A SIMPLE, RAPID AND EFFICIENT PROCEDURE FOR PURIFICATION OF CALMODULIN FROM HUMAN RED CELLS

S. MUALLEM and S. J. D. KARLISH
Biochemistry Department, The Weizmann Institute of Science, Rehovot, Israel

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1. Introduction

Calmodulin is a low molecular weight (16 700) Ca-binding protein found in a wide variety of tissues and involved in regulation of many Ca-dependent processes and enzymatic activities [1–5]. Calmodulin stimulates (Ca+Mg)ATPase [6] and the associated ATP-dependent Ca flux [7] in the human red cell. We have recently made a study of the mechanism of regulation of the Ca pump by calmodulin [8], using protein purified from red cell hemolysates by a method similar to [9]. During the course of that work it became clear that we required a more convenient procedure for obtaining the calmodulin then was then available, and the work in this paper was directed to that goal. Calmodulin has been purified to homogeneity from brain [10], heart [11] and red cells [9] and its properties studied in detail [12]. A striking feature of the protein is that a large fraction of the amino acids are acidic [13] and it shows a high Ca-binding affinity [14]. Another important property is heat stability [15].

We have made use of these features to develop a simple rapid and efficient method for purification of the calmodulin to homogeneity as judged by SDS–gel electrophoresis.

2. Experimental

DE-11 (Whatman) was precycled by pouring the resin into 15 vol. 0.5 M HCl and washing with water until the effluent was at pH 4. Then, the resin was resuspended in 0.5 M NaOH and washed with water until the pH of the effluent was near neutral. The resin was degassed, equilibrated in imidazole buffer 100 mM (pH 6.5) and washed with imidazole buffer 10 mM (pH 6.5) until the pH and the conductivity of the effluent was the same as that of the stock solution. Precycled DE-52 (Whatman) was equilibrated with imidazole 10 mM (pH 6.5) as described above.

Imidazole (Fluka) was recrystallized once from benzene and once from acetone. All the other chemicals were analytical grade. Red cells were washed and then lysed and the hemolysate obtained as in [9]. The preparation of calmodulin-deficient membranes and the assay of the (Ca+Mg)ATPase activity were as in [8]. SDS–acrylamide gel electrophoresis was performed using 10% acrylamide and 0.27% bisacrylamide as in [16]. The gels were stained and destained as in [17]. The protein concentration was determined as in [18].

3. Results and discussion

A summary of the purification procedure is given in table 1. At each stage of purification the ability of the protein fractions to stimulate (Ca+Mg)ATPase activity was assayed using the calmodulin-stripped membranes [8]. Figure 1 shows the SDS electrophoresis gels of proteins obtained at the major steps.

The calmodulin is highly acidic and at pH 6.5 it is bound very tightly to the DE-11 cellulose. The first purification stage involved mixing 1 l red cell hemolysate with 80 ml preswollen DE-11 equilibrated with

Abbreviation: SDS, sodium dodecyl sulphate

Table 1
Purification of calmodulin from human red blood cells

Purification stage	Total protein (mg)	Total units	Yield (%)	Purification (-fold)
Hemolysate	29 800	8.57×10^4	100	–
Conc. DE-11 extract	197.4	8.40×10^4	98	151
Boiling	56	8.25×10^4	96	532
Ca extraction	3.4	7.90×10^4	92	8673
Salt gradient	2.2	7.73×10^4	90	13 545

One activation unit is the protein amount which gives 50% of the maximum activation of the ATP-dependent (Ca+Mg)ATPase activity

10 mM imidazole (pH 6.5). The pH was readjusted to pH 6.5 with 1 M acetic acid, the resin was washed with 10 mM imidazole (pH 6.5) on a scinter glass funnel connected to a vacuum pump, until the A_{280} of the washings at 280 nm was less then 0.05. The protein loaded resin was then poured into a column and washed with a solution of imidazole 10 mM, pH 6.5, containing 200 mM NaCl until the A_{280} was <0.03 (6–8 h). Highly acidic proteins including calmodulin were eluted with imidazole (10 mM (pH 6.5) containing 600 mM NaCl (fig.2). Pooled fractions (140 ml) indicated by the bar in fig.2 were concentrated to 42 ml by ultrafiltration using the Diaflo apparatus with the PM-10 filter, dialyzed against 6 l water for 1 h and insoluble material was removed by centrifugation at 45 000 × *g* for 10 min.

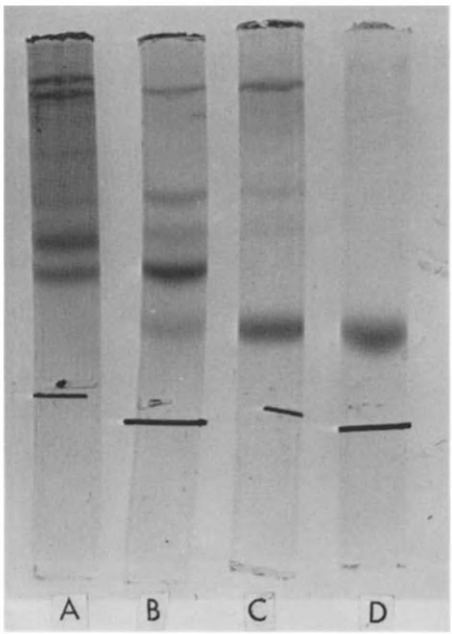

Fig.1. SDS gel electrophoresis pattern of various purification stages. SDS–polyacrylamide gel electrophoresis was performed as in section 2. Samples at various steps of purification were subjected to the gels (20 μg protein). (A) DE-11 fractions; (B) boiled supernatant; (C) 20 mM $CaCl_2$ elution fractions; (D) purified calmodulin after salt gradient.

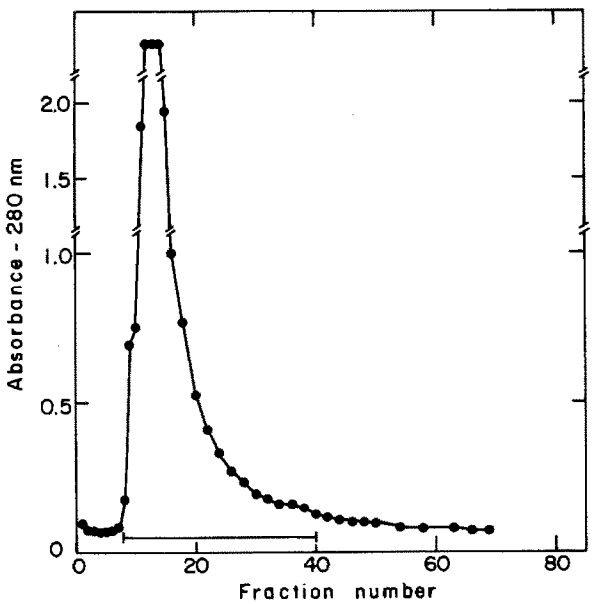

Fig.2. Elution profile of proteins from DE-11 cellulose column. The elution of proteins was as in the text. The column was developed at 60 ml/h and 4 ml fractions were collected, after which the A_{280} was measured. The horizontal bar indicates the fractions that were pooled for further purification.

The characteristic heat-stability of calmodulin allowed us to eliminate a large fraction of the irrelevant proteins by warming the supernatant from the previous stage to 70°C for 30 min, and removing the precipitated protein by centrifugation at 45 000 X *g* for 10 min. The remaining soluble proteins were concentrated to 10 ml using the Diaflo apparatus as above. The concentrated proteins were applied to a DE-52 cellulose column (1.2 X 12 cm) and the column was washed with 25 ml imidazole 10 mM (pH 6.5) (fig.3, arrow A) followed by 100 ml 10 mM imidazole (pH 6.5) containing 200 mM NaCl (fig.2, arrow B). Then the column was washed with 70 ml of a solution containing imidazole 10 mM (pH 6.5), 150 mM NaCl and 5 mM $CaCl_2$ (fig.2, arrow C). The latter wash eluted a small amount of a single protein which had no effect on the (Ca+Mg)ATPase activity (not shown). This protein has mol. wt ~26 000 and is always eluted with calmodulin unless removed by the above stage. Calmodulin was eluted with a solution containing imidazole 10 mM (pH 6.5), NaCl 150 mM and $CaCl_2$ 20 mM (fig.2, arrow D). The fractions indicated by the bar were pooled, dialyzed against 6 l water for 2 h and concentrated to 11.8 ml by the Diaflo apparatus with the PM-10 filter.

The concentrated proteins were then applied to a second DE-52 cellulose column (1.2 X 10 cm). The column was washed first with 25 ml imidazole 10 mM (pH 6.5) (fig.4, arrow A), then with 60 ml imidazole 10 mM (pH 6.5) containing NaCl 200 mM (fig.4, arrow B). The calmodulin was eluted with a linear salt gradient composed of 250 ml imidazole 10 mM (pH 6.5) and NaCl 200 mM as the initial buffer and 250 ml imidazole 10 mM (pH 6.5) and NaCl 500 mM as the final buffer (fig.4, arrow C). The A_{235} and the ATPase-stimulating activity of the fractions were monitored. The fractions containing the activity were pooled, dialyzed against 6 l water for 1 h, concentrated to 220 μg/ml by the Diaflo apparatus, Hepes buffer (pH 7.4) was added to

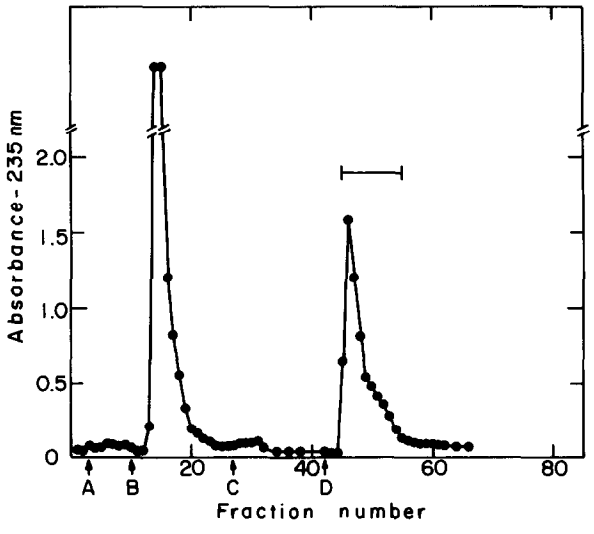

Fig.3. DE-52 cellulose chromatography of the calmodulin-step gradient. The column was developed at 40 ml/h by the following step gradient: (A) 25 ml imidazole 10 mM (pH 6.5); (B) 100 ml imidazole 10 mM (pH 6.5) and NaCl 200 mM; (C) 70 ml imidazole 10 mM (pH 6.5), NaCl 150 mM and $CaCl_2$ 5 mM; (D) imidazole 10 mM (pH 6.5), NaCl 150 mM and $CaCl_2$ 20 mM. Fractions (4 ml) were collected and the A_{235} determined.

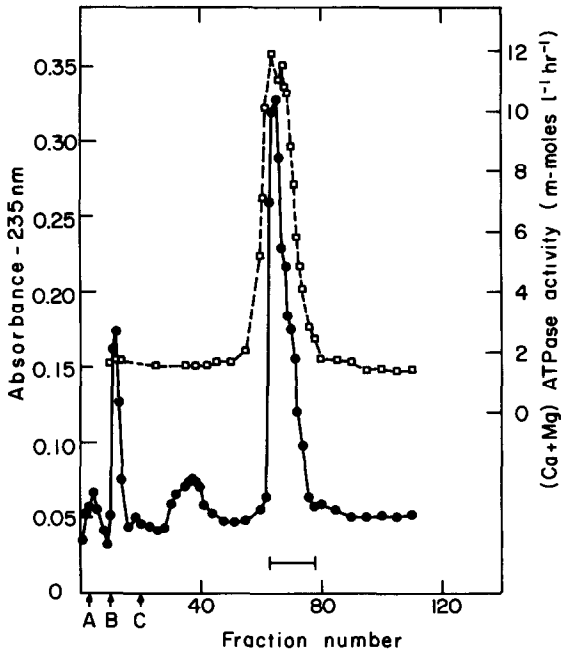

Fig.4. DE-52 cellulose chromatography of the calmodulin-step and linear salt gradient. The step gradient was composed of: (A) 25 ml imidazole 10 mM (pH 6.5); (B) 60 ml imidazole 10 mM (pH 6.5) and NaCl 200 mM. The linear salt gradient was as in the text. The column was developed at 40 ml/h and 3 ml fractions were collected. Activation of (Ca+Mg)ATPase by each fraction was measured using 2 μl effluent (□– – –□).

5 mM and the protein solution was frozen in liquid nitrogen and stored at −20°C.

The major advantages of the procedure described here are that the boiling stage increases the specific activity 3.52-times in comparison to the previous stage, while elution by Ca increased the specific activity 16.3-times and decreased the number of proteins contaminating the calmodulin. The final washing and elution of caldomulin on a salt gradient eliminate the need for further purification stages. Only 10% of the initial amount of the calmodulin is lost at the end of the procedure and the calmodulin was purified 13 545-fold.

The main differences between our method and that in [9] are:

(i) The use of an additional DE-52 cellulose column;
(ii) A specific purification and elution of the calmodulin with Ca rather than by gel filtration fractionation;
(iii) Substituting concentration by lyophilization of the pooled fractions by ultrafiltration using the Diaflo apparatus;
(iv) Avoiding ammonium sulfate precipitation after the final salt gradient.

These improvements decrease markedly the time needed for the purification of the calmodulin, simplify and economize the procedure and increase the efficiency by ~20% in comparison to the published method.

The method described should be useful for the purification of the calmodulin from other tissues and for study of its physiological roles.

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