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Short communication

Ultra-rapid preparation of milligram quantities of the purified polypeptide chains of human fibrinogen

S. Raut[a,*], P.H. Corran[b], P.J. Gaffney[a]

[a]*Division of Haematology, National Institute for Biological Standards and Control, South Mimms, Potters Bar, Hertfordshire, EN6 3QG, UK*

[b]*Division of Chemistry, National Institute for Biological Standards and Control, South Mimms, Potters Bar, Hertfordshire, EN6 3QG, UK*

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Abstract

In order to study the epitopes in fibrin towards which monoclonal antibodies are directed we needed the pure individual polypeptide chains of human fibrinogen in reasonable quantity. We report here a simplified, rapid method of separation of high-purity human fibrinogen chains. Following reduction and S-carboxymethylation of human fibrinogen, the sample was injected directly onto a column of the polymeric reversed-phase perfusion packing POROS 20-R2, and the chains were completely resolved in less than 3 min at a flow-rate of 10 ml/min. The capacity was equivalent to that of a similar sized conventional silica-based column. However the throughput was approximately five to ten times as high. The column was durable and robust in day-to-day use.

1. Introduction

Polymeric wide-pore reversed-phase columns have been used for rapid separations of proteins in such application as process monitoring [1,2]. Perfusion chromatography is a patented technique developed specifically for separations in which rapid mass transfer of solutes at high linear flow-rates is ensured by using ultra-wide pore polymeric packing under a regime where flow occurs through the pores as well as in the interstitial spaces between particles [1–9]. For example in Ref. [6] a mixture of proteins was separated in less than 1 min at mobile-phase velocities ten to thirty times higher than those used in conventional HPLC. Such rapid separations can also be exploited for preparative purposes [3,8], where short runs at high flow-rates can be used to achieve material throughput equivalent to preparative-scale runs on columns of conventional HPLC packing many fold larger.

A number of fibrin-specific monoclonal antibodies (MAbs) have been produced in our laboratory which react with crosslinked fibrin (XL-FN) but not with fibrinogen (Fg) or fibrin-(ogen) degradation products (FDPs) [10–12]. In order to determine the linear (or otherwise) epitopes towards which these MAbs are directed, milligram quantities of the three isolated polypeptide chains of human fibrinogen, Aα-67 kDa, Bβ-58 kDa and γ-47 kDa [13], were required. To date small quantities of these chains have been prepared by HPLC fractionation of the carboxymethylated chains [14] while milli-

* Corresponding author.

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gram quantities were prepared by ion-exchange chromatography in 8 *M* urea on a carboxymethyl cellulose (CMC) column [15,16]. The latter method was tedious, technically demanding and yielded individual chains of only moderate purity. We here report the application of perfusion chromatography in a rapid (3 min), technically simple procedure for the preparation of milligram quantities of high-purity polypeptide chains of fibrinogen.

2. Experimental

2.1. Column preparation

POROS 20-R2 (a 20-μm fimbriated reversed-phase perfusion packing based on a modified polystyrene–divinylbenzene support [6]) was purchased from PerSeptive Biosystems (Freiburg, Germany). It was suspended in 2-propanol and slurry-packed into a stainless steel HPLC column (100 × 5 mm I.D.) with a conventional HPLC pump (Kontron T414) (Kontron Instruments, Watford, UK) at a flow-rate of 10 ml/min using 80% methanol, 0.1% in sodium acetate as a follower. The column was evaluated using a mixture of seven proteins ranging from ribonuclease (10 kDa) to BSA (65 kDa), over a range of flow-rates from 1.25–10 ml/min using a constant gradient volume of 50 ml and a linear gradient of 20% to 60% acetonitrile in 0.1% trifluoroacetic acid (TFA) (v/v). Although the resolution at 1.25 ml/min was greatest, resolution at higher flow-rates was acceptable and at flow-rates greater than 2.5 ml/min there was no perceptible further reduction in resolution power. The Vydac C_4 Protein/Peptide RP column 214TP54 (5 μm particle size) (250 × 4.6 mm I.D.) was purchased from Hichrom (Reading, UK).

2.2. Apparatus

Separations were carried out with either of two HPLC systems, one consisting of a Spectra-Physics SP8800 gradient pump (Thermo Separations Products, Stafford, UK), a Polychrom (Model 9060) diode array spectrophotometer (Varian, Walton-on-Thames, UK) and a SP4270 integrator (Thermo Separations Products), with data captured to a PC running Winner On Windows (Thermo Separations Products). The other system consisted of an SP8700 solvent delivery system, an SP8500 dynamic mixer, a LC871 UV-Vis multiwavelength detector, a SP4270 integrator (all Thermo Separations Products) and a BioRad Model 2128 fraction collector (BioRad, Hemel Hempstead, UK). Both systems were operated at ambient temperature. Electrophoresis was carried out on a Hoefer SE 250-Mighty Small II slab gel electrophoresis unit (Hoefer Scientific Instruments, San Francisco, CA, USA).

2.3. Sample preparation for fractionation

Human fibrinogen (Grade L, KABI, Surrey, UK) in lyophilised form was reconstituted and reduced in the presence of 8 *M* urea and 5% (0.7 *M*) β-mercaptoethanol as described in Ref. [15]. Briefly, 40 mg of fibrinogen were dissolved in 3.6 ml of 1 *M* Tris buffer, pH 8.6–8 *M* urea–5% (0.7 *M*) β-mercaptoethanol and incubated overnight at 40°C to give mercaptolysed fibrinogen. An aliquot of this could be directly injected onto the HPLC. The remainder of this sample was carboxymethylated using the method of Crestfield et al. [17], by addition of an equal volume of 0.7 *M* iodoacetic acid (IAA) in 0.7 *M* NaOH, to give carboxymethylated fibrinogen. The mercaptolysed and S-carboxymethylated fibrinogen in urea did not require dialysis and could directly be injected onto the column. Problems with precipitation were experienced when using guanidinium chloride as a denaturant. Aliquots (100 μl) of the samples were snap-frozen in liquid nitrogen and stored at −40°C until ready for use.

2.4. Chromatographic procedures

Conventional reversed-phase HPLC: a modification of the procedure described by Kehl et al. [14] was used. These modifications are outlined in the legend to Fig. 1b. Optimum separation,

using the Vydac column, was achieved with a linear gradient from 39.2% to 49.6% acetonitrile in 0.1% TFA at a flow-rate of 1.5 ml/min over a 25-min period.

Perfusion chromatography: for the POROS column, fibrinogen chains were separated by injecting 20–100 μl of the reduced fibrinogen sample containing 0.2–1.0 mg protein. The best separation was achieved with a linear gradient from 34.4% to 47.2% acetonitrile in 0.1% trifluoroacetic acid. Several different flow-rates and gradient times were evaluated and a flow-rate of 10 ml/min with a gradient volume of 25 ml gave highest throughput. The effluent was measured at a wavelength of 210 nm for analytical separations and 278 nm for preparative scale up. The peaks were collected manually or automatically, using the Peak Detection Threshold option on the fraction collector, with the threshold set at approximately 10% peak height. The fractions collected were lyophilised and stored at −40°C for further analysis.

2.5. Gel electrophoresis

Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) was carried out according to the method of Schagger and Jagow [18] using 7.5% polyacrylamide mini gels (100 × 80 × 0.75 mm). Prior to electrophoresis the lyophilised samples were reconstituted in a buffer containing 1% SDS and heated at 90°C for 3–5 min. β-Mercaptoethanol 5% (0.7 *M*) was added for reduction to the individual polypeptide components. Electrophoresis was carried out at 30 mA constant current (15 mA/gel) for 1.5 h. The gels were then stained with Coomassie Blue (0.25%) in methanol–acetic acid–water (45:5:50, v/v).

3. Results

Mercaptolysed and S-carboxymethylated fibrinogen chains were separated on two different HPLC columns. Unreduced fibrinogen was also injected onto the columns in order to compare the elution behaviour with its individual polypeptide chain components. Fig. 1a shows the chromatograms of S-carboxymethylated fibrinogen on a perfusion chromatography (POROS 20-R2) column while Fig. 1b shows the elution profile on a conventional silica based reversed-phase C_4 (Vydac) column. In both cases near baseline separations of the fibrinogen chains were obtained. Each chromatogram also shows the elution pattern of the complete fibrinogen molecule.

The sample capacity was also investigated with the POROS column, and loadings up to 1.0 mg were found to yield excellent separations of the

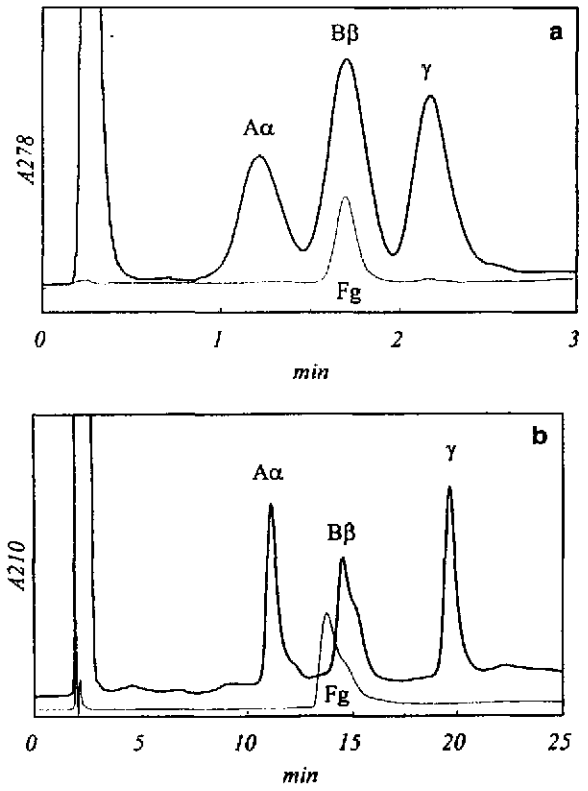

Fig. 1. HPLC separation pattern of 750 μg human S-carboxymethylated fibrinogen on POROS 20-R2 column using a linear gradient of 34.4%–47.2% acetonitrile at a flow-rate of 10 ml/min over 2.5 min (a) and 240 μg human S-carboxymethylated fibrinogen on Vydac C_4 Protein/Peptide RP column using a linear gradient of 39.2%–49.6% acetonitrile at a flow-rate of 1.5 ml/min over 25 min (b). The samples were acidified with acetic acid just prior to injection. In both cases fainter lines show the elution profile of whole unmodified fibrinogen (Fg).

polypeptide chains of fibrinogen. This capacity is similar to that of silica-based columns. Loadings greater than this, however, resulted in significant loss in resolution. The recommended maximum loading capacity for the Vydac column is 250 μg of protein.

The recovery of protein from the POROS column was evaluated by comparing the absorbance at 280 nm of the sample solution with that of the fractions following lyophilisation and reconstitution. The yield was found to be 74%, as compared to approximately 80% recovery observed with the conventional HPLC separation of fibrinogen chains [14].

The identities and purities of the fractions collected were confirmed by SDS-PAGE. In Fig. 2, preparations of carboxymethylated fibrinogen Aα, Bβ and γ chains purified by conventional HPLC (lanes 2–4) and perfusion chromatography (lanes 6–8) are compared with non-carboxymethylated fibrinogen (lanes 1 and 5) by SDS-PAGE. The purity of the isolated fibrinogen chains obtained by perfusion chromatography compared favourably with that of the chains isolated by conventional HPLC. The purity of the polypeptide chains isolated on the POROS column was further confirmed by both immunoblotting and reaction with individual antisera to the isolated polypeptide chains [19].

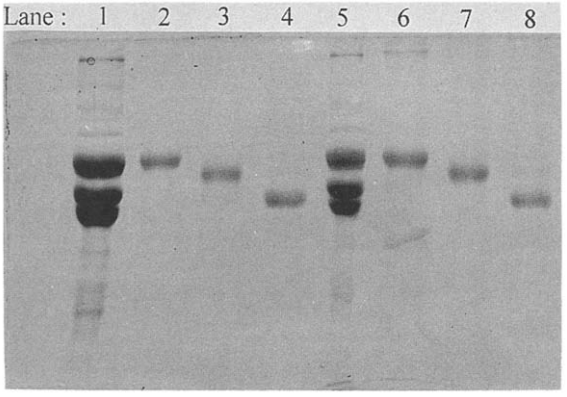

Fig. 2. SDS (7.5%)-polyacrylamide gel electrophoresis gels. Lanes 1 and 5: non-carboxymethylated fibrinogen; lanes 2–4: perfusion purified fractions corresponding to carboxymethylated Aα, Bβ and γ chains in Fig. 1a; lanes 6–8: fractions corresponding to carboxymethylated Aα, Bβ and γ chains in Fig. 1b.

4. Discussion

Our aim in undertaking this work was to develop a rapid and simple method of generating milligram quantities of the separated polypeptide chains of human fibrinogen. It is quite common in scaling up of chromatographic separations to have to sacrifice resolution or speed–often both, and this separation is no exception. The advantage of choosing to use perfusion chromatography is that the loss in resolving power is more than compensated for by the rapidity of the separation. The resolution with the POROS column (particle diameter 20 μm) was not as good as with the Vydac C_4 column (5 μm), nevertheless it was perfectly adequate for preparative purposes (Fig. 1). The reduction in resolution was not surprising in view of the difference in particle size compared to the Vydac column. Some of the difference may also be accounted for by differences in gradient profile (the gradient in the POROS column was about twice as steep in volume terms) and column dimensions. The slight discrepancy observed in the recovery of the protein material from the POROS column could possibly be due to incomplete solubilisation of fibrinogen chains following lyophilisation and perhaps also due to losses occurring by sorption to container surfaces during lyophilisation. However, the recovery of protein fractionated on the POROS column was satisfactory and comparable with that fractionated on conventional HPLC. The loading capacity of the POROS column, gave distinct advantage over the Vydac column. The overall throughput of the POROS column was equivalent to a silica-based column approximately 10-fold larger, with the added advantages that the POROS column can be prepared and used in conjunction with conventional HPLC apparatus. The technique is economical in its use of expensive bulk column packing and method development and optimisation are greatly simplified by the rapid turn-around time.

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