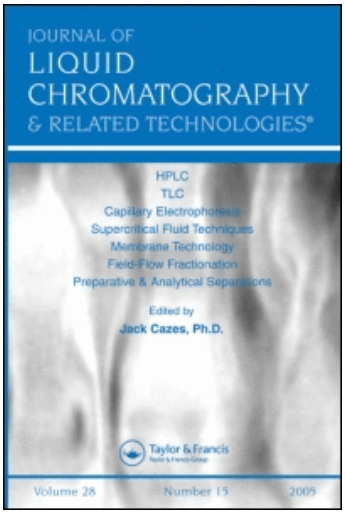

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ISOLATION AND CHARACTERIZATION OF AMYLOID PROTEINS USING MILLIGRAM AMOUNTS OF AMYLOID - CONTAINING TISSUE

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ABSTRACT

A new procedure is developed for extraction and purification of amyloid proteins from milligram amounts of amyloid - containing tissue. The procedure involves extraction of amyloid proteins with acidic aqueous acetonitrile and their purification by size-exclusion high-performance liquid chromatography (HPLC). The molecular weight and type of isolated amyloid proteins were determined by using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), followed by immunoblotting technique. The developed procedure is more rapid and requires significantly smaller amount of tissue material (about 10 mg), comparing with the conventional amyloid isolation technique performed by using gram amounts of autopsy specimen. The techniques applied presently may be useful for precise determination of amyloid type in biopsies.

INTRODUCTION

Amyloidosis comprises a heterogeneous group of diseases of diverse etiology characterized by the extracellular deposition of fibrillar proteins. Amyloid fibrils contain a

large number of proteins, proteoglycans and glycosaminoglycans (1). The major components of amyloid fibrils are low molecular weight proteins, different in their nature, that characterize the type of amyloid and relate it to different clinical forms of the disease.

In classical "primary idiopathic" and myeloma associated amyloidosis the major amyloid subunits derive from circulating monoclonal immunoglobulin light chains and comprise mainly a fragment of the variable region (AL type) (2). In reactive amyloidosis, as well as in familial Mediterranean fever (FMF), the subunit is the protein AA, which is related to acute phase protein, designated SAA (3). Major amyloid subunits related to transthyretin (TTH) were found in familial amyloidotic polyneuropathy and familial and senile amyloid cardiomyopathies (4,5). Novel forms of amyloidoses were described recently (5): cystatin C-, β-2 microglobulin-, apo AI- , apo AII-, and calcitonin - related amyloidosis.

Amyloidosis can be diagnosed by histological examination which depends on the demonstration of green birefringence of amyloid-containing tissue stained with Congo red and viewed under polarized light. However, for determination of amyloid type in amyloid - containing tissue, the chemical, immunochemical or immunohistochemical methods are needed. Immunohistochemical analysis of biopsy tissues is performed in several laboratories (6-8), but since amyloid deposits adsorb large amounts of plasma proteins, it may make immunohistochemical classification difficult (8). A

precondition for this type of diagnosis is the availability of suitable anti-amyloid antibodies, but the production of these antibodies is not an easy task (8). The most valuable information on the nature of amyloidosis is obtained by chemical and immunochemical examination of the major amyloid subunits, isolated from amyloid-containing tissues. The purified amyloid proteins may be typed immunochemically and also subjected to amino acid sequence analysis (9-13). Such studies, are usually performed by using autopsy specimens or amyloid-laden organs obtained by surgery. The techniques, commonly used for extraction (14) and purification (2,15) of amyloid proteins, require gram amounts of tissue material, which exceed significantly the amount of material available by biopsies. In this sense, the development of techniques based on microscale isolation and purification of amyloid proteins is important for exact determination of amyloid type in biopsies. Such information could be helpful to evaluate the response of treatment of patients suffering from different forms of amyloidosis.

In previous studies (16-18) we demonstrated the separation and purification of amyloid proteins by reverse phase and size-exclusion high performance liquid chromatography (HPLC), while using milligram amounts of amyloid fibrils. In the present report we describe a procedure for the extraction of amyloid proteins from milligram amounts of amyloid tissue, their purification by size-exclusion HPLC and further characterization by using immunoblotting technique.

EXPERIMENTAL

Chemicals and materials

Acetonitrile (Bio-Lab, Jerusalem, Israel) was of HPLC grade. Sodium dodecyl sulphate (SDS) and Amido black were obtained from BDH (Poole, UK). Trifluoroacetic acid (TFA), acrylamide, bromophenol blue, Coomassie brillant blue R (Coomassie blue), 2-mercaptoethanol, N,N,N',N' - tetramethylenediamine (TEMED) and a kit of SDS molecular mass markers (M_r=14300-66000) were purchased from Sigma (St. Louis, MO, USA). Rabbit antisera to λ-light chains, to κ-light chains and to prealbumin (transthyretin) were obtained from Behringwerke AG (Marburg, Germany). Goat anti-rabbit IgG peroxidase conjugate and 4-chloro-1-naphthol were purchased from Sigma. Protein AA and rabbit antisera to human protein AA were obtained in our laboratory by the techniques described earlier (2,14,15). The amyloid-containing tissues were obtained at autopsy from 11 patients with AA, AL and TTR amyloidosis (Table 1). Sections of all tissues were stained intensely with Congo red and showed characteristic green birefringence when examined under polarized light. The main amyloid proteins were isolated from these tissues in our laboratory by using conventional methods described elsewhere (2,14,15). Some of the amyloid proteins were characterized by amino acid sequence analysis and identified as AA proteins (COH, NOR, SIM and GAM) (9,11,12), AL proteins (GAB) (10) and ATTR protein (SSA) (13). The other amyloid proteins were supposed to represent AL proteins (RAM, SHOY, ADI, TESL, NAT)

TABLE 1

List of Amyloids Studied

Patient	Diagnosis	Organ	Amyloid protein and references
COH	FMF	Spleen	AA (12)
NOR	FMF	Thyroid	AA (11,12)
SIM	Reactive	Spleen	AA (12)
GAM	Idiopathic amyloidosis	Liver	AA (9,12)
RAM	Primary idiopathic amyloidosis	Spleen	AL (supposed)
GAB	Primary idiopathic amyloidosis	Liver	AL (10)
SHOY	Primary idiopathic amyloidosis	Spleen	AL (supposed)
ADI	Primary idiopathic amyloidosis	Spleen	AL (supposed)
TESL	Primary idiopathic amyloidosis	Spleen	AL (supposed)
NAT	multiple myeloma	Spleen	AL (supposed)
SSA	systemic senile amyloidosis	Heart	ATTR (13)

on the basis of the clinical manifestation of the disease and the electrophoretic analysis of the proteins.

Extraction of amyloid proteins

The samples of amyloid-containing tissues, ≤ 1.7mmx5mm (5-10mg, wet weight), were dispersed in 1.0-1.5 ml saline and incubated overnight at room temperature with a moderate shaking. The samples were centrifuged using Eppendorf centrifuge (Hamburg, Germany) for 10 min at 14000 r.p.m. The supernatant was discarded, the sediment was redispersed in saline and centrifuged. The obtained sediment was washed with distilled water and homogenized in 0.7-1.0 ml aqueous 20% acetonitrile solution containing 0.1% TFA. The obtained mixture was incubated at room temperature for several hours with a moderate shaking and centrifuged (Eppendorf centrifuge, 10 min, 14000 r.p.m.). The supernatant was collected and lyophilized. The sediment was redispersed in 20% acetonitrile solution (0.1% TFA) and the procedure was repeated two more times.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE (19) was performed on 17% polyacrylamide slab gels using mini Protean II vertical electrophoresis system (Bio-Rad Lab., Richmond, CA, USA). The samples were dissolved in 0.0625M Tris-HCl (pH 6.8) sample buffer containing 3% SDS, 5% 2-mercaptoethanol and 10% glycerol.

HPLC

The HPLC equipment consisted of Spectra-Physics 8700 solvent delivery system, 8500 dynamic mixer and 8750 organizer, coupled to a Jasco Uvidec 100-IV spectrophotometer with an 8µl cassette type cell (10mm pathway), a Hewlett-Packard 3390 A integrator and LKB 2211 Superrack fraction collector. Proteins were separated on a Bio-Sil TSK-125 (5µm) (Bio-Rad Lab.) column (600mmx7.5mm I.D.) and on TSK-gel G 3000 SW_{XL} (TosoHaas, Stuttgart, Germany) column (300mmx7.8mm I.D.). Two different eluant systems were used: (a) aqueous 20% acetonitrile in 0.1% TFA and (b) 0.05 M Tris-HCl buffer, pH 6.8, containing 0.1% SDS. The amyloid material extracted from the amyloid-containing tissue (as described above) was solubilized, correspondingly, in a) 200-300 µl aqueous 20% acetonitrile (0.1% TFA) and b) a sample buffer (50-70 µl) used for SDS-PAGE (see above). In the latter case the solubilized sample was diluted with the eluant (b), 1:3, vol/vol. The samples, prepared this way, were centrifuged (Eppendorf, 10 min, 14000 r.p.m.) and the obtained supernatants were filtered by using microfilterfuge tubes (Rainin, MA, USA) prior to their application on HPLC column.

Immunoblotting of amyloid proteins

The samples of amyloid proteins were run by SDS-PAGE (as described above). For the Western blots the proteins were electrotransffered from the gel to nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany) in Bio-Rad Trans-Blot electrophoretic transfer cell by using 25mM Tris/192 mM

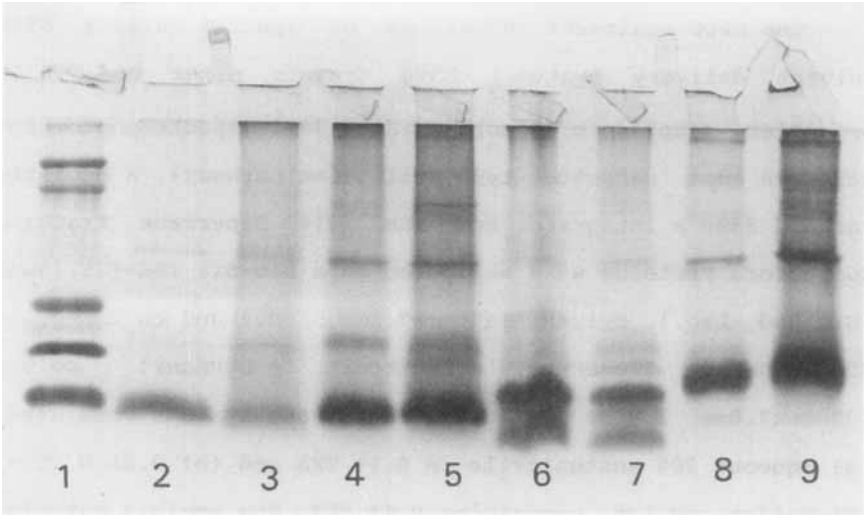

Figure 1. SDS-PAGE of amyloid proteins extracted from amyloid-containing tissues (1) molecular weight markers from top to bottom: bovine serum albumin (67000), ovalbumin (43000), β-lactoglobulin (17200), lysosyme (14600) and protein AA (9000); (2), (4), (6) and (8) amyloid fibrils isolated by the conventional procedure from samples GAM (2), COH (4), NOR (6) and RAM (8); (3), (5), (7) and (9) amyloid proteins extracted by the procedure described presently from samples GAM (3), CO (5), NOR (7) and RAM (9).

glycine buffer (pH 8.3)/20% vol/vol methanol (20). Unbound sites were blocked with 1% bovine serum albumin (BSA) in Tris-buffered saline (pH 7.5) containing 0.05% Tween -20.

The blots were stained with Amido Black stain. Rabbit antisera to λ-light chains, antisera to κ-light chains, antisera to prealbumin (transthyretin) and antisera to human protein AA were used as the first antibodies. Goat anti-rabbit IgG peroxidase conjugate was used as the second

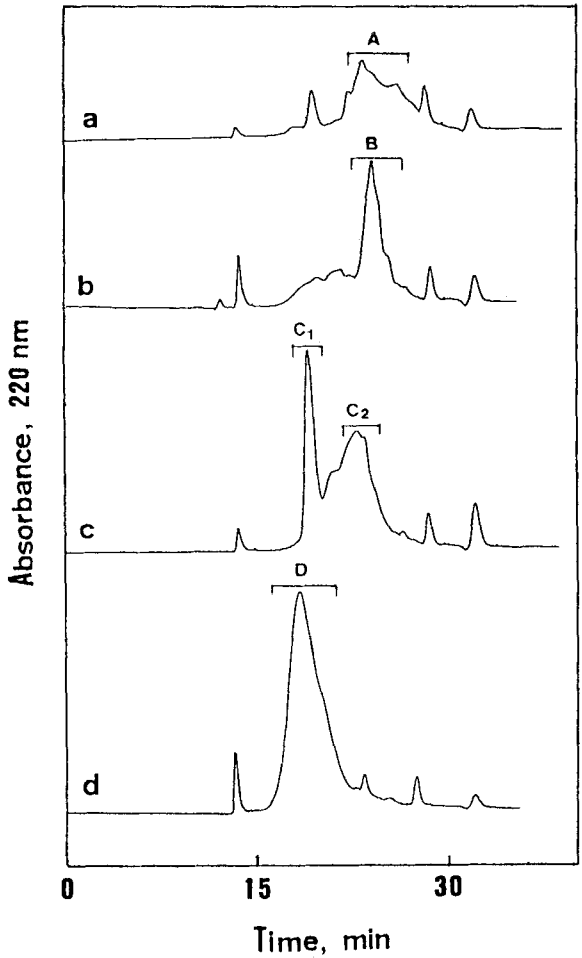

Figure 2. HPLC of the extracted amyloid proteins COH (a), GAM (b), NOR (c) and RAM (d) on Bio-Sil TSK-125 column in aqueous 20% acetonitrile (0.1% TFA) mobile phase. A flow-rate at 1.0 ml/min was used. The effluent was monitored by measuring the UV absorbance at 220nm, sensitivity 0.53 a.u.f.s.

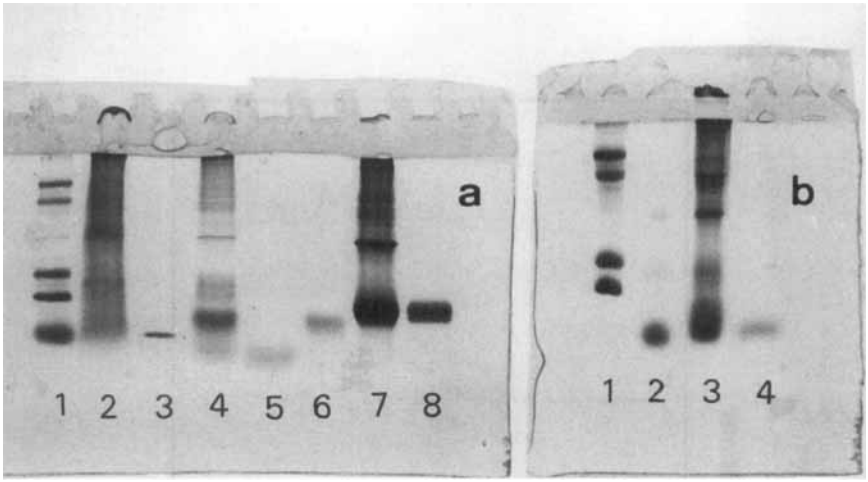

Figure 3. SDS-PAGE of major amyloid subunits isolated by HPLC in acidic aqueous acetonitrile (Fig. 2). A: (1) molecular weight markers (9000-67000); (2) amyloid proteins extracted from sample GAM; (3) HPLC fraction B (GAM) (Fig. 2b); (4) amyloid proteins extracted from sample NOR; (5) HPLC fraction C_1 (NOR) (Fig. 2c); (6) HPLC fraction C_2 (NOR) (Fig. 2c); (7) HPLC fraction D (RAM) (Fig. 2d). B: (1) molecular weight markers (14600-67000); (2) protein AA (CO) isolated by convetional procedure, mol. weight 9000; (3) amyloid proteins extracted from sample COH; (4) HPLC fraction A (COH) (Fig. 2a).

antibody. Immunoreactive bands were visualized by colour development with 4-chloro-1-naphtol and hydrogen peroxide.

RESULTS

Autopsy specimens, ~ 1.7mmx5mm (5-11mg wet weight) were applied to the extraction procedure, as described above. The amount of the extracted material ranged from 0.5 to 0.8 mg (dry weight). The electrophoregramms of the extracted proteins were similar to those of amyloid fibrils obtained

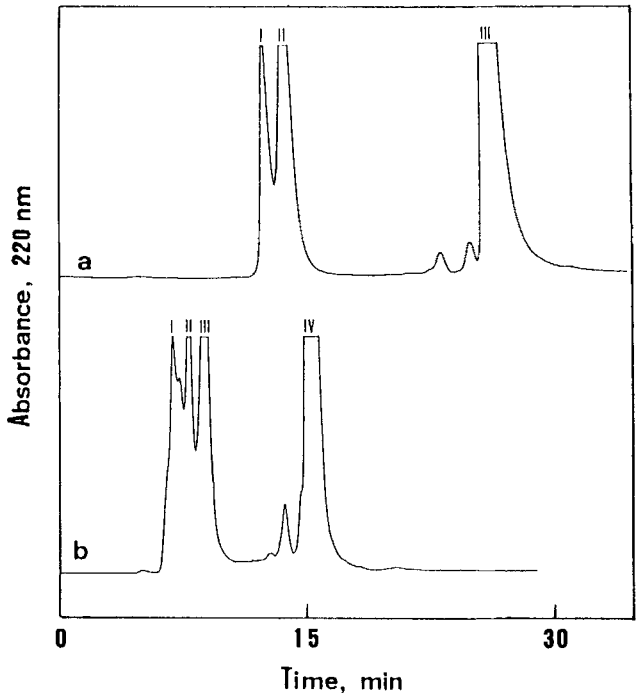

Figure 4. Typical HPLC profiles, obtained by separation of amyloid proteins on Bio-Sil TSK-125 (a) and TSK-Gel G 3000 (b) columns. Mobile phase: 0.05M Tris-HCl buffer (pH 6.8) containing 0.1% SDS. A flow-rate at 1.0 ml/min was used. The effluent was monitored by measuring the UV absorbance at 220nm, sensitivity 0.9 a.u.f.s.

conventionally from the corresponding autopsy specimens (Fig. 1).

Fig. 2 demonstrated HPLC separation of the extracted amyloid proteins using 20% acetonitrile (0.1% TFA) as eluant. The most prominent peaks A (COH), B (GAM), C_1 and C_2 (NOR) and D (RAM) contained electrophoretically pure major amyloid subunits (Fig. 3). Fig. 4 demonstrates HPLC profiles

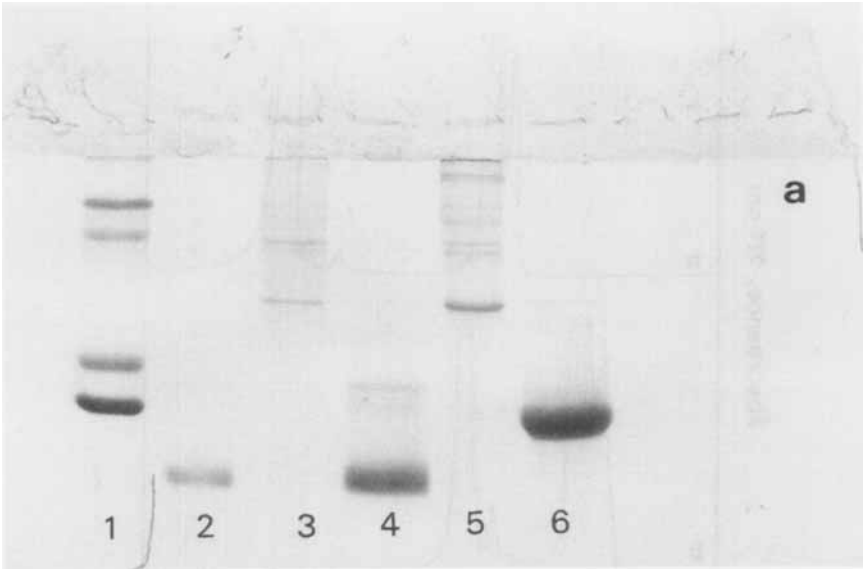

Figure 5. SDS-PAGE of amyloid proteins separated on Bio-Sil TSK-125 column (Fig. 4a). Mobile pahse: 0.05M Tris-HCl buffer (pH 6.8) containing 0.1% SDS.
A) (1) molecular weight markers (14600-67000); (2) protein AA (COH) isolated conventionally, mol. weight 9000; HPLC fractions (3) I (COH), (4) II (COH), (5) I (RAM) and (6) II (RAM).
B) (1) and (8) molecular weight markers (14600-67000); HPLC fractions (2) I (GAM), (3) II (GAM), (4) III (GAM), (5) I (NOR), (6) II (NOR) and (7) III (NOR).
C) (1) molecular weight markers (14600-67000); (2) amyloid proteins extracted from sample SHOY; HPLC fractions (3) I (SHOY), (4) II (SHOY) (5) III (SHOY); (6) amyloid proteins extracted from sample ADI; HPLC fractions (7) I (ADI), (8) II (ADI).

obtained by elution of amyloid proteins COH (4a) and TESL (4b) with 0.05M Tris HCl buffer (pH 6.8) containing 0.1% SDS. Very similar HPLC profiles (not shown in the picture) were obtained by the separation of all other amyloid samples. SDS-PAGE of the collected amyloid fractions is shown in Fig. 5 and 6. The major low molecular weight amyloid subunits were

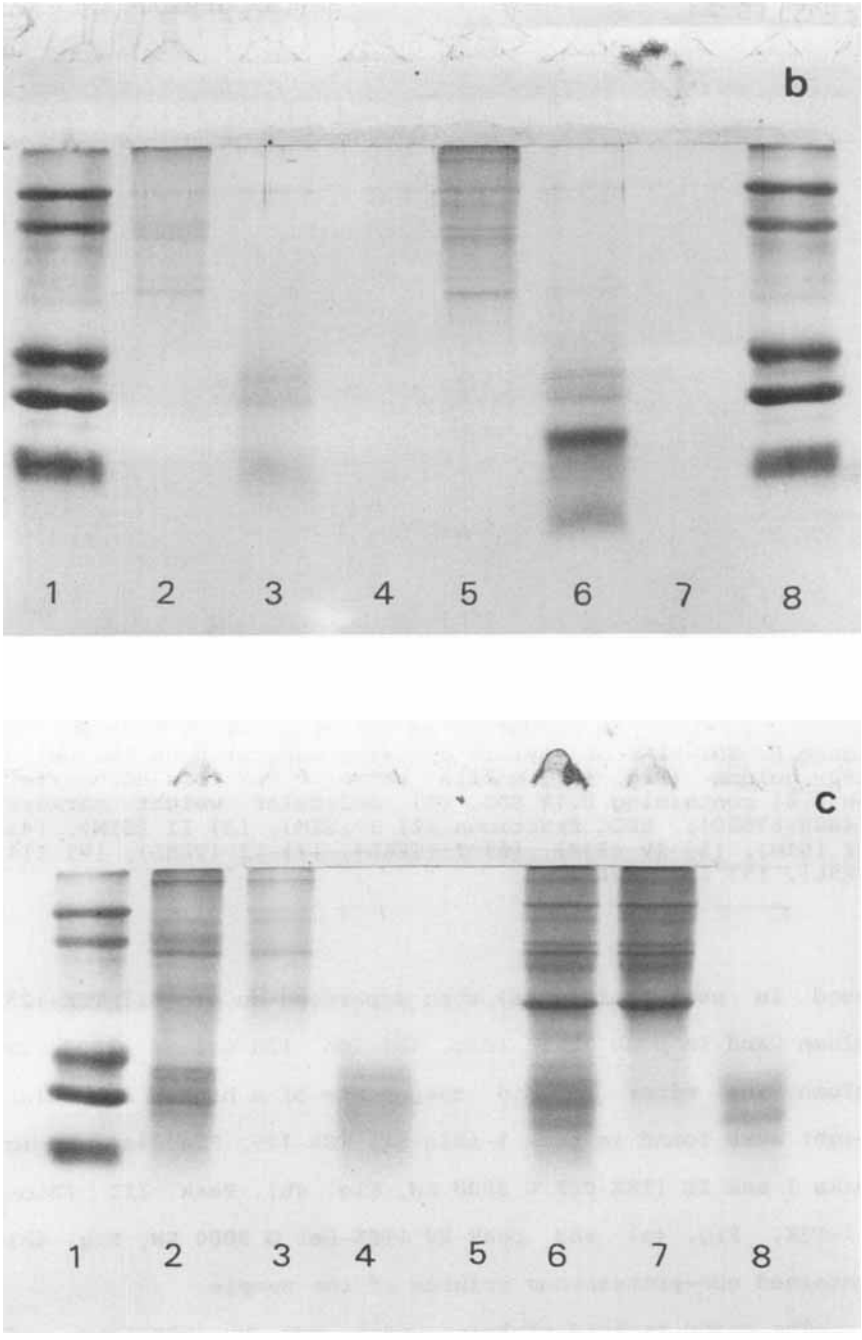

Figure 5 (continued)

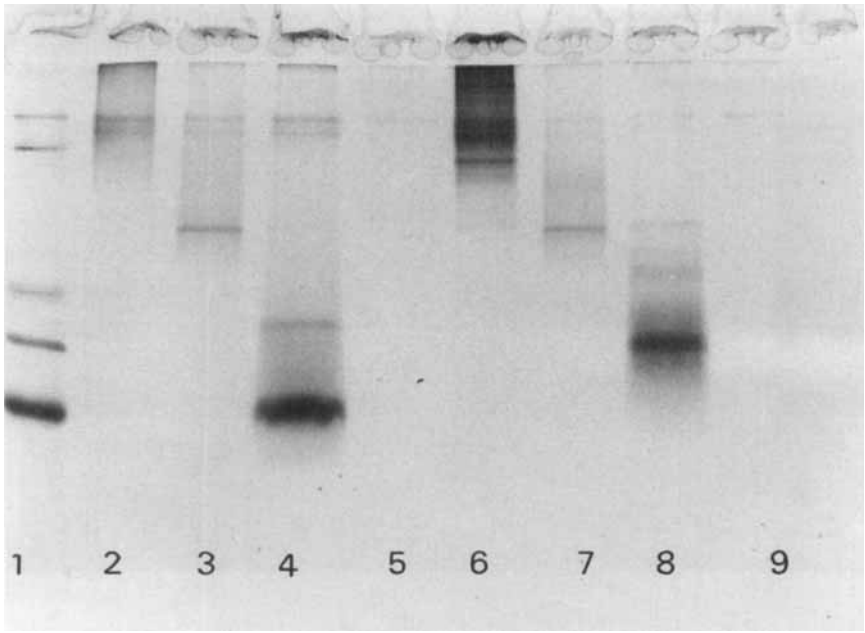

Figure 6. SDS-PAGE of amyloid proteins separated on TSK-Gel G 3000 column (Fig. 4b). Mobile phase: 0.05M Tris-HCl buffer (pH 6.8) containing 0.1% SDS. (1) molecular weight markers (14600-67000); HPLC fractions (2) I (SIM), (3) II (SIM), (4) III (SIM), (5) IV (SIM), (6) I (TESL), (7) II (TESL), (8) III (TESL), (9) IV (TESL).

found in peak II (Fig 4a) when separated on Bio-Sil TSK-125 column and in peak III (Fig. 4b) on TSK-Gel G 3000 SW column. The minor amyloid components of a higher molecular weight were found in peak I (Bio-Sil TSK-125, Fig. 4a) and in peaks I and II (TSK-Gel G 3000 SW, Fig. 4b). Peak III (Bio-Sil-TSK, Fig. 4a) and peak IV (TSK-Gel G 3000 SW, Fig. 4b) contained non-proteineous solutes of the sample.

The major amyloid proteins were run by SDS-PAGE and blotted. The amyloid proteins of samples COH, GAM, SIM (mol.

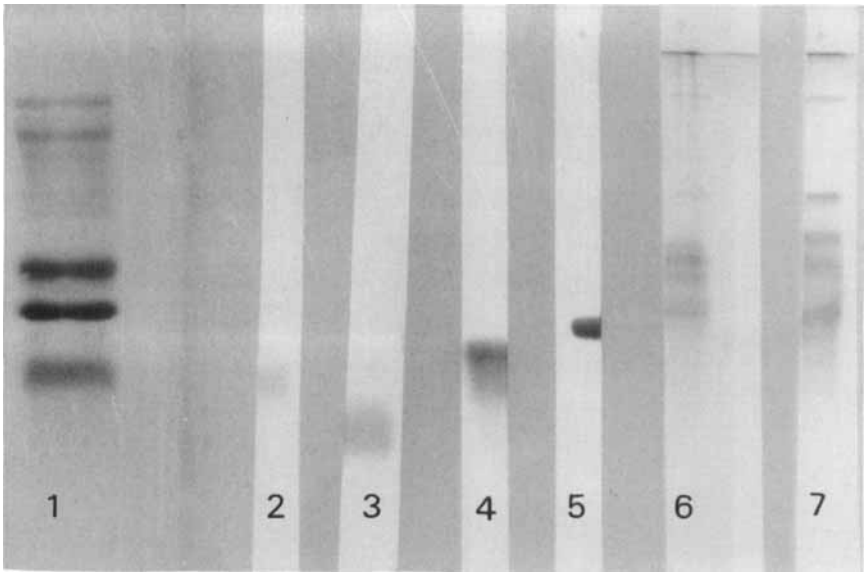

Figure 7. Immunoblots of major amyloid subunits purified by HPLC. (1) molecular weight markers (9000-67000) (blot stained with Amido black); HPLC fractions (2) B (GAM) (Fig. 3A, track 3), (3) C_1 (NOR) (Fig. 3A, track 4), (4) C_2 (NOR) (Fig. 3A, track 5), (5) II (RAM) (Fig. 5A, track 6), (6) II (SHOY) (Fig. 5C, track 4) and (7) II (ADI) (Fig. 5C, track 8). Immunoreactive bands were visualized using as the first antibodies rabbit antisera to human proteins AA (2-4) and antisera to λ - light chains (5-7).

weight about 9000) and NOR (5000 and 10000) were immunoreactive with rabbit antisera to human amyloid protein A; no immunoreactivity was found with rabbit antisera to λ- and to κ - light chains, and with antisera to prealbumin. The major amyloid proteins of samples RAM (about 11000), NAT (about 14000), SHOY, ADI and TESL (from about 14000 to 18000) were immunoreactive only when using rabbit antisera to λ - light chains (Fig. 7). The major amyloid proteins of sample

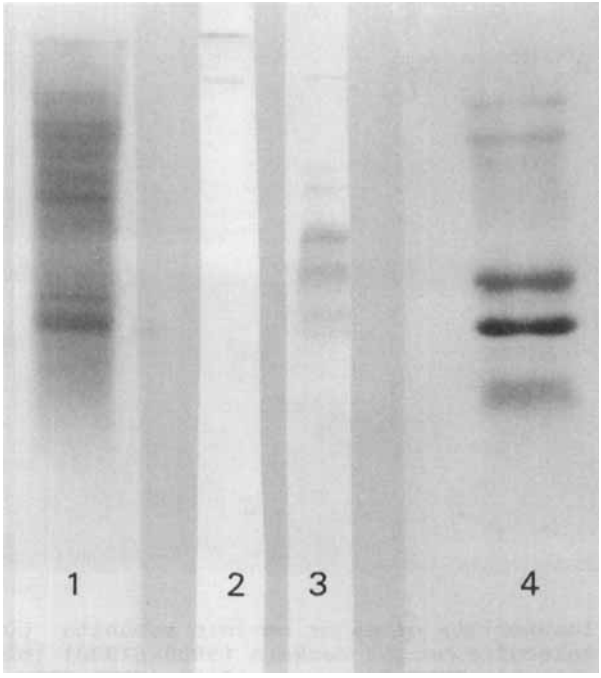

Figure 8. Immunoblots of major amyloid subunits of sample GAB. (1) amyloid proteins extracted from amyloid tissue GAB (blot stained with Amido black); major amyloid subunits (GAB) (purified by HPLC) were blotted and assayed with (2) rabbit antisera to λ-light chains and (3) κ-light chains; (4) molecular weight markers (9000-67000) (blot stained with Amido black).

GAB (from about 14000 to 23000) were immunoreactive only with rabbit antisera to κ-light chains (Fig. 8). Some of the minor amyloid proteins of a higher molecular weight (incompletely separated from the major low molecular weight proteins) were reactive with antisera to λ-, as well as to κ - light chains. The major amyloid protein of sample SSA (about 14000) was immunoreactive only with antisera to prealbumin.

DISCUSSION

The procedure conventionally used for isolation of amyloid proteins (9-15) involves: 1) extraction of amyloid fibrils from amyloid-laden organs with distilled water, 2) solubilization of the fibrils in 5M guanidine hydrochloride containing buffer and 3) separation of amyloid proteins on Sephadex or Sepharose columns. These techniques have been successfully applied for the isolation of many amyloid proteins from amyloid-containing tissues obtained at autopsy. However, this is a time - consuming procedure, which requires gram amounts tissue material and therefore can not be applied for examination of biopsy specimens.

A procedure developed presently includes extraction of amyloid proteins from amyloid tissues with acidic aqueous acetonitrile solution and their purification by size-exclusion HPLC. The electrophoretic and immunochemical characteristics of the isolated amyloid proteins corresponded to those obtained by using conventional amyloid extraction and purification procedure (9-13). Separation of amyloid proteins on Bio-Sil TSK-125 column in aqueous 20% acetonitrile (0.1% TFA) (Fig. 2) allowed to obtain the highly purified proteins AA (COH, GAM, NOR and SIM, mol. weight 5000-10000), as well as protein AL (RAM, mol. weight about 11000). However, the application of this technique was not effective for isolation of the larger amyloid proteins (mol. weight $\geq$ 14000, proteins AL of samples ADI, TESL, NAT, GAB and SHOY and protein ATTR of sample SSA). In our previous study we found that the larger proteins may be retained on

Bio-Sil TSK-125 column longer than the smaller proteins, when using aqueous organic mobile phase; the detection and recovery of high molecular weight amyloid components was complicated (18). Contrary to that, the major amyloid subunits of all amyloid samples studied presently were isolated on Bio-Sil TSK-125, as well as on TSK-Gel G 3000 SW columns when using SDS containing buffer, as a mobile phase (Fig. 4-6). The electrophoretic purity of the isolated proteins AA, however, was higher, when performing HPLC on Bio-Sil TSK-125 column with aqueous organic eluant (Fig. 2 and 3).

The procedure applied presently for extraction and purification of amyloid proteins is more rapid than the conventional technique (2,14,15) and may be performed by using several milligrams of tissue specimen. This amount is comparable with the amount of tissue taken by needle biopsy. In a preliminary study, the amyloid proteins were isolated from a rectal biopsy specimen (in a case of primary idiopathic amyloidosis) and typed immunochemically by using the procedure developed presently. This procedure is suggested to be utilized for the study of needle biopsies in order to determine accuratly the type of amyloidosis in patients during life.

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