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RAPID ION-EXCHANGE CHROMATOGRAPHY FOR PREPARATIVE SEPARATION OF PROTEINS

APPLICATION TO PORCINE AND BOVINE CARBONIC ANHYDRASES

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SUMMARY

A method of rapid ion-exchange chromatography of DEAE-cellulose for preparative purposes is described. Basically, the flow-rate is increased by applying an air pressure on the column. By this technique it is possible to purify gram quantities of protein in 2–4 h with acceptable resolution. In preparations of bovine and porcine carbonic anhydrases the elution times were reduced by a factor of about ten compared to those of conventional methods. The enzymes purified in this way showed a high degree of homogeneity. The method should be generally applicable in protein purification, and especially advantageous in purification of unstable proteins where time-consuming separations often give rise to low yields of active material.

INTRODUCTION

Ion-exchange chromatography is often employed for separation of proteins and other biomolecules. Excellent resolutions can be obtained in short times with the ion-exchange materials available for high-performance liquid chromatography. For preparative purposes, however, these columns are extremely expensive. Traditionally very time-consuming column chromatographic methods have been used in large-scale preparative purifications. Although the results obtained with the newly developed ion-exchange resins are often good, the long separation times are an obvious disadvantage. This is especially the case for unstable proteins and in large-scale applications. We have recently developed a considerably faster technique for the routine purification of various carbonic anhydrases. The equipment is very simple and easy to set up and allows separation of gram quantities of protein in 2–4 h with acceptable resolution.

Basically, air pressure is applied on a DEAE-cellulose column leading to an increased flow-rate. The technique is similar to the "flash chromatography" method, described by Still *et al.*[1] for separation of low-molecular-weight organic compounds on silica gel. The system has been optimized for the purification of porcine erythrocyte carbonic anhydrases, and has also been used for preparation of the corresponding bovine enzyme.

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EXPERIMENTAL

Ion-exchange resins

DEAE-cellulose DE-23 and DE-32 (Whatman), DEAE-Sephacel and DEAE-Sepharose CL 6B (Pharmacia) were used.

Purification of porcine carbonic anhydrases I and II

Citrated porcine blood was obtained from a slaughterhouse. Crude carbonic anhydrase was prepared using a modification of Method II of Keilin and Mann[2]. The erythrocytes were separated from plasma and leukocytes by centrifugation (2500 *g*, 20 min). The cells were then washed twice with an equal volume of cold isotonic sodium chloride solution (0.9%). Haemolysis was achieved by addition of half the volume of distilled water to the packed erythrocytes. Haemoglobin was removed in the cold room by selective denaturation with a chloroform–ethanol–water (33:27:40) mixture. To 1 l of haemolysate, 1.2 l of the mixture were added with vigorous stirring for 30 min. The haemoglobin precipitate was removed by centrifugation (1500 *g*, 20 min) and the slightly yellow supernatant solution containing the enzyme was filtered through glass wool to remove particles. The enzyme extract was dialyzed against distilled water. The subsequent purification conditions were similar to those described by Ashworth *et al.*[3].

Enzyme solution (400 ml) containing *ca.* 400 mg of protein was chromatographed on various DEAE ion-exchange resins equilibrated with 0.01 *M* Tris–HCl buffer, pH 8.7 (for description of the equipment, see below). The pH of the enzyme extract was adjusted to 8.7 by addition of 1 *M* Tris solution. The sample was applied on the column under pressure with the same flow-rate as during the chromatographic separation. Elution was achieved by a linear gradient in sodium chloride formed by 2 l of 0.01 *M* Tris–HCl + 0.2 *M* sodium chloride, pH 8.7 and 2 l of 0.01 *M* Tris–HCl + 0.6 *M* sodium chloride, pH 8.7.

Purification of bovine carbonic anhydrase II

The initial purification and the chromatographic conditions except for the flow-rate followed the method of Lindskog[4].

Rapid ion-exchange chromatography

Fig. 1 illustrates the equipment for rapid ion-exchange chromatography with gradient elution. A glass column (40 × 3.5 cm) was packed with ion-exchange resin (DEAE-cellulose, Whatman DE-23 for optimal results) to a height of 30 cm. Packing was performed under pressure in order to avoid compression of the resin during chromatography. This was accomplished by connecting the column directly to an air cylinder and applying a pressure corresponding approximately to the flow-rate that was later to be used in the chromatography (< 0.4 kp/cm^2*). Once the column had been packed, the resin was protected from disturbance by covering the top surface with a disc of glass wool.

In order to obtain a linear gradient elution, two 2.5-l plastic wide-mouth bottles with screw caps were connected to an air cylinder and, by means of a siphon, to each other. All connections were made with poly(vinyl chloride) (PVC) tubing (10 mm O.D. × 7 mm I.D.). Mixing in the second bottle was accomplished by magnetic stirring.

* kp/cm^2 = kilopond/cm^2 = kg/cm^2 = 98.07 kPa.

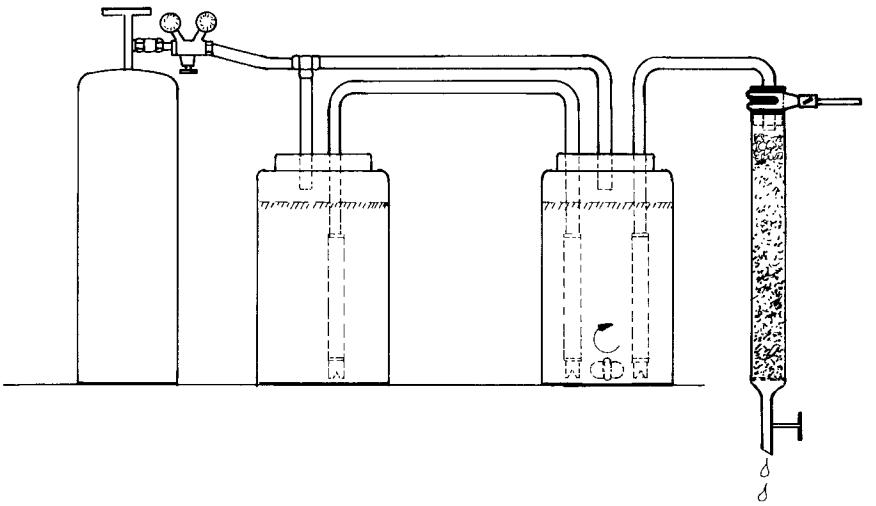

Fig. 1. Rapid ion-exchange chromatographic equipment for linear gradient elution. For details, see Experimental.

In the event of gradient elution being unnecessary, one of the plastic bottles, and thereby also the siphon, may be omitted. To avoid leakage, the 10-mm PVC tubing was pressed through 9-mm holes that had been drilled in the screw caps. Glass tubes were inserted into the PVC tubing ending in the buffer solutions in the bottles. To avoid flow-stop, short pieces of PVC tubing with V-shaped cuts were attached to the ends of the glass tubes. The siphon was easily filled by first closing the air line to the mixing bottle with a Hoffman clamp, and then applying a low pressure to the first bottle. When the siphon had been filled with buffer, the clamp was removed, and the tubing from the buffer solution in the mixing bottle was connected to the column by way of a glass tube inserted in a one-hole rubber stopper. A clamp was used to keep the stopper in place when the pressure was increased. The flow-rate was controlled by a pressure regulator; the needle valve was completely open. The eluent was gathered in a fraction collector at a rate of one fraction per minute.

Activity measurements

The carbon dioxide hydration activity was determined by the colorimetric method of Rickli *et al.*[5].

Protein concentration determinations

Protein concentrations were estimated spectrophotometrically using an $A^{1\%}_{280\ nm}$ of 18.5, 15.2 and 19.0 cm^{-1} for porcine carbonic anhydrase I (ref. 6), II (ref. 6) and bovine carbonic anhydrase II (ref. 7), respectively.

Polyacrylamide gel electrophoresis

This was carried out as described by Smith[8]. Gel concentrations of 7.5% were used and 0.095 *M* Tris–glycine buffer, pH 9.5 was employed in both gel and electrode reservoirs. Gels were stained with amido black in 10% trichloroacetic acid and destained in 10% acetic acid.

RESULTS

The flow properties of various ion-exchange resins were investigated as a function of applied pressure. The results are shown in Fig. 2. In this comparison DEAE-cellulose DE-23 was superior to the other materials and allowed flow-rates of *ca.* 30 ml/min (190 ml/h · cm²). If higher pressures than 1 kp/cm² were applied to the column, leakages occurred which set a limit to the flow-rate with our equipment.

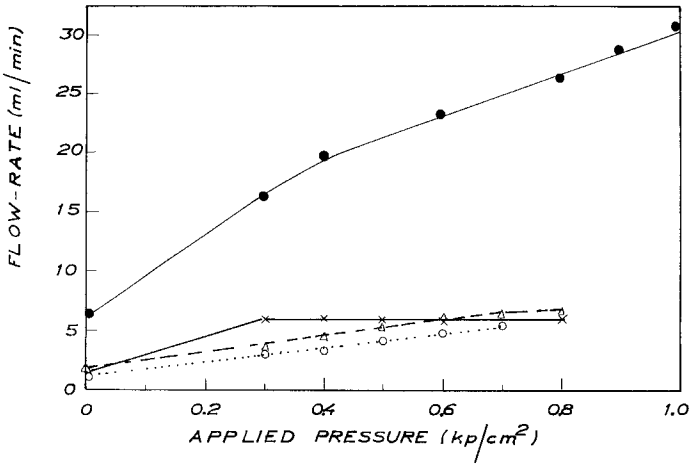

Fig. 2. The dependence of flow-rate on applied pressure for various ion-exchange resins. Gel bed dimensions: 30 × 3.5 cm. For further description of the equipment, see Experimental and Fig. 1. Resins: DEAE-cellulose DE-23 (●—●); DEAE-Sepharose (× — ×); DEAE-Sephacel (△---△); DEAE-cellulose DE-32 (○···○).

The crude carbonic anhydrase extract was then chromatographed with varying flow-rates on the DEAE-cellulose DE-23 column. Two chromatograms (Fig. 3A and B) are shown for the flow-rates 5 ml/min (31 ml/h · cm²) and 18.5 ml/min (115 ml/h · cm²), respectively. The yield of enzyme activity was 85% in the chromatography step at a flow-rate of 18.5 ml/min. The specific CO_2 hydration activities of the isoenzymes purified by the rapid ion-exchange chromatography method (Fig. 3B) were 7300 and 35,000 units/mg for porcine carbonic anhydrases I and II, respectively. The corresponding values from the slower chromatography (Fig. 3A) were 8100 and 40,000 units/mg. Similar specific activities were also obtained from preparations of the isoenzymes by chromatography on the high resolution resins DEAE-Sephacel, DEAE-Sepharose CL 6B and DEAE-cellulose DE-32 with a flow-rate of 5 or 1 ml/min. Average values were 7600 (enzyme I) and 33,000 units/mg (enzyme II).

The resolution in a chromatogram can be estimated in a number of ways. Since it is proportional to the square root of the number of theoretical plates, $\sqrt{N}$, we have chosen to analyze the $\sqrt{N}$ dependence on the flow-rate in our chromatographic series of the porcine carbonic anhydrases.

$\sqrt{N}$ was calculated according to $\sqrt{N} = 2.355 V_R / W_h$, where V_R is the retention

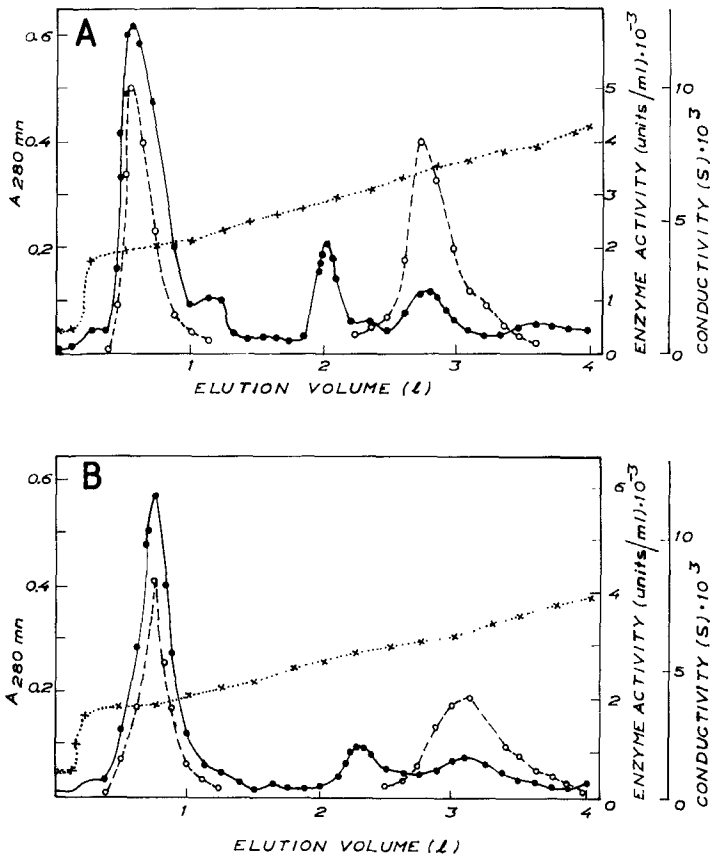

Fig. 3. Chromatography of crude porcine carbonic anhydrases on a DEAE-cellulose DE-23 column at different flow-rates. Gel bed dimensions: 30 × 3.5 cm. For further description of the equipment, see Experimental and Fig. 1. (A) Flow-rate: 5 ml/min (31 ml/h · cm²). No pressure applied. Fraction volumes: 25 ml. Temperature: 20°C. (B) Flow-rate: 18.5 ml/min (115 ml/h · cm²). Applied pressure: 0.3 kp/cm². Fraction volumes: 25 ml. Temperature: 20°C. ●—●, $A_{280\ nm}$; ○---○, carbon dioxide hydration activity; ×...×, conductivity.

volume and W_h is the peak width at half the peak height (in volume units). In Fig. 4 this dependence is illustrated for the porcine carbonic anhydrase II peak (the high-activity form), since it is the last significant peak in the chromatogram and most difficult to resolve. The contamination peak preceding this carbonic anhydrase peak could not be used in the resolution calculations, since the amount of the contaminants varied between the preparations.

It is also possible to load more material on the DEAE-cellulose DE-23 column without losing too much of its resolving power. In one experiment we applied 1.2 g of crude porcine enzyme solution and still obtained an acceptable separation of the isoenzymes at a flow-rate of 18 ml/min.

In a separate preparation of bovine carbonic anhydrase II, 3.2 l of crude enzyme extract were applied on the DEAE-cellulose column at a rate of 30 ml/min and

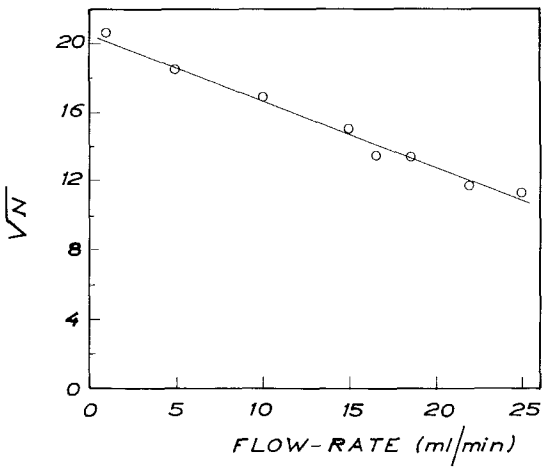

Fig. 4. Column efficiency in the separation of crude porcine carbonic anhydrases on DEAE-cellulose DE-23 as a function of flow-rate. $\sqrt{N}$, which is proportional to the resolution, is plotted *versus* the flow-rate, where N is the number of theoretical plates. The calculations are based on the UV trace of the high-activity form of carbonic anhydrase, which corresponds to the last peak containing enzyme activity in Fig. 3A and B.

the material was subsequently eluted at a rate of 20 ml/min. The total time for application and elution of the enzyme was about 3 h. The yield was 520 mg and had a specific carbon dioxide hydration activity of 12,300 units/mg, identical within experimental error to the activity of a parallelly chromatographed sample at a flow-rate of 1 ml/min. Moreover, comparisons of the purity of the two preparations by polyacrylamide gel electrophoresis did not reveal any detectable differences.

DISCUSSION

By using a DEAE-cellulose DE-23 resin it is possible to separate protein molecules at a very high flow-rate by applying a pressure on the column (Fig. 3B). The good flow properties of this ion exchanger compared to the others tested (Fig. 2) are probably due to the more open fibrous structure of the matrix. The column efficiency is of course decreased as a function of the flow-rate (Fig. 4), but surprisingly good separations are achieved at a flow-rate of about 20 ml/min (125 ml/h · cm^2) with our equipment (Fig. 3).

The gain in time by this method is considerable. An elution time of 45 h was used by Ashworth *et al.*[3] compared to 4 h for the separation shown in Fig. 3B to purify an equivalent amount of porcine carbonic anhydrases I and II. The obtained specific activities of the porcine and bovine carbonic anhydrases prepared by rapid ion-exchange chromatography indicate a high degree of purity and homogeneity of the enzymes. This is further confirmed by the polyacrylamide gel electrophoresis analysis of the bovine enzyme.

The described method should be applicable when maximal resolution is not required, such as for initial fractionation of a large volume of crude protein extract.

However, in many cases, as for the carbonic anhydrases investigated, the separation capacity should be sufficiently high to obtain a homogeneous preparation of gram quantities in a single step. The decrease in elution time with this technique during purification of unstable proteins should be especially advantageous. By using nitrogen gas it is also possible to perform the chromatography under anaerobic conditions to prevent oxidation.

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