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PURIFICATION OF BEEF HEART CYTOCHROME C BY CHROMATOGRAPHY ON AN AMBERLITE XE-64, POLYACRYLAMIDE BIO-GEL P-60 AND BIO-GEL P-300 "TANDEM" COLUMN*

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SUMMARY

A new chromatographic procedure for purification of beef heart cytochrome C is described. The method involves an efficient purification step leading to a high degree of purity by chromatography on an Amberlite XE-64, Bio-Gel P-60, Bio-Gel P-300 "tandem" column. Re-chromatography under the same conditions or re-chromatography on a Bio-Gel P-300 column gave a cytochrome C preparation with a still higher degree of purification. The purified beef heart cytochrome obtained had ratios A_{550} red/A_{280} = 1.24–1.29; A_{550} red/A_{550} ox. = 3.69 and showed a single band on polyacrylamide gel electrophoresis and a single peak on chromatography on a Sephadex G-100 column.

INTRODUCTION

Reviews on the subject of cytochrome C have been published recently by MARGOLIASH AND SCHEYTER[1] and PALEUS[2]. Cytochrome C (Cyt. C) has been purified by various chromatographic procedures[3–9], by molecular sieve chromatography on Sephadex G-75[10,11] or Sephadex G-100[12], and recently by ion exchange chromatography on carboxymethylcellulose[13].

The present paper describes a procedure consisting in a final purification step by chromatography on a column constituted of three different "tandem" layers.

MATERIALS

Cation-exchange resin Amberlite XE-64 (purchased from Serva, Heidelberg) in its ammonium form, and Bio-Gel P-60 (50–100 mesh) and Bio-Gel P-300 (50–150 mesh) (Bio-Rad Laboratories, purchased from Calbiochem) were used. All chemicals used were of analytical grade.

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METHODS

Protein was determined according to Lowry's method modified by MILLER[14] with bovine serum albumin (5 × cryst.) as standard. Nitrogen was determined by micro Kjeldahl and iron was determined by the sulphosalicylic method[15]. Spectrophotometric assays were performed in phosphate buffer (pH 6.8) using a Unicam recording spectrophotometer (SP 800 model). Absorbancy was determined at 550 mμ, 535 mμ and 280 mμ (quartz cells of 1 cm light path). The ratio A_{550} red/A_{280} was followed during the purification procedure. The absorbancy at 550 mμ was measured after solid dithionite was added until complete reduction was obtained. The catalytic capacity of the purified preparation of Cyt. C was assayed in a cytochrome oxidase system according to STOTZ[16].

Purification procedure

All procedures were carried out at 4°.

Step 1. Extraction of beef heart cytochrome C. Fresh beef heart muscles were trimmed of fat and connective tissue, minced, washed and passed through a meat grinder. The mince (3 kg) was stirred with cold water (2400 ml) and adjusted to pH 4.0 with 2.5 *N* H_2SO_4 (ref. 17). After standing for 1 h with occasional stirring the pH was adjusted to 6.5 by dropwise addition of 3 *N* NH_4OH with vigorous stirring. After standing for approximately 18 h the mince was filtered through muslin, re-extracted with cold water (300 ml/ kg) for 1 h and again filtered. The combined filtrate containing the crude Cyt. C was centrifuged and used for the further purification of Cyt. C.

Step 2. Adsorption on an Amberlite XE-64 column (NH_4^+ form) of the crude Cyt. C. Amberlite XE-64 resin (200–400 mesh) was treated and converted into its ammonium form as indicated by LOFTFIELD[18]. After equilibration with a 50 m*M* ammonium phosphate buffer (pH 6.4) referred to as the "adsorption buffer" the resin was introduced into a chromatographic column and washed with the same buffer until it had completely settled (6 cm high and 3 cm in diameter).

The crude Cyt. C solution was poured on the top of the column and washed in with the "adsorption buffer". The red Cyt. C solution is retained, adsorbed as a solid red band near the top of the resin. The column is washed with the same buffer until the eluates are colourless. The well-defined red resin band containing the adsorbed Cyt. C is carefully removed from the surface of the resin and poured into a small glass-fritted filter funnel. The adsorbed Cyt. C is eluted from the resin with the aid of a 401 m*M* ammonium phosphate buffer pH 7.5. This buffer will be referred to as "desorption buffer".

Step 3. Dialysis. The concentrated Cyt. C solution is dialysed until ammonium free, against large volumes of double distilled water. During the dialysis an abundant colourless precipitate is formed and is removed by centrifugation. The great majority of ballast proteins are thus eliminated.

Step 4. Chromatography on an Amberlite XE-64, Bio-Gel P-60, Bio-Gel P-300 "tandem column". The column used for this purification step is composed of three superimposed different layers as follows: the first one, at the bottom, is an Amberlite XE-64 layer (3 × 6 cm); the second one on top of the resin layer is a Bio-Gel P-60 layer (3 × 20 cm), the third above the Bio-Gel P-60 layer is a Bio-Gel P-300 layer

(3 × 3 cm). Each layer is allowed to settle until no further change in the height could be observed before the introduction of the next gel slurry. Care must be taken not to disturb the top surface of the lower layer during the introduction of the succeeding one. The entire column is equilibrated with the "adsorption buffer".

The dialysed Cyt. C preparation (step 3) is carefully layered onto the top of the column and washed in with the same buffer. Fractions (5 ml) of the eluent were collected (0.5 ml/min). Cyt. C moved slowly down the Bio-Gel P-300 and the Bio-Gel

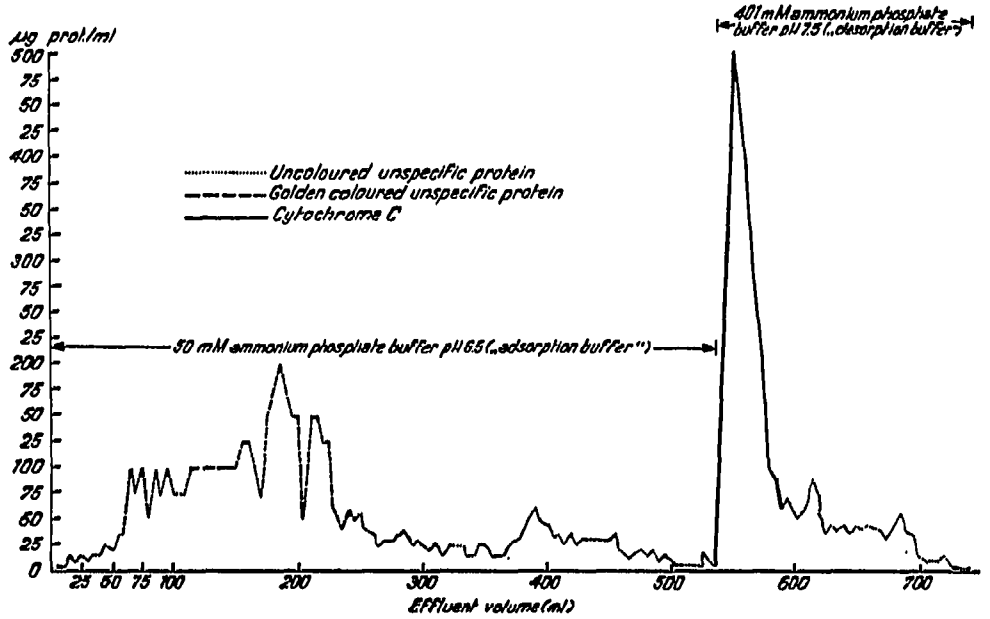


Fig. 1. The elution pattern when the crude Cyt. C preparation was subjected to chromatography on an Amberlite XE-64 (3 × 6 cm), Bio-Gel P-60, (3 × 20 cm), Bio-Gel P-300 (3 × 3 cm) "tandem" column equilibrated with the "adsorption buffer" (50 m*M* ammonium phosphate buffer pH 6.4). Development was with the same buffer but the final elution of Cyt. C from the resin layer was with the "desorption buffer" (401 m*M* ammonium phosphate buffer pH 7.5). 5 ml fractions were collected (0.5 ml/min).

P-60 layer and is retained by adsorption as a homogenous zone on the Amberlite XE-64 resin layer. When the adsorption is completed, the top two gel layers are removed from the column, and the remaining resin layer containing the adsorbed Cyt. C is washed with the "adsorption buffer" until the effluents are free of protein. The packed resin thus washed is transferred onto a glass filter funnel and the Cyt. C is displaced with the "desorption buffer" (401 m*M* ammonium phosphate buffer pH 7.5); 5 ml fractions were collected (0.5 ml/min). Each effluent fraction was analysed for protein and spectrophotometrically for A_{550} reduced, A_{280} and A_{535} values. The course of the chromatographic separation is illustrated in Fig. 1. Re-chromatography under the same conditions of the main peak yield a higher purification of the Cyt. C preparation (Fig. 2).

Step 5. Dialysis and re-chromatography. The effluent fractions containing the purest Cyt. C preparation were pooled and submitted to dialysis against a large volume of double distilled water, any precipitate formed was removed by centrifugation. The clear Cyt. C solution is submitted to re-chromatography on the "tandem" column under identical experimental conditions to those already described, or on a

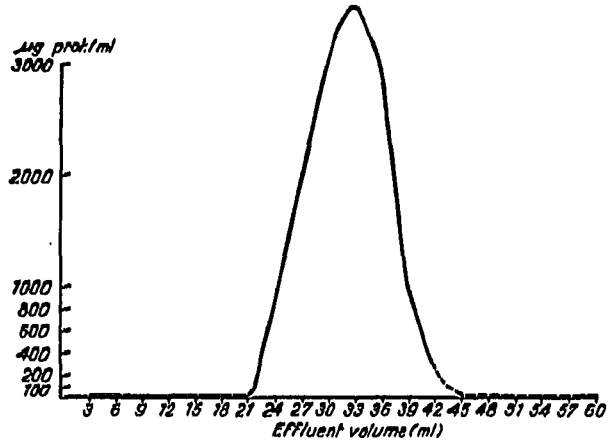


Fig. 2. Re-chromatography on Bio-Gel P-300 of the purified Cyt. C preparation. Equilibration and elution with the "adsorption buffer" (50 m*M* ammonium phosphate pH 6.4).

Bio-Gel P-300 column equilibrated and eluted with the "adsorption buffer". The fractions corresponding to the main peak are pooled and dialysed. For the biological assays on animals the Cyt. C solution can be sterilized by passing it through a bacteriological filter and can be stored after lyophilization as an intensely red powder.

RESULTS

Fig. 1 shows the elution pattern in a typical experiment when the partially purified Cyt. C (step 3) was subjected to chromatography on Amberlite XE-64, Bio-Gel P-60; Bio-Gel P-300 "tandem" column as described.

Under these special chromatographic conditions Cyt. C, on account of its low molecular weight, is delayed in its flow through the two gel layers of the column and is thus efficiently separated from the great majority of ballast proteins having higher molecular weights. These contaminants move ahead of the Cyt. C peak and are eluted when the "adsorption buffer" is used for development; at the same time the Cyt. C is adsorbed and retained on the top of the resin layer. The golden coloured impurities, which are very difficult to remove from the crude Cyt. C[11], as well as the colourless proteins are thus mostly eluted. After elution of these ballast proteins the gel layers are removed from the "tandem" column leaving behind only the resin layer containing the adsorbed Cyt. C. The resin, thoroughly washed with the "adsorption buffer" till the washings are protein free, retains only the almost pure Cyt. C.

On development with the "desorption buffer", the Cyt. C is eluted as a nearly symmetrical peak (Fig. 1). A small quantity of colourless proteins are eluted behind the Cyt. C.

The red coloured effluents are pooled together and dialysed against distilled water. Some uncoloured precipitate is formed and removed by centrifugation. The clear intensely red coloured solution of Cyt. C may be conserved as a lyophilized red powder.

For a higher purification re-chromatography on a "tandem" column or even on a Bio-Gel P-300 column can be successfully applied.

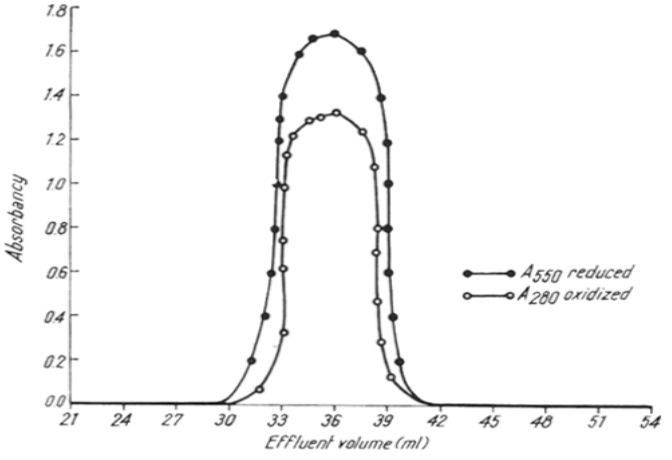
TABLE I

CHARACTERIZATION OF BEEF HEART CYTOCHROME C PURIFIED BY CHROMATOGRAPHY ON AN AMBERLITE XE-64, BIO-GEL P-60, BIO-GEL P-300 "TANDEM" COLUMN

Properties	*Values*
A_{550} red/A_{550} ox.	3.69
A_{550} red/A_{280}	1.24–1.29
A_{550} red/A_{535} red	4.00
Fe %	0.46
N %	16.00
Homogeneity	One band in polyacrylamide gel electrophoresis One peak on Sephadex G-100 and Sephadex G-200 respectively gel filtration

Characterization of the purified cytochrome C

Spectral properties. The ratio A_{550} red/A_{280} = 1.24–1.29 (Fig. 3), and the ratio A_{550} red/A_{550} ox. = 3.69, are comparable to those observed for the monomer form of Cyt. C purified by molecular sieve chromatography on Sephadex G-75[11] or Sephadex G-100[12]. The iron content = 0.46%; the nitrogen content = 16%. Electro-



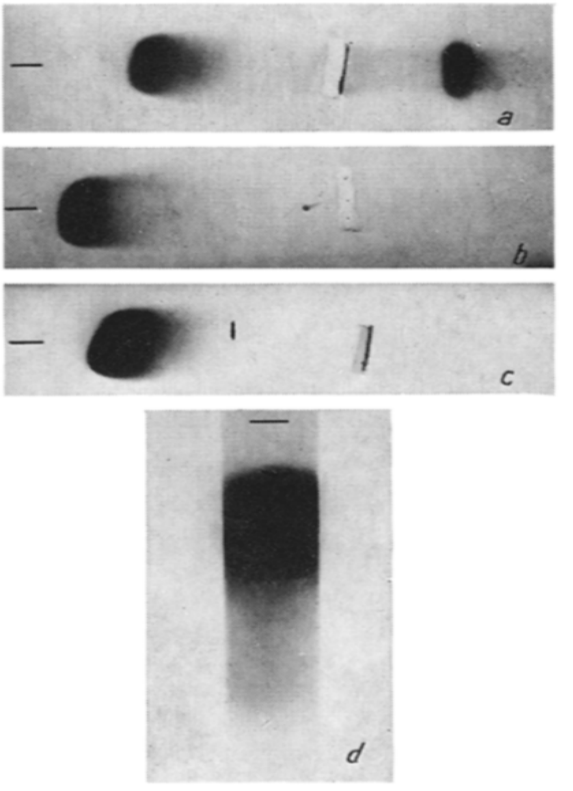


Fig. 3. Absorbancy at A_{550} red (●—●) and A_{280} (○—○) of the effluents of re-chromatographed Cyt. C on Bio-Gel P-300 column.

Fig. 4. Photograph of acrylamide gel slab and respectively disc electrophoresis of different purified preparations of Cyt. C: (a) partially purified on an Amberlite XE-64 column; (b) purified on a "tandem"column (Amberlite XE-64 Bio-Gel P-60 Bio-Gel P-300 column; (c) re-chromatographed Cyt. C on a Bio-Gel P-300 column; (d) disc electrophoresis of re-chromatographed Cyt. C.

phoresis on polyacrylamide gel using the slab method[19] and the disc method[20] show a single band for the purer preparation (Fig. 4).

As is shown in Fig. 4 (a, b, c, d) chromatography on a "tandem" column removes from the crude preparation of Cyt. C the great majority of contaminant proteins. Re-chromatography removes the last traces of the contaminants.

Chromatography on a Sephadex G-100 column of the pure Cyt. C carried out under experimental conditions described by FLATMARK[11] and HAVEZ *et al.*[12], as well as chromatography on a Sephadex G-200 column under the experimental conditions recommended for molecular weight determination[21], display a single peak.

The catalytic properties controlled in a cytochrome oxidase system[16] are maintained in the Cyt. C preparation purified by the method described.

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