Journal of Chromatography, 548 (1991) 165–178
Elsevier Science Publishers B.V., Amsterdam

CHROMSYMP. 2353

Characterization of synthetic macroporous ion-exchange resins in low-pressure cartridges and columns

Evaluation of the performance of Macro-Prep 50 S resin in the purification of anti-Klenow antibodies from goat serum

LEE DUNN*, MOHAMED ABOUELEZZ, LARRY CUMMINGS, MOHSEN NAVVAB, CHERYL ORDUNEZ, CHRISTOPHER J. SIEBERT and KENNETH W. TALMADGE

Bio-Rad Laboratories, CBU Research and Development, Chemical Division, 3300 Regatta Blvd., Richmond, CA 94804 (USA)

ABSTRACT

Three ion-exchange materials and one hydrophobic-interaction chromatography packing, based on a rigid macroporous polymer with large, relatively uniform pores, have been evaluated for low-pressure liquid chromatography of antibodies. These sorbents have high capacities for both small and large proteins and are mechanically, chemically, and thermally stable. Macro-Prep 50 S, CM and Q ion-exchange materials are strongly acidic, weakly acidic, and strongly basic, respectively. Protein binding and recovery, pressure–flow properties, and chemical and thermal stability were determined for each sorbent. A rapid, two-step method for the purification of anti-Klenow antibodies from goat serum was developed, based on the Macro-Prep™ 50 S strong-acid cation-exchange material and the Econo-Pac® HIC prepacked hydrophobic-interaction cartridge.

INTRODUCTION

The introduction of ion-exchange cellulose particles as a column packing material was a significant event in the history of protein purification [1,2]. Although physically weak and unstable, ion-exchange cellulose particles were very effective packings, because proteins could be freely bound and released from the complex outer surface regions of the particles, and non-specific adsorption was relatively low. Over the next three decades, improvements in the physical and chemical properties of ion exchangers led to the current, highly efficient materials, suitable for ion-exchange high-performance liquid chromatography (HPLC) of biopolymers [3,4]. The generally accepted use of particles $\leqslant 10$ μm to achieve high performance requires expensive high-pressure equipment. The need for reducing costs and increasing the scale of high-performance separations in processing techniques for biological products has placed new demands on chromatographic materials. This necessitates a better understanding of the physical and chemical properties of porous packing materials. The

0021-9673/91/$03.50

pharmaceutical and biotechnology industries need chromatographic materials with high chemical stability to withstand harsh sanitation conditions, with high mechanical strength to hold up under the pressures and flow-rates needed for large-scale separations, and high dynamic load capacities, giving high recoveries of biological activity.

This report describes a new macroporous methacrylate polymer (Macro-Prep™ 50) that meets the above-mentioned requirements. Three ion-exchange resins, as well as a hydrophobic-interaction packing, are discussed. All of these materials are characterized by a high percentage of pores in the range of 1000–1500 Å. To test this macroporous material, goat antibodies against the Klenow fragment of DNA polymerase from *Eschericia coli* [5] were purified on the Macro-Prep 50 S and Econo-Pac® HIC 5-ml cartridges.

MATERIALS AND METHODS

Materials

All chemicals used were of analytical-reagent grade. Guanidine–HCl was obtained from Aldrich (Milwaukee, WI, USA). Monobasic sodium phosphate, dibasic sodium phosphate, sodium acetate, thimerosal, 4-morpholinepropanesulfonic acid (MOPS), 4-morpholineethanesulfonic acid (MES) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffers, bovine serum albumin (BSA), bovine carbonic anhydrase B, cytochrome *c*, human hemoglobin, human immunoglobulin (IgG), human transferrin, lysozyme, myoglobin, and ribonuclease A were purchased from Sigma (St. Louis, MO, USA). Macro-Prep 50 CM, Q, and S packings, Econo-Pac HIC cartridges, ammonium sulfate, protein assay standard I (bovine γ-globulin) and II (BSA), sodium dodecyl sulfate (SDS), Tris–HCl, Coomassie Blue, Tween 20, horseradish peroxidase-conjugated protein G, Klenow DNA polymerase, and 3,3′,5,5′-tetramethylbenzidine (TMB) were obtained from Bio-Rad (Richmond, CA, USA). Goat serum (Bethyl Labs., Montgomery, TX, USA) was obtained from goats inoculated with Klenow DNA polymerase.

Polymer characterization

The pore structure analysis of the ion-exchange sorbents was carried out by mercury intrusion porosimetry, using the pore sizer, Model 9310 (Micromeritics Instruments, Norcross, GA, USA). In mercury porosimetry the volume of mercury intruded into the pores in a sample is measured as a function of pressure. From these measurements a variety of physical properties can be calculated, including pore diameter, pore volume, and total pore surface area. Particle-size distributions were determined by electrozone sensing, using an Elzone 80XY analyzer (Particle Data, Elmhurst, IL, USA) and by visual examination, using a light microscope.

The mechanical rigidity of the Macro-Prep 50 ion-exchange material was examined by measuring the backpressure (p.s.i.) as a function of the linear flow-rate (cm/h). Backpressure was measured with a 0–1000 p.s.i. electronic transducer (Sensym, Sunnyvale, CA, USA). Macro-Prep 50 CM, Q, and S resins were packed into Bio-Rex® MP columns (10 × 1 cm I.D.) under gravity and equilibrated with deionized water for 24 h at 1.0 ml/min, using a Model 1350 pump (Bio-Rad). The flow-rate was increased to 5.0 ml/min and maintained at this rate for 30 min before measuring

the backpressure. Subsequently, the flow-rate was increased in 5.0 ml/min increments and maintained for 30 min at each new flow-rate before measuring the backpressure.

The swelling and shrinking properties of the resins were determined by measuring changes in bed volume due to changes in pH (4.0–10.0), salt concentration (0–1.0 *M* NaCl), and organic solvents. Columns, 60 × 1.5 cm I.D., were filled to a height of approximately 11 cm with Macro-Prep resins. The bed height was determined before and after equilibration in the test solutions for 24 h, at a flow-rate of 1.0 ml/min.

The three Macro-Prep 50 ion-exchangers were submitted to an independent laboratory for a series of standard biological tests designed to detect potentially toxic contaminants leaching from the resins. The tests included; (a) USP systemic and intracutaneous injections of saline and cottonseed oil extracts of the resins into rabbits, (b) intraperitoneal injections of saline and cottonseed oil extracts of the resins into mice and guinea pigs, (c) cytotoxicity of extracts to L-929 mouse fibroblast cells in tissue culture, (d) Ames mutagenicity test.

Determination of ion-exchange capacity

Ionic capacities of the ion exchangers were determined by conductometric titrations with a YSI Model 32 Conductance Meter (Yellow Springs Instrument, Yellow Springs, OH, USA) and Model 3403 microcell (K = 1.0/cm). Using the Macro-Prep 50 S material as an example, 5.0 ml of resin was washed with deionized water, equilibrated with 0.5 *M* HCl for 1 h, and washed with deionized water until the conductivity was $\leqslant$ 2.0 μS/cm. Macro-Prep 50 S and CM packings were then titrated with 0.1 *M* NaOH. The Macro-Prep 50 Q packing was converted to the hydroxide form and titrated with 0.1 *M* HCl.

Determination of protein binding capacity

Static protein binding capacities for Macro-Prep 50 CM, Q, and S materials were determined by using an excess of protein. The conditions for the Macro-Prep 50 S resin are given as an example for these determinations. The Macro-Prep 50 S packing (0.5–1.0 ml) was washed with water, followed by 20 m*M* sodium acetate buffer, pH 5.0 (binding buffer). Next, the material was equilibrated for 24 h with 100 mg human IgG, dissolved in *ca.* 35 ml of binding buffer. The sorbent was washed with binding buffer to remove unbound material, and the bound protein was eluted with 20 m*M* Tris–HCl (pH 9.0), containing 1.0 *M* NaCl. The protein was quantitated using one of the following methods; absorbance at 280 nm, Bradford [6] reagent (Bio-Rad), BCA assay [7] (Pierce, Rockford, IL, USA). Protein-binding capacity, in mg IgG/ml resin, and percent recovery were calculated. The static protein-binding capacities for the Macro-Prep 50 CM and Q materials were performed similarly. For the CM packing, the proteins (100 mg) were bound in 15 ml of 10 m*M* sodium acetate buffer (pH 5.0), and eluted with 100 m*M* Tris–HCl buffer (pH 8.0). For the Q packing, the proteins (100 mg) were bound in 5.0 ml of 10 m*M* Tris–HCl buffer (pH 8.3), and eluted with 50 m*M* Tris–HCl buffer (pH 8.3), containing 1.0 *M* NaCl.

Chromatography

High-performance liquid chromatography was carried out on a HRLC® Model 800 chromatography system (Bio-Rad). Low-pressure liquid chromatography was

performed on an Econo System, consisting of a Model EP-1 Econo Pump, Model EM-1 Econo UV Monitor, and a Model ES-1 Econo System controller (Bio-Rad). Fractions were collected with a Model 2110 fraction collector (Bio-Rad). All buffers were filtered through 0.2-μm membrane filters (Gelman Sciences, Ann Arbor, MI, USA).

Column lifetime of the resins was determined by running 100 gradient cycles on a HRLC 800 gradient system, equipped with a Model AS-100 automatic sampler (Bio-Rad). Using the Macro-Prep 50 S support (10 × 1.0 cm I.D.) as an example, each cycle consisted of a 10-min gradient from 0–100% B, where buffer A was 10 m*M* sodium phosphate (pH 6.9), and buffer B was buffer A plus 1.0 *M* NaCl. Every tenth cycle, a Cationic Protein Standard (Bio-Rad) containing myoglobin, ribonuclease A, and cytochrome *c* was injected onto the column.

Enzyme-linked immunosorbent assay (ELISA)

Determination of specific antibody activity against the Klenow DNA polymerase was performed by ELISA. Wells of microtiter plates were coated overnight at 4°C with 50 μl of Klenow DNA polymerase at a concentration of 1.0 μg/ml in 50 m*M* sodium carbonate buffer (pH 9.6). Plates were washed one time with 200 μl of a solution consisting of 20 m*M* sodium phosphate (pH 7.2), 120 m*M* sodium chloride, 0.05% Tween 20, and 0.01% thimerosal (PTT). Wells were incubated for 2 h at room temperature with 200 μl PTT, containing 0.5% BSA (referred to as PTTB) to block non-specific binding sites on the plates. The plates were then washed once with 200 μl of PTT, blotted dry and sealed with parafilm before being stored at 4°C. Column fractions were assayed for antibody binding activity by adding 50–100 μl aliquots of the samples, diluted with PTTB, to the coated plates for 1–3 h at room temperature. The plates were washed twice with PTT and then incubated for 1 h at room temperature with 100 μl of horseradish peroxidase-conjugated protein G, diluted 1/10 000 with PTTB. The plates were then washed three times with PTT, and the enzyme activity was measured by adding 100 μl of TMB substrate for 5–10 min at room temperature before stopping the reactions with 100 μl of 0.5 *M* sulfuric acid. The plates were read at 450 nm, using a Model 3550 Microplate Reader (Bio-Rad).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoretic separations were carried out under reducing conditions by the method of Laemmli [8] on 12% pre-cast Mini-PROTEAN II® ready gels (Bio-Rad), 12% single percentage gel, 0.376 *M* Tris–HCl (pH 10), using the Mini-PROTEAN II cell and a Model 500/200 power supply (Bio-Rad). Staining was performed with Coomassie Blue.

RESULTS

Physical properties

Three macroporous ion-exchange derivatives were evaluated, a carboxylate weak-cation exchanger, a sulfopropyl strong-cation exchanger, and a quaternary ammonium strong-anion exchanger. The macroporous nature of the beads was confirmed by scanning-electron microscopy, as shown in Fig. 1. The bead shown is approximately 20 μm in size.

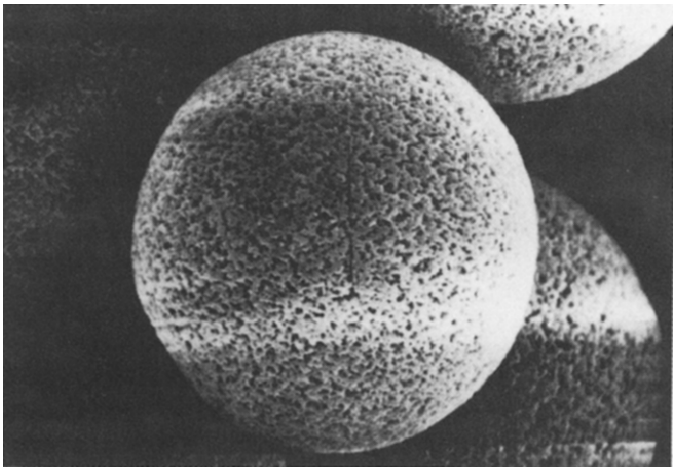

Fig. 1. Scanning.-electron micrograph of Macro-Prep 50 Q resin, obtained with an International Scientific Instrument, Model ISI SX-40. Magnification 2400×.

The surface and pore structure of the beads were characterized (Table I) by mercury intrusion porosimetry for pore size, pore volume, and surface area. The pore-volume distribution of the beads was plotted as a function of pore diameter for the Macro-Prep 50 Q packing. The results in Fig. 2 illustrate that most of the pore volumes were between 1000 and 1500 Å with only a low percentage of small pores.

The swelling and shrinking properties of the ion-exchange materials were different in aqueous and organic liquids. Macro-Prep 50 CM and Q packings exhibited less than 1% shrinkage or swelling in the pH range from 4–10, while the Macro-Prep 50 S packing exhibited less than 3% (Table I). In the range of 0.1–1.0 *M* NaCl, the Macro-Prep 50 CM and Q materials exhibited less than 5% shrinkage or swelling, and the Macro-Prep 50 S packing exhibited less than 9% (Table I). In organic solvents, such as acetonitrile and methanol, the packings swell 20–30% (unpublished results).

TABLE I

PHYSICAL AND CHEMICAL PROPERTIES OF MACRO-PREP 50 RESINS

	Macro-Prep 50 CM	Macro-Prep 50 Q	Macro-Prep 50 S
Type of exchanger	Weak cation	Strong anion	Strong cation
Functional group	$-COO^-$	$-N^+(CH_3)_3$	$-SO_3^-$
Ionic capacity	210 ± 30 μequiv./ml	190 ± 40 μequiv./ml	160 ± 40 μequiv./ml
Counter ion	Na^+	Cl^-	Na^+
Protein capacity	⩾ 25 mg hemoglobin/ml	⩾ 15 mg ferritin/ml	⩾ 35 mg IgG/ml
Mean particle size	50 μm	50 μm	50 μm
Pore size (nominal)	1200 Å	1200 Å	1200 Å
Surface area	18–22 m^2/g	18–22 m^2/g	18–22 m^2/g
Pore volume	⩾ 0.5 ml/g	⩾ 0.5 ml/g	⩾ 0.5 ml/g
Swell/shrink			
pH 4–10	<1%	<1%	<3%
0.1–1.0 *M* NaCl	<4%	<5%	<9%

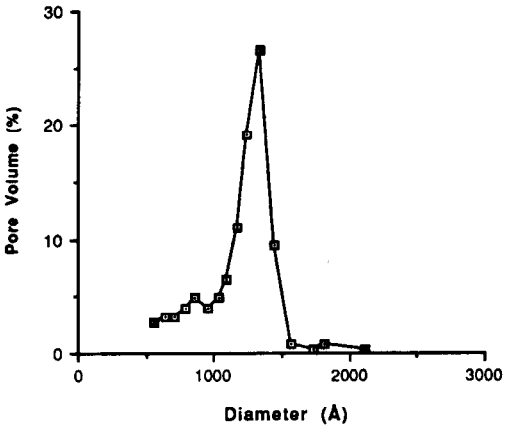

Fig. 2. Macro-Prep 50 Q material analyzed by mercury porosimetry, as described under Materials and Methods. Percent total pore volume is plotted as a function of the pore diameter.

The beads were mechanically strong in aqueous solutions, based on measurements of the backpressures at increasing flow-rates. The results of the pressure–flow-rate experiments for the three ion-exchange resins are shown in Fig. 3. The maximum linear velocity that could be reached was 3800 cm/h for the Macro-Prep CM material, 5700 cm/h for the Q material, and 4560 cm/h for the S material.

Ion-exchange properties

Ion-exchange capacity, static protein binding capacities, and protein recoveries for the three ion exchange materials are listed in Table II. Ion-exchange capacities of several lots of Macro-Prep ion exchangers were analyzed and the range for each is shown. The ionic capacities were found to vary by as much as 25% from the average value, determined for each material (data not shown).

The static protein capacity varied for each of the ion-exchange resins, depending upon the protein tested. Macro-Prep 50 CM material exhibited static protein-

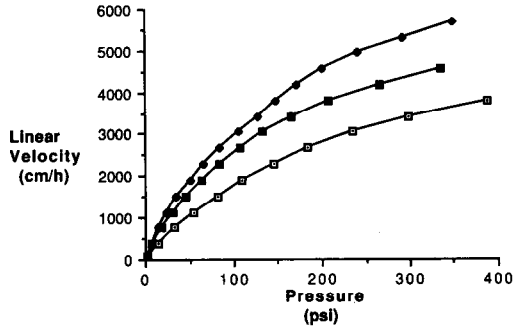

Fig. 3. Macro-Prep 50 CM (□), Macro-Prep 50 Q (◆) and Macro-Prep 50 S (■) materials were packed into 10 × 1.0 cm Bio-Rex MP columns. The backpressure, in p.s.i. was measured at increasing flow-rate, as described under Materials and Methods. The flow-rates (ml/min) have been converted to linear velocity (cm/h).

TABLE II

IONIC CAPACITY, PROTEIN-BINDING CAPACITY, AND RECOVERY

The ionic and static protein binding capacities were performed as described in the Materials and Methods. The percent recoveries were performed at loadings of 25% of the maximum capacity.

Materials	Ionic capacity (μequiv./ml resin)	Binding capacity (mg/ml resin)	Recovery (%)
Macro-Prep 50 CM	210 ± 40		
BSA		20	84
Cytochrome *c*		15	95
Ribonuclease A		15	108
Transferrin		26	108
Hemoglobin, human		35	79
Macro-Prep 50 Q	190 ± 40		
BSA		17	98
Ferritin		23	94
Thyroglobulin		24	88
Macro-Prep 50 S	160 ± 40		
Cytochrome *c*		35	103
IgG, human		57	102
Lysozyme		51	97
Ribonuclease A		62	102

binding capacities ranging from 15 to 35 mg/ml resin for five proteins examined. Similarly, static binding capacities of 17 to 24 mg/ml resin were observed of the Q material. The Macro-Prep 50 S material exhibited higher static protein-binding capacities, 35 to 62 mg/ml resin, depending upon the protein tested.

Protein recovery was good for all the proteins tested, as seen in Table II. Macro-Prep 50 S and Q resins showed 94 to 108% recoveries of the proteins tested, while the recoveries from the CM sorbent were also high, except for BSA (84%) and hemoglobin (79%).

TABLE III

CHEMICAL AND THERMAL STABILITY OF IONIC CAPACITY

The Macro-Prep 50 materials were suspended in the solutions and assayed at the indicated times for ionic capacity, as described under Materials and Methods.

Exposure	Ionic capacity (μequiv./ml resin)		
	Macro-Prep 50 CM	Macro-Prep 50 Q	Macro-Prep 50 S
Control	238	190	184
1% SDS, 24 h	241	168	194
8 *M* Guanidine–HCl, 24 h	234	195	189
1 *M* HCl, 7 days	249	189	192 (3 days)
1 *M* NaOH, 7 days	239	186	191 (3 days)
Autoclave, 121°C, 30 min	205	193	194

TABLE IV

CHEMICAL AND THERMAL STABILITY OF PROTEIN-BINDING CAPACITY

The Macro-Prep 50 materials were suspended in the solutions and assayed at the indicated times for the static protein-binding capacity, as described under Materials and Methods.

Exposure	Protein capacity (mg/ml resin)			
	Macro-Prep 50 CM[a]	Macro-Prep 50 Q[b]	Macro-Prep 50 Q[c]	Macro-Prep 50 S[d]
Control	40	20	17	40
1% SDS, 24 h	40	19	–	43
8 *M* Guanidine–HCl, 24 h	36	20	–	42
1 *M* HCl, 72 h	40	–	20	43
1 *M* NaOH, 72 h	40	–	16	41
Autoclave, 121°C, 30 min	40	–	17	45

[a] Hemoglobin (human).
[b] Ferritin.
[c] Different resin sample used for control.
[d] IgG (human).

Chemical properties

Treatment of Macro-Prep 50 packings with 1.0 *M* HCl, 1.0 *M* NaOH, 1% SDS, and 8 *M* guanidine–HCl can be carried out without substantial loss in ionic (Table III) or static protein-binding capacity (Table IV). Sanitization can be achieved with 1.0 *M* NaOH and sterilization by autoclaving at 121°C for 30 min. Except for losses in ionic capacities of the CM material after autoclaving (14%) and the Q material after treatment with SDS (11%), all materials were virtually unaffected by these treatments.

A major concern of the pharmaceutical and biotechnology industries is contamination of biological products by toxic materials coming from chromatographic resins. To address this concern a series of standard biological tests, including USP systemic and intracutaneous injections, tissue culture cytotoxicity, and the Ames mutagenicity test were performed on extracts of the three Macro-Prep 50 ion-exchange materials. All results were negative; no extract showed signs of toxicity, cytotoxicity, or mutagenicity in any of the tests.

Separation of standard proteins

The chromatographic performance of the Macro-Prep 50 ion-exchange packings was demonstrated by separating mixtures of proteins under gradient elution conditions (Fig. 4). Carbonic anhydrase, transferrin, and BSA are well resolved on the Macro-Prep 50 Q column (Fig. 4a) as are myoglobin, ribonuclease A, and cytochrome *c* on the Macro-Prep 50 S column (Fig. 4b). With the Macro-Prep 50 CM material (Fig. 4c), myoglobin, ribonuclease A, and cytochrome *c* are resolved. Cytochrome *c* was split into two peaks (presumably due to the oxidized and reduced forms of the protein) under the gradient conditions used.

Column lifetime studies over 100 gradient cycles on 10 × 1.0 cm columns indicated that the resins were robust. After 100 gradient cycles, with every tenth cycle

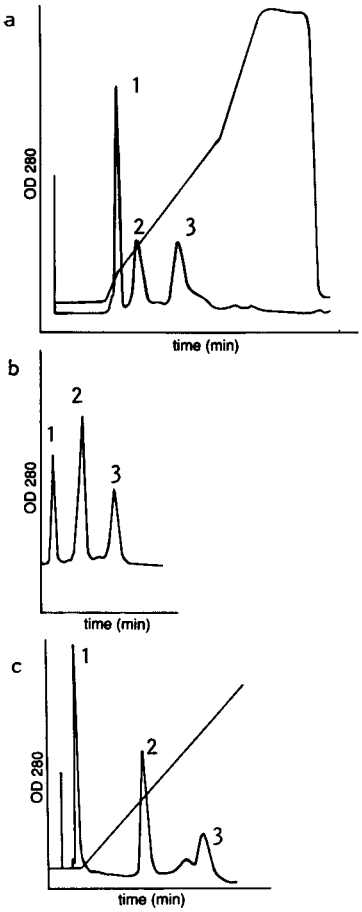

Fig. 4. Chromatography of standard protein mixtures on Macro-Prep 50 Q, S, and CM ion-exchange columns. (a) Separation of (1) bovine carbonic anhydrase B, (2) human transferrin and (3) BSA on a column (30 × 2.5 cm I.D.) of Macro-Prep 50 Q with mobile phase buffers of (A) 50 m*M* Tris–HCl (pH 8.6) and (B) 50 m*M* Tris–HCl (pH 8.6), containing 500 m*M* NaCl at a flow-rate of 4.0 ml/min. The proteins were eluted by a linear gradient of 0 to 50% B over 45 min, followed by a 30 min linear gradient to 100% B. (b) Separation of (1) myoglobin, (2) ribonuclease A, and (3) cytochrome *c* on a column (30 × 1.0 cm I.D.) of Macro-Prep 50 S with mobile-phase buffers of (A) 20 m*M* phosphate (pH 8.0) and (B) 20 m*M* phosphate (pH 8.0), containing 1.0 *M* NaCl. The proteins were eluted by a linear gradient of 0 to 70% B over 20 min at a flow-rate of 3.0 ml/min. (c) Separation of (1) myoglobin, (2) ribonuclease A and (3) cytochrome *c* on a column (20 x 1.0 cm I.D.) of Macro-Prep 50 CM with mobile-phase buffers of (A) 20 m*M* HEPES (pH 8.2) and (B) 20 m*M* HEPES (pH 8.2), containing 1.0 *M* NaCl. The proteins were eluted by a linear gradient of 0 to 60% B over 25 min at a flow-rate of 3.0 ml/min.

including an injection of a protein mixture, the performance was unchanged. The chromatograms in Fig. 5 for the first and hundredth cycle on the Macro-Prep 50 S column depict very similar profiles, demonstrating that the resin is stable under these conditions. Similar results, not shown, have been obtained for the Macro-Prep 50 Q and CM supports.

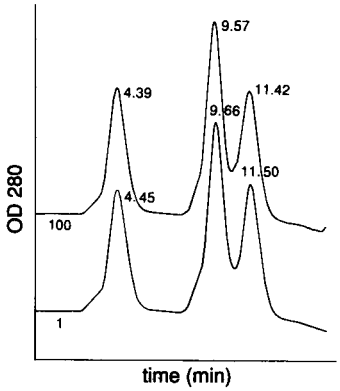

Fig. 5. A Macro-Prep 50 S column (10 × 1.0 cm I.D.) was subjected to 100 gradient cycles. Every tenth cycle, the protein standard (myoglobin, ribonuclease A, and cytochrome *c*) was injected onto the column. The chromatograms are shown for injection during the first and hundredth cycle.

Antibody purification from serum

The utility of these new chromatographic materials was demonstrated by the purification of antibodies against Klenow DNA polymerase from goat serum, using Macro-Prep 50 S, a strong-acid cation exchanger, as a first chromatographic step. Buffer and pH conditions were optimized for the purification of IgG on the Macro-Prep 50 S resin. In data not shown, similar separations were observed with several different buffers, including HEPES, MES, MOPS, and sodium phosphate.

The Macro-Prep 50 S material in 5.0 ml prepacked Econo-Pac cartridges provided a convenient way for separating IgG from goat serum, as shown in Fig. 6a. For

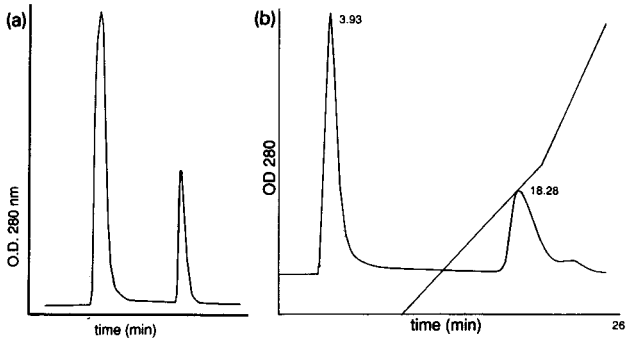

Fig. 6. Fractionation of goat serum on Macro-Prep 50S. (a) Dialyzed goat serum (2.0 ml) was fractionated on an Econo-Pac S cartridge, containing 5.0 ml of the Macro-Prep S material, equilibrated in 20 m*M* MOPS buffer (pH 6.8). The proteins were eluted by a 0–100% gradient over 20 min, using 20 m*M* MOPS buffer (pH 6.8), containing 1.0 *M* NaCl, at a flow-rate of 1.0 ml/min. (b) Goat serum, dialyzed against 20 m*M* MOPS (pH 6.8), was fractionated on a Macro-Prep 50 S column (10 × 1.0 cm I.D.), equilibrated in 20 m*M* MOPS buffer (pH 6.8). The column was washed for 10 min with the equilibration buffer, followed by a 0–50% gradient over 10 min with 20 m*M* MOPS buffer (pH 6.8), containing 1.0 *M* NaCl at a flow-rate of 1.0 ml/min.

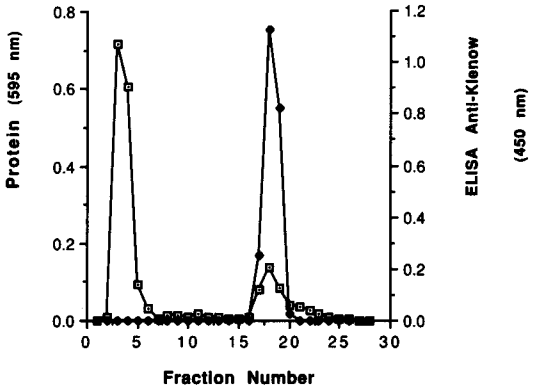

Fig. 7. Anti-Klenow activity from goat serum fractionation on Macro-Prep 50 S material. Fractions were collected from the separation shown in Fig. 6b and analyzed for Klenow binding activity (◆), as described under Materials and Methods. Protein (□) was determined on aliquots in microtiter plates with the Bio-Rad Protein Assay, using a Model 3550 Microplate Reader at 595 nm.

comparison, the same sample was chromatographed on a 10 × 1.0 cm column of the Macro-Prep 50 S packing (Fig. 6b). Identity of the two major peaks was confirmed with a Bio-Sil® SEC-250 gel filtration column (data not shown) and by SDS-PAGE (Fig. 8). Under these chromatographic conditions, most of the albumin is eluted in the first peak with 20 m*M* MOPS buffer (pH 6.8) while the IgG is bound and subsequently eluted with a salt gradient. Up to 10 ml of dialyzed goat serum was processed on the column (Fig. 6b) without loss in IgG purity; however, with higher loads some loss in recovery of IgG was noted. This step has been scaled up to purify

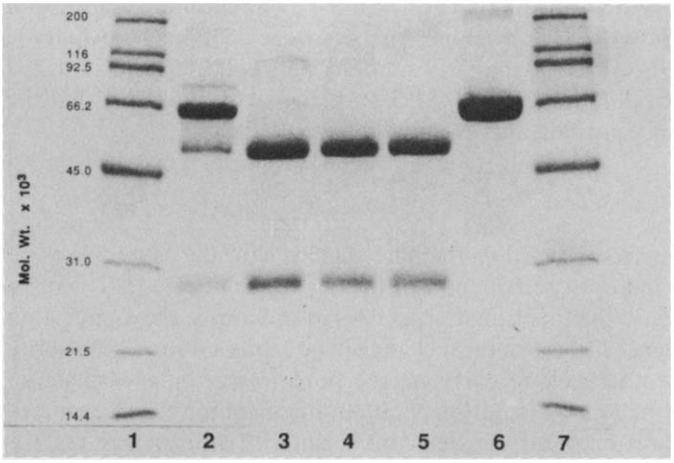

Fig. 8. SDS-PAGE analyses of goat serum fractions. SDS-PAGE on 12% gels under reducing conditions: low-molecular-weight Bio-Rad standard (lanes 1 and 7); dialyzed goat serum, (lane 2); IgG pool from Macro-Prep S column (lane 3); IgG pool from Econo-Pac HIC cartridge (lane 4); goat IgG standard (lane 5); goat albumin standard (lane 6).

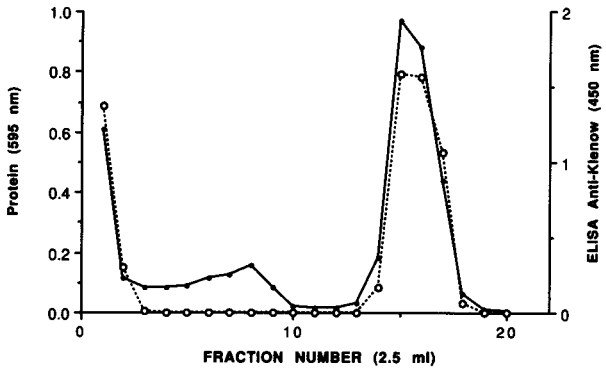

Fig. 9. Purification of IgG pool on Econo-Pack HIC cartridge. The IgG pool (1.5 ml) from the Macro-Prep 50 S chromatography was mixed with 1.5 ml of buffer A [20 m*M* phosphate (pH 6.8), containing 2.4 *M* $(NH_4)_2SO_4$] and added to an Econo-Pac HIC cartridge (5.0 ml), equilibrated in buffer A. The column was eluted at 1.0 ml/min for 8 min with 25% buffer B [20 m*M* phosphate (pH 6.8)], followed by a 20-min gradient from 25 to 100% B. Fractions were assayed for Klenow binding activity (○) and protein (●).

0.9 g of antibody, following chromatography of 100 ml of the dialyzed goat serum on a 20 × 2.5-cm column of Macro-Prep 50 S material. The profile obtained with this column was similar to those seen with the 5-ml cartridge and smaller column.

Fractions were collected from the Macro-Prep S column (Fig. 6b) and assayed for protein and specific anti-Klenow antibodies (Fig. 7). Klenow binding activity was present only in the second peak containing the IgG. The SDS-PAGE results in Fig. 8 show that the pooled IgG fraction from the Macro-Prep S column was relatively pure, with minor contaminants of albumin and other (unidentified) components. To purify the IgG from the Macro-Prep 50 S column further, a second step consisting of hydrophobic-interaction chromatography (HIC) was performed. The antibody fraction from the Macro-Prep S step was diluted 1:1 with 2.4 *M* ammonium sulfate and injected onto a 5.0-ml Econo-Pac HIC cartridge. This cartridge contains hydrophobic methoxy groups on the Macro-Prep 50 matrix. Fractions were collected and analyzed for protein and anti-Klenow binding (Fig. 9). SDS-PAGE (Fig. 8) indicated that the antibody fraction was highly purified and essentially free of albumin.

DISCUSSION

The geometric parameters concerning the pore structure for the Macro-Prep 50 resins were determined by mercury porosimetry of the dry beads (Table I). To correlate the porosimetry data with the functional properties of the resins, the assumption is made that the resins undergo little structural change in aqueous solutions. To verify this assumption, it will be necessary to correlate the porosimetry data with steric-exclusion chromatography in aqueous solution [9,10]. In the scanning electron micrograph, the Macro-Prep 50 resin appears as spherical beads with a distinctive macroporous structure on their surface and interior. The rigidity and mechanical strength of the resins were demonstrated by the minimal swelling and shrinking in aqueous solution between pH 4 and 10 and at salt concentrations up to 1.0 *M* (Table I), and the pressure–flow-rate study shown in Fig. 3. Linear flow-rates up to 3800 cm/h could be

obtained without collapse of the chromatographic bed. The column lifetime studies of the ion exchangers (Fig. 5) indicated no deformation or increase in backpressure, that would be indicative of collapse of the resin.

In protein separations, two important geometric parameters of a resin are the mean pore size and pore-size distribution. These determine the accessibility of the internal pore surface to different proteins. Unger *et al.* [11] have stated that the pore diameter must be a least five times the protein diameter to permit access of the protein to the total internal surface. They concluded that the pore diameters should be large and the particle diameters should be small to minimize the deleterious effects of pores on resolution. It has been estimated that a 1000-Å pore size would allow almost complete access to the internal surface for molecules of $> 10^5$ MW [12]. Sorbents with increasingly larger pore sizes have been developed for ion-exchange chromatography of proteins. However, beads with large pores typically exhibit broad pore-size distributions, including a significant fraction with small pores. Ritchie *et al.* [13] have demonstrated that in size-exclusion chromatography, resolution can be increased with a very narrow pore size distribution. This ensures that the sample is loaded in the minimum column volume. With large pore macroporous beads, more than 95% of the surface area occurs within the pore [3,13], placing significant performance requirements on the pore structure. In these macroporous packings the detailed structure and its effects on the microenvironment of the ionic groups is largely unknown, with uncertainties for the solute access being greatest for the smallest pores [10].

The Macro-Prep 50 beads exhibit an unimodal pore size distribution (Fig. 2) with an unusually high percentage of large pores of an average of 1200 Å. Only a minor fraction of small pores are present. In practical terms, this means that a large percentage of the functional groups on the internal surface is accessible for binding a wide molecular weight range of proteins. The experimental data to support this supposition are the relative independence of molecular weight of the static protein binding capacities for each of the ion-exchange resins (Table II). For example, the Macro-Prep 50 Q material exhibited a binding capacity of 24 mg/ml for thyroglobulin (660 000 MW), 23 mg/ml for ferritin (450 000 MW), and 17 mg/ml for BSA (68 000 MW).

The Macro-Prep 50 packings appear to be biologically safe for pharmaceutical applications. Extracts from the three ion-exchangers showed no sign of animal toxicity, tissue culture cytotoxicity, or mutagenicity in the Ames test. These ion-exchange resins show excellent chemical and thermal stability, as no decrease in protein-binding capacity was observed after 3 days with 1.0 *M* sodium hydroxide or 1.0 *M* hydrochloric acid. HIC resins are less chemically stable to base, but they can tolerate 0.1 *M* sodium hydroxide (data not shown). The resins can be autoclaved without loss in protein binding capacity. These results suggest that the materials can be easily and safely sanitized. The mechanical stability was further demonstrated in column lifetime studies. For example, the Macro-Prep 50 S material exhibited no change in the retention time or peak shape of injected protein standards after 100 gradient cycles (Fig. 5).

Macro-Prep 50 resins have been utilized in the purification of antibodies to Klenow DNA polymerase from goat serum. This purification was monitored by SDS-PAGE and ELISA for specific binding to the Klenow DNA polymerase. The initial evaluation was performed on 5.0-ml prepacked cartridges of Macro-Prep 50 S

(Econo-Pac S cartridge) with subsequent scale-up to 8- and 100-ml columns of Macro-Prep 50 S. The antibody fraction collected from the S resin was relatively pure as determined by SDS-PAGE. Further purification was achieved using a 5.0 ml Econo-Pac HIC prepacked cartridge to yield a highly purified IgG.

ACKNOWLEDGEMENTS

We would like to acknowledge the following individuals who contributed significantly to this investigation: David Cacciatore, Chris Moran, Harvard Morehead, Ted Tisch, Dr. Eberhardt Kuhn and Dr. Curt Munson.

REFERENCES

1 E. A. Peterson and H. A. Sober *J. Am. Chem. Soc.*, 78 (1956) 751.
2 S. R. Himmelhoch and E. A. Peterson *Anal. Biochem.*, 17 (1966) 383.
3 F. E. Regnier, *Anal. Biochem.*, 126 (1982) 1.
4 Y. Kato, K. Nakamura and T. Hashimoto, *J.Chromatogr.*, 245 (1982) 193.
5 C. M. Joyce and N. D. F. Grindley, *Proc. Natl. Acad. Sci. U.S.A.*, 80 (1983) 1830.
6 M. M. Bradford, *Anal. Biochem.*, 72 (1976) 248.
7 P. K. Smith. R. I. Krohn, G. T. Hermanson, A. K. Mallia, F. H. Gartner, M. D. Provenzano, E. K. Fujimoto, N. M. Goeke, B. J. Olson and D. C. Klenk, *Anal. Biochem.*, 150 (1985) 76.
8 U. Laemmli, *Nature (London)*, 227 (1970) 680.
9 K. Jerabek, *Anal. Chem.*, 57 (1985) 1595.
10 A. Guyot, *Reactive Polymers*, 10 (1989) 113.
11 K. K. Unger, R. Janzen and G. Jilge, *Chromatographia*, 24 (1987) 144.
12 G. Vanecek and F. E. Regnier, *Anal. Biochem.*, 121 (1982) 156.
13 H. J. Ritchie, P. Ross and D. R. Woodward, *Chromatography and Analysis*, October (1990) 9.