Journal of Chromatography, 448 (1988) 145–156
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 584

CHICK 28 000 M_r VITAMIN D-DEPENDENT CALCIUM-BINDING PROTEIN IN INTESTINE, KIDNEY AND CEREBELLUM

PURIFICATION USING CHROMATOFOCUSING

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(First received February 1st, 1988; revised manuscript received March 28th, 1988)

SUMMARY

A purification method has been developed for chick 28 000 M_r vitamin D-dependent calcium-binding protein, involving Blue Sepharose CL-6B column chromatography, heat treatment and chromatofocusing with a microparticulate anion exchanger (Mono P). It allowed the rapid and reproducible purification of milligram amounts of homogeneous calcium-binding protein with good yields from chick intestine, kidney and cerebellum. The calcium-binding proteins thus obtained have the same molecular weight of 28 000, heat stability, calcium binding capability and apparent isoelectric point of 4.0. These physico-chemical properties are in good agreement with those of proteins isolated by a previous procedure, which gave a low and variable yield of calcium-binding protein.

INTRODUCTION

Since a vitamin D-dependent calcium-binding protein (CaBP) was first shown to be present in chick duodenal mucosa[1], its possible function related to vitamin D-dependent calcium absorption has been extensively studied. However, its exact molecular action is still under discussion. Currently available evidence, in particular its high affinity specifically for calcium[2] and the dependence of the synthesis of its mRNA on vitamin D[3,4], suggest that this protein is involved in the molecular mechanism of intestinal calcium absorption. On the other hand, some investigators have suggested that CaBP is not involved directly in the initial calcium uptake process[5] and may act simply as a calcium buffering component[6–8]. This suggestion is supported by the presence of CaBP in tissues without a high calcium transporting capacity such as brain, pancreas, adrenals and retina of both birds[9] and mammals[10]. These problems are compounded by the observation that CaBP is apparently not vitamin D dependent at all these sites, having been identified and assayed merely on the basis of its

0021-9673/88/$03.50

immunological similarity to intestinal CaBP[9–11]. Clearly, more studies are needed at both the physiological and biochemical levels, not only on the chick intestinal CaBP but also on the CaBP-like activity found in the various tissues. Also, most of the physico-chemical properties of CaBP recorded so far have been made on the smaller mammalian CaBP (M_r = 10 000). Consequently, we wished to purify CaBP from several tissues but found that existing procedures[12–15] were not suitable for this purpose because of their poor recoveries and variabilities.

We have developed a new purification method for isolating CaBP using Blue Sepharose CL-6B and chromatofocusing. The proteins that we obtained from chick intestine, kidney and cerebellum with the use of this method showed an apparent homogeneity on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and high-performance liquid chromatography (HPLC) using a micro-particulate anion exchanger (Mono Q) with and without Ca^{2+} in the solvent. The usefulness of the proposed method was also compared with that of the existing method, which is mainly based on the intrinsic charge difference in the molecule of CaBP with and without bound Ca^{2+}.

EXPERIMENTAL

Materials

Crystalline vitamin D_3 was obtained from Sigma (London, U.K.). Blue Sepharose CL-6B and Sephadex G-100 were purchased from Pharmacia (Uppsala, Sweden). Both prepacked columns for HPLC, a Mono P column for chromatofocusing and a Mono Q column for ion-exchange chromatography were also obtained from Pharmacia. The enzyme inhibitors TPCK (N-tosyl-L-phenylalanine chloromethylketone), TLCK (N-tosyl-L-lysine chloromethylketone) and PMSF (phenylmethylsulphonyl fluoride) were supplied by Boehringer (Mannheim, F.R.G.). Rabbit antiserum to chick intestinal CaBP prepared previously by Spencer *et al.*[16] in our laboratory was used in this study. Protein molecular weight markers (Dalton Mark VII-L; Sigma) contained bovine serum albumin (66 000), ovalbumin (45 000), glyceraldehyde-3-phosphate dehydrogenase (36 000), carbonic anhydrase (29 000), trypsinogen (24 000), trypsin inhibitor (20 100) and α-lactalbumin (14 200). All other chemicals were of analytical-reagent grade.

Animals

One-day-old white leghorn chicks were housed in electrically heated tier brooders in an air-conditioned room from which sunlight was excluded. They were fed *ad libitum* on a vitamin D-deficient diet for 3–4 weeks[17] until they were assessed to be in a state of vitamin D deficiency by histological examination of the bones. Chicks recieved orally 12.5 μg of vitamin D in 0.2 ml of ethanol–2-propanol (1:1, v/v) and were killed after 72 h, when the production of CaBP in duodena had reached its maximum.

Preparation of tissue extracts

Duodena were split open lengthwise, rinsed with ice-cold Tris buffer A (13.7 m*M* Tris base, 150 m*M* sodium chloride, 5 m*M* potassium chloride, 1 m*M* 2-mercaptoethanol, pH adjusted to 7.4 with hydrochloric acid) and the mucosa scraped from underlaying serosal layers with a glass slide on ice, and then stored at −20°C before

extraction. Kidney and cerebellum were cut into small pieces with scissors and homogenized in an equal volume of ice-cold Tris buffer with a Polytron homogenizer for 30 s. The homogenates were then centrifuged at 100 000 *g* for 1 h at 4°C. The supernatant solutions were decanted and filtered through a glass filter (80–100 mesh) to eliminate membraneous fat-soluble materials floating on the surface of the supernatant solution and stored at −20°C.

Purification of CaBP

CaBP from chick intestine, kidney and cerebellum was purified by the following two procedures, one of which was a modification of that originally described by Hitchman and Harrison[12] (existing method), and the other our proposed method. All further steps were carried out at 4°C unless stated otherwise and the three tissues were treated in the same manner throughout. Unless indicated otherwise, all recoveries of CaBP were determined by rocket electroimmunoassay (see below).

Existing method. Approximately 20 ml of the supernatant solution were applied to an upward-flow column of Sephadex G-100 (90 × 2.5 cm I.D.) equilibrated with Tris buffer and eluted with the same buffer. The flow-rate was 40 ml/h, 4.5-ml fractions were collected and the CaBP concentration in every other tube was determined. Fractions containing CaBP were pooled and concentrated to a small volume using an Amicon stirred cell using an Amicon UM2 membrane filter under nitrogen pressure (40 kg/cm^2). Proteins recovered from the concentrator were placed in Visking tubing and dialysed against 500 ml of imidazole buffer (20 m*M* imidazole, 20 m*M* sodium chloride, 1 m*M* EDTA, 1 m*M* 2-mercaptoethanol, pH adjusted to 7.0 with hydrochloric acid) for 16 h. The dialysate was then centrifuged at 100 000 *g* in a refrigerated centrifuge for 30 min and the supernatant solution was applied to a DEAE-Sephacel column (30 × 1.5 cm I.D.) equilibrated with imidazole buffer. The column was washed with the same buffer until the eluate was free from protein, as determined by the ultraviolet absorbance (280 nm), and eluted with a 500-ml sodium chloride gradient from 20 to 700 m*M* in imidazole buffer. The flow-rate was 15 ml/h and 4.5-ml fractions were collected. All fractions were assayed for CaBP, which was detected as a single peak on the chromatogram in twelve fractions containing 280 m*M* sodium chloride as measured using a conductivity meter. These fractions were pooled and dialysed against 1000 ml of imidazole buffer containing 20 m*M* sodium chloride and 2 m*M* calcium chloride for 16 h. The dialysate was subsequently applied to another DEAE-Sephacel column (30 × 1.5 cm I.D.) equilibrated with the same buffer as used for dialysis. Development of this column proceeded as described for the preceding column except that 2 m*M* calcium chloride was added in imidazole buffer instead of EDTA. All fractions were assayed for CaBP, which was detected as a clear single peak on the chromatogram.

Proposed method. The supernatant was applied directly to a Blue Sepharose CL-6B column (40 × 1.5 cm I.D.) equilibrated with Tris buffer (13.7 mM Tris base, 20 m*M* sodium chloride, 5 m*M* potassium chloride, 1 m*M* 2-mercaptoethanol, pH adjusted to 7.4 with hydrochloric acid). Column elution was carried out in the downward direction at a flow-rate of 17 ml/h and 4.5-ml fractions were collected. All fractions containing CaBP were pooled, heated at 60°C for 20 min and immediately chilled to 4°C. After centrifugation at 100 000 *g* for 1 h at 4°C, proteins not precipitated from the pooled solution by heat treatment were recovered as the supernatant solution and placed in Visking tubing and dialysed against 500 ml of buffer B (20 m*M* N-methylpiperazine, 1

m*M* 2-mercaptoethanol, pH adjusted to 5.7 with hydrochloric acid) for 6 h with stirring. The dialysate was subsequently applied without concentration to a Mono P column for chromatofocusing.

The system used was a Waters Assoc. ALC/GPC Model 6000 liquid chromatograph equipped with an automated gradient controller, a U6K sample injector and a Model 441 variable-wavelength detector. Milli-Q water (Millipore, Bedford, MA, U.S.A.) with a measured resistance of 18 MΩ or less was used exclusively. The Mono P column was equilibrated with buffer B until the effluent showed the same pH value as this buffer. The sample solution, ranging from 2 to 8 ml in volume and containing up to 20 mg of protein, was applied to the column and washed with buffer B until the eluate was free from proteins, as determined by the ultraviolet absorbance (280 nm). Elution was carried out with Polybuffer 74 (pH 3.7) with a linear pH gradient from 5.7 to 3.7 at a constant flow-rate of 1 ml/min at room temperature. Fractions of 1 ml were collected and assayed for CaBP by rocket electroimmunoassay.

For complete purification, CaBP obtained from the first chromatofocusing was rechromatographed on the same column under the same conditions as those of the first chromatofocusing. The purified CaBP thus obtained was finally applied to a Sephadex G-100 column (90 × 1.5 cm I.D.) and eluted with Tris buffer A containing 2 m*M* calcium chloride to be preserved in the presence of Ca^{2+}, and also to remove unknown component(s) of Polybuffer 74 which show a positive interference in the measurement of protein by the method of Lowry *et al.*[18], although they do not affect the stability of CaBP on prolonged storage at −20°C.

Rocket electroimmunoassay for CaBP

This was carried out by a modification of that originally described by Laurell[19]. Chick intestinal CaBP antiserum was dissolved in 1% agarose (Koch-Light, Colnbrook, U.K.) in pH 8.6, 0.087 *M* barbital buffer at a final concentration of 0.1% at 55°C, and then transferred on to a glass plate to make a 1 × 100 × 200 mm slab of agarose containing antiserum. After standing for 30 min at room temperature to solidify, nineteen sample wells (2 mm) were made and 5 μl of the sample solutions were placed in each well. Electrophoresis at a constant voltage of 40 V per 10 cm was carried out for 6 h at room temperature. The electrode buffer was the same as used for the preparation of the agarose gel. After immunoelectrophoresis, the plate was dried and stained for protein with 0.05% Coomassie Brilliant Blue G. When the heights of the precipitin rockets were plotted against pure CaBP concentrations, a linear calibration graph was obtained over the range of 20–400 ng per 5 μl of sample solution.

SDS-PAGE

Approximately 20 μg of the purified CaBP from intestine, kidney and cerebellum were dissolved in a solution containing dithiothreitol (15 mg/ml), SDS (10 mg/ml) and Tris base (12 mg/ml) and heated at 70°C for 30 min for complete reduction to subunits. The electrophoresis was carried out at 3 mA per gel[20]. Staining of the gel was carried out at 70°C for 1 h in 0.05% Coomassie Brilliant Blue G in acetic acid–2-propanol–water (2:5:13, v/v). Destaining was carried out at 70°C for 15 min in the same solvent without Coomassie Brilliant Blue G and then the gel was left in 10% acetic acid at 30°C until destained. The identification of CaBP in the gel was also performed by the following immunological procedure.

After electrophoresis, the gel was immediately frozen using dry-ice and 1-mm slices of the gel were prepared using a Macrotome GTS (Yeda Research and Development). Each slice was placed on the same agarose gel plate containing antiserum as used in the rocket electroimmunoassay for CaBP and diffusion allowed to proceed in a humid atmosphere at room temperature. After diffusion for 24 h, the plate was dried, stained and destained and the diameter of the precipitin rings were measured.

Ion-exchange chromatography using HPLC

The same HPLC apparatus and equipment as used for chromatofocusing were employed in this experiment. Separation of CaBP was performed on a Mono Q column under the following conditions. Elution of proteins was carried out in a linear gradient of sodium chloride from 50 to 700 m*M* in piperazine buffer (25 m*M* piperazine, pH adjusted to 6.5 with hydrochloric acid) in the presence or absence of 2 m*M* calcium chloride at a constant flow-rate of 1 ml/min with the column pressure varying from 350 to 500 p.s.i.

Other method

Protein was determined by the method of Lowry *et al.*[18] with bovine serum albumin as a standard.

RESULTS

Chick 28 000 M_r CaBP was isolated from intestine, kidney and cerebellar homogenates by the procedure introduced by Hitchman and Harrison[12] in which the proteins in the homogenates were fractionated by a series of chromatographic steps: gel filtration chromatography and DEAE-Sephacel chromatography without and with Ca^{2+} as shown in Fig. 1 (Fig. 1Aa–c for intestine, Fig. 1Ba–c for kidney, Fig. 1Ca–c for cerebellum). After completion of the final chromatographic step, the protein purified from each tissue was subjected to SDS-PAGE to establish its homogeneity. As illustrated in Fig. 2, all proteins had one major and one faint minor band having the respective identical electrophoretic migration rates among tissues (as measured by electrophoresing the proteins in sets of two proteins on a single gel). The protein corresponding to the major band was apparently the native molecule of CaBP on the basis of its molecular weight (28 000), an intrinsic charge difference in the presence and absence of Ca^{2+} and immunoreactivity against CaBP antiserum. On the other hand, the protein corresponding to the minor band was thought to be a degradation, fragment of CaBP as it still had immunoreactivity against CaBP antiserum (as measured by immunodiffusion in 1% agarose gel plate containing anti-CaBP antiserum), but this protein has not been characterized. The overall recoveries of CaBP, as assayed by rocket electroimmunoassay, were from chick intestine 7–35%, kidney 11–32% and cerebellum 12–27%. These values are slightly lower than those reported by Hitchman and Harrison[12] and more variable.

The results of Blue Sepharose CL-6B chromatography used as the first step of our proposed method are shown in Fig. 3. Blue Sepharose CL-6B, having a wide range of binding affinity to a large number of serum proteins and enzymes, showed no interaction with CaBP and, of the total CaBP applied to the column, at least 80% was

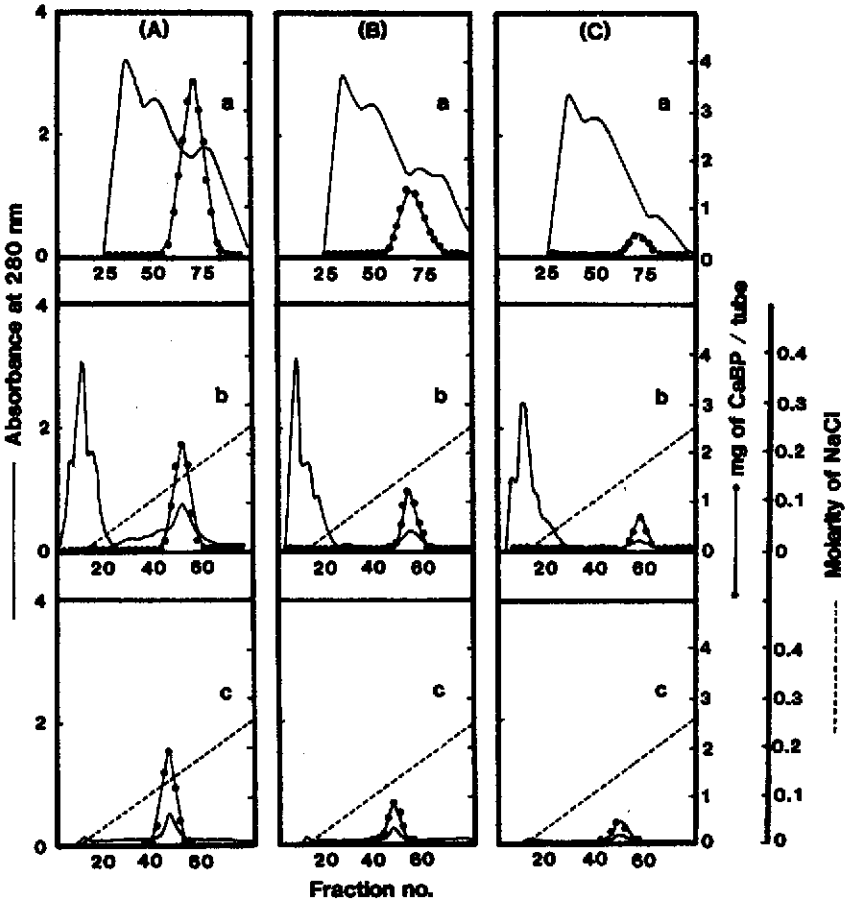

Fig. 1. Chromatograms for the purification of CaBPs from chick (A) intestine, (B) kidney and (C) cerebellum by successive gel filtration and DEAE-Sephacel chromatography in the absence or presence of calcium chloride. (a) Gel filtration on Sephadex G-100; (b) DEAE-Sephacel chromatography in the absence of calcium chloride; (c) DEAE-Sephacel chromatography in the presence of calcium chloride.

recovered. Further, this step provided a useful separation of CaBP from high-molecular-weight lipoproteins, peptides and salt. SDS-PAGE of the CaBP fraction also indicated that large amounts of proteins, including albumins and globulins, were effectively trapped in the column and thereby removed from the original homogenate.

Subsequently, heat treatment of the pooled CaBP solution at 60°C for 20 min was carried out with addition of 1 m*M* dithiothreitol to prevent the polymerization of CaBP. Approximately 30% of non-CaBP proteins in the original solution were denatured and precipitated after heat treatment and centrifugation without significant loss of CaBP. Heat treatment was therefore useful in removing the heat-labile non-CaBP proteins from the solution because CaBP is stable up to 70°C[21]. In addition, CaBP in the heat-treated solution was found to be relatively stable on prolonged storage at 4°C and at −20°C, although we have no evidence that the stability of CaBP attendant on heat treatment is due to the denaturation of protease(s) specific for CaBP.

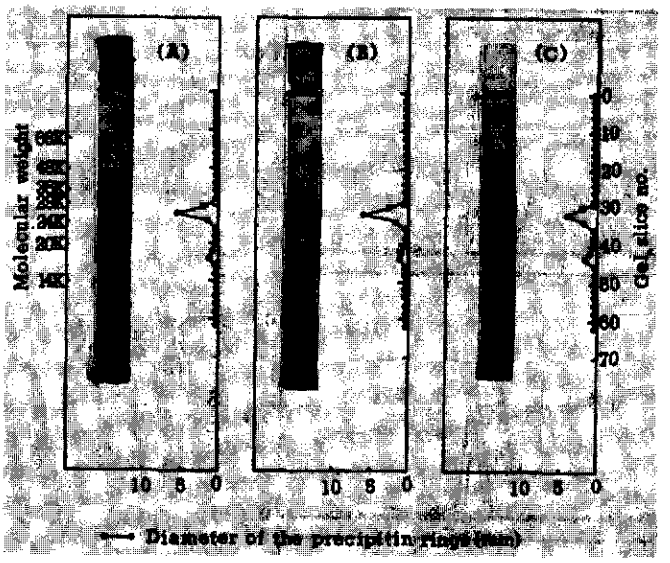

Fig. 2. SDS-PAGE of purified CaBPs isolated from chick (A) intestine, (B) kidney and (C) cerebellum by successive gel filtration and DEAE-Sephacel chromatography in the absence or presence of calcium chloride. Approximate 25 μg of protein were applied to each gel, electrophoresed and identified by staining and immunodiffusion as described under Experimental.

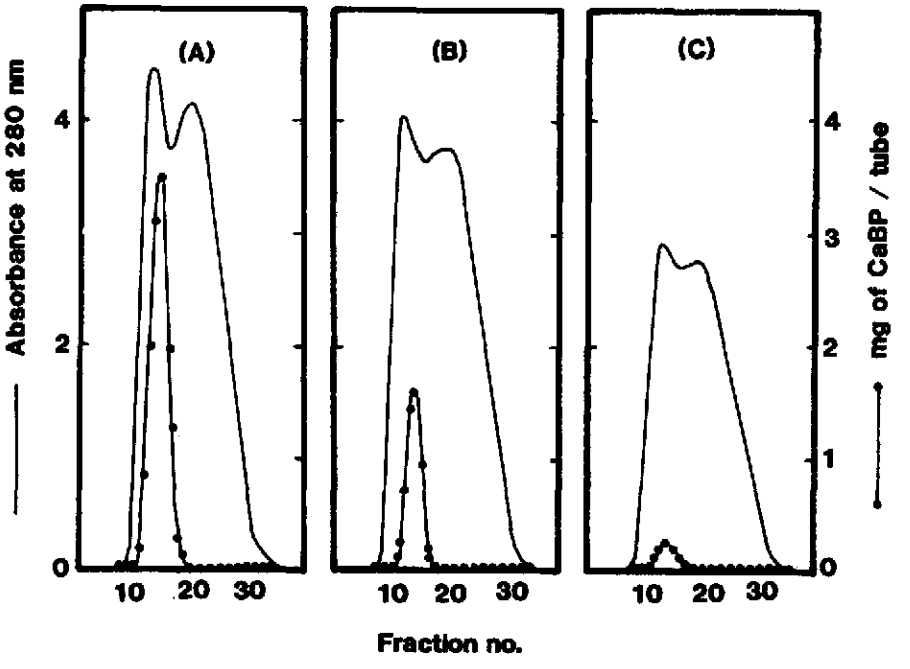

Fig. 3. Chromatography of chick (A) intestine, (B) kidney and (C) cerebellar homogenates on Blue Sepharose CL-6B.

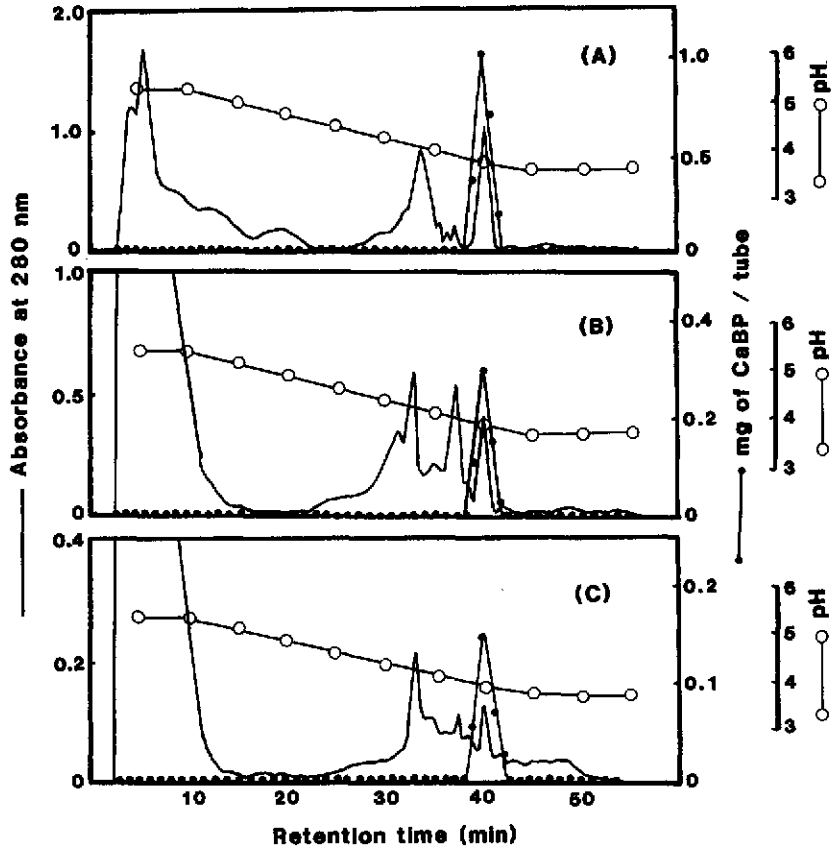

Fig. 4. Purification of chick (A) intestinal, (B) kidney and (C) cerebellar CaBPs by chromatofocusing. The Blue Sepharose CL-6B CaBP eluate was heated at 60°C for 20 min, centrifuged, then dialysed against 20 m*M* N-methylpiperazine (pH 5.7) and 2–8 ml were injected into a Mono P column.

The combination of Blue Sepharose CL-6B and heat treatment is a useful procedure for partially purifying CaBP, as there is a substantial decrease in the amount of protein to be applied to the chromatofocusing column. We also found that heat treatment was more effective than addition of protease inhibitors such as TLCK, TPCK and PMSF, particularly with intestine samples, to improve the stability of CaBP.

The characteristic separation of CaBP from other proteins in the heat-treated solutions from the three tissues by chromatofocusing on a Mono P column is shown in Fig. 4. The peak for CaBP was identified from the retention time (apparent pH value) by comparison with a standard intestinal CaBP and recoveries of CaBP from the column were determined by rocket electroimmunoassay. Chromatofocusing with a linear pH gradient from 5.7 to 3.7 was reproducible and provided a good separation without any detectable cross-contamination from other proteins in a given peak on the chromatogram (Fig. 5). As little as 10 μg of CaBP (as the pure protein) could be

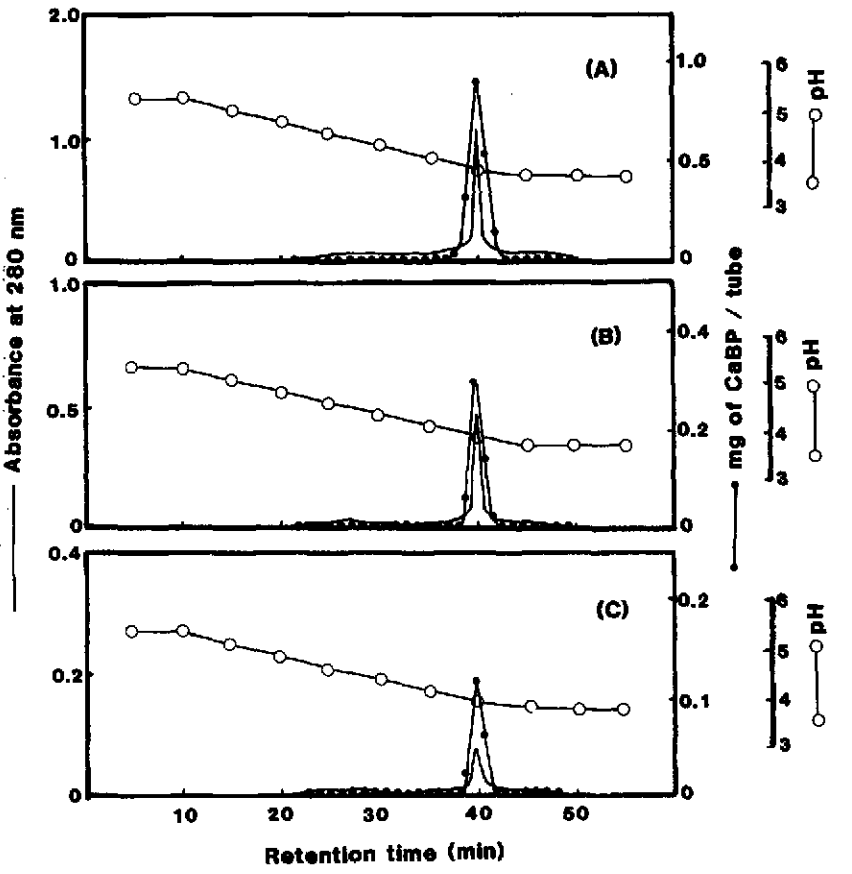

Fig. 5. Rechromatofocusing of chick (A) intestinal, (B) kidney and (C) cerebellar CaBPs obtained from the first chromatofocusing. The first chromatofocusing CaBP fraction was restored to pH 5.7 by passage through a Sephadex G-75 column (50 × 1.5 cm I.D.) equilibrated with 20 m*M* N-methylpiperazine buffer (pH 5.7), then applied to a Mono P column under the same conditions as before.

detected. The protein gave nearly a single band in SDS-PAGE (Fig. 6) and a single peak in anion-exchange chromatography (Mono Q) with and without Ca^{2+} (Fig. 7). Gel filtration chromatography calibrated with seven standard proteins showed that the CaBPs from chick intestine, kidney and cerebellum have the same molecular weight of 28 000. With this overall method, the recoveries of CaBP were from chick intestine 40–55%, from kidney 30–45% and from cerebellar homogenates 46–57%, as shown in Table I.

DISCUSSION

Recent developments in research into vitamin D-dependent CaBP have shown that this protein exists in two forms differing in their molecular weights (28 000 and 10 000)[1,22–24]. Current procedures[12–15] employed for the separation and purification of these proteins involve the use of gel filtration chromatography, ion-exchange

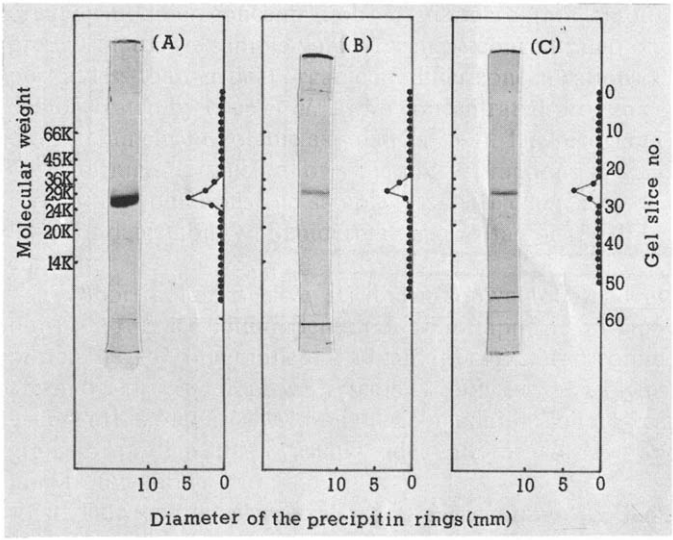

Fig. 6. SDS-PAGE of purified CaBPs isolated from chick (A) intestine, (B) kidney and (C) cerebellum by successive Blue Sepharose CL-6B chromatography, heat treatment and chromatofocusing. Approximately 20 μg of protein were applied to each gel, electrophoresed and identified by staining and immunodiffusion as described under Experimental.

chromatography and preparative discontinuous polyacrylamide gel electrophoresis. Ion-exchange column chromatography, introduced by Hitchman and Harrison[12], which takes advantage of an intrinsic charge difference in the molecule of CaBP with and without bound Ca^{2+}, has been widely accepted for the purification of either 28 000 or 10 000 M_r CaBP. As shown in Fig. 1, the combination of gel filtration chromatography and ion-exchange chromatography with and without Ca^{2+} is useful for purifying CaBP from chick intestine, kidney and cerebellum. However, we have found that this technique required several days for completion and also it had poor reproducibility and low yield. The recovery of the protein varied from 7 to 35% with chick intestine. This method is only effective when large amounts of proteins are available for purification. Therefore, brain and other tissues, having relatively small amounts of CaBP, need another effective purification procedure.

TABLE I

PREPARATION OF CaBPs FROM CHICK INTESTINE, KIDNEY AND CEREBELLUM

Step	*Yield of CaBP (mg)*		
	Intestine	*Kidney*	*Cerebellum*
Original supernatant	12.7	4.7	1.5
Blue Sepharose CL-6B	9.5–12.3	3.4–4.1	1.2–1.3
Heat treatment	7.7–9.2	2.3–2.7	1.1–1.2
1st chromatofocusing	6.4–7.7	1.7–2.1	0.8–1.0
2nd chromatofousing	5.1–7.0	1.4–2.1	0.7–0.9
Recovery (%)	40–55	30–45	46–57

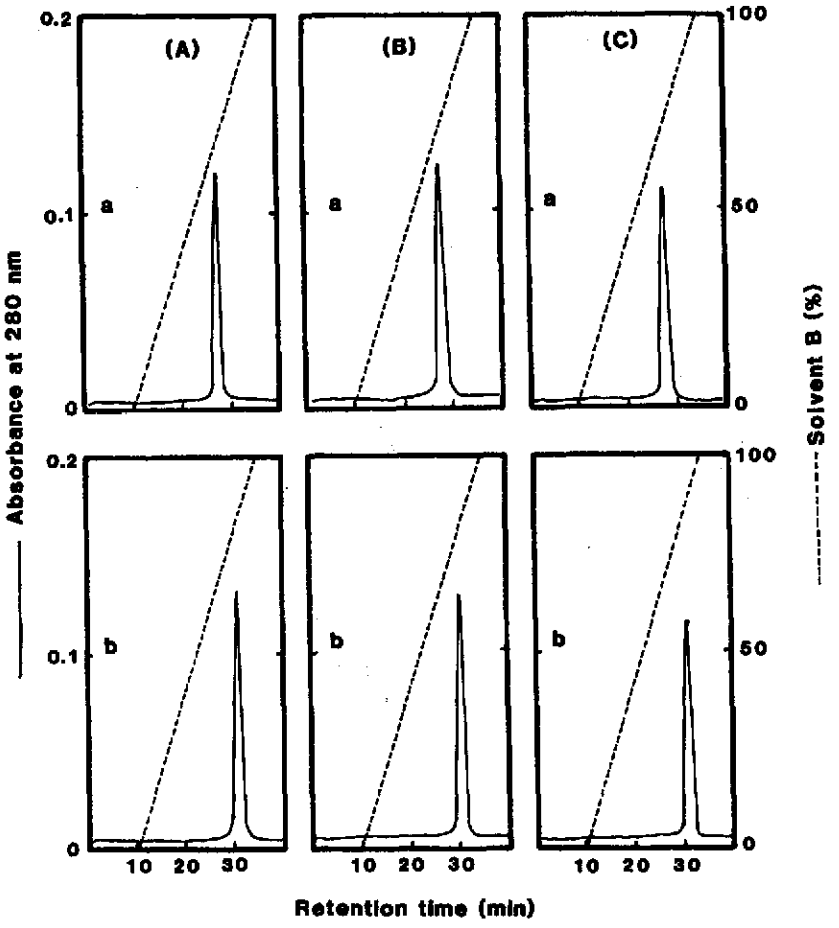

Fig. 7. Ion-exchange HPLC behaviour of (A) intestinal, (B) kidney and (C) cerebellar CaBPs in (a) the presence or (b) the absence of calcium chloride. Elution of proteins was carried out in a linear gradient of sodium chloride from 50 m*M* (solvent A) to 700 m*M* (solvent B) in piperazine buffer (25 m*M* piperazine, pH adjusted to 6.5 with hydrochloric acid) in the presence or absence of 2 m*M* calcium chloride at a constant flow-rate of 1 ml/min.

Our purpose in this study was to establish a simple purification method for CaBP that can be performed with good resolution and reproducibility, and involving minimal manipulation with routine apparatus. We used a combination of three purification: Blue Sepharose CL-6B chromatography, heat treatment and chromatofocusing. As the retention time (apparent pH value) of CaBP on the chromatofocusing trace was highly reproducible, it may be possible in future work to isolate CaBP from relatively crude extracts if care is taken to prevent proteolysis, *e.g.*, by heat treatment.

ACKNOWLEDGEMENTS

We thank Mrs. M. Harding and Mr. P. W. Wilson for the supply of chick CaBP antiserum and their thoughtful discussion and expert advice.

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