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Faster isolation of recombinant factor VIII SQ, with a superporous agarose matrix[1]

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Abstract

A superporous agarose matrix was compared with a corresponding homogenous matrix in the isolation of recombinant factor VIII SQ (r-VIII SQ) by immunoaffinity chromatography. As a reference, the commercially available Sepharose FastFlow, used for a similar purification in the industry, was also evaluated. Breakthrough curves are described for flows between 50 and 400 cm/h with pre-purified r-VIII SQ and with cell culture broth. The superporous gel gave the best performance and a 1000-fold purification was obtained in a one-step procedure. The superporous matrix made it possible to increase the throughput about four-fold, presumably due to its better mass transfer properties. The importance of the ligand distribution profile is discussed based upon immunofluorescence microscopy data.

Keywords: Immunoaffinity chromatography; Agarose, superporous; Ligand distribution; Factor VIII; Proteins

1. Introduction

Chromatography is an important unit operation during protein purification/isolation. Important factors that will limit the throughput in a chromatographic process are: (1) the binding capacity of the matrix, (2) the kinetics of ligand/ligate interaction, (3) the diffusion into, and inside the matrix and (4) the flow resistance/rigidity of the matrix.

In the development of an immunoaffinity isolation process, the ligand can at least to some extent be selected according to its binding properties (selectivity, binding strength and binding kinetics), but availability and cost are other important factors.

In most chromatography processes, diffusion is the limiting factor. If the diffusion distance between mobile phase and affinity sites could be shortened, the flow-rate could be increased, thereby giving a higher throughput. One way to shorten the diffusion distances is to make the gel beads smaller. This will unfortunately increase the flow resistance quite dramatically – the pressure drop is proportional to the inverse of the squared bead diameter. Hence, this is a solution only for very rigid matrices – soft gels would collapse under the pressure needed to get the desired flow.

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To solve this problem, new kinds of matrices have been developed [1–4]. Some of these matrices have, in addition to the normal diffusion pores, also ''highways'' of very large pores which transport molecules by convective flow into and out of the chromatography bead. In this way only very short distances are needed for diffusion, a situation that significantly improves the mass transfer in the bead. The benefits of this pore flow is particularly pronounced with large molecules like proteins which have low diffusion coefficients. This new concept of pore flow makes it possible to use large beads, which give a satisfactorily low backpressure and which still provide high efficiency due to short diffusion distances.

The target protein in this study is recombinant factor VIII SQ (r-VIII SQ) [5]. Factor VIII is a glycosylated blood protein of the haemostasis, which acts as a co-factor in the cascade of enzymatic reactions employed to generate a fibrin clot. Deficiency of this protein is the cause of haemophilia A, a hereditary bleeding disorder, which is treated by replacement therapy using plasma derived concentrates or recombinant DNA products [5–9]. r-VIII SQ, an example of the latter category, manufactured at Pharmacia and Upjohn, is a variant of the molecule, lacking the mid-part, i.e., the B-domain, for which no function is assigned. It is a dimer of two chains, M_r 80 000 and 90 000, held together by a Me^{2+} bridge. This protein is produced by Chinese hamster ovary (CHO) cells, expressed at low levels compared to the host cell proteins also secreted into the culture medium.

As is often the case with processes utilising animal cell culture, large volumes of medium have to be processed during isolation of the target protein. To preserve the integrity and the functional properties of the molecule, improved isolation methods are looked for, in particular methods that diminish the processing time.

Such rapid isolation procedures are not only important for product isolation at the end of the process, but also beneficial for biochemical/functional characterisations carried out repeatedly during the cell culture cycle, with the purpose of assessing the influence of various cultivation parameters on the product composition. Here methods for rapid isolation/enrichment of the often very diluted target molecule are necessary before the product can be analysed.

In this report we have investigated the rapid isolation of r-VIII SQ with immunoaffinity chromatography. The Sepharose FastFlow matrix was used as a reference, since it is presently used for a similar purification in the industry. An important criterion for improved performance was first of all a shortened isolation time, obviously with retained purity of the product. This latter aspect was emphasised by earlier tests [10] with a commercially available pore flow matrix (polystyrene based) and a commercially available conventional flow matrix (porous glass), both showing an unacceptable degree of non-specific binding of proteins, possibly via hydrophobic interactions. In contrast, the agarose-based FastFlow matrix showed a minimum of non-specific interactions. These observations prompted the use of an agarose-based support and a so-called superporous agarose matrix [2] was chosen. This support material has been used earlier also for affinity chromatography applications [11], although not for industrial applications.

2. Experimental

2.1. Materials

The recombinant factor VIII SQ (r-VIII SQ), M_r 170 000, containing one M_r 90 000 chain and one M_r 80 000 chain, held together by a Me^{2+} bridge [5,12,13] was produced at Pharmacia and Upjohn (Stockholm, Sweden). Both a highly purified fraction (purified by the four first chromatography steps of the process as described previously [12]) and a filtered fermentation broth was used. One unit (U) of r-VIII SQ corresponds to 70 ng of pure protein.

The monoclonal antibody (8A4), directed towards the M_r 90 000 chain, was developed by Pharmacia and Upjohn, and produced by Celltech (Slough, UK).

The fluorescein isothiocyanate (FITC) protein A used had an average of 6 mol of FITC/mol protein A, and was produced by Amersham Pharmacia Biotech (Uppsala, Sweden). BCA (bicinchoninic acid) reagent was obtained from Pierce (Rockford, IL, USA). Tween 80 (polyethulene sorbitan mono-

oleate) was purchased from Merch (Schuchardt, Germany) and Triton X-100 (*tert*.-octylphenoxypolyethoxyethanol) was from Merck (Darmstadt, Germany). Other chemicals were of analytical grade.

2.2. Matrices

Pre-activated Sepharose 4 FastFlow (45–165 μm) from Amersham Pharmacia Biotech was coupled with monoclonal anti-factor VIII antibodies at Pharmacia and Upjohn.

The homogenous and superporous agarose matrices were prepared as previously described [2] and were kindly supplied by Per-Erik Gustavsson (Department of Pure and Applied Biochemistry, Centre for Chemistry and Chemical Engineering, Lund, Sweden). Both matrices were prepared from 6% agarose solutions and were fractionated by wet sieving to 106–180 μm. The superporous agarose beads had a superpore volume of 40%.

2.3. CNBr activation and coupling

The superporous and homogenous gels were activated according to the following recipe: 10 ml (sedimented volume) of each of the gels were washed on a glass filter exceedingly with water, and then with about 100 ml of 2 *M* Na_2CO_3, The moist gels were then transferred to a 50-ml test tube with screw lid. A freshly prepared CNBr solution (0.5 g CNBr was dissolved in 0.5 ml acetonitrile and, immediately before activation, diluted with 2 *M* Na_2CO_3 to a volume of 10 ml) was added to the gels. After 2 min of constant mixing (end over end) the beads were washed on a glass filter with about 350 ml of 0.2 *M* $NaHCO_3$, 0.4 *M* NaCl, pH 8.2 [14]. All procedures were performed at room temperature.

2.4. Coupling of anti-factor VIII antibodies

The antibodies were diluted with 0.2 *M* $NaHCO_3$, 0.4 *M* NaCl, pH 8.2, to a concentration of 1.0 mg/ml, added to the gel to give a final concentration of 1.0 mg antibodies/ml sedimented gel, and incubated at room temperature on a rocking table for 2 h.

The remaining active groups were blocked with 1 *M* ethanolamine, pH 8.2 for 80 min and the gels were finally washed with 1 *M* NaCl.

The coupling yield was measured by an absorbance scan (230–320 nm) of the antibody solution before and after coupling, and was found to be over 80%.

The FastFlow gel was coupled in a similar way (carried out at Pharmacia and Upjohn).

2.5. Protein measurements

Besides the indirect and less precise determination of coupling yield described above, a more precise method [15] based on the BCA reagent was used for the determination of the amount of immobilised antibodies in the matrices. Procedure: 50 μl gel, sedimented volume, was mixed with 2 ml BCA reagent (Pierce), incubated on a rocking table for 2 h at room temperature, centrifuged, and the absorbance of the supernatant at 562 nm was measured. The results were compared with a standard curve for bovine serum albumin and the values corrected by dividing with 1.18 for immunoglobulin G (IgG) according to the instructions supplied by Pierce. Two samples from each gel were analysed.

The Bradford method [16] was used when the total protein concentration was measured in selected samples.

2.6. Factor VIII determination

Factor VIII activity was usually measured at Pharmacia and Upjohn using a two-step assay based on the co-factor function of factor VIII in the clotting cascade (factor VIII activates factor X, which cleaves a chromogenic substrate, releasing *p*-nitroaniline, which was measured spectrophotometrically [10]). The assay was adapted to a 96-well plate reader format. An error of 8% between samples on the same plate, and 14% between plates is to be expected from this method. The concentration in highly purified fractions was also measured spectrophotometrically at 280 nm.

2.7. Chromatography: equipment and running conditions

In the experiments with pre-purified r-VIII SQ, a fast protein liquid chromatography (FPLC) system containing a control unit LCC-500, pumps P500,

P50, detector UV-M, motor valves Mw 8 and Mw7 (Amersham Pharmacia Biotech) and a two-channel recorder was used together with C_{10} columns (Amersham Pharmacia Biotech). The control unit was programmed to run all experiments automatically, only the changing of columns and filling up of sample had to be done manually between runs. The absorbance at 280 nm was recorded. The running cycle included prewash with water, equilibration, sample application, wash with high ionic strength, low ionic strength, elution and finally a three-step sanitation. During elution the flow-rate was kept at 15 cm/h for all runs. The buffer components were chosen to give a low absorbance at 280 nm, in order not to interfere with the detection of r-VIII SQ.

A similar equipment was used for the experiments with unpurified protein. In these measurements, fractions were collected and stored frozen for determination of the coagulant activity at Pharmacia and Upjohn. Due to the masking by high concentration of non-target proteins the absorbance during sample application was not relevant.

Buffer compositions were as follows: the equilibration buffer contained 0.05 *M* NaCl, 0.1 *M* NH_4Ac and 0.005 *M* $CaCl_2$, pH 6.8; the high ionic strength wash buffer contained 0.8 *M* NaCl, 0.1 *M* NH_4Ac and 0.005 *M* $CaCl_2$, pH 6.8; the low ionic strength wash buffer contained 0.05 *M* $CaCl_2$ and 0.05 *M* histidine, pH 6.6 and the elution buffer contained 0.05 *M* $CaCl_2$, 0.05 *M* histidine and 50% ethylene glycol, pH 6.6. All buffers also contained 0.02% of detergent; Tween 80 for experiments with pre-purified r-VIII SQ and Triton X-100 for experiments with cell culture broth. Sanitation was performed using elution buffer, 0.5 *M* NaCl and finally 0.1 *M* HAc–20% ethanol. In the last portion of the last buffer, the pH was adjusted to 4.0.

2.8. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE [17] was performed at Pharmacia and Upjohn, with a gel thickness of 0.75 mm, a total polyacrylamide concentration of 4% in the stacking gel and 7.5% in the separating gel, and cross-linkage of 3.3%. Samples were reduced with 4% (v/v) 2-mercaptoethanol in 1.5% (w/v) SDS and heated for 7 min at 90°C. PAGE was run at 20 mA for 4 h. The electrophoresis was followed by silver staining of the gels [18].

2.9. Fluorescence labelling

FITC-protein A was used for the labelling of the affinity ligands (immobilised anti-factor VIII antibodies) in the matrices. Procedure: FITC-protein A was dissolved in 10 m*M* phosphate buffer, pH 8.0 containing 0.15 *M* NaCl, to a concentration of 0.12 mg/ml. One hundred μl of the solution was added to approximately 10 μl (settled volume) of each gel suspended in 300 μl of the above buffer in a microvial. To ensure equal staining conditions, some superporous beads were included in each vial as an internal reference. The following gels were labelled: superporous gel, homogenous gel and FastFlow gel with coupled ligands as well as the corresponding blank gels (activated and blocked with ethanolamine). To ensure complete equilibration the vials were left overnight at room temperature under gentle but thorough mixing. To remove the excess of FITC-protein A in a controlled way and without causing any significant dissociation of the FITC-protein A–antibody complex, each vial with gel was diluted 1:20 (in the above buffer) and after 7 h further diluted 1:10. The fluorescence of the labelled beads were checked at various stages during washing to ensure that the bound protein A was not washed away. The absorbance of the labelling solution was measured before and after labelling to ensure that FITC-protein A was present in excess.

2.10. Microscopy and CCD camera

An Eclipse E 400 microscope (Nikon, Tokyo, Japan) with an Y-FL Epi-fluorescence attachment with a mercury lamp was used. Epi-Fl filterblock N B-2A was used providing excitation between 450–490 nm and emission collection above 520 nm. The microscope was connected to an AT 200 charged coupled device (CCD) camera (Photometrics, Tucson, AZ, USA). Images were collected by the camera

and stored and treated in a computer by the PMIS software (Photometrics).

3. Results and discussion

One purpose of this investigation was to develop a rapid method for isolation of r-VIII SQ from cell culture broth, thereby making it possible to analyse the quality of the product during the progress of fermentation. An isolation time of a few hours was considered satisfactory in the view of that the whole cultivation cycle spans several weeks.

The more general purpose of the investigation was to compare superporous and homogenous agarose beads in order to clearly demonstrate the advantages and disadvantages related to the pore flow principle, as applied in affinity chromatography where both the ligand and ligate are large proteins. The investigation was carried out as a comparison between three matrices: (1) superporous agarose beads (diffusion pores and flow pores; prepared in the laboratory), (2) homogenous agarose beads (only diffusion pores; prepared in the laboratory) and (3) Sepharose 4 FastFlow (only diffusion pores; commercial product).

The superporous and homogenous beads were prepared to match each other (same bead size, agarose content and activation procedure etc.). The purpose of including also the Sepharose FastFlow material in spite of its less perfect matching properties was to have a reference that is commercially available and is a standard material for the kind of separations at hand.

Chromatography experiments were carried out both with pre-purified r-VIII SQ and cell culture broth with low r-VIII SQ content. The results from runs with pre-purified r-VIII SQ from which the dynamic capacity and yield were calculated, are first described. The sharpness and location of the breakthrough curves revealed the influence of flow-rates on the performance of each matrix. Next, the experiments with cell culture broth are presented, giving information on the degree of concentration and purification. The results also show the combined effect of impurities and low target molecule concentration on the performance. In the concluding part we address the ligand distribution within individual gel beads.

3.1. Studies with pre-purified r-VIII SQ

Breakthrough curves for pre-purified r-VIII SQ for the three studied matrices at flow-rates of 50, 100, 200 and 400 cm/h are shown in Fig. 1. The capacities, calculated from the adsorption phase of the 50 and 100 cm/h runs, were about 17 kU/ml sedimented gel for the superporous and homogenous matrix, and 7.5 kU/ml for the FastFlow matrix (all detected by UV measurements). The yield was found to be about 90% or higher.

A high flow-rate drastically impaired the performance with the very short columns used (2.5 cm). Thus, at a flow-rate of 400 cm/h, all matrices showed an immediate breakthrough. At 200 cm/h the column with superporous gel had a satisfactory retention up to and slightly beyond the addition of 6 kU/ml gel, whereas the other two still performed badly. With a flow-rate of 100 cm/h, the leakage started just after 3 kU with the FastFlow and the homogenous gel, and at 9 kU for the superporous gel. At 50 cm/h, the result was improved further to 4 kU, 5 kU and 11 kU, respectively.

The curve for superporous gel at 200 cm/h is almost identical to the curve for homogenous gel at 50 cm/h, showing that the superpores enable the matrix to be used at a four-times higher flow, without loss of performance. The FastFlow matrix had a surprisingly sharp breakthrough and low capacity. This will be further discussed.

3.2. Does it work with cell culture broth too?

The cell culture broth is a very complex solution, where r-VIII SQ only constitutes 0.03% of all protein present. To enable a detection of the breakthrough of r-VIII SQ, its coagulant activity was measured.

Breakthrough curves were recorded at 100 cm/h for all three matrices and at 400 cm/h for the superporous and FastFlow matrix (Fig. 2). The amount of functional r-VIII SQ that bound to the gel was only half of the amount found for pre-purified material. An explanation might be that non-functional r-VIII SQ molecules capable of binding to the

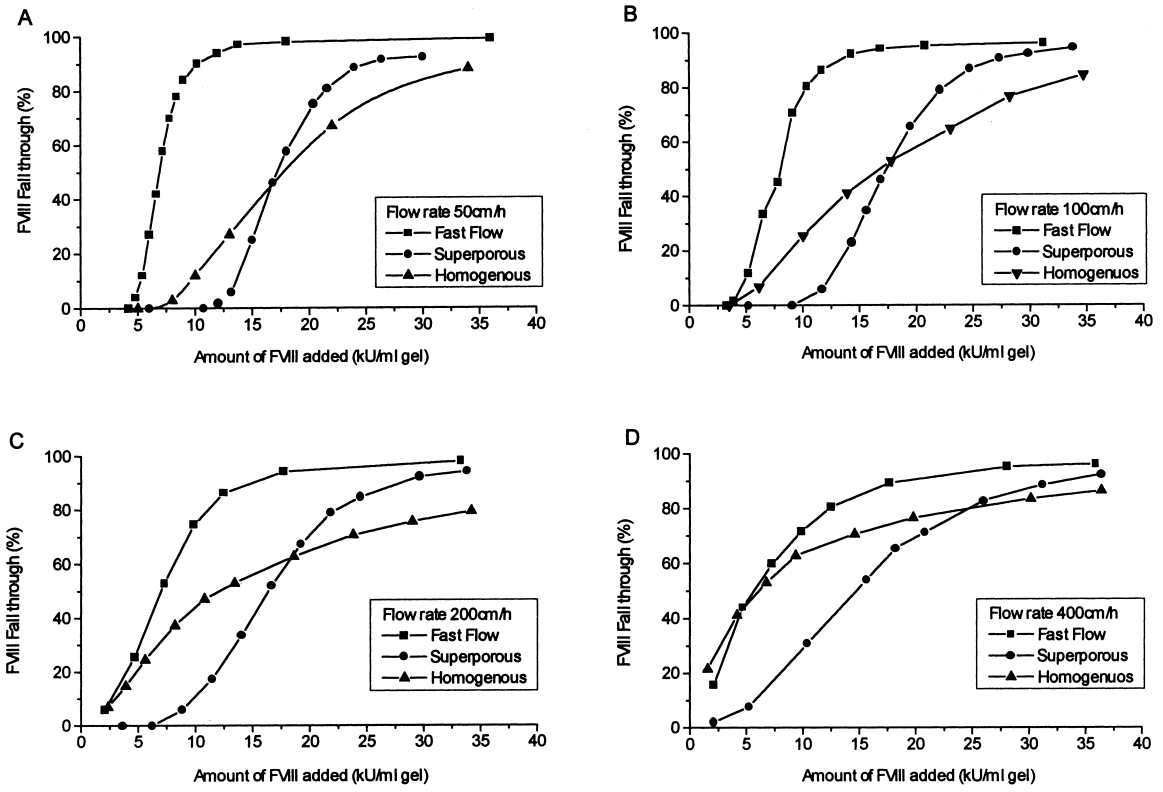

Fig. 1. Breakthrough curves for the three types of gel with pre-purified r-VIII SQ at different flow-rates monitored by UV detection. The affinity gels (anti-factor VIII antibodies bound to Pharmacia FastFlow gel, superporous agarose or homogeneous agarose) were packed in identical columns (25×10 mm) and attached to a FPLC system and run as described in Section 2.7.

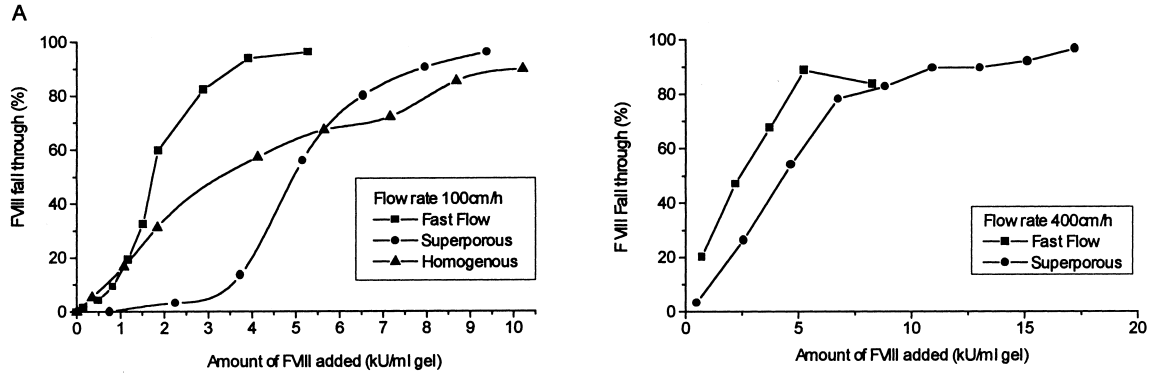

Fig. 2. Breakthrough curves for the three types of gels with cell culture broth at different flow-rates monitored by activity measurements. For further details see Fig. 1.

ligands would compete for the binding sites, but would not be detected by this assay and consequently diminish the observed capacity of the gel. Alternatively, the very complex cell culture broth contained other proteins/molecules that unspecifically interacted with the ligand or with the r-VIII SQ

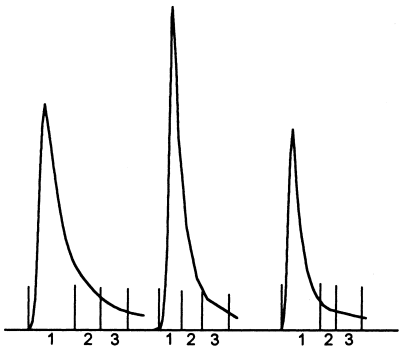

Fig. 3. Elution peaks for the three types of gels. The gels were loaded to saturation at 100 cm/h as shown in Fig. 2A, washed and eluted (at 15 cm/h) with ethyleneglycol–buffer as described in Section 2.7. The numbers denote collected fractions.

molecule in such a way that a diminished capacity was observed.

An otherwise straightforward explanation to the lowered capacity, namely the much lower r-VIII SQ concentration in the cell culture broth, is not believed to be important in the present case. Simple calculations using the equation

$$K_{\text{diss}} = \frac{[\text{antibody}][\text{r-VIII SQ}]}{[\text{antibody–r-VIII SQ complex}]} \quad (1)$$

showed that lowering the r-VIII SQ concentration 100-fold only lowered the total binding capacity of the gel with about 10%. The explanation to this excellent performance of the affinity material at low concentrations is the very low K_{diss} value for the antibody used. BIAcore measurements (preliminary results; Pharmacia and Upjohn) gave an estimated value for the dissociation constant of $3 \cdot 10^{-10}$ *M*. Even in the cell culture broth the r-VIII SQ concentration is more than 10-times higher than this value, thus securing an almost complete saturation of the binding sites at equilibrium.

The elution peaks (Fig. 3), corresponding to the breakthrough curves in Fig. 2, shows a sharp peak for the superporous and FastFlow matrices and a less sharp for the homogenous matrix. Even though the superporous and FastFlow matrices had the same sharpness of elution peaks, the concentration of r-VIII SQ in the pooled elution fractions (Table 1) differed significantly. This was due to the capacity difference, which resulted in a considerably higher product concentration for the superporous gel.

The purity of the eluted fractions was evaluated by SDS–PAGE (Fig. 4). No difference in purity was found. The total protein content was also measured with the Bradford method in some of the eluted fractions. The resulting purification factors are shown in Table 1.

In Fig. 5 the complete chromatogram for a typical run on the superporous matrix is shown. For the purpose of obtaining almost complete breakthrough curves, sample application was continued until nearly full saturation of the column. The figure shows that elution with 50% ethylene glycol was very efficient, and with a flow-rate of 15 cm/h, an extremely sharp elution peak was obtained. The loosely attached proteins were efficiently removed during the wash prior to elution. More then 40% of all protein in the eluted fraction was functional r-VIII SQ. Whether the impurities consisted of non-func-

Table 1
Characteristics of the superporous, homogenous and FastFlow matrices[a]

	Superporous gel	Homogenous gel	FastFlow
Concentration factor			
Elu1	177 times	118 times	63 times
Elu 1+2	110 times	66 times	33 times
Purification factor			
Elu 1	1109 times	1107 times	833 times
Elu 1+2	1051 times	n.d.	n.d.

[a] The size of the elution fractions are shown in Fig. 3. The concentration factor is calculated as [r-VIII SQ eluted]/[r-VIII SQ in cell culture broth].

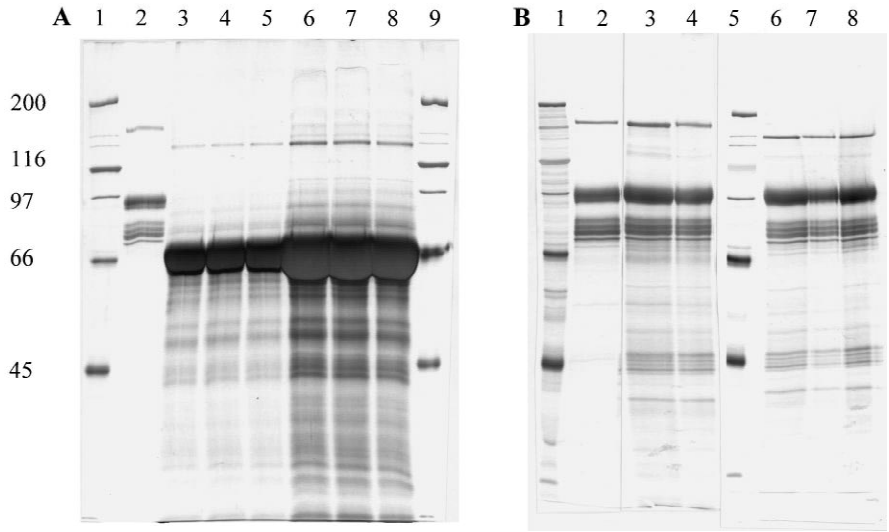

Fig. 4. SDS–PAGE of cell culture broth (A) and eluted r-VIII SQ (see Fig. 3) (B). The bands from left to right are: (A) M_r standard, highly purified r-VIII SQ, cell culture broth as sampled on three occasions, three lanes with 0.03 U/lane followed by three lanes with 0.13 U/lane and finally M_r standard. (B) M_r standard, highly purified r-VIII SQ, first and second elution fractions from the superporous matrix, M_r standard, first and second elution fractions from the homogenous matrix, and finally the first elution fraction from the FastFlow matrix. 4–10 U of r-VIII SQ was applied/well (10 for Elu 1 and 4–5 for Elu 2). For running conditions see Section 2.8. The M_r standard main components were 200 000, 116 000, 97 000, 66 000 and 45 000.

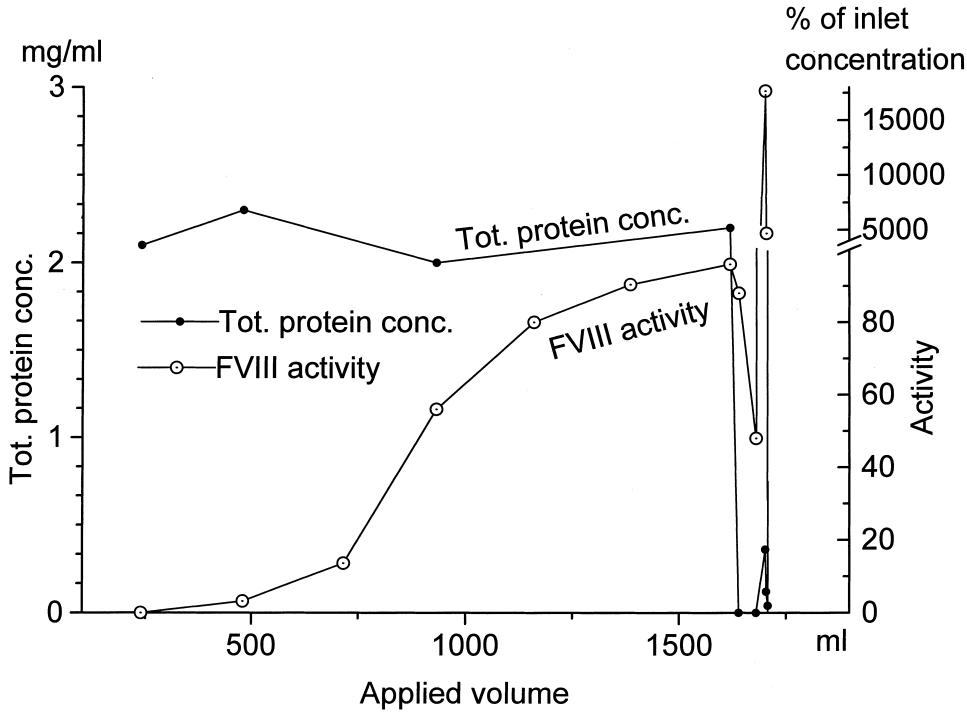

Fig. 5. Chromatogram for a superporous affinity gel showing total protein concentration and factor VIII activity during sample (cell culture broth) application (100 cm/h), washing (100 cm/h) and elution (15 cm/h) in a 25×10 mm column.

tional r-VIII SQ or protein from other origin was not determined.

3.3. Experiments to clarify unexpected gel performance

As discussed above, the FastFlow affinity gel had a dynamic capacity that was only 40% of that of the other two gels (superporous agarose and the corresponding homogeneous gel). This lower capacity of the gel was unexpected since all gels had been prepared with the intention of having similar binding capacities. Also the sharpness of the breakthrough obtained with the FastFlow gel came as a surprise, although it could be expected to be somewhat steeper than for the other homogenous matrix due to its smaller particle size. To investigate the reasons behind the behaviour some additional experiments were carried out.

First, the ligand concentration in the three matrices was determined additionally by a direct measurement of protein content on the gel by the BCA method. The results agreed well with the values obtained via UV measurements of the supernatant during the coupling step. It was found that the ligand concentration was slightly lower on the FastFlow gel. The superporous as well as the homogeneous agarose gel proved to have 1.0 mg ligand per ml of settled gel while the corresponding value for the FastFlow gel was 0.8 mg/ml, a moderate difference that not at all could explain the 2.5-times lower capacity observed with the FastFlow gel.

We then hypothesised that the unexpected behaviour could be due to an uneven distribution of ligand in the gel beads, and that the FastFlow gel might have a particularly uneven profile which could decrease the total binding capacity and at the same time improve the binding kinetics to a certain extent. Considering the fact that two of the gels were activated by CNBr in the laboratory while the third (Sepharose FastFlow) came preactivated from the manufacturer, such a hypothesis seemed reasonable to test.

According to earlier reports [19], ligands are often distributed unevenly in a gel bead. Both the activation step and the coupling step might act to give a higher concentration near the bead surface. In the activation step the reactive chemicals have to diffuse into the gel matrix and react before the alkaline medium destroys the reactants. Since the half-life of cyanogen bromide in the coupling medium is very short and dependent on the precise pH at hand, it is easy to anticipate an uneven activation profile of the bead, in which the highest activation would be at the gel surface. Furthermore, gels activated under different circumstances could easily result in different activation profiles.

In the coupling step the ligand must diffuse from the surrounding medium into the gel bead. If a gel has a much higher activation level near its surface than in its interior, the ligand density at the surface will be higher. Nominally the pores in all the three gels used in this investigation should have sufficient width for a fairly unhindered diffusion of an immunoglobulin ligand. However, as the coupling is proceeding the free pore diameter will diminish due to attached protein ligands. This will slow down the diffusion rate and further enhance the coupling of the ligands to the outer regions of the bead. Thus, it can easily be anticipated that under certain circumstances, a bead might obtain a highly uneven ligand density profile.

An aggravating factor, at least when the binding capacity is concerned, is that a high ligand density might lead to crowding, i.e., the antibody ligands are situated so close to each other that simultaneous binding of r-VIII SQ to all ligands is impossible for steric reasons. If such a crowding occurs the capacity of the gel must necessarily be reduced.

In a bead with crowding, the ligands could be expected to be concentrated in an outer layer of the bead for the reasons given above. This would give the bead faster kinetics than a bead with an even ligand distribution, since the diffusion distances from mobile phase to the affinity sites would be shorter.

Thus, if the manufacturing of the FastFlow beads had led to an uneven ligand distribution with ligand crowding, the anomalous behaviour of the gel could be explained.

To investigate this hypothesis several experiments with labelling and fluorescence microscopy were carried out with the three types of gel beads. FITC-labelled protein A was used as a fluorescent marker. The fluorescent conjugate was allowed to saturate the binding sites, and then studied in a fluorescence

microscope and images recorded with an attached CCD camera.

Fig. 6A and B shows the fluorescence images of the three types of gel and the corresponding intensity profiles registered by the CCD camera. The intensity profiles were obtained from a plane going approximately through the centre of each bead. Clearly, there are considerable differences between the gels, judged from the images as well as from the intensity profiles, suggesting different ligand concentration profiles.

To facilitate the interpretation of the intensity profiles, a number of ligand distribution profiles were constructed. A Microsoft Excel spreadsheet was then used to first calculate the fluorescence intensity in a large number of points (500 000) in a bead, and then to calculate how the CCD camera would pick up the fluorescence light from these points. Fig. 6D1–D3 gives three assumed distribution profiles and Fig. 6C1–C3 gives the corresponding fluorescence intensity profiles as the CCD camera was calculated to register them. Fig. 6C1 shows the results with an even ligand distribution, Fig. 6C2 with a somewhat surface-oriented distribution and Fig. 6C3 with a highly asymmetric surface-oriented distribution.

The fluorescence intensity profile simulated in Fig. 6C2 is reasonably similar to the profile obtained with the homogeneous bead (Fig. 6B2), which would indicate that it had ligands all through the bead, but with a higher concentration near the surface.

In Fig. 6C3 a very surface-oriented fluorescence intensity profile was simulated and, as can be seen, it fairly well resembles the profile actually observed with Sepharose FastFlow (Fig. 6A3), supporting the interpretation that the ligands in Sepharose FastFlow were mainly located to a thin outer layer. To extend the interpretation somewhat further, it could be argued that since a substantial part of the ligands in the Sepharose FastFlow bead were concentrated to a narrow zone, a situation that might lead to crowding effects, which could be the explanation of the rather low binding capacity observed with this gel. Detailed comparisons between the simulations and the actually observed profiles should be cautioned against, since the models used when doing the simulations should be regarded as rather crude. For instance, any corrections for light refraction phenomena, quenching or the fact that not the whole gel layer would be in focus was not carried out. On the other hand it should be pointed out that the fairly crude models used would not compromise the interpretation of the difference between the gels since the same, and probably rather small, errors should appear with all these gel types. In the same way it is reasonable to assume that the three gels will react very similarly with the labelling reagents even if the reagents are non-homogenous, in degree of fluorescine substitution and site of substitution, and even if the IgG molecules to be labelled are heterogeneous with regard to the site of attachment to the gel. The heterogeneity (reagents and molecule to be labelled) is the same for all the gels.

It could be argued that the observed ligand profile asymmetry is an effect of incomplete saturation/equilibration during labelling with FITC-protein A. To minimise that possibility, the staining was allowed to proceed for an extended time (overnight) in access of FITC-protein A, conditions that should allow equilibration even if the pores became partially blocked by bound FITC-protein A. Furthermore, some measurements were also carried out on sliced beads (microtome; 10 μm slices). The results agreed in the main features, although the asymmetric distribution observed with sliced beads was somewhat less pronounced than with whole beads. The reason for this is at present unclear but will be looked into. Also some reference experiments were carried out in another laboratory with confocal microscopy, which showed that analogous results could be obtained with that technique.

Concerning the superporous gel, a comparison between Fig. 6B1 and the simulations in Fig. 6C1–3 indicates that the ligand distribution was rather even with the obvious exception that there were no ligands in the space occupied by the superpores. Still, the full potential for obtaining an even profile with superporous beads was not used, since both CNBr activation and coupling was carried out in suspensions and not in a packed column. When the superporous beads are used in suspensions the pore flow occurring during mixing of the suspension will be considerably less than the pore flow through beads situated in a packed bed. Thus, it should be remembered that if the conditions call for extra effective distribution of activating agents, washing media and ligands a packed bed approach should be used.

In agreement with Subramanian et al. [19] we

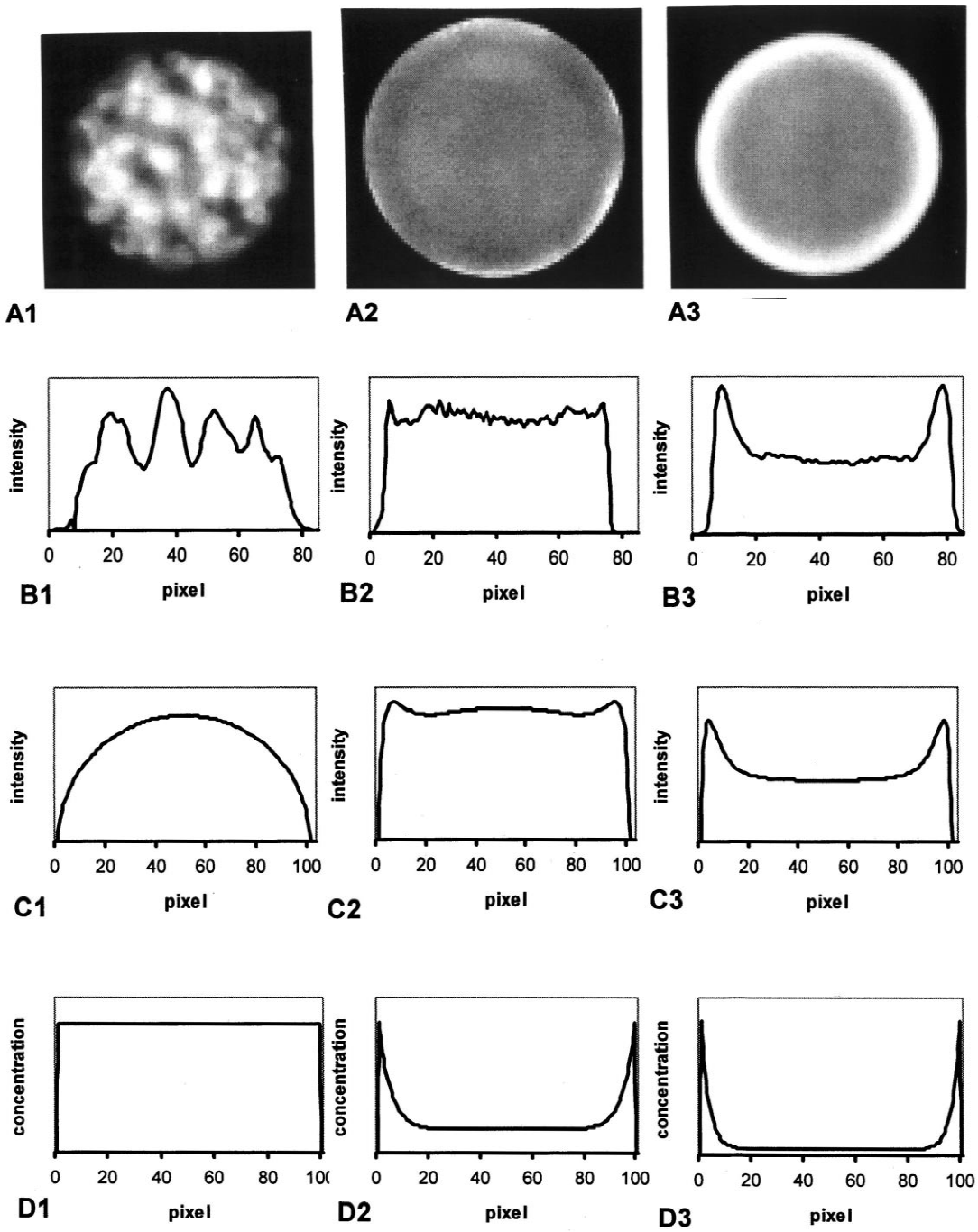

Fig. 6. Ligand distribution. (A) Photographs of three types of beads taken with a CCD camera fluorescense microscope which detected the fluorescent light from FITC-labelled protein A bound to the ligands (anti-factor VIII antibodies) located within the beads. 1 = Superporous bead, 2 = homogenous bead, 3 = FastFlow bead. (B) Intensity profiles obtained from a plane going approximately through the centre of the beads (in lane A). (C) Simulated fluorescense intensity profiles corresponding to the ligand distributions given in lane D. (D) Concentration profiles used for simulating the intensity profiles shown in lane C.

would like to underline the importance of knowing the ligand distribution in chromatographic matrices. In many instances the distribution may be fairly even and the performance of the matrix predictable. However, in case of asymmetric binding combined with crowding effects and hindered diffusion a matrix might behave quite atypical depending on the chromatographic conditions. Clearly, a firm knowledge of the involved profiles and parameters should be a valuable asset when describing the performance of the matrix in quantitative terms.

4. Conclusions

The present work shows that an efficient, direct isolation of r-VIII SQ from cell culture broth is indeed possible. Thus, in a one-step affinity procedure, a purification of 1000-fold was achieved. In comparison with standard-type chromatography gels the superporous agarose matrix showed a good chromatographic performance even at four-times higher flow-rate than the corresponding homogenous matrix, allowing much higher throughput. The rapid accessibility to the whole gel volume via the superpores for activation, ligand coupling and the subsequent affinity binding/elution, ultimately leads to a higher dynamic capacity, flow-rate and sharper elution peaks/higher product concentration.

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