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EVALUATION OF NEW SUPPORTS FOR HIGH-PRESSURE AQUEOUS GEL PERMEATION CHROMATOGRAPHY: TSK-GEL SW TYPE COLUMNS

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SUMMARY

New chemically modified silica gel based supports, TSK-GEL SW type columns, for aqueous gel permeation chromatography have been evaluated in detail. Adsorption or denaturation of proteins and enzymes on these supports is negligible, the recoveries of the proteins and enzyme activities being both almost quantitative. The applications of these supports for the study of proteins, enzymes, saccharides and water-soluble synthetic polymer have been investigated.

INTRODUCTION

In gel permeation chromatography (GPC) of biological compounds such as proteins, nucleic acids, enzymes and saccharides, soft gels such as dextran gel, agarose gel and polyacrylamide gel have been used. However, these gels have a serious disadvantage in that they cannot be used under high pressure because of their low mechanical strength. High-pressure supports for the aqueous mobile phase have therefore been developed.

Until now, two types of supports have been available. One type comprises the hydrophilic organic polymer gels such as poly(ethylene glycol dimethacrylate) gel (Merckogel PGM[1]), poly(2-hydroxyethyl methacrylate) gel (Spheron P[2,3]) and sulphonated polystyrene gel (TSK-GEL LS210, Aquapak). The other type consists of inorganic based supports such as controlled porous glass-based support (Glyceryl CPG[4,5]) and silica-based support (μBondagel[6]). However, few gels exhibit the desired properties of low adsorption and high resolution. Moreover, inorganic supports generally have high mechanical strength but show strong adsorption. Many attempts to diminish the strong adsorption have been made.

Recently, a new chemically modified silica gel based aqueous GPC support has been marketed by Toyo Soda (Yamaguchi, Japan) as the TSK-GEL SW type column. The characteristics and applications of this new support have now been investigated.

EXPERIMENTAL

Apparatus

The liquid chromatograph used was a Model HLC-802 UR (Toyo Soda) equipped with both refractive index (RI) and ultraviolet (UV) (280 nm) detectors.

Chromatographic packed columns and reagents

TSK-GEL SW consists of three grades, as shown in Table I. They are packed with distilled water into a stainless-steel column (600 × 7.5 mm I.D.). The characteristics of the three grades were investigated.

TABLE I

TSK-GEL SW SUPPORTS

Grade	*Particle size (μm)*	*Exclusive molecular weight*
G 2000 SW	10 ± 2	$2 \cdot 10^4$
G 3000 SW	10 ± 2	$1.5 \cdot 10^5$
G 4000 SW	13 ± 3	$6 \cdot 10^5$

Non-enzymatic proteins for molecular-weight markers were obtained from Becton-Dickinson, Toronto, Canada. Most of the proteins and enzymes used were the products of P-L Biochemicals (Milwaukee, Wisc., U.S.A.), ICN Pharmaceuticals (Cleveland, Ohio, U.S.A.) and Wako (Osaka, Japan). Standard dextrans were purchased from Pharmacia (Uppsala, Sweden). Saccharides and water-soluble synthetic polymers were obtained from Wako.

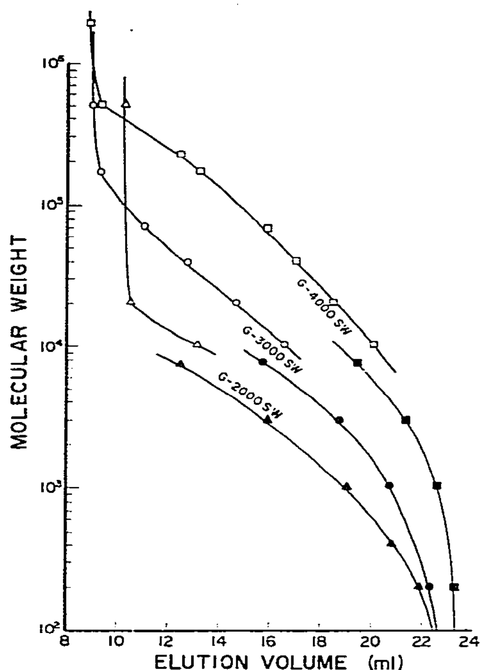
RESULTS AND DISCUSSION

Evaluation of TSK-GEL SW type columns

The calibration curves of the TSK-GEL SW type columns were constructed for standard dextran and polyethylene glycol (Fig. 1). From Fig. 1 it can be seen that the slopes of the curves are gentle compared with those obtained for other general GPC columns. Also, the ratio of the gel's inner volume (V_i) to void volume (V_0) is in general large compared with other general GPC columns. This is especially so in G 3000 SW and G 4000 SW.

It seems that for water-soluble compounds similar to dextran and polyethylene glycol, molecules having molecular weights from several hundreds to several hundred thousands can be separated by aqueous exclusion liquid chromatography.

Generally, the number of theoretical plates depends on the flow-rate: the slower the flow-rate, the larger is the number of theoretical plates. This relation was investigated for the supports G 3000 SW, and the relation between flow-rate and pressure drop was also examined. Ethylene glycol was used as the sample and the flow-rate was changed from 0.5 ml/min to 2.5 ml/min. The results are shown in Fig. 2. The number of theoretical plates decreased with increase of flow-rate but above 1 ml/min the degree of decrease was small. The pressure drop was proportional to the flow-rate, which shows the suitability SW type columns for high-pressure work.



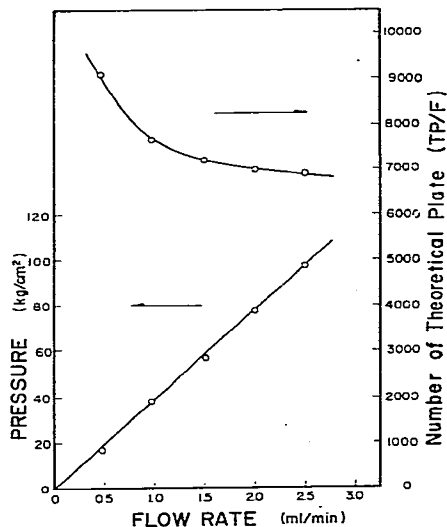


Fig. 1. Calibration curves of TSK-GEL SW type columns. Column: 600 × 7.5 mm I.D. Solvent: water. Flow-rate: 1 ml/min. Sample: dextran (△, ○, □) or polyethylene glycol (PEG) (▲, ●, ■). Detector: RI.

Fig. 2. Dependence of the number of theoretical plates and of the pressure drop on flow-rate. Column: G 3000 SW. Solvent: water. Sample: ethylene glycol, 1 %, 100 μl.

The number of theoretical plates of TSK-GEL SW type columns is guaranteed above 5000 TP/F, which seems to be high compared with other general aqueous GPC columns.

It is very important that an aqueous GPC support shows little adsorption of proteins and does not reduce enzyme activity. The recoveries of proteins and enzyme activity were measured by column chromatography on SW type supports.

For the recovery of proteins, a TSK-GEL G 4000 SW column and an eluent of 1/15 *M* phosphate buffer (pH 6.8) containing 0.1 *M* NaCl were used. About 0.1 mg of protein was charged on the column and the collected eluate examined with a UV detector at 280 nm. The following proteins were investigated and the recovery was almost quantitative in all cases: myoglobin, haemoglobin (bovine), γ-globulin (bovine serum), albumin (bovine serum), β-lactoglobulin, catalase, pepsin and cytochrome *c*.

The recovery of enzyme activity was measured using a G 3000 SW column and an eluent consisting of a 1/15 *M* phosphate buffer (pH 6.8) and 0.1 *M* NaCl. A thermolysin solution was charged on the column. The eluate was collected and the enzyme activity was measured by the casein-digestion method[7]. No decrease in the enzyme activity was observed. Similar results were obtained for trypsin, lysozyme and phosphofructokinase.

Next, the relation between the molecular weights of proteins and their elution

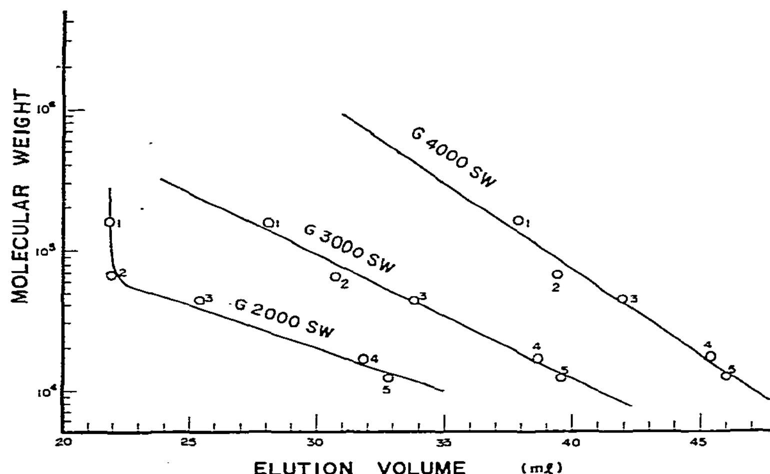
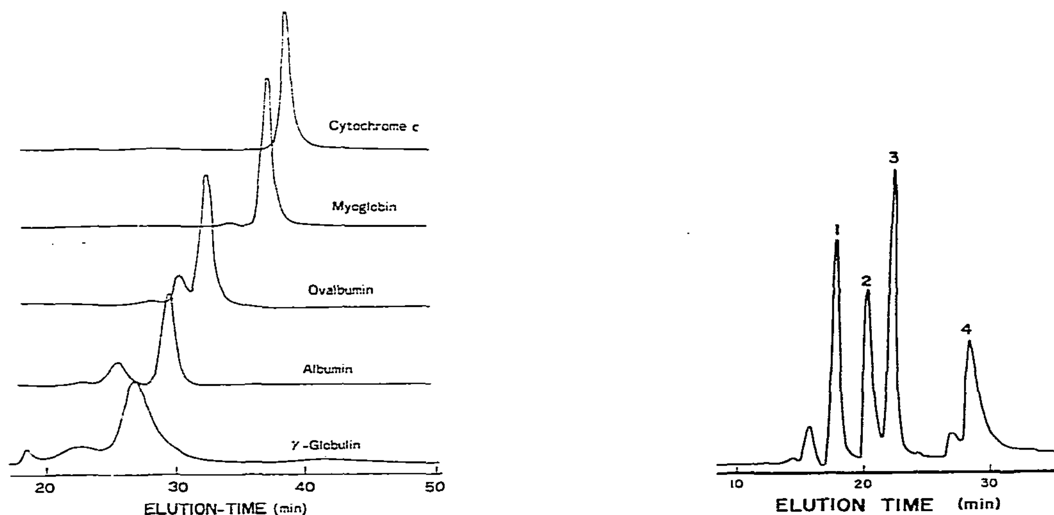


Fig. 3. The relation between molecular weight of proteins and elution volume for SW type columns. Column: SW type. Flow-rate: 1 ml/min. Sample: 1 = γ-globulin (human serum); 2 = bovine serum albumin; 3 = ovalbumin; 4 = myoglobin (sperm whale); 5 = cytochrome *c* (horse heart). Solvent: 1/15 *M* phosphate buffer (pH 6.8) containing 0.1 *M* NaCl.



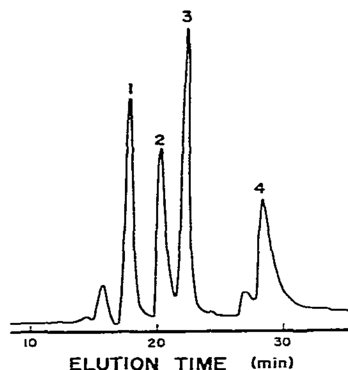


Fig. 4. Charts of standard proteins. Column: G 3000 SW. Pressure: 110 kg/cm^2. Sample concentration: 0.05%; charge 300 μl. Detector: UV 280 nm, 0.16 [ABS] (10 mV). Room temperature.

Fig. 5. Separation of commercial mixture of proteins. Sample: 1 = albumin (human serum); 2 = β-lactoglobulin; 3 = myoglobin; 4 = cytochrome *c*. Column: G 2000 SW. Solvent: 0.05 *M* Tris–HCl (0.1 *M* KCl) buffer. Pressure: 50 kg/cm^2. Flow-rate: 0.90 ml/min. Sample concentration: 0.1% for 1 and 3, 0.05% for 2 and 4; charge 300 μl. Detector: UV 280 nm, 0.16 [ABS] (10 mV). Room temperature.

volumes was investigated using standard proteins. The results for G 2000 SW, G 3000 SW and G 4000 SW are shown in Fig. 3. All the plots are almost on the line and delay due to affinity or adsorption is not recognized. On G 2000 SW, γ-globulin was eluted at the same elution volume as bovine serum albumin. This denotes the void volume of G 2000 SW. In case of G 3000 SW, chromatographic charts of standard proteins are shown in Fig. 4. Most proteins exhibited impurity peaks.

The results of Fig. 3 show that TSK-GEL SW type columns can be used to determine the molecular weights of proteins and enzymes.

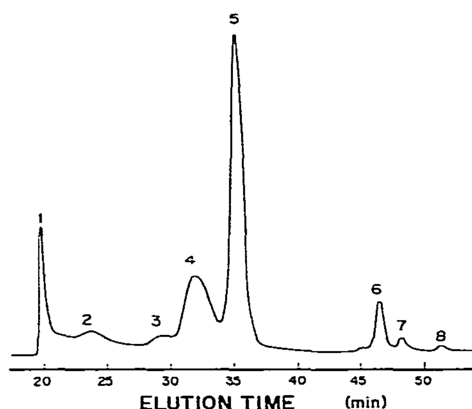
It may be concluded that TSK-GEL SW type columns are excellent for the high-speed preparative separation of biological compounds.

Applications

Applications of SW type columns were investigated for various substances.

A mixture of four commercial proteins, albumin (human serum), β-lactoglobulin, myoglobin and cytochrome C, was studied using a G 2000 SW column. The resulting chart is shown in Fig. 5. It seems that a good separation is easily performed in a very short time.

Human serum was examined using G 3000 SW columns. The resulting chromatography is shown in Fig. 6, which has about eight peaks. The fractions of peaks 1–5 were collected and investigated by cellulose acetate electrophoresis and agarose immunoelectrophoresis. The peaks were assigned as follows: 1 = α_1- and α_2-globulin; 2 = α_1-globulin; 3 = β-globulin; 4 = γ-globulin; 5 = albumin. Peaks 6–8 were not investigated. γ-Globulin and albumin are well separated in a short time. The data suggest that the SW type gel can be used in the preparative separation of human serum components.



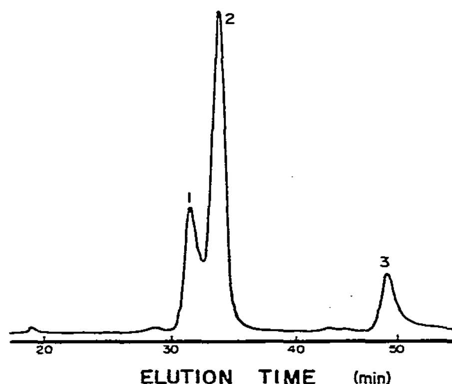


Fig. 6. Separation of human serum. Column: G 3000 SW. Solvent: 0.02 *M* acetate buffer (pH 6.0) containing 0.15 *M* NaCl. Pressure: 90 kg/cm^2. Flow-rate: 1.0 ml/min. Charge: 30 μl. Detector: UV 280 nm, 0.16 [ABS] (10 mV). Room temperature.

Fig. 7. Separation of egg white. Column: G 3000 SW. Solvent: 0.02 *M* phosphate buffer containing 0.15 *M* NaCl. Pressure: 80 kg/cm^2. Flow-rate: 1 ml/min. Sample concentration: 5%; charge 300 μl. Detector: UV 280 nm, 0.16 [ABS] (10 mV). Room temperature.

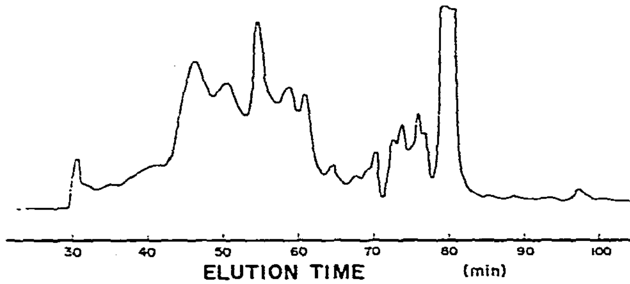


Fig. 8. Separation of rat liver extract. Column: G 3000 SW. Solvent: 1/15 M phosphate buffer (pH 6.8) containing 0.1 M KCl. Pressure: 110 kg/cm^2. Flow-rate: 0.95 ml/min. Sample concentration: 3.4 mg/ml; charge 500 μl. Detector: UV 280 nm, 0.16 [ABS] (10 mV). Room temperature.

Egg white was chromatographed using G 3000 SW columns (Fig. 7). Three of the peaks exhibited seem to be conalbumin (1), ovalbumin (2) and lysozyme (3), respectively. Rat liver extract, which has numerous components, was examined using three columns of G 3000 SW. The results shown in Fig. 8 show that the SW type column gives good resolution.

Next, three commercial enzymes were studied using G 3000 SW columns. The results are shown in Fig. 9. Each enzyme exhibits many peaks, which suggests that the SW type columns can be used for the purification of enzymes. TSK-GEL SW type columns were also used to follow an enzymatic reaction. Fig. 10 shows the time course of the decomposition reaction of β-lactoglobulin by α-chymotrypsin. These results suggest that SW type columns can be used for the study of enzymatic reactions.

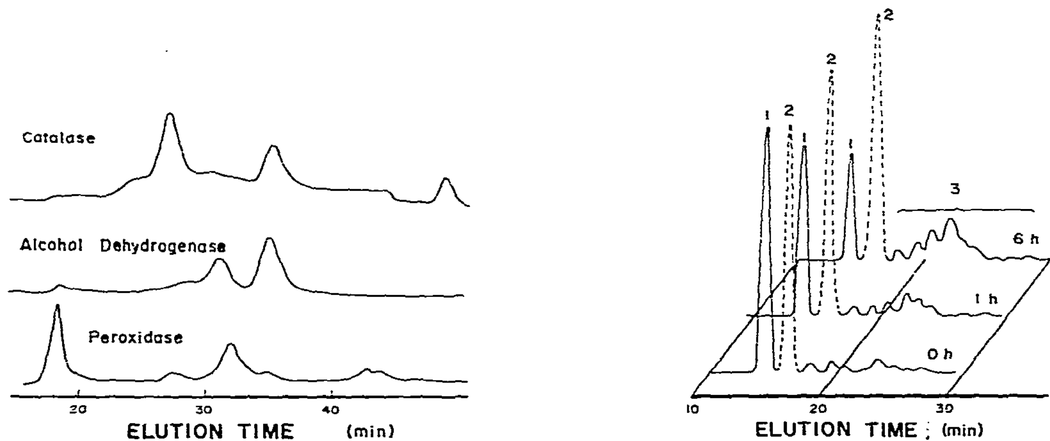


Fig. 9. Measurements of three commercial enzymes. Column: G 3000 SW. Solvent: 1/15 M phosphate buffer (pH 6.8) containing 0.1 M KCl. Pressure: 110 kg/cm^2. Flow-rate: 1.0 ml/min. Sample concentration: 0.05%; charge 300 μl. Detector: UV 280 nm, 0.16 [ABS] (10 mV). Room temperature.

Fig. 10. Pursuit of the time course of the decomposition reaction of β-lactoglobulin by α-chymotrypsin at 5°. Peaks: 1 = β-lactoglobulin; 2 = α-chymotrypsin; 3 = decomposition products. Column: G 3000 SW. Solvent: 1/15 M phosphate buffer (pH 6.8) containing 0.1 M KCl. Pressure: 30 kg/cm^2. Flow-rate: 1 ml/min; charge, β-lactoglobulin (150 μg), α-chymotrypsin (90 μg). Detector: UV 280 nm, 0.16 [ABS] (10 mV). Room temperature.

Several polysaccharides were then studied using SW type columns. Fig. 11 shows the chromatogram of standard dextran obtained on a G 4000 SW column. The molecular-weight distributions of standard dextrans are generally wide. Fig. 12 shows a chromatogram of inulin using a G 3000 SW column, and Figs. 13, 14 and 15 show the chromatograms of heparin sodium, three kinds of commercial sodium chondroitin sulphate and gum arabic, respectively, obtained using a G 4000 SW, columns. No adsorption or delay of the samples was observed. Oligosaccharides and glycosides such as steviocide and glycyrrhizin were also examined using a G 2000 SW column, and good results were obtained.

Water-soluble synthetic polymers were charged on the SW type columns. It was found that polymers such as polyethylene glycol, poly(vinylpyrrolidone), poly-(vinyl alcohol) and sodium polyacrylate could be measured by GPC. The results

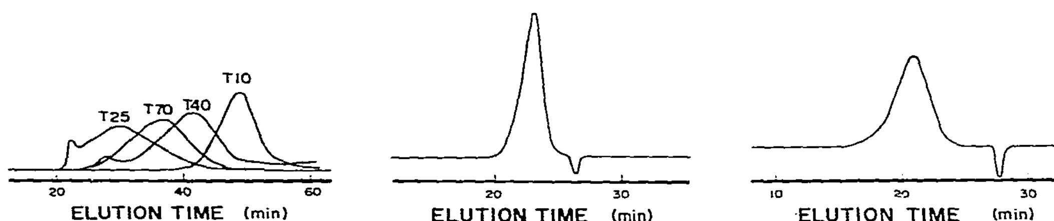


Fig. 11. Chromatogram of standard dextran. Sample: T 250 (—n = 234,000); T 70 (—n = 70,000); T 40 (—n = 40,000); T 10 (—n = 10,000). Column: G 4000 SW. Solvent: water. Pressure: 20 kg/cm^2. Flow-rate: 1.0 ml/min. Sample concentration: 0.4%; charge 300 μl. Detector: RI × 16. Room temperature.

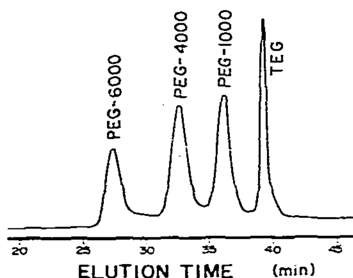
Fig. 12. Chromatogram of inulin. Column: G 3000 SW. Solvent: 0.1 *M* NaCl aqueous solution. Pressure: 40 kg/cm^2. Flow-rate: 1.0 ml/min. Sample concentration: 0.5%; charge 400 μl. Detector: RI × 16. Room temperature.

Fig. 13. Chromatogram of heparin sodium. Column: G 4000 SW. Solvent: 0.1 *M* NaCl aqueous solution. Pressure: 20 kg/cm^2. Flow-rate: 1 ml/min. Sample concentration: 0.5%; charge 500 μl. Detector: RI × 16. Room temperature.

Fig. 14. Chromatogram of three kinds of commercial sodium chondroitin sulphate. Sample: A = sodium chondroitin sulphate A (whale cartilage); B = sodium chondroitin sulphate B (pig skin); C = sodium chondroitin sulphate C (shark cartilage). Column: G 4000 SW. Solvent: 0.1 *M* NaCl aqueous solution. Pressure: 20 kg/cm^2. Flow-rate: 1.0 ml/min; charge: 500 μl. Detector: RI × 16. Room temperature.

Fig. 15. Chromatogram of gum arabic. Column: G 4000 SW. Solvent: 0.1 *M* NaCl aqueous solution. Pressure: 20 kg/cm^2. Flow-rate: 1.0 ml/min. Sample concentration: 0.5%; charge 500 μl. Detector: RI × 16. Room temperature.

for polyethylene glycol and sodium polyacrylate are shown in Fig. 16 and 17, respectively. However, for polymers such as polyacrylamide, sodium polystyrene sulphonate and polyethyleneimine, various extents of adsorption or delay were observed. Thus, SW type columns are not suitable for several water-soluble synthetic polymers.



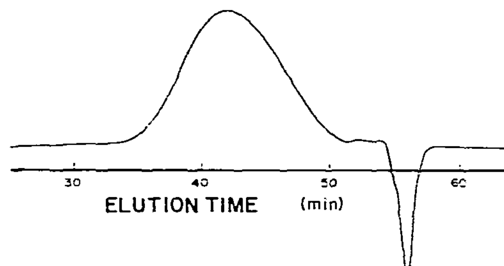


Fig. 16. Separation of polyethylene glycol mixture. Sample: PEG 6000 ($\bar{M}_n$ = 7500); PEG 4000 ($\bar{M}_n$ = 3000); PEG 1000 ($\bar{M}_n$ = 1000); TEG (tetraethylene glycol, $\bar{M}_n$ = 194). Column: G 3000 SW. Solvent: water. Pressure: 76 kg/cm². Flow-rate: 1 ml/min. Sample concentration: 0.2%; charge 300 μ. Detector: RI × 16. Room temperature.

Fig. 17. Chromatogram of sodium polyacrylate. Column: G 2000 SW + G 4000 SW. Solvent: 0.2 *M* sodium acetate. Pressure: 100 kg/cm². Flow-rate: 0.8 ml/min. Sample concentration: 0.2%; charge 900 μl. Detector: RI × 16. Room temperature.

CONCLUSIONS

From these measurements of the recoveries of proteins and enzyme activities, it has been found that SW type columns have very small adsorption for proteins and enzymes. The relation between the molecular weights of proteins and their elution volume has been investigated using standard proteins, and it has been shown that the SW type columns can be used to determine the molecular weight of unknown proteins and enzymes. Saccharides and several water-soluble synthetic polymers can be also be studied using SW type columns. It seem that TSK-GEL SW type columns are excellent for the high-speed preparative separation of biological compounds.

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