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# PREPARATIVE HIGH SPEED GEL PERMEATION CHROMATOGRAPHY OF PROTEINS ON TOYOPEARL HW55F

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SUMMARY: A method is described for the rapid separation of protein mixtures by high speed gel permeation chromatography using Toyopearl HW55F, a new semi-rigid hydrophilic polymer. Good resolution of protein mixtures according to molecular size can be achieved on this material with high flow rates and low column pressures. Molecular weight estimations in the range between $10^4$ and $10^6$ daltons can be performed within minutes. Large-scale enzyme purification (up to 1.6 gm of starting material with a 3.2 x 105 cm column) was achieved with 86-110% recovery of enzymatic activity. Data are presented on the optimum column length, flow rate, loading capacity and eluant ionic strength.

INTRODUCTION: A major advance in HPLC was recently achieved by the introduction of relatively rigid packing materials for permeation chromatography, such as Zorbax, Lichrospher, Porosil, Glycophase-GPC, Synchropak-GPC, Waters I-125, Shodex, and the two TSK-type gels, PW and SW (for recent reviews of gel permeation HPLC see ref. 1-3 and 4-11). The water-compatible TSK-SW gels have been used successfully for analytical and/or semi-preparative separation of protein mixtures (5-9). Using a 0.75 x 50 cm column of TSK-SW3000, we have been able to routinely estimate molecular weights of proteins in the range of $10^4$ to 5 x $10^5$ daltons within 16 minutes with 95% confidence limits of $\pm$ 14% for a single run (J. Germershausen and J. D. Karkas, unpublished observations). The major limitation of the TSK-SW gels is their high price; they are available, prepacked, in analytical-size columns and scaling up a procedure to a preparative level is prohibitively expensive. Additionally, the TSK material deteriorates at pH values above 9 and at temperatures above $45^{\circ}$.

We report here the use of a new material, Toyopearl, which has recently become available from Toyo Soda Co. in bulk quantities at reasonable prices. Using Toyopearl HW55F we have achieved high resolution of protein mixtures on a preparative scale. Also, according to the manufacturer, this material is compatible with a large array of detergents, protein denaturants and organic solvents and it can be used at virtually any pH, temperature or ionic strength.

MATERIALS AND METHODS: Toyopearl HW55F was supplied by Toyo Soda Co. (200 Park Ave., New York, N.Y.) as an aqueous suspension containing 0.02% $NaN_3$.

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All separations were performed on a Varian 5000 Liquid Chromatograph.

Stainless steel columns were fitted with stainless steel porous discs overlaid with circles of 10 u nylon screen (Small Parts Inc., Miami, Fla.). For packing, glass extensions of the same diameter were attached to the top of the column to visualize bed height. Buffer was pumped at a flow rate of 6 ml/min until the bed height remained stable, after which the glass extension was removed, the column was capped and further equilibrated with 2 column volumes of buffer. The entire operation of packing and equilibration was performed in less than 3 hours. Column pressures were corrected by subtracting the pressure required to overcome the resistance of the narrow diameter tubing leading into the column.

The protein standards, cytochrome C (M.W. 12,500), chymotrypsinogen (M.W. 25,000), ovalbumin (M.W. 45,000), bovine serum albumin (M.W. 69,000), aldolase (M.W. 158,000), catalase (M.W. 232,000), ferritin (M.W. 420,000) and thyroglobulin (M.W. 670,000) were purchased as a kit from Pharmacia Fine Chemicals. Calf thymus DNA was a product of Calbiochem Corp. and crystallized bovine serum albumin was from Pentex Corp.

DNA polymerase and DNase activity were measured according to (12) and (13), respectively. Mevalonate kinase and phosphomevalonate kinase were measured according to (14).

RESULTS AND DISCUSSION: The elution profiles of protein standards chromatographed individually on a 1.5 x 90 cm column at a flow rate of 2 ml/min are presented in Figure 1. As indicated in Figure 2, a plot of the log of molecular weight vs. retention volume gives a straight line. Using this standard curve, the molecular weight of an unknown protein in the molecular weight range of $10^4$ to $10^6$ daltons can be estimated within less than one hour.

As indicated in Figure 3, resolution was not affected significantly by column height. Using the same bed volume, flow rate and protein mixtures, while varying the bed height in three different columns resulted in almost identical elution patterns. This may be important for large scale applications where very long columns are not practical.

As expected, resolution was better at slower flow rates. This is illustrated in Figure 4 where the resolution of three protein standards was measured as a function of flow rate. Baseline separation of the mixture was achieved at 0.1 ml/min (Fig. 4A) and 0.2 ml/min (Fig. 4B) while resolution decreased progressively as the flow rate was increased from 0.4 ml/min to 4.0 ml/min (Figs. 4C-4F). Retention times decreased with increasing flow rates due to decreased permeation into the matrix, indicating that individual columns require standardization at the flow rate to be used in the analysis. Although resolution of the protein standards suffered at high flow rates, the actual separation of the three protein peaks changed very little as the flow rate was increased. Thus, fast flow rates can be used in certain situations such as the rapid determination of the approximate molecular weight of a protein or the determination of protein interconversions, dimerizations, breakdown, etc. Where high

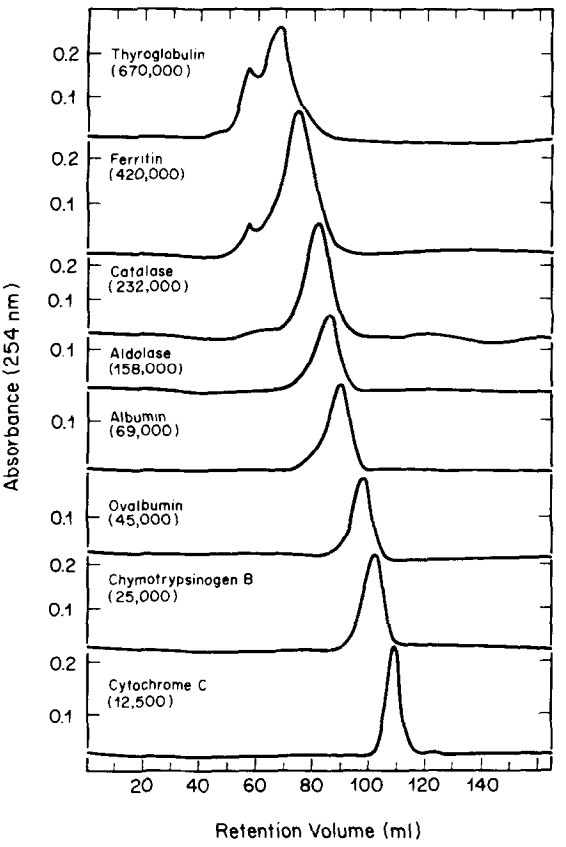
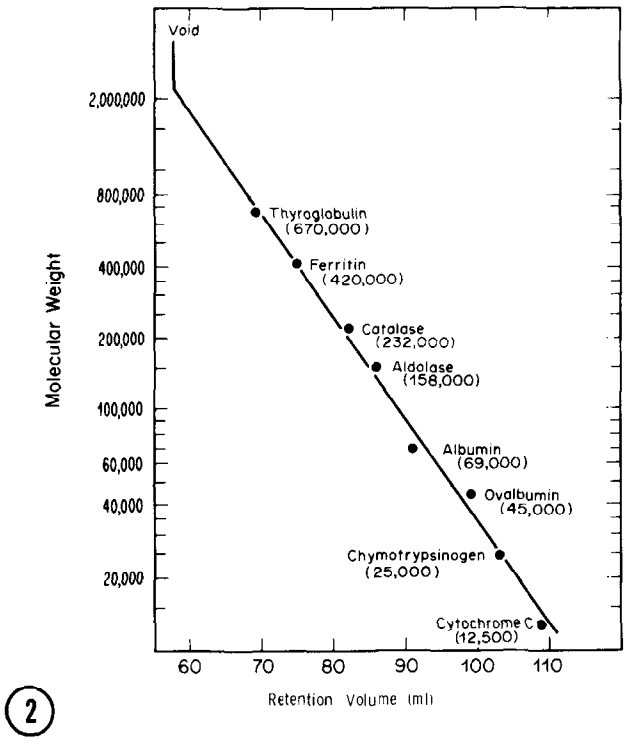



Figure 1. Elution profiles of protein standards. Protein solutions (0.2 ml) were injected onto a 1.5 x 90 cm HW55F column at room temperature and eluted with 0.025 $KHPO_4$ (pH 6.8) containing 0.05M KCl. The flow rate was 2.0 ml/min and the column pressure was < 2 atm. The amounts of protein injected were: cytochrome C, 0.4 mg; ferritin, 0.5 mg; chymotrypsinogen, 1 mg; all others, 2 mg.

resolution can be sacrificed as in the case of an unstable protein, for the sake of a rapid separation, a mixture can be partially resolved within minutes, as, for example, in Figure 4F, where the mixture was partially resolved at 4 ml/min within 25 minutes.

It should be noted, at this point, that these high flow rates can be achieved at very low column pressures. At 4 ml/min with a 2 x 51 cm column, the pressure was 2 atm.; at 10 ml/min the pressure was 4 atm. (data not shown). There was no detectable compression of the bed volume at these flow rates. The extreme rigidity of the gel makes it suitable for large scale separations which can be performed with standard pumps.

An unexpected feature of the separations on Toyopearl HW55 (also observed with the TSK-SW type gels) is that the sharpness of the peaks increases with increasing retention



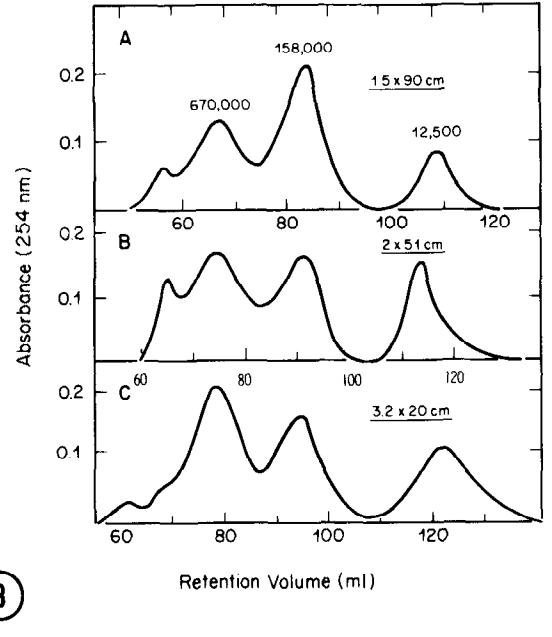




Figure 2. Standard curve. The data in Fig. 1 were used to plot the log M.W. versus retention volume. The void volume (58 ml) corresponding to a M.W. of $2 \times 10^6$ was determined with calf thymus DNA (data not shown in Fig. 1).

Figure 3. Effect of column length on resolution. Three columns were packed, each with 160 ml bed volume of HW55F. The dimensions were: (A) 1.5 x 90 cm; (B) 2 x 51 cm and (C) 3.2 x 20 cm. The protein mixture contained 2.4 mg each of thyroglobulin and aldolase and 0.6 mg of cytochrome C in 0.6 ml of elution buffer. The mixtures were resolved at room temperature in 0.025M $KHPO_4$ (pH 6.8) containing 0.05M KCl at a flow rate of 2 ml/min and a column pressure of <2 atm.

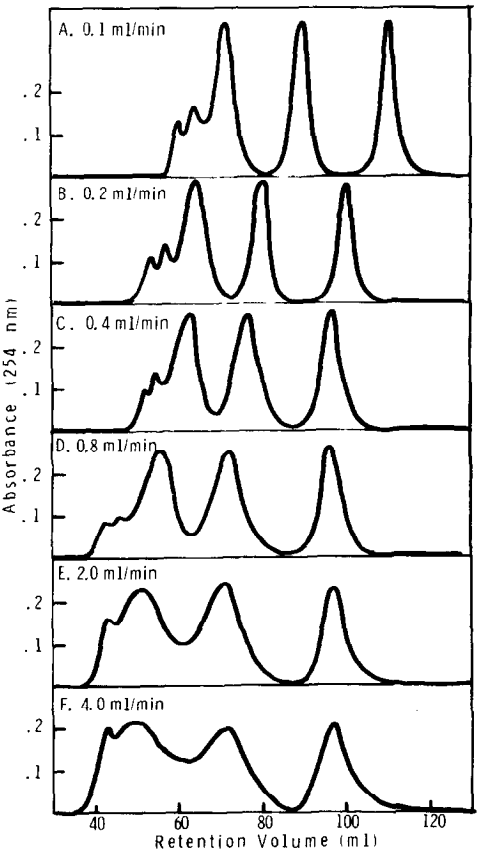



Figure 4. Effect of flow rate on resolution. A 2 x 51 cm column was loaded with 0.6 ml of a protein mixture containing 2.4 mg each of thyroglobulin and aldolase and 0.6 mg of cytochrome C. The mixture was resolved with 0.025M $KHPO_4$ (pH 6.8) containing 0.05M KCl at room temperature. The column pressures were (A-E), < 2 atm., (F), 2 atm. The flow rates were (A) 0.1 ml/min, (B) 0.2 ml/min, (C) 0.4 ml/min, (D) 0.8 ml/min, (E) 2.0 ml/min and (F) 4.0 ml/min.

volume (see Fig. 1). This is the opposite of what is observed with Sephadex type gels, and would seem to indicate a small but significant ion exchange effect. For this reason, the effect of the ionic strength of the eluting buffer on resolution was examined. Figure 5 indicates that there is no significant effect of ionic strength on the retention volumes of the large molecular weight markers (thyroglobulin; 670,000 and aldolase; 158,000) whereas the retention volume of the small molecular weight marker (cytochrome C; 12,500) was reduced by 5% as the molarity of KCl in the buffer was increased from 0.05M (Fig. 5A) to 0.15M (Fig. 5B). Increasing the ionic strength to 0.3M KCl (Fig. 5C) had no further effect. The use of 0.05M KCl in the eluting buffer provides a dual advantage in that the standard curve is linear (Fig. 2) and the smaller molecular weight proteins give sharper peaks (Fig. 1).

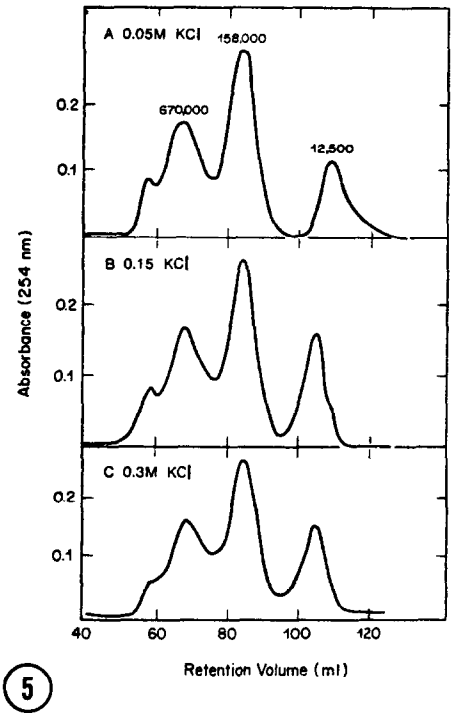
Figure 6 illustrates the results of two large-scale separations of enzymatic activities from relatively crude extracts. Both were performed on a 3.2 x 105 cm column at a flow rate of 0.8 ml/min at $4^{\circ}$. In the first (Fig. 6A), a crude extract of HeLa cells was applied and the effluent was tested for DNA polymerase activity. Three discrete peaks of activity were seen, corresponding to molecular weights of about 300,000, 100,000 and 45,000 daltons. The retention volumes of molecular weight standards are indicated at the top of the figure. A fourth peak of activity, eluting within the void volume, represents ribosome-bound polymerase(s).

In the second experiment (Fig. 6B), a 100,000 g supernatant from a rat liver homogenate was precipitated with ammonium sulfate and the redissolved pellet (1.6 gm in 10.5 ml) was applied to the column. The effluent was assayed for mevalonate kinase and phosphomevalonate kinase. These activities were separated (Fig. 6B) and in all cases (including the experiment described in Fig. 6A), the molecular weights determined by HW55F agreed with published molecular weights $\pm$ 15%.
Recoveries of enzymatic activities were quite high in both cases, 109% for DNA polymerase, 87% for mevalonate kinase and 88% for phosphomevalonate kinase. In a separate experiment (data not shown) the DNase activity of a crude HeLa extract was recovered after fractionation on the same column with 110% yield.

Enzyme activities were recovered without considerable dilution. In the experiment of Fig. 6B dilution was approximately 10-fold. In a separate experiment (data not shown), 500 mg of bovine serum albumin in 5 ml was applied to a 2 x 51 cm column and eluted at a flow rate of 2 ml/min. The albumin eluted with the expected retention volume and 95% of the protein was recovered in 30 ml of eluant. Thus, it appears that, even at very high loading levels, the dilution of the sample is not very large (6-10 fold) and that the loading capacity is, for all practical purposes, limited only by the volume in which the sample can be applied.

Finally, the material is not a substrate for bacterial growth (we have used the same columns for over 6 months at room temperature without any apparent bacterial contamination or column deterioration). This, coupled with the fact that short, large-diameter columns can be used with standard pumps, should make this material ideally suited for industrial-scale applications.

The properties of the Toyopearl HW55F gel described here, such as its high revolving power, low compressibility, high loading capacity and good enzyme recoveries, together with those properties described by the manufacturer and not tested directly in this work (compatibility with SDS, guanidine HCl, urea and organic solvents; stability to pH from 1 to 14 and temperatures up to $100^{\circ}$) suggest that this material should prove very useful both for rapid molecular weight determinations and for preparative-scale

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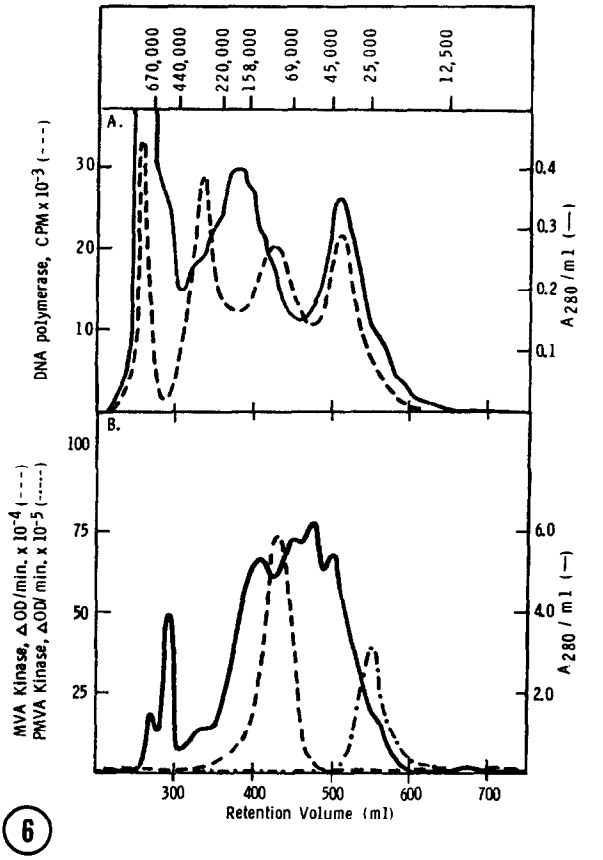


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Figure 5. Effect of buffer ionic strength on retention volumes. A 1.5 x 90 cm column was loaded with 0.6 ml of a protein mixture containing 2.4 mg each of thyroglobulin and aldolase and 0.6 mg of cytochrome C. The mixture was resolved at room temperature with 0.025M $KHPO_4$ (pH 6.8) containing (A) 0.05M KCl; (B) 0.15M KCl and (C) 0.3M KCl. The flow rate was 2.0 ml/min and the column pressure was <2 atm.

Figure 6. Enzyme purifications. A 3.2 x 105 cm column was used in both experiments and the buffer, 0.05M $KHPO_4$ (pH 7.4), 0.5 mM dithiothreitol, 5% glycerol, was pumped at 0.8 ml/min at 4° with <2 atm. column pressure. Fractions (10 ml) were collected and assayed for DNA polymerase (Fig. 6A) and both mevalonate kinase and phosphomevalonate kinase (Fig. 6B). Figure 6A; 123 mg of a crude extract from HeLa cells was applied in a volume of 6.5 ml. Figure 6B; 1.6 gm of a 0-65% ammonium sulfate precipitate from a 100,000 g supernatant of rat liver was applied in a volume of 10.5 ml. Protein was measured by absorbance at 280 nm and enzyme activities as indicated under Methods.

separations of macromolecules. We are at present studying the properties of Toyopearl HW-65 which can be used with a higher range of molecular weights, in the millions of daltons.

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