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Isolation and Characterization of Soluble β-Amyloid and Apolipoproteins from Cerebrospinal Fluid

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An original method of isolation and purification of soluble β-amyloid and apoproteins from the cerebrospinal fluid of healthy donors is developed. The method consists of purification of high density lipoproteins by centrifugation of cerebrospinal fluid and reverse phase high-performance liquid chromatography of isolated lipoproteins. The obtained β-amyloid and apoproteins from cerebrospinal fluid are characterized immunologically and by mass-spectroscopy.

Key Words: *β-amyloid; Alzheimer disease; high density lipoproteins; cerebrospinal fluid; protein purification*

β-Amyloid (Aβ) is a major component of amyloid deposits in the cerebral tissue in Alzheimer disease, Down syndrome, and normal aging. Since 1984, when this Aβ was first discovered, and until recently this protein was regarded as pathological. However, in 1992 Aβ in a soluble form (sAβ) was detected in conditioned media of some cell strains and in the plasma and cerebrospinal fluid (CSF) not only in patients but also in healthy subjects [3,10,12]. The concentration of sAβ in the plasma is about 1 ng/ml [10], while in CSF it varies from 4 to 20 ng/ml [9,11,12].

However, simple methods of quantitative isolation of native sAβ from biological fluids so far have not been developed, and therefore synthetic analogs are used for evaluating its structural and biological properties. Isolation of sAβ from human plasma and CSF by affinity chromatography and immunoprecipitation with anti-Aβ antibodies has been reported [10,12]. However, these protocols are suitable for analytic rather than preparative purposes. Moreover, these methods require expensive and not always available antibodies. In light of this, the development of a simple method of preparative isolation of sAβ is of great importance for understanding the structure and metabolism of this protein and its role in the pathogenesis of Alzheimer disease. It was found that sAβ in the plasma and CSF is associated with high density lipoproteins (HDL) [6], which served as the basis for the development of isolation and characterization of native sAβ from CSF HDL. Apart from sAβ, various apolipoproteins were isolated from HDL. Apolipoprotein composition of CSF is little studied, therefore, we identified these apoproteins using immunoblot analysis and mass-spectroscopy.

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TABLE 1. Isolation of sAβ from CSF of Health Donors

Stage of purification	Volume, ml	Total protein	sAβ, ng	Purification grade, arb. units
Whole CSF	60	21.25 mg	—	0
Ultracentrifugation of CSF at 1.25 g/ml (isolation of HDL)	6	0.25 mg	451	100
First RP-HPLC, 30-min gradient (five fractions 4+5)	2	0.5 μg	357	50000
Second RP-HPLC, 60-min gradient (fraction 7)	0.25	—	250	100000

Note. —) below sensitivity limits of the corresponding methods.

MATERIALS AND METHODS

Cerebrospinal fluid of normolipidemic donors without dementia was obtained from Clinical Laboratory of New York Medical Center. Aβ1-40 was synthesized by solid-phase method in the Yale University, USA [7]. The following antibodies were used: monoclonal anti-Aβ (6E10, Senetek) [5], anti-apolipoprotein (apo) A-I and polyclonal anti-apoA-II, anti-apoE, anti-apoC-II, and amyloid A antiserum (Calbiochem). Reverse-phase high-performance liquid chromatography (RP-HPLC) was carried out using an C4 analytic column (5 μ, 4.6×250 mm, Vidac) in a Waters HPLC system.

HDL were isolated from CSF by preparative ultracentrifugation (45,000 rpm, 60 h, 16°C) in a Ti 50.2 rotor (Beckman) [6]. The density was adjusted to 1.25 g/ml with dry KBr (39 g/100 ml CSF). Fraction of floating HDL (1/10 of initial volume) was dialyzed against water and lyophilized. Aliquots of HDL from CSF (about 50 μg protein) were dissolved in 200 μl 20% acetonitrile containing 0.1% trifluoroacetic acid and centrifuged for 30 sec in an Eppendorf microcentrifuge at 14,000 rpm. The supernatant was applied to the column [4] and eluted using a linear gradient of 20-80% acetonitrile in 0.1% trifluoroacetic acid for 30 or 60 min at flow rate of 0.5 ml/min and a detection wavelength of 214 nm. The obtained fractions were then lyophilized. Elution profile of the synthetic peptide Aβ1-40 (5 μg) was taken as a chromatography mobility standard of Aβ. Retention factor was specified as: $k' = (t_R \times t_S)/t_S$, where t_R is the retention time or time of elution and t_S is the t_R value for unbound substance [4]. Aliquots of each chromatographic fraction were separated by Tris-tricine-SDS 14% polyacrylamide gel electrophoresis under reducing conditions (200 mM dithiothreitol). The gels were stained with Coomassie or the proteins were transferred to Immobilon-P membranes at 400 mA for 2 h in 3-(cyclohexylamino)-1-propanesulfonic acid buffer (pH 11.0, Aldrich) containing 10% methanol. The membranes were incubated in 10 mM Na_2HPO_4/150 mM NaCl, pH 7.4, containing 5% dried milk and separated with different secondary antibodies for 16 h at 4°C. Secondary antimouse or antirabbit antibodies (Amersham) were diluted 1:3000 with phosphate buffer containing 0.3% Tween-20, 0.3% dried milk, and 10% glycerin. Fluorograms were prepared using a chemiluminescence reagent (DuPont). The concentration of Aβ was determined densitometrically (PDI Systems). Protein content was measured using Bio-Rad reagent [8]. All RP-HPLC fractions and synthetic Aβ1-40 peptide were analyzed by laser mass-spectroscopy [1].

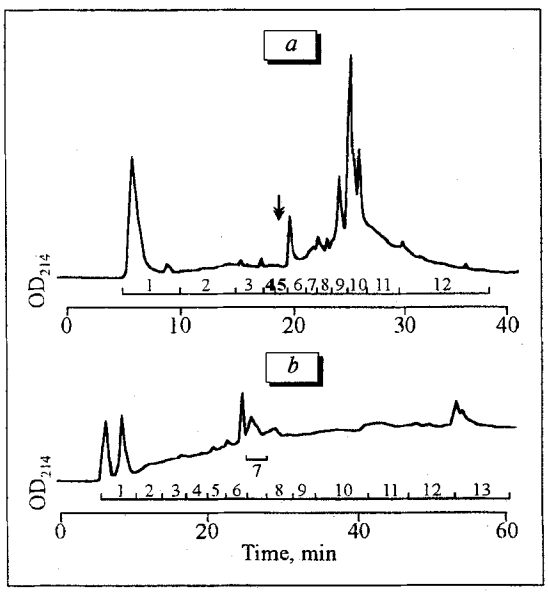

Fig. 1. Reverse-phase high-performance liquid chromatography of high density lipoproteins from cerebrospinal fluid with 30- (*a*) or 60-min (*b*) acetonitrile gradient (20-80%). Time of elution of 5 μg synthetic Aβ1-40 peptide (control) is indicated by an arrow. Fraction numbers are indicated under the curves.

RESULTS

Here we report an original, rapid, and simple method of isolation and purification of native sAβ and HDL apoproteins. Initially, an HDL fraction containing 1% total proteins and all sAβ was isolated from the

whole CSF (Table 1) [8]. Then HDL were fractionated by RP-HPLC under conditions of a 30-min acetonitrile gradient (Fig. 1, *a*). All chromatographic fractions were separated by polyacrylamide gel electrophoresis and stained with Coomassie for analyzing their protein spectrum (Fig. 2, *a*). Fractions *8-11* contained 2 main proteins corresponding to apoA-I and apoE. For specific identification of apoproteins and sAβ gel proteins were transferred to a membrane and immunocytochemically stained with different antibodies. Apoprotein-free fractions *4* and *5* contained about 80% sAβ, predominantly in the form of a monomer (Fig. 2, *b*, *4-5*; 1, *a*; *k'*=1.93-1.96). HDL apoproteins were primarily detected in fractions *8-11* (Fig. 1, *a*; *k'*=2.6-3.52). Isolated apoproteins were analyzed by immunoblot analysis and mass-spectroscopy (Table 2). Apoprotein-containing fractions *8-11* and albumin-containing fraction *6* (Fig. 1, *a*; *k'*=2.1) contained about 20% of sAβ monomer and dimer. For isolation of pure sAβ fractions *4-5* from 5 independent chromatographic runs were pooled, lyophilized, and separated by RP-HPLC under conditions of a 60-min acetonitrile gradient (Fig. 1, *b*; Table 1). Immunoblot analysis revealed the presence of the bulk of sAβ in fraction *7* (Fig. 3, *7*; 1, *b*; *k'*=2.96). This fraction was analyzed by mass-spectroscopy (Table 2); the molecular weight of sAβ strictly corresponded to a molecular weight of synthetic Aβ1-40 peptide, suggesting that sAβ1-40 is the main component of Aβ in HDL from CSF. Similarly to HDL-bound sAβ [8], sAβ isolated by us consisted only of monomers (Fig. 3, *7*). However, trace amounts of sAβ di- and monomers were also found in fractions *8-11* (Fig. 2, *b*, 3). Dimerization of sAβ in the course of RP-HPLC is probably a result of denaturation of HDL by organic solvents and impairment of physiological association between

TABLE 2. Mass-Spectroscopy of sAβ and HDL Apoproteins from CSF of Health Donors

HDL apoproteins	Experimental molecular weight, D (*n*=3)	Calculated mass, D
sAβ	4326.3±0.2	4329.8 (4326.6*)
ApoA-I	28075±4	28076
ApoA-II	17416±70	17414
ApoC-I	6635±33	6631
ApoA-III**	8704±40	8764
ApoE	34647±115	34183
AAA	11741±16	11683

Note. *n*) number of experiments; *experimental molecular weight of synthetic Aβ1-40 peptide: **apoC-II was characterized by immunoblot analysis; AAA) antiserum amyloid A.

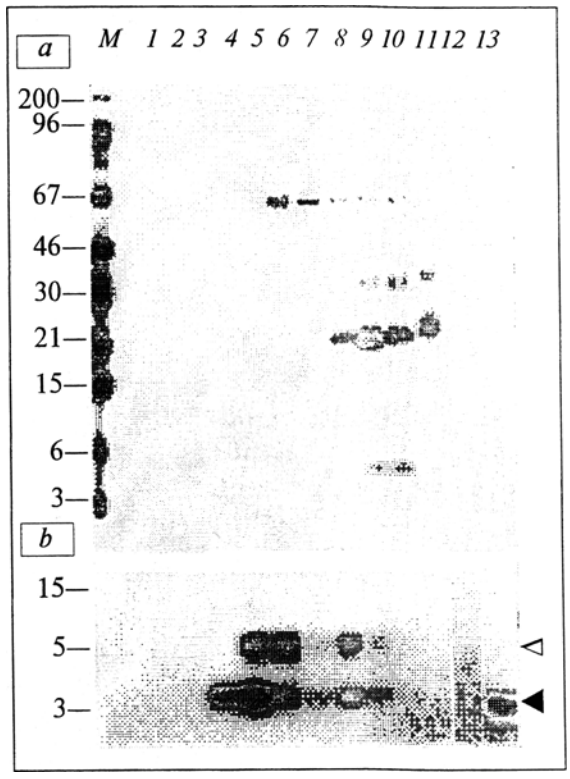

Fig. 2. Analysis of fractions obtained by reverse-phase high-performance liquid chromatography with a 30-min acetonitrile gradient (20-80%). *a*) electrophoresis of fractions *1-12* in a polyacrylamide gel. *b*) immunoblot analysis of these fractions with monoclonal anti-Aβ (6E10) antibodies. Run *13*: 25 ng synthetic Aβ1-40 peptide. Here and in Fig. 3: dark and light arrows indicates sAβ monomers and dimers, respectively. *M*: molecular weight standard, kD.

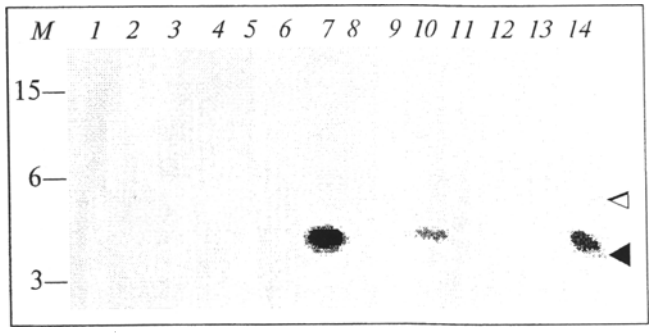

Fig. 3. Immunoblot analysis of fractions obtained by reverse-phase high-performance liquid chromatography with a 60-min acetonitrile gradient (20-80%). *1-13*: fractions; *14*: 100 ng synthetic Aβ1-40 peptide (positive control).

HDL and sAβ. This indicates that HDL integrity is important for maintenance of the sAβ conformation.

Thus, the proposed method is based on a combination of ultracentrifugation and RP-HPLC and allows rapid and effective isolation and characterization of sAβ and CSF apolipoproteins. This method can be used for preparative isolation of these proteins for a detailed study of their biological and physicochemical properties.

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