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POLY(ETHYLENE GLYCOL)- AND POLY(VINYL ALCOHOL)-SUBSTITUTED CARBOHYDRATE GELS FOR "MILD" HYDROPHOBIC CHROMATOGRAPHY*

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SUMMARY

The possibility of using polymers with intermediate hydrophobicity as ligands in hydrophobic interaction chromatography was studied. Serum albumin and haemoglobin were separated on a column using poly(vinylalcohol)–Sepharose. The retention of haemoglobin increased with increasing temperature or increasing ionic strength, indicating that the interaction with the stationary phase was of a hydrophobic nature. The retention of albumin was, however, relatively unaltered.

Glucose oxidase was modified by covalent attachment of monomethoxypoly(ethylene glycol) and conjugation with gentamicin. Two fractions were obtained when the product was run on monomethoxypoly(ethylene glycol)–Sephadex at high ionic strength. The hydrophobicities of the two fractions were measured by partitioning in an aqueous two-phase system.

INTRODUCTION

It is possible to separate successfully biomolecules chromatographically by exploiting their hydrophobic properties. Hydrophobic moieties such as alkyl and phenyl groups have been extensively examined as ligands in chromatography. Many proteins have been found to bind to these ligands, thus permitting chromatographic separation. However, negative side effects have also been noted. The binding is, in many cases, too strong to be useful in a chromatographic process and may even be practically irreversible[1–3]. Furthermore, in order to elute a strongly bound protein, chaotropic agents or organic solvents may be required, which can lead to denaturation. Ligands having an intermediate hydrophobicity would thus be of interest, since they would permit adequate binding strength without the above-mentioned disadvantages.

Weak hydrophobic interactions are, however, used in another separation

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method, *viz.* partitioning in an aqueous two-phase system. These systems are composed of two aqueous solutions, either two polymers or one polymer and a salt. Examples of such systems are poly(vinyl alcohol)–dextran, poly(ethylene glycol)–dextran, poly(ethylene glycol)–magnesium sulphate and methylcellulose–dextran. Aqueous two-phase systems have been utilized to separate many kinds of biomolecules[4]. As it is possible to get separation effects when there are comparatively small differences in hydrophobicity between the two aqueous phases, it seemed probable that separation could also be obtained if the same polymers were used as chromatographic materials.

The analogy between partitioning in aqueous two-phase systems and chromatographic procedures has previously been utilized in partition chromatography[5]. In this case, one of the two phases was merely adsorbed to the stationary phase and the experimental conditions were adjusted so that the adsorbed phase constituent was not dissolved.

We have used hydrocarbons substituted with hydroxy or ethergroups, such as poly(ethylene glycol) and poly(vinyl alcohol) covalently bound as ligands, in order to obtain "mild" hydrophobic chromatography. The material to be separated consisted of mixtures of pure proteins as well as derivatized proteins with slightly modified hydrophobicities.

MATERIALS AND METHODS

Bio-Gel P-4 was purchased from Bio-Rad Labs. (Richmond, CA, U.S.A.). Sephadex G-25, Sepharose 6B and epoxy-activated Sepharose 6B were obtained from Pharmacia (Uppsala, Sweden). Poly(ethylene glycol) (PEG-4000, new designation PEG-3350) came from Union Carbide (New York, NY, U.S.A.). Monomethoxypoly(ethylene glycol) (M-PEG) with a molecular weight of 5000 was a generous gift from Union Carbide. Poly(vinyl alcohol) (PVA), molecular weight 13000, was purchased from Serva (Heidelberg, G.F.R.). Bovine serum albumin, bovine haemoglobin, whale myoglobin, horseradish peroxidase (type VI) (E.C. 1.11.1.7), glucose oxidase (type V) (E.C. 1.1.3.4), N-hydroxysuccinimide, 4-aminoantipyrine and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) were obtained from Sigma (St. Louis, MO, U.S.A.). Gentamicin was a generous gift from Schering (Kenilworth, NJ, U.S.A.). All other chemicals used were of analytical-reagent grade.

Preparation of "mild" hydrophobic resins

M-PEG–Sephadex G-25 Medium. M-PEG was activated with epichlorohydrin to make a glycidyl ether which was then coupled to Sephadex[6,7].

PVA–Sepharose. PVA (4 g) in 0.1 *M* sodium carbonate (pH 11.0) (1 l) was coupled to epoxy-activated Sepharose 6B (9 ml) according to a procedure described by Pharmacia[8].

Modification of proteins with monomethoxy-PEG (M-PEG)

Activation of M-PEG with triazine was performed according to the method of Abuchowski *et al.*[9].

Glucose oxidase (1.5 mg) was dissolved in a solution of 0.1 *M* triethanolamine hydrochloride and 0.1 *M* sodium chloride (pH 9.4) and dialysed against the same

buffer. The enzyme was modified by the addition of 40 mg of activated M-PEG (in a total volume of 5 ml) and incubated for 1 h on a rocking table at room temperature. Then a conjugate between M-PEG–glucose oxidase and gentamicin was formed using a carbodiimide coupling procedure. N-Hydroxysuccinimide (1.15 mg) and EDC (1.9 mg) were added to the modified protein and after 15 min gentamicin (0.22 mg) was also added. Finally, glycine was added to give a concentration of 1.0 *M*.

Glucose oxidase activity was measured by a two-step enzymatic reaction where glucose oxidase oxidized glucose giving gluconic acid and hydrogen peroxide, which then acted as a substrate for peroxidase. The final concentrations were: 1.25 μg/ml horseradish peroxidase, 45.4 m*M* glucose, 1 m*M* 4-amino-antipyrine and 17.5 m*M* phenol. The enzyme activity was determined spectrophotometrically at 510 nm.

Two-phase systems. The protein solution (100 μl) was mixed with a well-mixed phase system (900 μl) to give a final concentration of 0.135 g/g PEG-4000, 0.135 g/g $MgSO_4 \cdot 7H_2O$ and 10 m*M* Tris–HCl (pH 7.50). The partitioning coefficient is defined as the ratio of the concentration in the top and bottom phase, respectively:

$$K_{part} = C_{top}/C_{bottom}$$

RESULTS AND DISCUSSION

The following characteristics have been proposed as criteria for hydrophobic interaction chromatography: (1) hydrophobic sites can be identified on the stationary phase; binding is promoted by (2) increased temperature and (3) increased ionic

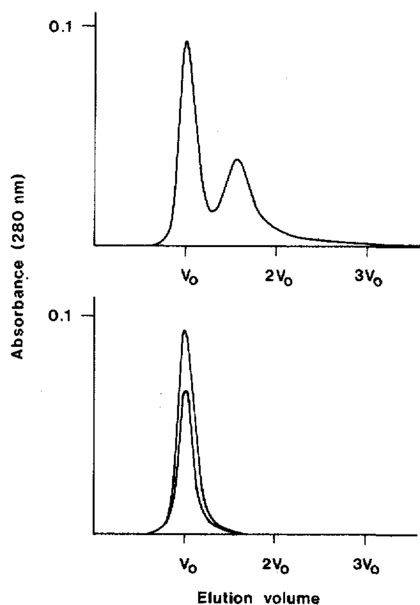

Fig. 1. (a) Separation of bovine serum albumin and bovine haemoglobin on a column (25 × 0.7 cm) of PVA–Sepharose at 26°C and 4.8 ml/h. The mobile phase consisted of 3 *M* sodium chloride and 10 m*M* Tris–HCl (pH 7.50). A 30-μl sample was applied, containing 17 mg/ml of albumin and 6 mg/ml of haemoglobin. (b) Same conditions as in (a) but using unsubstituted Sepharose.

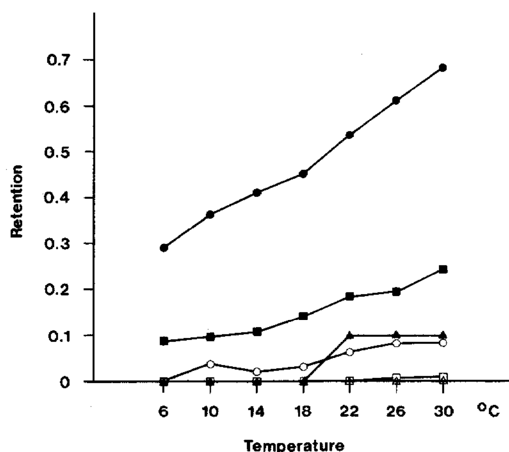

Fig. 2. Retention of serum albumin and haemoglobin as a function of temperature and ionic strength. Experimental conditions are given in Fig. 1a. For 0.15 *M* sodium chloride, the retention of both proteins is zero over the whole temperature range. Retention is defined as $(V_e - V_0)/V_0$, where V_e is the elution volume and V_0 is the void volume for the proteins. Symbols used: albumin at (△) 1 *M*, (□) 2 *M* and (○) 3 *M* sodium chloride; haemoglobin at (▲) 1 *M*, (■) 2 *M* and (●) 3 *M* sodium chloride.

strength[10]. All three points have to be met in order to establish the hydrophobic nature of the interactions.

The above criteria were used to evaluate the chromatographic separation of bovine serum albumin and bovine haemoglobin on PVA–Sepharose. It was found that the retention time for albumin did not depend very much on the ionic strength or the temperature, whereas haemoglobin was retarded when the ionic strength or temperature was increased implying that its interaction was of a hydrophobic nature. Fig. 1a shows an example of a chromatogram. The peaks were identified by making runs for each protein separately. The retention values of the two proteins are shown in Fig. 2 as a function of temperature and ionic strength. As a control, the two proteins were run on PVA–Sepharose at physiological ionic strength (0.15 *M*) and on unsubstituted Sepharose using 3 *M* sodium chloride at 26°C. In both cases a single symmetrical peak was obtained (Fig. 1b). In no case was material eluted when the ionic strength was decreased to 0.15 *M*, after a run with an ionic strength in the range 1–3 *M*.

The results differ markedly from those obtained on conventional hydrophobic interaction chromatography with, *e.g.*, octyl–Sepharose, where albumin is strongly retarded[3]. Serum albumin has binding sites for fatty acids and other hydrophobic ligands[11] which may be responsible for its binding to alkyl-gels. Such specific interactions are not expected when albumin is run on gels substituted with PVA or PEG. The results also demonstrate that aside from those specific hydrophobic regions, the molecules are hydrophilic in nature.

An important factor in hydrophobic interaction chromatography is the solubility of the proteins to be separated. It is generally known that adsorption on to a hydrophobic gel is expected to occur when the ionic strength is increased to a point rather close to that for salting-out precipitation of the protein. Since albumin has a good solubility even at 3 *M* sodium chloride, it may be expected that albumin would not be retarded on the PVA–Sepharose gel, a prediction that holds (Fig. 2).

TABLE I

RETENTION TIMES FOR THREE PROTEINS ON PVA-SEPHAROSE RUN AT 26°C AND 3 *M* SODIUM CHLORIDE

Myoglobin was eluted later than albumin and haemoglobin at low ionic strength, probably because of a gel filtration effect. In the corrected retention, the elution volume of myoglobin at 0.15 *M* sodium chloride was defined as the void volume.

Protein	*Relative retention*	*Corrected retention*
Bovine serum albumin	0.07	0.07
Bovine haemoglobin	0.62	0.62
Whale myoglobin	0.35	0.20

The other component in the mixture is a tetrameric protein, haemoglobin. This protein is known to dissociate into dimers at high ionic strengths[12]. The dimeric and tetrameric forms are in a rapid equilibrium, which can be followed by measuring the apparent molecular weight. When the ionic strength is increased from 0.15 to 3 *M* by addition of sodium chloride, the apparent molecular weight is slightly decreased. This effect is, however, too small to cause a separation based on gel filtration[13]. The fact that no separation occurs when the two proteins are run on unsubstituted Sepharose with 3 *M* sodium chloride also indicates that the separation is not dependent on the dissociation *per se*.

The solubility of haemoglobin is significantly lower than for serum albumin but high enough to show that the retention cannot be explained merely as an effect of limiting solubility. This is also shown by the fact that the same chromatographic behaviour was obtained when the protein solution was diluted ten times before application on to the column. Furthermore, myoglobin was run as indicated in Table I. Myoglobin is more soluble than both haemoglobin and albumin, but had an intermediate retention.

PEG and PVA are generally regarded as hydrophilic polymers, as they are soluble in water, in contrast to many other polymers. However, hydrophilicity and hydrophobicity are, in this context, no strict terms but merely relative notations. The

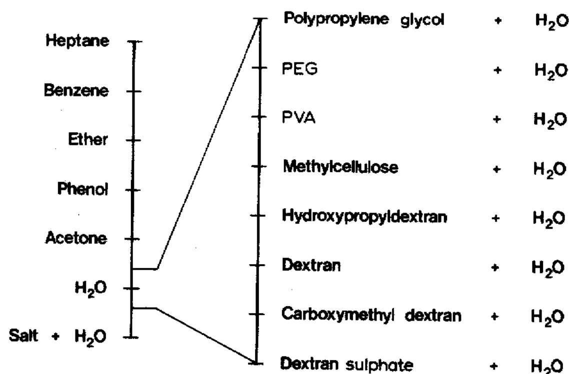

Fig. 3. Hydrophobic scale, where the different hydrophobicities of aqueous solutions are indicated for different polymers. (Reproduced from Albertsson[4] by kind permission.)

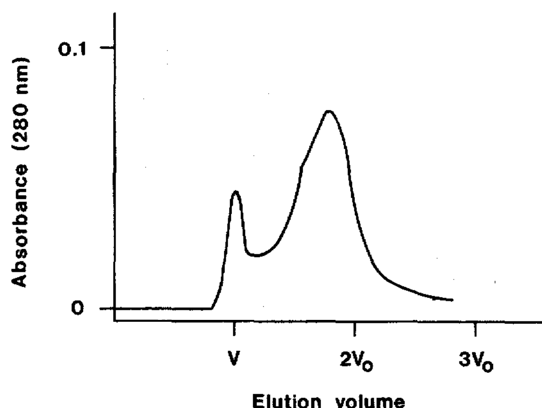

Fig. 4. Chromatogram showing the separation of M-PEG–gentamicin–glucose oxidase into two fractions on a M-PEG–Sephadex G-25 column (32 × 1.0 cm). The mobile phase consisted of 1.41 *M* magnesium sulphate and 10 m*M* Tris–HCl (pH 7.50).

fact that some polymers are water soluble does not prove that they cannot have hydrophobic properties. Aqueous solutions of different polymers can be ordered in a hydrophobic scale in the same way as organic solvents[4], as shown in Fig. 3.

The project for using these mild chromatographic systems was initiated from a need to separate native from modified proteins, which were prepared for use in partition affinity ligand assay[14]. At that time no useful chromatographic technique was available for separating proteins which differed only by the presence of absence of a few chains of covalently attached poly(ethylene glycol). For the above-mentioned approach to be useful, there must be a correlation between partition behaviour in the phase system and the corresponding chromatographic behaviour on the column. For this reason the hydrophobicity of poly(ethylene glycol) modified glucose oxidase was measured using these two methods and the two sets of results were compared subsequently.

We have previously attached PEG to proteins in order to govern their partitioning in aqueous two-phase systems. For example, native concanavalin A (Con A) partitions to the salt-rich bottom phase in the PEG–magnesium sulphate phase system (K_{part} = 0.031) whereas PEG–Con A partitions to the PEG-rich phase (K_{part} = 80)[14]. In this study, however, glucose oxidase was used. Native glucose oxidase had a K_{part} of 0.077 in the PEG–magnesium sulphate system as determined from the enzyme activity. The M-PEG-modified and gentamicin-conjugated glucose oxidase had a K_{part} of 0.064. This is lower than for the native enzyme, probably because of the hydrophilic nature of gentamicin.

The M-PEG-modified and gentamicin-conjugated glucose oxidase was run on M-PEG–Sephadex (Fig. 4) with 1.41 *M* magnesium sulphate and 10 m*M* Tris–HCl (pH 7.50) and was separated into two fractions. The K_{part} of the enzyme activity in the first peak was 0.012 whereas that for the retarded fraction was 0.11. Thus it was found that a material with an initial K_{part} of 0.064 in aqueous two-phase systems gives rise to two fractions from the hydrophobic column, having K_{part} values on either side of this value. This suggests that the mixture is resolved according to the same principles as those governing the partition behaviour. It was thus possible to separate strongly

PEG-modified enzyme molecules from unmodified or only weakly modified molecules by this chromatographic technique.

From the studies presented here it seems that use of weak hydrophobic substituents on chromatographic materials yields products with unique properties. It is therefore to be expected that such gels may be useful in the future for resolving mixtures of compounds that are not easily separated today. Separation based on multipoint attachment must, in order to be operable, be based on weak individual interactions. The use of sorbents as discussed in this paper may offer a useful alternative for the separation of particulate structures.

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