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Note

Operational variables in medium-performance ion-exchange chromatography of proteins on DEAE-Toyopearl 650

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High-performance liquid chromatography has developed very rapidly and now even supports with particle diameters as small as 3 μm have come into practical use. On the other hand, the importance of medium-performance liquid chromatography, in which supports of larger particle size are employed, has been emphasized recently[1]. Medium-performance liquid chromatography may become popular in preparative separations in the future and some supports for this purpose have become commercially available. For example, Toyopearl[2–4] and DEAE-Toyopearl 650[5] are supports of this type for gel filtration and ion-exchange chromatography, respectively. In this paper, operational variables in medium-performance ion-exchange chromatography of proteins on DEAE-Toyopearl 650, which is a weak anion exchanger prepared by introducing diethylaminoethyl groups into Toyopearl HW65, are discussed.

EXPERIMENTAL

DEAE-Toyopearl 650M (Toyo Soda, Tokyo, Japan) of particle diameter 44–88 μm was mainly used. DEAE-Toyopearl 650S of particle diameter 25–44 μm was also used in the studies of flow-rate and sample loading. Particles of 25–37, 37–44, 44–63 and 63–88 μm diameter obtained by further sieving the DEAE-Toyopearl 650S and 650M were used in the study of support particle size. Commercial glass columns (15 × 1.6 cm I.D.) (Amicon, Lexington, MA, U.S.A.) were usually used. Columns of different sizes were also used in the study of column size.

Packing was usually carried out by the constant-velocity method at flow-rates of 10–11 ml/min with a Model SJ-1211H peristaltic pump (Atto, Tokyo, Japan). Constant-velocity packing at different flow-rates and gravitational packing under a hydrostatic pressure of 250 cmH_2O were also performed in the study of the packing procedure. The packing procedure has been described elsewhere in detail[6,7]. A 0.05 *M* Tris–HCl buffer of pH 8.60 containing 0.5 *M* sodium chloride was used as the packing solvent. A slurry of 40% support by volume was prepared in the same buffer.

The ion-exchange chromatographic measurements were carried out with a Model HLC-803C high-speed liquid chromatograph equipped with a Model GE-2 gradient generator (Toyo Soda). The liquid chromatograph consisted of a reciprocating single-plunger pump, a valve loop injector and a variable-wavelength UV detector. The UV detector was operated at 280 nm. The gradient generator was a nozzle

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flapper type and can generate linear, convex or concave gradients of two solvents. Gradient times between 1 and 999 min can be selected.

Resolution was evaluated by measuring a mixture of equal amounts of ovalbumin (Seikagaku, Tokyo, Japan) and trypsin inhibitor (Sigma, St. Louis, MO, U.S.A.) with linear gradient elution from 0.05 *M* Tris–HCl buffer of pH 8.60 to 0.05 *M* Tris–HCl buffer of pH 8.60 containing 0.5 *M* sodium chloride. These proteins did not move along the column at all in the initial buffer. Gradient elution was started just after sample injection. The measurements were usually performed on 15 × 1.6 cm I.D. columns at a flow-rate of 2 ml/min with a gradient time of 150 min, which corresponds to a gradient volume of 300 ml. However, the flow-rate was varied in the studies of flow-rate and column size and the gradient time was varied in the study of gradient volume. Ovalbumin and trypsin inhibitor, 10 mg each in 2 ml of initial buffer, were injected on to columns of 1.6 cm I.D. and the protein concentration was varied in proportion to the column cross-sectional area for columns of different inner diameters. The protein concentration was also varied with the injection volume kept constant in the study of sample loading. All experiments were carried out at 25 ± 0.5°C.

RESULTS AND DISCUSSION

DEAE-Toyopearl 650M columns (15 × 1.6 cm I.D.) were prepared by the constant-velocity packing method at flow-rates of 5, 10, 14 and 17 ml/min in order to investigate the effect of packing flow-rate on column resolution. The results are shown in Fig. 1. The resolution for ovalbumin and trypsin inhibitor, R(OA,TI), defined by eqn. 1, was employed as a measure of column performance.

$$R(\mathrm{OA,TI}) = 2(V_{\mathrm{TI}} - V_{\mathrm{OA}})/(W_{\mathrm{OA}} + W_{\mathrm{TI}}) \quad (1)$$

where V_{OA}, V_{TI}, W_{OA} and W_{TI} are the elution volumes and baseline peak widths of ovalbumin and trypsin inhibitor, respectively. Columns of almost the same resolution were obtained over the whole range of packing flow-rates investigated. Although the resolution was independent of packing flow-rate above some critical value also in the packing of Toyopearl HW55[6,8], a wider range of packing flow-rates provided columns of the same resolution in the case of DEAE-Toyopearl 650.

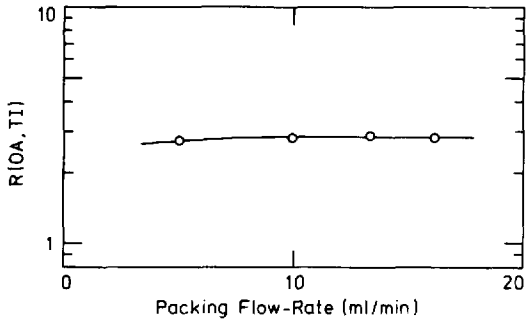

Fig. 1. Dependence of resolution on packing flow-rate in the packing of DEAE-Toyopearl 650M columns (15 × 1.6 cm I.D.) by the constant-velocity method.

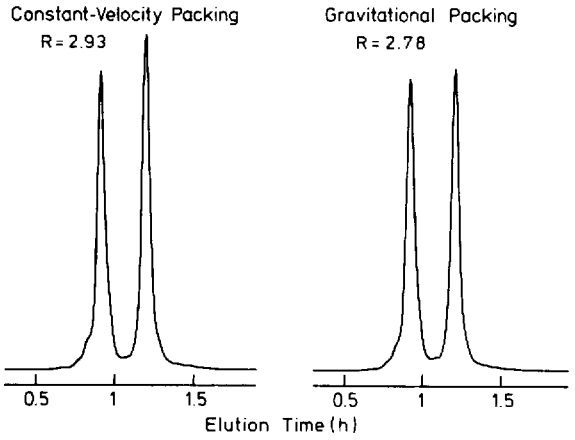

Fig. 2. Chromatograms of a mixture of ovalbumin and trypsin inhibitor obtained on DEAE-Toyopearl 650M columns prepared by constant-velocity packing at a flow-rate of 14 ml/min and by gravitational packing under a hydrostatic pressure of 250 cmH_2O.

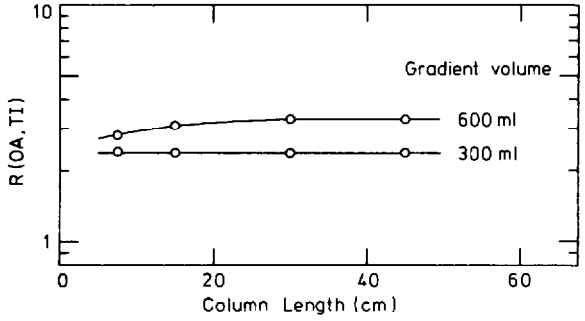

Fig. 3. Dependence of resolution on length of DEAE-Toyopearl 650M columns of 1.6 cm I.D.

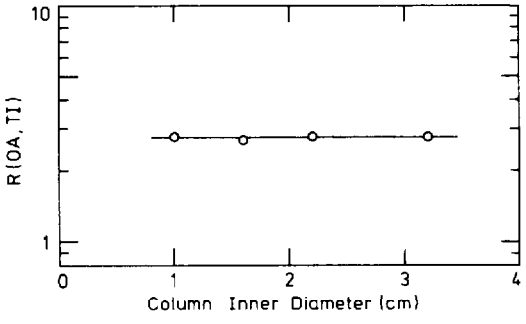

Fig. 4. Dependence of resolution on inner diameter of DEAE-Toyopearl 650M columns of length 30 cm.

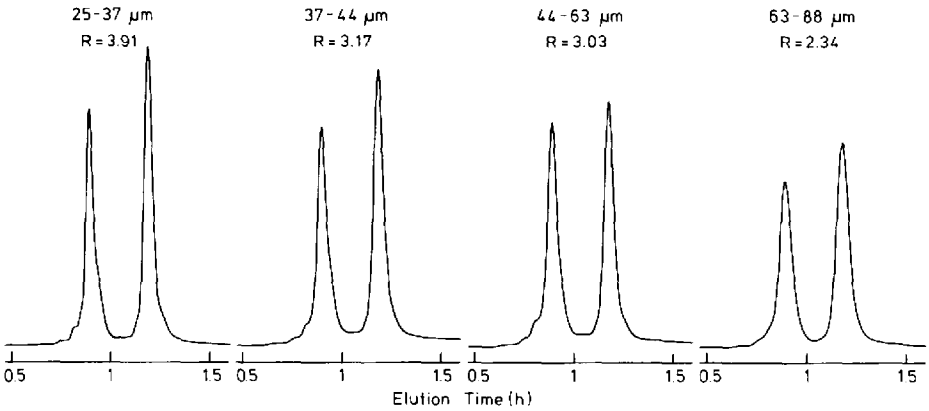

Fig. 5. Chromatograms of a mixture of ovalbumin and trypsin inhibitor obtained on 15 × 1.6 cm I.D. columns of DEAE-Toyopearl 650 of particle diameter 25–37, 37–44, 44–63 and 63–88 μm.

The gravitational packing under a hydrostatic pressure of 250 cmH_2O gave a column of only slightly lower resolution than constant-velocity packing, as shown in Fig. 2. Accordingly, gravitational packing without pump and high-speed packing with a pump are acceptable for obtaining good columns of DEAE-Toyopearl 650. In the gravitational packing, however, a hydrostatic pressure as high as possible is recommended in order to take advantage of the hardness of DEAE-Toyopearl 650.

The effect of column length on resolution was investigated by using DEAE-Toyopearl 650M columns of 1.6 cm I.D. and length 7.5, 15, 30 and 45 cm. The resolution of the columns was measured at a flow-rate of 2 ml/min with gradient volumes of 300 and 600 ml, which correspond to gradient times of 150 and 300 min, respectively. Fig. 3 shows the dependence of resolution on column length. The resolution was independent of column length over the range 7.5–45 cm with a gradient volume of 300 ml, while the resolution decreased slightly with a column length below 30 cm with a gradient volume of 600 ml. Therefore, resolution seems to be independent of column length with a steep gradient of salt concentration and to become dependent on column length as the gradient becomes small, probably owing to the approach to

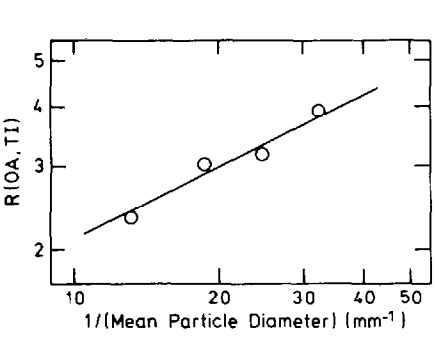

Fig. 6. Dependence of resolution on the reciprocal of mean particle diameter of DEAE-Toyopearl 650.

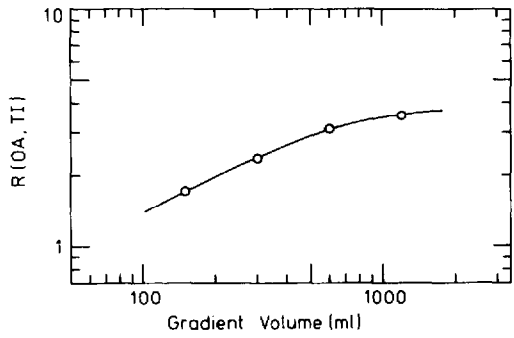

Fig. 7. Dependence of resolution on gradient volume on a DEAE-Toyopearl 650M column (15 × 1.6 cm I.D.).

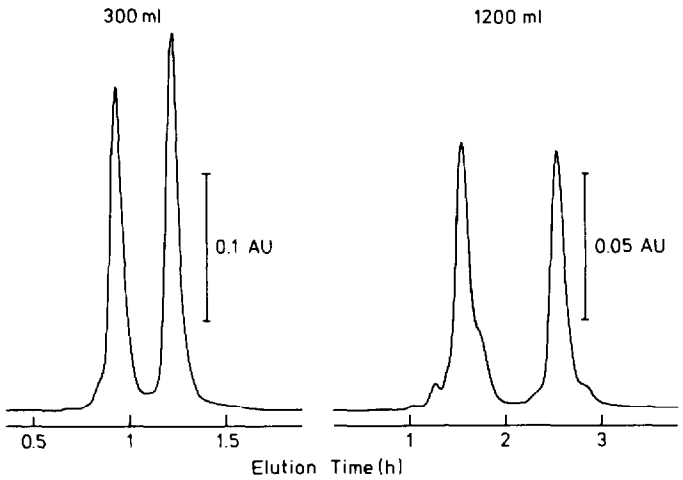

Fig. 8. Chromatograms of a mixture of ovalbumin and trypsin inhibitor obtained on a DEAE-Toyopearl 650M column (15 × 1.6 cm I.D.) with gradient volumes of 300 and 1200 ml.

isocratic elution. Although opposite situations in which resolution did[9] and did not[10,11] depend on column length have been observed in observed-performance ion-exchange chromatography, this seems to be due to differences in the magnitude of the gradient.

The effect of column inner diameter on resolution was investigated by using DEAE-Toyopearl 650M columns of 1.0, 1.6, 2.2 and 3.2 cm I.D. and length 30 cm. The flow-rate, gradient volume and sample loading were varied in proportion to column cross-sectional area in the measurements of resolution. The dependence of resolution on column inner diameter is shown in Fig. 4. Resolution was independent of column inner diameter over the range 1.0–3.2 cm. Consequently, columns of 1.0 cm I.D. can also be used without loss of resolution for DEAE-Toyopearl 650, although the use of narrow columns was not recommended for Toyopearl HW55 because columns of 1.0 cm I.D. provided lower resolutions than columns of 1.6 cm I.D. or greater[8].

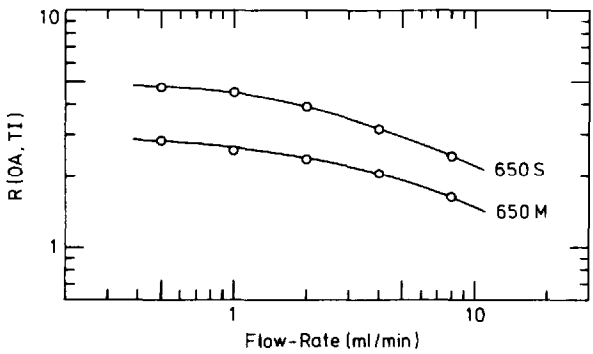

Fig. 9. Dependence of resolution on flow-rate with the gradient volume constant on DEAE-Toyopearl 650S and 650M columns (15 × 1.6 cm I.D.).

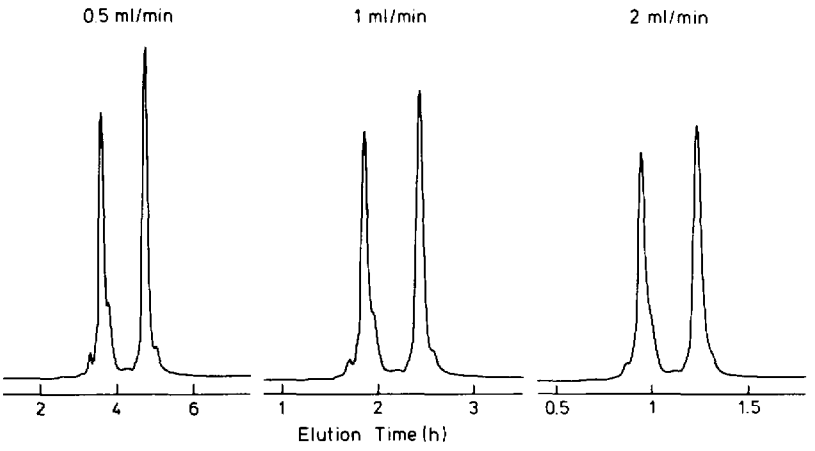

Fig. 10. Chromatograms of a mixture of ovalbumin and trypsin inhibitor obtained on a DEAE-Toyopearl 650S column (15 × 1.6 cm I.D.) at flow-rates of 0.5, 1 and 2 ml/min with a constant gradient volume of 300 ml.

The effect of ion-exchanger particle size on resolution was investigated by using 15 × 1.6 cm I.D. columns of DEAE-Toyopearl 650 of 25–37, 37–44, 44–63 and 63–88 μm particle diameter. Chromatograms of a mixture of ovalbumin and trypsin inhibitor obtained on these columns are shown in Fig. 5. Higher resolution could be achieved with ion exchangers of smaller particle size, as expected. Fig. 6 is a plot of resolution against the reciprocal of the mean particle diameter defined as the arithmetic mean of the two end values of the particle size range. As the slope of the line is 0.5, it can be concluded that the resolution is approximately proportional to the square root of the reciprocal of particle diameter.

The effect of gradient volume was investigated on a DEAE-Toyopearl 650M column (15 × 1.6 cm I.D.) by varying the gradient volume over the range 150–1200 ml at a constant flow-rate of 2 ml/min. The dependence of resolution on gradient volume is shown in Fig. 7. The resolution was considerably improved by increasing the

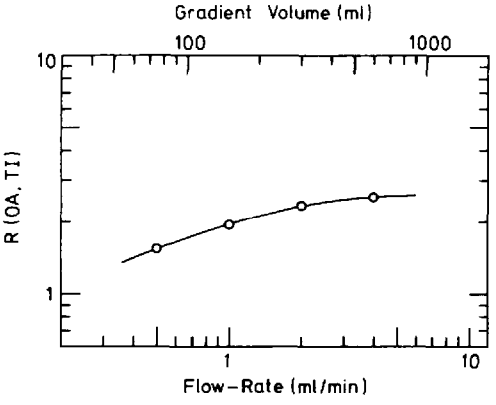

Fig. 11. Dependence of resolution on flow-rate with the gradient volume varying in proportion to the flow-rate on a DEAE-Toyopearl 650M column (15 × 1.6 cm I.D.).

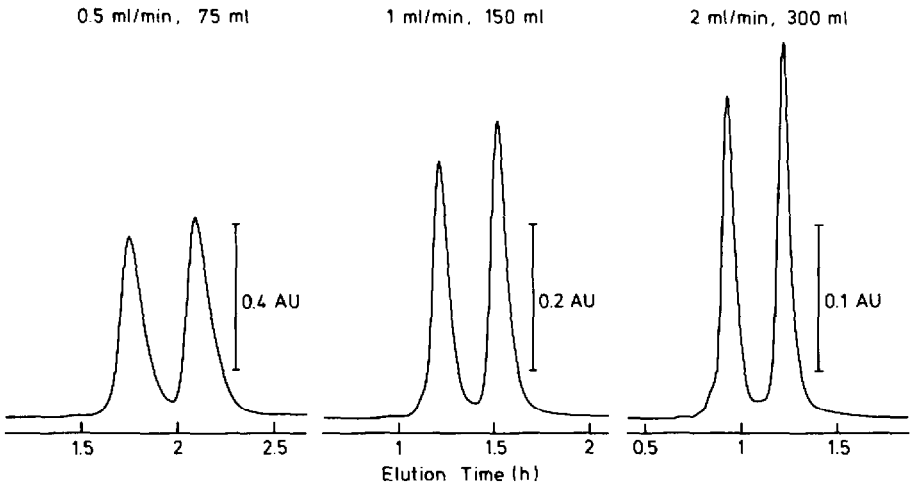

Fig. 12. Chromatograms of a mixture of ovalbumin and trypsin inhibitor obtained on a DEAE-Toyopearl 650M column (15 × 1.6 cm I.D.) at flow-rates of 0.5, 1 and 2 ml/min with gradient volumes of 75, 150 and 300 ml, respectively.

gradient volume. This observation is in agreement with that in conventional ion-exchange chromatography[12] or high-performance ion-exchange chromatography[11]. However, the resolution seems to have a tendency gradually to approach a constant value as the gradient volume increases. Further, the increase in gradient volume also results in a longer separation time and greater dilution of the sample, as shown in Fig. 8.

The effect of flow-rate was investigated on DEAE-Toyopearl 650S and 650M columns (15 × 1.6 cm I.D.) by varying the flow-rate over the range 0.5–8 ml/min with the gradient volume constant or varying in proportion to the flow-rate. The results are shown in Figs. 9–12. A decrease in flow-rate with constant gradient volume resulted in higher resolution, less dilution of the sample and longer separation time. On the other hand, an increase in flow-rate with gradient volume varying in propor-

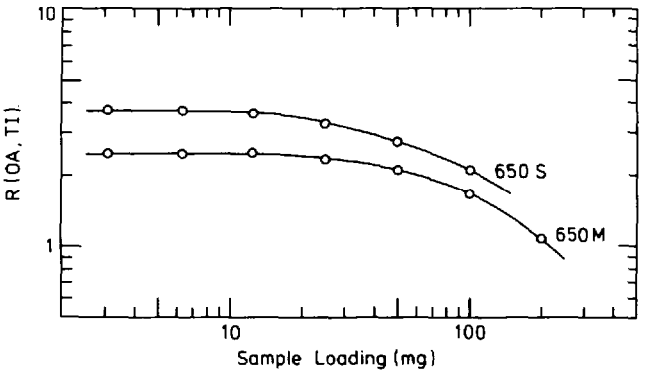

Fig. 13. Dependence of resolution on sample loading on DEAE-Toyopearl 650S and 650M columns (15 × 1.6 cm I.D.).

tion to flow-rate resulted in higher resolution, shorter separation time and greater dilution of the sample.

The effect of sample loading was investigated on DEAE-Toyopearl 650S and 650M columns (15 × 1.6 cm I.D.) by changing the sample loading over the range 3–200 mg. Fig. 13 shows the dependence of resolution on sample loading. The resolution was almost unchanged up to sample loadings of *ca.* 20 mg, which corresponds to 10 mg per cm^2 of column cross-sectional area, and then decreased with further increase in sample loading. The effect of sample loading on resolution was slightly greater on DEAE-Toyopearl 650S than 650M.

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