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Hydroxyethyl methacrylate-based sorbents for high-performance liquid chromatography of proteins

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ABSTRACT

TESSEK Separon HEMA sorbents are based on a copolymer of ethylene dimethacrylate and hydroxyethyl methacrylate, whose biocompatibility has been demonstrated by its widespread use in soft contact lenses. The copolymer has a high resistance to hydrolysis and microbial attack, high mechanical strength and a high surface hydroxyl group content. In addition to size-exclusion chromatography, a wide range of derivatives have been prepared for ion-exchange, hydrophobic interaction and affinity chromatography. New modifications for reversed-phase, immobilized metal affinity and protein A chromatography have increased the number of possibilities for separating proteins.

INTRODUCTION

In recent years there has been increased application of organic polymer-based supports in HPLC, especially in protein and other biopolymer separations. Demands for increased pH stability are outweighing the advantages of silica-based supports, *i.e.*, mechanical strength and efficiency. The hydroxyethyl methacrylate-based sorbents, introduced 20 years ago, belong to the few organic polymer supports suitable for high-pressure applications of HPLC of proteins. In 1973 Čoupek *et al.* [1,2] described a spherical macroporous copolymer of 2-hydroxyethyl methacrylate and ethylene dimethacrylate (Fig. 1). The original patented technology of suspension polymerization of water-soluble monomers permits the preparation of spherical particles with size and porosity controlled over a wide range. The subsequently hydrophilized HEMA-BIO series of sorbents exhibit pressure resistance up to 20 MPa, long-term stability in the pH range 2–12 and compatibility with most organic solvents; 1 *M* hydrochloric acid and 1 *M* sodium hydroxide solution can be used for short term regeneration and purification. The highly hydrophilic surface results in a high protein recovery and activity of isolated proteins. The hydrophilic sorbent, first used and characterized by size-exclusion chromatography (SEC) of oligosaccharides, was later derivatized by ion-exchange groups or used for immobilization of both low- and high-molecular-mass ligands for affinity chromatography. More recently, modifications for reversed-phase, hydrophobic interaction and immobilized metal affinity chromatography were added. The sorbents are commercially available under the trade name TESSEK Separon HEMA (Spheron) from Tessek.

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MATRIX PROPERTIES

The sorbent is prepared by suspension copolymerization, in which the monomeric components are soluble in a mixture of the solvents used. The copolymer formed separates as fine, submicron particles (nodules). The nodules agglomerate in the course of reaction to form major particles and are connected on the contact

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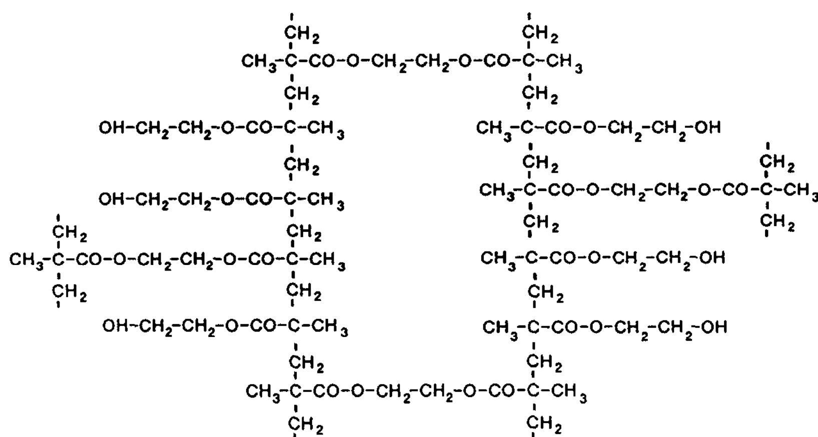

Fig. 1. HEMA structure.

surfaces by covalent bonds. On completion of the copolymerization, hard, spherical microparticles with pores resulting from internodular spaces are formed. The whole bead thus represent one giant macromolecule. The microspheres are characterized by a high degree of cross-linking, corresponding to the X15–X25 cross-linking degree used for polystyrene–divinylbenzene-based sorbents. This high cross-linking is the condition underlying the hardness and strength of the particles formed and the low swelling observed in all solvents. The repeating unit of polymer-bound trimethylacetic acid adds to the polymer resistance to hydrolytic attack, pivalate esters being the most difficult to hydrolyse.

The composition of inert solvents and variation of the polymerization conditions allow sorbents to be prepared with different pore and particle sizes. The large inner surface area (60–300 m^2/g) covered with a large number of hydrophilic groups gives the sorbent hydrophilicity and also can readily be chemically modified by a number of common methods. However, the long C–C polymer backbone chains cause a certain degree of hydrophobicity of the material. It shows mixed hydrophilic–hydrophobic properties and can be used without modification for hydrophobic interaction chromatography [3,4].

It is not possible to increase further the cross-linking of the nodules during the polymerization and the nodules exhibit decreasing density from the centre. The surface is then characterized by a layer of relatively loose polymer chains. Saturation of the surface with monomers and additional secondary cross-linking [5] result in increased rigidity (such sorbents are designed HEMA-S). Different methods have been attempted to reduce the hydrophobicity of the matrix to make it more suitable for the SEC of proteins. The binding of saccharides (Separon HEMA 1000 Glc) was effective [6]; treatment with epichlorohydrin and subsequent hydrolysis proved to result not only in the desired hydrophilicity towards proteins, but also in increased pressure stability (Fig. 2). Instead of the expected doubling of hydroxyl groups by addition of epichlorohydrin and subsequent hydrolysis, some decrease in the number of available hydroxyl

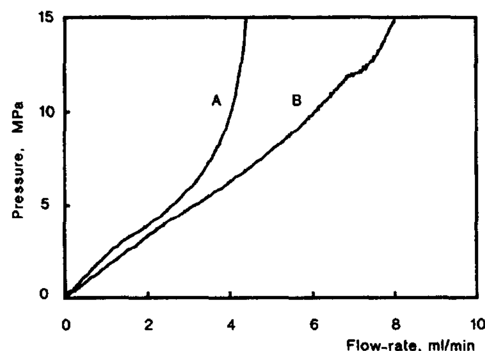

Fig. 2. Comparison of pressure stability of HEMA-S and HEMA-BIO sorbents. Column, stainless steel (80 × 8 mm I.D.); eluent, water; flow-rate, gradient from 0 to 10 ml/min in 60 min. (A) HEMA-S 1000 C_{18}, 10 μm; (B) HEMA-BIO 1000 C_{18}, 10 μm.

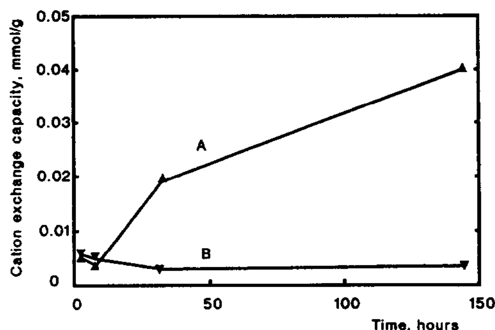

Fig. 3. Sorbent stability in 0.5 *M* KOH. Sorbent was suspended in 0.5 *M* KOH and left to stand at room temperature with occasional mixing. Cation-exchange capacity was measured from breakthrough curves in 150 × 3 mm I.D. CGC compact glass columns using 0.1 m*M* nitric acid with conductimetric detection. (A) HEMA 1000; (B) HEMA-BIO 1000.

groups was observed. Apparently, cross-linking occurs in the polymer surface, with more hydroxyl groups oriented towards the mobile phase. They increase the hydrophilicity towards large molecules, whereas the hydrophobicity towards small molecules, those which can penetrate into the matrix, is not significantly changed with either the Glc or BIO modifications. The base HEMA matrix contains a small measurable amount of unreacted double bonds, originating from the cross-linking agent ethylene dimethacrylate, which can hydrolyse under strongly alkaline conditions. The result is a small amount of carboxylate groups and possible leaching of methacrylic acid. During the preparation of HEMA-BIO (and also some other modifications) those groups are removed and the material does not show an increase in carboxylic groups under alkaline conditions (Fig. 3).

SIZE-EXCLUSION CHROMATOGRAPHY

The application of SEC for preparative purposes has many advantages, but also serious drawbacks. The advantages are the use of a simple, isocratic mobile phase, low retention volumes and easy prediction of the resulting separation. The drawbacks are limited resolution and low sample capacity (easy overloading). The only way to speed up the SEC separation of proteins with the same efficiency is to decrease the particle size of the sorbent, otherwise unacceptable band spreading will occur. Even then, low flow-rates are usually necessary to obtain the required efficiency.

The selectivity of separation in SEC is determined solely by the distribution of pores in the column packing material. For practical separations this distribution is best characterized by a calibration graph. V_0, the excluded volume, is equal to the volume of mobile phase between particles of the sorbent. Molecules larger than pores of the sorbent are eluted in this volume, usually in a narrow peak. V_t, the void volume, is equal to the sum of V_0 and V_i, the volume of mobile phase in the pores of the sorbent. In the void volume small, non-retained molecules are eluted. In a pure SEC mechanism, all molecules are eluted within V_0 and V_t.

Three main types of sorbents are used for the SEC of proteins. Classical, soft or semi-rigid gels (Sephadex, Sepharose, agarose), also called microporous or homogeneous, are formed by a cross-linked network, which swells in solvents to have pores of a nearly uniform size. This results in a calibration graph with a small slope, but a limited working range. Hence the gel will efficiently resolve molecules of similar size, but within a limited range. For preparative purposes, the pore size is usually selected so that one protein is excluded, others are in the working range and smaller molecules are fully penetrating (eluting in the void volume). Their low-pressure stability precludes their use in high-performance (HP) SEC.

Silica-based gels have a very rigid matrix backbone and can be prepared with relatively uniform pore size. The calibration graphs then usually have a similar shape to those with the previous gels, but slightly steeper. Efficient, small-particle sorbents are well suited for the preparative HPSEC of proteins. Their only drawbacks are low chemical stability at high pH and deterioration of the bonded phase, which ultimately results in uncovering of reactive silanol groups, thus spoiling the separation. TSK gels of the SW series are the most often used. A typical calibration graph is shown in Fig. 4a.

Rigid, macroporous polymers are formed by agglomerates of small, highly cross-linked par-

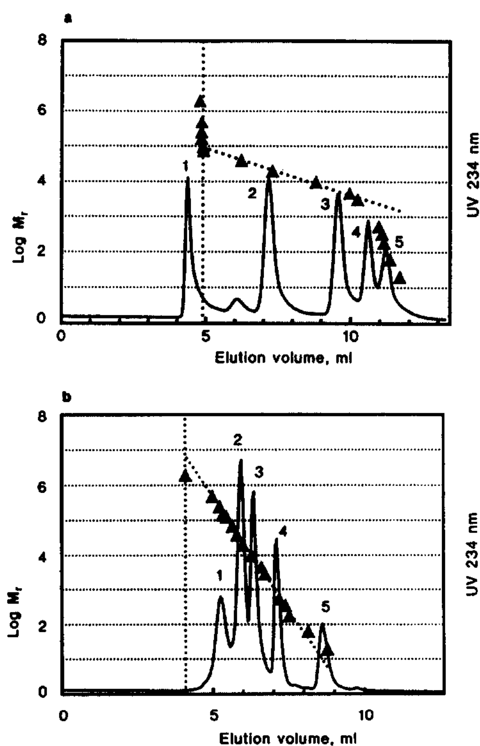

Fig. 4. (a) Size-exclusion chromatography on silica-based sorbent. Column, stainless steel (300 × 7.5 mm I.D.) BIO-Sil TSK 250, 10 μm (Bio-Rad); eluent, water; flow-rate 1.0 ml/min; detection, refractive index; standards: ^2H_2O, glucose, saccharose, raffinose, dextrans 3000 and 4500 and Pharmacia D10, D20, D40, D70, D110, D150, D250, D500 and D2000. Protein separation: eluent, 0.1 *M* phosphate buffer–0.15 *M* NaCl (pH 7.0); flow-rate, 0.5 ml/min; detection, UV at 234 nm. Peaks: 1 = thyroglobulin (M_r 660 000); 2 = bovine serum albumin (67 000); 3 = cytochrome *c* (12 400); 4 = cytidine-5-monophosphate (340); 5 = low-molecular-mass impurity. (b) Size exclusion chromatography on HEMA-based sorbent. Column, stainless steel (250 × 8 mm I.D.) HEMA-BIO 1000, 10 μm; other conditions and peaks as in (a).

ticles (nodules). This structure results in a wide pore distribution. The calibration graph is usually linear, or nearly linear, from the exclusion limit down to low molecular masses. A typical calibration graph is shown in Fig. 4b. This is an advantage for the characterization of the molecular mass distribution of polymer samples, but the slope is steep, resulting in a lower selectivity. This is the structure of HEMA or TSK gels of the PW series. For preparative purposes, they are usually applicable only for simple mixtures of proteins with sufficiently differing sizes. The best results are achieved if the exclusion limit is selected so that the larger molecule is excluded and the smaller one is well in the working range. Even if better separation can be achieved on the first two types of sorbents, the HEMA type of sorbent may be the only alternative for the HPSEC of proteins if purification of the column with hydroxide solution is required according to the US Food and Drug Administration protocols. Owing to the hydrophobicity of HEMA for small molecules, good separations can be sometimes obtained by a non-purely SEC mechanism, as was demonstrated for the isolation of DNA [7]. The DNA was fully excluded, the proteins emerged within V_0 and V_t and the phenol used for extraction was strongly retained.

ION-EXCHANGE CHROMATOGRAPHY

Ion-exchange chromatography (IEC) is one of the most commonly used techniques for the separation of proteins in both analytical and preparative chromatography. A gradient of increasing salt concentration or a pH gradient is used for elution. The separation can to some extent be predicted on the basis of the p*I* values of the proteins to be separated. All the commonly used ion-exchange modifications have been prepared on a HEMA matrix: medium-basic diethylaminoethyl [8], strongly basic quaternary amine [9], weakly acidic carboxymethyl (CM) [10], medium-acidic phospho [11] and strongly acidic sulpho [12] derivatives. Structures of currently produced derivatives are depicted in Fig. 5. The DEAE modification usually contains some quaternized functional groups and can also be used for chromatofocusing in a limited range of pH. Although aqueous mobile phases are usually used in IEC, the rigidity of the HEMA matrix allows the use of organic solvents, as demonstrated by the preparative isolation of gangliosides in non-aqueous media (Fig. 6).

The loading capacity of HEMA-BIO 1000 DEAE sorbent is shown in Fig. 7 for a model separation of haemoglobin and serum albumin, where the column separates 25 mg of mixture without apparent signs of overloading. Although the IEC mode is widely applied in the preparative HPLC of proteins, several other modes offering different selectivity are available.

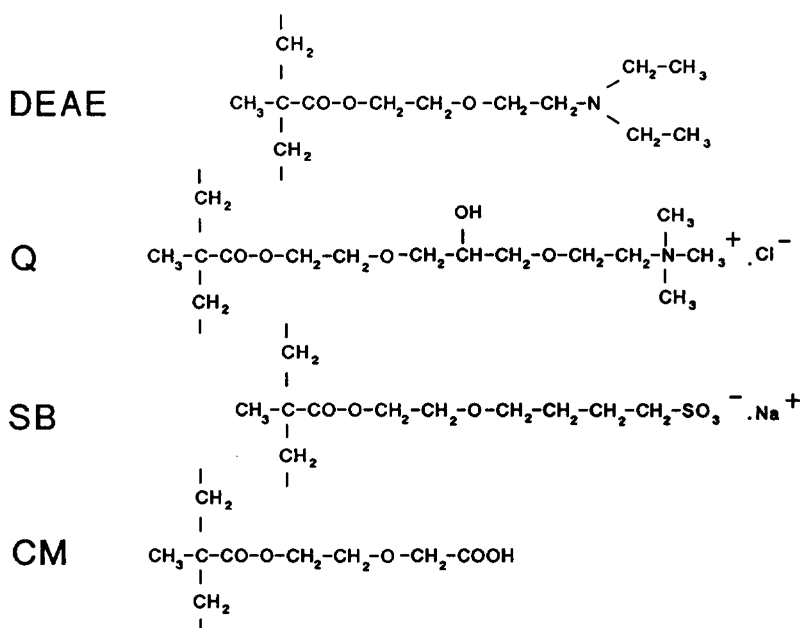

Fig. 5. Functional group structures of currently produced HEMA-BIO ion exchangers.

HYDROPHOBIC INTERACTION CHROMATOGRAPHY

Hydrophobic interaction chromatography (HIC) separates proteins on the basis of their different hydrophobicities. A descending gradient of an antichaotropic salt (most commonly ammonium sulphate) is used for elution. Such a process usually ensures low denaturation and high activity of recovered proteins. Hydrophilic sorbents with a low content of attached hydrophobic groups are preferably used. An unmodified HEMA or HEMA-S matrix can be used for HIC separations [3,4], but better results are achieved on a specially designed HEMA-BIO 1000 phenyl sorbent [13]. The low-content

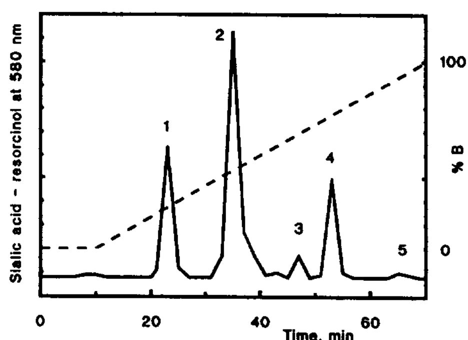

Fig. 6. Preparative separation of gangliosides. Column, titanium (250 × 25 mm I.D.) Tessek Separon HEMA-BIO 1000 DEAE, 10 μm; eluent, A = methanol, B = 0.15 *M* ammonium acetate in methanol, gradient 10 min 100% A, 60 min linear gradient to 100% B; flow-rate, 20 ml/min; detection by resorcinol reaction in collected fractions; sample, 100 mg of gangliosides in 6 ml of $MeOH–CHCl_3–H_2O$ (1:1:1). Peaks: 1 = monosialo-; 2 = disialo-; 3 = trisialo- (GT1a); 4 = trisialo- (GT1b); 5 = tetrasialogangliosides.

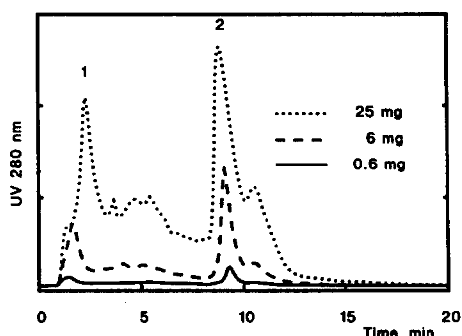

Fig. 7. Separation of bovine haemoglobin and bovine serum albumin. Column, stainless steel (80 × 8 mm I.D.) HEMA-BIO 1000 DEAE, 10 μm; eluent, A = 20 m*M* Tris (pH 7.8), B = 20 m*M* Tris–0.5 *M* NaCl (pH 7.8), gradient from 0 to 100% B in 20 min; flow-rate, 2 ml/min; detection, UV at 280 nm; sample, mixture of bovine haemoglobin (Sigma; recrystallized twice) and bovine serum albumin (Sigma, fraction V.) (2:3). Peaks: 1 = haemoglobin; 2 = bovine serum albumin.

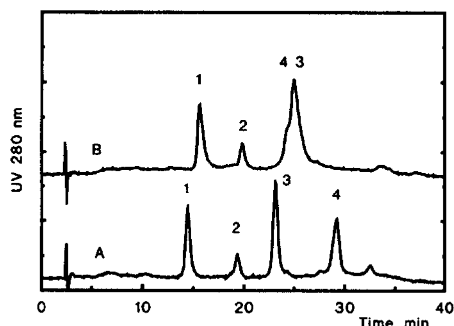

Fig. 8. Hydrophobic interaction chromatography of protein standard mixture. Column, stainless steel (80 × 8 mm I.D.); eluent, A = 0.1 *M* phosphate–2.2 *M* ammonium sulphate (pH 7.0), B = 0.1 *M* phosphate (pH 7.0), gradient from 0 to 100% B in 30 min; flow-rate, 1 ml/min; detection, UV at 280 nm; injection volume, 250 μl. Peaks: 1 = myoglobin (1 mg/ml); 2 = ribonuclease A (1 mg/ml); 3 = lysozyme (0.4 mg/ml); 4 = chymotrypsin (1 mg/ml). Sorbent: (A) HEMA-BIO 1000 phenyl, 10 μm; (B) HEMA-BIO 1000 butyl, 10 μm.

butyl modification was also tested, giving different selectivities for some proteins; *e.g.*, the elution order of lysozyme and chymotrypsin is reversed, but the peaks are not resolved (Fig. 8). As HIC separations can tolerate high salt concentrations in a sample, they are advantageously used after ammonium sulphate precipitation or IEC in the separation scheme.

REVERSED-PHASE CHROMATOGRAPHY

For separations of proteins, reversed-phase chromatography gives the best efficiency, but the conditions used for elution denature many proteins. It is predominantly used in the analytical mode, but it can also be applied to the preparative chromatography of smaller proteins and peptides, which are not so susceptible to denaturation. Many active peptides and proteins can be isolated and purified by RP-HPLC, such as insulin or bee venom constituents (Fig. 9a). In this example, 4 mg of highly active and pure phospholipase A-2 were isolated in a single run from 20 mg of bee venom on an 80 × 8 mm I.D. column. The purity of the recovered phospholipase A-2 was verified by rechromatography under analytical conditions (Fig. 9b).

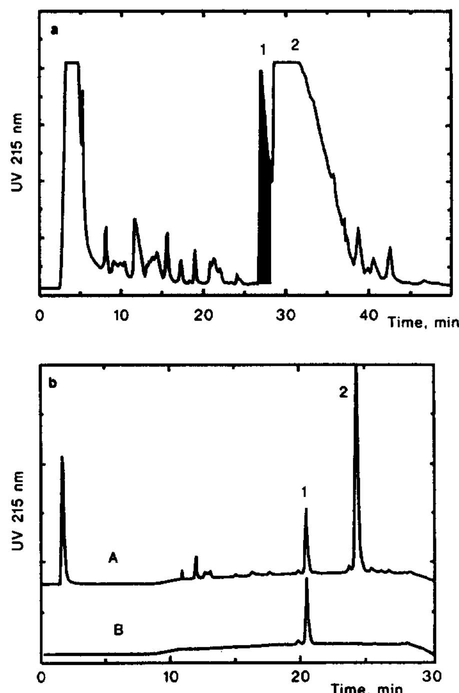

Fig. 9. (a) Preparative isolation of phospholipase from bee venom. Column, stainless steel (80 × 8 mm I.D.) HEMA-S 1000 C_{18}, 10 μm; eluent, A = 0.22% trifluoroacetic acid in water, B = 0.2% trifluoroacetic acid in acetonitrile, gradient 0–50% B in 40 min, 50–100% B in 10 min; sample loop, 2.5 ml; detection, UV at 215 nm, 2 a.u.f.s; injection, 20 mg of bee venom in 2 ml. (b) Analytical chromatography of bee venom. Column, CGC compact glass cartridge (150 × 3 mm I.D.) HEMA-BIO 1000 C_{18}, 10 μm; eluent, A = 0.22% aqueous trifluoroacetic acid, B = 0.2% trifluoroacetic acid in acetonitrile, gradient 0–50% B in 20 min, 50–100% B in 5 min; sample loop, 2.5 ml; detection, UV at 215 nm, 0.5 a.u.f.s. (A) 5 μl of bee venom (2.0 mg/ml); (B) 5 μl of isolated phospholipase (lyophilized) (0.4 mg/ml).

AFFINITY CHROMATOGRAPHY

For the separation of proteins, affinity chromatography employs very selective interactions with other low- or high-molecular-mass compounds. The most commonly used affinity ligands for enzymes are substrates, substrate analogues or inhibitors. Owing to their high hydroxyl group content, HEMA sorbents can be easily utilized for the immobilization of different ligands by commonly used methods with, *e.g.*, cyanogen bromide [14], glutaraldehyde [15] or

benzoquinone [16]. Stable preactivated affinity supports containing epoxy [17] or vinyl sulphone [18] groups with different activation levels are commercially available. The application of HEMA sorbents as supports for affinity chromatography and their comparison with other commercially available matrices were presented by Taylor and Marenchic [19,20]. They demonstrated that the application of pressure-stable, efficient HEMA supports can speed up the separations by a factor of five.

Immobilized proteins can be utilized for the isolation of other proteins such as immunoglobulin G (IgG) on protein A (Fig. 10). Low non-specific binding is demonstrated by injection of excess of bovine serum albumin (BSA) (line A in Fig. 10a), while the purity of isolated immunoglobulins is verified by rechromatography (line B in Fig. 10b). Immobilized active enzymes can be used for the chemical modification of other substances. HEMA-immobilized alcohol oxidase [21] or acetylcholinesterase, choline oxidase, catalase and peroxidase [22] have been used in detection systems, but other enzymes can also be used in production processes, *e.g.*, glucoseisomerase in the manufacture of D-fructose from D-glucose [23].

IMMOBILIZED METAL AFFINITY CHROMATOGRAPHY

Immobilized metal affinity chromatography (metal interaction chromatography) [24] employs selective interactions of certain metal ions with specific residues on the protein surface. The sorbent contains chelating functional group (usually iminodiacetic acid), which can be loaded with the desired metal ion. The metal ion can subsequently be removed by flushing with EDTA solution and the column can then be used for another metal. Without the metal loaded, those sorbents usually exhibit ion-exchange and hydrophobic properties and can be used in those modes. A hydroxyethyl methacrylate-based sorbent with immobilized iminodiacetic acid was developed by Šmídl *et al.* [25]. The retention is governed by mixed interactions including ionic and hydrophobic types and is generally difficult to predict. Immobilized copper ions interact preferentially with hystidine and tryptophan residues, whereas immobilized iron (III) ions prefer phosphate residues (Fig. 11), as demonstrated by

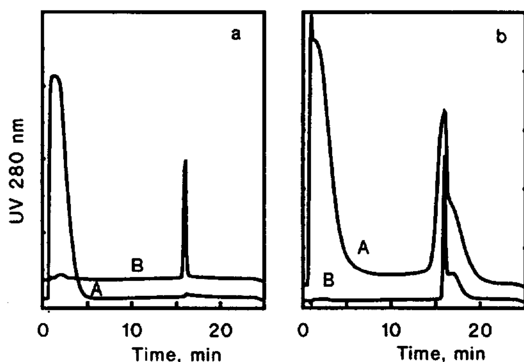

Fig. 10. (a) Protein A affinity chromatography. Column, CGC compact glass cartridge (70 × 3 mm I.D.) HEMA-BIO 1000 VS–protein A, 10 μm; eluent, A = 0.75 *M* glycine–1.5 *M* sodium chloride (pH 8.0), B = 0.1 *M* glycine (pH 3.0), gradient 3 min 100% A, 10 min 0–100% B, 7 min 100% B; flow-rate, 0.5 ml/min; detection, UV at 280 nm. (A) BSA, 10 mg in 250 μl; (B) human IgG, 300 μg in 1 ml. (b) Protein A affinity chromatography with conditions as in (a). (A) pig serum, 1 ml diluted 1:4; (B) isolated immunoglobulins, 1 ml of collected immunoglobulins fraction diluted 1:1 with doubly concentrated eluent A.

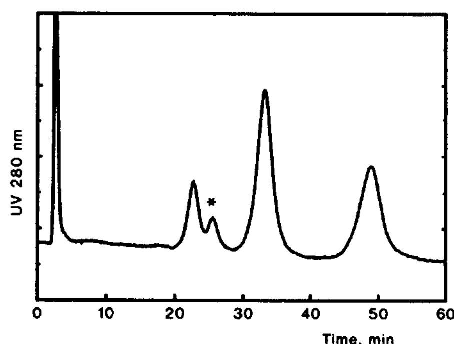

Fig. 11. Immobilized metal affinity chromatography of ovalbumin. Column, CGC compact glass cartridge (150 × 3 mm I.D.) HEMA-BIO 1000 IDA-Fe^{3+}, 10 μm; eluent, A = 0.025 *M* sodium acetate–0.5 *M* NaCl (pH 5.5), B = 0.025 *M* morpholinoethanesulphonate buffer–0.5 *M* sodium chloride (pH 7.5), gradient from 0 to 100% B in 60 min; flow-rate, 0.5 ml/min; detection, UV at 280 nm; injection volume, 50 μl; ovalbumin (Sigma) 9.0 mg/ml. The peak marked with an asterisk corresponds to conalbumin present as an impurity.

separation of ovalbumin constituents. Although not much used yet in preparative chromatography, different selectivities can be obtained on the same column by varying the conditions and hence the same column may be employed for different tasks.

CONCLUSIONS

In this paper we have surveyed the properties and some possible applications of hydroxyethyl methacrylate-based sorbents of the Separon HEMA type in analytical and preparative separations of proteins, and have emphasized that techniques other than the ion-exchange chromatography can be successfully applied to preparative separations of proteins. Hydroxyethyl methacrylate copolymers provide a chemically and mechanically rugged matrix for the preparation of wide range of derivatives suitable for protein separations. A proper selection of the chromatographic mode enables the desired proteins to be obtained in high yield and with high activity.

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