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Controlled mixed-mode interaction chromatography on membrane adsorbers

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Abstract

Membrane adsorbers (MAs) are used for protein separation in controlled mixed-mode interaction chromatography. The strong anion- and cation-exchange MAs used are made either from a synthetic copolymer or from modified cellulose membranes. The affinity MAs (Cibacron Blue) are also made from the copolymer membranes. Standard protein mixtures, whey proteins, and biotechnological culture supernatants are separated. The influence of the flow-rate, the ratio of cation- and anion-exchange MAs inserted in the stack, and the pattern, i.e. the comparative worth of an alternating vs. a two-consecutive-stacks arrangement, on the separation is investigated. While the flow-rate shows no influence, both the pattern of arrangement and the ratio of the two types of ion exchanger do. Compared to single-mode MA chromatography, a broader range of proteins, e.g. in terms of the isoelectric points, can be separated in a single chromatographic procedure. Whey proteins (β-lactoglobulin, α-lactalbumin, BSA, IgG) are separated at pH 6, using a mixed-mode ion-exchange system. Here however, a two-stack approach is used to allow for module-uncoupling before elution, to prevent IgG and α-lactalbumin from coeluting. By using a mix of anion-exchange and Cibacron Blue affinity MAs, recombinant human antithrombin III (rh-AT III) can be separated in a single run from the major protein impurities present in the fermenter supernatant, namely transferrin and BSA.

Keywords: Mixed-mode stationary phases; Membranes; Stationary phase, LC; Proteins

1. Introduction

Save for a few exceptions, such as hydroxyapatite chromatography [1,2] or the Bakerbond ABX-phases (Baker, USA), that were especially developed for antibody purification [3], mixed-mode interactions are avoided in liquid chromatography. On the other hand, single-stage chromatography is seldomly sufficient for preparative protein separation, and multi-stage procedures, where several types of interaction are exploited in series, are the norm. Controllable mixed-mode phases may thus yield columns with unique separation features [4]. The advantages of mixed-mode stationary phases in low- to medium-pressure separations of biopolymers have been recognized [5]. With conventional HPLC (silica-based) packing materials, however, such mixed-mode phases are difficult to produce in a homogeneous, reproducible and concomitantly flexible manner. Ion exchangers, that also show a pronounced hydrophobic character stemming from the support material [6], as well as mixed anion/cation exchangers [4] are some of the few examples discussed in that area. The situation with regard to the preparation of the mixed-mode stationary phase is quite different in membrane adsorber (MA) chromatography, where stacks of

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functionalized interactive membranes are used as stationary phase. In this case the 'column' can be constructed from different types of membrane, which in addition can be arranged in the desired pattern.

The advantages and disadvantages of the so-called membrane adsorbers (MAs), i.e. filter membranes that have been functionalized by the linkage of interactive groups to their surface, as stationary phase in liquid chromatography have been discussed in the pertinent literature for some time now [7–16]. Fluid-dynamic and mass-transfer properties as well as handling, scale-up and hardware requirements appear to be superior in MAs compared to conventional HPLC/FPLC columns. Short MA stacks (typically of no more than some millimeter of height with a diameter of several centimeter) have been substituted for columns in HPLC and FPLC systems for the fast and efficient isolation of biotechnological products such as blood factors, monoclonal antibodies, and technical enzymes [17–23].

Since the mass transport in MAs phases occurs predominantly by convection rather than diffusion, there is little deterioration of the separation efficiency at elevated flow-rates [14,20,24–26]. Since the backpressure caused by the MA stacks is often quite low, high flow-rates and concomitantly high throughputs and fast separations become possible. Just as conventional column chromatography, however, MA chromatography usually relies on a single molecular feature, such as the size, the charge density, the hydrophobicity, etc., for substance separation, i.e. is carried out as single-interaction-mode chromatography. Under these circumstances, the separation can be related straightforwardly to the parameter in question and the theoretical understanding is facilitated. Moreover, MAs are often used for 'specific filtration' rather than true chromatography, i.e. for applications under conditions that favour the retention of only the target molecule on the MA, while the other components are allowed to break through unretained. Multi-stage separations where different types of interactions are used consecutively to bring about the separation are still extremely rare in MA chromatography [23,27].

In this paper, strong ion-exchange MAs (sulfonic acid groups and quaternary ammonium groups) as well as affinity MAs (Cibacron Blue ligands) are investigated for controlled mixed-mode interaction chromatography of standard protein mixtures as well as for the separation of whey proteins and biotechnological culture supernatants.

2. Experimental

2.1. Chemicals

All proteins were from Sigma (Desienhofen, Germany), except for the human antithrombin III, which was obtained from the Behring-Werke (Marburg, Germany) (tradename Kybernin). Bulk chemicals were from Fluka (Neu-Ulm, Germany). Buffers were prepared with deionized water and passed through a 0.2-μm filter (Sartorius, Göttingen, Germany) before use. Cell culture supernatants were kindly donated by B. Rössler, Cell Culture Technology Research Group (Dr. G. Kretzmer) at our institute.

2.2. Instrumentation

The FPLC system (Pharmacia, Uppsala, Sweden) consisted of two P 500 pumps, the UV detector 2141, the fraction collector Superrac, and the injection valve MV7, all controlled by the controller LCC 500. The data were analyzed by FPLC software on a PS/2 computer.

2.3. Membrane adsorber

The MAs had the functional groups attached to a support of a synthetic copolymer or modified cellulose. The average thickness was 180 μm, the average pore size 0.45 μm in case of the copolymer-based MAs. The data were >250 μm and 5.0 μm for the thickness and the pore size, respectively, in case of the cellulose-based MAs. Strong cation-exchange MAs (sulfonic acid groups), strong anion-exchange MAs (quaternary ammonium groups), and affinity MAs (Cibacron Blue ligands) were used. The affinity MAs are under development at Sartorius. The cellulose-based strong ion-exchange MAs are commercially available (Q15, S15: 15 cm^2 total area, 3 layers of 5 cm^2 each; Q100: 100 cm^2 total area, 5 layers of 20 cm^2 each; Sartorius). Strong ion-exchange MAs based on the synthetic copolymer used in this work, were remainders of a line now dis-

continued by Sartorius. The MAs are stable within a pH range 2–12. A pressure limit of 7 bar is given by the manufacturer for the commercially available units.

MAs were also available as cut-outs from sheets. For the integration of the cut-out MAs into the chromatographic system the re-usable filter holder SM 16517 (Sartorius) made from polycarbonate was used, which could hold stacks of up to 10 MAs, each with an effective filtration area of 3.4 cm^2. The pressure limit given by the manufacturer for the filter holder is 7 bar. The filter holder is connected to the FPLC system via Luer connectors.

Prior to the first use, MAs are stored dry at room temperature in the dark. Between use, the MAs are kept in an appropriate buffer with a bacteriostatic agent added. When blocking or a significant decrease in capacity is observed, the ion-exchange MAs are routinely regenerated by washing with 1 *M* NaOH followed by 1 *M* HCl. In case of the ion-exchange MAs based on the synthetic copolymer, rinsing with 0.5 *M* NaOH at 50°C for 30 min may be used alternatively. The affinity MAs are rinsed with 0.1 *M* NaOH for 10 to 30 min to remove the contaminants.

2.4. Methods

Protein concentrations in solution were established by the BCA assay (Sigma, Deisenhofen, Germany) and the Micro-Lowry Assay (Pierce, Rockford, IL, USA) according to the manufacturers' instructions.

The isolation of rh-AT III from a Chinese hamster ovary (CHO) cell culture supernatant on the strong anion-exchange MAs was done as follows: buffer A was 50 m*M* phosphate, pH 8.5, and buffer B 50 m*M* phosphate, pH 8.5, containing an additional 1 *M* of NaCl. The flow-rate was 1 ml/min. The culture supernatant was diluted 1:1 with deionized water prior to application to the MA. A module containing five 20-cm^2 layers of copolymer-based strong anion-exchange MA was used.

The separation of AT III and BSA by Cibacron Blue affinity MA was done as follows: buffer A was 20 m*M* phosphate, pH 7.0, containing 0.8 *M* NaCl, and buffer B was 20 m*M* phosphate, pH 7.0, containing 0.5 *M* NaSCN. The sample consisted of 7.5 ml solution containing 1 mg of both AT III and BSA. The flow-rate was 1 ml/min. A module containing five 20-cm^2 layers of Cibacron Blue modified affinity MA based on the synthetic copolymer was used.

The separation of AT III, transferrin and BSA by immobilized metal affinity MA (IMA-MA) was done as follows: buffer A was 20 m*M* phosphate, pH 7.0, containing 0.5 *M* NaCl, and buffer B was 20 m*M* phosphate, pH 7.0, containing 0.5 *M* of NaCl and 20 m*M* Imidazol. The sample consisted of 1.0 ml solution containing 1 mg of AT III, 1 mg of transferrin and 2 mg of BSA. The flow-rate was 1 ml/min. A module containing five 20-cm^2 layers of imminodiacetic acid (IDA) MA (based on the synthetic copolymer) loaded with Cu(II) ions was used.

Mixed-mode chromatography was carried out as described below.

3. Results and discussion

Membrane adsorbers are interesting stationary phases, especially for preparative protein chromatography, since they combine excellent fluid-dynamic and mass-transfer properties and thus a compatibility with high mobile phase flow-rates, short separation times and high throughputs, with good chemical and mechanical stability and easy handling. By adapting the principles of filtration theory, the scale-up is easily accomplished

For some reasons, MAs are not commonly employed in multi-stage chromatography, even though this has been shown to be possible and even quite efficient [23,27]. The utilization of various forms of interaction is on the other hand quite often required in the downstream process of a biotechnological high-value product, in order to meet the purity requirements. Multi-stage chromatography, based on the consecutive use of several chromatographic columns, is a most flexible and versatile way to realize such a purification procedure based on several interaction modes.

MAs might, however, constitute a unique possibility for producing controlled mixed-mode interaction chromatographic stationary phases in a single column (MA stack). By alternating active MAs with 'inert' filter membranes and comparing the chromatograms thus obtained with chromatograms recorded for MA stacks of a similar number of active

MAs but lacking the inert ones, it can be shown that an inert 'filter' membrane does little to disturb the residence time distribution of the substances retained on the active MA [28]. By the same argument it should be possible to separate a mixture of proteins on a mixed-mode MA, utilizing different interactions. One consequence of using, e.g., mixed-mode ion-exchange chromatography would be that a separation could be carried out at a physiological pH, rather than going for the extremes just to ensure that the net charge of all proteins has the same sign.

3.1. Separation of standard proteins by mixed-mode ion-exchange MA chromatography

In order to investigate the potential of mixed-mode strong anion/cation-exchange chromatography for protein separation, a mixture of standard proteins varying in their isoelectric points (p*I*) was designed: β-lactoglobulin (p*I* 5.1), conalbumin (p*I* 6.8), α-chymotrypsinogen (p*I* 9.6), lysozyme (p*I* 10.5) and cytochrome C (p*I* 11.2).

The separation of these five standard proteins on 5 Q- and 5 S-MA (all based on the synthetic co-polymer), that were placed alternatingly in the re-usable filter holder, is shown in Fig. 1 together with the chromatograms of the single substances on the same 'column'. The separation is carried out at pH 7.0 in linear NaCl-gradient at a flow-rate of 2 ml/min. The resolution is excellent. The β-lactoglobulin peak, e.g., shows a shoulder, which can be ascribed to a differentiation between the two genetic variants of this milk protein. Obviously the mixed-mode MA stack combines the features of anion and cation exchangers, since all proteins are retained. Otherwise at pH 7.0 β-lactoglobulin is retained on the Q-MA,

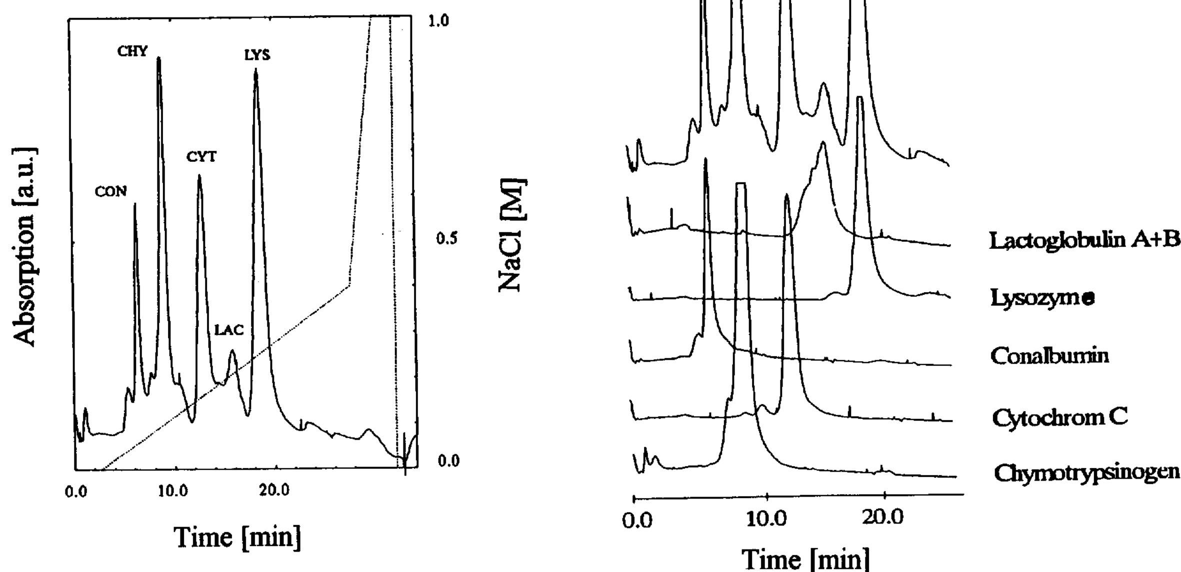

Fig. 1. Left: separation of five standard proteins on a mixed-mode ion-exchange MA. Right: chromatograms of the single substances under similar conditions. Buffer A: 20 m*M* Tris, pH 7.0. Buffer B: 20 m*M* Tris, pH 7.0, plus 1.0 *M* NaCl. Sample: 50 μl, containing conalbumin, lysozyme, α-chymotrypsinogen, cytochrome C, β-lactoglobulin A and B (each 2 mg/ml). Flow-rate: 2 ml/min. Module: re-usable filter holder, containing (alternatingly) five layers of 3.4 cm^2 of Q-MA and five layers 3.4 cm^2 of S-MA (both based on the synthetic co-polymer).

and α-chymotrypsinogen, lysozyme and cytochrome C on the S-MA, while only conalbumin is retained on both.

Next the ratio of the two types of MAs in the stack was varied, keeping all other conditions constant (Fig. 2). During these experiments the principle of alternating the two types of MAs was maintained as closely as possible. Resolution appears to be best in the case of 7 Q-MAs and only 3 S-MAs. This is somewhat surprising, since the majority of the proteins included in the standard mixture were shown to interact preferentially with the S-MA. The reason for this behaviour can presently only be speculated about. For instance, protein separation on two MA modules connected in series by Luer Look connectors, may give a better resolution than the separation of the same protein mixture on a single MA with the same capacity (see Ref. [29]). In this case it did not matter whether the capacity in the single MA was provided by the same number of membrane layers but with a larger diameter, or whether the single MA module contained the same number of membrane layers as the two modules combined. In that particular case we ascribed the better resolution in the two-module system to the fact that a certain amount of re-mixing may occur between the two modules. In the mixed-mode MAs the Q-MA might play a similar role with respect to the S-MA.

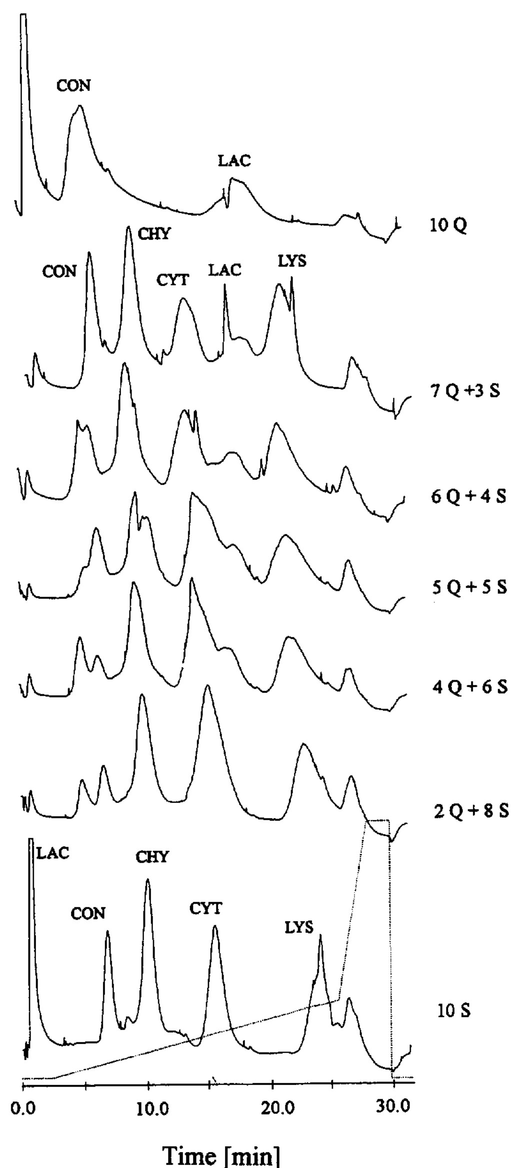

Fig. 2. Influence of the ratio of S- and Q-MAs on the separation of standard proteins. Save for the number of Q- and S-MAs in the module, conditions were as given in Fig. 1.

Inserting the 10 MAs in the form of two consecutive stacks within the module, while keeping all other conditions as described for the experiment depicted in Fig. 1, is, however, not recommended (Fig. 3). In the case of the stacked MAs the five S-MAs were placed on 'top' and the five Q-MAs at the 'bottom' of the column. A direct comparison shows that the separation is more efficient in case of the module containing alternating membrane layers.

The efficiency of MA chromatographic separations in general tends to show little dependency on the flow-rate. Deviations of this rule have so far mainly been observed for slow bioaffinity-based interactions, not for ion-exchange MAs [22,25]. Also in mixed-mode ion-exchange MA chromatography, the mobile phase flow-rate appears to be restricted by the pressure limits of the chromatographic system (an FPLC in our case) and the MA-modules, respectively, but – at least in the range accessible to us within these boundaries – not by the flow-rate per se (Fig. 4). The gradient volume and shape were kept constant during these experiments. Save for the flow-rate, all other conditions were identical to those given for the experiment depicted in Fig. 1. The

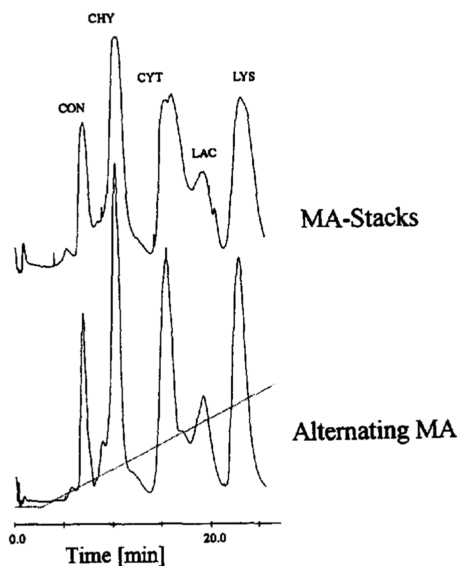

Fig. 3. Alternating vs. 'two-stacks' mixed-mode MA-chromatography. Save for the arrangement of the MAs in the module, conditions were as given in Fig. 1.

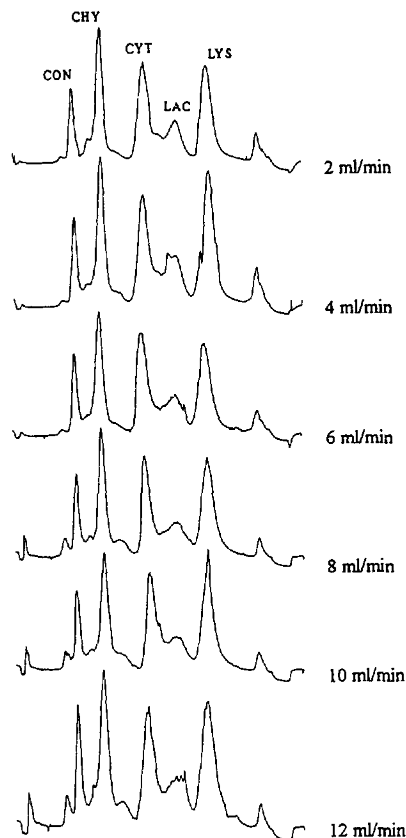

Fig. 4. Influence of mobile phase flow-rate on duration and resolution of mixed-mode ion-exchange MA chromatography. Save for the flow-rate, conditions were as given in Fig. 1.

increase of the flow-rate from 2 ml/min to 12 ml/min, on the other hand, decreases the separation time from 25 min to 1.6 min.

3.2. Separation of whey proteins

In recent years cow milk has increasingly been discussed as a source not only of nutrients but also of raw material for the cosmetics, detergent and pharmaceutical industries [30,31]. The caseins constitute 80% of the protein fraction of cow milk; the remaining 20% are whey proteins, i.e. mainly α-lactalbumin, β-lactoglobulin, BSA and the immunoglobulins. Cheap and efficient isolation of the latter would be prerequisite to their broader utilization. Currently whey constitutes a 'raw material' that is being produced at several hundreds of tons per day in a modern dairy.

The separation of the major whey proteins at near-neutral pH in a single chromatographic run is best accomplished by mixed-mode ion-exchange MA chromatography. At pH 6.0 the immunoglobulins, e.g., are retained on the cation exchanger, while the other whey proteins are retained on the anion exchanger. Working at elevated pH to ensure the retention of all proteins on the Q-phase is not advisable for whey protein separation, since especially the β-lactoglobulin is known to be rather sensitive to elevated pH values.

The MA 'column' used in the case described here consisted of two Q15-MAs and one S15-MA (note that cellulose-based MAs were used here). The loading was done on the connected system. Prior to elution, however, the Q and the S units had to be disconnected and eluted separately in a NaCl gradient to prevent the immunoglobulins from coeluting at the same ionic strength with the α-lactalbumin (Fig. 5). The use of two rather than one Q-MA as well as the complex gradient shown in Fig. 5, were necessary to ensure good separation of the BSA from the other whey proteins as well as for the adequate

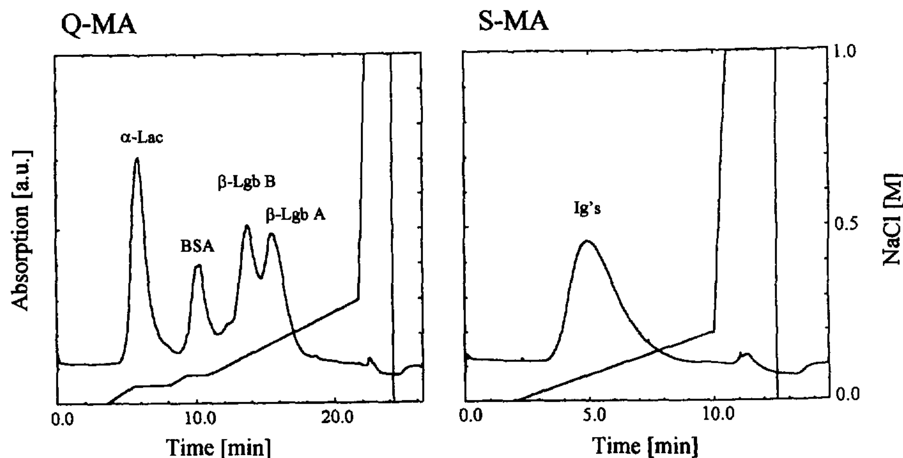

Fig. 5. Separation of immunoglobulin, BSA, α-lactalbumin and the genetic variants of β-lactoglobulin by MA chromatography on strong anion- and cation-exchange MAs. Buffer A: 20 m*M* piperazine, pH 6.0. Buffer B: 20 m*M* piperazine, pH 6.0, plus 1.0 *M* NaCl. Sample: 8 ml (BSA, 0.125 mg/ml; IgG, 0.05 mg/ml; α-lactalbumin, 1 mg/ml; β-lactoglobulin, 1 mg/ml). Module: 2 Q15+1 S15 in series.

resolution of the genetic variants of β-lactoglobulin. Using these conditions, α-lactalbumin is found to elute first, followed by BSA and the two β-lactoglobulins. The B variant was less strongly retained than the A variant in that particular case.

3.3. Mixed-mode anion exchange/affinity vs. single-mode MA chromatography

The anticoagulant antithrombin III is a important blood factor [32]. Its considerable pharmaceutical value has already led to expressing this glycoprotein, e.g., in recombinant Chinese hamster ovary (CHO) cells [33]. In rh-AT III production with CHO cells, the protein fraction contains mainly BSA and transferrin besides the product itself. It has been shown that the separation of rh-AT III from these two contaminants requires at least two chromatographic steps based on different interaction principles [23]. BSA may, e.g., be removed by Cibacron Blue affinity MA (Fig. 6), while transferrin can be removed – due to the difference in p*I* – on a strong anion-exchange MA (Fig. 7). MAs based on the synthetic copolymer were used in all mixed-mode anion-exchange/affinity experiments.

AT III and BSA will not be separated on the anion-exchange MA, while the separation of transferrin and AT III, on the other hand, is not possible by Cibacron Blue affinity chromatography [23]. The only single-mode MA system investigated by us which shows some resolution of all three proteins, is an IMA-MA loaded with Cu(II) ions (Fig. 8). In this

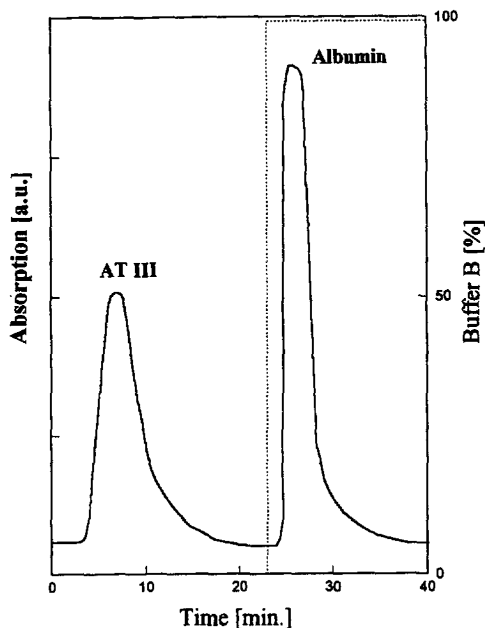

Fig. 6. Separation of AT III and BSA on a Cibacron Blue affinity MA. Buffer A: 20 m*M* phosphate, pH 7.0, plus 0.8 *M* NaCl. Buffer B: 20 m*M* phosphate, pH 7.0, plus 0.5 *M* of NaSCN. Sample: 7.5 ml (1 mg AT III and BSA). Flow-rate: 1 ml/min. Module: containing 5 layers of 20 cm^2 of Cibacron Blue modified MA based on the synthetic copolymer.

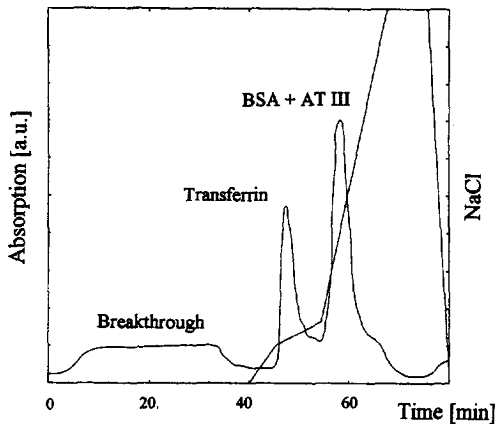

Fig. 7. Separation of a CHO cell culture supernatant containing mainly AT III, BSA and transferrin on a strong anion-exchange MA. Buffer A: 50 m*M* phosphate, pH 8.5. Buffer B: 50 m*M* phosphate, pH 8.5, plus 1 *M* of NaCl. Flow-rate: 1 ml/min. The culture supernatant was diluted 1:1 with deionized water prior to application to the MA. Module: containing 5 layers of 20 cm^2 of a strong anion-exchange MA based on the synthetic copolymer.

case, however, AT III activity is found in the breakthrough as well as in one of the later eluting fractions. Moreover, in the downstream processing of biotechnological products, IMA-MA has been shown

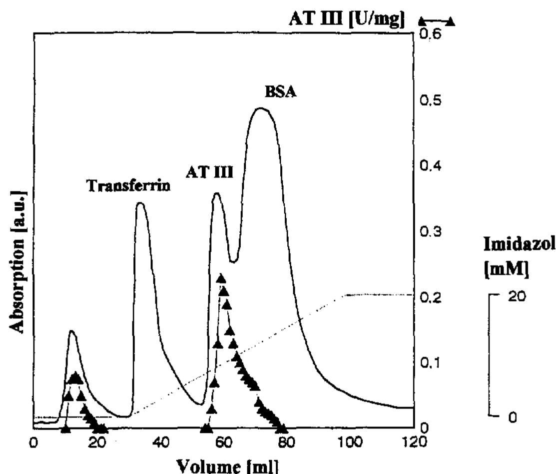

Fig. 8. Separation of AT III, BSA and transferrin on a Cu(II)-IMA-MA. Buffer A: 20 m*M* phosphate, pH 7.0, plus 0.5 *M* NaCl. Buffer B: 20 m*M* phosphate, pH 7.0, plus 0.5 *M* NaCl and 20 m*M* imidazol. Sample: 1.0 ml (1 mg AT III, transferrin, 2 mg BSA). Flow-rate: 1 ml/min. Module: containing 5 layers of 20 cm^2 of imminodiacetic acid (IDA-)MA (based on the synthetic copolymer) loaded with Cu(II) ions.

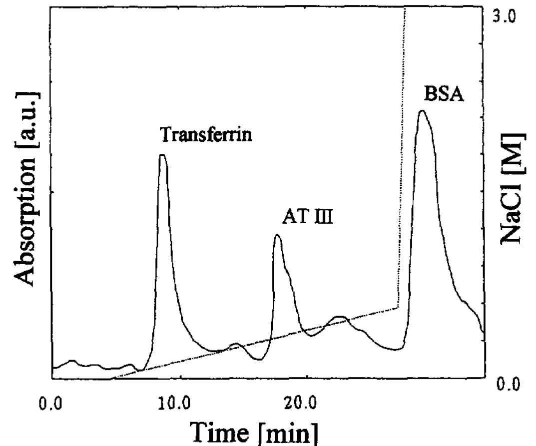

Fig. 9. Separation of a CHO cell culture supernatant containing mainly AT III, BSA and transferrin on a mixed-mode anion-exchange/Cibacron Blue affinity MA. Buffer A: 20 m*M* Tris, pH 7.0, plus 20 m*M* NaCl. Buffer B: 40 m*M* Tris, pH 7.0, plus 0.5 *M* NaCl. Buffer C: 40 m*M* Tris, pH 7.0, plus 3 *M* NaCl. Sample: 250 μl supernatant (AT III, transferrin, BSA: 50 μg/ml). Flow-rate: 1 ml/min. Module: re-usable filter holder, containing (alternatingly) 5 layers of 3.4 cm^2 of the strong anion-exchange MA, and 5 layers of 3.4 cm^2 of the affinity MA (both based on the synthetic copolymer).

to cause some problems with respect to long-term stability, ligand leakage and interaction kinetics [22].

By simply using controlled mixed-mode anion-exchange/affinity interaction, on the other hand, the separation of AT III, BSA and transferrin in a single chromatographic experiment at pH 7.0 is easily accomplished (Fig. 9). Since BSA may be expected to interact mainly with the affinity MAs under these circumstances, it is not surprising that a comparatively high NaCl step gradient (3 *M* NaCl) is required for elution of that particular protein. AT III and transferrin, on the other hand, which should adsorb mainly onto the anion-exchange MAs may be eluted in a linear NaCl gradient running only up to 1 *M* NaCl.

4. Conclusion

MAs enable the controlled preparation of mixed-mode stationary phases for liquid chromatography. Protein mixtures may be resolved on these 'columns' due to their interaction with one or both types of MA

present. If the retention is governed by a single type of interaction process, the presence of the other MA type has no influence on the overall residence time distribution of the substance in question and thus chromatographic conditions may easily be optimized. As shown for the separation of rh-AT III from other culture supernatant proteins, a mixed-mode MA may be substituted for a multi-column (MA) chromatography and achieve a similar resolution. We thus expect mixed-mode MA to find its place in preparative protein chromatography.

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