Chapter 10

Microbial Production of High-Performance Fibers from Muscle Protein Titin

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Abstract

Nature has produced a variety of proteinaceous materials, each with a set of mechanical properties tuned by evolution to adapt to particular environments. While these advantageous properties have also made many of these materials well-suited to various human needs, few protein materials can be harvested from their natural hosts at scale. To meet the demand for these materials using scalable biomanufacturing processes, our lab has developed tools and a biopolymerization platform for the microbial synthesis and processing of nature-derived, high-molecular-weight protein polymers. In this chapter, we describe the application of this platform for polymerizing a segment of the muscle protein titin and processing the resulting polymer into high-performance, muscle-mimetic fibers with a unique combination of desirable mechanical properties.

Key words Protein materials, Synthetic biology, In vivo polymerization

1 Introduction

As one of the key building blocks in biology, protein can be found in a wide range of biomaterials, with applications both in nature and society. Well-known examples include strong and tough spider silks [1, 2], underwater adhesive mussel byssus [3–5], and the stiff, thermoplastic suckerin proteins from cephalopods [6]. In addition to advantages of renewability and biodegradability, these materials also often outcompete many petroleum-based materials with regards to mechanical performance [1, 4, 7]. While the utility of these protein materials is obvious, many cannot be harvested from their natural sources by economically feasible means [1].

Whether made from protein or petroleum, many high-performance materials are composed of high molecular weight (HMW) polymer chains. HMW proteins are difficult to recombinantly express due to metabolic burden, recombination of highly repetitive gene sequences, instability of long mRNAs, translational pausing, and biased amino acid compositions [8, 9]. Drawing

Cassandra Callmann (ed.), *Biomedical Nanotechnology*, Methods in Molecular Biology, vol. 2902,
https://doi.org/10.1007/978-1-0716-4402-7_10,

inspiration from polymerization strategies used to create synthetic polymers, the Zhang laboratory has engineered biosynthetic strategies for polymerizing easily expressed, low molecular weight (LMW) proteins into HMW protein chains inside living bacteria [10, 11]. These strategies employ natural protein domains called split inteins (SIs). SIs can be genetically fused to proteins of interest to spontaneously ligate them via a peptide bond, cleaving the SIs and leaving only a few residual "scar" residues behind [12, 13]. By fusing N- and C-terminal SIs to the termini of a target LMW protein, this strategy creates reactive LMW "monomers" that ligate together in vivo to yield HMW polymers (*see* Fig. 1). This protein polymerization approach, therefore, overcomes many of the challenges of directly expressing HMW material proteins.

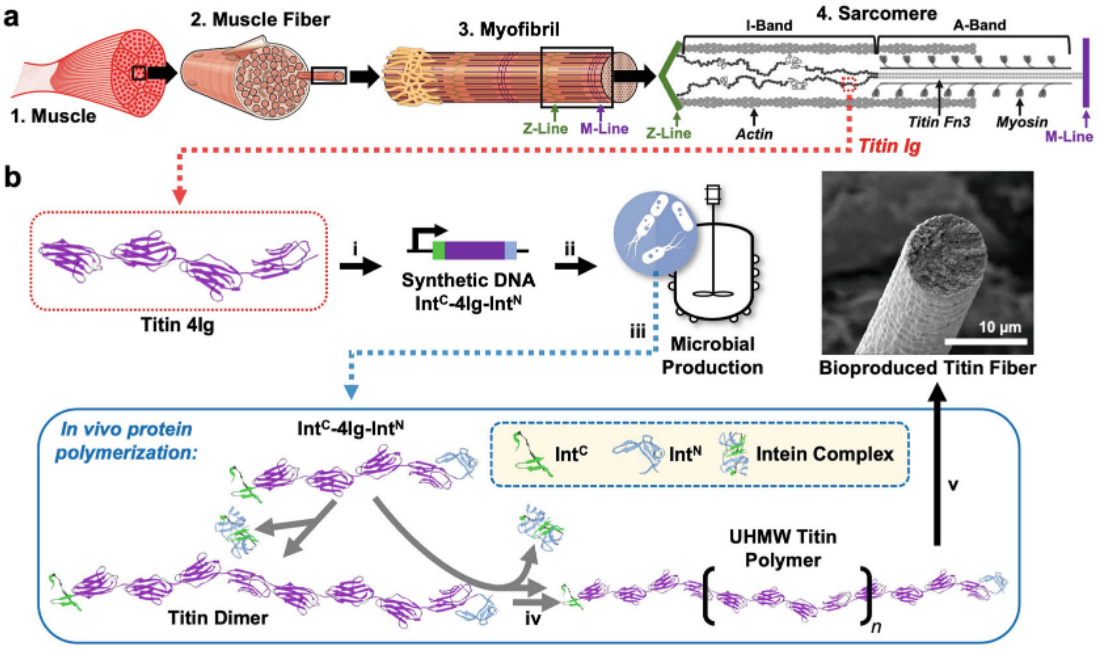

Fig. 1 Schematic representation of SI-driven, microbial biopolymerization of the muscle protein titin. (**a**) Animal muscle (1) is made of elongated cells known as muscle fibers (2), which in turn are composed of proteinaceous myofibrils (3). Myofibrils are made up of contractile elements called sarcomeres (4), which are composed primarily of the proteins actin, myosin, and titin. A single titin molecule stretches half the length of the sarcomere and consists of hundreds of immunoglobulin domains, which enable its unique mechanical properties. (**b**) In this embodiment of the biopolymerization platform, the titin coding sequence for four immunoglobulin domains is flanked by the coding sequences for complimentary SI halves (Int^C and Int^N) (i). The resulting gene is introduced to an engineered *E. coli* host (ii), which is then used to express the reactive titin monomer protein (iii). This protein polymerizes intracellularly via SI ligations to yield ultra-HMW (UHMW) protein chains (iv). These proteins are then purified and processed to create titin fibers with unique material properties (v). (This image is taken without modifications from Bowen et al. [10] under a Creative Commons Attribution 4.0 International License (https:/creativecommons.org/licenses/by/4.0/))

While this work presents the application of this platform to the microbial production of the I67–I70 segment of the rabbit soleus muscle protein titin [14], it can also be used in the biosynthesis of other protein polymers. The genetic tools created during the development of this platform make it easy to swap other proteins in and out in a "plug and play" fashion characteristic of synthetic biology [15]. Regardless of the material protein of choice, the general strategy remains the same (*see* Fig. 1). Using standard cloning techniques, the gene of interest, in this case the titin coding sequence, is genetically flanked by reactive SI groups. The resulting genetic construct is expressed in a bacterial host, yielding a bi-reactive protein monomer that spontaneously polymerizes inside the cell. This polymer is then purified using chemical solubility, affinity chromatography, dialysis, and lyophilization. The resulting protein powder is dissolved to a high concentration in an organic solvent and wet-spun into macroscopic fibers in water (*see* Fig. 2). While details of the downstream analysis of the structure and properties of these fibers can be found elsewhere [10, 16, 17], the production and processing of these titin polymers into fibers is outlined as follows.

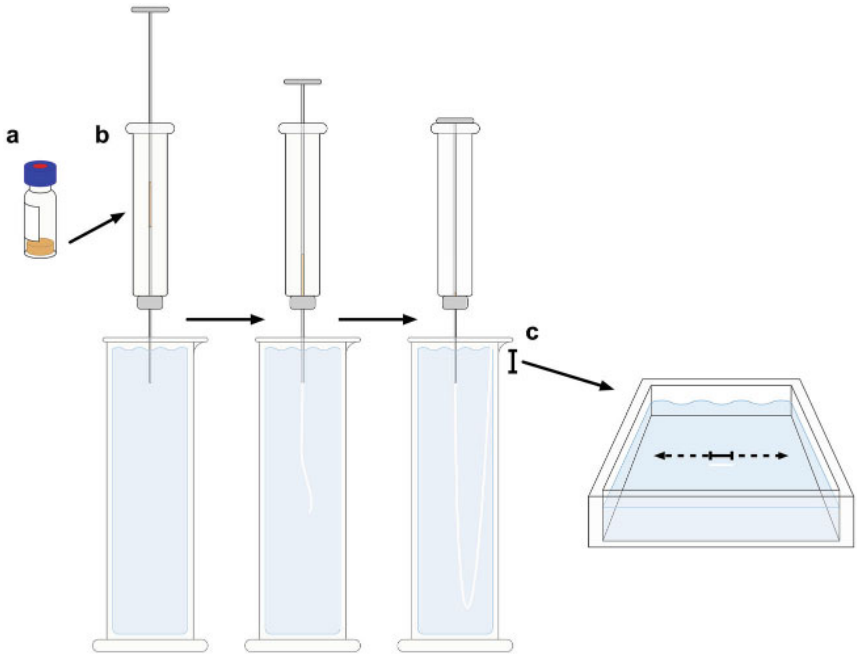

Fig. 2 Schematic of the aqueous wet spinning of titin polymer fibers. (**a**) Purified, lyophilized titin polymer protein is dissolved in high concentration in HFIP. (**b**) The resulting protein dope is then loaded to a narrow-gauge syringe and extruded into the water extrusion bath by the syringe pump until all dope has been extruded as visible fibers. (**c**) Short segments of the fibers are carefully extended to approximately five times their original length to facilitate axial polymer chain alignment for improved mechanical performance

2 Materials

2.1 Cloning of Reactive Titin Monomer Plasmids (Alternative Cloning Methods May Be Used—See Note 1)

1. Computationally optimized titin coding sequence (*see* **Note 2**).
2. SI-Bricks vector [2] with gp41-1^{C} and gp41-1^{N} SIs (the C- and N-terminal halves of the gp41-1 SI) [18]; P_{BAD} or P_{LacO1} promoter; and C-terminal His-tag [10].
3. Restriction digest enzymes (KpnI, Kpn2I).
4. Standard materials for restriction digest:
   (a) Substrate DNA to be digested.
   (b) Vendor-supplied restriction enzyme buffer appropriate for the desired restriction enzymes.
   (c) Microcentrifuge or polymerase chain reaction (PCR) tube.
   (d) Heat bath/block.
5. Standard materials for gel electrophoresis:
   (a) Agarose.
   (b) Microwave.
   (c) Ethidium bromide or other dyes for DNA visualization.
   (d) Gel casting tray and well combs.
   (e) Gel running box and voltage source.
   (f) UV lamp to visualize dye-stained DNA.
6. Standard materials for gel extraction:
   (a) Heat bath/block.
   (b) Vendor-supplied gel extraction kit typically containing the following:
      (i) DNA binding centrifugal columns.
      (ii) Binding buffer.
      (iii) Wash buffer.
      (iv) Elution buffer.
7. Standard materials for ligation:
   (a) Heat bath/block.
   (b) Digested and gel-extracted vector and insert DNA.
   (c) Vendor-supplied ligation kit for sticky-end ligation (e.g., T4 ligase), typically containing the following:
      (i) T4 DNA ligase.
      (ii) T4 DNA ligase buffer.
8. Standard materials for transformation:
   (a) Vendor-supplied chemically competent cells.
   (b) Ligated DNA.

(c) Heat bath/block for heat shock transformation.

(d) Luria-Bertani broth (LB) agar plate with antibiotic selection corresponding to the selection marker in the DNA vector.

(e) 37 °C incubator.

9. *Escherichia coli* New England Biolabs (NEB) 10-beta (NEB10β).

2.2 Shake Flask Protein Production (see Note 3)

1. *E. coli* NEB10β chassis transformed with SI-flanked titin expression cassette (p-1-4XT-1_B).
2. Terrific broth (TB):
   (a) 24 g/L yeast extract.
   (b) 20 g/L tryptone.
   (c) 0.4% v/v glycerol.
   (d) 17 mM KH_2PO_4.
   (e) 72 mM K_2HPO_4.
   (f) For this work, include antibiotics (50 μg/mL kanamycin) for strain selection and plasmid maintenance.
3. 250 mL Erlenmeyer flask.
4. Incubation chamber at 37 °C.
5. Orbital shaker.
6. 2 L Erlenmeyer flask.
7. 20% arabinose.
8. Cell collection containers.
9. High-speed centrifuge.

2.3 Bioreactor Protein Production

1. *E. coli* NEB10β chassis transformed with SI-flanked titin expression cassette (p-1-4XT-1_L).
2. Terrific broth (TB).
3. 250 mL Erlenmeyer flask.
4. Incubation chamber at 37 °C.
5. Orbital shaker.
6. M9 glucose medium with tryptone supplement:
   (a) 2% w/v glucose.
   (b) 1× M9 salts.
   (c) 75 mM MOPS pH 7.4.
   (d) 12 g/L tryptone.
   (e) 5 mM sodium citrate.
   (f) 2 mM $MgSO_4 \cdot 7H_2O$.

(g) 100 μM $FeSO_4 \cdot 7H_2O$.
(h) 100 μM $CaCl_2 \cdot 2H_2O$.
(i) 3 μM thiamine.
(j) 1× micronutrients:
(i) 40 μM $ZnSO_4 \cdot 7H_2O$.
(ii) 20 μM $CuSO_4 \cdot 5H_2O$.
(iii) 10 μM $MnCl_2 \cdot 4H_2O$.
(iv) 4 μM H_3BO_3.
(v) 0.4 μM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$.
(vi) 0.3 μM $CoCl_2 \cdot 6H_2O$.
(k) For this work, include antibiotics (50 μg/mL kanamycin) for strain selection and plasmid maintenance.

7. 2 L Bioflo120 heat-blanketed bioreactor (Eppendorf).
8. Antifoam 204.
9. Sterile substrate feed:
(a) 20% w/v glucose.
(b) 48 g/L tryptone.
(c) 10 g/L $MgSO_4 \cdot 7H_2O$.
10. 1 M IPTG.
11. Cell collection containers.
12. High-speed centrifuge.

2.4 Protein Purification

1. Urea lysis buffer (pH 7.4):
(a) 8 M urea.
(b) 300 mM NaCl.
(c) 10 mM imidazole.
(d) 20 mM KH_2PO_4.
2. Ice.
3. Probe sonicator.
4. Collection containers.
5. High-speed centrifuge.
6. 0.45 μm polyethersulfone (PES) filter.
7. HisTrap HP 5 mL columns.
8. Software-controlled FPLC system.
9. Urea lysis buffer (pH 7.4) plus 50 mM imidazole.
10. Urea lysis buffer (pH 7.4) plus 300 mM imidazole.
11. 5 mM ammonium bicarbonate.
12. 10 kDa MWCO snakeskin dialysis tubing.

13. 3 L beaker.
14. Incubation chamber at 4 °C.
15. Stir plate.
16. Magnetic stir bar.
17. Liquid nitrogen.
18. Freeze dryer.

2.5 Fiber Spinning

1. Glass gas chromatography (GC) vial.
2. Microbalance.
3. Hexafluorisopropanol (HFIP).
4. 100 μL Hamilton gastight syringe (Hamilton Robotics).
5. 23 s gauge (116 μm inner diameter, 4.34 cm length) needle.
6. Syringe pump.
7. 500 mL Graduated cylinder (or similarly tall, narrow, and stable glass vessel).
8. Deionized water.
9. Scissors.
10. 9 in × 9 in glass dish.

3 Methods

3.1 Cloning of Reactive Titin Monomer Plasmids

1. Synthesize computationally optimized coding sequence for immunoglobulin domains I67-70 of rabbit soleus titin flanked with KpnI and Kpn2I restriction sites [2, 10, 14] (*see* **Note 2**).
2. Using KpnI and Kpn2I restriction enzymes and standard restriction digestion protocols, digest synthesized coding sequence and SI-Bricks plasmid containing gp41-1^{C} and gp41-1^{N} SIs; a P_{BAD} or P_{LacO1} promoter; and a C-terminal His-tag.
3. Isolate-digested fragments of interest using gel electrophoresis and gel extraction kits.
4. Ligate titin coding sequence fragment to digested SI-Bricks vector, yielding p-1-4XT-1_B, and p-1-4XT-1_L (with P_{BAD} or P_{LacO1} promoter, respectively).
5. Transform ligation products into *E. coli* NEB10β host.

3.2 Shake Flask Protein Production

1. Pick single colonies from p-1-4XT-1_B transformation plate to inoculate 50 mL TB medium in 250 mL Erlenmeyer flask.
2. Incubate 50 mL seed cultures overnight at 37 °C with shaking.
3. Use seed cultures to inoculate 500 mL TB in 2 L Erlenmeyer flasks at OD_{600} of 0.08.