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Short communication

Combined use of micro-preparative gel electrophoresis and reversed-phase high-performance liquid chromatography for purification of amyloid β peptides deposited in brains of Alzheimer's disease patients

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Abstract

A new micro-technique is developed for purification of amyloid β peptides (Aβ) extracted from brain tissues of patients with Alzheimer's disease (AD). It includes SDS–polyacrylamide gel electrophoresis of the extracted brain tissue material, electroblotting onto supporting membranes, and reversed-phase HPLC of the proteins eluted from membranes. By this technique, the extracted Aβ are first separated electrophoretically from the higher and lower molecular mass tissue components, and then purified by reversed-phase HPLC from the contaminants having similar molecular masses, but different retention times on the column. In contrast to the common large-scale isolation procedures employing density gradient centrifugation, enzymatic digestions and size-exclusion chromatography, the developed micro-technique might be applied for biochemical analysis of Aβ contained in small AD brain tissue specimens.

Keywords: Amyloid β peptides; Peptides

1. Introduction

Alzheimer's disease (AD) is one of the most common disorders affecting nearly 5% of the population over the age of 65. AD is characterized by the extracellular deposition of the fibrillar amyloid β peptides (Aβ) found within neuritic plaques in the cortical region of the brain, as well as in the walls of cerebrovasculature. Early studies showed that neuritic plaques contain Aβ consisting of 42 amino acid residues; these peptides were extremely insoluble even when using strong solubilization agents, such as guanidinium hydrochloride, sodium dodecyl sulfate (SDS) or urea [1]. The 40-residue Aβ found in cerebrovasculature were more soluble and could be extracted from blood vessel walls with guanidinium hydrochloride [2]. Further examination showed that Aβ comprise a much larger number of species differing in solubility and structure. In addition to the highly insoluble species, AD brain accumulates the more soluble Aβ which could be extracted from tissue homogenates with SDS and even with the detergent-free aqueous buffers [3,4]. Beside the major 1–40 and 1–42 Aβ forms, Aβ

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peptides were found to have ''ragged'' N-termini, starting from Asp1 to Glu11 residue. Recent studies demonstrated extensive chemical modification of Aβ peptides, including racemization of aspartyl and seryl residues, cyclization of N-terminal glutamyl residue, and isomerization of aspartyl residues [5]. Since different Aβ structures vary in their ability to form fibrils, as well as in their neurotoxicity [5,6], the analysis of content and distribution of different Aβ species in the brain is important to understand the AD pathogenesis. Unfortunately, the data from Aβ analysis vary in different studies, being probably affected by differences in the extraction and fractionation procedures applied, and by variability in peptide solubility and yield [5]. Structural analysis of Aβ is especially complicated in the cases when only small tissue specimens are available. In fact, the classical Aβ isolation methods involve physical separation of cortical plaques/vessels by density gradient centrifugation prior to Aβ extraction and chromatography procedures [7]. It allows essential concentration of Aβ and removal of many tissue contaminants which facilitate subsequent purification of these peptides. However, this technique requires large amounts of starting tissue material and is not suitable in studies of small amounts of available autopsy material, biopsy specimens, or tissue samples taken from small experimental animals. In fact, only a few studies employing small-scale purification protocols are reported; in such studies, Aβ are extracted from crude (non-separated) tissue homogenates [8,9]. In order to minimize Aβ contamination by other co-extracted tissue proteins, the tissue homogenates can be subjected to proteolytic digestions and/or washing with SDS containing aqueous buffers [8]. However, the enzymatic procedures could lead to artifactual generation of ragged N- and C-termini [8], whereas treatment with SDS could result in the losses of the detergent-soluble Aβ species [4,9].

In our previous studies [10,11], a simple microtechnique was developed for extraction of Aβ from minute amounts of AD brain cortex homogenates by employing aqueous acetonitrile-trifluoroacetic acid solution and formic acid. To minimize either in vitro degradation of Aβ or the loss of some more soluble Aβ species, tissue treatments by enzymes and detergents were omitted in our protocol. In the present study, we describe a new small-scale technique for purification of Aβ extracted from the AD brain tissues homogenates. This technique includes SDS–polyacrylamide gel electrophoresis (SDS–PAGE), followed by blotting to and elution from the supporting membranes, and finally, reversed-phase high-performance liquid chromatography (RP-HPLC). By this combined micro-technique, amyloid β peptides were first separated electrophoretically from both the higher and lower molecular mass tissue components, and then purified by RP-HPLC from the contaminants having similar molecular masses, but different retention times on the column.

2. Experimental

2.1. Brain tissues

Brain tissues were obtained at autopsy from five AD patients and two normal persons. The AD diagnosis was confirmed by the histopathological examination of the autopsy specimens. For the biochemical studies, the unfixed tissue specimens were snap-frozen and stored at −70 °C until used.

2.2. Extraction of Aβ proteins

The extraction procedure described previously [10,11] was adapted. Cortical tissue samples (1–3 g wet wt.) were washed three times by homogenization in PBS in the presence of protease inhibitor cocktail (Complete TM, Boehringer Mannheim GmbH, Germany) followed by centrifugation at 14 000 *g* for 10 min. The resulting pellet was washed with deionized water and suspended in 20% acetonitrile containing 0.1% TFA. The mixture was incubated at room temperature for 1 h with moderate stirring and then centrifuged. The incubation and centrifugation steps were repeated two times, and the supernatants obtained were pooled and lyophilized (Ac-TFA extracted material). The pellet was lyophilized, incubated with formic acid (20 mg/ml) for 0.5 h, centrifuged, and the resulting supernatant was dried

(FA extracted material) in Speed Vac apparatus (Speed Vac SC 100, Savant, Farmingdale, NY).

2.3. SDS–PAGE and electroblotting

Protein samples were dissolved in SDS–PAGE sample buffer (pH 6.9) containing 6% SDS, 125 m*M* Tris, 4 m*M* Na_2EDTA, 6 *M* urea, 0.2 *M* sucrose (final concentration, 20–25 μg/μl). Electrophoresis was performed in the NuPAGE Electrophoresis System on 10% Bis-Tris gels (1 mm thick, 2D well). Protein samples (at a volume about 300 μl) were applied to 2D sample slot and run with MES SDS running buffer containing 0.05 *M* 2-(*N*-morpholino)ethane sulphonic acid, 0.05 *M* Tris Base, 3.415 m*M* SDS and 1.025 m*M* EDTA (Novex, San Diego, CA, USA). Rainbow colored molecular mass (MW) markers, ranging from 4 to 46 kDa (Amersham, Buckinghamshire, UK), were used in each run. The electrophoresed samples were electroblotted onto Sequi-Blot PVDF membranes (Bio Rad, Hercules, CA, USA) in a Novex X Cell II blotting apparatus with a NuPAGE transfer buffer (pH 7.2) containing 25 m*M* bicine, 25 m*M* Bis-Tris and 1.025 m*M* EDTA according to the instructions of the manufacturer (Novex).

2.4. Elution of proteins from PVDF membranes

Elution of the blotted proteins was performed as described previously [12]. After the completion of the electroblotting, the PVDF membrane was rinsed with bi-distilled water, incubated in 40% methanol/1% acetic acid solution for about 5–10 min in order to remove SDS, and finally washed with water. The membrane areas containing proteins with M_r of about 4000 were excised, cut into small pieces and incubated in TFA/acetonitrile/water (3:4:3) for 30–40 min (at a volume of about 300 μl per excised membrane piece from one blot). The eluted material was dried in Speed Vac apparatus. The elution procedure was repeated two times. Notable, in case the eluted samples were prepared for the subsequent HPLC separation (described below), the elution from PVDF was carried out using the unstained membranes, and the location of separated tissue proteins was determined by the prestained molecular mass markers.

2.5. RP-HPLC

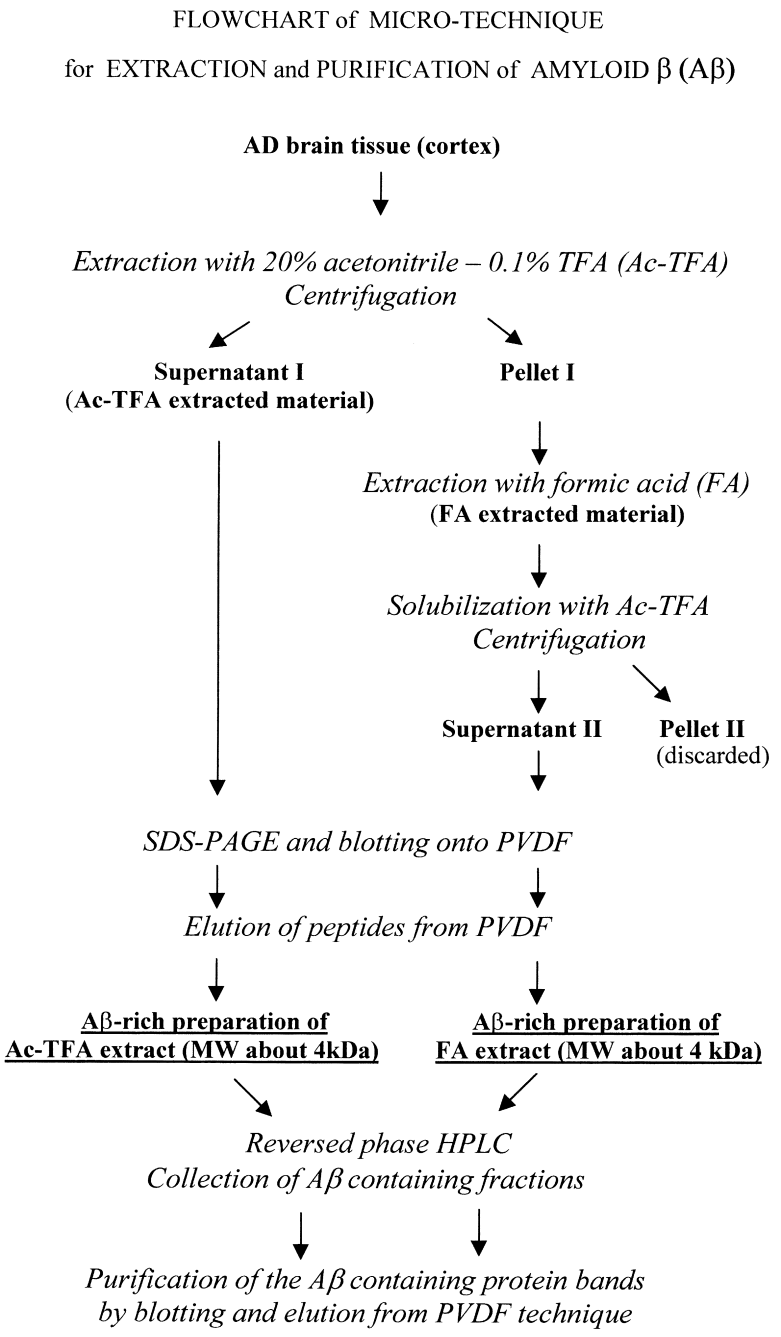
The peptides recovered from four or five PVDF blots were re-dissolved in about 100 μl formic acid and applied to a Vydac 214TP54 (Alltech, Deerfield, IL, USA) reversed-phase C_4 column (250×4.6 mm, I.D., pore diameter 300 A, particle size 5 μm). The HPLC equipment consisted of a Spectra-Physics 8700 solvent delivery system, a 8500 dynamic mixer and 8750 organizer, coupled to a Jasco Uvidec 100 IV spectrophotometer with an 8 μl cassette type cell, and a Hewlett-Packard 3390 A integrator. Isocratic elution with 20% acetonitrile in 0.1% TFA (10 min) was followed by a linear gradient from 20 to 80% acetonitrile in 0.1% TFA over 30 min, and finally by an isocratic elution with 80% acetonitrile in 0.1% TFA for an additional 10 min. A flow-rate of 1.0 ml/min was maintained. The elution of proteins was monitored by UV absorbance at 220 nm. The eluted fractions were collected and lyophilized.

2.6. Western blotting

For the immunodetection of Aβ proteins, samples were run on 10% Bis-Tris gels (1 mm thick, 10 wells) and blotted on nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) in the NuPAGE Electrophoresis System as described above. Monoclonal mouse 6E10 (Senetec, MO) recognizing the residues 1–17 of Aβ were used as primary antibodies. Horseradish peroxidase conjugated sheep F(ab′)2 fragment antimouse IgG (Amersham, Arlington, IL) was used as a secondary antibody. Aβ were visualized by enhanced chemiluminescence (ECL) Western blotting detection system (Amersham).

2.7. Protein recovery from SDS-gels

To evaluate the efficiency of amyloid transfer from SDS-gel to PVDF membrane, the samples of crude tissue extracts (Ac-TFA and FA) and the preparations of the purified Aβ (Scheme 1) were electrophoresed and blotted onto two sequentially placed PVDF membranes. After completion of the



Scheme 1.

blotting procedure, the gel was stained with 0.25% (w/v) Coomassie Brilliant Blue G250 in methanol/acetic acid/water, 5:1:4 (v/v); the membranes were stained with 0.1% Coomassie Brilliant Blue G250 in methanol/acetic acid/water, 40:1:59.

To determine the efficiency of Aβ elution from

PVDF membrane, the samples with known amounts of synthetic Aβ 1–40 peptide, and the preparations of purified Aβ were electrophoresed, blotted onto and eluted from PVDF as described above. The eluted peptides and the corresponding starting preparations were subjected to Western blot analysis. The immunostained blots were scanned as described previously [11], and the staining intensity values of eluted peptides were referenced to those of known amounts of starting amyloid material. The linear increase in staining values resulting from application of 5–40 ng of synthetic Aβ peptide was used as a basis for calculations.

3. Results

Gray matter specimens from normal and AD brains were subjected to the extraction procedure (Scheme 1) by using sequentially aqueous acetonitrile–TFA (Ac-TFA) solution and formic acid (FA). The Ac-TFA and FA soluble material was dried and re-dissolved in sample buffer containing 6% SDS and 6 *M* urea and applied to electrophoretic and Western blot analyses (Fig. 1). No differences were found in the electrophoretic patterns of proteins extracted from normal and AD brains (Fig. 1A). However, the 4-kDa bands immunoreactive with 6E10 antibodies were revealed in the Ac-TFA and FA material extracted from the AD tissues, but not from the normal brain tissues (Fig. 1B).

We found that Ac-TFA extracted material was readily soluble in the SDS and urea containing sample buffer, while the FA material was only partially solubilized resulting in considerable amounts of the insoluble residue. This insoluble FA residue is presumed to contain no Aβ, since treatment with formic acid renders Aβ soluble in SDS and urea solutions [1]. Further, we found that Aβ species insoluble in 20% acetonitrile–0.1% TFA, might be solubilized in this solvent after their pretreatment with formic acid. In fact, when the Ac-TFA insoluble material (pellet I, Scheme 1) was dissolved with formic acid, dried and then re-extracted with Ac-TFA (as described above), the solubilized material (supernatant II, Scheme 1) contained 4-kDa bands strongly immunoreactive with 6E10 antibodies (Fig. 2). In contrast, no immuno-

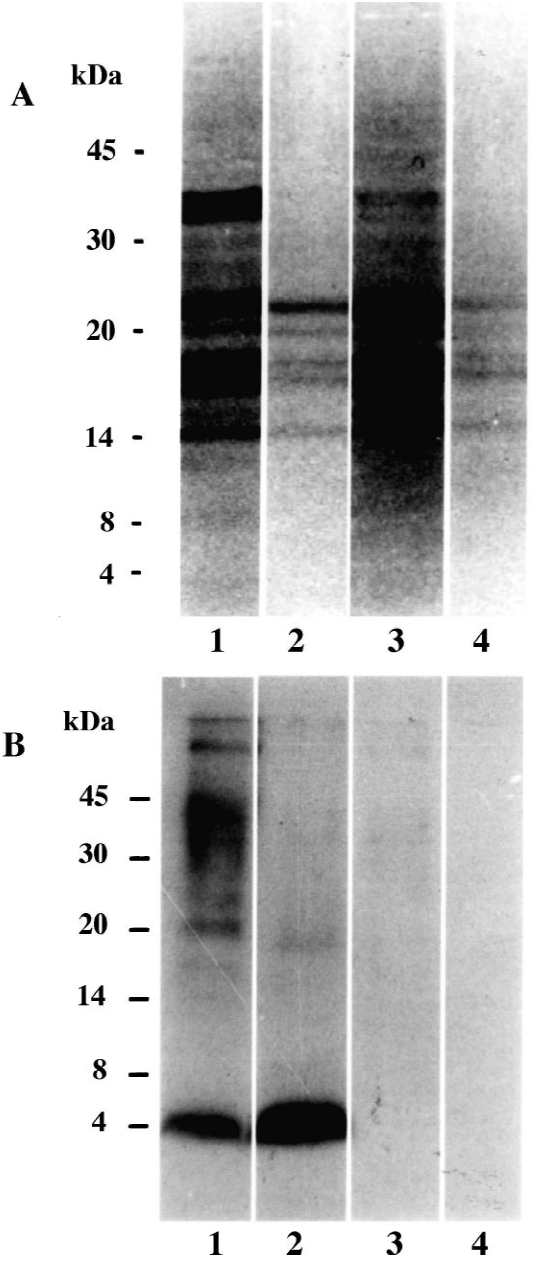


Fig. 1. SDS–PAGE (A) and Western blot (B) analyses of AD (1, 2) and normal (3, 4) tissue extracts obtained using sequentially 20% acetonitrile–0.1% TFA (1, 3) and formic acid (2, 4). Samples were run on 10% Bis-Tris polyacrylamide gels. Coomassie Blue was used for total protein staining. Immunostaining was performed with monoclonal mouse antibodies against Aβ (6E10, Senetec).

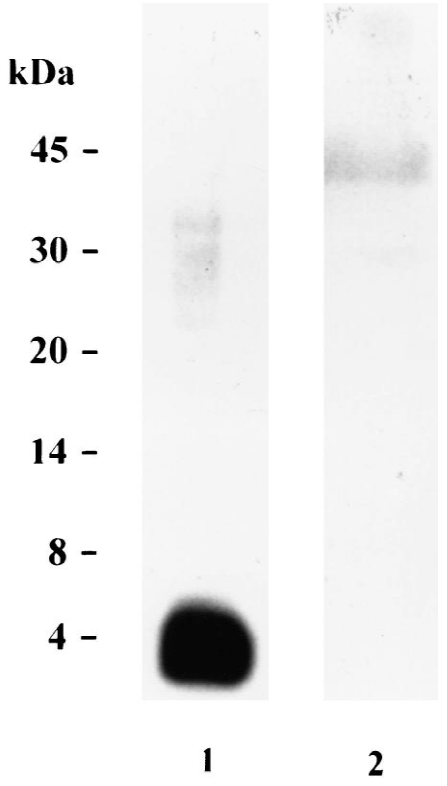


Fig. 2. Western blot analysis of the formic acid (FA) tissue extracts treated with 20% acetonitrile–0.1% TFA. FA extract (obtained from the AD brain tissue) was incubated with acetonitrile–TFA and then centrifuged, as described in text. (1) Aβ-positive tissue material soluble in acetonitrile–TFA (supernatant II, Scheme 1); (2) the insoluble residue containing no Aβ (pellet II, Scheme 1). Immunostaining was performed with monoclonal mouse antibodies against Aβ (6E10, Senetec).

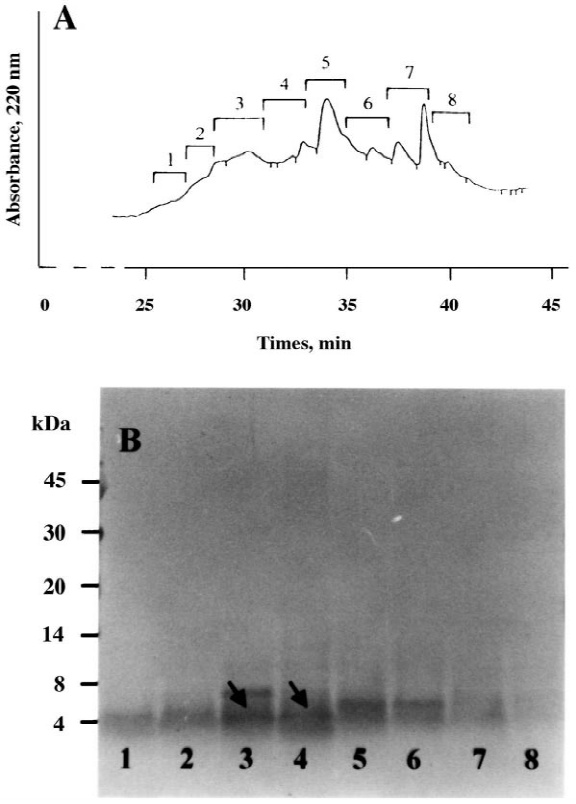


Fig. 3. RP-HPLC fractionation of the Aβ rich preparation obtained from Ac-TFA tissue extract (MW about 4 kDa). HPLC was performed on a Vydac 214 TP54 column (A). Isocratic elution with 20% acetonitrile in 0.1% TFA (10 min) was followed by a linear gradient from 20 to 80% acetonitrile in 0.1% TFA over 30 min, and finally by an isocratic elution with 80% acetonitrile in 0.1% TFA for an additional 10 min. The effluent was monitored by UV absorbance at 220 nm, sensitivity 0.53 a.u.f.s. The elution time of the unretained solute (ammonia solution), t_o=3.2, was determined and used for the calculation of the k' values. Fractions 1–8 were collected and subjected to the SDS–PAGE analysis on 10% Bis-Tris polyacrylamide gels by using Coomassie Blue staining (B). Arrows indicate protein bands (fractions 3 and 4) immunoreactive with 6E10 antibodies.

reactivity was found in the insoluble residue (pellet II, Scheme 1). Thus, solubilization of the FA extracted material by acetonitrile–TFA appears to be a useful Aβ concentration step and was therefore employed in our purification procedure (Scheme 1).

As follows from Scheme 1, the AD brain material extracted sequentially with Ac-TFA and FA was run on SDS gels and blotted; the membrane area containing the 6E10 immunoreactive peptides (M_r of about 4000) was eluted and separated by RP-HPLC (Figs. 3A and 4A). While all the HPLC fractions (peaks 1–8) contained protein bands with M_r of about 4000, Western blot analysis showed that only some of these bands were immunoreactive with 6E10 antibodies (Figs. 3B and 4B). The latter were revealed in the HPLC fractions 3 and 4 (k' range 10.36–12.20) from the Ac-TFA tissue extracts (Fig. 3) and in the fraction 5 (k' range 12.20–13.00) from the FA extracts (Fig. 4). These Aβ positive HPLC fractions were collected, electrophoresed and blotted;

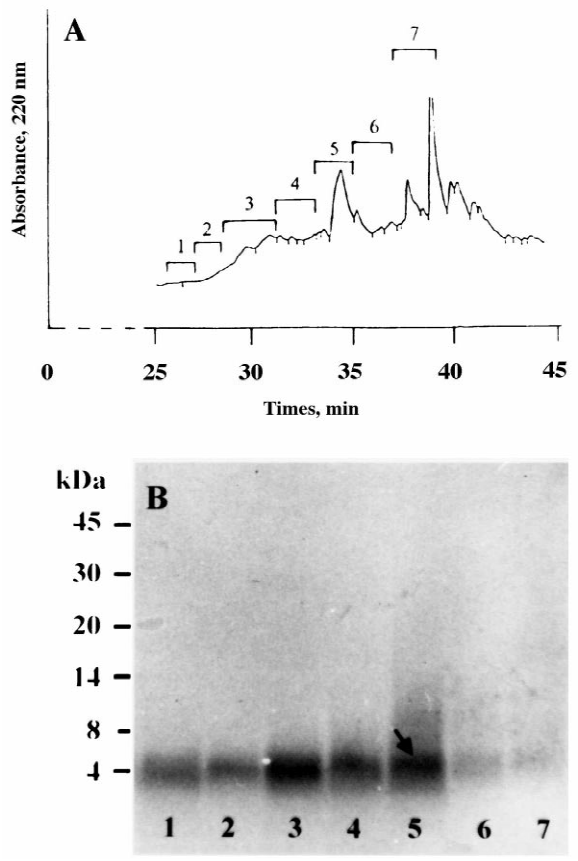


Fig. 4. RP-HPLC fractionation of the Aβ-rich preparation obtained from the FA tissue extract (MW about 4 kDa). HPLC (A) and the electrophoretic analysis of the HPLC fractions (B) was performed as described in Fig. 3. Arrow indicates protein band (fraction 5) immunoreactive with 6E10 antibodies.

the Aβ-containing membrane area was excised and eluted.

We found that the protocols used allowed highly efficient transfer of Aβ peptides from the SDS-gel to PVDF membrane, as well as the effective elution of these peptides from the membranes. The results obtained using AD samples of crude tissue extracts (Ac-TFA and FA), as well as the purified Aβ, showed that essentially all the proteins with M_r below 15 000–20 000 were completely transferred and blotted on the first membrane. Further, more than 90% of the PVDF blotted Aβ (initial amount 45 ng and higher) was recovered from the membrane following the elution of these peptides with TFA/acetonitrile/water solvent (3:4:3).

4. Discussion

Combined chromatographic and electrophoretic methods play an important role in studies of proteins and peptides, including amyloid research. In most studies, SDS–PAGE is a final separation step, which is used as an analytical tool to check the protein purity or molecular mass, or used in the preparation of a sample for microsequencing or mass spectrometry. Although SDS–PAGE is extremely efficient in achieving a high level of protein purity that would otherwise require multiple size-exclusion chromatography procedures, the latter technique is usually preferable over preparative SDS–PAGE. It might be explained by the difficulties of SDS removal from the electrophoresed proteins, which complicate subsequent fractionation of proteins by either ion-exchange, reversed-phase HPLC or hydrophobic interaction chromatography. In fact, the insufficient removal of SDS affects RP-HPLC by decreasing the resolution and retarding the elution of proteins and peptides from the column [13–15].

In our previous studies, we employed sequentially slab gel SDS-electrophoresis and RP-HPLC for purification of amyloid A (AA) [16] and for isolation of serum amyloid A (SAA) [17]. In contrast to the commonly used purification methods, the developed technique included the analytical-scale SDS–PAGE, as a first micro-preparative separation step. The electrophoretically pure proteins were eluted from the gel, purified from SDS by a specially developed chromatographic procedure, and then separated using RP-HPLC [18–20].

In our recent study [12], an alternative methodology was used to purify amyloid proteins AA, AL and ATTR: the electrophoresed proteins were blotted onto supporting membranes (PVDF), eluted from these membranes, and then fractionated by RP-HPLC. In this micro-technique, SDS is partially dissociated from the electrophoresed proteins in the course of electrotransfer from gel to the membrane; further, SDS is removed during staining/destaining of the blots [14]. We found that the recovery of amyloid proteins from Sequi-Blot PVDF membranes

(Bio Rad) was highly efficient when using TFA/acetonitrile/water (3:4:3) elution solvent. The exposure of amyloid proteins to this solvent was compatible with the subsequent separation of amyloids by RP-HPLC [12].

Our present research was focused on the development of micro-techniques providing efficient extraction of amyloid β peptides from small AD brain tissue specimens, and their further purification from other co-extracted proteins and peptides. We believe that the micro-extraction procedure used was effective for recovering essentially all the insoluble Aβ species accumulating in the AD affected brain. First, washing of tissue homogenates was carried out in absence of detergents, thus avoiding the losses of the detergent-soluble Aβ species. Second, no detectable Aβ was found in the insoluble residue following the extraction of Aβ with acetonitrile/TFA and formic acid. We found that application of slab gel SDS–PAGE, followed by blotting and elution of the 4-kDa tissue components from PVDF allowed effective concentration of Aβ containing material prior to its subsequent fractionation by RP-HPLC. Finally, the Aβ-rich material recovered from PVDF blots was fractionated by RP-HPLC. It allowed separation of Aβ from the contaminating tissue components of similar molecular masses, but different hydrophobicities. It is remarkable that Aβ species differing in their solubility properties, i.e. Ac-TFA and FA extracted amyloid β, demonstrated different retention times on the RP-HPLC column.

The developed micro-purification technique combines the resolution power of analytical SDS–PAGE with the high separation speed of HPLC. We believe that combination of the simple and inexpensive slab gel electrophoresis and the rapidly developing RP-HPLC techniques is a promising methodological approach for small-scale purification and biochemical analysis of amyloid β peptides.

Acknowledgements

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