Journal of Chromatography, 397 (1987) 313–320
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1237

COMBINATION OF ZETAPREP MASS ION-EXCHANGE MEDIA AND HIGH-PERFORMANCE CATION-EXCHANGE CHROMATOGRAPHY FOR THE PURIFICATION OF HIGH-PURITY MONOCLONAL ANTIBODIES

A. JUNGBAUER*, F. UNTERLUGGAUER, F. STEINDL, F. RÜKER and H. KATINGER
Institute of Applied Microbiology, University of Agriculture, A-1190 Vienna (Austria)

SUMMARY

A procedure involving diafiltration, mass ion exchange on a QAE Zetaprep disk, gel chromatography and cation-exchange chromatography was used for the purification of mouse monoclonal antibodies from hybridoma culture supernatant. Prior to the separation steps, the starting solution was adjusted to the desired pH and conductivity. Diafiltration was used for this purpose in order to keep the volume constant or even to reduce the volume of sample. A QAE Zetaprep disk was used to remove the main protein contaminants from the culture supernatant. After washing unbound proteins out of the Zetaprep disk, slightly bound protein was eluted with a buffer solution containing 50 m*M* sodium chloride. The monoclonal antibody was eluted with a solution containing 150 m*M* sodium chloride. The purity of the eluted antibody was 50%, and was increased to 99% by subsequent high-performance cation-exchange chromatography. The purity was determined by means of sodium-dodecyl sulphate polyacrylamide gel electrophoresis and silver staining.

The advantage of the two high-performance techniques, mass ion exchange and high-performance cation-exchange chromatography, are the high-flow-rates and the high resolution that can be obtained. These techniques are suitable for the production of injectable therapeutic preparations.

INTRODUCTION

There are numerous publications on purification procedures for monoclonal antibodies (mAbs) based on ion-exchange chromatography, all of them being modifications of a procedure published by Svasti and Milstein[1] for the purification of a mouse immunoglobulin G_1, produced by the plasmacytoma cell line MoPC 21. The immunoglobulin was isolated from serum by precipitation with 40% saturated ammonium sulphate, followed by chromatography on a column of DEAE-cellulose with a linear gradient of sodium phosphate. In some instances, further purification was performed on a column of CM-cellulose with a linear gradient of sodium phosphate. If hybridoma culture supernatant is used as starting material, it is necessary to introduce a concentration step. However, if modern separation techniques (such as mass ion exchange) are used, the initial precipitation step is not necessary[2].

0021-9673/87/$03.50

Holborow *et al.*[3] have proposed a general purification procedure for mAbs. Anion-exchange chromatography should be used as the first step, and the sample should be loaded onto the column at pH 8.0 and an ionic strength of about 0.1. These are optimal conditions for a rigid purification process, and the process is suitable for a broad range of mAbs. The isoelectric point (p*I*) of mAbs can be in the range 5.0–7.8. In a modern purification process, pH and ionic strength are adjusted by diafiltration. Precipitation and dialysis, as reported by Jonak[4], are only convenient for samll-scale operations, and the method has many disadvantages compared with diafiltration, redilution and titration. Gel chromatography of the crude antibody solution has also been suggested[5], but these methods do not reduce the volume of sample or remove impurities; the sample volume must be brought back to its original value, in order to adjust the hybridoma culture supernatant to the optimum ionic strength for sample application.

Conventional growth media for hybridomas are Dulbecco's minimal essential medium (DMEM) and Rosevelt Park Memorial Institute Medium 1640 (RPMI), with additives such as ethanolamine, transferrin and sera; serum-free media are also used[3]. An osmolarity of about 280 mOsmol, equivalent to physiological conditions, corresponds to solutions of 175 m*M* potassium phosphate or 150 m*M* sodium chloride. For this reason, Jungbauer *et al.*[2] introduced the method of diafiltration for the adjustment of sample application conditions, instead of dilution and other techniques. Diafiltration is very well suited to the treatment of large volumes, and maintains the volume of the sample at a moderate level.

After exact adjustment of the hybridoma culture supernatant to the loading conditions, the sample can be further purified by ion-exchange chromatography or mass ion exchange. QAE, DEAE ion-exchange media or QAE Zetaprep products are used to remove the main protein contaminants from the hybridoma culture supernatant. The reduced capacity of ion-exchange chromatography, due to the binding of a large amount of protein at pH 8.0, is often quoted as one of the disadvantages of this technique. Cation-exchange chromatography used as a first step at pH 5.0–5.2 will separate the main protein contaminants, fetoin and albumin, from the mAb, if the p*I* of the mAb is higher than 5.2. A decrease in pH of the culture supernatant to 5.0 will cause precipitation of the proteins and activation of proteases; at neutral and slightly alkaline pH the latter are inhibited by natural protease inhibitors present in the added serum. Precipitation and protease activation lead to reduced yields of antibody.

Final purification of the mAbs is often performed by cation-exchange high-performance liquid chromatography (HPLC). High-performance gel-permeation chromatography, which has also been used as the final step, is suitable only for small-scale operations[6]; reasons for this include the low capacity of the columns and the fact that the overload technique cannot be applied. Columns of SP-TSK-5PW, DEAE-TSK-5PW, Mono Q, Mono S and HPHT[7–9] are reported to work well in the final purification of mAbs from partially purified samples.

In this paper, we describe a purification scheme based on diafiltration, mass ion exchange and HPLC that is suitable for the pilot-scale and large-scale purification of mAbs under strictly aseptic conditions. The aim of the schjeme is the production and purification through to the final product in a single, closed processing line.

EXPERIMENTAL

Mouse/mouse hybridomas, producing anti-urokinase antibodies (U0208), were cultivated in an airlift fermenter on DMEH H21 (Gibco, U.K.), supplemented with 5% foetal calf serum (PAA Labs., Linz, Austria). The harvested culture supernatant had a conductivity of about 11 mS and a pH of 7.2. Cells and debris were removed by cross-flow microfiltration in a Pellicon system (Millipore, Bedford, MA, U.S.A.), using Durapore 0.45-μm membranes.

The clarified culture supernatant was adjusted by diafiltration to pH 8.0 and a conductivity of 3–4 mS. The diafiltration buffer corresponds to the equilibration buffer in the mass ion-exchange step. Hollow fibres with a cut-off of $5 \cdot 10^4$ (Amicon, Danvers, U.S.A.) were used in an Amicon DC-10 ultrafiltration apparatus.

A QAE Zetaprep 15 disk (LKB, Bromma, Sweden) was connected to a MicroPerpex S peristaltic pump. The disk was wquilibrated with 20 m*M* Tris–HCl buffer (pH 8.0) and regenerated with 20 m*M* Tris–HCl buffer (pH 8.0) containing 1 *M* sodium chloride. The adjusted culture supernatant was pumped through the equilibrated disk, and non-bound proteins were washed out with equilibration buffer. Slightly bound proteins were eluted with equilibration buffer containing 50 m*M* sodium chloride. mAb was eluted with equilibration buffer containing 150 m*M* sodium chloride.

UV absorbance, pH and conductivity were monitored continuously with a flow-through photometer (Uvicord II; LKB) and a pH/ion monitor (LKB), respectively. Fractions were collected according to their UV absorbance.

The buffer, containing the partially purified mAbs eluted from the Zetaprep disk, was replaced with 20 m*M* sodium citrate buffer (pH 5.0) by means of gel chromatography on Trisacryl GF 05. The mAbs were then further purified on a column of DEAE-TSK-5PW, equilibrated with 20 m*M* sodium citrate buffer (pH 5.0) and eluted with 20 m*M* Tris–HCl buffer (pH 7.2) containing 250 m*M* sodium chloride; the column was regenerated with 20 m*M* Tris–HCl buffer (pH 7.2) containing 1 *M* sodium chloride.

Proteins were determined by the Lowry method[10]. Immunoglobulin G (IgG) was determined by enzyme-linked immunosorbent assay (ELISA), using sheep anti-mouse Fc as the first antibody and goat anti-mouse γ-chain as the second antibody[11]. The purity of the eluted mAbs was checked by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli[12]. The proteins were detected in the gel by a silver-staining technique[13]. Isoelectric focusing was performed in a 1% agarose gel (pH 3.5–9.5). The markers used were mouse IgG (affinity purified), human transferrin and bovine serum albumin (all from Sigma, St. Louis, MO, U.S.A.) and the low-p*I* marker kit (Pharmacia, Uppsala, Sweden).

RESULTS

Optimization of separation conditions for mass ion exchange

Five QAE Zetaprep disks were each wquilibrated with a different loading buffer. Hybridoma culture supernatants were adjusted to the corresponding pH and ionic strength and loaded onto the disks. Proteins were eluted by step gradients of 50 and 150 m*M* sodium chloride in the loading buffer, and two pools of eluent were

TABLE I

OPTIMIZATION OF LOADING CONDITIONS FOR QAE ZETAPREP 15 DISK

IgG eluted with 50 and 150 m*M* sodium chloride was collected in pool 1 and pool 2, respectively.

pH of applied sample	*Amount of IgG eluted (mg)*		*IgG eluted (%)*	
	Pool 1	*Pool 2*	*Pool 1*	*Pool 2*
6.5	12.7	3	74	18
7.0	4.9	7.3	38	56
7.5	3.6	8.9	23	59
8.0	1.4	14.3	8	86
8.5	0	7.9	0	74

collected from each disk. Table I shows the IgG present in each pool. At pH 8.0, the mAbs were eluted only in pool 2 (see Table I), whereas at other pH values the IgG was found in both pools 1 and 2. Therefore, elution at pH 8.0 was chosen for the full-scale fractionation experiments. Table I shows the amount of IgG eluted as a percentage of the IgG in the material applied to the disk.

Purification of mAbs

Five unit operations were fitted to a purification process, consisting of microfiltration, diafiltration, mass ion-exchange chromatography, gel chromatography and HPLC.

In order to obtain an optimal purification process, each step must fit in with the previous one. From each purification step the maximum resolving power must be utilized. If practicable, the unit operations have to be fitted together in a manner that avoids time- and buffer-consuming operations. In this particular purification procedure, diafiltration is more convienent than gel chromatography as a first step. An extensive concentration by diafiltration is not necessary, because the volume *per se* is not limiting and also will be reduced in the subsequent mass ion-exchange chromatography. The pre-purified mAbs are more sensitive than the crude solution to shear. Therefore, gel chromatography was choosen to change the mass ion-exchange eluate to the appropriate buffer for the subsequent high-performance cation-exchange step.

The harvested culture supernatant was cooled, clarified by microfiltration and adjusted to pH 8.0 and 3–4 mS by diafiltration. After diafiltration, the solution was slightly opalescent; the opalescence could not be removed by centrifugation at 10 000 *g* or by filtration through a 0.22-μm sterile filter. No coalescence was noted in the fractions after elution from the Zetaprep disk.

A 100-ml aliquot of the adjusted supernatant was pumped through a QAE Zetaprep disk at a flow-rate of 4 ml/min, and the disk was washed at the same rate until the UV absorbance returned to the baseline. The first protein contaminants were removed by elution with 50 m*M* sodium chloride in equilibration buffer. IgG was eluted with equilibration buffer containing 150 m*M* sodium chloride (Fig. 1). Prior to further purification, the buffer was exchanged by gel chromatography. A

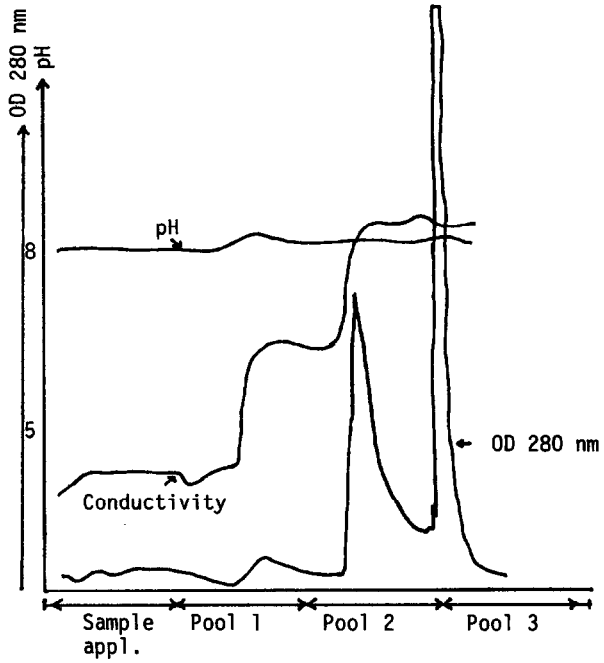

Fig. 1. Fractionation of culture supernatant by mass ion exchange on a QAE Zetaprep 15 disk. Eluted proteins were collected in four pools: sample application, pool 1, pool 2 and pool 3.

sample was applied in several portions to a 18 × 5 cm I.D. column of Trisacryl GF 05 at a flow-rate of 12 ml/cm per hour. Multiple application to the gel column was preferred for economic reasons only.

Up to 40 ml of the partially purified antibody solution was applied to an SP-TSK-5PW column (7.5 × 0.75 cm I.D.) at a flow-rate of 1 ml/min. After washing out non-bound proteins and impurities, the IgG was eluted with 20 m*M* Tris–HCl buffer (pH 7.2) containing 250 m*M* sodium chloride (Fig. 2). Purity and homogeneity

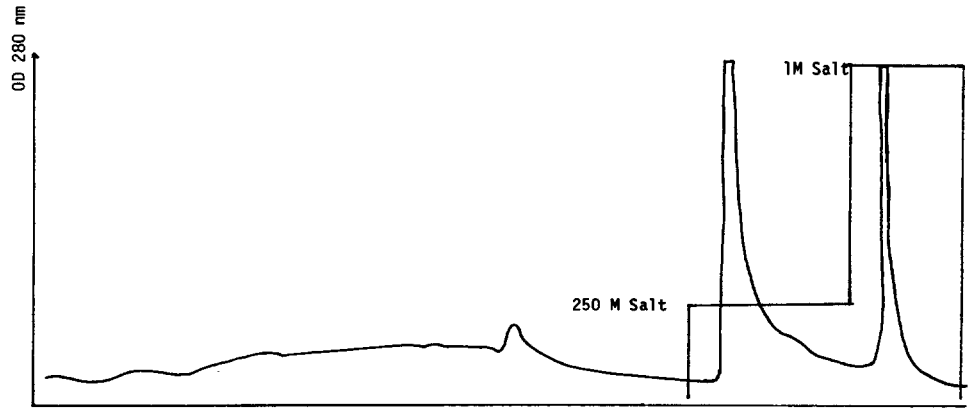

Fig. 2. Fractionation of mAbs by high-performance cation-exchange chromatography.

TABLE II

SUMMARY OF YIELDS AND PURITIES FROM THE DIFFERENT PURIFICATION STEPS

n.d. = Not determined.

Purification step	*Purity (%)*	*IgG concentration (mg/ml)*	*Yield (%)*
Hybridoma culture supernatant	4	0.1	100
Adjusted hybridoma culture supernatant	4	0.1	95
QAE Zetaprep 15 disk	50	0.15	79.8
Gel chromatography	n.d.	n.d.	n.d.
Cation-exchange chromatography	99	4.0	71.8

were checked by SDS-PAGE (Fig. 3). Table II shows the yield, purity and enrichment of mAbs after HPLC. A final yield of 70% with a purity of > 99% could be obtained.

The isoelectric point of the purified U0208 antibody was determined (Fig. 4), and was found to be similar to that of transferrin.

DISCUSSION

In order to construct an optimal, continuous process from several individual steps, it is necessary for each step to fit in with the previous one. Equilibration, buffer exchange, removal of precipitates, etc., are not usually regarded as purification steps. This may not be a problem on a laboratory scale, as small volumes of sample can be handled easily and quickly. In pilot- and large-scale operations, however, it is not just the chromatographic steps that which require special equipment and processing

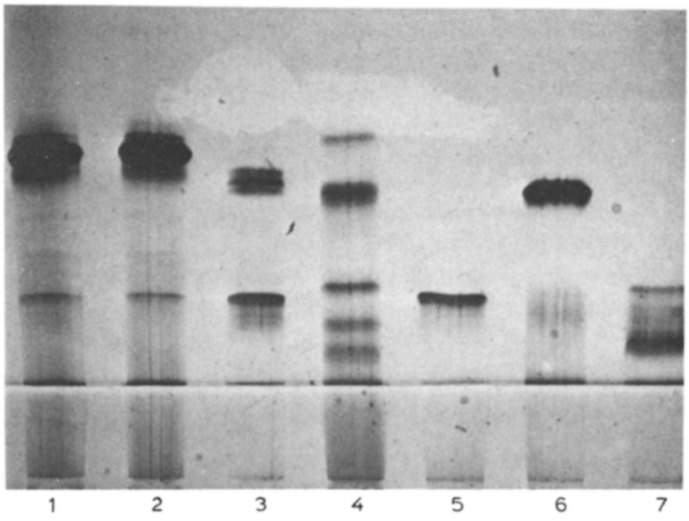

Fig. 3. SDS-PAGE from different steps of the purification process. Lane 1 = starting material; lane 2 = diafiltered hybridoma culture supernatant; lane 3 = partially purified mAbs after mass ion exchange; lane 4 = after gel chromatography; lane 5 = purified mAb after high-performance cation-exchange chromatography; lane 6 = bovine transferrin; lane 7 = affinity-purified polyclonal antibody.

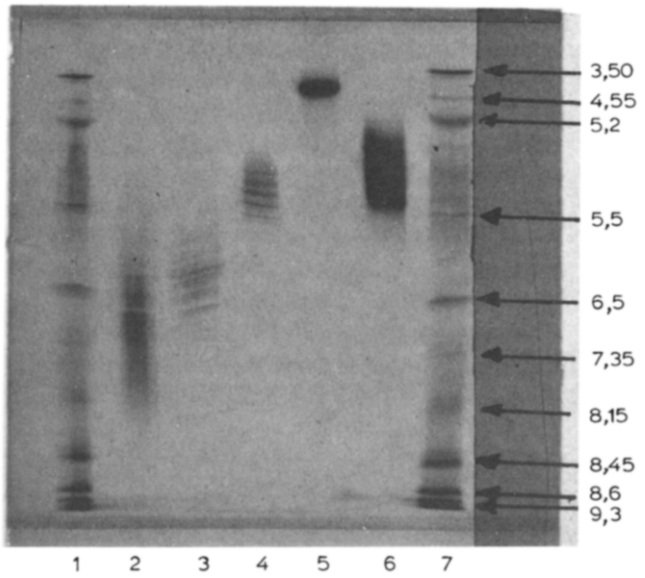

Fig. 4. Determination of isoelectric point of the purified monoclonal antibody. Lanes 1 and 7 = isoelectric-point marker (Pharmacia); lane 2 = polyclonal mouse IgG (Sigma); lane 3 = purified mAb against superoxide dismutase; lane 4 = purified mAb; lane 5 = bovine serum albumine (Sigma); lane 6 = human transferrin (Sigma). Isoelectric points of the markers (lanes 1 and 7) are indicated on the right.

time. This is the reason why we consider the whole process to constitute more than just the three chromatographic steps: they are the major steps, but the steps between treatments are also necessary for binding the chromatographic steps together.

Gel chromatography is often used for adjusting the sample to the best loading conditions at the beginning of a process. Malm[5] used this method as the starting point of a purification scheme. However, gel chromatography results in at least 1.5-fold dilution of the sample. We prefer diafiltration, as it allows the sample volume to be kept constant or even to be reduced; in theory, a volume of buffer equal to 4.6 times the volume of sample is sufficient to remove 99% of an unwanted substance, assuming no rejection of the substance by the ultrafiltration membrane.

After conditioning the culture supernatant, the main contaminants are removed. In this instance, they are albumin, fetoin and transferrin from the foetal calf serum. Albumin and fetoin are removed very easily on the QAE Zetaprep disk. Transferrin was purified together with the mAb, probably because of the similarity of the p*I* values of transferrin and mAb.

Isoelectric focusing shows that other proteins are more or less absent. Bruck *et al.*[14] reported that the mAb they purified by affinity chromatography on DEAE Affi-Gel Blue also contained transferrin. However, the use of DEAE Affi-Gel Blue cannot be recommended for the preparation of therapeutic preparations, because the immobilized ligand, Cibacron Blue FG3A, is very toxic and a carcinogen, and the possibility of ligand leakage cannot be excluded[15]. Up to the mass ion-exchange chromatography, the procedure was scaled up (pilot scale). With the QAE Zetaprep 800 (flow-rate 7 l/h) the same results could be achieved (data not shown).

The subsequent high-performance cation-exchange step gave a homogeneous and >99% pure antibody at a high concentration (up to 20 mg/ml). Other purification techniques, such as protein A affinity chromatography and hydroxyapatite chromatography, also require preliminary purification in order to achieve 99% purity (one band in SDS-PAGE with silver staining). Protein A affinity chromatography has the same susceptibility to ligand leakage as DEAE Affi-Gel Blue.

We can conclude that our process, consisting of diafiltration, mass ion exchange with Zetaprep products, gel chromatography and high-performance cation-exchange chromatography, is well suited for the production of injectable monoclonal antibody solutions. Diafiltration, gel chromatography and HPLC materials are often used in the preparation of therapeutic preparations. The Zetaprep matrix is non-toxic, in accordance with USP XX systematic injection and *in vivo* biocompatibility and safety tests. It is also non-mutagenic in the *Salmonella*/microsome assay (Ames test). Housing materials meet the FDA Code of Federal Regulations.

ACKNOWLEDGEMENTS

We thank LKB Produkter (Bromma, Sweden) and LKB-Instruments (Vienna, Austria) for supporting this work.

REFERENCES

1 J. Svasti and C. Milstein, *Biochem. J.*, 126 (1972) 837.
2 A. Jungbauer, F. Unterluggauer, F. Steindl, E. Wenisch and H. Katinger, presented at the *3rd Conference on Recovery on Bioproducts, Uppsala, May 11–15, 1986.*
3 E. Holborow, V. Nussenzweig and F. Borek, *J. Immunol. Methods*, 39 (1984) 1.
4 Z. Jonak, in *Monoclonal Antibodies*, Plenum Press, New York, 1980.
5 B. Malm, presented at *Biotech 85 USA, Washington, DC, October 21–33, 1985.*
6 G. Berry, J. Jackson, T. McDonald, D. Crouse and J. Sharp, *Prep. Biochem.*, 14 (1985) 431.
7 K. Tasaka, K. Kobayashi and T. Tanaka, *Acta Histochem. Cytochem.*, 17 (1984) 283.
8 P. Clezardin, J. L. McGregor, M. Manach, H. Boukerche and M. Dechavanne, *J. Chromatogr.*, 319 (1985) 67.
9 G. Smith, R. McFarland, H. Reiser and G. Hudson, *Anal. Biochem.*, 141 (1986) 432.
10 O. Lowry, N. Rosebrough, A. Farr and J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
11 E. Engvall, K. Jonsson and P. Perlman, *Biochim. Biophys. Acta*, 251 (1971) 427.
12 U. Laemmli, *Nature (London)*, 227 (1970) 680.
13 R. Switzer, C. Merril and C. Shifrin, *Anal. Biochem.*, 98 (1979) 231.
14 C. Bruck, D. Protetelle, C. Glineur and A. Bollen, *J. Immunol. Method*, 53 (1982) 313.
15 P. D. G. Dean and D. H. Watson, *J. Chromatogr.*, 165 (1979) 301.