Chapter 7

Preparation of Tau Oligomers After the Protein Extraction from Bacteria and Brain Cortices

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Abstract

Oligomerization of soluble tau protein is attracting the attention of an increasingly larger number of scientists involved in research on Alzheimer's disease and other tauopathies. A variety of methods have been developed for the purification of proteins from biological tissues and bacterial cells. Various types of high performance liquid chromatography (HPLC) and affinity tags represent the most common techniques for isolating proteins. Here, we describe a procedure for extracting recombinant tau protein from bacterial cells, utilizing a 6×His affinity tag, or endogenous tau from brain cortices using acid extraction followed by fast protein liquid chromatography (FPLC). Additionally, we introduce a method for oligomerization based on reduction and oxidation of cysteine residues. Our preparation assures high yield of tau protein, while preserving its physiological function.

Key words Recombinant tau, Tau extraction, Tau oligomerization, Affinity tag purification, Fast protein liquid chromatography, Alzheimer's disease, Tauopathy

1 Introduction

While originally characterized as a microtubule binding protein, subsequent research has identified additional intra- and extracellular activities for tau and its aggregates. These discoveries and the need to further understand the biological actions of normal and pathological forms of tau have created the need for an efficient and cost effective tau preparation method.

Protein purification refers to a multistep process aiming to isolate a protein of interest from a diverse mixture utilizing unique physicochemical characteristics of the target protein including mass, net charge, isoelectric point, solubility, and ligand/metal binding properties [1]. There are a variety of protocols for protein purification in modern biochemistry. However, the development of an appropriate procedure for isolating the given protein while preserving its physiological properties and ensuring high yield is challenging. In general,

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common procedures for protein purification comprise the following steps: (1) extraction from the biological tissue or bacterial culture, (2) cell lysis, removal of cell debris and non-protein components, (3) enrichment process, including concentration and precipitation, (4) intermediate purification, usually accomplished by exploiting the binding properties of the desired protein, (5) polishing, in order to achieve higher level of purity alongside with high yield. Common techniques for the purification include HPLC and affinity-tag purification for recombinant proteins. Different types of HPLC could be used depending on the properties of the protein and the level of purification including reversed-phase chromatography, ion-exchange chromatography, size-exclusion chromatography, and affinity chromatography [2–5]. When it comes to the isolation of recombinant proteins, utilization of affinity protein tags is a common strategy. Maltose-binding protein (MBP), glutathione-S-transferase (GST), and poly-His (6×His) tag represent the most widely used affinity tags [6–8].

In this chapter, we describe in detail a method to isolate tau protein from bacterial cells and cortices of Alzheimer's disease (AD) patients or mice. In the protocol for recombinant tau preparation, bacterial cells were lysed in Triton X-100 phosphate buffered saline with protease inhibitor. Further purification of the tau construct was performed with a nickel column with His-bind resin, and eluted with phosphate buffer containing imidazole. For the human Tau, homogenization with a buffer containing perchloric acid (PCA) followed by further enrichment by anion exchange chromatography. An advantage of the acidic homogenization with PCA is that it allows the removal of DNA and other proteins, while preserving tau phosphorylation. Moreover, this method permits the enrichment of soluble oligomeric tau species from human or mouse brain samples. In both the cases, purification was completed with several steps of buffer exchange via centrifugal concentrators.

After the final step of purification, isolated soluble tau was oligomerized. There are several methods for tau oligomerization such as addition of heparin, heparin sulfate, polyunsaturated fatty acids, RNA or quinones, and Aβ seeding [9–14]. Although the above-mentioned methods are effective for producing tau oligomers, they result in a variety of species with controversial toxicity or no toxicity [15]. Indeed, aggregation kinetics differ among methods, resulting in tau species that are not necessarily equivalent in terms of both size and biophysical properties [15]. In our preparation, tau oligomerization was achieved via introduction of disulfide bonds after overnight incubation with 1 mM H_2O_2. Even though the species that induce the toxicity could not be identified, our protocol for producing tau oligomers proved to affect efficiently key features of AD, including synaptic plasticity and memory [16]. We propose using the protocol described in this manuscript as a standardized method for the preparation and oligomerization of tau.

2 Materials

2.1 Over-Expression and Purification of Recombinant Tau

1. LB agar plates containing ampicillin.
2. Expansion and overexpression broth.
3. 10% Triton X-100: Mix 10 ml of 100% Triton X-100 with 90 ml ddH_2O in constant stirring. Store at 4 °C (*see* **Note 1**).
4. 50 ml of 1 M $MgCl_2$: Add 2.61 g $MgCl_2$ in a final volume of 50 ml.
5. 25 ml lysis buffer: Mix 5 ml of 10% Triton X-100, 50 μl of 1 M $MgCl_2$, 5 μl of 5000 U/μl benzonase, three tablets of cOmplete ULTRA, EDTA-free protease inhibitor and fill in the volume with ddH_2O (*see* **Note 2**). Prepare fresh lysis buffer before each preparation and keep it on ice.
6. Streptomycin sulfate. Store it at 4 °C.
7. 20 ml 20 K MWCO protein concentrators.
8. Ultra-pure DNase/RNase-free distilled water.
9. His-spin protein miniprep kit.
10. Tris-(2-Carboxyethyl)phosphine hydrochloride (TCEP-HCl).
11. 1% Perchloric acid (PCA).
12. Ethylenediaminetetraacetic acid (EDTA).

2.2 Oligomerization Lysis Buffer for Recombinant Tau

1. 500 ml of 5 M NaCl: Add 146.1 g of NaCl in a final volume of 500 ml.
2. 500 ml of 1 M Na_2HPO_4: Add 70.98 g Na_2HPO_4 in a final volume of 500 ml.
3. 500 ml of 1 M NaH_2PO_4: Add 78 g NaH_2PO_4 in a final volume of 500 ml.
4. 75 ml of 1 M Na_3PO_4 buffer (pH 7.2): Add 51.3 ml of 1 M Na_2HPO_4 and 23.7 ml of 1 M NaH_2PO_4.
5. 1.5 L oligomerization dialysis buffer: Add 30 ml of 5 M NaCl, 75 ml of 1 M $NaPO_4$ buffer (pH 7.2) and fill in the volume with ddH_2O. Store it at 4 °C.
6. 30% H_2O_2. Store it at 4 °C.
7. Anti-tau primary antibody, western blotting reagents and equipment.

2.3 Extraction, Purification, and Oligomerization of Tau from Human and Mouse Brain

1. Artificial cerebrospinal fluid (ACSF) consisting of in mM: 124 NaCl, 4.4 KCl, 1 Na_2HPO_4, 25 $NaHCO_3$, 2 $CaCl_2$, 2 $MgCl_2$, and 10 glucose, chilled on ice and bubbled with 95% oxygen/5% carbon dioxide.
2. Lysis buffer: 20 mM histidine, 1% perchloric acid pH 1, supplemented with Roche PhosSTOP phosphatase inhibitor and

cOmplete ULTRA protease inhibitors at 3× the manufacturer's recommended concentration. Crush phosphatase and protease inhibitor tablets to facilitate their dissolution. Filter buffer using a 0.2 μm syringe filter and chill to 4 °C prior to use.

3. Tissue Tearor.
4. Slide-a-lyzer cassette (15 ml 10,000 MWCO).
5. Protein concentrators (Sartorius, Vivaspin Turbo 15, 10,000 MWCO).
6. AKTA FPLC device and frac-950 fraction collector connected to a PC running Unicorn software v5.31.
7. LC/MS grade water.
8. Nalgene rapid flow disposable filter unit with PES membrane and 0.2 μm pore size.
9. Buffer A for FPLC: 20 mM histidine, pH 6.5, filtered (*see* **item 8**).
10. Buffer B for FPLC: 1 M NaCl in 20 mM histidine pH 6.5, filtered (*see* **item 8**).
11. Glass culture tubes (13 × 100 mm).
12. 20% EtOH for FPLC, filtered (*see* **item 8**).
13. 0.5 M NaOH filtered (*see* **item 8**) for column cleanup.
14. Anti-tau primary antibody and western blotting reagents.
15. Oligomerization Buffer: 100 mM NaCl in 1× PBS, filtered (*see* **item 8**).
16. 2.2 ml Eppendorf protein LoBind tubes.
17. 30% H_2O_2 (Alfa Aesar, 29–32% w/w aq. Soln.).

3 Methods

For the preparation of recombinant tau in our protocol, a C-terminal His-tag tau 4R/2N construct (gift of Dr. Furukawa; University of Yokohama, Japan) was prepared in expression vector pET29a and transfected into the E. coli bacterial strain BL21 for protein expression. The day before starting overexpression of recombinant tau, bacteria were streaked in LB agar ampicillin plates and left overnight to grow at 37 °C. The plates were stored at 4 °C. The procedures were carried out at room temperature unless otherwise stated in the procedure. Disposal of cultured medium containing bacteria followed the disposal regulation of hazardous materials imposed by the university.

3.1 Overexpression and Recovery of Recombinant Tau

1. Inoculate a single colony in culture medium (100 ml expansion broth + 300 ml overexpression broth) at 37 °C overnight at 200–250 rpm (*see* **Note 3**).
2. Separate the culture in two bottles and harvest the bacterial cell by centrifugation at 6000 × *g* for 30 min in a GS3 rotator at 4 °C.
3. Discard the supernatant and resuspend the two pellets in 25 ml of ice-cold lysis buffer (*see* **Note 4**).
4. Add 0.5 g streptomycin sulfate to precipitate DNA, incubate 5 min on ice and sonicate.
5. Boil for 15 min, spin for 20 min at 15,000 × *g* at 4 °C, and collect the supernatant in new tubes (*see* **Note 5**).
6. Add Tris-(2-Carboxyethyl)phosphine hydrochloride (TCEP-HCl) to obtain a final concentration of 5.73 mg/ml and 1/10 volumes of 1% PCA.
7. Neutralize pH with 1 N NaOH (*see* **Note 6**).

3.2 Isolation of Recombinant Tau

1. Clean the 20 K MWCO protein concentrators by adding ultrapure distilled water and centrifuge for 20 min at 40,000 × *g* at 4 °C. Repeat the step three times. Each time discard the flow-through and fill up the column entirely with water.
2. Transfer the supernatant to the concentrators and dialyze it in the binding buffer from the His-spin protein miniprep kit by centrifuging at 40,000 × *g* at 4 °C (*see* **Note 7**).

   The following steps are carried out in the His-spin protein miniprep kit:

3. Assemble the columns by placing the green columns containing the filter in 1.7 ml collecting tubes.
4. Add 300 μl of His-affinity gel to each Zymo-spin column (*see* **Note 8**), spin at 15,000 × *g* for 10 s at 4 °C, and place the columns to new collecting tubes.
5. Add 360 μl of lysate to each column and mix by flicking the tubes for 5 min, spin columns for 10 s at 15,000 × *g* at 4 °C (*see* **Note 9**), and save the flow-through (*see* **Note 10**).
6. Wash the columns with 250 μl binding buffer, mix for 5 min, spin for 5–10 s, and save the flow-through (×2).
7. Add 200 μl of His-elution buffer, shake for 5 min, spin for 5–10 min, and collect the flow-through containing recombinant tau (*see* **Note 11**). At this step, recombinant tau could be visualized by Western Blot (Fig. 1).

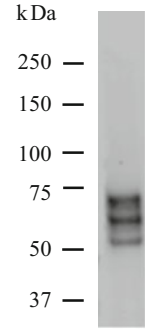

Fig. 1 Immunoblot for recombinant tau preparation using anti-tau antibody. The lane represents the flow-through after the isolation of recombinant tau from the His-affinity columns (Subheading 3.2, **step 7**). Multiple bands are likely to arise from degradation, proteolysis, or prematurely terminated translation

3.3 Extraction of Tau from Human and Mouse Brain

1. Tissue Isolation.
   - For human brain use ~2 g of tissue.
   - For mouse brains:
     - Rapidly remove brain from mouse, and rinse and chill in ice cold ACSF or PBS.
     - Dissect cortices and place one cortex from each hemisphere in a 1.5 ml Eppendorf.
     - Snap freeze in liquid nitrogen and store at −80 °C until ready for homogenization and tau extraction.
     - Repeat for a total of 6–10 mice per preparation.
2. Homogenization.
   - Pool cortices from 6–10 mice in one 50 ml disposable tube on ice.
   - Add 10 ml ice-cold lysis buffer.
   - Homogenize on ice using a Tissue Tearor until a homogeneous lysate is obtained.
   - Seal the tube and incubate at 4 °C for 30 min on a rotator.
   - Transfer lysate to 2 ml Eppendorf tubes on ice.
   - Centrifuge tubes in a refrigerated microfuge at 21,000 × *g* for 10 min at 4 °C.
   - Transfer supernatants to a single 15 ml disposable tube on ice.
3. Buffer Exchange.
   - Precool 3 L of buffer A for dialysis.
   - Prewet slide-a-lyzer (15 ml 10,000 MWCO) as per the manufacturer's instructions in buffer A.

- Inject homogenate supernatant into slide-a-lyzer cassette.
- Place cassette in a 2 L beaker with 1 L of buffer A.
- Dialyze for 18–24 h at 4 °C with two buffer changes.
- Rinse protein concentrator (Sartorius, Vivaspin Turbo 15, 10,000 MWCO) by adding ~15 ml LC/MS grade water and centrifuging at 3900 × *g* but do not allow all of the water to pass through the filter to prevent it drying out.
- Repeat rinse two more times.
- Add dialyzed sample to protein concentrator and centrifuge at 3900 × *g* to achieve a concentrated volume of ~5 ml (this is the capacity of the column and sample loop). pH should be ~6.

4. FPLC.
   - Change filter and connect sample loop to FPLC device.
   - Flush machine with water.
   - Connect HiTrap Q HP 5 ml column being careful not to introduce bubbles.
   - Run water through both the pumps until conductivity and UV are almost 0.
   - Place intake for pump A into buffer A and intake for pump B into buffer B.
   - Run wash protocol twice consisting of:
     - 1 column volume gradient from 100% buffer B to 100% buffer A.
     - 1 column volume wash in 100% buffer A.
     - 1 column volume gradient from 100% buffer A to 100% buffer B.
     - 2 column volume wash in 100% buffer B.
     - 1 column volume gradient from 100% buffer B to 100% buffer A.
     - 1 column volume gradient from 100% buffer A to 10% buffer B.
     - 7 ml wash in 15% buffer B.
   - Place clean glass culture tubes (13 × 100 mm) into a fraction collector.
   - Place labeled protein LoBind 1.5 ml Eppendorf tubes into tubes 11 through 32.
   - Filter unpurified protein sample with a 0.2 μm syringe filter before loading.
   - Run anionic exchange protocol consisting of:

  - Preconfigured pump wash.
  - 5 column volume equilibration in 100% buffer A.
  - Manual sample loading followed by 15 ml sample injection in 100% buffer A collected in 3 × 5 ml fractions.
  - 7 column volume wash of unbound sample in 100% buffer A collected in 1 ml fractions.
  - 6 column volume gradient segment 1 from 100% buffer A to 50% buffer B collected in 1 ml fractions.
  - 5 column volume gradient segment 2 from 50% to 1000% buffer B collected in 2 ml fractions.
  - 2 column volume wash in 100% buffer B collected in 2 ml fractions.
- At the loading step, manually fill sample loop with buffer A, then load sample into sample loop using a syringe through a two-way stopcock that has been filled with water to prevent injecting air into the system. Make sure the injection valve on the FPLC device is set to load during this process.
- Check that the pressure is stable during chromatography and remains below 0.28 (0.30 is maximum for the column).
- After the completion of anion exchange protocol, transfer 10 μl from each fraction into Eppendorf tubes for western blot analysis to identify tau-containing fractions (Fig. 2).
- Store remainder of fractions at −20 °C until western blot analysis has been completed.
- Clean column by:
  - Running wash protocol outlined above.
  - Running 5 column volumes of 0.5 M NaOH through column.

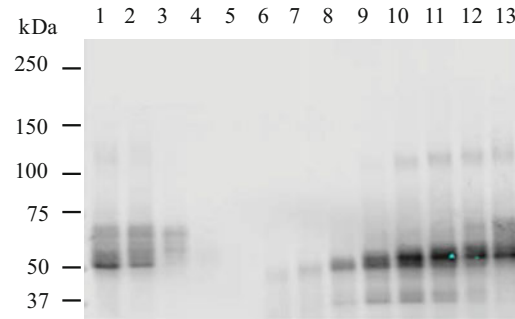

Fig. 2 Western blot for tau performed on fractions obtained during the isolation of endogenous mouse tau. Lanes 1–3 tau immunoreactivity present unbound sample. Lanes 4–13 tau immunoreactivity present in fractions obtained during elution with increasing concentrations of FPLC buffer B (20 mM histidine pH 6.5) (Subheading 3.3, **step 4**)

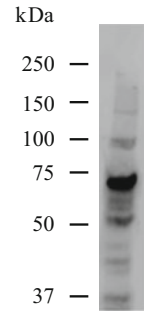

Fig. 3 Immunoblot for recombinant tau oligomers using anti-tau antibody. The lane shows the final product of tau preparation after isolation and oligomerization (Subheading 3.4, **step 8**). The bands above 100 kDa represent various order oligomeric tau. Multiple bands below 50 kDa are likely to arise from degradation, proteolysis, or prematurely terminated translation

- Running 5 column volumes of water through column.
- Running 5 column volumes of 20% Ethanol through column for storage (use both the pumps).

3.4 Oligomerization of Recombinant Tau Monomers

1. Add TCEP to the eluate to a final concentration of 0.287 mg/ml.
2. Add EDTA to a final concentration of 5 mM.
3. Incubate at 37 °C for 1 h.
4. Clean the 20 K MWCO protein concentrators with ultra-pure distilled water as described before, transfer the eluate to the column, and buffer exchange it with the oligomerization buffer by centrifuging at 40,000 × *g* at 4 °C (*see* **Note 12**).
5. Collect the eluate from the filter and add 30% H_2O_2 to obtain a final concentration of 1 mM.
6. Incubate at room temperature for 20 h in constant rotation for introducing disulfide bonds.
7. Dialyze again against oligomerization buffer as in **step 4** (*see* **Note 13**).
8. Determine tau protein concentration from the absorption at 280 nm with a nanodrop spectrophotometer (*see* **Note 14**) and additionally visualize tau oligomers by Western Blot (Fig. 3) (*see* **Note 15**).

3.5 Oligomerization of Mouse and Human Tau and Sample Preparation

1. Load 5 μl from each fraction on each well of a 26-well 1 mm 10% Tris-glycine gel.
2. Perform western blotting according to standard procedures and probe using anti-tau primary antibody to identify tau-containing fractions.

3. Thaw and pool tau containing fractions and load on protein concentrator (Sartorius, Vivaspin Turbo 15, 5000 MWCO).
4. Use concentrator to perform buffer exchange by repeatedly spinning at 3900 × *g* until ~5 ml of sample remains followed by the addition of oligomerization buffer to a volume of 15 ml until a total of 50 ml of oligomerization buffer has been applied.
5. Allow the final centrifugation step to proceed until the sample has been concentrated to a volume of approximately 2 ml.
6. Place samples into 2.2 ml Eppendorf protein low-bind tubes and add 1.1 μl of 30% H_2O_2.
7. Place the tube on a rotating mixer for 20 h at room temperature.
8. Repeat the buffer exchange procedure to remove H_2O_2—containing buffer by transferring the sample to a protein concentrator (Sartorius, Vivaspin Turbo 15, 5000 MWCO), and repeatedly adding fresh oligomerization buffer until a total of 50 ml has been applied.
9. Allow the final centrifugation step to proceed until the sample volume has again reached approximately 2 ml.
10. Determine protein concentration of final solution by using a Nanodrop spectrophotometer as described in Subheading 3.4, **step 8**.
11. Presence and oligomeric state of tau can also be assessed by western blot under nonreducing conditions.

4 Notes

1. Since Triton X-100 is viscous, cutting the tip of the pipette will facilitate its aspiration.
2. Grind the protease tablets with mortar and pestle in order to expedite the process of dissolving them in the lysis buffer.
3. The velocity of rotation is essential for bacterial growth. In case that the incubator does not reach the 250 rpm speed, you should increase the time of incubation. Bacteria overgrowth can be easily distinguished since the culture media is turning from clear to hazy.
4. Resuspending the bacterial pelettes could be time consuming. In order to break up the pellets you could pipette up and down and vortex the bottles. Usually at the end of this step we end up with ~30 ml of lysate.
5. We divide the above lysate equally into 24 tubes and place them in a water bath at 70 °C. After 15 min of boiling you should see

separation of lysate in two phases. Subsequent centrifugation at 15,000 × *g* will cause precipitation of one phase. In case that your centrifuge does not reach the above speed, you should increase the time of centrifugation until achieving complete precipitation. We usually collect ~24 ml of supernatant.

6. After adding the TCEP the pH should be acidic (pH ~4–5). Caution should be taken when you adjust the pH with 1 N NaOH since a single drop could cause dramatic shift in the pH from acidic to basic. Therefore, we suggest adding a single drop, mix well and measuring each time the pH before adding the next drop.
7. We mix the supernatant containing recombinant tau (~24 ml) with 45 ml of binding buffer and perform several steps of centrifugation—each lasting 20 min—until collecting ~50 ml of flow-through and ~9 ml from the lysate in the column. At each centrifugation step, we collect the flow-through in a new tube and fill up the column again with the above mixture. Progressively the volume that passes through the column is decreasing and the procedure could take time to be completed. When you collect the supernatant from the column at the very last step, avoid aspirating the viscous brown precipitant that formed at the bottom.
8. We recommend to vortex or shake the His-affinity gel each time before pipetting the appropriate amount to the columns; otherwise, the resin containing in the gel will precipitate. Additionally, make sure that after the centrifugation step the His-affinity gel is completely drained. It is possible that in some tubes the His-affinity gel of the columns will not be completely dry. In that case, repeat the step only for the above tubes in order to avoid over-drying the rest of the tubes due to the excess centrifugation.
9. Mixing the lysate with the gel and the following materials from the kit is essential; otherwise, the recombinant tau will pass through the column.
10. Along the same lines, we save the flow-through in the steps conducted with the kit in order to detect possible loss of our protein.
11. At this step we expect to collect ~5 ml of sample. Due to time limitation, we usually freeze the sample at −20 °C and proceed with the rest of the steps the following day.
12. As in the previous step we mix the eluate (~5 ml) with 47 ml of oligomerization buffer and perform several steps of centrifugation—each last 20 min—until collecting ~50 ml of flow-through and 1.5–2 ml of the eluate in the column.

13. This step is carried out as before. After cleaning the protein concentrators, we mix the eluate from the previous step with oligomerization buffer and we perform several steps of centrifugation. We want to collect 50 ml in the flow-through and 1–2 ml of the sample in the column. Adjusting the volume that you will finally collect from the column could result in more or less concentrated sample. Nevertheless, we do not recommend collecting less than 1 ml since at the final step it is difficult to predict the volume that will pass through the filter and it entails the danger of losing the protein.
14. For NanoDrop measurement we use molecular weight of 45,900 kDa and extinction coefficient of 7450 cm^{-1} m^{-1}. Oligomerization buffer is used for the blank measurement.
15. For visualization with Western Blot, 7 μg of the sample was mixed with loading buffer without reducing agent and boiled for 5 min at 100 °C. Reducing agent was excluded because the latter breaks down disulfide bonds and in that case we won't be able to visualize high order oligomers formed by disulfide bonds, but only monomers. The samples were loaded to 10% Tris-Acetate gels that transferred on nitrocellulose membrane, following a common Western Blot protocol. For immunoblotting we used an anti-tau antibody diluted to a final concentration according to the manufacturer's instructions. After developing the membrane you should be able to detect bands above 100 kDa that represent tau oligomers. The band at ~50 kDa represents the monomeric form.

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