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Authors

Bitan, Gal
Teplow, David B

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Preparation of Aggregate-Free, Low Molecular Weight Amyloid-β for Assembly and Toxicity Assays

Gal Bitan and David B. Teplow

Summary

More than 20 diseases have been identified which are caused by the deposition of amyloid. Natural and chemically synthesized amyloidogenic proteins are used widely to study the structure, assembly, and physiologic effects of both oligomeric and fibrillar forms of these proteins. In many cases, conflicting results arise in these studies, in part owing to difficulties in reproducibly preparing amyloidogenic proteins in a well-defined assembly state. To avoid these problems, several methods have been devised that provide reliable means of preparing amyloid-forming proteins for experimental use. Here, we discuss methods that have been used successfully to prepare one such protein, the amyloid β-protein (Aβ), involved in Alzheimer's disease.

Methods for reproducible preparation of Aβ in a well-defined assembly state include isolation of low molecular weight (LMW) Aβ by size exclusion chromatography, filtration through LMW cut-off filters, and solubilization/lyophilization in the presence of reagents which facilitate disassembly of Aβ. These reagents include strong bases and acids, and fluorinated alcohols. These methods, which were originally developed for Aβ, are generally applicable to amyloidogenic peptides and proteins. In this chapter, we describe the preparation of LMW Aβ using size exclusion chromatography and filtration. The advantages and disadvantages of each method are discussed.

Key Words: Aggregation; solubility; size exclusion chromatography; filtration; amyloid β-protein.

1. Introduction

In vitro studies using synthetic amyloidogenic proteins have been highly important for understanding the mechanisms by which these proteins assemble into neurotoxic species and thus may be involved in aberrant protein folding diseases. A significant difficulty in these studies has been obtaining reproducible data. For example, in studies of the amyloid β-protein (Aβ), significant differences in assembly kinetics and neurotoxicity have been observed using

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synthetic Aβ from different manufacturers or even using different lots from the same manufacturer *(1–4)*. This irreproducibility likely resulted from the presence of preexisting aggregates in the peptide stocks. These seeds must be removed or dissociated in order to improve reproducibility. An "aggregate-free" protein solution contains only monomer and small oligomers *(5)* and is termed low molecular weight (LMW) protein solution. Removal of preexisting aggregates is performed based on the size difference between the aggregates and the LMW protein solution. The methods for Aβ illustrated here are generally applicable to other amyloidogenic proteins. LMW Aβ has been isolated by using size exclusion chromatography (SEC) *(6)*, or filtration through a low molecular weight cutoff (MWCO) filter *(7)*. Alternately, dissociation of aggregates can be accomplished by treatment of the lyophilized Aβ stock with strong acids *(8)*, bases *(7)*, or polyfluorinated alcohols *(8,9)*. An important advantage of mechanical, rather than chemical, elimination of the aggregates is that heterogeneous seeds, which have been shown to induce rapid aggregation *(10)*, are also eliminated. In addition, side reactions such as oxidation or racemization may result upon treatment of the protein with strong acids or bases, respectively. An additional advantage of SEC, relative to the other methods, is that the separation of the LMW protein from preexisting aggregates is monitored online, providing a means to assess the quality of the preparation. Here we describe methods for preparation of LMW Aβ by either SEC or filtration through a 10 kDa MWCO filter.

2. Materials

1. Waters (Milford, MA) FPLC system, including a Rheodyne (Ronnert Park, CA) 9725i injector, 1 mL injection loop (*see* **Note 1**), Waters 650 system controller or Waters 515 pump, and Waters 486 tunable absorbance UV detector set to 254 nm (*see* **Note 2**).
2. Branson (Danbury, CT) 1200 bath sonicator.
3. Eppendorf (Westbury, NY) 5415 C microcentrifuge.
4. Amersham Biosciences (Piscataway, NJ) 30/10 Superdex 75 HR column.
5. 10 m*M* Sodium phosphate buffer, pH 7.4 (*see* **Note 3**).
6. 20 m*M* Sodium phosphate buffer, pH 7.4 (*see* **Note 4**).
7. Dimethyl sulfoxide (DMSO).
8. 2 m*M* NaOH.
9. 1 N NaOH.
10. Millipore (Bedford, MA) Microcon-10 filters.

3. Methods

The methods described here outline preparation of LMW Aβ by using SEC and preparation of LMW Aβ by filtration through a 10 kDa MWCO filter. Both methods give comparable results for Aβ40 *(5)*, whereas the oligomer size

distributions obtained for Aβ42 differ. Both filtration and SEC yield similar low order Aβ42 oligomers (up to octamer) but high order oligomers (~30–60 kDa) are detected only when LMW Aβ42 is isolated by SEC ***(11)***.

3.1. Preparation of LMW Aβ by SEC

1. Prepare 10 m*M* sodium phosphate buffer and adjust the pH to 7.4 using solutions of phosphoric acid or NaOH.
2. Filter the resulting solution through a 0.22 μm filter (Corning [Corning, NY] sterilizing filter system, part no. 430517) to eliminate contamination by bacteria or dust.
3. Using this buffer, equilibrate the Superdex 75 HR column at a flow rate of 0.5 mL/min. Do not prepare the peptide solution until the column is equilibrated and the system is ready for injection (*see* **Note 5**).
4. Dissolve 350–400 μg of lyophilized Aβ in DMSO at 2 mg/mL (*see* **Note 6**).
5. Sonicate this solution for 1 min in a bath sonicator (*see* **Note 7**).
6. Centrifuge for 10 min at 16,000*g* to pellet large aggregates.
7. Inject 160–180 μL of the solution onto the Superdex 75 column.

A typical chromatogram would display 2 to 3 peaks (**Fig. 1**). A small void volume peak is observed in all samples, as is a major peak in the included volume that corresponds to LMW Aβ. A protofibril peak is consistently observed in Aβ42 samples but not in all Aβ40 samples. The top third of the LMW peak is collected for further experiments. The LMW fraction contains small to medium oligomers (**Fig. 2**). It should be used immediately after its isolation to ensure minimal aggregation.

3.2. Preparation of LMW Aβ by Filtration

For preparation of LMW Aβ by filtration, the peptide may be prepared using either of two methods. In both methods, the peptide is dissolved in the presence of a strong base. Using one method, the peptide is re-lyophilized. With the other method, the peptide is diluted into buffer and filtered immediately.

3.2.1. Preparation of LMW Aβ for Filtration With Re-Lyophilization

1. Dissolve lyophilized Aβ (normally, a TFA salt) at nominal concentration 1 mg/mL in 2 m*M* NaOH. The final pH of the solution should be ≥10.5 (*see* **Note 8**).
2. Sonicate this solution for 1 min in a bath sonicator and lyophilize.
3. Dissolve this lyophilizate at 2 mg/mL and filter (*see* **Subheading 3.2.3.**).

3.2.2. Preparation of LMW Aβ for Filtration Without Re-Lyophilization

1. Dissolve lyophilized Aβ in deionized water (*see* **Note 9**) at 4 mg/mL (*see* **Note 10**).
2. Add 1 *M* NaOH to adjust the pH to ≥10.5 (*see* **Note 11**).
3. Add 20 m*M* sodium phosphate, pH 7.4, to dilute the Aβ concentration to 2 mg/mL.
4. Sonicate this solution for 1 min in a bath sonicator and continue directly to filtration (*see* **Subheading 3.2.3.**).

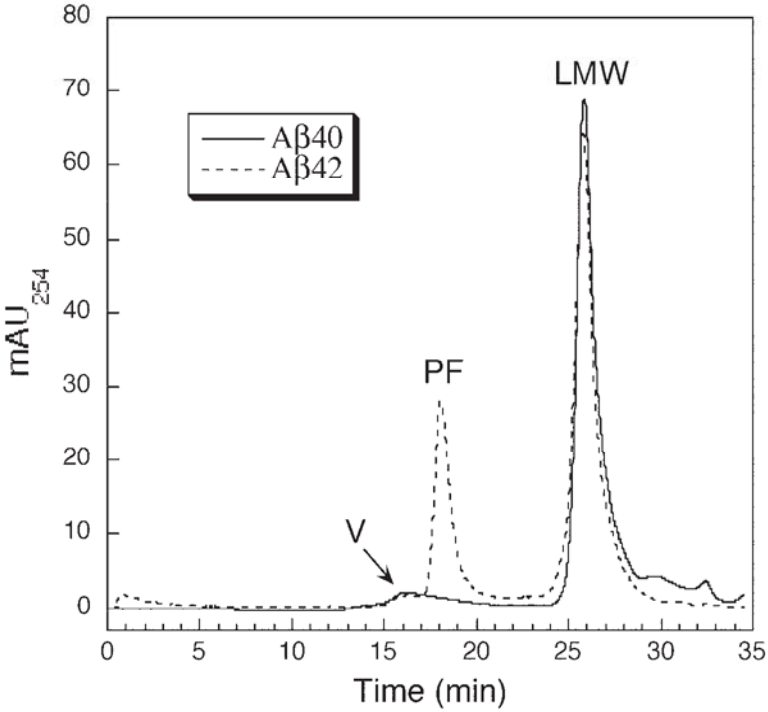

Fig. 1. Preparation of LMW Aβ by SEC (adapted with permission from *[11]*). Aβ40 (solid line) and Aβ42 (dotted line) fractionated by SEC using a Superdex 75 matrix and 10 m*M* sodium phosphate, pH 7.4, as the mobile phase. A small void volume peak (V) is observed in both samples, as is a major peak (LMW) in the included volume which corresponds to LMW Aβ. A protofibril peak (PF) is consistently observed in Aβ42 samples but not in all Aβ40 samples. This peak is always larger for Aβ42 than for Aβ40, likely reflecting the faster rate of fibrillogenesis of the longer Aβ alloform.

3.2.3. Filtration

1. Prepare the appropriate number of Microcon-10 filters (MWCO 10 kDa) (*see* **Note 12**) by washing each filter twice with 200 µL of 10 m*M* sodium phosphate buffer. The washing is done by centrifugation at room temperature for 20–25 min at 16,000*g*.
2. Transfer the filters into new collecting tubes.
3. Apply the Aβ solution prepared by either method in **Subheadings 3.2.1.** or **3.2.2.** and centrifuge at room temperature for 30 min at 16,000*g*.

The filtrate contains the LMW fraction. The LMW fraction contains small oligomers (**Fig. 2**). It should be used immediately after its isolation to minimize aggregation.

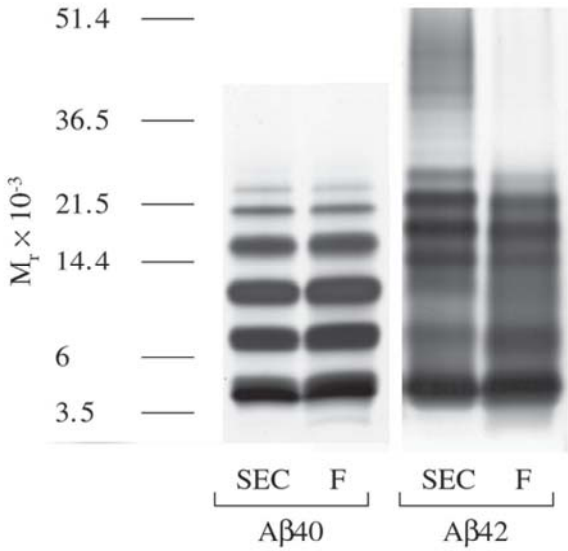

Fig. 2. Oligomer size distribution of LMW Aβ40 and Aβ42 isolated by SEC or filtration (adapted with permission from ***[5]*** and ***[11]***). LMW Aβ40 and Aβ42 were isolated either by SEC or by filtration through a 10,000 MWCO filter. The peptides were photochemically crosslinked to produce a quantitative "snapshot" of the oligomer size distribution ***(5)*** and the products were analyzed by SDS-PAGE. The mobilities of molecular mass markers are shown on the left. The oligomer size distributions obtained using the two methods are identical for Aβ40 but differ for Aβ42 ***(11)***.

4. Notes

1. A PEEK injector and loop, or equivalent non-metallic fluid path, should be used because stainless steel injectors and loops facilitate Aβ aggregation and tend to get clogged.
2. Comparable, isocratic FPLC systems equipped with all nonmetallic tubing are suitable for this procedure. Other wavelengths may be used for detection. For example, tryptophan- or tyrosine-containing proteins may be detected at 279 nm. Proteins without chromophores can be detected at 214 nm.
3. Other buffers may be used. However, buffers containing a high amount of salt (such as PBS) accelerate Aβ aggregation.
4. In case a different buffer is desired, this is a 2X buffer corresponding to the buffer in item 5.
5. A flat UV trace indicates that the column is equilibrated.
6. The peptide content (% peptide by weight) should be taken into account when calculating the volume of DMSO used for preparing this solution.
7. Sonication is an efficient way to break apart loosely attached aggregates. However, sonication for longer than 1 min may induce aggregation and should be avoided.

8. The pH may be adjusted using 0.1 *M* NaOH if necessary.
9. Deionized water of the highest quality should be used at all steps, including preparation of all buffers. Water of conductivity 18.2 MΩ is considered good quality.
10. Dissolution may take 1–2 min. Tap the tube gently to dislodge air bubbles but do not vortex or mix by pipetting up and down. Aβ40 normally gives a clear solution, whereas Aβ42 preparations tend to be turbid.
11. Approximately 1.5 µL of 1 *M* NaOH per 1 mL of Aβ solution is required.
12. Each filter will hold up to 550 µL. Since some of the peptide adsorbs to the filter membrane, higher final concentrations of LMW Aβ are obtained when higher volumes are used.

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