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Note

Manipulation of the mobile phase to achieve multiple step protein (calmodulin) purification using the same chromatographic material (a weak anion exchanger)

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The ability to change chromatographic selectivity by changing the mobile phase is a powerful approach in achieving the desired resolution in a given chromatographic separation. Indeed the application of this approach finds wide use in the chromatography of relatively small molecules[1,2]. Classically, however, in protein purification selectivity is manipulated by changing the separation mechanism at the chromatographic surface in order to probe differential affinities between the protein of interest and contaminants. This is carried out by changing the mode of chromatography, *e.g.* from ion-exchange to hydrophobic interaction or from size-exclusion to ion-exchange etc. Hence by cascading several chromatography runs each of which uses a different separation mechanism a highly purified protein can be isolated from a complex biological matrix.

With the recent introduction of new rigid wide-pore chromatography media appropriate for use on high-performance liquid chromatography (HPLC) instrumentation very rapid chromatographic separations of proteins has become feasible[3]. This capability of high chromatographic speed opens avenues of investigation for achieving separation of proteins which were previously too time consuming and therefore too impractical to pursue with conventional chromatography materials. One such avenue which in the past has received insufficient attention is the modulation of protein chromatographic surface interactions by using different buffer components and pH[4–7]. Manipulation of the chromatographic selectivity in this manner offers the possibility of using the same chromatography media in more than one step to achieve the purification of a given protein[8]. In this paper we have utilized this approach to achieve the rapid purification of calmodulin (CaM, a calcium binding protein) from crude brain extract in two chromatographic steps using the same weak anion exchanger.

EXPERIMENTAL

Material

Bovine brain acetone powder extract and serum albumin was obtained from Sigma (St. Louis, MO, U.S.A.). Biochemical grade Tris, sodium dodecyl sulfate (SDS), potassium phosphate, ammonium sulfate and HPLC grade sodium acetate were obtained from J. T. Baker (Phillipsburg, NJ, U.S.A.).

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Crude brain supernatant

Crude brain supernatants in 25 m*M* Tris, pH 7.0, were prepared as previously reported[9]. Similar supernatants in 25 m*M* Tris containing 0.1 *M* ammonium sulfate were also prepared. All samples were centrifuged in a Sorvall type SS-34 rotor at 17000 rpm for 30 min at 3°C prior to conducting chromatography.

Chromatography

Analytical 50 × 4.6 mm and preparative 250 × 10 mm high-performance weak anion-exchange (HPWAX) columns containing either 5- or 15-μm Bakerbond wide-pore (300 Å) polyethyleneimine (PEI) silica were obtained from J. T. Baker. All chromatography was conducted on a Beckman chromatography system previously described[9] at room temperature using 280 nm wavelength light to monitor column effluent.

Electrophoresis and protein concentration

SDS-polyacrylamide gel electrophoresis (PAGE) was carried out on 12% Laemmli[10] slab gels and stained with Coomassie Brilliant Blue 250R. Total protein concentration of samples were determined using the Bio-Rad Labs. (Richmond, CA, U.S.A.) protein assay which is based on the method of Bradford[11] using bovine serum albumin as a protein standard.

RESULTS AND DISCUSSION

The ability to conduct rapid and highly resolving analytical chromatographic runs on proteins using short HPLC columns[12] makes it practical to investigate the effects of a wide range of experimental variables including the effects of different buffer conditions on a given protein chromatographic separation. This is illustrated in Fig. 1A–C. Here large differences are revealed in the resulting chromatography of crude brain extract on the same HPWAX column. In particular the retention time of CaM under these chromatographic conditions was changed as shown by the arrow in each chromatogram. The ability to alter the selectivity of this chromatography media for CaM relative to the other proteins present in the sample offers the possibility of using this exchanger in more than one step in the purification of this protein. Conditions indicated in Fig. 1C, which gave the best separation of CaM from the other components present in the crude brain extract, was further improved by increasing the initial ionic strength of the applied sample by adding ammonium sulfate to a concentration of 0.1 *M* (this was achieved by homogenizing the brain acetone powder extract with 25 m*M* Tris containing 0.1 *M* ammonium sulfate, pH 7.0). The addition of ammonium sulfate significantly reduces the binding capacity of this exchanger for contaminating material during sample loading (Fig. 1D), while still maintaining quantitative binding of CaM. Conditions used in Fig. 1D were scaled up on a preparative anion-exchange column packed with 15-μm silica having the same surface chemistry (PEI) as present on the 5-μm media. The results from such a preparative run are shown in Fig. 2. Chromatographic characterization of the pooled CaM fractions on a short (5-μm) HPWAX column using conditions indicated in Fig. 1A revealed the non-homogeneity of the isolated material (Fig. 3), and demonstrates the ability of a different buffer system to further purify CaM. Optimization of the chro-

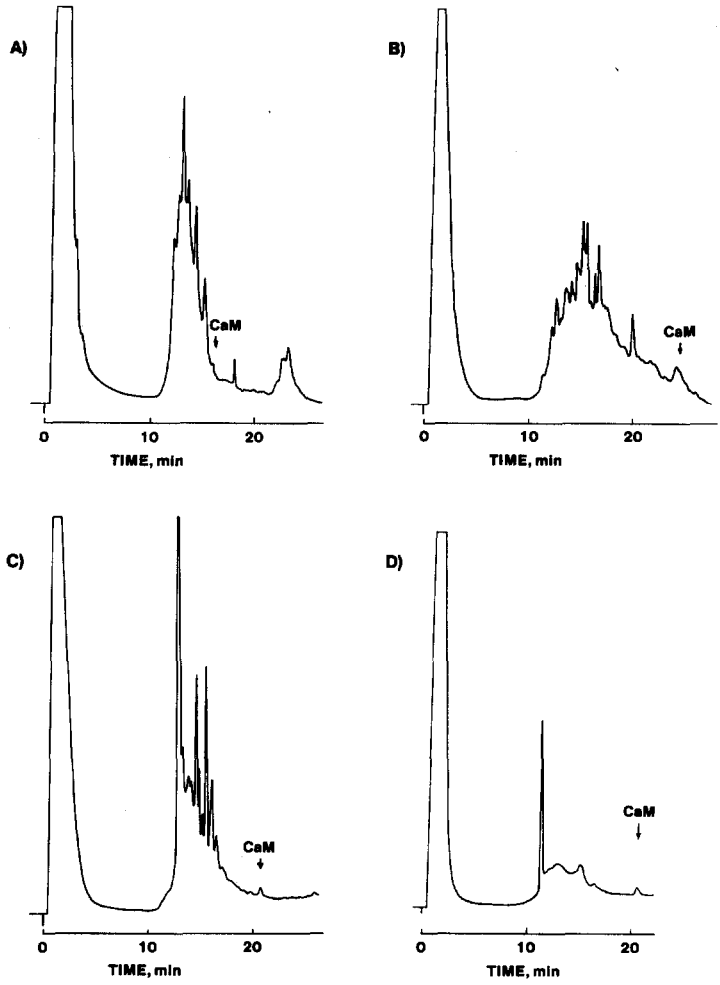

Fig. 1. Changes in the chromatography of crude brain extracts on a weak anion exchanger resulting from the use of different buffer components and pH. The following are the mobile phases used during gradient elution: (A) buffer A = 25 m*M* potassium phosphate, pH 7.0 and buffer B = 25 m*M* potassium phosphate, 1.0 *M* ammonium sulfate, pH 7.0; (B) buffer A = 25 m*M* Tris, pH 7.0 and buffer B = 2.0 *M* sodium acetate, pH 7.0; (C) buffer A = 25 m*M* Tris, pH 7.0 and buffer B = 2.0 *M* sodium acetate, pH 5.0; (D) same as C but the ionic strength of the sample loaded on the column was increased by the addition of ammonium sulfate to a concentration of 0.1 *M*. The following experimental conditions were used for all chromatography generated in this figure; column: 50 × 4.6 mm packed with 5-μm Bakerbond PEI; gradient conditions: 0–100% B in 15 min; flow-rate: 1.0 ml/min; sample volume injected: 1.0 ml (protein concentration 8.0 mg/ml).

matography in phosphate buffer allowed the pooled CaM fractions from the preparative run in Fig. 2 to be further purified (Fig. 4), to give highly purified CaM. The purity of CaM obtained from the second PEI run is indicated by the presence of a single band on SDS-PAGE analysis shown in Fig. 4.

The ability to alter the binding selectivity of an ion exchanger for proteins by

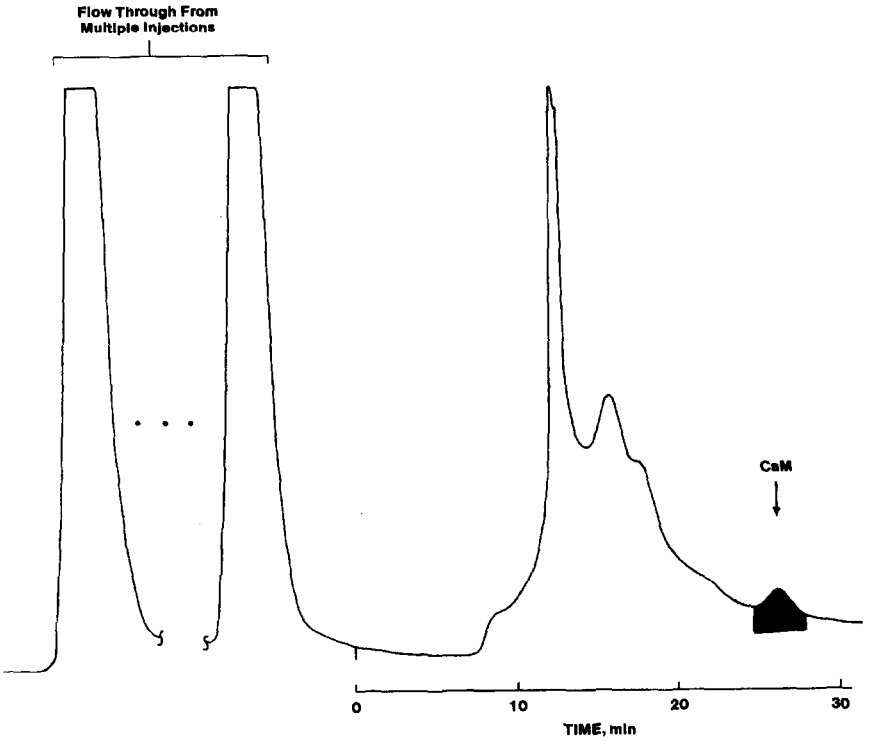

Fig. 2. Preparative fractionation of crude brain extract on a weak anion exchanger (first step in purification of CaM). Experimental conditions used; column: 250 × 10 mm packed with 15-μm Bakerbond PEI; mobile phase: buffer A = 25 m*M* Tris, pH 7.0 and buffer B = 2.0 *M* sodium acetate, pH 5.0; gradient condition: 0–100% B in 30 min; flow-rate: 4.0 ml/min; sample volume injected: 39 ml (protein concentration 8.0 mg/ml).

manipulating the buffer components and pH of the mobile phase has been investigated by others[4–8], but is rarely exploited effectively. One of the main reasons for this is the time consuming aspects associated with conducting investigative work on conventional (soft gel) liquid chromatography media. In this paper the significance of this capability is demonstrated by achieving the rapid purification of CaM, an intensely studied protein of enormous importance in molecular biology[13,14], from crude brain extract using only a weak anion exchanger. Although numerous purification procedures exist for this protein[14–16] they are either time consuming, due to the number of steps involved and the use of conventional chromatography, or involve the use of highly specialized affinity chromatography media. The present procedure is extremely fast and simple requiring non prior sample purification such as isoelectric or ammonium sulfate precipitation step, and employs only one general purpose chromatographic medium (a weak anion exchanger).

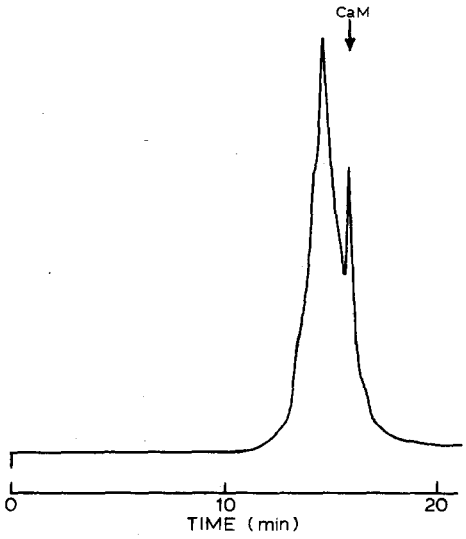

Fig. 3. Chromatographic characterization of the pooled CaM fraction obtained from the preparative run shown in Fig. 2. Experimental conditions used; column: 50 × 4.6 mm packed with 5-μm Bakerbond PEI; mobile phase: buffer A = 25 mM potassium phosphate, pH 7.0 and buffer B = 25 mM potassium phosphate, 1.0 M ammonium sulfate, pH 7.0, gradient conditions: 0–100% B in 30 min; flow-rate: 1.0 ml/min; sample volume injected: 3.0 ml of pooled CaM fractions + 2.5 ml of 1 M sodium hydroxide (used to adjust pH to 7.0) + 15 ml of buffer A.

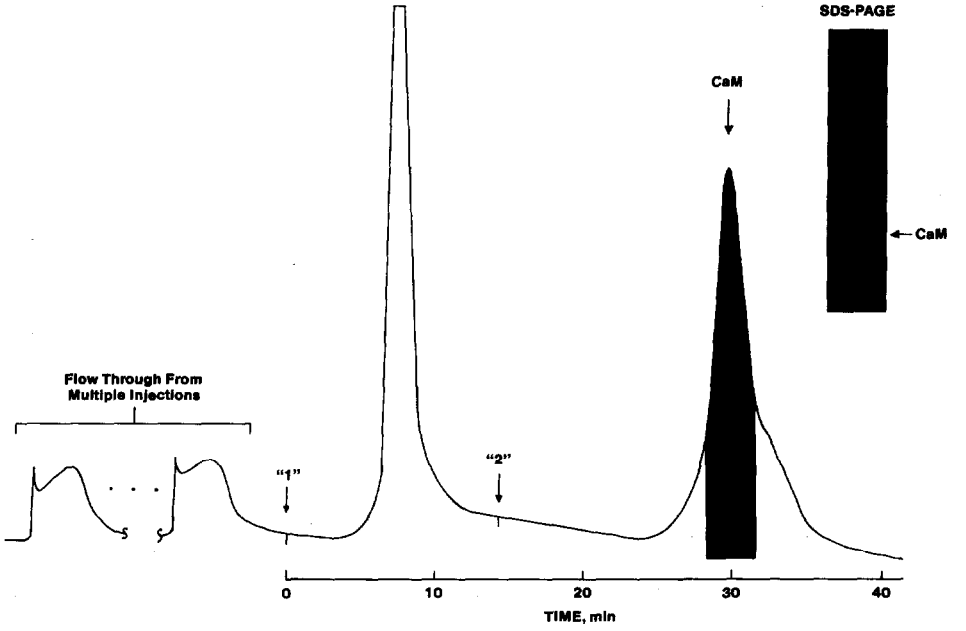

Fig. 4. Further purification of the pooled CaM peak (obtained from the preparative run shown in Fig. 2) using the same weak anion exchanger as indicated in Fig. 2, but a different mobile phase (which is the same as that used in Fig. 3, but further optimized in terms of starting solvent strength and gradient conditions to improve resolution). Experimental conditions used; column: 50 × 4.6 mm packed with 15-μm Bakerbond PEI; mobile phase: A = 25 mM potassium phosphate pH 7.0 and buffer B = 25 mM potassium phosphate, 1.0 M ammonium sulfate pH 7.0; gradient conditions: at "1" mobile phase was changed from 0 to 10% B, at "2" a gradient from 10 to 40% B in 15 min was started; sample volume injected: 10 ml of pooled CaM fractions + 8.5 ml of 1 M sodium hydroxide (used to adjust pH to 7.0) + 50 ml of buffer A.

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