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PURIFICATION OF PLANT CALMODULIN BY FLUPHENAZINE-SEPHAROSE AFFINITY CHROMATOGRAPHY

Harry Charbonneau and Milton J. Cormier

Bioluminescence Laboratory, Department of Biochemistry

University of Georgia, Athens, GA 30602

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SUMMARY: The calcium-dependent binding of phenothiazine drugs to calmodulin (Levin, R. M. and Weiss, B. (1977) Mol. Pharmacol. 13, 690-697) has been utilized to develop a rapid purification procedure for calmodulin based on fluphenazine-Sepharose affinity chromatography. Calmodulin from plants, a fungus, porcine brain and the coelenterate, Renilla reniformis, were easily purified by the calcium-dependent binding of calmodulin to fluphenazine-Sepharose.

Calmodulin, a low molecular weight Ca^{2+} binding protein (Mr = 16,700), was first discovered as an activator of cyclic nucleotide phosphodiesterase (1,2). More recently calmodulin has been found to activate several other enzymes including brain adenylate cyclase (3,4), (Ca^{2+} + Mg^{2+}) ATPase (5,6,7), several protein kinases (8,9,10), NAD kinase from plants (11), and phosphorylase kinase (12), and to participate in the control of cellular processes such as the phosphorylation of membrane proteins (13), and disassembly of microtubules (14). Calmodulin activity has been found in many species from both the plant and animal kingdoms (11,15,16,17) suggesting that this protein is ubiquitous among the eucaryotes. Since calmodulin possesses the ability to modulate a large number of cellular functions many investigators have suggested that calmodulin may be a common element in the control of a wide variety of Ca^{2+}-mediated cellular events in eucaryotes.

It has recently been reported that the antipsychotic phenothiazine drugs have the ability to inhibit several calmodulin activated systems (18,19,20,21) through the Ca^{2+}-dependent binding of these drugs to calmodulin. Levin and Weiss (19,22) have shown that this binding is highly specific for calmodulin and that trifluoperazine binds to two Ca^{2+}-dependent high affinity sites (K_d = 1 μM) and to approximately 24 low affinity Ca^{2+}-independent sites (K_d = 5 mM).

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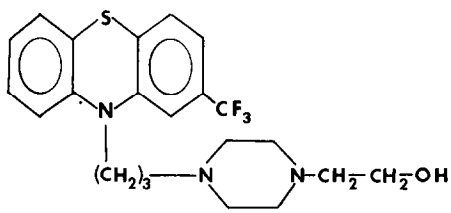
We report here the utilization of the phenothiazine, fluphenazine (see structure below), in an affinity chromatography application for the isolation of calmodulins from a variety of tissue sources including plants and fungi. A similar affinity chromatography procedure is reported by Jamieson and Vanaman (23) in the accompanying paper.



MATERIALS AND METHODS: The bisoxirane, 1,4-butanediol diglycidyl ether was obtained from Aldrich Chemical Co. Baker reagent grade urea was deionized immediately before use by passing a 10 M solution over a column of Rexyn 300 ion exchange resin (Fischer Scientific Co.). Other reagents used were the best grades commercially available. Fluphenazine·2HCl was kindly provided by E. R. Squibb and Sons, Inc. Trifluoperazine was a generous gift of Smith, Kline and French Laboratories.

Shelled peanut seeds (Arachis hypogea) and fresh mushrooms (Agaricus bisporus) were obtained from local venders. Pea seedling (Pisum satavum L.) calmodulin was obtained in partially purified form utilizing the method of Anderson and Cormier (11). Porcine brains were obtained from local slaughter houses and were stored at -80°C. Pure bovine brain calmodulin was a gift of Dr. T. C. Vanaman, Duke University.

Calmodulin was assayed on the basis of its ability to stimulate the activity of calmodulin-dependent porcine brain phosphodiesterase using the firefly bioluminescence assay described by Matthews and Cormier (25). One unit of calmodulin was defined as the quantity of protein giving 50% maximal stimulation of calmodulin dependent-phosphodiesterase. In the absence of calmodulin, the phosphodiesterase solution (pH 8.0) hydrolyzed 35 picomoles of cAMP per min at room temperature. Calmodulin-dependent phosphodiesterase used in these assays was prepared from porcine brain by the procedure of Egrie (26).

SDS gel electrophoresis was performed on 15% slab gels according to the procedure of Laemmli (27) except that the sample contained 15 mM EGTA and all buffers contained 1.5 mM EGTA. Alkaline discontinuous gel electrophoresis was done on 15% slab gels employing the method described by Gabriel (28). All gels were stained in a solution of 0.2% Coomassie Blue R-250 in 50% methanol, 10% acetic acid for 1 hr and destained in 20% ethanol, 10% acetic acid.

Preparation of Fluphenazine-Sepharose - Fluphenazine was coupled to Sepharose 4B using the bisoxirane method described by Sundberg and Porath (29). Utilizing this method fluphenazine was attached to the agarose matrix via a 12 atom spacer provided by the diglycidyl ether coupling reagent. The following is a description of the activation and coupling procedures used in these studies: Sepharose 4B was washed with deionized water on a glass filter and suction-dried under vacuum before use. The activation reaction mixture was prepared by mixing 100 g of

suction-dried Sepharose 4B with 100 ml of 0.6N NaOH containing 3 mg/ml sodium borohydride and 100 ml of diglycidyl ether. This mixture was incubated at room temperature with gentle stirring. After 8 hr, the gel was washed with 6-8 liters of deionized water. Immediately following washing, the coupling reaction was performed by mixing the activated Sepharose with 160 ml of 0.1 M carbonate buffer, pH 11.0 containing 404 mg of Fluphenazine-2HCl. This reaction mixture was heated with gentle stirring for 48 hr at 70°C. Since fluphenazine is relatively insoluble in aqueous solution above pH 6-7, the reaction mixture described above was a cloudy suspension containing insoluble drug. Efforts to achieve more effective coupling with either 90% DMF or 50% acetone were not successful. After coupling, the Sepharose mixture was washed on a glass funnel with 1-2 liters of acetone to remove any unreacted fluphenazine. The acetone wash was followed by a wash with one liter of absolute ethanol. During this wash, the gel was exposed to absolute ethanol for at least 30 min to allow any excess oxirane groups to react with the ethanol. During the coupling procedure, the reaction mixture containing fluphenazine was covered with aluminum foil whenever possible in order to reduce exposure to light since the compound is photosensitive.

Quantitative estimates of fluphenazine binding were based on the measurement of fluphenazine by its visible fluorescence. Known quantities of fluphenazine-Sepharose were hydrolyzed in 6N HCl for 24 hr. The supernatant was adjusted to pH 10 with NH_4OH and the fluphenazine was extracted with methylene chloride. After drying, the samples were dissolved in 2N HCl and the fluorescence emission at 485 nm (uncorrected) was determined. These measurements produced an estimate of approximately 5-10 μmoles of fluphenazine coupled per gram of suction-dried gel.

Fluphenazine-Sepharose suspended in 1 mM sodium azide was stored at 4°C in the dark for several months without any apparent degradation or loss of bound drug. To regenerate fluphenazine-Sepharose for repeated chromatographic use, the resin was washed thoroughly with 1 M NaCl, 10 mM EGTA, pH 8-9 followed by several volumes of acetone and deionized water. Although there was usually some discoloration and possible decomposition of the bound fluphenazine when crude homogenates were used, there were only minor losses in gel capacity after multiple uses. We have found that fluphenazine immobilized on Sepharose is quite stable providing precautions are taken to protect the gel from prolonged light exposure.

Purification of Peanut Calmodulin - One kilogram of shelled peanuts was homogenized for 2 min at maximum speed on a Tekmar model SD-45 in 2 liters of 50 mM Tris, pH 8.0. This extract was filtered under vacuum through a layer of cheese cloth on top of Mirracloth (Chicopee Mills) on a large funnel. The residue remaining on the cheese cloth was squeezed by hand to remove any remaining fluid. This filtered material was immediately heated to 85°C for 2 min, cooled, and centrifuged for 20 min at 7500 x g at 4°C.

The supernatant from this extraction was then fractionated with (95%) ethanol. This crude extract was mixed with ethanol (1.1 volume per volume of extract) and stirred at 4°C for approximately 10 min before centrifugation at 7500 x g for 20 min. The supernatant was collected and 1.45 volume of ethanol per volume of supernatant was added. After stirring for 10 min in the cold, this mixture was centrifuged at 7500 x g for 20 min. This pellet was suspended in 10 mM Hepes, 0.5 mM $CaCl_2$, pH 7.0 and dialyzed extensively against the same buffer at 4°C. The precipitate formed during dialysis was removed by centrifugation at 13,000 x g for 30 min at 4°C.

This dialyzed sample was loaded on a 150 column (3 x 19.8 cm) containing fluphenazine-Sepharose equilibrated with 10 mM Hepes, 0.5 mM $CaCl_2$, pH 7.0.

After loading, the column was washed with approximately 100 ml of column buffer. This wash was followed with 0.5 M NaCl, in column buffer (approximately 2.5-3.5 liters). This salt wash was continued until the absorbance at 280 nm was less than 0.005. Peanut calmodulin was eluted with a pulse of 10 mM Tris, 5 mM EGTA, pH 8.0. All fluphenazine-Sepharose chromatography was performed at room temperature. Tris, Hepes, and Mes buffers were all used successfully as column buffer at a concentration of 10 mM over a pH range of 6.5 to 7.2

Purification of calmodulin from other sources - Modifications of the fluphenazine-Sepharose purification procedure described above have also been used for the purification of calmodulin from porcine brain, a coelenterate, Renilla reniformis, and a fungal source, the mushroom (Agaricus bisporus). Mushroom calmodulin was purified using the following modifications: a) In the first step, mushrooms (1 kg/2 liters) were homogenized in buffer pre-heated to 100°C and centrifuged as described. b) Before the ethanol precipitation was performed the supernatant was treated with 50% ammonium sulfate, pH 4.0 and centrifuged. The precipitate was suspended and dialyzed against 50 mM Tris, pH 8.0. The only modification used for porcine brain preparations was the use of 700 ml of 9 M Urea (deionized), 50 mM Tris, 15 mM 2-mercaptoethanol, pH 8.0 as the extraction buffer for 100 g of tissue and the deletion of the heat step. Renilla reniformis calmodulin was purified by performing the first 3 steps described in the purification procedure of Jones et al. (24) and then subjected to fluphenazine-Sepharose chromatography as described.

A simple procedure for the preparation of porcine brain calmodulin under non-denaturing conditions has also been developed. An extract of porcine brain was prepared by homogenizing 100 g of brain in 500 ml of 50 mM Hepes, 0.5 M NaCl, 5 mM $CaCl_2$, pH 7.0. The soluble supernatant was obtained by centrifugation of the homogenate at 100,000 x g for 1 hr in a Beckman L-2 ultracentrifuge (4°C). This extract was loaded directly onto a 150 ml fluphenazine-Sepharose column and washed with 1.5 liters of 0.5 M NaCl in column buffer prior to elution of homogenous calmodulin with 10 mM Tris, 10 mM EGTA pH 8.0.

RESULTS AND DISCUSSION: In the presence of appropriate buffers containing 0.5-1 mM $CaCl_2$ both crude and homogenous preparations of calmodulin were bound to fluphenazine-Sepharose columns. Sources of calmodulin tested have included purified bovine brain and partially purified extracts of porcine brain, peanuts, mushrooms, pea seedlings, and Renilla reniformis. With all samples tested, there was complete binding of all calmodulin present as evidenced by the absence of calmodulin activity in the column eluate. With pure porcine brain calmodulin, fluphenazine-Sepharose had a total capacity of 3 mg/ml of packed gel. With crude or partially purified preparations, the column was washed with a large volume of column buffer containing 0.5-1.0 M NaCl in order to remove all non-specifically bound contaminating proteins. Following this high salt wash, calmodulin was eluted with 10 mM Tris, 5-10 mM EGTA, pH 8.0.

Figure 1 illustrates a typical elution profile for the chromatography of calmodulin on fluphenazine-Sepharose. As shown some calmodulin activity elutes

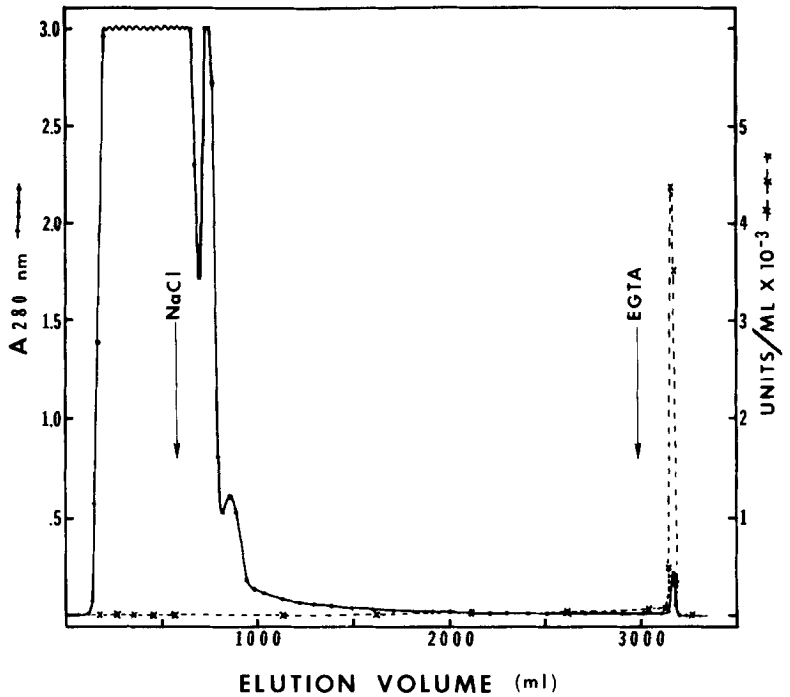


Figure 1. Fluphenazine-Sepharose chromatography of peanut calmodulin. A partially purified sample of peanut calmodulin from step 3 was chromatographed on a 150 ml column (3 x 19.8 cm) as described in the text. Fractions were collected immediately following loading of the sample and assayed for calmodulin activity.

during the salt wash. In both the porcine brain and peanut calmodulin preparations approximately 10-20% of the calmodulin loaded was found in this salt wash. In the case of the porcine preparation about 55% of the total calmodulin activity loaded was recovered in the fractions eluted with EGTA. With peanut calmodulin, only 20-30% of the total activity was found in the EGTA fraction. Thus approximately 75% of the porcine and approximately 50% of the peanut calmodulin activity was accounted for after fluphenazine-Sepharose chromatography.

These results suggest that some fraction of the calmodulin loaded was irreversibly bound to the column or that there were materials other than calmodulin in these extracts which are capable of stimulating porcine phosphodiesterase. Another explanation could involve the aggregation of calmodulin. Aggregation has been observed in preparations of peanut and all other calmodulins utilized in our laboratory and could account for the variations observed in the specific activities

for peanut calmodulin which have been found to range from approximately 10,000 units/mg to 60,000 units/mg. If the aggregated forms of calmodulin are of relatively low activity, then preparations with a large portion of aggregates would be expected to exhibit low specific activity. The fact that preparations showing maximal specific activity have all had aggregates removed by gel filtration is consistent with this possibility. Incomplete recoveries, based on activity, would be expected if the extent of aggregation was greater in the EGTA fractions than in the crude samples loaded.

Attempts to purify peanut calmodulin by conventional methods presented many problems. Thus the affinity chromatography technique reported here greatly facilitated efforts to purify calmodulin from plants. As shown in Table I, this protein was purified 830-fold with an overall recovery of 16%. Based on the specific activity of 8,000 units/mg, the heat-treated crude extract contains approximately 42 mg of calmodulin per kg of dry peanut seed. Preparations of protein obtained by this procedure were considered homogenous because they migrated as a single band on both alkaline discontinuous and SDS polyacrylamide gels. This protein is considered to be a true calmodulin because it possesses physical and chemical properties such as molecular weight, acidic nature, heat stability, amino acid composition, absorption spectrum and biological activity (J. M. Anderson, et al., unpublished observations) which are similar to those of mammalian calmodulins (30,31). In addition, a highly purified protein with properties similar to peanut calmodulin has also been obtained from crude samples of pea seedlings by fluphenazine-Sepharose chromatography.

Fluphenazine-Sepharose chromatography has also been employed for the purification of calmodulin from a fungus (Agaricus bisporus), porcine brain, and a coelenterate Renilla reniformis. As outlined under Methods, porcine brain calmodulin has been extracted under both non-denaturing and denaturing conditions and purified to homogeneity by this procedure. Calmodulin from all these sources were bound to fluphenazine-Sepharose and eluted in exactly the same manner as peanut calmodulin. All preparations of calmodulin prepared by fluphenazine-

Table I. Purification scheme for calmodulin isolated from 5 kg of peanuts

Step	Volume ml	Protein mg	Total Activity x 10^{-5} units	Specific Activity units/mg	Purification	Yield
1. Heat-treated Crude Extract	4730	172,170[a]	16.6	9.6	--	100%
2. Ethanol Precipitation	275	2,650[a]	12.8	480	50	77
3. Concentration and Dialysis	440	2,336[a]	10.4	450	47	63
4. Fluphenazine-Sepharose	76	33.9[b]	2.7	8000	830	16

[a]Protein determined by biuret assay employing BSA as a standard (32).

[b]With homogenous preparations, protein values measured by biuret were low by a factor of 1.9 as determined from fringe displacement measurements (33). Thus this biuret value was accordingly corrected.

Sepharose chromatography showed physical and chemical properties which were similar to previously characterized mammalian calmodulins (30,31) including the ability to activate porcine brain phosphodiesterase. Both porcine brain and fungal calmodulin preparations showed a single band on SDS gels. In addition, porcine brain calmodulin prepared in this manner possessed an absorption spectrum and amino acid composition nearly identical to that published by Klee (30). Similar preparations of Renilla calmodulin (see Methods) contained one major contaminant which was seen as a more slowly migrating species on SDS gels. This unknown contaminant was separated from calmodulin by ion exchange chromatography.

The observations described above suggest that fluphenazine-Sepharose chromatography can be employed for the purification of calmodulin from any tissue source. This affinity chromatography method should facilitate efforts to purify calmodulin from tissues where it is present in relatively small quantities. Since the purification procedures described here can be accomplished in 2-3 days, it seems likely that many of the published purification schemes for calmodulin can be simplified through the use of fluphenazine-Sepharose chromatography.

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