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Affinity purification of Car9-tagged proteins on silica matrices: Optimization of a rapid and inexpensive protein purification technology

Jessica Soto-Rodríguez, Brandon L. Coyle, Ariana Samuelson, Kannan Aravagiri, François Baneyx*

Department of Chemical Engineering, University of Washington, Seattle, WA 98195, United States

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ABSTRACT

Car9, a dodecapeptide identified by cell surface display for its ability to bind to the edge of carbonaceous materials, also binds to silica with high affinity. The interaction can be disrupted with L-lysine or L-arginine, enabling a broad range of technological applications. Previously, we reported that C-terminal Car9 extensions support efficient protein purification on underivatized silica. Here, we show that the Car9 tag is functional and TEV protease-excisable when fused to the N-termini of target proteins, and that it supports affinity purification under denaturing conditions, albeit with reduced yields. We further demonstrate that capture of Car9-tagged proteins is enhanced on small particle size silica gels with large pores, that the concomitant problem of nonspecific protein adsorption can be solved by lysing cells in the presence of 0.3% Tween 20, and that efficient elution is achieved at reduced L-lysine concentrations under alkaline conditions. An optimized small-scale purification kit incorporating the above features allows Car9-tagged proteins to be inexpensively recovered in minutes with better than 90% purity. The Car9 affinity purification technology should prove valuable for laboratory-scale applications requiring rapid access to milligram-quantities of proteins, and for preparative scale purification schemes where cost and productivity are important factors.

1. Introduction

Affinity tags have transformed protein purification by converting time-intensive, multistep chromatography processes into single-step schemes that yield fairly pure polypeptides [1–4]. The primary function of a genetically (or chemically) appended affinity tag is to endow a protein of interest with the ability to reversibly bind to a ligand coupled to a stationary phase, or to directly interact with the stationary phase itself. Tags may also function as solubility enhancers, help facilitate the detection of proteins to which they are fused, and are often excisable if the fusion joint is engineered to contain elements supporting endoproteolytic or self-cleavage events [5].

The polyhistidine tag (*a.k.a.* His-tag) is one of the most extensively used affinity tag. It relies on the ability of histidines to form coordination bonds with immobilized transition metal ions (typically Ni^{2+} or Co^{2+}) and on the competitive disruption of this interaction with imidazole [6]. One of the primary advantages of the His tag is its small size which leads to little interference with the target. However, His-tagging has been reported to cause protein insolubility [7–9], alter structure and function [10], and lead to product inhomogeneity [11]. In addition, the stripping of protein-coordinated metal ions by imidazole can induce protein misfolding [12], and His-tagging remains expensive at the laboratory scale due to the cost of the resin [1].

Silica (SiO_2) is one of the most abundant and inexpensive materials on earth and underivatized silica has shown promise as a stationary phase for the preparative scale purification of proteins whose interactions with the matrix is well understood [13]. There has also been interest in the development of more versatile technologies that rely on the use of polypeptides with high affinity for silica as affinity tags [14,15]. For instance, Ikeda and coworkers have shown that fusion of the 273-residues long ribosomal protein L2 (*a.k.a.* Si-tag) to the C-terminus of *Staphylococcus aureus* protein A, allows for recovery of the chimera at better than 85% purity when it

* Corresponding author.
E-mail address: baneyx@uw.edu (F. Baneyx).

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is released from the matrix with 2 M $MgCl_2$ [16]. Similarly, fusion of the 171 amino acids-long *Bacillus cereus* CotB protein to mCherry with an intervening SUMO recognition sequence supports batch-mode binding of the fusion protein on silica beads, and intact mCherry can be released with high purity after 3 h incubation with SUMO protease [17]. The large size of L2 and CotB is a disadvantage of both approaches, as is the elution strategy (high concentrations of $MgCl_2$ for L2 and lengthy and costly SUMO protease processing for CotB). And while Abdelhamid and coworkers have found that a 14 residues-long peptide derived from the C-terminus of CotB (CotBp, SGRARAQRQSSRGR) supports the binding of mCherry to silica, SUMO protease treatment remains required for target protein release [17].

Previously, we reported that Car9 (DSARGFKKPGKR), a dodecapeptide originally isolated for its ability to bind to the edge of carbonaceous materials [18] exhibits high affinity for silica [19]. Capitalizing on this finding and on the fact that Car9 can be competitively released from silica by addition of 1 M lysine or arginine, we further demonstrated that C-terminal Car9 extensions support the microcontact printing of proteins on glass substrates [20], their self-immobilization and dynamic release from silica sol-gels [21], the assembly of functional multi-material architectures [22], and affinity protein purification on unmodified silica gels [19].

In this study, we show that Car9 is functional and tobacco etch virus (TEV) protease-excisable when fused to the N-terminal of target proteins and that it remains capable of binding to silica under denaturing conditions, although it does so with lower affinity. Additionally, we harness the current understanding of how proteins and small molecules interact with SiO_2 to optimize a simple kit that enables rapid and inexpensive affinity purification of Car9-tagged proteins at the laboratory scale on a disposable silica phase.

2. Materials and methods

2.1. Plasmids and DNA manipulations

Plasmids pET24a(+)-sfGFP-Car9 and pET24a(+)-mCherry-Car9 which encode sfGFP-Car9 and mCherry-Car9 under transcriptional control of the IPTG-inducible T7 promoter, and plasmid pBLN200-MBP-Car9, which encodes a MBP-Car9 fusion protein under transcriptional control of the arabinose-inducible P_{BAD} promoter have been described [19,23]. Plasmid pNC9, a vector suitable for inserting gene products as *Nco*I-*Xho*I or *Nco*I-*Hind*III fragments downstream of a N-terminal Car9 tag followed by a flexible GGGS linker and TEV protease cleavage site was constructed by inserting an *Nde*I-*Nco*I cassette encoding tag, linker and TEV site in the same sites of pET-22b(+). An *Nco*I-*Xho*I cassette encoding TEM1-β-lactamase was amplified by PCR using primer pair 5′-CCGTAGCCATGGATCACCCAGAAACGCTGGTG-3′ and 5′-GACCGGCTCGAG TTACCAATGCTTAATCAGTGAGGC-3′ and plasmid pET-22b(+) as a template. An *Nco*I-*Hind*III cassette encoding sfGFP was amplified using primer pair 5′- GGGCCATGGGGCGTAAAGGCGAAGAGCTGTTC-3′ and 5′- GCCAAGCTTTTAT TTGTACAGTTCATCCATACC-3′ and plasmid pET24a(+)-sfGFP-Car9 [19] as a template. Both genes were inserted into the same sites of pNC9 to produce plasmids pET-22b(+)-Car9-β-lactamase and pET-22b(+)-Car9-sfGFP. The Car9-β-lactamase gene was transferred to pET-24a(+) by inserting the small *Nde*I-*Xho*I fragment of pET-22b(+)-Car9-β-lactamase into the same sites of that vector.

2.2. Optimizing elution pH and silica matrix

Seed cultures (25 mL) of BL21(DE3) cells (Novagen) harboring plasmid pET24a(+)-sfGFP-Car9 were used to inoculate 500 mL of LB medium supplemented with 50 μg/mL of kanamycin. Cells were grown to mid exponential phase (A_{600} ~ 0.5) at 37 °C and protein synthesis was induced by addition of 1 mM IPTG. After 3 h of cultivation at 37 °C, cells were harvested by centrifugation at 7000*g* for 10 min, resuspended in 35 mL of 20 mM Tris-HCl, pH 7.5, 2 mM EDTA (lysis buffer), and disrupted by six round of sonication for 3 min at 30% duty cycle using a Branson sonifier. Insoluble material was removed by centrifugation at 10,000*g* for 10 min. To test the effect of pH, clarified lysate (1.5 mL) was contacted with 1.5 mL of silica slurry (Sigma Merck Grade 7734, 6 nm pore size, 60–200 μm particle size) for 5 min on a rotary mixer operated at room temperature. The slurry was loaded on a 0.5 cm ID glass column (Bio-Rad), and 10 mL of 20 mM Tris-HCl, pH 7.5 was added to the top of the bed. A 5 mL syringe was connected to the outlet of the column and the fluid was collected by aspiration to remove non-specifically bound proteins. Bound sfGFP-Car9 was eluted by aspiration of successive 0.5 mL fractions using 20 mM Tris-HCl, pH 6.0–9.0 supplemented with 1 M L-lysine. The influence of silica particle dimension and pore size was additionally tested using Davisil 646 (15 nm pore size, 250–500 μm particle size), Davisil 636 (6 nm pore size, 250–500 μm particle size) and Davisil 643 (15 nm pore size, 35–70 μm particle size) as above except that protein elution was only conducted with 20 mM Tris-HCl, pH 8.25 supplemented with 1 M L-lysine. All silica powders were extensively washed in lysis buffer before use.

2.3. Optimizing small-scale purification

Seed cultures (5 mL) were used to inoculate 25 mL of LB medium supplemented with 50 μg/mL kanamycin or 100 μg/mL carbenicillin. Mid-exponential phase cultures were treated with 1 mM IPTG (pET derivatives) or 0.2% arabinose (pBLN200 derivatives) and recombinant proteins were allowed to accumulate for 3 h at 37 °C, or for 5 h at 30 °C in the case of Car9-β-lactamase due to its lower solubility. Culture samples (5 ml) were subjected to centrifugation at 7000*g* for 10 min. The paste was resuspended in 3 mL of 20 mM Tris-HCl, pH 7.5, 2 mM EDTA (lysis buffer), and cells were disrupted with a French press operated at 10000 psi. Lysates were clarified by centrifugation at 10000*g* for 10 min. To optimize operational parameters, 1 mL of silica slurry and 3 mL of clarified lysate from cells expressing sfGFP-Car9 were added to conical 0.8 × 4 cm polypropylene disposable columns (BioRad). After 10 min of mixing at 23 °C on a rotary mixer, a stopcock and syringe were connected to the outlet of the column and contaminating proteins were removed by aspirating 15 mL of 20 mM Tris-HCl, pH 7.5 through the bed. Successive elution fractions (0.5 mL) were collected by aspiration after supplying the column with 5 mL of 20 mM Tris-HCl, pH 8.25 supplemented with 0.5 M or 1 M L-lysine. The effect of glycerol and NaCl was tested by adding 10% glycerol or 250 mM NaCl to the lysis, wash and elution buffer. For these experiments, elution was conducted with 20 mM Tris-HCl, pH 8.25 supplemented with 0.5 M lysine. The effect of Tween 20 was tested by preparing clarified extracts of sfGFP-Car9, Car9-β-lactamase, MBP-Car9 and mCherry-Car9 as above and adding 0.1 or 0.3% (v/v) of the surfactant to the lysis buffer. Target proteins were eluted as above.

2.4. Removal of N-terminal Car9 tags with TEV protease

Car9-β-lactamase and Car9-sfGFP purified using the small-scale system under optimized conditions were dialyzed against 2 L of 20 mM Tris-HCl, pH 7.5, and concentrated with 10-kDa cutoff ultracentrifugal filters (Amicon). Protein concentration was determined using a BCA Assay (Thermo Scientific) with BSA as a standard. TEV protease with an N-terminal His tag [24] was purified by Ni-NTA affinity chromatography. The protease (1.5 μg) was mixed with 25 μg of target proteins (0.5 μM TEV to ~8 μM protein)

in the presence of 5 mM 2-mercaptoethanol. All digestions were conducted for 16 h at 23 °C. For experiments performed in the presence of lysine, 94 μL of the second eluted fraction was mixed with 1 μL of 1.7 mg/mL TEV protease and 5 μL of 20 mM Tris-HCl, 0.5 M L-lysine and 5 μL of 100 mM 2-mercaptoethanol in the same buffer for a final concentration of 5 mM.

2.5. Purification of Car9-β-lactamase under denaturing conditions

Car9-β-lactamase was expressed as above except that the temperature was raised to 37 °C to promote aggregation (~65% insoluble). Culture samples (5 ml) were subjected to centrifugation at 7000*g* for 10 min. The paste was resuspended in 3 mL of 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, and cells were disrupted with a French press operated at 10000 psi. Lysates were centrifuged at 1500*g* for 10 min to remove unbroken cell and the supernatant was subjected to centrifugation at 10000*g* for 10 min. The pellet was washed in 3 mL of 20 mM Tris-HCl, pH 7.5, 2 M urea and 0.5% Tween 20 and with 20 mM Tris-HCl, pH 7.5 with intervening cycles of centrifugation at 10000*g* for 10 min. Washed inclusion bodies were solubilized in 20 mM Tris-HCl, pH 7.5, 8 M urea and 0.3% Tween 20 for 1 h at 23 °C with gentle shaking. The solution was centrifuged at 10000*g* for 10 min to remove remaining aggregates. Unfolded proteins were contacted with silica slurry and subjected to small-scale purification as above, except that all buffers were supplemented with 8 M urea.

3. Results and discussion

3.1. Car9 is functional as an N-terminal affinity tag

We previously reported that fusion of the Car9 sequence to the C-termini of sfGFP, MBP and mCherry enables efficient affinity purification of these proteins on silica gel when 1 M L-lysine or L-arginine is used as an eluent [19]. To determine if the tag would remain functional in an N-terminal position, we fused the Car9 extension to the N-terminus of sfGFP, inserting a flexible GGGS linker and a TEV protease cleavage site between the two domains (Fig. 1A). Clarified extracts from cells overexpressing the Car9-sfGFP fusion protein were loaded on a Merck 7734 silica column equilibrated in Tris-HCl buffer, pH 7.5. The bound material was eluted with 1 M L-lysine after contaminating proteins had been removed by washing. This operation reproducibly led to the recovery of 60–75% pure Car9-sfGFP depending on the fraction (Fig. 1B), proving that Car9 is fully functional as an N-terminal affinity tag. Under the same experimental conditions, sfGFP-Car9 was recovered with a purity of 75–95% and with a slightly different pattern of contaminants (Fig. 1C–D). Considering that both fusion proteins are expressed at comparable levels (about 40% of the total cell protein and thus at an effective 40% purity), this result suggests that the orientation in which the Car9 sequence of amino acids is presented to silica, and/or the local structural or chemical context experienced by the tag, influences silica binding affinity. Of note, although both Car9-sfGFP and sfGFP-Car9 continued to elute after two column volumes of buffer had flowed through, it was possible to achieve quantitative release of sfGFP-Car9 by increasing the lysine concentration to 2 M (Fig. S1). We therefore embarked on a campaign of buffer and stationary phase optimization to maximize the purity and yields of Car9-tagged proteins.

3.2. Alkaline conditions improve the efficiency of sfGFP-Car9 elution

Silica gels are amorphous materials with variable porosity in which silicon atoms are tetrahedrally coordinated to oxygen atoms through siloxane bonds (Si—O—Si). The surface of silica contains

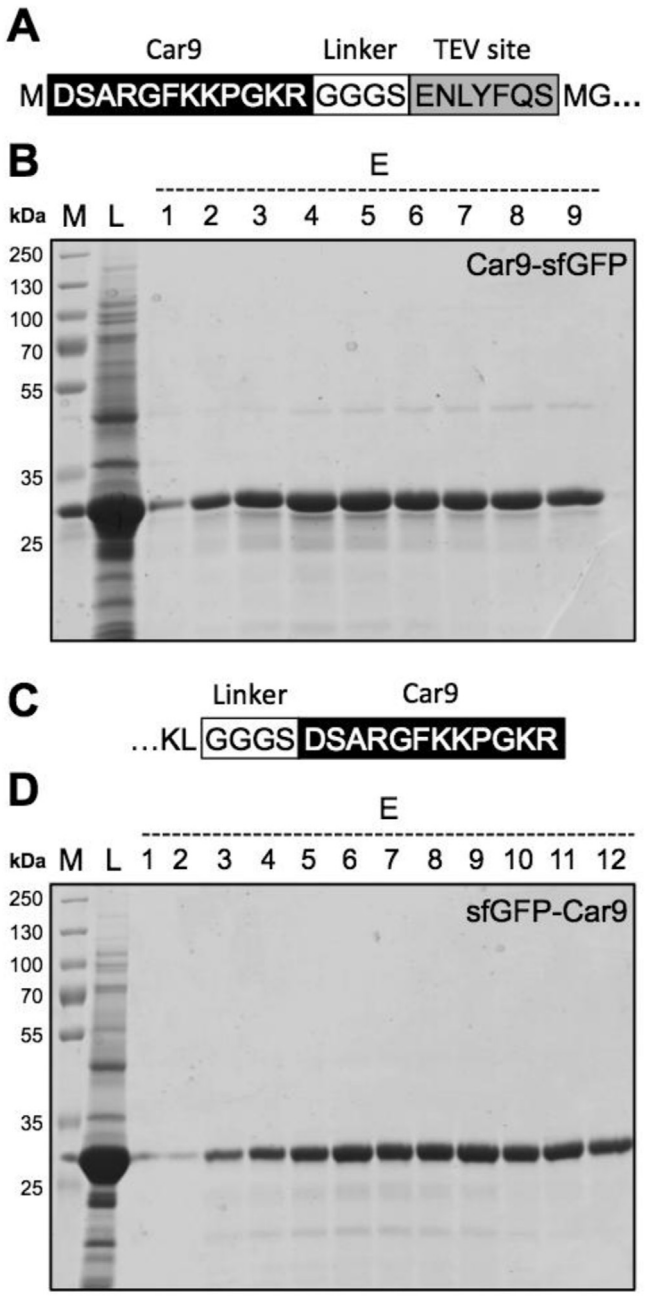

Fig. 1. (**A**) Structure of the N-terminal Car9 extension. The sfGFP sequence starts with Met-Gly. (**B**) Purification of Car9-sfGFP on Merck 7734 silica with 1 M lysine elution at pH 7.5. (**C**) Structure of the C-terminal Car9 fusion. The sfGFP sequence ends with Lys-Leu. (**D**) Purification of sfGFP-Car9 on Merck 7734 silica with 1 M lysine elution at pH 7.5. Lanes: M, markers; L, load; E, successive elution fractions.

both hydrophobic siloxane sites [25], and unreacted, hydrophilic hydroxyls called silanols (Si—OH). These silanols can be found in isolated (single), geminal (silanediol) or vicinal (H-bonded) forms. The concentration and distribution of these species depends on the mode of silica preparation and influences adsorption properties [26]. In addition, deprotonation of silanol groups at pH higher than ~3 (the point of zero charge for silica) leads to their ionization to SiO^-, and to the development of an increasingly negative surface charge as the solution becomes more basic [27–30]. For instance, the degree of ionization of 80 nm SiO_2 nanoparticles increases from 9% at pH 5, to 18% at pH 7.4, reaching a maximum of 50% at a pH

somewhat above 8.5 [28,30].

The nonspecific binding of proteins to silica is dominated by a combination of electrostatic and hydrophobic interactions, although H-bonding, van der Waals interactions and structural flexibility also play significant roles [13,31–34]. Similar mechanisms appear to control the binding of combinatorially-selected silica-binding peptides to SiO_2 [28,35]. In these specific adsorption schemes, binding affinity can be influenced by the extent of surface ionization. As an example, molecular dynamics (MD) simulations have revealed that a silica-binding peptide of sequence KLPGWSG strongly adsorbs to SiO_2 through electrostatics and H-bonding under conditions of high silanol ionization, but exhibits much weaker affinity for less ionized surfaces [30]. Not surprisingly, the adsorption of L-lysine to silica also increases with the solution pH, and therefore with the extent of surface ionization [36]. On the basis of these reports and our empirical observation that the release of Car9-tagged proteins entrapped within silica sol-gels improves under basic conditions [21], we reasoned that lysine might be most effective at disrupting Car9-silica interactions under conditions of high pH where electrostatics dominate.

To test this hypothesis, clarified extracts from cells over-expressing sfGFP-Car9 were loaded onto Merck 7734 silica, and the matrix was washed to remove nonspecifically bound proteins as above. Lysine elution (1 M) was conducted on replicate samples over a range of 3 pH units. Fig. 2A shows that elution under neutral or acidic conditions significantly reduced the recovery yields of sfGFP-Car9, with low amounts of protein eluting over many fractions. Conversely, increasing the pH of the elution buffer to 8.0–8.5 led to quantitative release of sfGFP-Car9 in a small number of highly concentrated and fairly pure fractions (Fig. 2B). To minimize potential problems with protein unfolding under alkaline conditions, we selected an elution pH of 8.25 for all follow-on experiments.

3.3. Small size, large pore silica particles are optimal for the purification of Car9-tagged proteins

A wide variety of inexpensive silica gels with different particle and pore sizes are commercially available. While the experiments of Figs. 1 and 2 were conducted with 63–200 μm particles with 6 nm pores (Merck 7734), we used three additional silica gels to further investigate the influence of size distribution and porosity on sfGFP-Car9 recovery.

Fig. 3A shows that the elution profile of sfGFP-Car9 from Davisil 636 was similar to that observed with Merck 7734 silica (Fig. 2A, pH 8), except that less protein was present in successive fractions. Considering that the two silica gels have the same porosity, this result is well explained by a decrease in surface area available for sfGFP-Car9 binding on the larger Davisil 636 particles. Conversely, increasing the pore size to 15 nm while maintaining particle size at 250–500 μm provided additional binding sites for the Car9 extension, and led to more concentrated fractions (Fig. 3B). Finally, and as expected from the comparison of Merck 7734 and Davisil 636 matrices, we observed a further improvement in yields with Davisil 643 whose particles have the same 15 nm pore size as Davisil 646 but are an order of magnitude smaller (Fig. 3C). Although the use of such small particle size, large pore silica was accompanied by an increase in nonspecific protein binding and a shift to higher elution volumes, the Davisil 643 matrix provided the best outcome (defined here as a high concentration of pure protein over a small number of fractions) and was selected for subsequent experiments.

3.4. Optimization of the small-scale Car9 purification technology

We previously demonstrated that the Car9 affinity purification technology can be implemented in a small scale, disposable format that enables rapid and inexpensive protein purification [19]. We improved on our original design as described in Materials and Methods, building a kit that uses 1 mL of Davisil 643 slurry. In an effort to minimize the amount of costly L-lysine used in the elution step, we first investigated how reducing the concentration of this amino acid from 1 M to 0.5 M would impact sfGFP-Car9 elution at pH 8.25. Fig. 4 shows that the use of the lower lysine concentration led to a 0.5 mL delay in the onset of sfGFP-Car9 elution and to a slight broadening of the elution peak. A likely explanation for this result is that too few lysine molecules are initially available to occupy all Car9 binding sites in the region of the bed that is first

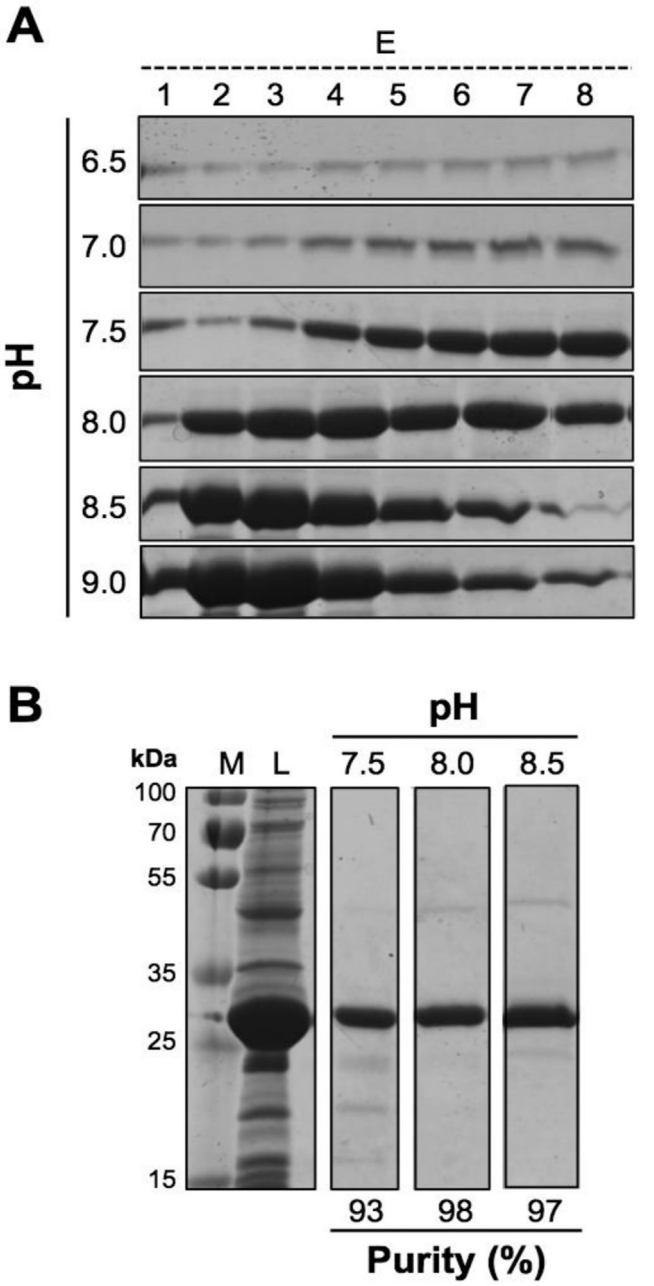

Fig. 2. (**A**) SDS-PAGE analysis of sfGFP-Car9 fractions eluted with 1 M lysine at the indicated pH. Successive elution fractions (E) are shown. (**B**) Comparison of the purity of eluted fractions at comparable sfGFP-Car9 concentrations, as determined by videodensitometric analysis. Lanes: M, markers; L, load.

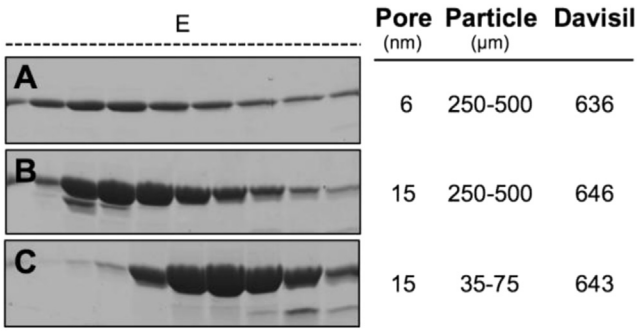

Fig. 3. Clarified extracts from cells overexpressing sfGFP-Car9 were loaded onto Davisil 636 (**A**), Davisil 646 (**B**) or Davisil 643 (**C**). The fusion protein was eluted with 1 M lysine in 20 mM Tris-HCl, pH 8.25. Successive elution fractions (E) are shown, as are the characteristics and commercial names of the silica gels.

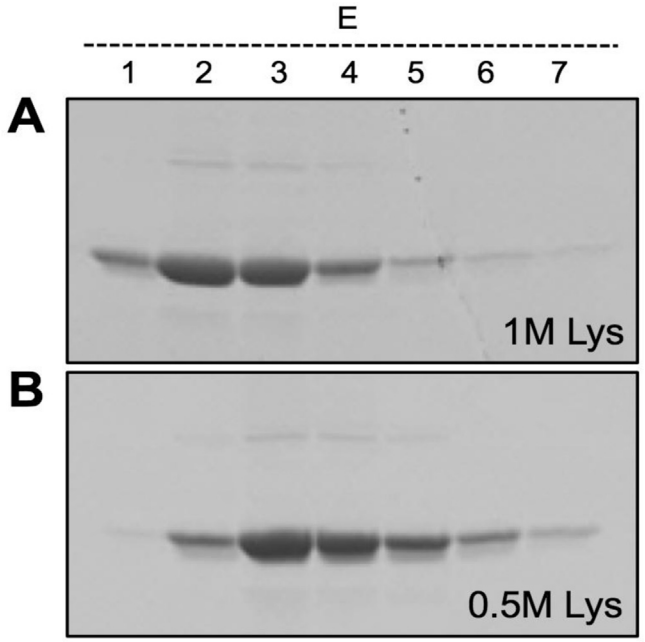

Fig. 4. Influence of the lysine concentration on the elution of sfGFP-Car9 in a small-scale purification system. Clarified extracts from cells expressing sfGFP-Car9 were loaded onto 1 mL of Davisil 643 slurry and the protein was eluted with 1 M (**A**) or 0.5 M lysine (**B**) in 20 mM Tris-HCl buffer pH 8.25. Successive elution fractions (E) are shown.

percolated by the eluent. As the mobile phase brings in additional lysine molecules, competition for site occupancy increases and Car9 fusion protein rebinding events are progressively eliminated in the local zone. The phenomenon repeats itself down the length of the bed, causing a lag in elution. Making use of smaller silica particles similarly delays sfGFP-Car9 breakthrough because it increases the number of Car9 binding sites that require lysine passivation (Fig. 3B–C). Bearing in mind that the economic benefits of a lower lysine concentration far outweigh the inconvenience of collecting more fractions, we conducted all subsequent optimization steps using an elution buffer supplemented with 0.5 M L-lysine.

We next turned our attention to the suppression of nonspecific binding events. As mentioned earlier, proteins adsorb to silica nonspecifically through a combination of electrostatic and hydrophobic interactions that are supplemented by H-bonding and van der Waals interactions [13,31,32]. We therefore explored how the presence of NaCl, a charge-screening agent that modulates protein-protein and protein-silica interactions [37,38], and that of glycerol, a co-solvent that increases protein compactness [39] and interacts with large patches of contiguous surface hydrophobicity [40], would impact sfGFP-Car9 purity.

Supplementing the lysis, wash and elution buffers with 250 mM NaCl had little impact on sfGFP-Car9 purity. However, inclusion of 10% glycerol improved the purity of both sfGFP-Car9 (Fig. 5A) and MBP-Car9 (Fig. S2). By contrast, while a Car9-β-lactamase fusion protein efficiently bound to silica (confirming that Car9 is functional as an N-terminal tag), glycerol did not appreciably suppress nonspecific protein binding and the protein eluted with very low purity (Fig. 5B). We attribute this outcome to the fact that sfGFP-Car9 and MBP-Car9 represent 30–40% of the total protein in clarified cell lysates while Car9-β-lactamase only accounts for ~15%. Because they are present at higher molarities, sfGFP-Car9 and MBP-Car9 will occupy 2-to-3 times as many Car9 binding sites as Car9-β-lactamase will, and possibly more since tethered moieties might block neighboring sites via steric hindrances. Under such conditions of high silica surface coverage, glycerol effectively suppresses residual interactions between host proteins and silica. The beneficial effect of the additive is however lost when fewer Car9-β-lactamase molecules bind to the surface as there are more opportunities for host proteins to adsorb/unfold at the silica interface. In agreement with this hypothesis, reducing the number of Car9 binding sites by making use of smaller pore, larger size Davisil 636 particles significantly improved the purity of Car9-β-lactamase (Fig. S3A). The use of this matrix was however not pursued since it led to an unacceptable reduction in binding capacity (about 50% for sfGFP-Car9 based on fluorescence; Fig. S3B).

Tween 20 is a nonionic polysorbate surfactant that is commonly used as a blocking agent in the immunological detection of proteins and to prevent non-specific binding in protein purification schemes [41,42]. Because it lacks a charged head group, Tween 20 has a much lower critical micelle concentration that anionic detergents, and because it cannot reach a high enough monomer concentration to cooperatively bind to proteins, it does not promote their denaturation [43]. At concentrations lower than ~0.4% (v/v), Tween 20 strongly binds to silica through its polyol head, producing a hydrophobic surface studded with progressively denser alkyl chains as the concentration increases [44].

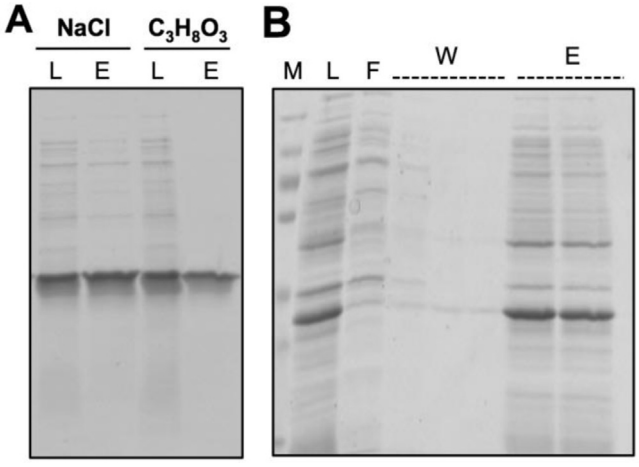

Fig. 5. Influence of NaCl and glycerol on nonspecific protein binding. (**A**) Clarified extracts from cells expressing sfGFP-Car9 were loaded onto 1 mL of Davisil 643 slurry and the target protein was eluted in 20 mM Tris-HCl buffer pH 8.25 supplemented with 0.5 M lysine and 250 mM NaCl (NaCl) or 10% glycerol ($C_3H_8O_3$). (**B**) Clarified extracts from cells expressing Car9-β-lactamase were loaded and eluted as above using buffers supplemented with 10% glycerol. Lanes: M, markers; L, load; W, wash; E, elution fractions.

To determine if the surfactant would improve the purity of Car9-β-lactamase, we processed cell paste in the presence of 0.1 or 0.3% (v/v) Tween 20 and contacted clarified lysates with silica slurry as above. The surfactant was not included in the wash and elution buffers to minimize its carry-over concentration in eluted fractions. Fig. 6A shows that whereas the use of 0.1% Tween 20 caused a slight improvement in Car9-β-lactamase purity, a significant amount of contaminating proteins still eluted with the target protein. By contrast, using 0.3% Tween 20 yielded an ~90% pure target. Importantly, the surfactant did not interfere with Car9-β-lactamase binding, as little protein was found in the flow-through (Fig. 6A). The observed improvement in purity may be related to the effective passivation of silica at high Tween concentrations through the formation of irreversible complexes between hydrophobic regions of host proteins and projecting surfactant brushes. The remaining contaminant at ~43-kDa is likely to be an RNA-binding protein (a class of polypeptides prone to nonspecific interactions with silica [34]) that is abundant enough to compete with both Tween molecules and Car9 fusion proteins for binding to silica.

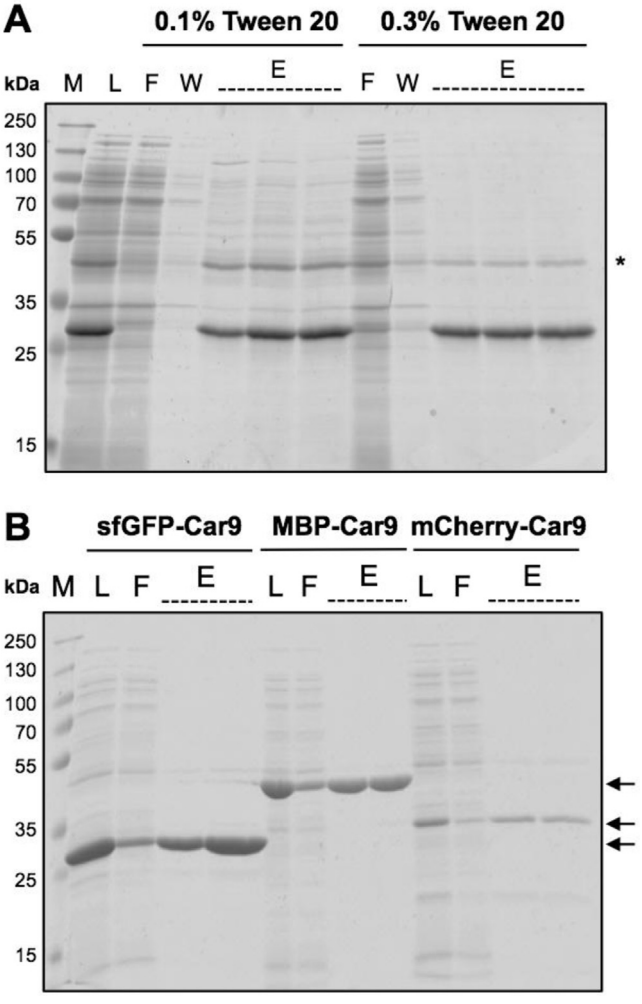

Fig. 6. Influence of Tween 20 on nonspecific protein binding. (**A**) Clarified extracts from cells expressing Car9-β-lactamase were prepared in lysis buffer supplemented with 0.1% or 0.3% (v/v) Tween 20. Proteins were loaded onto 1 mL of Davisil 643 slurry and elution was conducted with 0.5 M Lysine in 20 mM Tris-HCl, pH 8.25. The star denotes the main contaminant. (**B**) Clarified extracts from cells expressing sfGFP-Car9, MBP-Car9 or mCherry-Car9 were prepared in lysis buffer supplemented with 0.3% Tween 20 and loaded and eluted as above. Arrows indicate the migration positions of the purified proteins. Lanes: M, markers; L, load; F, flow through; E, elution fractions.

Finally, we verified that supplementation of the lysis buffer with 0.3% Tween 20 did not interfere with the recovery of highly expressed proteins (sfGFP-Car9 and MBP-Car9), and demonstrated that it was beneficial to the purification of another low-expressing protein (mCherry-Car9). As shown in Fig. 6B, all targets could be recovered with purities ranging from 93 to 99%, as judged by videodensitometric analysis of the gels. However, we found by measuring GFP fluorescence in the various fractions that inclusion of 0.3% Tween reduced the binding of the high-expressing sfGFP-Car9 to silica by 23% and that an additional 9% of the material was lost during column wash, reducing overall yields by ~35% (from about 1 mg to 0.63 mg for a 5 mL culture).

3.5. The Car9 tag is processed by the TEV protease in the absence and presence of lysine

Removal of affinity tags is necessary if a protein with (near) native sequence is required, or if the tag interferes with structure or function [2]. Previously, we have shown that it is possible to excise a C-terminal Car9 tag preceded by paired basic residues by incubating purified fusion protein with whole *E. coli* cells expressing the outer membrane protease OmpT on their surface [19]. Although this approach may reintroduce contaminating proteins in the preparation and may lead to nonspecific cleavage, it is effective, inexpensive, and only extends the target protein C-terminus by one basic residue.

For the removal of N-terminal tags, the TEV protease is often preferred because of its stringent specificity, and the fact that it only adds a single serine residue to the N-terminus of the target [5]. To determine if TEV protease would process the Car9 extension, we purified Car9-sfGFP and Car9-β-lactamase (which both contain a TEV recognition sequence downstream of Car9; Fig. 1A) using small-scale purification under optimized conditions. Fig. 7 shows that the TEV protease processed about 80–90% of Car9-β-lactamase and 65% of Car9-sfGFP under our experimental conditions whether or not digestion was conducted in the presence of 0.5 M lysine.

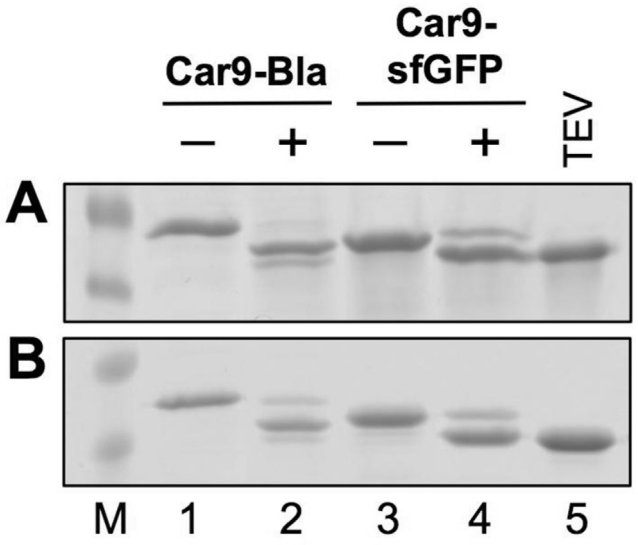

Fig. 7. Excision of Car9 tags by TEV protease before (**A**) or after (**B**) removal of lysine by dialysis. Samples were supplemented (+) or not (–) with 0.5 μM of TEV protease and digested for 16 h at room temperature. Note that the TEV protease and processed Car9-sfGFP migrate at the same position (lanes 4). However, the contribution of the protease signal, which corresponds to the faint lower bands in lanes 2, is minimal. Lanes 5 contains overloaded TEV protease as a marker.

3.6. The Car9 tag retains partial functionality under denaturing conditions

A relatively small number of affinity tags support protein purification under denaturing conditions, either because the tag does not require an ordered conformation to bind to immobilized ligands (e.g., polyhistidine [41], polyarginine [45] and polylysine [46] tags), or because it remains structured under conditions where most polypeptides unfold (e.g., cellulose-binding domain [47]). The Si-tag, which is believed to be disordered [48], has also been found to function under denaturing conditions [42], encouraging us to explore the possibility that Car9 might be useable in the presence of high concentrations of chaotropic agents.

To test this hypothesis, we unfolded Car9-β-lactamase inclusion bodies in 8 M urea and applied the solubilized material to a small-scale silica column, conducting all steps in the presence of the same concentration of denaturant. Videodensitometric analysis of the various fractions revealed that about 70% of the protein was lost during the load and wash steps (Fig. 8). However, the remainder of the material bound to the matrix and could be eluted free of contaminants with 0.5 M lysine (Fig. 8). Because high urea concentrations do not prevent the Si-tag from binding to silica [42], the inefficient capture of Car9-β-lactamase under denaturing conditions is unlikely to be due to silica-chaotrope interactions. Rather, we believe that high-affinity binding of the Car9 dodecapeptide requires that it adopts a specific conformation at silica surfaces.

4. Conclusions

Building on a previous study [19], we have shown that Car9 is a versatile affinity tag that supports protein purification on unmodified and inexpensive silica gels. The tag may be appended to the C- or N-termini of target proteins. It is excisable from the latter position via TEV protease digestion, and it retains partial silica-binding affinity under denaturing conditions. We have further demonstrated that capture of Car9-tagged proteins can be enhanced by appropriate selection of the silica matrix, and that efficient elution can be achieved at reduced lysine concentration and under alkaline conditions. Finally, we have determined that inclusion of 0.3% Tween 20 in the lysis buffer leads to product purities higher than 90%. An optimized small-scale purification kit incorporating the above embellishments enables the recovery of high-purity Car9-tagged proteins in minutes and at very low cost. The Car9 affinity purification technology should prove particularly valuable for laboratory-scale purification from small culture samples (5 mL), and for commercial scale purification since both processes are sensitive to cost and time.

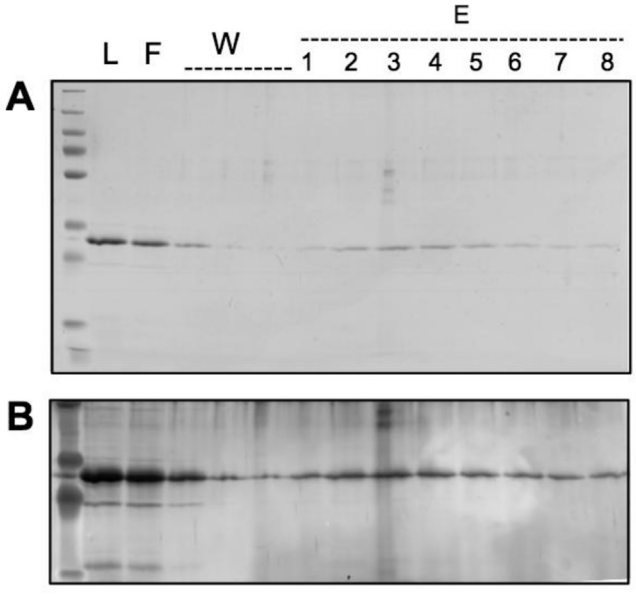

Fig. 8. Coomassie blue- (**A**) and corresponding silver-stained (**B**) minigel of various fractions collected during the affinity purification of Car9-β-lactamase under denaturing conditions. Lanes: L, load; F, flow through; W, wash; E, successive elution fractions (0.5 mL). All steps were conducted in the presence of 8 M urea.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.pep.2017.05.003.

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