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MICROCOLUMN HIGH-PERFORMANCE SIZE-EXCLUSION CHROMATOGRAPHIC SEPARATION OF PROTEINS

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SUMMARY

Size-exclusion chromatographic separation of proteins is demonstrated by using a microcolumn high-performance liquid chromatograph. Column preparation procedures and the apparatus used are described. A 2-m microcolumn resolved more peaks than the commercially available columns.

INTRODUCTION

High-performance liquid chromatography (HPLC) has been of great use for the analysis of biological samples. Proteins have been separated by reversed-phase chromatography, ion-exchange chromatography and size-exclusion chromatography (SEC). This last method gives us information regarding the molecular size of peaks in the chromatogram, which is an added advantage of SEC. It is for this reason that SEC has been employed for the separation of proteins, even though it has lower peak capacity compared with that of other chromatographic separation modes. Peak capacity of an SEC column is given by the following equation[1]:

$$n \approx 1 + 0.2\,N^{1/2} \quad (1)$$

where n is the peak capacity and N is the theoretical plate number. The peak capacity in SEC can be improved by increasing the column length, *i.e.*, producing larger theoretical plates. Microcolumn HPLC facilitates the use of a long column and its high resolution has been appreciated by some workers[2–6]. Scott and Kucera[2] have examined long silica-gel microbore columns for SEC separation of alkyl benzenes, while we have employed fused-silica microcolumns packed with styrene–divinyl benzene copolymer gels for SEC separation of oligomers[7]. Furthermore, increased mass sensitivity in microcolumn HPLC is advantageous for valuable samples such as biological samples. Micro-scale reversed-phase chromatographic separations of proteins have been reported[8,9], in which microbore columns with 1–2 mm I.D. have been used.

This paper will describe the preparation procedures of SEC columns with *ca.* 0.35 mm I.D. for the separation and resolution of proteins.

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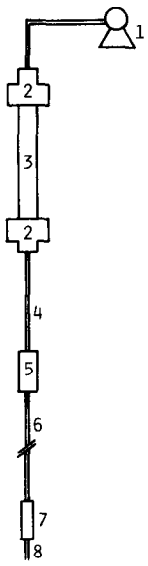
EXPERIMENTAL

Preparation procedures of columns

Fused-silica capillary tubing with *ca.* 0.35 mm I.D. (Gasukuro Kogyo, Tokyo, Japan) was selected as the column material. Polyimide ferrules (Vespel) with 0.5 mm I.D. (Valco, Houston, TX, U.S.A.) were used to connect the fused-silica tubing directly to stainless-steel unions. A 1/16 in. × 1/32 in. zero-dead-volume reducing union (Valco) was attached to the inlet of the capillary and 1/32-in. zero-dead-volume union was attached to the outlet. A schematic diagram of the equipment for packing is shown in Fig. 1. Stainless-steel tubing of 10 cm × 4.6 mm I.D. × 1/4 in. O.D. (1.7 ml in volume) was employed as the packer, which would be fitted with the slurry solution. After passing an adequate volume of the packing solution into the capillary, a stainless-steel frit (2 μm) was placed in the outlet union, as shown in Fig. 2. The frit was prepared from a thin plate (0.3 mm thick) in the laboratory. Stainless-steel tubing of 0.51 mm I.D. × 0.81 mm O.D. was connected to the outlet of the union so that the position of the frit could be fixed.

Two types of slurry solvent were examined in this work: a methanolic solution of 10% (w/w) glycerol and a 0.1 *M* phosphate solution (pH 7) of 0.2 *M* sodium chloride. In the former case, methanol was employed as the packing solvent, while the buffer solution was employed as the packing solvent in the latter case. For a 1-m column, *ca.* 80 mg of the packing material were weighed in a 2–5 ml vial, followed



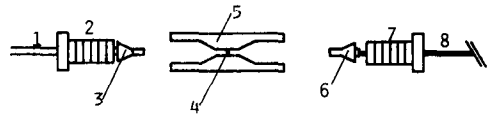


Fig. 1. Schematic diagram of the packing equipment. 1 = Pump; 2 = 1/4 in. × 1/16 in. reducing union; 3 = packer, 10 cm × 4.6 mm I.D. × 1/4 in. O.D.; 4 = stainless-steel tubing, 10 cm × 0.8 mm I.D. × 1/16 in. O.D.; 5 = 1/16 in. × 1/32 in. zero-dead-volume union; 6 = fused-silica capillary column; 7 = 1/32 in. zero-dead-volume union; 8 = stainless-steel tubing, 0.51 mm I.D. × 0.81 mm O.D.

Fig. 2. Schematic diagram of the outlet of the column. 1 = Stainless-steel tubing, 0.51 mm I.D. × 0.81 mm O.D.; 2 = 1/32 in. nut; 3 = 1/32 in. stainless-steel ferrule; 4 = stainless-steel frit; 5 = 1/32 in. zero-dead-volume union; 6 = 0.5 mm I.D. × 1/32 in. polyimide ferrule; 7 = 1/32 in. nut; 8 = fused-silica column.

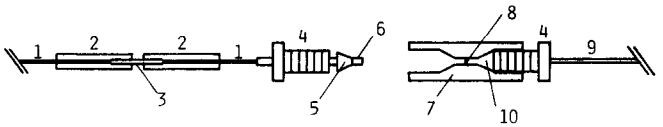


Fig. 3. Schematic diagram of the connecting part between the column and the detector. 1 = Fused-silica tubing, 55 μm I.D. × 0.24 mm O.D.; 2 = PTFE tubing, 0.2 mm I.D. × 2 mm O.D.; 3 = stainless-steel tubing, 0.13 mm I.D. × 0.31 mm O.D.; 4 = 1/32 in. nut; 5 = 1/32 in. stainless-steel ferrule; 6 = stainless-steel tubing, 0.25 mm I.D. × 0.8 mmm O.D.; 7 = 1/32 in. zero-dead-volume union; 8 = stainless-steel frit; 9 = fused-silica column; 10 = 0.5 mm I.D. × 1/32 in. polyimide ferrule.

by the addition of 1.8 ml of the above slurry solvent. The packer was detached from the flow line and its contents removed. The prepared slurry solution was vibrated for a few minutes and immediately placed in the packer by using a 1-ml gas-tight syringe. After connecting the packer tightly, packing was started by using a high-pressure pump. Familic-300S (Jasco, Tokyo, Japan) and CCPM (Toyo Soda, Tokyo, Japan) pumps were employed in the constant-pressure mode for packing. The pressure was increased at the rate of 10 atm/5 s (in 10-atm increments) up to 350 or 470 atm, and kept at the maximum pressure for 30 min–2 h. When methanol was employed as the packing solvent, distilled water and the mobile phase had to be passed into the column after packing. A laboratory-prepared 1/16-in. stainless-steel frit was finally placed in the inlet union of the column and the column was connected to the injector and the detector. A schematic diagram of the connecting part between the column and the detector is shown in Fig. 3.

Apparatus

Familic-300S, CCPM and microMetric metering pumps (LDC/Milton Roy, Riviera Beach, FL, U.S.A.) were employed for supplying the mobile phase. The former two pumps were employed in the constant-pressure mode, while the third pump was employed in the constant-flow mode. The flow-rate was measured by using a gas-tight syringe connected to the outlet of the detector.

An ML-422 valve injector (Jasco) and a UVIDEC-100V detector (Jasco) were employed. The flow cell was modified in the laboratory according to the previous work[10]. Injection and detection volumes were 0.02 μl and 0.05–0.1 μl, respectively. The connecting tube between the column and the injector is composed of stainless-steel tubing of 6–8 cm × 0.25 mm I.D. × 1/16 in. O.D., in which stainless-steel tubing of 80 μm I.D. × 0.2 mm O.D. was fixed with silver solder.

Reagents and materials

MW (molecular weight)-Marker was obtained from Oriental Yeast (Tokyo, Japan). The content in a vial was dissolved in 0.1 ml of the mobile phase and kept in the refrigerator. Hexokinase was obtained from Wako (Osaka, Japan). Catalase and aldehyde dehydrogenase were supplied by Sigma (St. Louis, MO, U.S.A.). These enzyme protein samples were dissolved in the mobile phase. Other reagents were obtained from Wako, unless otherwise stated.

AF-102 (10 μm; Jasco), TSKgel 3000SW (10 μm; Toyo Soda) and TSKgel 3000SW$_{XL}$ (5 μm; Toyo Soda) were employed as the packing material.

Stainless-steel tubing was supplied by Gasukuro Kogyo or Hakkoshoji (Tokyo, Japan). Fused-silica tubing of 55 μm I.D. was obtained from SGE (Melbourne, Australia) and PTFE tubing was supplied by Gasukuro Kogyo.

RESULTS AND DISCUSSION

The effect of the slurry solvent on column performance was examined by using AF-102 as the packing material. When the column is packed using methanol as the packing solvent and 10% glycerol in methanol as the slurry solvent, distilled water and the mobile phase (0.1 *M* phosphate buffer–0.2 *M* sodium chloride, pH 7) should be passed into the column in this order, prior to the separation. When washing the column with distilled water, we observed that the dead-space was produced at the top of the column, due to shrinkage of the gel. Thus, in order to avoid this, it is recommended that washing with distilled water and the mobile phase is performed while the column is attached to the packer. On the other hand, when the column was prepared with phosphate buffer, proteins were retarded on the column and no peak was eluted from the AF-102 column. However, reasonable chromatograms were obtained when the column was washed with distilled water, methanol and distilled water, in this order, subsequent to packing. In this case, the problem of shrinkage during washing with the above solvents was slight, compared with the former case.

Fig. 4 shows chromatograms of MW-Marker obtained with the above packing procedures. The difference in elution pattern is distinct. There are peaks that cannot be ascertained in the methanol chromatogram, while five components of proteins were identified in the phosphate buffer chromatogram. It is found that column performance is better when phosphate buffer is used as both the slurry and the packing solvent, followed by washing with distilled water and methanol.

Washing with distilled water and methanol subsequent to packing with phosphate buffer gave good results in the case of TSKgel 3000SW and 3000SW$_{XL}$. But,

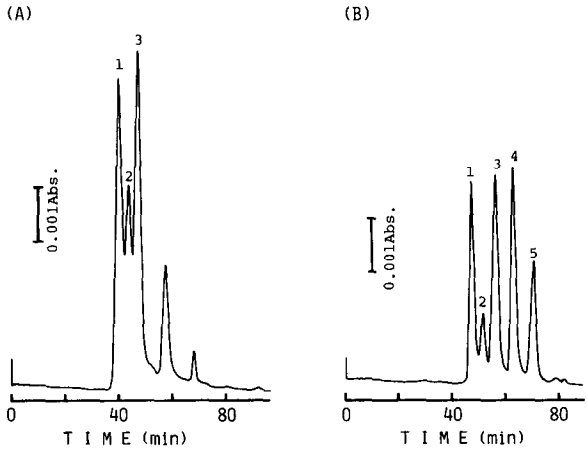


Fig. 4. Separation of MW-Marker on AF-102 columns. Columns: AF-102, (A) 94 cm × 0.35 mm I.D.; (B) 106 cm × 0.36 mm I.D. Mobile phase: 0.1 *M* phosphate buffer–0.2 *M* sodium chloride, pH 7. Flow-rate: 1.0 μl/min. Wavelength of UV detection: 220 nm. Sample: MW-Marker, 1 = glutamate dehydrogenase (MW = 290 000); 2 = lactate dehydrogenase (MW = 142 000); 3 = enolase (MW = 67 000); 4 = adenylate kinase (MW = 32 000); 5 = cytochrome *c* (MW = 12 400).

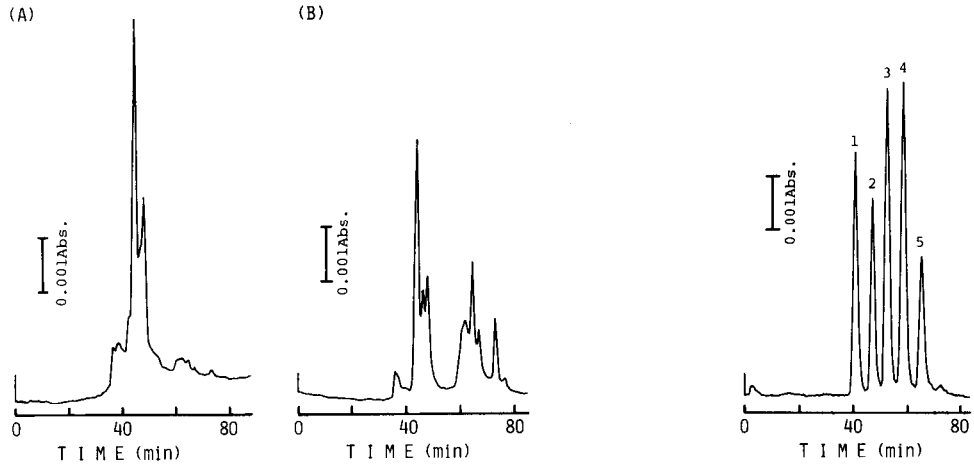


Fig. 5. Size-xclusion chromatograms of hexokinase. (A) Before washing, (B) after washing with distilled water and methanol. Column: TSKgel 3000SW$_{XL}$, (A) 82.5 cm × 0.35 mm I.D.; (B) 81 cm × 0.35 mm I.D. Flow-rates: (A) 0.96 μl/min; (b) 0.94 μl/min. Sample: 1% hexokinase. Mobile phase and wavelength of UV detection as in Fig. 4.

Fig. 6. Separation of MW-Marker on a TSKgel 3000SW column. Column: TSKgel 3000SW, 94 cm × 0.35 mm I.D. Other operating conditions and peaks as in Fig. 4.

constituents of MW-Marker were eluted from these columns. Fig. 5 compares chromatograms of hexokinase. It is found that elution of lower-molecular-weight constituents is improved by washing with distilled water and methanol. Although the washing volume is little, *viz.*, 20 μl of distilled water, 90 μl of methanol and 20 μl of distilled water, the washing effect is distinct.

Trumbore *et al.*[11] have noted that there could be severe losses of sample on surfaces of stainless-steel, glass and PTFE capillary tubing in the HPLC of proteins. The system reported here employs capillary tubing for the whole passage, including

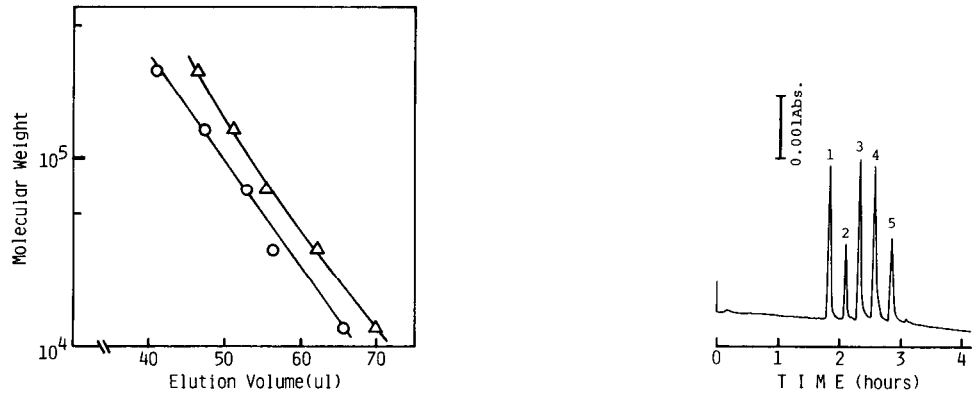


Fig. 7. Calibration curves. Columns: ○ = TSKgel 3000SW, 94 cm × 0.35 mm I.D.; △ = AF-102, 106 cm × 0.36 mm. Solute: as in Fig. 4.

Fig. 8. Separation of MW-Marker on a TSKgel 3000SW$_{XL}$ column. Column: TSKgel 3000SW$_{XL}$, 203 cm × 0.35 mm I.D. Flow-rate: 0.76 μl/min. Other operating conditions and peaks as in Fig. 4.

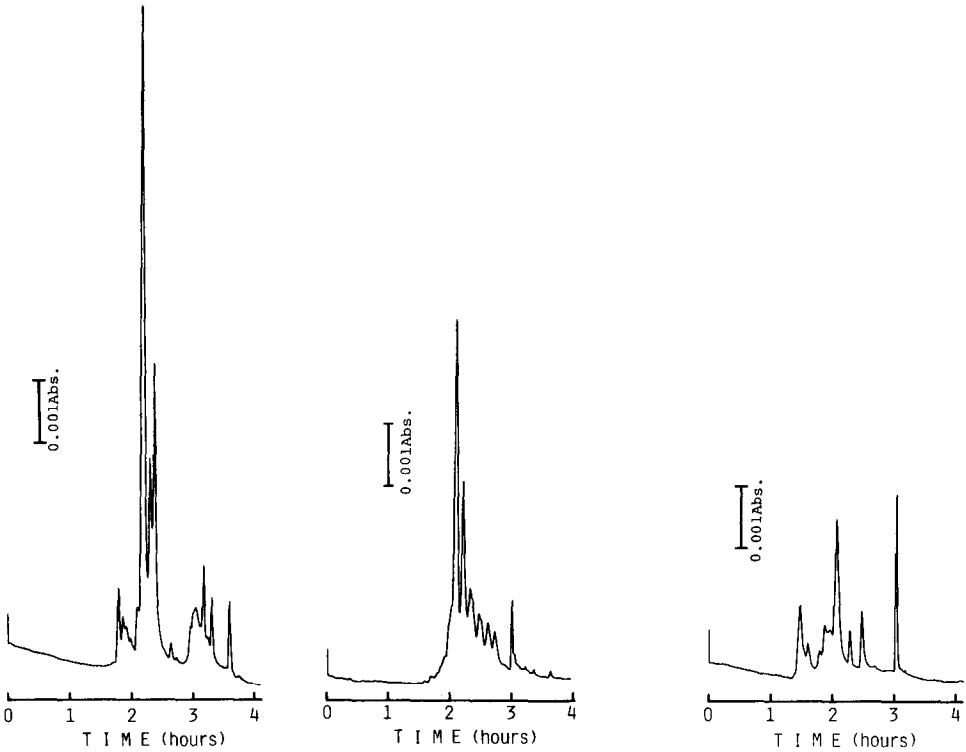


Fig. 9. Size-exclusion chromatogram of hexokinase on a TSKgel 3000SW$_{XL}$ column. Sample: 2% hexokinase. Other operating conditions as in Fig. 8.

Fig. 10. Size-exclusion chromatogram of catalase on a TSKgel 3000SW$_{XL}$ column. Sample: 2% catalase. Other operating conditions as in Fig. 8.

Fig. 11. Size-exclusion chromatogram of aldehyde dehydrogenase on a TSKgel 3000SW$_{XL}$ column. Sample: 5% aldehyde dehydrogenase. Other operating conditions as in Fig. 8.

the column. Thus, the system may lose sample, according to Trumbore *et al.*[11]. If the pump was stopped for a while after repeated injection of the sample, we observed many peaks eluting from the column as the mobile phase passes through the column again. It is not certain whether these peaks are based on sample that has been coated on the capillary wall or impregnated (or adsorbed) solutes in the gel. In addition, most of the passage in this system is composed of fused-silica tubing and loss of protein on the surface of such material has not been investigated.

Fig. 6 shows the separation of MW-Marker on a TSKgel 3000SW column and Fig. 7 illustrates calibration curves for AF-102 and TSKgel 3000SW columns. A slight difference in calibration curves is found, although a strict comparison cannot be performed owing to the difference in column dimensions.

Figs. 8–11 demonstrate chromatograms of MW-Marker, hexokinase, catalase and aldehyde dehydrogenase obtained by a 2-m column packed with TSKgel 3000SW$_{XL}$. It is found that resolution is improved by increasing the column length and using fine particles (5 μm). Although it takes longer, this column resolves more constituents compared with commercially available columns.

CONCLUSION

Microcolumns for the separation of proteins were prepared and their resolution was shown to be comparable or superior to that of commercially available columns. This system will be of great use for the analysis of biological samples.

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REFERENCES

1 J. C. Giddings, *Anal. Chem.*, 39 (1967) 1027.
2 R. P. W. Scott and P. Kucera, *J. Chromatogr.*, 169 (1979) 51.
3 P. Kucera and G. Manius, *J. Chromatogr.*, 216 (1981) 9.
4 F. J. Yang, *J. Chromatogr.*, 236 (1982) 265.
5 D. Ishii and T. Takeuchi, *J. Chromatogr.*, 255 (1983) 349.
6 Y. Hirata and K. Jinno, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 6 (1983) 196.
7 T. Takeuchi, D. Ishii and S. Mori, *J. Chromatogr.*, 257 (1983) 327.
8 R. van der Zee and G. W. Welling, *J. Chromatogr.*, 325 (1985) 187.
9 E. C. Nice, C. J. Lloyd and A. W. Burgess, *J. Chromatogr.*, 296 (1984) 153.
10 T. Takeuchi, D. Ishii, M. Saito and K. Hibi, *J. Chromatogr.*, 295 (1984) 323.
11 C. N. Trumbore, R. D. Tremblay, J. T. Penrose, M. Mercer and F. M. Kelleher, *J. Chromatogr.*, 280 (1983) 43.