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Señor

SUPERINTENDENTE DE INDUSTRIA Y COMERCIO

DIVISIÓN DE NUEVAS CREACIONES

E. _____ S. _____

REGISTRO NACIONAL DE PATENTES Y COMERCIO



No. 06-048228- -00004-0000

FECHA DE RECEPCIÓN: 17/10/2009
DIRECCIÓN: PATENTES Y COMERCIO - LVA 378 FASE NACIONAL
400 444 RUMOR STARLÓQUE - Folios: 27

Re: PI.-WYETH.-Solicitud de Patente de Invención en Fase Nacional PCT para
"ELIMINACIÓN DE AGREGADOS DE ALTO PESO MOLECULAR
UTILIZANDO CROMATOGRAFÍA DE HIDROXIAPATITA".-Clase C07K
16/06.-Expediente número 06-048.228.-

1. Me refiero al auto dictado por ese Despacho notificado por fijación en lista el 30 de Octubre de 2009.

2. De acuerdo con el Concepto Técnico No. **3698** se tiene que:

"A efectos del examen de patentabilidad y como requerimiento de esta oficina, el solicitante deberá proporcionar, en un plazo que no excederá de 3 meses, los siguientes documentos relativos a la solicitud extranjera correspondiente a la misma invención que se examina y tales documentos son los que a continuación se enumeran:

1. **BIOTECHNOLOGY AND BIOENGINEERING, Vol. 73, No. 6, de 20 de Junio 2001 (2001-06-20), pags. 522-529, titulado: "Isolation of a recombinant antibody from cell culture supernatant: Continuous annular versus batch and expanded-bed chromatography"; autores: GIOVANNINI ROBERTO ET AL.**
2. **BIOSEPARATION. Vol. 9, No. 6, (año 2000), pags 359-368, titulado: "Comparison of different types of ceramic hydroxyapatite for the chromatographic separation of plasmid DNA and a recombinant anti-rhesus D antibody", autores: GIOVANNINI R ET AL.**
3. **HPLC BIOLOGICAL CHEMISTRY HOPPE-SEYLER., Vol. 372, No. 3, pages 149-156, (1991-marzo); titulado: "Purification of monoclonal antibodies by hydroxylapatite HPLC and size exclusion", autores: JOSIC D. ET AL.**

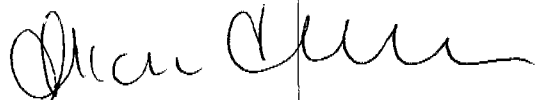
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3. Para responder a los requerimientos del señor Examinador hechos de conformidad con el Artículo 46 de la Decisión Andina 486 de 2000, anexo me permito presentar a ese Despacho copia de los siguientes documentos solicitados:

1. BIOTECHNOLOGY AND BIOENGINEERING, Vol. 73, No. 6, de 20 de Junio 2001 (2001-06-20), pags. 522-529, titulado: "Isolation of a recombinant antibody from cell culture supernatant: Continuous annular versus batch and expanded-bed chromatography"; autores: GIOVANNINI ROBERTO ET AL.
2. BIOSEPARATION. Vol. 9, No. 6, (año 2000), pags 359-368, titulado: "Comparison of different types of ceramic hydroxyapatite for the chromatographic separation of plasmid DNA and a recombinant anti-rhesus D antibody", autores: GIOVANNINI R ET AL.
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4. Ruego a usted tomar atenta nota de lo anterior y proceder de conformidad.

Señor Superintendente,



ALICIA LLOREDA RICAURTE

T.P. 53.215

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Isolation of a Recombinant Antibody from Cell Culture Supernatant: Continuous Annular Versus Batch and Expanded-Bed Chromatography

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Abstract: Annular chromatography represents a cross-flow approach to chromatographic separations, that allows the continuous separation of multicomponent mixtures. The potential of the method for continuous bioseparation has been discussed for some time, however, we demonstrate for the first time the processing of a complex feed (cell culture supernatant) taken from an actual (bio)process. Moreover, while previously published applications of annular chromatography concentrated on noninteractive (gel filtration) or nonspecific (ion exchange) chromatography, we show the possibility of continuous annular affinity chromatography. In particular, a commercially available preparative continuous annular chromatography (P-CAC) system was used to purify a recombinant antibody (human IgG₁-κ) from CHO cell culture supernatants by (pseudo)affinity chromatography on hydroxyapatite (HA) and rProtein A. Methods developed using small (2 mL) batch columns could be directly transferred to the P-CAC, where they yielded similar results in terms of final product quality. Yields were between 87% and 92% in the case of HA and between 77% and 82% in the case of rProtein A chromatography. DNA removal was nearly quantitative in all cases. Concomitantly, the antibody fraction of the total protein content was raised by one order of magnitude in HA and by a factor of 50 by rProtein A chromatography. In addition, a novel HA material (particle diameter ~120 μm) was investigated, which was compatible with expanded-bed applications. However, the final purity of the antibody thus obtained and also the yields (<70%) were less than satisfactory. © 2001 John Wiley & Sons, Inc. *Biotechnol Bioeng* 73: 522–529, 2001.

Keywords: hydroxyapatite; Rhesus factor; bioseparation; cell culture; P-CAC

INTRODUCTION

Product isolation and purification plays an important role in many bioproduction processes and can contribute quite heavily to the overall production costs. Especially in the biopharmaceutical industry, where products must be not

only purified to a high degree, but concomitantly need to be concentrated by several orders of magnitude, efficient downstream processes tend to rely on the principle of “chromatography” to achieve the purification goal. The majority of the corresponding chromatographic separations to date are performed in the batch mode. From a process-engineering point of view, continuous production would be preferable, especially at larger scale and when the related regulatory problems (e.g., definition of a “batch”) can be resolved. One possibility for continuous chromatography is the simulated moving bed (SMB) (Nicoud, 1993). The main inconvenience of SMB systems stems from the fact that one unit is suited only to the separation of a binary mixture and thus $N-1$ units are necessary to separate N compounds. In addition, the question of how to downscale such systems is not completely solved.

The continuous separation of a multicomponent system in a single unit requires a crossflow approach. The possibility of continuous annular chromatography (CAC) to achieve this was already discussed by Martin in 1949 and the theoretical feasibility demonstrated in 1962 by Giddings. Also in 1962, Dinelli built a rotating gas chromatograph consisting of 100 columns (Dinelli et al. 1962). The first true CAC-system was built in 1969 (Fox et al. 1969). This system already looked very much like modern CAC-units. In the late 90, Prior Engineering (Götzis, Austria) started to provide commercial CAC-instruments first for the separation of precious metals and lately also for application in the bioseparation area.

In a CAC-system, the “stationary” phase is packed in the form of an annular bed between two concentric cylinders. For chromatography, the annulus is slowly rotated around its axis, while feed and eluent(s) are introduced on the top of the bed through fixed inlets. In the case of isocratic elution or gel filtration, a suitable mobile phase is distributed evenly over the cylinder. The CAC-system used for the experiments described in this article allows in addition, for the introduction of steps of different (specific) eluents, which can be introduced from nozzles placed around the circumference of the column (Fig. 1). Similar to batch chro-

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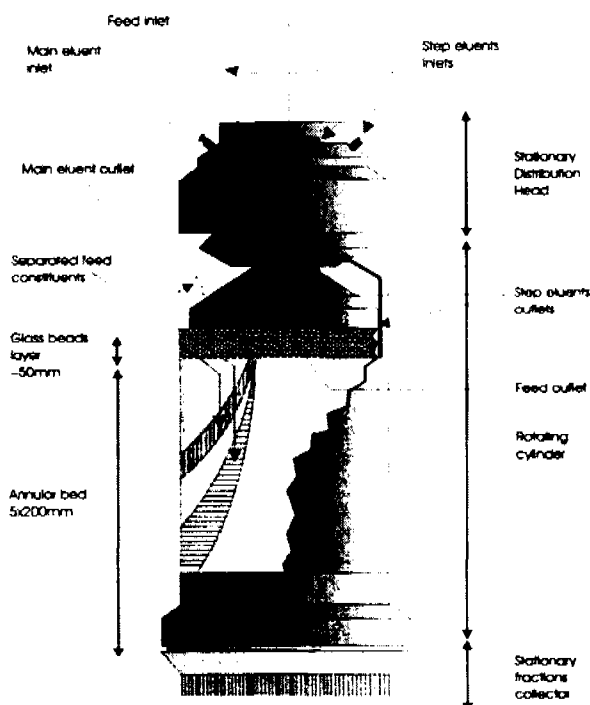


Figure 1. Schematic drawing of the P-CAC system commercialized by Prior Engineering (Götzis, Austria).

matography, in CAC separation takes place along the column axis in the vertical direction: As a result helical bands develop because of the simultaneous rotation of the cylinder. Once the steady state is obtained, the various components of the mixture leave the column at fixed outlet points. The inlet/outlet-angle is directly determined by the substances' affinity for the stationary phase, i.e., weakly retained substances elute only a few degrees away from the inlet point, while strongly retained substances appear "later" in space. In fact, a direct relation exists between the retention time, t , in a batch column and the retention angle, α , in a CAC-column (Wankat, 1977):

$$\alpha = \omega \times t$$

with ω : rotation speed [$^{\circ}/S$].

A vast variety of different substances have been separated by CAC, including metals (Begovich and Sisson, 1982; Begovich et al., 1983; Canon and Sisson, 1978; Carta et al., 1989; Sun et al., 1994), amino acids (DeCarli II et al., 1990; Kitakawa et al., 1997; Takahashi and Goto, 1991a,b; Yonemoto et al., 1993) and sugars/oligosaccharides (Bart et al., 1996; Byers et al., 1990; Howard et al., 1988; Takahashi and Goto, 1994; Wolfgang et al., 1997). Separation of a nonretained and a retained protein was demonstrated by Bloominburg using a cation exchanger (Bloominburg et al., 1991). Takahashi showed the use of the CAC for the

separation of myoglobin and hemoglobin (Takahashi and Goto, 1992). Recently, Porcine lipase was continuously purified by annular chromatography (Genest et al., 1998). It has also been shown that the CAC-approach can be used as a chromatographic reactor/separator (Cho et al., 1980).

The majority of the CAC-application to date describes separations, which do not require a change in mobile phase, i.e., either isocratic elution or gel filtration. However, biomacromolecule separations encountered in real life often require some sort of gradient elution. Moreover, from the viewpoint of efficient bioseparation one of the most attractive possible uses of the CAC-approach seems to be for the processing of large volumes of complex, low titer product streams. Affinity chromatography is a method of choice in such cases, which has so far not been used in the CAC mode. In this article we investigate the compatibility of the CAC with affinity chromatography, step-gradient elution and continuous-column regeneration. The isolation of a recombinant antibody from CHO cell culture supernatants by rProtein A and hydroxyapatite (affinity) chromatography is taken as pertinent example.

MATERIALS AND METHODS

Chromatographic Systems

The CAC-system used in the investigations was a prototype (Preparative continuous annular chromatograph, P-CAC) provided by Prior Separation Technology (Götzis, Austria). The outer cylinder of the system is made of glass, while the inner cylinder is made of polypropylene. The annulus between the two cylinders has a width of 5 mm and an outside diameter of 150 mm. The chromatographic resin is slurry-packed into the annulus at a height between 10 and 20 cm. The lower end of the bed is supported by a porous PTFE filter (11 μm). On top of the bed a layer (~5 cm high) of glass beads (150–250 μm) is packed. This layer ensures uniform flow distribution over the entire annulus. Up to 90 exit holes exist at the column outlet, each fitted to a short section of Tygon tubing for fraction collection.

The annular system can be rotated over a wide range of rates (0–5000 $^{\circ}/h$), controlled by a digital speed drive system. Feed, main eluent, and step-eluent are introduced to the column through seven inlets spaced 45 $^{\circ}$ apart from each other. The nozzles of the step-eluent penetrate into the glass bead layer, thus reducing the mixing of the step-eluent with the main eluent. A wide inlet nozzle covering about 90 $^{\circ}$ of the annulus is used for feed introduction.

The chromatographic system for the batch experiments consisted of a 6-ports valve (Valco, Schenkon, Switzerland), two HPLC pumps (Irica Σ 871, ERC, Alteglofsheim, Germany), a mixing chamber, and a UV-detector S-3702 (Soma Optics Ltd., Tokyo, Japan) with the wavelength set at 214 nm. The data were processed with a Chromatography Station for Windows software program (Data Apex Ltd., Techlab Braunschweig, Germany). Batch columns were

packed in a stainless steel column 25 × 4 mm (length × diameter) (Macherey Nagel, Düren, Germany). Expanded-bed experiments were performed using the Streamline™ 25 system (Pharmacia). Fluidization of the bed was achieved with a Gradifrac pump (Pharmacia). Elution took place using a FPLC system (Pharmacia), consisting of two P-500 syringe pumps and an LCC-500 plus gradient programmer, coupled with an UV-Vis spectrophotometer (VWM 2141, Pharmacia) set at 214 nm. Data recording was made with a plotter (Pharmacia).

Materials

High-purity water was used throughout. Chemicals were from Fluka (Buchs, Switzerland), Sigma (St. Louis, MO), Merck (Darmstadt, Germany) or Adros Organics (Geel, Belgium). The hydroxyapatite type I stationary phase was donated from Bio-Rad (Hercules, CA; 20-μm and 120-μm diameter particles). The latter material was used for the expanded-bed experiments. rProtein A Sepharose™ Fast Flow was from Pharmacia. Polyacrylamide gels and gel electrophoresis equipment were from Bio-Rad. Supernatants from CHO cell cultures containing between 2 and 10 mg/L of recombinant human antibody were donated by members of the cell culture technology group (Prof. F. Wurm) of the Center of Biotechnology. Cells had been grown for 4–6 days in DMEM/F12 (Gibco Life Technologies, Paisley, Scotland) or in FEME (fortified DMEM/F12) culture medium without calf serum in roller bottles (Falcon, Becton Dickinson Labware, Lincoln Park, NJ) or in cell-cubes (Corning, Shipol Rijk, Netherlands). Supernatants were stored at –20°C with 0.02% sodium azide added. Supernatants were used without further preparation for expanded-bed applications. Prior to fixed-bed or P-CAC experiments, supernatants were clarified by centrifugation for 20 min at 4000 rpm (5200g) and at 20°C. Afterwards, the supernatants were directly applied to the rProtein A columns. For hydroxyapatite chromatography, supernatants were, in addition, diluted (1:3) in the appropriate buffer.

Methods

Purity of the isolated antibody was analyzed by SDS-PAGE using 7.5% polyacrylamide resolving gels (unless indicated otherwise) and nonreducing and reducing conditions as well as silver staining for detection (Silver Stain plus Bio-Rad kit). Estimation of the purity was done by comparison with a highly purified antibody standard. Quantification of the antibody was done by ELISA as described previously (Jordan et al., 1999). Total protein content was measured according to the protocol of the BioRad protein assay according to the manufacturer's instructions. A calibration curve was established with highly purified antibody. Residual DNA was quantified by a Pico-Green assay (Molecular Probes, Eugene, OR) following the manufacturer's protocol. Samples were diluted 10–20 times in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) prior to measurements to

diminish the interfering effects of other contaminants. Concentrations were calculated using a highly purified linear dsDNA as standard. Phosphate concentrations were measured by a colorimetric method (APHA 1976). Samples were diluted 500–5000 times with 0.01 M HCl prior to transfer into a 96-well plate (Immulon4 HBX, Dynex Technologies, Chantilly, VA). 10 μL of ethanol were added as well as 10 μL of a solution containing 2.5 M of H₂SO₄, 66 mM of K(SbO)C₄H₄O₆, 33 mM of (NH₄)₆Mo₇O₂₄ and 0.1 M of ascorbic acid in water. After 5–10 min of reaction the absorbance of the blue complex was measured at 710 nm using an Elisa reader (SPECTRA max 340, Molecular Devices, Sunnyvale, CA). The assay was calibrated with 80, 40, and 20 μM phosphate standards.

RESULTS AND DISCUSSION

Development and Optimization of Hydroxyapatite Chromatography of Recombinant Antibodies

Hydroxyapatite (HA) is a calcium phosphate-based stationary phase, well known for its complex ("pseudo-affinity") type of interaction with proteins. Positively charged ("basic") proteins are retained on the surface due to electrostatic interactions of their amino groups with the phosphate moieties (P-sites) of the HA. The interaction is reminiscent of ion-exchange chromatography and elution can often be enforced by gradients of increasing ionic strength, i.e., by anions like PO₄^{3–} or Cl[–] as well as by certain bivalent cations like Mg²⁺ or Ca²⁺. Acidic proteins are retained via coordination bonds with the calcium cations (C-sites) of the HA-surface and such proteins are eluted by agents with high calcium affinity, such as PO₄^{3–} (Gorbunoff, 1984a, 1984b; Gorbunoff and Timasheff, 1984). Another biomacromolecule with high affinity for HA is DNA (Yamasaki, 1989). Most proteins, and hence, most proteinaceous contaminants of a recombinant product have an isoelectric point in the acidic range (Gianazza and Righetti, 1980). For the antibody considered here, an isoelectric point of 8.3 had been determined previously by isoelectric focusing (Freitag et al., unpublished results). Conditions are therefore envisionable, where most contaminating proteins have a lower affinity for HA than the product (antibody), while DNA has a much higher affinity. The latter point is especial importance under the given circumstances, because the supernatants provided to us contained at least as much DNA in mg/L as antibody (Freitag et al., unpublished results).

The composition of the binding buffer proved to be the most important aspect during method development. The standard binding buffer in HA chromatography is a low-concentration phosphate buffer with a pH between 6 and 7. Elution occurs in a gradient of increasing phosphate concentration, with 400 mM phosphate being the usual maximum value. When 5 mL of clarified supernatant were injected onto a fixed-bed column (25 × 4 mm, HA type I, 20 μm particles) equilibrated with 1 mM phosphate buffer pH

6.0 and subsequently eluted with 400 mM phosphate pH 6.0, the antibody peak contained several impurities (Fig. 2A). This became even more obvious when a supernatant spiked with an additional 40 µg of purified antibody was processed under otherwise identical conditions (Fig. 2B). When a high saline buffer (1 mM phosphate pH 6.0 + 1M NaCl) was used instead as binding buffer, however, the antibody was separated from the main impurities.

Because the interaction of basic proteins with the HA surface is assumed to follow an ion-exchange mechanism, the retention of an antibody was a priori unlikely in a high salt buffer. However, an exception to this rule has been observed before in certain cases (Hjertén, 1959; Gorbunoff 1984a, 1984b), i.e., mainly for molecules that contain a sufficient number of carboxylic acid groups on the surface to still allow interaction with the C-sites in addition to the electrostatic interaction with the P-sites. Figure 3 shows the isolation of the antibody from 10 mL of a clarified supernatant diluted 1:3 in high salt-binding buffer (1 mM phosphate pH 6.0 + 1M NaCl) in a linear phosphate gradient from 0% to 15% buffer B (400 mM phosphate pH 6.0). The SDS-PAGE of the antibody-containing fraction showed that the antibody was still contaminated by other protein (Fig.

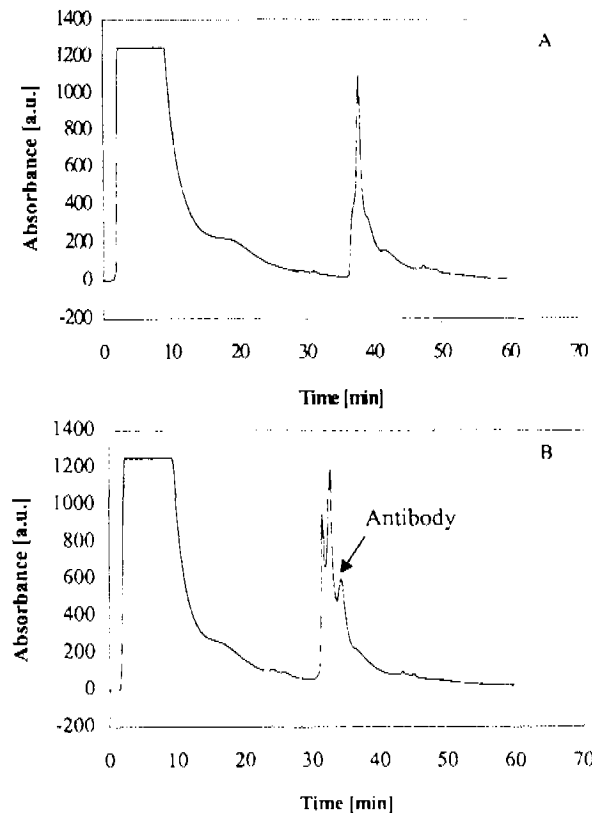


Figure 2. Antibody-purification by IIA-chromatography using a conventional phosphate buffer system. Sample: 5 mL of clarified cell-culture supernatant, buffer B: 400 mM phosphate pH 6.0, flow rate: 1 ml/min. (A) Buffer A 1 mM phosphate pH 6.0, cell culture supernatant; (B) Buffer A 1 mM phosphate pH 6.0, supernatant spiked with 40 µg antibody.

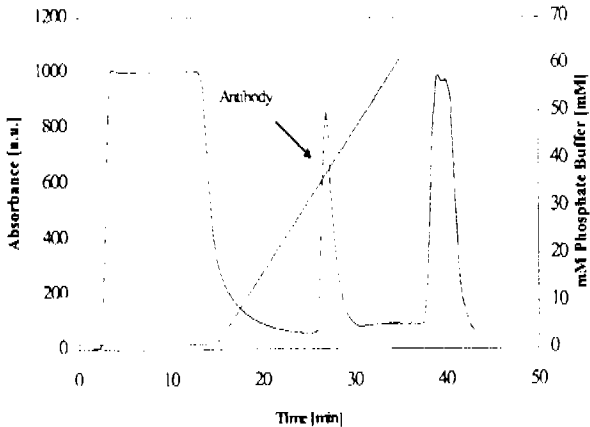


Figure 3. Antibody-purification by HA-chromatography using a high salt binding buffer. Sample: 10 mL of clarified cell-culture supernatant diluted 1 : 3 in buffer A, flow rate: 1 ml/min, buffer A 1 mM phosphate pH 6.0 + 1M NaCl, buffer B: 400 mM phosphate pH 6.0, gradient from 0% to 15% B in 20 minutes, regenerant pure buffer B.

4). However, the antibody fraction in the total protein content was increased from 4.3% in the feed to 40% in the eluate from the HA column (determined by comparing ELISA and total protein assay results). Yields, calculated as the amount (in %) of antibody recovered after purification compared to the amount originally present in the feed, were consistently between 87% and 92%. The Pico-Green assays showed that the antibody was essentially free of DNA. Concomitantly, 98% of the nucleic acids previously detected in the feed were found to elute during column regeneration with pure buffer B.

Continuous Annular Hydroxyapatite Chromatography

It was expected that the necessity to change from the linear to a step gradient might pose an obstacle to the transfer of

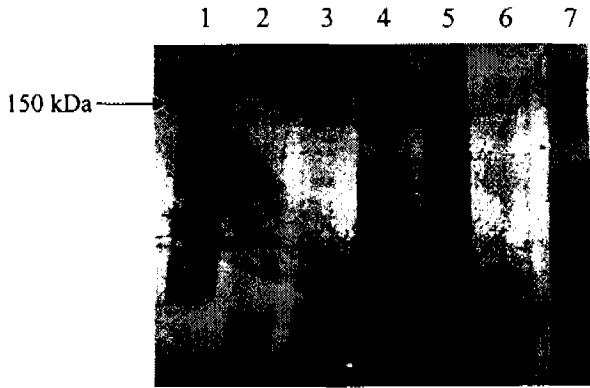


Figure 4. SDS-PAGE gel (7.5% polyacrylamide) of the antibody fractions obtained by IIA chromatography. 1: Antibody standard; 2: antibody from P-CAC; 3: antibody from batch column; 4: antibody from expanded bed; 5: strongly retained impurities; 6: flow-through; 7: cell culture supernatant.

the separation protocols developed and optimized for a small batch column to the P-CAC (presently only a step-gradient is compatible with the P-CAC design). However, as the subsequently performed experiments showed, this was not the case. Data collected from small columns operated with linear gradients can be used directly to determine the conditions for a P-CAC separation of similar quality. Table I summarizes the condition chosen for the continuous purification of the recombinant antibody. The clarified supernatant (as before, diluted 1:3 in binding buffer) was pumped in the system with a flow rate of 16 mL/min. Fractions corresponding to sectors of 4° were collected when the steady-state had been reached, i.e., after one complete revolution. The column was continuously regenerated using a 400 mM phosphate buffer (pH 6.0). The antibody was eluted in an eluent step composed of 15% regenerant and 85% binding buffer.

Figure 5 shows the UV-absorbencies as well as the mobile-phase phosphate concentrations of the collected fractions as a function of the rotation angle. The antibody eluted, as expected, in the step zone and most contaminants either did not bind at all or eluted in the regeneration zone. The placement of the peaks depends on the rotation speed, the feed/step-eluent flow rates and the position of the inlet nozzles. To maximize the throughput, conditions were chosen in such a way that the eluted antibody peak just touched the feed zone. Yields were in a similar range for the P-CAC as for the batch column, i.e., between 87% and 92%. According to the SDS-PAGE (Fig. 4) a similar purity was also obtained. The antibody fraction within the total protein was concomitantly increased from 9% in the feed to 26% in the eluate of the affinity column. According to the Pico-Green assays, the DNA removal was also of equal efficiency.

The P-CAC approach hence yielded a product of similar quality to that of the batch column. Under the chosen experimental conditions, the buffer consumption (calculated per L of feed) was somewhat lower (10 vs. 17 L) for the continuous compared to the batch approach. The throughput of the P-CAC (calculated as mL of feed per min and mL of stationary phase) was limited by the maximum pressure applicable to the system (3 bars), which reduced the applicable flow rate to values below the ones possible for the

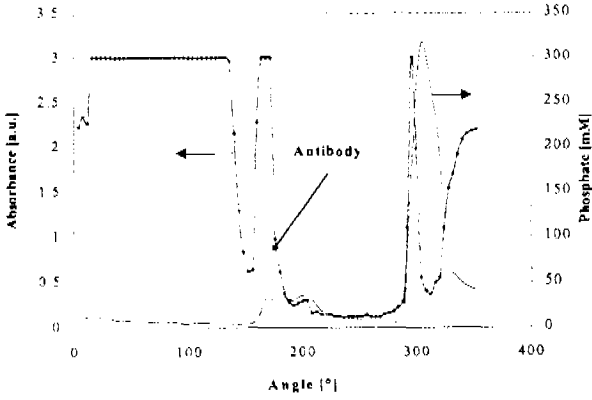


Figure 5. Absorbance and phosphate concentration of the fractions collected from the P-CAC under steady-state conditions during the antibody purification. Chromatographic conditions as given in Table I.

batch column. However, by substituting the glass with a metal cylinder this drawback may, in principle, be overcome.

Expanded-Bed Hydroxyapatite Chromatography

The expanded-bed technology has been suggested as an alternative to conventional column chromatography, especially for early product capturing steps from cell culture supernatants (Anspach et al., 1999). Bio-Rad made a novel type of hydroxyapatite available to us for this investigation, which is compatible to the expanded-bed approach. The particles have a diameter of 120 μm, but correspond otherwise to the 20 μm HA type I particles used in the batch and P-CAC columns. For expanded-bed HA chromatography, the Streamline™ 25 system was slurry-packed to a height of 12 cm with the 120-μm beads. Antibody-containing supernatants were again diluted 1:3 with binding buffer. The feed-flow rate was set at 25 mL/min. The ensuing expanded bed measured 25 cm in height and was visually stable. The elution was done by a linear gradient identical to the one used in the batch column (0% to 15% buffer B, linear flow rate 1.6 cm/min corresponding to 8 mL/min). However, the yield was only 77% in this case and the SDS-PAGE (Fig. 4) showed a lower purity of the obtained antibody, while the DNA removal was again almost quantitative. The lower yields obtained with the expanded bed may be due to the short contact time and could then be improved by increasing the bed length. The particle size may be responsible for the low purity of the antibody produced by expanded-bed HA chromatography.

Batch and Continuous Protein A Chromatography for Antibody Purification

The interaction between certain antibodies and microbial surface receptors such as Protein A has been widely used in the affinity purification (Jiskoot et al., 1991). Binding of the

Table I. P-CAC parameters for the antibody purification by hydroxyapatite chromatography.

Feed rate	16 mL/min
Elution step rate	8 mL/min
Regenerant rate	3 mL/min
Binding-buffer flow rate (main buffer)	16 mL/min
Flow rate within the column	1.89 cm/min
Rotation speed	180°/h
Feed sector	134°
Step sector	67°
Regenerant sector	25°
Bed height	195 mm

receptor occurs to the Fc fragments of the antibodies and each receptor molecule can bind several antibody molecules (Forsgren and Sjoquist, 1966). Though efficient, Protein A chromatography also has drawbacks, especially in the purification of therapeutic antibodies, because the complete removal (<10 ppm per dose) of the ligand has to be assured for the final product. Especially in the case of human(ized) antibodies, problems with reliable and reproducible detection of residual ligands have been reported (Steindl et al., 2000). However, because the hydroxyapatite chromatography did not yield a sufficiently pure antibody, Protein A affinity chromatography was studied as an alternative.

Figure 6 shows the isolation of the recombinant antibody using a rProtein A Sepharose FF batch column. Five milliliters of the clarified supernatant were injected into a column previously equilibrated with phosphate buffer 50 mM pH 7.4 + 120 mM NaCl (buffer A). The flow rate was set at 1 mL/min (7.96 cm/min). The antibody was eluted by a glycine buffer step (100 mM pH 3.0). The glycine is also responsible for the baseline difference before and after the antibody-peak in Figure 6. Yields were between 75% and 85%. Both reduced and nonreduced SDS-PAGE showed that a pure antibody fraction was obtained (Fig. 7), while a comparison of the results from the antibody specific ELISA assay and the total protein assays showed that the antibody fraction within the total protein improved by a factor of 52 in this case. The Pico-Green assay showed that > 99% of the nucleic acids were removed from the product zone in the process.

The step-gradient could be transferred directly to the P-CAC, see Table II for details. The flow rate within the P-CAC column was 3.51 cm/min, i.e., only half as high as in the batch column for the already mentioned reason of a restriction in applicable pressure to 3 bar. Fractions corresponding to sectors of 4° were collected when the steady

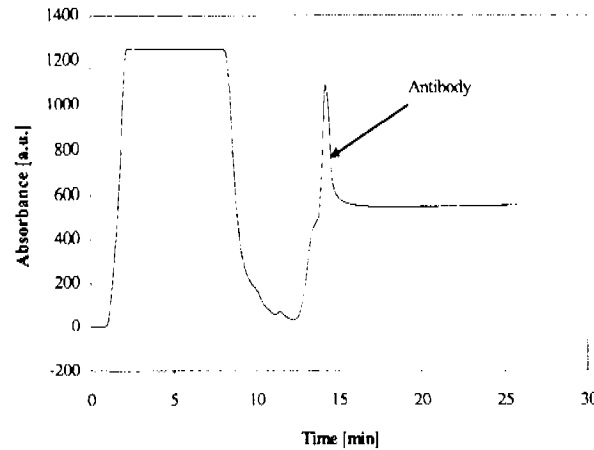


Figure 6. Antibody purification by batch column affinity chromatography with rProtein A as affinity ligand. Sample: 5 mL of clarified cell culture supernatant, binding buffer: 50 mM phosphate pH 7.4 + 120 mM NaCl, elution buffer: 100 mM glycine pH 3.0, step elution after 10 min, flow rate 1 mL/min.

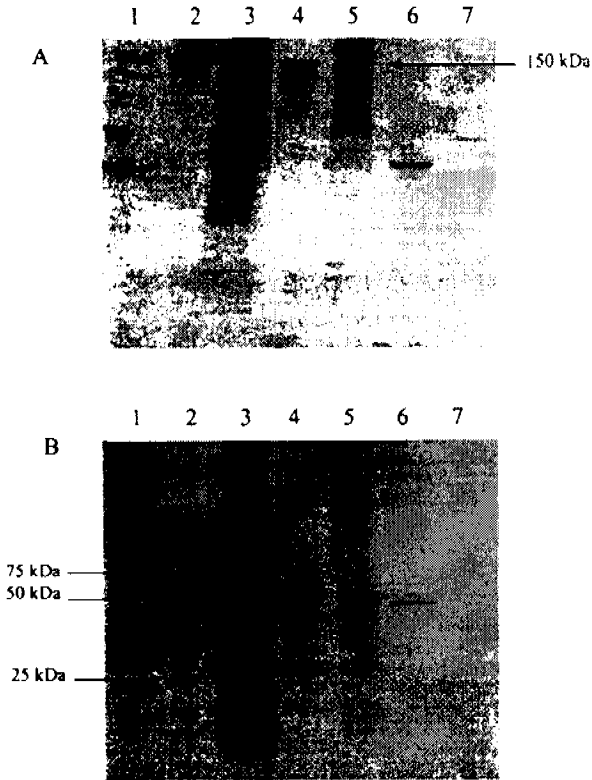


Figure 7. SDS-PAGE gel (4–20% polyacrylamide) of the fractions obtained by rProtein A affinity chromatography. 1: Molecular mass marker; 2: antibody from P-CAC; 3: cell culture supernatant for P-CAC; 4: antibody from batch column; 5: cell culture supernatant for batch column 6. Protein A standard (Sigma); 7: rProtein A standard (Pharmacia). (A) Nonreduced gel; (B) reduced gel.

state was reached (usually after one revolution) and their pH and absorbance (214 nm) was measured (Fig. 8). As in the batch column, the antibody eluted just in the front of the glycine step. Yields were 77% in this case. According to the SDS-PAGE (Fig. 7) the antibody preparation obtained with the P-CAC was of equal purity to the one obtained with the batch column. Concomitantly, the purity (antibody fraction of the total protein content) was increased by a factor of 50. Contrary to the situation in hydroxyapatite chromatography, P-CAC and batch column hence showed similar performance in purifying the antibody.

Table II. P-CAC parameters for the antibody purification by rProtein A chromatography.

Feed rate	16 mL/min
Step rate	30 mL/min
Binding buffer	34 mL/min
Flow rate within the column	3.51 cm/min
Rotation speed	180°/h
Feed sector	72°
Step sector	135°
Bed height	195 mm

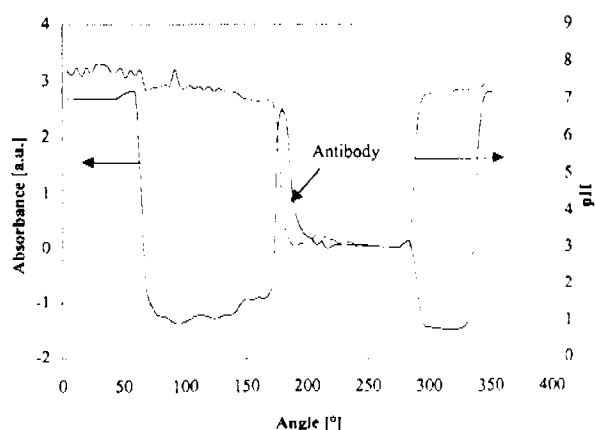


Figure 8. Absorbance and pH of the fractions collected from the P-CAC under steady-state conditions during continuous antibody purification by rProtein A affinity chromatography. Chromatographic conditions as given in Table II.

CONCLUSIONS

The need of the pharmaceutical and life science-based industry for high-resolution separation techniques is expected to continue to grow at considerable speed compared to separation technology in general. In this context, the purification of labile high-value compounds such as recombinant proteins is especially challenging, because chromatographic multicomponent separations are almost inevitable in their case. The aim of this contribution was to demonstrate that continuous annular chromatography, a principle known for over 50 years, presents a viable option for the continuous chromatographic separation of the desired protein from a complex mixture such as a cell culture supernatant.

Despite the basic difference, that is of continuous vs. batchwise operation, and in contrast, for example, to the simulated moving bed, continuous annular chromatography behaves similarly to conventional batch-column chromatography. In fact, the direct scale up of a method developed for a 25 × 4 mm column to a CAC (bed height: 15 cm, thickness of the annulus: 5 mm, outer diameter of the annulus: 150 mm) is usually possible without any problems or major subsequent adjustment and optimization. The potential of the CAC approach was demonstrated here taking (pseudo-) affinity chromatography as an example. However, we propose that a similar straightforward transfer of methodology from a batch to a CAC-column is possible for any type of step-elution protocol. Chromatographic performance (retention, resolution, yield, product quality) will be similar when the CAC is used, while throughput and buffer consumption should improve.

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Comparison of different types of ceramic hydroxyapatite for the chromatographic separation of plasmid DNA and a recombinant anti-Rhesus D antibody

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Abstract

The chromatographic properties of three types of ceramic hydroxyapatite (HAP) are compared. All three materials were prepared by sintering the original precipitate, albeit at different temperatures (400, 700 and 1000°C for type I, II and III HAP, respectively). The three materials differed in pore size and pressure limits (both lowest for type I and highest for type III). Type I and II HAP had an average particle diameter of 20 μm . The particle size of the type III material was 40 μm . HAP-beads were slurry-packed into 4 $\times$ 25-mm stainless steel columns and investigated for the chromatographic isolation of plasmid DNA from clarified *E. coli* lysates and of a recombinant human antibody from CHO cell culture supernatants respectively. The chromatographic performance of the three types of HAP showed significant differences, which were correlated to the binding capacities of the materials for (linearized) plasmids of different size (4.7, 10.3 and 11.4 kb) and proteins of different isoelectric point (lysozyme, $pI = 10.5$; anti RhD antibody, $pI = 8.3$; β -lactoglobulin, $pI = 4.9$). The accessibility of the adsorptive surface (pore size) but also the types of binding sites on the HAP-surface (P/C-site ratio) are proposed as determining factors for the chromatographic behavior.

Introduction

Recombinant proteins have for some time now been the high-value products of the modern pharmaceutical biotech industry. The developments in gene therapy have recently also increased the demand for large amounts of pharmaceutical-grade plasmid DNA (Ledley, 1995; Marcel and Grausz, 1997). In both cases, chromatography has been playing a major role in the purification of such biomacromolecules to the required level (Chandra et al., 1992; Ferreira et al., 1998; Jiskoot et al., 1991; Prazeres et al., 1998, 1999). A vast body of literature exists in regard to possible purification strategies, and the question of the relative worth of affinity versus multi-step conventional chromatography has been raised (Sadana et al., 1994). In this context, a calcium phosphate-based stationary phase, namely hydroxyapatite (HAP), has been

discussed for both protein and DNA purification (Aoyama and Chiba, 1993; Bernardi, 1971; Jungbauer et al., 1989; Tiselius et al., 1956). In contrast to most other chromatographic phases, HAP is an inorganic material, usually assumed to have the formula $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$. Especially for the purification of biomacromolecules, HAP shows some unique separation properties, together with high selectivity and resolution.

Retention mechanisms for biomacromolecules on HAP are complex, since two types of interaction with the surface are possible (Deppert and Lukacin, 2000; Gorbunoff, 1984; Kawasaki et al., 1990). Substances may, for example, interact with the phosphate moieties (P-sites) on the HAP-surface; this interaction requires either positive charges or groups with high phosphate affinity on the retained molecule. The interaction of negatively charged molecules, such as acidic proteins

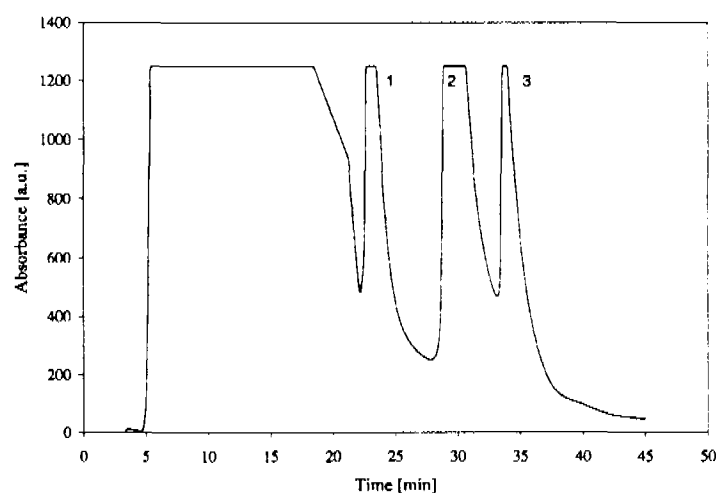


Figure 1. Isolation of plasmid pEAX 39 LH1 on HAP Macro-Prep[®] type I. Feed volume, 5 ml; step gradient, from 0 to 10 min, 100% A; from 10 to 28 min, 60% A; from 28 to 45 min, 0% A. Buffer A, 1 mM phosphate, pH 6.2; buffer B, 400 mM phosphate, pH 6.2; flow rate, 1 ml/min. Peak 1, impurities (such as proteins, oligonucleotides); peak 2, RNA; peak 3, plasmid DNA. The corresponding agarose gel is shown in Fig. 2.

and DNA, is also possible on HAP (Kawasaki, 1990). In this case, an interaction of calcium-chelating or -binding groups on the retained molecule with the calcium-based C-sites on the HAP-surface is assumed to be responsible for the retention. In the case of DNA, the interaction is mediated by the phosphate groups in the molecule's backbone. Since the P-site interaction is assumed to be due to an electrostatic type of interaction, while the C-site interaction involves chelating of the calcium groups, a gradient of increasing phosphate concentration is usually employed to elute all interacting substances from an HAP-column. The P-site bound molecules are eluted because of charge screening by the increasing salt concentration and the C-site bound ones due to the specific interaction of the phosphate ions in the elution buffer with the C-sites.

While the interaction between DNA and HAP appears to be rather straightforward, complex mechanisms involving both P- and C-site interaction have been observed for large proteins (e.g., some antibodies) due to the inhomogeneity of their charge distribution (Gorbunoff, 1985). Such proteins bind in a mixed mode manner to the HAP-surface, which allows the fine-tuning of a separation strategy to a given purification problem. Examples include the double gradient elution proposed by Hjertén et al. (1988) or more recently a purification protocol for a recombinant antibody from cell culture supernatant (Giovannini and Freitag, 2001).

In the early days, only crystalline HAP was available for chromatographic purposes. The mechanical stability of these stationary phases was less than satisfactory, which made HAP-chromatography unsuitable for many biochromatographic applications. In order to overcome this handicap, ceramic HAP has been developed by BioRad Laboratories. Some stationary phases are already commercially available under the name of Macro-Prep[®]. In this paper three HAP variants, the commercially available type I and type II Macro-Prep and a prototype HAP, termed type III, are compared for the purification of a recombinant human antibody from cell culture supernatants and the isolation of plasmid DNA from clarified *E. coli* lysates.

Materials and methods

Materials

Chemicals were from Fluka (Buchs, Switzerland), Sigma (St. Louis, MO, USA), Merck (Darmstadt, Germany) or Acros Organics (Geel, Belgium). High-purity water was used throughout. The HAP (types I, II, and III) stationary phases were donated by BioRad (Hercules, CA, USA). Average particle diameter of the type I and II beads was 20 μm , the type III beads had an average diameter of 40 μm (all values according to the manufacturer). Clarified cell culture

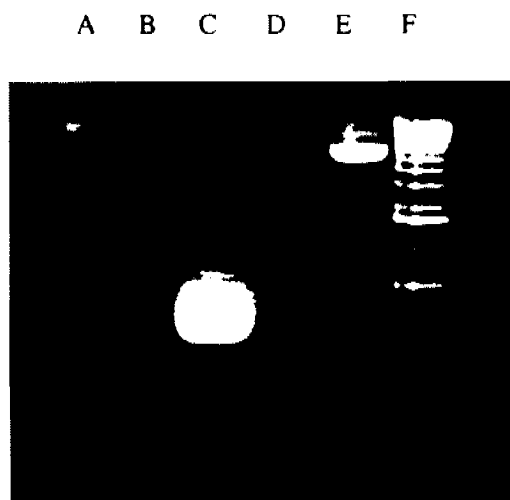


Figure 2. Agarose gel (0.8%) of the peaks identified in Fig. 1. Lanes: (A, F) 1-kb DNA ladder; (B) peak 1; (C) peak 2; (D) peak 3; (E) pEAX39 LH1 standard.

supernatants of CHO cells expressing a recombinant anti-Rhesus D-antibody (Amstutz et al., 1999) were donated by the Laboratory for Cellular Biotechnology (LBTC), Center of Biotechnology, Swiss Federal Institute of Technology (EPFL), Lausanne, Switzerland. The 1-kb DNA ladder used in the agarose gels and the restriction enzyme *Bam*HI were from Life Technologies (Paisley, UK). Pure plasmid standards were obtained using the Nucleobond AX cartridges (Macherey-Nagel, Düren, Germany), according to the manufacturer's recommendations.

Instrumentation and methods

The chromatographic system consisted of a six-port valve (Valco, Schenk, Switzerland), two HPLC pumps (Irica Σ 871, ERC, Alteglofsheim, Germany), a mixing chamber, and a UV detector S-3702 (Soma Optics, Tokyo, Japan). Detection wavelengths of 260 and 214 nm were used for DNA and protein separations, respectively. Data were processed with a Chromatography Station for Windows software program (Data Apex, Techlab, Braunschweig, Germany). All HAP-materials were slurry-packed in 4 × 250-mm stainless steel columns for the elution chromatography and 4 × 50-mm stainless steel columns (all Macherey Nagel) for the frontal experiments. 0.8% agarose gels (8 cm) containing 0.1 μl/ml ethidium bromide were run in a horizontal gel electrophoresis unit (MWG Biotech, Ebersberg, Germany) coupled to a BioRad

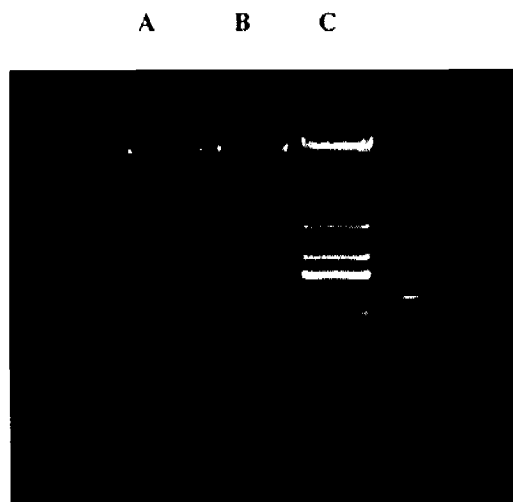


Figure 3. Comparison of the HAP-purified plasmid DNA with the standard after linearization with *Eco*RI. Gel, 0.8% agarose. Lanes: (A) HAP-purified plasmid pEAX39 LH1; (B) plasmid standard; (C) 1-kb DNA ladder.

PowerPac 300 power supply. The running buffer was a 40 mM Tris-acetate buffer (pH 8.3) containing 1 mM EDTA. The electrophoresis was carried out at 90 V for ca. 45 min.

Cell growth and lysis

Escherichia coli XL1 Blue bacteria harboring plasmid pEAX 39 LH1 (10.3 kb) encoding for the light chain of the recombinant anti-Rhesus D antibody were made available to us by the Laboratory for Cellular Biotechnology (LBTC), Center of Biotechnology, Swiss Federal Institute of Technology (EPFL), Lausanne, Switzerland. The bacteria were grown in LB medium (Life Technologies) containing 100 mg/l of ampicillin. One-liter shake flask cultures were each inoculated with 3 ml of an 8-h pre-culture and incubated overnight in a shaker incubator (120 rpm, 37°C). Cells were harvested by centrifugation at 4000 rpm (5200 g) for 15 min (25 °C). Afterwards the cells were resuspended in 25 mM Tris-HCl buffer (pH 8) containing 50 mM glucose and 10 mM EDTA. Per liter of fermentation broth 50 ml of resuspension buffer were used. The plasmid was subsequently liberated by alkaline lysis. For this purpose, 50 ml of lysis buffer (0.2 M NaOH, 1% SDS) were used per liter fermentation broth. After 5 min of lysis, the pH was shifted to 5 by adding sufficient amounts of a 3 M acetate buf-

Table 1. Properties of the three types of ceramic HAP investigated.

Support	HAP Type I	HAP Type II	HAP Type III
Functional group	$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$	$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$	$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$
Particle size	20 μm	20 μm	40 μm
Sintering temperature	400 °C	700 °C	1000 °C
Pore size (nominal)	700 Å	800 Å	1000–2000 Å
Maximum pressure	60 bar	100 bar	n.a.
Protein capacity (lysozyme)	55 mg/g	21 mg/g	9 mg/g
Protein capacity (β -lactoglobulin)	67 mg/g	30 mg/g	18 mg/g
PDNA capacity (pEAX 41 LH2, 11.4 kb)	59 $\mu\text{g/g}$	38 $\mu\text{g/g}$	4 $\mu\text{g/g}$
PDNA capacity (pEGFP-N1, 4.7 kb)	149 $\mu\text{g/g}$	99 $\mu\text{g/g}$	14 $\mu\text{g/g}$
PDNA capacity (linearized pEAX41 LH2)	446 $\mu\text{g/g}$	80 $\mu\text{g/g}$	20 $\mu\text{g/g}$

fer. The ensuing potassium dodecyl sulfate precipitate was removed by centrifugation ($5200 \times g$, 30 min, 4°C), followed by a filtration step. Cell debris, genomic DNA, and most of the proteins are eliminated by this precipitation step. The resulting clarified lysate contains mostly RNA and the plasmid DNA in the supercoiled and the relaxed (open circular) form.

Escherichia coli XL1 Blue bacteria harboring plasmid pEAX 41 LH2 (11.4 kb) and *Escherichia coli* DH5 α bacteria harboring plasmid pEGFP-N1 (4.7 kb) were grown and lysed following the same procedure. In the latter case, the ampicillin in the medium was replaced by 50 mg/l kanamycin.

Assays

Double-stranded DNA (dsDNA) was quantified by a Pico-Green assay (Molecular Probes, Eugene, OR, USA) following the manufacturer's protocol. Samples were diluted 10–20 times in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) prior to measurement in order to diminish the interfering effect of contaminants. Highly purified linear dsDNA standards were used for calibration. Fluorescence was measured with a CytoFluor 4000 (Framingham, MA, USA). The excitation wavelength was 485 nm and the emission wavelength 530 nm. The antibody was quantified by a specific ELISA following a protocol described previously (Jordan et al., 1999).

Results and discussion

The three types of ceramic HAP stationary phase investigated in this study were prepared at different sintering temperature (Table 1). With increasing sintering temperature, the original HAP-crystals show a more pronounced degree of melting. As a result with increasing temperature, ceramic structures with increasingly large pore size, better mechanical stability (higher pressure limits), but also somewhat lower surface area (binding capacity) are produced. The P-site/C-site ratio of the surface may also change, which would have different consequences for the respective binding behavior of P-site and C-site interacting substances. This aspect was subsequently investigated taking the isolation of a negatively charged biomacromolecule (plasmid DNA) and a protein with a positive net-charge (antibody) as examples. All three HAP types were compared for the purification of the two biologicals from their typical complex environment, a bacterial lysate in the case of the plasmid DNA and a cell culture supernatant in the case of the antibody.

The chemical stability of the three HAP types is very similar. Apparently, the sintering temperature is of little influence in this context. Prolonged exposure of HAP of any kind to a pH below 5.5 should be avoided, since the material will eventually dissolve and the stability of the column bed usually will deteriorate rapidly. On the other hand, HAP in general is stable up to a pH of 14 (or 2 M NaOH), which

363

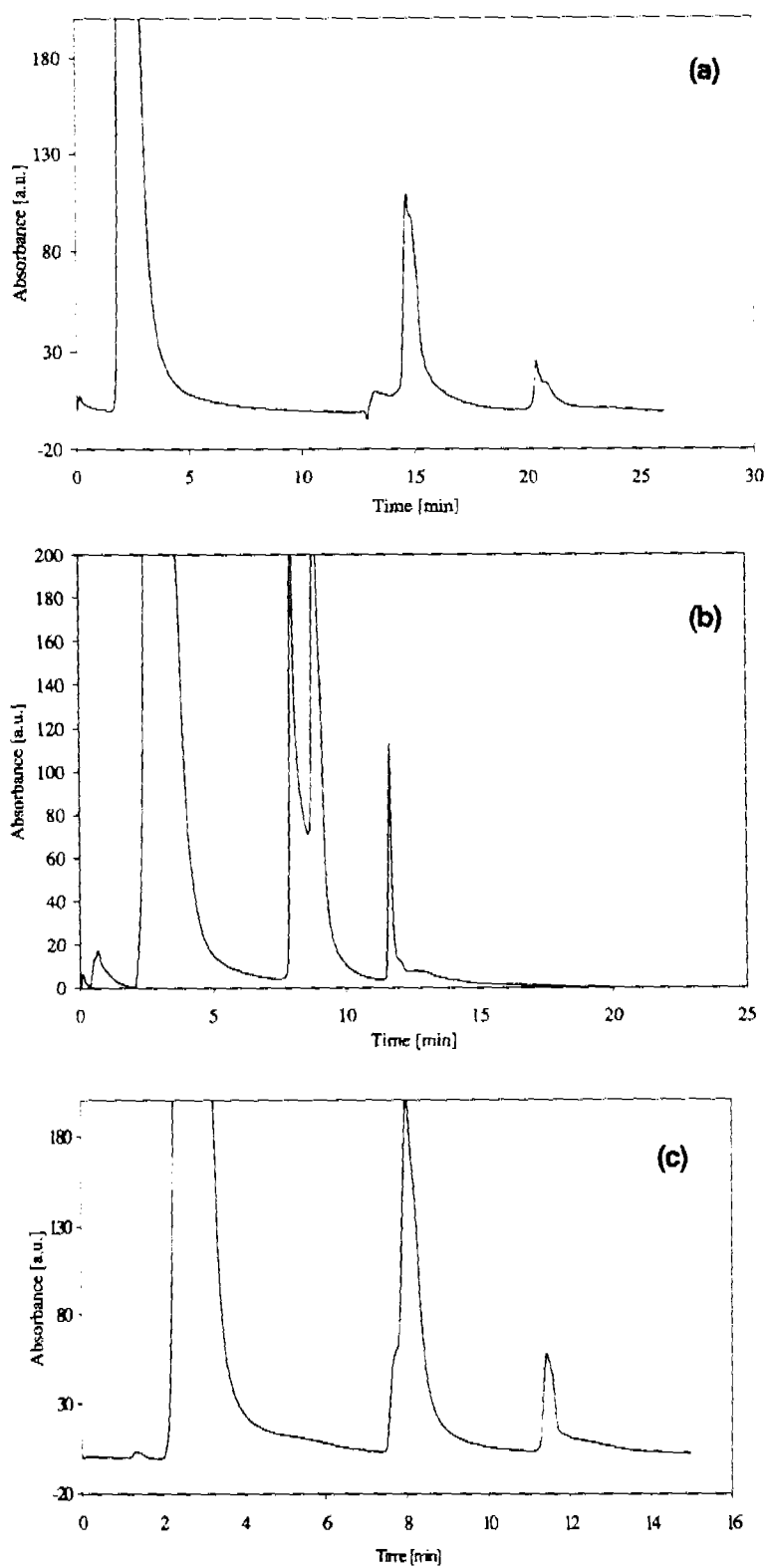


Figure 4.

Figure 4. (a) Purification of pEGFP-N1 from clarified lysate using a HAP Macro-Prep® type I-column: 100 μ l of clarified lysate were injected. Step gradient, from 0 to 10 min, 100% A; from 10 to 18 min, 60% A; from 18 to 25 min, 0% A. Buffer A, 1 mM phosphate, pH 6.2; buffer B, 400 mM phosphate, pH 6.2; flow rate, 1 ml/min. (b) Purification of pEGFP-N1 from clarified lysate using a HAP Macro-Prep® type II-column: 100 μ l of clarified lysate were injected. Step gradient, from 0 to 10 min, 100% A; from 10 to 18 min, 60% A; from 18 to 25 min, 0% A. Buffer A, 1 mM phosphate, pH 6.2; buffer B, 400 mM phosphate, pH 6.2; flow rate, 1 ml/min. (c) Purification of pEGFP-N1 from clarified lysate using a HAP Macro-Prep® type III-column: 100 μ l of clarified lysate were injected. Step gradient, from 0 to 10 min, 100% A; from 10 to 18 min, 60% A; from 18 to 25 min, 0% A. Buffer A, 1 mM phosphate, pH 6.2; buffer B, 400 mM phosphate, pH 6.2; flow rate, 1 ml/min.

facilitates the cleaning and sanitation of the column after exposure to biological matrices. High concentrations of urea (8 M), guanidine/HCl (6 M) and organic solvents such as alcohols or acetonitrile (100%) could also be used with all investigated materials.

Purification of plasmid DNA

Nucleic acids are assumed to bind to HAP exclusively through (very strong) coordinative interactions between the phosphate moieties of the polynucleotide backbone and the C-sites of the HAP-surface. While large polynucleotides tend to bind strongly to HAP, the opposite is usually observed for small oligonucleotides, which do not bind well to HAP. Nucleic acids such as plasmid DNA (pDNA), genomic DNA, or RNA are, on the other hand, easily eluted from the column in a gradient of increasing concentration of a calcium chelating agents, i.e., most often a phosphate buffer of increasing strength. Standard separation methods for (plasmid) DNA purification on HAP call for a binding buffer containing between 1 and 10 mM phosphate and an elution step with up to 500 mM phosphate. A low pH supports the interaction. Given the restriction in the pH-stability of HAP, most of the applications use a buffer pH between 6 and 7 (Deppert and Lukacin, 2000).

Figure 1 shows the purification of plasmid pEAX 39 LH1 (10.3 kb) using a column packed with HAP type I. Five ml of clarified lysate were injected into a column previously equilibrated with a 1 mM phosphate buffer, pH 6.2 ('buffer A'). After 10 min the phosphate concentration was increased to 160 mM. Finally the concentration was raised to 400 mM phosphate ('buffer B') before the column was reequilibrated with pure buffer A. The chromatogram shows three well-spaced peaks after a large flow through frac-

tion, which were subsequently collected and analyzed by agarose gel electrophoresis (Fig. 2). The first peak (corresponding to lane B in the gel), did not contain any nucleic acids but rather some oligonucleotides and the proteinaceous impurities (analytical data not shown). The RNA was eluted in the 160 mM phosphate step (lane C in Fig. 2, corresponding to the peak 2 in Fig. 1), while the pure plasmid DNA eluted at the highest phosphate concentration used in the experiment, i.e., 400 mM (lane D in Fig. 2, corresponding to the peak 3 in Fig. 1). During sample loading and pDNA elution the pressure drop over the column was found to increase significantly.

The comparison between the gel of the HAP purified plasmid pEAX 39 LH1 and a high purity standard plasmid pEAX 39 LH1 obtained by using a Macherey-Nagel kit and protocol (lanes D and E in Fig. 2, respectively), shows that both preparation contained the plasmid in two forms, with the upper of the two bands corresponding to the open circular and the lower one to the supercoiled configuration. The plasmid obtained by HAP chromatography is equally distributed between the relaxed and the supercoiled form while the plasmid obtained through the standard method is mainly supercoiled. The slight differences in migration speed between the two plasmid DNA preparations were mainly due to differences in the sample matrix (salt content). Once the plasmids were linearized, both DNA molecules showed identical migration behavior (Fig. 3).

When the chromatographic experiments were repeated with type II and type III HAP, similar chromatograms were recorded. The nucleic acids bound well to the HAP columns, while the majority of the contaminants including the oligonucleotides were found in the flow through. The RNA was eluted by the 160 mM phosphate step, while the elution of the (pure) plasmid DNA required 400 mM phosphate.

The plasmid DNA binding performance of the three HAP types was subsequently investigated in more detail and especially in regard to yield using the following approach. Ten ml of a clarified lysate containing plasmid pEGFP-N1 (4.7 kb) were processed by a given column using the previously described chromatographic conditions. Lysates were in addition treated with RNase in order to remove the RNA competing for the binding sites and study the binding of the plasmid DNA alone. In this case, i.e., with a smaller plasmid, the increase in pressure drop during sample loading and plasmid elution is less pronounced, showing a clear influence of the plasmid size on viscosity.

Table 2. Summary of the yields obtained for the recovery of the pEGFP-N1 plasmid and of a recombinant human antibody for the three types of HAP investigated. The percentages given correspond to the amount of pDNA in relation to the amount in the original sample, which elutes in 'peak 3', i.e., in the 400 mM phosphate step.

HAP type	Yield (%)	
	pDNA	Antibody
I	73	87-92
II	75	78
III	10	5-10

The RNase eluted in this case in the 160 mM step (data not shown). The peaks corresponding to the flowthrough, the first elution step (160 mM), and the second elution step (400 mM) were subsequently analyzed with the Pico-green assay for DNA contents. Yields are taken as the ratio between the pDNA eluted during the second elution step and the total amount of pDNA present in the sample:

$$Y\% = 100 \times \frac{C_{peak}[\mu\text{g/ml}] \times V_{peak}[\text{ml}]}{C_{feed}[\mu\text{g/ml}] \times V_{feed}[\text{ml}]}$$

where C_{peak} is the eluted peak concentration, V_{peak} the peak volume, C_{feed} the feed concentration and V_{feed} the injected volume.

Figure 4a – 4c and Table 2 summarize the results obtained for the different HAP materials. In case of HAP type I, a yield of 73% was found. 10% of the pEGFP N1 eluted in the first elution peak (160 mM) and 15% were not recovered. Yields were comparable in case of HAP type II but decreased considerably for HAP type III, where values of 10% were found, Table 2. In the case of the type III material, 85% of the pDNA were eluted in the first elution peak (160 mM) together with the RNase. In the chromatogram obtained for the lysate on the type III (Fig. 4c) and especially the type II (Fig. 4b) HAP column the 160 mM fraction is better resolved than in case of the type I material (two peaks for type II, a shoulder for type III). This behavior is reproducibly observed and taken as a first hint towards difference in the chromatographic properties of the HAP surface produced at different sintering temperatures, i.e. most likely the C/P-site ratio.

Purification of proteins (recombinant antibody)

While the interaction of polynucleotides with HAP columns is comparatively straightforward due to the

presence of only negative charges on the biopolymer, the situation becomes more complex in the case of proteins. In spite of a negative or positive net-charge, proteins will almost always bear both negatively and positively charged groups on their surface. It is therefore an oversimplification to expect negatively charged ('acidic') proteins to interact with the C-sites and positively charged ('basic') proteins to interact exclusively with the P-sites. The type I material is nevertheless often considered the best choice among the different HAP types for the purification of proteins, and especially basic proteins. However, the situation may be more complex in the case of glycosylated, basic proteins such as antibodies.

In this study, all three HAP types available were investigated for the purification of a recombinant antibody against the Rhesus D antigen. The antibody in question belongs into the immunoglobulin subclass IgG₁-κ. A molecular mass of 150 000 Da and an isoelectric point of 8.3 were determined by MALDI-MS and isoelectric focussing respectively. When a general buffer screening was performed, the antibody was found to bind to HAP even in the presence of 1 M NaCl. This is unusual for a basic protein, where normally 1 M NaCl is sufficient for elution by charge screening. The antibody could subsequently be eluted in a simple gradient from a 10 mM phosphate buffer containing 1 M NaCl (buffer A, pH 6) to a 400 mM phosphate buffer without NaCl (buffer B, pH 6).

For a comparison of the binding behavior of the antibody to the different HAP types, 10 ml of a diluted (1:3 in binding buffer A) cell culture supernatant of recombinant CHO cells were injected into a given HAP-column, preequilibrated with the binding buffer A. The antibody was eluted by a 20 min linear gradient from 100% buffer A to 85% buffer A. The elution step was followed by a regeneration step using 100% buffer B. The resulting chromatograms for all HAP types are depicted in Figs. 5a – c. The yields were determined by ELISA as published previously (Jordan et al., 1999). In case of HAP type I, yields were consistently between 87 and 92%. In case of HAP type II this value dropped to 78%, while almost no antibody retention was observed in case of HAP type III material (5 – 10%). In this case up to 95% of the antibody were instead recovered in the flowthrough fraction, i.e. in this case almost no binding took place.