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Displacement chromatography in biotechnological downstream processing

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Abstract

Displacement chromatography was used for the isolation and purification of recombinant antithrombin III (rh-AT III), an anticoagulant of high pharmaceutical interest, which is usually isolated from human blood plasma. A fully automated system was developed, where the preparative chromatographic unit is continuously monitored by fast analytical HPLC. High purities and high concentrations were achieved in a single step. Macroporous hydroxyapatite (HA, diameter 2 μm, pore size 1000 Å) was used to pack the displacement column. Adsorption isotherms were recorded for various proteins. The influence of the column length, the mobile phase composition and the conditioning of the adsorptive surface on the separation by displacement of both basic and acidic proteins was investigated. Much attention was paid to the design of a suitable protein displacer. Cytochrome *c* and the polycation PEI (polyethyleneimine) were used for the displacement of basic proteins. Better results were achieved for the cytochrome *c*. A number of polyacids, proteins and Ca^{2+} complexing agents were investigated as putative displacers of acidic proteins from HA. While the polyacids, PA (polyacrylic acid, M_r 6000) and PGA (polyglutaminic acid, M_r 12 000), were unsuited to the task, the carboxylate cluster-carrying protein β-casein was more successful. The best results were obtained with complexing agents such as the commercially available EGTA [ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid, M_r 380.4] and IDA–PEG (polyethylene glycol carrying iminodiacetic acid end-groups, $M_{r\,PEG}$ 1000), synthesized for the purpose of protein displacement. Short reversed-phase columns packed with non-porous beads were used for the fast monitoring of the effluent of the displacement column. By working at 60°C the mobile phase flow-rate could be increased to an extent where the time required for a single analysis was reduced to 20 s. Including column regeneration, an analytical frequency of 2 min^{-1} could be maintained. As the flow-rates in the preparative column were chosen such (usually 100 μl/min), that a displacement including column regeneration required ca. 1 h, this analytical frequency is high enough to allow adequate monitoring and documentation of the purification and to permit automatic collection of the product-containing fractions.

1. Introduction

The importance of chromatographic separations for the isolation and purification of the more valuable products of modern biotechnology is unsurpassed [1–3]. At present, non-linear elution chromatography dominates the field, although neither the stationary nor the mobile phase are used efficiently in such processes and the superiority of displacement chromatography in terms of throughput, recovery, final product concentration and waste production has been recognized [4–10]. Other than in, e.g., affinity chromatography, several substances can be iso-

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lated simultaneously in displacement chromatography and concomitantly concentrated.

In displacement chromatography, the substance mixture to be separated is loaded on to the preparative column until saturation. Subsequently the displacer, a substance with an exceptionally high affinity for the stationary phase, is introduced at high concentration. Owing to the competition for the adsorptive sites ensuing between the various substances and the displacer, the so-called "displacement train" is formed. Consecutive zones of the pure substance move through the column at the speed of the displacer front. When suitable conditions are chosen, the final substance concentration in the respective zones will be considerably higher than that in the feed. While the theory of displacement chromatography is more involved than that of elution chromatography, it is now possible to predict the effect of column length, sample volume and concentration and also the displacer concentration on mass transport and separation efficiency [11–14].

Displacement chromatography is rarely used in biotechnological or pharmaceutical downstream processing. The lack of suitable protein displacers is one of the reasons for this [6,15–20]; the lack of adequate means for the automatic monitoring of the progress of a separation may also contribute. As the purified proteins appear consecutively in highly concentrated zones, at the column outlet, monitoring of the composition of the column effluent by means other than simple UV detection becomes necessary. Usually this is being done by laboriously collecting and analysing fractions. Besides some model separations using reversed-phase chromatography (RPC), hydrophobic interaction chromatography (HIC) and most often ion-exchange chromatography (IEC), only a few applications of displacement chromatography have been reported [6,8,21]. The antibiotic Cephalosporin C has been isolated from culture supernatants [6] and the enzyme alkaline phosphatase has been recovered from the periplasm of *Escherichia coli* [22]. Polyclonal IgG has been isolated from serum [23] and monoclonal IgG from ascites [7]. The number of protein displacers described in the literature is limited. Since 1979, Torres and Peterson have used carboxymethyldextranes to separate proteins in ion-exchange displacement chromatography [7,22,24,25]. The anionic carbohydrate chondroitin sulfate [17], carboxymethyl starch [15], Nacolyte [18] and the polycation polyethyleneimine (PEI) [6] have also been used. Good results were obtained for polyvinylsulfonic acid [20] and dextran sulfates [26], and also with portentous protein displacers [19].

Hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$, HA], a ceramic substance, is an interesting support for the chromatographic purification of high-molecular-mass biological substances [27–31]. Well defined biological macromolecules (proteins, DNA, etc.) are retained on HA, whereas low-molecular-mass substances and denatured proteins are less well retained and are thus easily removed. As HA stabilized the proteins during recovery, the yields of active product tend to be high. The exact mechanism of protein retention on HA is still under discussion, but conditions for adsorption and elution depend on the net charge of the protein in question. Acidic proteins such as bovine serum albumin (BSA), commonly added to most cell culture media, do not interfere with the isolation of a more basic product and vice versa. Several interactions may contribute to protein retention of HA, depending on whether the phosphate or the calcium sites at the HA surface are involved. Often a phosphate buffer is used as mobile phase, which causes the adsorption of more phosphate groups on the HA surface and concomitantly a negative net charge. At near neutral pH, basic proteins will be retained by unspecific electrostatic interaction of their positive net charge with the negative net charge of the HA surface. Such proteins may consequently be eluted by the addition of anions to the mobile phase or by cations with a high affinity for phosphate such as Ca^{2+}. Acidic proteins, on the other hand, interact by complexing the calcium sites with their carboxylate groups. At the same time they are repelled by the negative net charge of the HA surface. Desorption may be achieved by ions that form insoluble calcium salts such as F^- and PO_4^{2-}. Neutral proteins are also adsorbed on HA and

they can be eluted with PO_4^{2-}, Mg^{2+} and F^- but not Ca^{2+}.

2. Experimental

2.1. Materials

Proteins and fine chemicals were obtained from Sigma (Deisenhofen, Germany) and bulk chemicals for buffer and eluent purification from Fluka (Neu-Ulm, Germany). AT III derived from human blood (Kybernin) was donated by the Behring Werke (Margburg, Germany). AT III-containing cell culture supernatants of CHO (Chinese hamster ovary) cells were donated by the Cell Culture Technology Group (Dr. G. Kretzmer) of our Institute. Polymeric displacers and precursors thereof were from Polyscience (Warrington, PA, USA). The HA stationary phases were obtained from Asahi Optical (Tokyo, Japan). The particles were of 2- and 10-μm diameter with average pore size of 1000 Å. Non-porous 2-μm Micropell C_{18} beads (Glycotech, Hamden, CT, USA) were used to pack the analytical RPC columns.

2.2. MALDI-MS

Matrix-assisted laser desorption/ionization mass spectrometric (MALDI-MS) measurements were kindly carried out by Dr. U. Bahr (Institut für Medizinische Physik und Biophysik, University of Münster, Münster, Germany) using a Vision 2000 laser time-of-flight mass spectrometer (Finnigan MAT, Bremen, Germany). Samples were dissolved in water at a concentration of 5 g/l. The matrix was a 10 g/l solution of 2,5-dihydroxybenzoic acid in water–ethanol (9:1). Volumes of 0.5 μl of sample and 2 μl of matrix solution were applied to a silver target and allowed to dry. A post-acceleration voltage of 10 kV was used during the measurements.

2.3. Preparative chromatography

The preparative chromatographic system was assembled from a Gyncotech (Germering, Germany) Model 300 CS pump, a Shodex pulse damper (Showa Denko, Tokyo, Japan) and a Valco (Houston, TX, USA) ten-port valve. A 1-ml sample loop was used for sample injection, while the displacer was introduced from a preparative sample loop (Knauer, Berlin, Germany), that could hold up to 11 ml. Preparative columns were packed at 200 bar. Unless indicated otherwise, the column dimensions were 100 mm × 4.6 mm I.D. A flow-rate of 100 μl/min was used in the displacements. The effluent from the displacement column was analysed twice per minute by analytical RPC or 50-μl fractions were collected and analysed likewise by analytical RPC. For column regeneration, 400 m*M* phosphate buffer (pH 6.8) was used. In the case of protein displacements by Ca^{2+} chelating agents, 100 m*M* $CaCl_2$ solution was used instead for column regeneration.

2.4. Analytical chromatography

The high-pressure gradient system for the analytical HPLC was assembled from two IRICA S 871 pumps (ERC, Alteglofsheim, Germany) controlled by an Autochrom gradient controller (ERC) and a 250-ml dynamic mixing chamber (Knauer, Berlin, Germany). An SF 757 UV detector (ABI, Applied Biosystems, Foster City, CA, USA) equipped with a 0.5-ml/1-mm micro flow cell (0.1-s filter time) with the detection wavelength set at 214 nm was used. For sample injection (5 μl), either an electrically driven EC6W valve (Valco) or a pressure-driven Model 7010 valve (Rheodyne, Cotati, CA, USA) was used. The electrically driven valve caused a high-pressure pulse during injection. The column dimensions were 30 mm × 4.6 mm I.D. Buffer A consisted of deionized water with 0.1% (v/v) of trifluoroacetic acid (TFA) added, and buffer B of acetonitrile with 0.08% (v/v) of TFA added. The gradient was run from 30% to 70% B. By using a flow-rate of 4 ml/min and an operating temperature of 60°C, an analysis was completed within 20 s. Data collection and interpretation were carried out with a Spectra-Physics (now Thermo-Separation Products) (Darmstadt, Germany) SP4270 integrator or a PC using the

CAFCA (computer-assisted flow control and analysis) software developed by Dr. B. Hitzmann and co-workers. (Institut für Technische Chemie, University of Hannover, Hannover, Germany).

2.5. Isotherm measurements

The isotherms were determined as described in Ref. 32. A mobile phase flow-rate of 100 μl/min was used in all instances. The column dimensions were 50 mm × 2 mm I.D. and the detection wavelength was 280 nm. For the basic proteins, 10-μm HA particles (pore size 1000 Å) and 10 m*M* phosphate buffer (pH 6.8) were used. For the acidic proteins, 5 m*M* phosphate buffer (pH 6.8) containing 0.4 m*M* $CaCl_2$ was used as the mobile phase and 2-μm HA particles (pore size 1000 Å) were used to pack the 50 mm × 2 mm I.D. column. For comparison, the basic protein lysozyme (p*I* 10.5) was included in both groups of isotherm measurements.

3. Results and discussion

Many of the highly valuable products of modern biotechnology are produced by mammalian cells. The natural and environmental requirements of such cells are complex. Many culture media contain, e.g., significant amounts of foetal calf serum (FCS). The product is therefore contaminated by a large number of proteins, peptides and other substances, many of which have never been isolated and characterized. BSA (bovine serum albumin, p*I* 4.9), b-IgG (bovine immunoglobulin G, p*I* 7.3) and b-transferrin (p*I* 5.5) must be expected as major product contaminants [33]. The so-called serum-free media usually contain some BSA and b-transferrin. As explained above, depending on its net charge, a protein interacts preferably with the phosphate or the calcium sites on the HA surface. Consequently, acidic proteins such as BSA do not interfere with the isolation of a more basic product, and vice versa. The separation, e.g., of immunoglobulins (p*I* 7.2–7.5) from other more acidic serum proteins therefore poses no problem. Most proteins [34] and consequently most biotechnological products belong, however, to the group of acidic proteins. In such cases the removal of BSA and b-transferrin, but not b-IgG, is necessary and HA displacement chromatography will be most valuable.

For some time now, the aim of our group has been the development of chromatographic separations in the displacement mode for the isolation of pharmaceutically applicable substances (e.g. recombinant h-AT III, monoclonal antibodies) from mammalian cell culture supernatants. The monitoring of the progress of a separation tends to be a problem in displacement chromatography. Usually volume fractions of the displacement train are collected at the column outlet and analysed. Consequently, the time required for documentation may be considerably longer than that necessary for the separation proper. This handicaps process development and prevents the formulation of an on-line process algorithm. We developed a fully automated system instead, where the preparative unit is quasi-continuously monitored by an extremely fast analytical HPLC system (Fig. 1).

3.1. Monitoring of the displacement

The achievement of short analysis times necessitates high mobile phase flow-rates. Following a suggestion of Kalghati and Horvath [35], the viscosity of the mobile phase was lowered by raising the temperature, thus making high flow-rates comparable to a still sustainable pressure drop. A number of macroporous and non-porous RPC stationary phases and macroporous IEC resins were evaluated with regard to their suitability for providing such fast analytical columns. The best results in terms of resolution and long-term stability at high flow-rates were achieved with columns packed with non-porous 2-μm Micropell C_{18} resin.

By using a temperature of 60°C and a flow-rate of 4 ml/min, the composition of the effluent of the displacement column could be analysed within 20 s. Including column regeneration, an analytical frequency of 2 min^{-1} could be maintained. This frequency allowed the quasi-on-line monitoring and documentation of the purification and made accurate control of the fractiona-

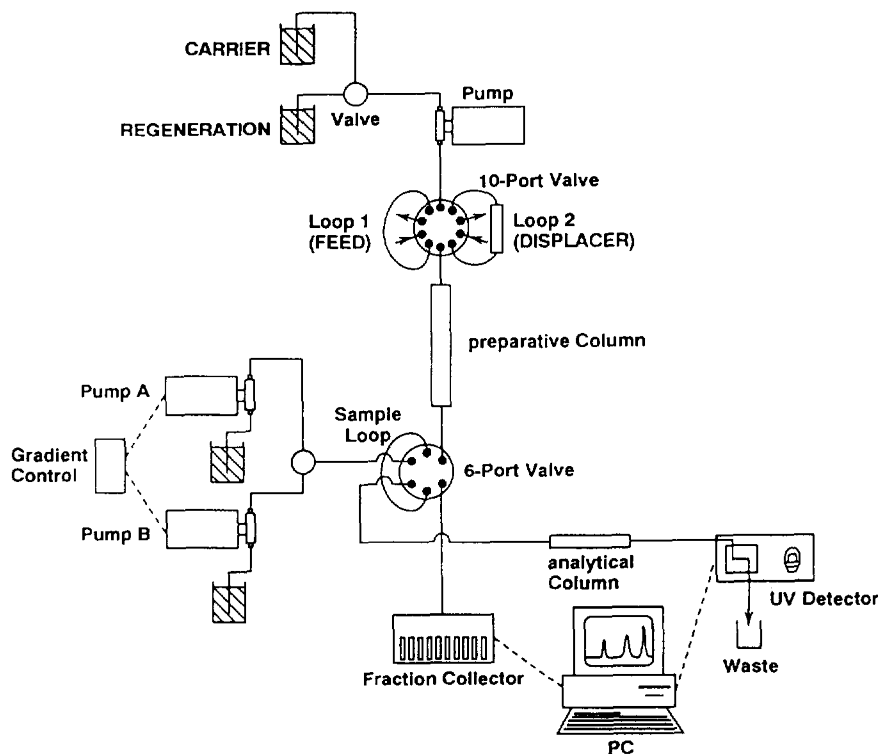


Fig. 1. Schematic diagram of the chromatographic system. The preparative unit is continuously monitored by fast analytical HPLC.

tion possible. The increase in temperature had the beneficial side-effect of improving the resolution at a given flow-rate, perhaps owing to an improvement in the diffusional mass transport at that temperature. In Fig. 2, examples are given of analytical runs performed on the feed and at two points during the displacement of basic (lysozyme, myoglobin; displacer, cytochrome *c*) and acidic (AT III, BSA, b-transferrin; displacer, polyglutaminic acid) proteins. The sample volume was 5 μl in the standard assay but might be decreased to 1 μl by using a microinjection loop; little product was therefore lost in the analysis.

3.2. Displacement of basic proteins

In Fig. 3, the adsorption isotherms of several basic proteins on HA are shown; 10-μm HA particles and 10 m*M* phosphate buffer (pH 6.8) were used. The availability of a suitable displacer is most important in displacement chromatography. In Fig. 4a, the polycation PEI (polyethyleneimine) is used to displace lysozyme (p*I* 10.5) and cytochrome *c* (p*I* 11.2). In Fig. 4b, cytochrome *c* serves as displacer of lysozyme and myoglobin (p*I* 7.4). Not only is the separation better in the latter instance, but also the concentration in the "product" is zones higher. Less contamination of these zones by the displacer is found in the case of protein displacement by cytochrome *c*. Moreover, the removal of cytochrome *c* from the displacement column posed no problem, as flushing with 400 m*M* phosphate buffer sufficed, whereas the removal of the PEI was extremely difficult and time consuming.

In Fig. 5, the influence of the column length on the displacement of lysozyme and myoglobin by cytochrome *c* is demonstrated. The separation quality is clearly improved when a 100- rather than a 50-mm column is used. A further increase in the column length, e.g., 250 mm did not

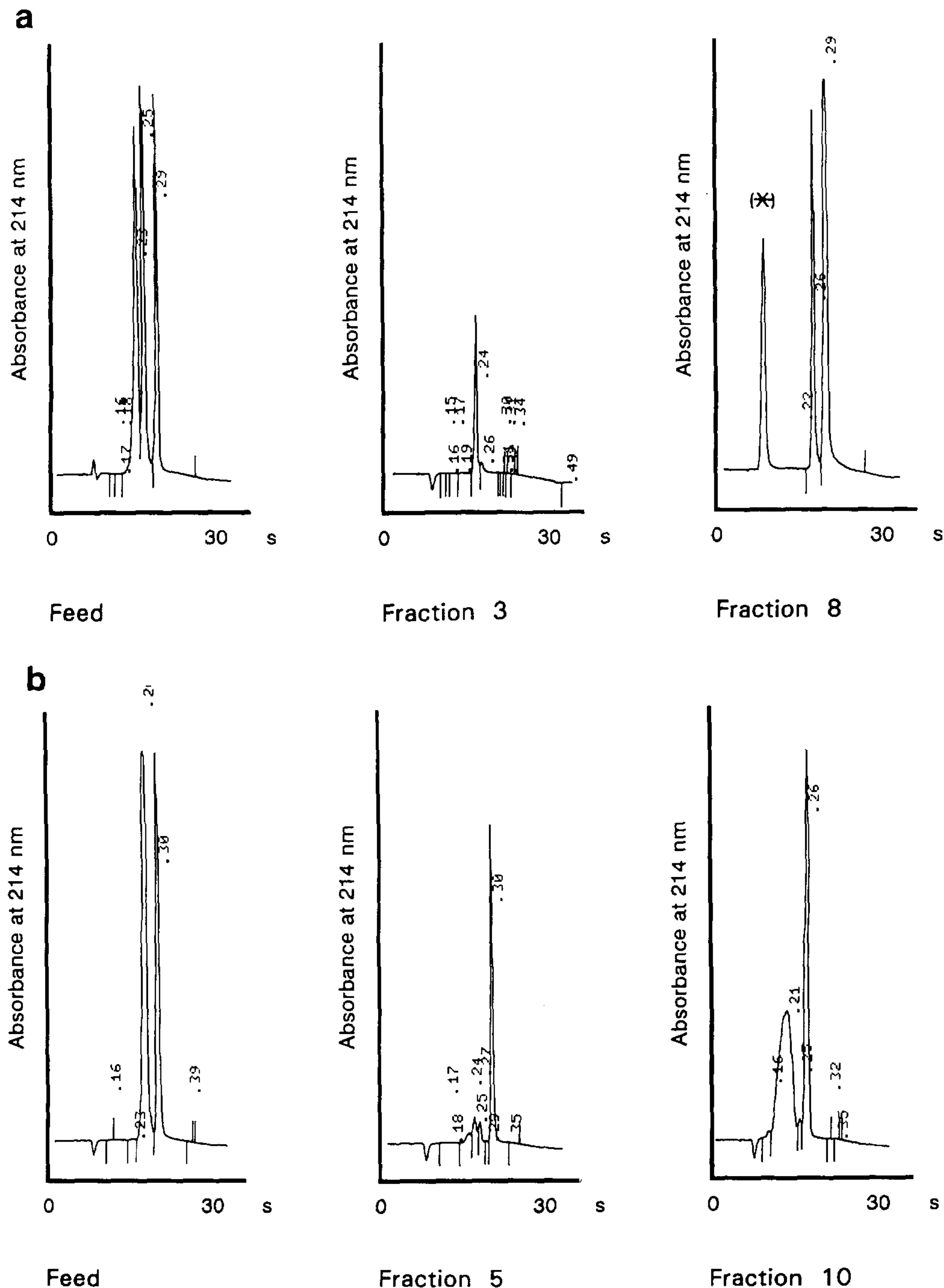


Fig. 2. (a) RPC analysis of the feed and at two points during the separation of the acidic proteins b-transferrin (retention time 0.24 min, p*I* 5.5), BSA (retention time 0.26 min, p*I* 4.9) and AT III (retention time 0.29 min, p*I* 4.6–5.1) by displacement chromatography, using polyglutaminic acid (*) as displacer. (b) RPC analysis of the feed and at two points during the separation of the two basic proteins lysozyme (retention time 0.26 min, p*I* 10.5) and myoglobin (retention time 0.30 min, p*I* 7.4) by displacement chromatography using cytochrome *c* (retention time 0.21 min) as displacer.

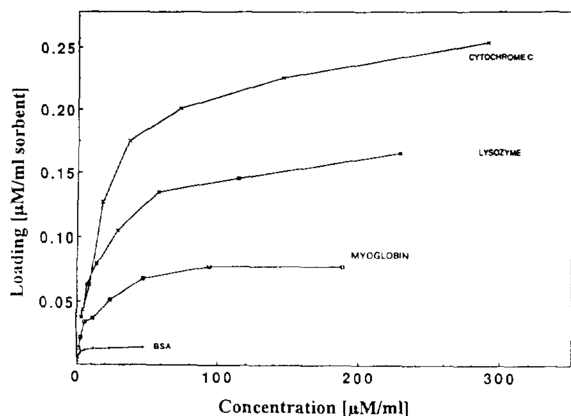


Fig. 3. Selected adsorption isotherms of basic proteins on HA.

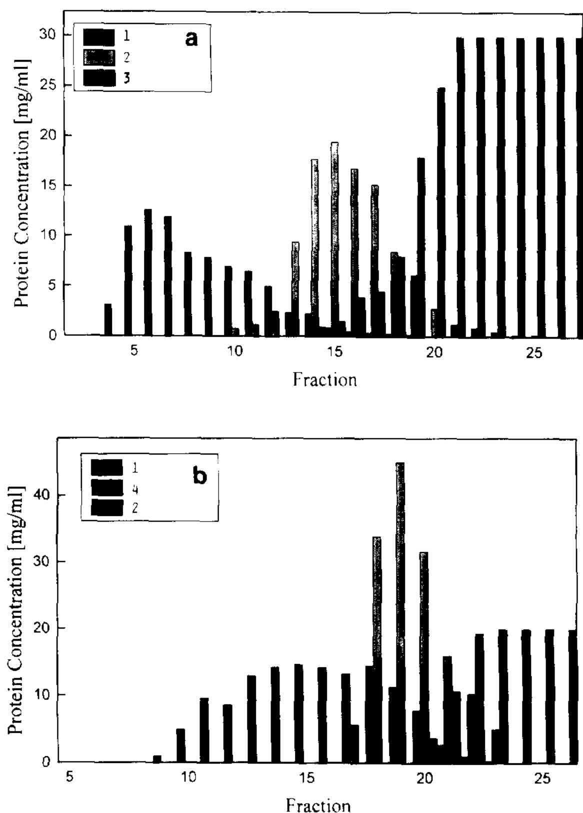


Fig. 4. (a) Displacement of lysozyme and cytochrome c (both 10 mg/ml) by PEI (30 mg/ml, M_r 60 000). (b) Displacement of myoglobin and lysozyme (both 10 mg/ml) by cytochrome c (30 mg/ml). 1 = Lysozyme; 2 = cytochrome c; 3 = PEI; 4 = myoglobin.

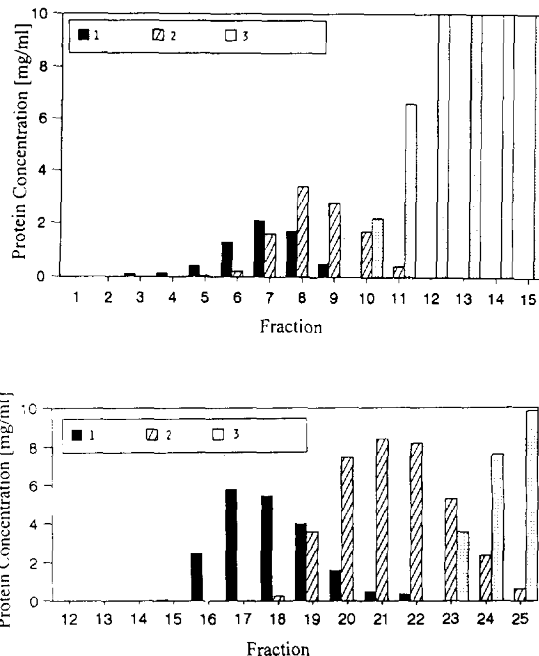


Fig. 5. Influence of the column length on a displacement of myoglobin (1.1 mg/ml) and lysozyme (1.7 mg/ml) by cytochrome c (10 mg/ml). Column dimensions: (top) 50 mm × 4 mm I.D.; (bottom) 100 mm × 4.6 mm I.D. 1 = Myoglobin; 2 = lysozyme; 3 = cytochrome c.

improve the separation any further, however, while necessarily increasing the time required for the experiment. Thus, when 10 mM phosphate buffer (pH 6.8) is used, myoglobin and lysozyme can be well separated when displaced by cytochrome c. Presumably, however, the conditioning of the HA surface by the phosphate ions of the buffer is necessary, as a displacement carried out in Tris buffer of similar pH and concentration (10 mM, pH 7.0) yielded less satisfactory results (Fig. 6). When 1 mM $CaCl_2$ were added to the Tris buffer (Fig. 6), lysozyme, but not myoglobin, was hardly retained, even though this protein showed a higher affinity to HA than myoglobin in most other instances.

3.3. Displacement of acidic proteins

To improve protein retention and capacity by decreasing the repelling effect of the surface and increasing the number of adsorptive sites, HA

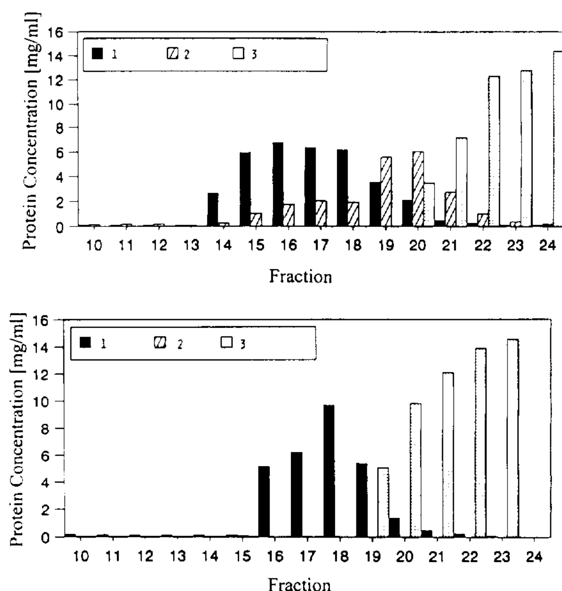


Fig. 6. Displacement of myglobin (1.1 mg/ml) and lysozyme (1.7 mg/ml) by cytochrome *c* (10 mg/ml) using as mobile phase (top) 10 m*M* Tris buffer (pH 7.0) and (bottom) 10 m*M* Tris buffer (pH 7.0) with 1 m*M* $CaCl_2$. 1 = Myoglobin; 2 = lysozyme; 3 = cytochrome *c*.

columns were conditioned by a wash with 2 ml of 200 m*M* $CaCl_2$ solution (in Tris buffer) prior to any displacement of acidic proteins. In addition, Tris rather than phosphate buffers were used as mobile phases in the displacement experiments. In Fig. 7, the adsorption isotherms of acidic proteins are shown. For comparison, the adsorption isotherm of the basic lysozyme as obtained under these conditions is also included. Even under conditions favourable to the adsorption of acidic proteins, the HA material shows a higher capacity for the basic protein than for any of the acidic proteins. Whereas with basic proteins the elution order usually corresponds to the saturation capacities, this is not necessarily so for the acidic proteins.

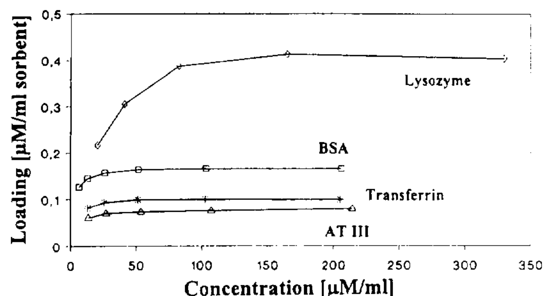


Fig. 7. Selected adsorption isotherms of basic proteins on HA. The isotherm of lysozyme is included for comparison.

Acidic proteins are assumed to interact with the Ca^{2+} sites of HA via their deprotonated carboxylate groups. Therefore, the displacement of acidic proteins such as BSA (p*I* 4.9), AT III (p*I* 4.6–5.1) and b-transferrin (p*I* 5.5) was initially attempted with certain polyacids such as polyacrylic acid (PAA, M_r 6000) and polyglutaminic acid (PGA, M_r 12 000) (Fig. 8a–8c). Neither displacer substance gave satisfactory results. Hardly any protein separation took place, regardless of column length, mobile phase composition or type and average molecular mass of the displacer. Significant amounts of polymeric displacer were found in the protein zones.

Carboxylate cluster-carrying proteins are known for their exceptional HA affinity. In some instances basic carboxylate cluster-carrying proteins have been observed to behave similarly to acidic proteins in their adsorption and elution behaviour, e.g., in their resistance to elution by Ca^{2+}. Here one of these proteins, namely β-casein, was investigated as a protein displacer (Fig. 9). Even though neither the column length nor the buffer composition was ideal for the displacement of acidic proteins, the results achieved with the β-casein displacer are distinctly superior to those obtained with the polyacids. Apparently, not only the number but also the arrangement of the carboxylate groups is of consequence.

As the arrangement of the carboxylate groups appears to be of importance, it is not surprising that the best results were found when a Ca^{2+}-chelating agent was used to displace acidic proteins. Here EGTA [ethyleneglycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid, M_r 380.4], a high-molecular-mass analogue of EDTA, was used (Fig. 10). However, when EGTA was used to purify AT III in the presence of both BSA and b-transferrin, hardly any separation between AT III and BSA took place. This is a general problem in the isolation of AT III

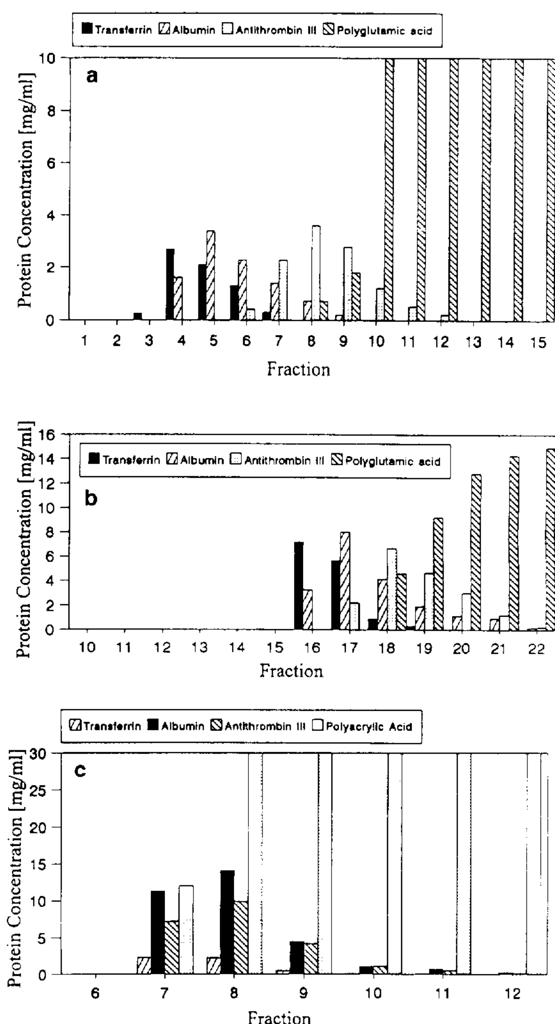


Fig. 8. (a) Displacement of transferrin (0.7 mg/ml), BSA (0.8 mg/ml) and AT III (0.9 mg/ml) by PGA (15 mg/ml, M_r 12 000). Mobile phase, 5 mM phosphate (pH 6.8) with 0.3 mM $CaCl_2$; column, 50 mm × 4 mm I.D. (b) Displacement of transferrin (0.8 mg/ml), BSA (1.8 mg/ml) and AT III (1.2 mg/ml) by PGA (15 mg/ml, M_r 12 000). Mobile phase, 2 mM phosphate (pH 6.8) with 1 mM $CaCl_2$; column, 100 mm × 46 mm I.D. (c) Displacement of transferrin (0.8 mg/ml), BSA (01.4 mg/ml) and AT III (1.0 mg/ml) by PAA (30 mg/ml, M_r 6000). Mobile phase, 10 mM Tris (pH 7.5) with 0.3 mM $CaCl_2$; column, 100 mm × 4.6 mm I.D.

from cell culture supernatants [33], since BSA is part of almost any cell culture medium recipe. The physiological function of BSA as a transport protein requires close adherence to the various blood factors, including, unfortunately, AT III.

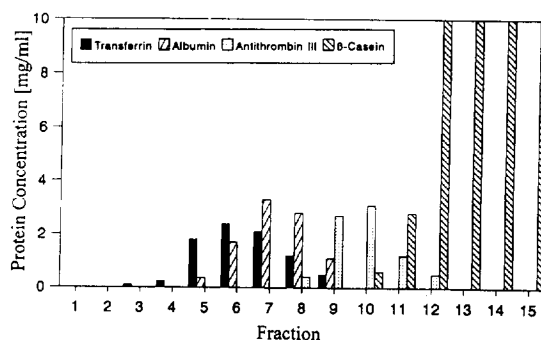
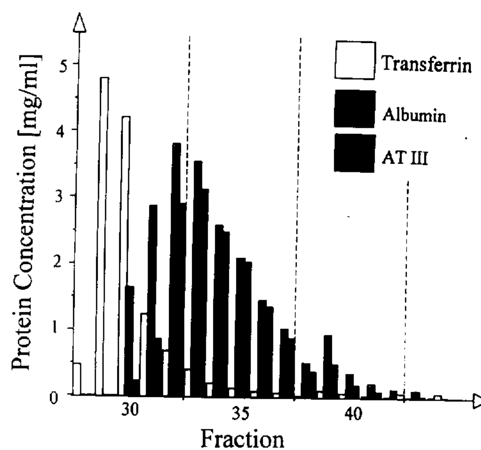


Fig. 9. Displacement of transferrin (0.8 mg/ml), BSA (0.8 mg/ml) and AT III (0.6 mg/ml) by β-casein (15 mg/ml). Mobile phase, 5 mM phosphate (pH 6.8) with 0.3 mM $CaCl_2$; column, 50 mm × 4 mm I.D.



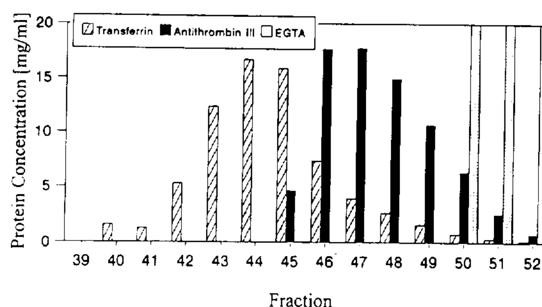


Fig. 10. Top: displacement of transferrin (4.2 mg/ml), BSA (2.8 mg/ml) and AT III (3.1 mg/ml) by EGTA (20 mg/ml, M_r 380.4. Mobile phase, 20 mM Tris (pH 9.0). Bottom: displacement of transferrin (4.2 mg/ml) and AT III (3.1 mg/ml) by EGTA (20 mg/ml, M_r 380.4). Mobile phase, 20 mM Tris (pH 9.0).

If the BSA is removed at the earliest possible moment, e.g., by Cibacron Blue affinity chromatography [33], a much improved separation is found (Fig. 10, bottom).

Polymeric displacers usually show a broad molecular mass distribution and also tend to vary in structure. As a consequence, the affinity for the stationary phase tends to be inhomogeneous and often less well retained displacer molecules contaminate the preceding protein zones. With EGTA, on the other hand, a sharp displacer front is observed, as all displacer molecules are alike in mass and structure. The final AT III concentration in the displacement fractions is several times higher than that in the original feed. The yields were generally better than 98%. With a time requirement of 30 min, displacement chromatography is fast compared with the multi-stage chromatographic systems used before [33]. When the biological activity of the recovered AT III was tested using a heparin co-factor activity assay (Boehringer, Mannheim, Germany) [36], displacement chromatography was found to be not only a fast but also a mild means for protein purification.

3.4. Synthesis and application of a polymeric displacer of acidic proteins

Starting from a highly homogeneous (molecular mass standard) PEG (polyethylene glycol, M_r 1000), a novel protein displacer was synthesized (Fig. 11), by adapting procedures given in Refs. [37] and [38]. The displacer carries IDA (iminodiacetic acid) groups at both ends, which are assumed to form strong chelates with the calcium sites of the HA. When the PEG educt and the final displacer were investigated by MALDI-MS, the expected shift in molecular mass was clearly seen (Fig. 12). The resulting polymer was used in a displacement experiment (Fig. 13) and appeared to be an excellent displacer of acidic proteins from HA. High protein concentrations are found. A sharp displacer front is again observed. Moreover, other than in the case of the PEI used for the displacement of basic

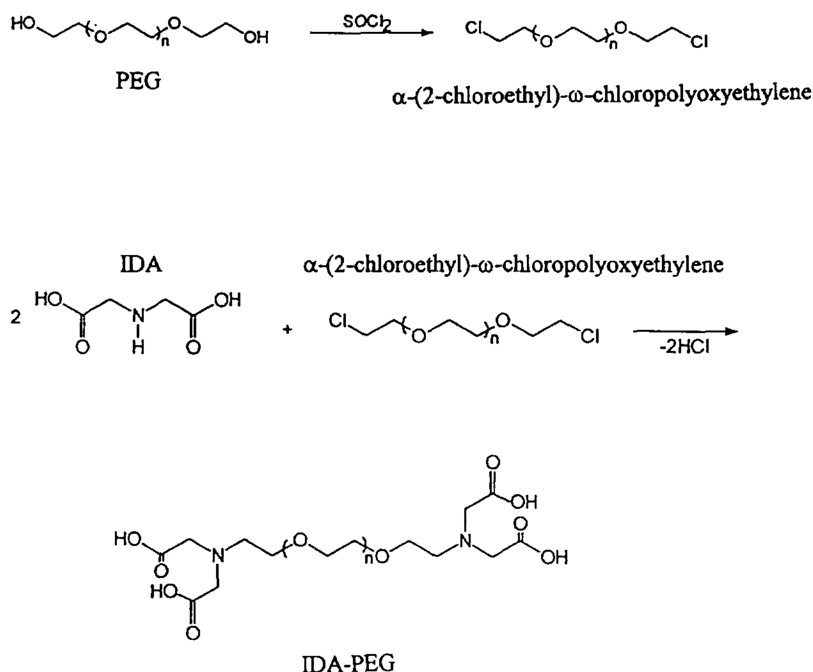


Fig. 11. Synthesis of a polymeric protein displacer according to Refs. [37] and [38].

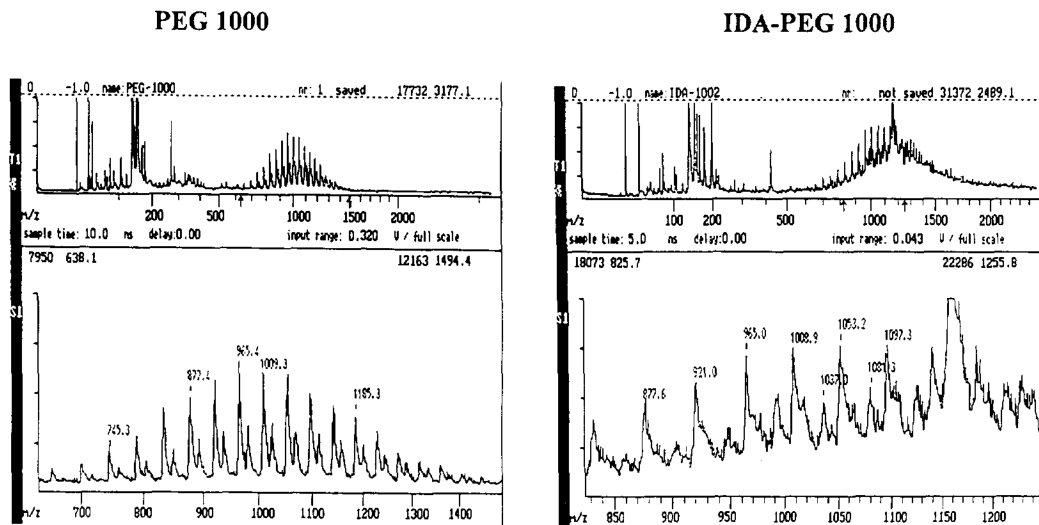


Fig. 12. MALDI-MS measurements of the PEG 1000 molecular mass marker and the synthesized IDA–PEG.

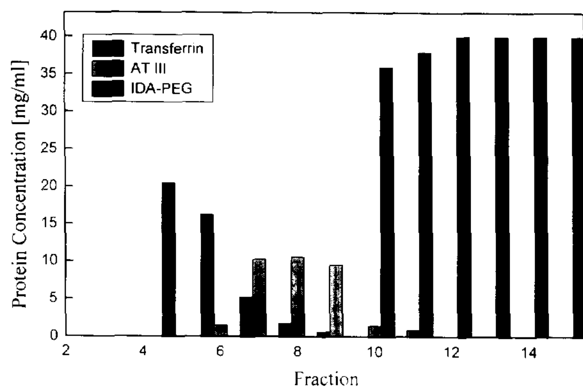


Fig. 13. Displacement of transferrin (2.4 mg/ml) and AT III (2.1 mg/ml) by IDA–PEG (40 mg/ml). Mobile phase, 20 m*M* Tris (pH 9.0).

proteins, the IDA–PEG displacer could easily be removed from the HA column, e.g., by washing with 100 m*M* $CaCl_2$ solution in Tris buffer.

Acknowledgement

Dr. Ute Bahr is thanked for the MALDI-MS measurements.

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