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Development of a downstream process for the isolation and separation of monoclonal immunoglobulin A monomers, dimers and polymers from cell culture supernatant

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Abstract

The isolation and separation of the molecular variants of monoclonal IgA from cell culture supernatants is possible using several filtration and ion-exchange chromatography steps, followed by size-exclusion chromatography for the actual separation of the molecular variants. The latter step is especially time consuming and laborious. This report presents possible improvements of the procedure. Use of the displacement rather than the elution mode may render the ion-exchange step more productive (higher product concentrations and space–time yield). For the final separation of the molecular variants, hydroxyapatite (HA) elution chromatography can serve as an alternative to size-exclusion chromatography. By using an optimized, complex phosphate gradient, the IgA dimers can be separated quantitatively from the monomers and higher oligomers. It may in individual cases be necessary to use a size-exclusion polishing step to reach the required final degree of purity, however, the amount of material to be processed is reduced to such an extend by the HA-step, that the overall process is still more productive. Buffer pH and flow-rate as well as the stationary phase material used were additional factors considered during the optimization of the HA elution chromatography. HA-displacement chromatography resulted only in a concentration of the overall IgA fraction, but not in a separation of the molecular forms.

Keywords: Preparative chromatography; Hydroxyapatite; Stationary phases, LC; Immunoglobulins; Monoclonal antibodies

1. Introduction

Immunoglobulin type A (IgA) is typically found in external secretions of mammals and can be considered a major weapon of their mucosal immune system. Monoclonal IgA may be used for passive immunization or active therapy against certain viral infections [1,2]. For a long time the role of IgA in immunoprotection of the host was poorly understood, mainly because typically monomeric and oligomeric IgA molecules of the same type are produced in vivo and the separation of the individual biologically active forms is difficult [3,4]. IgA production in myeloma or hybridoma cells also results in a mixture of monomeric, dimeric and polymeric molecules [5,6].

Recently, a method for the purification of IgA forms from human myeloma plasmas has been published, which is mainly based on repetitive size-exclusion chromatography (SEC) [7]. In 1996 some of us reported on a successful preparation of milligram quantities of the individual molecular forms of a given monoclonal IgA, which also involved SEC [4]. These preparations allowed, among other things,

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the study of the antigen binding properties of the different molecular types. Polymerization was found to be indispensable for a fully functional IgA molecule in these studies. When it comes to their application as a therapeutic, monoclonal IgAs should be purified to an extent that the recipient is only challenged with biologically active molecules. In our case, this would call for the removal of a nonantigen binding monomeric IgA. In the end, this may require the preparation of pure IgA dimers.

While the most pronounced difference in the molecular variants of the IgA molecule is their size and while SEC is capable of separating these forms, an alternative method was needed to develop a pilot scale production process. SEC can be used at process scale. However, in the discussed case, the processing of 25 to 30 mg required a 200×2.6 cm column and took 36 h. The separation of dimers and higher oligomers was, nevertheless, incomplete. With this approach, the preparation of a theoretical 1 g of active dimeric IgA would require 200 runs. Thus, the size-exclusion approach is not feasible for an industrial IgA downstream process. Hydroxyapatite (HA) chromatography seemed to present an alternative, since a small scale —albeit incomplete— separation of IgA molecular forms on such columns has been shown possible [8].

Hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] has been discussed as putatively interesting stationary phase material for liquid chromatography (LC) of biopolymers since the 1950s [9]. The fragile nature of the material has in the past caused problems. Recently, however, a high-performance liquid chromatography (HPLC) compatible material has become commercially available. Protein adsorption on the apatite surface is a complex and hitherto not yet fully understood process. It has been reported that only biopolymers of well defined structure interact with apatite, while denatured or ill-structured ones, such as ovomucoid, show an unusually low stationary phase affinity [10]. HA chromatography is able to separate even closely related molecular species. This has been ascribed to the fact, that apatite as a ceramic material presents a more structured arrangement of interaction points than, e.g., a conventional chromatographic adsorber [11]. Thus, slight differences in the structure of the molecules to be adsorbed result already in noticeable differences of the binding strength. Due to the complexity of the involved mechanisms, protein separations on apatite columns remain difficult to predict.

2. Materials and methods

2.1. Materials

Proteins and fine chemicals were from Sigma, bulk chemicals for buffer and eluent preparation were from Fluka. Polyacrylic acid was from Polysciences (Warrington, PA, USA). The ceramic HA beads used in the investigations were from Asahi Optical (Tokyo, Japan) and Bio-Rad Labs. (Munich, Germany), Asahi's European distributor, respectively. Unless indicated otherwise, 10 μm porous particles (average pore size: 100 nm) and 2 ml Macro-Prep columns (52×7 mm) were used. The ''type I'' material was used, which according to the manufacturer (Bio-Rad) has a high binding capacity for proteins and particularly for acidic ones. For the RPC analysis of the displacement fractions, a Hytach C_{18} column (30×4.6 mm) (Glycotech, USA) filled with nonporous particles was used.

2.2. Cell line and culture supernatants

The murine hybridoma cell line ZAC3 is a fusion of a lymphocyte from Peyers patches of a BALB/c mouse orally immunized with *Vibrio colerae* Inaba strain with the myeloma cell line Sp/2.0. The cell line was kindly provided by Prof. J.-P. Kraehenbuhl (Swiss Institute for Experimental Research, Lausanne, Switzerland). ZAC3 secretes IgA antibodies corresponding to the human allotype A2m(1) [12]. Hybridoma cell culture supernatants containing IgA were donated by T. Stoll, A.P.-A. Ruffieux and M. Huser, all Swiss Federal Institute of Technology, Lausanne, Switzerland. The hybridomas were cultivated in different types of bioreactor, which has been established previously to influence the product quality and quantity [13].

2.3. Chromatographic hardware

The displacement system was assembled from an ERC HPLC pump 64 (ERC, Alteglofsheim, Ger-

many) and a Valco 10-port valve (Valco, Houston, TX, USA). A 1-ml loop was used for sample injection, the displacer was introduced from preparative sample loops (Knauer, Berlin, Germany), that could hold 5 and 11 ml, respectively. A Biologic LC-system controlled by BioLogic software, both from Bio-Rad was used for preparative elution chromatography. For analytical HA chromatography, a HPLC system assembled from a ERC degasser 3112, a binary gradient pump (Techlab Chromatographie, Braunschweig, Germany), a Spark Holland Basic Marathon autosampler, and an UV–Vis detector (Shimadzu SPD-10A) was used.

2.4. Sample preparation

Cell-free cell culture supernatant from the fluidized bed reactor was ultrafiltrated (Scan, Switzerland, M_r cut off: 100 000) and diafiltrated (Scan, Switzerland, M_r cut off: 100 000) followed by two passages through a DEAE–Sepharose Fast Flow column (Pharmacia, Uppsala, Sweden), as described previously [4]. The harvest from the continuous stirred tank reactor was ultrafiltrated and diafiltrated and passed once over the DEAE–Sepharose Fast flow column. The cell-free harvest from the hollow fiber reactor was used without pretreatment.

In addition IgA dimer and polymer samples were prepared from pure materials derived from a continuous stirred tank reactor, as described previously [4]. Before application to HA-columns, all samples were passed over a PD 10 (Pharmacia) gel binding column for desalting and buffer equilibration to the binding conditions desired for HA chromatography.

2.5. Analytical methods

Overall protein concentration were estimated by UV_{280} adsorbance (factor 1). Gel electrophoresis of proteins was carried out in a mini-Protean II apparatus (Bio-Rad), according to the method of Laemmli [14]. Polyacrylamide gel electrophoresis (PAGE) was performed under nonreduced denaturing conditions (1% SDS). For separation, a gradient from 4 to 12% acrylamide was used.

For immunoblotting of IgA heavy chains, nonspecific binding sites on nitrocellulose membranes (Bio-Rad) were saturated for 1 h at room temperature by incubating in a blocking buffer made of PBS, 10% BSA (Fluka) and 0.05% Tween 20 (Sigma). The membrane was probed for 2 h at room temperature with biotinylated goat-anti mouse IgA heavy chain antibody (Amersham) diluted 1:1000 in PBS–0.05% Tween 20. Bound antibodies were detected using streptavidin coupled to horse radish peroxidase using the enhanced chemiluminescence kit from Amersham for visualization.

2.6. Analytical HA chromatography

The IgA samples (0.5 ml) were desalted as above and equilibrated to loading conditions (0.005 *M* potassium phosphate buffer, pH 6.8). Buffer A was a 0.005 *M* potassium phosphate buffer, pH 6.8. Buffer B was a 0.5 *M* potassium buffer, pH 6.8. A linear gradient (volume 20 ml) was run from 0% to 100% with a constant flow-rate of 1 ml/min.

2.7. Preparative HA elution chromatography

Samples (1 to 10 ml) were desalted and equilibrated to 0.005 *M* potassium phosphate buffer, pH 6.8, just before HA chromatography to avoid possible protein degradation in the low salt buffer. Buffer A was a 0.005 *M* potassium phosphate buffer, pH 6.8. Buffer B was a 0.5 *M* potassium buffer, pH 6.8. Different elution protocols were compared.

Program 1: 100% A (10 ml), linear gradient 0 to 30% B (20 ml), 30% B (4 ml), 100% B (5 ml), 100% A (10 ml).

Program 2: 100% A (10 ml), linear gradient 0 to 20% B (20 ml), 30% B (4 ml), 100% B (5 ml), 100% A (10 ml).

Program 3: 100% A (10 ml), 10% B (10 ml), 15% B (10 ml), 20% B (10 ml), 30% B (10 ml), 100% B (5 ml, at 2 ml/min), 100% A (20 ml, at 2 ml/min).

Program 4: 100% A (10 ml), 10% B (10 ml), linear gradient to 20% B (20 ml), 30% B (4 ml), 100% B (5 ml), 100% A (10 ml).

A flow-rate of 1 ml/min was adjusted, unless indicated otherwise. Fractions of 1 ml were collected. Usually the fraction representing the ‘‘peak maximum’’ was analyzed by gel electrophoresis.

2.8. HA chromatography with HA-Ultrogel

In case of the HA-Ultrogel, 200 ml culture supernatant from the hollow fiber reactor were passed over a Sephadex G25 gel-filtration column (500×50 mm, i.e., 25 ml) for buffer exchange to 0.1 *M* NaCl in 0.01 *M* potassium phosphate buffer, pH 7.3. The sample was then passed twice over a DEAE–Sepharose FF column. The IgA containing fractions were desalted and equilibrated to 0.005 *M* potassium phosphate, pH 6.8 and loaded on a 70×25 mm HA-Ultrogel column (Sepracore, Villeneuve la Garenne, France). Elution program 4 was adapted. In particular, a flow-rate of 3 ml/min was used and fractions of 5 ml were collected.

2.9. Displacement chromatography

Unless mentioned otherwise, a 0.02 *M* Tris buffer at the indicated pH and a flow-rate of 0.1 ml/min were used. For column regeneration a 0.4 *M* sodium phosphate buffer, pH 6.8, was used. Polyacrylic acid (PAA, M_r: 6000 g/mol) and EGTA [ethylenglycol bis(β-aminoethylether)-N,N,N′,N′-tetraacetic acid, M_r: 380.4 g/mol] were used as displacers, usually at a concentration of 20 mg/ml. Displacement separations were monitored by collecting fractions twice per minute. Fractions were analyzed by native gradient gel. The displacers were quantified by analytical HPLC as described previously [15].

3. Results and discussion

The hybridoma cell line ZAC3 has previously been shown to produce specific monoclonal mouse IgA against *V. colerae* lipopolysaccharide (LPS) as a mixture of monomeric, dimeric and polymeric forms [4]. The separation of this heterogeneous population into fractions containing the pure molecular forms is desirable for a human therapeutic. HA chromatography has been investigated in this context as an alternative to SEC. The material has been shown capable of separating even of the molecular variants of those immunoglobulins that typically show oligomerization (IgA/IgM) [8]. HA chromatography may thus conceivably be used to replace the size-exclusion step or perhaps even both the ion-exchange and the SEC, i.e., be used after the ultra- and diafiltration.

3.1. Optimization of the gradient elution protocol

Proteins are usually adsorbed to apatite in a low-strength phosphate buffer at near neutral pH and eluted in a gradient of increasing phosphate strength. The separation is also influenced by the pH of the mobile phase. The paper by Aoyama and Chiba [8] suggests, that the separation of IgA oligomers (ACmAb in their case) by HA chromatography is possible under standard conditions in a linear phosphate gradient. According to them, the IgA monomers can be separated from the oligomeric forms which themselves are only incompletely separated. When we tried a similar separation of the IgA mixtures produced by ZAC 3 in different bioreactors, using a linear potassium phosphate gradient from 0.005 to 0.5 *M*, the IgA monomers were not retained on the column at all, while a mixture of dimers and polymers eluted in peak showing two maxima, (data no shown). While the ACmAb oligomers required a phosphate concentration between 0.25 and 0.4 *M* for elution, the ZAC 3 mAb oligomers were eluted between 0.05 and 0.15 *M* phosphate. Thus the ZAC 3 mAb binding affinity to HA seems to be considerably lower than that of the ACmAb. Since Aoyama and Chiba used only standard reduced SDS–PAGE gels for analysis of their fractions, little can be said on the purity achieved by them of the individual IgA preparations.

From the view point of an IgA purification, the failure of the ZAC 3 IgA monomers to bind to the HA-column is a positive aspect, since this presumably facilitates the removal of this biologically nonactive molecular variant from the final product. Before the HA-step could be used on a process scale to substitute for the size-exclusion step, however, the separation of the dimer fraction from the higher oligomers needed to be improved considerably. This was achieved by gradient optimization experiments. Unless mentioned otherwise IgA produced in a fluidized bed reactor was used, which had been partially purified (filtration and ion-exchange steps).

Since, the IgA considered here seems to show a comparatively low affinity to HA, a less steep gradient than the classical one, i.e., from 0.005 to

0.15 *M* potassium phosphate was tried (protocol 1), Fig. 1a. The monomer was not retained under these conditions, while the oligomers eluted in a complex peak showing a shoulder (1) and two maxima (2 and 3). According to the SDS-gels, little oligomer separation was achieved, since all fraction contained dimer and higher oligomers, albeit at different ratios. At lower phosphate concentration dimers were preferentially eluted, whereas at higher phosphate concentration the higher oligomers were found. Protocol 2, Fig. 1b, gave better results in terms of the separation of the higher oligomers. Four peaks were observed in this case. As before, peaks 1 and 2 contained mainly dimers contaminated by higher oligomers, while for the first time trimers (3) and tetramers (4) were enriched in individual zones. Still, the separation was not satisfactory.

The resolution of the peaks could be improved by choosing a three step gradient elution protocol (protocol 3), Fig. 1c. Under these conditions, however, a part of the IgA dimer did not bind to the column. The first peak in Fig. 1c (0.05 *M* phosphate) peak could not be analyzed, because the protein concentration was too low. Mainly trimers were found in peak 2 (0.075 *M* phosphate). Tetramers were eluted in peak 3 (0.1 *M* phosphate). Since our acknowledged aim was the separation of the dimers from the other IgA modifications, protocol 4 was used to improved the separation of the dimer peak from the higher oligomers, Fig. 1d. In this case a linear gradient was run between 0.05 and 0.1 *M* phosphate. The linear gradient was preceded by a step from 0% (10 ml) to 10% (10 ml) buffer B. Once more, a certain IgA dimer fraction did not bind to the column, while another portion could be eluted by a step from 0% to 10% B (peak 1 in Fig. 1d). The

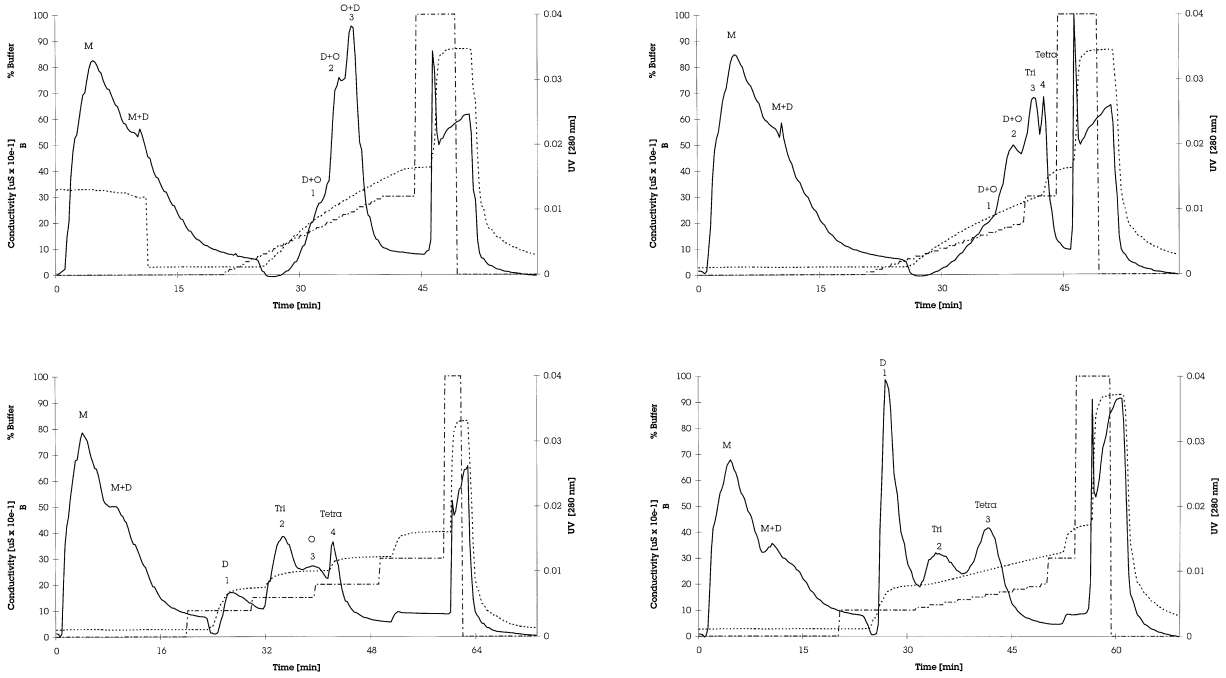


Fig. 1. Optimization of the phosphate gradient used in elution of IgA molecular variants from hydroxyapatite. The goal was to optimize the separation of IgA dimers (D) from the monomers (M) and the higher oligomers (P). The true gradient was monitored by conductivity detection (———). The preprogrammed gradient shape is indicated by a broken line (- - -). Five-ml samples originally produced in a fluidized bed reactor were applied. Samples were desalted and equilibrated to 0.005 *M* potassium phosphate buffer, pH 6.8, just before HA chromatography. Buffer A was a 0.005 *M* potassium phosphate buffer, pH 6.8. Buffer B was a 0.5 *M* potassium buffer, pH 6.8. (a) Elution by continuous gradient from 5 to 150 m*M* phosphate, pH 6.8. (b) Elution by continuous gradient from 5 to 100 m*M* phosphate, pH 6.8. (c) Elution by step gradient of phosphate, pH 6.8. (d) Elution by combination of step and linear gradient of phosphate, pH 6.8.

eluted dimer fraction was contaminated by traces of trimers. Peak 2 was enriched in trimeric IgA and peak 3 in tetrameric IgA.

The fact that some IgA dimers are not able to bind to HA was observed here for the first time. Some evidence can be presented that IgA dimers produced by ZAC3 can be separated into a HA binding and a HA nonbinding fraction, since rechromatography of the nonbinding fraction again showed no adsorption.

3.2. Influence of the buffer pH

The buffer pH is known to influence protein separation on HA to a great extent. The effect of the pH was investigated between 6.5 and 7.5. The lower limit was determined by the stability of the HA and the upper limit by the stability of the IgA. We found the separation to be very sensitive to changes in the buffer pH between 6.8 and 7.0, Fig. 2. Within this range the resolution deteriorated rapidly. At pH 7 a considerably smaller fraction of the IgA dimers was bound to the column matrix. The separation of the dimers from the higher oligomers was much worse. Higher oligomers (mainly trimers) were found in the dimer fraction eluting from the column. An increase of the buffer pH to 7.5 had no further influence. On the other hand, chromatograms recorded for buffer pH values between 6.5 and 6.8 showed great similarity in terms of separation efficiency and resolution. It is known that HA chromatography is very sensitive to protein denaturation. A possible cause for the observed behavior may therefore lie in the fact that IgA denaturation begins at a pH of 6.8.

3.3. Other types of HA resins

Early experiments using HA resins from Fluka (HA high-resolution and fast flow, Fluka) showed

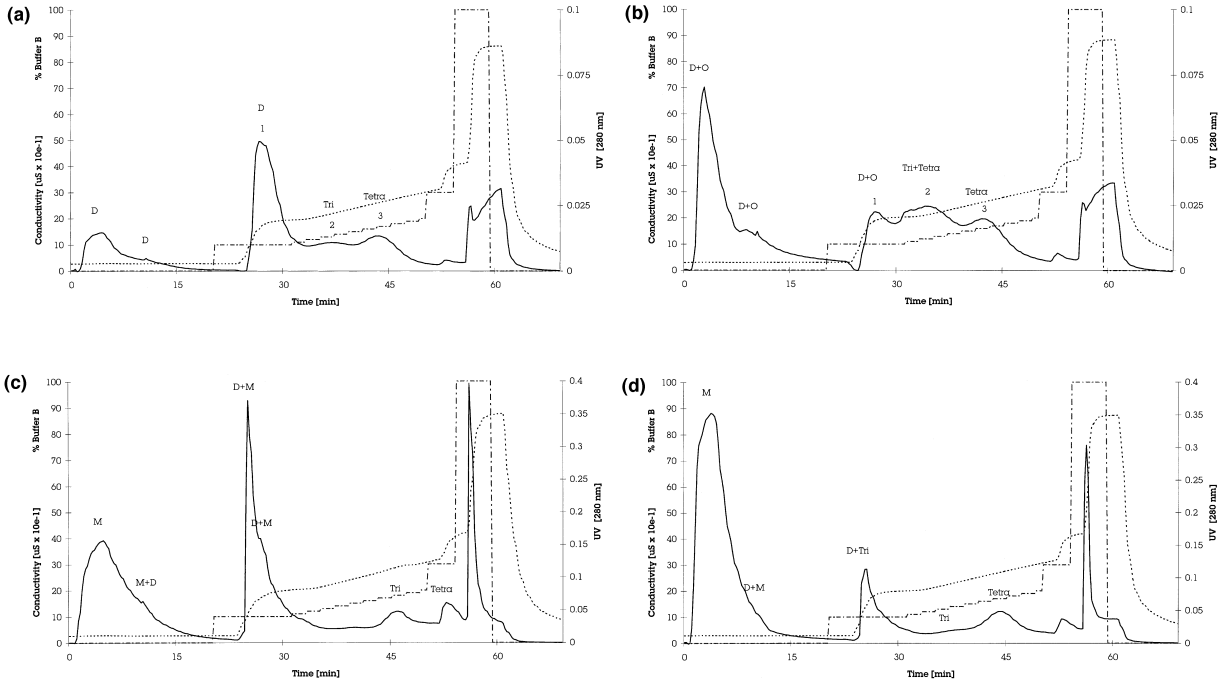


Fig. 2. Influence of the buffer pH on HA elution chromatography of IgA variants. The combination of step and linear phosphate gradient was used. In (a) and (b) 1 ml of a sample containing solely IgA dimer and higher oligomeric molecules but no monomers or other impurities was applied. In (c) and (d), the sample was 5 ml of a culture supernatant concentrate originally produced in a hollow fiber reactor. Samples were desalted and equilibrated to 0.005 *M* potassium phosphate buffer of the indicated pH, just before HA chromatography. Buffer A was a 0.005 *M* potassium phosphate buffer, buffer B was a 0.5 *M* potassium buffer, both of the indicated pH. (a) Elution at pH 6.8. (b) Elution at pH 7.0. (c) Elution at pH 6.8. (d) Elution at pH 7.0.

similar results, when program 3 was used. A major portion of the IgA dimer could be eluted in a step gradient to 10% buffer B (corresponding to 0.005 *M* potassium phosphate), whereas the higher IgA oligomers were eluted at 0.1 *M* phosphate. The Fluka HA-resins were very fragile and the columns tended to clog after a couple of separations. Concomitantly, very low flow-rates (5 to 15 cm/h) had to be used. On the other hand, the 2 ml column prepacked with the new ceramic type of HA used in the above experiments was operated for 60 cycles with no change in column properties or performance at flow-rates of 200 cm/h. This material seems to have overcome the disadvantages in the conventional resins and can be used in larger columns.

A column packed with HA-Ultrogel (Biosepra), which is composed of crosslinked agarose beads into which microcrystals of HA are incorporated, was not able to resolve the IgA modifications. This negative result was repeatedly observed for IgA samples from different bioreactors.

3.4. Consequences for a future IgA production process

The elution program with a combination step and linear gradient using columns packed with pressure compatible HA thus led to the best resolution of the molecular IgA variants. Depending on the actual protocol, only IgA dimers can be separated from the higher oligomers or IgA trimers and tetramers can also be enriched albeit at the price of reduced purity of the individual fractions. The substitution of the size-exclusion step in the preparation of a therapeutic IgA by HA chromatography is not possible at present, since, according to our gels, the dimer fraction is always somewhat contaminated by other IgA modifications. However, since HA chromatography succeeded in both enriching and concentrating the individual IgA modifications, SEC can now be used as a highly effective polishing step to remove the final traces of the contaminants and obtain homogeneous, pure fractions of IgA-dimers, IgA-trimers and IgA-tetramers on a much smaller scale then previously necessary.

It is also not possible to use HA chromatography at an earlier stage in the isolation procedure. Direct loading of filtrated culture supernatants from hollow fiber reactors onto the HA column allowed no separation of the IgA dimers from the IgA monomers. Only the higher oligomers were once more resolved, data not shown.

The attempted scale-up from a 2 ml column run at 1 ml/min to a 20 ml column run at 2.5 ml/min, caused a concomitant reduction in the linear flow-rate from 2.6 cm/min to 1.4 cm/min. The separation was further improved by this reduction in the mobile phase velocity, Fig. 3.

3.5. Displacement chromatography

Displacement chromatography has been discussed as a specific mode of preparative chromatography, which is prior to high throughputs, results in superior product concentrations, uses the stationary phase capacity more efficiently than conventional preparative elution chromatography [16]. The separation is based on the displacer-enforced competition of the sample molecules for a limited amount of binding sites on the stationary phase. Displacement chromatography has been repeatedly shown to be a useful technique in biotechnical downstream processing [17].

Since HA obviously has the power to resolve the molecular variants of the IgA in principle, HA-displacement chromatography was tried alternatively to the elution approach. Two advantages were hoped for, higher product concentration and higher recoveries. A 2-ml BioScale column, similar to the one used in the elution experiments was used. EGTA was

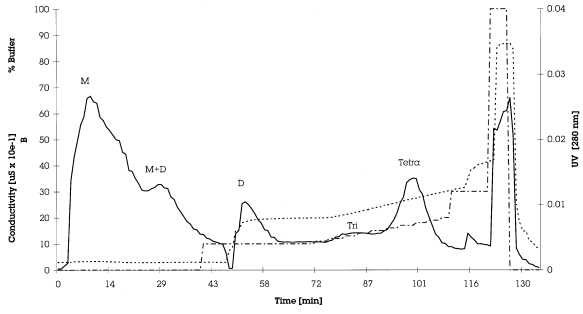


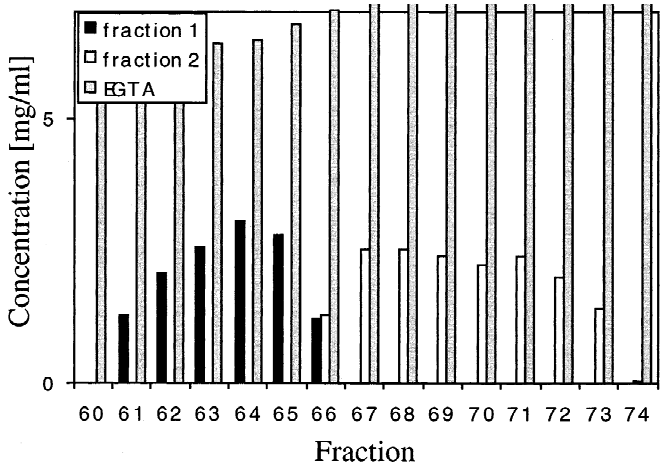
Fig. 3. Elution obtained after reduction of the mobile phase flow-rate from 2.6 cm/min to 1.4 cm/min. The column was concomitantly scaled up from 2 ml to 20 ml. A combined step and linear phosphate gradient was used.

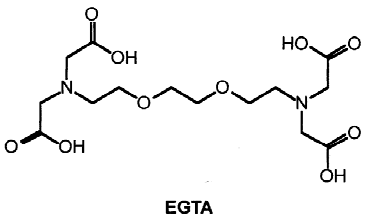
used as displacer, since it had previously yielded good results with protein displacement on HA [18].

A first attempt to separate a sample mixed from previously purified IgA dimer and higher oligomers preparation was carried out using a standard 0.005 *M* phosphate buffer, pH 6.8, as carrier, Fig. 4. As observed before with the HA–EGTA system, the displacer front shows a pronounced tendency to advance through the protein fractions [19]. Due to the large difference in size between the displacer and the IgA molecules, a separation could easily be achieved. However, according to the SDS–PAGE analysis, the collected protein fractions contained mainly degraded IgA molecules. A reason for this occurrence cannot be given at present. However, pH values of 7.0 to 7.5 were determined in the fractions by pH paper dots. The behavior of the IgA molecules in HA elution chromatography at pH 7.0 and higher has already led to some speculations concerning the pH stability of the molecules. Perhaps here too we observed a beginning denaturation of the molecules as cause for both the failure to be separated on the HA column in the displacement mode and the detection of large amounts of degradation products in the gels. The result was reproducible. Further experiments were carried out in 0.02 *M* Tris buffer as carrier, as described previously.

The results of the ensuing influence of the carrier pH on the separation using Tris buffer as mobile phase differed from those found in case of the elution chromatography. When a pH of 6.5 was adjusted, EGTA was not able to displace the IgA from the column. At a pH of 6.8 both IgA dimers and higher IgA oligomers were found in the collected fractions, Fig. 5a. At a pH of 7.5, the recovery as well as the concentration in the fraction was improved, Fig. 5b. The first IgA containing fraction was number 37 compared to number 32 as found for a carrier pH of 6.8. A further increase of the carrier pH to 8.0 lowered the binding strength further (Fig. 5c). IgA appeared for the first time in fraction 10. Concomitantly, the EGTA front tended to overtake the substance zones to a considerable extend when a carrier pH of less than 8.0 was adjusted, while this was not observed when the carrier pH was 8.0. However, none of these experiments resulted in a separation of the molecular variants of IgA. In displacement experiments with either just the pure IgA dimer or a mixture of the higher IgA oligomers as sample, all IgA molecules were found to show identical behavior regardless of size. Thus, the displacement mode can not be used to substitute for the elution mode for the final separation of the IgA modifications.

Additional displacement experiments were done on a Bio-Rad Q2 column (52×7 mm, strong anion-exchange groups). Both polyacrylic acid and EGTA were investigated as displacers. An experiment with PAA as displacer of a mixture of IgA oligomers verified that an concentration of the total IgA by

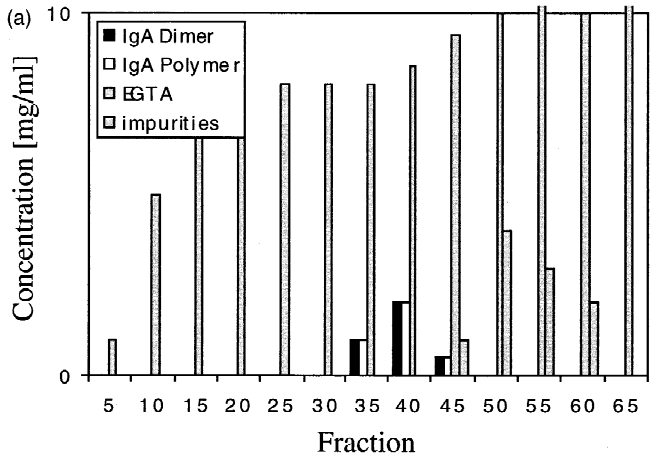


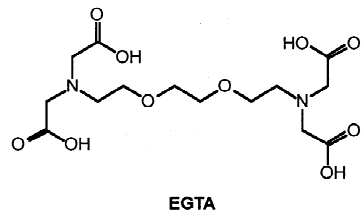


EGTA

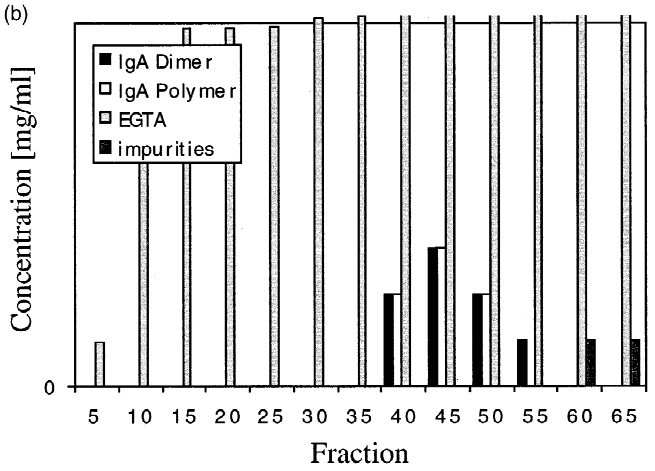
Column: Bio-Scale (2 ml)
Stationary Phase: 10 µm HA (Typ I)
Carrier: 0.005 M phosphate, pH 6.8
Flow Rate: 0.1 ml/min

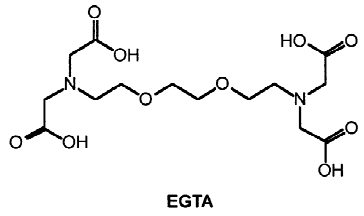
Fig. 4. HA displacement chromatography of IgA variants using a 0.005 *M* phosphate buffer as mobile phase.



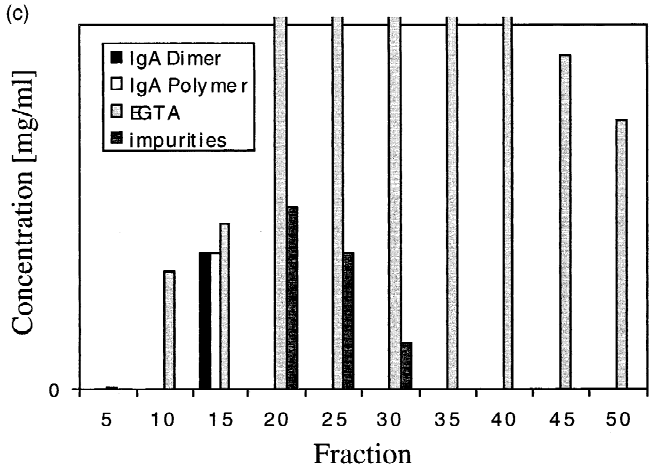


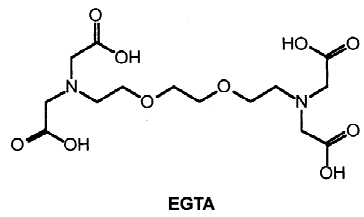
Column: Bio-Scale (2 ml)
Stationary Phase: 10 µm HA (Typ I)
Carrier: 0.02 M TRIS/HCl pH 6.8
Flow Rate: 0.1 ml/min





Column: Bio-Scale (2 ml)
Stationary Phase: 10 µm HA (Typ I)
Carrier: 0.02 M TRIS/HCl pH 7.5
Flow Rate: 0.1 ml/min





Column: Bio-Scale (2 ml)
Stationary Phase: 10 µm HA (Typ I)
Carrier: 0.02 M TRIS/HCl pH 8
Flow Rate: 0.1 ml/min

Fig. 5. HA displacement chromatography of IgA variants using a 0.02 *M* Tris buffer of the indicated pH. (a) Buffer pH of 6.8. (b) Buffer pH of 7.5. (c) Buffer pH of 8.0.

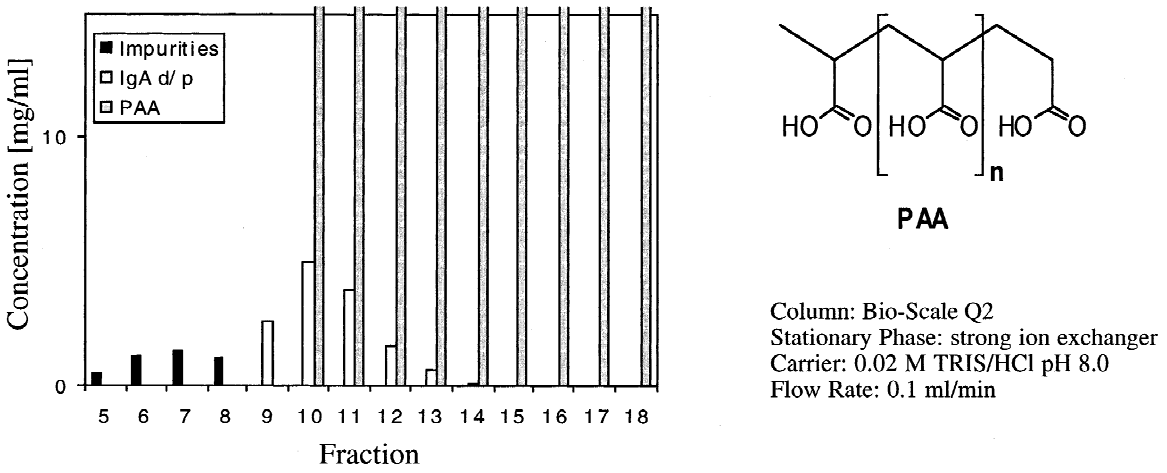


Fig. 6. Ion-exchange displacement chromatography of IgA variants using polyacrylic acid as displacer and a 0.02 *M* Tris buffer, pH 8.0, as mobile phase.

displacement chromatography is indeed possible, but also that no separation of the individual molecular forms can be achieved. A similar experiment using 1 ml of culture supernatant concentrate (hollow fiber reactor), collected after the filtration steps but before the standard DEAE ion-exchange step, yielded IgA enriched fractions separated form fractions containing the majority the impurities. Both PAA and EGTA could be used as displacers to achieve this goal. Once more, however, the EGTA front tended to overrun the IgA fractions, even though a pH of 8.0 was used, so further experiments with EGTA were abandoned at this point.

The overall protein concentration of the pooled IgA fractions was around 2.5 mg/ml in the culture supernatant concentrate. 1.8 mg of pure IgA were obtained with PAA as displacer. Concomitantly, the contaminants and/or degraded IgA molecules present in the original sample were removed by the IEX-DC-step, Fig. 6. The IgA concentration in the pooled fractions 3.5 mg/ml, so some concentration had taken place. Compared to the concentration achievable by DEAE elution chromatography, the higher fraction concentration together with the smaller scale should constitute an advantage and displacement chromatography may conceivably be implemented in the final downstream process at this point.

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