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Fibulin-1 purification from human plasma using affinity chromatography on Factor H-Sepharose

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Abstract

A method is reported to purify Fibulin-1 from human plasma resulting in a 36% recovery. The steps involve removal of the cryoglobulin and the vitamin K dependent proteins followed by polyethylene glycol and ammonium sulfate precipitations, DEAE-Sephadex column chromatography and finally Factor H-Sepharose affinity purification. The procedure is designed to be integrated into an overall scheme for the isolation of over 30 plasma proteins from a single batch of human plasma. Results from mass spectroscopy, SDS-PAGE, and Western blotting indicate that human plasma Fibulin-1 is a single chain of the largest isotype. Functional binding assays demonstrated calcium ion dependent interaction of Fibulin-1 for fibrinogen, fibronectin, and Factor H. The procedure described is the first to our knowledge that enables a large scale purification of Fibulin-1 from human plasma.

Keywords

Fibulin-1; Complement Factor H; Human Plasma; Purification; Affinity Chromatography

Introduction

Fibulin-1 (Mr~80,000) is a member of a family of 6 proteins that are associated with elastic fibers and extracellular matrices (for reviews see: [1] [2] [3]). Fibulin-1(P23142) (FBLN1_HUMAN) is a single chain polypeptide, which including the signal sequence, consists of 703 amino acids. The protein is crosslinked by 35 disulfide bridges and contains three asparaginyl-linked oligosaccharide side chains. The molecule is constructed of a series of 12 modules terminating in a unique domain. The three N-terminal modules are homologous to the complement anaphylatoxins, and these are followed by one typical Epidermal Growth Factor¹(EGF) module, in turn linked to eight calcium binding EGF

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(cbEGF) repeats. The largest isotype of the molecule ends in a domain of 125 amino acids that has homologous counterparts in other members of the Fibulin family [4] [5].

Fibulins are interesting from both a structural and biological perspective. Fibulin-1 is a calcium ion binding protein, and presumably all other members of the family are as well [6] [7]. The trains of cbEGF modules are of special importance as these are calcium ion binding units. A single cbEGF module from Fibulin-4 and pairs of recombinant cbEGF modules from fibrillin-1 were deciphered structurally using nmr spectroscopy (RCSB data base entries: 2KL7, 1EMO & 1LMJ) [8] [9]. The results demonstrate how these units bind calcium ion resulting in a stabilization of modular pairs thereby maintaining them in an extended rigid conformation. Each module of cbEGF is formed from about 40 amino acids containing four short β -strands crosslinked by three disulfide bonds creating an extended structure of ~ 30 Å in length [8] [10] [9].

In the Fibulins calcium ion is chelated by a conserved consensus of amino acids that includes three acidic residues at the N-terminus of each cbEGF module [11]. Interestingly a modified amino acid, β -hydroxyaspartic acid, was identified in the cbEGF module of factor IX of blood coagulation. β -Hydroxyaspartate has been suggested to contribute to calcium ion binding, and this modified amino acid may be common to other cbEGF units [12] [13]. However, it is currently not known whether plasma derived Fibulin-1 contains β -hydroxyaspartic acid.

The mosaic nature of Fibulin-1 creates an elongated molecule as visualized by electron microscopy after rotary shadowing. Recombinant Fibulin-1 is seen to have a dumbbell shape of ~ 330 Å in length [14].

From a biological perspective the Fibulins are of interest because they are constituents of the Extracellular Matrix (ECM), and consistent with this Fibulin-1 has important functions for vascular development, wound healing, elastogenesis, angiogenesis, and hemostasis [1] [2] [15]. The biological importance of Fibulin-1 may be inferred from knock-out mice and human subjects with genetic abnormalities within the Fibulin-1 gene. Mice deficient in Fibulin-1 usually die perinatally from vascular disease as a consequence of aberrant vascular development [16]. Human subjects with a haploinsufficiency or mutant forms of Fibulin-1 exhibit limb abnormalities exhibiting phenotypes similar to Marfan syndrome as well as synpolydactyly [17] [18].

A moderate affinity is reported for tropoelastin and Fibulin-1, and Fibulin-1 has been located by immuno-histochemistry to be in the cores of elastic fibers [19] [20]. Fibulin-1 has

¹Abbreviations used: AMD, Age related Macular Degeneration
 cbEGF, calcium ion binding Epidermal Growth Factor (module)
 CIG, Cold Insoluble Globulin
 ECM, Extracellular Matrix
 EGF, Epidermal Growth Factor (module)
 Hepes, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
 HRP, Horseradish Peroxidase
 Mes, (N-Morpholino)ethanesulfonic acid
 Mops, (N-Morpholino)propanesulfonic acid
 PEG, Polyethylene Glycol
 TMB, 3,3',5,5'-Tetramethylbenzidine

other interaction partners most of which function for hemostasis, ECM integrity and repair including: fibronectin, aggrecan, and versican, Extracellular Matrix Protein-1 (ECM-1) and the $\beta 1$ subunit of the fibronectin receptor [21] [22] [23].

Fibrinogen binding by Fibulin-1 also was demonstrated, and Fibulin-1 was found associated with fibrin within blood clots [24]. In this context Fibulin-1 was reported to mediate platelet adhesion indirectly by forming cross bridges with fibrinogen that was linked to the platelet integrin $\alpha IIb\beta 3$ [25].

Fibulin-1 may play a role in pathology. In a mouse model Fibulin-1 was found associated with vasculature calcification, and Fibulin-1 was found incorporated in human coronary artery atherosclerotic lesions. Fibulin-1 may also be involved in valvular heart pathology and atrial fibrillation [26] [27]. Fibulin-1 synthesis was detected in breast carcinomas, although a pathological role for Fibulin-1 in human breast cancer remains under study [28].

Because Fibulin-1 is an important multifunctional component that is of interest for structural biology, function, and pathology, it is of value to have a facile method to purify it from human plasma. This source of the protein is of special advantage because in contrast to other members of the family, Fibulin-1 is the only one that is found in a soluble form above a trace level in normal human plasma [15]. We have exploited the ability of Fibulin-1 to bind complement Factor H-Sepharose in a calcium ion dependent fashion enabling affinity purification of Fibulin-1 from human plasma in a good yield.

Methods & Materials

Frozen recovered human plasma was obtained from volunteer donors from the San Diego Blood Bank. DEAE-Sephadex and Sephacryl S300 were from GE Healthcare (Little Chalfont, UK). 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), Polyethylene Glycol 4000 (PEG), (N-morpholino)ethane sulfonic acid (Mes), (N-morpholino)propanesulfonic acid (Mops), Horseradish Peroxidase (HRP), 3,3',5,5'-tetramethylbenzidine (TMB) and 3-amino-9-ethylcarbazole were from Sigma-Aldrich Chemical Corp. (St. Louis, MO). Luminata forte chemoluminescent HRP detection reagent for ELISA was from EMD/Millipore (Billerica, MA). A murine mAb (clone B-5) directed against the amino terminal modules of human Fibulin-1 was purchased from Santa Cruz Biotechnology (Dallas, TX). Goat anti-rabbit IgG coupled to HRP was purchased from Life Technologies (Carlsbad, CA). Factor H was purified from human plasma similarly to a published method using the following steps: 4–12% PEG precipitation of barium citrate treated plasma, DEAE-Sephadex column chromatography, gel filtration through Sephacryl S300, and column chromatography on dextran sulfate Sepharose [29]. Factor H-Sepharose was prepared by conjugating Factor H (10 mg/ml) in 0.15 M NaHCO_3 pH 8.2 to Sepharose 6B using the cyanogen bromide reaction [30].

GC-MS Identification of proteins bound to Factor H-Sepharose

Protein samples were alkylated with iodoacetamide and were subjected to trypsin digestion. The resultant peptides were analyzed by high resolution high accuracy LC-MS/MS consisting of a Michrom HPLC, a 15-cm Michrom Magic C18 column, a low-flow

ADVANCED Michrom MS source, and a LTQ-Orbitrap XL (Thermo Fisher Scientific). A gradient was applied within 60 min ranging from 10–30% of (0.1% formic acid, 100% acetonitrile) to separate the peptides. The LTQ-Orbitrap XL was set to scan precursors in the Orbitrap followed by data-dependent MS/MS of the top 10 precursors. The LC-MSMS raw data were submitted to Sorcerer Enterprise v.3.5 release (Sage-N Research Inc.) with SEQUEST algorithm as the search program for peptide/protein identification.

Western blotting & ELISA

Fibulin-1 was identified by Western blotting using either a murine mAb (200 µg/ml) to the amino-terminal modules (diluted 1/1500) or a rabbit polyclonal antibody (5 mg/ml) diluted 1/400. The detection substrate was 3-amino-9-ethylcarbazole.

A double antibody sandwich ELISA was used to quantify Fibulin-1. Microtiter wells were coated at 4° with a rabbit polyclonal antibody (0.1 mg/ml) against human Fibulin-1 in 50 mM sodium carbonate buffer pH 9.5. After blocking with 10 mM Mops pH 7.0/0.15 M NaCl/2% BSA/0.05% Tween 20, serially diluted samples were added and left for 1 h at room temperature. After washing a solution of a murine mAb against human Fibulin-1 (0.7 µg/ml) was allowed to combine with the bound protein for one h at room temperature. Subsequently a solution of an HRP-conjugated goat anti-murine IgG (0.7 µg/ml) was added for 1 h at room temperature. After washing the chromogenic substrate TMB was added to the wells in order to measure HRP activity using a Molecular Devices ELISA microplate reader.

Purification of Fibulin-1 from human plasma

Frozen recovered human plasma was thawed and centrifuged at 6000 x g for 20 min to remove the Cold Insoluble Globulin (CIG). To three liters of cryo-supernatant was added 120 ml of 1 M BaCl₂, resulting in a barium citrate precipitation that extracted the vitamin K dependent proteins. The insoluble material was removed after centrifugation at 6000 x g for 20 min. Subsequent slow addition of a 50% suspension of PEG to a final concentration of 4% PEG resulted in a precipitate enriched for Fibulin-1. The precipitate was centrifuged at 6000 x g for 30 min, and the pellet was suspended in 800 ml of buffer B (20 mM imidazole pH 6.2/75 mM NaCl/5 mM EDTA). The suspension was subjected to two successive precipitations with ammonium sulfate at 35% of saturation. The ammonium sulfate precipitated protein was dialyzed against buffer B, and afterwards a small amount of euglobulin was removed by centrifugation. Then the suspension was applied to a DEAE-Sephadex column (3 x 26 cm). After rinsing in buffer B, a linear gradient of 500 ml each of 0 to 0.4 M NaCl was applied to the column. The Fibulin-1 containing fractions were detected by Western blotting. These were pooled, dialyzed against 10 mM Mes pH 6.0/0.15 M NaCl/3 mM Ca⁺² and applied to a column of Factor H-Sepharose (4 x 20 cm). After rinsing until the optical density at 280 nm was nearly zero, Fibulin-1 was eluted in 10 mM Mes pH 6.0/0.15 M NaCl/5 mM EDTA.

Plate binding assays

HRP (3 mg) in 1 ml of 25 mM sodium acetate pH 5.0 was oxidized with 4 mM sodium meta periodate for 30 min on ice, and subsequently 3 ml of Fibulin-1 (0.3 mg/ml) in 50 mM

sodium phosphate pH 7.4 was added. After 1 h sodium cyanoborohydride was added to a final concentration of 0.3 M. After 15 h at 4° the protein was dialyzed against 10 mM Hepes/0.15 M NaCl. Fibulin-HRP was subsequently purified on an 8 ml column of Factor H-Sepharose. Uncoupled HRP did not adsorb to Factor H-Sepharose.

Wells within a white polystyrene plate were coated with either casein, fibronectin, fibrinogen, or Factor H at a concentration of 100 µg/ml for 15 h at 4°. After blocking the wells with BSA (20 mg/ml) and washing with 10 mM Hepes/0.15 M NaCl, 5 µl of Fibulin-HRP 5 (20 µg/ml) in 10 mM Hepes pH 7/0.15 M NaCl was added in buffer containing either 5 mM Ca⁺² or no additional divalent metal ions. After 1 h the plate was drained, washed once rapidly in buffer with or without 5 mM Ca⁺², and to each well was added 50 µl of Luminata Forte. The reactions were measured in a Flexstation plate reader.

Results

We developed a purification procedure for human Fibulin-1 based on an observation that it has a capacity to bind to Factor H-Sepharose. Human Factor H is a single chain plasma glycoprotein (Mr ~155,000) that is present in two major variants, one of which has a tyrosine and the other a histidine at amino acid position 402 {the numbering includes the signal sequence}. Several separate studies have demonstrated that Factor H variant H402 is associated with a two fold higher frequency of Age Related Macular Degeneration (AMD) per copy [31] [32].

Because the side chain of histidine frequently is observed in divalent metal ion chelation [33] [34], we suspected that the high risk variant of Factor H might have an augmented capacity for divalent metal ion binding. Furthermore, because it also had been published that excessive lead in the retina can accompany AMD [35], we queried as to whether any protein(s) in human plasma would couple to Factor H-Sepharose selectively in the presence of trace concentrations of lead ion. Two identical columns (8 ml) of Factor H-Sepharose were used for comparison, and these were equilibrated using two different conditions. In one case no extra divalent metal ions were included in the buffer (10 mM Mops pH 7.4/0.15 M NaCl), and in the second case the buffer contained 10 µM lead ion. To one sample of five ml of barium citrated treated human plasma that had been dialyzed in 10 mM Mops pH 7.4/0.15 M NaCl no additional metal ion was included, and in another sample PbCl₂ was added to a final concentration 10 µM.

The columns were washed in buffer until no more optical density at 280 nm was detected, and then 0.5 M NaCl in 10 mM Mops pH 7.4 was used to remove electrostatically bound proteins. After the optical density of the effluent was near zero, 10 mM Mops pH 7.4/150 mM NaCl/5 mM EDTA was used to elute any components adsorbed by divalent metal ion interaction. As seen in Figure 1, in the presence of 10 µM Pb⁺², one plasma protein (Mr~80,000) was eluted specifically with EDTA from Factor H-Sepharose. Similar results were observed when 3 mM Ca⁺² was in the buffer, but this condition differed from the situation of Pb⁺² addition in that some of the Factor H binding protein could be eluted in 0.5 M NaCl.

LC-MS after trypsin digestion was employed in order to identify Factor H binding proteins, and the results are shown in Table I. Predictably some peptides originated from IgG and complement C3, but the majority identified Fibulin-1 D-isotype as the interactive species. This determination of Fibulin-1 was confirmed by Western blotting.

Because our Factor H-Sepharose was prepared from bulk Factor H purified from plasma of multiple donors, we could not determine whether any variant played a more consequential role in trapping Fibulin-1. However, the application of using Factor H-Sepharose as an affinity adsorbent for Fibulin-1 purification was obvious. Accordingly we established a purification procedure for this protein from this basis.

Several fractionation steps were developed inclusive of PEG and ammonium sulfate precipitations (Fig. 2) followed by DEAE-Sephadex anion exchange chromatography (Fig. 3). Because Fibulin-1 is acidic with an estimated pI of 5.0, it predictably elutes near the back of a salt gradient applied to this anion exchange column. Specific elution from Factor H-Sepharose resulted in pure Fibulin-1 (Fig. 4) with a respectable recovery of 36% and yield of 6.8 mg from three liters of starting plasma (Table II).

SDS-PAGE patterns indicate only a single chain that migrates more slowly and broadens after reduction evidently as a consequence of a large number of disulfide bonds that were broken by the reducing agent (Fig. 5). Both SDS-PAGE and Western blotting showed only a single band, suggesting that it is exclusively the D-isoform as indicated by LC-MS (Table I).

Because Fibulin-1 is not an enzyme a functional assay of the purified protein relied on its specific binding to matrix proteins (Fig. 6). As seen Fibulin-1 interactions with all binding partners (fibrinogen, fibronectin, and Factor H) were amplified in the presence of calcium ion.

Discussion

We report a novel procedure for the affinity purification of Fibulin-1 from human plasma. A previous publication described the purification of Fibulin-1 from human placental extracts using an affinity column of Sepharose to which was coupled a peptide corresponding to a sequence found in the cytoplasmic domain of the $\beta 1$ subunit of the fibronectin receptor [6]. In another report a method was presented for the isolation of murine recombinant Fibulin-1 from cell culture extracts using DEAE-cellulose chromatography and gel filtration through Superose 6 [14]. A purification of Fibulin-1 from human plasma was reported using a mAb conjugated to Sepharose, but this procedure required 8 M urea for recovery, and the eluted material also contained fibrinogen [24].

As a consequence of interrogating the effect of toxic metal ions such as Pb^{+2} for interaction of plasma proteins with Factor H, a complement regulatory protein ($Mr \sim 155,000$), we made the observation that Fibulin-1 can bind to Factor H-Sepharose with specificity in the presence of Pb^{+2} , and we extended that observation to show that Ca^{+2} also enables Fibulin-1 interaction with Factor H-Sepharose (Fig. 1). The identification was made by LC-MS and Western blotting (Table I). The utility of this observation for affinity purification of Fibulin-1 was clear. Accordingly we established an isolation procedure from human plasma

based on affinity chromatography using Factor H-Sepharose. The steps included: removal of the CIG, barium citrate adsorption of the vitamin K-dependent proteins, 4% PEG precipitation, two 35% ammonium sulfate precipitations, DEAE-Sephadex column chromatography, and finally Factor H-Sepharose affinity chromatography (Figs. 2–4). The measured yield was 6.8 mg from 3 liters of human plasma reflecting a 36% recovery (Table II).

Reported concentrations of Fibulin-1 from human plasma vary from 620 ng/ml to 33 µg/ml [36] [5]; however, using an ELISA we measured an intermediate value of 6.3 µg/ml (Table II). About 20% of this component fractionates into the CIG, apparently associated with fibrinogen [24], whereas, most of it remains soluble in the cryo-supernatant where it can be precipitated with 4% PEG. Accordingly we used the 4% PEG pellet as source material.

The purification scheme that we developed has been integrated with other published procedures for the fractionation of human plasma proteins yielding over 30 proteins purified from a single lot of human plasma inclusive of fibronectin and fibrinogen (localized in the CIG) the vitamin K dependent proteins of blood coagulation (extracted in the barium citrate pellet), the fibrinolytic components plasminogen and plasma carboxypeptidase B2, and all the complement proteins [37] [38] [39] [40] [41] [42] [43] [44] [45] [46] [47] [48] [49]. Given that human plasma is a precious resource, establishing such an integrated procedure for fractionation and purification has clear value.

The adaptive biological role of an affinity of Factor H for Fibulin-1 needs to be discovered, but the possibility that this interaction is consequential in AMD is a major point for further examination. Pertinently Factor H is also reported to be a binding partner for Fibulin-3, and an expressed construct of modules 6-through-8 containing the His(402) variant of Factor H bound to Fibulin-3 better than the Tyr(402) form [50]. Thus Factor H interacts with at least two members of the Fibulin family, and it is possible it binds also with the other Fibulins.

Both Factor H and Fibulin-1 have multiple interaction partners, and the consequent networks are complicated making functional interpretations problematical. A non-inclusive listing of Fibulin-1 binding proteins is: fibrinogen, fibronectin, the β -subunit of integrin $\alpha v \beta 1$, ECM-1, and the proteoglycans, aggrecan and perlecan (through their lectin domains) [24] [51], [6] [52] [23]. Similarly diverse, Factor H has been reported to bind with high affinity and specificity to C3b, CRP, DNA, the integrin CD11b/CD18, the von Willebrand protein, factor I, and andromedullin [53] [54] [55] [56] [57] [58] [59] [60] [61].

Accordingly, Fibulin-1 and Factor H have multiple functions, and surely it is going to take considerable experimental work to decipher the biological significance of the combined interaction networks of Fibulins and Factor H; and it is for this very reason that it is important to have available a purification method for Fibulin-1 that results in sufficient yield enabling experiments at a pure biochemical level. Thus our contribution is important for future research. But also it is of special interest to our laboratory to attain sufficiently homogeneous Fibulin-1 for crystallization, which would enable the decipherment of the tertiary of this protein by X-ray diffraction. Because the tertiary structure of no member of the Fibulin family has been attained to date, a structural determination of Fibulin-1 at the

atomic resolution would be a milestone in structural biology, and would be of major medical value for understanding wound healing, angiogenesis, and development at a molecular level.

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Highlights

1. A method has been established to purify human Fibulin-1 from plasma.
2. The last step is affinity chromatography on Factor H-Sepharose.
3. The purification procedure can be integrated with several other published methods enabling the purification of at least 30 plasma proteins from a single lot of human plasma.
4. Fibulin-1 binding to Factor H, fibronectin and fibrinogen is augmented appreciably by calcium ion.
5. At least two Fibulins have a binding capacity for Factor H.

Fig. 1.

Demonstration that Fibulin-1 in human plasma binds to Factor H-Sepharose dependent on divalent metal ions. Pools of human plasma (5 ml) were applied to a pair of Factor H-Sepharose columns (1 x 8 cm) in 10 mM Mops pH 7.4/0.15 M NaCl containing either no additional divalent metal ions or 10 μ M Pb²⁺ (left panel) or 3 mM Ca²⁺ (right panel). After rinsing in buffer 10 mM Mops pH 7.4/0.15 M NaCl was applied to remove proteins adsorbed by ionic interaction followed by 10 mM Mops pH 7.4/0.15 M NaCl/5 mM EDTA. The eluted protein bands were visualized by SDS-PAGE after silver staining.

Fig. 2.

Detection of Fibulin-1 from early purification steps by Western blotting. Left panel) CIG recovered from frozen and thawed human plasma along with successive PEG precipitations were examined by Western blotting for Fibulin-1. Right panel) The 4% PEG precipitate was subjected to serial ammonium sulfate (AS) precipitations. The results show Fibulin-1 fractionates selectively in the CIG and 4% PEG precipitates, and 35% ammonium sulfate further precipitates this protein from the 4% PEG extract.

Western blot

Fig. 3.

Fractionation of plasma proteins containing Fibulin-1 on DEAE-Sephadex. A Fibulin-1 enriched fraction after 4% PEG, and two successive 35% ammonium sulfate precipitations was dialyzed vs. 20 mM imidazole pH 6.2/75 mM NaCl/5 mM EDTA and applied to a DEAE-Sephadex column (3 x 26 cm) equilibrated in the same buffer. A linear gradient of 0.5 liters each of 0 to 0.4 M NaCl in the starting buffer was used to elute Fibulin-1. The fractions containing this protein were identified after Western blotting. The bar indicates the pooled material.

Fig. 4.

Affinity purification of Fibulin-1 on Factor H-Sepharose. The pool from the DEAE-Sephadex chromatography stage was dialyzed vs. 10 mM Mes pH 6.0/0.15 M NaCl/3 mM CaCl_2 and applied to a Factor H-Sepharose column (4 x 20 cm). After all non-adsorbed proteins passed through the Sepharose, a solution of 10 mM Mes pH 6.0/0.15 M NaCl/5 mM EDTA was used to elute Fibulin-1 selectively as detected by Western blotting. The lanes of SDS-PAGE labeled 1, 2, 3 refer to the material before addition to Factor H-Sepharose, non-binding fractions, and the protein specifically eluted using 5 mM EDTA, respectively.

Fig. 5. SDS-PAGE & Western blotting for human Fibulin-1. Left) SDS-PAGE patterns of Fibulin-1 either unreduced (-SH) or reduced with 2-mercaptoethanol (+SH). Right) Western blots of Fibulin-1 developed with either a murine mAb against the amino-terminal modules or rabbit polyclonal antibodies against the entire protein.

Fig. 6.

Calcium ion dependent binding of Fibulin-1 to fibrinogen, fibronectin, and Factor H. Wells of a white microtiter plate were coated with casein, fibrinogen, fibronectin or Factor H. After blocking with BSA, HRP-Fibulin-1 was added in 10 mM Hepes pH 7.3/0.15 M NaCl containing 5 mM calcium ion or no additional divalent metal ions for 1 h at room temperature. After one rapid washing, a luminescence detection reagent was added, and the results measured using a Flexstation plate reader.

Table I

Results of LC-MS identification of human proteins bound to Factor H-Sepharose in buffer with divalent metal ions and eluted with EDTA.

Protein ID	Description	Spectral Count	Number unique peptides
IP100889740	Fibulin-1	99	18
IP100296534	Fibulin-1 D-domain	79	6
IP100783987	Complement C3 fragment	26	19
IP100399007	IGHG2	22	4
IP100892870	IGHM	17	10
IP100887169	IGLV1-44	16	4
IP100168728	IGHG3	15	4
IP 100220327	KRT1 Keratin type II	12	7
IP 100939442	IGHG4	12	4
IP 100939442	ApoAI	6	4

Table II

Purification steps for Fibulin-1 from human plasma. Protein concentration was estimated using an extinction coefficient at 280 nm of 10 OD cl/mg cm for all stages except the last one in which an extinction coefficient at 280 nm of 5.4 OD cl/mg cm was employed for purified Fibulin-1. The cryo-supernatant was used for further purification.

Step	Volume (ml)	Protein Concentration (mg/ml)	Fibulin Concentration (µg/ml)	Total Fibulin (mg)	Recovery %
Plasma	3000	70.0	6.3	18.9	100
CIF	310	12.5	12.0	3.7	19
Cryo-supernatant	3000	46.6	5.2	15.6	82
4% PEG Precipitation	800	21.6	17.0	13.6	72
35% AS Precipitation	330	14.5	33.0	10.9	58
DEAE-Sephadex	120	0.7	62.5	7.5	40
Factor H-Sephadex	68	0.1	100.0	6.8	36