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PURIFICATION AND MEASUREMENT OF CALPROMOTIN, THE CYTOPLASMIC PROTEIN WHICH ACTIVATES CALCIUM-DEPENDENT POTASSIUM TRANSPORT

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Summary: A simple procedure is described for the purification of calpromotin, a protein from the cytoplasm of red blood cells which is capable of activating calcium-dependent potassium transport. The purification steps involve a salt gradient elution from an anion exchange column (Whatman DE-52) followed by a potassium phosphate gradient elution from a column of hydroxyapatite (HA Ultrogel). These steps result in a 54% yield with a 161 fold purification. The calpromotin is estimated to be 99% pure as determined by densitometry of the protein profile on an SDS polyacrylamide gel. A competitive enzyme-linked immunosorbent assay (ELISA) using rabbit anti-human calpromotin antibodies, is described for measuring levels of calpromotin in the 5 to 100 ng range.

Erythrocytes and other cells contain a mechanism for potassium transport which is dependent on a rise in cytoplasmic calcium. This calcium-dependent potassium transport was first described in red blood cells about 30 years ago by Gardös (1). It is now known that the calcium concentration required to activate the potassium efflux is in the low micromolar range and the channel or carrier is highly selective for potassium and rubidium over cesium, lithium and sodium (2,3). To date, neither the mechanism of calcium activation nor the biochemical identification of the potassium channel or carrier is known. Previous reports (4,5) implicate a cytoplasmic protein and its association to the inner surface of the membrane in the presence of calcium with potassium transport.

This manuscript provides a simple procedure for purifying this cytoplasmic protein, which we have named calpromotin, and for establishing a standard curve by competitive ELISA to measure levels in unknown samples.

MATERIALS AND METHODS

Materials

Pre-swollen microgranular DEAE-cellulose (DE-52) was purchased from Whatman and HA-Ultrogel (hydroxyapatite) was purchased from IBF Biotechnics. For the ELISA

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procedure, biotinylated goat anti-rabbit IgG and streptavidin conjugated horseradish peroxidase were obtained from Bethesda Research Laboratories. The substrate o-phenylenediamine was ordered from Sigma Chemical Company. All reagents used for SDS-polyacrylamide gel electrophoresis were bought from Bio-Rad Laboratories.

Preparation of Hemolysate

After obtaining informed consent from normal healthy volunteers, 50 ml of fresh human blood was centrifuged at 7,000 g for one minute in a Sorvall RC-5B centrifuge using an SA-600 rotor. The plasma and buffy coat were removed. The packed erythrocytes were washed four times more with isotonic saline with continued removal of white cells (i.e. the buffy coat). The 20 ml of packed red cells were then hemolyzed with 10 volumes of 50 mM Tris buffer pH 8.0 containing 0.2 mM EDTA and 5 mM β-mercaptoethanol. The lyzed suspension was centrifuged at 15,000 g to pellet the membranes and the hemolysate carefully removed for fractionation.

Fractionation Over DE-52 and HA Ultrogel Columns

About 200 ml of hemolysate were loaded onto a DE-52 column (2.5 cm x 40 cm, bed volume 196 ml) which had been equilibrated with 50 mM Tris buffer pH 8.0 containing 0.2 mM EDTA and 5 mM β-mercaptoethanol. The sample was eluted from the column using a linear gradient of sodium chloride ranging from 0 to 300 mM in the column buffer. The total volume of the gradient was 1 liter. One hundred fractions, 10 ml volume each, were collected. The absorbance at 280 nm of the eluate was monitored continuously during the fractionation. Samples (25 μl) from the various fractions were analyzed by SDS-polyacrylamide gel electrophoresis (see below) to identify the protein profiles of the fraction samples containing calpromotin. Also, 5μl of each fraction was analyzed for calpromotin using the competitive ELISA procedure. These fractions were pooled and concentrated by ultrafiltration over an Amicon PM-10 filter. The concentrate was resuspended with 15-20 volumes of 20 mM potassium phosphate buffer pH 7.4 containing 0.2 mM EDTA and 5 mM β-mercaptoethanol and then concentrated to about 15 ml.

The DE-52 concentrate was loaded onto an HA-Ultrogel column (2.5 cm x 11 cm, bed volume 54 ml) which had been equilibrated with the 20 mM phosphate buffer described above. A linear gradient formed from 250 ml of 20 mM potassium phosphate pH 7.4, 0.2 mM EDTA, 5 mM mercaptoethanol and 250 ml of 300 mM potassium phosphate pH 7.4, 0.2 mM EDTA, 5 mM mercaptoethanol was used to elute the sample from the column. One hundred fractions, 5 ml volume each, were collected. Again, fractions were analyzed by SDS-polyacrylamide gel electrophoresis and competitive ELISA. The samples enriched in calpromotin were pooled and concentrated by ultrafiltration as described above. Separate PM-10 filters were used for concentrating DE-52 fractions and HA-Ultrogel fractions. The final concentrate was diluted with 50 mM Tris buffer pH 8.0, 0.2 mM EDTA, 5 mM mercaptoethanol and then concentrated. This final concentrate was stored at -75°C. Under these conditions the protein remains stable for many months. Purification of the calpromotin was assessed by measuring the different samples for total protein by the Bradford procedure (6) and calpromotin by the competitive ELISA described below.

SDS-Polyacrylamide Gel Electrophoresis

Proteins within the fractions obtained from each column were analyzed by SDS-polyacrylamide gel electrophoresis according to the procedure of Laemmli (7). A slab gel consisting of 12% acrylamide in the separating gel and 3% acrylamide in the stacking gel was used. Following electrophoresis, the proteins in the gel were fixed and stained with 50% methanol, 10% acetic acid, 0.025% Coomassie brilliant blue R-250. Gels were destained in 10% acetic acid. The densitometric scan shown in figure 5 was obtained using an ISCO gel scanner, model number 1312, linked to an ISCO UA-5 detector. The

maximal absorbance of the calpromotin peak was set at 80 units on the recorder paper; full scale deflection of the paper was 100 units.

Measurement of Calpromotin by Competitive ELISA

One hundred ng of calpromotin in 100 μl of 0.l M sodium bicarbonate pH 9.6 was placed into each well of a 96 well plate (E.I.A. microtitration plate, #76-381-04 Flow Laboratories). After 15 hours, the solution was removed and the wells washed three times with 100 μl of PST buffer (10 mM phosphate pH 7.4, 150 mM NaCl, 0.5% Tween 20). Unreacted sites in the well were blocked with 100 μl of PSTA buffer (PST buffer containing 1% albumin) for 2 hours. During this time, tubes were prepared with dilutions of standard calpromotin - 1, 2, 5, 10, 20, 50, 100, 200, 500, 1000 ng per 100 μl in 1 ml total volumes or different dilutions of unknown samples. Thirty minutes prior to the removal of the PSTA buffer, rabbit anti-human calpromotin antiserum final dilution of 1:500, was added to the tubes containing the calpromotin or hemolysate fractions. Upon removal of the blocking buffer (PSTA), the wells were washed three times with PST buffer and the calpromotin antiserum solutions added to the plate, each dilution to one column of wells. These solutions were allowed to stay in the wells for 3 hours, after which they were removed and the wells washed four times with PST buffer. Biotinylated goat anti-rabbit IgG was diluted a thousand fold in PSTA buffer to a final concentration of 1 μg/ml. One hundred microliters of this secondary antibody solution was added to each well of the plate and allowed to incubate (R.T.) for 2 hours. The secondary antibody was removed and washed three times with PSTA buffer. Streptavidin-horseradish peroxidase conjugate diluted 500 fold in PSTA buffer was then added to the wells and allowed to react for 30 minutes. Upon removal of the enzyme-conjugate solution, the plate was washed four times with PSTA buffer and twice with PST buffer. Color was initiated by the addition of 100 μl of the substrate solution (0.2 M dibasic sodium phosphate, 0.1 M citric acid, 0.012% hydrogen peroxide and a 30 mg tablet of o-phenylenediamine in a total volume of 75 ml). The reaction was allowed to proceed for 15 to 30 minutes, after which the absorbance at 450 nm was taken using a Multiscan MKII microtiter plate reader (Flow Laboratories). A standard curve was prepared from plotting the absorbances against standard amounts of calpromotin preincubated with the primary antiserum. This procedure has been performed also with a goat anti-human calpromotin antiserum and a biotinylated-goat anti-rabbit IgG. Concentrations of the calpromotin bound to the plate, primary antibody, secondary antibody and SA-horseradish peroxidase used in this procedure have been optimized.

RESULTS AND DISCUSSION

Purification of Calpromotin

The elution profile of the hemolysate sample from the DE-52 column as monitored by absorbance at 280 nm is shown in Figure 1. Typically, there is an initial small peak containing a pure protein of 30,000 MW (possibly carbonic anhydrase) followed by a very large peak, which contains most of the hemoglobin, and then four minor peaks. Analysis of these fractions using the competitive ELISA procedure showed that the calpromotin (23,000 MW) elutes along the trailing edge of the hemoglobin peak (Figure 1). Every other fraction from 36 to 68 was analyzed for the presence of calpromotin on SDS-polyacrylamide gels (Figure 2). Although there are still many proteins which contaminate the calpromotin fractions, most of the hemoglobin is removed. After calpromotin fractions 50 to 62 from the DE-52 column were pooled and concentrated, further purification is

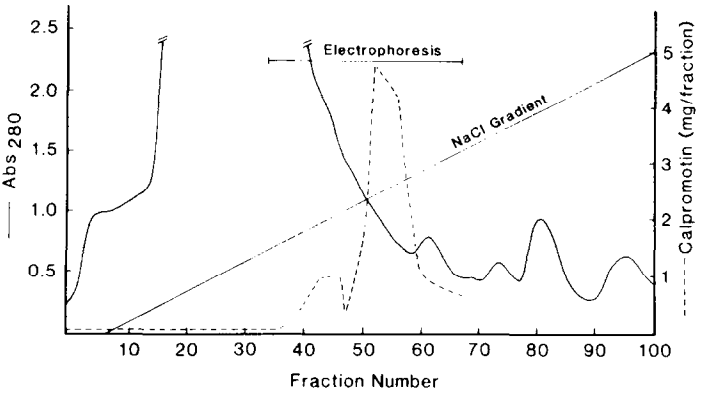

Fig. 1 Separation of calpromotin by DE52 ion exchange chromatography. Details regarding the preparation, loading and elution of the hemolysate are provided in the Materials and Methods section. The sodium chloride gradient was increased from 0 to 300 mM.

obtained by hydroxyapatite (Figure 3). From the HA-Ultrogel column, almost all of the hemoglobin and contaminating proteins elute at the beginning of the gradient as one peak followed by a broad peak (fractions 45 to 75) which consists of highly purified calpromotin.

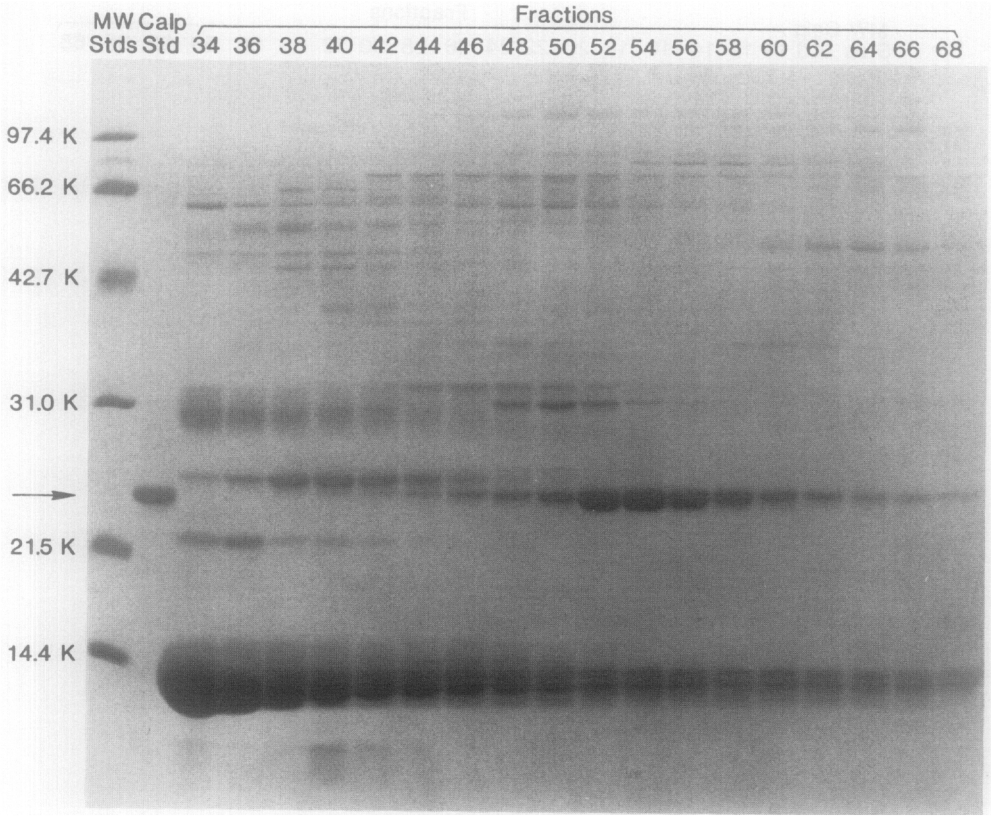

Fig. 2 SDS-polyacrylamide gel electrophoresis of hemolysate fractions 34 through 68 obtained from Fig. 1. The arrow at the left indicates the calpromotin band at 23,000 da MW.

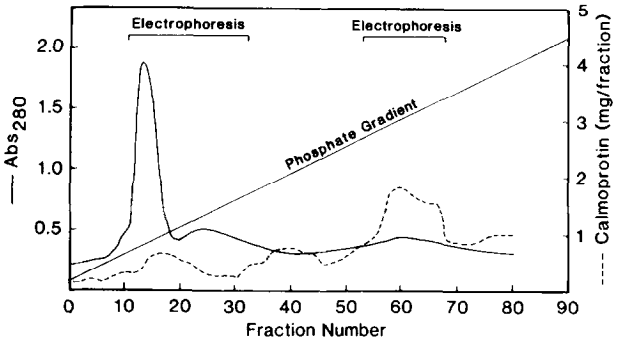

Fig. 3 Separation of calpromotin from the pooled DE-52 fractions by hydroxyapatite chromatography. The potassium phosphate gradient increased from 20 to 300 mM over fractions 0 to 100.

An SDS-polyacrylamide gel analysis of these fractions is presented in Figure 4. Most often, there is a trace contamination of a protein at 60,000 MW which cannot be removed by carboxymethyl cellulose or gel filtration.

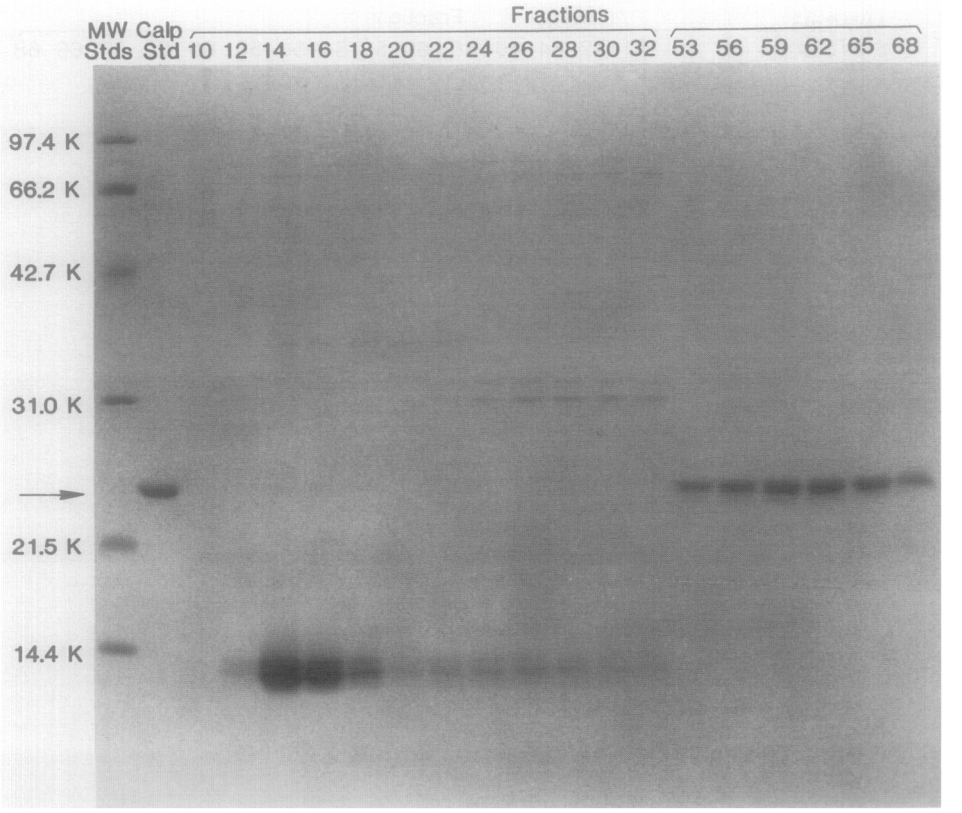

Fig. 4 SDS-polyacrylamide gel electrophoresis of fractions 10 through 32 and 53 through 68 eluted from the hydroxyapatite column. Again, the arrow at the left indicates the calpromotin band at 23,000 da MW.

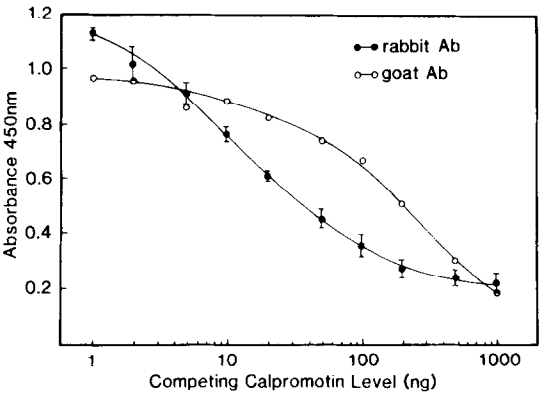

Fig. 5 Competitive enzyme-linked immunosorbent assay (ELISA) for calpromotin. Details for the procedure are described in the Materials and Methods section.

Measurement of Calpromotin Standards and Hemolysate Samples By Competitive ELISA

Standard curves for calpromotin using rabbit and goat antisera are shown in Figure 5. Note that at low amounts of calpromotin, there is a high absorbance which decreased with increasing levels of calpromotin. The most sensitive range for measuring calpromotin is 5 to 100 ng using the rabbit antiserum and 50 to 1000 ng using the goat antiserum. Assays of unknown samples were carried out using the rabbit antiserum.

Analyses of the hemolysate and concentrates from the DE-52 and HA-Ultrogel columns for total protein and calpromotin are shown in Table 1. The purification procedure results in a 54% yield of calpromotin which is purified 161 fold. The estimate of calpromotin by the competitive ELISA is slightly higher than the actual protein measured but this is within the limits of the logarithmic scale of the ELISA procedure. To verify the

TABLE 1 CALPROMOTIN PURIFICATON

FRACTION	TOTAL PROTEIN mg	TOTAL CALP mg	%YIELD	FOLD PURIFICATION
Hemolysate	**9,576**	**59.0**	**100**	**1**
DE-52 Fraction	**108**	**45.2**	**77**	**68**
HA-Ultrogel Fraction	**29.1**	**31.8**	**54**	**160**

Summary of calpromotin purification procedure showing percent yield was calculated by Total calpromotin after fractionation/Total calpromotin in hemolysate. The fold purification was calculated from the following equation:

$$\frac{\text{total calpromotin column/total protein column}}{\text{total calpromotin hemolysate/total protein hemolysate}}.$$

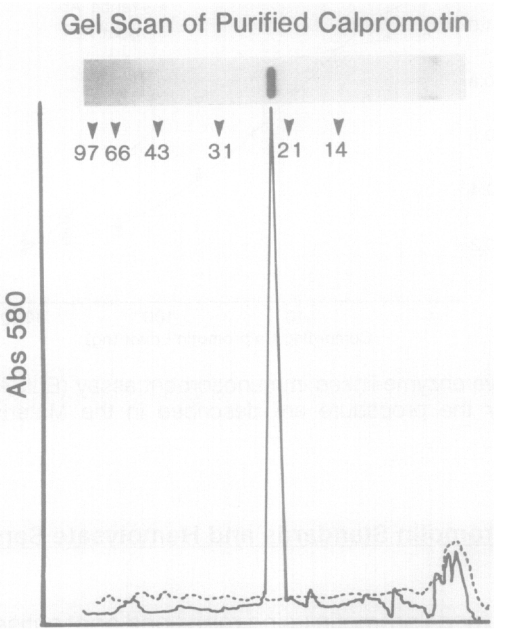

Fig. 6 Scan of a Coommassie blue stained SDS-polyacrylamide gel containing purified calpromotin. Calpromotin fractions from the hydroxyapatite column were pooled and concentrated by Amicon ultrafiltration (PM10 filter). A sample of the concentrated calpromotin fraction was run on an SDS-polyacrylamide gel (12% acrylamide). The Coommassie blue stained gel was stained with an ISCO gel scanner using a 580 nm filter. —calpromotin fraction, ---- background gel scan.

purity of the calpromotin, the final HA-ultrogel fractions containing this protein were concentrated and rerun on an SDS-polyacrylamide gel and then scanned quantitatively. The results in Fig. 6 show that the calpromotin is greater than 99% pure. A faint band at a molecular weight of about 46,000 daltons, which can be seen on the gel although not detected by the scanner, is most likely a residual amount of unreduced dimer of this protein. In real terms, about 30 mg of calpromotin which is 99% pure can be obtained from 50 ml of blood.

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