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Published

2017

Journal Title

Biotechnology and Bioengineering

Version

Accepted Manuscript (AM)

DOI

[10.1002/bit.26079](https://doi.org/10.1002/bit.26079)

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**Non-Chromatographic Bioprocess Engineering of a Recombinant
Mineralizing Protein for the Synthesis of Silica Nanocapsules**

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Running title: Chromatography-Free Mineralizing Protein Production

Abstract

Inspired by Nature, synthetic mineralizing proteins have been developed to synthesize various structures of silica-based nanomaterials under environmentally-friendly conditions. However, the development of bioprocesses able to assist in the translation of these new materials has lagged the development of the materials themselves. The development of cost-effective and scalable bioprocesses which minimize reliance on chromatography to recover biomolecules from microbial cell factories remains a significant challenge. This paper reports a simplified purification process for a recently-reported recombinant catalytic modular (D4S2) protein (M(DPSMKQLADS-LHQLARQ-VSRLEHA)₄EPSRKKRKKRKKRKKGGGY; *M* 13.3 kDa; *pI* 10.9), which combines a variant of the established designer biosurfactant protein DAMP4 with a new biomimetic sequence (RKKRKKRKKRKKGGGY), providing for a bi-modular functionality (emulsification and biosilicification). The four-helix bundle structure of the protein has been demonstrated to remain stable and soluble under high temperature and high salt conditions, which confers simplified bioprocessing character. However, the high positive charge on the biosilification sequence necessitates removal of DNA contaminants from crude cell-extract at an early stage in the process by adding poly(ethyleneimine) (PEI). In this process, cellular protein contaminants were selectively precipitated by adding Na₂SO₄ to the protein mixture up to a high concentration (1 M) and mixed at high temperature (90°C, 5 min) where D4S2 remained stable and soluble due to its four-helix bundle structure. Further increase of the Na₂SO₄ concentration to 1.8 M precipitated, thus separated, D4S2 from residual PEI. The overall yield of the protein D4S2 was 28.8 mg per 800 mL cells (final cultivation OD₆₀₀ ~2) which gives an approximate 79% D4S2-protein yield. In comparison with the previously-reported chromatographic purification of D4S2 protein (Wibowo et al., 2015), the final yield of D4S2 protein is increased fourfold in this study. The bio-produced protein D4S2 was proved to retain its emulsification and biosilicification functionalities enabling the formation of oil-core

silica-shell nanocapsules at near-neutral pH and room temperature without the use of any toxic organic solvents, confirming no adverse effects due to bioprocess simplification. This work demonstrates that, through proper bioprocess engineering including the removal of critical contaminants such as DNA, a more efficient, simple and scalable purification process can be used for the high-yield bio-production of a recombinant templating protein useful in the synthesis of bio-inspired nanomaterials. This simplified process is expected to be easily adapted to recover other mineralizing helix bundle-based functional proteins from microbial cell factories.

Keywords: selective precipitation; non-chromatography; recombinant; mineralizing proteins; silica; nanocapsules

Introduction

Advances in genomics and proteomics have enabled the identification of genes, peptide and protein sequences enabling silica biomineralization hence forming endogenous silica on, for example, spicules of sponges (Cha et al., 1999) and cell walls of diatoms (Kröger et al., 1999). Although silica only provides structural support and protection for the organisms, it is the inherent functionality of the mineralizing proteins to catalyze nucleation and growth of silica under physiological conditions (i.e., biosilicification) that has garnered attention from academic and industrial communities (Chen & Rosi, 2010; Dickerson et al., 2008; Sarikaya et al., 2003). This green process to synthesize controlled silica structures in biological systems is in contrast to those traditional silica-formation methods *in vitro* which usually require chemical catalysts and organic solvents at extreme pH and/or temperature. Fundamental investigations on biomineralizing silicatein- α (Cha et al., 1999; Zhou et al., 1999) and silaffin (Kröger et al., 1999) proteins extracted from sponges and diatoms, respectively, demonstrated that nucleophilic and cationic amino acid residues played key roles in biosilicification. In turn, numerous studies have utilized proteins or peptides with tailored amino acid sequences to advance bio-inspired synthesis of silica-based nanomaterials having various structures *in vitro*, e.g., nanoparticles, nanocapsules and nanotubes, for applications in optical (Brott et al., 2001), sensing (Graf et al., 2011), delivery (Wibowo et al., 2014b), biocatalysis (Betancor & Luckarift, 2008; Chien & Lee, 2008; Luckarift et al., 2004), tissue regeneration (Mieszawska et al., 2010) and living-cell encapsulation (Park et al., 2015).

There is an increasing demand for developing feasible and economical methods for producing mineralizing peptides or proteins. Two methods are commonly employed: chemical and biotechnological syntheses. Chemical synthesis based on solid-phase chemistry (Merrifield, 1963) is rapid and effective for the production of short-chain peptides in small quantities. It is not generally cost-effective for large-scale production, especially for sequences

of over 35 amino acids, and it also poses sustainability problems as it employs toxic chemicals (Andersson et al., 2000; Latham, 1999). Biotechnological synthesis based on recombinant protein production in microbial cell factories, such as *Escherichia coli* (*E. coli*), offers sustainability and ease of process scale-up using established bioprocess unit operations, thus avoiding those problems highlighted in chemical synthesis routes. However, production of peptides in microbial expression systems is generally difficult as they can self-associate to form insoluble aggregated materials (Kyle et al., 2009) or else intracellularly degrade by proteases (Itakura et al., 1977) due to their small size and lack of tertiary structure. In addition, as mineralizing peptides or proteins normally have high cationic charge density, their strong interactions with anionic cellular compounds such as nucleic acids (Olins et al., 1967) or membrane lipopolysaccharides (Rietschel et al., 1994) under neutral pH render their recovery and isolation challenging. These problems are usually addressed by the fusion of the mineralizing biomolecules to carrier proteins, e.g., poly(histidine) (Sano et al., 2010; Tahir et al., 2004; Wong Po Foo et al., 2006; Zhou et al., 2015), maltose-binding protein (Zhou et al., 1999), and ketosteroid isomerase (Zerfaß et al., 2015) tags, to facilitate downstream processing reliant mainly on chromatography. The fusion tags can either be subsequently cleaved with further purification or be left combined with the target biomolecules as long as retention does not compromise intended functionality. Despite their wide utility, most chromatographic supports have associated capacity and diffusional limitations, and especially high cost related to media replacement cost and relatively long cycle times (Azevedo et al., 2009). Alternative methods that minimize the use of chromatography and enable the cost-effective, rapid and scalable manufacture of mineralizing biomolecules are urgently needed.

Four-helix bundle proteins have attracted considerable attention (Baltzer et al., 1999) because of their high molecular stability under extreme conditions, e.g, high temperature (Akanuma et al., 2010) and concentrated chemical denaturants (Regan & Degrado, 1988). Our

laboratory has developed a new class of four-helix bundle proteins as protein surfactants i.e., DAMP4 (MD(PSMKQLADS-LHQLARQ-VSRLEHAD)₄) that can be expressed at high levels of stability and solubility in recombinant *E. coli* and then recovered from *E. coli* and purified either by using a chromatographic method (Middelberg & Dimitrijevic-Dwyer, 2011) or by using a thermal cell-breakage and precipitation-based purification method (Dwyer et al., 2014; Zhao et al., 2015). These unique properties have allowed versatile utilities of DAMP4 protein including as a biosurfactant itself (Dimitrijevic-Dwyer et al., 2012; Middelberg & Dimitrijevic-Dwyer, 2011) and as a carrier protein to produce an antimicrobial peptide pexiganan (Zhao et al., 2015).

Recently, we modularized a DAMP4 protein variant (M(DPSMKQLADS-LHQLARQ-VSRLEHA)₄EPS) at the DNA level with a Si peptide (RKKRKKRKKRKKGGGY) derived from SurSi peptide (Wibowo et al., 2014a) to develop a recombinant mineralizing D4S2 protein (M(DPSMKQLADS-LHQLARQ-VSRLEHA)₄EPSRKKRKKRKKRKKGGGY) (*M* 13.3 kDa; *pI* 10.9) (Wibowo et al., 2015). The modular combination of surface-active DAMP4 protein variant module and biosilicification-active Si peptide module have allowed the D4S2 protein to stabilize nanoemulsions and concurrently catalyze formation of silica shells encapsulating the nanoemulsion droplets under environmentally-friendly conditions, hence forming oil-core silica-shell nanocapsules. The D4S2 protein expressed in recombinant *E. coli* was purified from the cell lysate using a chromatographic method.

In this work, we aim to develop a chromatography-free purification method for cost-effective, scalable and high-yield bio-production of D4S2 protein from recombinant *E. coli*. The dual functionalities (emulsification and biosilicification) of the purified D4S2 protein will be investigated for making oil-core silica-shell nanocapsules. The chromatography-free downstream processing method reported in this work has the potential to be generalized to other helix bundle protein designs comprising various kinds of functionalities.

Materials and Methods

Materials

Poly(ethyleneimine) (PEI) 50% (w/v) in H₂O was obtained from Sigma-Aldrich (Cat. no. P3143). A stock solution of 5% (w/v) PEI in H₂O was prepared at pH 8 by adding concentrated HCl followed by filtration through a 0.45 µm filter then stored at 4°C until further use. Miglyol 812 purchased from Caesar & Loretz GmbH (Hilden, Germany) was passed through heat-activated silica gel (Sigma-Aldrich) prior to use. Other chemicals were of analytical grade purchased from either Sigma-Aldrich or Merck and used as received unless otherwise stated.

Protein Expression

The expression vector generated by inserting a nucleotide sequence encoding D4S2 protein in the pET-48b(+) plasmid (Novagen Merck Biosciences, Germany) was provided by the Protein Expression Facility at The University of Queensland. The expression vector was transformed into chemically competent *E. coli* strain BL21(DE3) which was then cultivated as previously described (Wibowo et al., 2015). Briefly, a single colony selected from a freshly streaked Luria Bertani (LB) agar plate (10 g/L NaCl, 10 g/L tryptone, 5 g/L yeast extract, 15 g/L agar, water) was inoculated into 5 mL LB media (10 g/L NaCl, 10 g/L tryptone, 5 g/L yeast extract, water) and incubated at 30°C, 180 rpm for overnight (OD₆₀₀ ~2.5). Subsequently, 800 µL of the overnight culture was seeded into a 2.5 L-baffled shake flask containing 800 mL of 2× yeast extract and tryptone (2YT) media (5 g/L NaCl, 16 g/L tryptone, 10 g/L yeast extract, water), and incubated at 37°C, 180 rpm until OD₆₀₀ reached approximately 0.5. Protein expression was induced by adding 1 mM isopropyl β-D-1-thiogalactopyranoside (Astral Scientific, Australia), and the cells were further incubated at 37°C for 4 h. A final OD₆₀₀ of approximately 2 was routinely obtained. The cells were harvested by centrifugation (5,000×g, 4°C, 20 min), and the cell pellet was stored at –80°C until further processing. All media for cultures were supplemented with 15 µg/mL kanamycin sulfate (Life Technologies, CA, USA).

Protein Purification Optimization

Cells from an 800 mL culture were resuspended in 40 mL of lysis buffer (25 mM Tris-HCl, pH 8) containing NaCl. Cells were lysed by sonication using Branson Sonifier 250 ultrasonicator (Branson Ultrasonics, CT, USA) at energy output of 60 W for four burst of 30 s to obtain crude cell-extract. Clarified cell-extract was obtained after centrifugation of crude-cell extract (48,000×g, 4°C, 20 min). Both crude- and clarified- cell-extract were then subjected to DNA removal test.

To determine the optimal conditions for DNA removal, two parameters were examined: (1) concentrations of NaCl in the lysis buffer; and (2) concentrations of PEI added into either the crude- or clarified- cell-extract. Effect of NaCl concentrations on DNA precipitation was studied by adding clarified cell-extract (in 25 mM Tris-HCl, pH 8) into tubes containing weighed amounts of NaCl with final concentrations from 0.2 to 2 M, followed by addition of PEI into the suspension to a final PEI concentration of 0.5% (w/v) with stirring at 4°C for 60 min. To study the effect of PEI concentration, aliquots of PEI solution were added into either crude- or clarified- cell-extract (in 25 mM Tris-HCl, 1 M NaCl, pH 8) to give final PEI concentrations from 0.05 to 0.5% (w/v) with stirring at 4°C for 60 min.

Following DNA removal from crude cell-extract (in 25 mM Tris-HCl, 1 M NaCl, pH 8) using PEI 0.5% (w/v), the protein samples were centrifuged (48,000×g, 4°C, 20 min) and solid Na₂SO₄ was added into aliquots of the supernatants (1 mL) to a final concentration of 1 M, followed by incubation at 30–90°C for 5–30 min to allow precipitation of protein contaminants.

Suspension obtained after the protein-contaminant precipitation step was centrifuged (48,000×g, 25°C, 20 min), and the D4S2 protein was then isolated from the supernatants by selective precipitation using Na₂SO₄. Solid Na₂SO₄ were added into aliquots of the supernatants (3 mL) to give final Na₂SO₄ concentrations from 1.2 to 2.2 M, followed by stirring at 30°C for 60 min.

Subsequent to the isolation of D4S2 protein, the resulting D4S2 precipitate was washed in rinsing buffer (25 mM Tris-HCl, 1 M NaCl, pH 8) containing Na₂SO₄ at a concentration that retained D4S2 protein as precipitate. Following centrifugation (48,000×g, 30°C, 20 min), the D4S2 precipitate was resuspended in the solubilizing buffer (25 mM Tris-HCl, 1 M NaCl, pH 8) and then buffer-exchanged against sodium 4-(2-hydroxyethyl)-1-piperazine ethanesulfonate (HEPES) buffer (25 mM, pH 7.5) for five batch cycles using centrifugal filter (4,000×g, 4°C, 20 min) with cellulose membrane's size of 3.5 kDa (Merck Millipore, Merck KGaA, Germany). The resulting D4S2 protein in HEPES buffer was subjected to functionality tests: emulsification and biosilicification.

Functionality Assessment

Emulsification and biosilicification functionalities of the purified D4S2 protein were tested to prepare nanoemulsion and silica nanocapsules, respectively. To prepare nanoemulsion containing 10% (v/v) oil, Miglyol 812 was added into D4S2 solution (1.53 g/L) in HEPES buffer (25 mM, pH 7.5) to a final volume of 1 mL and the mixture was subsequently homogenized using a Branson Sonifier 250 ultrasonicator at an energy output of 30 W for four bursts of 30 s and interspersed in an ice bath for 60 s. To synthesize silica nanocapsules, an aliquot of the resulting nanoemulsion (400 µL) was transferred into a 4-mL glass vial containing a silica precursor tetraethoxysilane (TEOS, 32 µmol), and then stirred at room temperature for 36 h.

Analytical Characterization

DNA concentration was assayed by fluorescence intensity measurement using Infinite M200 Pro plate reader (Tecan, Switzerland). The samples were prepared by mixing a protein sample (100 µL) with SYBR Safe fluorescent dye (100 µL, 1× concentration) (Life Technologies, Australia) in a 96-well plate (Greiner Bio-One, Germany) for 5 min in a dark room, followed by fluorescence measurement at excitation and emission wavelengths of 502 and 530 nm,

respectively. To calculate the concentrations of DNA, a calibration curve was constructed using the pET-48b(+) plasmid with inserted D4S2 nucleotide sequence as the standard DNA solution (0–50 ng/mL).

Qualitative analysis of protein samples was performed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using NuPAGE 4–12% Bis-Tris Precast Gels mounted in a Bio-Rad XCell 3 system (Bio-Rad, CA, USA) with 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer and Novex Benchmark protein ladder (Life Technologies, Australia), as described by the manufacturer.

Quantitative measurement of D4S2 concentration was conducted using reversed-phase high-performance liquid chromatography (RP-HPLC) equipped with a Jupiter C₁₈ column (5 μm; 300 Å; 150 mm × 4.6 mm) (Phenomenex, CA, USA) and connected to an LC-10AVP series HPLC system (Shimadzu, Japan) with mobile phases A (0.1% (v/v) trifluoroacetic acid (TFA) in water) and B (90% (v/v) acetonitrile, 0.1% (v/v) TFA in water). A linear gradient from 30 to 65% B in 35 min at a flow rate of 1 mL/min was used, and a detection wavelength was set at 214 nm.

To characterize nanoemulsions and nanocapsules, their size distribution and zeta potential were determined using dynamic light scattering (DLS) using Malvern Zetasizer Nano ZS (Malvern Instrument, UK) at a scattering angle of 173° and a temperature of 25°C. The sample was diluted 100-fold prior to the measurement to avoid multiple scattering effects. Morphology of the silica nanocapsules was visualised using transmission electron microscopy (TEM) using a JEOL 1010 (JEOL, Japan) operated at 100 kV. The sample (2 μL) was deposited onto Formvar-coated copper grids (ProSciTech, Australia), and the size was analyzed using iTEM software (version 3.2, Soft Imaging System GmbH).

Results and Discussion

DNA Removal

During the purification of recombinant proteins from a microbial cell factory, it is critical that residual host material such as cellular DNA is substantially removed from the final product. DNA contamination could lead to a dramatic increase in sample viscosity that reduces the efficiency of the subsequent downstream processing steps. Moreover, our previous result for the purification of D4S2 protein (Wibowo et al., 2015) indicated that DNA contaminants, even after being removed by 95%, shielded the positive charge of D4S2 protein during emulsification causing charge reversal of the resultant nanoemulsions (i.e., negative zeta potential at neutral pH) most likely through an unfortunate layer-by-layer complexity, and, as a consequence, negated biosilicification activity of the D4S2 protein to react with anionic silica species hence unable to form silica shell. Further removal of DNA contaminants by >99% using chromatography was necessary to restore the D4S2 protein functionalities enabling the synthesis of silica nanocapsules (Wibowo et al., 2015).

In this work, the purification process of D4S2 protein was re-engineered to avoid costly chromatographic steps. Cellular DNA contaminants were removed by adding PEI, a cheap and commonly used cationic polymer that has shown to be effective in DNA precipitation through the formation of charge neutralization complexes (Burgess, 1991; DeWalt et al., 2003). Initially, crude cell-extract containing D4S2 protein was obtained using the thermal cell-breakage and precipitation-based purification method (Dwyer et al., 2014; Wibowo et al., 2015) that disrupted the cells (in 25 mM Tris-HCl buffer, 1 M NaCl, pH 8) and precipitated protein-contaminants by incubation at 90°C in the presence of 1 M Na₂SO₄ where D4S2 protein remained soluble. After mixing the resultant supernatant with PEI 0.05–0.5% (w/v), however, no precipitation occurred. It was likely due to the high concentration of Na⁺ that was sufficient to screen the opposite charges of PEI and DNA. Monovalent ions such as Na⁺ have been demonstrated able to associate with DNA backbone phosphate oxygens, and NaCl (at 1.04 M)

could induce fluctuations and loosening to the already formed cationic polymer–DNA complex (Antila et al., 2015).

Based on this preliminary test, sonication was conducted to obtain crude cell-extract (in 25 mM Tris-HCl, pH 8) and then centrifuged to obtain clarified cell-extract in which PEI 0.5% (w/v) and NaCl at different concentrations (0–2 M) were mixed and the resulting supernatants were examined (Figure 1). It can be seen from the SDS-PAGE gel that the amount of soluble protein-contaminants in the absence of NaCl was lower than that in the presence of NaCl, and increasing NaCl concentrations from 0 to 1 M resulted in a gradual increase of the amount of soluble protein-contaminants (Figure 1A). Meanwhile, the DNA concentration decreased when increasing the NaCl concentration from 0 to 1 M, and achieved the lowest at 1 M NaCl, but further increase to 2 M NaCl rather significantly increased the DNA concentration (Figure 1B). This result demonstrated that PEI could electrostatically interact and form a complex (as precipitates) with protein-contaminants (Figure 1A) and DNA (Figure 1B), and this complexation strongly depended on NaCl concentration. Interestingly, the concentration of D4S2 protein in the supernatant in the absence of NaCl was 29% lower than that in the presence of 1 M NaCl (Figure 1C) indicating that in the absence of NaCl D4S2 protein could bind to DNA then co-precipitate along with the DNA–PEI complex, whereas in the presence of 1 M NaCl the D4S2 protein–DNA interaction was sufficiently prevented and D4S2 was salted-in, thus allowing the formation and then precipitation of predominantly PEI–DNA complex. High NaCl concentrations (> 1 M) could have an adverse effect for DNA precipitation as it screened most of the DNA charge thus lowered the amount of PEI–DNA precipitate.

Figure 1

Figure 2 demonstrates the effect of PEI concentration in the presence of 1 M NaCl on DNA precipitation and its comparison between crude- and clarified- cell-extract. It can be seen that increasing PEI concentration from 0.05 to 0.5% (w/v) decreased the relative DNA

concentration in both crude- and clarified- cell-extract. Substantial DNA removal began at 0.2% (w/v) PEI, and the concentration of residual DNA in both crude- and clarified- cell-extract continued to decrease gradually to 19.1 and 17.4 ng DNA/mg D4S2 which account for 99.1% and 99.0% of DNA being removed, respectively, at 0.5% (w/v) PEI. This result demonstrated that a final PEI concentration of 0.5% (w/v) was sufficient to remove >99% of DNA contaminant from the extracted protein mixture. The protonated amine group of PEI can bind to the negatively-charged phosphate group of DNA on the backbones, then form charge neutralization complexes as precipitates which can then be removed by centrifugation. Furthermore, the result suggests that a cell clarification step after cell lysis was not necessary as the surface potential of cell debris in the presence of 1 M NaCl has no significant effect on the PEI–DNA interactions.

Figure 2

Protein-Contaminant Precipitation

Kosmotropic salts such as Na_2SO_4 are well-known salting-out agents for proteins having exposed or buried hydrophobic residues (Shih et al., 1992). Selective precipitation of protein-contaminants either in the absence or presence of Na_2SO_4 (1 M) was tested by heating the protein mixture, obtained after the DNA-removal step, at different temperatures (30, 50, 70 and 90°C) and incubation times (5, 15 and 30 mins). The resultant protein mixtures were centrifuged, and then each of the supernatants was diluted in water at the same dilution factor for SDS-PAGE analysis (Figure 3). Protein-contaminants began to precipitate by addition of Na_2SO_4 and incubation at 30°C from 5 to 30 mins, as compared to the sample incubated without the addition of Na_2SO_4 at the same temperature and time period. The effect of Na_2SO_4 in precipitating protein-contaminants was more pronounced at increasing temperatures (50–90°C) and a much better impurity precipitation was effectively achieved at 90°C with incubation time for only 5 min. Heating the protein mixtures (50–90°C) for 30 min in the absence of Na_2SO_4

did not completely precipitate protein contaminants (Figure 3). This is consistent with our previous result in which some protein contaminants were still present as a soluble form even when protein mixtures were heated to 90°C in the absence of Na₂SO₄ for 30 min (Wibowo et al., 2015). Additionally, our previous study using circular dichroism spectroscopy showed that the four-helix bundle protein was able to retain the helical structure up to 110°C in the presence of Na₂SO₄ (Dwyer et al., 2014). The synergistic effect of Na₂SO₄ (1 M) and heating (90°C) exposed the hydrophobic patches in protein contaminants by removing highly structured water which usually covers these patches in solution and, as a result, these hydrophobic residues could interact with one another eventually leading to aggregation and hence precipitate formation. In contrast, D4S2 protein remained soluble under these conditions (Figure 3) because of the four-helix bundle structure of D4S2 protein interlocking its hydrophobic domain at its core which contributes to the very high energy of structural unfolding at 90°C in the presence of 1 M Na₂SO₄.

Figure 3

D4S2-Protein Isolation

Subsequent to protein-contaminant precipitation, the concentrations of Na₂SO₄ in the supernatant were adjusted (1.2–2.2 M), knowing that the maximum solubility of Na₂SO₄ in water is 2.6 M at 30°C (Okorafor, 1999), to study its effect on the precipitation of D4S2 protein at 30°C and the results were presented in Figure 4. From the SDS-PAGE, D4S2 protein began to precipitate at 1.2 M Na₂SO₄ and D4S2 protein could be precipitated thoroughly using Na₂SO₄ concentrations from 1.8 to 2.2 M as there were no soluble D4S2 proteins in the aqueous phase (Figure 4A). This was also confirmed using RP-HPLC where D4S2 protein could only be detected in the precipitates formed at Na₂SO₄ concentrations of 1.8 M and higher (Figure 4B). The results demonstrated that increasing concentrations of Na₂SO₄ (>1 M) reduced the amounts of water molecules forming hydrogen bonds with the hydrophilic residues of D4S2

protein as they were associated with the salt molecules thus resulting in fewer water molecules being available for dissolving the protein, and the concentration of Na_2SO_4 at 1.8 M was sufficient to isolate most D4S2 protein from the bulk solution through precipitation. The D4S2-protein precipitate can be solubilized entirely in a buffer composed of 25 mM Tris-HCl, 1 M NaCl, pH 8. The D4S2-protein isolation step could further decrease the DNA concentration in the protein solution to 1.9 ng DNA per mg of D4S2 protein which is comparable to the relative DNA concentration obtained using a previously-reported chromatography-based D4S2-protein purification method (Wibowo et al., 2015). Subsequently, centrifugal filtration was conducted for buffer exchange against the buffer used for emulsification and biosilicification tests (25 mM HEPES buffer pH 7.5). For process scale-up, a faster and more buffer-efficient diafiltration process can be utilized (van Reis et al., 1997), which utilizes a similar buffer-exchange mechanism to the laboratory centrifugal filtration process used here.

Figure 4

D4S2-Protein Preparation

Results from the preceding trials of optimizing the purification parameters suggested that an efficient protein recovery process generally involves (Figure 5): (1) cell lysis by sonication (or, at process scale, homogenization (Middelberg, 1995)); (2) DNA removal from the crude cell-extract using PEI (0.5% (w/v)) and incubated at 4°C for 60 min; (3) protein-contaminant precipitation using 1 M Na_2SO_4 and incubated at 90°C for 5 min; (4) D4S2-protein isolation by addition of Na_2SO_4 to a final concentration of 1.8 M with incubation at 30°C for 60 min; and (5) buffer exchange by centrifugal filtration against 25 mM HEPES buffer pH 7.5.

Figure 5

The protein mixtures obtained after each step of the processes were qualitatively analyzed using SDS-PAGE (Figure 6A). The band at the bottom of the SDS-PAGE gel (lanes 2–4) can possibly be attributed to residual PEI remaining after DNA removal (lane 2) since this band

was not present in the cell-extract after cell lysis (lane 1). This band was not detected after D4S2-protein precipitation process as can be seen either on the rinsed precipitate sample (lane 5) or on the solubilized precipitate sample (lane 6). Importantly, the samples obtained either after solubilization of protein precipitate (lane 6) or after buffer exchange (lane 7) suggested that D4S2 protein was mostly present in the SDS-PAGE gel (Figure 6A). The purified D4S2 protein was also characterized using RP-HPLC on which the result shows two peaks: (i) main peak and (ii) additional small peak at retention times 27.2 and 28.1 min, respectively (Figure 6B). Both the main peak and the additional small peak were also detected on the RP-HPLC result of cell-extract sample after cell lysis (Supporting Information, Figure S1). To determine the molecular weight of the peaks shown in RP-HPLC, the purified D4S2 protein was analyzed using liquid chromatography–mass spectrometry (LC–MS) (Supporting Information, Figure S2). It was shown that the molecular weight (M) corresponds to the main peak is 13,307.46 Da (Figure S2A) which is close to the M of D4S2 protein purified using chromatography (13,308.2) (Wibowo et al., 2015). On the other hand, the additional small peak corresponds to M 13,337.19 Da (Figure S2B) which is 29.73 Da higher than the M of D4S2 protein. The M increase can probably be attributed to the additional oxygen in D4S2 protein as thioether group (–S–) in the methionine (M) residue (at the N -terminal of D4S2 protein) which could easily undergo oxidation to either sulfoxide (–SO) or sulfone (–SO₂) group (Berlett & Stadtman, 1997; Ignasiak et al., 2013).

Figure 6

Table 1 presents the mass balance of the non-chromatographic purification method developed in this study. Using a standard laboratory shake-flask culture method with a volume of 800 mL, a final cultivation OD₆₀₀ of approximately 2 was routinely obtained and a final yield of 28.8 mg of D4S2 protein which accounts for 78.6% of D4S2-protein recovery can be achieved from the bacterial expression system. The final yield of D4S2 protein from this newly

developed process was four times higher than that obtained from chromatographic purification process (Wibowo et al., 2015).

Table 1

Emulsification and Biosilicification Functionalities

The purified D4S2 protein was tested for making nanoemulsions as well as forming oil-filled silica nanocapsules. A mixture of Miglyol 812 oil and D4S2 protein solution in HEPES buffer was homogenized through sonication. The D4S2 protein demonstrated its emulsification functionality as it was able to facilitate disruption of the oil phase into small droplets and concurrently stabilize the resultant nanoemulsions which had a hydrodynamic diameter of 200 ± 26 nm, with a polydispersity index (Đ) of 0.153 ± 0.021 (Figure 7A) and a zeta potential of 52 ± 3 mV. The nanoemulsions then reacted with a silica precursor tetraethoxysilane (TEOS, 32 μmol) at pH 7.5 and room temperature for 36 h without the use of any toxic catalysts. The reaction yielded silica nanocapsules having outer diameter and shell thickness of 462 ± 18 and 76 ± 5 nm, respectively, as measured by using TEM (Figure 7B) on 100 silica nanocapsules. The emulsification activity of D4S2 was due to the amphiphilic character of the D4S2 protein's surface-active module that allowed D4S2 to unfold upon interfacial adsorption, thus oriented the hydrophobic residues toward the oil phase while the hydrophilic residues faced toward the bulk phase. On the other hand, the biosilicification activity of D4S2 protein resulted from the catalytic role of the Si module, which comprises densely packed cationic arginine (R) and lysine (K) residues projecting toward bulk aqueous phase, as well as from polar residues of the surface-active module, that induced hydrolysis of TEOS and polycondensation at the oil–water interfaces forming silica shell encapsulating the oil core. The results demonstrated that the optimized purification process has no adverse effects on the functionalities of D4S2 protein for stabilizing nanoemulsions as well as for making oil-core silica-shell nanocapsules.

Figure 7

Conclusions

In this work, we developed a chromatography-free purification method to recover mineralizing D4S2 protein from recombinant *E. coli* BL21(DE3) for the synthesis of oil-filled silica nanocapsules. The method was based on selective thermochemical precipitation and consists of sequential steps including: (1) cell lysis by sonication; (2) DNA removal from the crude cell-extract using poly(ethyleneimine) (4°C, 60 min); (3) protein-contaminants precipitation using 1 M Na₂SO₄ (90°C, 5 min) in which D4S2 protein remained soluble due to its four-helix bundle structure in bulk interlocking its hydrophobic residues at its core; (4) D4S2-protein isolation using 1.8 M Na₂SO₄ (30°C, 60 min); and (5) buffer exchange using centrifugal filtration. Cellular DNA contaminants could be reduced by >99% to a level comparable to that attained in a previously-reported chromatographic purification of D4S2 protein. Furthermore, the final yield of the non-chromatographic purified D4S2 protein, i.e., 28.8 mg per 800 mL cells (final cultivation OD₆₀₀ ~2) which accounts for 78.6% of D4S2-protein recovery, is 400% of that obtained from the chromatographic purification method. Emulsification and biosilicification tests demonstrated that D4S2 protein purified using this non-chromatographic method was able to stabilize nanoemulsions and concurrently mineralize silica shells that surround the nanoemulsion droplets forming oil-core silica-shell nanocapsules, confirming no adverse process-related effects. This work utilized fundamental knowledge of high thermal and chemical stability of a four-helix bundle protein as compared to other cellular protein contaminants to co-engineer both the recombinant mineralizing D4S2 protein and the bioprocess while eliminating expensive chromatographic processing steps which hinder the commercial development of bio-inspired nanomaterials. We expect that this non-chromatographic purification method can be used as a cost-effective, scalable and high-yield purification tool for peptide or protein fusions having high isoelectric point where the recombinant peptides or proteins genetically appended to four-helix bundle protein tags.

Acknowledgments

This research was supported by the Australian Research Council (ARC) under Discovery Project (DP150100798). C.-X.Z. acknowledges financial support from the award of the ARC Future Fellowship (FT140100726). We acknowledge the facilities, and the scientific and technical assistance, of the Australian Microscopy & Microanalysis Research Facility at the Centre for Microscopy and Microanalysis, The University of Queensland. This work was performed in part at the Queensland Node of the Australian National Fabrication Facility – a company established under the National Collaborative Research Infrastructure Strategy to provide nano and microfabrication facilities for Australia’s researchers. We thank Andrea Schaller for computationally modelling the D4S2 protein. The University of Queensland (UQ) filed patent on the use of mineralizing proteins for making silica nanocapsules. A.P.J.M., C.-X.Z., and D.W. are named inventors on this patent and through their employment with UQ hold an indirect interest in this intellectual property. The other author declares no conflict of interest.

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Table Legends

Table 1. Mass balance of the non-chromatographic purification of recombinant mineralizing D4S2 protein.

Figures Legends

Figure 1. Effect of NaCl concentrations (0–2 M) on the DNA precipitation using poly(ethyleneimine) (PEI) at a concentration of 0.5% (w/v) in Tris-HCl buffer (25 mM, pH 8). (A) SDS-PAGE analysis. Concentrations of (B) DNA and (C) D4S2 protein remaining in the solution after PEI addition.

Figure 2. Effect of poly(ethyleneimine) (PEI) concentrations (0.05–0.5% (w/v)) on the DNA precipitation in Tris-HCl buffer (25 mM, pH 8) containing 1 M NaCl. Insert: the magnification region of the relative DNA concentration in the solution after adding PEI from 0.2 to 0.5% (w/v).

Figure 3. Effect of temperature and incubation time on the protein-contaminant precipitation in buffers (25 mM Tris-HCl, 1 M NaCl, pH 8) with the absence and presence of 1 M Na₂SO₄.

Figure 4. Effect of Na₂SO₄ concentrations (1.2–2.2 M) on the solubility of D4S2 proteins in aqueous phase. (A) SDS-PAGE analysis (S: supernatant; P: precipitate). (B) D4S2-protein concentrations in both the supernatants and the solubilized precipitates.

Figure 5. Process flow diagram of the non-chromatographic bioprocess engineering of recombinant mineralizing D4S2 protein developed in this study. *E. coli* BL21(DE3) was

used to express D4S2 protein in 800 mL of 2YT media supplemented with 1 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) and 15 μ g/mL kanamycin sulfate (final cultivation $OD_{600} \sim 2$), and the final yield of D4S2 protein after purification was 28.8 mg which accounts for 78.6% D4S2-protein recovery. *Lysis buffer*: 25 mM Tris-HCl, 1 M NaCl, pH 8. *Solubilizing buffer*: 25 mM Tris-HCl, 1 M NaCl, pH 8. *Rinsing buffer*: 25 mM Tris-HCl, 1 M NaCl, 1.8 M Na_2SO_4 , pH 8. PEI: poly(ethyleneimine). Pptn: precipitation.

Figure 6. Characterization of D4S2 protein. (A) SDS-PAGE analysis of the supernatant samples obtained after: (1) cell lysis; (2) removal of DNA using poly(ethyleneimine) (PEI) 0.5% (w/v); (3) precipitation of protein-contaminants using 1 M Na_2SO_4 (90°C, 5 min); (4) precipitation of D4S2-protein using 1.8 M Na_2SO_4 (30°C, 60 min); (5) rinsing of D4S2-protein precipitate; (6) solubilization of D4S2-protein precipitate; and (7) buffer exchange using centrifugal filtration. M: Marker. (B) Reversed-phase high-performance liquid chromatography (RP-HPLC) profile of the sample obtained after buffer-exchange step.

Figure 7. Functionality assessment of D4S2 protein produced from the non-chromatographic method developed in this study for making nanoemulsion-templated silica nanocapsules. (A) Size distribution of nanoemulsions (—) and silica nanocapsules (—) obtained by using dynamic light scattering (DLS). (B) Morphology of the silica nanocapsules taken using transmission electron microscopy (TEM).