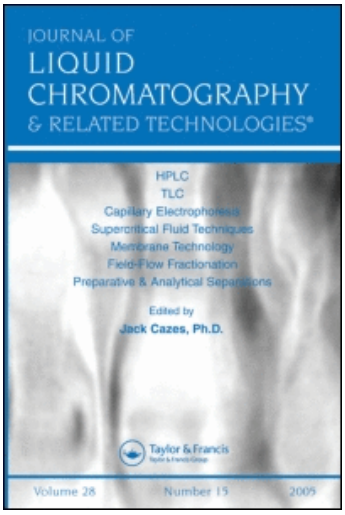

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Separation of Amyloid Proteins by Size-Exclusion Chromatography in Aqueous Organic Mobile Phase

B. Kaplan[a]; M. Pras[a]
[a] Heller Institute of Medical Research, Chaim Sheba Medical Center, -hashomer, Israel

To cite this Article Kaplan, B. and Pras, M.(1992) 'Separation of Amyloid Proteins by Size-Exclusion Chromatography in Aqueous Organic Mobile Phase', Journal of Liquid Chromatography & Related Technologies, 15: 14, 2467 — 2486
To link to this Article: DOI: 10.1080/10826079208017196
URL: http://dx.doi.org/10.1080/10826079208017196

JOURNAL OF LIQUID CHROMATOGRAPHY, 15(14), 2467-2486 (1992)

SEPARATION OF AMYLOID PROTEINS BY SIZE-EXCLUSION CHROMATOGRAPHY IN AQUEOUS ORGANIC MOBILE PHASE

B. KAPLAN AND M. PRAS
Heller Institute of Medical Research
Chaim Sheba Medical Center
52621, Tel-Hashomer, Israel

ABSTRACT

Separation of amyloid proteins was performed by size - exclusion chromatography using acidic aqueous 20% acetonitrile, as a volatile mobile phase. Pure major amyloid constituents (proteins AA and AL) were isolated on a Fractogel TSK HW-55 and a Bio-Sil TSK-125 columns. The isolation procedure used is simpler and more rapid comparing with the traditional separation of amyloid proteins (size - exclusion chromatography on Sephadex type columns in 5M guanidine - HCl). The technique described allows to avoid the use of strong denaturants and may be applied for HPLC separation of amyloid proteins on a microgram scale.

INTRODUCTION

Amyloid fibrils are complex biological compounds containing a large number of proteins, proteoglycans and glycosamines (1). The major components of amyloid fibrils are low molecular weight proteins; marked differences in their nature were revealed in the different clinical forms of amyloidosis. In reactive amyloidosis as well as in familial

Mediterranean fever (FMF) the major amyloid subunit is the protein AA, which is related to acute phase reactant, designated SAA (2). In idiopathic and myeloma associated amyloidosis the major subunits (proteins AL) derive from circulating monoclonal immunoglobulin light chain and comprise mainly a fragment of the variable region (3). Major amyloid subunits related to transthyretin (proteins AP) have been found in familial amyloidotic polyneuropathy (4).

The development of effective methods for isolation and characterization of major amyloid subunits is important for the understanding of amyloidogenesis and may contribute to more precise diagnostics of the disease. The traditionally used method of isolation and purification of amyloid proteins is size-exclusion chromatography (3,5). Since amyloid fibrils are not soluble in aqueous buffers, the denaturants, such as urea or guanidine-HCl, are used for their solubilization and fractionation; most researchers use Sephadex type columns (2-5). However, this is a time-consuming procedure: Sephadex columns cannot withstand high pressures and the elution of proteins is slow (2-4 days). The long-term dialysis (3-4 days) is also needed in order to remove the denaturants from the eluted proteins. In addition, Sephadex deteriorates in the presence of solutions containing guanidine-HCl (6-9). Finally, this procedure is not always effective, especially for separation of proteins of a similar molecular weight.

In our previous search for more rapid and effective amyloid separation techniques we found that amyloid proteins are soluble in an acidic aqueous acetonitrile and, thus, may

be fractionated by reversed phase high performance liquid chromatography (HPLC) (10). Highly purified proteins AA were obtained by using consecutively sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and reversed phase HPLC (9). The present study demonstrates a simpler and more rapid isolation of major amyloid proteins on size - exclusion columns by using acidic aqueous 20% acetonitrile, as a volatile mobile phase. This technique alows to avoid the use of strong denaturants and detergents (urea, guanidine-HCl or SDS) and may be applied for amyloid separation on a microgram scale.

EXPERIMENTAL

Chemicals and materials

Acetonitrile (Bio-Lab, Jerusalem, Israel) was HPLC grade. Trifluoroacetic acid (TFA), acrylamide, bromophenol blue, Coomassie brilliant blue R, 2-mercaptoethanol, N,N,N',N' - tetramethylenediamine, a kit of SDS molecular weight markers (M_r 14300-66000), insulin and myoglobin were purchased from Sigma (Poole, UK). Amyloid fibrils were obtained in our laboratory according to the method of Pras et al (11) from liver and spleen of patients CO and GAM (FMF), RAM (idiopathic amyloidosis), and MARZ (amyloid arthropathy). Protein AA was obtained in our laboratory (12) by the technique described earlier (3,5).

HPLC equipment

The HPLC equipment consisted of a Spectra-Physics 8700 solvent delivery system, 8500 dynamic mixer and 8750

organizer, coupled to a Jasco Uvidec 100-IV spectrophotometer with an 8 µL cassette-type cell (10 mm pathway), a Hewlett-Packard 3390 A integrator and a LKB 2211 Superrack fraction collector. Proteins were separated on a Bio-Sil TSK-125 (5 µm) (Bio-Rad Lab., Richmond, CA, USA) size-exclusion HPLC column (600 mm x 7.5 mm I.D.). A Vydac 218TP (5 µm) (Alltech, Deerfield, IL, USA) column (250 mm x 4.6 mm I.D.) was used for reversed phase HPLC.

Separation on a Fractogel TSK HW-55 column

The samples of amyloid fibrils (35-70 mg) were suspended in 5-9 ml of aqueous 20% acetonitrile solution containing 0.1% TFA, and stirred gently for 0.5-2h at room temperature. The mixtures were centrifuged with an Eppendorf 5415 centrifuge (Hamburg, Germany) at 13700g for 10 min. The obtained supernatants were lyophilized, redissolved in 2-3 ml of aqueous 20% acetonitrile (0.1% TFA) and applied to the Fractogel TSK HW-55 (Merck, Darmstadt, Germany) column (920 mm x 20 mm I.D.), equilibrated with the same solvent. A flow-rate of 0.3 ml/min was maintained. The elution of proteins was monitored by UV absorbance at 220 or 280 nm. The fractions of eluted proteins were pooled and lyophilized.

The samples of standard proteins (1.7-3 mg/ml) were chromatographed in the same manner. Total permeation volume was determined by the elution volume of riboflavin.

Separation on a Bio-Sil TSK-125 HPLC column

Amyloid fibrils were solubilized in an aqueous 20% acetonitrile solution containing 0.1%. TFA (6-8 mg/ml) as

described above. The mixtures were centrifuged, and the obtained supernatant (60-100 µl) was applied to Bio-Sil TSK-125 column, equilibrated with the same solvent. A flow-rate of 1.0 ml/min was maintained. The elution of proteins was monitored by UV absorbance at 220 nm. The fractions of eluted proteins were pooled and lyophilized.

The samples of protein standards (0.5-5 mg/ml) were chromatographed in the same manner. Total permeation volume was determined by the elution time of TFA.

Reversed phase HPLC

Reversed phase HPLC was performed on a Vydac 218TP column using a linear gradient from 20 to 47.5% acetonitrile in 0.1% TFA (6 min) followed by a linear increase of acetonitrile concentration to 75% (0.1% TFA) over 84 min (9). A flow-rate of 1.0 ml/min was maintained. The elution of proteins was monitored by UV absorbance at 220 nm.

SDS-PAGE

The identity and purity of the proteins eluted from the columns was checked by SDS-PAGE according to Laemmly (13) on 17% polyacrylamide gels.

RESULTS

Separation of protein standards on a Fractogel TSK HW-55 column in aqueous 20% acetonitrile (0.1% TFA) is shown in Figure 1 (top). The eluted proteins were identified

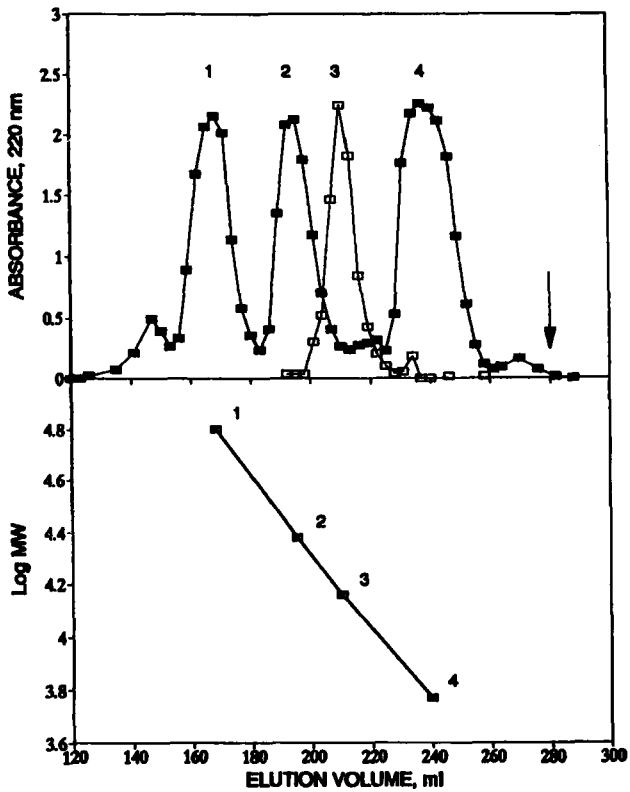

Figure 1. Size-exclusion chromatography of protein standards on a Fractogel TSK HW-55 (920 mm x 20 mm I.D.) column; mobile phase, aqueous 20% acetonitrile in 0.1% TFA, flow-rate 0.3ml/min. Top: elution profiles of a mixture of (1) bovine serum albumin, (2) tripsinogen and (4) insulin (—■—) and of (3) lysozyme (—□—). Bottom: plot of log molecular weight versus retention time of the protein standards. Arrow denotes the total permeation volume of the column.

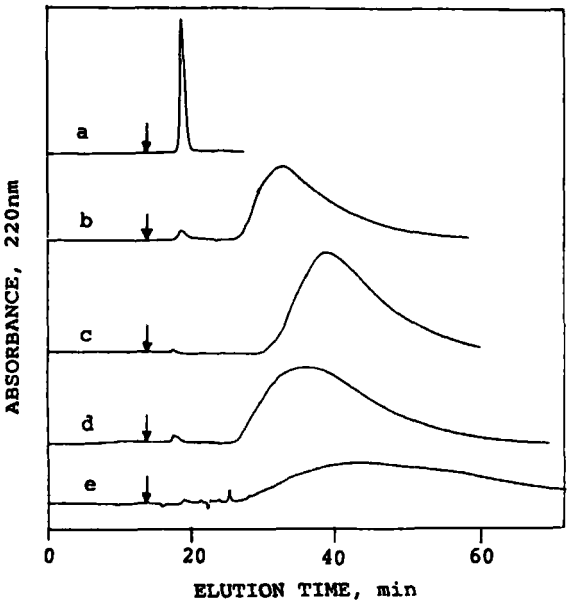

Figure 2. Elution profiles of protein standards obtained on a Bio-Sil TSK-125 (600 mm x 7.5 mm I.D.) HPLC column; mobile phase, aqueous 20% acetonitrile in 0.1% TFA, flow-rate 1.0 ml/min. (a) insulin, (b) ribonuclease, (c) lysozyme, (d) β-lactoglobulin and (e) myoglobin. Arrow denotes the elution time for the total permeation volume of the column.

electrophoretically; their recovery corresponded to 93-98% of the initial protein amount applied to the column. The linear character of log MW versus protein elution time plot was obtained using this column (Figure 1, bottom).

Figure 2 demonstrates the chromatographic profiles of protein standards, separated on a Bio-Sil TSK-125 column in aqueous 20% acetonitrile (0.1% TFA). The deviation from size-exclusion mechanism was observed. Insulin, the smallest

protein, was eluted first, while the larger proteins emerging as very broad peaks were eluted later. The identities of the eluted proteins were confirmed by their electrophoretic analysis. All these proteins were retained on Bio-Sil TSK-125 column longer than the elution time for the total permeation volume of this column (denoted by arrow).

Amyloid fibrils were partially solubilized in aqueous 20% acetonitrile containing 0.1% TFA (40-50% of their initial amount). The solubilized samples (CO, GAM and RAM) were separated on Fractogel TSK HW-55 (Figure 3) and Bio-Sil TSK-125 (Figure 4) columns. Amyloid fractions obtained using Fractogel TSK HW-55 column contained a large number of protein subunits of a different molecular weight, ranging from high M_r (protein bands localized at the top to the gel) to low M_r values (about 9000) (Figure 5). Amyloid fractions obtained using Bio-Sil TSK-125 HPLC column, contained low molecular weight amyloid components (M_r < 11000) (Figure 5 (track 9), 6 (tracks 2 and 5), and 7 (tracks 4-8)). The major amyloid constituents, representing the low molecular weight proteins, were effectively purified when using Fractogel TSK HW-55, as well as Bio-Sil TSK-125 columns (Figures 5-7). The electrophoretic mobility of the major proteins obtained from samples CO and GAM corresponded to that of protein AA, M_r about 9000 (Figure 6). The major subunit constituent of amyloid RAM is a protein of M_r about 11000 (Figure 5).

Separation of sample MARZ on Bio-Sil TSK-125 column is shown in Figure 8 (chromatogram A). The obtained fractions I-

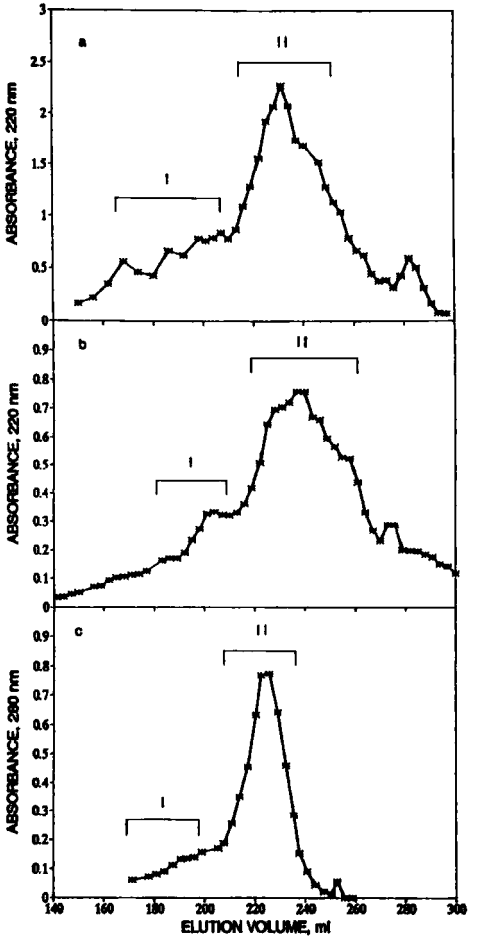

Figure 3. Size-exclusion chromatography of amyloid samples on a Fractogel TSK HW-55 (920 mm x 20 mm I.D.) column; mobile phase, aqueous 20% acetonitrile in 0.1% TFA, flow-rate, 0.3 ml/min. The samples (a) CO (40 mg), (b) GAM (32 mg) and RAM (72 mg) were solubilized in the same solvent (as described in EXPERIMENTAL); the dissolved amyloid material was applied to the column.

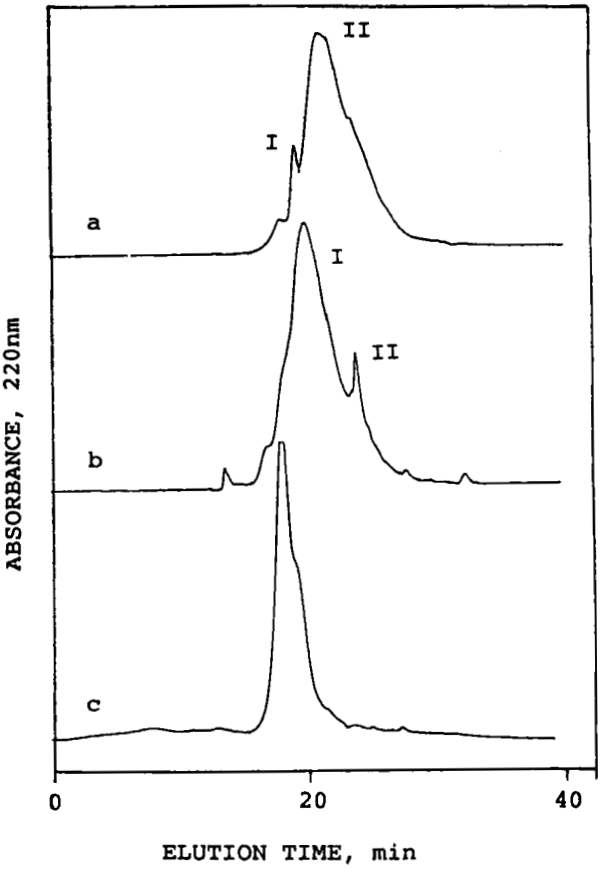

Figure 4. Elution profiles of amyloid samples obtained on a Bio-Sil TSK-125 (600 mm x 7.5 mm I.D.) HPLC column mobile phase, aqueous 20% acetonitrile in 0.1% TFA, flow-rate 1.0 ml/min. The samples (a) CO, (b) GAM and (c) RAM were solubilized in the same solvent (6-8 mg amyloid fibrils / ml); 60-100 µl of the dissolved amyloid material was injected.

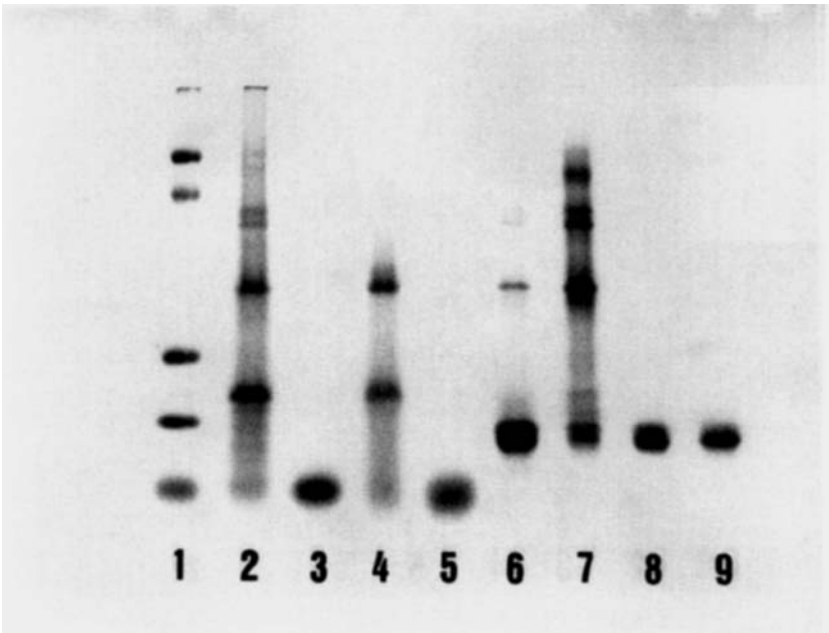

Figure 5. SDS-PAGE of amyloid components separated on Fractogel TSK HW-55 and Bio-Sil TSK-125 columns, (1) from top to bottom: molecular weight markers, M_r 67000, 43000, 17200, 14600, 9000 (bovine serum albumin, ovalbumin, β-lactoglobulin, lysozyme and protein AA, (2) sample CO, fraction I (Fig. 3a), (3) sample CO, fraction II (Fig. 3a), (4) sample GAM, fraction I (Fig. 3b), (5) sample GAM, fraction II (Fig. 3b), (6) sample RAM material dissolved in aqueous 20% acetonitrile (0.1% TFA), (7) sample RAM, fraction I (Fig. 3c), (8) sample RAM, fraction II (Fig. 3c), (9) sample RAM, HPLC fraction (Fig. 4c).

V were rechromatographed and analysed by SDS-PAGE (Figure 9). Only fraction III contained a single electrophoretically pure protein, M_r about 11000; other fractions were insufficiently resolved and contained two or more protein subunits (M_r $\lesssim$ 11000). Figure 8 demonstrates the elution

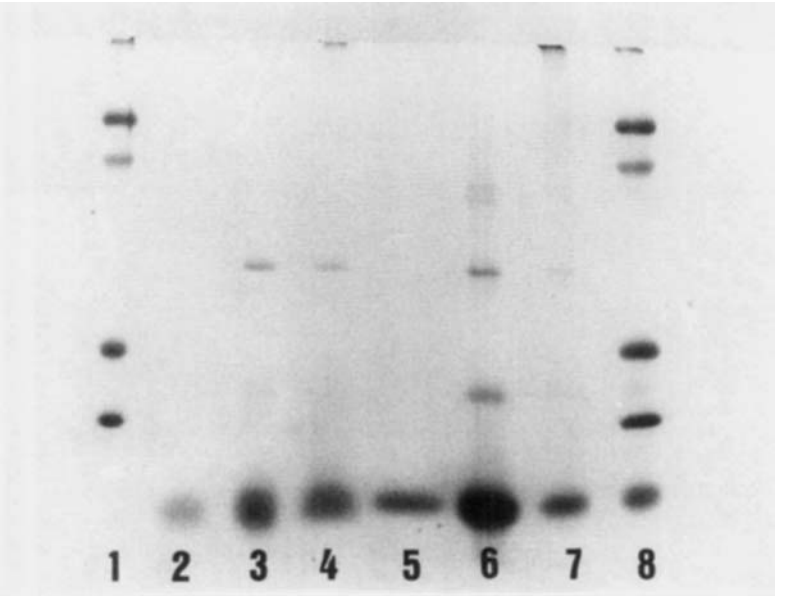

Figure 6. SDS-PAGE of major amyloid components (samples CO and GAM) isolated on Bio-Sil TSK-125 HPLC column. (1) from top to bottom: bovine serum albumin, ovalbumin, β-lactoglobulin, and lysozyme, (2) sample GAM, HPLC fraction I (Fig. 4b), (3) sample GAM material dissolved in aqueous 20% acetonitrile (0.1% TFA), (4) sample GAM (before solubilization), (5) sample CO, HPLC fraction II (Fig. 4a), (6) sample CO material dissolved in aqueous 20% acetonitrile (0.1% TFA), (7) sample CO (before solubilization), (8) from top to bottom: bovine serum albumin, ovalbumin, β-lactoglobulin, lysozyme and protein AA.

profiles of fractions II (chromatogram BII) and III (chromatogram BIII) obtained by reversed phase HPLC. The major amyloid subunits (peak 2, k' = 4.97, and peak 3, k'= 5.24, chromatogram BII) were effectively resolved; these proteins varied only slightly in their molecular weight (Figure 10). Peak 1 found in chromatograms BII and BIII

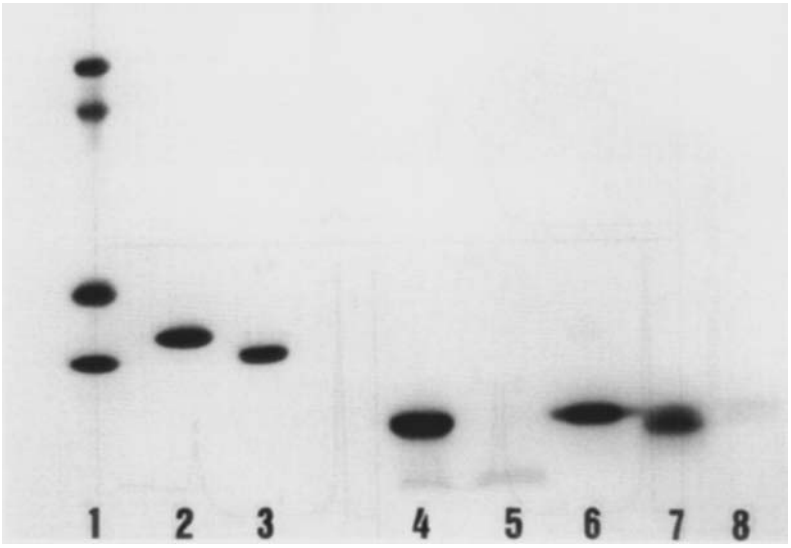

Figure 7. Electrophoretic demonstration of minor low molecular amyloid components (samples CO and GAM), separated on size-exclusion Bio-Sil TSK-125 HPLC column. (1) from top to bottom: bovine serum albumin, ovalbumin, β-lactoglobulin, lysozyme, (2) ribonuclease, (3) lysozyme, (4) sample CO, fractions I+II (Fig. 4a), (5) sample CO, fraction I (Fig. 4a), (6) sample CO, fraction II (fig. 4a), (7) sample GAM, HPLC fractions I+II (Fig. 4b), (8) sample GAM, fraction II (Fig. 46).

(Figure 8) corresponded to non-proteinous impurities of the samples (Figure 10, tracks 2 and 5).

DISCUSSION

Separation of proteins and peptides by an ideal size-exclusion mechanism occur only in absence of ionic or hydrophobic interactions between the macromolecules and matrix. These interactions may by minimized by adjusting pH,

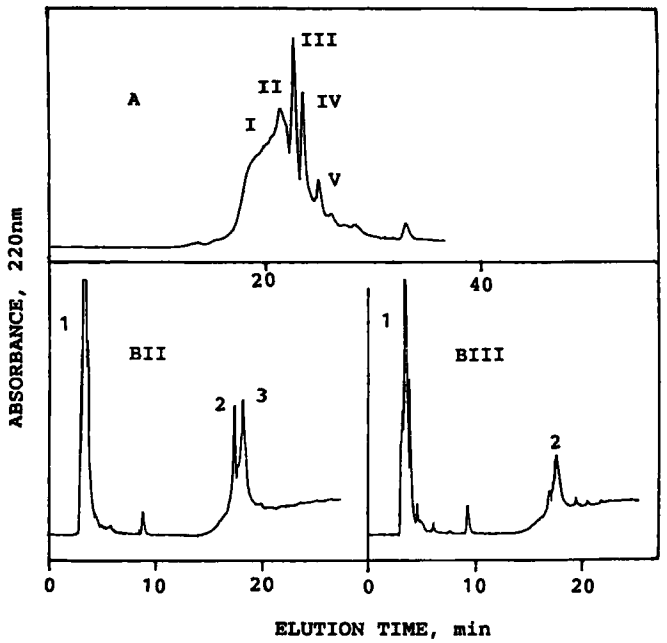

Figure 8. Separation of amyloid sample MARZ using consecutively size-exclusion (Bio-Sil TSK-125) and reversed phase (Vydac 218TP) HPLC columns. Amyloid sample was solubilized in aqueous 20% acetonitrile containing 0.1% TFA (11 mg/ml) and separated on Bio-Sil TSK-125 column, equilibrated with the same solvent (chromatogram A). The obtained fractions II and III were separated by reversed phase HPLC (chromatograms BII and BIII, correspondingly); a linear gradient from 20 to 47.5% acetonitrile in 0.1% TFA (6 min) was followed by a linear increase of acetonitrile concentration to 75% (0.1% TFA) over 84 min.

addition of salts or denaturants to the mobile phase (14,15). As a rule, these compounds must be removed from the isolated proteins or peptides, that requires additional and sometimes complicated manipulations.

Search for more effective mobile phase systems reached a new phase with the development of new mechanically and

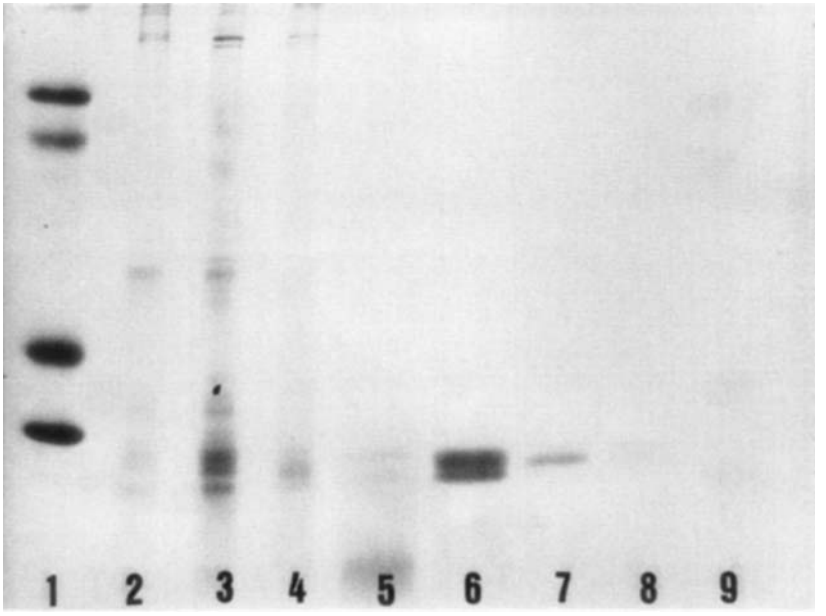

Figure 9. SDS-PAGE of amyloid components (sample MARZ) obtained on size-exclusion HPLC column (Bio-Sil TSK-125). (1) from top to bottom: bovine serum albumin, ovalbumin, β-lactoglobulin, lysozyme, (2) sample MARZ, (3 and 4) sample MARZ material dissolved in aqueous 20% acetonitrile (0.1% TFA), (5) sample MARZ, fraction I (Fig. 8A), (6) fraction II (Fig. 8A), (7) fraction IV (Fig 8A), (8) fraction 4 (Fig. 8A, (9) fraction V (Fig. 8A).

chemically stable chromatographic media designed both for open-column chromatography and HPLC. Several researchers showed the utility of adding organic solvents (acetonitrile, trifluoroethanol) to 0.1% TFA for separation of proteins and peptides (15-18). This volatile mobile phase system was relatively transparent to short wavelength light, decreased non specific hydrophobic adsorbtion of peptides on matrix and increased the overall solubility of proteins. These features

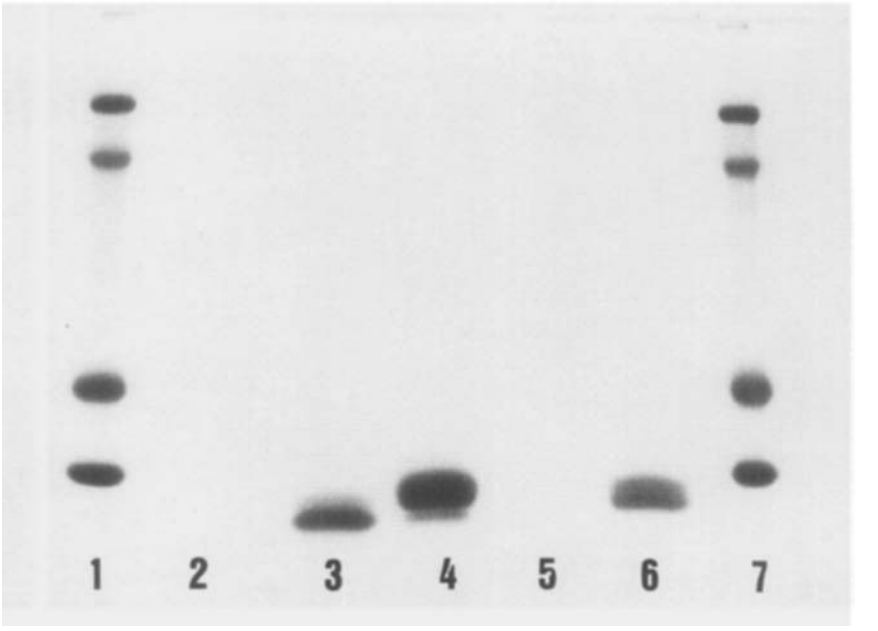

Figure 10. SDS-PAGE of main amyloid components (sample MARZ), isolated using consecutively size-exclusion and reversed phase HPLC columns. (1 and 7) from top to bottom: bovine serum albumin, ovalbumin, β-lactoglobulin, lysozyme, (2) sample MARZ fraction 1 (Fig. 8 BII), (3) fraction 2 (Fig. 8 BII), (4) fraction 3, (Fig. 8 BII), (5) fraction 1 (Fig. 8 BIII), (6) fraction 2 (Fig. 8 BIII).

make such a mobile phase more versatile than that containing detergents or high salt concentrations. Linear relationship between log MW of protein/peptide standards and their retention time was observed using volatile organic solvents and silica-based Waters PAC 1-125 column (14). Similar results were obtained using silica-based TSK G 3000 (18), TSK G 3000 PW (15,16) and agarose-based Superose 12 (19) columns. In other studies, however, the hydrophobic peptides showed

non-ideal behaviour on size-exclusion chromatography (TSK G 3000 SW) in 0.1% aqueous TFA containing 50% acetonitrile (17). Separation of peptides on a silica based SynChropak GPC 60 column also exhibited non-ideal size-exclusion mechanism when using 0.1% aqueous TFA, containing 35 or 60% acetonitrile (20). Nevertheless, it was stated that non-ideal size-exclusion behaviour may even be adventageous for effective separation of peptides (20).

In the present study an ideal size-exclusion behaviour of protein standards was demonstrated using Fractogel TSK HW-55 in aqueous 20% acetonitrile (0.1% TFA) (Figure 1). Amyloid proteins were also separated according to size-exclusion mechanism on this column (Figures 3 and 5). Contrary to that, both protein standards and amyloid proteins deviate from ideal size-exclusion behaviour on Bio-Sil TSK-125 column (Figures 2,4 and 8A). Obviously, the interactions (ionic and/or hydrophobic) between proteins and the support of this column are not suppressed when using aqueous 20% acetonitrile (0.1% TFA), as a mobile phase. In fact, an ideal size-exclusion behaviour of protein standards was obtained on the same column when using the mobile phase recommended by manufacturer (0.05 M Na_2CO_4-0.02 M Na H_2PO_4, pH 6.8).

Despite the non-ideal size-exclusion mechanism demonstrated on Bio-Sil TSK-125 column, the effective separation of major amyloid components may be obtained. Moreover, the minor low molecular weight components (which usually are not detected by the traditional amyloid separation method (3,5)) were revealed by the present

technique (Figure 7). However, the use of Bio-Sil TSK-125 column and the aqueous organic mobile phase was not effective for the separation of the larger amyloid proteins (MW>11000). These proteins are obviously strongly retained on Bio-Sil TSK-125 column and were not detected even applying the very prolonged elution times. It is also possible, that the detection of the relatively small amounts of high molecular weight amyloid components is complicated due to zone broadening effect (observed in separation of the higher molecular weight protein standards (Figure 2)).

For separation of amyloid proteins of a very similar molecular weight the use of different separation modes may be promising. Since the mobile phase used presently in size-exclusion chromatography is one of the most popular solvents in reversed phase HPLC, these two methods may be combined in a very simple way. In fact, a consecutive use of size-exclusion and reversed phase HPLC columns allowed simple, rapid and effective separation of amyloid proteins (Figure 8) which varied only slightly in their molecular weight (Figure 10).

Finally, most reports on size-exclusion chromatography in aqueous organic mobile phase describe separation of standard proteins or peptides (14-20); the possibility to separate complex biological mixtures by this technique is less studied. The present report shows the utility of this technique for isolation of pure major amyloid constituents from a large number of protein subunits comprising amyloid fibrils. The isolated proteins demonstrated the same

electrophoretic mobility as the major amyloid components obtained by a traditional method from the corresponding amyloid samples (CO (12) and GAM (21) (protein AA), MARZ (22) (protein AL) and RAM (protein AL, unpublished data)). The presently used isolation procedure is simpler and more rapid comparing with the traditional method (3,5) and is performed in absence of strong denaturants (urea, guanidine-HCl or SDS). A microgram scale separation of amyloids on Bio-Sil TSK-125 column (HPLC) is performed during 25-30 min. The use of a volatile mobile phase (aqueous 20% acetonitrile in 0.1% TFA) allows to avoid the time-consuming dialysis, which is needed in the traditional amyloid isolation procedure.

REFERENCES

1. R. Kisilevsky, "Common elements in amyloidosis", in Amyloid and Amyloidosis, J.B. Natvig, Q. Forre, G. Husby, A. Huseekk, B. Skogen, K. Sletten, P.Westermark, eds., Cluwer Acad. Publsh. London, 1990, pp. 251-253.

2. M. Levin, E.C. Franklin, B. Frangione, M. Pras, Clin. Investig., 51, 2773-2776 (1972).

3. G.G. Glenner, W. Terry, M. Harada, C. Isersky, D. Page, Science, 172: 1150-1511 (1971).

4. M. Pras, F. Prelli, E.C. Franklin, B. Frangione, Proc. Natl. Acad. Sci. USA, 80: 539-542 (1983).

5. M. Pras, B. Frangione, E.C. Franklin, "Isolated giant hepatomegaly - a clinical marker of AL-kappa amyloidosis", in Amyloid and Amyloidosis, G.G. Glener, P.P. E Costa, A.F. De Freitas, eds., Excerpta Medica, Amsterdam - Oxford - Princenton, 1980, p.p. 249-252.

6. K.D. Ganchev, God. Vissh. Khim-Tekhnol. Inst. Sofia, 20: 295-302 (1972).

7. A.A. Ansari, R.G. Mage, J. Chromatogr., 140: 98-102 (1977).

8. D. Mahuran, J. Chromatogr., 172: 394-396 (1979).

9. B. Kaplan, M. Pras, M. Ravid, J. Chromatogr., 573: 17-22 (1992).

10. B. Kaplan, M. Pras, Clin. Chim. Acta, 163: 199-205 (1987).

11. M. Pras, M. Schubert, D. Zucker-Franklin, A. Rimon, E.C. Franklin, J. Clin. Invest., 47: 924-933 (1968).

12. M. Pras, J. Gafni, E.C. Franklin, "Deviations from expected patterns in AA-amyloidosis", in Amyloid and Amyloidosis, G.G. Glenner, P.P.E Costa, A.A. De Freitas, eds., Excerpta Medica, Amsterdam-Oxford-Princenton, 1980, p.p. 211-214.

13. U.K. Laemmly, Nature, 227: 680-682 (1970).

14. J.E. Rivier, J. Chromatogr., 202: 211-222 (1980).

15. G.D. Swergold, C.S. Rubin, Anal. Biochem., 131: 295-300 (1983).

16. G.D. Swergold, D.M. Rosen, C.S. Rubin, J. Biol. Chem., 257: 4207-4215 (1982).

17. A.K. Taneja, S.Y.M. Lau, R.S. Hodges, J. Chromatogr., 317: 1-10 (1984).

18. S.Y.M. Lau, A.K. Taneja, R.S. Hodges, J. Chromatogr., 317: 129-140 (1984).

19. R.G. Feick, J.A. Shiozawa, Anal. Biochem., 187: 205-211 (1990).

20. C.T. Mant, J.M.R. Parker, R.S. Hodges, J. Chromatogr., 397: 99-112 (1987).

21. M. Pras, J. Zaretzky, E.C. Frangione, Am. J. Med., 68: 291-294 (1980).

22. M. Pras, M. Itzchaki, F. Prelli, L. Dollberg, B. Frangione, Clin. Exp. Rheum. 3: 327-331 (1985).