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Characterization of synthetic macroporous packing materials in low-pressure cartridges and columns

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ABSTRACT

Three ion-exchange packing materials, Macro-Prep 50 S, Macro-Prep 50 CM and Macro-Prep 50 Q (strong cation, weak cation and strong anion, respectively) were characterized with respect to dynamic protein-binding capacities and chemical stability and resolution following exposure to 0.1 and 1.0 *M* NaOH. The pore-size distribution in the hydrated state was determined by size-exclusion chromatography on a Macro-Prep *t*-Butyl hydrophobic interaction (HIC) sorbent. Isolation of anti-Klenow antibodies from goat serum was performed on a 1-l Macro-Prep 50 S column. Specific anti-Klenow antibodies were affinity purified on a semi-preparative scale using Klenow coupled to an Affi-Prep 10 (NHS activated) sorbent. Recombinant Klenow polymerase from *Escherichia coli* was purified in one chromatographic step on an Econo-Pac heparin 5-ml cartridge.

INTRODUCTION

The introduction of ion-exchange cellulose particles as a column packing material in the 1950s and 1960s was a significant event in the history of protein purification [1,2]. During that period, polysaccharide-based chromatographic packing materials [3] were introduced for use in a broad range of applications, including ion exchange [4]. Over the next three decades, improvements in the physical and chemical properties of ion-exchange sorbents led to the current, highly efficient materials suitable for the high-performance liquid chromatography (HPLC) of biopolymers [5].

Enhanced resolution was accomplished by going to smaller and smaller particle sizes. The use of particles with diameters $\leqslant 10 \ \mu$m as packing materials in HPLC required expensive high-pressure equipment, columns and hardware designed to withstand these pressures. These high-performance sorbents were primarily designed for analytical separations and not as sorbents in preparative chromatography. Hence there was a need for new packing materials. Reducing downstream-processing costs and increasing the scale of high-performance separa-

tions placed new demands on chromatographic materials. This necessitated a better understanding of the physical and chemical properties of sorbents used in process chromatography.

Pharmaceutical and biotechnology industries need chromatographic materials with sufficient chemical stability to withstand harsh sanitation conditions, good mechanical strength to hold up under the pressures and flow-rates required for large-scale separations, and high dynamic loading capacities, with high recoveries of biological activity. To meet these rigorous standards, a new macroporous methacrylate polymer, Macro-Prep 50 sorbent, was developed for process chromatography. The physical and chemical properties of these macroporous sorbents have been described [6], including pore structure, ionic capacities, static protein binding capacities and chemical and thermal stability. Some of the studies were performed on Econo-Pac cartridges (prepacked devices for low-pressure chromatography). These cartridges are easy to use and come in a convenient size before scale-up to larger preparative columns.

In continuation of this characterization, data are presented in this paper on the size-exclusion proper-

ties, dynamic protein-binding capacities, chemical stabilities and resolution of Macro-Prep 50 sorbents. To explore the potential of the new Macro-Prep sorbents in biological applications, a Klenow/anti-Klenow antibody system has been studied. Klenow, which is the fragment representing the carboxyl-terminal two-thirds of the DNA polymerase 1 [7], has been cloned and overproduced in *Escherichia coli* [8]. Klenow is an important enzyme in molecular biology, and is used in dideoxy methods for DNA sequencing. The purification of anti-Klenow from goat serum on Macro-Prep 50 S sorbent has been demonstrated [6]. This application is expanded to include (a) preparative isolation of goat antibodies on a 1-l Macro-Prep 50 S column, (b) purification of the specific anti-Klenow antibodies on an Affi-Prep 10–Klenow affinity column and (c) semi-preparative purification of recombinant Klenow, from *E. coli*, using an Econo-Pac heparin cartridge.

EXPERIMENTAL

Materials

Goat serum (Bethyl Labs., Montgomery, TX, USA) was obtained from goats inoculated with Klenow DNA polymerase. Affi-Prep 10, Macro-Prep *t*-Butyl HIC, Macro-Prep 50 S sorbent, Econo-Pac S, CM, Q, and heparin cartridges, Coomassie Brilliant Blue R-250, MAPS binding buffer, protein assay standard I (bovine γ-globulin), protein dye reagent, protein cation and anion HPLC standards, sodium dodecyl sulfate (SDS), dithiothreitol (DTT), Tween 20, horseradish peroxidase-conjugated protein G, Klenow DNA polymerase and TMB substrate (3,3′,5,5′-tetramethylbenzidine) were obtained from Bio-Rad Labs. (Richmond, CA, USA). Bovine serum albumin (BSA), cytochrome *c*, blue dextran, ferritin, human immunoglobulin G (IgG), *Staphylococcus aureus*, thimerosal and thyroglobulin were obtained from Sigma (St. Louis, MO, USA). Ovalbumin was obtained from Calbiochem-Boehring (La Jolla, CA, USA). Glycine, ethanolamine and isopropanol were obtained from Aldrich (Milwaukee, WI, USA). All other chemicals were of analytical-reagent grade.

Chromatography

HPLC was performed with an HRLC Model 800 chromatography system (Bio-Rad Labs.). Low-pressure liquid chromatography was performed with 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 Labs.). Fractions were collected with a Model 2110 fraction collector (Bio-Rad Labs.). All buffers were filtered through 0.2-μm membrane filters (Gelman Sciences, Ann Arbor, MI, USA).

Size exclusion

A column (30 × 1 cm I.D.) was packed under gravity with Macro-Prep *t*-Butyl HIC sorbent to a height of 16.7 cm. The column was equilibrated with phosphate-buffered saline (PBS: 20 mM phosphate + 140 mM NaCl, pH 7.2) and run at a linear velocity of 15.3 cm/h (0.2 ml/min). The calculated column volume was 13.1 ml. *Staphylococcus aureus* was used to determine the interstitial volume, V_o (5.6 ml), potassium chromate was used to determine the volume of solvent in the column, V_t (10.3 ml), and V_e were determined (in duplicate) for blue dextran (2·10^6 dalton), thyroglobulin (670 000 dalton), ferritin (440 000 dalton), bovine serum albumin (BSA) (68 000 dalton), ovalbumin (44 000 dalton) and cytochrome *c* (12 000 dalton).

Chemical stability of Macro-Prep 50 ion exchangers

The chemical stability of Macro-Prep 50 CM, Q and S sorbents in 5-ml Econo-Pac cartridges was investigated using the following protocol. One hundred gradient cycles were run from 0 to 100% B in 10 min at a flow-rate of 1.0 ml/min on a Model 800 HRLC gradient system, equipped with a Bio-Rad Labs. Model AS-100 autosampler. The buffers used in the gradient cycles were buffer A, 20 mM phosphate (pH 6.9), and buffer B, A + 1.0 M NaCl. Each gradient cycle was followed by a 35-ml wash with 0.1 M NaOH (1.0 ml/min). Protein standards were injected onto the cartridges at the end of the wash cycle and the chromatograms were compared with those obtained for a cartridge not previously exposed to base.

The CM, Q and S sorbents were also tested by washing with 1 M NaOH at 1.0 ml/min using a peristaltic pump for periods of up to 72 h. The Econo-Pac cartridges were removed periodically, equilibrated in 25 mM phosphate buffer (pH 8.0) and connected to a Model 800 HRLC gradient system, equipped with a Bio-Rad Labs. Model AS-100 au-

tosampler. The protein cation standard was analyzed on the Econo-Pac 50 CM and S sorbents and the protein anion standard on the Q sorbent.

Dynamic protein-binding capacity

Protein samples were loaded onto Macro-Prep 50 sorbents packed in 5.0-ml Econo-Pac cartridges in the indicated buffers at flow-rates of 0.50, 1.0, 5.0 and 10.0 ml/min until the absorbance at 280 nm of the effluent reached 50% of the absorbance of the starting protein solution. The cartridge was washed free from unbound protein, at a flow rate no greater than that used for loading, until the absorbance of the effluent dropped to ≤2% of the maximum absorbance of the starting protein solution. Bound protein was eluted using the buffers indicated below. BSA (3.0 mg/ml) was bound to the CM sorbent in 20 mM sodium acetate (pH 5.0) and eluted with this buffer containing 1.0 M NaCl. BSA (3.0 mg/ml) and ferritin (0.1 mg/ml) were bound to the Q sorbent in 50 mM Tris (pH 8.3) and eluted with this buffer containing 2.0 M NaCl. IgG (0.5 mg/ml) and cytochrome c (1.0 mg/ml) were bound to the S sorbent in 20 mM sodium acetate (pH 5.0) and eluted with this buffer containing 1.5 M NaCl. Protein concentrations were determined from absorbance values measured at 280 nm.

Isolation of the IgG fraction from goat serum

The IgG fraction containing the anti-Klenow antibodies was isolated from goat serum on the Macro-Prep 50 S sorbent packed in a column (23 × 7.5 cm I.D.). After equilibrating the column in buffer A [20 mM MOPS (4-morpholinepropanesulfonic acid), pH 6.8)], goat serum (1 l; 122 g of protein) dialyzed against buffer A was applied to the column at a flow-rate of 40 ml/min. Proteins were eluted using a linear salt gradient from 0 to 50% buffer B (buffer A + 1.0 M NaCl) in 100 min. Fractions were collected and analyzed for Klenow binding and protein.

Protein assay

Protein assays were generally performed in microtiter plates using the Bio-Rad Labs. protein assay [9]. Calibration graphs were prepared using bovine γ-globulin (standard 1). Typically, wells were filled with 5–40 μl of sample. The total volume in the wells was adjusted to 40 μl with sample buffer

and 250 μl of fourfold diluted protein dye concentrate were added to each well. The plates were incubated at room temperature for 10 min and then read at 595 nm using a Model 3550 microplate reader (Bio-Rad Labs.).

SDS–polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoretic separations were performed under reducing conditions by the method of Laemmli [10] on 7.5% or 12% pre-cast Mini-PROTEAN II ready gels (Bio-Rad Labs.), using the Mini-PROTEAN II cell and a Model 500/200 power supply (Bio-Rad Labs.). Staining was done with Coomassie Brilliant Blue and quantification of the gels was performed using a Model 620 video densitometer (Bio-Rad Labs.).

Enzyme-linked immunosorbent assay (ELISA)

Antibody detection. Determination of specific antibody activity against Klenow was performed by coating wells of microtiter plates (Bio-Rad Labs.) overnight at 4°C with 50 μl of Klenow at a concentration of 1.0 μg/ml in 50 mM sodium carbonate buffer (pH 9.6). Plates were washed once with 200 μl of PTT [20 mM sodium phosphate (pH 7.2) containing 120 mM sodium chloride, 0.05% Tween 20 and 0.01% thimerosal]. Wells were incubated for 2 h at room temperature with 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 storing at 4°C. Samples were assayed for antibody-binding activity by adding duplicate 50-μl aliquots diluted in PTTB to the Klenow-coated plates for 1–3 h at room temperature. The plates were washed twice with PTT and incubated for 1 h at room temperature with 100 μl of horseradish peroxidase-conjugated protein G diluted 1/10 000 in PTTB. The plates were then washed three times with PTT and the enzyme activity was measured by adding 100 μl of the 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.

Klenow detection. For the detection of Klenow, plates were coated overnight at 4°C with 50 μl of goat anti-Klenow antibodies at a concentration of

46 µg/ml in 50 mM sodium carbonate buffer (pH 9.6). The incubations and wash steps were similar to those described above for the antibody ELISA, except that the enzyme conjugate used was horseradish peroxidase conjugated to the goat anti-Klenow antibody. The conjugate was prepared according to the procedure of Wilson and Nakane [11].

Coupling of Klenow to Affi-Prep 10

Affi-Prep 10 is a N-hydroxysuccinimide (NHS)-activated macroporous polymeric sorbent [12] used for the covalent coupling of proteins via their free amino groups. Affi-Prep 10 (12 ml of a 50% suspension in isopropanol) was transferred to a 15-ml coarse sintered-glass funnel and rapidly washed with three 5-ml aliquots of cold deionized water, acidified with acetic acid to pH 4. Immediately following these washes, 4.0 ml (2.17 mg/ml) of purified Klenow (Bio-Rad Labs.) in 100 mM sodium carbonate (pH 8.3) were added carefully to the top of the 6-ml sorbent bed and allowed to drain through the bed over a 6-min period. The sorbent was washed with 8.0 ml of 100 mM sodium carbonate (pH 8.3) and the effluent collected and saved for protein assay. The sorbent was then washed with 8 ml of 1.0 M ethanolamine (pH 8.5) to block any unreacted NHS sites, and washed with 15 ml of deionized water, 15 ml of MAPS binding buffer, pH 9.0 (Bio-Rad Labs.), 15 ml of deionized water, 15 ml of 100 mM glycine (pH 2.5) and 15 ml of PBS. A total of 4.2 mg of Klenow was coupled to 5.9 ml of Affi-Prep 10, as determined by the difference between the total protein added and the unbound protein.

Affinity purification of anti-Klenow antibody

The IgG fraction from the Macro-Prep 50 S column (Fig. 3) containing anti-Klenow antibodies was dialyzed against PBS. An aliquot (4.0 ml) containing 36 mg of protein was injected onto 5.9 ml of Affi-Prep 10–Klenow affinity sorbent (7.5 × 1.0 cm I.D.) equilibrated in PBS and recycled over this column for at least 2 h at 0.5 ml/min. The column was then washed with PBS until the absorbance at 280 nm of the effluent was ≤0.05. The bound anti-Klenow antibody (4.2 mg) was eluted from the affinity sorbent at 0.5 ml/min using 100 mM glycine (pH 2.5). Immediately after elution, the pH of the antibody fraction was adjusted between 6 and 8 with 1.0 M Tris base (pH 10.6) and the sample was dialyzed against PBS.

Isolation of recombinant Klenow from E. coli

Recombinant Klenow was obtained from *E. coli* cells. The first steps of the purification were similar to those described by Joyce and Grindley [8]. Lysed cells were centrifuged at 10 000 g and the supernatant was treated with polyethyleneimine (0.2%) and sodium chloride (0.2 M) to precipitate nucleic acids and some proteins. The supernatant was adjusted to 60% ammonium sulfate, centrifuged and the supernatant dialyzed against 10 mM potassium phosphate (pH 7.0) containing 1 mM DTT. The Klenow was then purified on an Econo-Pac heparin 5-ml cartridge, which was equilibrated with buffer A [10 mM potassium phosphate (pH 7.0) + 1 mM DTT]. Samples of the supernatant were loaded onto the cartridge at a flow-rate of 2.0 ml/min. After washing with buffer A to remove unbound proteins, Klenow was eluted from the cartridge using a 30-min linear salt gradient from 0 to 100% B (A + 0.5 M NaCl). Fractions were assayed for protein and binding to anti-Klenow antibodies by ELISA.

RESULTS AND DISCUSSION

Physical properties and stability

In protein separations on macroporous sorbents, two important geometric parameters to consider are the mean pore size and pore-size distribution. These physical characteristics determine the accessibility of the internal pore surface of the bead to proteins. The surface and pore structure of the Macro-Prep beads have been characterized by mercury intrusion porosimetry [6]. These beads have a mean particle size of 50 µm, surface area 18–22 m²/g, pore size 1200 Å and pore volume ≥ 0.5 ml/g. A unique feature of these sorbents is that most of the pore volume is found between 1000 and 1500 Å [6].

One of the limitations of mercury intrusion porosimetry is that the analysis must be done on a dry powder. As chromatography is done on a hydrated bead, the porosimetry results may not accurately reflect the microenvironment of the beads. To obtain a pore-size profile of the hydrated beads, size-exclusion chromatography [13] was performed on a Macro-Prep *t*-Butyl HIC packing material in PBS.

As this experiment was done under low salt conditions (140 mM NaCl), hydrophobic interaction effects, which are maximized at high salt concentrations, should be minimal. Hence, the relative elution order should not be influenced by the HIC functionality.

The average distribution coefficients, K_{ave}, can be calculated from $K_{ave} = (V_e - V_o)/(V_t - V_o)$, where V_o is the interstitial volume, V_t is the total volume of solvent in the column and V_e is the elution volume of the protein sample. *Staphylococcus aureus* was used to determine the interstitial volume (estimated molecular weight *ca.* 10^9 dalton). The volume of solvent within the sorbent is given by $V_i = V_t - V_o$. From these calculations, the working volume of the Macro-Prep sorbents is 36%. The plot of K_{ave} *versus* log (molecular weight) for a number of proteins is shown in Fig. 1. If the mid-point between blue dextran (10^6 dalton) and thyroglobulin ($6.7 \cdot 10^5$ dalton) represents the mean exclusion limit for the Macro-Prep sorbent, then proteins up to 10^6 dalton should be able to penetrate the pores. From the mercury porosimetry data, a mean pore size of 1200 Å was calculated for the Macro-Prep sorbent [6]. These experimental data are consistent with the prediction made by Vanecek and Regnier [14] that a 1000 Å pore should allow free access to proteins $> 10^5$ dalton. In addition, Unger *et al.* [15] predicted that the pore diameter must be at least five times the protein diameter to permit access of the protein to the internal surface. In order for thyroglobulin, which has a diameter of 172 Å [16], to permeate the sorbent, the minimum pore size must be 860 Å. Hence the size-exclusion data presented here are

Fig. 1. Size-exclusion study on Macro-Prep butyl HIC sorbent packed in a Bio-Rex MP column (16.7 × 1.0 cm I.D.).

consistent with the earlier reported porosimetry results [6].

Mechanical strength is an important prerequisite for any cost-effective chromatographic matrix for downstream processing in the biotechnology industry. Mechanical strength of the Macro-Prep sorbents in aqueous solutions has been demonstrated by measuring back-pressures at increasing flow-rates. The maximum linear velocity that could be reached is 3800 cm/h for the CM, 5700 cm/h for the Q and 4560 cm/h for the S sorbents [6].

As reported previously [6], no changes in performance of Macro-Prep ion-exchange sorbent were observed during 100 gradient cycles in 10 × 1.0 cm I.D. columns. In addition, there was little or no decrease in ionic or protein binding capacity after prolonged treatment with acid, base or detergents. Stability to NaOH is particularly important because this reagent is commonly used to sanitize chromatographic materials. With this in mind, column lifetime and stability studies were performed by subjecting the Macro-Prep 50 CM, Q and S sorbents in Econo-Pac 5-ml cartridges to 100 gradient cycles, with each cycle containing a 35-min wash with 0.1 M NaOH at 1.0 ml/min. Protein standards were injected onto the columns every tenth cycle to evaluate performance parameters. For each of the three ion-exchange sorbents very similar profiles were obtained (data not shown), demonstrating the lifetime stability of these sorbents. To take this investigation one step further, the chemical stability was examined by comparing the separation of protein mixtures in 5-ml cartridges before and after exposure to 1.0 M NaOH. After 4, 24, 48 and 72 h, the sorbents were washed free of NaOH, equilibrated in buffer, and protein standards were injected to evaluate performance. Retention times and symmetry (B/A at 10% peak height) of the protein peaks for the initial injection and after 4, 48 or 72 h exposure were used to measure column performance. The results are shown in Table I. The symmetry factor, B/A, is used to measure peak fronting or tailing and is one criterion used to determine packing efficiency [13].

The values for the CM and S sorbents remained unchanged after 72 h of exposure. The Macro-Prep 50 Q sorbent underwent noticeable changes in retention time after 4 h, and these changes became even more pronounced after 48 h of exposure.
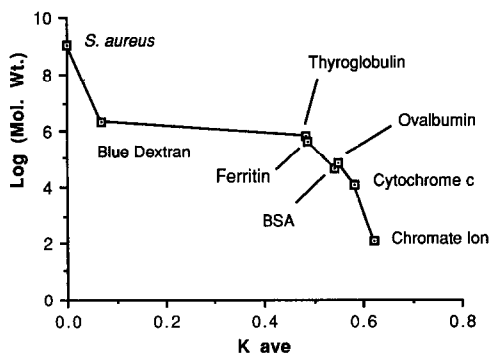
TABLE I

STABILITY OF MACRO-PREP 50 CM, Q AND S SORBENTS IN ECONO-PAC CARTRIDGES IN 1 *M* NaOH

Macro-Prep 50 CM, Q and S sorbents packed in Econo-Pac 5-ml cartridges were washed with 1.0 *M* NaOH. The indicated protein standards were injected before and after exposure for up to 72 h. as described under Experimental. The results are expressed as retention times (RT) and peak symmetry (Sym) (*B* and *A* are measured from the apex perpendicular to the curve at 10% above the baseline).

Protein	Time (h)	CM		Q		S	
		RT (min)	Sym (*B/A*)	RT (min)	Sym (*B/A*)	RT (min)	Sym (*B/A*)
Myoglobin	0	3.9	1.29	5.6	1.09	4.2	1.58
	4	–	–	5.3	1.02	–	–
	48	3.9	1.32	4.8	1.19	4.1	1.02
	72	3.9	1.31	–	–	4.3	0.86
Ribonuclease A	0	7.5	0.64	–	–	6.5	0.70
	3	7.4	0.57	–	–	6.5	0.52
	48	7.4	0.57			6.7	0.47
Cytochrome *c*	0	8.9	1.90	–	–	7.6	2.92
	48	8.7	2.22	–	–	7.5	3.40
	72	8.7	2.25			7.6	3.83
Conalbumin	0	–	–	8.2	0.93	–	–
	4	–	–	7.7	0.89	–	–
	48	–	–	7.4	1.07	–	–
Ovalbumin	0	–	–	10.7	1.11	–	–
	4	–	–	10.2	1.20	–	–
	48	–	–	9.6	1.13	–	–
Trypsin inhibitor	0	–	–	15.2	1.21	–	–
	4	–	–	15.0	0.44	–	–
	48	–	–	14.1	1.05	–	

However, the data in Table I indicate that no significant decrease in resolution of the proteins on the Macro-Prep Q sorbent occurred with the decrease in retention time. The symmetry value of 0.44 for trypsin inhibitor cannot be readily explained, as the symmetry value increases to 1.05 after 48 h. The results of the chemical stability studies indicate that the Macro-Prep CM and S sorbents can be sanitized with 1.0 *M* NaOH and the Q sorbent can be safely sanitized with 0.1 *M* NaOH.

Dynamic protein-binding capacity

Static binding capacities of the three Macro-Prep ion-exchange sorbents have been reported [6]. A more meaningful measure of a packing material's performance is the dynamic binding capacity, which measures the capacity of a sorbent to bind proteins under solvent flow conditions. Dynamic protein-binding capacities of ion-exchange sorbents

(Fig. 2) in 5-ml Econo-Pac cartridges were measured at flow-rates of 0.5, 1.0, 5.0 and 10.0 ml/min. When the flow-rate was increased from 0.5 to 10.0 ml/min, the BSA-binding capacity decreased by 29% for the CM sorbent. For the Q sorbent, the BSA binding decreased by 9.4% and the ferritin binding capacity decreased by 42%. For the S sorbent, the IgG capacity decreased by 55%, whereas the cytochrome *c* capacity increased slightly but was within experimental error. For the CM, Q and S ion-exchange sorbents, the protein-binding capacities of smaller proteins, such as cytochrome *c* (12 000 dalton) and BSA (68 000 dalton), were essentially unaffected by flow-rates as high as 10.0 ml/min. However, the binding capacities for larger proteins such as IgG (150 000 dalton) and ferritin (440 000 dalton) were substantially reduced as the flow-rate was increased.
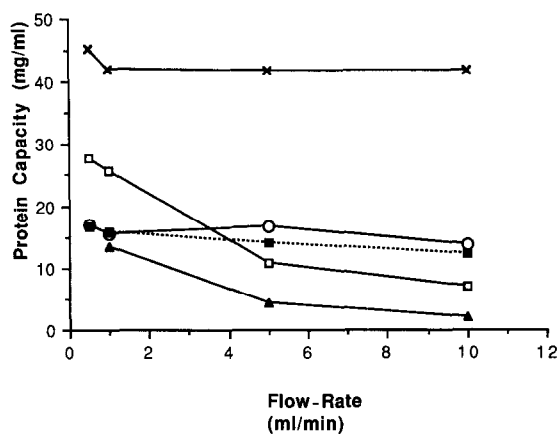
Fig. 2. Dynamic protein binding capacities of Macro-Prep 50 CM, Q and S sorbents *versus* flow-rate. The binding of BSA (■) to the Macro-Prep 50 CM sorbent, BSA (○) and ferritin (▲) to the Macro-Prep Q sorbent and cytochrome *c* (×) and IgG (□) to the Macro-Prep 50 S sorbent, were carried out in Econo-Pac cartridges at the indicated flow-rates as described under Experimental.

Fig. 3. Fractionation of goat serum on Macro-Prep 50 S sorbent. Goat serum (1.0 l), dialyzed against 20 m*M* MOPS buffer (pH 6.8), was applied at 40 ml/min to the column (23 × 7.5 cm I.D.) equilibrated in 20 m*M* MOPS buffer (pH 6.8). The proteins were eluted with a 0–50% gradient over 100 min, using 20 m*M* MOPS buffer (pH 6.8) containing 1.0 *M* NaCl. Fractions of 400 ml were collected and assayed for Klenow binding (○) and protein (▲) as described under Experimental.

Purification of antibodies against Klenow from goat serum

The isolation of an IgG fraction from goat serum using Macro-Prep 50 S sorbent in an Econo-Pac 5-ml cartridge described by Dunn *et al.* [6] was scaled up to a 1-l column (Fig. 3). This column was used to process 1 l of serum to yield 8.8 g of IgG which was >96% pure by SDS-PAGE (Fig. 5, lane 3). Hence this IgG purification demonstrated the scale-up capabilities from a 5-ml Econo-Pac cartridge to a 1-l column.

Specific anti-Klenow antibodies were purified on a Klenow affinity sorbent, prepared by coupling purified Klenow to Affi-Prep 10, a pressure-stable polymer derivatized with a N-hydroxysuccinimide (NHS) ester [12]. Binding of the specific anti-Klenow antibodies to the affinity matrix was achieved by recycling the IgG fraction or goat serum over the affinity sorbent. In one experiment, 4 ml (36 mg of total protein) of the purified IgG fraction from the Macro-Prep 50 S column were loaded onto the column and 100-μl aliquots were withdrawn from the recycling buffer at 0, 1.5, 3 and 16 h. These aliquots were analyzed for Klenow binding acitvities by ELISA. After 1.5 h, no activity was found in the recycled antibody solution, indicating that all of the anti-Klenow antibodies were bound to the sorbent.
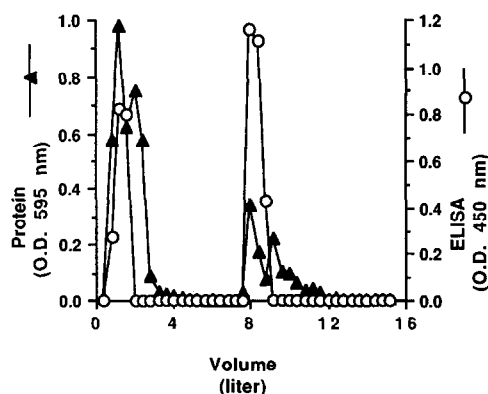
The bound Klenow antibodies (4.2 mg) were eluted from the column using 100 m*M* glycine (pH 2.5). Under conditions of antibody excess, the affinity column had a maximum capacity to bind 6.9 mg (46 nmol) of Klenow antibody. Based on the binding of one antibody molecule per Klenow molecule, this represents an 84% binding efficiency of the affinity sorbent. The purification of anti-Klenow on the affinity sorbent was confirmed in ELISA titrations (data not shown). In these experiments, serial three-fold dilutions of each of the three fractions (goat serum, IgG fraction from the Macro-Prep 50 S column and affinity-purified anti-Klenow antibodies) were assayed for Klenow-binding activity by ELISA. Based on the protein required to attain 50% of maximum activity, the affinity-purified anti-Klenow exhibited a three-fold higher specific activity than the IgG fraction, which in turn had a twelve-fold higher activity than the starting goat serum.

Purification of recombinant Klenow from E. coli

Recombinant Klenow was initially purified as described by Joyce and Grindley [8] to obtain a 60% ammonium sulfate supernatant and was further purified by passing 15 ml of the crude supernatant over an Econo-Pac heparin cartridge (Fig. 4). He-
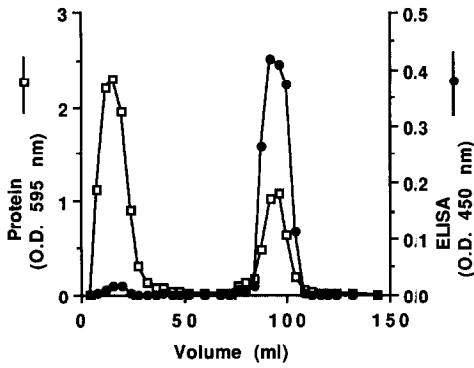
Fig. 4. Purification of Klenow on an Econo-Pac heparin cartridge. An aliquot of 15 ml of the ammonium sulfate supernatant (2.4 mg/ml) was applied to an Econo-Pac heparin 5-ml cartridge equilibrated in 10 mM potassium phosphate buffer (pH 7.0) containing 1 mM DTT (loading buffer). Bound proteins were eluted with a 30-min linear gradient of 0 to 0.5 M NaCl in 10 mM potassium phosphate buffer (pH 7.0) containing 1 mM DTT at 2.0 ml/min. Fractions of 4.0 ml were collected and assayed for binding to anti-Klenow using ELISA (●) and protein (□) as described under Experimental.

parin is a linear glycosamine–glycan composed of mostly sulfated 1,4-linked glycosamine and glucuronic acid residues. Proteins bind to heparin ionically or by more specific interactions. The capacity of the heparin cartridge was 1.3 mg/ml. The Klenow was assayed by ELISA using the affinity-purified anti-Klenow described above, conjugated to horseradish peroxidase. Most of the protein did not bind to the heparin sorbent, whereas all the Klenow was bound and eluted as a single peak in the salt gradient. The SDS-PAGE analysis (Fig. 5, lanes 5, 6 and 7) of the proteins in the crude cell extract fraction and the bound and unbound fractions indicated that the bound protein was a single band which migrated the same as the purified Klenow standard.
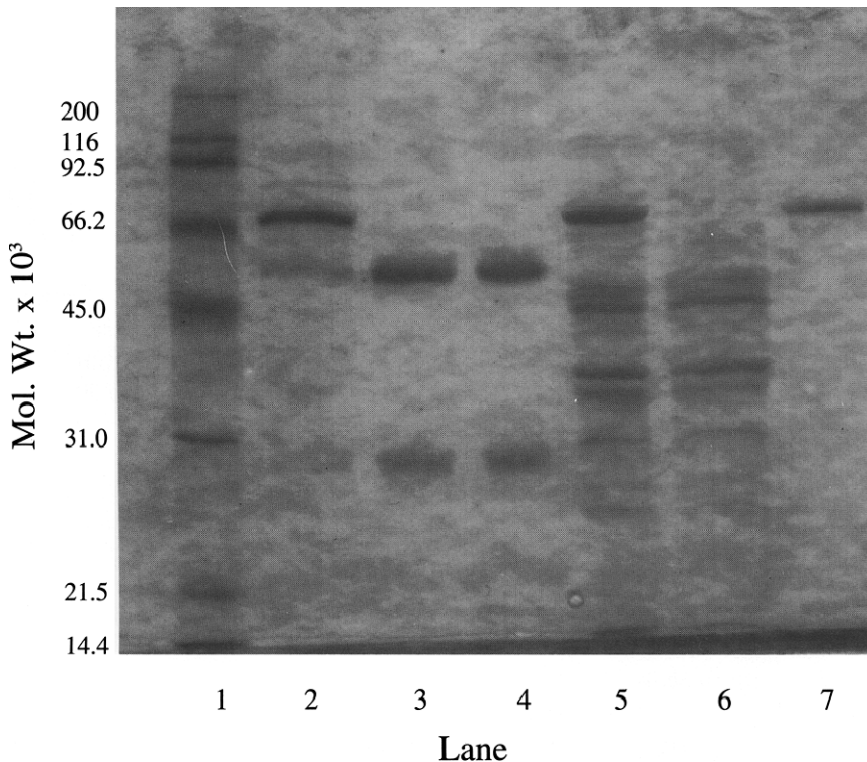

Fig. 5. SDS-PAGE analysis of proteins on 12% gels under reducing conditions. Samples: high- and low-molecular-weight Bio-Rad Labs. standards (lanes 1), dialyzed goat serum (lane 2), IgG pool from Macro-Prep 50 S column (lane 3), goat IgG standard (lane 4), crude cell extract containing Klenow (lane 5), unbound fraction from crude cell extract on Econo-Pac heparin cartridge (lane 6) and bound fraction (Klenow) from Econo-Pac heparin cartridge (lane 7).

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