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HIGH-PERFORMANCE GEL FILTRATION OF WATER-SOLUBLE SAMPLES ON POLYVINYL ALCOHOL COLUMNS

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SUMMARY

The chromatographic behaviour in aqueous solution of pullulans, polyethylene glycols, peptides and proteins with different molecular weights on newly developed polyvinyl alcohol columns (Asahipak GS column series: GS-310, GS-320, GS-510 and GS-520) has been investigated. Pullulans and polyethylene glycols eluted according to the gel filtration mode, so both series of compounds produced almost equal calibration curves for GS-310 and GS-320 and for GS-510 and GS-520; the exclusion limits on GS-310 and GS-320 and on GS-510 and GS-520 were found to be *ca.* 40,000 and 300,000, respectively. Peptides and proteins were found to adsorb slightly on the columns. However, the plots of the elution volumes against the logarithm of the molecular weights for many of the substrates tested gave rise to the linear calibration curves. Recovery of several crude proteins was also studied for the columns and high values were obtained (81–100%). The effects of changes in the flow-rate, temperature and concentration of electrolytes added to the eluents, on the retention of all four types of compound are also reported.

INTRODUCTION

As one of the most powerful and useful analytical methods in biological science, gel filtration chromatography has played a significant role in the separation and purification of biologically important substrates as well as in the determination of the molecular weights of the samples.

The method began with the introduction of cross-linked dextran gel by Porath and Flodin[1]. Because of the increasing requirements of more precise and accurate separation, many packing materials, which can be used in aqueous systems, have

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been since developed, e.g. polyacrylamide, polyethylene glycol dimethacrylate[2,3] and polyacryloylmorpholine[4,5]. These materials, however, were mechanically weak or semirigid, resulting in tedious and time-consuming experiments. This meant that high-speed gel filtration chromatography could not be achieved, and also that the separation caused denaturation of biological samples at ambient temperature.

The silica-[6–8] and polymer-[9–12] based chromatographic gels that have recently become commercially available overcome the above problems. However, although high-performance gel filtration chromatography of complex biological samples is possible on such columns, chromatographic materials which have improved performance or chromatographic behaviour different from that of currently available columns are still needed for the separation of samples that cannot be resolved satisfactorily by the methods available at present.

Recently, columns consisting of rigid polyvinyl alcohol (PVA) gels have been developed for gel filtration chromatography and marketed by Asahi (Tokyo, Japan). In the present study, characteristics of these PVA columns were examined using standard samples (polyethylene glycols, pullulans, peptides and proteins) in order to evaluate their applicability to biological samples.

TABLE I

MOLECULAR WEIGHTS OF COMPOUNDS USED

Compound	*Mol. wt.*	*Source**
P-800	758,000	A
P-400	338,000	A
P-200	194,000	A
P-100	95,400	A
P-50	46,700	A
P-20	20,800	A
P-10	12,000	A
P-5	5300	A
PEG 4000	4000	A
PEG 1000	1000	A
PEG 600	600	A
PEG 300	300	A
EG	62	A
PEO	25,000	A
IgM	900,000	B
Haptoglobin	760,000	B
IgA	400,000	B
IgG	150,000	B
Transferrin	90,000	C
Albumin	65,000	C
Ovalbumin	45,000	C
Chymotrypsinogen A	24,500	C
Myoglobin	17,000	C
Lysozyme	14,000	C
Ribonuclease	13,800	C
Cytochrome *c*	13,000	C
Insulin	6000	C
Oxytocin	1007	C

* (A) Showa Denko; (B) The Green Cross Corp.; (C) Sigma.

EXPERIMENTAL

Pullulans (PL) and polyethylene glycols (PEG) were purchased from Showa Denko (Tokyo, Japan) and standard peptides and proteins were from Sigma (MO, U.S.A.). Molecular weights of these compounds are listed in Table I. Pronase E was purchased from Kaken Kagaku (Tokyo, Japan). Other chemicals were obtained from The Green Cross Corporation (Osaka, Japan). These samples were used without further purification. Silkworm serum powder was prepared in our laboratory according to the method described previously[13]. Water purified by a Mili R/Q (Millipore, U.S.A.) was used.

An HLC-830D high speed liquid chromatograph (Toyo Soda, Japan) equipped with a UV-8 Model II spectrometer (Toyo Soda) and an RI-8 differential refractometer (Toyo Soda) was used in this study. A combination of an HPLC-803 high-speed liquid chromatograph (Toyo Soda) with UVIDEC 100 UV spectrometer (Jasco, Japan) was also employed. Refractive index (RI) detection was used for PL and PEG. Standard peptides, proteins and pronase E were monitored at 210 nm and silkworm serum samples at 280 nm.

Prepacked PVA gel columns (50 cm × 7.6 mm I.D.), GS-310, GS-320, GS-510 and GS-520, were obtained from Asahi. The mean particle size of the column material is *ca.* 9 μm.

RESULTS AND DISCUSSION

According to the manufacturer, the four columns are classified into two groups: GS-310 and GS-510 in one and GS-320 and GS-520 in the other. Therefore, differences in chromatographic behaviour between the two types of column in the same group and between the two groups was first investigated. Samples used in this experiment were PL and PEG. PL was chosen as high-molecular-weight sample. Purified water was used as the eluent.

PL and PEG were first chromatographed with water on GS-310 and GS-510 columns. The retention volumes were plotted against the logarithm of the molecular weights. The calibration curves obtained from the observed values (Fig. 1a) indicate

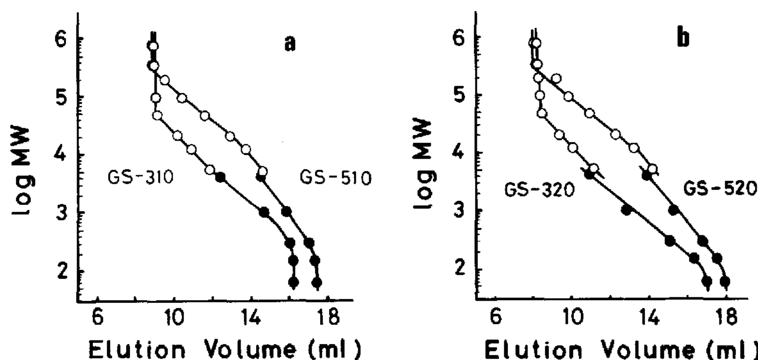


Fig. 1. Calibration curves for (a) GS-310 and GS-510 and (b) GS-320 and GS-520. Samples: ○ = pullulan; ● = polyethylene glycol. Conditions: eluent, water; flow-rate, 1 ml/min; sample concentration, 0.1%; sample volume, 50 μl; detection, RI; temperature, ambient.

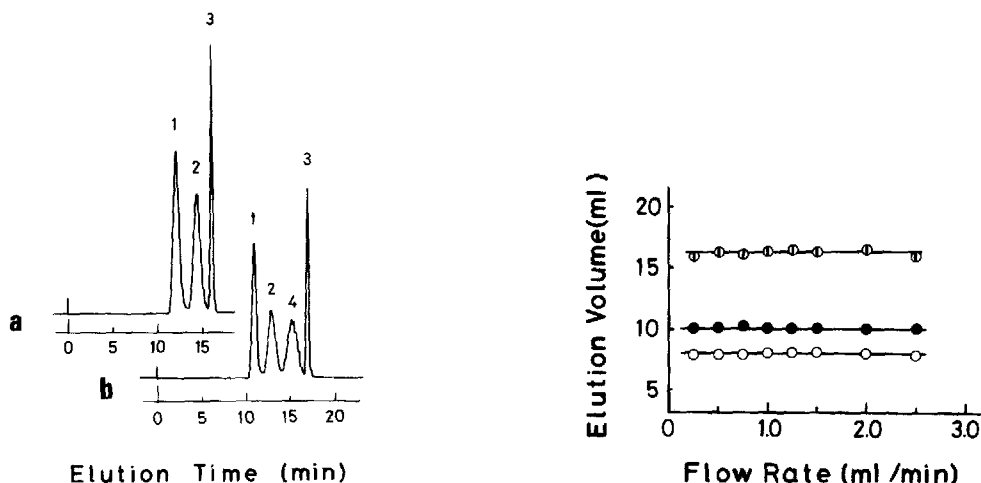


Fig. 2. Separation of a mixture of polyethylene glycols on (a) GS-310 (b) GS-320. Peaks: 1 = PEG 4000; 2 = PEG 1000; 3 = EG; 4 = PEG 300. Conditions: eluent, water; flow-rate, 1 ml/min; sample concentration, 0.1% of each solute; sample volume, 50 μl; detection, RI; temperature, ambient.

Fig. 3. Effect of a change in the flow-rate on the elution volumes observed for GS-320. Samples: ○ = PEO; ● = PEG 400; ◑ = EG. Conditions: eluent, water; sample concentration, 0.1%; sample volume, 20 μl; detection, RI; temperature, ambient.

that the GS-310 column separates molecules with molecular weights ranging from 300 to 46,700, and the GS-510 column detects molecular weights from 300 to 300,000. Also, smaller molecules were found to be resolved better by GS-310. It should also be noted that the calibration curves for PL and PEG are almost identical on the two columns. This may indicate that compounds such as carbohydrates found in biological samples can be studied according to the calibration curves obtained in this work.

Similar experiments were carried out using GS-320 and GS-520 columns. As shown in Fig. 1b, calibration curves analogous to those in Fig. 1a were obtained, except that both the curves in Fig. 1a were a little steeper than those in Fig. 1b. This implies that GS-320 and GS-520 columns have a better separation performance for the PL and PEG tested than GS-310 and GS-510. This comparison is represented in Fig. 2 by showing the difference in the separation profiles of a mixture of PEGs on GS-310 and GS-320. It is clear that GS-320 has a higher resolution than GS-310. Also, from the plots in Fig. 1b, the exclusion limits of GS-320 and GS-520 were found to be close to the values obtained for GS-310 and GS-510.

The possibility of fast separation on PVA columns was examined using GS-320 with the same eluent. The samples, polyethylene oxide (PEO) (MW 25,000), PEG 400 and ethylene glycol (EG), were already known to be eluted in gel filtration under such conditions. The effect of a change in the flow-rate on the retention volumes is depicted in Fig. 3: they were found to be constant for flow-rates in the range 0.25–2.5 ml/min. This implies that the GS-320 column could be used for the rapid purification of biological materials. Practical separations at different flow-rates are shown in Fig. 4 for PEG 4000, PEG 1500, PEG 600 and EG on GS-320. The substrates are separated without significant loss of chromatographic resolution even at a flow-rate of 2.0 ml/min (Fig. 4c). At a flow-rate of 0.25 ml/min, each component of PEG 600,

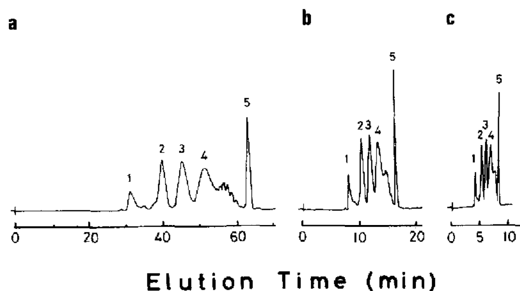


Fig. 4. Separation of a mixture of PEGs on GS-320 at different flow-rates. Peaks: 1 = PEO; 2 = PEG 4000; 3 = PEG 1500; 4 = PEG 600; 5 = EG. Conditions as in Fig. 3.

which was less resolved in Figs. 4b and 4c, could be separated (Fig. 4a).

The retention behaviour of peptides and proteins on the GS-320 and GS-520 columns was investigated. Standard proteins were chromatographed with an eluent (pH 7.0) including sodium phosphate (0.1 *M*) and sodium chloride (0.3 *M*). Plots of the retention volumes against the logarithm of the molecular weights gave straight lines for both GS-320 and GS-520 (Figs. 5 and 6, respectively). However, lysozyme and chymotrypsinogen A were found to deviate from the calibration curves obtained, and bovine albumin eluted so slowly on GS-520 that it did not fit the curve. Of the samples chromatographed, lysozyme travelled most slowly in both the columns. These three compounds may weakly adsorb on the columns. However, straight calibration curves for other peptides and proteins indicate that these columns can be applied to the approximate determination of molecular weight of peptides and proteins that do not adsorb on the columns. As an example, the Fig. 7 shows the separation of ovalbumin, myoglobin and oxytocin on GS-320 within 16 min. The effect

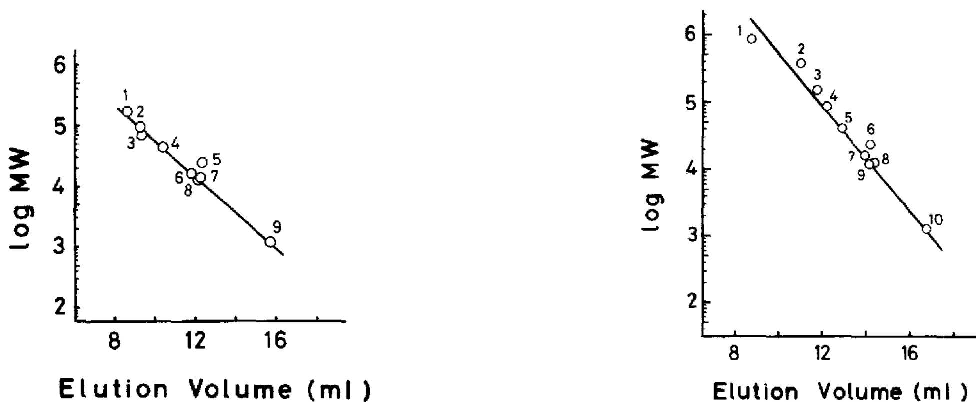


Fig. 5. Calibration curve for standard proteins on GS-320. Proteins: 1 = γ-globulin; 2 = transferrin; 3 = albumin (bovine); 4 = ovalbumin; 5 = chymotrypsinogen A; 6 = myoglobin; 7 = ribonuclease; 8 = cytochrome *c*; 9 = oxytocin. Conditions: eluent, 0.1 *M* sodium phosphate–0.3 *M* sodium chloride (pH 7.0); flow-rate, 1 ml/min; sample concentration, 0.1%; sample volume, 20 μl; detection, UV 210 nm; temperature, ambient.

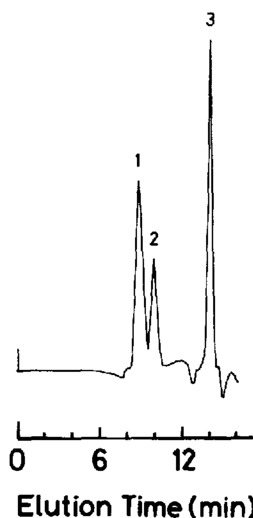
Fig. 6. Calibration curve obtained standard proteins on GS-520. Proteins: 1 = IgM; 2 = IgA; 3 = IgG; 4 = transferrin; 5 = ovalbumin; 6 = chymotrypsinogen A; 7 = myoglobin; 8 = ribonuclease; 9 = cytochrome *c*; 10 = oxytocin. Conditions as in Fig. 5.

of a change in the flow-rate on the retention volumes of these samples on GS-320 was also investigated. The values obtained with flow-rates in the range 0.5–2 ml/min are shown in Fig. 8. The constant retention volumes obtained suggest that this PVA column can also be applied to the fast separation of proteins and peptides.

Another important factor that may influence rapid separation of enzymes is the sample loading capacity of the column. Accordingly, we examined the change in the height equivalent to a theoretical plate (HETP) for different loadings on two column systems: a single GS-520 column and four GS-520 columns connected in series. Transferrin, which was found to elute in gel filtration, was used as a sample. The injection volume was 50 μl for the former system and 100 μl for the latter. Samples with different concentrations were chromatographed in 0.1 *M* sodium phosphate–0.3 *M* sodium chloride at a flow-rate of 1 ml/min (Fig. 9). For a system consisting of a GS-520 column only, it was found that samples up to 1 mg can be loaded without disturbing the separation. With the four GS-520 columns in series, the HETP value was almost constant until the amount reached 3 mg.

The temperature can also be an important factor which may change the retention of samples driving chromatography. We therefore investigated the effect of temperature on the HETP values for the standard proteins, using a GS-520 column. As seen in Fig. 10, the degree of the effect was found to be dependent on the samples. The values of the standard proteins decreased linearly as the temperature increased, whereas that of albumin did not. This temperature-dependent decrease may be due to a decrease in the viscosity of the mobile phase caused by the rising temperature.

It was demonstrated previously[11,12,14] that the addition of salt to a mobile phase results in a change of the retention of the solutes. An analogous test was carried out on GS-320 column by varying the concentration of the sodium chloride solution.



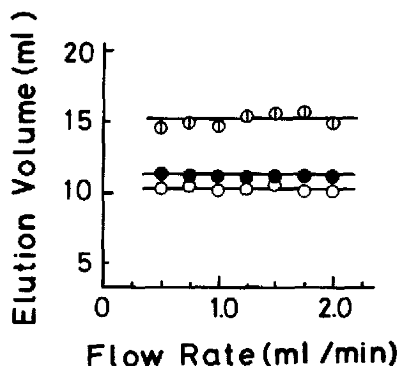


Fig. 7. Separation of a mixture of standard proteins on GS-320. Peaks: 1 = ovalbumin; 2 = myoglobin; 3 = oxytocin. Conditions as in Fig. 5.

Fig. 8. Effect of a change in the flow-rate on the elution volumes of proteins observed GS-320. Samples: ○ = ovalbumin; ● = myoglobin; ⦶ = oxytocin. Conditions as in Fig. 5.

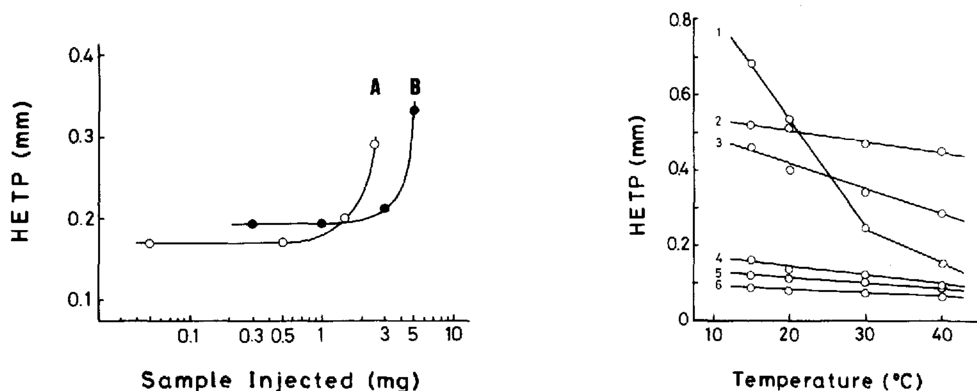


Fig. 9. Effect of sample amount on HETP (A) on a single GS-520 column (50 cm) and (B) on four linked GS-520 columns (200 cm). Conditions: eluent, 0.1 *M* sodium phosphate plus 0.3 *M* sodium chloride (pH 7.0); flow-rate, 1 ml/min; sample volume, 50 μl (A) or 100 μl (B); detection, UV 210 nm; temperature, ambient.

Fig. 10. Effect of a change in temperature on HETP of proteins observed for GS-520. Samples: 1 = albumin (bovine); 2 = IgA; 4 = ovalbumin; 5 = chymotrypsinogen A; 6 = cytochrome *c*. Conditions as in Fig. 5 except for temperature.

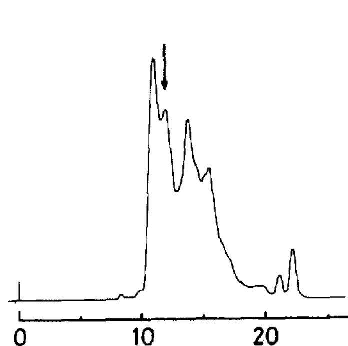
The changes in the retention volumes of two compounds (albumin and myoglobin) at different salt concentrations are summarized in Table II. The use of water as a mobile phase resulted in small retention volumes for the proteins, indicating that the solutes are excluded from the gel matrix by electrostatic repulsion. When salt was added to the eluent, the retention increased. This may be due to the reduction of the ion exclusion. In the salt concentration range from 0.1 *M* to 0.3 *M*, the retention

TABLE II

EFFECT OF ADDED SALT ON THE RETENTION OF PROTEINS ON GS-320

Conditions: Flow-rate, 1 ml/min; sample volume, 20 μl; Detection, UV 210 nm; temperature, ambient.

Mobile phase	*Elution volume (ml)*	
	Albumin	*Myoglobin*
Water	7.44	7.74
0.2 *M* Sodium phosphate	9.76	10.88
0.2 *M* Sodium phosphate + 0.1 *M* sodium chloride	9.68	10.78
0.2 *M* Sodium phosphate + 0.2 *M* sodium chloride	9.52	10.64
0.2 *M* Sodium phosphate + 0.3 *M* sodium chloride	9.48	10.90
0.2 *M* Sodium phosphate + 0.4 *M* sodium chloride	9.48	10.60
0.2 *M* Sodium phosphate + 0.5 *M* sodium chloride	9.60	10.80



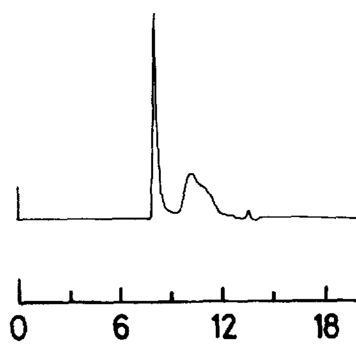


Fig. 11. Separation of crude pronase E on GS-320. Conditions: eluent, 1/15 *M* KH_2PO_4 plus 1/15 *M* Na_2HPO_4 (pH 7.4); flow-rate, 1 ml/min; sample volume, 20 μl; detection, UV 210 nm; temperature, ambient.

Fig 12. Separations of silkworm serum extract on GS-320. Conditions: eluent, 0.1 *M* sodium phosphate plus 0.1 *M* sodium chloride (pH 7.0); flow-rate, 1 ml/min; sample volume, 20 μl; detection, UV 280 nm; temperature, ambient.

volumes of the proteins were constant, but they increased at concentrations higher than 0.3 *M*, suggesting perhaps that hydrophobic interactions are responsible for the large retention volumes. The desirable salt concentration seems to be 0.1–0.3 *M*.

As a practical application, Fig. 11 shows the separation of crude pronase E was performed on GS-320 with phosphate buffer (1/15 *M* KH_2PO_4 plus 1/15 *M* Na_2HPO_4, pH 7.4). The eluates giving peaks were fractionated and examined for enzymatic activity by the Folin–Lowry method[15]. The enzymatic activity was found

TABLE III

CHROMATOGRAPHIC RECOVERY OF PROTEINS

Conditions: eluent, 0.1 *M* sodium phosphate plus 0.3 *M* sodium chloride (pH 7.0); flow-rate, 1 ml/min; Sample concentration, 0.1%; sample volume, 50 μl; detection, UV 210 nm; temperature, ambient.

Compound	*Column*	
	GS-310	*GS-320*
Ovalbumin	92%	91%
Myoglobin	93%	91%
Lysozyme	90%	86%
Cytochrome *c*	89%	89%
	GS-510	*GS-520*
IgM	88%	90%
Haptoglobin	90%	98%
IgA	81%	91%
IgG	88%	91%
Transferrin	98%	100%
Albumin	83%	95%
Lysozyme	90%	95%

to be highest in the fraction indicated by the arrow in Fig. 11. The recovery of the total protein in the chromatogram was measured by the cut-and-weight method, where the peak area obtained was compared with that obtained by passing the same sample through an empty column. The resulting recovery value was *ca.* 85%.

Another application was a separation of crude silkworm (larvae) serum powder on a GS-320 column (Fig. 12). The mobile phase used was 0.1 *M* phosphate buffer including 0.3 *M* sodium chloride. In a previous paper[13], it has been reported that silkworm serum, which was studied by use of Sephadex G-150 gel, contained a glucoprotein with a molecular weight of 130,000 and a group of concomitant proteins with molecular weights from 23,000 to 74,000. The molecular weights of the proteins in the peaks in Fig. 12, which were estimated from the calibration curves shown in Fig. 5, were *ca.* 150,000 (first peak) and 50,000 (second peak), in reasonable agreement with those obtained previously by using G-150. Also, by the cut-and-weigh method, the recovery of silkworm serum proteins was determined to be 78%. The recoveries of various crude proteins obtained on GS columns are listed in Table III. Such high values suggest efficient separation and purification by this column.

CONCLUSION

Pullulans and polyethylene glycols, which have only hydroxyl functional groups were found to elute in gel filtration mode on these newly developed PVA columns. Proteins and peptides were also found to travel in the columns as a function of their molecular weights, although a few of them adsorbed slightly on the gel matrix. A salt concentration of 0.1–0.3 *M* was found to be desirable to eliminate hydrophobic adsorption of the solutes. High recovery values of various proteins were obtained. The PVA columns tested appear to have a significant potential for the separation of biological compounds.

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