Journal of Chromatography, 316 (1984) 125–132
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 512

PREPARATIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PROTEINS ON ION-EXCHANGE COLUMNS WITH CARBOXYMETHYLDEXTRANS AS DISPLACERS

ANTHONY R. TORRES*, BRUCE E. DUNN and STEPHEN C. EDBERG
Department of Laboratory Medicine, Yale University School of Medicine, New Haven, CT 06510 (U.S.A.)
and
ELBERT A. PETERSON
Laboratory of Biochemistry, National Cancer Institute, Bethesda, MD 20205 (U.S.A.)

SUMMARY

It is shown that carboxymethyldextrans can be used to displace proteins from high-performance ion-exchange columns. The high resolving power of the method is demonstrated by the total separation of the A and B genetic variants of the β-lactoglobulins, which have only 0.1 pH difference in their isoelectric points. The separation of up to 12.8 mg of the β-lactoglobulins on a 150-μl column demonstrates the potential for high capacity in displacement chromatography.

INTRODUCTION

For many applications it is desirable to obtain purified proteins in a fast manner. Columns packed with highly substituted microparticulate rigid beads have been shown to offer high capacity, excellent resolution and short elution time in the ion-exchange elution chromatography of proteins[1]. These columns include not only silica-based adsorbents, but also the more recently developed synthetic polymer beads[2]. The advantages of these high-performance liquid chromatography (HPLC) ion-exchange columns have not been utilized in separating proteins by displacement chromatography, as this method has been developed with conventional ion-exchangers.

The purification of proteins by displacement chromatography on DEAE-cellulose and DEAE-agarose with specially prepared carboxymethyldextrans (CM-Ds) as displacers has been described in several previous manuscripts[3–7]. The separation is achieved by the introduction of CM-Ds, which space the protein components according to column affinities. After application of the spacer CM-Ds, the train of contiguous, successively higher-affinity bands is driven through the column by the addition of a CM-D (final displacer), which has a higher column affinity than any of the previous components.

The separation of the A and B genetic variants of the β-lactoglobulins, which differ only by 0.1 pH unit in their isoelectric points, has demonstrated the potential for resolution[4,5]. The high capacity, the convenience of analyzing fractions directly

0021-9673/84/$03.00

by high-resolution two-dimensional gel electrophoresis[6], and the ease of purifying a protein from a complex mixture have been demonstrated by the purification of Gc-2 globulin from human serum[8]. A simple method, utilizing a cation-exchange column for removing the CM-Ds, has also been demonstrated[8].

We show, with a simple mixture of the A and B forms of the β-lactoglobulins and CM-D displacers, that a fast, high-resolution displacement system can be established on HPLC ion-exchange columns. Two silica-based columns and a synthetic polymer column are compared with regard to displacer effectiveness and protein capacity. The high capacity obtainable in displacement chromatography is demonstrated by the total separation of up to 12.8 mg of the above proteins on a 150-μl column.

EXPERIMENTAL

A mixture of the A and B forms of β-lactoglobulin as well as the individual proteins were purchased from Sigma (St. Louis, MO, U.S.A.). Stainless-steel columns (45 × 2.1 mm) were purchased from Rainin (Woburn, MA, U.S.A.) and packed with either Synchropak AX-300 (Synchrom, Linden, IN, U.S.A.), DEAE Spherogel-TSK (Altex, Berkeley, CA, U.S.A.), or a polymer-based column (DEAE-5PW, Waters Assoc., Milford, MA, U.S.A.). Beckman supplied a Model 421 controller, equipped with two Model 112 pumps, a Model 165 detector and a Kipp and Zonen dual-pen recorder (courtesy of Beckman Instruments, Brea, CA, U.S.A.). A 2.0-ml stainless-steel sample loop was used with an Altex 210 sample injector and the column effluent was collected in an ISCO Cygnet fraction collector (Lincoln, NE, U.S.A.). The microcombination pH electrode (MI-411A) was furnished by Microelectrodes (Londonderry, NH, U.S.A.). The preparation of narrow-range CM-Ds has been described previously[5].

Column chromatography

The silica-based beads were packed in 100% methanol under 5000 p.s.i. and the synthetic polymer beads were packed in water under pressure rising to 3000 p.s.i. A helium-degassed buffer, 20 m*M* potassium phosphate (pH 6.0), was used in all experiments. The spacer CM-Ds as well as the final displacer CM-D were used as 1% solutions in the 20 m*M* potassium phosphate buffer. The concentration is not a critical factor as similar results are obtained with lower or higher concentrations of CM-Ds. All samples, displacers, and buffers were filtered through 0.45-μm filters. The flow-rate was 0.1 ml/min, and 2-min fractions were collected in 1.8-ml polypropylene centrifuge tubes. After each chromatographic experiment, the column was washed with 0.25 ml of 0.5 *M* potassium chloride and 0.5 ml of 0.2 *M* potassium dihydrogen phosphate in order to remove displacer and residual protein. Before injecting samples, the column was thoroughly equilibrated to pH 6.0 with the degassed buffer.

Electrophoresis

The discontinuous Tris–glycine buffering system of Davis[9] was used in a vertical polyacrylamide slab gel (Mini-Slab, Idea Scientific, Corvallis, OR, U.S.A.). Since the proteins migrate very closely to the leading ion front in the 7.5% gel, it is im-

portant that the pH of the acrylamide mixture for the sieving gel be no higher than 8.9. All protein-containing chromatographic fractions were thoroughly examined by gel electrophoresis for purity. The gels were used to confirm the separation, but documentation has not been included, since only two proteins are in question. Samples (0.2–1.0 μl) from the chromatographic fractions were mixed with 5 μl of 20% sucrose, applied to a slot in the slab gel, and subjected to electrophoresis at 100–125 V for 2 h. Standards contained 2 μg of each β-lactoglobulin.

The gels were stained for protein with Coomassie Brilliant Blue R-250[10]. After

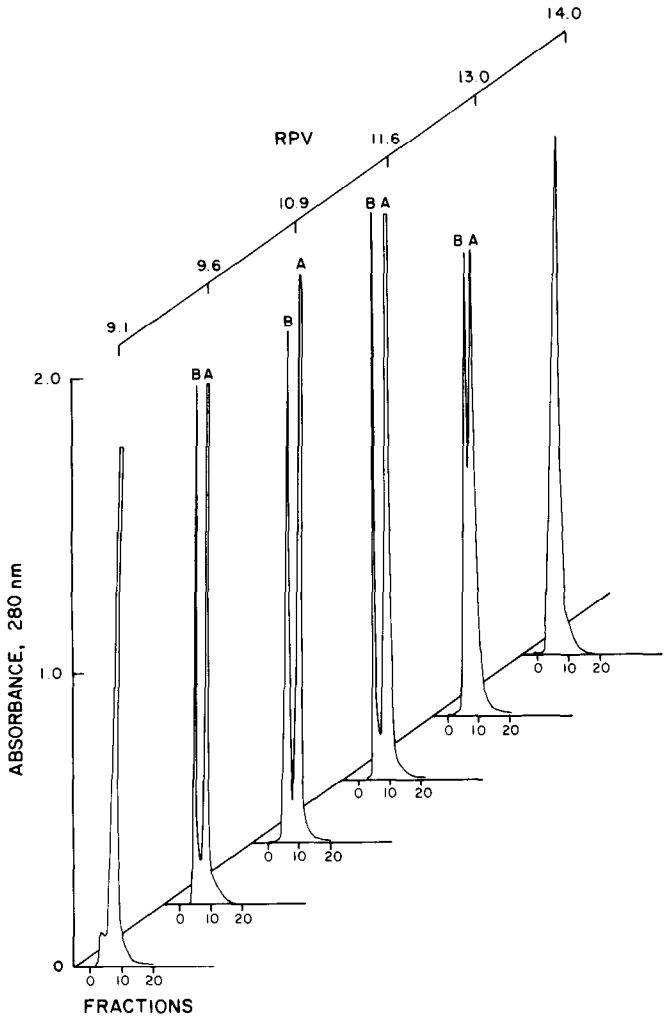

Fig. 1. The effects of different affinity CM-Ds in spacing the β-lactoglobulins. In each of the six experiments, 0.8 mg of β-lactoglobulin A and B in 0.4 ml were injected into a 45 × 2.1 mm (150-μl) DEAE Spherogel-TSK column, equilibrated in 20 m*M* potassium phosphate, pH 6.0. Application of the protein was followed by 0.75 ml of a 1% solution of a CM-D having the affinity shown by the RPV values. At point zero, 1.1 ml of a 1% solution of a high-affinity CM-D (RPV 28.6) displaced the train from the column. The samples and displacer all contained 20 m*M* potassium phosphate (pH 6.0). Solutions were pumped at 0.1 ml/min, fractions were collected at 2-min intervals, and the chart speed was 0.1 cm/min.

destaining in 7% acetic acid, the gels were photographed with a Polaroid MP-3 camera using Polaroid type 55 film and a yellow Wratten No. 8 filter.

RESULTS AND DISCUSSION

The effects of following the protein sample on a DEAE Spherogel-TSK column with two different concentrations of the same CM-D were first examined to determine whether displacement was occurring. No effort was made to fractionate protein but only to show that the position and the concentration of the protein in the effluent are determined by the concentration of the displacer. In both experiments, 4.8 mg of β-lactoglobulin A in 0.6 ml was applied to a 45 × 2.1 mm (150 μl) DEAE Spherogel-TSK column, equilibrated with 20 m*M* potassium phosphate (pH 6.0). A decrease in the concentration of the final displacer from 0.05% to 0.025% decreased the protein concentration in the effluent by one-half. In another experiment, a predicted plateau appeared at the same protein concentration when the 0.025% CM-D displaced twice as much protein (9.6 mg) from the column.

These results are the same as previously observed with CM-D-induced protein displacement from DEAE-cellulose[4] and agree with the criteria established by Tiselius[11] when he observed the displacement of small, reversibly adsorbed molecules.

The results of using CM-Ds of different affinity as displacers in an attempt to identify the appropriate spacer for the A and B forms of the β-lactoglobulins on DEAE Spherogel-TSK are shown in Fig. 1. The affinity values of the CM-Ds have been discussed previously[5,7] and will not be covered here, except to point out that the column affinity of the CM-Ds increases with the reciprocal of the pellet volume (RPV) value. For these experiments it was necessary to try different CM-Ds in a hit-or-miss fashion, since there is, as yet, no affinity index applicable to such small fractions. Although the present assay makes it possible to determine the RPV value of a spacer CM-D after a single application of a combination of several CM-Ds, 2.0-ml fractions are required.

It is apparent in Fig. 1 that the displacers with the lowest and the highest RPV values did not separate the proteins. The CM-Ds with RPV values of 9.6, 10.9, and 11.6 had appropriate affinities to displace or space form B form A β-lactoglobulin. The CM-D with the lowest RPV was unable to displace either protein, whereas the highest RPV displacer had sufficient affinity to displace both proteins. The intermediate CM-Ds had column affinities between those of the proteins and therefore served as effective spacers. One CM-D (RPV 10.9) appeared most effective for the DEAE Spherogel-TSK and the Synchropak AX300 columns whereas a lower affinity CM-D (RPV 9.1) gave optimal spacing on the polymer (DEAE-5PW) column.

Fig. 2 shows the total separation of the β-lactoglobulins on three commercially available columns. In each experiment, protein was injected into the column and followed by spacer CM-D, buffer and final displacer. The amount of spacer CM-D applied was sufficient to displace the B form of the protein completely from the colum. The A form of the protein was removed from the column by the final displacer.

To determine the relative quantity of β-lactoglobulin A and B that could be separated by using each of the three packing materials, we arbitrarily started at 0.8 mg of proteins. If complete separation of the A and B forms was achieved, the amount of protein was doubled. The amounts of β-lactoglobulin A and B used in Fig. 2 represents the highest amount separated with this protocol.

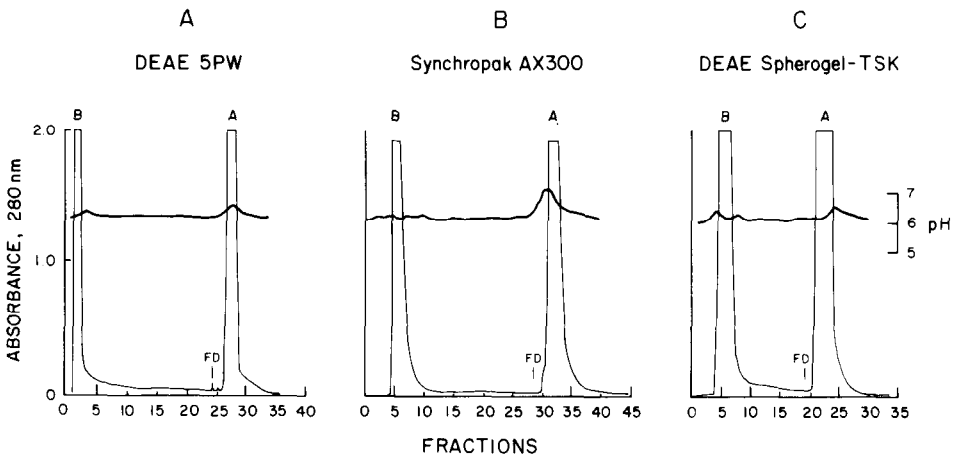

Fig. 2. Separation of the β-lactoglobulin (A and B) on three different columns. Column volumes of 150 μl (45 × 2.1 mm) were used in all three experiments. Different amounts of the β-lactoglobulins A and B were applied to the respective columns: 3.2 mg in 1.6 ml to the DEAE-5PW in A, 6.4 mg in 0.8 ml to the Synchropak AX-300 in B, and 12.8 mg in 1.6 ml to the DEAE Spherogel-TSK in C. In all cases, the indicated amount of a 1% solution of CM-D, applied at time zero, displaced β-lactoglobulin B completely from the columns; in A, 0.5 ml of RPV 9.1; in B, 0.75 ml of RPV 10.9; and in C, 1.25 ml of RPV 10.9. β-Lactoglobulin A, in each experiment, was completely displaced from the columns by the application (Final Displacer) of 1.1 ml of a 1% solution of final CM-D (RPV 28.6). The columns were equilibrated in 20 m*M* potassium phosphate (pH 6.0), and all solutions contained this buffer. This standard buffer was also applied between the spacer and final displacers.

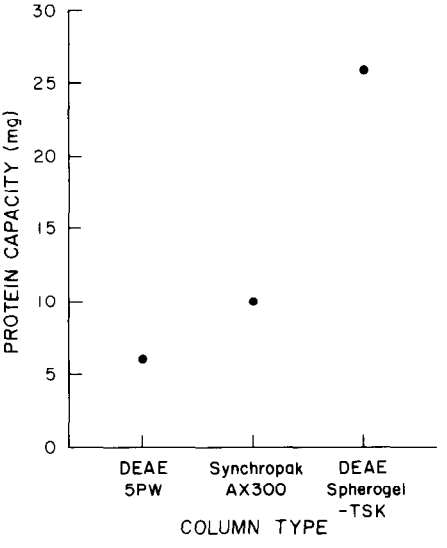

Fig. 3. The capacity for the β-lactoglobulins (A and B) on three different columns, as determined by frontal analysis. Standard column volumes of 150 μl (45 × 2.1 mm) with a flow-rate of 0.1 ml/min. In each case, a 2-ml solution of the β-lactoglobulin (A and B); (4 mg/ml to the DEAE-5PW column, 8 mg/ml to the Synchropak AX-300 column and 20 mg/ml to the DEAE Spherogel-TSK Column) was applied to the column from a 2-ml sample loop. All contained 20 m*M* potassium phosphate, pH 6.0. The capacity for the β-lactoglobulin was determined by the volume at which a rise in UV absorbance (280 nm) appeared in the column effluent at the protein concentration of the influent solution.

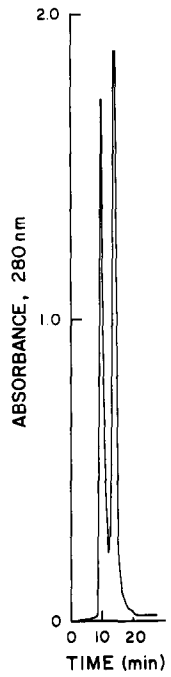

Fig. 4. The effects of applying the spacer CM-D to the column before the sample. In this experiment 0.75 ml of a 1% solution of a spacer CM-D (RPV 10.9) were applied to a 150-μl (45 × 2.1 mm) DEAE Spherogel-TSK column. After re-equilibrating the column with 20 m*M* potassium phosphate (pH 6.0), 400 μl, containing 0.8 mg of a mixture of the β-lactoglobulins (A and B), were applied (point zero). The protein solution was immediately followed by 1.1 ml of a 1% solution of a high-affinity CM-D (RPV 28.6) in the 2-ml sample loop. The sample and both CM-Ds contained 20 m*M* potassium phosphate.

There is quite a wide variation in the amount of protein that can be separated on the different columns. The amount separated on the DEAE-polymer column (3.2 mg) was less than that accommodated by the polyethyleneimine Synchropak AX300 (6.4 mg) or the DEAE Spherogel-TSK (12.8 mg) columns. Although these values do not represent the absolute upper limit, the amounts are close to one-half of what it takes to saturate the different columns with the β-lactoglobulins (Fig. 3). The high capacity of these columns can be attributed not only to the degree of substitution but also to the band-sharpening effects induced by the CM-D displacer, as complete separation was accomplished on columns which were 50% saturated with two very similar proteins.

Each of these chromatographic experiments was completed in less than an hour, but we have not attempted to determine the limit. For example, the maximal flow-rate for a particular column has not been determined.

It is also possible to decrease the time by saturating the column with the spacer, CM-D, before applying the protein sample (Fig. 4). The distance between the two proteins peaks increases as the amount of CM-D is increased up to 7.5 mg. Additional spacing was not achieved when more CM-D was applied as the column appeared completely saturated with 7.5 mg of spacer displacer. However, a good separation of the 0.8 mg protein load was accomplished, and the concept of applying the spacer

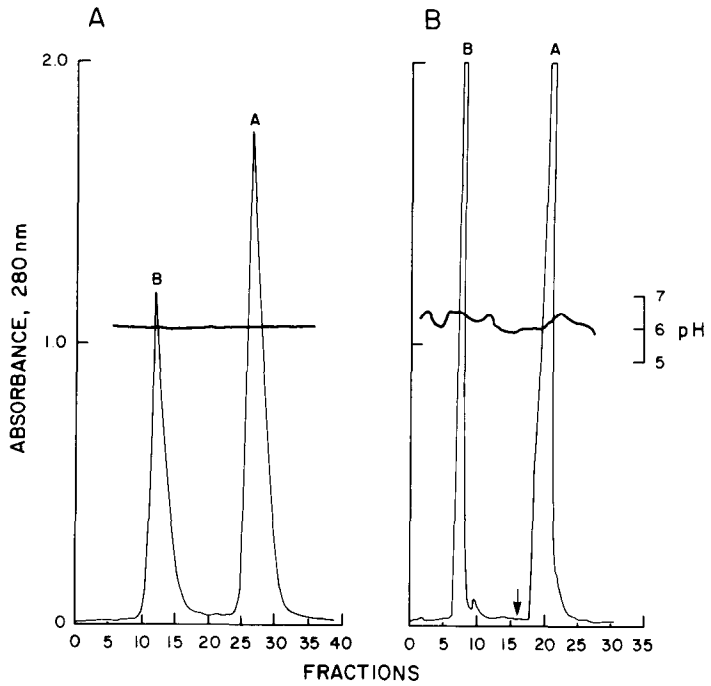

Fig. 5. Comparison of the separation of the β-lactoglobulins by elution (A) and displacement (B) chromatography. In each experiment, 2.4 mg of the proteins in 0.6 ml were applied to a DEAE Spherogel-TSK 150-μl column (45 × 2.1 mm). In A, the sample was followed by 20 m*M* potassium phosphate (pH 6.0), containing 0.1 *M* potassium chloride until the pH had become stabilized at 6.0. At point zero, a 65-min potassium chloride gradient from 0.1–0.14 *M* in the 20 m*M* buffer was begun. In B, after column equilibration with 20 m*M* potassium phosphate, the sample was applied, followed with 1.5 ml of a 1% solution of spacer CM-D (RPV 11.6). After complete displacement of β-lactoglobulin B from the column by the spacer CM-D, 1.1 ml of a 1% solution of the high-affinity CM-D (RPV 28.6) was applied (arrow) to displace β-lactoglobulin A.

displacer before the sample was demonstrated. This 15-min separation was accomplished with the standard column, flow-rate and CM-D concentration. Substantial reduction of the protein load should allow a faster flow-rate and provide a better analytical separation in a shorter time.

In an effort to show the potential for high resolution, we decided to compare gradient elution with displacement chromatography. In Fig. 5A, 2.4 mg of the β-lactoglobulins (A and B) were completely separated by elution with a shallow (0.1–0.14 *M* potassium chloride) salt gradient over 65 min. Steeper salt gradients resulted in sharper peaks; however, complete separation was not accomplished.

In Fig. 5B, the same amount of protein was completely separated by displacement chromatography by using the standard conditions previously described. In both experiments, electrophoretic evaluations confirmed the purity of the peaks. Although a good separation was accomplished by elution, it is apparent that displacement gave better results as the peaks are sharper and about twice as high. The spacing of the peaks was determined by the amount of spacer CM-D employed.

In conclusion, we have shown that ion-exchange protein displacement chro-

matography is readily adaptable to HPLC systems with attendant high capacity and high resolution. Although the DEAE Spherogel-TSK packing material had the highest capacity for the β-lactoglobulins at pH 6.0, we are presently using the DEAE-5PW column, since the polymeric material is stable at higher pH values. Higher values up to about pH 8.6 allow better adsorption of many proteins to anionic exchangers. The advantages of high-performance displacement chromatography demonstrated in this article are currently being applied to the separation of enzymes from complex biological mixtures.

ACKNOWLEDGEMENTS

We wish to thank Dr. Csaba Horváth for thoughtful discussion and Miss Lourdes Rivera for excellent technical assistance.

REFERENCES

1 F. E. Regnier and K. M. Gooding, *Anal. Biochem.*, 103 (1980) 1.
2 V. J. Britton, L. C. Beadling and A. Barry, *LC*, 1 (1983) 176.
3 E. A. Peterson, *Anal. Biochem.*, 90 (1978) 767.
4 A. R. Torres and E. A. Peterson, *J. Biochem. Biophys. Methods*, 1 (1979) 349.
5 E. A. Peterson and A. R. Torres, *Anal. Biochem.*, 130 (1983) 271.
6 A. R. Torres, G. G. Krueger and E. A. Peterson, *Clin. Chem.*, 28 (1982) 998.
7 E. A. Peterson and A. R. Torres, *Methods Enzymol.*, 104 (1984) 113.
8 A. R. Torres, G. G. Krueger and E. A. Peterson, *Anal Biochem.*, (1985) in press.
9 B. J. Davis, *Ann. N.Y. Acad. Sci.*, 121 (1964) 404.
10 A. H. Reisner, P. Nemes and C. Bucholtz, *Anal. Biochem.*, 64 (1975) 509.
11 A. Tiselius, *Ark. Kemi Mineral. Geol.*, 16A (1943) n18.