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Purification of MBP fusion proteins using engineered DARPin affinity matrix

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ABSTRACT

Maltose binding protein (MBP) has a long history as an expression tag with the ability to increase the solubility of fused proteins. A critical step for obtaining a sufficient amount of the MBP fusion protein is purification. Commercially available amylose matrix for the affinity purification of MBP fusion proteins has two main issues: (i) low (micromolar) affinity and (ii) the limited number of uses due to the cleavage of polysaccharide matrix by the amylases, present in the crude cell extract. Here, we present a new affinity purification approach based on the protein-protein interaction. We developed the affinity matrix which contains immobilized Designed Ankyrin Repeat Protein off7 (DARPin off7) – previously identified MBP binder with nanomolar affinity. The functionality of the DARPin affinity matrix was tested on the purification of MBP-tagged green fluorescent protein and flavodoxin. The affinity purification of the MBP fusion proteins, based on the MBP-DARPin off7 interaction, enables the purification of the fusion proteins in a simple two-steps procedure. The DARPin affinity matrix - easy to construct, resistant to amylase, insensitive to maltose contamination, and reusable for multiple purification cycles - provides an alternative approach to commercially available affinity matrices for purification of proteins containing the MBP tag.

1. Introduction

Production of recombinant proteins in bacterial systems is often accompanied by the accumulation of overexpressed proteins in the form of insoluble and biologically inactive aggregates [1]. One way how to address this issue is the expression of the protein of interest (POI) as a fusion protein with the tag or domain which can enhance its solubility or even promote its proper folding. The most popular solubility tags are the maltose binding protein (MBP) [2,3], glutathione S-transferase (GST) [4], and thioredoxin (TRX) [5]. Although the use of each tag has its advantages and disadvantages [6], it has been shown that MBP possesses chaperone-like properties and is a better solubilizing agent than the GST or TRX [7–10]. Moreover, the natural periplasmic localization of MBP provides a suitable expression strategy for toxic proteins [11], antibody fragments [12–15] or membrane proteins [16,17]. All these positive effects of MBP on its fusion partners, along with the possibility of MBP to enhance the formation of crystal contacts, the fact that the MBP structure is known and may be used for solving the phase problem by molecular replacement, make MBP the most successful crystallization chaperone [18,19]. The number of crystal structures of polypeptides fused to MBP has grown dramatically during the past decade, which resulted in more than one hundred solved structures available in the Protein Data Bank [20].

On the other hand, the major disadvantage of using MBP as an expression tag is the tedious purification of the MBP fusion proteins. Native MBP can be isolated from *E. coli* by its binding to amylose [21]. *New England Biolabs* developed vectors with multiple cloning sites for expression and purification of cytosolic as well as periplasmic MBP fusion proteins [2,3]. Later, to allow alternative purification strategies, Nallamsetty et al. [22,23] designed constructs containing combinations

Abbreviations: CD, circular dichroism; CV, column volume; DSC, differential scanning calorimetry; IMAC, immobilized metal-ion affinity chromatography; IPTG, isopropyl-β-D-thiogalactopyranoside; ITC, isothermal titration calorimetry; GFP, green fluorescent protein; GST, glutathione S-transferase; FLD, flavodoxin; MBP, maltose binding protein; POI, protein of interest; TRX, thioredoxin; SPR, surface plasmon resonance.

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of His-tag and MBP-tag. Despite the improvements made in this field, the weak affinity of MBP to amylose (K_D of 1.2 μM to the disaccharide maltose, a constituent of amylose [24]) remains the main obstacle of this purification strategy. Another limitation of the amylose matrix is related to the presence of the enzyme amylase and the maltose in the cell lysate. While the amylose matrix degradation by amylases causes a decrease in binding capacity of the matrix as well as reduces the number of matrix regeneration cycles, the presence of maltose in the cell lysate causes elution of the MBP fusion proteins from the amylose matrix [25,26].

Therefore, we aimed to develop a new affinity matrix for the purification of MBP fusion proteins, based on protein-protein interaction. We adopted Designed Ankyrin Repeat Protein off7 (DARPin off7), previously evolved to bind MBP with high affinity (K_D of 4.4 nM) [27]. DARPin off7 binds to a site on the MBP surface distant from the maltose binding cleft and exhibits a high specificity to MBP [27]. Despite all that, the utilization of the DARPin off7 as a ligand for the purification of MBP fusion proteins has not yet been exploited. In this paper, we investigate the conformational and binding properties of both DARPin off7 and MBP in order to determine basic characteristics of the matrix, including temperature stability, pH stability or reproducibility. Further matrix optimization and the binding capacity determination were performed on the purification of MBP-tagged green fluorescent protein (GFP) and flavodoxin (FLD). The purification method established in the paper provides: (i) a novel purification approach for the purification of MBP fusion proteins in a single-step procedure; (ii) optimized affinity matrix with repetitive regenerations without affecting the binding capacity of the matrix, and (iii) a rapid and generic protocol that can be adapted for a particular protein. The presented DARPin affinity matrix represents an alternative way for the purification of MBP fusion proteins with a higher yield of a purified protein and better ability of regeneration than currently available polysaccharide affinity matrices.

2. Materials and methods

2.1. MBP fusion proteins cloning

MBP fusion proteins were expressed using a modified pQE-30 vector containing the open reading frame of MBP, which was extended with a human rhinovirus 3C protease cleavage motif (LEVLFQGP). The open reading frames encoding GFP and FLD were amplified by PCR with introducing *Bam*HI and *Hind*III sites at the N- and C- terminal sequences, respectively. The used primers are listed in Table 1. The resulting PCR amplicons were digested with *Bam*HI and *Hind*III (New England BioLabs, Ipswich, Massachusetts, USA), and then ligated with the *Bam*HI/*Hind*III vector backbone of pQE-30 to create the final expression vector. The nucleotide sequences were verified experimentally. Both nucleotide and amino acid sequences of the full-length MBP-GFP and MBP-FLD are included in supporting information.

2.2. Site-directed mutagenesis

The plasmid encoding DARPin off7 was a gift from Profesor Plückthun from the University of Zurich. Defined point mutations (K17R, K144R and K147R) were introduced in the binding interface of DARPin off7 using the QuikChange site-directed mutagenesis kit (Agilent Technologies, Santa Clara, California, USA). The synthesized oligonucleotide primers (Microsynth AG, Switzerland) containing the desired mutations (Table 2) were used in consecutive PCR reactions to generate different DARPin off7 mutants. After temperature cycling, the PCR products were treated with *Dpn*I and the nicked plasmids containing the desired mutations were then transformed into homemade XL1-Blue competent cells.

2.3. Protein expression

All studied proteins were expressed in the *E. coli* strains XL1-Blue, except for 3C protease which was expressed in BL21. 2xYT medium containing 100 μg/ml ampicillin (35 μg/ml kanamycin in the case of the 3C protease) and 0.4% glucose was inoculated to OD_{600} ~ 0.1 with an overnight pre-culture harbouring a corresponding expression plasmid. Protein expression at 37 °C was induced with 1 mM IPTG upon reaching OD_{600} ~ 0.6 and continued for the next 4 h. After incubation, the cells were harvested by centrifugation (15 min, 5000*g*, 4 °C) and frozen in liquid nitrogen till they further proceeded.

2.4. Purification of DARPins, MBP and 3C protease

All the following steps were carried out on the ice and with cooled (4 °C) buffers. The cell pellet was resuspended in TBS lysis buffer pH 8.0 (4 ml/g) containing 50 mM Tris-HCl, 400 mM NaCl and lysozyme (1.5 mg/ml). Subsequently, the resuspended cells were incubated for 30 min with 1 mM EDTA and 1 μl of protease inhibitors (5 μg/ml of leupeptin and 1 μg/ml of pepstatin) per 1 ml of the resuspended pellet. Small amounts (on a spatula tip) of DNase I and 6 mM $MgCl_2$ were added and the suspension was incubated for another 30 min. The cells were disrupted using sonification (Branson sonifier 450, Fisher Scientific) and cell debris was removed by centrifugation (35 min, 17,000*g*, 4 °C). The filtrated supernatant was applied to a pre-equilibrated IMAC column (Ni-NTA Superflow resin, Qiagen) and washed with 10 column volumes (CV) of TBS washing buffer pH 8.0 (50 mM Tris-HCl, 400 mM NaCl pH, 20 mM imidazole and 10% glycerol), 10 CV of TBS low-salt buffer pH 8.0 (50 mM Tris-HCl, 20 mM NaCl, 20 mM imidazole and 10% glycerol), 10 CV of TBS high-salt buffer pH 8.0 (50 mM Tris-HCl, 1 M NaCl pH, 20 mM imidazole and 10% glycerol) and again 10 CV of TBS washing buffer. Finally, the proteins were eluted with TBS elution buffer pH 7.4 (50 mM Tris-HCl, 400 mM NaCl pH, 250 mM imidazole and 10% glycerol), frozen in liquid nitrogen and stored at -80 °C.

2.5. Preparation of DARPin off7 affinity matrix

NHS-activated Sepharose 4 Fast Flow (GE Healthcare, 2 ml of slurry) was poured into an empty PD-10 column and washed immediately with 10 ml of ice-cold 1 mM HCl. Subsequently, 1 ml of 20 mg/ml of the purified DARPin off7 in coupling buffer (0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3) was added and incubated for 3 h at room temperature. After the coupling reaction was completed, the column was drained and a flow-through was further tested with a Biuret assay in order to determine the coupling efficiency. To block unreacted NHS groups, the column was incubated overnight with 0.1 M Tris-HCl, pH 8.5 at 4 °C. Finally, the column was washed 3-times with 0.1 M CH_3COONa, 0.5 M NaCl pH 4.5 and 0.1 M Tris-HCl pH 8.5 in an alternative manner. To prevent microbial contamination, the column was stored in 20% ethanol.

Table 1
List of primers used for cloning of MBP fusion proteins.

MBP-GFP	
Forward	5′-GGAGGAGGATCCGGAGCAAGCAAAGGAGAAGAACTTTTCACTGG-3′
Reverse	5′-TCCTCCAAGCTTTTTGTAGAGCTCATCCATGCCATGTG-3′
MBP-FLD	
Forward	5′-GGAGGAGGATCCGGAGCAATGAGTAAGGTACTGATTGTTTTTGG-3′
Reverse	5′-TCCTCCAAGCTTCAGCTGCTTGAGCACATCCTC-3′

Table 2
List of primers used for site-directed mutagenesis.

K17R mutation	
Forward	5′-CCGACCTGGGTAGG**AGA**CTGCTGGAAGCTGCTCGTGCTGG-3′
Reverse	5′-CCAGCACGAGCAGCTTCCAGCAG**TCT**CCTACCCAGGTCGG-3′
K114R and K147R double mutations	
Forward	5′-CGCTCAGGAC**AGA**TTCGGT**AGG**ACCGCTTTCGACATCTCCATCG-3′
Reverse	5′-CGATGGAGATGTCGAAAGCGGT**CCT**ACCGAA**TCT**GTCCTGAGCG-3′

2.6. Circular dichroism (CD)

The experiments were performed using a Jasco J-810 spectropolarimeter (Tokyo, Japan). Ellipticity in the far-UV region was measured at room temperature with a 1 mm pathlength quartz cuvette. The experiments were performed in 50 mM glycine (pH 3.5 and pH 2.7) or in 50 mM phosphate buffer (pH 7.0) with the protein concentrations of 10 μM.

2.7. Isothermal titration calorimetry (ITC)

ITC experiments were performed using a MicroCal iT200 system from Malvern. In the experiments related to the study of the interaction recovery, the proteins were dialyzed against a buffer containing 50 mM glycine pH 3.5 and then against 50 mM phosphate buffer pH 7.0. All other experiments were performed with PBS pH 7.0. The protein solutions were degassed prior to titration. In all cases, DARPin off7 from a 500 μM stock solution was titrated into a 50 μM solution of MBP. The calorimetry cells were thermostated at 23 °C and each injection was separated by 180 s. The baseline, calculated as an average of the normalized heat per injection value of the extra injections at the end of the experiment, was subtracted and data were fit with the Origin software provided with the instrument.

2.8. Differential scanning calorimetry (DSC)

DSC measurements were performed using a VP-Capillary DSC system (Microcal Inc., acquired by Malvern Instruments Ltd.). The proteins were dialyzed against the corresponding buffers in the same manner as in the ITC experiments. The protein concentrations were adjusted to 23 μM and all samples were degassed prior to measurement. The samples were heated from 25 °C to 90 °C with a scan rate of 0.5 K/min. Thermograms were corrected by subtraction of the so-called chemical baseline, i.e., the sigmoidal curve connecting the signal of excess heat capacity of the native and denatured states and normalized to the molar concentration of the protein.

2.9. Surface plasmon resonance measurements (SPR)

SPR experiments were performed using BIAcore 3000 (GE Healthcare). DARPin off7 was modified by substoichiometric coupling of an amine-reactive biotin reagent to achieve preferential immobilization via His-tag on an SA chip (GE Healthcare). The measurement was carried out at 25 °C with degassed buffers containing 0.005% Tween 20. The reversibility of the interaction between immobilized DARPin off7 and 50 nM MBP was measured in 50 mM phosphate pH 7.0, followed by regenerating the immobilized DARPin with 100 mM glycine pH 3.5, and then reapplying fresh MBP in 50 mM phosphate pH 7.0 containing 50 nM MBP.

3. Results

The existence of a specific interaction with the high affinity between DARPin off7 and MBP at neutral pH has been demonstrated previously [27]. However, the determination of conditions under which this interaction is perturbed in a reversible manner is inevitable for repetitive use of the DARPin affinity matrix. In general, such perturbation can be achieved, for example, by extreme pH or chemical denaturants, including urea or guanidium chloride. Because of the high resistance and conformational stability of DARPins against chemical denaturants [28], we decided to examine the effect of an acidic pH on the stability and reversibility of conformational states of both proteins.

3.1. Conformation and binding properties of DARPin off7 and MBP at different pH

Conformational properties of DARPin off7 and MBP under different pH conditions were investigated by CD and DSC. While an ellipticity in the far-UV region reflects the protein secondary structure, the protein thermal stability determined by DSC is related to the changes of the protein tertiary structure. CD spectra and DSC thermograms were collected within a broad pH region, from pH 2.7 to pH 10.0 (Figs. S1 and S2). The largest structural changes were observed at acidic pHs (Fig. 1).

The most significant ellipticity changes in both proteins occur below pH 3.0 and reflect a large decrease of the protein secondary structures. The absence of a significant change in the ellipticity of DARPin off7 in the far-UV spectral region and on the other hand, a decrease of its transition temperature by more than 30 °C at pH 3.5 clearly indicate significant perturbation of its tertiary structure already at this pH value. In the case of MBP, a decrease of about 7.5 °C of transition temperature after the transition from pH 7.0 to 3.5 suggests a smaller destabilization of MBP conformation at this pH. We showed that observed conformational changes if both proteins at pH 3.5 are sufficient to induce complete dissociation of the complex DARPin off7-MBP.

The exposure of DARPin off7 and MBP to acidic pH in the matrix regeneration process may be accompanied by irreversible changes in the protein structures. Therefore, it is necessary to determine whether the observed structural changes in the proteins are reversible and the interaction between them is fully recovered. The structure recovery of both proteins, as well as their complex, was determined after a 4-h incubation at pH 3.5 with a subsequent transition back to pH 7.0 (Fig. 2). DSC transition curves were virtually identical, demonstrating that the structural changes under acidic conditions are entirely reversible.

The DARPin off7/MBP complex formation can be followed also by DSC. In fact, the binding of DARPin off7 to MBP leads to a 5.5 °C shift of the DARPin off7/MBP complex transition temperature in comparison to free MBP at pH 7.0. This shift indicates a strong interaction between these proteins. Using Brandts' theoretical model [29], which allows determining the binding affinity of two interacting proteins from DSC measurements, we were able to determine the dissociation constant of the DARPin off7-MBP interaction in the low nanomolar range, $K_D \sim 1.5$ nM, consistent with the previously measured affinity by SPR [27].

The influence of acidic pH on the interaction between the DARPin off7 and MBP was also analyzed by SPR (Fig. 3A) and ITC (Fig. 3B) methods. The obtained results from both SPR and ITC techniques confirmed a strong interaction at pH 7.0, reflected by $K_D = 6.4 \pm 1.3$ nM ($k_{ON} = 9.66.10^5$ M^{-1} s^{-1}; $k_{OFF} = 6.15.10^{-3}$ s^{-1}) and 47 ± 3 nM, respectively. No binding at pH 3.5 was detected by these methods. Both techniques showed almost identical binding curves obtained after neutral-acidic-neutral exposure as before pH transition. These results support the ability of the proteins to refold properly back into their native states after their exposure to the acidic pH = 3.5.

MBP-tagged recombinant proteins are commonly eluted from an amylose column by 10 mM maltose solution. Moreover, the maltose itself can be present in the cell lysate. Therefore, the influence of maltose on the DARPin off7 and MBP complex formation was examined. The formation of the DARPin off7/MBP complex was examined by ITC in the presence of two different maltose concentrations, 250 mM and 400 mM (Fig. S3). The similarity of the binding curves strongly indicates that maltose does not affect the affinity as well as other thermodynamic parameters of the interaction between DARPin off7 and MBP (Table S1). This is not an unexpected result because maltose and DARPin off7 binding sites on the MBP are distant from each other. However, it was important to find out whether maltose may affect the DARPin off7-MBP interaction due to the relatively large conformational change of MBP upon maltose binding [30,31].

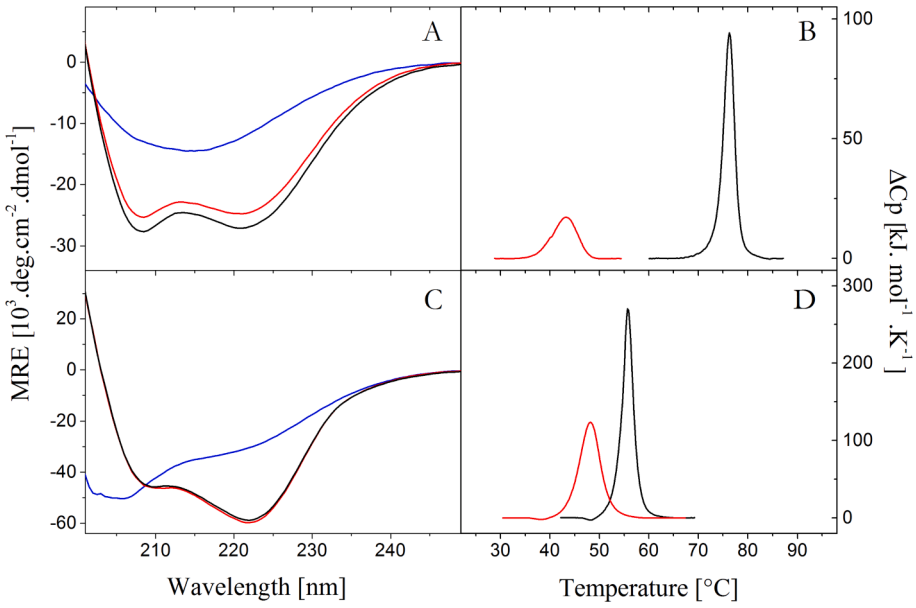

Fig. 1. CD spectra in the far-UV spectral region and DSC thermograms of DARPin off7 (A, B) and MBP (C, D), respectively. pH 7.0 (black), pH 3.5 (red) and pH 2.7 (blue).

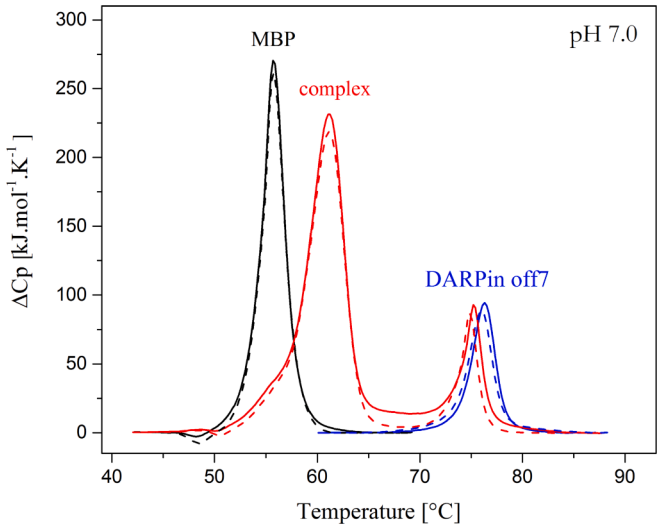

Fig. 2. DSC thermograms showing the thermal stability of MBP (black), DARPin off7 (blue) and DARPin off7/MBP complex (red). Solid lines represent the original thermal transitions of the proteins at pH 7.0. Dashed lines represent thermal transitions of the proteins in a neutral environment at pH 7.0 after their previous 4-h incubation in an acidic environment of pH 3.5.

3.2. Isolation of MBP fusion proteins

In order to test the DARPin off7 affinity matrix, we constructed two different MBP fusion proteins. Green fluorescent protein (GFP, MW: 27.3 kDa) and flavodoxin (FLD, MW: 16.3 kDa) were fused to MBP (MW: 43.1 kDa) through the linker containing a 3C protease cleavage site and expressed in *E. coli*. The MBP fusion protein bound to the DARPin off7 matrix was either eluted with 100 mM glycine buffer of pH 3.5 or was incubated for 2 h at room temperature with 3C protease and then the flow-through was collected. The application of 100 mM glycine of pH 3.5 led to elution of the intact MBP-POI (Fig. 4A) while the 3C protease cleavage directly eluted the purified POI (Fig. 4B).

Neither elution by acidic pH nor elution by 3C protease affects the structural properties of the studied proteins (Fig. S5). The fact that 3C

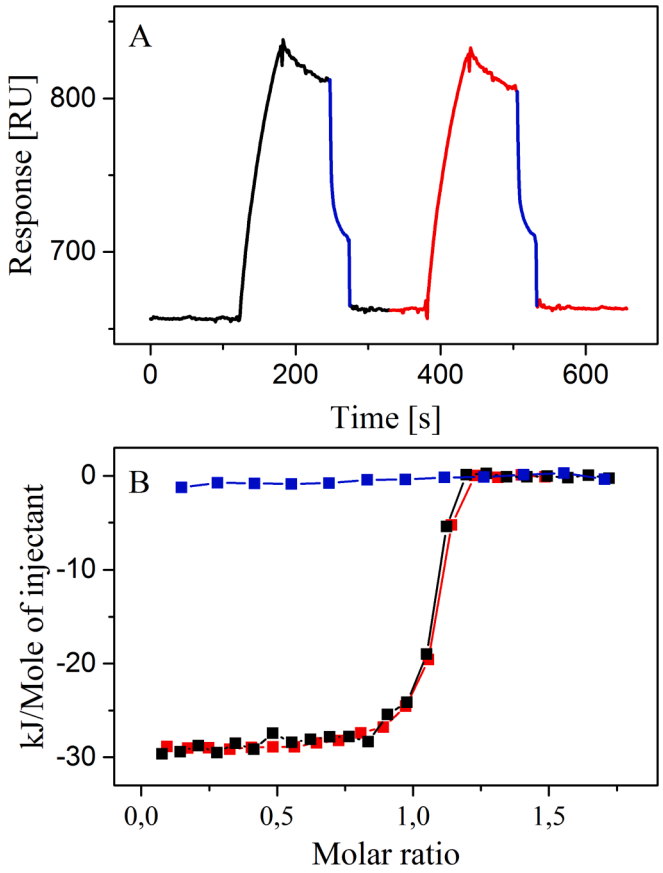

Fig. 3. pH dependence of the interaction between DARPin off7 and 50 nM MBP. (A) SPR experiment: first binding of MBP to immobilized off7 at pH 7.0 and dissociation (black), regeneration by dissociation of bound MBP at pH 3.5 (blue), and the second run at pH 7.0 (red). (B) ITC experiment: binding events at pH 7.0 (black), at pH 3.5 (blue) and at pH 7.0 after shifting back from acidic to neutral condition (red). Representative raw ITC data of interaction between DARPin off7 WT and MBP are shown in Fig. S4.

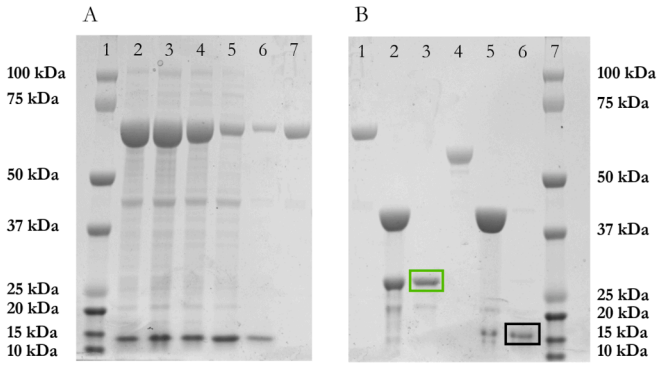

Fig. 4. Isolation of MBP fusion proteins by DARPin off7 affinity column. (A) Whole process of MBP-GFP isolation: 1 – molecular weight standard, 2 – cell lysate containing a large excess of the fusion protein, 3 – flow-through, 4 – 1st CV of washing buffer, 5 – 2nd CV of washing buffer, 6 - 3rd CV of washing buffer, 7 – MBP-GFP elution by 100 mM glycine of pH 3.5; (B) cleavage of MBP fusion proteins: 1 – MBP-GFP, 2 – MBP-GFP cleavage by 3C protease, 3 – purified GFP (green frame), 4 – MBP-FLD, 5 – MBP-FLD cleavage by 3C protease, 6 – purified FLD (black frame), 7 – molecular weight standard.

protease was added directly onto the column was responsible that this enzyme was collected together with desired recombinant protein (weak band at 22 kDa in Fig. 4B line 3). A 3C protease amount of a total of 2% *w*/w of target protein was sufficient to efficiently cleave all MBP fusion proteins. To remove the 3C protease, the protein mixture was further purified by reverse IMAC and, finally, polished by size exclusion chromatography, yielding a pure and homogeneous preparation (Fig. 5).

3.3. Binding capacity optimization

The DARPin off7 molecules are covalently conjugated to the column matrix through the amino groups of lysine residues, even though a coupling through the N-terminal amine may also occur. Since DARPin off7 contains lysine residues on both sides of the molecule (Fig. 6) it may bind to the sepharose resin in a random manner. An attachment of DARPin off7 through Lys17, Lys144 or Lys 147 located in the N- and C-terminal capping repeat, respectively, would sterically inhibit the interaction of DARPin off7 with MBP mediated by the β-turn and the first α-helix of the central ankyrin repeat modules (Fig. 6). To improve the

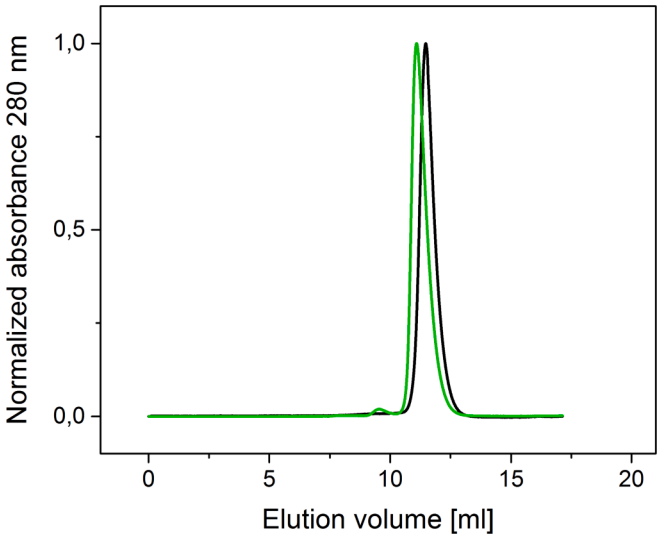

Fig. 5. Size exclusion chromatography of the purified FLD (black) and GFP (green) detected by Sepharose Increase 75 in PBS, pH 7.0. The chromatograms of GFP and FLD consist of single peaks demonstrating a high purity and homogeneity of the proteins.

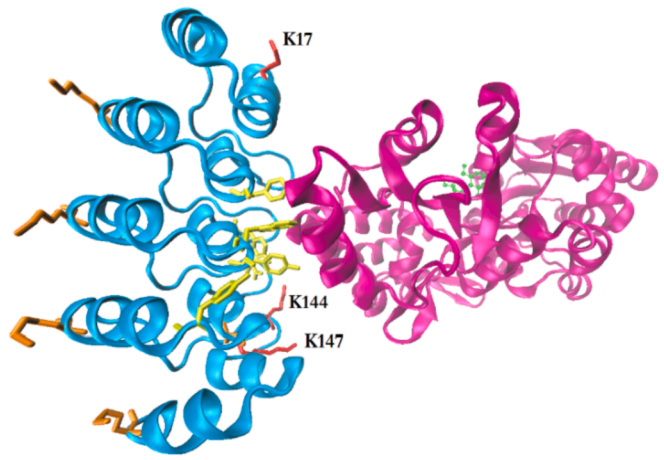

Fig. 6. Structure of DARPin off7/MBP complex with highlighted Lys residues and a maltose molecule bound to MBP (superposition of the crystal structures PDB ID: 1SVX and 1ANF). DARPin off7 is shown in light blue, MBP in purple, maltose in green, Lys residues able to covalently bind the resin in sticks, Lys residues (K17, K144, and K147) that were replaced with Arg are shown in red. The DARPin off7 residues responsible for the interaction with MBP are shown in yellow.

binding capacity of the DARPin affinity matrix by preventing the reaction of these lysine residues with the resin, they were replaced by mutation to arginine. Lysine 122 was selected from randomized residues during DARPin off7 evolution [27], therefore it was not replaced. DARPin off7 mutants containing various combinations of K17R, K144R or K147R were expressed and purified through IMAC (Fig. S6): (i) M1 containing the single mutation K17R, (ii) M2 containing two mutations, K144R and K147R, and (iii) M3 containing all three mutations, K17R, K144R and K147R. Expression of all DARPin off7 variants resulted in more than 100 mg of the purified proteins per litre of a medium.

The SDS-PAGE of the purified variants of DARPin off7 did not show any contaminants, but size exclusion chromatograms of all the variants consist of one major peak constituting the monomer and one smaller peak (Fig. S7). The position of the smaller peak strongly suggests the presence of a dimeric form in the DARPin off7 population which increases with an increasing number of mutations.

3.4. Binding properties of DARPin off7 variants

The binding properties of the DARPin off7 variants were determined by ITC (Fig. 7). The analysis of the binding curves revealed the effect of

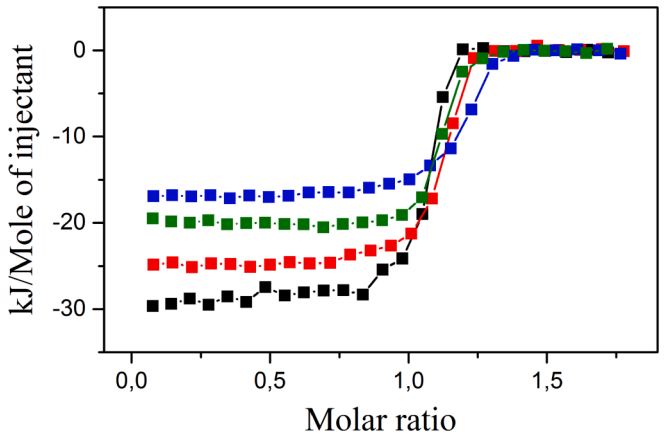

Fig. 7. ITC experiments showing the interaction of DARPin off7 variants with MBP. The DARPin off7 variants: WT (black), M1 (red), M2 (blue), and M3 (green).

the mutations on the thermodynamic parameters of the protein-protein interaction (Table S2). Although the mutated residues on DARPin off7 are distant from the site that participates in the interaction with MBP, the changes in the affinity of the variants were apparent. The weakest binding affinity was observed for the M2 variant, $K_D = 114 \pm 2$ nM. The variants M1 and M3 are characterized by comparable affinity to MBP as the wild type with $K_D = 65 \pm 4$ nM and $K_D = 41 \pm 2$ nM, respectively. We note that the value of K_D for MBP wild type obtained by ITC are higher than previously obtained data by surface plasmon resonance [27] likely due to limitations of ITC at such high-affinity interactions. However, we believe that the determined K_D values for the MBP wild type and its mutants obtained by ITC correctly express relative affinities of the MBP-off7 interaction.

3.5. The binding capacity of the columns containing the DARPin off7 wt and its variants

In order to determine the binding capacity of the matrix containing different DARPin off7 variants, 20 mg of the DARPin off7 variants were separately immobilized to 1 ml of sepharose resin. Prepared columns were used for the purification of MBP-FLD and MBP-GFP fusion proteins (Fig. 8).

A comparison of the binding capacity of the DARPin off7 variants revealed that the binding capacity for MBP itself is larger than for MBP fusion proteins (Table 3).

This phenomenon is commonly observed and it is probably caused by a blocking of additional MBP binding site by the size of the fusion protein [25]. Moreover, the binding capacity of the column does not correlate with the number of mutations. In the case of the purification of MBP itself, the column binding capacity decreases with the increasing number of mutations. On the other hand, in the case of MBP-GFP and MBP-FLD purification, the highest binding capacity is observed for the matrix containing mutant M1; mutants M2 and M3 have a similar capacity as the wild type. On the other hand, amylose resin has a noticeably lower capacity for MBP but comparable capacity as DARPin off7 matrix for fusion proteins. This suggests modified steric properties of the binding site on MBP with DARPin off7 due to fused proteins.

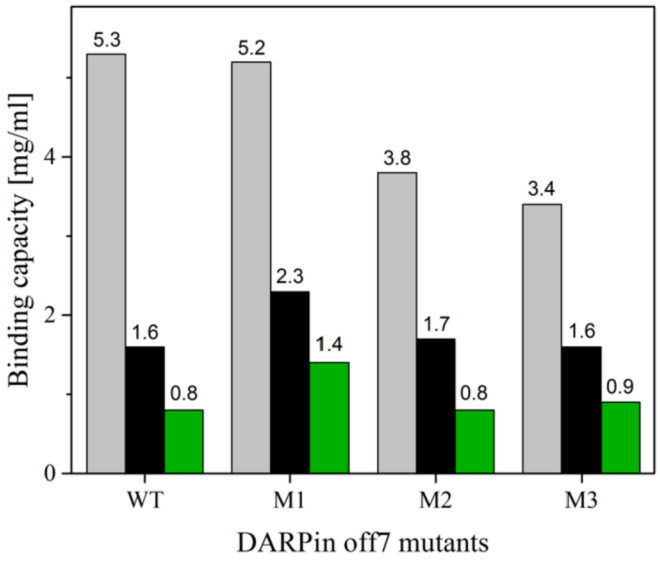

Fig. 8. Comparison of the binding capacity of the columns containing different DARPin off7 variants. The amount of the purified proteins per ml of the column matrix is listed at the corresponding columns. The purified proteins were: MBP (gray), MBP-FLD (black), and MBP-GFP (green). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Comparison of the yields of MBP and MBP fusion proteins after their purification on DARPin affinity matrices and amylose resin.

Matrix	MBP	MBP-FLD	MBP-GFP
DARPin off7 WT	5.3	1.6	0.8
DARPin off7 M1	5.2	2.3	1.4
DARPin off7 M2	3.8	1.7	0.8
DARPin off7 M3	3.4	1.6	0.9
Amylose	3.2	2.1	1.2

4. Discussion

Due to its excellent solubilizing properties, MBP is often used for the purification of recombinant proteins that are not able to fold properly or that have to be expressed in the oxidizing environment of the periplasm. Purification of MBP fusion proteins by DARPin off7 affinity matrix can result in purified POI alone or the intact MBP-POI molecule (Fig. 9). The pure POI has usually utilized in the subsequent conformational and/or functional studies. The MBP-POI fusion can be used as an alternative approach in protein crystallography when a standard way does not work [20], or in further immobilization of the POI if a solid phase is needed for particular assays.

4.1. Comparison of DARPin and amylose matrix

MBP is considered as one of the best solubility tags, but on the other hand as a relatively poor affinity tag with respect to purification on the amylose matrix. Several attempts were made to improve the purification strategy of MBP fusion proteins. Marvin and Hellinga [32] increased the binding affinity of MBP to maltose by specific point mutations. Nallamsety et al. [22] used an additional His-tag to avoid purification over amylose resin. While these attempts were aimed at modification of the MBP tag properties, we focused on an improvement of the matrix by using DARPin off7 as an affinity ligand. The DARPin off7 was evolved by using ribosome display [33] to bind MBP with very high affinity and specificity [27]. To dissociate the DARPin off7/MBP complex, one may use chemical denaturants or extreme pHs. In our hands, the most efficient method was acidic pH $\leq$ 3.5. From a practical point of view, the ability of DARPin off7 to recover its native structure and binding properties after exposure to acidic pH is crucial for column regeneration (Fig. 3A). As a result, the DARPin off7 matrix can be used repeatedly without a significant decrease of binding capacity towards MBP. In fact, after five consecutive purifications, the capacity of the DARPin off7 column decreased by ~ 15% (Table S3). On the other hand, the number of amylose matrix regeneration is typically limited to 3-5 times due to enzymatic degradation of amylose [25].

Buffers with pH $\leq$ 3.5 are the most commonly used elution buffer for

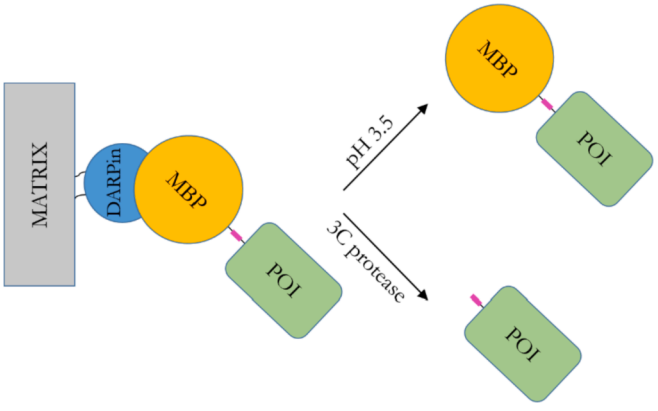

Fig. 9. Schematic representation of MBP fusion protein purification by DARPin off7 affinity column. Cleavage site of 3C protease is shown in pink.

affinity purification based on protein-protein interaction [34]. However, such pH may have a deleterious effect on some less stable proteins. One may avoid the elution of POI by acidic pH by performing on-column enzymatic cleavage by 3C protease. Application of a high-performance protease (e.g., 3C protease) leads to only small contamination of the used enzyme in eluted fraction, nonetheless, further purification steps will become necessary. In our case, 2% (*w*/w) 3C protease was sufficient to separate all POI from matrix-bound MBP. In contrast, affinity purification using an amylose matrix does not allow efficient digestion of fusion proteins directly on the column, and therefore, a standard purification protocol consists of several steps [26]. Firstly, bound MBP-POI is eluted with maltose, then it is enzymatically cleaved and finally, the POI is separated from MBP in the second affinity purification step. In addition, before repeated binding of MBP to amylose matrix can be carried out, proper removal of maltose from cleaved MBP is indispensable [25].

In order to improve this time-consuming purification, Nallamesty et al. [9,22] designed a His_6-MBP tag that allows purification of MBP fusion proteins through IMAC. Although it has been shown that a His-tag at N-terminus of MBP does not influence MBP's solubilizing properties, its low specificity causes the presence of contaminants in the purified POI. Therefore, most of the His_{tag}-MBP fusion protein purification methods include a combination of IMAC and amylose media or other purification procedures [6,15,20].

It is well known that MBP undergoes a relatively large conformational change upon maltose binding [30,31]. This structural reorganization can lead to complications. For example, Cherry et al. [35] obtained a different crystal structure of the SUFU-MBP in the presence and in the absence of maltose. In another case, Matsumoto and his colleagues [36] were able to obtain protein crystals only in the absence of maltose. Therefore, our single-step purification not only saves time but also avoids obstacles related to maltose elution in the purification of the intact MBP-POI fusion proteins for crystallographic purpose.

4.2. Purification of MBP fusion proteins by DARPin matrix

In order to improve the binding capacity of our matrix, we introduced specific mutations at Lys residues to optimize the spatial orientation of the DARPin off7 with respect to the matrix (Fig. 10).

An affinity of DARPin off7 to MBP, which is almost a thousand times higher than the affinity of MBP – sugar complexes [37], allows for a more efficient MBP purification (Fig. 8). While a commercially available amylose matrix has a binding capacity of 3.2 mg/ml of MBP, 1 ml of matrix containing 20 mg of DARPin off7 can bind about 5 mg of MBP (Table S4). On the other hand, the yields of MBP fusion proteins after purification on the DARPin off7 affinity matrix and amylase matrix are comparable (Table 3). The observed decrease in the yield of MBP-GFP and MBP-FLD could be further improved by optimization of the linker connecting MBP and fusion protein. It has been shown that the length of the linker can significantly enhance the structural properties of the fusion protein [38].

5. Conclusions

A newly developed DARPin off7 affinity matrix allows efficient purification of MBP fusion proteins. In the effort to achieve an oriented binding of DARPin off7 to the matrix and thus optimize its affinity to MBP, we prepared several mutants of DARPin off7. We showed that the relative affinity of protein-protein interaction can be assessed also by DSC using Brandt's analysis. The optimized chromatographic purification method based on the interaction between MBP and DARPin off7 provides a rapid and effective alternative approach for the purification of MBP fusion proteins. DARPin off7 expression and purification through IMAC, along with the matrix preparation, is a simple and inexpensive procedure that can be easily accomplished within three days and at a large scale. Moreover, due to the high stability of DARPin off7, the prepared matrix can be stored in a refrigerator for several weeks (and probably much longer) without affecting its binding properties.

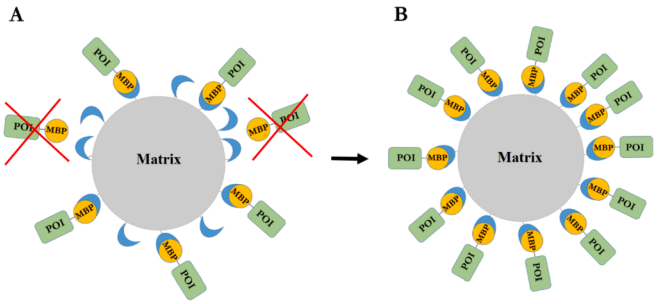

Fig. 10. Schematic representation of spatial optimization of DARPin off7 matrix after the substitutions of lysine residues located in the capping repeats of DARPin off7.

CRediT authorship contribution statement

Michal Nemergut: Investigation, Formal analysis, Writing – Original Draft; **Rostislav Škrabana**: Investigation, Formal analysis; **Martin Berta**: Investigation; **Andreas Plückthun**: Conceptualization, Writing – Review & Editing; **Erik Sedlák**: Funding acquisition, Formal analysis, Writing – Review & Editing.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2021.07.117.

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