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Purification of Fibronectin from Human Plasma by Affinity Chromatography under Non-Denaturing Conditions

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Fibronectin was purified from human plasma by affinity chromatography under non-denaturing conditions. The method was based on the previously known binding of fibronectin to gelatin. The novel features of our method are the use of arginine in the elution of fibronectin from immobilized gelatin [Vuento & Vaheri (1978) *Biochem. J.* **175**, 333–336] and the use of arginine–agarose as second affinity step. The purified protein was homogeneous as judged by polyacrylamide-gel electrophoresis, analytical ultracentrifugation and two-dimensional immunoelectrophoresis. The yield was 60%. We propose that the method would be useful in preparation of fibronectin for studies on its biological activities, where it is important that the protein is obtained in a native state.

Fibronectin [also known as 'LETS protein', 'cold-insoluble globulin'; see recent reviews by Vaheri & Mosher (1978) and Yamada & Olden (1978)] is a glycoprotein with a mol.wt. of 450000. The concentration of fibronectin in human plasma is about 300mg/litre. Fibronectin interacts with heparin (Stathakis & Mosesson, 1977) and fibrin in the cold (Ruoslahti & Vaheri, 1975) and binds to collagen (Engvall & Ruoslahti, 1977; Dessau *et al.*, 1978). During blood-clotting, it may be cross-linked to fibrin by transglutaminase (Mosher, 1976), and it is a substrate for the plasma proteinases plasmin (Jilek & Hörmann, 1977) and kallikrein (Iwanaga *et al.*, 1978). Fibronectin is also present in connective tissue and in the pericellular matrix of fibroblasts grown *in vitro*, possibly bound to other macromolecules (Linder *et al.*, 1978; Hedman *et al.*, 1978). It has been recently suggested that fibronectin might have a functional role in the reticuloendothelial system as an opsonizing protein that enhances the uptake of collagenous particles by phagocytic cells (Blumenstock *et al.*, 1978).

To study the various molecular interactions of fibronectin, a method for purification of native fibronectin with high yield is important. Fibronectin has been purified from human plasma by methods combining precipitation steps, molecular-exclusion chromatography and ion-exchange chromatography (Mosesson & Umfleet, 1970; Mosher, 1975; Chen & Mosesson, 1977). These methods are generally time-consuming and the yield of purified protein is low. Fibronectin has also been purified by affinity chromatography on immobilized antibody (Vuento *et al.*, 1977) or on immobilized gelatin (Engvall & Ruoslahti, 1977; Dessau *et al.*, 1978). These methods appeared to have some advantages, but strongly denaturing elution buffers were used. We have found that complexes between fibronectin and gelatin can be dissociated with arginine at pH 7.5 (Vuento & Vaheri, 1978), and, on the basis of this finding, describe in the present paper a non-denaturing affinity-chromatography method for purification of fibronectin from human plasma. A second affinity-chromatography step on arginine–agarose was used to complete the purification scheme.

Abbreviation used: SDS, sodium dodecyl sulphate.

Experimental and Results

Materials

All reagents used were of analytical grade and obtained from commercial suppliers unless otherwise indicated. We used pooled citrate-treated human plasma, obtained from Finnish Red Cross Transfusion Service, Helsinki, Finland; benzamidine was added to a concentration of 5mM and the plasma samples were centrifuged at 20000*g* for 1h to remove any insoluble materials.

Preparation of affinity columns

Sepharose 4B gel (Pharmacia, Uppsala, Sweden) was activated with CNBr by the method described for 'intermediately activated 1–8% agarose gels' (Porath & Kristiansen, 1975). Gelatin (type I; Sigma, St. Louis, MO, U.S.A.) was coupled to the activated gel in 0.1M-$NaHCO_3$/1M-NaCl by incubation of an excess of gelatin (1mg/ml) with the gel with mixing at 22°C for 16h. Approx. 0.5–1.0mg of gelatin was

bound per ml of gel, as judged from the A_{280} of the gelatin solution before and after incubation with the activated gel. Gelatin–Sepharose was washed with an acidic (0.1M-sodium acetate, pH4.5) and basic buffer (0.1M-Tris/HCl, pH8.5), and before use it was additionally washed with 1M-arginine, pH7.5. Arginine–Sepharose was prepared by incubating the activated gel with excess of 1M-arginine, pH7.5, with mixing at 22°C for 16h. The gel was washed with acidic and basic buffers as above. Non-substituted Sepharose 4B gel was used after a thorough washing with 0.05M-Tris/HCl buffer, pH7.5. After its use for removing agarose-binding proteins from plasma, it was regenerated by washing with 8M-urea.

Determination of fibronectin

Fibronectin was determined by a solid-phase 'sandwich' enzyme immunoassay as described by Engvall & Ruoslahti (1977), with the modifications that antibody-coated polystyrene tubes were used instead of microtitre plates, and incubations of the coated tubes with sample and with labelled antibody were carried out at 20 instead of 4°C. Purified fibronectin was used as a standard.

Determination of protein

Protein was determined by the method of Lowry *et al.* (1951). Concentrations of solutions of purified fibronectin were determined by assuming $A^{1\%}_{1cm,\ 280}$ 12.8 (Mosesson *et al.*, 1975).

SDS/polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis in the presence of SDS was carried out in a slab-gel apparatus by using a discontinuous buffer system (Laemmli, 1970). The acrylamide concentration of the stacking gel was 3.3% (w/v). In the running gel a linear polyacrylamide gradient of 5–15% (w/v) was used. The samples were reduced by incubation with 5% (v/v) of 2-mercaptoethanol at 96°C for 4min. The gel slabs were stained with Coomassie Blue. The molecular-weight markers were reduced plasma fibronectin (mol.wt. 220000), α_2-macroglobulin (170000), phosphorylase *a* (93000), human serum albumin (68000), ovalbumin (43000) and pancreatic ribonuclease (13800).

Immunoelectrophoresis

Two-dimensional immunoelectrophoresis was carried out by the method of Clarke & Freeman (1968) with 2% agarose in sodium barbiturate buffer, pH8.6 (*I* 0.02). In the second dimension, the gel contained 10% (v/v) of a polyspecific anti-human serum (Orion Diagnostica, Helsinki, Finland). Anti-(human fibronectin) serum (3%, v/v) was included, since the above serum reacted only weakly with fibronectin. The specificity of the anti-fibronectin serum has been documented (Hedman *et al.*, 1978).

Analytical ultracentrifugation

Sedimentation-velocity analyses were performed at 20°C in Beckman model E analytical ultracentrifuge equipped with either a photoelectric-scanning system or schlieren optics. For calculation of the sedimentation coefficient, the partial specific volume of the protein was assumed to be 0.72ml/g (Mosesson *et al.*, 1975).

Amino acid analysis

Amino acid composition was determined with a Beckman model 120C amino acid analyser after hydrolysis with 6M-HCl at 110°C for 24h. In some experiments the samples were oxidized with performic acid before hydrolysis (Hirs, 1956), and cysteine and methionine were determined as cysteic acid and methionine sulphone respectively. Tryptophan was calculated from the known tyrosine/tryptophan ratio (Vuento *et al.*, 1977).

Purification procedure

All operations were carried out at 22°C, except the dialysis steps, which were done at 4°C. The plasma sample (500ml) was applied to a column (8cm × 20cm) of Sepharose 4B equilibrated with 0.05M-Tris/HCl, pH7.5, containing 5mM-benzamidine and 0.02% (w/v) sodium azide. The column was eluted with this buffer, and proteins not bound to Sepharose were applied on a column (4cm × 12cm) of gelatin–Sepharose equilibrated with the above buffer. Gelatin–Sepharose was first washed with the equilibrating buffer (1000ml), then with 1M-NaCl in the above buffer (1000ml) and finally with 0.2M-arginine buffered with 0.05M-Tris/HCl, pH7.5 (400ml). Fibronectin was eluted with 1M-arginine in 0.05M-Tris/HCl, pH7.5. Fractions of 50ml were collected, and those containing fibronectin were pooled and dialysed extensively against 0.05M-Tris/HCl, pH7.5. After dialysis the fibronectin solution (150ml) was centrifuged at 10000*g* for 20min to remove possible precipitates, and applied on a column (2.5cm × 10cm) of arginine–Sepharose equilibrated with 0.05M-Tris/HCl, pH7.5. After washing the arginine–Sepharose column with the equilibrating buffer (250ml), fibronectin was eluted with 0.1M-NaCl in the above buffer. When used repeatedly, the capacity of the columns of gelatin–Sepharose and arginine–Sepharose to bind fibronectin gradually decreased. This was apparently due to a binding to the columns of protein material, which was not eluted by the elution buffers used. The same column of gelatin–Sepharose could

be successfully used in ten purification operations, and a column of arginine–Sepharose in five purifications, after which new columns were prepared.

Removal of proteins binding to the affinity matrix

The SDS/polyacrylamide-gel electrophoresis pattern of plasma did not change appreciably when plasma was passed through Sepharose 4B (Figs. 1*a* and 1*b*). Fractions eluted from Sepharose gel during its regeneration with 8M-urea showed a polypeptide pattern different from that of plasma (Fig. 1*c*). The pattern indicated that several plasma components, but not albumin, bound to Sepharose. Fibronectin assays showed that 3–5% of the fibronectin present in plasma were retained non-specifically by the Sepharose gel.

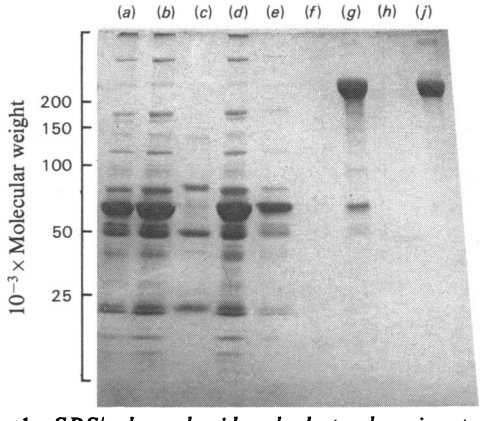

Fig. 1. *SDS/polyacrylamide-gel electrophoresis at the different stages of the purification procedure*
(*a*) Human plasma, 0.5 μl (17.5 μg of protein); (*b*) plasma passed through Sepharose 4B, 1 μl (26 μg); (*c*) material eluted from Sepharose 4B with 8M-urea, 4 μg; (*d*) plasma passed through Sepharose 4B and gelatin–Sepharose, 31 μg; (*e*) material eluted from gelatin–Sepharose with 1M-NaCl, 22.5 μg; (*f*) material eluted from gelatin–Sepharose with 0.2M-arginine, 3 μg; (*g*) material eluted from gelatin–Sepharose with 1M-arginine, 18 μg; (*h*) material from partially purified fibronectin preparation not binding to arginine–Sepharose, 2.5 μg; (*j*) fibronectin eluted from arginine–Sepharose with 0.1M-NaCl, 20 μg. Migration is from top to bottom. The scale on the left extends from the top of the running gel to the dye front.

Adsorption of gelatin-binding proteins to gelatin–Sepharose

A polypeptide band corresponding to fibronectin was strongly reduced in plasma after it was passed through gelatin–Sepharose. In the SDS/polyacrylamide-gel electrophoresis of plasma samples, two closely spaced polypeptide bands were seen in the 220000-dalton area. These are not clearly resolved in Fig. 1. After passage of the plasma through gelatin–Sepharose, the slower-migrating band was lost. No other major polypeptide bands were significantly decreased. However, the resolution of the electrophoresis method used did not allow for detection of possible binding of minor plasma components to gelatin–Sepharose. Under the experimental conditions used, about 99% of immunoreactive fibronectin was bound to gelatin–Sepharose.

Elution of fibronectin from gelatin–Sepharose by arginine

Washing of the gelatin–Sepharose column with 1M-NaCl resulted in the elution of several polypeptides, but very little fibronectin was eluted with this buffer (Fig. 1*e*). Subsequent washing with 0.2M-arginine buffered with 0.05M-Tris/HCl, pH7.5, removed some minor polypeptides, but again only very little fibronectin was eluted (Fig. 1*f*). Fibronectin was eluted from gelatin–Sepharose with 1M-arginine. The protein emerged from the column with the front of the buffer containing arginine. It eluted as a tailing peak. Usually 75–80% of fibronectin applied to the column was recovered in 50ml. Part of the protein bound to the column so strongly that it could not be eluted by the buffer used. As

Table 1. *Purification of fibronectin from human plasma*
The results from ten purification operations are summarized below.

Step	Volume (ml)	Protein (mg)	Fibronectin (mg)	Purity (%)	Yield (%)
Plasma	500	35×10^3	125	—	100
Sepharose 4B	1000	35×10^3	120	—	96
Gelatin–Sepharose (elution by 1M-arginine)	150	95	90	95*	76
Arginine–Sepharose	50	77	77	100†	61

* Estimated from SDS/polyacrylamide-gel electrophoresis.
† Estimated from SDS/polyacrylamide-gel electrophoresis, immunoelectrophoresis and analytical ultracentrifugation.

determined from the SDS/polyacrylamide-gel electrophoresis, the preparations were about 95% pure at this stage (Fig. 1*g*).

In some experiments we used an elution with a gradient of 0–2M-arginine in 0.05M-Tris/HCl, pH7.5. Fibronectin was eluted as a broad peak starting at about 0.4M-arginine. The purification was not significantly more effective than when stepwise elution with 1M-arginine was used.

Chromatography of fibronectin on arginine–Sepharose

When partially purified fibronectin was applied on a column of arginine–Sepharose, the protein was

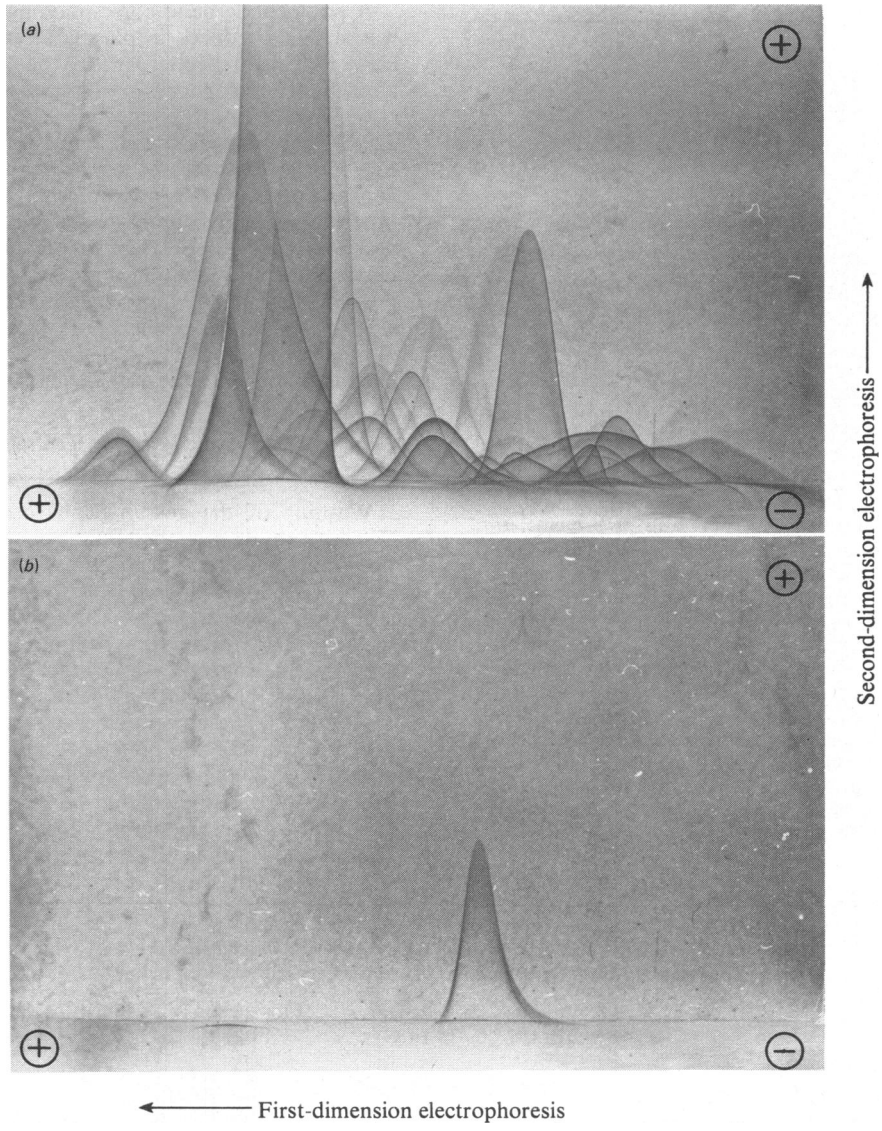

Fig. 2. *Two-dimensional immunoelectrophoresis of normal human plasma* (*a*) *and of purified fibronectin* (*b*)
In the first dimension the samples, 3μl of plasma or 3μg of purified fibronectin, were electrophoresed in agarose for 100min at 180V (anode at the left). The sample well is seen as a white circle on the right. In the second dimension the proteins were electrophoresed for 18h at 50V (anode at the top) into gel containing antisera against normal human serum and fibronectin. The purified preparation gives a single precipitation arc.

effectively bound to the column. Washing with the equilibrating buffer (0.05M-Tris/HCl, pH7.5) resulted in a slow release of protein material that consisted of contaminants together with trace amounts of fibronectin (Fig. 1*h*). Fibronectin typically constituted 10–20% of the protein content of these fractions. Fibronectin was eluted from the column as a sharp peak with 0.1M-NaCl in 0.05M-Tris/HCl, pH7.5. Further increase in the ionic strength of the elution buffer did not bring out significantly more protein. The recovery of protein from the arginine–Sepharose column was usually nearly complete. The recovery of fibronectin in the peak eluting with 0.1M-NaCl was 80–90%. Table 1 summarizes the results from ten separate purification operations. We typically obtained 70–80mg of fibronectin from 500ml of plasma, which corresponds to a yield of about 60%.

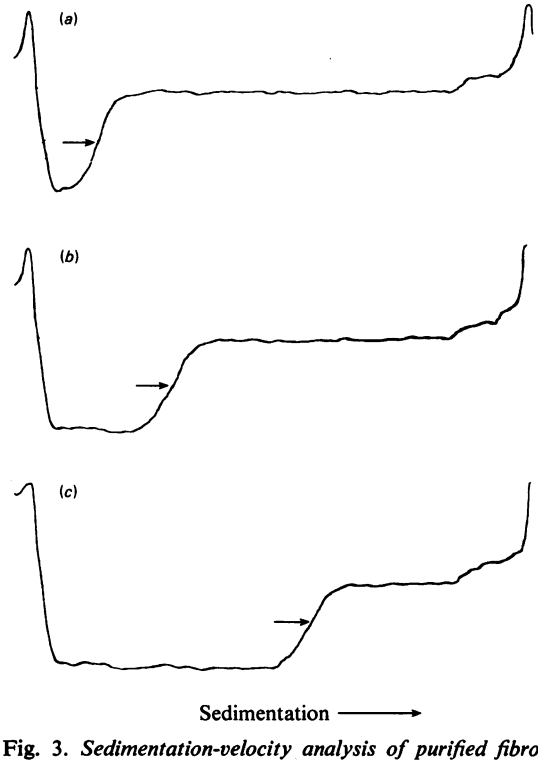

Fig. 3. *Sedimentation-velocity analysis of purified fibronectin*

Fibronectin (0.5mg/ml in 0.05M-Tris/HCl, pH7.5) was analysed in a Beckman model E analytical ultracentrifuge at 20°C. The scans shown were made at 8 (*a*), 20 (*b*), and 40 (*c*) min after reaching a constant rotor speed of 44770rev./min. The direction of sedimentation is from left to right. A single symmetrical sedimenting boundary corresponding to a sedimentation coefficient ($s_{20,w}$) of 15.0S is shown by the arrows.

Homogeneity and properties of the purified protein

In SDS/polyacrylamide-gel electrophoresis with high loads of protein, the purified fibronectin showed a single polypeptide band, indicating size homogeneity of the reduced preparation (Fig. 1*j*). On immunoelectrophoresis (Fig. 2) the purified protein formed a single immunoprecipitation arc with a mixture of antibodies against fibronectin and human serum proteins. Thus a single antigenic species was detected in the preparation. Sedimentation-velocity analysis of the purified protein showed a single symmetrical concentration gradient (Fig. 3). The sedimentation coefficients ($s_{20,w}$) of fibronectin measured in 0.05M-Tris/HCl, pH7.5, at protein concentrations of 0.5mg/ml and 3mg/ml were 15.0 and 14.7S respectively. The amino acid composition of purified fibronectin is shown in Table 2.

The purified protein was usually stored in concentrations of 1–3mg/ml in glass vials at 4°C in 0.05M-Tris/HCl, pH7.5, containing 0.1M-NaCl and 0.005% (w/v) sodium azide. Fibronectin stayed in solution without difficulty for periods up to 1 month under

Table 2. *Amino acid composition of fibronectin purified from human plasma*

The analyses were performed after hydrolysis with 6M-HCl at 110°C for 24h. Values for tryptophan were calculated assuming the ratio of tyrosine to tryptophan to be 1.6. Cysteine and methionine were determined as cysteic acid and methionine sulphone respectively, after performic acid oxidation and acid hydrolysis of the protein. The results are expressed as mol % and represent a mean value from five analyses (I). The values in columns II and III are from Vuento *et al.* (1977) and Mosesson *et al.* (1975) respectively, and represent amino acid compositions of fibronectin purified from plasma by other methods.

	Composition (mol %)		
Amino acid	I	II	III
Tyr	3.8	4.3	4.5
Phe	2.3	2.6	2.7
Trp	2.4	2.7	2.8
Lys	3.6	4.4	3.7
His	1.9	1.8	2.1
Arg	5.2	4.5	5.2
Asx	9.8	10.1	9.3
Thr	10.0	10.4	9.7
Ser	6.2	8.6	6.8
Glx	12.5	11.8	11.6
Pro	8.0	7.0	7.6
Gly	8.4	8.3	8.0
Ala	4.3	5.1	4.3
Cys	2.7	2.1	2.6
Val	8.3	6.0	8.1
Met	1.2	1.1	1.1
Ile	4.3	3.1	4.4
Leu	5.3	6.2	5.7

these conditions. Fibronectin was quite stable at 4°C, even in 5mM-Tris/HCl, pH7.5. Freezing and thawing of fibronectin solutions resulted in loss of material as fibrous aggregates. These aggregates also formed when solutions of fibronectin were passed over various surfaces, e.g. when transferring fibronectin solutions by means of glass pipettes. Dialysis against distilled water resulted in heavy precipitation, and if freeze-dried, fibronectin could only partially be redissolved in neutral iso-osmotic buffers, but better in, e.g., 0.1M-NaOH.

Discussion

It has been established by other workers that fibronectin represents a major fraction of gelatin-binding proteins of human plasma (Engvall & Ruoslahti, 1977; Dessau *et al.*, 1978). In the present experiments we used our previous finding that fibronectin–gelatin complexes can be dissociated by specific cations to develop a non-denaturing affinity-chromatography method for purification of fibronectin from plasma. We consider that this is an important improvement to the previously published methods (Engvall & Ruoslahti, 1977; Dessau *et al.*, 1978) using gelatin–agarose. Since fibronectin is a large and apparently structurally complex molecule, its exposure to agents such as urea or chaotropic ions is likely to give rise to denaturation, which would make studies on the biological activities on fibronectin difficult.

When eluting fibronectin from gelatin–Sepharose with 1M-arginine, pH7.5, we obtained a recovery of 75–80% of fibronectin bound to the column from plasma. At this purification stage the preparations were about 90–95% pure fibronectin; the rest was made up by a heterogeneous group of proteins (Fig. 1*g*), some of which may represent other gelatin-binding proteins. Preliminary experiments showed that these contaminants are immunogenic, giving rise to multispecific antisera when rabbits were immunized with fibronectin purified by gelatin–Sepharose only. These contaminants were effectively removed by chromatography on arginine–Sepharose. Fibronectin was bound to the gel, whereas the contaminating proteins were eluted with the equilibrating buffer.

The binding of fibronectin to arginine–Sepharose may probably to a large extent be attributed to ion-exchange effects. In preliminary experiments, fibronectin also bound to lysine–Sepharose and spermidine–Sepharose. Arginine was also tested for its ability to elute fibronectin from arginine–Sepharose at pH7.5; it proved to be no better than NaCl in eluting fibronectin from the gel.

After the final step of chromatography on arginine–Sepharose, the product was homogeneous as judged by SDS/polyacrylamide-gel electrophoresis, immunoelectrophoresis and analytical ultracentrifugation. Several immunizations of rabbits have been carried out with the final product, and these have resulted in the production of monospecific anti-fibronectin antisera. The sedimentation coefficient we obtained is close to that obtained by other workers (Mosesson *et al.*, 1975). The amino acid composition of the purified protein (Table 2) is very similar to that of fibronectin preparations obtained by other purification methods (Mosesson *et al.*, 1975; Vuento *et al.*, 1977).

Our fibronectin preparations remained soluble at 4°C for long periods of time, even when stored in 5mM-Tris/HCl buffer. However, some observations suggested that fibronectin has a peculiar capacity to form a specific type of aggregate. Studies on purified fibronectin by electron microscopy with negative staining have revealed that a minor portion of freshly prepared fibronectin was always present as very thin, long fibrillar polymers (M. Vuento, M. Saraste, C.-H. von Bonsdorff & A. Vaheri, unpublished work). The proportion of these polymers appeared to increase slowly during storage of fibronectin. Bundles of fibrillar polymers having a variable diameter and a length of up to several micrometres were also formed. Interestingly, even larger fibrillar structures, visible by phase-contrast light-microscopy, were observed, especially when solutions of fibronectin were passed over surfaces, e.g. over glass. It is possible that these observations reflect a capacity of fibronectin to form specific fibrillar structures by self-assembly.

By the method described in the present paper, a high degree of purification was achieved together with high yield and without exposure of the protein to denaturing conditions. We propose that the method will prove useful in studies on structure and function of fibronectin where it is necessary to use the native protein.

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