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Unveiling Brain A β Heterogeneity Through Targeted Proteomic Analysis

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Abstract

Amyloid β (A β) is the major constituent of the brain deposits found in parenchymal plaques and cerebral blood vessels of patients with Alzheimer's disease (AD). Besides classic full-length peptides, biochemical analyses of brain deposits have revealed high degree of A β heterogeneity likely resulting from the action of multiple proteolytic enzymes. This chapter describes a sequential extraction protocol allowing the differential fractionation of soluble and deposited A β species taking advantage of their differential solubility properties. Soluble A β is extracted by water-based buffers like phosphate-buffered saline—PBS—whereas pre-fibrillar and fibrillar deposits, usually poorly soluble in PBS, are extractable in detergent containing solutions or more stringent conditions as formic acid. The extraction procedure is followed by the biochemical identification of the extracted A β species using Western blot and a targeted proteomic analysis which combines immunoprecipitation with MALDI-ToF mass spectrometry. This approach revealed the presence of numerous C- and N-terminal truncated A β species in addition to A β 1–40/42. Notably, the more soluble C-terminal cleaved fragments constitute a main part of PBS homogenates. On the contrary, N-terminal truncated species typically require more stringent conditions for the extraction in agreement with their lower solubility and enhanced aggregability. Detailed assessment of the molecular diversity of A β species composing interstitial fluid and amyloid deposits at different disease stages, as well as the evaluation of the truncation profile during various pharmacologic approaches will provide a comprehensive understanding of the still undefined contribution of A β truncations to AD pathogenesis and their potential as novel therapeutic targets.

Keywords

Amyloid- β ; Posttranslational modifications; Proteolytic cleavage; N-terminal truncations; C-terminal truncations; Immunoprecipitation; Mass spectrometry

1 Introduction

Alzheimer's disease (AD), the most common form of dementia in humans over the age of 65 affecting more than 50% of individuals 85 or older, constitutes one of the major public health concerns in all developed countries. Neuropathological hallmarks of the disease are the presence of intraneuronal neurofibrillary tangles (NFT)—deposits of hyperphosphorylated protein tau in the form of paired helical filaments—and the existence of parenchymal extracellular deposits composed of both diffuse pre-amyloid lesions as well as compact amyloid plaques. Although its significance was ignored for decades, together with these parenchymal lesions, the amyloid deposition is also commonly observed in

medium-sized and small cerebral vessels, a feature known as cerebral amyloid angiopathy (CAA).

Cerebrovascular and parenchymal amyloid deposits are composed of self-aggregates of the amyloid- β (A β) peptide which is generated by proteolytic cleavage of a larger amyloid precursor protein (APP) by the β and γ secretases [1–3]. In contrast to β -secretase, the multiprotein γ secretase complex has broader specificity and is able to cleave APP at multiple sites within its trans-membrane domain generating A β peptides ranging in length from 38 to 42 residues [4]. Nearly 90% of A β generated ends at residue 40, whereas A β 1–42 accounts for only <10%, and peptides ending at residues 38 are minor components [5]. Notably, the pattern and distribution of these species varies among the different topographical lesions. Parenchymal deposits consist of A β 1–42 as the major component, whereas vascular A β —particularly in the large leptomeningeal vessels—is primarily composed of A β 1–40 species organized in large concentric sheets and replacing the smooth muscle cell layer [6]. Amyloid associated with arterioles and small cortical arteries contains a mixture of A β 1–40 and A β 1–42, while deposits affecting the capillary network are mainly composed of A β 1–42 [7]. The reasons for this selectivity as well as its importance for the pathogenesis of the disease remain unclear.

Recent evidence indicates that the complexity of amyloid deposits extends well beyond the A β 1–40/A β 1–42 dichotomy and is further expanded by the presence of multiple posttranslational modifications. Indeed, these types of protein modifications play in general a key role in functional proteomics by dramatically increasing the proteome diversity and modulating many aspects of cell function [8]. Among the posttranslational modifications reported for A β , the most studied include isomerization and racemization of aspartates, cyclization of N-terminal glutamates to form pyroglutamate, oxidation of methionine residues, as well as abundant N- and C-terminal truncations [2, 9]. In some cases, like the formation of N-terminal pyroglutamate, one of the most studied protein modifications present in truncated forms of A β starting at positions E3 and E11, the loss of a negative charge as a result of the cyclization dramatically changes the peptide structure. The presence of pyroglutamate increases the β -sheet content and alters the molecule hydrophobicity increasing A β aggregation propensity and resistance to enzymatic degradation [10, 11] suggesting a contribution of these species to the disease process. In this sense, pE-modified forms of A β have been shown to be more neurotoxic than full-length, unmodified A β [12–15] and to be more abundant in the AD brain compared to cognitively intact age-matched controls, further highlighting the biological relevance of these A β species [16–20].

Recent evidence indicates that the molecular heterogeneity of A β deposits is significantly more complex than originally anticipated and that in addition to the abundance of pE-, iso-Asp and oxidized A β species, numerous N- and C-terminal truncations significantly contribute to the complexity of the amyloid profile. Besides the intact A β peptides generated by the combined action of BACE1 and γ -secretase—starting at the aspartate residue at position 1 and ending at amino acids 38/40/42—different truncated A β species have been identified in cellular and animal models as well as in AD patients [11, 21–29], likely generated by the action of a number of A β -degrading enzymes, among them neprilysin, insulin degrading- and endothelin converting-enzymes, plasmin and matrix metalloproteases

[30–35]. Reduced levels and/or decreased catalytic activity of these A β -degrading enzymes as a result of age, genetic factors, and specific disease conditions have been proposed to affect A β accumulation, an issue well documented in murine models, in which gene deletion of different proteases translate into increased levels of A β deposition [32, 33, 36]. Evidence of a genetic association of these proteases with AD has only been reported for a few enzymes albeit no consensus exists at the moment with regard to the importance or reproducibility of these associations in the general AD population [37]. Nevertheless, in spite of this elusive information, the relevance of these enzymatic processes for brain clearance is supported in part by studies highlighting the higher solubility and non-toxic characteristics of most of the resulting truncated species [34] as well as by recent findings demonstrating the faster preferential elimination of C-terminal truncated species to the CSF, all features that point out to a general beneficial role of A β enzymatic processing for AD pathogenesis [29].

Based on the diversity of A β species existing in Alzheimer's disease cases and recapitulated in animal models, the potential differences in the clearance proclivity of the different fragments, and/or their pro-amyloidogenic properties, it is clear that the evaluation of A β —either in biological fluids or extracted from tissue deposits—needs to provide a complete information regarding all the species constituting the A β peptidome profile and not be limited to the mere evaluation of the A β 1–40/A β 1–42 pair. Only the detailed assessment of the molecular diversity of all species composing the deposits at different stages of the disease and their relative ratios, as well as the evaluation of changes in the truncation profile with different treatment strategies will provide a comprehensive understanding of the still undefined contribution of N- and C-terminal truncations to the pathogenesis of the disease. The present work describes a sequential extraction methodology allowing the differential fractionation of all soluble and deposited A β species taking advantage of their differential solubility. Soluble A β is extracted by water-based buffers like phosphate-buffered saline—PBS—whereas pre-fibrillar and fibrillar deposits, usually poorly soluble in PBS, are extractable in 2% sodium dodecyl sulfate (SDS) or 70–99% formic acid (FA), respectively (Fig. 1). The extraction procedure is followed by the biochemical identification of the extracted A β species using Western blot and a targeted proteomic analysis which combines immunoprecipitation with MALDI-ToF mass spectrometry. Through these approaches it is possible to unveil the heterogeneity of A β deposits both in terms of the degree of A β oligomerization/aggregation as well as in the divergent biochemical/solubility properties exhibited by the different species composing A β peptidome. The composition of the PBS fraction depicted in Fig. 2a illustrates the presence of numerous A β species, predominantly truncated at the C-terminus, together with A β 1–40, the more soluble of the full-length peptides. Using the more stringent conditions SDS and FA, extracts are enriched in A β 1–40 and A β 1–42. Notably, two fragments truncated at Phe4—A β 4–40 and A β 4–42—are frequently extracted together with the full-length peptides (Fig. 2b–d). This is in agreement with our previous findings demonstrating remarkably similar populations of posttranslationally cleaved A β forms in AD and aged squirrel monkeys [38], which also included the N-terminally truncated and pyroglutamate modified species A β 3–42 and A β 11–42, strongly suggesting that N-terminal degradation negatively affects peptide solubility. Supporting these findings, truncated peptides at Phe4 were recently demonstrated

to primarily localize at dense plaque cores through the use of specific antibodies recognizing species starting at this position 4 [39]. Overall, the targeted proteomic analysis in AD brain indicates that, although exhibiting some variability in the A β peptidome among the patients studied, the general profile predominantly consisting of C-terminally truncated A β species in water-based extraction conditions and the more insoluble A β 1–42 and N-terminally truncated derivatives retrieved in SDS or FA extracts is a constant finding. Whether the differential composition in N- and C-terminal truncated species and their relative ratio to full-length peptides found in different patients relates to the magnitude, complexity, or composition of the deposits, to the duration of the disease, or to other clinical phenotypes is currently being studied and remains to be elucidated.

2 Materials

2.1 Tissue Homogenization and Sequential Amyloid Extraction

2.1.1 Reagents

1. Brain tissue: Frozen brain tissue obtained at autopsy from AD patients with short post-mortem delay, preferable 4–8 h (ideally <2–4 h) to minimize the mostly enzymatically driven protein modifications occurring after death (*see* Note 1).
2. Specific buffers and solutions.
 - a. Milli-Q water (Purified water with resistivity values at 25°C < 18.2 Mho/cm).
 - b. PBS (Phosphate-Buffered Saline): 10 mM phosphate pH 7.4, containing 2.7 mM KCl and 137 mM NaCl. Dissolve one tablet of Phosphate-Buffered Saline (Sigma, St. Louis, MO) in 200 ml of deionized water. Scale up as needed.
 - c. PBS/SDS: PBS containing 2% SDS.
 - d. FA 70%: prepared by diluting 99% formic acid with deionized water.
 - e. FA 99%: Formic acid undiluted.
 - f. Protease inhibitors cocktail (Complete, Roche Applied Science, Indianapolis, IN), prepared in accordance with the manufacturer's specifications.
3. Miscellaneous materials.
 - a. Dissecting instruments.
 - b. Dounce glass homogenizer.
 - c. 13.5 ml polycarbonate tubes.
 - d. 300- and 70- μ m Spectra/Mesh nylon filters (Spectrum Laboratories, Inc.; Rancho Dominguez, CA).

2.1.2 Instrumentation

1. XL100K ultracentrifuge (Beckman Coulter, Fullerton, CA) equipped with a Beckman 70.1 Ti rotor, or equivalent.
2. 5417 microcentrifuge (Eppendorf, Westbury, NY), or equivalent.
3. Savant SpeedVac concentrator (Global Medical Instrumentation, Inc.; Ramsey, MN).

2.2 Immunoprecipitation of A β from Brain Extracted Fractions

2.2.1 Reagents

1. Antibodies: 4G8 and 6E10 monoclonal anti-A β antibodies (Biolegend, San Diego, CA).
2. Paramagnetic beads: Magnetic beads coated with goat anti-mouse IgG (Dynabeads M-280, Dynal/ThermoFisher, Waltham, MA).
3. Specific buffers and solutions.
 - a. Blocking buffer: PBS containing 0.1% (w:v) Bovine Serum Albumin (BSA).
 - b. Neutralizing solution: 0.5 M Tris-base, pH 11.0.
 - c. SDS-OUT (Pierce Biotechnology, Inc., Rockford, IL).
4. Miscellaneous materials.
 - a. Eppendorf tube-rack equipped with a removable slide-out magnet (Dynal Magnetic Particle Concentrator MPC-S, Dynal/ThermoFisher Scientific).
 - b. Micro-reverse-phase chromatography tips (Zip-Tip C18, EMD Millipore, Mahopac, NY).

2.2.2 Instrumentation

1. Savant SpeedVac concentrator.
2. Mini LabRoller dual format rotator or equivalent.

2.3 Western Blot Analysis

2.3.1 Reagents

1. Acrylamide Stock: 40% Acrylamide/Bis solution, 29:1 (3.3% C; BioRad).
2. TEMED (*N,N,N,N*- Tetra-methyl-ethylendiamine).
3. Tris-tricine sample buffer.
4. Nitrocellulose membranes for chemiluminescence (Hybond ECL; GE Healthcare Life Sciences).
5. Monoclonal anti-A β antibodies, 4G8 and 6E10 (Biolegend).

6. HRP-labeled F(ab')₂ anti-mouse IgG (Amersham Pharmacia).
7. Western Blotting ECL Detection Substrate (SuperSignal West Pico, Pierce Biotechnology, Rockford, IL).
8. Film for chemiluminescence.
9. Specific buffers and solutions.
 - a. Cathode buffer: Tris-Tricine-SDS buffer 10× concentrate (Sigma) [1 M Tris-HCl, 1 M Tricine, 1% (w:v) SDS, pH 8.2].
 - b. Anode Buffer: 10× concentrate: 2 M Tris-HCl, pH 8.9.
 - c. Gel Buffer: 3 M Tris-HCl, 0.3% (w:v) SDS pH 8.45.
 - d. 10% (w/w) Ammonium persulfate (APS).
 - e. Transfer buffer: 10 mM 3-cyclohexylamino-1-propane-sulfonic acid (CAPS, Sigma), pH 11.0 containing 10% v: v methanol.
 - f. Blocking solution: 5% nonfat dry milk in PBS containing 0.1% Tween 20 (Sigma).
 - g. Tris-buffered saline (Fisher Scientific) containing 0.1% Tween 20 (TBST).

2.3.2 Miscellaneous Materials and Instrumentation

1. Hoeffer MiniVE Mini Vertical Electrophoresis Unit.
2. Hoeffer TE22 Mini Tank Blotting Unit.
3. Power Station 300 plus (Labnet International, Inc.; Wood-bridge, NJ) or equivalent.
4. Orbital Shaker.
5. M35A X-OMAT Film Processor (Eastman Kodak Company, Rochester, NY) or equivalent.

2.4 Mass Spectrometry Analysis

2.4.1 Reagents

1. Trifluoroacetic acid (TFA).
2. Acetonitrile.
3. α -Cyano-4-hydroxycinnamic acid (CHCA) matrix, 6.2 mg/ml solution in 36% (v/v) methanol and 56% (v/v) acetonitrile (Agilent part number G2037A) (*see* Note 16).
4. Calibration standards for internal and external calibration: human adrenocorticotrophic hormone peptide (ACTH) 18–39 (average mass = 2465.68 Da) and bovine insulin (average mass = 5733.49 Da). Synthetic A β peptides can also be used for external calibration. Stock solutions of standards at 5 nmol/ml

can be prepared in 0.1% (v/v) TFA and 30% (v/v) acetonitrile and stored at -80°C until use.

2.4.2 Miscellaneous Materials and Equipment

1. Bruker Daltonics MTP 384 massive target T aluminum plate.
2. Bruker Daltonics Autoflex MALDI-ToF mass spectrometer.

3 Methods

3.1 Tissue Homogenization and Sequential Amyloid Extraction

Tissue homeostasis is significantly altered post-mortem and changes in the enzymatic activity may translate in alterations of the $\text{A}\beta$ profile. Thus, a key aspect to take into consideration when analyzing the composition of truncated and full-length species is the post-mortem delay of the specimen which ideally should not exceed 4 h. It is also imperative that the tissue is maintained frozen at -80°C until processing and that it is not subjected to freezing/thawing cycles. Any further modifications during processing of the specimen are avoided by performing all tissue processing and homogenization steps at 4°C , on ice, and in the presence of protease inhibitors. The high insolubility of the amyloid deposits has challenged investigators for many years and prevented a thorough characterization of the proteins constituting the lesions and their posttranslational modifications. The methods typically employed consisted of various combinations of sieving through different size-meshes to eliminate microvascular components, density centrifugation to enrich preparations in heavier components— including mature plaques and plaque cores—, addition of different detergents and chaotropic agents, heating and sonication in attempts to solubilize the materials [40–46]. The protocol we have selected is based on a sequential extraction procedure that benefits from the differential solubility of different $\text{A}\beta$ species. The methodology has proved useful in the identification of pre-amyloid and amyloid components in sporadic and familial Alzheimer's disease cases, as well as in the characterization of the molecular heterogeneity found in amyloid deposits in familial Danish dementia [9], a disorder with striking clinical and neuropathological phenotypes to Alzheimer's disease, in which the disease-specific deposits of ADan amyloid coexist with $\text{A}\beta$ deposition [47].

1. Remove leptomeninges and large blood vessels. Separate gray from white matter with the aid of dissecting tools (scalpel, tweezers, small spatula). Finely mince the gray matter (~2.5 g) with a razor blade, add 12.5 ml of ice-cold PBS containing 2 $\times$ protease inhibitor cocktail and homogenize in a Dounce glass homogenizer immersed in ice until no large pieces are visible (*see Note 2*).
2. After homogenization, eliminate tissue debris via filtration through a 300 μm mesh nylon filter followed by subsequent removal of small vessels by filtration through a 70 μm nylon mesh; the vessel fraction may be reserved for further analysis of vascular amyloid, if desired [48]. Subject the filtrate of the 70 μm -filter to ultracentrifugation in a XL100K ultracentrifuge with a Beckman 70.1 Ti rotor at 112,000 $\times$ g for 1 h at 4°C and save the PBS-extracted supernatant enriched in soluble $\text{A}\beta$ species for further analysis (*see Note 3*).

3. Resuspend the PBS-insoluble pellet, containing amyloid and pre-amyloid materials, in 12.5 ml of 20 mM Tris, pH 7.4 containing 2% SDS (*see* Note 4).
4. Ultracentrifuge samples at 112,000× g for 1 h at 10 °C, as above, and save the SDS-extracted supernatant rich in pre-amyloid species for further analysis (*see* Note 5).
5. Resuspend the pellet in 1 ml 70% formic acid, vortex for 2 min. and centrifuge at 20,800× g in a 5417 microcentrifuge for 15 min at 4°C. The resulting supernatant (70% FA fraction) contains solubilized amyloid-rich fraction for further analysis (*see* Note 6).
6. Resuspend the remaining pellet in 1 ml 99% formic acid, vortex for 2 min. and spin-down at 20,800× g in a 5417 microcentrifuge for 15 min at 4°C, as above. The resulting supernatant (99% FA fraction) also contains solubilized amyloid-rich fraction for further analysis (*see* Note 7).

3.2 Immunoprecipitation of A β from Brain Extracted Fractions

Immunoprecipitation is a widely used methodology to enrich a preparation in a given protein, allowing subsequent elution of the precipitated protein and further analysis by gel electrophoresis, mass spectrometry, western blotting, amino acid sequence analysis or any other method for identifying protein constituents in a sample. Different methods are available for the immobilization of the formed antigen-antibody complex, among them those based on its capture by immobilized Protein A or Protein G. More recently, new methods have been developed based on the use of paramagnetic beads precoated with secondary antibodies which can be easily coupled with the desired primary antibodies. After immunoprecipitation and subsequent washes, paramagnetic beads are rapidly and efficiently collected with the aid of a magnet, and specifically bound protein retrieved typically with high recoveries.

3.2.1 Coating Paramagnetic Beads with Anti-A β Antibodies

1. Resuspend the mouse-IgG-coated paramagnetic beads by inverting several times the vial. Transfer 50 μ l of the suspension to a 1.5 ml-Eppendorf tube and position the tube in the rack equipped with a removable slide-out magnet (*see* Note 8).
2. Wash the beads three times with 1 ml PBS. For this, remove the tube from the magnetic rack, add the PBS, resuspend by inverting the tube, position it back in the magnet-equipped rack, and remove the supernatant after beads have compacted down. Repeat two-times.
3. Incubate pelleted beads with 3 μ g each of anti-A β 4G8 and 6E10 overnight, at 4°C with bi-directional rotation in a Mini LabRoller rotator, or equivalent, in a final volume of 100 μ l (*see* Note 9).
4. Collect the beads by placing the tube on the magnet rack for 2 min; remove and discard the supernatant.
5. Block the beads by resuspension in 1 ml PBS containing 0.1% BSA and subsequent three washes with the same solution. Once blocked beads can be

safely stored at 4°C for up to a week or used immediately for immunoprecipitation as indicated below.

3.2.2 Immunoprecipitation

1. Pretreatment of the brain extracts: The PBS extracts, containing the most soluble A β species, may be used directly for the immunoprecipitation steps described below. In contrast, SDS- and FA-extracted fractions require pretreatment steps to allow for a successful immunoprecipitation. SDS extracts should be treated with SDS-OUT reagent, following the manufacturer's instructions to eliminate SDS. Formic acid extracts require neutralization using 0.5 M Tris-base pH 11 (*see* Note 10).
2. Incubate anti-A β coated beads with neutralized amyloid- or SDS-free pre-amyloid rich- extracts overnight in a 4°C cold-room and with bi-directional rotation, as above (*see* Note 11).
3. Wash the magnetic beads three times with PBS.
4. Elute the material bound to the paramagnetic beads in accordance with the assays that will be performed subsequently on the eluted material, as in **steps 5 and 6**.
5. For Western blot analysis of the immunoprecipitated brain extracts, resuspend the beads in 10 μ l of Tris-Tricine SDS sample buffer containing 10% β -mercaptoethanol and directly load onto 16% Tris-Tricine gels for SDS-PAGE and Western blot analysis, as described in detail below (*see* Note 12).
6. For MALDI-ToF mass spectrometry analysis of the immuno-precipitated brain extracts, wash the beads three times with Milli-Q water and dry them in a SpeedVac concentrator system. Elute bound A β peptides with 5 μ l of a 4:4:1 mixture of isopropyl alcohol/water/formic acid [49], and store the eluate at -80°C until mass spectrometry analysis.

3.3 Western Blot Analysis

Mounting evidence continues to highlight the complexity of the amyloid deposits underscoring the diversity of aggregation conformers ranging from dimeric to highly oligomeric assemblies as well as pre-fibrillar components present together with the final stage fibrillar elements. Through the use of Western blot analysis, crucial information is obtained regarding the degree of oligomerization of the extracted amyloid, a crucial piece of information taking into consideration current knowledge supporting the key relevance of these species for the disease pathogenesis. Analysis of tissue extracted amyloid by Western blot is a commonly used method to assess the degree of A β aggregation and has also been extensively employed to characterize amyloid oligomerization/fibrillization properties of synthetic peptide homologues [50, 51]. The methodology we are describing below is based on the electrophoretic separation of tissue extracts by SDS-PAGE, followed by electro-transfer to nitrocellulose membranes, and end-point evaluation of A β species by chemiluminescence. For the electrophoretic separation we have selected 16%

polyacrylamide gels which are easier to perform and provide a good separation of aggregated species particularly those consisting of monomeric and low molecular mass oligomeric assemblies. Depending on the information sought and the specific composition of the respective amyloids in different specimens, it may be important to perform gradient-gels, typically 5–20% acrylamide, as well as native, non-SDS gels which preserve all aggregated species including those formed by SDS-sensitive oligomers [50]. Chemiluminescent substrates have been steadily gaining in popularity throughout the past decade to become the detection method of choice in most protein laboratories due to their several advantages over other detection methods. ECL not only allows multiple exposures to obtain the best representative image but also through stripping away the detection reagents this methodology allows reprobing the entire blot to optimize detection conditions, test different antibodies, or visualize other proteins, including control and house-keeping proteins for normalization purposes. The chemiluminescence large linear response range allows detection and quantitation over a large range of protein concentrations. Most importantly, it yields the greatest sensitivity of any currently available detection method with detection limits in the low picogram range.

3.3.1 Preparation of Slab Gels for Tris-Tricine SDS-PAGE [52]

Running gel: For the preparation of two 16% gels (12 × 10 × 1.5 mm) combine.

12.4 ml Acrylamide stock solution.

10 ml Gel buffer.

3.2 ml glycerol.

4.5 ml deionized water.

After mixing the above solutions, add 150 µl 10% APS and 15 µl TEMED and pour immediately between the two previously cast glass plates. Lay with isopropanol and leave at room temperature until polymerization. Once polymerized, remove the isopropanol layer, and pour the stacking gel prepared as follows.

Stacking gel: 1.6 ml Acrylamide stock solution.

3 ml Gel buffer.

7.4 ml deionized water.

Add 80 µl 10% APS and 14 µl TEMED to the combined reagents, pour on the top of the running gel, and carefully insert the comb avoiding the formation of bubbles.

3.3.2 Electrophoresis

1. Resuspend the samples to be analyzed—either purified and lyophilized amyloid extracts or paramagnetic beads containing the immunoprecipitated material—in ~20 µl Tris-Tricine sample buffer.

2. Boil for 5 min and load onto the gel previously assembled into the electrophoresis unit with the cathode buffer in the upper chamber and the anode buffer in the lower chamber.
3. Run electrophoresis at ~45 V (20 mA) for 15 min to 1/2 h until samples have entered the running gel; at this point, voltage can be increased to 120–150 V until completion, typically within ~2–3 h (*see* Note 13).
4. Remove the gel, equilibrate in transfer buffer for a few minutes, and assemble the transfer cassette positioning the pre-wetted nitrocellulose membrane carefully on the top of the gel. Avoid touching gel, nitrocellulose, as well as transfer sponges and cassettes without gloves. Make sure there are no air bubbles trapped between the gel and the nitrocellulose which will prevent successful transfer.
5. Electrotransfer 45 min at 400 mA in a Hoeffer TE22 Mini Tank Blotting Unit, or equivalent (*see* Note 14).
6. Carefully disassemble the transfer cassette and remove the nitrocellulose sheet. Rinse the membrane with PBS, and block with blocking solution for 3 h at 4°C while rocking in a rotating platform. Blocking time is not critical and it can be extended overnight or even performed during weekend depending on individual schedules.
7. Remove the blocking solution and incubate with a combination of 4G8 and 6E10 (1 µg/ml of each antibody in TBST) for 3 h at room temperature, while on shaker.
8. Wash the membrane three times for 10 min with TBST while rocking vigorously.
9. Incubate with the secondary antibody at 1:10,000 dilution for 1 h at room temperature.
10. Wash the membranes three-times for 10 min with TBST while rocking vigorously.
11. Prepare the two-component ECL substrate, consisting of a stable luminol solution with an enhancer and a stable peroxide solution, by mixing the two components together at a one-to-one ratio. Once prepared, the ECL substrate working solution is stable for a minimum of 24 h at room temperature.
12. Immerse the nitrocellulose sheet in the ECL substrate working solution and incubate for 2 min. Assemble the autoradiography cassette positioning the drained nitrocellulose membrane between regular plastic sheet protectors making sure there are no bubbles remaining on the top of the nitrocellulose membrane.
13. In a dark-room, assemble the autoradiography cassette by putting a film on top of the sheet protector sandwich containing the transferred membrane covered by the ECL substrate. Close the cassette and exposed the membrane for ~30 s to 1 min (*see* Note 15).
14. Develop the film in a M35A X-OMAT Film Processor, or equivalent.

3.4 Mass Spectrometry Analysis

The advent of mass spectrometry methodologies provided invaluable tools for the analysis of the components of extracted material from tissue deposits and was instrumental in the assessment of posttranslational modifications associated with amyloid molecules. In the case of A β , mass spectrometry corroborated the presence of cyclic pyroglutamate-modified fragments, exclusively associated with deposited N-terminally truncated A β species not detected in biological fluids, and demonstrated an abundance of soluble N-, and C-terminal degraded species likely representing the action of an array of diverse proteases and clearance mechanisms [9, 47, 53, 54].

Mass spectrometry is a very effective method for the identification and relative quantitation of A β species after enrichment by immunoprecipitation. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-ToF) MS is especially well suited for most of these analyses because of its simplicity, speed of MS analysis (usually less than 5 min per sample), and ability to analyze highly hydrophobic or poorly soluble A β species without potential bias due to differential behavior of different species during the HPLC associated with most electrospray ionization (ESI) mass spectrometry techniques. Another advantage of this approach is that MALDI-ToF is able to simultaneously measure several dozens of A β species without the need for species-specific antibodies (Fig. 2) (*see Note 16*). While fractional proportions of different A β species can be compared between samples based on relative MS signal intensities (ion counts), absolute amounts of each species from different samples can be measured and compared using stable isotope-labeled synthetic A β peptides spiked into the A β -containing samples before immunoprecipitation [34]. Linear ion mode is usually preferred because it is more sensitive and accurate for relative quantitation than reflectron mode. The relative simplicity and purity of A β after the IP methods described earlier allow the unambiguous identification of the species by mass, even in linear ion mode. If unanticipated A β species are observed, they can be identified with the help of more accurate reflectron mode MALDI-ToF, or by MALDI or ESI tandem mass spectrometry for peptide sequencing and/or determination of posttranslational modifications such as N-terminal pyroglutamate formation. Here, we describe a method for relative quantitation of A β species by MALDI-ToF MS in linear ion mode.

3.4.1 Spotting of Samples on MALDI Plates

1. Mix the immunoprecipitated brain extract sample containing A β and CHCA matrix solution 1:1 (v/v) (*see Notes 17 and 18*).
2. Apply 1 μ l sample/matrix solution to a MALDI target plate well and allow it to dry at room temperature for approximately 10 min.
3. For external calibration, apply μ l standard/matrix solution containing 100 fmol of each standard peptide to a MALDI target plate well adjacent to a well containing A β sample mixture.
4. If internal peptide mass calibration standards are used, adjust the concentration of standards so that the signal intensity of the standards is similar to that of the most abundant A β peptide species.

5. After external calibration, confidently identified A β species can be used for internal calibration.

3.4.2 MALDI-ToF Analysis

1. Acquire MALDI-ToF mass spectra of peptide standards in positive ion, linear mode in order to perform external calibration of A β samples on sample wells adjacent to the standards. Then acquire spectra for the A β samples. Standard instrument settings optimized for peptide analysis can be used, varying laser intensity to obtain optimal ion signals. In most cases up to 400 laser shots are summed for each spectrum. If higher mass accuracy is needed for peptide identification, reflectron mode can be used for the same samples.
2. MALDI-ToF data are processed using FlexAnalysis software from Bruker. When linear mode is used, lack of isotopic resolution in most cases necessitates use of average masses for calculations. If isotopic resolution is achieved, for example when reflectron mode is used, monoisotopic masses can be used.
3. Major peaks within the spectra can be identified with the help of freely available software such as ExPASy's FindPept software (<http://us.expasy.org/tools/findpept.html>).
4. Relative quantitation of A β species within each sample is estimated from ion intensity peak heights (*see* Note 19). Because A β species are not chemically identical and therefore may have different ion signal response characteristics, relative intensities can only be taken as estimates of relative quantities. Likewise, quantitative comparisons between samples should only be attempted with the use of stable isotope-labeled synthetic A β peptides.

4 Notes

1. The magnitude of the neuropathological lesions is highly variable not only among different AD cases but also among different regions of the same brain. Confirmation of the presence and intensity of the amyloid deposits in the selected area should be assessed by histopathological or immunohistochemical standard protocols [55] prior to the isolation protocol.
2. All solutions should be ice-cold and procedures should be carried out on ice or inside a 4°C-cold room. A useful alternative to the Dounce glass homogenizer, particularly when many samples are processed at once, is the use of a mechanical homogenizer equipped with disposable probes for soft tissues (e.g., Omni International, Marietta, GA).
3. PBS supernatant typically contains soluble brain proteins and a minor proportion of soluble A β species (primarily monomeric/dimeric species of high solubility in water-based buffers). To assure removal of undesirable water-soluble proteins and extract most of the soluble A β , the homogenization/centrifugation step should be repeated at least two times and the resulting supernatants either combined or analyzed separately as the PBS fraction.

4. Before the addition of the SDS-containing buffer the samples should be brought at 10°C to avoid SDS crystallization.
5. Depending on the pre-amyloid load of the specimen, **steps 3 and 4** should be repeated 2–3 times not only to increase pre-amyloid yield in the SDS-extracts but to minimize contamination of the fishould amyloid to be extracted in **step 5**.
6. Depending on the load of dense plaques in the specimen, it might be necessary to add an additional extraction (**step 6**) using 99% FA to maximize the yield of solubilized amyloid. Use the initial immunohistochemical analysis of the tissue to assess amyloid load and compact plaque density.
7. Regardless of the compact plaque density, proceed with **step 6** if the pellet after **step 5** is still significant. Typically, after 99% FA the remaining pellet is minimal or not existent.
8. It is very important to use specially designed racks which are equipped with potent magnets allowing the compacting of the beads at the bottom of the tube for volumes of 0.5 µl–2 ml. The strong magnetic field permits total recovery of the beads without losses of material in the subsequent steps of the immunoprecipitation.
9. Coating with primary antibodies is also successfully performed by incubation for 2–3 h at room temperature. The proportions indicated in the procedure detailed above are enough for one immunoprecipitation; if more samples are to be processed, the protocol should be scaled up accordingly.
10. Incomplete neutralization or lack of SDS removal will result in partial or total failure to immunoprecipitate Aβ from the brain extracts. Immunoprecipitation is based on the specific antigen-antibody reaction, in this case the interaction of Aβ species with the immobilized anti-Aβ 4G8 and 6E10 antibodies. Extreme pH values and presence of detergents are among the known elements that disrupt antigen-antibody interactions.
11. The volumes of the tissue-extracted fractions to be incubated with the beads depend on the Aβ load of the different cases, as well as on the detection method that follows. Typically, immunoprecipitation of ~4% of the formic-acid- and ~20% of the SDS-extracts yield enough material for subsequent Western blot and mass spectrometry analyses.
12. β-Mercaptoethanol may be substituted by 5 µl of 1 M dithiothreitol.
13. The electrophoresis may also be run overnight, if desired. In this case, after the sample has entered the running gel, lower the voltage to ~20 V (<10 mA) for overnight run.
14. To analyze degree of Aβ oligomerization in pre-amyloid extracts increase the transfer time up to 2 h to allow proper electrotransference of higher molecular mass assemblies.

15. Exposure times should be determined empirically and range from 3–5 s to 10–20 min depending on the intensity of the signals which in turn are related to the protein load on the gel, the concentration of A β of the samples analyzed, as well as the strength of the primary antibody. It is recommended to start with a medium time, ~30 s to 1 min, each time developing the film as below; depending on the quality of the image obtained, either decrease or increase the exposure time, accordingly.
16. Numerous truncated forms of A β have been detected in brain tissues from sporadic and familial AD cases although there are some differences among the fragments identified in the different reports [9, 39, 56]. Factors contributing to these dissimilar results may rely on differences among the brain areas studied, age of the individuals, or stages of the disease together with variations in the tissue extraction protocols and the methodologies employed for analyzing the different peptide variants. In addition to the full-length A β 1–40 and A β 1–42, the generally the dominating species in FA extracts are peptides truncated at Phe4—either A β 4–40 or A β 4–42—concomitantly with pyroglutamate A β 3–42 [39, 57]. Earlier reports have described the presence of A β 17–40/42 in brains with extensive diffuse amyloid deposition in AD and Down's syndrome patients [58, 59]. Nevertheless, these fragments were not identified in the current studies as well as in those from other groups [56] suggesting that either these species are minor constituents, or that their presence is not a common feature among different cases.
17. Other CHCA matrix solutions may be used, for example, 10 mg/ml CHCA in 0.1% TFA and 50% (v/v) acetonitrile.
18. The A β peptide samples can be dissolved in most salt-free acidic solutions containing for example methanol, water, isopropanol, water, formic acid, and/or TFA. High pH can prevent matrix crystallization during spotting of MALDI target plates, and non-volatile salts can interfere with MALDI-ToF analysis. All reagents should be of the highest purity available to minimize MALDI-ToF background.
19. In our experience A β oligomers dissociate during sample preparation and/or MALDI analysis so only monomeric A β peptides are observed. Therefore, MALDI-ToF is not a good method for relative quantitation of A β oligomerization status [9].

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Fig. 1. Schematic representation of the sequential extraction protocol of the different Aβ species. The diagram illustrates the consecutive steps required for the sequential isolation of deposited Aβ species in AD brain tissue for subsequent biochemical and mass spectrometry studies

Fig. 2.

Biochemical analysis of Aβ deposits in AD specimens. Brain tissues were sequentially extracted in PBS, SDS-containing buffer, and formic acid; the different fractions were subsequently immunoprecipitated with paramagnetic beads coated with 4G8/6E10 anti-Aβ antibodies and analyzed by MALDI-TOF MS. (a) MS profile obtained from the PBS homogenate (top panel), SDS extract (central panel) and FA extract (bottom panel) in an AD brain specimen highlights the heterogeneity of the Aβ peptidome composed of numerous truncated Aβ species in addition to the full-length Aβ1–40, and Aβ1–42 peptides. (b–d) MS pattern of PBS and FA extracts in different AD cases illustrates the presence of N-terminally truncated Aβ species, particularly at Phe4, in addition to full-length Aβ, in agreement with our recent reports depicting the high heterogeneity and abundance of posttranslationally modified Aβ forms in AD and aged squirrel monkeys [38, 39]. Whether the differences in the composition of the brain Aβ deposits among the different cases relate to the duration of the disease, any association with known genetic, vascular, or metabolic risk factors, or to other clinical phenotypes remains to be elucidated.