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Protein Downstream Processing

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https://doi.org/10.1007/978-1-0716-0775-6_29

Chapter 29

(Hyper)Thermophilic Enzymes: Production and Purification

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Abstract

The discovery of thermophilic and hyperthermophilic microorganisms, thriving at environmental temperatures near or above 100 °C, has revolutionized our ideas about the upper temperature limit at which life can exist. The characterization of (hyper)thermostable proteins has broadened our understanding and presented new opportunities for solving one of the most challenging problems in biophysics: how are structural stability and biological function maintained at high temperatures where "normal" proteins undergo dramatic structural changes? In our laboratory, we have purified and studied many thermostable and hyperthermostable proteins in an attempt to determine the molecular basis of heat stability. Here, we present methods to express such proteins and enzymes in *E. coli* and provide a general protocol for overproduction and purification. The ability to produce enzymes that retain their stability and activity at elevated temperatures creates exciting opportunities for a wide range of biocatalytic applications.

Key words Thermozymes, Thermal stability, Heterologous production, Protein purification, His-tag, Immobilized metal affinity chromatography (IMAC), Size exclusion chromatography (SEC), Biocatalysis

1 Introduction

Living organisms can be grouped into four main categories as defined by the temperature range in which they thrive: psychrophiles (−15 to 20 °C), mesophiles (20–45 °C), thermophiles (45–80 °C), and hyperthermophiles (≥80 °C) [1]. The origin of extremophilic microorganisms has long been debated. One of the theories suggests that (hyper)thermophilic microorganisms actually appeared on Earth before mesophilic microorganisms [2]. Intuitively, this is in agreement with the environmental conditions on the surface of Earth when life emerged. According to this theory, all biomolecules evolved so as to be functional and stable at elevated temperatures, and subsequently adapted to lower temperature environments. However, another theory suggests that (hyper) thermophiles arose from mesophiles via adaptation to

Nikolaos E. Labrou (ed.), *Protein Downstream Processing: Design, Development, and Application of High and Low-Resolution Methods*, Methods in Molecular Biology, vol. 2178, https://doi.org/10.1007/978-1-0716-0775-6_29,

high-temperature environments. One of the arguments of the latter hypothesis is based on the supposition that RNA (the genetic material in emerging cellular life) is unstable at elevated temperatures [3, 4].

The first hyperthermophilic bacterium (*Thermus aquaticus*) was discovered in 1969 in hot acidic springs in Yellowstone National Park [5]. Since then, over 70 hyperthermophiles (both bacteria and archaea) have been isolated from the environments of extreme temperatures: near or above 100 °C. Examples of environments that, until recently, were considered as being hostile to life include volcanic areas rich in sulfur and "toxic" metals and hydrothermal vents in the deep sea (~4 km below sea level) of extremely high pressure [6]. Interestingly, hyperthermophilic microorganisms do not grow below temperatures of 50 °C and, in some cases, do not grow below 80–90 °C [7]. Yet, they can survive at ambient temperatures for prolonged times; the same way we can preserve mesophilic organisms in the fridge for prolonged times. Thermozymes and hyperthermozymes, in particular, are essentially inactive at moderate temperatures, but gain activity as temperatures increase [8].

Hyperthermozymes are not only active and stable at high temperatures, but are generally also more resistant to organic solvents, detergents, and high concentrations of chemical denaturants (e.g., GdHCl and urea), compared to their mesophilic counterparts [9]. These features may enable their use in a plethora of biotechnological applications. Therefore, it is important to develop technologies that allow the large-scale production and purification of such proteins. Purification of (hyper)thermophilic proteins expressed in a mesophilic host, such as *Escherichia coli*, is greatly facilitated by their intrinsic thermal stability. This generally allows a single-step purification, which involves removal of the vast majority of the host-cell proteins through heat-induced denaturation and subsequent aggregation and precipitation, while the structural integrity and function of the expressed (hyper)thermostable proteins remain intact. After heat treatment, the cell-free extract generally consists of about 90% of the expressed (hyper)thermophilic protein, which is often sufficient for most purposes including biochemical characterization and applications in biocatalysis. Although one or two additional chromatographic steps are usually sufficient to obtain pure protein, nowadays most recombinant proteins are expressed with an N- or C-terminal tag for affinity chromatography and protein detection. A commonly used tag is a polyhistidine (His-tag) tail for a single-step purification using immobilized metal affinity chromatography (IMAC).

In this chapter, we describe general procedures to produce (hyper)thermophilic proteins on the lab scale and to purify at least several mg of His-tagged (hyper)thermophilic protein. The described method has been developed in our lab during the last

20 years and has been successfully applied for the production and purification of various (hyper)thermozymes belonging to a number of enzymatic classes and subclasses, such as hydrolases [10–14], kinases [15–17], isomerases [18, 19], carboxylesterases [20, 21], aldolases [22, 23], dehydrogenases [24, 25], catalase-peroxidases [26], and oxidases [27].

2 Materials

Prepare all solutions using deionized water and analytical grade reagents. The materials are intended for the expression of a gene cloned in a pET24d vector (kanamycin resistant) in frame with a C-terminal His-tag and produced in BL21(DE3) cell (*see* **Note 1**).

2.1 Transformation

1. Electrocell manipulator system ECM 600 (BTX) and 2 mm gap electroporation cuvettes (Bio-Rad Laboratories).
2. pET24d (Novagen) plasmid DNA harboring the gene of interest, for example, the sequence encoding for a hyperthermostable esterase (pET24d-EstD) (*see* Subheading 3).
3. Electro-competent *E. coli* BL21(DE3) cells (Novagen) (*see* **Note 2**).
4. Tris–HCl and EDTA (TE) buffer: 10 mM Tris–HCl and 1 mM EDTA, pH 8.5. Sterilize by filtration through a 0.22-μm filter. Store at room temperature.
5. Super Optimal Broth with Catabolite repression (SOC) medium: 2% w/w tryptone, 0.5% w/w yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 20 mM glucose, and pH 7.0. Dissolve 2 g of tryptone and 0.5 g of yeast extract in approximately 90 mL of water and transfer to a 100 mL graduate cylinder. Add 1 mL of 1 M NaCl, 0.25 mL of 1 M KCl, and 2 mL of 2 M $MgCl_2$. Mix and adjust the pH at 7.0 if necessary. Bring to a final volume of 100 mL with water. Autoclave the solution. Then, add 1 mL of filter-sterilized 2 M glucose. SOC medium can be stored in 5 mL aliquots at −20 °C.
6. Luria Bertani (LB) medium: 1% w/w peptone, 0.5% w/w yeast extract, 1% w/w NaCl, and pH 7.0. Dissolve 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl in 900 mL of water. Mix and adjust the pH at 7.0 if necessary. Bring to a final volume of 1000 mL. Autoclave the solution.
7. Luria Bertani (LB) agar: LB medium with 1.5% w/w agar. Add 7.5 g of agar to 500 mL of the LB medium and autoclave the solution.

8. Kanamycin stock solution (1000×): 50 mg/mL in water. Dissolve 500 mg of kanamycin in 10 mL of water and sterilize through a 0.22 μm syringe filter. Store at −20 °C (*see* **Note 3**).
9. LB-agar plates. Dispense the LB-agar medium supplemented with 50 μg/mL of kanamycin in Petri dishes.

2.2 Growth Medium and Inducer

1. Dispense 500 mL of the LB medium in four 2.5 L Erlenmeyer flasks and autoclave the solution.
2. Isopropyl-β-D-thiogalactopyranoside (IPTG) solution: 1 M solution in water. Sterilize through a 0.22 μm filter. Store the solution at −20 °C.

2.3 Solutions for Protein Preparation and FPLC Purification

1. Deoxyribonuclease I (DNaseI): prepare a solution containing 10 U/μL in lysis buffer.
2. Lysis buffer: 50 mM Tris–HCl, 300 mM NaCl, and pH 7.5. Dissolve 1.21 g of Tris and 3.5 g of NaCl in approximately 190 mL of water and transfer to a 250 mL graduate cylinder. Mix and adjust the pH at 7.5 with 6 M HCl. Bring to a final volume of 200 mL with water. Store at room temperature.
3. FPLC-IMAC Buffer A: 50 mM Tris–HCl, 300 mM NaCl, and pH 7.5. Dissolve 3.03 g of Tris and 8.77 g of NaCl in 480 mL of water and transfer to a 500 mL graduate cylinder. Mix and adjust the pH at 7.5 with 6 M HCl. Bring to a final volume of 500 mL with water. Filter the solution through a 0.45 μm filter and store at room temperature.
4. FPLC-IMAC Buffer B: 50 mM Tris–HCl, 300 mM NaCl, 500 mM imidazole, and pH 7.5. Dissolve 1.52 g of Tris, 4.39 g of NaCl, and 8.51 g of imidazole in 240 mL of water and transfer to a 250 mL graduate cylinder. Mix and adjust the pH at 7.5 with 6 M HCl. Bring to a final volume of 250 mL with water. Filter the solution through a 0.45 μm filter and store at room temperature.
5. FPLC-desalting column and FPLC-Size exclusion chromatography (SEC) buffer: 50 mM Tris–HCl, 150 mM NaCl, and pH 7.5. Dissolve 1.52 g of Tris and 2.2 g of NaCl in 240 mL of water and transfer to a 250 mL graduate cylinder. Mix and adjust the pH at 7.5 with 6 M HCl. Bring to a final volume of 250 mL with water. Filter the solution through a 0.45 μm filter and store at room temperature.

3 Methods

3.1 Gene Cloning into Expression Vector

The genes encoding the proteins of interest may be amplified from the microbial genomic DNA by the polymerase chain reaction (PCR). This approach is simple and inexpensive but does not allow the introduction of modifications in the coding sequence.

Although hyperthermozymes can be easily expressed in a mesophilic host, such as *E. coli*, sometimes the different codon usage between the native species and the production organism may result in poor protein expression. Currently, due to the decrease of the cost of synthetic DNA constructs, it is possible to design a gene with codons optimized for good expression in the host cell of choice. Most genes of interest are expressed after cloning in a pET series vector (Novagen) in frame with an N- or C-terminal His-tag for affinity chromatography and protein detection. These expression vectors are suitable for protein expression in *E. coli* strains, which express T7 RNA polymerase under control of the *lac* promoter, e.g. BL21(DE3), using IPTG as an inducer (see below).

3.2 E. coli BL21(DE3) Electrotransformation

1. Thaw the electrocompetent *E. coli* BL21(DE3) cells (which were stored at −80 °C) on ice (*see* **Note 4**).
2. In an ice cooled 1.5 mL polypropylene tube, mix 50 μL of the cell suspension with 1–2 μL of solution containing 1–2 μg of plasmid DNA in a low-ionic buffer such as TE buffer (*see* **Note 5**). Mix well and allow equilibration on ice for ~1 min.
3. Set the electro cell manipulator apparatus at 25 μF/2.5 kV. Set the pulse controller to 200 μΩ. Add the mixture of cells and plasmid to an ice-cooled 0.2 cm and make sure that the suspension is at the bottom of the cuvette. Push the cuvette in the safety chamber slide.
4. Apply one pulse using the above settings. This should produce a pulse with a time constant of ~5 μs (field strength should be 12.5 kV/cm). (Settings depend on the type of apparatus and therefore, optimization may be necessary).
5. Remove the cuvette from the chamber and immediately add 1 mL of 37 °C prewarmed SOC medium to the cuvette and gently mix by pipetting (*see* **Note 6**).
6. Transfer the cell suspension to a 14-mL polypropylene tube and incubate at 37 °C for 1 h while shaking the tube at 225 rpm to increase the recovery of the transformants.
7. Plate the cells on the LB agar medium supplied with 50 μg/mL kanamycin and incubate the plates overnight at 37 °C (*see* **Note 7**).

3.3 Protein Production and Purification

1. In a sterile atmosphere (flow chamber, or close to a flame), transfer a single colony of *E. coli* BL21(DE3) harboring the plasmid pET24d-EstD from the plate to a sterile tube containing 3 mL of the LB medium supplied with kanamycin. Incubate overnight in a shaking incubator at 37 °C.

2. The next day, again in a sterile atmosphere, transfer 0.5 mL of the overnight culture into each flasks containing 0.5 L of LB medium supplied with 50 μg/mL of kanamycin. Incubate in a shaking incubator (180 rpm) at 37 °C for 6 h (or until a cell suspension optical density at 600 nm of about 1.2–1.5).
3. Remove and store 1 mL culture sample before the induction for sodium dodecyl sulfate polyacrylamide gel electrophoresis gel analysis (SDS-PAGE) (*see* **Note 8**). Induce protein expression by adding, in a sterile atmosphere, IPTG to a final concentration of 0.5 mM. Incubate at 37 °C while shaking (150 rpm) for at least 16 h (*see* **Note 9**).
4. Harvest cells by centrifugation at 4 °C, 10,000 × *g* for 10 min. Resuspend the cell pellet in 25 mL of cell lysis buffer.
5. Pass the cell suspension twice through a French press at 110 MPa to lyse the cells. Add 1 μL of the DNaseI solution (10 U) to the crude cell extract and incubate for 20 min at room temperature to degrade the DNA and reduce the viscosity. Centrifuge the DNaseI-treated crude cell extract at 4 °C, 43,000 × *g* for 30 min. Transfer the supernatant (cell-free extract (CFE)), to a new tube and discard the pellet, which contains the cell debris. Incubate the CFE for 30 min at 70 °C to denature *E. coli* proteins (*see* **Note 10**). When correctly folded and present in the soluble fraction, this step should not affect the heterologously produced (hyper)thermostable protein. Centrifuge at 4 °C, 43,000 × *g* for 30 min to remove denatured biomolecules, which precipitate in the pellet. Transfer the supernatant, which represents the heat stable cell-free extract (HSCFE), to a new tube (*see* **Note 11**).
6. Filter the HSCFE through a 0.45 μm syringe filter, and use an ÄKTA FPLC protein purification system (GE Healthcare) to apply it to a 1 mL HisTrap column (GE Healthcare) equilibrated with Buffer A. Wash with Buffer A until the absorbance trace at 280 nm has returned to the baseline (*see* **Note 12**), and subsequently apply a linear gradient of 0–500 mM imidazole (Buffer B). All fractions are collected. Pool fractions containing the protein of interest (*see* **Note 13**).
7. Apply the collected fractions to a HiPrep 26/10 desalting column (GE Healthcare) equilibrated with 50 mM Tris–HCl buffer (pH 7.5) and 150 mM NaCl to remove the excess of imidazole.
8. Run a SDS-PAGE with samples collected during all the extraction and IMAC purification steps and measure the total protein content using for instance the Bradford assay [27] (*see* **Note 14**).

9. Apply the collected fractions to a HiPrep 26/10 desalting column (GE Healthcare) equilibrated with 50 mM Tris–HCl buffer (pH 7.5) and 150 mM NaCl to remove the excess of imidazole.
10. Apply the protein preparation to a Superdex-200 gel filtration column (GE Healthcare) equilibrated in 50 mM Tris–HCl buffer (pH 7.5) containing 150 mM NaCl to determine the native molecular weight of your protein.

3.4 Troubleshooting

1. Possible reasons for low protein yield:
   (a) IPTG in the stock solution may be degraded or the protein is toxic for the host. Protein is not properly folded with the consequent formation of inclusion bodies.
   (b) Incomplete resuspension of the pellet before the French Press processing, which may have resulted in the presence of significant amounts of protein in the pellet after centrifugation.
   (c) Incomplete lysis of the *E. coli* cells in the French Press.
   (d) Protein loss during chromatographic separation due to the nonaccessibility of the affinity tag.
   (e) No protein expression due to different codon usage bias.
2. Possible solutions for increasing the protein yield.
   (a) Use freshly made IPTG. Reduce the amount to a micromolar concentration to decrease the expression level and facilitate the correct protein folding.
   (b) Reduce the growth temperature during induction to 28 °C or 20 °C to facilitate correct protein folding and prevent the formation of inclusion bodies. At a lower temperature, the protein synthesis rate is lower giving more time to the neo synthetized protein to fold correctly and this may reduce protein aggregation.
   (c) To induce the expression of endogenous *E. coli* chaperone proteins, proteins that bind to unfolded proteins avoiding protein aggregation and facilitating protein folding, it is also possible to perform a cold shock before the IPTG induction, e.g. from 37 °C to 4 °C for a duration of 20–30 min.
   (d) If the protein is produced as stable and soluble at 37 °C only for a short time before aggregation, the induction time could also be reduced to 3–4 h although this step may result in reduced protein yield production. Consider to express your protein fused to a highly soluble protein, like Maltose Binding Protein (MBP) to facilitate the folding and to avoid the formation of inclusion bodies.

(e) Although proteins from (hyper)thermophilic organisms can be easily expressed in *E. coli* sometimes, the different codon usage bias between organisms can lead to failure in protein production. In this case, it is advisable to use *E. coli* strains containing a tRNA helper plasmid encoding several rare tRNAs to overcome the difference in codon usage.

4 Notes

1. Several affinity tags are available on different vectors from different suppliers. According to the type of vector, promoter/inducer combination, affinity tags, and antibiotic resistance gene may vary. For an overview of bacterial expression vector features, *see* Ref. 28.
2. *E. coli* BL21(DE3) strain expresses the T7 RNA polymerase under control of the lac promoter, using IPTG as an inducer. Several *E. coli* BL21(DE)3 strains are available for protein production, those may include strains containing plasmid coding for rare tRNA (BL21(DE3)pRIL), to overcome difference in codon usage bias, or for T7 lysozyme (BL21(DE3) pLys,) to reduce the background expression of toxic proteins.
3. It is important to apply antibiotic selection during all the stages of growth according to the resistance gene carried on your expression vector. Working concentrations of commonly used selection's antibiotics are: 100 μg/mL for ampicillin (Na-salt) and 50 μg/mL for kanamycin, streptomycin, and tetracycline-HCl.
4. Commercial electrocompetent cells gave the best results but several protocols are available for competent cell preparation. For a detailed general protocol, *see* Ref. 29.
5. DNA stored at −20 °C should be in low ionic strength buffer such as TE. A high ionic strength solution, containing a high concentration of salts, is conductive and can cause arcing under the high voltage conditions used in electroporation.
6. Quick addition of the SOC medium to the electroporation cuvette directly after the electric pulse is very important to maximize the recovery of the transformants.
7. Only the *E. coli* cells that contain the plasmid with the antibiotic resistance gene will survive.
8. For the SDS-PAGE protocol, *see* Ref. 30.
9. *E. coli* growth conditions may influence protein expression. When a low amount of soluble protein is produced or the protein aggregates as insoluble precipitates (inclusion bodies,

IB), culturing *E. coli* under suboptimal conditions (e.g. lower temperature and minimal medium) may improve protein expression. The amount of inducer and the duration of induction may also be changed to improve protein expression.

10. As standard conditions, a temperature of 70 °C and a duration time of 30 min are used. However, it is best to test different temperatures and incubation times using small portions of the CFE. Depending on the (hyper)thermozyme, better purification can be obtained optimizing the temperature and incubation time.
11. A biological assay should be used for monitoring the activity of the (hyper)thermozyme of interest during the extraction and the purification steps. When a specific assay is not available, it is possible to detect the presence of the protein of interest through immunoblotting techniques using anti-His-tag antibodies.
12. Optionally, the column can be washed with 10–20 mM imidazole (2–4% Buffer B) in the same Buffer A, and subsequently proteins can be eluted with a linear gradient of 10/20–500 mM imidazole. Washing may be necessary to remove contaminant proteins bound to the nickel column.
13. Generally, the presence of the affinity tag is enough to guarantee high purity. When contaminant proteins are present, it is possible to increase purity using other chromatographic techniques like hydrophobic interaction chromatography (HIC) and/or ion exchange chromatography (IEC).
14. SDS-PAGE analysis and measurements of the activity of the protein of interest can be used for the construction of a purification table.

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