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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PROTEINS ON N-METHYLPYRIDINIUM POLYMER COLUMNS

ATSUSHI SUGII*, KUMIKO HARADA, KOICHI NISHIMURA, RYOSUKE HANAOKA and SEIICHI MASUDA

Faculty of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Kumamoto 862 (Japan)

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SUMMARY

Two types of 4-methylpyridinium polymers (4VP–DVB–Me and 4VP–EG–Me, cross-linked with divinylbenzene and ethylene glycol dimethacrylate, respectively) were employed for the analysis of proteins in ion-exchange high-performance liquid chromatography. These polymers had different physical properties in the dry state, but showed similar retentions in size-exclusion chromatography using carbohydrate standards. Generally, the 4VP–EG–Me column was superior to the 4VP–DVB–Me column with regard to separation and recovery of proteins.

INTRODUCTION

Of the available techniques for the purification and analysis of proteins, high-performance liquid chromatography (HPLC) in various modes has been widely used. Ion-exchange chromatography has the advantage that the chromatography can be carried out in an aqueous buffer solution without an organic solvent. Therefore, it is possible to prevent the denaturation of proteins caused by organic solvents.

Among the column packings for the ion-exchange HPLC of proteins, organic polymer-based supports[1–4] have the advantages over surface-modified silica gels of chemical stability in alkaline solutions, higher sample loading capacities and a longer column life. In general, improvements in these matrices occur with hydophilic polymers such as poly(vinyl alcohol) gel[1–3] and glycol methacrylate gel[4].

In this work, two types of N-methylpyridinium polymers, cross-linked with either hydrophobic divinylbenzene (DVB) or hydophilic ethylene glycol dimethacrylate (EG), were applied to the ion-exchange HPLC of proteins, and the effect of cross-linkages on the retention behaviour was studied. Various anion-exchange polymers containing diethylaminoethyl (DEAE)/quaternary ammonium groups acting as the functional groups have been applied to the separation of proteins by HPLC However, polymers having quaternary pyridinium groups intended for protein analysis have not previously been studied.

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EXPERIMENTAL

Apparatus and materials

Chromatographic measurements were carried out with a Hitachi 655A-11 high-performance liquid chromatograph equipped with a Hitachi L-5000 gradient generator and a Hitachi L-4000 variable-wavelength UV monitor or a Hitachi L-3300 refractive index detector.

The specific surface area was measured with a Monosorb instrument (Yuasa Battery, Osaka, Japan) and the pore volume and pore diameter with a mercury porosimeter (Carlo Erba, Milan, Italy).

All the proteins employed were commercial products. Bovine serum albumin (BSA), soybean trypsin inhibitor (STI), chicken egg albumin (CEA), bovine milk β-lactoglobulin (β-LG), bovine milk β-lactoglobulin A (β-LGA), bovine milk β-lactoglobulin B (β-LGB), bovine γ-globulin (γ-G), bovine pancreatic α-chymotrypsin (α-CHT) and horse heart cytochrome *c* (CYC) were obtained from Sigma (St. Louis, MO, U.S.A.) and chicken egg lysozyme (LY) from Merck (Darmstadt, F.R.G.).

All other chemicals were of analytical reagent grade from Wako (Osaka, Japan) and Tokyo Kasei Kogyo (Tokyo, Japan).

Preparation of column packings

Macroporous 4-vinylpyridine polymer cross-linked with DVB (4VP–DVB) was prepared as described[5] previously and macroporous 4-vinylpyridine polymer cross-linked with EG (4VP–EG) was prepared in the same manner. Both polymers (particle size 10–15 μm) were converted by methylation with methyl bromide into N-methylpyridinium polymers[6], which are abbreviated to 4VP–DVB–Me and 4VP–EG–Me, respectively.

Chromatography

The bromide forms of 4VP–DVB–Me and 4VP–EG–Me were packed into a stainless-steel column (25 cm × 4 mm I.D.) and conditioned with 0.05 *M* Tris–HCl buffer (pH 7.0) containing 0.5 *M* sodium chloride or 0.05 *M* phosphate buffer (pH 7.0) containing 0.5 *M* sodium chloride. These columns showed a pressure drop of 30–40 kg/cm^2 at a flow-rate of 0.5 ml/min. There was no problem with the use of high pressure (150 kg/cm^2).

Sample proteins were eluted with a 30-min linear gradient from 0 to 0.5 *M* sodium chloride in 0.05 *M* Tris–HCl buffer (pH 7) or 0.05 *M* phosphate buffer (pH 7) at a flow-rate of 0.5 ml/min. The chromatographic procedure was performed at room temperature, with detection at 280 nm. Unless specified otherwise, 60–80 μg of protein were injected onto the column.

The pore size of the polymers in the wet state was evaluated by measuring the relationship between the molecular weight and the elution time in size-exclusion chromatography (SEC) with standard samples of the dextrans T10, T40, T70 and T2000 (Pharmacia, Uppsala, Sweden) and the oligosaccharides xylose and lactose (Wako), maltotriose and maltoheptaose (Sigma). The amount of sample injected was 2–20μg. The hold-up volume of a column was measured with heavy water (^2H_2O). The retention behaviour of the carbohydrates was measured on the same column with 0.5 *M* sodium chloride solution as the eluent and by using the refractive index detector.

Recovery of proteins

The recovery of proteins during chromatographic operation was evaluated by injection of the proteins (0.2 mg) into the column equilibrated with 0.05 *M* Tris–HCl buffer (pH 7.0) and by eluting with 0.05 *M* Tris–HCl (pH 7.0) containing 0.5 *M* sodium chloride at a flow-rate of 0.5 ml/min. Spectrophotometric determination at 225 nm of the proteins in the column effluent, which was pooled for 20 min after protein injection, gave the recovery[2].

RESULTS AND DISCUSSION

Characterization of the polymer

Ion-exchange chromatography requires an electrostatic interaction between the solutes and the functional groups on the support surface. Therefore, it is generally desirable that the ion exchangers are macroporous in order to permit the penetration of proteins into the ion-exchange group.

It has been reported that the pore size of the support markedly affects the resolution and recovery of proteins in reversed-phase chromatography[7–9]. Similar results were obtained by Vanecek and Regnier[10,11] with ion-exchange HPLC systems. Table I shows the physical properties of the dried polymers used in this experiment. The characteristic surface properties of both polymers substantiate a porous structure. However, the most common pore radius of 4VP–DVB–Me is larger than that of 4VP–EG–Me. Fig. 1 shows molecular-weight calibration graphs obtained from the retention behaviour of carbohydrates. The exclusion limits of both polymers for the carbohydrates used were about 500 daltons.

Although 4VP–DVB–Me has a larger pore diameter than 4VP–EG–Me in the dry state, the exclusion limits of both polymers are similar and small. The pore volumes of the polymers in the wet state were determined by comparing the elution volume of an excluded molecule (T2000) with that of an included molecule (^2H_2O)[12]. The pore volumes (ml per ml of polymer) were 0.38 ml (4VP–DVB–Me) and 0.34 ml (4VP–EG–Me), *i.e.* nearly identical, indicating that the pore size distribution of both polymers in the wet state is different from that in the dry state. This may suggest that the proteins used scarcely penetrate into the pores and interact only on the limited surface of the polymers. Recently, non-porous polymer packings with covalently coupled functional groups on the surface (ion-exchange capacity 0.1–0.15 mequiv./ml) have been used for the separation of proteins in ion-exchange HPLC[12–14]. It was suggested that the interaction related to the retention of proteins occurs only on the

TABLE I

CHARACTERIZATION OF POLYMERS IN THE DRY STATE

Polymer	*Elemental analysis (%)*		*Specific surface area* (m^2/g)	*Pore volume* (*ml/g*)	*Most frequent pore radius* (*nm*)
	N	*Br*			
4VP–DVB–ME	4.62	27.90	23.90	0.287	27.5
4VP–EG–ME	3.87	22.28	9.49	0.077	8.7

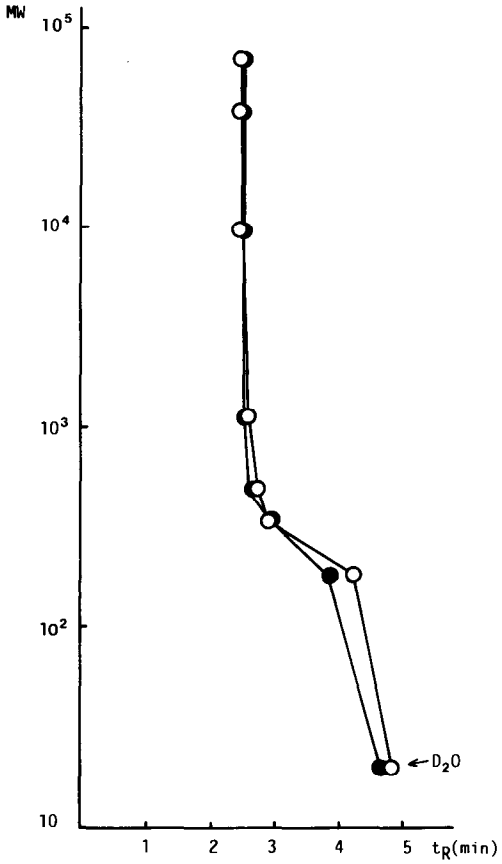

Fig. 1. Plots of molecular weights against retention time (t_R) for carbohydrates. ○, 4VP–DVB–Me; ●, 4VP–EG–Me. Column, 25 cm × 4 mm I.D.; eluent, 0.5 *M* NaCl; flow-rate, 0.5 ml/min; detection, refractive index. Samples: xylose (20 μg), lactose (20 μg), maltotriose (2 μg), maltoheptaose (2μg), dextrans T10 (2 μg), T40 (2 μg) and T70 (2 μg).

outer surface of the polymers. In the proposed pyridinium polymers, a similar behaviour in the retention of proteins is expected.

Anion-exchange HPLC of proteins

Table II shows the retention times of proteins obtained using a 30-min linear gradient from 0 to 0.5 *M* sodium chloride in Tris-HCl buffer (pH 7.0). The retention times of the proteins in both polymer columns are approximate. Proteins having lower isoelectric points (p*I*) than the pH of the mobile phase (pH 7) were retained on the columns, but those having p*I* > 7.0 were eluted in the void volume of the column. The retention times of the proteins varied with the pH of the buffer, as shown in Fig. 2. Considerable changes in the retention of proteins were observed at pH values near the p*I* valvues of the proteins. The major retention process of a protein having an overall negative charge may be governed by ionic interactions in the present system.

TABLE II

RETENTION TIMES (t_R) OF PROTEINS ON 4VP–DVB–ME (I) AND 4VP–EG–ME (II) COLUMNS IN TRIS–HCl BUFFER SYSTEM

The proteins were eluted using a 30-min linear gradient of sodium chloride from 0 to 0.5 *M* in 0.05 *M* Tris–HCl (pH 7) at a flow-rate of 0.5 ml/min. Sample loading, 60 μg. Column, 25 cm × 4 mm I.D.

Protein	*pI*	t_R *(min)*	
		I	*II*
BSA	4.7–4.9	15.82	17.2
		16.50	17.98
		16.89	18.40
β-LGA	5.2	21.4	23.34
β-LGB	5.1	19.12	21.32
CEA	4.6	15.63	17.1
STI	4.3	20.62	21.44
γ-G	8.0	2.42	2.40
LY	11.4	2.45	2.40
CYC	11.0	2.42	2.40
α-CHT	8.1	2.57	2.48

However, it is also necessary to take into account the effect of other interactions, such as hydrophobic and π–π interactions, on the retention of proteins.

The kind of buffer solution used markedly affected the retention of proteins as reported with other ion-exchange HPLC systems[15,16]. When gradient elution with sodium chloride in a phosphate buffer system was used, the protein peak became broad and poor peak resolution was observed compared with that in the Tris–HCl system.

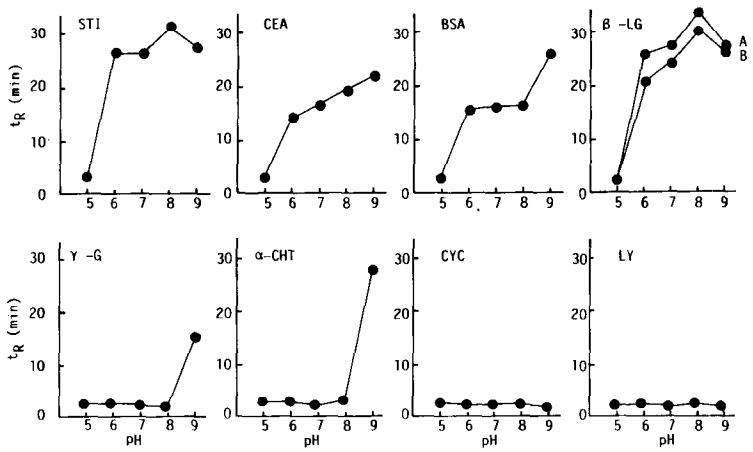

Fig. 2. Relationship between pH of mobile phase and retention time (t_R) on 4VP–DVB–Me column. Eluent, 30-min linear gradient of NaCl from 0 to 0.5 *M* in 0.05 *M* phosphate buffer (pH 5–9); flow-rate, 0.5 ml/min.

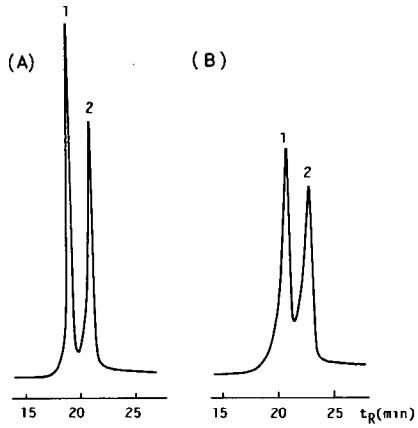

Fig. 3. Chromatograms of β-LG on (A) 4VP–DVB–Me and (B) 4VP–EG–Me columns. Peaks: 1 = β-LGB; 2 = β-LGA. Chromatographic conditions as in Table II. Sample loading, 80 μg.

The resolution of β-LGA and β-LGB in the Tris–HCl system is shown in Fig. 3. Good separation of these proteins was achieved on the 4VP–DVB–Me column. On the TSK Gel IEX-645 DEAE column, for which the exclusion limit of proteins is much higher than 10^6 (ref. 2), similar resolutions have been reported. Hence it is concluded that a polymer with a small pore size that excludes large molecules can also be applied to packing materials for protein analysis in ion-exchange chromatography.

The loading capacity of the 4VP–DVB–Me columns was examined with β-LG. Good resolution of β-LGA and β-LGB was obtained with up to 0.5 mg of sample but it decreased with increasing sample load (Fig. 4). In the investigations with the non-porous polymer packing, β-LG (60 μg) was loaded onto the non-porous MA7P column (10-μm particle diameter, 5 cm × 4.6 mm I.D.)[12] and a crude lipoxidase (100–

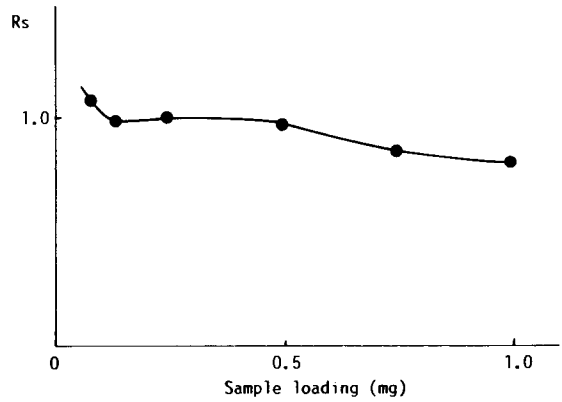

Fig. 4. Dependence of resolution (R_s) on sample load in the separation of β-LGA and β-LGB on the 4VP–DVB–Me column. (25 cm × 4 mm I.D.). Chromatographic conditions (except for sample loading) as in Table II.

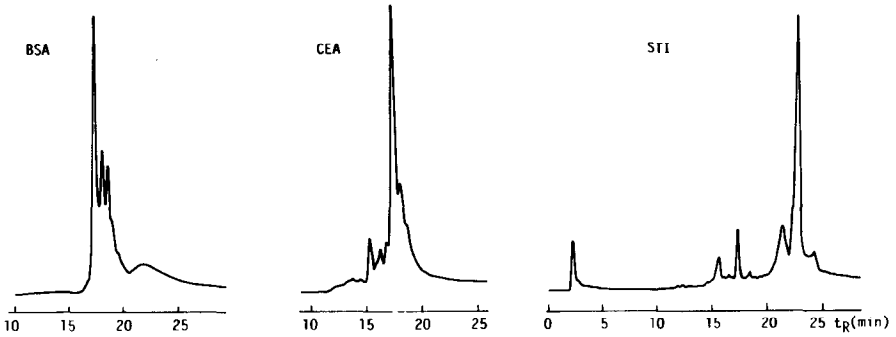

Fig. 5. Chromatograms of proteins on the 4VP–EG–Me column. Chromatographic conditions as in Table II.

200 μg) was applied to the TSKgel DEAE-NPR column (2.5-μm particle diameter, 3.5 cm × 4 mm I.D.)[13]. The loading capacity of the 4VP–DVB–Me column was higher than those of the non-porous polymer columns.

On the other hand, the chromatograms of other proteins show slightly better separations on the 4VP–EG–Me column than on the 4VP–DVB–Me column, as shown in Fig. 5. For BSA, multiple peaks were observed, owing to the intrinsic heterogeneity of BSA; serum albumin consists of several components, such as mercaptoalbumin, non-mercaptoalbumin and fatty acid binding albumin[17]. Identification of the peaks is now under investigation.

The recovery of proteins is shown in Table III. The recovery on the 4VP–EG–Me column was almost quantitative for all the proteins investigated, whereas that on the 4VP–DVB–Me column was poor. A hydrophobic interaction between the protein and the cross-linking part of the polymer may affect the non-specific adsorption of the protein.

In order to examine the stability of the column packings in alkaline solution, the columns were treated with 30 ml of dilute sodium hydroxide solution containing

TABLE III

RECOVERY OF PROTEINS FROM 4VP–DVB–ME (I) AND 4VP–EG–ME (II) COLUMNS

Protein	*Recovery (%)*[a]	
	I	*II*
BSA	85.9	101.2
β-LG	74.6	92.5
CEA	70.1	93.8
STI	63.6	89.3
γ-G	84.3	104.6
LY	97.8	103.1
CYC	95.1	102.2
α-CHT	95.9	100.9

[a] Recovery was obtained from absorbance at 225 nm as described under Experimental. Sample loading, 0.2 mg.

0.2 *M* potasium chloride (pH 12) at a flow-rate of 0.5 ml/min. The resolution of proteins on the columns did not change with this treatment. No significant deterioration of the column was observed even after continuous use for 3 months.

In conclusion, the porous 4VP polymers studied here are useful for protein analysis by anion-exchange HPLC, despite their low-molecular-weight exclusion limits. 4VP–EG–Me prepared by using a hydrophilic cross-linker is particularly promising for high protein recoveries.

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