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Simplified Expression and Production of Small Metal Binding Peptides

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Abstract. Phage display for discovery of metal binding peptides is an innovative way to engineer metalsorptive biological structures with a broad spectrum of applications in geobiotechnology, for example in reusable filter modules in biosorption processes. Using state-of-the-art cloning techniques we developed an easy-to-use cloning and expression system, allowing the fast production of identified peptides.

Introduction

Interactions between metals and biological matter are as old as life. Today, 30 % of all known proteins are estimated to be metalloproteins, making peptide moieties the largest library of metal interacting biological molecules, evolved through billions of years [1]. Peptides involved in the interactions with manifold materials can be easily identified using phage surface display (PSD), where randomized exogenous peptides are expressed and displayed on the surface of phage. This technology has long been used for medical or pharmaceutical applications e.g. cancer therapy research [2]. In the process large phage libraries are screened to identify sequences that specifically bind to the target material. Recently, PSD has also entered material sciences and geobiotechnology to assess the interaction of PSD derived peptides with metals. Peptides that selectively bind specific metal ions or other critical elements may be used for biosorption, -flotation and -remediation, allowing completely new biomining approaches [2,3]. In this work, peptide sequences able to bind to cobalt- and nickel ions, previously identified using PSD, were used in heterologous expression systems. Simple and inexpensive production of such peptides would allow for ecologically friendly and economically feasible biological sorption solutions.

However PSD technology is also prone to identify multiple eligible peptides as well as false positive, target-unrelated sequences. Additionally, identified sequences need to be transferred form phage libraries to a usable peptide form using molecular biology or expensive synthesis. Direct expression and purification of these small peptides however, is limited by fast product proteolysis and low recovery yields. To overcome this issue, fusion proteins may be used. Protein expression using fusion partners, self-splicing inteins [5] and affinity tags avoids proteolysis [6] and can furthermore be beneficial as it protects the host organisms from toxicity, simplifies purification [6,7] and promotes product solubility [9].Hence, for further characterization a simple cloning and expression method is needed for the production of selected peptides.

In this study we describe the development of a fast and reliable cloning and expression system, allowing the transfer of pre-identified peptide sequences into small peptides. The functionality of this expression system is considered a very important step for an efficient upscaling process in view of industrial applications. Peptide sequences were taken from ongoing PSD experiments. IMPACT^{TM} Kit (New England Biolabs) was combined with Gibson Assembly [10] to simplify the method and avoid restriction enzyme-based cloning. For demonstration a particular peptide was

overexpressed in *E. coli* and subsequently purified. The developed system can be rapidly adapted to other phage display libraries, making it a versatile tool for the characterization of the interactions of many PSD derived peptide sequences.

Materials

The polymerase used for PCR and Gibson assembly was Phusion® High-Fidelity DNA Polymerase by New England Biolabs. Gibson assembly was performed as described elsewhere [10]. Primer DNA was ordered from Eurofins Genomics. Enzymes used in Gibson Assembly were ordered from New England Biolabs. Sequencing was performed using Lightrun by GATC Biotech. Protein expression was induced with 0.1 mM IPTG and overnight cultivation in LB medium at 20 °C and 100 rpm in shaker flasks. Cell were lysed in lysis buffer (20 mM Tris, 500 mM NaCl, 0,5 g L^{-1} lysozyme, 100 U DNAseI) for 2 h at 4 °C after subsequent sonification Branson Sonifier® W-250 D and 30 % amplitude. SDS gel electrophoresis was performed according to Laemmli [11]. Protein purification was done using ÄKTA avant 25 from GE Healthcare and chitin resin from New England Biolabs. The following buffers were used: column buffer (20 mM Tris, 500 mM NaCl, pH 8.5) for equilibration and elution, cleavage buffer (20 mM Tris, 500 mM NaCl, 100 mM DTT) for induction of intein cleavage and stripping buffer (300 mM NaOH) for strpping remaining intein and chitin binding domain of the column. Protein concentration was determined using Roti® quant universal and Mithras² LB 943 from Berthold. Chemicals were ordered from Sigma-Aldrich or Carl Roth. All cloning experiments were performed in *Escherichia coli* 2566 by New England Biolabs.

Cloning Strategy. The structure of the M13 minor coat protein PIII used in PSD needed to be taken into consideration. The insertion site for the displayed randomized peptide is located on the N-terminal site of the protein, however the protein structure is more complex and consists of the three mayor domains N1, N2 and CT, which are connected with glycine-rich linker regions [12], [13]. Binding behavior of peptides, previously identified via PSD, may be strongly connected to adherent domains as well as the leader peptide and its subsequent cleavage. Therefore we included both surrounding regions, leader peptide and N1 domain to avoid misfolding. Furthermore the self-splicing protein GyrA Mxe from *Mycobacterium xenopi* [14] was added. For controlled cleavage we used an engineered variant, which splices in presence of thiol reagents such as dithiothreitol [15]. For on-column purification and cleavage, a chitin binding domain was included. The plasmid pTXB1 from New England Biolabs was used as template for the plasmid backbone. Gibson Assembly [10] was used to combine the generated PCR products, allowing seamless assembly. Attention must be drawn in generating these, as careful consideration is needed to ensure In-frame cloning of fusion proteins and successful usage of existing DNA elements such as promoters. The following primer sequences were used to amplify pTXB1 (New England Biolab) for subsequent Gibson Assembly: pTXB1_GA_rev (5'-3', TATCTCCTTC TTAAAGTTAA ACAAAAT), pTXB1_GA_fwd (5'-3', CTCGAGGGCT CTTCCTGCAT C). The reverse primer binds in the Shine Dalgarno sequence, ensuring that ribosomal binding site, promoter and operator for expression remains untouched. Primer sequences for amplification of the randomized peptide sequence were: M13_GA_rev (5'-3', *CAACTAGTGC ATCTCCCGTG ATGCAGGAAG AGCCCTCGAG* ACCCTCAGAG CCACCACCCT CATTTTCAG), M13_GA_fwd (5'-3', *TCCCCTCTAG AAATAATTTT GTTTAACTTT AAGAAGGAGA TAA*TGAAAAA ATTATTATTC GCAATTCCTT TAG). The forward primer included the new start codon, which replaced the original gtg start codon of M13KE phage *pIII* gene. Italic letters mark overlapping sequences, necessary for Gibson Assembly.

Results

Generation of DNA Fragments. Both, vector backbone and M13KE randomized library sequence were successfully amplified via PCR and assembled together to create the pTXB1 || M13KE *pIII* || gyrA || CBD expression vector. In Fig. 1 (A) amplification of the M13KE phage *pIII* gene with the

randomized peptide sequence and the vector backbone (B) is shown. The vector map shows the combined sequence of both, vector and M13KE insert after Gibson Assembly. The resulting sequence of the generated expression vector has been verified by sequencing.

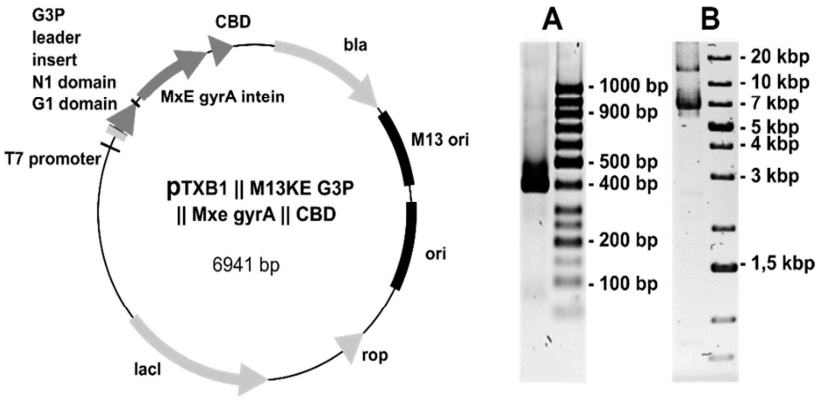

Fig. 1. Illustration of the resulting plasmid after assembly of pTXB1 vector backbone PCR product and the library insert out of M13KE. The plasmid codes for a fusion protein, containing PIII leader, N1 domain, Ph.D.™-C7C library insert, MxE gyrA intein and a chitin binding domain. Gel electrophoresis pictures show the amplification of M13KE library insert (A, 400 bp) and pTXB1 vector backbone (B, 6665 bp)

Expression and purification. After transformation and heterologous expression, cells were disrupted and lysis supernatant was used for purification with fast protein liquid chromatography (FPLC). Expression and purification were controlled with SDS polyacrylamide gel electrophoresis (SDS PAGE). FPLC results show a high protein concentration in the lysis fraction, followed by decreasing protein concentration when the column is washed. Elution after overnight incubation with DTT shows a single peak for the desired peptide. When stripping the column after elution, the intein and chitin binding domain, which remained at the column after DTT intein cleavage, are eluted, as shown in Fig. 2 A. SDS PAGE shows, that a single, small peptide can be identified in the elution fraction. The particular peptide, which was expressed in this work, has a theoretical molecular weight of 8.4 kDa, however limitations of SDS PAGE may account for the observed differences.

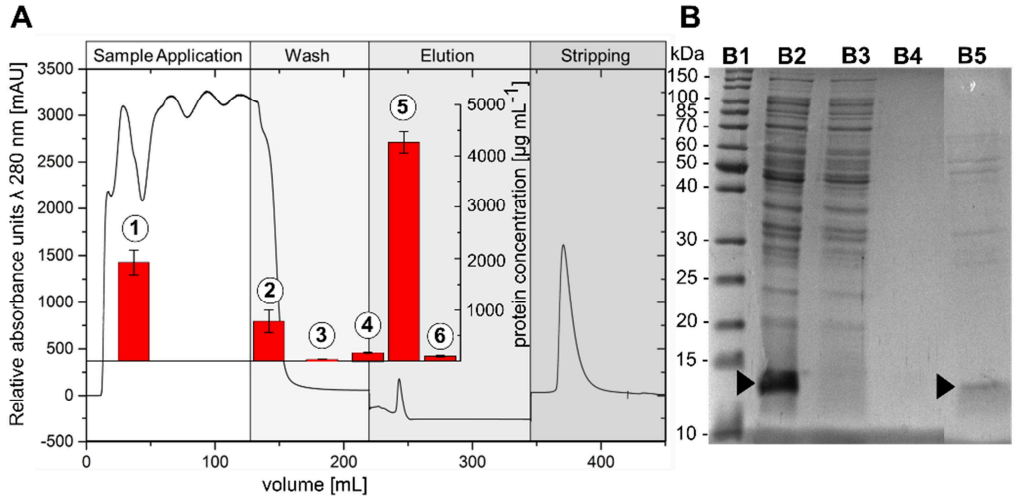

Fig. 2. Chromatogramm of FPLC (A) and SDS PAGE (B) of expression and purification of the peptide of interest. (A) Shown is the relative absorbance at 280 nm in the different purification steps as well as the protein concentration for chosen fractions, indicating successful protein purification (1 lysate 1927 ± 240 µg mL^{-1}; 2 1st wash fraction 786 ± 224 µg mL^{-1}, 3 2nd wash 15 ± 5 µg mL^{-1}, 4 1st elution fraction 176 ± 12 µg mL^{-1}, 5 2nd elution fraction 4275 ± 210 µg mL^{-1}, 6 3rd elution fraction 100 ± 15 µg mL^{-1}). (B) SDS PAGE of (lane B2) supernatant of cell lysis, (B3) 1st wash fraction, (B4) 2nd wash fraction, (B5) 2nd elution fraction, lane B1 Pageruler™ unstained protein ladder. The heterologously expressed peptide in marked with black arrows.

Conclusions

Despite great progress in PSD. and discovery of hundreds of peptides for specific purposes, the subsequent expression of identified peptide sequences still poses a major challenge. However it is as important as the identification. Especially in geobiotechnology and bioprocessing operational scale is normally in the range of tons rather than kilograms. Costs for synthesized peptides therefore impede its widespread use, while presently used cloning strategies are time-consuming and need to be adapted individually for each sequences. Our system allows fast und multifunctional cloning for heterologous expression in *Escherichia coli.* Subsequent expression and purification are greatly simplified as affinity tags used for purification can be easily cleaved off during the process, resulting in a small peptide, which may be used for further characterization or application. Heterologously expressed metal-binding peptides are potent building blocks for metal recovery in bioprocessing, usable immobilized in filter modules for re-usable purposes as well as continuously produced for remediation applications.

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