Published in final edited form as:
Methods Mol Biol. 2012 ; 849: 213–223. doi:10.1007/978-1-61779-551-0_15.

Search for Amyloid-Binding Proteins by Affinity Chromatography

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Abstract

'Amyloid binging proteins' is a generic term used to designate proteins that interact with different forms of amyloidogenic peptides or proteins and that, as a result, may modulate their physiological and pathological functions by altering solubility, transport, clearance, degradation, and fibril formation. We describe a simple affinity chromatography protocol to isolate and characterize amyloid-binding proteins based on the use of sequential elution steps that may provide further information on the type of binding interaction. As an example, we depict the application of this protocol to the study of Alzheimer's amyloid β (Aβ) peptide-binding proteins derived from human plasma. Biochemical analysis of the proteins eluted under different conditions identified serum amyloid P component (SAP) and apolipoprotein J (clusterin) as the main plasma Aβ-binding proteins while various apolipoproteins (apoA-IV, apoE, and apoA-I), as well as albumin (HSA) and fibulin were identified as minor contributors.

Keywords

Affinity chromatography; Alzheimer disease; Amyloid peptides; Amyloid-binding proteins

1. Introduction

Amyloid-binding protein is a generic term used to group together a heterogeneous collection of proteins that interact with different structural assemblies (monomeric, oligomeric, and fibrillar) of amyloidogenic peptides or proteins. These interactions have been described to modulate physiological and pathological functions of the respective amyloid subunits by altering their solubility, transport, clearance, degradation, and fibril formation.

Extensive immunohistochemical analysis have demonstrated that, *in vivo*, complex mixtures of unrelated molecules, collectively referred in the field as amyloid-associated proteins, colocalize with all types of amyloid deposits albeit not being a structural part of the final fibril (reviewed in ref. 1). Serum amyloid P component (SAP), α1-antichymotrypsin (ACT), apolipoprotein E (apoE), apolipoprotein J (apoJ) or clusterin, complement components, vitronectin, glycosaminoglycans, interleukins, and extracellular matrix proteins are among the many amyloid-associated components so far described coexisting with all forms of cerebral and systemic amyloidosis (2–11). To the moment, it is still unclear whether these molecules are innocent bystanders or whether their presence is related to the mechanism of amyloidogenesis. Several lines of investigation favor the latter notion, at least for some of them. For example, apoE and SAP have been found in all amyloid light chain (AL) fibrillar deposits, whereas their presence could not be demonstrated in the nonfibrillar Congo red negative immunoglobulin deposits in cases of light chain deposition disease (12, 13). Similar findings have been reported in cerebral forms of amyloidosis in which SAP and

activation-derived products of the complement system are present in amyloid deposits but consistently absent in nonfibrillar preamyloid lesions (8, 12). Mice knockout for either SAP or apoE exhibit fewer amyloid lesions and delay in their onset although neither SAP nor apoE gene ablation prevents the formation of amyloid deposits (14, 15).

Most of the published reports dealing with amyloid-binding proteins are limited to those found associated with amyloid β (Aβ), the major constituent of the fibrils deposited into senile plaques and cerebral blood vessels of patients with Alzheimer's disease (AD) (16). Aβ extracted from senile plaques of AD patients is mainly 42–43 amino acids long (17), while vascular amyloid is two residues shorter at the C-terminus (18). A soluble form of Aβ (sAβ), homologous to the amyloid protein extracted from cerebrovascular lesions (19), has also been identified in culture media supernatants from untransfected and β amyloid precursor protein (βAPP)-transfected cells, as well as in cerebrospinal fluid (CSF), plasma, urine and brain parenchyma from normal subjects and AD patients (19–23). Notably, several proteins have been shown to interact with sAβ in physiological fluids (24–26) and in vitro experiments have demonstrated that the presence of plasma or CSF prevents the fibril formation of synthetic peptides homologous to sAβ (27, 28), likely reflecting the result of the peptide's interaction with the biological fluid components. In this sense, many of the amyloid-associated proteins have also the ability to modulate the formation of Aβ fibrils *in vitro*. Some of them (e.g., complement component C1q, apoE4, SAP, ACT) enhance Aβ fibril formation (27, 29–32), while others (e.g., apoJ) contribute to the peptide solubility precluding fibrillogenesis *in vitro* (11, 33). In the latter, this protecting effect has been proposed to contribute to the enhanced production of slowly sedimenting Aβ-derived diffusible ligands (ADDLs) highly toxic to neurons in culture at nanomolar concentrations (34).

The present work describes a simple methodology to identify amyloid-binding proteins by using affinity chromatography (see Note 1). This methodology is readily applicable to the study of different amyloidogenic proteins and their respective interactions with components of different physiological fluids. As an example, we describe the application of this protocol to the study of human plasma proteins with Aβ-binding properties by affinity chromatography using sequential elution with buffers of different characteristics. Detailed biochemical analysis and quantitation of the proteins eluted in the different fractions indicated that SAP and apoJ (clusterin) are the main plasma Aβ-binding proteins, while other minor components were identified as apoA-IV, apoE, and apoA-I, as well as HSA and fibulin (see Fig. 1). The distribution of these proteins within the different elution protocols is indicative of the distinct nature of the physicochemical interactions involved.

2. Materials

2.1. Reagents

1. Plasma samples were obtained from normal healthy subjects (age 25–40 years) after 12 h fast with adequate understanding and written consent of subjects.
2. Peptide DAEFRHDSGYEVHHQKLVFFAEDVGSNKGA IIGLMVVGGV ($Aβ_{1–40}$), homologous to residues 672–711 of β PP_{770} was synthesized at W.M. Keck facility at Yale University using tBOC (*N-tert*-butyloxycarbonyl) chemistry.
3. All buffers and solutions were freshly prepared using water provided by a Milli-Q system (18 MΩ/cm at 25°C, Millipore Corp., Bedford, MA). All reagents were of

[1]Affinity chromatography is one of the most powerful chromatographic methods for purification of specific molecules from a complex mixture based on the interaction with the affinity matrix. Sequential elution may allow purifying proteins that interact by different mechanism, providing further information on the type of binding interaction.

the highest purity available and were purchased from Sigma-Aldrich, unless otherwise noted.

4. *Specific buffers*.
    a. Tris-buffered saline (TBS):10 mM Tris–HCl, 150 mM NaCl, pH 7.4.
    b. TBS-Ca: TBS containing 2.5 mM $CaCl_2$.
    c. TBS-E: TBS containing 10 mM EDTA.
    d. TBS-HS: TBS containing 2 M NaCl.
    e. TBS-T: TBS containing 1% Triton X-100 (v/v).
    f. AcH: Acetic acid 1 M solution. Prepare by adding 57.5 ml of glacial acetic acid (Sigma-Aldrich, # 537020) to 942.5 ml of Milli-Q water to obtain 1 l of 1 M acetic acid solution, pH ~2.2. Do not adjust the pH.
    g. SDS/DTT buffer: 5% SDS and 1 M DTT in TBS buffer.
5. *Other reagents*.
    a. CNBr-activated Sepharose 4B (GE HealthCare, # 17-0430-01).
    b. CAPS (3-cyclohexylamino-1-propanesulphonic acid) transfer buffer: 10 mM CAPS, 10% methanol. Prepare 1 l of 10× CAPS stock (100 mM CAPS) by adding 22.13 g of CAPS (Sigma-Aldrich, #C2632) to 950 ml of Milli-Q water, adjust to pH and complete volume to 1 l. Prepare CAPS transfer buffer (1×) with 100 ml of CAPS 10× solution, 100 ml of methanol, and 800 ml of Milli-Q water.
    c. DC Protein Assay (Bio-Rad, #500-0111).
    d. Tricine Sample Buffer (Bio-Rad #161-0739).

2.2. Instrumentation

1. Peristaltic pump. Single channel laboratory peristaltic pump for use in liquid chromatography (GE-Healthcare, #18-1110-91).
2. Fraction Collector (GE-Healthcare, #18-1177-40).
3. Spectrophotometer UV-Vis.
4. Polystyrene 20-ml volume chromatographic columns including upper bed supports, end caps, tip closures (Econo-Pac Chromatography Columns, Bio-Rad #732-1010).
5. Appropriate column holders and clamps for attaching columns and tubing to the system.

3. Methods

3.1. Immobilization of Amyloid β Peptides to Chromatography Medium

3.1.1. Selection of Affinity Matrix Support and Coupling Chemistry—The nature of the affinity matrix support (solid support onto which ligand is immobilized) is an important factor that has to be considered when planning an affinity chromatography experiment. Detailed discussion on how to select an appropriate matrix is discussed in depth elsewhere (35). For the particular case of coupling hydrophobic amyloidogenic peptides, it is very important to select a hydrophilic, non-charged supports that minimize nonspecific hydrophobic and ionic interactions (see Note 2).

Depending on the nature of the peptide or protein to be coupled (amino acid composition, presence of carbohydrates, sulphydril groups, etc.) and the aim of the experiment, different chemistries can be used for coupling the ligand to the chromatographic media (35). Activated chromatography media with carboxyl esters such as *N*-hydroxysuccinimide (NHS) or cyanogen bromide (CNBr) represent two of most convenient methods for covalent binding of short peptides. Both NHS or CNBr moieties react with free primary amino groups from peptides or proteins to form stable covalent links, most typically through lysine side chains. In this protocol, we used CNBr-activated Sepharose (GE HealthCare) for binding of Aβ peptides.

CNBr-activated Sepharose 4B is a commercially available preactivated medium for immobilization of ligands containing primary amines with a very high coupling efficiency. It provides a very convenient way to immobilize proteins and peptides. The coupling reaction is spontaneous, rapid and easy to carry out (see Note 3). Buffers containing primary amines (e.g., Tris and glycine) should not be used for coupling reactions.

3.1.2. Ligand Preparation and Coupling—Amyloid peptides have an intrinsic tendency to oligomerize and aggregate. Therefore, proper handling of peptide solutions is a key aspect to be controlled to obtain an affinity matrix with a defined ligand composition. For this protocol, we aimed at coupling to the matrix support $A\beta_{1–40}$ peptide mainly as monomers or small oligomers. Coupling of the peptide to CNBr-activated Sepharose was performed following the manufacture's instruction with one important exception regarding the coupling buffer where the ligand is dissolved. In brief, 2.3 μmol (10 mg) of $A\beta_{1–40}$ peptide was slowly dissolved in 20 ml of 0.1 M carbonate/bicarbonate buffer, pH 9.0 without NaCl. After centrifugation at 16,000 × *g* at 4°C, this peptide solution was allowed to interact with 2 ml of activated Sepharose matrix for 16 h at 4°C on a rotating wheel. After washing, any remaining active groups were blocked by washing with 0.1 M Tris–HCl buffer, pH 8.0.

The $A\beta_{1–40}$ peptide coupled affinity matrix (2 ml) was packed into a polystyrene chromatographic column (Bio-Rad #732-1010) and washed extensively by three cycles of alternating pH with 0.1 M acetic acid/sodium acetate, pH 4.0 containing 0.5 M NaCl, and 0.1 M Tris–HCl, pH 8 containing 0.5 M NaCl.

3.2. Affinity Chromatography with Sequential Elution

This procedure can be performed in an FPLC system, or conveniently in an in-house chromatography system composed of a column containing the packed affinity media, a peristaltic pump to provide flow circulation, a fraction collector, and a UV-Vis spectrophotometer for later analysis of fractions.

3.2.1. Protocol for Affinity Chromatography with Sequential Elution

1. Equilibrate the affinity matrix by flowing 10 volumes of TBS-Ca (see Note 4). For a bed volume of 2 ml, use at least 20 ml of TBS-Ca.
2. Drain remaining TBS-Ca buffer, stop the flow, and load the sample of interest into the column (see Notes 5 and [6]). Connect the exit tubing to the top of the column

[2]Sepharose from GE-HealthCare and Affigel from Bio-Rad are agarose-derived matrices that fulfill these criteria. Cellulose or synthetic supports such as polyacrylamide beads, Sephacryl, or Ultragel are also good options. Hydrophobic polystyrene beads and negatively charged silica supports are not generally recommended for this purpose.

[3]This type of attachment presents also some drawbacks, including partial leaking of ligands, and potential steric hindrance due to the absence of linker. Additionally, attachment of ligands through free primary amino groups may alter their interaction with other proteins.

[4]A regular flow from 0.2 to 0.5 ml/min is recommended for most applications.

and recirculate the sample through the column with a slow flow (0.2 ml/min) for 1 h at 37°C, 3 h at room temperature, 16 h at 4°C, or any other suitable setting (see Note 7).

3. Collect the pass-through sample in one single fraction that contains unbound proteins.
4. Remove additional unbound material by extensive washing with at least 10 bed volumes of TBS-Ca, or until the absorbance of the eluent at 280 nm is negligible.
5. Elute Ca^{2+}-dependent binding proteins with TBS-E. Carefully add 5 volumes of TBS-E to the drained ligand matrix, restart the flow and take fractions of about one-forth of the bed volume (see Note 8).
6. Wash EDTA excess with 5 volumes of TBS.
7. Elute electrostatically bound proteins with TBS-HS. Carefully add to the drained matrix bed 5 volumes of TBS-HS, restart the flow and take fractions of about one-forth of the bed volume.
8. Wash salt excess with 5 volumes of TBS.
9. Elute hydrophobically bound proteins with TBS-T. Carefully add 5 volumes of TBS-T to the drained matrix bed, restart the flow and take fractions of about one-forth of the bed volume.
10. Wash Triton X-100 detergent with 5 volumes of TBS.
11. Elute pH-dependent binding proteins bound proteins with 1 M acetic acid (AcH). Carefully add 5 volumes of AcH to the drained matrix bed, restart the flow, and take fractions of about one-forth of the bed volume.
12. Wash AcH with 5 volumes of TBS.
13. Analyze remaining bound proteins by taking a small aliquot of affinity matrix and elute by boiling the sample for 5 min in the presence of 5% SDS and 1 M DTT in TBS (SDS/DTT buffer) (see Note 9).
14. To determine the fractions containing the eluted proteins at the different steps, read the absorbance at 280 nm of each fraction against a blank reference buffer, or by a suitable protein quantitation method such as the Bio-Rad DC Protein Assay (Bio-Rad, #500-0111) (see Note 8).
15. Pool fractions containing protein for each elution step, dialyze extensively against 50 mM ammonium bicarbonate, and store at −80°C until used.

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[5]Do not allow to matrix to get dry at any point during the procedure, because this will adversely affect the performance of the column.
[6]Viscous samples, such as plasma and serum, should be diluted at least 1:1 with TBS-Ca. Additionally, before loading the sample into the column, samples should be centrifuged for 15 min at >4,000 × *g*, or filtered through a 0.23-μm filter to remove debris.
[7]Incubation at 37°C mimics physiological conditions; however, for labile proteins this setup may result unsuitable. Adjust temperature and time for binding according to experimental needs. In the experiment depicted in this work (see Fig. 1), 2 ml of the $A\beta_{1–40}$ peptide matrix was allowed to interact with 10 ml of normal plasma for 3 h at room temperature with continuous recirculation.
[8]For a ligand matrix bed volume of 2 ml, take 0.5 ml fractions. The bulk of protein should elute within the last two fractions of the first bed volume.
[9]Since this procedure may render the affinity matrix unusable, it is important that you analyze only a small fraction (20–50 μl) by this procedure. The rest of the affinity matrix can be reused several times with optimal performance, provided proper care of column has been taken. For general cleaning and regeneration, wash the column with 3 volumes of alternating high pH (0.1 M Tris–HCl, 0.5 M NaCl, pH 8.5) and low pH (0.1 M sodium acetate, 0.5 M NaCl, pH 4.5) buffers. To remove precipitated or denatured substances, wash with 2 column volumes of 6 M guanidine hydrochloride, and wash immediately with 5 column volumes of TBS or other suitable buffer. To remove hydrophobically bound substances, wash with 3 column volumes of 70% ethanol or 1% Triton X-100, and immediately with 5 column volumes of TBS. For storage, keep the column at 4°C in 20% ethanol at neutral pH.

3.3. Downstream Analysis

3.3.1. Identification—Proteins bound to the affinity column and eluted with different buffers could be easily identified via either amino acid sequence or mass spectrometry analysis. To obtain rapid *N-terminal sequence* data without the need of complex and time-consuming fractionation procedures (e.g., high performance liquid chromatography separation of the individual components of the eluate), electrophoretic methodology usually provides enough resolution and sufficient amount of material to successfully carry out protein identification. Samples are separated on 10% tris-tricine SDS-PAGE and transferred to polyvinylidene difluoride membranes (Immobilon P, Millipore, Mildford, MA) using CAPS (3-cyclohexylamino-1-propanesulphonic acid) pH 11, containing 10% methanol. After transfer, the membrane is stained with Coomassie Blue (see Note 10) to visualize the components of the mixture, procedure that in our example render a series of components of different molecular mass (see Fig. 1). The protein bands are excised from the membrane and used directly to obtain N-terminal sequence (in our example, proteins were identified on a 477 A protein sequencer, Applied Biosystems, Foster City, CA). An alternative identification methodology that has greater sensitivity is *mass spectrometry*, an approach highly recommended for samples with low abundance components. The technique, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF), requires an additional step in the sample preparation; samples separated on SDS-PAGE are stained with mass spectrometry compatible Silver stain (SilverQuest, InVitrogen) following the manufacturer's guidelines. Bands are excised from the gel, subjected to proteolytic degradation with TPCK-trypsin and the resulting peptide mix resolved in the mass spectrometer using the protocols recommended by the manufacturer. Searching the mass data against a database of tryptic peptides will allow the identification of the unknown components (36).

3.3.2. Data Validation—Further validation of the data obtained via N-terminal sequence or mass spectrometry can be obtained via *Western blot analysis* of the different eluates using specific antibodies for the identified proteins. The technique is highly sensitive and will provide direct visualization of each of the specific components of the sample. The use of complementary *immunohistochemical* techniques will provide additional confirmation of the topographical location of the identified amyloid-binding proteins in the tissue deposits.

Acknowledgments

This work was supported by grants from the Fundación CIEN-ISCIII (MPY 1308/08) and CIBERNED to MC and from the National Institutes of Health NS051715 to AR and AG030539 to JG.

Abbreviations

ACT	α1-Antichymotrypsin
AD	Alzheimer's disease
apoA-I	Apolipoprotein A-I
apoA-IV	Apolipoprotein A-IV
apoE	Apolipoprotein E
apoJ	Apolipoprotein J (clusterin)

[10]Use the high-quality reagents for the preparation of the Coomassie Blue staining and distaining solutions. Use 1% acetic acid as fixative in the solutions instead of 5–10% commonly used in Coomassie Blue staining standard protocols to facilitate downstream analysis by N-terminal sequencing.

CAPS	3-Cyclohexilamino-1-propanesulphonic acid
CNBr	Cyanogen bromide
ELISA	Enzyme-linked immunosorbent assay
HSA	Human serum albumin
NHS	*N*-hydroxysuccinimide
SAP	Serum amyloid P component
Vn	Vitronectin

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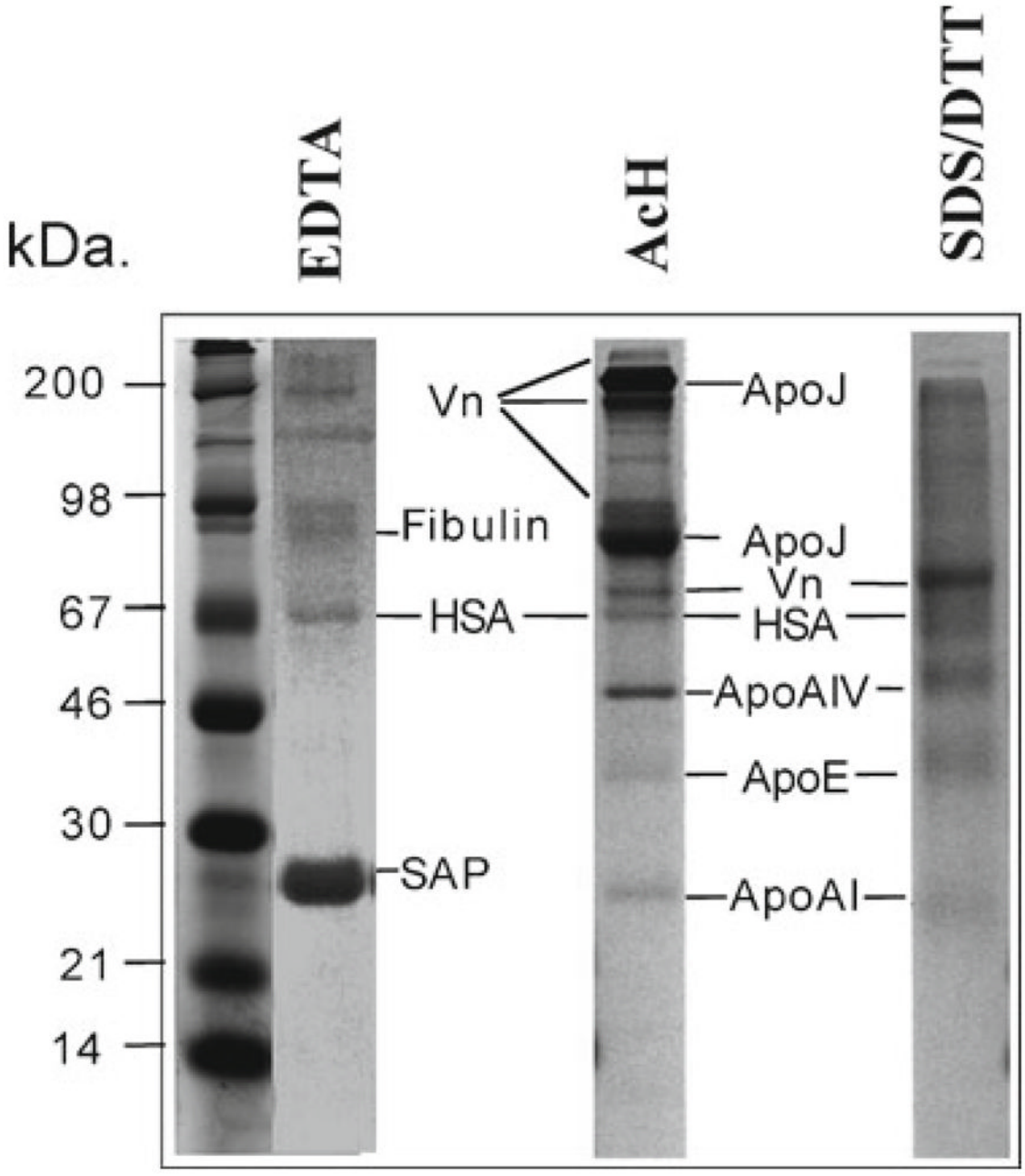

Fig. 1.

Binding of human plasma proteins to an Aβ-affinity matrix. Normal human plasma was incubated for 3 h at room temperature with $A\beta_{1-40}$ affinity matrix equilibrated in TBS containing physiological concentrations of Ca^{2+}. After extensive washing to eliminate non-specific binding, bound proteins were eluted from the Aβ-matrix sequentially with 10 mM EDTA (EDTA), 2 M NaCl, 1% (v/v) Triton X-100, 1 M acetic acid (AcH), and 5%/1 M SDS/DTT (SDS/DTT). Aliquots of the eluted fractions were separated on 10% tris-tricine SDS-PAGE, transferred to PVDF and the membranes stained with Coomassie Blue. The identity of the eluted components, determined by amino-terminal amino acid sequence, is indicated. Affinity chromatography of normal human serum revealed an array of Aβ-binding

proteins that interact with immobilized Aβ. The major Ca^{2+}-dependent binding component observed in the EDTA eluate corresponded to amyloid P component (SAP), while other minor bands were also observed and identified as albumin and fibulin. Under acidic conditions (AcH elution), we observed the presence of two major bands corresponding to apoJ (monomer and dimer), several vitronectin isoforms, as well as other minor bands identified as apolipoprotein A-IV, apolipoprotein A-I, apolipoprotein E, and albumin (see Note 11). Under denaturing conditions (SDS/DTT fraction), we observed the elution of vitronectin, apoE, apoA-IV, apoA-I, and albumin (HSA).

[11]In some Aβ-affinity chromatography experiments, we have found a significant presence of IgG (0–10%) in the Triton X-100 and acetic acid fractions. This fact may be associated to a specific interaction of bound apoJ with IgG molecules (37).