ELSEVIER

Journal of Chromatography A, 752 (1996) 111–122

JOURNAL OF CHROMATOGRAPHY A

Isolation of monoclonal antibodies from cell containing hybridoma broth using a protein A coated adsorbent in expanded beds

Jörg Thömmes*, Andrea Bader, Markus Halfar, Andreas Karau, Maria-Regina Kula

Institut für Enzymtechnologie, Heinrich-Heine Universität Düsseldorf, P.O. Box 2050, 52404 Jülich, Germany

Received 22 March 1996; revised 24 May 1996; accepted 24 May 1996

Abstract

A novel expanded bed adsorbent carrying a recombinant protein A ligand was used for the isolation of monoclonal antibodies from cell containing hybridoma fermentation broth. The untreated effluent from a continuous hybridoma cultivation was applied to the stable expanded adsorbent (Streamline rProteinA), which proved to have a high capacity for the MAb studied (14 mg MAb per ml of adsorbent). A clarified and highly concentrated (up to 50 fold) eluate of high purity was obtained. A scale up of the MAb purification is demonstrated from lab scale (250 mg MAb per purification cycle) to a small pilot scale (2 g MAb per cycle). Low product concentration in the broth in combination with the high capacity of the adsorbent caused long sample application cycles (10–11 h). Experimental problems arising from these long cycle times are discussed with regard to a large scale application of the method.

Keywords: Immunoadsorbents; Fluidized bed adsorption; Expanded bed adsorption; Protein A; Monoclonal antibodies

1. Introduction

The purification of protein products from mammalian cell culture processes is usually performed starting from a very dilute broth containing suspended particles. Therefore the initial step of the downstream process should deliver a particle free solution which can be purified by chromatographic methods. Clarification of culture broths may be performed by centrifugation or filtration methods and although not without problems both methods have been applied successfully in numerous cases for the clarification of mammalian cell broths [1,2]. A point of concern, however, is the fact that these initial steps are only the beginning of a long sequence of downstream operations which all contribute to the overall time requirements for the product purification and which all have their inherent product losses. Therefore the overall cost of the downstream process, which has been described to contribute up to 80% to the total production costs [3–5], is closely correlated with the number of steps involved. Reducing the number of sequential operations by combination of originally independent unit operations can significantly contribute to an increased performance of the whole sequence. A second point of importance is the velocity of processing of the original culture broth considering the stability of the product. Sensitive molecules may be subject to secondary processing by proteases or glycosidases, which are not removed from the product during the initial clarification step. Chemical modification by oxidation or deamidation is a time and concentration dependent process which also is not prevented by centrifugation

*Corresponding author.

0021-9673/96/$15.00
PII S0021-9673(96)00504-3

and micro- or ultrafiltration. Therefore a reduced number of unit operations may result in an increased yield of active product of consistent quality. These considerations are especially important for pharmaceutical proteins suffering from a loss of biological activity due to secondary processing [6]. As an alternative to the standard procedure, purification technologies which tolerate the presence of particles may be used. Thus the clarification step is combined with an initial product capture and concentration thus minimising the process time. One of the alternative strategies is the use of fluidised adsorbents for product isolation from cell containing broths [7]. The increased particle distance in a fluidised bed permits processing of particle containing solutions without the risk of blocking the bed. Choosing adsorbent particles of specific weight larger than that of the solid material present in the broth combines clarification by solid–solid separation with product capture directly from the culture broth. After elution from the adsorbent a clarified and initially purified product concentrate is obtained. Using classified fluidised beds with reduced axial mixing, capacity and resolution of the purification can be increased in the sense of a chromatographic separation [8–12]. To distinguish well mixed fluidised beds for protein adsorption from the use of classified beds, the latter are called expanded beds. Since the early use of well mixed fluidised bed adsorption in the purification of antibiotics [13], the isolation of proteins from cell containing broth has been shown using this technology in many cases. Together with the increasing number of applications the theoretical background regarding the major influences on the performance of fluidised/expanded bed adsorption is also improved [14–16].

The demand for monoclonal antibodies (MAb) is steadily increasing due to their large potential in therapeutic and diagnostic applications. Therefore monoclonals are a very good example of a bulk product, which has to be purified efficiently in order to make its production economically competitive. In a few publications the purification of MAbs from whole Hybridoma broth has been described using fluidised or expanded adsorbents. Ericsson et al. employed protein A coated glass spheres in a well mixed fluidised bed [17] and Batt et al. demonstrated the usefulness of a commercial expanded bed cation-exchanger (Streamline SP) for the purification of a recombinant antibody from CHO cells [18]. In an earlier work we compared glass based and commercial agarose based cation-exchangers (Streamline SP) for the isolation of IgG2a [15,19,20]. Starting from cell containing hybridoma culture broth we found a fourfold purification and a fourfold concentration of an anti-EGFR IgG2a and a fairly low capacity between 160 and 2000 μg MAb per ml of adsorbent depending on the hydrodynamics in the expanded beds. The reduced capacity was due to the fact that we performed the adsorption from undiluted culture broth (conductivity 13 mS/cm) at pH 5.5. Under these conditions the capacity was similar to a standard fixed bed procedure with undiluted culture supernatant. The clarification was very efficient in the expanded bed mode, so the eluates could be transferred to chromatographic fine purification in packed columns without further treatment. This procedure was successfully applied in an integrated purification where a fluidised bed production of IgG2a was directly coupled to an expanded bed process for product capture employing the cation-exchange procedure described above. A clarified, concentrated, and initially purified product solution was obtained from this coupled production–purification process [21].

Irrespective of the success of the MAb capture from cell containing broth the results were only partially satisfying. The major drawback was the low capacity obtained, a result from the isoelectric point of the MAb and the high conductivity of the original medium. A more salt tolerant purification step was needed to obtain a higher capacity of the adsorbent employed. As protein A ligands have been used successfully in a multitude of IgG purifications, protein A coated expanded bed adsorbents seemed to be well suited for antibody purification from cell containing broth. Pharmacia BioTech (Uppsala, Sweden) supplied us with a developmental sample of a protein A coated agarose for expanded bed adsorption (Streamline rProteinA). Contrary to the commercial Streamline ion-exchangers containing crystalline quartz the specific weight of the agarose adsorbents was increased by incorporating stainless steel particles. The characteristics of the classified fluidisation were retained.

In the studies described below we evaluated the

IgG purification from untreated culture broth with regard to operating conditions, capacity, selectivity, and clarification efficiency of the new adsorbent. Starting from a very small scale, a 10-fold scale-up of the purification was performed in order to show that adsorption on protein A coated expanded bed matrices provides a valuable method of purification of bulk amounts of monoclonal antibodies in the future.

2. Experimental

2.1. Equipment and biological system

The monoclonal antibody used belongs to the IgG2a subclass and is produced in continuous culture using immobilised hybridoma cells as described elsewhere [22].

Four different columns were employed: The small scale fixed bed experiments were performed in a CC 10 column (Pharmacia Biotech, Uppsala, Sweden) of 1 cm diameter using 1 ml of packed adsorbent. Expanded bed studies were conducted on three scales: 20 ml of matrix were used in a XK 16/40 column (Pharmacia Biotech) with 1.6 cm diameter. To improve flow distribution the column inlet was modified: A 1-cm bed of non-porous glass ballotini (3 mm diameter) was packed in the inlet zone of the column, the upper end of the distributor bed was sealed with a steel net of 60 μm width which ensured that no adsorbent particles entered the distribution zone but allowed cellular material to pass in an upward direction of flow. For scale-up two commercially available columns for expanded bed adsorption were used, the Streamline 25 (SL 25) and the Streamline 50 column (SL 50, Pharmacia Biotech). Flow distribution in these columns is optimised by a special distributor plate at the bottom end of the column which is covered by a 70-μm steel net. The details of the expanded bed columns used as well as the respective operating conditions are provided in Table 1.

The adsorbent used (Streamline rProteinA) consisted of a composite material from crosslinked agarose and steel beads and had a particle size distribution of 80–165 μm (130 μm average particle size). The mean particle density was 1400 kg/m^3.

2.2. Hydrodynamic studies

2.2.1. Fluidisation behaviour

Settled Streamline rProteinA matrix (12 ml) was packed into the modified XK 16/40 column. Fluidisation was performed in a recycled mode using deionised water or cell containing hybridoma fermentation broth ($1 \cdot 10^6$ cells per ml) at pH 7.0. The liquid flow-rate was varied from 2 to 14.1 ml/min representing linear flow-rates U from $1.7 \cdot 10^{-4}$ to $11.7 \cdot 10^{-4}$ m/s. The results were plotted as $\ln U$ versus $\ln \varepsilon$ according to the Richardson–Zaki relationship [9,14,18,19].

$$U = U_t \cdot \epsilon^n \tag{1}$$

Linear regression of the double logarithmic plot yielded the terminal settling velocity U_t and the Richardson–Zaki parameter n. The voidage of the bed ϵ was calculated according to Eq. (2) ($\epsilon_0 = 0.4$):

$$\epsilon = 1 - (1 - \epsilon_0) \cdot \frac{H}{H_0} \tag{2}$$

where H and H_0 denote the expanded and settled bed height, respectively.

2.2.2. Residence time distributions

Residence time distributions were performed in the modified XK 16/40 column. Settled Streamline

Table 1
Columns for expanded bed adsorption used in the study

Column	Cross sectional area	Amount of adsorbent used	Sample size	Flow-rate
XK 16/40	2 cm^2	20 ml	5 l	10–14 ml/min
Streamline 25	5 cm^2	50 ml	20 l	30–35 ml/min
Streamline 50	20 cm^2	150 ml	60 l	120–140 ml/min

adsorbent (12 ml) was filled into the column and were fluidised using 50 m*M* potassium phosphate buffer pH 7.0 at various flow-rates. After bed stabilisation 0.1 ml of a tracer solution (4 g/l dextrane blue in deionised water) was injected as a pulse and the output signal was detected using the UV adsorption at 280 nm in a flow through cell. The data were transferred to a personal computer and processed for the residence time distribution. The moments of the RTD (mean residence time τ and standard deviation σ^2) were determined and analysed according to the diffusion model as well as the tanks in series model [23]. The whole reactor was defined as a closed system and the Bodenstein number *Bo* was calculated according to Eq. (3):

$$\sigma_{\Theta}^2 = \frac{2}{Bo} - \frac{2}{Bo^2} \cdot (1 - \exp(-Bo)) \quad (3)$$

The particle Peclet number *Pe* was calculated according to Eq. (4):

$$Pe = \frac{U \cdot d_p}{D_{ax}} \quad (4)$$

with the linear velocity U, the particle diameter d_p, and the coefficient of axial dispersion D_{ax}, which is obtained from the Bodenstein number according to Eq. (5) (H = expanded bed height).

$$D_{ax} = \frac{U \cdot H}{Bo} \quad (5)$$

2.3. Chromatographic procedure

2.3.1. Frontal analysis in packed bed

A CC 10 column was packed with 1 ml of Streamline rProteinA adsorbent and was equilibrated with 50 m*M* potassium phosphate buffer at different pH values. Hybridoma fermentation broth was clarified by centrifugation (10 000 *g*) and microfiltration (0.45 μm). A 500-ml volume of the particle free solution was adjusted to the pH of the equilibration buffer and applied to the column at 1 cm/min. After sample application the column was washed with equilibration buffer until no protein could be detected in the effluent. The antibody bound was eluted with 100 m*M* sodium citrate buffer at different pH values. Adsorbent regeneration was performed using 2 column volumes of 2 *M* urea and 2 column volumes of 1 *M* acetic acid. Based on the amount of antibody eluted under the different conditions the optimum binding and eluting conditions were chosen.

2.3.2. Frontal analysis in an expanded bed

The principle of fluidised/expanded bed adsorption and the respective experimental details has been summarised in previous publications [9,10,12,19]. Therefore only the specific details of the protocol with the protein A material will be described. Depending on the column chosen a defined amount of Streamline rProteinA adsorbent was filled into the column and equilibrated in an upward flow with 50 m*M* potassium phosphate buffer pH 7.0 at defined linear flow-rates. The equilibration was performed with an upward direction of flow until the fluidised bed appeared optically stable, at least with 10 expanded bed volumes. Subsequently the sample was adjusted to pH 7.0, applied at identical linear flow-rate, and the effluent was collected in fractions. After sample application the column was washed with equilibration buffer until no protein and no particles could be detected in the effluent. The antibody bound was eluted with 100 m*M* sodium citrate buffer at pH 4.0. The eluate was collected in a vessel containing 1 *M* Tris–HCl, pH 8.0 for direct neutralisation. For elution the bed was allowed to settle and elution was performed in a fixed bed mode at 1.5 cm/min linear flow-rate. Alternatively the antibody was eluted in an expanded mode at the same linear flow-rate as during sample application. Regeneration of the matrix was performed as in the fixed bed experiments.

2.4. Analyses

Protein content was measured by the method of Bradford [24], the antibody content was analysed by a sandwich ELISA technique as described by Ray et al. [25]. SDS-PAGE was performed under reducing conditions according to standard protocols, the protein bands were visualised by silver staining.

Particle size and concentration analysis was performed using a Coulter Counter (Coulter Electronics, Krefeld, Germany). Fractions to be analysed were diluted with isotonic solution (Isoton II, Coulter Electronics) to a total particle content of max. $6 \cdot 10^4$/ml and treated according to the standard protocol of

the manufacturer. A sample volume of 0.5 ml was used per count employing a tube with 140 μm orifice diameter.

3. Results and discussion

3.1. Fluidisation studies

Fluidisation studies were performed with deionised water and cell containing hybridoma fermentation broth ($1 \cdot 10^6$ cells/ml). As can be taken from Fig. 1, fluidisation characteristics did not vary significantly between the two liquid media. Determination of the coefficients of fluidisation after Richardson and Zaki by linear regression of the ln ϵ vs. ln U plot shows a standard picture. Due to the similar physicochemical properties (viscosity and density of hybridoma fermentation broths at $1 \cdot 10^6$ cells per ml are only slightly higher than the values observed with pure water) the terminal settling velocity remained unchanged within the error of measurement. The dependency of the Richardson–Zaki exponent n from viscosity and density of the liquid medium still is unclear. In the present study a small increase of n was determined. Table 2 summarises the results obtained.

Table 2
Results of the linear regression of the Richardson–Zaki plot

	Water	Cell culture broth ($1 \cdot 10^6$ cells/ml)
Richardson–Zaki coefficient n	3.4	3.95
Terminal settling velocity U_t (m/min)	0.165	0.183

3.2. Residence time distribution (RTD)

Experiments describing the residence time distribution in stable expanded beds with the Streamline rProteinA material were performed at different linear flow-rates in the modified XK 16/40 column. The results indicated, that a certain minimum linear flow-rate is required for the development of the classified fluidised bed as desired for an adsorption process with a low degree of axial mixing in the solid and liquid phase. At lower flow-rates such as $5.7 \cdot 10^{-4}$ m/s and $7.6 \cdot 10^{-4}$ m/s axial mixing was increased as can be seen from the low Bodenstein and particle

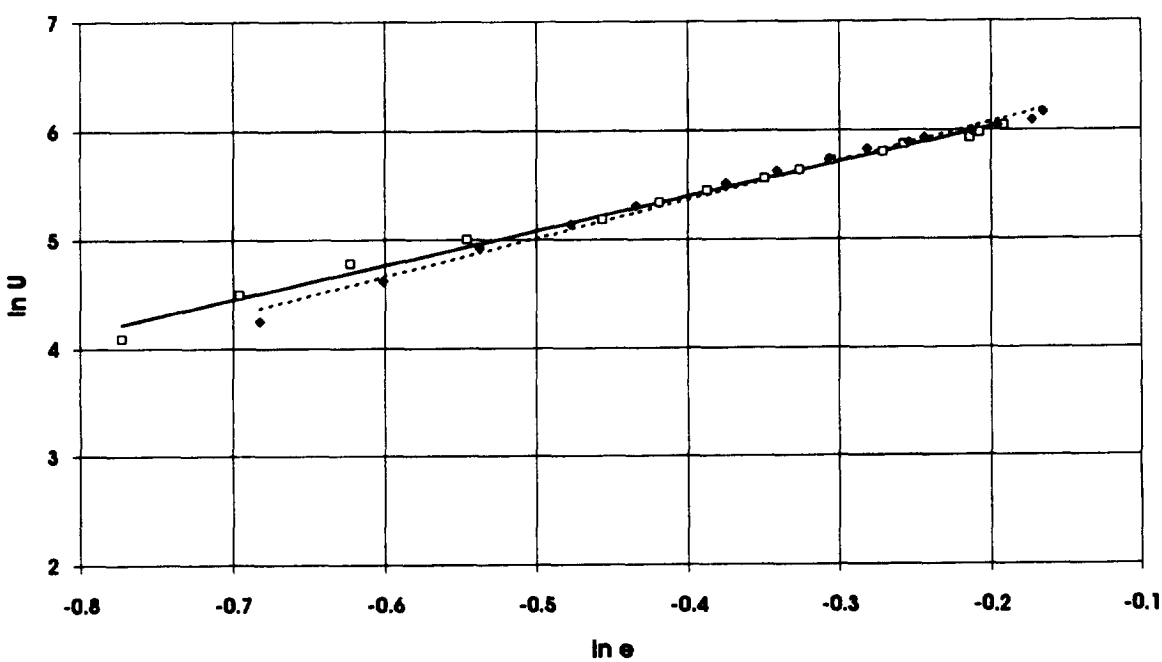

Fig. 1. Richardson–Zaki plot of Streamline rProteinA fluidised in the modified XK 16/40 column in deionised water (◆) and cell containing hybridoma culture broth ($1 \cdot 10^6$ cells /ml □).

Peclet numbers. Comparing the values obtained at $10.4 \cdot 10^{-4}$ m/s to literature data obtained with cation-exchange Streamline adsorbents we find the same order of magnitude of axial mixing (D_{ax} of $6.4 \cdot 10^{-6}$ m^2/s compared to $2 \cdot 10^{-6}$ m/s as reported by Batt et al. [18] and $8–9 \cdot 10^{-6}$ m^2/s as found by Lindgren et al. [26]). Table 3 summarises our results from the RTD measurements. To ensure that liquid flow in the modified XK 16/40 column was comparable to commercial columns we additionally conducted RTD measurements with commercial Streamline SP material in the column. Under standard conditions (linear flow-rate $8.3 \cdot 10^{-4}$ m/s, 20 ml of Streamline SP) we found Bodenstein numbers of 50 corresponding to coefficients of axial mixing of $3.3 \cdot 10^{-6}$ m^2/s, which fits literature data (see above) and values obtained in the commercially available expanded bed columns in our laboratory (SL 25 and SL 50, data not shown). This allows the use of the column for evaluation studies reducing the amounts of adsorbent and especially the amounts of sample needed for the experiments. The data on capacity and resolution can be well compared to data obtainable with the larger columns, so a scale up can be planned based on the XK 16/40 experiments.

3.3. Optimisation of binding and elution conditions

Prior to adsorption of IgG2a from whole broth to expanded protein A adsorbents the binding and elution conditions were optimised by varying the pH of the equilibrating buffer and of the sample as well as the pH during the elution cycle. During these experiments a packed bed of adsorbent was completely overloaded with IgG2a from clarified hybridoma fermentation broth and the percentage of challenge eluted was taken as a relative measure for the success of the purification. Table 4 demonstrates that binding at a pH of 7.0 and elution at pH 4.0 were the optimum conditions for the IgG2a used in this study. The conditions found here were identical to the optimum conditions obtained with this antibody for standard packed bed protein A adsorbents (protein A Sepharose FastFlow, Pharmacia Biotech), so experiences already existing from packed bed experiments with standard matrices could be transferred to the expanded bed procedure.

Table 3
Results of the RTD measurements with Streamline rProteinA (mean values from three repetitive measurements)

Flow (m/s)	*Bo*	D_{ax} (m^2/s)	*Pe*
$10.4 \cdot 10^{-4}$	33	$6.4 \cdot 10^{-6}$	0.024
$7.9 \cdot 10^{-4}$	15	$9.2 \cdot 10^{-6}$	0.012
$5.7 \cdot 10^{-4}$	11	$7.4 \cdot 10^{-6}$	0.011

Table 4
Optimisation of binding and elution conditions in a packed bed

pH of equilibration buffer and sample	% eluted at pH 4.0[a]
6.0	44
6.5	62
7.0	71
7.5	54
pH of elution buffer (sample applied at pH 7.0)	**% eluted[a]**
3	44
4	71
5	49

1 ml of packed adsorbent was overloaded with 500 ml of clarified hybridoma culture broth (50 mg MAb/l).
[a] The total amount of MAb applied to the column (500 ml of supernatant at 50 mg/l) is defined as 100%.

3.4. Expanded bed adsorption

After characterising the hydrodynamic behaviour of the fluidised adsorbent and defining the process conditions for IgG2a purification, expanded bed adsorption was conducted with real culture broth.

In initial experiments 20 ml of Streamline rProteinA were expanded in the XK 16/40 column under the optimised adsorption conditions as described above. Both 5- and 4.2-l volumes of cell free hybridoma supernatant were applied in an expanded mode at 6 cm/min linear flow-rate. The MAb concentration in the feed was 59.3 mg/l, so the total challenge applied was 14.8 and 12.5 mg/ml of matrix, respectively. In the two independent experiments no IgG2a could be detected in the flow-through fractions, 98 and 105% of the total challenge could be eluted. From these data capacities of 14.5 and 13.2 mg IgG2a per ml of adsorbent could be calculated. The MAb concentrations in the eluate

Table 5
Results from the experiments with the modified XK 16/40 column

Experiment No.	MAb feed concentration (mg/l)	Sample volume (l)	Challenge (mg MAb/ml matrix)	MAb eluate concentration (mg/l)	Yield (%)	mg MAb purified per cycle
1	59.3	5	14.8	2591	98	290
2	59.3	4.2	12.5	3288	105	263
3	15.2	5	3.8	1216	102	78
4	15.2	5	3.8	1179	108	82
5	18.9	10	8.8	2500	126	222
6	13.8	10	6.9	1200	87	120

Experiments No. 1 and 2 were conducted with cell free supernatant, in experiments 3–6 hybridoma cells at an average concentration of $1 \cdot 10^6$ cells/ml were present.

were high, the concentration factors achieved were 44 and 56.

In further experiments cell containing broth (average cell concentration $1 \cdot 10^6$ cells/ml) was applied to 20 ml of adsorbent under otherwise identical conditions. Due to different MAb concentrations in the feed, the challenges were different from the experiments with cell free supernatant. In four experiments no Mab could be detected in the flow-through fractions and the antibody could be eluted with very high yields. Table 5 summarises the data obtained with the XK 16/40 column, the variations in yield are an expression of the experimental error of the ELISA method employed for MAb detection rather than variations in the specificity of the adsorption process.

As a first step of the scale up the Streamline 25 column was charged with 50 ml of adsorbent. To apply challenges comparable to the experiments on the small scale we had to use a sample size of 20 l cell containing culture broth. The results of three experiments are summarised in Table 6. We applied samples of similar concentration at two different linear flow-rates ($10 \cdot 10^{-4}$ m/s and $11.7 \cdot 10^{-4}$ m/s). The results are plotted in Fig. 2 as flow-through concentration versus effluent volume. As had to be expected for an adsorption process, which is residence time controlled at high linear flow [15,16], an increase in linear flow-rate results in an earlier antibody breakthrough and therefore in a lower useful capacity of the matrix. The amount of MAb eluted as well as the eluate concentration was comparable to the experiments on a small scale (eluted amount of antibody 13.3, 16, and 16.9 mg/ml of adsorbent) thus confirming the successful operation at increased scale. The varying eluate concentrations reported are caused by different modes of elution. In experiment number one elution was performed in an expanded mode of operation at the same linear flow-rate as during sample application,

Table 6
Results of the MAb adsorption on increased scale: Experiments No. 1–3 were conducted in the SL 25 column, experiments 4 and 5 in the SL 50 column

Experiment No.	MAb feed concentration (mg/l)	Linear flow rate (10^{-4} m/s)	Challenge (mg/ml matrix)	MAb eluate concentration (mg/l)	Yield (%)	mg MAb purified per cycle
1	34	10	13.6	1199	95	646
2	50	10	20	2084	78	780
3	50	11.7	20	2509	84	840
4	40	10	16	1197	83	1992
5	29	10	11.6	962	82	1427

The elution was performed in an expanded mode of operation in experiments No. 1, 4, and 5 at $10 \cdot 10^{-4}$ m/s and in experiments No. 2 and 3 in a packed bed mode at $2.5 \cdot 10^{-4}$ m/s.

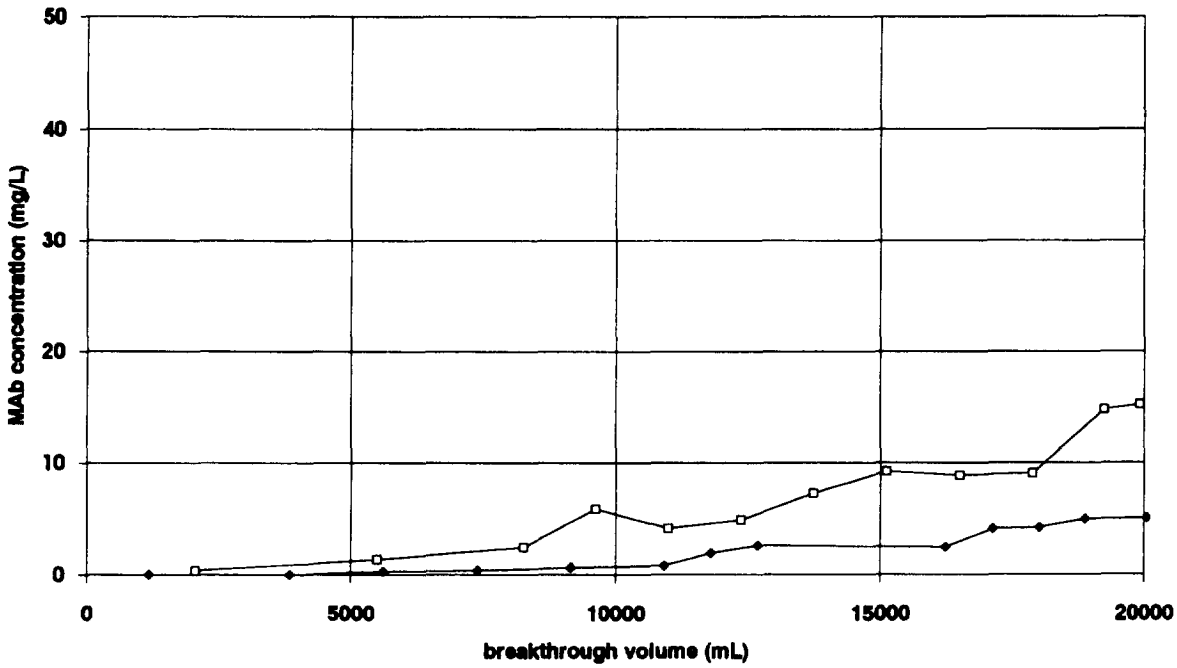

Fig. 2. Breakthrough of IgG2a on Streamline rProteinA in the SL 25 column (50 ml of adsorbent), linear flow rate $10 \cdot 10^{-4}$ m/s (◆) and $11.7 \cdot 10^{-4}$ m/s (□).

whereas in the subsequent experiments (2 and 3, compare Table 6) the standard mode of elution in a packed bed was chosen. Due to the increased void volume of the expanded bed this resulted in a higher dilution of the eluate in experiment No. 1. As can be taken from the residence time distributions the liquid flow in the expanded bed is close to plug flow as it is the case in a packed bed, so no additional dilution of the peak as a consequence of increased dispersion during expanded bed elution was to be expected. An advantage of an expanded bed elution is the fact that the top adapter can remain in its original position without the need of downward movement to the top of the settled bed. This may be a beneficial effect as moving parts may present an operational risk which can thus be eliminated.

In the next step of the scale up 150 ml of adsorbent were placed in the Streamline 50 column. Again the antibody challenge should be similar to the small scale experiments, so 60 l of cell-containing broth were applied to the adsorbent. The results are shown in Table 6. In these runs elution was performed in an expanded mode of operation as well, so the reduced antibody concentration in the eluate can be explained accordingly. The yield was comparable to the experiments with the Streamline 25 column confirming the successful increase in scale of the adsorption process (amount of IgG2a eluted 13.1 and 9.51 mg/ml of adsorbent).

Summarising the scale-up experiments during this study Fig. 3 depicts the overall yield obtained with the different column configurations in relation to the MAb concentration in the feed and the challenge applied per ml of matrix.

3.5. Experimental problems

Using an affinity matrix for direct capture of monoclonals from cell containing broth solves the problem of the low capacity of an ion-exchange step at high salt concentration in the broth. Comparing the capacity of Streamline SP ion-exchange resin for the IgG2a from whole broth (160 μg/ml of matrix from undiluted broth [19]) shows nearly a 100 fold increase in capacity. This significant improvement allows the application of very large samples to small

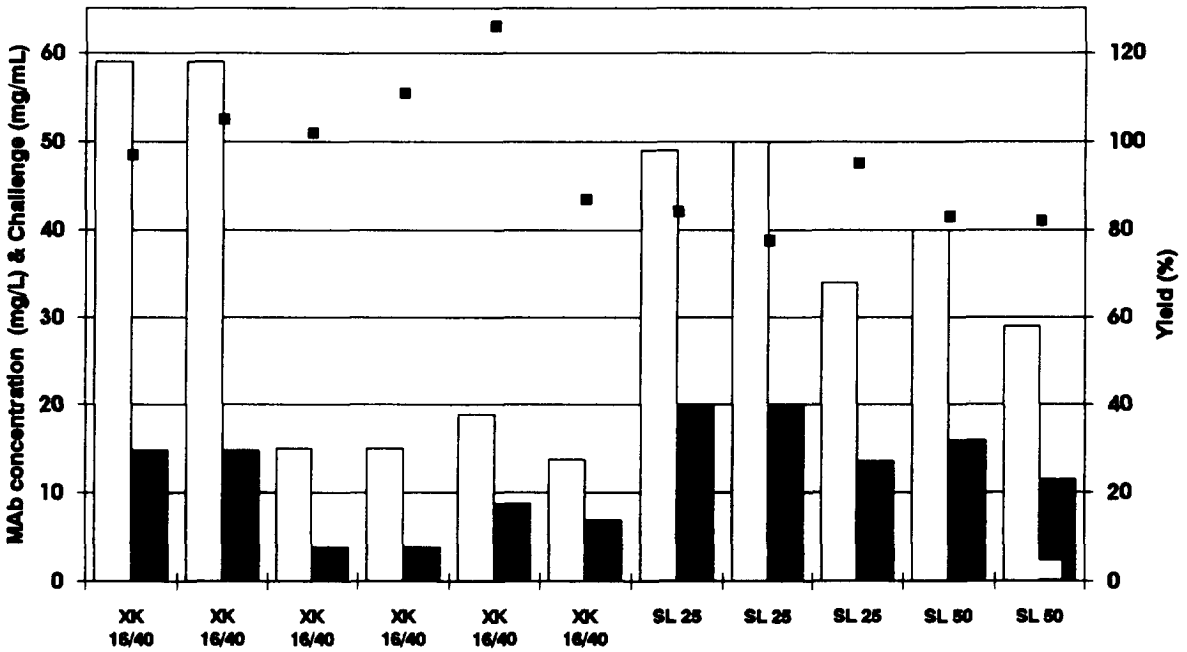

Fig. 3. Comparison of the performance of Streamline rProteinA during increase in scale from 20 to 150 ml of matrix (Challenge: black bars; MAb-concentration: white bars; Yield: black squares).

columns. In our case 60 l of sample were applied to 150 ml of adsorbent in a 5 cm diameter column. Linear flow-rates of 6 cm/min allowed processing of 120 ml of sample per minute, so the total time of sample application was 8.3 h in this column. During this time several experimental problems may occur which mostly are caused by the long storage time of cell containing broth. Long storage of mammalian cells allows partial settling of the cells in the feed tank, which may lead to substantial formation of cell aggregates. If these aggregates enter the expanded bed column they may clog the net at the column inlet and cause a flow maldistribution and thus an unevenly expanded bed with reduced chromatographic performance. Even worse, they may settle at the bottom of the expanded bed in a dead zone at the column inlet and may serve as a focal point of the formation of larger clogs. In the worst case they may lead to the complete breakdown of the expanded bed due to severe channelling or the formation of aggregates from cells, cell debris, and adsorbent particles. To avoid these consequences, the feed vessel was carefully stirred to prevent the formation of initial aggregates. Alternatively the cells may be allowed to settle completely before sample application as described by Batt et al. [18]. In this case the total cell load of the sample is significantly reduced. As expanded bed adsorption is meant to be a fast method of direct recovery from cell containing broth a gravity settling overnight is in contrast to the fast product recovery intended in order to minimise secondary processing of the protein product. Therefore it is strongly recommended to avoid dead zones at the column inlet where initial cell/adsorbent aggregates are formed. This may be achieved by a careful control of the classified fluidisation of the bed with a homogeneous flow distribution at the column inlet. Secondly a steady flow of process liquid at constant linear flow-rate has to be provided, using a flow control system is advisable. A thorough cleaning in place step is strongly recommended after passage of a large amount of cell culture broth to ensure that cell debris or denatured protein, which may have been adsorbed to the matrix, does not initiate aggregate formation in subsequent cycles. In the experiments performed in this study five column volumes of 2 *M* urea followed by five column volumes of 1 *M* acetic acid were used.

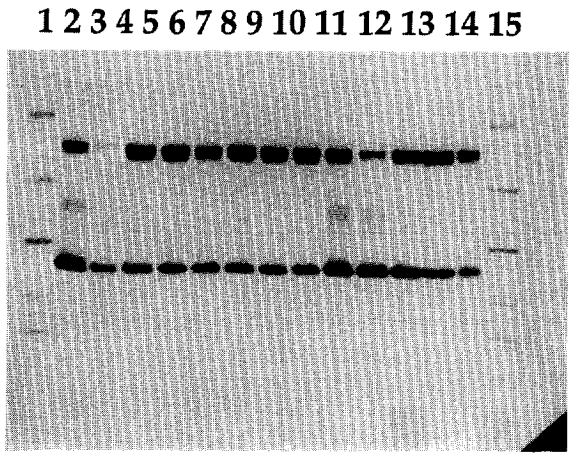

Fig. 4. SDS-PAGE (silver stained, reducing conditions) of different eluates from IgG2a purification. Lane 1: Molecular mass standards of $14.4 \cdot 10^3$, $26.6 \cdot 10^3$, $39.2 \cdot 10^3$, $66.2 \cdot 10^3$, $97.4 \cdot 10^3$. Lane 2: Eluate from protein A Sepharose Fast Flow. Lanes 3,4,6–9: Eluates from fixed bed adsorption on Streamline rProteinA. Lane 5: Eluate from expanded bed adsorption in the SL 25 column (cell containing broth). Lanes 10–12: Eluates from expanded bed adsorption in the XK 16/40 column (cell free supernatant). Lanes 13 and 14: Eluates from expanded bed adsorption in the XK 16/40 column (cell containing broth). Lane 15: Standards.

3.6. Purity of the eluted fractions

The purity of the eluted fractions was examined by SDS PAGE. Fig. 4 shows a silver stained gel of eluted fractions from different experiments: In lane 2 IgG2a standard purified using a commercial affinity matrix (protein A Sepharose Fast Flow) is shown as a reference. Lane 3, 4 and 6–9 show purifications starting from cell free supernatant using the Streamline rProteinA matrix in a small fixed bed. Lanes 10–12 show the eluates from expanded bed adsorption conducted in the modified XK 16/40 column starting from cell free supernatant. Lanes 13 and 14 show results from a purification in the same column starting from cell containing broth and in lane 6 the eluate from one of the experiments in the SL 25 column is shown, where cell containing broth had been applied as well. As can be seen clearly from this gel, all eluates contained the IgG2a in very high purity. No difference can be detected between the results from cell free or cell containing broth and the results are equal to a standard affinity matrix, which has long been recognised to allow a very selective adsorption of IgG.

3.7. Analysis of particle content of the fractions collected

As expanded bed adsorption is an integrative technology combining clarification, concentration, and chromatographic purification in a single operation, we were interested in the performance of this technology in the clarification of particle containing solutions. Therefore fractions of the sample applied, the washing step and the final eluate were analysed for particle content by measurements with a Coulter Counter. The results are shown in Fig. 5. Comparing the particle concentration of the sample to the concentration in the flow-through fractions shows, that after an initial retardation all the particles contained in the feed leave the column with the flow-through, thus confirming that solid–solid separation is possible by choosing expanded beds for the processing of cell containing solutions. At this point it must be noted, that the 140-μm orifice employed in the Coulter Counter measurements has a detection limit of 1.5 μm. All particles smaller than this size were not detected by the method. A severe retardation of cell debris smaller than 1.5 μm may have happened without coming to our notice. This problem is in the focus of ongoing research in our group. The total particle concentration in the eluate was only 0.8% of the concentration in the sample, so a more than 100 fold clarification was achieved in addition to the very high purity of the protein in the eluate and the significant volume reduction.

4. Conclusions

In these experiments we have demonstrated that it is possible to increase the selectivity of a first integrated downstream operation by using an affinity ligand in an expanded bed adsorption. Employing a new adsorbent with a protein A ligand coupled to a weighted agarose matrix the isolation of IgG2a of very high purity was achieved. The dynamic capacity of the matrix reached 9 to 16 mg/ml of matrix depending on the MAb concentration in the feed and the linear flow-rate employed. Employing a standard

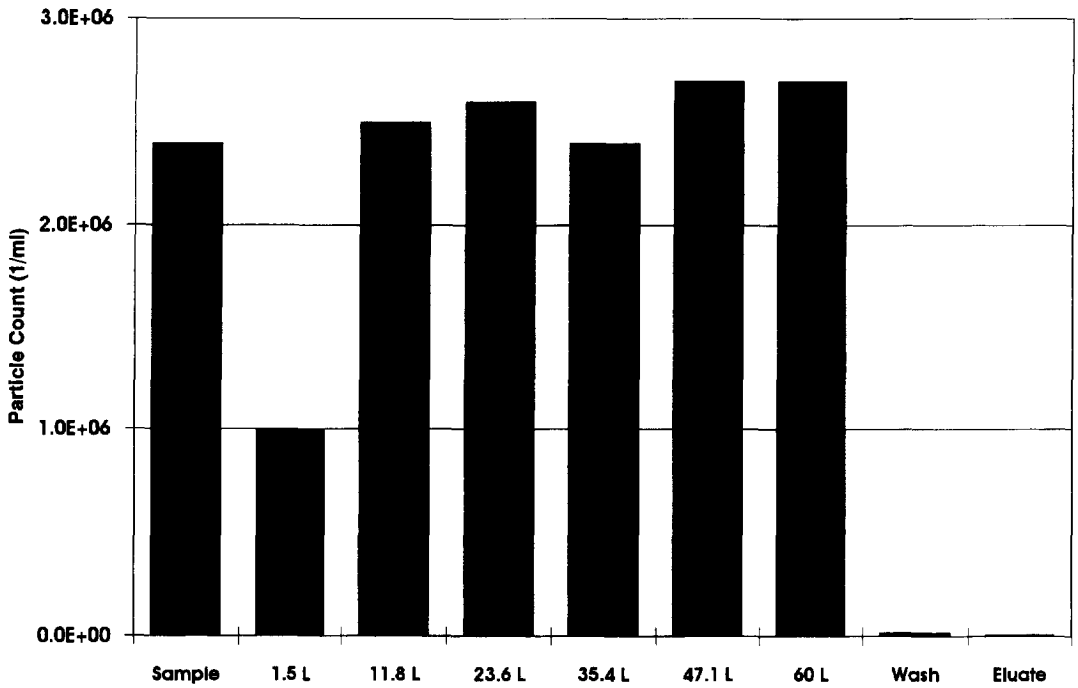

Fig. 5. Total particle concentration in various fractions of an expanded bed adsorption process starting with 60 l of cell containing culture broth in the SL 50 column (150 ml of adsorbent, $10 \cdot 10^{-4}$ m/s linear flow-rate).

mode of packed bed elution the antibody was concentrated 50 fold in the eluate, using an expanded bed mode of operation during elution the antibody concentration in the eluate was 30 times higher than in the feed. Elution in an expanded bed removes the need to change the position of the column head to the top of the settled bed, the top adaptor may remain in the position attained during sample application. Thus a moving part, which sometimes is a point of concern in large scale production, is no longer necessary. The clarification efficiency of the process is very high, a more than 100 fold reduction in particle concentration was achieved. Using expanded bed adsorption for the purification of IgG2a from cell containing culture broth in fact allows the combination of clarification, concentration, and purification in a single step. The experiments also demonstrated, that initial evaluation on a very small scale (20 ml of matrix in a modified column for packed bed chromatography) may serve as a rational basis for scale up of the method. In the final experiments 2 g of IgG2a were purified in a single cycle employing 150 ml of expanded bed adsorbent. Exploiting the full potential of the Streamline 50 column used in the final step of the scale up presented here (600 ml of adsorbent [20]) would allow the application of 170 L of cell containing culture broth at 50 mg/l MAb concentration. Eluting this challenge (14 mg MAb per ml of adsorbent) at 85% yield, as it has been possible in our experiments, would result in the purification of 7.1 g of antibody in a single run at more than 30 fold reduction in volume and more than 100 fold reduction of the particle load. The majority of the time requirement for this procedure will result from sample application: Processing 170 l at 120 ml per min (6 cm/min) will take 23 h, the additional time for equilibration, washing, elution, and cleaning in place can be estimated to another 4 h, so 27 h must be assumed as total process time. It is important to note, that most of the time no hand-on coverage is necessary because sample application can be controlled by an automatic device. Therefore expanded bed adsorption of monoclonals from cell containing broth may be an interesting alternative to the standard sequence of operation employing centrifuga-

tion/microfiltration, ultrafiltration, and packed bed chromatography.

Acknowledgments

The authors thank the cell culture group of the Institute of Biotechnology of the Forschungszentrum Jülich GmbH (M. Biselli) for their effort in providing several hundred litres of cell culture broth necessary for these studies. Supply with the prototype expanded bed adsorbent Streamline rProteinA and of the Streamline 25 column by Pharmacia Biotech (Uppsala, Sweden) is greatly appreciated.

References

[1] R. van Reis, L.C. Leonard, C.C. Hsu and S.E. Builder, Biotechnol. Bioeng., 38 (1991) 413.
[2] R. Kempken, A. Preissmann and W. Berthold, Biotechnol. Bioeng., 46 (1995) 132.
[3] R. Datar, Process Biochem., 21 (1986) 19.
[4] B.J. Spalding, BIO/TECHNOLOGY, 9 (1991) 229.
[5] M. Zlokarnik, Chem. Biochem. Eng. Q., 2 (1988) 173.
[6] S. Li, C. Schöneich and R.T. Borchardt, Biotechnol. Bioeng., 48 (1995) 490.
[7] H.A. Chase, TIBTECH, 12 (1994) 296.
[8] Y.K. Chang and H.A. Chase, Biotechnol. Bioeng., 49 (1996) 512.
[9] Y.K. Chang and H.A. Chase, Biotechnol. Bioeng., 49 (1996) 204.
[10] A.-K. Barnfield-Frej, R. Hjorth and A. Hammarstroem, Biotechnol. Bioeng., 44 (1994).
[11] Y.K. Chang, G.E. Mc Creath and H.A. Chase, Biotechnol. Bioeng., 48 (1995) 355.
[12] M. Hansson, S. Stahl, R. Hjorth, M. Uhlen and T. Moks, BIO/TECHNOLOGY, 12 (1994) 285.
[13] C.R. Bartels, G. Kleimann, J.N. Korzun and D.B. Irish, Chem. Eng. Progr., 54 (1958) 49.
[14] L. de Luca, D. Hellenbroich, N.J. Titchener-Hooker and H.A. Chase, Bioseparation, 4 (1994) 311.
[15] J. Thömmes, M. Weiher, A. Karau and M.R. Kula, Biotechnol. Bioeng., 48 (1995) 367.
[16] R. Hjorth, S. Kämpe and M. Carlsson, Bioseparation, 5 (1995) 217.
[17] J.C. Erickson, J.D. Finch and D.C. Greene, in B. Griffiths, R.E. Spier and W. Berthold (Editors), Animal Cell Technology: products for today, prospects for tomorrow, Butterworth and Heinemann, Oxford, 1994, p. 557.
[18] B.C. Batt, V.M. Yabannavar and V. Singh, Bioseparation, 5 (1995) 41.
[19] J. Thömmes, M. Halfar, S. Lenz and M.-R. Kula, Biotechnol. Bioeng., 45 (1995) 203.
[20] J. Thömmes, M. Weiher and M.-R. Kula, DOWNSTREAM, 17 (1994) 5.
[21] C. Born, J. Thömmes, M. Biselli, C. Wandrey and M.-R. Kula, Bioproc. Eng., 15 (1996) 21.
[22] J. Thömmes, J. Gätgens, M. Biselli, P. Runstadler and C. Wandrey, Cytotechnol., 13 (1993) 29.
[23] O. Levenspiel, Chemical Reaction Engineering, Wiley, New York, 1972.
[24] M.M. Bradford, J. Anal. Biochem., 72 (1976) 248.
[25] N.G. Ray, S.B. Karkare and P.W. Runstadler, Biotechnol. Bioeng., 33 (1989) 724.
[26] A. Lindgren, S. Johannson and L.-E. Nyström, in B. Henon (Editor), BED-The seventh Bioprocess Engineering Symposium, The American Society of Mechanical Engineers, 1993, p.27.