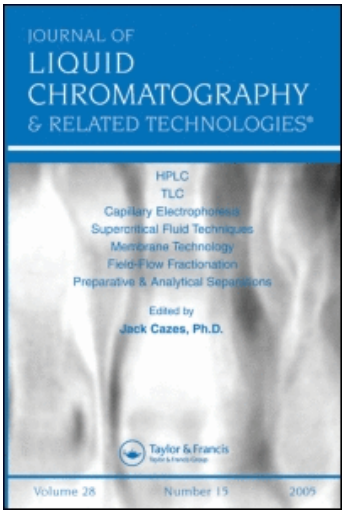

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MODIFIED MACROPOROUS COPOLYMERS OF GLYCIDYL METHACRYLATE WITH ETHYLENE DIMETHACRYLATE AS SORBENTS FOR ION-EXCHANGE AND HYDROPHOBIC INTERACTION CHROMATOGRAPHY

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ABSTRACT

Copolymers glycidyl methacrylate-ethylene dimethacrylate which proved to be well suited for size exclusion chromatographic separations in water solutions were provided with hydrophobic alkyl chains containing 4 or 8 carbon atoms or with hydrogen sulfonate groups by simple chemical modifications. Separations of model protein mixtures document the suitability of these sorbents for hydrophobic interaction and ion-exchange chromatography and suggest ways for fine adjustment of the chromatographic conditions assuring the optimal result.

INTRODUCTION

Interaction high-performance liquid chromatography (HPLC), which includes ion-exchange (IEC), hydrophobic interaction (HIC), and affinity chromatography (AC) can at present be regarded as an important tool in both

analytical and preparative separation of proteins. The first two modes have become particularly popular as the chromatographic process takes place under mild conditions, which guarantee that the physiological activity of the protein does not deteriorate. The unifying feature of all modes of interaction chromatography is that their mechanism consists in a selectively controlled desorption of the protein following the sorption process, which first takes place at the inlet part of packing in the chromatographic column. Desorption in the column packing occurs depending on the extent of the protein-sorbent interaction energy within a narrow interval of eluent properties, such as pH, ionic strength, etc.

To achieve the separation, the separation media have to meet a number of requirements. The preferred shape is a bead. The porous structure of the beads has to ensure the accessibility of all groups located on the inside surface of the beads to reagents which provide the surface with interacting groups. From the chemical point of view, this means that the surface should be modified "homogeneously", the density of active groups is expected to be identical throughout the bead. All the active groups (ligands) have to be available to the compounds undergoing separation, and the interaction between the groups located at any site of the sorbent and a compound must be equal as far as the interaction energy is concerned. To meet all the requirements, the separation media are supposed to have a narrow pore size distribution with a maximum of the distribution curve exceeding 30 nm, while pores smaller than about 8 nm are completely absent.

In our preceding papers we described a procedure controlling the pore size distribution of polymeric separation media based on the copolymer glycidyl metha-

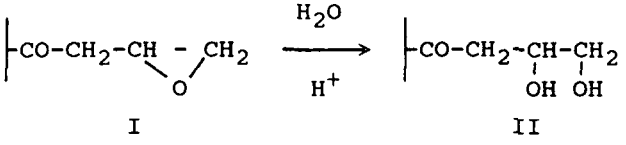
crylate - ethylene dimethacrylate (GMA-EDMA) and their use in size exclusion chromatography (SEC) (1,2). This media fit the above properties adequately. In this study we report results obtained in the separation of proteins in the IEC and HIC mode using GMA-EDMA copolymers modified by ionogenic or hydrophobic groups.

EXPERIMENTAL

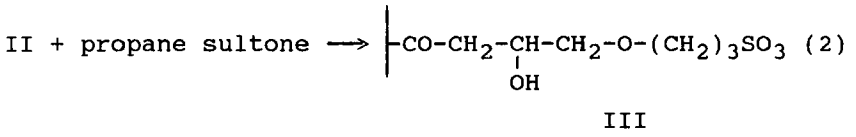
Separation media

The macroporous polymer G-60 (I) was prepared by the radical suspension copolymerization of 60 vol.% glycidyl methacrylate with 40 vol.% ethylene dimethacrylate in the presence of cyclohexanol as a porogen initiated by azobisisobutyronitrile described in detail elsewhere (3). The properties of the plain matrix were determined from a chromatographic measurement (2): specific surface area 146 m^2/g, specific pore volume 1.48 ml/g, average pore diameter calculated from the specific surface area (D_S) 16.6 nm, average pore diameter calculated from the pore volume (D_V) 49.3 nm, the ratio D_V/D_S characterizing the width of the pore size distribution is 2.97. For the modification and subsequent column packing a fraction of beads having the size in range 5 + 1 μm was separated from the raw polymerization product using Alpine Zig Zag Sichter (Alpine, Augsburg, Germany) (1).

The strong acidic ion exchanger containing 1.0 mmol/g hydrogen sulfonate groups was prepared via a sequence of reaction shown schematically in equations 1 and 2:



I II (1)

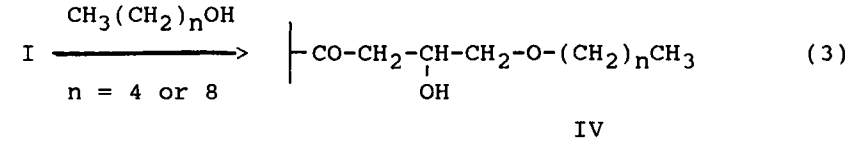


II + propane sultone ⟶ $\left|\text{-CO-CH}_2\text{-CH(OH)-CH}_2\text{-O-(CH}_2\text{)}_3\text{SO}_3\right.$ (2)

III

and described in detail elsewhere (4), i.e. the acid catalyzed hydrolysis of the epoxide group and a reaction with propane sultone.

Hydrophobization was achieved by a direct reaction of an alcohol with pendant epoxide groups of copolymer I. The beads were immersed in a 0.01 mol/l sodium hydroxide solution in butanol or octanol (equation 3) at room temperature for 24 hours and then washed with methanol and water until the supernatant liquid remained neutral:



$$\text{I} \xrightarrow[n = 4 \text{ or } 8]{\text{CH}_3(\text{CH}_2)_n\text{OH}} \left|\text{-CO-CH}_2\text{-CH(OH)-CH}_2\text{-O-(CH}_2\text{)}_n\text{CH}_3\right. \quad (3)$$

IV

The reaction products contain approximately 0.02 mmol/g butyl or octyl residues according to a semiquantitative determination from IR spectra.

On completion of the reaction, the residual epoxide groups, which could react with proteins and thus interfere with the separation, were subjected to acid catalyzed hydrolysis according to equation 1.

Chromatography

Chromatographic experiments were carried out in a microcolumn liquid chromatograph KHZH 1311 provided with a fluorimetric detector (Science and Technology Corp., Academy of Science of the USSR, Leningrad). Proteins (Sigma, St Louis, USA) were detected using the

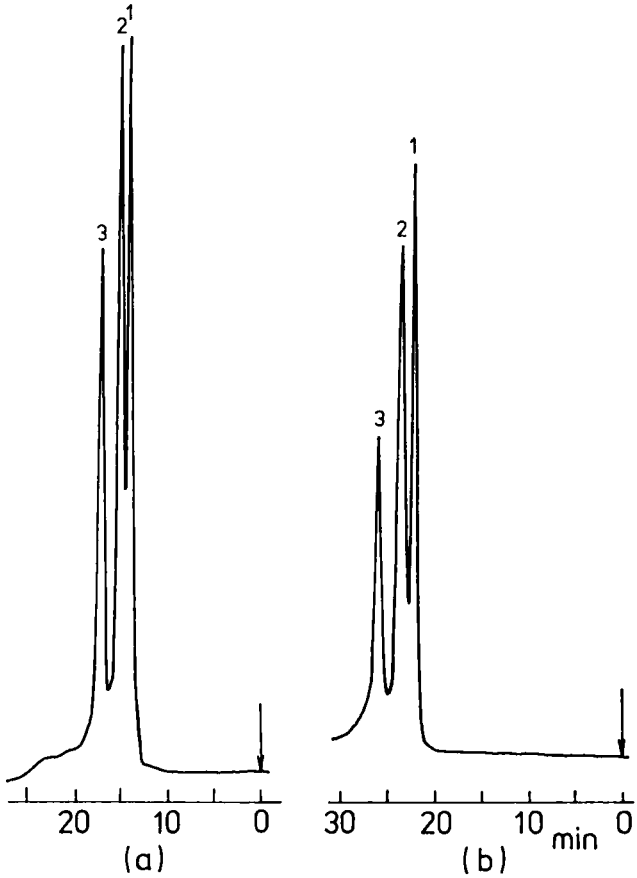


FIGURE 1. Effect of the flow rate on HPLC separation of a protein mixture in hydrophobic interaction mode. Packing G-60-C8, 4-6 μm, Teflon column 14 cm x 0.5 mm i.d., mobile phase A 2 mol/l ammonium sulfate in 0.025 mol/l phosphate buffer pH 6.8, mobile phase B buffer only, gradient from 1 to 90 % B in A in 10 min, flow rate 10 μl/min (a) or 5 μl/min (b); 1 ovalbumin, 2 lysozyme, 3 chymotrypsinogen.

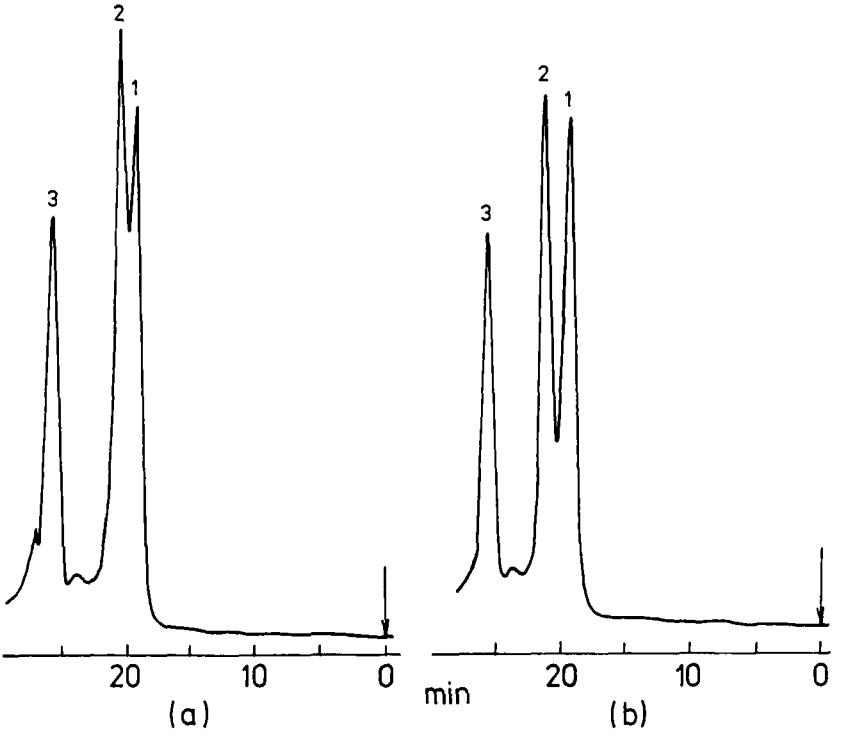


FIGURE 2. Effect of the flow rate on HPLC separation of a protein mixture in hydrophobic interaction mode. All conditions as in Figure 1 except gradient from 1 to 90 % B in A in 20 min.

fluorescence of tryptophan residues excited with light (230 nm). The columns used were made from Teflon. The concentration of the individual protein standards in testing solutions and mixtures was 0.5 mg/ml and the injected volume amounted to 0.5 μl.

RESULTS AND DISCUSSION

Chromatograms of model protein mixtures separated in a column packed with the C_8 modified sorbent are

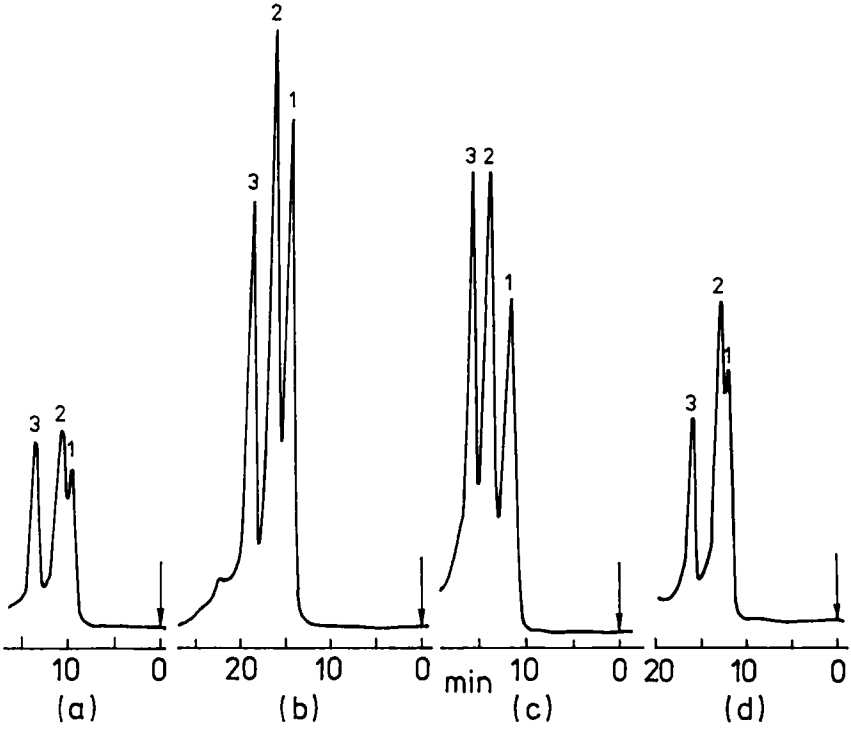


FIGURE 3. Effect of the flow rate and column length on HPLC separation of a protein mixture in hydrophobic interaction mode. Packing G-60-C4, 4-6 μm, Teflon column 14 cm x 0.5 mm i.d.(a,b,c) or 6.5 cm x 0.5 mm i.d. (d), mobile phase A 2 mol/l ammonium sulfate in 0.025 mol/l phosphate buffer pH 6.8, mobile phase B buffer only, gradient from 1 to 90 % B in A in 20 min, flow rate 20 μl/min (a), 10 μl/min (b,c) or 5 μl/min (d); 1 ovalbumin, 2 lysozyme, 3 chymotrypsinogen.

shown in Figures 1 and 2. It is evident that a slow down in the elution rate improves the separation of ovalbumin and lysozyme, while lysozyme and chymotrypsinogen remain virtually unseparated, although the latter usually exhibits strong adsorption on the surface of chromatographic packings. Retardation of the rate of change in the composition of the mobile phase

FIGURE 4. Effect of the gradient steepness on HPLC separation of a protein mixture in hydrophobic interaction mode. Packing G-60-C4, 4-6 μm, Teflon column 14 cm x 0.5 mm i.d., mobile phase A 2 mol/l ammonium sulfate in 0.025 mol/l phosphate buffer pH 6.8, mobile phase B buffer only, gradient from 1 to 90 % B in A in 20 min, flow rate 5 μl/min (a), isocratic elution by B (b); 1 ovalbumin, 2 lysozyme, 3 chymotrypsinogen.

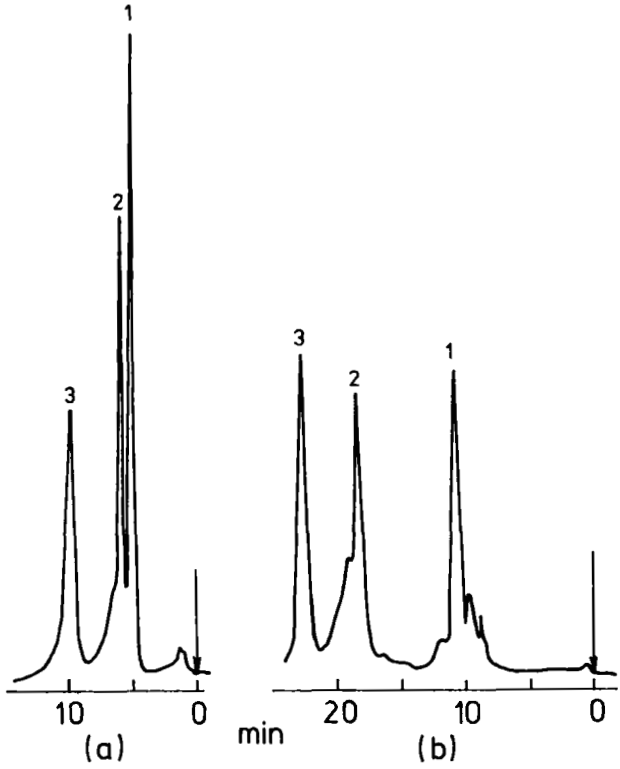


FIGURE 5. Effect of the column length and gradient steepness on HPLC separation of a protein mixture in ion exchange mode. Packing G-60-SP, 4-6 μm, Teflon column 14 cm x 0.5 mm i.d.(a) or 7.5 cm x 0.5 mm i.d. (b), mobile phase A 0.025 mol/l phosphate buffer pH 6.8, mobile phase B 0.5 mol/l sodium chloride in the buffer, gradient from 1 to 99 % B in A in 5 min (a) or 1 - 20 % B in 15 min and 20 -99 % B in 5 min (b), flow rate 20 μl/min; 1 trypsinogen, 2 chymotrypsinogen, 3 lysozyme.

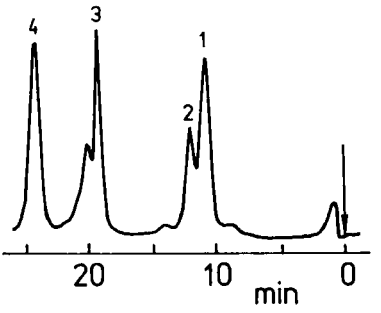


FIGURE 6. HPLC separation of a protein mixture in ion exchange mode. Packing G-60-SP, 4-6 μm, Teflon column 14 cm x 0.5 mm i.d., mobile phase A 0.025 mol/l phosphate buffer pH 6.8, mobile phase B 0.5 mol/l sodium chloride in the buffer, gradient 1 - 20 % B in 15 min and 20 -99 % B in 5 min, flow rate 20 μl/min; 1 ribonuclease, 2 trypsinogen, 3 chymotrypsinogen, 4 lysozyme.

(gradient steepness) also positively affects the separation. The improved selectivity of separation in this case may be attributed to the effect of the size exclusion mechanism (5).

The separation presented in Figure 3 confirms the complexity of the separation mechanism in which the hydrophobic absorption/desorption process interferes with the size exclusion of some component of the mixture after desorption when passing through the column. A comparison between the chromatograms obtained with beads bearing butyl (C_4) groups shown in Figure 3 reveals again the positive effect of column length and slow elution rate on selectivity. On shortening the column length from 140 mm to 65 mm the separation of lysozyme from ovalbumin deteriorates markedly. The size exclusion is quite significant in the case of ovalbumin which has a large molecule and, simultaneously, its sorption is weak. An isocratic elution (Figure 4)

yields only two peaks; the first is again that of ovalbumin.

Figures 5 and 6 show separation of a mixture in a column packed with the polymeric ion-exchange stationary phase (IV). They demonstrate, that by varying conditions of the chromatographic process, it is possible to achieve selective separation not only of the proteins, but also of impurities present in them. In this case the shortening of the column does not affect the separation of the peaks, as it is in the case with HIC. Since the effect of size exclusion on the peak spreading after desorption cannot be ruled out, it may be inferred that this affects all the proteins present to the same extent and does not disturb the separation itself (6).

The series of separations confirms that polymeric separation media obtained by a chemical modification of the copolymer glycidyl methacrylate-ethylene dimethacrylate can be successfully applied not only in SEC (1,2) but also in both high-performance HIC and IEC of proteins. The optimal separation conditions can be tuned up for each mixture of proteins by varying the mode, column length, flow rate, and gradient steepness.

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