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Chapter 6

Recombinant Expression and Purification of "Virus-like" Bacterial Encapsulin Protein Cages

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Abstract

Ultracentrifugation, particularly the use of sucrose or cesium chloride density gradients, is a highly reliable and efficient technique for the purification of virus-like particles and protein cages. Since virus-like particles and protein cages have a unique size compared to cellular macromolecules and organelles, the rate of migration can be used as a tool for purification. Here we describe a detailed protocol for the purification of recently discovered virus-like assemblies called bacterial encapsulins from *Thermotoga maritima* and *Brevibacterium linens*.

Key words Virus-like assemblies, Protein cages, Virus purification, Bacterial encapsulins, Nanotechnology

1 Introduction

Protein cages such as ferritins [1–9], viruses [5, 10–12], bacteriophages, bacterial encapsulins [13, 14], and the recently discovered bacterial microcompartments [6–8, 14–19] are hollow protein-based assemblies of various sizes that are found in nature and play a crucial role in biological catalysis, mineralization, molecular storage, detoxification, and gene delivery. Icosahedral capsids are assembled according to the Caspar-Klug *quasi*-equivalence theory in which 60 N subunits (where N is the triangulation (T) number) are symmetrically arranged as pentamers and hexamers to form the final icosahedron [20]. The smallest icosahedral virus is composed of 60 protein subunits arranged as 12 pentamers to form a $T = 1$ capsid assembly. Owing to their diverse role in nature, viruses and virus-like assemblies are increasingly used in materials science, engineering, and nanotechnology, as tools and building blocks for controlled catalysis, nanoparticle synthesis, and electronics and as molecular cargo delivery systems [4, 10, 21–24]. In the past, prokaryotes were thought to lack subcellular organization and compartmentalization; however, the recent discovery of bacterial

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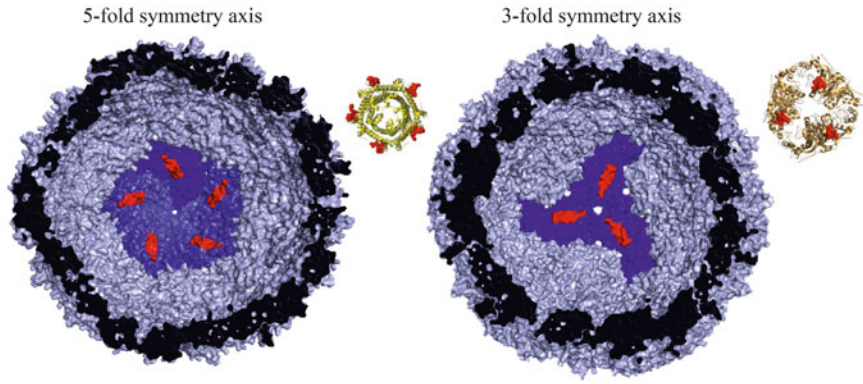

Fig. 1 Electron density surface mapping of the encapsulin from (*left*) *Thermatoga maritima* and (*right*) *Brevibacterium linens*. The native cargo proteins, ferritin-like protein and dye-decolorizing peroxidase, respectively, are shown as insets

encapsulins and microcompartments, or so-called primitive organelles, has since provided first evidence that subcellular organization is important for even simple organisms [6–8]. For example, the 24 nm bacterial encapsulins from *Thermotoga maritima* and *Brevibacterium linens* are similar in morphology and size diameter to plant-based viruses (Fig. 1) [14]. The interior cavity of the *B. linens* bacterial encapsulin contains a dimer of trimers of a dye-decolorizing peroxidase (DyP), whereas the bacterial encapsulins from *T. maritima* contain a dimer of pentamers of a ferritin-like protein (Flp) and are involved in bacterial catalysis and/or mineralization, respectively. However, although morphologically similar to small icosahedral plant-based viruses, both structural and gene homology studies suggest that encapsulins are of non-viral origin, due to the absence of any genes of viral origin within the vicinity of the encapsulin gene [14, 25]. Bacterial encapsulins are much more stable against temperature, pH, ionic strength, and chaotropes than their virus-based counterparts and therefore exhibit enormous potential for future applications of nanotechnology.

This chapter provides a detailed description for the recombinant expression and purification of bacterial encapsulins in *Escherichia coli* (Fig. 2) [3]. For both non-viral and viral assemblies, the use of ultracentrifugation has significantly improved the purification and isolation of such assemblies [3]. Ultracentrifugation takes advantage of the relative size to density migration of virus-like particles and protein cages through a gradient of viscous liquid, typically sucrose or cesium chloride [3]. Since virus-like particles and protein cages have a unique size compared to cellular macromolecules and organelles, the rate of migration can be used as a tool for purification.

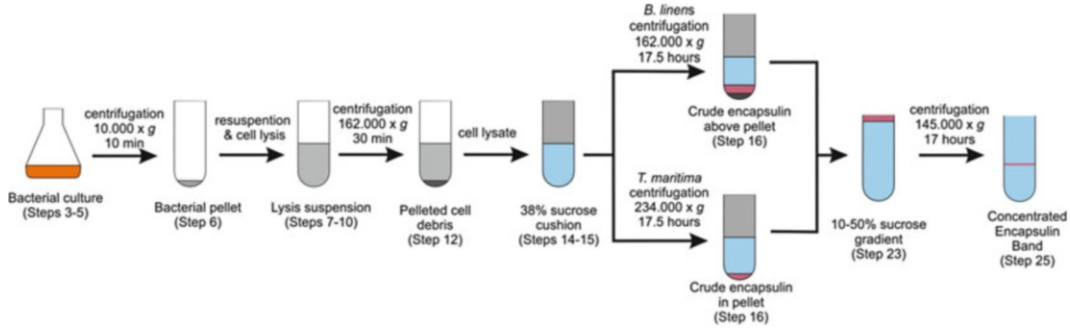

Fig. 2 Schematic diagram for the recombinant expression and purification of bacterial encapsulins

2 Materials

Antibiotics: Stock 100 mg/mL ampicillin sterilized by 0.2 μm filtration in ultrapure sterile H_2O. Stock 34 mg/mL chloramphenicol prepared in pure ethanol.

Bacterial growth medium: Autoclave 10 g bacterotryptone, 5 g yeast extract, 5 g NaCl made up to 1 L deionized water at 120 °C for 20 min.

Encapsulin buffer: 20 mM Tris-Cl, 150 mM NH_4Cl, 20 mM $MgCl_2$, 1 mM β-mercaptoethanol, pH 7.5.

Specialist equipment: Thermo Scientific Sorvall WX80 ultracentrifuge T 865 rotor (30 mL polycarbonate tubes) and Surespin 630/36 rotor (38.5 mL polyclear tubes).

3 Methods

1. Inoculate 5–7 mL of sterile LB medium with *E. coli* Rosetta (DE3) cells containing pET21a plasmid vector expressing either *B. linens* or *T. maritima* (*see* **Note 1**). Add ampicillin (stock 100 mg/mL, 5–7 mL) and chloramphenicol (stock 34 mg/mL, 5–7 mL) and grow 18 h (overnight) at 37 °C with continuous shaking.
2. Prepare 0.5 L of sterile LB medium in 2 L culture flasks for bacterial expression the next step.
3. The next morning, add the overnight cultures to inoculate 0.5 L sterile LB medium containing ampicillin (stock 100 mg/mL, 50 mL) and chloramphenicol (stock 34 mg/mL, 50 mL) and grow at 37 °C with continuous shaking until OD_{600} reaches 0.6–0.8.
4. Allow the bacterial cells to cool slowly to 22 °C with continuous shaking (*see* **Note 2**).

5. Once the bacterial cells are cooled, add isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM and grow 18–22 h (overnight) at 22 °C with continuous shaking.
6. The following day, pellet the bacterial cells by centrifugation at 10,000 × *g* for 15 min at 4 °C.
7. Resuspend the bacterial cells in 10 mL encapsulin buffer (*see* **Note 3**).
8. Transfer the resuspended cells to a 50 mL Falcon tube.
9. Keeping the cells cooled in an ice box, insert an ultrasonic probe into the suspended bacterial cell mixture and lyse the bacterial cells at full amplitude with full power for 2 min.
10. Add DNAse and RNAse (10 μL of each nuclease) to the lysed cells and incubate for 2 h at 4 °C with gentle mixing.
11. Transfer the lysed cells into ultracentrifuge tubes, weigh, and tare to <10 mg difference (*see* **Note 4**).
12. Centrifuge the lysed cells at 162,000 × *g* for 15 min to pellet the cell debris (*see* **Note 5**).
13. Prepare 38 % w/v sucrose in encapsulin buffer (10 mL per sample).
14. For each sample, transfer 10 mL 38 % w/v sucrose to clean ultracentrifuge tubes (*see* **Note 6**).
15. Carefully layer the supernatant from **step 13** on top of the 38 % w/v sucrose cushion.
16. Centrifuge at 162,000 × *g* for 17 h (*see* **Note 7**).
17. Carefully collect the bottom 3 mL of sample from the ultracentrifuge tube (avoiding the pellet) and transfer to a clean spin-filtration unit (MWCO 100,000 Da) (*see* **Note 8**).
18. Concentrate the crude encapsulins by centrifugation for 30 min.
19. Rinse by adding encapsulin buffer to the concentrated crude encapsulins and repeat **step 18**.
20. Repeat the previous step 4–5 times to remove the sucrose and concentrate to a final volume of 1.0 mL.
21. Prepare two separate beakers containing 10 % sucrose and 50 % sucrose dissolved in encapsulin buffer (17 mL of each per sample required).
22. Prepare 10–50 % sucrose gradient in encapsulin buffer (17 mL of each) in ultracentrifuge tubes (*see* **Note 9**).
23. Gently add the crude encapsulins from **step 21** (1.0 mL) on top of the 10–50 % sucrose gradient, weigh, and tare to <10 mg difference (*see* **Note 10**).

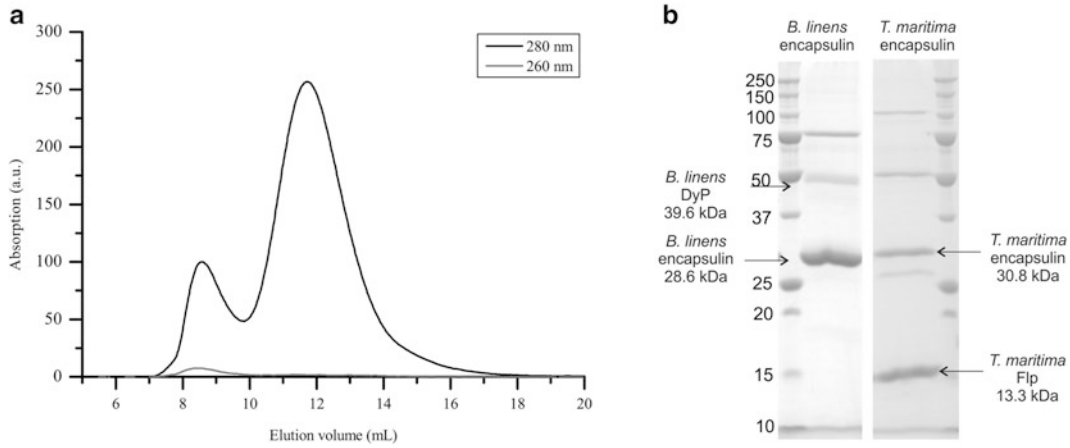

Fig. 3 (**a**) Purification of the *T. maritima* bacterial encapsulin by size exclusion chromatography (after sucrose gradient, **step 12**), monitoring at $\lambda = 280$ nm (*black trace*) and 260 nm (*grey trace*), (**b**) SDS-PAGE analysis of (*left*) the purified *B. linens* bacterial encapsulin as the monomer (28.6 kDa) and its native dye-decolorizing peroxidase (DyP) cargo (39.6 kDa) and (*right*) the purified *T. maritima* encapsulin as the monomer (30.8 kDa) and its native ferritin-like protein (Flp) cargo (13.3 kDa). The gels were stained with Coomassie Blue stain

24. Centrifuge at 12,300 × *g* for 17.5 h (*see* **Note 11**).
25. Collect sucrose gradient in fractions: 0–10, 10–15, 15–20, 20–23, 23–26, 26–29, 29–32, and 32–35 mL.
26. Purify fractions containing desired bacterial encapsulins using size exclusion chromatography (Fig. 3, *see* **Note 12**).

4 Notes

1. *E. coli* Rosetta (DE3) cells expressing *B. linens* or *T. maritima* encapsulin can be stored as glycerol stocks at −80 °C.
2. While protein expression is typically slower and lower yielding at lower temperatures, the lowered temperature allows proper folding of the encapsulin protein. We recommend not adding IPTG until the bacterial cultures are cooled to approximately 22 °C.
3. We do not recommend using commercially available chemical lysis solutions (such as BugBuster). In our experience, the use of commercial lysis buffers, particularly those containing surfactants, interferes with the efficiency of the sucrose cushion and often leads to either loss of the encapsulins or insufficient purification in the subsequent steps. However, if the use of commercially available buffers cannot be avoided, we strongly recommend an additional step in which the clarified cell supernatant is buffer exchanged to the encapsulin buffer prior to the sucrose cushion steps (between **steps 13** and **14** of the described protocol).

4. We recommend using 30 mL polycarbonate tubes for this step together with the T-865 rotor from Thermo Scientific.
5. The speed and duration are provided based on a T-865 rotor. These parameters may vary depending on the rotor used. Adjust the time and speed parameters according to manufacturer and suppliers recommendations.
6. Again, we recommend using 30 mL polycarbonate tubes for this step. If unavailable, we suggest using the same type of ultracentrifuge tubes used in **step 11** in Subheading 3.
7. The speed and duration are provided for *B. linens* encapsulins. For *T. maritima* encapsulins, centrifuge at 234,000 × *g* for 17 h. The speed and duration are provided based on a T-865 rotor. These parameters may vary depending on the rotor used. Adjust the time and speed parameters according to manufacturer and suppliers recommendations.
8. The crude encapsulins from *B. linens* are found in the last 3 mL above the pellet. For encapsulins from *T. maritima*, the crude encapsulins are found in the pellet at the bottom of the ultracentrifuge tube. Dialysis tubes can be used instead of spin-filtration tubes; however, an additional centrifugation step is necessary to concentrate the crude encapsulins for the next steps.
9. We recommend using 38.5 mL polyclear tubes together with the Surespin 630/36 rotor from Thermo Scientific.
10. Take extra care not to disturb the sucrose gradient when layering the crude encapsulins. If necessary, add encapsulin buffer to balance the tubes, taking gain take care not to disturb the gradient.
11. The speed and duration are provided based on a Surespin 630/36 rotor. These parameters may vary depending on the rotor used. Adjust the time and speed parameters according to manufacturer and suppliers recommendations.
12. The encapsulins isolated from the ultracentrifugation gradient are typically found in fractions 20–23, 23–26, and 26–29. We recommend using a Superose 6 column for the final purification step by size exclusion chromatography (SEC).

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