



Chapter 11

Preparation of Amyloidogenic Aggregates from EF-Hand β -Parvalbumin and S100 Proteins

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Abstract

Proteins containing EF-hand helix-loop-helix-binding motifs play essential roles in calcium homeostasis and signaling pathways. These proteins have considerable structural and functional diversity by virtue of their cation-binding properties, and occur as either Ca^{2+} -bound or Ca^{2+} -free states with distinct aggregation propensities. That is the case among β -parvalbumins and S100 proteins, which under certain conditions undergo Ca^{2+} -dependent self-assembly reactions with the formation of oligomers, amyloid-type aggregates and fibrils. These phenomena may be particularly relevant in human S100A6 protein and in fish Gad m 1 allergenic protein, which are implicated in human disease processes. Here, we describe detailed methods to generate and monitor the formation of amyloidogenic assemblies and aggregates of these two EF-hand proteins in vitro.

Key words Calcium, S100A6, Gad m 1, Fish allergens, Amyloids, EF-hand

Abbreviation

Gad m 1	Allergome nomenclature of Atlantic cod β -parvalbumin with UniProtKB sequence A51783
ThT	Thioflavin T

1 Introduction

Globular proteins acquire the competence for amyloid formation upon the exposure of aggregation prone segments. This process involves the perturbation of protein native structures such as that caused by the release of bound ligands or changes in pH [1]. Among the distinct ligand-binding site configurations, the Ca^{2+} -binding EF-hand motif constitutes an example of how ligand occupancy modulates conformational malleability [2, 3]. This motif is

characterized by a canonical sequence of about 30 residues defining a helix-loop-helix structure and usually appears in adjacent pairs [2, 3]. S100A6 and Gad m 1 represent two cases of proteins containing EF-hand motifs for which amyloid aggregation has been described [4–8].

The S100 proteins are a protein family that constitutes the largest subgroup of EF-hand Ca^{2+} -binding proteins with roles in Ca^{2+} buffering and/or transduction in vertebrates, which are engaged in a variety of intra and extracellular processes and disease states including cancer and neurodegeneration [9–11]. S100 are small (10–12 kDa) proteins consisting of two so called pseudo-EF-hand motifs that occur mostly as dimers that bind four Ca^{2+} ions at micromolar affinity. The conformational properties and function of S100 proteins are modulated by metal ion binding. This involves binding of Ca^{2+} to EF-hand type domains which triggers conformational changes that mediate functional interactions and in some cases (e.g., S100B, S100A8/A9, or S100A6), binding of Zn^{2+} to additional sites. Oligomerization is determinant to S100 function and is also regulated by metal-ion binding: S100 proteins spontaneously self-assemble up to functional octamers and have been recently implicated into amyloidogenic aggregation processes, with the first case reported for S100A8/A9 amyloids in the ageing prostate tissue [8], denoting potential physiopathological implications. The β -aggregation propensity of S100 proteins was later correlated with the exposure of aggregation prone regions upon changes in tertiary and quaternary interactions [5, 12] that can be triggered by acidification. For S100A6 we established that the protein also forms amyloid fibrils under near physiological conditions (pH 7.0 and 37 °C) upon longer incubation. However, the aggregation lag phase is substantially decreased under non-native conditions (pH 2.5 and 57 °C) [4]. Interestingly, Ca^{2+} binding represses the formation of S100A6 aggregates. Of these assemblies, S100A6 oligomers but not fibrils display neurotoxicity in cell culture [4]. On the contrary, S100A6 fibrils potentiate in vitro the aggregation of superoxide dismutase-1 that forms the cytoplasmic deposits in ALS-affected motor neurons [4].

On the other hand, Gad m 1 is one of the distinct β -parvalbumin chains from Atlantic cod for which the binding of the IgE of fish allergic patients has been found [13, 14]. As for other fish β -parvalbumins, the 109 residues chain of Gad m 1 contains three tandem EF-hand domains (so called AB, CD, and EF). Of them only the CD and EF are functional cation ion-binding sites with high Ca^{2+} affinity (K_D in the nM range) and moderate Mg^{2+} affinity [15–19]. As S1006, Gad m 1 forms amyloid oligomers and fibrils in the absence of Ca^{2+} [6, 7]. In this case, fibrillation under conditions mimicking the transit along the gastrointestinal tract provides the protease resistance that features type I food allergens [6].

2 Materials

2.1 S100A6

Expression and Purification

1. pGEMEX-Human S100A6 plasmid (UniProtKB P06703).
2. *E. coli* BL21(DE3) competent cells.
3. LB agar ampicillin plates.
4. DYT medium supplemented with 0.2% glucose and 100 µg/mL ampicillin.
5. 1 M IPTG.
6. Cooled incubator.
7. Resuspension buffer: 50 mM Tris-HCl pH 7.6 + 5 mM MgCl₂ + 0.5 mM PMSF + DNaseI.
8. PhenylSepharose Buffer A: 50 mM Tris-HCl pH 7.6 + 5 mM CaCl₂ + 0.5 mM DTT.
9. PhenylSepharose Buffer B: 50 mM Tris-HCl pH 7.6 + 10 mM EDTA.
10. SEC buffer: 20 mM Tris-HCl pH 7.6 + 150 mM NaCl.
11. Ultracentrifuge and benchtop centrifuge.
12. PhenylSepharose High Sub column (1.6 cm × 15 cm).
13. Superdex 75 16/600 prep grade column.
14. Amicon Ultra-4 Centrifugal filters, cutoff 3 kDa.
15. Bradford assay kit and UV-visible spectrophotometer.

2.2 Demetallation of S100A6

1. 50 mM Tris pH 7.6.
2. 50 mM Tris-HCl pH 7.5 + 0.1 M NaCl.
3. 100 mM EDTA.
4. 1 M DTT.
5. Chelex 100 resin.
6. Superdex 75 10/300 Tricorn column (GE Healthcare).
7. Amicon Ultra-4 Centrifugal Filters, cutoff 3 kDa.
8. Benchtop centrifuge.
9. Bradford assay kit and UV-visible spectrophotometer.

2.3 Gad m 1

Expression and Purification

1. pET15b-Gad m 1 (plasmid containing the synthetic gene of Gad m 1 under NdeI/BamHI targets).
2. *E. coli* BL21(DE3) competent cells.
3. LB agar ampicillin plates.
4. LB medium supplemented with 100 µg/mL ampicillin.
5. 1 M IPTG.
6. Incubator.

7. Resuspension buffer: 10 mM Tris-HCl, pH 7.5 containing freshly added 0.5 mM PMSF.
8. CaCl_2 stock.
9. Ni^{2+} -loaded IMAC Sepharose 6 Fast Flow column (25 mL).
10. IMAC Buffer A 2 $\times$: 40 mM Tris-HCl pH 7.5 + 0.2 M NaCl + 5 mM imidazole.
11. IMAC Buffer B: 20 mM Tris-HCl pH 7.5 + 0.1 M NaCl + 15 mM imidazole.
12. IMAC-Buffer C: 20 mM Tris-HCl pH 7.5 + 0.1 M NaCl + 0.6 M imidazole.
13. Q-Sepharose Fast Flow column (25 mL).
14. Q-Sepharose Buffer A: 20 mM Tris-HCl pH 7.5 + 10 mM NaCl.
15. Dialysis tubing of 8 kDa pore.
16. Amicon Ultra Centrifugal Filters, cutoffs of 10 and 30 kDa.
17. Thrombin CleanCleave kit.
18. Sonicator.
19. Centrifuge.
20. SDS-PAGE gels, electrophoresis power supply and image analysis.
21. Bradford assay kit and spectrophotometer.

2.4 Preparation of S100A6 Aggregates

2.4.1 Under Non-native Conditions

1. Spectrofluorometer with temperature control.
2. 5 mM CaCl_2 .
3. 100 mM EDTA.
4. 50 mM Tris-HCl pH 7.4, containing 50 mM TCEP.
5. 1 cm quartz cell.
6. 5 mM ThT stock solution.
7. 1.5 mL low binding microtubes (*see Note 1*).

2.4.2 Under Physiological Conditions

1. Spectrofluorometer with temperature control.
2. Thermo-Shaker.
3. 5 mM CaCl_2 .
4. 100 mM EDTA.
5. 50 mM Tris-HCl pH 7.
6. 1 cm quartz cell.
7. 5 mM ThT stock solution.
8. 1.5 mL low binding microtubes.

2.5 Preparation of Gad m 1 Amyloid Aggregates

1. Gad m 1 stock solution (>2.5 mg/mL protein concentration in ddH₂O).
2. Benchtop centrifuge and ultracentrifuge.
3. $5\times$ Ca²⁺-buffers: 250 mM Tris-HCl + 0.5 M NaCl + 25 mM CaCl₂ for pH 6.5–7.5, MES for pH 5.0–6.5, NaAc/AcH for pH 3.5–5.0 and Gly for pH 2.0–3.5.
4. $5\times$ EDTA-buffers: 250 mM Tris-HCl + 0.5 M NaCl + 25 mM EDTA for pH 6.5–7.5, MES for pH 5.0–6.5, NaAc/AcH for pH 3.5–5.0 and Gly for pH 2.0–3.5.
5. $5\times$ ThT buffers: $5\times$ Ca²⁺-buffers and $5\times$ EDTA buffers containing 50 μ M ThT.
6. 96-well fluorescence plates and their sealing foil.
7. 3 mm glass-balls.
8. Fluorescence microplate reader with temperature control, bottom reading, 450 nm excitation and 480 nm emission filters and time-dependent measurement option.
9. 1.5 and 2.0 mL low binding microtubes.
10. Incubator.

3 Methods

3.1 S100A6 Expression and Purification

S100A6 is produced using E. coli as expression host:

1. Transform *E. coli* competent BL21(DE3) cells with pGEMEX-S100A6 plasmid according to the instructions of the manufacturer.
2. Spread transformed cells on LB agar plates containing ampicillin (100 μ g/mL) and incubate overnight at 37 °C.
3. Inoculate a single colony in 50 mL DYT medium containing ampicillin (100 μ g/mL) and 0.2% glucose and incubate overnight at 37 °C and 150 rpm.
4. The overnight culture is used to inoculate the expression culture that is incubated at 37 °C and 150 rpm. Allow culture grow until reaching OD_{600nm} = 0.6.
5. Induce the expression of S100A6 by addition of 1 mM IPTG.
6. The culture is harvested by centrifugation 4 h after induction. Cell pellets are harvested by centrifugation 10 min at 11,000 $\times g$ and may be frozen at –80 °C.

S100A6 purification:

1. Thaw frozen cell pellets on ice and suspend the cells in 50 mM Tris-Cl pH 7.6 with DNase I and 2 mM MgCl₂.

2. Break the cells with two passages through a French pressure cell set at 320 MPa.
3. Ultracentrifuge the cell lysate at $100,000 \times g$ for 1 h (e.g., 40,000 rpm in 45TiRotor, Beckman).
4. Separate the supernatant and dilute threefold with Phenyl Sepharose Buffer A and incubate for 30 min under gentle stirring (*see Note 2*).
5. Load the resulting mixture onto the Phenyl Sepharose column, previously equilibrated with Phenyl Sepharose Buffer A.
6. Wash the column with Phenyl Sepharose Buffer A until absorption at 280 nm is restored to baseline.
7. Elute bound S100A6 with Phenyl Sepharose Buffer B (*see Note 3*).
8. Pool fractions containing S100A6 protein and concentrate the solution by ultrafiltration with 3 kDa Amicon filters.
9. Apply the concentrated S100A6 to Superdex 75 column equilibrated in 20 mM Tris-HCl, 150 mM NaCl, pH 7.6.
10. Combine eluted fractions containing S100A6 protein, concentrate by ultrafiltration and store at -80°C until further use.

3.2 Demetallation of S100A6

Unless otherwise noted, operations must be performed at 4°C to prevent protein precipitation.

1. Add 300-fold molar excess of DTT and 0.5 mM EDTA to the S100A6 stock and incubate for 2 h at 37°C without agitation.
2. Elute in a Superdex 75 Tricorn in 50 mM Tris pH 7.5 at 1 mL/min to remove excess DTT and EDTA. Concentrate the eluted protein by ultrafiltration using Amicon ultracentrifugal filters.
3. Quantify S100A6 spectrophotometrically using the molar extinction coefficient $\epsilon_{280\text{nm}} = 8940 \text{ M}^{-1} \text{ cm}^{-1}$ (S100A6 dimer) or the Bradford assay.

3.3 Gad m 1 Expression and Purification

Gad m 1 is produced using E. coli as expression host:

1. Transform *E. coli* competent BL21(DE3) cells with pET15b-Gad m 1 according to the instructions of the manufacturer.
2. Spread transformed cells on LB agar-ampicillin plates and incubate overnight at 37°C .
3. Inoculate single colonies in 200 mL of LB-ampicillin medium and incubate overnight at 37°C and 150 rpm. The overnight culture is used to inoculate 1.8 L of expression culture containing ampicillin that is incubated at 37°C and 150 rpm. Allow culture grow until reaching $\text{OD}_{600\text{nm}} = 1$ (*see Note 4*).

4. Induce Gad m expression during 4 by the addition of 0.5 mM IPTG, and harvest the cells by a 10 min centrifugation at $11,000 \times g$ and store at -80°C until use.

Gad m 1 purification:

1. Thaw frozen cells on ice and suspend in 10 mM Tris pH 7.5 (7 g of cells in 40 mL of buffer) containing 1 mM PMSF.
2. Break the cells by sonication in ice (for a Vibracell sonicator use 20% potency and 30 s on/30 s off for 5 min) and then centrifuge for 30 min at 4°C and $13,200 \times g$ (*see Note 5*).
3. Make the supernatant 50 μM in CaCl_2 and incubate in ice for 10 min with gentle agitation.
4. Dilute the supernatant with IMAC-Buffer A $2\times$ and load it on a Ni^{2+} -IMAC column, previously equilibrated in IMAC-Buffer A $1\times$.
5. Wash the column with 2 V of IMAC- buffer A $1\times$ followed by 2 V of IMAC-buffer B. Bound Gad m 1 is eluted with IMAC-Buffer C (*see Note 6*).
6. Pool the fractions containing Gad m 1, load them onto a Q Sepharose column previously equilibrated in Q Sepharose buffer A, wash with 2 V of Q Sepharose buffer A. Collect the flow-through containing the protein (*see Note 7*).
7. Pool the fractions containing the protein and filter through a 30 kDa Amicon.
8. Dialyze the filtrate against ddH₂O using 8 kDa-pore dialysis tubing and concentrate using a 10 kDa-Amicon device (*see Note 8*).
9. Determine the protein concentration using the Bradford assay, and store in aliquots (>3 mg/mL protein concentration) at -80°C until further use.
10. Remove the N-terminal tag using the Thrombin CleanCleave kit following the manufacturer's indications and monitoring its yield by SDS-PAGE (Fig. 2A) (*see Note 9*).
11. Verify the quality of the product (*see Note 10*).

3.4 Formation of S100A6 Amyloidogenic Aggregates

All water used in buffers and metal ion solutions should passed through a Chelex column to remove trace metal ions.

3.4.1 S100A6 Aggregates Formed Under Non-native Conditions

1. Prepare one tube with 800 μL of S100A6 for each condition with 10 mM TCEP in 50 mM Tris-HCl pH 7.4 (*see Note 11*). CaCl_2 may be added as 50-fold molar equivalents over S100A6. Alternatively, 0.5 mM EDTA is added as a control.

2. Homogenize the solution gently and transfer to the cuvette (*see Note 12*).
3. Record for 30 h the turbidity change as increase in the 90° angle light scattering intensity setting excitation and emission wavelengths to 450 nm, the temperature to 55 °C, agitation to 800 rpm.
4. Plot the turbidity increase as a time function (Fig. 1a).
5. Determine the ThT-binding properties of the sample by recording the emission spectrum (460–650nm) upon excitation at 440 nm in the presence of 10 µM ThT (Fig. 1b).

3.4.2 S100A6 Aggregates Formed Under Native Conditions

1. In a 1.5 mL tube prepare S100A6 at a final concentration of 3 mg/mL (295 µM) in Tris-HCl buffer pH 7.0. CaCl₂ may be added as tenfold molar equivalents over S100A6. Alternatively, 0.5 mM EDTA is added as a control. Lastly, 75 µM ThT was added (*see Note 13*).
2. Amyloid formation is promoted by incubation at 37 °C under orbital agitation at 1000 rpm for more than 60 days. Record ThT fluorescence (480 nm) at different time points upon excitation at 440 nm.
3. Plot the ThT fluorescence intensity increase as time function (Fig. 1c).
4. Remove an aliquot at the end-time point for analysis of Ca²⁺ effects, e.g., by TEM imaging as described in [4] (Fig. 1d, e).

3.5 Formation of Gad m 1 Amyloid Aggregates

For kinetic monitoring of amyloid formation under varying pH and Ca²⁺ binding:

1. Centrifuge Gad m 1 solution at 13,500 × *g* for 20 min at 4 °C to remove undesired aggregates and readjust the protein concentration to 2.5 mg/mL.
2. Prepare the fluorescence 96-well by inclusion of one 3 mm glass ball per well.
3. Mix Gad m 1 solution (160 µL/well) with 5×-buffers containing 50 µM ThT (40 µL/well) in triplicate and transfer 190 µL per well.
4. Samples for background baselines (no protein) must be also included.
5. Place the plate in the fluorescence reader, set measurement parameters (temperature, agitation, etc.), and record the emission at 480 nm using 450 nm for excitation wavelength. For long measurements as 70–120 h, best reproducibility is achieved with readings every hour.

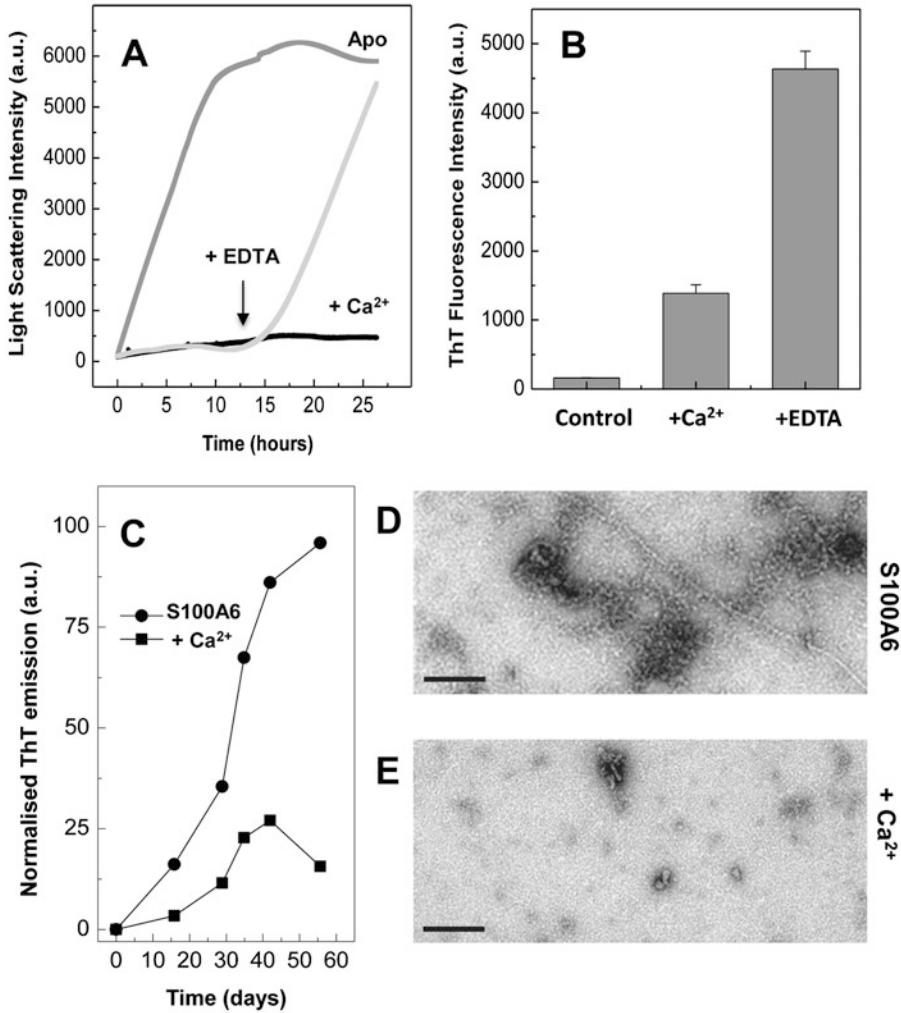


Fig. 1 Formation of S100A6 amyloidogenic aggregates. (a) Scattering data acquired during aggregation assay of 20 μ M apo S100A6 dimer at pH 7.4, 55 $^{\circ}$ C and in the presence of 50-fold CaCl_2 or EDTA. Ca^{2+} considerably reduce the amyloid aggregates formation in this time scale, however, Ca^{2+} chelation by EDTA (EDTA addition indicated by arrow) restored amyloidogenesis. (b) ThT fluorescence intensity at the end-point of aggregation kinetic performed at pH 7.4, 55 $^{\circ}$ C ($t = 25$ h). (c) ThT aggregation kinetics of 295 μ M apo and Ca^{2+} -S100A6 performed at pH 7, 37 $^{\circ}$ C under 1000 rpm orbital agitation [4]. (d) TEM analysis of S100A6 aggregates formed at the end-point of aggregation kinetics (65 days) performed in (c) in the absence of calcium. Scale bars: 200 nm. (e) TEM analysis of S100A6 aggregates formed at the end-point of aggregation kinetics (65 days) performed in (c) in the presence of calcium. Scale bars: 200 nm

- Construct the kinetic plots by averaging the triplicates and subtracting the corresponding baselines. Use the end points of the fluorescence intensity increment to analyze the effect of ligand and pH on amyloid formation (Fig. 2B).

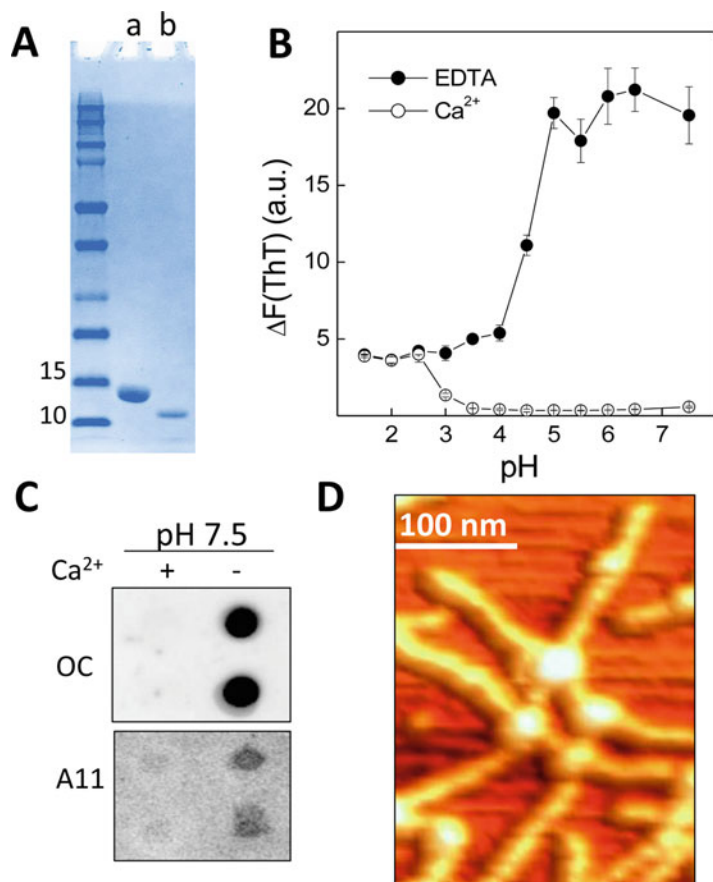


Fig. 2 Formation of Gad m 1 amyloid aggregates. **(A)** Analysis of Gad m 1 before (a) and after (b) His-tag cleavage by Coomassie blue stain of SDS-PAGE gels. **(B)** Effect of pH on the ThT fluorescence intensity enhancement of Gad m 1 under both Ca^{2+} -loaded (open circles) and Ca^{2+} -depleted (solid circles) conditions. Protein concentration was 2 mg/mL. Fluorescence intensity values were taken after at 120 h of reaction. **(C)** Dot-blot analysis of the recognition of Gad m 1 incubated for 120 h at 37 °C in 25 mM Tris-HCl, 0.1 M NaCl containing either 5 mM CaCl_2 (+) or 5 mM EDTA (–) by anti-amyloid fibril (OC) and anti-oligomer (A11) antibodies. Blotting was performed as described [6]. **(D)** 3D representation of AFM images of Gad m 1 fibrils. Averaged fibril height amounts to 5 nm [6]

For the preparation of quantities of amyloid aggregates:

1. Centrifuge Gad m 1 solution at $13,500 \times g$ for 20 min at 4 °C to remove undesired aggregates and readjust the protein concentration to 2.5 mg/mL.
2. Mix in duplicates 1.6 mL of Gad m 1 solution with 0.4 mL of 0.25 M Tris-HCl, 0.5 M NaCl pH 7.5 containing 25 mM EDTA (*see Note 14*).
3. Incubate at 37 °C for a minimum of 72 h with no agitation.
4. Pool the reaction mixtures; remove and freeze a 20 μL aliquot (total) and ultracentrifuge at $100,000 \times g$ for 1 h at 4 °C.

5. Remove the supernatant and resuspend the pellet in 199 μL of 50 mM Tris-HCl, 0.1 M NaCl pH 7.5 containing 5 mM EDTA.
6. Determine the aggregation yield from the comparison of protein concentrations in the total (removed aliquot) and in the supernatant fraction by either Bradford assay or SDS-PAGE.
7. Determine the amyloid properties of the aggregates using either the binding of conformational antibodies, their morphology or both (*see* Fig. 1c, d) [6, 20].

4 Notes

1. Low binding surface tubes should be used to avoid adsorption of aggregates to the surface of the eppendorf.
2. S100 proteins expose a hydrophobic patch upon Ca^{2+} binding responsible for the interaction with partner proteins. This property is used for Ca^{2+} -dependent affinity chromatography on hydrophobic matrices, such as Phenyl Sepharose. This conformational change is fully reversible when bound calcium is released by EDTA chelation.
3. After elution the column should be washed with 1 M NaOH and/or 70% ethanol to remove precipitated proteins and contaminants hydrophobically bound to the matrix and equilibrated in 20% ethanol for long-term storage.
4. Protein production is performed using a 2 L-MiniForce fermenter which allows optimal air exchange, temperature, and pH-control. Using this setup allows high reproducibility of cell yield (9 g/2 L of culture) and purified protein (35 mg tagged protein/2 L of culture).
5. Gad m 1 cell lysates produced either at higher sonication potency or by enzymatic digestions with lysozymes and DNases decreases the binding yield to Ni^{2+} -IMAC Sepharose.
6. Gad m 1 chain does not contain any aromatic residue for $\text{Abs}_{280\text{nm}}$ reading. Therefore, all purification must be followed by SDS-PAGE. Notwithstanding, UV-Vis absorption spectrum of protein pools must be recoded to check the potential presence of undesired nucleic acids ($\text{Abs}_{260\text{nm}}$), proteins ($\text{Abs}_{280\text{nm}}$) and aggregates ($\text{Abs}_{350\text{nm}}$).
7. This step works as a filtration to remove polyanions from the protein solution. In some preparations we observed some retention (about 10% of the total protein) in the column. The bound protein can be eluted increasing NaCl concentration.
8. For best preservation on freezing-thawing operations final protein concentration should be >3 mg/mL

9. Tag-removal using the thrombin CleanCleave kit is performed at 1 mg/mL Gad m 1 for 3 h and requires additional dialysis (removal of the cleaved peptide and the excess of CaCl_2) and concentration step reducing the protein yield to about 50% (*see* Fig. 2A).
10. To determine the quality of the isolated protein, before their use all purification batches are characterized by SDS-PAGE (loading proteins in the presence and absence on β -mercaptoethanol), dynamic light scattering and circular dichroism spectroscopy [6, 7].
11. In the aggregation assays working concentration of S100A6 is 20 μM . Preparing a S100 stock solution of at least 100 μM allows diluting the solution with additives such as metal ion solutions.
12. Mixture of all components should be fast and performed on ice.
13. The order of the addition of component into final solution is important. ThT should be the last component to be added. The initial fluorescent can be changed when metal ions are added.
14. As opposed to S100A6, demetallation of Gad m 1 triggers its aggregation and therefore isolation of its cation-free chain is impeded. On the other hand, as fish β -parvalbumin, Gad m 1 contains the conserved C19 which if oxidized as in long incubations can lead to disulfide bonded dimers. Inclusion in the reaction media 5 mM TCEP yields similar results of incubations prepared in its absence.

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