Chem. Pharm. Bull. 26(6)1915—1918(1978)

UDC 547.964.05 : 611.36.08

Purification of Calcium Binding Substance from Soluble Fraction of Normal Rat Liver

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(Received November 2, 1977)

A calcium binding substance has been isolated from the soluble fraction of normal rat liver. The calcium binding substance was purified by means of gel filtration on Sephadex G-75 and G-50 followed by ion-exchange chromatography on diethylamino-ethyl-cellulose. By these methods, the calcium binding activity from the soluble fraction has been purified several 100-fold and the homogeneity of the calcium binding substance has been established by polyacrylamide gel electrophoresis.

Keywords——liver calcium; calcium binding protein in liver; isolation of calcium binding substance; normal rat liver; calcium metabolism

Introduction

It is well known that a calcium binding protein is synthesized in the intestine of mammals when exposed to vitamin D.[2,3] Also, calcium binding protein has been identified in three other tissues which there is a large flux of calcium, the kidney,[4] parathyroid glands,[5] and eggshell gland of laying hens.[6] The physiological function of calcium binding protein remains unknown, but its high concentration in the gut, kidney, parathyroid and eggshell glands is suggested that it may be involved in transcellular transport and storage of calcium.[7]

Recently, on the basis of fact that the calcium concentration in the liver of rats is increased by calcitonin which has a hypocalcemic effect,[8] we found that a calcium binding activity is exhibited in the soluble fraction from normal rat liver.[9] Presumably it was suggested that calcium binding protein may be involved in the transport of calcium in the liver cells. This paper describes the isolation of a calcium binding substance in the soluble fraction from a normal rat liver.

Materials and Methods

Animals——Male Wistar strain rats, weighing approximately 100 g, were obtained from the Nippon Bio Supp. Center Co., Tokyo. They were kept at room temperature of 25° ± 1° and fed lab. chow (containing 7.4% carbohydrate, 1.1% Ca, and 1.1% P, Oriental Test Diet Co., Tokyo) and tap water freely. After one week on this diet animals were killed by decapitation.

Preparation of Soluble Fraction——The livers were perfused with Tris-HCl buffer (pH 7.4, containing 0.1 M Tris, 0.12 M NaCl, 0.004 M KCl, cooled to 4°). The liver was removed, cut into small pieces, suspended 1: 4 in Tris-HCl buffer (pH 7.4) and homogenized in a Potter–Elvehjem homogenizer with a Teflon pestle. The homogenate was spun at 1800 × *g* in a refrigerated centrifuge for 10 min and the supernatants were

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spun at 105000×*g* for 60 min. The supernatants from the latter was collected and heated at 60° for 10 min. The solution was then cooled and recentrifuged at 38000×*g* for 20 min. The resulting supernatant fraction contained essentially all the calcium binding activity measured in the homogenate. All extraction and subsequent isolation procedures, including assays for calcium binding activity, were carried out at 4°.

Gel Filtration——The supernatant fraction was concentrated to less than 20 ml by the method of frozen dry for 12 hr. The concentrated material was clarified by centrifugation and was then chromatographed on Sephadex G-75 (superfine grade). Fractions were assayed for calcium binding activity and for protein by the method of Lowry *et al.*[10] with crystalline bovine serum albumin as standard. Fractions with a calcium binding activity greater than 15% ^{45}Ca bound/ml were pooled, and then chromatographed on Sephadex G-50 (superfine grade).

Ion-Exchange Chromatography——Fractions having calcium binding activity greater than 10% ^{45}Ca bound/ml of fraction after gel filtration on Sephadex G-50 were pooled and desalted by passage through a 2.5×80 cm column of Sephadex G-25 equilibrated with 0.01 M Tris-HCl (pH 7.3). The desalted protein fraction was then applied to a column of diethylaminoethyl (DEAE)-cellulose and was eluted by the linear gradient of Tris-HCl (pH 7.3) concentration.

Polyacrylamide Disc Gel Electrophoresis——Disc gel electrophoresis was performed according to Davis.[11] The electrophoretic system was as follows: 10% acrylamide stacking gels (pH 6.7); 30% acrylamide separating gels (pH 8.9); and a Tris-glycine electrode buffer (pH 8.3). Samples were layered on top of the stacking gels in volumes of 0.2 ml as 20% sucrose solutions. Electrophoresis was carried out at 2 mA/tube for 45 min. Upon completion of the run, the gels were stained in 1% Amido Schwarz in 7% acetic acid and destained in 7% acetic acid. Gels were photographed in 12×110 mm Pylex culture tubes.

Calcium Binding Assay——Assay for calcium binding activity was based on the competitive-binding Chelex-100 method of Wasserman and Taylor.[2] Samples were added to test tubes in volumes of 1.0 ml, followed by 0.2 ml of Chelex in suspension. $^{45}CaCl_2$ (0.5 μCi) was added, the contents of the tubes were vigorously mixed for 20 sec, and the tubes were then centrifuged at 600×*g* for 10 min. The ^{45}Ca radioactivity in 0.2-ml aliquots of the supernatant was determined by liquid scintillation spectrophotometry.[12] Channel ratio values indicated that quench corrections were not required. Then the calcium binding activity, in units of % supernatant ^{45}Ca, could be calculated from the expression $[(S)-(B)]100\%/(T)$ where S, B, and T are the supernatant, blank, and total ^{45}Ca, respectively.[13]

Results and Discussion

The calcium binding activity existed in the soluble fraction from a normal rat liver was increased approximately 4-fold by heat treatment.[9] By gel filtration on Sephadex G-75, the supernatant after heat treatment was resolved into three components having calcium binding activity, after the bulk of the total soluble protein (Fig. 1). The component having the highest specific calcium binding activity appeared in a region which eluted at 1.5 void volumes. The calcium binding activity of the peak fractions ranged between 15 and 25% ^{45}Ca bound ml of fraction, and these fractions were pooled.

A second gel filtration, on Sephadex G-50, of the calcium binding substance fraction obtained by chromatography on Sephadex G-75 resulted in a slight increase in specific calcium binding activity (Fig. 2). Maximal calcium binding activity fraction eluted on the leading edge of the protein peak was collected and desalted by passage through a Sephadex G-25 column.

The protein fractions having the highest specific calcium binding activity obtained from several separate extractions were pooled and applied to a column of DEAE-cellulose. The calcium binding substance was eluted by the linear gradient of Tris-HCl (pH 7.3) concentration (0.01—0.10 M). Figure 3 shows a typical elution profile of calcium binding activity from the DEAE-cellulose column. The third peak having the calcium activity was eluted after an increase to approximately 0.05 M.

On disc gel electrophoresis of the protein fraction applied to the DEAE-cellulose column and of the eluted protein peak having the highest specific calcium binding activity, the calcium

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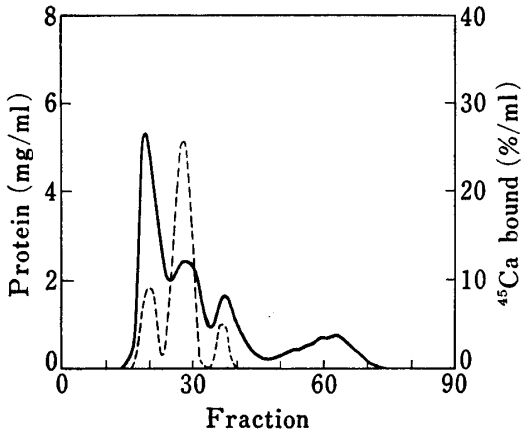

Fig. 1. Sephadex G-75 Chromatogram of Concentrated Tris-Soluble Fraction of Rat Liver Homogenate

Column size, 2.5×60 cm; buffer eluate, Tris-NaCl-KCl; flow rate, 0.46 ml/min; fraction size, 5.0 ml. Protein concentration (———), calcium-binding activity, % ^{45}Ca bound/ml as measured in the Chelex-100 assay (------) (see Methods).

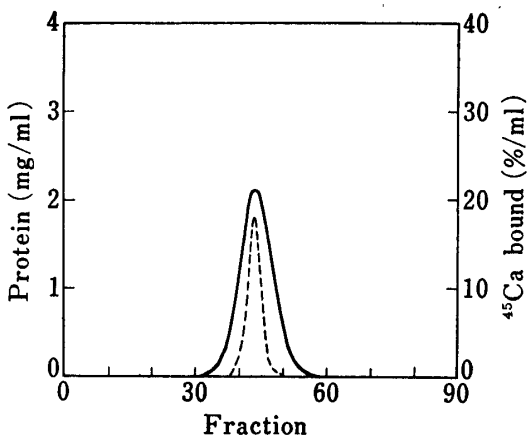

Fig. 2. Sephadex G-50 Chromatogram of Pooled Fractions from Sephadex G-75 Chromatography (Fig. 1)

Column dimensions, buffer eluate, and flow rate (0.12 ml/min) as described in Fig. 1; ordinate and abscissa are marked as in Fig. 1.

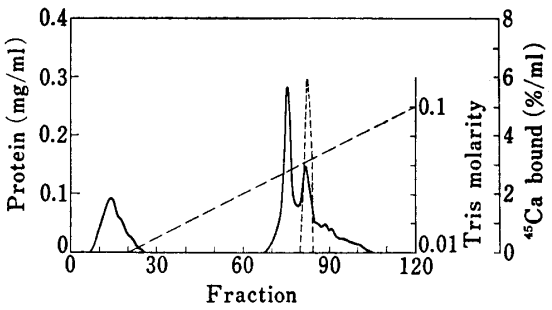

Fig. 3. DEAE-cellulose Chromatogram of Calcium-Binding Substance Fraction obtained by Sequential Gel Filtration on Sephadex G-50 and Sephadex G-75 Fine

Column size, 1×20 cm; flow rate, 0.25 ml/min; fraction size, 2.0 ml. Sample was applied in a volume of 30 ml. The adsorbed protein was eluted from the column with a 0.01—0.1 M linear Tris-buffer (pH 7.3) gradient. Ordinate and abscissa are marked as in Fig. 1.

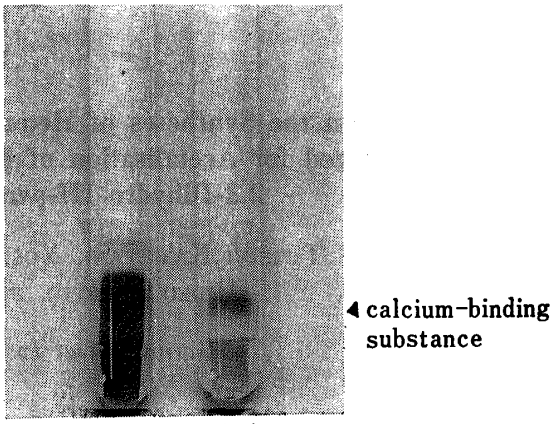

Fig. 4. Polyacrylamide Disc Gel Electrophoresis of Calcium-Binding Substance in Fraction Applied to DEAE-cellulose and of Fraction Eluted

Arrow indicates probable calcium-binding substance band. Migration was toward the cathode (bottom).
(A); applied fraction, 118.3 μg.
(B); calcium-binding fraction (Number 82), 39.6 μg.

binding substance appeared to be essentially homogeneous (Fig. 4). The calcium binding substance was migrated to the *Rf* range of 0.17.

The increase in specific calcium binding activity and recoveries of calcium binding activity per step achieved during the purification of the calcium binding substance are summarized in Table I. The overall recovery of calcium binding activity present in the initial soluble extract after heat treatment was approximately 17%. The relative specific calcium binding activity increased about 278-fold.

In the present study, a calcium binding substance has been purified from the soluble fraction of normal rat liver. Chromatography on DEAE-cellulose permitted isolation of calcium binding substance in an essentially homogeneous form. The highly purified calcium

Table I. Increase in Specific Activity of Calcium-Binding Substance with Fractionation[a)]

Fraction	Specific activity[b)]	Relative specific activity	Calcium-binding activity (% recovery/step)
Heated supernatant	0.89	1.0	100
Sephadex G-75 (superfine)	10.62	11.9	50.2
Sephadex G-50 (superfine)	13.00	14.6	41.5
DEAE-cellulose	247.69	278.3	17.2

a) Fractions were assayed by the Chelex method described in text; for each chromatogram, the activity in the peak tube was used for the calculation of specific activity. The percentage recovery per step is based on each preceding step.
b) As % ^{45}Ca bound/mg of protein.

binding substance in rat liver has a specific calcium binding activity comparable to that of the calcium binding protein isolated from rat intestinal mucosa.[13)] What possible physiologic role the calcium binding substance may play in the liver remains to be investigated. It seems likely that the calcium binding substance is involved in calcium transport in the liver cells.

[Chem. Pharm. Bull. 26(6)1918—1922(1978)]

UDC 547.756.04 : 547.299.04

Studies on the Syntheses of Heterocyclic Compounds. DCCLIV.[1)] A Novel Method for Acetalisation of Formyl Group at the C_3-Position of 2,3-Dihydro-1H-pyrrolo[1,2-*a*]indole Skeleton

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(Received November 4, 1977)

Reaction of 2,3-dihydro-7-methoxy-6-methyl-1H-pyrrolo[1,2-*a*]indole-9-carboxaldehydes (**2**), (**3**), (**4**), (**5**), and (**6**) with thiolacetic acid in the presence of 6 N sulphuric acid at room temperature gave 9-diacetylthiomethyl-2,3-dihydro-7-methoxy-6-methyl-1H-pyrrolo[1,2-*a*]indoles (**12**), (**13**), (**14**), (**15**), and (**16**), respectively. The same reaction of the compound (**2**) at 0° afforded 1-acetoxy-9-diacetylthiomethyl-2,3-dihydro-7-methoxy-6-methyl-8-nitro-1H-pyrrolo[1,2-*a*]indole (**11**). Successive treatment of the compound (**11**) with absolute methanol in the presence of sodium methoxide gave 2,3-dihydro-1-hydroxy-7-methoxy-9-dimethoxymethyl-6-methyl-8-nitro-1H-pyrrolo[1,2-*a*]indole (**17**).

Keywords——acetalisation; 1H-pyrrolo[1,2-*a*]indole-9-carboxaldehydes; thioacetic acid; diacetylthiolation; mitomycins

Regarding the synthesis of the mitomycins[3)] it is necessary to develop a general method for the protection of a formyl group at the C_3-position of an indole skeleton, because the formyl

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