Performance of WorkBeads™ 17/100 SEC prepacked size-exclusion column

ABSTRACT

A new cross-linking method has made it possible to modify agarose and produce small spherical beads for high performance size-exclusion chromatography of biomolecules, WorkBeads™ SEC. In this application, separation of an artificial protein mixture consisting of common proteins will be shown and resolution factors (R_s), efficiency (N) and partition coefficients (K_{av}) reported. In addition, the influence of flow rate on resolution for the protein mixture was studied.

- new agarose prepacked column
- fast, high performance size-exclusion chromatography
- compatible with biologic samples

INTRODUCTION

WorkBeads™ 17/100 SEC is produced from highly purified agarose according to a new emulsification and cross-linking procedure (1) that makes it possible to decrease the pore size and particle size while keeping the matrix volume low.

Because of the small particle size (d_{50}- 17 μm), a high flow rate and high pressure are required for packing the gels into the columns. WorkBeads™ is prepacked in a specially developed 8 x 300 mm, glass column to enable optimal performance.

WorkBeads™ 17/100 SEC is designed for analytical and semi- preparative separations of proteins in the 10,000 to 100,000 molecular weight range. The exclusion limit is approximately 200,000. To illustrate the separation range and show the high resolution capability of WorkBeads™ 17/100 SEC , along with the

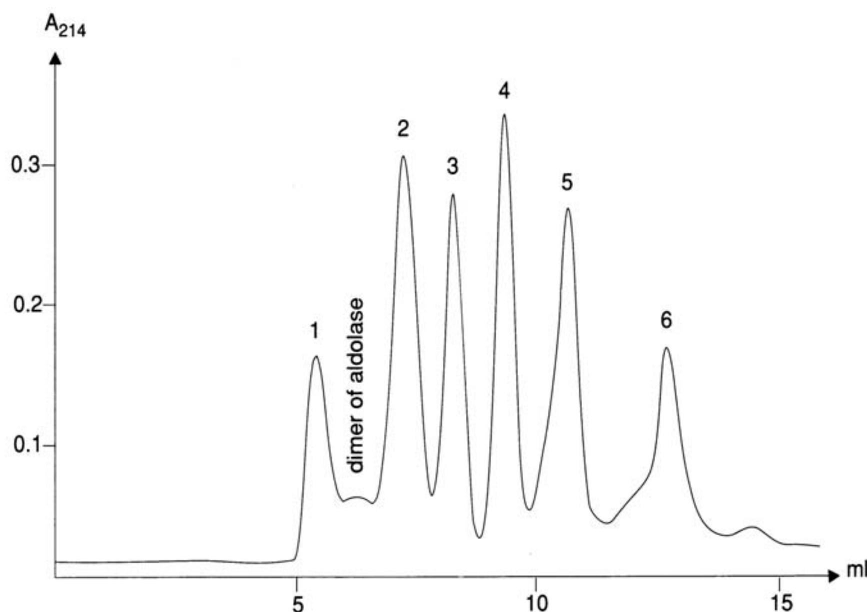

Figure 1. An analytical run of 80 μl of the protein mixture. 1. Thyroglobulin (22.0 μg), 2. Aldolase (16.0 μg), 3. Ovalbumin (20.6 μg), 4. Carbonic anhydrase (20.6 μg), 5. Ribonuclease A (40.0 μg), 6. Insulin (28.6 μg). Flow rate 0.05 ml/min. Buffer 0.05 M sodium phosphate and 0.15 M sodium chloride.

Bio-Works

Application No. 301

influence of flow rate and column length on efficiency and resolution, a protein mixture consisting of aldolase, ovalbumin, carbonic anhydrase, ribonuclease A and insulin was prepared. Thyroglobulin and acetone were used as markers for the void volume (V_0,) and total volume (V_t), respectively.

Determinations of efficiency (N), partition coefficients (K_{av}) and resolution factors (R_s) were carried out for a single column and for two columns coupled in series. The columns were easily connected in series with negligible dead volume.

EXPERIMENTAL

Test samples
Thyroglobulin, bovine, No. T-1001. Sigma, MW= 669,000. Aldolase, rabbit muscle, No. A-1893, Sigma, Mw = 158,000. Ovalburnin, No. A-2512, Sigma, Mw = 45,000. Carbonic anhydrase, bovine erythrocytes, No. C-7500, Sigma, Mw = 29,000. Ribonucleas A, bovine pancreas, No. R-5503, Sigma, Mw = 13,700. Insulin, bovine, No. 44061, Biochemical BDH, Mw = 6,000. Acetone, No. C 2501, Lab-Scan. The protein mixture used in all the separations consisted of a mixture of 24.6 μg of thyroglobulin, 24.6 μg of aldolase, 12.3 μg of ovalbumin, 12.3 μg of carbonic anhydrase, 24.6 pg of ribonuclease and 24.6 μg of insulin dissolved in 80 μl buffer.

Buffer
0.05 M sodium phosphate buffer, pH 6.67, containing 0.15 M sodium chloride.

Column
WorkBeads™ 17/100 SEC, 8 x 300 mm prepacked column, bed volume 15ml.

Instrumentation
HPLC System, GE Healthcare

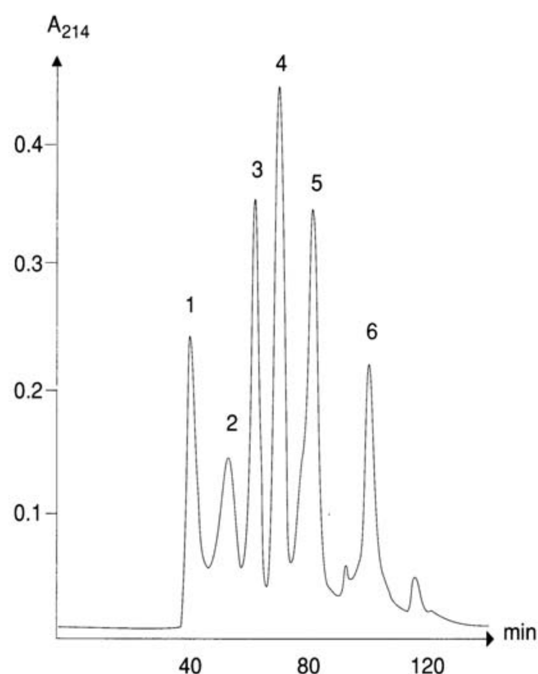

Figure 2. Protein mixture. Flow rate 1.0 ml/min.

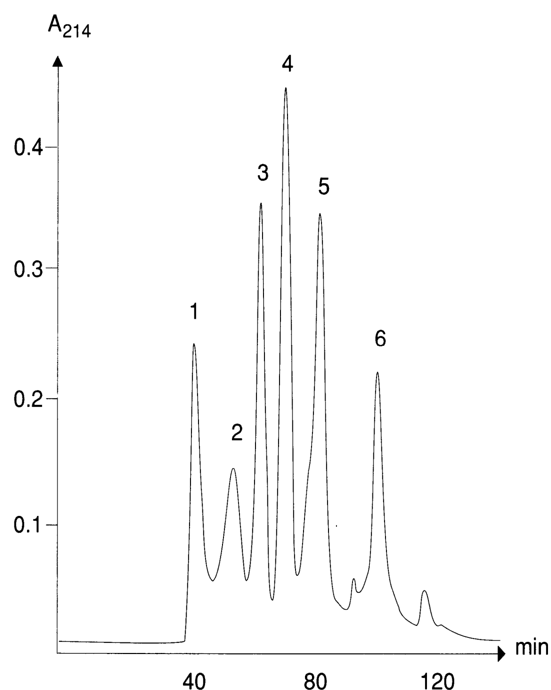

Figure 3. Protein mixture. Two columns coupled in series. Flow rate 0.25 ml/min

RESULTS AND DISCUSSION

Due to the low diffusion coefficients involved, protein separation requires comparatively low flow rates to give high resolution in size exclusion chromatography. This is especially true when the sample is highly heterogeneous. We deliberately selected the proteins in our sample to represent a narrow range molecular weight distribution, thereby creating a difficult separation problem.

Fig. 1 shows an analytical separation of five different proteins on WorkBeads™ 17/100 SEC . The molecular weights of the five proteins eluting between V_o and V_t range from 6,000 to 160,000. For this complex mixture, a low flow rate was needed to obtain base line separation. V_t was determined using acetone. Acetone is excluded from the protein sample because of the large volume demanded for monitoring the UV trace at 214 nm. A further reason was that acetone in larger volumes can cause protein denaturation and precipitation.

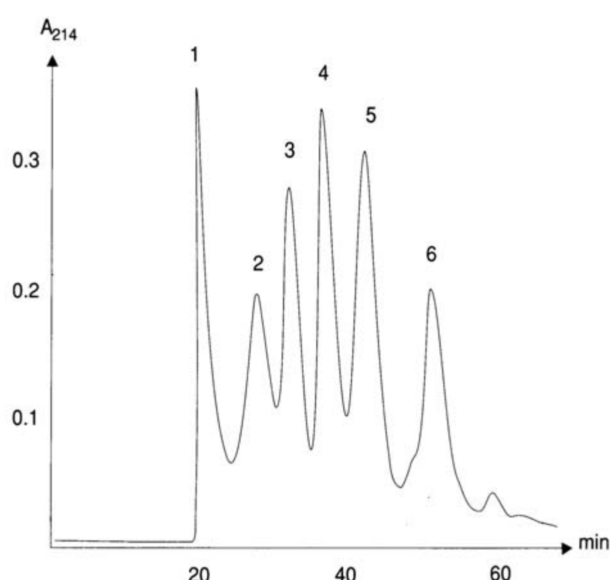

Figure 4. Protein mixture. Flow rate 0.25 ml/min.

The influence of flow rate and column length on resolution is shown in Figures 1-6. With a reduced flow rate (0.05 ml/min, 0.1 cm/min), a higher resolution was obtained (Fig. 1). At this low flow rate, even the dimer of aldolase was separated from the monomer. Data on resolution and efficiency are compiled in Table 1.

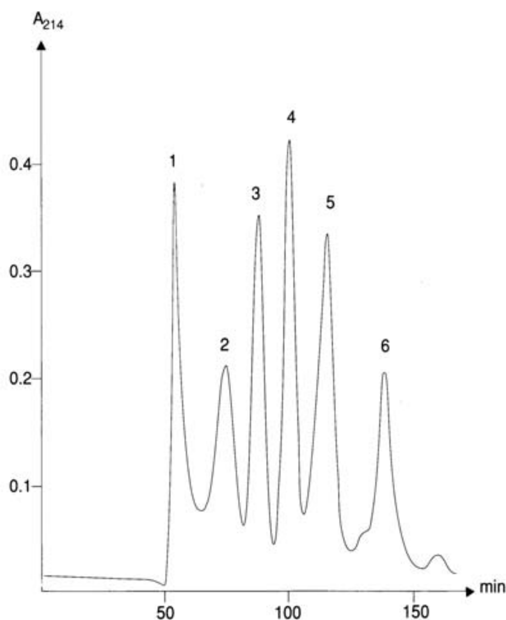

Figure 5. Protein mixture. Flow rate 0.1 ml/min.

Table 1. Data of resolution of proteins.

Figure Number	Flow rate (ml/min)	Resolution between successive peaks 2,3	3,4	4,5	5,6	Efficiency[a] (plates/m)
2	1.00	0.44	0.56	0.71	1.08	1420
6	0.50	0.54	0.79	0.91	1.16	2900
4	0.25	0.77	0.96	1.11	0.89	4267
5	0.10	0.96	1.30	1.46	1.88	8113
1	0.05	1.11	1.34	1.57	2.00	8317
3	0.25[b]	0.89	1.38	1.59	2.40	4327

[a] calculated on peak number four, carbonic anhydrase
[b] two columns

Normally, good resolution is obtained at high flow rates. In Fig. 2, the flow rate was increased to 1 ml/min (2 cm/min), allowing a run to be completed in 15 minutes. Although resolution was reduced, it remained sufficient to provide information on separation (Table 1) and optimization of the chromatographic conditions.

The column allows easy serial column connection. In Figure 3, where two columns were used, a flow rate of 0.25 ml/min (0.5 cm/min) gave high resolution in 120 minutes. This separation was compared with one achieved using a single column at the same flow

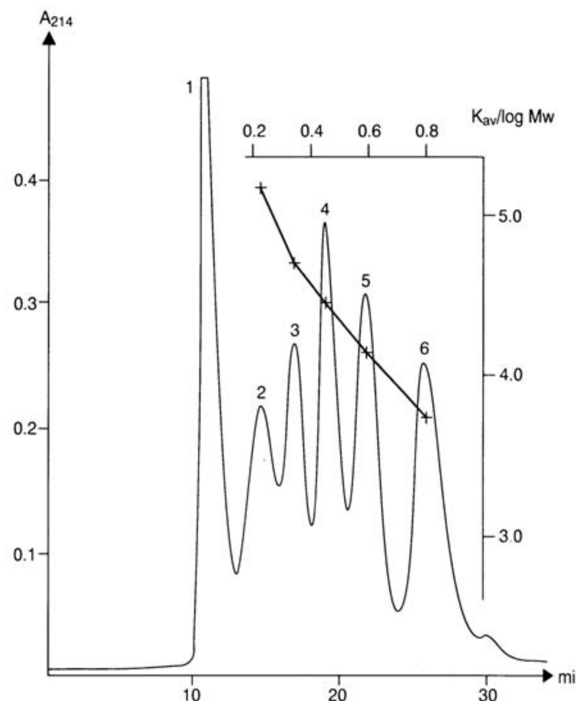

Figure 6. Protein mixture. Flow rate 0.50 ml/min. K_{av}-values 0.21 (2), 0.33 (3), 0.45 (4), 0.60 (5) and 0.80 (6) respectively.

rate (Fig. 4, 0.25 ml/min) and also with a separation for which a single column was used at a lower flow rate (Fig. 5. 0.1 ml/min). The separation with two columns compared well with the single-column separation at a lower flow rate. The resolution of the peaks, Table 1, is improved by a factor of 1.4 when two columns are used, as compared with one column run at the same flow rate. If a separation is extremely difficult it is advisable to use two (or more) columns in series at a low flow rate.

Impurities, which always are of interest, were also detected when two columns were used. As shown in Figure 3, when the resolution of peaks 5 and 6 was improved, the previously hidden impurity appeared.

In Figure 6 the K_{av}-values for the different proteins in the mixture are calculated and plotted against the logarithm of their molecular weight. Good linearity is shown in the recommended separation range.

Table 1 summarizes the efficiency of carbonic anhydrase (peak number four) at different flow rates. Decreasing the flow rate clearly demonstrates the slower diffusion rate for larger molecules. The efficiency nearly doubles when the flow rate is reduced by half. This is particularly true at higher flow rates (> 1 cm/min). At lower flow rates, the gain in efficiency is smaller, but the resolution is improved (see Table 1, Figs. 1 and 2). For a small molecule, e.g. acetone, the efficiency is greater than 25,000 plates/m. This corresponds to a reduced plate height close to 2 when calculated at a flow rate of 0.75 ml/min (1.5 cm/min) using water as the mobile phase and a particle size distribution of a d_{50} (by volume) of 17.5 um.

CONCLUSIONS

The WorkBeads™ 17/100 SEC prepacked column (8 x 300 mm) was shown to provide superb separation of a protein mixture selected to give a narrow molecular weight distribution.

Although a reduced flow rate further improved both the efficiency and the resolution, good resolution was obtained at high flow rates.

The narrow particle size distribution of the WorkBeads™ 17/100 SEC column was shown to give a reduced plate height close to 2, demonstrating high packing efficiency. It was also demonstrated that two columns in series would improve resolution. The increased efficiency showed that the dead volume was negligible.

REFERENCES

1. The data was produced in the research laboratory at Inovata AB in Stockholm, Sweden.

WorkBeads is a trademark of Bio-Works Company Limited.

Bio-Works

Bio-Works Company Limited.
Visit us at www.bio-works.net
Email: info@bio-works.net