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Evaluation of displacement chromatography for the recovery of lactate dehydrogenase from beef heart under scale-up conditions

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ABSTRACT

A complex mixture of proteins was subjected to displacement development from a Tris Acryl DEAE anion-exchange column. Lactate dehydrogenase was used as the target protein in the evaluation of the resolution and separation under scale-up conditions. The conditions of operation were scaled up in terms of column size and protein load. Column length was found to play an important role in resolving the mixture. The performance of the displacement mode run was compared with a conventional ion-exchange elution run in terms of fraction purity and specific activities. In general, displacement chromatography of the complex mixture yielded better results.

INTRODUCTION

The recovery and purification of proteins from crude mixtures is a multi-stage process in which a number of different unit operations in downstream processing can be involved. Chromatography is one such separation method and is a powerful tool for separation towards the end stages. Chromatographic separation can be based on ionic charge, hydrophobicity, molecular size and biospecific affinity, among others. The majority of operations have been conducted in the elution mode.

The use of displacement development in preparative-scale chromatography has been carried out by a number of researchers in recent years for the purification of peptides [1–4], amino acids [2,5–10] and antibiotics [3,11,12]. These applications have involved the use of both reversed-phase (C_8 and C_{18}) and ion-exchange high-performance liquid chromatographic (HPLC) columns. Good resolutions have been obtained with loadings higher than those applied on analytical scale. The systems consisted mainly of binary and ternary mixtures. The small particle sizes, in the range of 5–10 μm, presumably played an important role in the separations.

Protein purifications by displacement chromatography have also been tried and the capacity and power of such a process were demonstrated through the separation of A and B forms of β-lactoglobulin by Torres *et al.* [13] and later by Liao *et al.* [14] using two different types of displacers. Preparative amounts of these two protein forms, differing in p*I* by only 0.1 unit, were separated on HPLC anion exchangers.

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However, the application of displacement chromatography to protein purification from complex mixtures has been limited, although the potential has been shown by the work done on alkaline phosphatase purification from *Escherichia coli* periplasmic space protein [15], GC-2 globulin from human serum [16] and monoclonal antibody purification by complex displacement chromatography [17].

Although the above approaches have yielded promising results, the general practice has been to use relatively simple protein mixtures on small-scale HPLC columns operating at relatively low flow-rates. Through the work outlined in this paper, it was attempted to show the possibility of running displacement chromatography using a conventional low-pressure column with a complex protein mixture and comparing the performance *vis-à-vis* conventional elution. Purification of lactate dehydrogenase (LDH) from beef heart proteins was attempted. The problems encountered with such complex systems have been addressed by Peterson [18] using serum proteins and the importance of these non-ideal considerations were apparent in this work.

EXPERIMENTAL

The displacer carboxymethylstarch (CMS) was obtained as a gift from Reppe Glykos (Räppe, Sweden) and was used as a 20 g/l solution in 0.01 *M* Tris–HCl (pH 8.5). β-NADH and sodium pyruvate used in the LDH activity assay were purchased from Sigma (St. Louis, MO, USA). The column gel Tris Acryl DEAE M was supplied by IBF France.

Preparation of beef heart homogenate

The homogenate was prepared by first blending in a kitchen mixer 100 g of beef heart with 100 g of ice and 100 ml of 0.05 *M* sodiumphosphate buffer containing 1 m*M* β-mercaptoethanol (pH 7.0). The homogenized mass was mixed well with an additional 50 ml of the buffer and then centrifuged at 20 000 *g* for 10 min. The supernatant was filtered over glass-wool to remove any fat, then ammonium sulphate was added to 30% saturation. This was followed by a second centrifugation at 20 000 *g* for 5 min, the supernatant from which was made up to 65% saturation. A final centrifugation was then carried out at 20 000 *g* for 10 min. The pellet obtained from the last step was dissolved in water and dialysed against the column equilibrating buffer to obtain the homogenate in its ready to use form.

Column chromatography

Tris Acryl DEAE M, a weak anion exchanger, was used as the column gel material. Prior to packing, it was washed several times with the column equilibrating buffer (0.01 *M* Tris–HCl, pH 8.5) and degassed gently with constant stirring. The gel was slurry packed into a Bio-Rad Labs. column under a hydrostatic pressure head of about 85–100 cm. Solutions were pumped through an LKB 2132 Microperpex peristaltic pump via a manually operated valve system. UV detection at 280 nm was carried out with an LKB 2138 Uvicord-S detector and the column effluent was sampled using an LKB 2112 Redirac fraction collector. The detector output was recorded on an LKB 2210 single-channel chart recorder.

LDH activity measurement

Lactate dehydrogenase (E.C. 1.1.1.27) activity was measured as proportional to the initial rate of decrease of absorbance at 340 nm of NADH due to its oxidation to NAD^+ in the reaction converting pyruvate to lactate. A 10-μl volume of the sample was added to a reaction mixture containing 2.8 ml of 0.01 *M* Tris–HCl (pH 7.3), 0.1 ml of 6.6 m*M* NADH in buffer and 0.1 ml of 30 m*M* sodium pyruvate in buffer. Absorbance changes were measured with a Shimadzu UV-260 spectrophotometer.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)

Some of the output fractions were subjected to SDS-PAGE using a Bio-Rad Labs. mini gel slab. A 3% stacking and 12.5% running gel were used. The best running times were found to be 30 min at 50 V followed by 45 min at 200 V. The gels were fixed in 40% methanol–10% acetic acid and stained using a 0.1% Kenacid Blue R solution in the fixative base. The gels were finally destained in 40% methanol–10% acetic acid solution. The gels were then soaked in 25% acetic acid containing 4% of glycerol to prevent them from cracking on drying.

RESULTS AND DISCUSSION

The work done on the displacement chromatography of peptide mixtures [1–4] and simple protein samples [3,13,14,19–21] using HPLC columns has yielded very positive results regarding the preparative-scale capacity of this operating mode. Its application to systems of a more complex nature has been limited, although the work done so far [15–17] has demonstrated its feasibility. We chose a system consisting of proteins from beef heart and investigated the separation and recovery of LDH by displacement from an anion exchanger.

An important aspect of the work was the displacer itself. The choice of carboxymethylstarch (CMS) was based on the use of similar substituted polymers [carboxymethyldextrans (CMDs) and carboxymethylcellulose] by other workers [13,15–17]. From titration analysis, its pK_a value was observed to be *ca.* 5.8 and it had a hydroxyl group equivalent of $4.7 \cdot 10^{-4}$ mequiv. NaOH/mg polymer. Carboxymethyl-substituted dextrans synthesized using the method of Peterson [18] were observed to have values higher than this, even the relatively weak affinity ones. However, the displacement worked well with the CMS, indicating that the dextran-based displacers would have been over-effective. The use of CMDs has been extensively studied by other workers [13,15–17]. The CMDs have different degrees of substitution and have been used both as spacers and as a final displacer.

Effect of column length

A displacement run was made on a 9.1 × 1.0 cm I.D. column (run I). The profile for the absorbance at 280 nm and LDH activity at the column outlet are shown in Figure 1. LDH activity higher than 1.0 ($\Delta A/\Delta t$) appears over one column volume of eluent, which can be considered reasonable given the size of the column and the load. However, the resolution was poor in that no recognizable plateaux typical of displacement chromatography were observed. In order to improve upon this, a run was carried out under identical conditions except that a 25.0 × 1.0 cm I.D. column was used (run II). The corresponding absorbance and activity profiles are

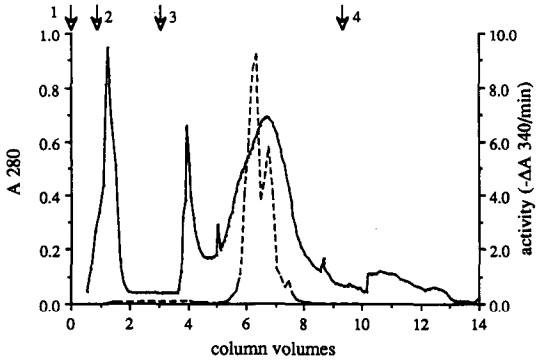

Fig. 1. Column outlet profile for displacement run on a 9.0 × 1.0 cm I.D. column of Tris Acryl DEAE M. The equilibrating buffer was 0.01 *M* Tris–HCl (pH 8.5) and all solutions were prepared using this. Displacer, 20 g/l CMS; protein load, 6 ml of homogenate. Arrows: (1) start protein load; (2) stop protein load, start buffer wash; (3) stop buffer wash, start displacer flow; (4) stop displacer flow, start column wash. Solid line, absorbance at 280 nm; dashed line, activity.

shown in Fig. 2. The one large peak in the previous run containing the LDH is resolved into four smaller peaks with the LDH being maintained within about one column volume of the eluent.

The LDH activity profile undergoes some kind of transition between the two column runs and also shows a drop in the maximum observed value. It is difficult to give any conclusive reason for the differences. It is plausible that some type of interactions between the two predominant isoenzyme forms in the heart, H_4 and H_3M, could be of importance.

The purities of the fractions can be compared from the SDS-PAGE of the active fractions from runs I and II. These are shown in Figs. 3 and 4, respectively, and a marked improvement can be observed. A high-molecular-weight contaminant from the smaller column is removed to a large extent in the second run. LDH forms the major component.

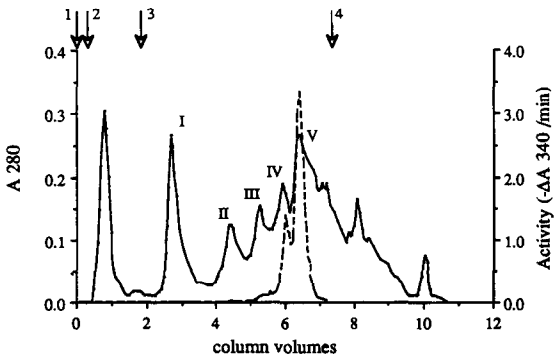

Fig. 2. Column outlet profile for displacement run on a 25.0 × 1.0 cm I.D. column of Tris Acryl DEAE M. Conditions, arrows and lines as in Fig. 1.

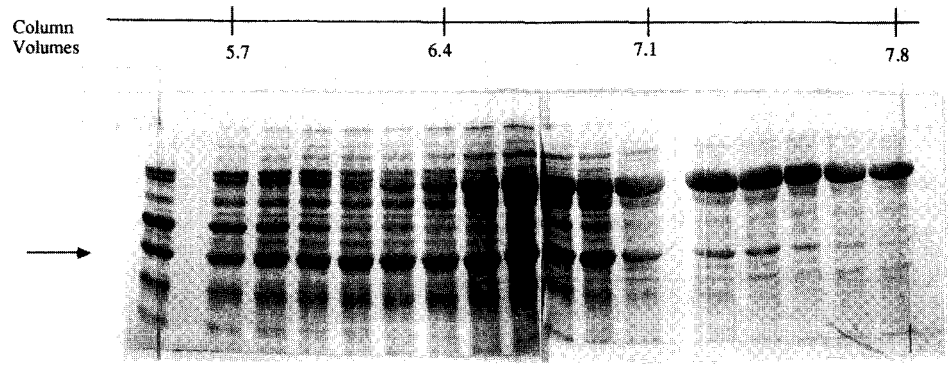

Fig. 3. SDS-PAGE of some of the LDH-active fractions between column volumes 5.5 and 8.0 from the displacement run in Fig. 1. Position of LDH isoenzyme H4 is depicted by the arrow.

The absorbance profile in Fig. 2 under displacement (between arrows 3 and 4) shows the formation of peaks of increasing height (peaks II–V), although there are no sharp step profiles as dictated by the theory [22,23]. This non-ideality can be attributed mainly to the relatively larger particle sizes (40–80 μm) of the column gel and to non-homogeneity in size. This compares poorly with HPLC-based material used by other workers, which yielded better resolutions. However, in spite of the drawbacks, the system presented here has great operational simplicity and is capable of reasonable fractionation of a relatively large protein load.

Effect of displacer solution pH

An important point addressed was that of the pH of the displacer solution. The column equilibrating buffer used in the runs was 0.01 *M* Tris–HCl (pH 8.5). A 20 g/l solution of the displacer in this buffer decreased the pH to 3.8. Under these conditions any number of ionic effects could have an influence and the observed behaviour results from a combination of displacement and pH changes as in conventional elu-

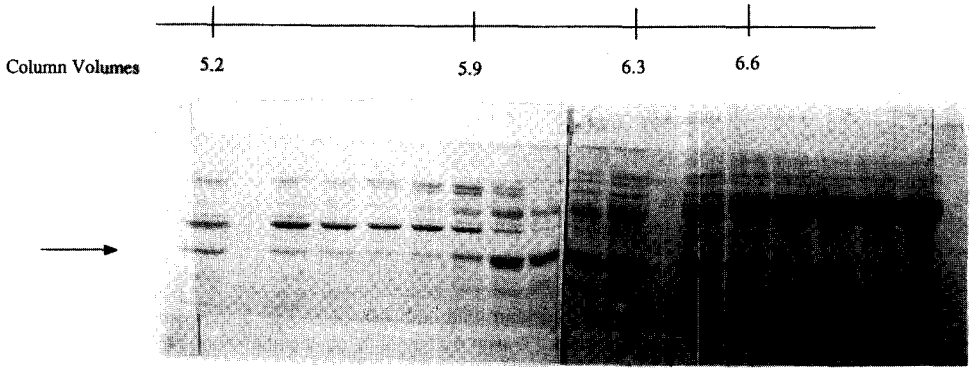

Fig. 4. SDS-PAGE of some of the LDH-active fractions between column volumes 5.5 and 7.0 from the displacement run in Fig. 2. Position of LDH isoenzyme H4 is depicted by the arrow.

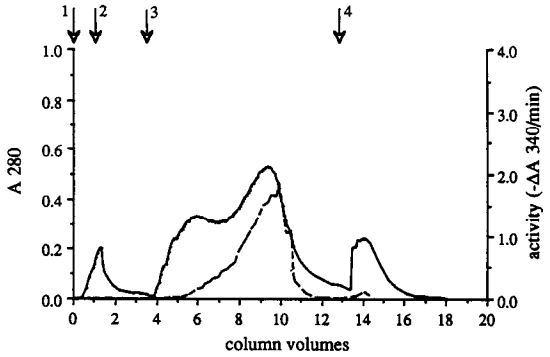

Fig. 5. Column outlet profile for a displacement run on an 8.0 × 1.0 cm I.D. column of Tris Acryl DEAE M under conditions in which the pH of the displacer solution in the buffer base was not adjusted. Conditions, arrows and lines as in Fig. 1, except displacer solution, 20 g/l in buffer.

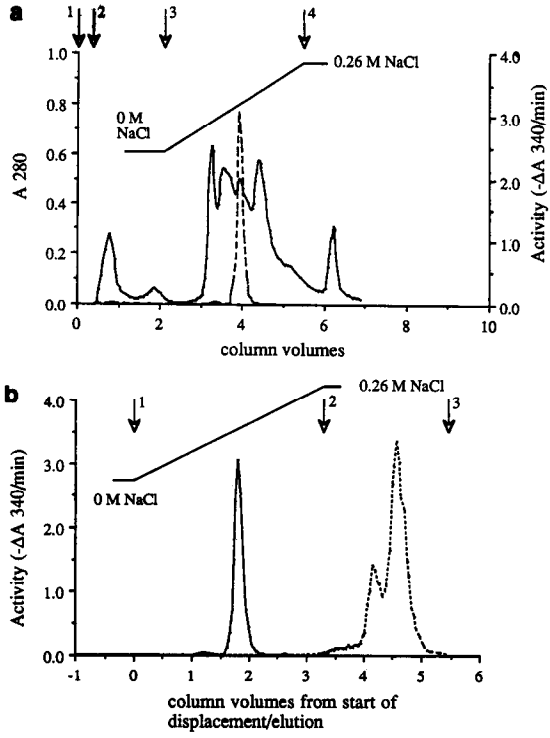

Fig. 6. (a) Column outlet profile for ion-exchange run with salt elution on a 24.0 × 1.0 cm I.D. column of Tris Acryl DEAE M. Equilibrating buffer, protein load and lines as in Fig. 1. Arrows: (1) start protein load; (2) stop protein load, start buffer wash; (3) stop buffer wash, start salt elution; (4) stop salt elution, start column wash. (b) Comparison of the appearance of the LDH-active peaks from the start of displacement/elution. Arrows: (1) start of displacement/elution; (2) end of gradient elution; (3) end of displacement.

tion. Hence, adjustment of the pH was always carried out prior to the runs. However, a displacement run was conducted on an 8.0 × 1.0 I.D. column without pH adjustment and the absorbance and activity profiles are shown in Fig. 5. It can be said that at a lower pH the CMS would possess less negative charge but its effectiveness as a displacer need not necessarily be affected drastically, as the solute system would also undergo some kind of a charge distribution, correspondingly reducing its affinity for the column. The overall profile and activity profiles are, however, much poorer, suggesting the importance and need for proper pH control.

Comparison with conventional ion exchange

A comparative evaluation of the performance under displacement was made with a conventional elution run on a 24 × 1.0 cm I.D. column (Fig. 6a). Elution was carried out using a salt gradient from 0 to 0.5 *M* sodium chloride in buffer in 120 min. The gradient was operated for 62 min. The activity profile obtained, shown in Fig. 6b from the start of elution, was much sharper than that from the displacement run and appears over a smaller eluent volume. However, analysis of the active fractions by SDS-PAGE (Fig. 7) indicates a much lower degree of purification. This is also evident from the specific activity plots in Fig. 8. The separation could be improved through the use of a shallower gradient, but then a higher dilution effect would result.

The recovery of LDH from beef heart proteins can be compared in terms of complexity of the protein system with the purification of alkaline phosphatase from periplasmic space protein [15], Gc-2 globulin from human serum [16] and monoclonal antibody purification by complex displacement chromatography [17]. CMDs were used as spacers and the final displacers in the first two separations. The protein loads were comparatively large although two displacement steps were incorporated for recovery of the target proteins. HPLC was used in the separations. In the mAb purification, a slightly different methodology was used. The displacer (carboxymethylcellulose) bound to the protein of interest while actually displacing the impurities. Final removal was carried out with saline solution.

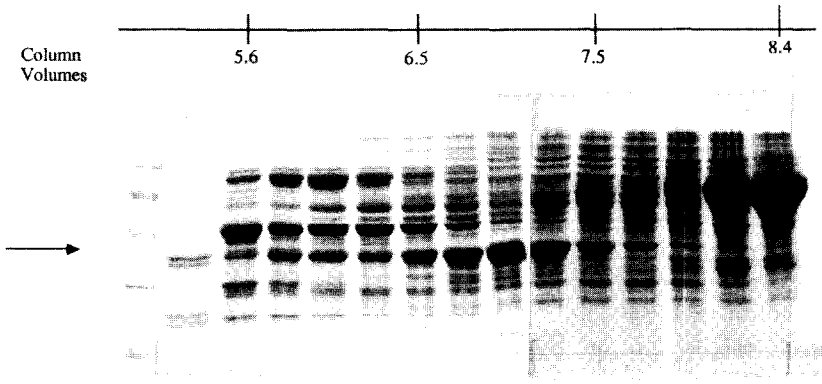

Fig. 7. SDS-PAGE of some of the LDH-active fractions between column volumes 3.5 and 4.5 from the elution run in Fig. 6a. Position of LDH isoenzyme H4 is depicted by the arrow.

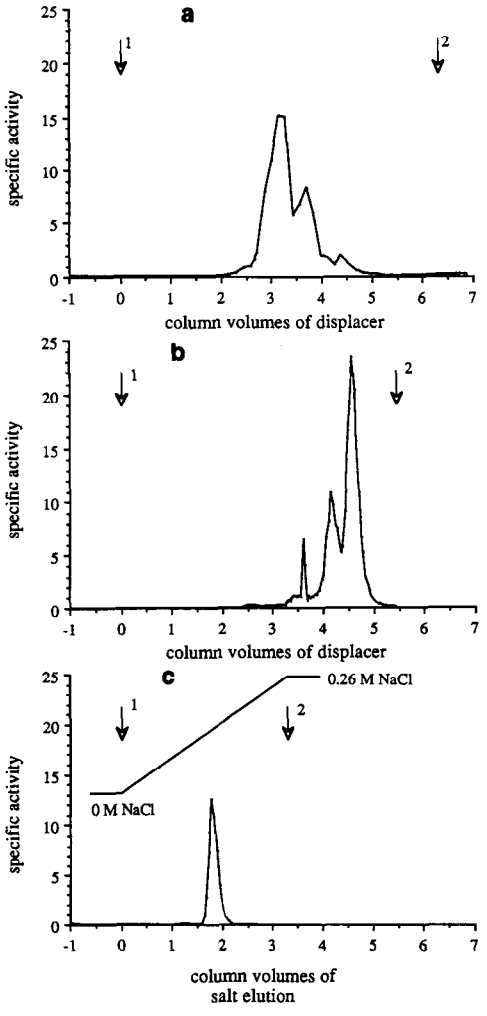

Fig. 8. Specific activity profiles for the following cases: (a) displacement on a 9.0 × 1.0 cm I.D. column; (b) displacement on a 25.0 × 1.0 cm I.D. column; (c) salt elution on a 24.0 × 1.0 cm I.D. column. Arrows 1 and 2 depict the part of the chromatogram where displacement/salt elution was carried out.

CONCLUSIONS

Adsorption chromatography is a widely used and powerful tool for the separation and recovery of proteins and other biomolecules. The mode of recovery has been mostly elution with salt and/or pH gradients in ion-exchange systems and changes in mobile phase composition for reversed-phase separations. Operation in the displacement mode has shown considerable advantages for preparative-scale purification. When applied to complex protein mixtures as presented in this work, there are distinct advantages over conventional ion exchange in terms of purity of product

and overall yields. It is not justified at this stage to generalize for all systems, especially where non-idealities and more fundamental molecular interactions could play an important role, as in most biological systems. The results do, however, agree well with the expectations already established.

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