Journal of Chromatography, 503 (1990) 93–102
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM 22 127

Preparative affinity chromatography of proteins

Influence of the physical properties of the base matrix

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(First received August 7th, 1989; revised manuscript received October 31st, 1989)

SUMMARY

Several silica-based high-performance affinity media differing in pore size and surface area were synthesized for the immobilization of proteins containing primary amino groups. Surface characterization of these silica-based media was carried out by mercury intrusion porosimetry and the pore surface area and pore diameters were compared. The intrinsic binding capacities for proteins of different molecular weights were determined by static binding studies. Concanavalin A was covalently immobilized on these media and its dynamic binding capacity was determined in the affinity mode by frontal uptake studies. The studies show that binding capacity increases with increasing pore size, then decreases as the pore size becomes sufficiently large to significantly decrease the surface area. Thus, an ideal affinity chromatographic support would have sufficiently large pores accessible for proteins to penetrate, yet would preserve the maximum surface area for binding. These studies suggest that a final bonded phase pore diameter of at least 200 Å is desirable for the preparative affinity chromatography of proteins of molecular weight in excess of 150 000 daltons.

INTRODUCTION

As early as 1970, Cuatrecasas[1] pointed out that an affinity sorbent containing a very high concentration of the ligand, much of which is inaccessible to the protein, is not very efficient for chromatography. It was later observed that staphylococcal nuclease, a small protein of molecular weight of 17 000, had to be purified on a very porous support[1]. It is now very well accepted that before a protein can efficiently bind to a porous support, it has to diffuse into the pores[2–4]. Based on these observations, it follows that if the size of the pores is insufficient for the protein to rapidly diffuse, the efficiency of the column will be diminished even if the other characteristics of the medium are suitable for the binding of the protein to the affinity ligand. Physical characterization of the pore structure of the chromatographic supports is therefore necessary for establishing an efficient, cost-effective purification protocol[5].

0021-9673/90/$03.50

The pore structure parameters most commonly used to characterize chromatographic supports include the following: the mean or average pore diameter (Å), the specific pore surface area (m^2/g) and the specific pore volume (ml/g). We have investigated the influence of these parameters with new affinity ligand using silica as a chromatographic support because of its desirable characteristics for high-resolution and high-performance chromatography.

The use of silica for high-performance liquid chromatography (HPLC) is well documented[6–9]. The speed and resolution combined with the ability to obtain high flow-rates and the rigid surface have attracted more and more scientists to use silica-based sorbents for preparative HPLC of proteins[10–12]. Among the various attributes of silica, the most attractive in the context of affinity chromatography is the range of porosities, particle sizes and surface areas that are commercially available from the manufacturers of silica. This feature makes it possible to design a support optimized for a specific purification protocol which is based on the properties of the protein to be isolated as well as the properties of those contaminating the crude extract. Although the above-mentioned factors play a crucial role in any purification method, on a preparative scale the support must also possess good binding capacity in order to be efficient and cost effective.

Several reports have dealt with the effect of particle size, pore size and surface area in HPLC[13–16]. Walters[17] studied the efficiency of diol-bonded silica of varying pore size and concluded that in affinity chromatography two factors were responsible for poor binding capacities at intermediate pore size: restricted diffusion and slow adsorption–desorption kinetics. Horstmann *et al.*[18] observed the binding of proteins on Sepharose-based affinity sorbents of varying particle sizes and reported that the maximum capacities obtained increased with decreasing particle size in Sepharose-based affinity sorbents.

Rounds *et al.*[2] demonstrated that in anion-exchange chromatography, the binding of proteins is dependent on accessible surface area (that is the total pore surface area excluding the external particle surface) rather than the total surface area of the support indicating that wide pores in addition to high surface areas provide maximum capacity. In 1987, Kopaciewicz *et al.*[19] reported that both pore and particle size influence the frontal uptake of proteins in anion-exchange chromatography. They also observed that frontal uptake of proteins was inversely related to flow-rate and particle size of the adsorbent. A ligand density study was conducted by Wu and Walters[20] on silica-based affinity supports of pore size ranging from 300 to 4000 Å and they concluded that the optimum pore size of the silica for protein immobilization was 300–1000 Å. Recently Forster and Anderson[21] studied the effect of pore size on the capacity and efficiency of Protein A-derivatized silicas. Results presented indicated that binding capacity was related to surface area and the ability of the solute to diffuse in and out of the porous silica. The authors suggested improvement in the peak symmetry if the pore size either totally includes or excludes the solute. All these studies have contributed to our understanding of how the surface characteristics of a sorbent influence the binding of molecules, be it in the ion-exchange or affinity mode.

Although the intrinsic binding capacity (that is the amount of affinity ligand bound) of an adsorbent can provide information while choosing an affinity support, it does not always indicate the efficiency or the loading capacity (the amount of protein bound to the affinity ligand) of the adsorbent. The amount of ligand coupled or the

ligand concentration is the first step in affinity chromatography while the amount of protein that will bind to the ligand is the crucial factor that determines the success and efficiency of the purification protocol. Loading capacity in affinity chromatography thus depends on the ligand concentration, the pore size, the particle size and the surface area available for the binding of multiple molecules.

This work deals with systematic evaluation of a newly developed affinity sorbent, Glutaraldehyde-P, for HPLC. We report here the studies conducted on surface characterization of Glutaraldehyde-P preparations on silicas of varying pore sizes and surface areas and attempt to accurately interpret the effect of these physical properties on the binding of proteins of different molecular weights by static adsorption and by frontal uptake measurements in the affinity mode.

MATERIALS AND METHODS

Materials

Proteins used in these studies were purchased from Sigma (St. Louis, MO, U.S.A.). The protein assay reagent was from Biorad. Glutaraldehyde and sodium cyanoborohydride were from Aldrich. All other chemicals were products of J. T. Baker.

Instrumentation

HPLC was conducted on two high-performance pumps (Beckman Instruments), a variable-wavelength detector (Beckman Instruments) and a two-channel recorder (Kipp and Zonen). Pore structure analysis was performed by mercury intrusion porosimetry by using an Autoscan-33 from Quantachrome (Syosset, NY, U.S.A.).

Methods

Activation of the affinity sorbent. Silica was first treated with a hydrophilic polymer according to our patented chemistry, to which glutaraldehyde was covalently attached[22,23]. The amount of aldehyde linked in each case was carefully controlled by optimizing the reaction conditions. The aldehyde content was determined by the procedure of Narayanan *et al.*[22] and Parkinson and Wagner[24].

Sample preparation for mercury porosimetry studies. In order to obtain a representative sample for mercury porosimetry studies, a rotary microriffler (Quantachrome, Syosset, NY, U.S.A.) was used to reduce the sample size to approximately 0.35 g. The sample was dried at 80°C overnight in a vacuum oven, cooled in a desiccator and the weight was accurately determined.

Pore structure analysis. The pore structure analysis of the Glutaraldehyde-P affinity sorbent was carried out by mercury intrusion porosimetry. Assuming the pores to be cylindrically shaped, the basic principle of mercury porosimetry is expressed by the Washburn equation in the following manner

$$Pd = -4\gamma \cos \theta \tag{1}$$

which allows one to determine the diameter d of the pores into which mercury will intrude as a function of the applied pressure P. If the surface tension γ of mercury and its contact angle θ are taken as 480 dynes/cm and 140° (this was experimentally

determined for silica) respectively, eqn. 1 can be simplified to the following

$$Pd = 213.4 \text{ p.s.i. } \mu\text{m} \quad (2)$$

Scanning porosimetry provides a means of measuring the volume of mercury intruded into the pores in a sample as a function of the pressure, which is continuously increased from below ambient to 33 000 p.s.i. The volume of mercury is monitored by means of a capacitance bridge, as the quantity of mercury in the stem of the sample cell decreases as filling of the pores occurs. For example, one can calculate from the Washburn eqn. 1 that at initial pressures of 0.5 p.s.i., pores and interparticle voids having a diameter of 426 μm will fill with mercury. The lower pore size limit of the instrument is determined by the maximum pressure achievable in the porosimeter and is 64.6 Å at 33 000 p.s.i.

A variety of physical properties of the silica can be determined from these pressure–volume measurements:

(1) pore diameter distribution and the average (mean) or median pore diameter;
(2) intruded volume of mercury;
(3) $D_v(d)$ volume distribution function as a function of diameter d;
(4) $D_s(d)$ surface area distribution function as a function of diameter d;
(5) cumulative pore surface area.

Calculations were carried out as described by Lowell and Shields[25].

Static coupling of proteins. Glutaraldehyde-P preparations were washed with an excess of 2 *M* sodium chloride and then with 0.1 *M* potassium phosphate buffer, pH 7.4. An amount of 50 mg of each bonded phase sample was carefully weighed into a test tube and treated with 2.5 ml of 0.1 *M* potassium phosphate buffer, pH 7.4 containing 15 mg protein. Sodium cyanoborohydride was added (final concentration 0.1 *M*) in small amounts and the reaction was left undisturbed overnight at 4°C. The bonded phase was then washed extensively with 0.5 *M* sodium chloride and then with 0.1 *M* potassium phosphate, pH 7.4. The amount of protein adsorbed was determined by relating the loss of protein in the reaction mixture to the weight of bonded phase. Concanavalin A (Con A) was immobilized on Glutaraldehyde-P as outlined in an earlier report[22].

Column packing. The silica preparations activated by glutaraldehyde and coupled with Con A were packed into 50 × 4.6 mm I.D. columns by slurry packing by using 0.1 *M* potassium phosphate buffer, pH 6.0 containing 0.5 *M* sodium chloride, 1 m*M* calcium chloride and 1 m*M* manganese chloride.

Frontal uptake studies. Con A immobilized on silica of varying pore size was packed into 50 × 4.6 mm I.D. columns and equilibrated with 0.025 *M* Tris–HCl, pH 6.8 containing 0.2 *M* sodium chloride, 1 m*M* calcium chloride and 1 m*M* manganese chloride. Horseradish peroxidase (5 mg/ml) was injected at 1 ml per minute until the break-through peak was visible. The buffer was changed to 0.02 *M* Tris–HCl, pH 6.8 containing 0.2 *M* sodium chloride, 1 m*M* calcium chloride, 1 m*M* manganese chloride and 0.025 *M* methyl-α-D-glucopyranoside and the amount of peroxidase released was monitored at 406 nm.

RESULTS AND DISCUSSION

Fig. 1 gives the pore volume distribution as a function of diameter of one

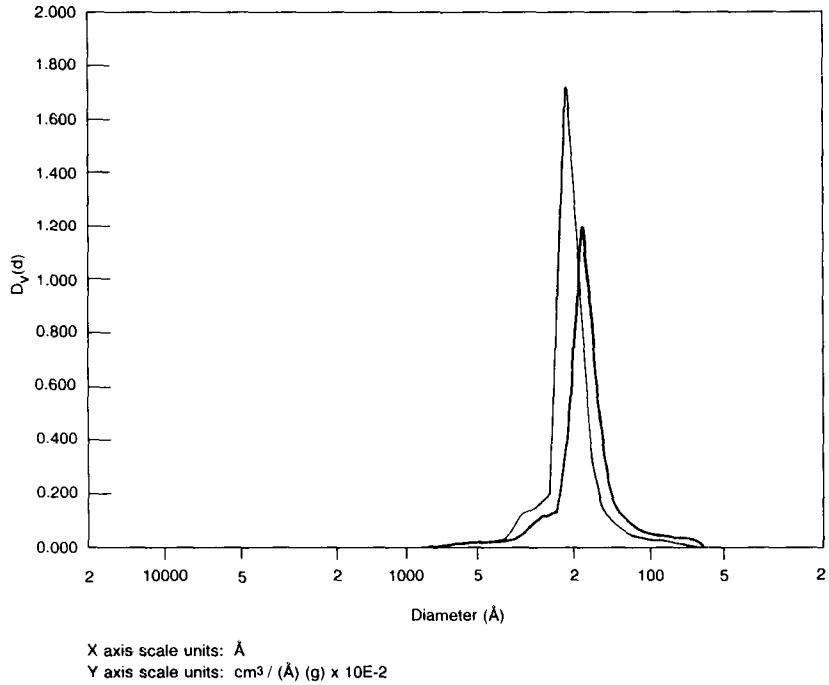

Fig. 1. Volume distribution curve for Glutaraldehyde-P (preparation 2) before (line) and after (bold line) treatment with glutaraldehyde. $D_v(d)$ and diameter are calculated by mercury intrusion porosimetry as described in Materials and Methods. $D_v(d)$ in $cm^3/Å \cdot g \times 10^{-2}$.

particular Glutaraldehyde-P affinity matrix (preparation 2, see Table I) before and after it is activated by glutaraldehyde. The volume distribution $D_v(d)$ for intrusion per unit change in pore diameter d is plotted against diameter d. The figure illustrates a shift and a decrease in pore volume distribution of the bonded phase after it is treated with glutaraldehyde. Similar shifts are observed in the case of the pore surface area distribution of the support material when it is clad with the polymer (Fig. 2). In this case the surface area distribution $D_s(d)$ for intrusion per unit change in pore diameter d is shown. In an ideal support, the pore size distribution is unimodal and preparation 2 fulfills this requirement (Fig. 1). A heterogeneous or bimodal distribution of pores

TABLE I

PORE STRUCTURE ANALYSIS OF GLUTARALDEHYDE-P (PREPARATION 2)

The physical properties were determined by mercury intrusion porosimetry and corrected for interparticle void. See Materials and Methods for details.

Sample	*Pore volume (ml/g)*	*Pore surface area (m^2/g)*	*Median pore diameter (Å)*
Starting material	0.95	182	211
After polymer treatment	0.76	150	204
After glutaraldehyde treatment	0.66	143	190
After binding of Con A	0.55	122	183

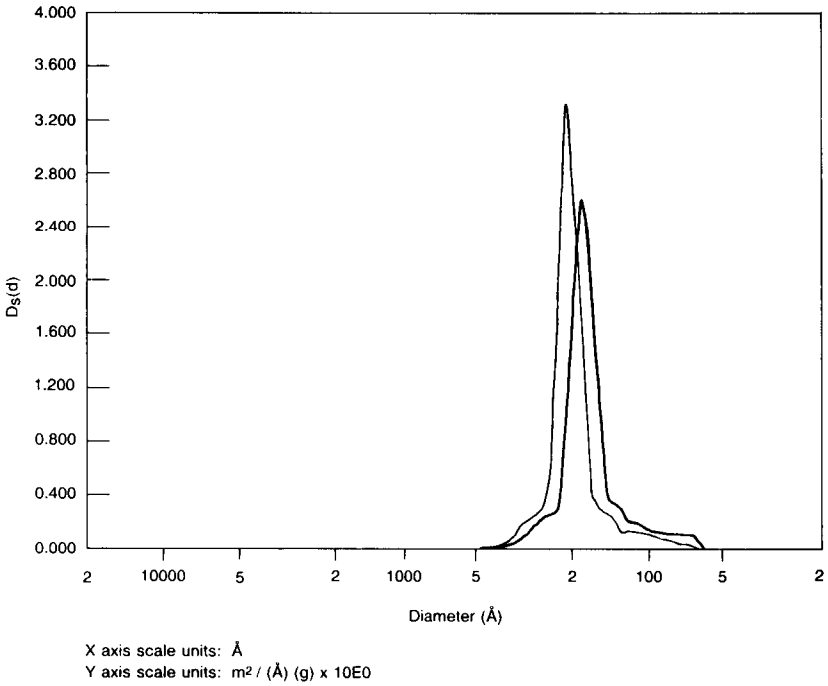

Fig. 2. Pore surface area distribution of Glutaraldehyde-P (preparation 2) before (line) and after (bold line) treatment with glutaraldehyde. $D_s(d)$ and diameter are calculated as described in Materials and Methods. $D_s(d)$ in $m^2/Å \cdot g$.

would affect the chromatographic resolution. The fact that the pore volume and the pore surface area distribution shifted towards smaller pore diameter when silica was treated with the polymer suggests that the pores were evenly coated in the interior and not blocked by the polymer.

Table I lists the physical properties of preparation 2 (Table II) before and after it is clad with glutaraldehyde. The data has been corrected for interparticle void volume

TABLE II

SURFACE CHARACTERIZATION AND PROPERTIES OF GLUTARALDEHYDE-P PREPARATIONS

Pore volume, pore surface area, and pore diameter were determined by mercury intrusion porosimetry and corrected for interparticle void.

Preparation	*1*	*2*	*3*	*4*
Property				
Pore volume (ml/g)	0.26	0.66	0.83	0.77
Pore surface area (m^2/g)	130	143	85	46
Pore diameter (median) (Å)	88	190	408	776
Carbon coverage (m^2/g)	385	424	460	280
Aldehyde ($\mu mol/m^2$)	1.3	1.8	1.53	1.3
Con A (mg/g)	80	140	104	86

in each case. After the bonded phase was treated with glutaraldehyde there is a 42% reduction in pore volume, 35% reduction in pore surface area and 13% reduction in the average pore diameter. Binding of Con A (a large protein of molecular weight 102 000) did not show any substantial change in the pore diameter.

Table II gives the pore diameter and surface area of Glutaraldehyde-P preparations used in our study. The carbon surface coverage determined by elemental analysis correlates fairly well with the experimentally determined amount of glutaraldehyde (μmol/m^2) which in turn correlates with the ligand binding capacity of the matrices for Con A (Fig. 3), indicating that the functional groups on the activated matrices are generally accessible to the protein.

Fig. 4 compares the binding capacities of glutaraldehyde-activated silica preparations of different pore diameters and surface areas for proteins of various molecular weights. In order to compare accessible surface areas of sorbents with different pore diameter and surface area, the protein binding capacity of the affinity sorbent was divided by the molecular weight of the protein and expressed as μmoles of protein per gram of the support. A large protein like thyroglobulin (molecular weight 670 000) is totally excluded from preparation 1 (average pore diameter 88 Å) while a medium sized protein like peroxidase (molecular weight 40 000) binds less to preparation 4 despite the large pore size (average pore diameter 776 Å).

Recently, Regnier[26] postulated that steric phenomena play a significant role in the interaction of large molecules in chromatography, be it in an ion-exchange, hydrophobic interaction or affinity chromatographic process. Though the binding mechanism of a protein to an affinity support is not based singularly on the molecular size of the protein, there seems to be a fairly good correlation between the binding capacity and the surface area and pore size of the support.

Consequently, silica packings of low surface area and large pore diameters are not necessarily the best candidates for chromatographic supports. Surface area and pore size should be optimized for protein size classes in order for the affinity support to be most efficient and cost effective. This is illustrated in Fig. 5. Although the ligand

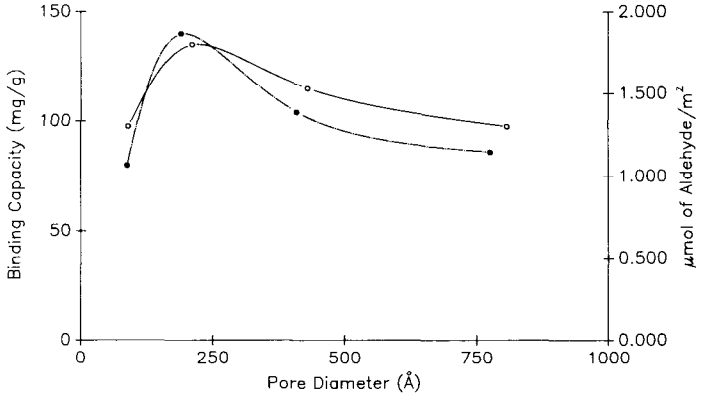

Fig. 3. The effect of pore diameter on the amount of glutaraldehyde (○) and Con A bound (●) to Glutaraldehyde-P affinity matrices. Con A concentration was determined by Bradford assay and glutaraldehyde content was estimated as described in Materials and Methods. An amount of 50 mg Glutaraldehyde-P was treated with 15 mg protein and immobilization was carried out as described in Materials and Methods.

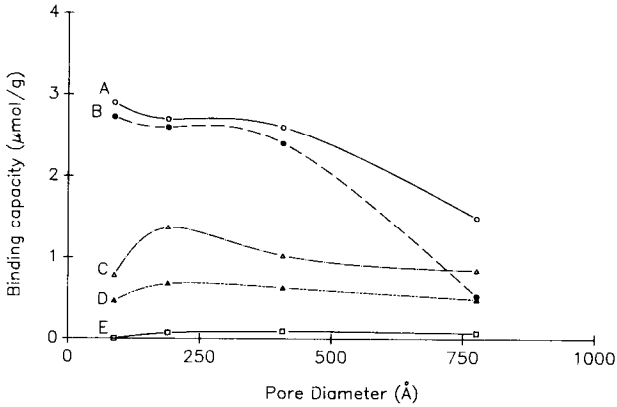

Fig. 4. The effect of pore diameter on the specific binding capacity of Glutaraldehyde-P affinity media for different proteins. (A) Lactoglobulin A; (B) horseradish peroxidase; (C) Con A; (D) glyceraldehyde-3-phosphate dehydrogenase; (E) thyroglobulin. The binding capacity of these supports for each protein was calculated by dividing the total binding capacity by the molecular weight of the respective protein. See Materials and Methods for more details.

concentration of preparation 3 (mean pore diameter 408 Å) is comparable to preparation 2, the surface area of the support is half of that of preparation 2; the loading capacity for horseradish peroxidase on Con A–Glutaraldehyde-P is higher in the case of preparation 2 (Table I and Fig. 5). Preparation 1 has a similar binding capacity for Con A as preparation 2 but due to its limited pore diameter, it does not bind the same amount of peroxidase onto the Con A bonded surface. Preparation 2 is an ideal matrix with both pore size and surface area optimal for maximum binding capacity for proteins of a wide molecular weight range (Table I and Fig. 5). In general, an average pore diameter of at least 200 Å may be desirable for chromatography of large proteins of molecular weight in excess of 150 000.

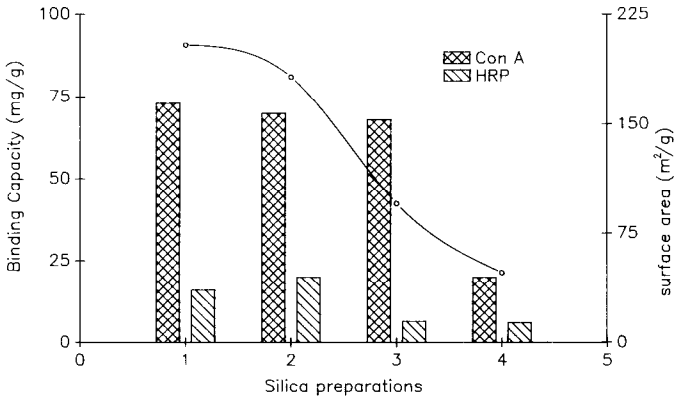

Fig. 5. The effect of pore diameter and surface area on the binding capacity of Glutaraldehyde-P affinity preparations for covalently bound Con A. Horseradish peroxidase (HRP) was bound to Con A in the dynamic mode as described in Materials and Methods. An amount of 2 g of Glutaraldehyde-P was allowed to react with 200 mg of protein and immobilization was carried out as described in Materials and Methods.

The above data exemplifies the importance of the physical properties of the support material for affinity chromatographic separations of proteins. It highlights the importance of choosing the right support to make the affinity medium. In this way, the binding and loading capacity can be greatly controlled, and lead to a more efficient, optimized purification.

Use of the same support for affinity separation of small molecules shows that the pore diameter is much less important for binding. The chromatographic separation of closely related *p*-nitrophenyl sugar derivatives on the same samples of Con A–Glutaraldehyde-P is given in Fig. 6. All three preparations showed similar resolution in the analytical range.

In conclusion, these experimental results illustrate the importance of understanding the surface and physical characteristics of the support material for optimum application of affinity chromatography. More and more preparative-scale purifications are being based on biological recognition, where maximum loading of the

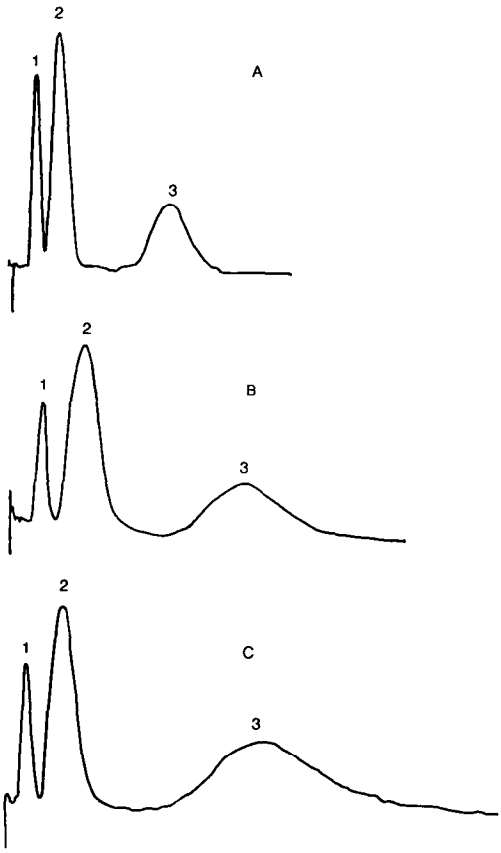

Fig. 6. Separation of *p*-nitrophenyl sugar derivatives on Glutaraldehyde-P affinity media. (A) Preparation 1; (B) preparation 2; (C) preparation 3. Peaks: 1 = *p*-nitrophenyl-β-D-glucoside; 2 = *p*-nitrophenyl-α-D-glucoside; 3 = *p*-nitrophenyl-α-D-mannoside. Con A–Glutaraldehyde-P was equilibrated with 0.025 *M* Tris–HCl, pH 6.8 containing 0.2 *M* NaCl, 1 m*M* $MnCl_2$ and 1 m*M* $CaCl_2$ at 2 ml/min. Absorbance was monitored at 305 nm.

column is well above the analytical range. A better understanding of how to increase loading without affecting the specificity and stability of the medium may well make affinity-based separation procedures lses costly and should considerably expand the use of affinity chromatography on a process scale.

ACKNOWLEDGEMENTS

We are grateful to Dr. S. Kakodkar for synthesizing the bonded phases and to Drs. D. Nau, S. Berkowitz and M. Henry for their valuable suggestions in the preparation of this manuscript.

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