Chapter 20

Purification of Human Plasma/Cellular Fibronectin and Fibronectin Fragments

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Abstract

A method is described for the purification of plasma fibronectins based on a combination of gelatin- and arginine-Sepharose chromatography steps. Cellular fibronectin can be purified from an osteosarcoma fibroblast cell line by affinity chromatography using a monoclonal antibody anti-fibronectin as ligand. Furthermore, we also provide a protocol for the purification of fibronectin domains obtained by fractionation of thermolysin-digested plasma fibronectin on ion-exchange/gel filtration chromatography columns. Assessment of the fibronectin purity is performed by SDS-PAGE, while the ligand binding activities of specific fibronectin domains are determined by ELISA.

Key words Fibronectin, Fibronectin purification, Affinity chromatography, Fibronectin domain, Ion-exchange chromatography, Gel filtration chromatography, SDS-PAGE, ELISA

1 Introduction

Fibronectin (Fn) is a multifunctional glycoprotein found in the extracellular matrix (ECM) and body fluids of higher organisms [1]. In the ECM, Fn plays a critical role in numerous processes such as cell adhesion, cell spreading, migration, growth, and differentiation. Other important functions attributed to Fn include a role in physiological processes such as embryogenesis, wound healing, hemostasis, and thrombosis [2]. Fn exists in two main forms: cellular Fn, which is localized and assembled in a fibrillar matrix in tissues, and soluble Fn synthesized and secreted by hepatocytes and found at 300 μg/ml in human plasma [3].

Human Fn is composed of two polypeptides (α and β chains) with a molecular weight of 220–250 kDa each and joined by two disulfide bonds near the carboxyl termini to form a dimer. The chains are primarily made up of three different repeating modules of <100 amino acids, termed type I, II, and III repeats. In addition, sets of adjacent modules concur to the formation of a variety of discrete globular domains connected by flexible segments (Fig. 1).

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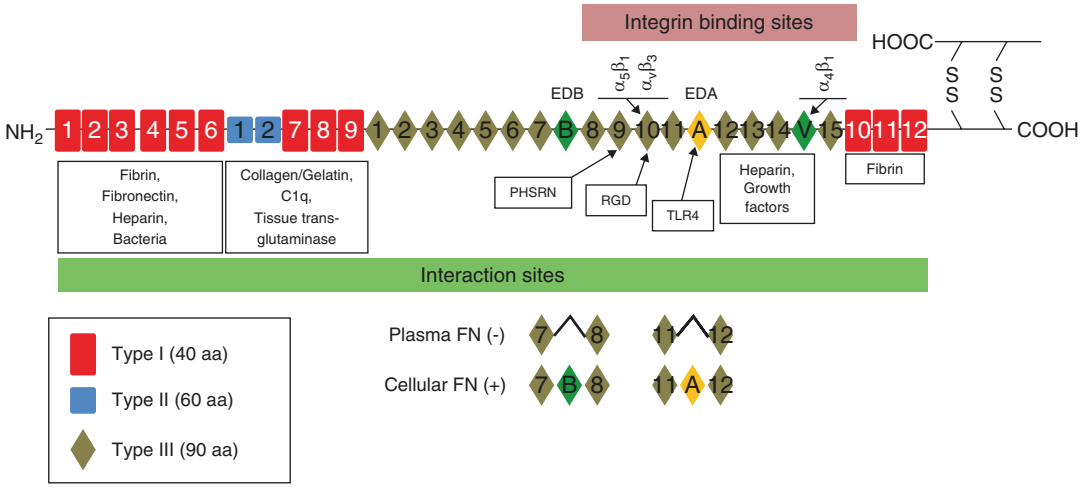

Fig. 1 Schematic representation of fibronectin. The scheme shows a representation of a Fn dimer and its interaction with specific ligands. The different types of homologies (12 type I, two type II, and 15 type III) are also reported. Numbering of type III homologies does not include EDA and EDB subdomains. Type I, II, and III domains are constituted of 40, 60, and 90 amino acids, respectively. Constitutive (RGD) and synergy (PHSRN) sites are indicated, together with their integrin receptors

Partial protease digestion of Fn was used to isolate these globular domains that retain their function and shown to be useful to map the location of major structural features of the protein. The different domains specifically interact with various biological molecules including heparin, fibrin, collagens/gelatin, and glycosaminoglycans of the ECM and integrins on the surface of the cells. Fn also binds to a wide variety of microorganisms, most particularly bacteria. The N-terminal domain (modules I_{1-5}) can be released and isolated as an early fragment of 25–30 kDa by a variety of proteases. It binds among others to heparin, fibrin, and Gram-positive bacteria (*S. aureus* and streptococci) and participates in folding of Fn in matrix assembly and can be cross-linked via factor XIII to collagen and bacteria [2]. Adjacent to the N-terminal domain is the 40–45 kDa glycosylated, intrachain disulfide bond-rich gelatin-binding domain [4]. This domain also binds the collagen-like domain of the complement factor C1q [5]. The region following the gelatin-binding site is made up of 15 constitutive type III repeating units and serves as a natural ligand for several integrins [6].

Alternative spliced type III repeats extra domains A (FnIII EDA) and B (FnIII EDB) are expressed during embryogenesis [7], wound healing [8], and tumor progression [9] and at sites of chronic inflammation such as psoriatic lesions [10]. One of the most interesting functions attributed to FnIII EDA is its ability to act as ligand for the pattern recognition toll-like receptor (TLR) 4 [11] that leads to the activation of NF-kB. Through TLR4 signaling FnIII EDA promotes chronic cutaneous fibrosis in conditions such as scleroderma [12]. A segment connecting two other type

III repeats, called type III connecting segment (IIICS) or variable V region, also exists between the 14th and 15th type III module that contains the binding site for α4β1 integrin [13]. It is present in most cellular fibronectin, but only one of the two subunits in a plasma fibronectin dimer contains a V-region sequence. Modules $III_{9–10}$ correspond to the "cell-binding domain" of Fn. The RGD sequence (Arg-Gly-Asp) located in III_{10} is the site of cell attachment via α5β1 and αVβ3 integrins on the cell surface [6]. Some integrins require a "synergy sequence" (PHSRN, Pro-His-Ser-Asn) in repeat III_9 for maximal interaction of integrins with Fn [14]. This central domain contains also sites for fibrin ($III_{10–12}$), heparin, and syndecan binding ($III_{12–14}$). Small fragments (21–37 kDa) comprising three repeats of type I disulfide-bonded loop structure are released from plasma Fn by thermolysin or plasmin and show a fibrin-binding activity [1].

Plasma Fn has been purified through standard methods of protein chemistry including precipitation steps, gel filtration chromatography, and ion-exchange chromatography. These procedures produce reasonably pure Fn, although the yields are relatively low and Fn purified in this way usually contains contaminants. Presently, the most used procedure is based on the gelatin-Sepharose affinity chromatography method developed by Engvall and Ruoslahti [15, 16]. This procedure allows isolation of large quantities of Fn, although several contaminants such as fibrinogen and plasminogen co-elute with the protein. A development of the above strategy includes a combination of gelatin-affinity column with heparin affinity chromatography. With this procedure, Fn is initially purified on gelatin-Sepharose column and then adsorbed on heparin-Sepharose column pre-equilibrated with 4 M urea. The Fn is then eluted from the column by 0.5 M NaCl [17]. In this work, we propose plasma Fn purification protocol based on gelatin-affinity chromatography combined with arginine-affinity chromatography, as previously reported by Vuento and Vaheri [18]. The yield of purified Fn obtained by this method is about 60%. We also report on a rapid analytical method to isolate cellular Fn by immunoaffinity chromatography. Furthermore, we describe a protocol for the purification of Fn domains following digestion of the protein with thermolysin and fractionation on a hydroxyapatite column as described by Zardi et al. [19]. Assays to test the functionality of the purified fragments, based on their ability to recognize appropriate ligands, are also described.

2 Materials

All buffers should be prepared using double distilled and sterile filtered (0.2 μm) water and analytical grade reagents. Prepare and store all concentrated reagents at 4 °C for 2–3 weeks and dilute to 1X before use.

2.1 Preparation of Human Plasma

1. 0.2 M PMSF (phenyl-methyl-sulfonyl fluoride) in 95% (v/v) ethanol. PMSF will be used at a final concentration of 1 mM (1:200 dilution).
2. Citrate phosphate dextrose (CPD) solution (we use the one from Sigma).
3. Human citrated plasma (*see* **Notes 1** and **2**).

2.2 Chromatography Analyses

Different types of chromatography equipment can be used, from automated systems to simpler alternatives such as peristaltic pump connected to an absorbance measuring unit that can follow protein elution and a fraction collector.

2.3 Affinity Columns

1. Glass columns: size: 3 × 7.5 cm; volume: ~ 50 ml.
2. Vacuum pump.
3. Sintered glass filter.
4. End-over-end mixer.
5. Ice-cold 1 mM HCl.
6. Ultrafiltration apparatus.
7. Washing solution for gelatin treatment: 3 M NaCl, 10 mM EDTA.
8. Porcine skin gelatin (*see* **Note 3**).
9. 200 mM EDTA stock solution (*see* **Note 4**).
10. L-Arginine.
11. CNBr-activated Sepharose 4B Fast Flow.
12. Coupling buffer: 0.1 M $NaHCO_3$, 0.5 M NaCl, pH to 8.3 with 1 M HCl.
13. 0.2 M glycine solution, pH 8.0.
14. 0.1 M sodium acetate, 0.5 M NaCl, pH 4.
15. Tris buffer saline (TBS): 50 mM Tris/HCl, 0.15 M NaCl, pH 7.4.
16. 95% (v/v) ethanol in ultrapure water (*see* **Note 5**).
17. 0.2% (w/v) sodium azide stock solution in ultrapure water (*see* **Note 6**).
18. Bradford protein assay reagents (we use the BCA kit from Bio-Rad).
19. Anti-plasma Fn mouse monoclonal antibody (we use clone IST-9 from Abcam) (*see* **Note 7**).
20. Cellular Fn eluting buffer: 20 mM CAPS (*N*-cyclohexyl-3-aminopropanesulfonic acid), 0.15 M NaCl, 10 mM EDTA, pH 11.
21. Dialysis tubes, buckets, and beakers.

2.4 Fibroblast Culture

1. Osteosarcoma fibroblasts MG-63 cell line (*see* **Note 8**).
2. Eagle minimum essential medium.
3. Fetal bovine serum.
4. Nonessential L-amino acids.
5. Penicillin.
6. Streptomycin.

2.5 ELISA

1. EIA/RIA microtiter plates.
2. Bovine serum albumin (BSA).
3. Heparin.
4. Porcine gelatin (*see* **Note 3**).
5. Recombinant human α5β1 integrin (we use the one from R&D Systems).
6. Human fibrinogen.
7. Thrombin (purified from human plasma).
8. Immobilization buffer: 50 mM Na_2CO_3, pH 9.5.
9. Phosphate buffer saline (PBS).
10. Washing solution (PBST): PBS containing 0.5% (v/v) Tween 20.
11. Blocking solution: 2% (v/v) bovine serum albumin (BSA) in PBS.
12. *o*-Phenylenediamine dihydrochloride (OPD) solution: mix four tablets with 12 ml of distilled water and 5 μl of 30% hydrogen peroxide (*see* **Note 9**).
13. Anti-human fibrinogen antibody.
14. Anti-human plasminogen antibody.
15. Anti-human Fn antibody.
16. Horseradish peroxidase (HRP)-conjugated secondary antibody (*see* **Note 10**).
17. Stop solution: 0.5 M H_2SO_4.

2.6 Purification of Fn Fragments

1. Thermolysin (protease type X) (we use the one from Sigma).
2. Thermolysin digestion buffer: 25 mM Tris buffer containing 0.5 mM EDTA, 50 mM NaCl, and 2.5 mM $CaCl_2$, pH 7.6.
3. Hydroxyapatite DNA grade Bio-Gel.
4. Gel equilibrating buffer: 0.5 mM sodium phosphate buffer pH 6.8, with 0.1% (w/v) sodium azide.
5. Bio-Gel P-30 (fractionation range: 2,500–40,000 Da).
6. Superdex 75 (fractionation range: 3,000–75,000 Da).
7. Sephadex G-150 Superfine (fractionation range: 4,000–150,000 Da).

2.7 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. Electrophoresis unit.
2. Gel casting system.
3. Water bath.
4. Benchtop centrifuge.
5. Reducing loading buffer: 50 mM Tris/HCl, pH 6.8, 20% (v/v) glycerol, 0.1% bromophenol blue, 2% (w/v) sodium dodecyl sulfate (SDS), and 20 mM dithiothreitol (DTT).
6. Running buffer: dissolve 3 g Tris base, 14.04 g glycine, and 1 g SDS in 1 l of distilled water.
7. Coomassie blue staining solution: dissolve 0.4 g Coomassie brilliant blue R250 in 60 ml 2-propanol, 40 ml of glacial acetic acid, and 100 ml distilled water.
8. Protein molecular weight standard (full range, MW 10–250 kDa).
9. Destaining solution: methanol/water/glacial acetic acid (45:45:10, v/v/v) (*see* **Notes 5** and **11**).

3 Methods

3.1 Coupling of Gelatin and Arginine to Sepharose 4B Fast Flow and Preparation and Packing of Affinity Columns

1. Suspend 15 g of freeze-dried powder of CNBr-activated Sepharose 4B Fast Flow in 3 l of 1 mM ice-cold HCl.
2. Wash the slurry on a sintered glass filter with 1 mM HCl for 20 min.
3. Transfer slurry to a beaker and equilibrate with 80–100 ml of coupling buffer.
4. Add 100 ml of gelatin or arginine dissolved in coupling buffer (2–3 mg/ml).
5. Incubate the gel suspension at room temperature in an end-over-end mixer for 2 h.
6. To block remaining reactive groups, centrifuge the mixture at 1,000 *g* for 5 min; resuspend the slurry with a 0.2 M glycine solution, pH 8.0; and incubate for 2 h.
7. Wash the gel with 250 ml coupling buffer followed by 250 ml of 0.1 M sodium acetate buffer, pH 4, containing 0.5 M NaCl (*see* **Note 12**). Repeat this two-step wash for a total of three times to wash away excess of uncoupled ligand in the slurry.
8. Resuspend and equilibrate the slurry in TBS and store at 4 °C until use.
9. To prepare and pack the affinity columns (gelatin- or arginine-Sepharose 4B Fast Flow), allow the slurries in TBS to equilibrate at room temperature for at least 2 h.
10. Degas the suspension under vacuum.

11. Slowly pour the slurry into a column and permit settling of the matrix bed under gravity.
12. Pack the column by running TBS at a flow rate of 2–3 ml/min for at least 60 min.

3.2 Purification of Plasma Fibronectin on Affinity Columns

3.2.1 Gelatin-Sepharose Chromatography

1. Pour 100 ml of cleared plasma through a 50 ml column of gelatin-Sepharose Fast Flow, previously equilibrated with TBS containing 1 mM PMSF and 0.02% (w/v) sodium azide (*see* **Note 13**).
2. *Important*: For each step below, set aside 50–100 µl of sample from each flow through obtained from column washes and elutions for later SDS-PAGE and other analyses.
3. Wash the column with TBS until no material appears in the eluent, as monitored by absorption at 280 nm (*see* **Note 12**) (Fig. 2a).
4. Wash sequentially the slurry with 100 ml of 1 M NaCl and 100 ml of 0.2 M arginine.
5. Elute bound Fn from the column with 1 M arginine.
6. Dialyze pooled fractions against ~5 l of TBS for 48 h at 4 °C.
7. Clarify the above dialyzed solution by centrifugation at 10,000 *g* for 10 min.
8. Collect supernatant and concentrate by ultrafiltration.

3.2.2 Arginine-Sepharose Fast Flow Chromatography

1. Apply the concentrated, clarified material to a column containing 50 ml arginine-Sepharose Fast Flow equilibrated with TBS.
2. Wash the column with 150 ml of TBS.
3. Elute bound Fn with TBS containing 0.3 M NaCl (Fig. 2b).
4. Dialyze Fn at 4 °C against 5 l of TBS.
5. Determine the concentration of purified protein by UV measurement at 280 nm, based on a value of 12.8 for the absorbance of a 1% Fn solution. Alternatively, protein concentration can be determined by BCA protein assay kit, using bovine serum albumin as reference protein.
6. Purified Fn can be aliquoted and stored in polyethylene tubes at 4 °C for several weeks without apparent degradation. For long-term storage, freeze aliquots in containers at −20 °C or colder. To thaw Fn samples, place the containers at room temperature with occasional agitation. Where formation of cryoprecipitates is observed in the Fn solution, it is advisable to subject the Fn solution to centrifugation (10,000 *g* for 10 min).

3.3 Culturing of Human Osteosarcoma Fibroblasts MG-63 Cell Line

Grow MG-63 fibroblast cell line in Eagle minimum essential medium supplemented with 10% heated (56 °C, 30 min) fetal bovine serum, nonessential amino acids, penicillin (200 U/ml), and streptomycin (100 µg/ml) using standard cell culture methods.

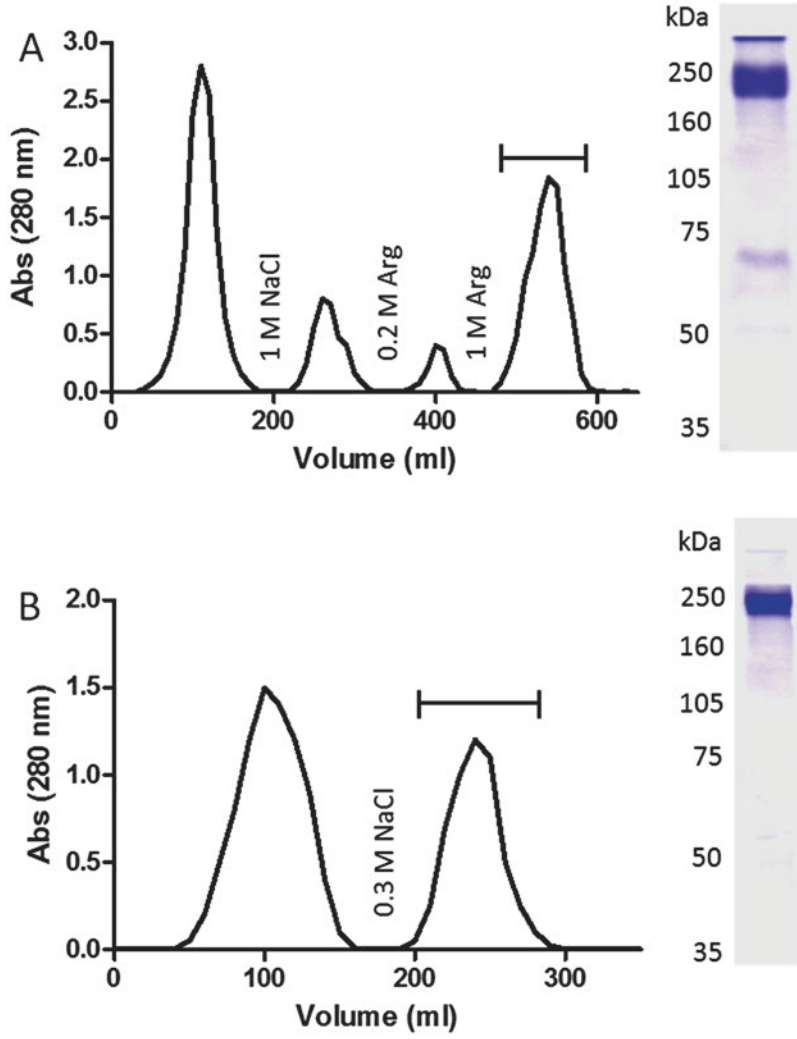

Fig. 2 Purification steps of fibronectin from human plasma. (**a**) Chromatography on gelatin-Sepharose column. Human plasma is loaded on gelatin-Sepharose column. After removing nonspecifically adsorbed proteins to the slurry by washing with equilibrating buffer, the column is sequentially washed with 1 M NaCl and 0.2 M arginine. Fn is eluted from the column with 1 M arginine. (**b**) Affinity chromatography on arginine-Sepharose column. The material obtained from gelatin-Sepharose column is dialyzed and after concentration by ultrafiltration applied to a column of arginine-Sepharose. The column is washed with equilibrating buffer and fibronectin eluted from the gel with 0.3 M NaCl. The pooled fraction are dialyzed and quantitated for protein content. In the *insets*, the 7.5% SDS-PAGE analysis under reducing conditions of Fn eluted with 1 M arginine from gelatin-Sepharose column (**a**) and with 0.3 M NaCl from arginine-Sepharose (**b**) column is reported. On the *left* of each gel, molecular masses (in kDa) of standard proteins are indicated

3.4 Purification of EDA (+) Fn

EDA(+) Fn can be purified on IST-9 mouse monoclonal antibody conjugated to Sepharose 4B Fast Flow from the culture medium of human osteosarcoma fibroblasts MG-63 cell line.

1. Prepare a 1–2 ml column of anti-Fn monoclonal antibody IST-9 coupled to Sepharose 4B.

2. Equilibrate the column with 10 ml TBS containing 1 mM PMSF and 0.02% (w/v) sodium azide.
3. Clear 100 ml of conditioned cultured medium by centrifugation at 5,000 *g* for 5 min.
4. Collect supernatant and pour it onto the equilibrated column.
5. Wash the column with TBS until no material appears in the eluent, as monitored by absorption at 280 nm (*see* **Note 12**).
6. Elute EDA(+) Fn from the affinity column with 10 ml elution buffer.
7. Dialyze against 2 l TBS containing 0.5 mM PMSF at 4 °C for 24 h.
8. Store the dialysate at −20 °C.

3.5 Isolation of Plasma Fn Fragments by Ion-Exchange Chromatography Column

Plasma Fn can be cleaved into five domains [20], and we refer to these discrete domains as N-terminal heparin/fibrin-binding domain-1 (HBD-1/FBD-1), gelatin-binding domain (GBD), cell-binding domain (CBD), heparin-binding domain-2 (HBD-2), and C-terminal fibrin-binding domain-2 (FBD-2).

3.5.1 Digestion of Fibronectin

1. Digest approximately 40 mg of intact fibronectin at 1 mg/ml with 2.5 μg/ml thermolysin at room temperature for 4 h in thermolysin digestion buffer.
2. Add 200 mM EDTA stock solution (to 5 mM final concentration) to terminate the digestion. This treatment inactivates thermolysin [20].

3.5.2 Fractionation of Fibronectin Proteolytic Fragments on Hydroxyapatite Chromatography Column

Fractionation of Fn fragments is performed as previously reported by Zardi et al. [19].

1. Hydrate 20 g of dry powder of Bio-Gel hydroxyapatite with 120 ml of 0.5 mM sodium phosphate buffer, pH 6.8, containing 0.1% (w/v) sodium azide with gentle swirling. Avoid the use of magnetic stir bars or stirring rods, as these will damage the powder.
2. Allow the slurry to settle for 30 min at room temperature.
3. Decant the fine particles present at the top of the settled bed.
4. Degas the suspension under vacuum and pack the gel in a 20 × 1.6 cm glass column.
5. Equilibrate the column with gel equilibrating buffer.
6. Dialyze the total thermolysin Fn digest (~ 40 mg in 40 ml thermolysin digestion buffer) against 4 l of gel equilibrating buffer at 4 °C for 48 h.
7. Load the whole fibronectin thermolysin onto the hydroxyapatite column.

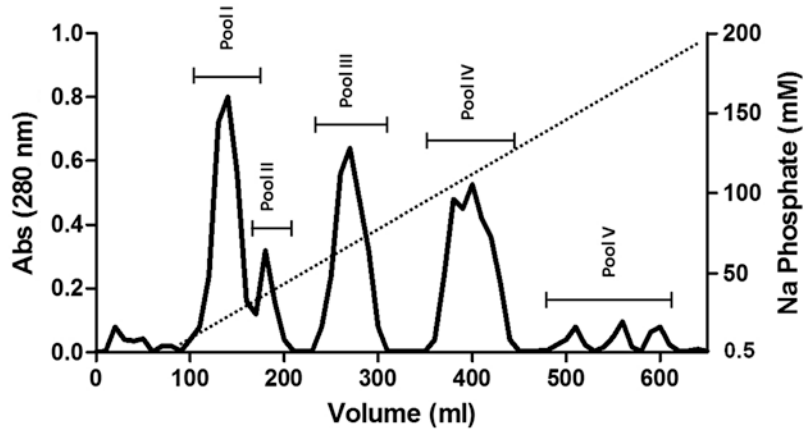

Fig. 3 Elution profile of thermolysin digest of Fn from hydroxyapatite chromatography column. Forty milligrams of Fn subjected to thermolysin digestion and equilibrated with 0.5 mM Na phosphate buffer, pH 6.8, are loaded onto a chromatography column of hydroxyapatite. Fragments of Fn were sequentially eluted with a linear Na phosphate (0.5–200 mM) gradient. Five main peaks are observed in the eluate of hydroxyapatite column, and the pooled fractions were analyzed for molecular weight on a 4–20% SDS-PAGE gradient. The main pooled factions are pool I, 40 kDa; pool II, 20 kDa; pool III, 110 kDa; pool IV, a mixture of 150 + 28 kDa polypeptides; and pool V, 38/30 kDa

8. Wash the column with 60 ml of the above buffer.
9. Elute bound fragments with 600 ml of a linear gradient of 0.5–200 mM sodium phosphate buffer, pH 6.8, at room temperature with a flow rate of 16 ml/h.
10. Check the concentration of the fragments in the eluent buffer by absorbance at 280 nm measurements (Fig. 3).

3.5.3 Purification of Fibronectin Binding Domains by Gel Filtration Chromatography

Each pool obtained from the ion-exchange chromatography of hydroxyapatite can be further fractionated by gel filtration chromatography as follows:

1. Suspend Bio-Gel P-30, Superdex 75, and Sephadex G-150 Superfine slurries in gel equilibration buffer. Fine particles can be removed by decantation.
2. Degas the suspensions under vacuum.
3. Mount 90 × 2 cm columns on a laboratory stand, and pour the well-mixed gel suspension into the vertical mounted column using a glass road. All the gel required should be poured in a single step.
4. Load the individual pools on the appropriate column and elute with the gel equilibration buffer.
5. Pool I (40 kDa, gelatin-binding domain) is purified on Superdex 75 (Fig. 4a).
6. Pool II (20 kDa, fibrin-binding domain) is fractionated on Bio-Gel P30 chromatography column (Fig. 4b).

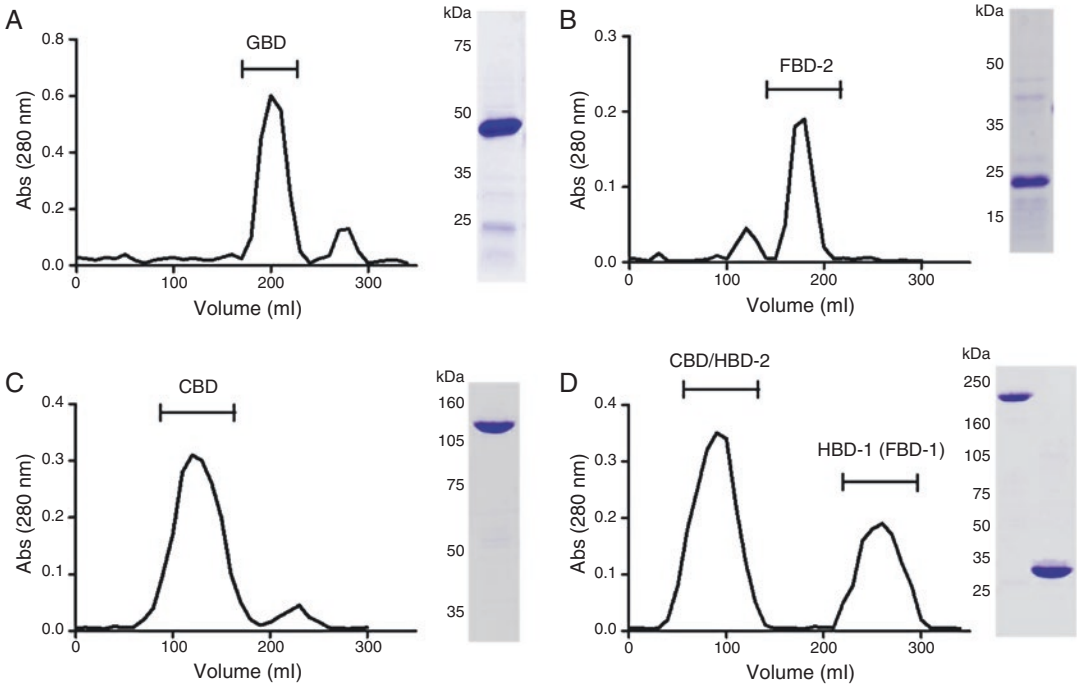

Fig. 4 Purification of fibronectin fragments by gel filtration chromatography. (**a**) Elution profile of hydroxyapatite pool I from a Superdex 75 chromatography column (100 × 2.5 cm). (**b**) Elution profile of hydroxyapatite pool II obtained from a Bio-Gel P30 chromatography column. (**c**) Elution profile of hydroxyapatite pool III resulting from a Sephadex G-150 Superfine chromatography column. (**d**) Elution profile of hydroxyapatite pool IV from a Superdex 75 chromatography column. Pooled fractions obtained by gel filtration chromatography columns were concentrated, analyzed for protein content, and subjected to 10% SDS-PAGE under unreduced conditions (*insets*). Each fragment is also identified by ELISA on the basis of the binding properties for specific ligands (*see* Table 1)

7. Pool III (110 kDa, cell-binding domain) is purified on Sephadex G-150 Superfine chromatography column (Fig. 4c).
8. Pool IV loaded on a Superdex 75 chromatography column separate 150 kDa Cell/hep-2 region from a 29 kDa fragment (Fig. 4d).
9. The heparin-binding domain-2 (HBD-2) (pool V) elutes as a pure polypeptide directly from the hydroxyapatite column and does not need to be further fractionated by gel filtration chromatography.

3.6 Analysis of Fn and Fn Fragments by SDS-PAGE

The purity of all preparations (full-length Fn and Fn fragments) is checked by electrophoresis analysis, using SDS-PAGE.

1. Pour a gel-casting system with a 7.5% (for full length Fn) or 12% (for Fn fragments) acrylamide separating gel and a 4–5% acrylamide stacking gel.

2. Remove the well-forming comb and fill the sample wells with running buffer. Place the gel into the electrophoresis unit and fill the unit with 900 ml of running buffer.
3. Mix 10 μg (at 1 μg/μl) of the appropriate fraction obtained from purification steps with 10 μl reducing loading buffer, and incubate at 99 °C for 3 min in a water bath, and centrifuge for 20 s at 10,000 *g* on a benchtop centrifuge.
4. Load the samples into the wells of the stacking gel within the running buffer.
5. Load 5 μl of molecular weight standard into one lane.
6. Close the tank with its lid, and carry out the electrophoresis at a constant voltage of 100 V for 10 min to allow uniform penetration of the samples in the separating gel.
7. Increase the voltage to 125 V until the dye front reaches the bottom of the gel.
8. Turn off the power; disconnect the cassette from the electrodes.
9. Gently remove the gel from the glass plates and transfer into Coomassie blue stain to visualize protein bands. Stain under gentle agitation for 30 min.
10. Discard the stain and wash 3–4 times with tap water for 10 min each.
11. Destain by immersing gel in 20 ml destaining solution. Maintain gentle agitation until the background staining is reduced and the protein bands appear clear.
12. Transfer gel into tap water for storage.

3.7 ELISA of Purified Fibronectin and Fragments

To validate the absence of trace amounts of fibrinogen and/or plasminogen in the final purified Fn preparation, the arginine-Sepharose purified Fn samples are subjected to ELISA-type assay.

3.7.1 Assessment of Fn Purity

1. Coat microtiter wells with 1 μg/well of isolated Fn in immobilization buffer.
2. Prepare standards in parallel: one "zero" well with immobilization buffer alone and five additional wells with pure Fn (range between 50 and 1,000 ng/well).
3. Incubate the plate overnight at 4 °C.
4. Discard the coating solution by reverting the plate, and wash the plates with 200 μl/well of PBST. Discard the washing solution. Repeat wash for a total of three washes.
5. Block additional protein-binding sites in the wells by treatment of the plates with 200 μl/well of blocking solution for 1 h at room temperature.
6. Discard blocking solution.

7. Incubate the plates for 1 h with 100–200 μl of 5 μg/ml primary anti-Fn, anti-fibrinogen, or anti-plasminogen antibody.
8. Discard antibody and wash the plates three times with PBST.
9. Incubate the plates for 1 h with 100 μl of 1:1000 dilution HRP-conjugated secondary antibody.
10. Discard antibody and wash the plates three times with PBST.
11. Add 100 μl of OPD solution to each well and follow development of yellow color.
12. When a suitable color has developed (~1–5 min), stop the enzymatic reaction by adding 100 μl of stop solution.
13. Quantify the binding of the secondary antibody measuring the absorbance at 490 nm in an ELISA plate reader.
14. Use the standard curve to translate OD value to Fn concentration.

3.7.2 Identification of the Isolated Fn Fragments by Assessment of the Binding Properties

Single isolated pools obtained by the combination of hydroxyapatite chromatography and gel filtration chromatography columns are analyzed for the binding to surface-coated ligands heparin, gelatin, fibrin (*see* **Note 14**), and $\alpha_5\beta_1$ integrin as it follows:

1. Coat microtiter wells with 1 μg/well of the appropriate Fn ligand diluted at 10 μg/ml in immobilization buffer at 4 °C overnight.
2. Wash the plates with 200 μl/well of PBST.
3. Block additional protein-binding sites in the wells by treatment of the plates for 1 h at room temperature with blocking solution.
4. Discard blocking solution.
5. Incubate the plates for 1 h with 1 μg of each isolated Fn fragment (typically in 100 μl).
6. Discard the samples and wash the plates with 200 μl PBST per well.
7. Add 100–200 μl of 5 μg/ml anti-Fn primary antibody in 1% (w/v) BSA to the wells, and incubate for 90 min at room temperature.
8. Wash the plates with PBST.
9. Add 100 μl of 1:1,000 dilution HRP-conjugated secondary antibody, and incubate for 1 h at room temperature.
10. The quantification of the secondary antibody binding to the wells can be determined as reported in **step 13** of Subheading 3.7.1.
11. On the basis of molecular weight determined by SDS-PAGE and the binding properties determined by ELISA (Table 1), a correlation between the isolated pools and the identity of each purified fragment can be established (*see* **Note 15**).

Table 1
Analysis of binding properties of Fn fragments

	Ligand			
Fn fragment	**Heparin**	**Gelatin**	**α5β1**	**Fibrin**
GBD		+		
CBD			+	
CBD/HBD-2	+		+	
HBD-1 (FBD-1)	+			
FBD-2				+
HBD-2	+			

Microtiter wells are coated overnight at 4 °C for 1 μg of the heparin, gelatin, α5β1 integrin in 0.1 M sodium carbonate, and pH 9.5. For binding analysis of Fn fragments to fibrin, fibrinogen was immobilized and cleaved with thrombin as reported in **Note 14**. After washing with 0.5% Tween 20 in PBS (PBST), the wells are treated for 1 h at 22 °C with 2% BSA dissolved in PBS. The wells are then incubated with the 1 μg of the appropriate Fn fragment. After several washings with PBST, 1 μg of anti-Fn rabbit IgG in BSA is added to the wells and the mixture incubated for 90 min. After washing, the plates are incubated for 1 h with goat HRP-conjugated anti-rabbit IgG. After washing, o-phenylenediamine dihydrochloride is added to the wells and the absorbance at 490 nm determined using an ELISA plate reader

4 Notes

1. Human blood is potentially hazardous, so treat any sample according to health and safety standards.
2. Human plasma can be obtained by centrifugation of whole blood. The usual starting material for Fn purification is outdated human plasma, although the fresh citrated plasma is better in order to avoid some degree of proteolysis. To prevent Fn degradation, plasma should be stored at −20 °C, and all purification steps have to be carried out at 4 °C. Protease inhibitors such PMSF should be also included during the process of purification, particularly in the early steps of purification when proteases are still present in the crude preparations. It is recommended to use as starting material citrated plasma (15 ml CPD for 100 ml of blood) which has been supplemented with PMSF (add 1/200 of blood volume of 0.2 M stock solution to the plasma with stirring to reach a final PMSF concentration of 1 mM).
3. To prepare and solubilize porcine skin gelatin, wash 100 mg of dry gelatin with several changes of distilled water at 4 °C over a period of 24 h, with occasional shaking and a final centrifugation at 1,000 *g* for 10 min. Then, wash the gelatin for 1 h

with 3 M NaCl solution containing 10 mM EDTA, followed by three washes with distilled water. The protein is finally solubilized in gelatin-dissolving solution and by heating at 65 °C under continuous and vigorous stirring for 1 h. The concentration of the proteins is estimated using a value for absorbance at 235 nm of $E_{1mg/ml} = 0.8$.

4. EDTA is difficult to dissolve when the pH is below 7.0. To ensure an easy dissolution, mix 29.22 g of EDTA and 12.5 g of NaOH pellets in 500 ml of deionized water, which yield a 200 mM solution, pH 7.8.
5. Ethanol, methanol, and propanol are highly flammable. Store and handle according to appropriate health and safety requirements
6. Sodium azide (NaN_3) is very toxic. If contact with skin occurs, wash immediately with tap water. The stock solution (0.2%, w/v) should be prepared under ventilated hood.
7. Anti-Fn antibody IST-9 is a monoclonal antibody raised against a region in the EDA domain of human cellular fibronectin.
8. It is important to ascertain the origin and health of the cell line. Purchasing a new vial at a cell culture depository (such as the American Tissue Culture Collection (ATCC)) and keeping track of passages will ensure good cell culture practices.
9. OPD tablets are light sensitive. Store the vial containing the tablets at 4 °C protected from light with the lid tightly closed.
10. Repeated freezing and thawing of antibody solutions reduces the activity of the proteins. Thawed solutions that are not completely used can be kept at 4 °C for several weeks.
11. Glacial acetic acid is strongly corrosive and may cause severe burns. Ensure safety glass or face mask and nitrile gloves are worn.
12. To wash the gel, resuspend in appropriate buffer, homogenize, and centrifuge at 1,000 *g* for 5 min.
13. Due to the tendency of gelatin-Sepharose column to gel in the cold, it is advisable that this step is performed at room temperature.
14. To analyze the binding of purified fragments to immobilized fibrin, microtiter 96-well plates are coated with 100 µl of 0.34 g/l fibrinogen for 1 h at room temperature. Fibrin clot in the wells is generated by adding 0.05 U/ml thrombin in 20 mM Tris–HCl, pH 7.4, 150 mM NaCl, and 5 mM $CaCl_2$ overnight at room temperature.
15. The identification of the isolated domains can be also achieved assessing the immunological reactivity of each fragment for specific monoclonal or polyclonal antibodies, where available, by ELISA-type assay.

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