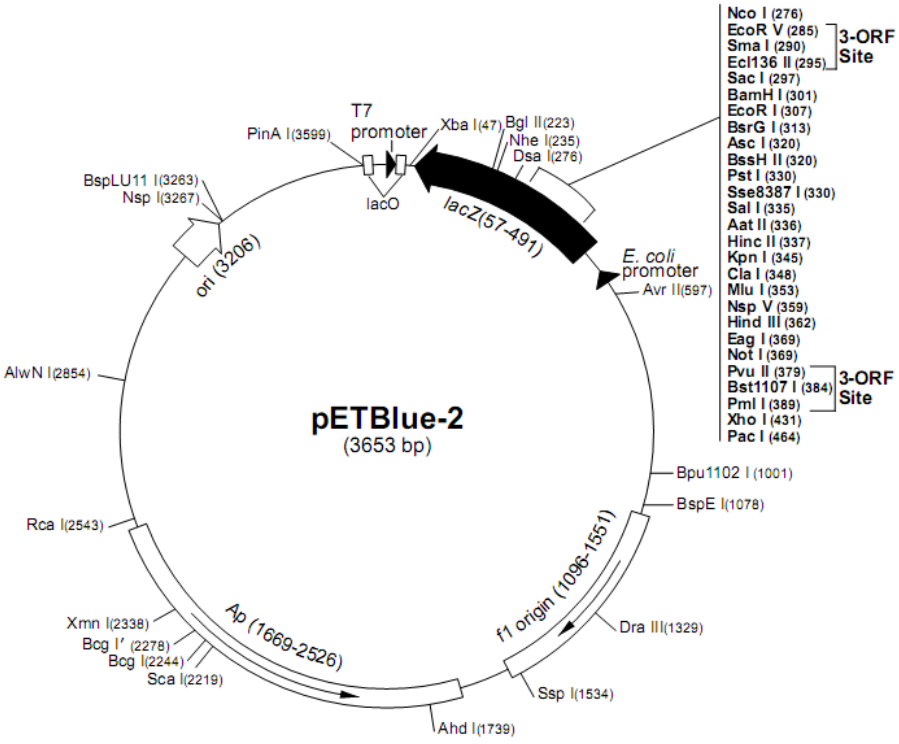


Figure A.1 : Multiple Cloning Sites (MCS) on pETBlue2 vector.

APPENDIX A.2 :

Sequence Landmarks of pCR®2.1-TOPO® Vector

Element	**Position (bp)**
LacZα fragment	1-547
M13 reverse priming site	205-221
Multiple cloning site	234-357
T7 promoter/priming site	364-383
M13 Forward (-20) priming site	391-406
F1 origin	548-985
Kanamycin resistance ORF	1319-2113
Ampicillin resistance ORF	2131-2991
pUC origin	3136-3809

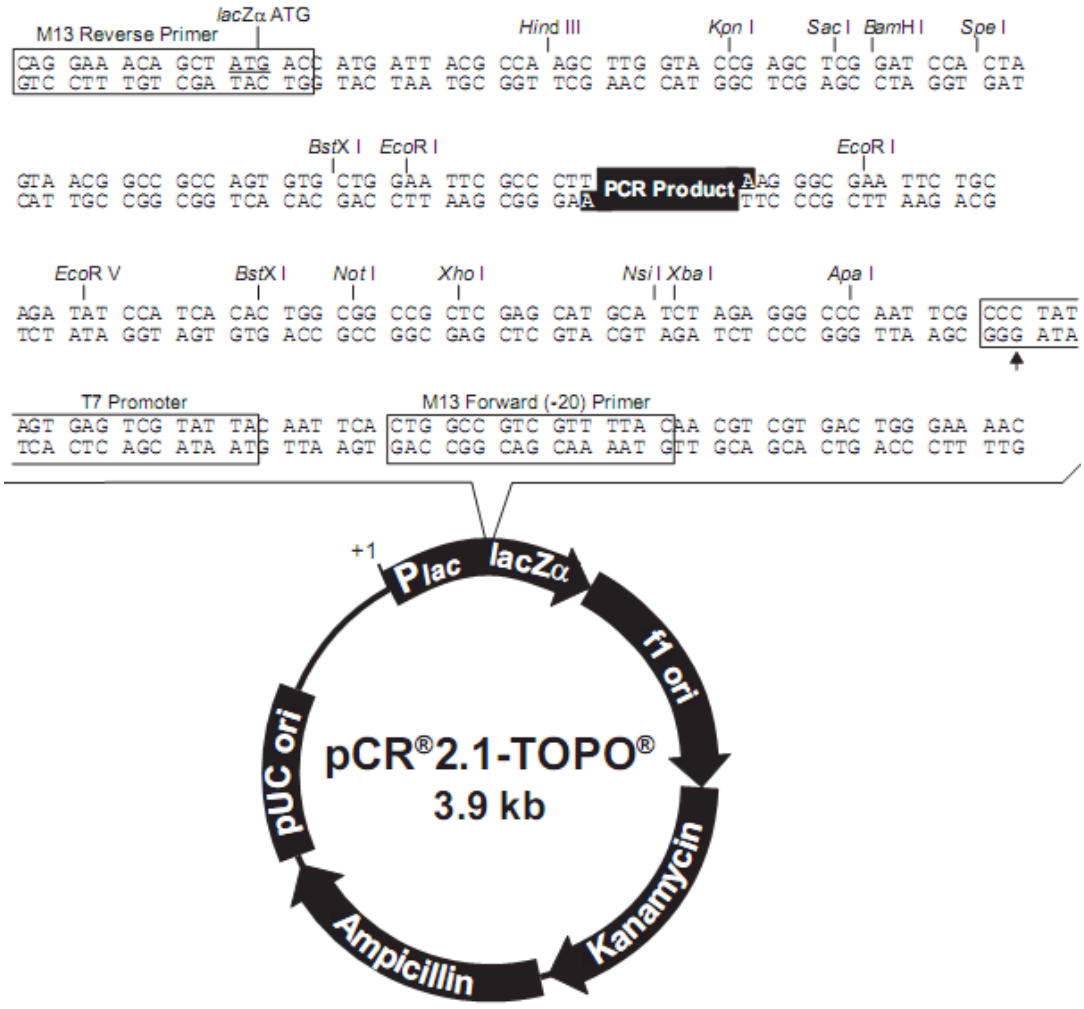


Figure A.2 : Multiple Cloning Sites (MCS) on pCR2.1-TOPO vector.

APPENDIX A.3 :

Sequence Landmarks of pPICZ-B Vector

5′ end of *AOX1* mRNA — 5′ *AOX1* priming site

```
811  AACCTTTTTT TTTATCATCA TTATTAGCTT ACTTTCATAA TTGCGACTGG TTCCAATTGA

871  CAAGCTTTTG ATTTTAACGA CTTTTAACGA CAACTTGAGA AGATCAAAAA ACAACTAATT
```

Sfu I — *Eco*R I — *Pml* I — *Sfi* I — *Bsm*B I *Asp*718 I *Kpn* I *Xho* I

```
931  ATTCGAAACG AGGAATTCAC GTGGCCCAGC CGGCCGTCTC GGATCGGTAC CTCGAGCCGC
```

Sac II *Not* I — *Xba* I — *myc* epitope

```
991  GGCGGCCGCC AGCTT TCTA GAA CAA AAA CTC ATC TCA GAA GAG GAT CTG
                        Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu
```

Polyhistidine tag

```
1040 AAT AGC GCC GTC GAC CAT CAT CAT CAT CAT CAT TGA GTTTGTAGCC TTAGACATGA
     Asn Ser Ala Val Asp His His His His His His ***

1096 CTGTTCCTCA GTTCAAGTTG GGCACTTACG AGAAGACCGG TCTTGCTAGA TTCTAATCAA
```

3′ *AOX1* priming site

```
1156 GAGGATGTCA GAATGCCATT TGCCTGAGAG ATGCAGGCTT CATTTTTGAT ACTTTTTTAT
```

3′ polyadenylation site

```
1216 TTGTAACCTA TATAGTATAG GATTTTTTTT GTCATTTTGT TTC
```

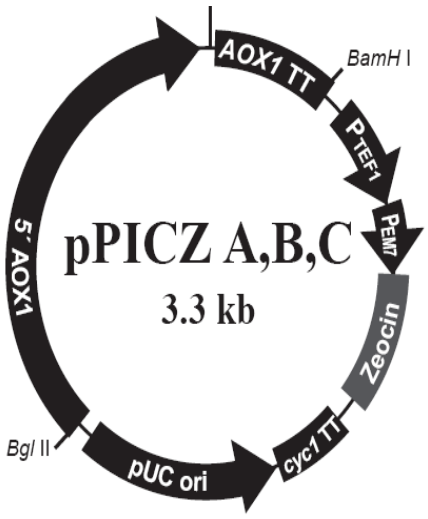


Figure A.3 : Multiple Cloning Sites (MCS) on pPICZ-B vector.

APPENDIX A.4 :

Sequence Landmarks of pGEM-T Easy Vector

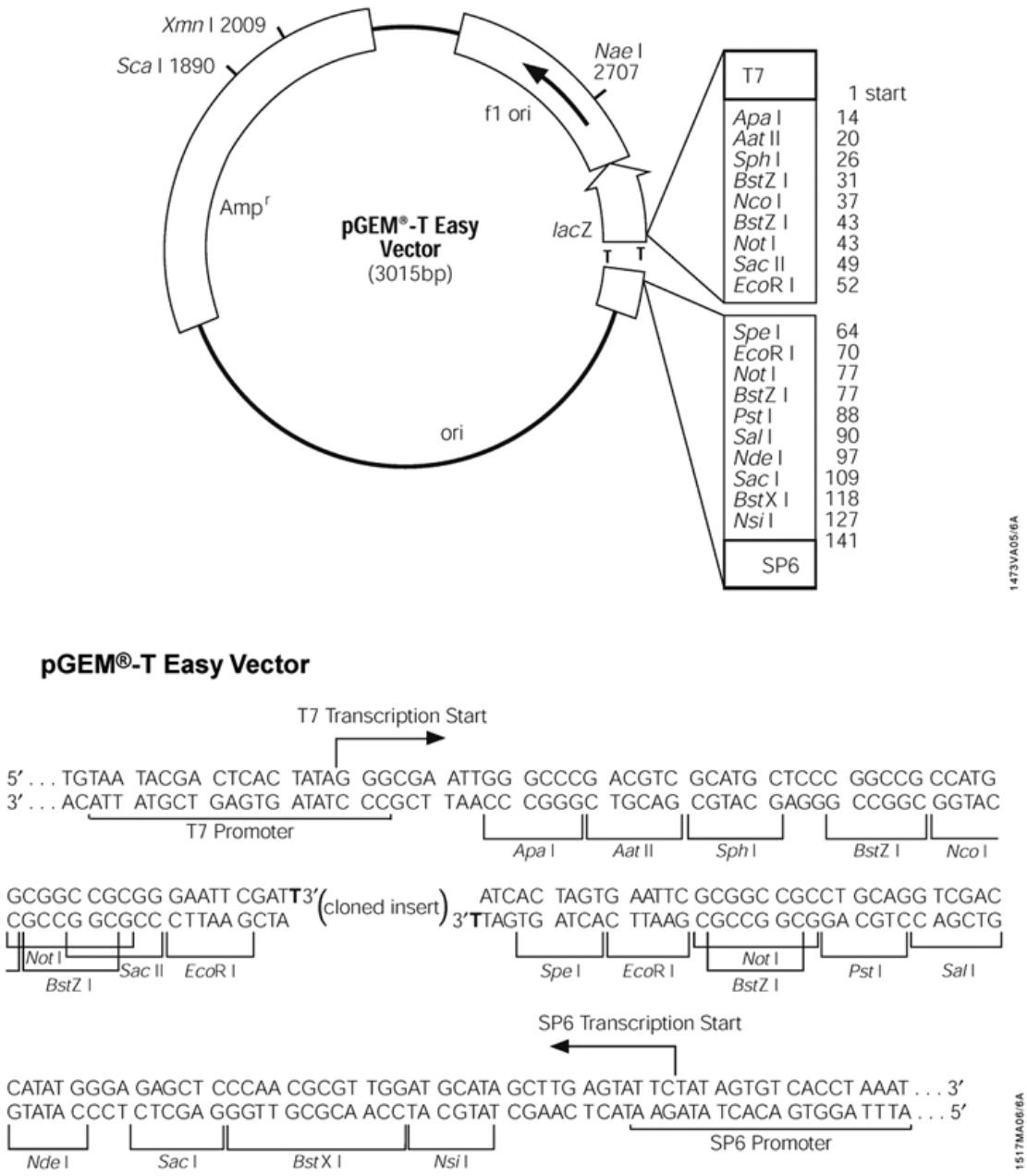
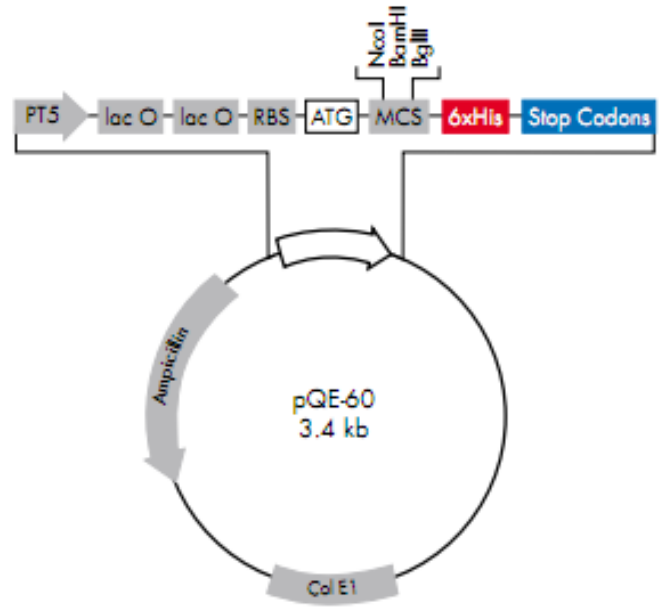


Figure A.4.: pGEM®-T Easy Vector Map and Sequence Reference Points.

APPENDIX A.5 :

Sequence Landmarks of pQE-60 Vector



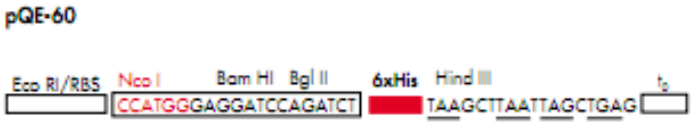


Figure A.5.: pQE-60 Vector Map and Sequence Reference Points.

APPENDIX B.1 :

Laccase (lcc1) mRNA sequence from *Pycnoporus sanguineus* (1557 bp)

5’-
ATGGGCTCCGGTCTTTTCAGCATCTTCGTCACCATCGCGGCCATCTCTGG
CAGCCTCGCTGCCATCGGGCCCAAGGCGGACCTCGTCATCTCGGACGCTG
TCGTCAATCCTGATGGCACGCCCCGAGCTGCCGTCGTCGTTAATGGCGCA
TTCCCTGGCCCCCTCATCTCTGGGAAGAAGGGTGATCACTTCCAGCTCAA
CGTGATCAACAAGTTGACCAACCACACTATGCTGAAGACGACCAGTATA
CACTGGCACGGACTTTTCCAGGAACACACTAACTGGGCTGACGGTCCCGC
TTTCGTCAATCAATGTCCCATTGCTTCTGGACACTCCTTCCTCTACGACTT
CCATGTGCCCGATCAAGCCGGCACATACTGGTACCACAGCCATCTTTCCA
CGCAGTACTGCGACGGATTGAGAGGGCCGCTTGTCGTGTACGACCCCCAC
GATCCTCAGGCGCATCTGTATGATGTTGACAACGATGACACTGTCATCAC
TTTGGCGGATTGGTATCATGTCGCGGCCAAGCTAGGCCCGCAATTCCCGA
GGGGCGCAAACTCTACGCTCATCAACGGCCTTGGACGAGCGGCGACTGA
TAGCACTTCCGATCTCAGTGTCATTACCGTTGAGCATGGGAAGCGCTATC
GTTTCAGGCTTGTATCCATCTCTTGTGATCCGAACCACACCTTCAGCATCG
ATGGCCACAACATGACCATCATCGAAGTCGATGGCGTCAACAGCAAGCC
CCTCACCGTCGACTCCATCCAGATTTTCGCAGCCCAGCGCTACTCCTTCGT
GTTGAATGCTAACCAACCGGTGGACAACTACTGGATTCGTGCGAATCCGA
GTGGCGGAACCGTGGGTTTCGAGGGCGGCATCAACTCTGCCATTCTCCGA
TACAAGGGTGCGCCGGATGCCGAGCCCACGAACACGACCGCGCCGACAT
CTGTCATTCCTCTGGTGGAGACGAATTTGCACCCCCTCAAGCCGATGCAA
GTGCCCGGCCGGTCTGGTGTCGGTAACGTTGATTATGCGAAGACACTCAA
TTTCAACTTCAACGGCACCAACTTTTTCATCAACAATGCGACGTTCACCC
CGCCCACAGTCCCCGTCCTCCTCCAGATCCTGAGCGGAGCGCACAACGCG
CAGGACCTCCTCCCCGCCGGGTCTGTTTACACTCTTCCGCCGCACAGCGC
CATCGAGATTACCATGCCGGCTACTACCCTAGCCCCGGGATCTCCCCACC
CCTTCCACTTGCACGGGCACGTCTTCGCTGTCGTACGCAGCGCCGGCAGC
ACCGAGTACAACTACCACGACCCCATCTTCCGCGACGTCGTGAGCACCGG
CCAGCCCGGCGACAGCGTCACGATCCGGTTCATGACGGACAACCCGGGT
CCGTGGTTCCTCCATTGCCACATCGACTTCCATCTCGAGGCCGGCTTCGCC
ATCGTGTTTGCCGAGGACGTGAACGATATCAAGTATGCGAACCCGGTCCC
GCCGTCGTGGTCGGAGCTTTGCCCCATCTACGACAAGCTCCCGGAGTCCG
ACCAT**TAG-**3’

APPENDIX B.2 :

Alkaline phosphatase DNA sequence (1434 bp):

5’-
GTGAAACAAAGCACTATTGCACTGGCACTCTTACCGTTACTGTTTACCCC
TGTGACAAAAGCCCGGACACCAGAAATGCCTCTGCAGTAGGCGCTCGAG
GTTCTGGAAAACCGGGCTGCTCAGGGCGATATTACTGCACCCGGCGGTGC
TCGCCGTTTAACGGGTGATCAGACTGCCGCTCTGCGTGATTCTCTTAGCG
ATAAACCTGCAAAAAATATTATTTTGCTGATTGGCGATGGGATGGGGGAC
TCGGAAATTACTGCCGCACGTAATTATGCCGAAGGTGCGGGCGGCTTTTT
TAAAGGTATAGATGCCTTACCGCTTACCGGGCAATACACTCACTATGCGC
TGAATAAAAAAACCGGCAAACCGGACTACGTCACCGACTCGGCTGCATC
AGCAACCGCCTGGTCAACCGGTGTCAAAACCTATAACGGCGCGCTGGGC
GTCGATATTCACGAAAAAGATCACCCAACGATTCTGGAAATGGCAAAAG
CCGCAGGTCTGGCGACCGGTAACGTTTCTACCGCAGAGTTGCAGGATGCC
ACGCCCGCTGCGCTGGTGGCACATGTGACCTCGCGCAAATGCTACGGTCC
GAGCGCGACCAGTGAAAAATGTCCGGGTAACGCTCTGGAAAAAGGCGGA
AAAGGATCGATTACCGAACAGCTGCTTAACGCTCGTGCCGACGTTACGCT
TGGCGGCGGCGCAAAAACCTTTGCTGAAACGGCAACCGCTGGTGAATGG
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GACCGAAAGCAACGTACCATGGCAATATCGATAAGCCCGCAGTCACCTG
TACGCCAAATCCGCAACGTAATGACAGTGTACCAACCCTGGCGCAGATG
ACCGACAAAGCCATTGAATTGTTGAGTAAAAATGAGAAAGGCTTTTTCCT
GCAAGTTGAAGGTGCGTCAATCGATAAACAGGATCATGCTGCGAATCCTT
GTGGGCAAATTGGCGAGACGGTCGATCTCGATGAAGCCGTACAACGGGC
GCTGGAATTCGCTAAAAAGGAGGGTAACACGCTGGTCATAGTCACCGCT
GATCACGCCCACGCCAGCCAGATTGTTGCGCCGGATACCAAAGCTCCGG
GCCTCACCCAGGCGCTAAATACCAAAGATGGCGCAGTGATGGTGATGAG
TTACGGGAACTCCGAAGAGGATTCACAAGAACATACCGGCAGTCAGTTG
CGTATTGCGGCGTATGGCCCGCATGCCGCCAATGTTGTTGGACTGACCGA
CCAGACCGATCTCTTCTACACCATGAAAGCCGCTCTGGGGCTGAAATAA-
3’

APPENDIX B.3 :

Mutant Green Fluorescent Protein (GFPuv) DNA Sequence (717 bp):

5’-
ATGAGTAAAGGAGAAGAACTTTTCACTGGAGTTGTCCCAATTCTTGTTGA
ATTAGATGGTGATGTTAATGGGCACAAATTTTCTGTCAGTGGAGAGGGTG
AAGGTGATGCAACATACGGAAAACTTACCCTTAAATTTATTTGCACTACT
GGAAAACTACCTGTTCCATGGCCAACACTTGTCACTACTTTCTCTTATGGT
GTTCAATGCTTTTCCCGTTATCCGGATCATATGAAACGGCATGACTTTTTC
AAGAGTGCCATGCCCGAAGGTTATGTACAGGAACGCACTATATCTTTCAA
AGATGACGGGAACTACAAGACGCGTGCTGAAGTCAAGTTTGAAGGTGAT
ACCCTTGTTAATCGTATCGAGTTAAAAGGTATTGATTTTAAAGAAGATGG
AAACATTCTCGGACACAAACTCGAGTACAACTATAACTCACACAATGTAT
ACATCACGGCAGACAAACAAAAGAATGGAATCAAAGCTAACTTCAAAAT
TCGCCACAACATTGAAGATGGATCCGTTCAACTAGCAGACCATTATCAAC
AAAATACTCCAATTGGCGATGGCCCTGTCCTTTTACCAGACAACCATTAC
CTGTCGACACAATCTGCCCTTTCGAAAGATCCCAACGAAAAGCGTGACCA
CATGGTCCTTCTTGAGTTTGTAACTGCTGCTGGGATTACACATGGCATGG
ATGAGCTCTACAAATAA-3’

APPENDIX C.1

1) **Single Strand DNA oligo including Enzyme Cutting Sites+QBP1+14 aa linker+ Factor Xa Cutting Site:**

5'-
GCTGACGTTGGAAGATCTTCAAGGAGATATACCATGCCGCCGCCGTGGCT
GCCGTATATGCCGCCGTGGAGCGGATCCCCGCCGCCGGGCCCGCCGGGC
CCGCCGCCGGGCCCGCCGCCGATTGAAGGCCGTGGAATTCATGGCATCG-
3'

Primers for ssDNA amplification:

Forward Primer: 5'- GCTGACGTTGGAAGATCTTCAAGGAGA-3'

Reverse Primer: 5'-CGATGCCATGGAATTCCACGGCCTTCA -3'

2) **Oligo DNA Primer without linker:**

5'-
TACCATGGCGCCGCCGCCGTGGCTGCCGTATATGCCGCCGTGGAGCATTG
AAGGCCGTGGAATTCAG -3'

Reverse Primer: 5'- AACAAGCTTTTATTTCAGCCCCAGAGCGG -3'

3) **Primers for Alkaline phosphatase amplification from pSB2991;**

Forward Primer:
5'-GCTGGAATTCAGGTGAAACAAAGCACTATTGCACTGGC-'3

Reverse Primer:

5'- AACCTGCAG-TTATTTCAGCCCCAGAGCGG -3'

4) **Primers for QBP1-Alkaline Phosphatase amplification:**

Forward Primer:

5'-CCATGGTGAAACAAAGCACTATTGC-3'

QBP1a primer:

5'-CCACGGCGGCGGGAATTCACGGCCTTCAATTTTCAGCCCCAGAGCGG-
3'

QBP1b primer:

5’-
AAGCTTTTATTAGCTCCACGGCGGCATATACGGCAGCCACGGCGGCGGG
AATTC-3’

5) Primers for QBP1-GFPuv fusion molecule.

Forward Primer:
5’-CCA CGG CGG CAT ATA CGG CAG CCA CGG CGG CGG CAT-3’
Reverse Primer:
5’-CTG CCG TAT ATG CCG CCG TGG AGC CCG CCG CCG GG-3’

6) Primers for QBP1-lcc1 fusion molecule.

Forward Primer:

5’-

GAATTCAGCATGCCACCACCATGGTTGCCATATATGCCACCATGGTCTAT

TGAAGGTAGAGGCTCCGGTCTTTTC-3’

Reverse Primer:

5’-AACGCGGCCGCCCTAATGGTCGGACT -3’

Appendix D.

Molecular Weight Markers

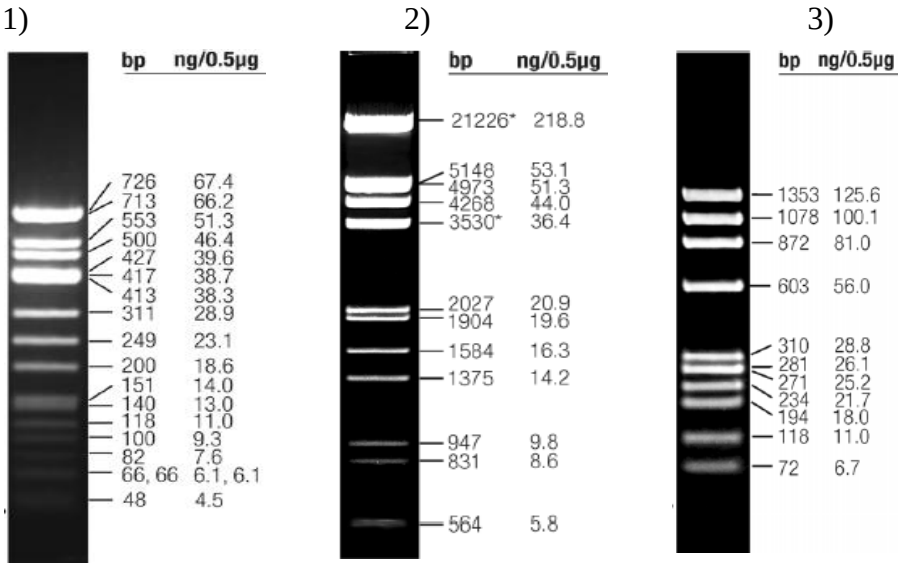

Figure A. 6.: DNA Molecular Weight Markers.

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

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

3) ΦX174 DNA *Bsu*RI digested marker-9, 1.7% agarose, 0.5µg/lane, 8 cm length gel, 1X TBE, 5V/cm. (Catalog # SM0251, NBI Fermentas).

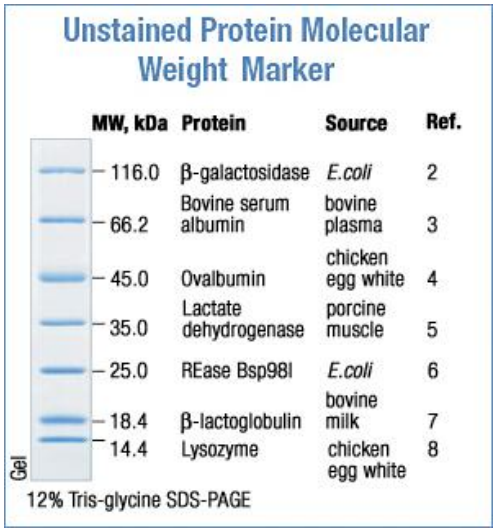

Figure A. 6.: Unstained Protein Molecular Marker.

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Publications:

- E.Emre Oren, Candan Tamerler, **Deniz Sahin**, Marketa Hnillova, Urartu O. S. Seker, Mehmet Sarikaya, Ram Samudrala. A Novel Informatics-Based Approach to Design Inorganic Binding Peptides, Bioinformatics, 24 (21) 2007.

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