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Note

Biospecific affinity chromatography of plasma fibronectin on cross-linked gelatin microbeads

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Fibronectin (FN) is a high-molecular-mass glycoprotein (M_r=440 000) involved in cell–cell and cell–substrate adhesions, in cell motility and chemotaxis (for a review see ref. 1). It also promotes binding and ingestion of certain materials by macrophages and other reticuloendothelial cells. These biological functions of FN give some idea of the pathological situations in which plasma levels of FN may be altered and may be of clinical importance. Thus plasma FN is increased in some inflammatory diseases, but it is reduced in shock, in general septicaemia, in severe abdominal infections and in malignant tumours [2].

Besides its endogenous occurrence, FN can be used as a drug in situations where its blood level is decreased, and then its plasma level must be monitored so that a proper dosage can be administered [3].

Currently, the methods for FN estimation are based on immunochemical reactions, such as Laurell's immunoelectrophoresis or laser nephelometry of the antibody–antigen complex. Chromatographic techniques have been used mainly for preparative purposes, and their analytical application was hindered by some problems of weak binding during affinity separations [4]. Biospecific affinity chromatography, however, appears to be the method of choice for preparative purposes. Here, the specific binding abilities of FN to collagen [5,6] and fibrin [7] have been explored. Hydrophobic chromatography represents another route for the isolation of FN [8]. Certainly, the most frequent procedure for FN separation from other blood proteins involves binding it to the gelatin–agarose bed [5,6,9]. Bound FN can be eluted by chaotropic agents [9,10] or under milder

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conditions by eluents containing free amino or guanidino groups [6] (for a review see ref. 1), e.g. solutions of spermine, spermidine, putrescine, arginine and various amino sugars. For quantitative purposes, application of chaotropic agents appears most promising, as the elution strength of such solutions is high, yielding good recoveries. Nevertheless, the conventional gelatin–agarose sorbent has the disadvantage of some non-specific binding through the polysaccharide matrix of agarose gel [1].

The aim of this study was to investigate the binding of FN to glutaraldehyde-cross-linked gelatin microbeads with a view to using this sorbent for either the plasma FN assay or for the preparation of large amounts of FN.

EXPERIMENTAL

Materials

All chemicals were of reagent grade and were purchased from Lachema (Brno, Czechoslovakia). Gelatin of commercial grade was obtained from Deutsche Gelatine Fabriken Stoess (Eberbach/Baden, F.R.G.). Sepharose 4B and cyanogen bromide-activated Sepharose 4B were purchased from Pharmacia (Uppsala, Sweden). Fibronectin standard was prepared in the laboratory by gelatin–Sepharose bioaffinity chromatography according to Ruoslahti and Engvall [11]. Outdated human plasma (3.8% sodium citrate) was acquired from the Institute of Haematology and Blood Transfusion (Prague, Czechoslovakia), divided into 5-ml aliquots and stored frozen in glass vials at −18°C. For FN plasma level estimation blood samples were obtained from patients with osteoarthrosis and with rheumatoid arthritis hospitalized in the Rheumatism Research Institute in Prague.

Preparation of gelatin microbeads

Microbeads of cross-linked gelatin (fraction 100–160 μm in diameter) were prepared essentially according to the method described earlier [12]. The modification comprised treatment of glutaraldehyde-cross-linked gelatin microbeads with an excess (ca. ten-fold per volume) of 0.5 *M* glycine buffer (pH 7.5) to eliminate unchanged glutaraldehyde and free aldehyde groups within the gelatin gel matrix. The microbeads were thoroughly washed before use with 6 *M* urea–50 m*M* Tris–HCl (pH 7.5) in order to remove any extractable or unlinked material.

Preparation of gelatin–Sepharose 4B

Gelatin was coupled to cyanogen bromide-activated Sepharose 4B (150 mg per 15 ml of swollen gel) as described in the instruction booklet from Pharmacia [13].

Biospecific affinity chromatography

Biospecific affinity chromatography on glutaraldehyde-cross-linked gelatin microbeads was compared with the classical procedure of Ruoslahti and Engvall [11]. In both approaches a water-cooled column (12 cm × 1.6 cm I.D.) was used. In all experiments the flow-rate was kept constant at 30 ml/h using the Vario-

perpex II-2120 pump from LKB Produkter (Bromma, Sweden). Plasma samples (5 ml) were introduced to the columns immediately after thawing.

Procedure with gelatin microbeads

The column packed with gelatin microbeads prepared as described above was equilibrated with an initial buffer, 1 *M* sodium chloride–50 m*M* Tris–HCl (pH 7.5). After the sample had been loaded, elution with the initial buffer was continued until zero absorbance was achieved (ca. 200 ml). The effluent was monitored with a UV–VIS SP8-200 Pye-Unicam spectrophotometer (Cambridge, U.K.) set to 280 nm. The eluting buffer, 3 *M* urea–1 *M* sodium chloride–50 m*M* Tris–HCl (pH 7.5), was used to remove bound FN from the column.

All buffers contained 0.02% of sodium azide to prevent microbial growth.

Quantitative analysis

The FN peak was collected, and its concentration in the effluent was evaluated by assuming the extinction 1.28 to be equal to 1 mg/ml FN [14]. Linearity from 100 to 800 μg/ml was ascertained.

Procedure with gelatin–Sepharose

This followed in principle the method of Ruoslahti and Engvall [11]. However, a preliminary purification step was necessary when gelatin–Sepharose was used: in this procedure the plasma sample was passed through underivatized Sepharose 4B (12 cm × 1.6 cm I.D.; eluting buffer: 1 *M* sodium chloride–50 m*M* Tris–HCl, pH 7.5). The column was connected in series with the second column packed with gelatin–Sepharose. Elution of the system was started with the above buffer. When the absorbance at the outlet of the second column decreased to zero, the first column (Sepharose 4B) was disconnected and FN was eluted with the 3 *M* urea–1 *M* sodium chloride–50 m*M* Tris–HCl (pH 7.5) buffer.

Electrophoretic characterization

About 30 ml of the effluent from the affinity column were concentrated using an ultrafiltration chamber, Model 52 with PM10-Diaflo ultrafilter, from Amicon (Lexington, MA, U.S.A.). Urea was washed off using 5 ml of 1.5% Tris–HCl–0.2% sodium dodecyl sulphate (SDS), pH 6.8, and concentrated nearly to dryness. The material collected on the filter was then dissolved in 0.5 ml of sample buffer consisting of 0.063 *M* Tris–HCl–2% SDS–10% glycerol–0.01% bromophenol blue, pH 6.8.

Gradient discontinuous SDS polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli [15] (7.5–15.0% gel, 100 V, 10 h) was used when needed for the characterization of isolated substances.

RESULTS AND DISCUSSION

Choice of the eluting buffer

It was proved in the preliminary experiments that the eluting buffer, 4 *M* urea–50 m*M* Tris–HCl (pH 7.5), which is commonly used for removing FN from

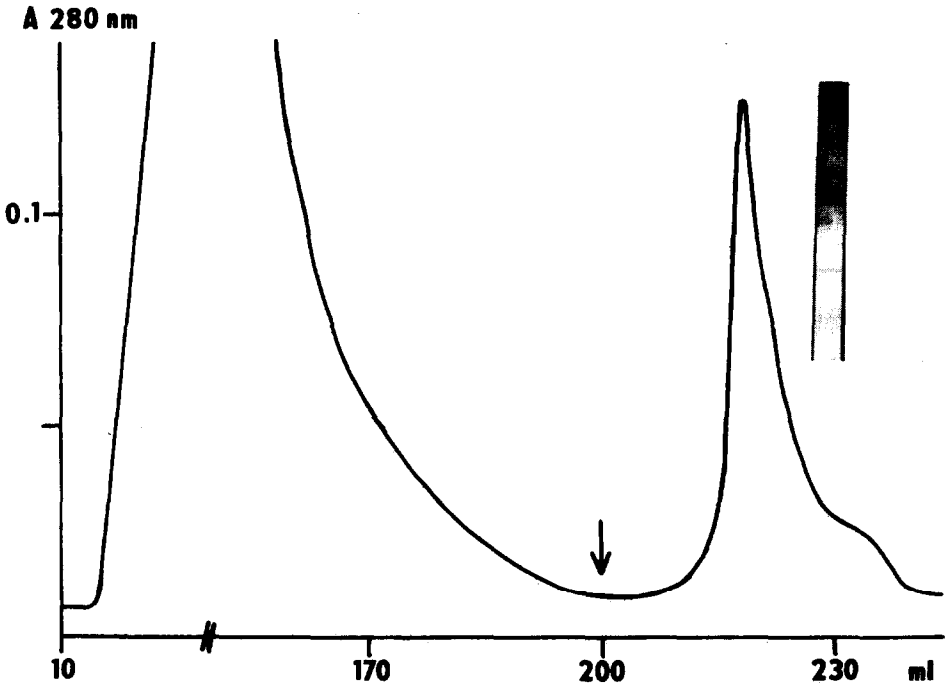

Fig. 1. Affinity chromatography profile of plasma FN on a gelatin–Sepharose column (12 cm×1.6 cm I.D.). Equilibration buffer, 1 *M* sodium chloride–50 m*M* Tris–HCl (pH 7.5); elution buffer, identical with the equilibration buffer but 3 *M* with respect to urea. Polyacrylamide gel characterization of the peak is shown in the insert.

the gelatin–agarose affinity column, cannot be applied to the new sorbent because the microbeads of cross-linked gelatin swelled and the column back-pressure increased excessively. In order to prevent this, we used 3 *M* urea–1 *M* sodium chloride–50 m*M* Tris–HCl (pH 7.5) as the eluting buffer. This buffer was compatible with the gelatin microbeads and was capable of quantitative elution of FN from the affinity column (recovery 98.2±1.3%). Moreover, the flow-rate was constant and no increase in the back-pressure was observed.

Comparison of the affinity separation on gelatin–Sepharose 4B and gelatin microbeads

A typical chromatographic separation obtained with gelatin–Sepharose 4B is presented in Fig. 1. In the first peak all the proteins not retained on the column are eluted. The second peak eluted with the urea-containing buffer was characterized by SDS-PAGE and was shown to contain FN, as indicated in the insert. The presence of FN in this peak was further proved by the immunoprecipitation test (not shown); however, the FN fraction in this case was heavily contaminated, so the product could not be quantitated.

The procedure involving gelatin microbeads yields a pure product with a high recovery, and is simplified in that it does not require a preliminary purification step on the Sepharose 4B column; the plasma sample is loaded directly on the

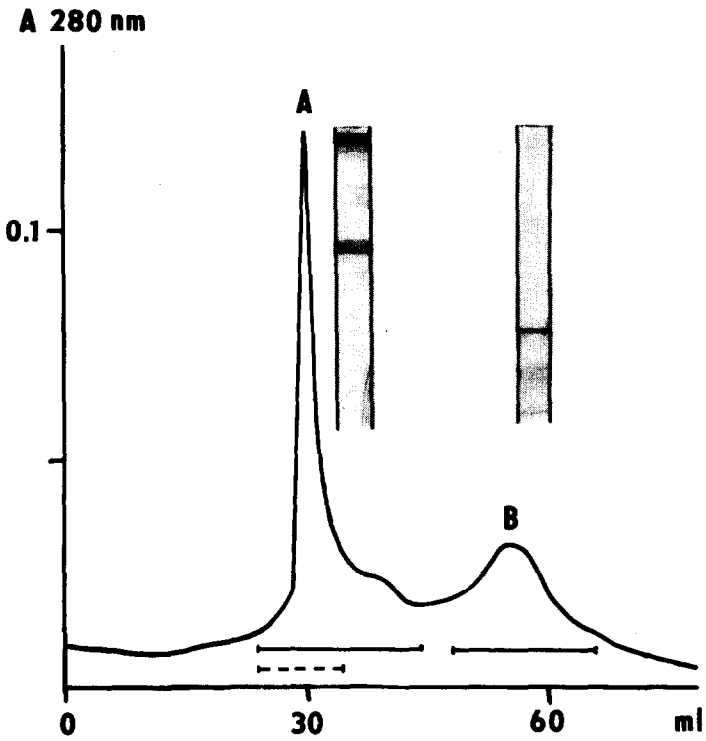

Fig. 2. Elution profile of FN on a gelatin microbeads column (12 cm × 1.6 cm I.D.). Equilibration and elution buffers were the same as in Fig. 1. The bulk of unretained proteins is not shown: recording starts after the elution buffer is introduced. Peaks: A = FN; B = FN degradation products. Polyacrylamide gel characterization of the proteins present in peaks A and B is shown in the insert. Citrate plasma (5 ml) was loaded into the column. The solid lines in the bottom of the figure indicate the eluate collected for use in electrophoresis. The dashed line indicates the eluate used for FN quantitation.

column. The elution profile (Fig. 2) obtained after the introduction of the urea-containing buffer exhibits two different peaks (the peak of unretained proteins is not shown). The main peak of these two clearly contains FN, as revealed by SDS-PAGE (see insert) and its immunoprecipitation test (not shown). The minor peak, which has a considerably longer retention time, must contain material with a specific binding ability to gelatin. In SDS-PAGE it shows the presence of low-molecular-mass entities. According to their immunoreactivity this material contains proteins (or peptides) with the same determinants as FN, and it is feasible to conclude that it represents FN fragments that are smaller than those in the shoulder of the first peak. The presence of such fragments is understandable when one takes in consideration the proteolytic lability of the parent FN molecule.

In the method for FN separation that involves gelatin bound to Sepharose 4B, it is necessary to remove the bulk of blood plasma proteins that bind to the carbohydrate–agarose matrix and that would otherwise contaminate the FN peak when the specific FN–gelatin bond-breaking agent (the urea-containing buffer) is applied. It has to be remembered that in the gelatin-modified Sepharose bed

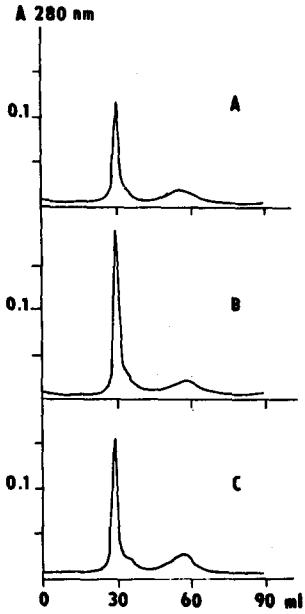

Fig. 3. Elution profiles of plasma FN in samples obtained from a healthy individual (A), the same spiked with purified FN (B) and plasma of a patient with rheumatoid arthritis (C). Plasma volume applied to the column was 5 ml in all cases.

the proportion of bound gelatin is rather small (1.5–3.0% per dry mass of the sorbent), and therefore the chance of non-specific binding is quite high. Such a procedure is thus unlikely to be applied for quantitative purposes. From the preparational point of view the problems to be countered are those of obtaining a contaminated product that needs further purification.

Clearly, the advantage of the newly proposed sorbent is in the absence of the carbohydrate matrix. The treatment of the gelatin microbeads with the glycine-containing buffer during their preparation removes practically all free aldehydic groups, which are converted into the corresponding aldimines. These are sufficiently stable under the conditions used (pH 7.5) for an interchange with free protein amino groups to be unlikely. However, this treatment introduces some extra carboxy groups, which make the sorbent more anionic. The aldimine linkage is fairly stable, particularly at neutral or basic pH, but the application of acidic buffers has to be avoided.

Quantitative analysis of plasma FN

The applicability of bioaffinity chromatography to the determination of human plasma FN was studied. A comparison of two patients, in one of which a rather high plasma FN level was expected because of rheumatoid arthritis (an inflammatory disease, sedimentation rate 76/102), while the other with osteoarthrosis was expected to have a lower plasma FN level, typically revealed plasma FN concentrations of 440 and 273 μg/ml, respectively. Plasma samples obtained from healthy individuals revealed FN levels in the range 150–200 μg/ml (average 185.8,

S.D. 19.57; R.S.D. 10.53%). Results obtained from plasma samples spiked with purified FN are presented in Fig. 3.

As shown previously, gelatin–Sepharose sorbents are unsuitable for quantitative evaluation since at their best they offer only FN-enriched fractions. The original procedure of Ruoslahti and Engvall [11] takes into account the fact that the bulk of the material exhibiting affinity to the carbohydrate matrix of Sepharose has to be removed because it distorts FN isolation. On the other hand, our procedure with glutaraldehyde-polymerized microbeads at present requires too much starting material and takes too long. Such inconvenience is likely to be overcome by using high-performance bioaffinity miniature columns. Such work is in progress.

This is particularly stressed here because the productivity with other, non-chromatographic procedures for plasma FN estimation is much higher. On the other hand, however, in the non-chromatographic methods based on immunoreactivity, various breakdown products are apparently assayed along with the intact FN molecules. The biological activity of FN fragments differs according to their nature, i.e. according to the binding domains preserved. Hence, if these fragments are assayed with intact FN, the applicability of the results is necessarily limited. Therefore the advantage of the present method is seen in its ability to separate intact FN and FN fragments, as both these differ in their affinity towards gelatin; naturally such a method offers the possibility of evaluating separately the plasma concentration of intact FN and FN fragments where needed.

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