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PREPARATIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PROTEINS ON AN ANION EXCHANGER USING UNFRACTIONATED CARBOXYMETHYLDEXTRAN DISPLACERS

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SUMMARY

It has been previously demonstrated that carboxymethyldextran (CMD) displacers fractionated on an ion-exchange column can be used for the resolution and high capacity displacement chromatography of proteins on high-performance ion-exchange columns. It is shown, in this manuscript, that high resolution and high capacity are obtainable using unfractionated CMD displacers. Up to 25 mg of a mixture of ovalbumin, α-lactalbumin, and soy-bean trypsin inhibitor along with many impurities were separated on a 3.3-ml Altex DEAE-5PW column using three unfractionated CMD displacers and a final CMD displacer. A method for removing the displacers from proteins, on a hydrophobic interaction column, is also described.

INTRODUCTION

The relatively new high-performance liquid chromatography (HPLC) ion-exchange columns used for protein purification offer much better resolution than classical ion-exchange columns because of the smal, uniform size and close packing of the particles[1]. However, their capacities, like those of classical columns, are not fully utilized under standard elution conditions. We have shown in a series of manuscripts that displacement chromatography utilizing carboxymethyldextrans (CMDs) as displacers offers higher capacity than elution chromatography[2–7]. Although initially demonstrated on DEAE-cellulose and DEAE-agarose, this displacement method has been extended to anion-exchange HPLC columns with similar results[8]. The separations are achieved by applying a series of previously fractionated displacers to the columns after application of the sample. A train of contiguous bands, successively higher in affinity and including the separated proteins, is driven through the column by the addition of a saturating amount of final displacer, which has the highest

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affinity for the adsorbent. The high capacity and high resolving power of these displacement systems has been demonstrated by the separation of Gc-globulin from human serum on DEAE-cellulose[7] and by the HPLC separation of 13 mg of a mixture of the A and B genetic variants of the β-lactoglobulins on a 150-μl column[8]. In the latter case, a total separation of the closely related β-lactoglobulins was accomplished on columns that were approximately 50% saturated with protein[8]. To achieve such separations, narrow-range CMD were used. These were made by fractionating the initial, very heterogeneous preparations by displacement chromatography on preparative DEAE-cellulose columns. The narrow range CMDs make it possible to focus resolving power on a region of the chromatogram that is of particular interest, adequately spacing proteins that are so similar in affinity that they would otherwise be poorly separated.

However, in the present manuscript we show that unfractionated displacers can give excellent resolution at high capacity on a commercial HPLC column. Up to 25 mg of a moderately complex protein mixture containing commercial preparations of ovalbumin, α-lactalbumin, and soy bean trypsin inhibitor, along with many impurities, was separated using three unfractionated spacers and a final displacer on a standard Altex DEAE-5PW column. The quantitative removal of the displacers from the above proteins on a hydrophobic interaction column is also demonstrated.

EXPERIMENTAL

The proteins (ovalbumin, grade VI; α-lactalbumin, type 1; and soy bean trypsin inhibitor, type 1-S) were purchased from Sigma (St. Louis, MO, U.S.A.). The ion-exchange column (DEAE-5PW, 75 × 7.5 mm I.D.) and the hydrophobic interaction column (Spherogel-CAA-HIC, 100 × 4.6 mm I.D.) were furnished by the Altex Division of Beckman Instruments (San Ramon, CA, U.S.A.). A gradient HPLC with two Model 112 pumps and a Model 165 variable dual pen recorder was also provided by Beckman. A 5.0-ml stainless-steel sample loop was used with an altex 210 sample injector, and fractions were collected in an ISCO Cygnet fraction collector (Lincoln, NE, U.S.A.).

Carboxymethyldextran preparation

Procedures for preparing the unfractionated CMDs from Dextran T-10 (Pharmacia, Uppsala, Sweden) have been described previously[2,4]. However, the CMDs used in this work were purified by a simpler method utilizing ion-exchange resins that wil be reported elsewhere.

Column chromatography

A helium-degassed buffer, 20 m*M* Tris–phosphate (pH 8.0), was used in all ion-exchange displacement experiments. The spacer CMDs and the final displacer CMD were used as 1% solutions in this buffer. All samples, displacers, and buffers were filtered through 0.45-μm filters. The flow-rate was 0.5 ml/min and 1-min fractions were collected in 1.8-ml polypropylene centrifuge tubes. After each experiment, the column was washed with 2 ml of 0.5 *M* sodium chloride in order to remove bound displacers and any residual protein. The column was thoroughly equilibrated to pH 8.0 with the degassed buffer before injecting another sample.

Removal of displacers

To demonstrate the removal of CMDs from protein samples, mixtures of unfractionated protein and displacers were chromatographed on an HPLC hydrophobic interaction column. In each of the three experiments, 5 mg of ovalbumin, α-lactalbumin, or soy bean trypsin inhibitor was mixed with 2.0 ml each of 1% solutions of a spacer and a final displacer. The displacer–protein mixture was then taken to 30 ml with 25 ml of 3 *M* ammonium sulfate containing 0.15 *M* ammonium phosphate, pH 6.2. The ammonium phosphate buffer was substituted for the ammonium acetate buffer recommended by the manufacturer since it offers better buffering above pH 6.0. This sample was then pumped into a column equilibrated with the same ammonium sulfate–phosphate solution. After the absorbance at 220 nm had dropped to baseline, the displacer-free protein was eluted in a sharp peak with 0.15 *M* ammonium phosphate, pH 6.2. Small portions (50 μl) of the fractions from the hydrophobic interaction column were reacted with anthrone and read at 625 nm (ref. 9) to provide a measure of the CMD carbohydrate concentration.

RESULTS AND DISCUSSION

A mixture of ovalbumin, α-lactalbumin, and soy bean trypsin inhibitor (2:1:2) was selected as a sample for these experiments in order to provide a wide range of affinities. Less α-lactalbumin was used because its specific absorbance at 280 nm was higher than those of the other two proteins. Although each protein preparation contained several impurities as judged by HPLC and gel electrophoresis, there was a major component in each case. Ovalbumin is known to be very heterogeneous[4,10].

Preliminary tests with the polymer-based DEAE-5PW column showed that ovalbumin was subfractionated as well as separated from the other two major proteins by narrow-range CMDs with RPV (reciprocal of the pellet volume[6]) values of 7–10, in agreement with previous results on DEAE-cellulose[4]. Further trials indicated that an RPV of *ca.* 11 was needed to separate α-lactalbumin form soybean trypsin inhibitor. The heterogeneity of unfractionated CMDs covers a much wider range and compensates for errors in the RPV selection. Indeed the selection of appropriate unfractionated CMDs can be made directly with those preparations, but the assignment of RPV values to critical regions of the chromatogram would be more difficult at this small scale. Three unfractionated CMD preparations, with RPV values of 7.7, 9.5, and 11.7, respectively, were selected to provide the desired affinity range for spacing. Another preparation, with an RPV value of 21.2, served as the final displacer.

The fractionation of 6.25 mg, 12.5 mg, and 25 mg of the mixture are shown in Fig. 1a, b, and c, respectively. In each case, the major ovalbumin component emerged first, followed by α-lactalbumin, and soy bean trypsin inhibitor. All the conditions were the same except for the increase in sample load and a doubling of the amount of each spacer solution to 10 ml in Fig. 1b and c; at the higher sample loads, separation of the peaks was not adequate with 5 ml of spacer solution. A displacement train developed, driven by the higher-affinity components of the spacer CMD, but the most tightly bound protein did not move appreciably and was not part of the train until the final displacer was applied. Approximately the same amount of the latter was required in each case.

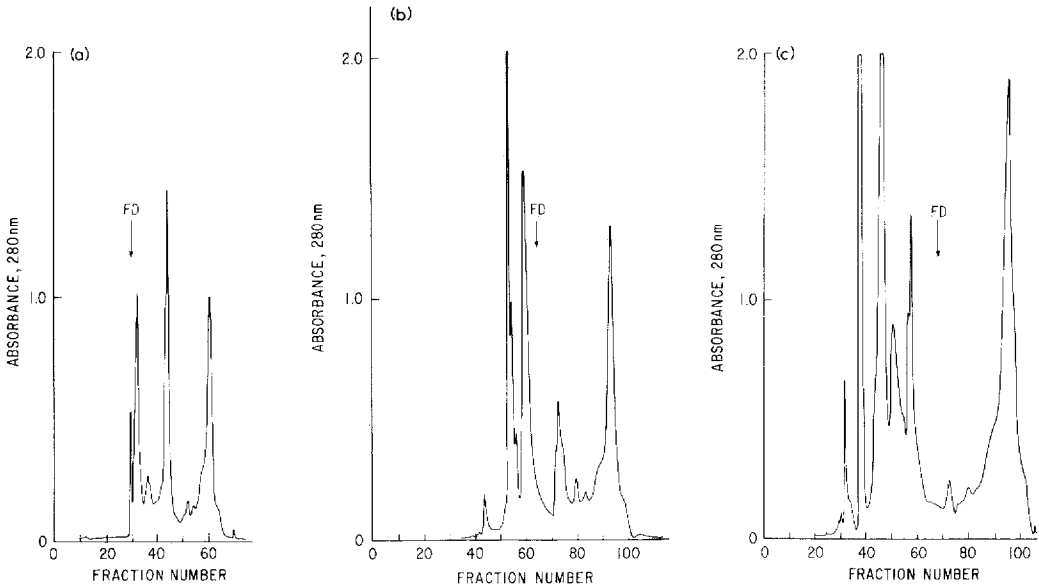

Fig. 1. Fractionation of ovalbumin, α-lactalbumin, and soy bean trypsin inhibitor on DEAE-5PW. The column (75 × 7.5 mm I.D.) was equilibrated with 20 m*M* Tris–phosphate, pH 8.0, and all the solutions contained this buffer. A mixture of 6.25 mg of protein containing 2.5 mg of ovalbumin, 2.5 mg of soy bean trypsin inhibitor, and 1.25 mg of α-lactalbumin served as the sample in section (a). Samples for (b) and (c) contained twice (12.5 mg) and four times (25 mg) as much of the same mixture, respectively. The sample in (a) was followed consecutively by 5 ml of each of the three spacer displacers (RPV values of 7.7, 9.5, and 11.7) and 15 ml of the final displacer (FD) (PRV = 21.2) (arrow), all as 1% solutions in the buffer. Conditions were the same in (b) and (c), except that 10 ml of each spacer solution was used. The flow-rate was 0.5 ml/min, the chart speed was 0.1 cm/min, and 1-min fractions were collected. For peak identification see text.

The extra peaks represent impurities in the samples as well as multiple forms of the named proteins. We have shown in a previous manuscript[4] that the same high-purity ovalbumin can be fractionated on DEAE-cellulose into six peaks which reflect differences in carbohydrate and phosphate composition. Impurities in all three protein preparations are also apparent by gel electrophoresis and displacement chromatography when single proteins are examined. It was not intended that all the impurities be separated from one another but rather to demonstrate a general fractionation over a wide range of affinity. To expand any particular region, additional displacers of appropriate affinity would be added. Electrophoretic characterization of alternate fractions from Fig. 1b is shown in Fig. 2.

Although the CMDs are generally innocuous to proteins and do not interfere with subsequent chromatographic or electrophoretic steps, at times their removal from the purified protein is necessary. This has been accomplished previously by adjusting pooled fractions to pH 5.0–5.5 and passing the solution through CM-cellulose equilibrated at the same pH. Under these conditions, most of the proteins so far dealt with are bound and the CMDs pass through, unadsorbed. The bound proteins can be subsequently eluted sharply with salt and/or an increase in pH. In the present study, this method was not effective because the capacity of the HPLC cation

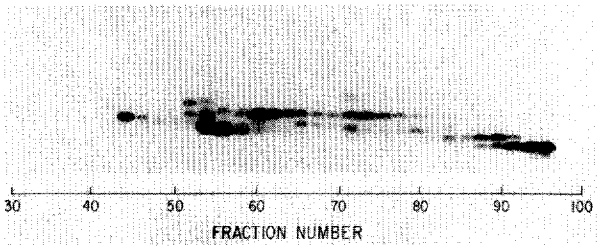

Fig. 2. Electrophoretic evaluation of fractions from Fig. 1b. The discontinuous Tris–glycine buffer system of Davis[11] was used in a 35-place vertical 7.5% polyacrylamide slab gel (25 × 10 × 0.2 cm) and 30 μl of each even-numbered fraction was mixed with 4 μl of 30% sucrose before application to the gel. Electrophoresis was carried out at 200 V for 3 h. The gel was stained with Coomassie Brilliant Blue R-250 (ref. 12).

exchanger employed (CM-Spherogel TSK) was not adequate for these particular proteins under these conditions. However, "salting out" the protein on a hydrophobic interaction column provides another approach. The displacers pass unadsorbed through the column, and the bound protein can then be eluted sharply by lowering the salt concentration. Fig. 3 confirms that whereas the displacers pass through the

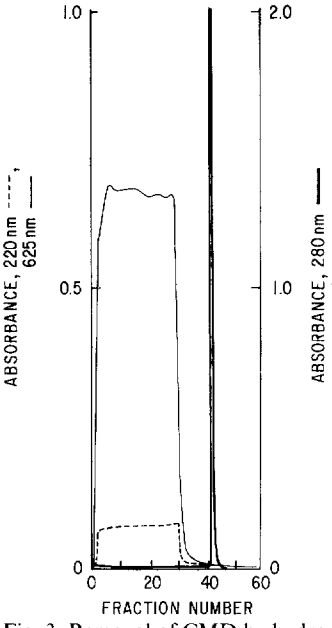

Fig. 3. Removal of CMD by hydrophobic interaction chromatography on an Altex Spherogel-CAA-HIC column (100 × 4.6 mm I.D.). The sample consisted of 1 ml of α-lactalbumin solution (5 mg/ml) and 2 ml of a 1% solution of each of two displacers (RPV values of 8.7 and 30) added to 25 ml of 3 *M* ammonium sulfate containing 0.15 *M* ammonium phosphate, pH 6.2. The column was equilibrated with the same salt solution. At fraction 38, 0.15 *M* ammonium phosphate, pH 6.2, (without the ammonium sulfate) was initiated to elute the protein. The broken line shows the absorbance at 220 nm of CMD carboxyl groups and peptide bonds. The thin line indicates absorbance at 625 nm after reaction of 50-μl samples with anthrone as a measure of CMD concentration. The heavy line represents absorbance at 280 nm as a measure of protein concentration.

hydrophobic interaction column, α-lactalbumin is bound in the presence of 3 *M* ammonium sulfate and can be subsequently eluted by a low-salt buffer. The anthrone reaction (for glucose residues from hydrolyzed dextran) shows that the protein peak is free of dextran. Similar results were obtained for ovalbumin and soy bean trypsin inhibitor. This procedure should be applicable to many different proteins.

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