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AFFINITY PURIFICATION OF HUMAN PLASMA FIBRONECTIN ON IMMOBILIZED GELATIN

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SUMMARY

Several problems are associated with the biospecific affinity purification of plasma fibronectin on gelatin-Sepharose. Large-scale development of this purification procedure requires optimization of adsorption and elution conditions. The adsorption capacity depends on the amount of gelatin coupled to the Sepharose, the residence time, the temperature and the amount of fibronectin loaded on the adsorbent. Elution of adsorbed fibronectin with 3 *M* urea leads to incomplete recovery. The elution yield was found to vary with both the gelatin concentration and the amount of adsorbed fibronectin. Despite the incomplete elution, the adsorption capacity did not decrease after twelve consecutive isolation procedures. Under optimized conditions, the method described here provides a rapid, single-step and convenient way for the isolation of pure and functional fibronectin, either for analytical or large-scale preparative purposes.

INTRODUCTION

Fibronectin (Fn) is a multifunctional, polymorphous adhesive protein found in the blood cells and circulating in blood plasma. The various functions attributed to Fn during biological processes, i.e. cell adhesion and proliferation, tissue inflammation and repair, opsonization of bacteria and other particles, haemostasis, etc., all rely on the affinity of Fn for cell surfaces and a wide variety of macromolecules, including collagen, fibrin, heparin, DNA, actin, the Clq component of the complement (see ref. 1 for review).

A substantial decrease in the plasma level of Fn has been observed during certain medical and surgical states (septicemia, trauma, burns, respiratory difficulty) or during neoplastic processes. An exogenous source of Fn supplied to the organism should have some therapeutic value and should also provide a means of improving the organism's defence mechanisms. Clinical trials have generally been carried out using Fn-enriched plasma fractions, and the results are somewhat

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debatable (see ref. 2 for review). In addition, since the properties of Fn enhance cell adhesion, the protein is an essential ingredient in certain cell cultures.

Fn purification is therefore doubly useful, from both a therapeutic and a biological point of view. Various sources of Fn can be used: fresh plasma, cryoprecipitate supernatant, by-products from factor VIII preparation, plasma fractionation by-products using either Cohn's method or chromatographic techniques. The technique chosen for Fn purification must comply with several requirements. In particular, as Fn is considered to be sensitive to proteolytic enzyme action, there is a certain advantage in using a fast and, if possible, single-step method for retaining the physical and functional integrity of the molecule. Further, the technique used to obtain a therapeutic concentrate must be suitable for large-scale production.

Precipitation methods have been suggested for purifying Fn [3,4], but the best technique is affinity chromatography on immobilized gelatin [5], which provides Fn of high purity. This technique exploits one of the essential properties of Fn: its capacity for specifically recognizing a ligand, denatured collagen. The technique poses a certain number of problems, though, depending on the conditions used. Incomplete elution of the adsorbed Fn on the gelatin has been reported [6]. Thus both adsorption and elution conditions must be optimized.

If the purification technique using affinity chromatography on immobilized gelatin is optimized, it should be possible to work both on the laboratory scale, under standardized conditions for obtaining high-purity Fn with known physical and functional properties, and on an industrial scale for obtaining large amounts of Fn for therapeutic purposes.

EXPERIMENTAL

Preparation of affinity gels

Gelatin (Type I, Sigma) was covalently coupled to Sepharose CL-4B (Pharmacia) activated with cyanogen bromide [7]. A volume of a 10% solution of cyanogen bromide in distilled water was added to an identical volume of packed Sepharose, and the pH was maintained at 11.0 with 10 *M* sodium hydroxide. After extensive washing at 4°C using distilled water and a 0.1 *M* sodium bicarbonate buffer–0.5 *M* sodium chloride (pH 8.5), the gelatin solution in the same buffer was added to the activated Sepharose and the mixture was left overnight at room temperature under gentle agitation. Various concentrations of the gelatin solution ranging from 1 to 8 mg/ml were used to study the influence of the amount of immobilized gelatin on the gel. All subsequent experiments were carried out using a 5 mg/ml gelatin solution. The amount of immobilized gelatin was then ca. 3 mg/ml of gel. The excess protein was eliminated by washing with the sodium bicarbonate buffer. The amount of gelatin linked to the gel was assessed by measuring the protein content of the solutions before and after coupling and washing, by the method of Lowry et al. [8], using gelatin as standard. The excess reagent groups were blocked by incubating with 1 *M* ethanolamine (pH 8) for 2 h. After washing with 3 *M* sodium thiocyanate–0.1 *M* sodium acetate (pH 6) to eliminate possible protein–protein interactions, the gels were stored at 4°C in buffer A,

which consisted of 0.05 *M* Tris–HCl buffer, 0.05 *M* ϵ-amino-*n*-caproic acid (EACA), 0.02 *M* sodium citrate and 0.2 g/l sodium azide (pH 7.5).

Purification of human plasma Fn

Fn was purified from citrated human plasma. Fresh plasma was centrifuged for 30 min at 4000 *g*, then filtered (3-μm Sartorius filter) and frozen at −70°C. Before use, the plasma was thawed for 1 h at 37°C, the temperature at which the fibronectin–fibrinogen interactions are labile. After the plasma had passed through, columns were washed using buffer A until the absorbance reached zero. The column was then washed with 1 *M* sodium chloride in the same buffer. Fn was eluted with 3 *M* urea in Buffer A. After concentration to ca. 1 mg/ml by means of dialysis against poly(ethylene glycol) 20 000, Fn was dialysed against 0.15 *M* sodium chloride–0.05 *M* Tris–0.001 *M* calcium chloride (pH 9). Fn was stored in this buffer at −30°C.

Analytical methods

Fn levels were assessed by means of the immunoelectrophoresis method of Laurell [9]. The antiserum used was a specific anti-Fn rabbit anti-serum prepared in our laboratory. Standard plasma (OTFI Behring) was used. For pure Fn solutions, the Fn level was assessed by spectrophotometric assay at 280 nm using $A^{1\%}_{1\,cm} = 12.8$ [3].

The purity of the prepared Fn was ascertained by immunoelectrophoresis, electrophoresis in polyacrylamide gel and fast protein liquid chromatography (FPLC)-type analytical chromatography (mono-Q column, Pharmacia).

The following tests were undertaken to monitor the integrity of the functional properties of Fn: its capacity for gelatin binding by means of an enzyme-linked immunosorbent assay (ELISA)-type technique, developed in our laboratory [10]; its cell adhesion capacity according to the technique described by Ruoslahti et al. [11]; its opsonization capacity using the technique described by Van de Water III et al. [12].

RESULTS

The parameters that have an influence on adsorption and those with an influence on desorption were studied.

Parameters influencing adsorption capacity

Amount of immobilized gelatin. Adsorbents were prepared with gelatin solutions at different concentrations. It was observed that the fixation yield of the ligand on the matrix decreased as the gelatin concentration increased. This could be accounted for by the lack of solubility of the concentrate solutions at room temperature. Consequently, it is difficult to couple much more than 5 mg of gelatin per ml of gel. Three adsorbents were prepared with, respectively, 0.7, 1.35 and 4.3 mg of gelatin per ml of Sepharose. A 100-ml sample of plasma (25 mg of Fn, i.e. more than the amount required) was applied to each 3-ml adsorbent (4.7×0.9 cm) at a linear flow-rate of 118 cm/h (residence time between plasma

Fig. 1. Influence of the gelatin density (mg/ml of gel) on adsorption capacity. Plasma (100 ml) was applied to each adsorbent (4.7×0.9 cm) at a linear flow-rate of 118 cm/h. The amount of adsorbed Fn was calculated as the difference in plasma Fn content before and after passage through the column.

and adsorbent = 144 s). As shown in Fig. 1, the amount of Fn adsorbed increases with the concentration of the gelatin immobilized on the gel.

All subsequent experiments were carried out using adsorbents prepared by immobilizing the gelatin at 3 mg/ml.

The influence of the composition of the buffer used for washing the column was studied. Two parallel experiments were carried out with the same plasma passed through two identical adsorbents. Washing (until zero absorbance) was carried out with buffer A, on the one hand, and buffer B (50 m*M* Tris–HCl, 100 m*M* sodium chloride, 2 m*M* benzamidine, 0.1 m*M* phenylmethylsulphonyl fluoride (PMSF) and 0.2 g/l sodium azide, pH 7.4) on the other.

With buffer A, 83% of the Fn in the plasma sample was adsorbed, whereas with buffer B, 69% of the Fn was adsorbed.

Residence time between Fn and adsorbent. A 100-ml sample of plasma was applied to various 1-ml adsorbents (1.6×0.9 cm) at linear flow-rates ranging from 3 to 315 cm/h, equivalent to a residence time varying from 1920 to 18 s. The residence time is defined by the ratio of the adsorbent volume to the flow-rate. The adsorption capacity of gels increases until it reaches a plateau (Fig. 2). Saturation level occurs when the amount of adsorbed Fn is ca. 15 mg per ml of gel and when the residence time is ca. 780 s.

Further, the amount of Fn recovered after elution was always less than the amount of Fn adsorbed.

Amount of Fn applied to the adsorbent. In order to avoid saturation conditions, a linear flow-rate of 118 cm/h was chosen for all the experiments (the ascending part of the curve shown in Fig. 2). Each experiment was carried out using a fresh adsorbent. A 5–300 ml aliquot of plasma, i.e. 1.78–83.75 mg of Fn, was applied to 3-ml adsorbents (3 mg of gelatin per ml of gel) (4.7×0.9 cm). The amount of Fn adsorbed increases with the amount of Fn applied to the gel (Fig. 3). The increase

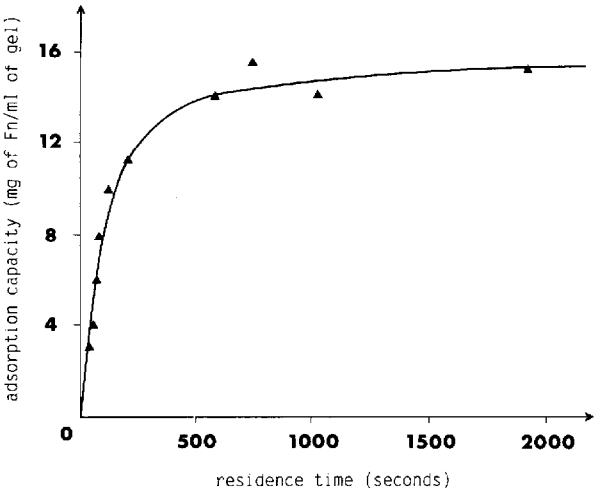

Fig. 2. Effect of the residence time on adsorption capacity. Plasma (100 ml) was applied to the adsorbent (1.6×0.9 cm) at linear flow-rates ranging from 3 to 315 cm/h. A 10-ml volume of gelatin-Sepharose (3 mg of immobilized gelatin per ml of gel) was prepared and each experiment was performed on an unused column. Residence time was expressed as the ratio of adsorbent volume to flow-rate.

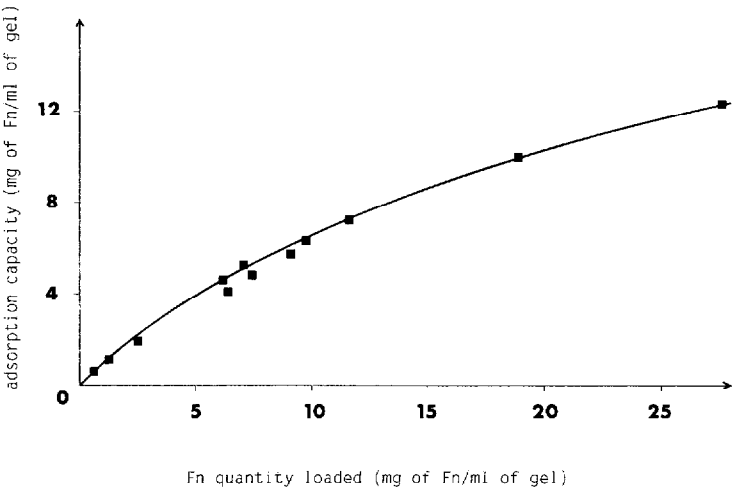

Fig. 3. Effect of the amount of Fn applied to the adsorbent on adsorption capacity. Plasma (5–300 ml) was applied to the adsorbent (4.7×0.9 cm) at a linear flow-rate of 118 cm/h. Each experiment was carried out using a fresh gelatin-Sepharose gel (3 mg of gelatin per ml of gel).

is not linear, consequently the fixation yield (the ratio of the amount of Fn adsorbed to the amount of Fn applied) decreases. In fact, the fixation yield reaches 100% only when the amount of Fn applied is less than 1 mg per ml of gel.

Temperature. Experiments were carried out in thermostatted columns at different temperatures. A 100-ml sample of plasma (i.e. ca. 26 mg of Fn) was loaded on 4-ml adsorbents (2×1.6 cm) at a linear flow-rate of 50 cm/h (residence time = 144 s). The amount of Fn adsorbed varies significantly depending on the

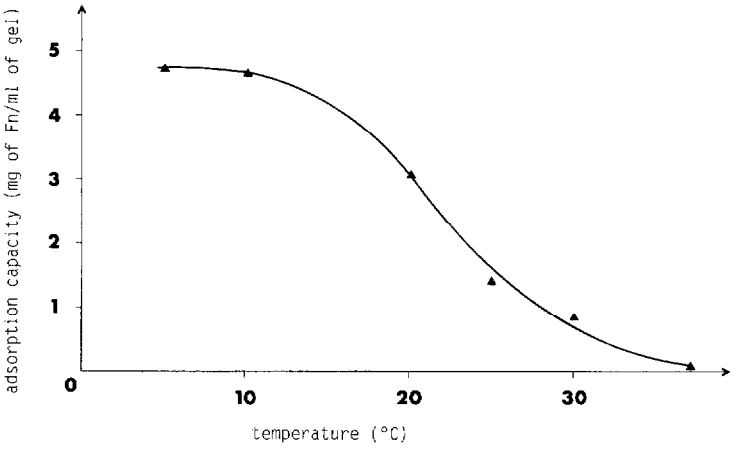

Fig. 4. Effect of temperature on adsorption capacity. Plasma (100 ml) was applied to the adsorbent (2×1.6 cm) at a linear flow-rate of 50 cm/h. The column was thermostatted and all solutions passed through were placed in a water-bath. Each experiment was performed with an unused gelatin-Sepharose gel (3 mg of gelatin per ml of gel).

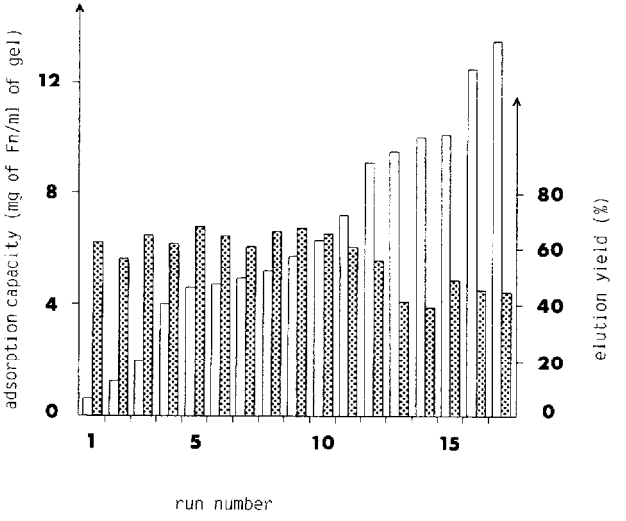

Fig. 5. Variations of elution yield with the amount of adsorbed Fn. Plasma (5–300 ml) was applied to the adsorbent (4.7×0.9 cm) at a linear flow-rate of 118 cm/h. Each experiment was carried out using a fresh gelatin-Sepharose gel (3 mg of gelatin per ml of gel). (□) Adsorption capacity; (▒) elution yield.

temperature (Fig. 4). Maximum adsorption occurs at temperatures below 10°C and the amount adsorbed decreased as the temperature rises. At 37°C the amount of Fn adsorbed is insignificant.

Parameters influencing desorption

The elution yield (the ratio of the amount of Fn desorbed to the amount of Fn adsorbed on an adsorbent containing 3 mg of gelatin per ml of gel) remains con-

TABLE I

EFFECT OF GELATIN CONCENTRATION AND Fn QUANTITY LOADED ON ADSORPTION CAPACITY AND ELUTION YIELD

Each experiment was carried out at room temperature using a fresh 3-ml adsorbent. The residence time was 144 s.

Gelatin concentration on the adsorbent (mg/ml)	Amount of Fn (mg/ml of gel)		Specific adsorption capacity (mg of Fn adsorbed/mg of gelatin)	Elution yield (%)
	Applied	Adsorbed		
0.7	3.8	1.60	2.28	100
	8.3	2.13	3.04	63
	16.5	4.70	6.68	42
1.35	3.8	2.76	2.05	89
	8.3	3.22	2.39	75
	16.5	6.23	4.62	55
4.3	2.45	1.93	0.45	65
	7.35	4.90	1.14	61
	11.48	7.16	1.66	60
	27.9	13.48	3.13	44

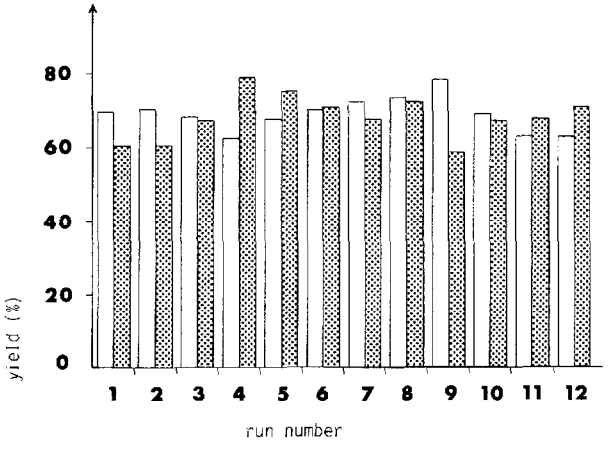

Fig. 6. Regeneration of adsorbents. Each run was achieved by applying 100 ml of plasma to the adsorbent (6.3 × 0.9 cm; 3 mg of gelatin per ml of gel) at a linear flow-rate of 79 cm/h. (□) Adsorption yield; (▒) elution yield.

stant at ca. 65% when the amount of Fn adsorbed per ml of gel is less than 6 mg (Fig. 5). For amounts of Fn larger than 6 mg per ml of gel the elution yield decreases. Even when low amounts of Fn are adsorbed, Fn elution is incomplete.

Adsorbents containing 0.7, 1.35 and 4.3 mg of gelatin per ml of gel were studied (Table I). For all three adsorbents, the elution yield is observed to decrease as a function of the increase in the amount of Fn adsorbed. Moreover, the results

indicate that when the gelatin concentration on the gel increases, and for equivalent amounts of Fn adsorbed, there is a decrease in elution yield.

Regeneration of adsorbents

The possibility of using the gels several times was studied. Twelve runs of 100 ml of plasma were performed on a 4-ml adsorbent column (6.3×0.9 cm) at a linear flow-rate of 79 cm/h. As the plasma Fn concentration varied (0.24–0.3 mg/ml), the amount of Fn applied to the gel was not identical every time and resulted in small differences in the amount of Fn adsorbed. The results are therefore given as the adsorption yield (Fig. 6). No decrease in adsorption capacity was observed during the different runs, although the desorption yield for each of the twelve tests is only ca. 70%.

Quality of the Fn obtained

Immunoelectrophoretic analysis of Fn, purified by the above method, confirms the absence of contaminants. In 3.5% polyacrylamide gel, a major 480 000 band is observed. After reduction, just one band is found at 250 000. Analysis using FPLC reveals just one symmetrical peak, eluted at a molarity of 0.35 *M* in sodium chloride pH 7.7 (Fig. 7).

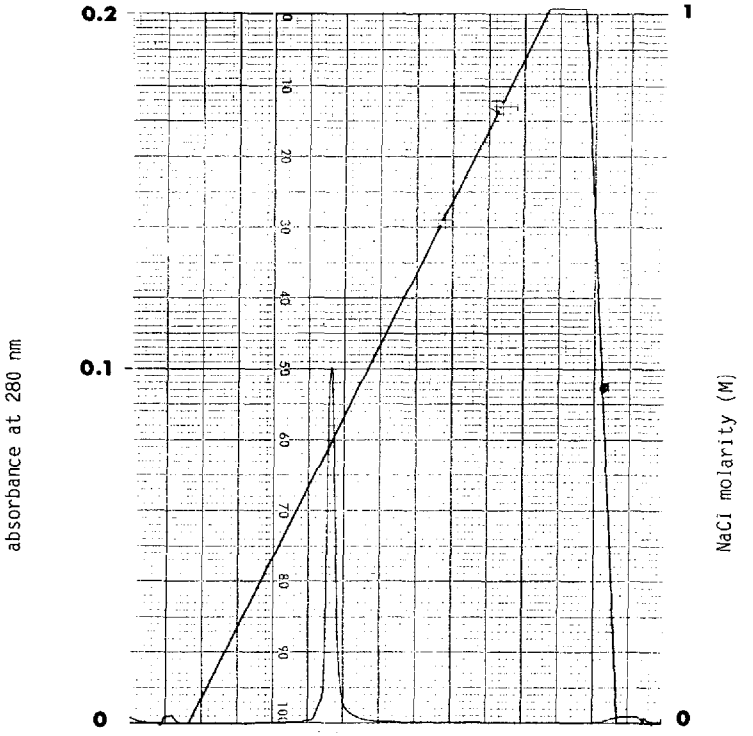

Fig. 7. Fast protein ion-exchange liquid chromatography. Purified Fn (50 μg) was applied to a mono-Q HR 5/5 column equilibrated in 20 m*M* triethanolamine (pH 7.7) at a flow-rate of 1 ml/min. Proteins were eluted with a linear gradient of sodium chloride (0–1 *M*). The effluent was monitored at 280 nm.

Quantitative tests were carried out to measure the functional properties of Fn (adhesion to gelatin, cell adhesion, opsonization capacity). The results show that the functional properties of the Fn obtained by the above method are similar to those of a standard preparation obtained by other means [10].

DISCUSSION

Human Fn, that is to be used for studying the potential value of this protein for therapeutic applications, must be obtainable in large amounts and with a sufficiently high level of purity. The precipitation methods described previously [3,4] do not appear to provide Fn of adequate quality. Affinity chromatography on immobilized gelatin is the technique most commonly applied [5,6,11,13–20].

The work described in this paper was aimed at finding the best conditions for obtaining and utilizing adsorbents, in order to define the technique that could be adapted to large-scale production. A survey of various parameters indicated that a certain number have a significant influence on the adsorption capacity of the adsorbent. In order to minimize the possible influence of proteolytic enzymes, some authors have suggested adding enzyme inhibitors to the plasma [21]. The study described in this paper has shown that the presence of inhibitors such as benzamidine and PMSF did influence the adsorption capacity of the adsorbents. The full investigation was undertaken in a buffer that did not contain enzyme inhibitors.

The amount of Fn adsorbed on the gels increases with an increase in the following parameters: gelatin concentration on the gel; residence time between plasma and adsorbent; and total amount of Fn applied to the gel. The amount of Fn adsorbed levels out after residence times of ca. 780 s. This parameter should provide the means of assessing the best flow-rates for use during purification. Even though the amount of Fn adsorbed increases with increasing concentration of gelatin immobilized on the gel and with increasing amount of Fn applied, it is not clear, as we shall see below, that the best results are obtained by setting the parameters to produce the maximum adsorption capacity.

One parameter is of particular interest: the temperature at which purification is carried out. Maximum yield is obtained below 10 °C, and the yield then drops at a uniform rate to reach zero at 37 °C. Structural changes probably take place, and the fixation sites on the immobilized gelatin are probably altered. This alteration in Fn at temperatures between 4 and 37 °C has been reported previously [22]. Adsorbents with a high gelatin concentration (3–4 mg/ml of gel) are an advantage, as a maximum amount of Fn is adsorbed under these conditions. In contrast, the results indicate that the elution yield by 3 *M* urea (ratio of the amount of eluted Fn to amount of adsorbed Fn) remains constant at ca. 70% for amounts of up to 6 mg/ml of adsorbed Fn. Over and above 6 mg/ml, elution yields decrease. Elution is never complete, whatever the experimental conditions. No rational explanation has yet been found for this phenomenon, which has been reported by other authors [6]. Trials using [^{125}I]Fn were undertaken to try to account for it but were unsuccessful. Indeed, ^{125}I-labelled Fn loses its capacity for fixing onto gelatin [11]. Moreover, the results showed that the elution yield de-

creases as a function of two factors: the gelatin concentration on the gel and the amount of adsorbed Fn. It appears that various types of Fn–gelatin binding are involved and that the weakest affinity bindings occur when the two above-mentioned factors are diminished.

In spite of the incomplete elution, which may be caused by types of binding other than Fn–gelatin binding, the results have shown that the adsorbents could be used several times over, under the same conditions, without any notable loss of activity.

The quality of the preparations obtained under optimized conditions has been assessed. Tests revealing the physical and functional properties of the product have shown that the Fn obtained was free from contaminants and that its functional activities remained unchanged.

By taking into account the different parameters studied above, it should be possible to scale-up the process of Fn purification by affinity chromatography on immobilized gelatin.

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