Heterogeneity of water-soluble amyloid β-peptide in Alzheimer's disease and Down's syndrome brains

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Abstract Water-soluble amyloid β-peptides (sAβ), ending at residue 42, precede amyloid plaques in Down's syndrome (DS). Here we report that sAβ consists of the full-length $Aβ_{1-42}$ and peptides truncated and modified by cyclization of the N-terminal glutamates, $Aβ_{3(pE)-42}$ and $Aβ_{11(pE)-42}$. The $Aβ_{3(pE)-42}$ peptide is the most abundant form of sAβ in Alzheimer's disease (AD) brains. In DS, $sAβ_{3(pE)-42}$ concentration increases with age and the peptide becomes a dominant species in the presence of plaques. Both pyroglutamate-modified peptides and the full-length Aβ form a stable aggregate that is water soluble. The findings point to a crucial role of the aggregated and modified sAβ in the plaque formation and pathogenesis of AD.

Key words: Amyloid beta-peptide; Alzheimer's disease; Down's syndrome; Pyroglutamate

1. Introduction

The amyloid plaques, an invariant neuropathologic feature of AD affected brains, are primarily composed of ~4.5 kDa Aβ peptide and its derivatives that are proteolytically cleaved from a large transmembrane precursor protein — APP [[1], for a recent review see [2]]. Complex processing of APP along the two major pathways — secretory and endosomal/lysosomal — generates a set of intermediates of various length and stability. Aβ is proteolytically derived only from those larger carboxy-terminal fragments of APP that carry its intact sequence [3]. Cellular processing of APP generates a monomeric Aβ that is secreted by cells in culture [4–6]; the peptide is also detectable intracellularly [7,8]. Consistent identification in cultured cells of the secreted Aβ peptides beginning at residues 1 and 17, and ending at residue 40, indicates that Aβ is generated proteolytically by putative proteases, dubbed 'α- and β-secretases', cutting at those positions; the third protease, 'γ-secretase', cuts at the C-terminus which normally resides within the membrane [9–12]. However, protein-chemical analyses of AD brain amyloid show that the processing might be quite different in the brain. The presence of Aβ ending at residue 42 ($Aβ_{1-42}$) as the dominant form and of numerous N-terminally truncated Aβ peptides in plaques has been established [13–16]. Moreover, immunocytochemical studies have shown a temporal sequence of $Aβ_{42}$ and $Aβ_{40}$ appearance in the amyloid plaques with $Aβ_{42}$ being the initial and dominant peptide in all forms of AD and DS [17–20]. We have recently extended these observations and found that Aβ which can be extracted from brain parenchyma as a set of water-soluble peptides [21–25], ending at residue 42, precedes amyloid plaque formation in DS brains that inevitably develop AD pathology [24].

The identity and sequence of appearance in plaques of Aβ with defined C-terminal residues is now evident [26]; however, there is no consensus as to the form of the N-terminally truncated Aβ in the brain. Aβ processing is additionally complicated by the post-translational, post-secretory modifications such as isomerization or racemization of aspartate and cyclization of the N-terminal glutamate [14,27–29]. Such modifications are usually attributed to long-lived proteins which is consistent with the time required for senile plaque formation.

Here, we characterize the three electrophoretic sAβ forms [21,24] in DS brains with and without amyloid plaques as well as sporadic and familial AD brains. The full-length sAβ consists of a mixture of non-modified, racemized and isomerized $Aβ_{1-42}$, while the other two forms are truncated with the N-terminal glutamates cyclized. In DS brains the $Aβ_{3(pE)-42}$ increases with age and is present many years prior to plaque formation. The appearance of the N-terminally truncated and modified sAβ appears to be the first marker of pathologic Aβ accumulation in the brain.

2. Materials and methods

2.1. Source of tissue

Cerebral cortex was obtained at autopsy from subjects in which the diagnosis of DS had been established by chromosomal analysis or from neuropathologically verified cases of sporadic and familial AD (linked to APP V717I mutation and to presenilin 1 mutation C410Y). For controls the tissue was obtained from subjects affected by a variety of neurological and non-neurological conditions but in which DS and AD had been excluded with clinical and autopsy examinations including immunohistochemical analyses.

2.2. Antibodies

A rabbit polyclonal antibody R3659 was raised against unconjugated synthetic $Aβ_{1-40}$. The antibody is specific for the N-terminus of the peptide. A polyclonal PC421, specific for residue 42, was raised against a synthetic C-terminal fragment of Aβ, GLMVGGVVIA. N-terminally specific antibodies against the truncated Aβ peptides with and without modifications: anti-N3(E), anti-N3(pE), anti-N11(E), anti-N11(pE), and anti-N17(L), as well as antibodies recognizing the N-terminal aspartate at position 1, either non-modified, racemized or isomerized, are described elsewhere [30]. Monoclonals 4G8 and 6E10 were kindly supplied by Dr. K.S. Kim [31].

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Abbreviations: Aβ, amyloid β peptide; AD, Alzheimer's disease; APP, amyloid precursor protein; DS, Down's syndrome; pE, pyroglutamate; sAβ, water-soluble Aβ

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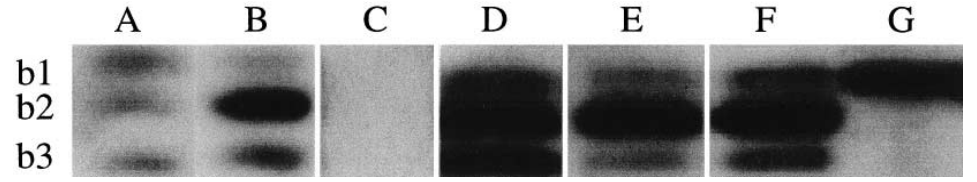

Fig. 1. Three electrophoretic forms, b1–b3, of water-soluble Aβ peptides are present in AD and DS brains. The peptides were extracted from DS brains without (A) and with immunodetectable plaques (B); control, plaque-free brain (C); presenilin 1 mutation (C410Y) carrier (D); APP V717I mutation carrier (E), and sporadic AD (F), resolved on Tris–Tricine gels, blotted to PVDF membranes and detected by chemiluminescence with a monoclonal antibody 4G8. Synthetic Aβ1–42 was loaded in lane G as a reference.

2.3. Extraction from brain and immunoprecipitation of sAβ

These were carried out as described previously [24]. For immunoprecipitation the antisera R3659, PC421, and anti-N3(pE) were used.

2.4. Isolation and extraction of insoluble Aβ

After buffer extraction and centrifugation, the resultant pellets were washed twice with 10% SDS, twice with water and finally extracted with 75% formic acid and spun down. The supernatants, after neutralization, were further processed by chromatography and electrophoresis or analyzed for the presence of Aβ by immuno-blotting.

2.5. Electrophoresis and Western blotting

The Protein A–agarose beads were boiled in the electrophoresis sample buffer for 5 min. Different aliquots of the immunoprecipitated peptides were separated on 10% Tris–16.5% Tricine gels [32], and electroblotted to PVDF or nitrocellulose membranes at 90 V for 2 h. The resolved peptides were visualized with the enhanced chemiluminescence system (ECL, Amersham) after immunodetecting with the poly- or monoclonal antibodies specific for different parts of Aβ. ECL films were densitometrically scanned at 42μ resolution. Synthetic Aβ peptides were used as standards.

2.6. Pyroglutamate aminopeptidase treatment

Antibodies used for Aβ detection on PVDF membranes were stripped with 0.2 M glycine, pH 2.85. The membranes were washed 4 times with phosphate-buffered saline (PBS), pH 7.4, containing 0.1% Tween-20, and incubated for 3 h at 37°C with 10 U pyroglutamate aminopeptidase (EC 3.4.19.3, Sigma), in 3 ml of PBS, pH 8.0, con-

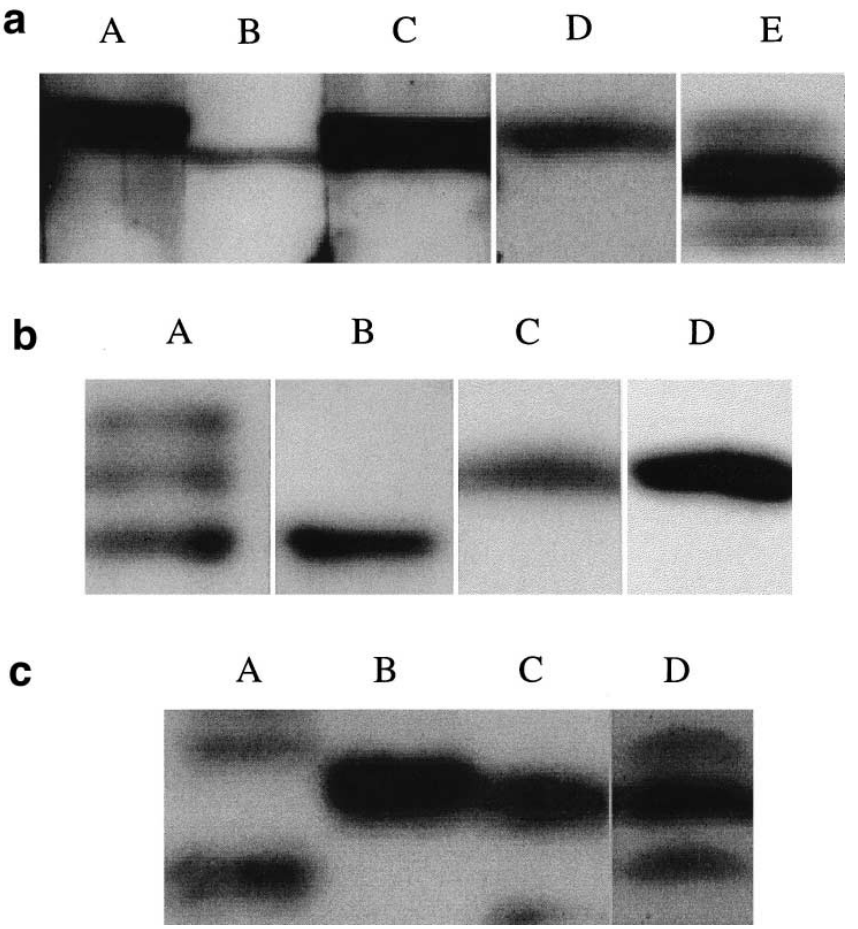

Fig. 2. a: Heterogeneity of sAβ peptides demonstrated with various N-terminally specific antibodies raised against: (A) Aβ with non-modified Asp-1; (B) Aβ with isomerized Asp-1; (C) Aβ with racemized Asp-1. Synthetic Aβ1–42, and sporadic AD sAβ, loaded in lanes D and E, respectively, were detected with monoclonal 4G8 and used as a reference. b: sAβ peptides immunoprecipitated from sporadic AD (lanes A–C) and DS (lane D), brain extracts were separated by electrophoresis and detected with monoclonal 4G8 (lane A); polyclonals anti-N11(pE), (lane B), and anti-N3(pE), (lanes C and D). c: Synthetic Aβ peptides migration versus an AD sample. Thirty nanograms of each peptide was loaded on gels, electrophoresed, blotted to PVDF membrane and visualized by chemiluminescence with monoclonal 4G8: (A) $A\beta_{1-42}$ and $A\beta_{11(pE)-40}$; (B) $A\beta_{3(pE)-40}$; (C) $A\beta_{3-40}$ and $A\beta_{17-42}$; (D) sporadic AD extract.

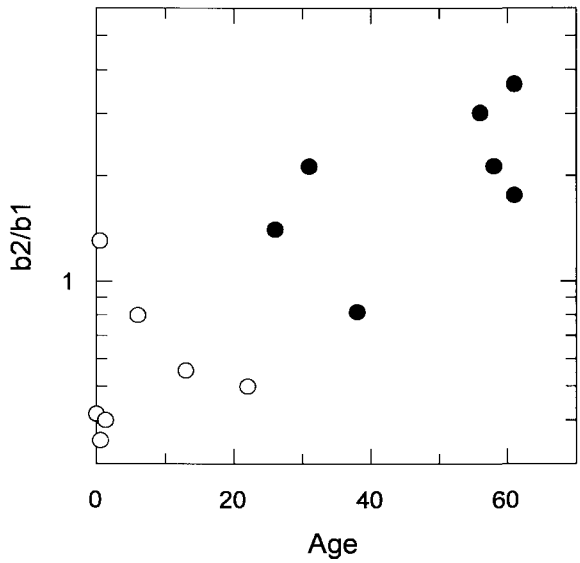

Fig. 3. The ratio of $A\beta_{3(pE)-42}$ to the full-length $A\beta_{1-42}$ increases with age in Down's syndrome. The figure shows the densitometrically measured ratio of band 2 ($A\beta_{3(pE)-42}$) to band 1 (full-length Aβ) detected on Western blots of the soluble brain fraction after staining with a monoclonal 6E10. ○, DS without immunodetectable amyloid plaques; ●, DS with plaques. The original data were taken from [24].

taining 5 mM dithiothreitol, 10 mM EDTA. After replacing the enzyme solution, digestion continued for an additional 8 h. The membrane was then washed extensively with water, equilibrated with PBS–Tween solution and used for Aβ immunodetection.

2.7. Immunohistochemistry

Immunohistochemistry was carried out in formalin-fixed paraffin-embedded blocks of cerebral cortex from the lobe used for quantitation of the soluble Aβ and from other lobes, as well from ~1 mm thick slices obtained from the same tissue blocks used for the biochemical determination [24]. The analysis was carried out with antibodies 4G8, anti-N3(pE) and PC421.

2.8. Chromatography

Immunoprecipitated peptides captured on Protein A–agarose were dissociated from the beads by adding either 1 M glycine, pH 2.0, to obtain final concentration of 0.2 M or by using 0.1 M triethylamine acetate, pH 11.5 (final concentration). After gentle vortexing and spinning down the beads, the dissociation step was repeated twice and the supernatants combined. The samples were applied on BioGel P-6 or P-10 columns equilibrated with the same solvents. For HPLC, a polymer column — PLRP-S (Polymer Laboratories, UK) — was used. 20–60% acetonitrile/2-propanol (9:1) gradient in 0.05 M Tris, 0.01 M betaine, pH 8.9, was used for the elution of Aβ peptides. Either synthetic Aβ standards or Aβ previously added to the extraction mixtures were used. Whenever possible samples were prepared in an organic solvent to ensure full solubility. The HPLC elution was monitored at 220 nm and the collected fractions were analyzed for the presence of Aβ by immunodetection by the dot-blot technique with monoclonal 4G8 or a respective N-terminally specific antibody.

3. Results

3.1. Immunochemical characterization of the N-terminal modification of sAβ peptides

Buffer extraction of AD brain grey matter, immunoprecipitation with anti-Aβ antibodies R3659, PC421 or anti-N3(pE), and detection with a monoclonal antibody 4G8, results in the separation of three distinct bands on Tris–Tricine SDS–PAGE. A similar pattern is observed in sporadic AD, familial AD linked to presenilin 1 and APP mutations or DS (Fig. 1). Reactivity of the Aβ peptides with the N- and C-terminally specific antibodies indicates that the top band, b1, contains the full-length Aβ starting with aspartic acid and ending at residue 42 [21,24]. Heterogeneity of b1 was demonstrated with a set of the N-terminal-specific antibodies [30]; the full-length sAβ consists of the unmodified peptide and peptides with either racemized or isomerized aspartate (Fig. 2a). The presence of $A\beta_{1-40}$ is only evident in advanced AD that is accompanied by amyloid angiopathy [24].

On the basis of the relative mobility we initially assumed that the middle band, b2, differs from b1 by at least 5 residues and that b3 begins at residue 17 [6,24] (Fig. 1). The presence of $A\beta_{3(pE)-42}$ in b2 was established with an antibody specific to the N-terminal pyroglutamate (Fig. 2b). Moreover, a polyclonal anti-N3(E), specific to uncyclized glutamate at the third position of Aβ, did not react with any of the peptides extracted from AD brain, indicating that all sAβ truncated at the third residue has its N-terminal glutamate cyclized. The fastest migrating peptide (b3), which we originally assumed to comprise $A\beta_{17-42}$, was identified as $A\beta_{11(pE)-42}$ by using an antibody specific for $A\beta_{11(pE)-X}$ (Fig. 2b). It should be noted that both pE–Aβ antibodies are highly specific not only on the basis of the recognition of synthetic peptides [29] but also because they do not cross-react and do not react with other unrelated peptides containing N-terminal pyroglutamates (not shown). The apparent absence of $A\beta_{17-42(40)}$ was confirmed with an anti-N17(L) antibody. To corroborate these results we compared the electrophoretic migration of AD derived peptides to their synthetic counterparts (Fig. 2c). As predicted the full-length synthetic Aβ peptides comigrated with b1, $A\beta_{3(pE)-40}$ and $A\beta_{3-40}$ with b2, and $A\beta_{11(pE)-40}$ with b3, while $A\beta_{17-42}$ migrated, faster than b3.

All electrophoretic forms of sAβ were consistently present, although at different ratios, in extracts from all sporadic and

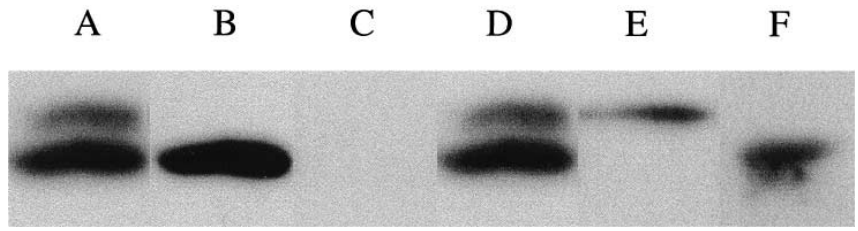

Fig. 4. Identification of $A\beta_{(pE)-42}$ in the AD brain extract. A: Electrophoretically separated Aβ peptides detected with a monoclonal antibody 6E10. B: Peptides from the same brain sample detected with the anti-N3(pE) antibody. C: PVDF membrane shown in (B) treated with pyroglutamate aminopeptidase and re-probed with the anti-N3(pE) antibody. D: The same membrane re-probed with 6E10. E: Synthetic $A\beta_{1-42}$ detected with 6E10. F: Synthetic $A\beta_{3(pE)-40}$ detected with the anti-N3(pE) antibody.

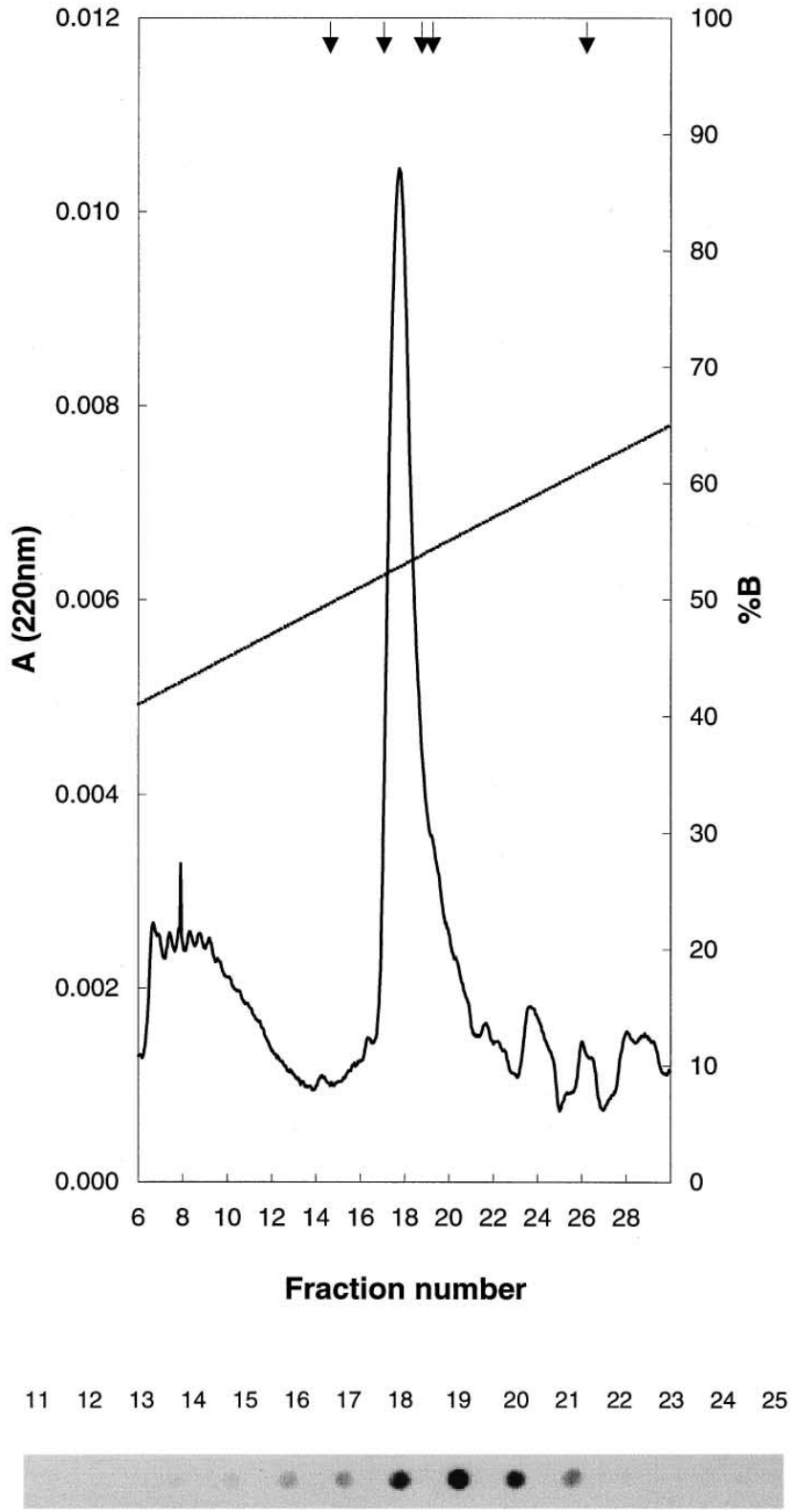

Fig. 5. HPLC analysis of brain sAβ. Immunoprecipitated sAβ was dissolved in 75% formic acid and loaded onto the PLRP-S column. Peptides were eluted with a linear gradient of 20–60% acetonitrile: 2-propanol (9:1) in 0.05 M Tris, 0.01 M betaine, pH 8.9. Elution positions of synthetic Aβ peptides are shown by arrows, from left to right: $Aβ_{1-40}$, $Aβ_{3(pE)-40}$, $Aβ_{1-42}$, $Aβ_{11(pE)-42}$ and $Aβ_{17-42}$. The HPLC fractions were analyzed by the dot-blotting with monoclonal 4G8 (bottom panel).

familial AD and DS brains studied, and the modified form, $Aβ_{3(pE)-42}$, is dominant. Although this form is abundantly present in Aβ deposits [29], it is also recoverable in the water-soluble fraction. When DS brains of different ages were extracted with buffer and detected on blots with monoclonal antibody 6E10 [24], a striking relationship between age

and the ratio of b2/b1 was observed (Fig. 3). In young DS brains which did not contain amyloid plaques in the cortex the average ratio was below 1, i.e. there was more full-length $sA\beta_{1-42}$ than $A\beta_{3(pE)-42}$. In the brains with plaques the ratio increased dramatically with age.

3.2. Enzymatic and chromatographic characterization of sAβ with the N-terminal pyroglutamate

The identity of $A\beta_{3(pE)-42}$ was confirmed by incubating the membrane-immobilized peptide with pyroglutamate aminopeptidase, the enzyme removing the N-terminal pyroglutamates (Fig. 4). Reprobing the membrane after digestion with a monoclonal 6E10 showed that the lack of signal with the anti-N3(pE) antibody was not due to the loss of the peptide (Fig. 4). This experiment demonstrated both the peptide identity and the antibody specificity. These analyses clearly demonstrate that b2 detectable by 6E10 is not an artifact as previously thought [16]. We have also examined if $A\beta_{3(pE)-40}$ or $A\beta_{3(pE)-42}$ can be generated in vitro by exposure of the synthetic full-length peptides $A\beta_{1-40}$ and $A\beta_{1-42}$, respectively, to heat or extremely low pH which promote glutamate cyclization. Neither boiling the peptides for 10 min nor dissolving in 70% formic acid followed by neutralization and SDS–PAGE caused the appearance of any additional bands.

To further analyze the modified Aβ peptides we applied a purification protocol based on immunoprecipitation of sAβ and chromatography at alkaline conditions [33]. The synthetic $A\beta_{1-40}$, $A\beta_{3(pE)-40}$, $A\beta_{1-42}$, $A\beta_{11(pE-42)}$ and $A\beta_{17-42}$ were eluted with the acetonitrile gradient in this order (Fig. 5), confirming their theoretically predicted hydrophobicity. The synthetic peptides could also be resolved if loaded on the column as mixtures. In contrast, brain-derived peptides could not be separated into the forms detectable on Tris–Tricine gels, although they were identifiable by immuno-blotting with respective antibodies in different HPLC fractions (not shown). This suggests that brain-derived Aβ peptides easily reaggregate during the HPLC separation, despite the fact that they were applied in 75% formic acid [33].

3.3. Brain water-soluble Aβ is aggregated but distinct from insoluble Aβ

We also performed a series of experiments aiming at the separation of monomeric and aggregated sAβ. The peptides were extracted with buffer and, after immunoprecipitation, resolved on a non-denaturing gel filtration column (BioGel P-6 or P-10, not shown). Chromatography demonstrated that the bulk of sAβ elutes in the void volume indicating that, although water-soluble, the peptides are present as an aggregate consisting of all three electrophoretic forms. That sAβ is extractable from brain as an aggregate was also evident from a series of immunoprecipitation experiments followed by electrophoresis and chromatography. Thus, the polyclonal, N-terminal-specific anti-$A\beta_{1-40}$ antibody R3659, recognizing only the full-length peptide on Western blots, precipitated all three Aβ electrophoretic forms from brain extracts, i.e. those Aβ forms were aggregated. Moreover, immunoprecipitation with the anti-N3(pE) antibody, which targets only one truncated peptide form, also resulted in the detection of all three electrophoretic bands in the immunoprecipitate. The same result was also obtained when the C-terminal-specific antibody — PC421 — was used.

Finally, to assess whether sAβ is directly related to insoluble Aβ, we analyzed the latter in brains from control subjects which were plaque-free and in which sAβ was undetectable and in DS brains with and without immunodetectable deposits [24]. We found no significant difference between insoluble Aβ levels in control group ($n=9$, 157 ± 46 ng/g of tissue) and plaque-free DS brains ($n=7$, 137 ± 56 ng/g of tissue), despite the fact that sAβ was elevated in all DS brains (20 ng/g of tissue) and undetectable in controls [24]. This result suggests that sAβ is present as a distinct pool in plaque-free brains [25].

4. Discussion

In search ot the molecular events initiating plaque formation in AD, we have investigated the heterogeneity of sAβ peptides extracted from human brain. We demonstrate that: (a) all three major sAβ peptides are N-terminally modified having their N-terminal aspartate residues racemized or isomerized (aspartate at position 1), and cyclized (glutamate at positions 3 and 11); (b) $A\beta_{3(pE)-42}$ is a dominant species of sAβ in all forms of AD and in DS; (c) in DS brain $A\beta_{3(pE)-42}$ concentration increases with age; (d) in the brain sAβ exists as a stable aggregate. Our results suggest that pyroglutamate-modified Aβ peptides might be unique forms present only in the diseased brain as a result of the impaired catabolism and clearance of Aβ. The sAβ aggregate formation may impose protease resistance by a steric hindrance as described for synthetic Aβ aggregates [34]. It is also likely that $A\beta_{3-42(40)}$ and $A\beta_{11-42(40)}$ are generated by limited proteolysis due to the restricted access imposed by the peptide rearrangement upon aggregation. A local environment, with possible expulsion of water, would favor the subsequent cyclization of glutamates. Whether lack of proteolytic cleavage at glutamate residues is caused by a reduction in glutamyl aminopeptidase activity [35], or is a consequence of a specific arrangement of Aβ in the aggregate remains to be elucidated.

It is apparent that in DS brains the amount of $A\beta_{3(pE)-42(40)}$ increases relatively to the full-length Aβ with age and that the peptide is an invariant component of all AD brains including familial cases. Whatever the mechanism of $A\beta_{3(pE)-42}$ formation, it appears that the peptide is the earliest marker of aggregation and its amount in the brain reflects the progress of the pathologic process. A highly insoluble Aβ is present in both young DS and normal brains that are free of plaques. In contrast, sAβ level is increased in plaque-free DS brains but undetectable in control brains [24]. The nature of the insoluble Aβ in plaque-free brains remains unclear.

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