Isolation and Characterization of Calmodulin and a Calmodulin-dependent Phosphodiesterase Inhibitor from Plants

A Thesis presented to
the Faculty of Graduate Studies
of the University of Manitoba

In Partial Fulfillment of
the Requirements for the Degree
of Master of Science

William A. Taylor

ISOLATION AND CHARACTERIZATION OF CALMODULIN AND A CALMODULIN-DEPENDENT PHOSPHODIESTERASE INHIBITOR FROM PLANTS

BY

WILLIAM A. TAYLOR

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ABSTRACT

Calmodulin, a Ca^{2+}-binding protein with multiple regulatory activities has been suggested to be a general Ca^{2+}-mediator in Eukaryotes. The wide distribution of calmodulin in plants is demonstrated in the present study on the basis of the following observations: (1), Among thirteen plant extracts examined, all except those of a fern (Nephrolepis exaltata) and a conifer (Larix decidua) were found to be capable of activating a calmodulin-dependent cyclic nucleotide phosphodiesterase from bovine brain. (2), The activation of phosphodiesterase was Ca^{2+}-dependent and reversible. (3), Several of the plant extracts were also found to activate rabbit skeletal muscle myosin light chain kinase. (4), Activation of these calmodulin dependent enzymes by the plant extracts could be reversed by the addition of calmodulin binding protein I; a bovine brain protein capable of undergoing Ca^{2+}-dependent and specific association with calmodulin. (5), For each plant extract that was subjected to disc gel electrophoresis, a single activity band was obtained with a relative mobility similar to that of mammalian calmodulin. These observations suggest that calmodulin is widely distributed in plants, and bears similar biological and molecular properties to vertebral and invertebral calmodulins.

Calmodulin has been purified from cabbage, spinach, cucumber and alfalfa. Each of these plant calmodulins are similar to the mammalian calmodulin from bovine brain on the

basis of gel electrophoresis in the presence of Ca^{2+}, amino acid composition, UV absorption spectrum, cyclic nucleotide phoshodiesterase activation, and cyanogen bromide peptide mapping. Some notable changes are observed in each of the aforementioned criteria, however.

Evidence is presented for a calmodulin dependent cyclic nucleotide phosphodiesterase inhibitor from fern (Polypodium aureum areolatum) leaves. The inhibitor has been partially purified by glycine-Sepharose 4B affinity chromatograhy. It is eluted from the affinity column using 6M urea, 1M NaCl, 20 mM Tris-HCl buffer (pH 7.0). It is not bound by CM-cellulose but binds very strongly to DEAE-cellulose. It is resistant to trypsin, pronase, DNase, and RNase treatment. Heating the inhibitor factor(s) at neutral pH or at 1 N HCl does not destroy the inhibitor activity while heating at 1 N NaOH results in a slight loss of activity. The inhibitor is stable to organic solvents such as ethanol, and chloroform, and is non-dialyzable.

The inhibitor demonstrates potent inhibition of the calmodulin activated form of the calmodulin-dependent cyclic nucleotide phosphodiesterase. Furthermore, the inhibition is not reversed by calmodulin.

The inhibitor is also a potent inhibitor of the trypsin-activated enzyme and the lysophosphatidyl choline-activated enzyme at low concentrations of lysophosphatidyl choline. At high concentrations of lysophosphatidyl choline (130 μM) , the inhibitor exhibits 2-fold stimulation of the enzyme. In addition, higher

concentrations of lysophosphatidyl choline (220 μM) can reverse the inhibition of the trypsin activated enzyme.

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1. INTRODUCTION

Cyclic nucleotide and Ca^{2+} are considered to be second messengers whose primary function is to regulate various cellular processes in response to external stimuli. It is now generally accepted that the effects of cAMP are mediated in animal cells by the action of a cAMP dependent protein kinase: an ubiquitous enzyme having multiple regulatory activities (1.2). On the other hand, Ca^{2+} appears to be mediated by a number of different Ca^{2+} binding proteins. For example, the regulation of skeletal or cardiac muscle contraction by Ca^{2+} is mediated by troponin (3) whereas the Ca^{2+} action in smooth muscle contraction appears to be regulated by the Ca^{2+} dependent myosin light chain kinase (4).

Within the last 10 years, a Ca^{2+} binding protein called calmodulin has been shown to be involved in the regulation of a number of mammalian enzymes, including a Ca^{2+} dependent cyclic nucleotide phosphodiesterase (5-7), brain adenylate cyclase (8,9), (Ca^{2+}, Mg^{2+})-ATPase from erythrocytes (10,11), myosin light chain kinase (12,13), a membrane Ca^{2+}-activated kinase (14) and phosphorylase kinase (15), and others (for review see references 16-19). In addition, the protein has been implicated in the regulation of microtubule assembly (20). Thus, this protein functions in a variety of cellular processes such as muscle contraction, cell motility, Ca^{2+} and cyclic nucleotide metabolism and glycogen metabolism. These

results strongly suggest that calmodulin is a general Ca^{2+} mediatory protein and that it plays a central role in the second messenger system in mammalian cells.

In addition to being a general Ca^{2+} mediatory protein in mammalian cells, calmodulin may also play central roles in the mediation of Ca^{2+} effects in lower phylogenetic forms. Waisman et al (21) have examined extracts of more than ten different invertebrates and found that they all contain a protein factor which is capable of Ca^{2+} dependent activation of the mammalian cyclic nucleotide phosphodiesterase. Such a protein factor from earthworm has been purified to a homogeneous state and chemical and physical characterizations have indicated that this Ca^{2+}-dependent protein is very similar to mammalian calmodulin (22). Although calmodulin-like activity is abundant in many of the invertebrates examined, Ca^{2+} dependent cyclic nucleotide phosphodiesterase does not appear to be present, which may infer the presence of other calmodulin regulated enzymes (21,22).

The presence of calmodulin in prokaryotes is not as well documented as in Eukaryotes. Iwasa et al (23) discovered a heat stable, trypsin-labile, calmodulin-like factor from the soluble fraction of Escherichia coli which had been partially purified by ion-exchange chromatography (DEAE-cellulose). This factor was able to activate mammalian Ca^{2+}- and calmodulin-dependent enzymes including bovine brain cyclic nucleotide phosphodiesterase, human erythrocyte (Ca^{2+},Mg^{2+})-ATPase, and rabbit myosin light chain kinase. In

one prokaryote system, mammalian brain calmodulin has been demonstrated to activate the adenylate cyclase from Bordetella pertussi (24).

In addition to prokaryotes, calmodulin has been isolated from the protozoans including Paramecium tetraurelia (25,26), Trypanosoma cruzi (27), Trypanosoma brucei (28), Tetrahymena pyriformis (29-37), Chlamydomonas reinhardii (38), and Volvox carteri (39). Calmodulin is also present in the fungi including the yeasts, Candida albicans and Saccharomyces cerevisiae (40), Physarum polycephalum (41), Phycomyces blakesleeanus (42), Neurospora crassa (43,44) and Dictyostelium discoideum (45-48).

The second messenger system is not as well established in plants as in the mammalian system. Ashton and Polya (49) have found cAMP concentration in axenic rye grass endosperm cell cultures to range from 2 to 12 pmol/g fresh weight. Previous studies on plant cyclic necleotides had raised questions concerning possible cAMP production through microbiological contamination. Brown et al (50) have shown the presence of a cAMP binding protein in barley seedlings Hordeum vulgare, with a K_d for cAMP of 8nM which is comparable to that for the mammalian cAMP binding-protein (2-3 nM). Amrhein (51) has summarized current research on cAMP in higher plants and has emphasized the lack of definitive proof concerning cAMP in plants.

While the evidence for cyclic nucleotides and their role in plants is limited (51,52), the Ca^{2+}-mediated processes are well established. Cormier, Charbonneau and Jarrett (53) have

compiled a list of these Ca^{2+}-dependent events including: "1. a phytochrome mediated uptake of extracellular Ca^{2+}, 2. a Ca^{2+}-dependent stimulation of chloroplast rotation in Mougeotia, 3. a Ca^{2+} and phytochrome-dependent depolarization of Nitella cells, 4. a Ca^{2+}-dependent inhibition of cytoplasmic streaming in Nitella, 5. a Ca^{2+}-dependent regulation of directional growth that involves elevated levels of Ca^{2+} in the growing tips of plant cells, 6. a Ca^{2+}-dependent cyclic nucleotide phosphodiesterase in Phaseolus vulgaris, 7. a Ca^{2+}-dependent inhibition of chloroplastic fructose bisphosphatase, 8. a Ca^{2+}-dependent activation of glutamic dehydrogenase, and 9. the regulation of Ca^{2+} fluxes across the plasma and mitochondrial membranes of plant cells by phytochrome." Other Ca^{2+}-dependent processes in plants have been listed by Dieter and Marme (54), who have reported on the mitochondrial and microsomal active Ca^{2+}-transport systems in Zea mays.

During the study on the phylogenetic distribution of calmodulin, it was observed that potatoes and other plants also contain a similar protein factor (22,55). The present communication reports on the distribution of calmodulin in various plant species. The results suggest that calmodulin is widely distributed in plants. During the course of this work, various reports have appeared concerning the presence of calmodulin in plants. Anderson and Cormier (56) demonstrated the existence of calmodulin in pea seedlings, mung bean, spinach and wild carrot. Chafouleas et al (57) have employed a radioimmunoassay for the detection of

calmodulin in peanut seeds and Charbonneau and Cormier (58) utilized a fluphenazine-Sepharose affinity chromatography procedure for the purification of calmodulin from pea seedlings, peanut seeds and mushrooms. Grand et al (59) have purified calmodulin from the fruiting bodies of higher and barley. More recently, Van Eldik et al (60) and Watterson et al (61) have isolated and characterized calmodulin from spinach leaves.

Circumstantial evidence for calmodulin in plants has been based on results of physiological studies using calmodulin-Ca^{2+} complex inhibitors, i.e. the phenothiazines. Lado et al (62) have shown that trifluoperazine and chlorpromazine promoted proton secretion with an accompanying hyperpolarization of the transmembrane electrical potential in maize coleoptile, maize root and pea stems. In addition, Barr et al (63) demonstrated inhibition of the electron transport in Photosystem II of spinach chloroplasts by treatment with chloropromazine, phenothiazine and trifluoperazine.

Mammalian tissues contain a cyclic nucleotide phosphodiesterase requiring Ca^{2+} and calmodulin for full activity (5,64,65). In addition to calmodulin, the enzyme can be activated by phospholipids (66,67), free fatty acids (68), gangliosides (69) and limited proteolysis(5).

A variety of inhibitors of the enzyme have been described including those which compete for the substrate binding site (e.g. theophylline) on the enzyme (70) and those which bind to the Ca^{2+}-calmodulin complex (e.g.phenothiazines, other

calmodulin binding proteins) preventing activation of the enzyme (70-73).

A number of plant species have been examined for calmodulin activity on the basis of calmodulin-dependent cyclic nucleotide phosphodiestrase activation. Among those examined all except those of a fern (Nephrolepis exaltata), and a conifer (Larix decidua) were found to be capable of Ca^{2+} dependent activation of the enzyme from bovine brain. Further examination of a crude homogenate from another fern species (Polypodium aureum arolatum) led to the discovery of an inhibitor preventing calmodulin activation of the enzyme. Enzyme inhibition kinetics using the partially purified inhibitor showed preferential inhibition of the calmodulin and trypsin activated forms of the enzyme. Preliminary characterization of the inhibitor revealed a water soluble, chemically stable, non-protein, non-dialyzable organopolymer.

2. EXPERIMENTAL

2.1 Protein Preparations

Bovine brain calmodulin and calmodulin deficient cyclic nucleotide phosphodiesterase were prepared according to the procedure of Wang and Desai (71). Calmodulin binding protein 1 (calcineurin) was purified according to the method of Sharma et al (74). Rabbit skeletal muscle myosin light chain kinase was prepared according to Waisman (75). Calmodulin independent cyclic nucleotide phosphodiesterase from bovine heart was isolated using the method of Ho et al (76). The 5'-nucleotidase (Crotalus atrox venom) and the Type II histone were obtained from Sigma.

2.2 Preparation of Crude Plant Extract

Two methods were used. With the first method, frozen plants were chopped and homogenized for three minutes with a polytron in three volumes of buffer A (100 mM Tris-HCl, 2 mM EDTA, pH 7.5). The slurry was centrifuged at 750 x g for 45 minutes and the supernatant strained through cheesecloth, adjusted to 75% ammonium sulfate, allowed to stir slowly for 30 minutes, and then centrifuged at 10,000 x g for 30 minutes. The precipitated protein was solubized and dialyzed against buffer B (40 mM Tris-HCl, pH 7.5, 1 mM Mg^{2+}, 50 µM Ca^{2+}) overnight, then heated in a boiling water bath for five

minutes. The precipitated protein was discarded following centrifugation.

In a second method, frozen plant samples wre chopped, and homogenized in buffer A using a Waring blender. The slurry was sonicated, incubated in a boiling water bath for five minutes, centrifuged at 10,000 x g for 20 minutes and then filtered. Calmodulin was isolated from the supernatant by DEAE-cellulose chromatography. After elution of calmodulin from the anion exchange column with buffer B (containing 0.5 M NaCl) the eluate was dialyzed against distilled water and then lyophilized.

2.3 Phosphodiesterase Activation Assay

The activity of the calmodulin dependent and the calmodulin independent cyclic nucleotide phosphodiesterase was measured according to a previously described procedure (43). The procedure involved coupling the phosphodiesterase reaction to a 5'-nucleotidase reaction followed by quantitation of the released inorganic phosphate. One unit of phosphodiesterase activity is defined as the amount of enzyme which when fully activated hydrolyzes 1 μmole cAMP per minute at 30°C. One unit of inhibitor activity is defined as the amoount causing half-maximal inhibition of the phosphodiesterase reaction.

2.4 Purification of Plant Calmodulin

2.4.1 Buffers:

Buffer C: 20 mM Tris-HCl, 1 mM Mg^{2+}-acetate, 1 mM imidazole 50 μM Ca^{2+}, pH 7.0

Buffer D: 20 mM Tris HCl, 1 mM Mg^{2+}-acetate, 1 mM imidazole 50 μM EGTA, pH 7.0

Buffer E: 50 mM Tris-HCl, 2 mM EDTA, 1 mM 2-mercapto-ethanol, 0.15 M NaCl, pH 7.0

Buffer F: 20 mM Tris-HCl, 0.1 mM EGTA, 1 mM 2-mercapto-ethanol, 0.15 M NaCl, pH 7.0

Buffer G: 20 mM Tris-HCl, 1 mM Mg^{2+}-acetate, 1 mM imidazole, 0.5 mM $CaCl_2$, pH 7.0

Buffer H: 20 mM Tris-HCl, 1 mM Mg^{2+}-acetate, 1 mM imidazole, 5 mM EGTA, pH 7.0

2.4.2 Purification of Calmodulin from Cabbage

For a more definite characterization of the plant-type calmodulin, calmodulin has been purified from cabbage, a plant rich in this protein. The purification procedure was a modified version of that employed by Teo et al for the bovine heart calmodulin (93). Five kilograms of frozen, finely cut cabbage was homogenized in buffer C and strained through cheesecloth. The homogenate was mixed with 400 ml of DEAE-cellulose (previously equilibrated with buffer C) and poured into a large column and washed with buffer C

containing 0.2M NaCl. Calmodulin was then eluted from the column using buffer C containing 0.6 M NaCl. The active fractions were pooled and dialyzed against 18 l of buffer C for 12 hours. The dialyzed solution was applied to a 300 ml DEAE-cellulose column and the column was washed with 1 l of buffer D containing 0.2 M NaCl, and then eluted with a two liter 0.2-0.6 M NaCl gradient. Fractions in this region were pooled, dialyzed against 50 μM Ca^{2+} and lyophilized. The lyophilized crude calmodulin was dissolved in a minimal volume of buffer C, subjected to heat treatment (1 minute in boiling water) and further purified using Sephadex G-100 gel chromatography.

During recent experiments, fluphenazine-Sepharose 4B affinity chromatography has been employed as an alternative to gel filtration for the purification of plant calmodulins.

2.4.3 Preparation of fluphenazine-Sepharose 4B:

Fluphenazine-Sepharose was prepared according to the method of Charbonneau and Cormier (58). Briefly, 100 g of Sepharose 4B was washed with deionized water on a glass filter funnel, and then suction-dried under vacuum. The Sepharose was activated by mixing with 100 ml of 0.6 N NaOH containing 3mg/ml of sodium borohydride and 100 ml of diglycidyl ether, incubated at room temperature with gentle stirring for 8 hours, and washed with 6 to 8 l of deionized water. Next, the activated Sepharose was mixed with 160 ml of 0.1 M carbonate buffer, (pH 11.0) containing 400 mg fluphenazine-2HCl and heated with gentle stirring for 48

hours at 70°C. The fluphenazine-coupled Sepharose was washed with 1 to 2 l of acetone to remove unreacted Fluphenazine, and then with 1 l of absolute ethanol, exposing the gel to the alcohol for 30 minutes. Finally, the gel was washed with deionized water and column buffer prior to use.

2.4.4 Purification Procedure: Using affinity chromatography calmodulin was purified from plants (fresh or frozen) according to the following method described for cucumber. Finely cut cucumber (2.4 kg), homogenized in 4 l of buffer E was strained through cheescloth and centrifuged at 10,000 x g for 20 minutes. The crude homogenate supernatant was passed through a 280 ml DEAE-cellulose column previously equilibrated with buffer F. The column was washed with 400 ml of buffer F, then a gradient consisting of 800 mls each of 0.15 M NaCl and 0.7 M NaCl in buffer F was applied. The fractions containing the calmodulin activity were pooled (total volume of 1 l), and mixed with 500 ml of buffer G. Calcium chloride solution (100 mM, pH 7.0) was added to a final concentration of 0.5 mM, the solution was stirred for 1 hour and then applied to a 50 ml fluphenazine-Sepharose column, pre-equilibrated with buffer G. The fluphenazine-Sepharose column was washed with buffer G containing 0.5 M NaCl. Calmodulin was eluted with buffer H, containing 0.5 M NaCl, the fractions pooled, dialyzed against buffer G and then lyophilized.

2.5 Protein Determination

The protein concentration was determined with a dye reagent concentrate from Bio Rad laboratories which employs the Coomassie blue protein dye binding principle developed by Bradford (77). Bovine serum albumin was used as the protein standard.

2.6 Protein Kinase Assay

The filter paper method (78) was used for the assay of proten kinase. Myosin light chain kinase was assayed in a reaction mixture containing 1.0 mg/ml of histone, 20 mM Tris-HCl, 0.25 mM [^{32}P]ATP of specific activity 20 to 100 cpm/pmol, 40 mM 2-mercaptoethanol, either 0.8 mM EGTA or 0.4 mM $CaCl_2$ plus 20 µg/ml of purified calmodulin, and 10 mM Mg-acetate.

2.7 Acrylamide Gel Electrophoresis

Sodium dodecyl sulfate gel electrophoresis was performed in tube gels using the method described by Weber and Osborn (79). Discontinuous gel electrophoresis was carried out according to Davis (80) using 12% and 15% polyacrylamide tube gels. To localize calmodulin on the gel, gels were sliced after the electrophoresis and each slice was extracted with 100 µl of 20 mM Tris-HCl buffer, pH 7.5, then assayed for Ca^{2+}-dependent phosphodiesterase activation.

Sodium dodecyl sulfate slab gel electrophoresis was performed according to the method of Laemmli (81) using 15% acrylamide gels. Protein standards employed were ovalbumin, myoglobin, and cytochrome C. The electrophoresis gels were stained with Coomassie blue.

2.8 Amino Acid Analysis

The purified calmodulins were hydrolyzed according to the method of Howard and Pierce (82). Approximately 500 µg samples were hydrolyzed in 6 N HCl containing 50 µl of 5% (w/v) aqueous phenol and 2 µl of mercaptoacetic acid per ml, at 110^0C in sealed, evacuated tubes for 22 hours. The bovine brain and cabbage calmodulin were also subjected to 48 and 72 hour hydrolysis times. Amino acid analysis was performed using a Spinco 121M amino acid analyzer as outlined in the Spinco manual (Beckman). Methylated amino acids for the bovine brain and cabbage calmodulins were determined on a Spinco 120/139 amino acid analyzer using the method of Zarkadas (83).

2.9 Cyanogen Bromide Cleavage and Analysis by High Pressure Liquid Chromatography

Purified brain, spinach and cucumber calmodulins were digested with cyanogen bromide according to the method of Lawson et al (84) as modified by Steers et al (85). One half milligram of calmodulin in 50 µl of H_2O was mixed with 2 mg

of CNBr and 1 ml of 70% formic acid. The reaction mixture was kept at room temperature in the dark for 40 hrs, 10 ml of H_2O was added, frozen, then lylphilized. Ten milliliters of H_2O was added to the lyophilized sample, the sample was again lyophilized and the procedure was repeated.

High pressure liquid chromatography was performed with a Model 322 liquid chromatograph (Beckman), using a buffer system of 0.1% H_3PO_4 with a gradient of 0 to 70% CH_3CN in 40 minutes with a flow rate of 1 ml/min. The system was run using a Beckman ultrasphere-ODS 5 μM column (25 cm x4.6 mm I.D.).

2.10 Ultraviolet Absorption Spectrum

The UV absorption spectrum of the purified cabbage calmodulin sample was determined using a Pye Unicam (SP8-100), UV Spectrophotometer at the wavelengths from 320 to 240 nm.

2.11 Preparation of Glycine-Sepharose 4B

Thirty grams of Sepharose 4B was activated with divinyl sulfone in the presence of 0.5 M Na_2CO_3 (pH 11.0) as previously described (86). After washing alternatively with water and acetone, the activated gel was mixed with 0.7 g glycine in 0.2 M $NaHCO_3$ buffer (pH 9.5) overnight. The gel was then washed with column buffer (buffer D) and poured into a column (32.7 x 3.0 cm). One milliliter of the gel binds to

90 units of the inhibitor.

2.12 Preparation of Antisera to Calmodulin

Calmodulin was oxidized by the method of Hirs et al (87) as described subsequently: Performic acid reagent was prepared by mixing five volumes of 30% H_2O_2 with 95 volumes of 99% formic acid, and was allowed to react for 120 minutes at 25°C. Approximately 17 mg of lyophilized calmodulin was dissolved in 6.0 ml of a solution composed of 5.0 ml of 99% formic acid and 1.0 ml methanol. This protein solution was kept at 5°C for 30 minutes. Ten milliliters of the performic acid reagent was mixed with the 6.0 ml of the protein solution and the reaction proceeded at 5°C for 150 minutes. Then, the performic acid treated protein was diluted with 400 ml of ice water, lyophilized, redissolved in 100 ml of water and again lyophilized.

New Zealand, white, female rabbits were immunized with 1.0 mg of the oxidized calmodulin using a water in oil emulsion with Freund's incomplete adjuvant (complete adjuvant was employed for the first injection). Bleeding from the ear was alternated with immunization on a weekly basis. Antiserum was obtained by allowing the blood to clot overnight, and centrifuging at 1,000 x g to remove the clot. Sodium azide was added as a preservative and the antiserum was kept at 4°C.

2.13 Antiserum Dilution curve and Radioimmunoassay

Tritium labelled calmodulin was prepared according to the procedure of Tack et al (88). Approximately 500 mg of pure brain calmodulin was dissolved in 0.5 ml of deionized water and dialyzed in 0.2 M borate buffer (pH 8.9) overnight. The dialyzed calmodulin was mixed with 60 ml of formic acid (0.3M) and 180 ml ^{3}H $NaBH_4$ in 0.01 M NaOH (100 mCi) and kept in a fume hood for 60-70 minutes. The labelled calmodulin was then applied to a fluphenazine-Sepharose column and purified as described in section 2.4. The purified ^{3}H labelled-calmodulin was dialyzed against buffer G. The specific activity of the labelled calmodulin was 152,800 cpm/µl at a protein concentration of 64 µg/ml.

The radioimmunoassay (RIA) is a modification of the procedure described by Chafouleas et al (89) for the R.I.A. of calmodulin using radioiodinated calmodulin. Six microliters of ^{3}H-labeled calmodulin was diluted with phosphate buffered saline (PBS), pH 7.4 to prepare a working solution with 0.1 mg/ml bovine serum albumin, 1mM EGTA and 384 mg of ^{3}H-labeled calmodulin. The working solution was prepared by diluting 200 ml of the stock solution to 1 ml with PBS-1mM EGTA and bovine serum albumin (0.1 mg/ml). Fifty milliliters of antiserum (diluted with PBS-1mM EGTA 1:10, 1:20, 1:40, 1:60, 1:80, 1:100, 1:500, and 1:1000) was mixed with ^{3}H-labeled calmodulin (50 mls of the working solution) in 0.5 ml Brinkmann Eppendorf micro test tubes, and incubated at 30°C for 30 minutes, followed by the addition of

50 µl Pansorbin (10% solution of Staphylococcus aureus cells) with a further 30 minutes incubation period at 30°C. After the incubation with Pansorbin, the reaction mixture was centrifuged for 30 seconds in an Eppendorf Centrifuge 3200, washed two times with 200 ml of PBS containing 0.05% Tween 20 (polyoxyethylene sorbitan monolaurate), and the pellet dissolved in PBS (200 ml). The supernatant from the reaction mixture is pooled with the supernatants from the two washing steps and placed in a scintillation vial to which 10 ml of aquasol is added. The same procedure is followed for the dissolved pellet, and the amount of radioactivity is determined in each fraction. The results are reported as the percent radioactivity which has bound to the Pansorbin.

For the competition curve with purified unlabeled calmodulins, a 1:20 dilution of antiserum was chosen. Fifty microliters of diluted antiserum was mixed with 50 µl of the ^{3}H-labeled calmodulin and 50 µl of unlabeled purified brain and plant calmodulins in the following quantities (4,6,10,25,50,75,100,250,500, and 1,000 ng). Incubations proceeded as described for the antiserum dilution curve and the percent radioactivity bound to the Pansorbin was determined.

2.14 Ophiobolin A Inhibition of Calmodulin Activity

Ophiobolin A, which was purified from the fungus, Helminthosporium maydis, was obtained as a gift from Pak C. Leung, Department of Biochemistry and Biophysics, Iowa State

University in Ames, Iowa. Five hundred micrograms of ophiobolin A was dissolved in 400 ml of methanol and then brought to a volume of 2,400 ml with deionized water to make a working solution of 450 mM. The calmodulin-dependent cyclic nucleotide phosphodiesterase reaction was performed as described (90) using approximately 4 units of purified brain and spinach calmodulin in the presence of ophiobolin A from 10 to 100 mM.

2.15 Isolation of Cyclic Nucleotide Phosphodiesterase Inhibitor

Ca^{2+}-dependent activation of the enzyme can be achieved with heat treated dialyzed crude extracts from a variety of plant sources (75). During a calmodulin distribution survey in plant tissues, all plants examined, with the exception of a fern (Nephrolis exaltata) and a conifer (Larix decidua) exhibited the ability to activate the enzyme. Further attempts to demonstrate calmodulin like activity from N. exaltata and another fern, Polypodium aureum areolatum by direct fluphenazine Sepharose chromatography of the crude homogenate showed minimal activity. The possibility of a calmodulin binding protein analogous to those characterized from mammalian sources (71-74) which would block calmodulin-dependent activation was examined.

2.16 Stability of the Inhibitor

The inhibitor factor was examined for stability to urea (3 M) and guanidine-HCl (3 M) denaturants. The crude homogenate was incubated in the presence of 3 M urea containing 0.5 M NaCl, and in the presence of 3 M guanidine-HCl for 1.5 hours. A control sample was incubated in buffer C. The treated samples were then dialyzed against buffer C overnight and then analyzed for inhibitor activity.

The inhibitor was also tested for stability to acid and base treatment. Two samples of the fern crude homogenate were brought to 1 N HCl and 1 N NaOH with concentrated solutions of HCl and NaOH. The two treated samples and a third control sample (untreated) were heated in a boiling water bath for five minutes, centifruged at 10,000 x g for 30 minutes and dialyzed against buffer B. Each sample was analyzed for inhibitor activity.

2.17 Routine Inhibitor Purification

One hundred grams of fern leaves were washed, chopped and homogenized in 450 ml of buffer G (etc.). The homogenate was filtered through cheesecloth and centrifuged at 10,000 x g for 30 minutes. One hundred and fifty milliliters of the homogenate was applied to a glycine-Sepharose 4B column (90 ml bed volume). The column was washed with buffer G until no protein emerged from the column. The inhibitor was eluted from the column using buffer G containing 3 M or 6 M urea and

1 M NaCl. The protein peak emerging from the glycine-Sepharose 4B column was pooled, dialyzed against 20 l of distilled, deionized water for 24 hours and then lyophilized. The lyophilized inhibitor was dissolved in 4 ml of distilled, deionized water and dialyzed for another 24 hours in 4 l of distilled, deionized water. This sample was employed for characterization of the chemical and physical properties and the inhibition kinetics on the calmodulin-dependent phospphodiesterase.

2.18 Protease Treatment

Forty units of the fern inhibitor was incubated with 30 µg trypsin in the presence of 90 mM Tris-HCl, 90 mM imidazole and 10 mM Mg^{2+} for 20 minutes at 30°C. Another 40 units was incubated with 640 µg pronase in the presence of 20 mM Tris-HCl, 1 mM imidazole and 1 mM Mg^{2+} for the same time period. The samples were then heated for five minutes in a boiling water bath, dialyzed overnight against buffer B and then analyzed for inhibitor activity.

2.19 Organic Solvent Treatment

The inhibitor was treated separately with equal volumes of the following solvents: water, ethanol, 2-propanol, and chloroform. After thorough mixing the samples were analyzed for inhibitor activity.

3. RESULTS

3.1 Activation of Cyclic Nucleotide Phosphodiesterase by Plant Extracts

Among the various regulatory activities of calmodulin, the Ca^{2+}-dependent activation of mammalian cyclic nucleotide phosphodiesterase has been characterized most extensively (5-7). Thus, to investigate the possible existence and distribution of calmodulin in plants, extracts of many plants were tested for their ability to activate a bovine brain calmodulin dependent cyclic nucleotide phosphodiesterase. The calmodulin-dependent phosphodiesterase may be activated by, in addition to calmodulin, phospholipids (66,67) fatty acids (68) or limited proteolysis (5). However, only the enzyme activation by calmodulin is both Ca^{2+}-dependent and reversible. The activation of cyclic nucleotide phosphodiesterase by lipids is independent of Ca^{2+} (66,67). The enzyme activation by limited proteolysis is irreversible. The activation of bovine brain cyclic nucleotide phosphodiesterase by plant extracts appears to be Ca^{2+}-dependent. In the presence of excess EGTA to chelate Ca^{2+}, no activation of the enzyme could be demonstrated even at saturating amounts of the plant extracts (75). The reversibility of the activation of cyclic nucleotide phosphodiesterase by plant extracts and Ca^{2+} was also established (75). The activation of bovine brain phosphodiesterase by the potato extract was completely

suppressed upon the addition of EGTA. When Ca^{2+} was then added to a concentration in excess of EGTA, the activation of cyclic AMP phosphodiesterase was restored (75). These observations, along with the demonstration of phosphodiesterase stimulating activity in crude extracts, strongly suggest the existence of calmodulin in plants. In the present study in which more than a dozen plants were examined for calmodulin activity, only the extracts of a fern and a conifer did not significantly activate bovine brain nucleotide phosphodiesterase. These results again suggest that calmodulin is widely distributed in both animal and plant cells.

In a previous study (22), gel electrophoretic analysis of animal extracts indicated that cyclic nucleotide phosphodiesterase activating activity could be located on the electrophoretic gel as a single band with R_f values ranging from 0.59 to 0.62. Similarly, for the study described herein, when several plant extracts were analyzed by disc gel electrophoresis on 15% polyacrylamide gels, phosphodiesterase activating activity was observed as a single band on the gel. The activity bands of the various extracts showed similar electrophoretic mobilities. Their R_f values ranged from 0.61 for the potato extract to 0.65 for the mushroom extract. These values are similar to that of purified bovine brain calmodulin (Fig. 1). Since the mobility of proteins on analytical disc gel electrophoresis depends on both the size and the charge properties of the proteins, the results of Figure 1 suggest that calmodulins from plants and animals

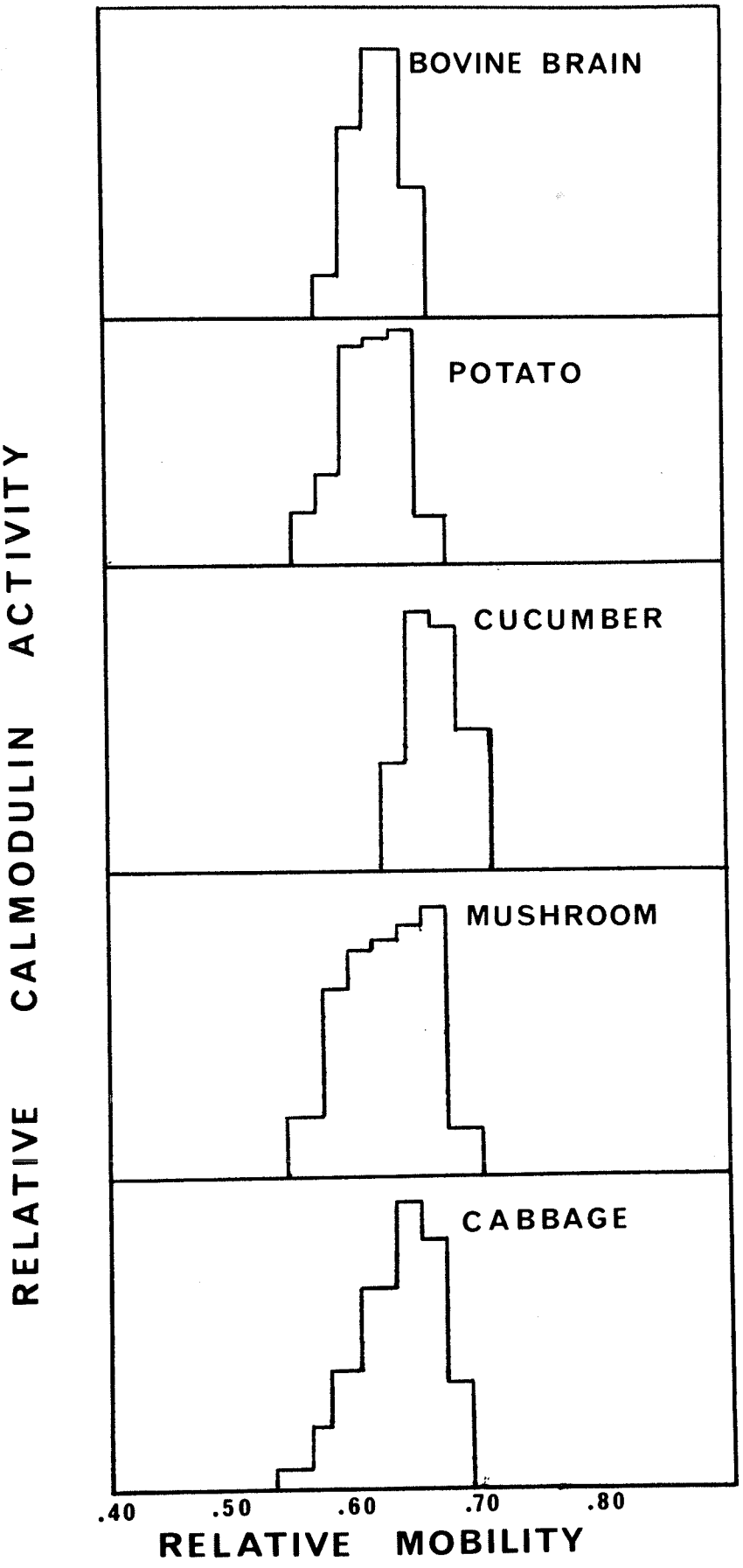

BOVINE BRAIN
POTATO
CUCUMBER
MUSHROOM
CABBAGE
RELATIVE CALMODULIN ACTIVITY
.40
.50
.60
.70
.80
RELATIVE MOBILITY

FIGURE 1

Discontinuous gel electrophoresis of crude bovine brain extract (to frame) and extracts of several plants as indicated according to the procedure of Davis (26) on 15% polyacrylamide gels. After electrophoresis, gels were sliced, extracted and assayed for Ca^{2+}-dependent activation of bovine brain cyclic nucleotide phosphodiesterase (see Materials and Methods). Plant extracts were prepared according to the second method (see Materials and Methods).

have similar physical-chemical properties.

3.2 Other Calmodulin-like Activities

Bovine brain contains a heat labile protein which specifically counteracts the activation of cyclic nucleotide phosphodiesterase by bovine brain or bovine heart calmodulin (71). The protein has been purified to homogeneity and shown to exert its inhibitory activity by associating with calmodulin in the presence of Ca^{2+} (73,74,91,92). The possible interaction of plant calmodulin with calmodulin binding protein 1 (calcineurin) was examined. Figure 2 shows that the activation of bovine brain cyclic nucleotide phosphodiesterase by the extracts of cabbage and broccoli could be completely abolished by increasing concentrations of calmodulin binding protein 1. Thus, it is demonstrated that plant calmodulins are also capable of interacting with bovine brain calmodulin binding protein 1.

Three plant extracts were also examined for their ability to activate rabbit skeletal muscle myosin light chain kinase. Table I shows that they could activate the protein kinase to approximately the same extent which is similar to that achieved by mammalian calmodulin. As is the case of the activation of cyclic nucleotide phosphodiesterase, activation of the protein kinase by the plant extracts depends on the presence of Ca^{2+} (Table I).

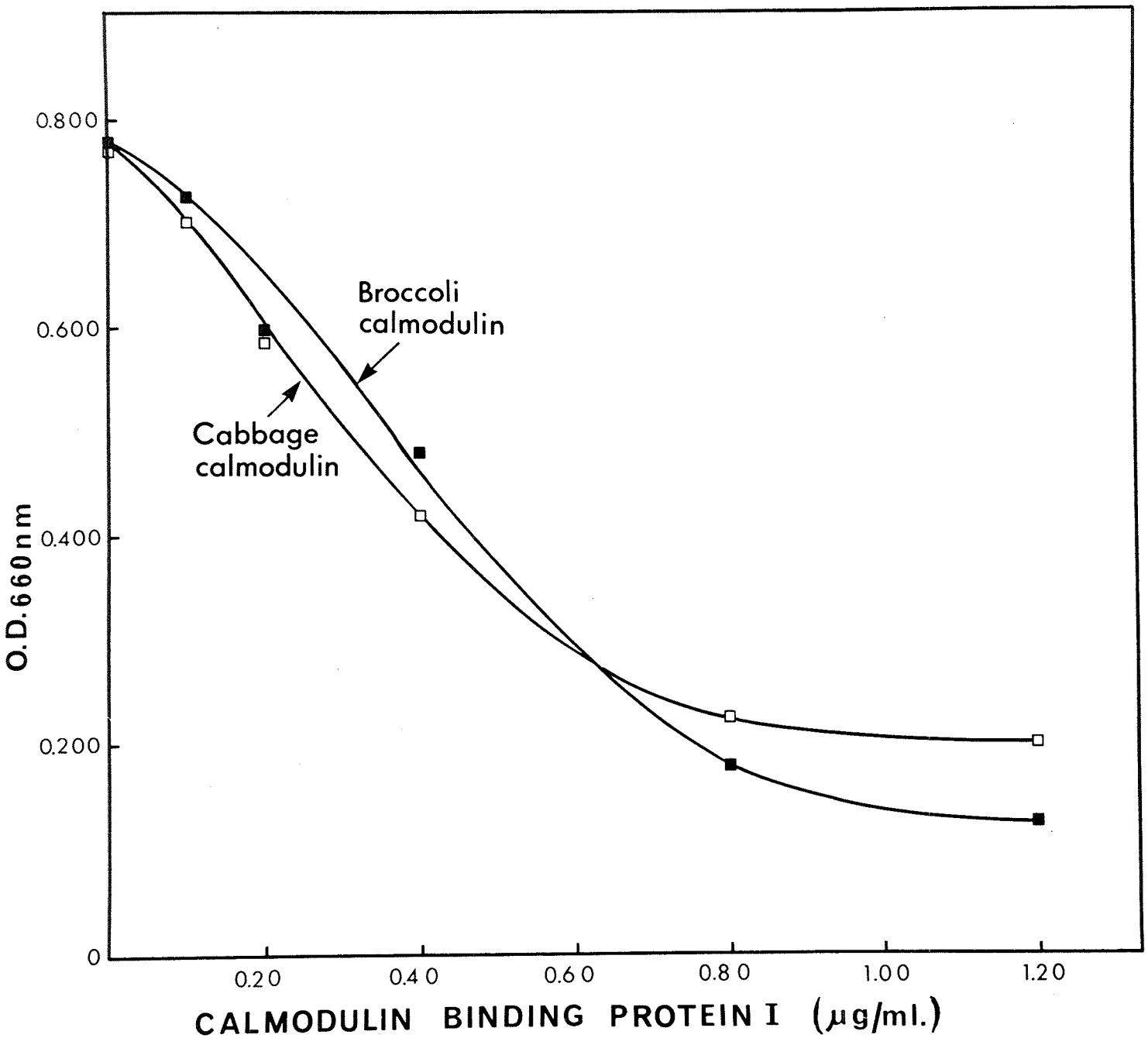

0.800
0.600
0.400
0.200
0
O.D.660nm
Broccoli
calmodulin
Cabbage
calmodulin
0.20
0.40
0.60
0.80
1.00
1.20
CALMODULIN BINDING PROTEIN I (μg/ml.)

FIGURE 2

Effect of bovine brain calmodulin binding protein 1 on the activation of phosphodiesterase by extracts of cabbage and broccoli. Amount of plant extracts used in the assay corresponded to about 4 units of calmodulin activity.

TABLE I

ACTIVATION OF MYOSIN-LIGHT CHAIN KINASE

PLANT EXTRACTS[a]	PROTEIN KINASE ACTIVITY (pMol/Assay)		
	+ EGTA and Extract	+ Ca^{2+}	+ Ca^{2+} and Extract
CABBAGE	10.7	13.8	89.0
BROCCOLI	13.6	14.3	84.9
CUCUMBER	9.8	15.8	83.0

[a]Plant extracts were prepared by the second method (Experimental Sec. 2.2)

3.3 Purification of Cabbage Calmodulin

When applied to DEAE-cellulose, the maximal calmodulin activity occurred at calmodulin activity occurred at approximately 0.3M NaCl (Fig. 3). When the calmodulin isolated by anion exchange chromatography is subjected to heat treatment and Sephadex G-100 gel chromatography, a single peak with calmodulin activity is obtained in fraction nos. 77 to 88 with a corresponding protein peak in this region (Fig. 4).

3.4 Purity of the Cabbage Calmodulin

From the titration curve for the activation of bovine brain phosphodiesterase (Fig. 5), the purified cabbage calmodulin appears to have similar but slightly less potency than the bovine brain calmodulin. Usually 3.3 to 4.6 pmoles per 900 µl of the purified cabbage calmodulin is required for half-maximal activation of the bovine brain phosphodiesterase. When the Sephadex G-100 fraction of the cabbage sample was subjected to discontinuous gel electrophoresis on 12% polyacrylamide gels, a single major protein with an identical mobility (R_f=0.71) to that of bovine brain calmodulin was obtained. An identical gel for the cabbage sample which was sliced and tested for calmodulin activity, yielded a calmodulin activity pattern coinciding with the stained protein band (Fig. 6). Furthermore, when the cabbage calmodulin sample was subjected to 10% and 15%

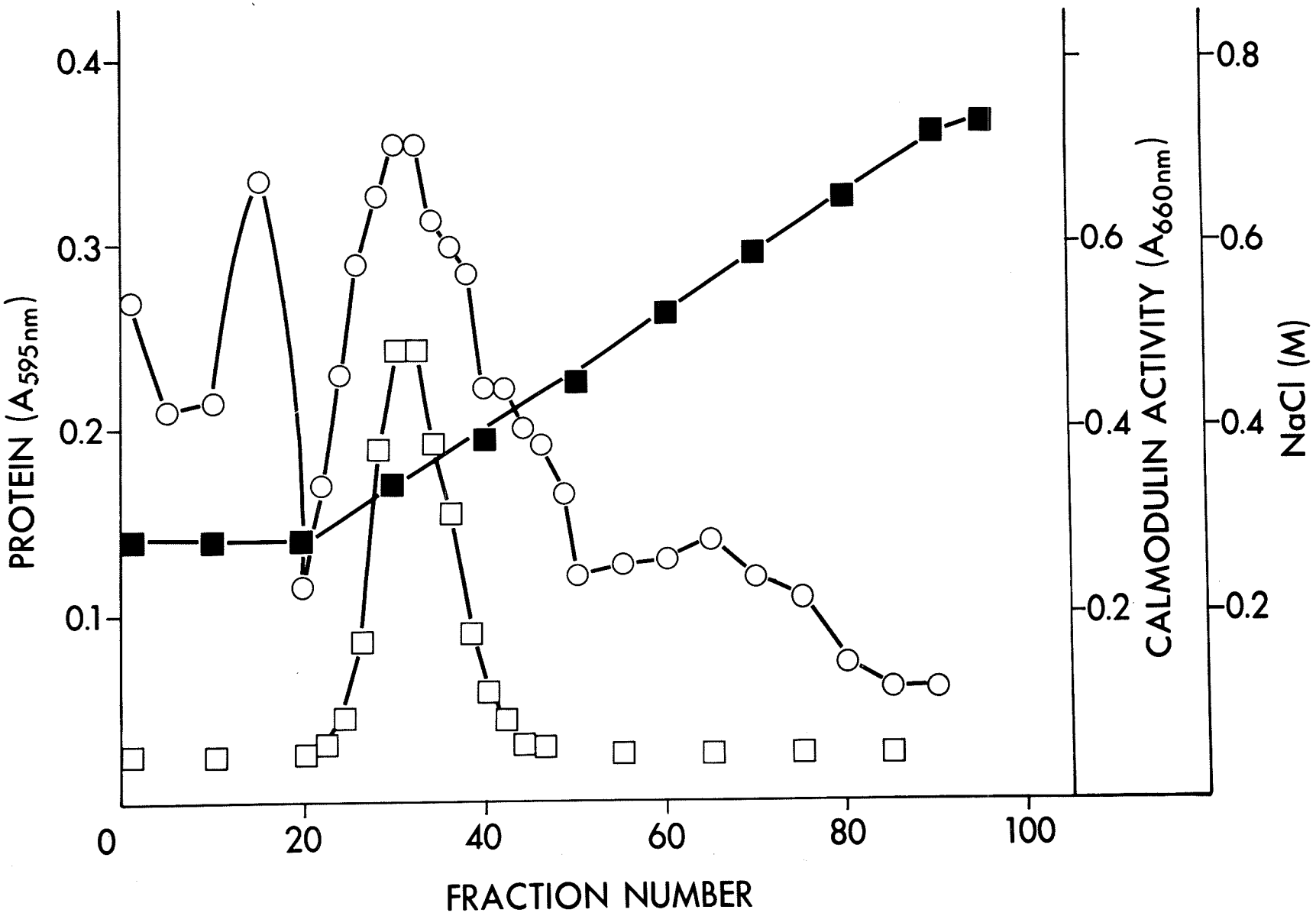

0.4
0.3
0.2
0.1
PROTEIN (A_{595nm})
0.8
0.6
0.4
0.2
CALMODULIN ACTIVITY (A_{660nm})
0.6
0.4
0.2
NaCl (M)
0
20
40
60
80
100
FRACTION NUMBER

FIGURE 3

DEAE-cellulose column chromatography of the crude cabbage calmodulin. Thirty eight hundred milliliters of extract from batch-wise DEAE-cellulose was applied to a 300 ml DEAE-cellulose column (section 2.4.1). The column was washed with one liter of buffer C containing 0.2 M NaCl. Calmodulin was eluted with a two liter 0.2 - 0.6 M NaCl gradient. Fractions were examined for protein (-o-o-), calmodulin (-□-□-) and NaCl concentration (-■-■-). Fractions 26 to 38 were pooled for further purification.

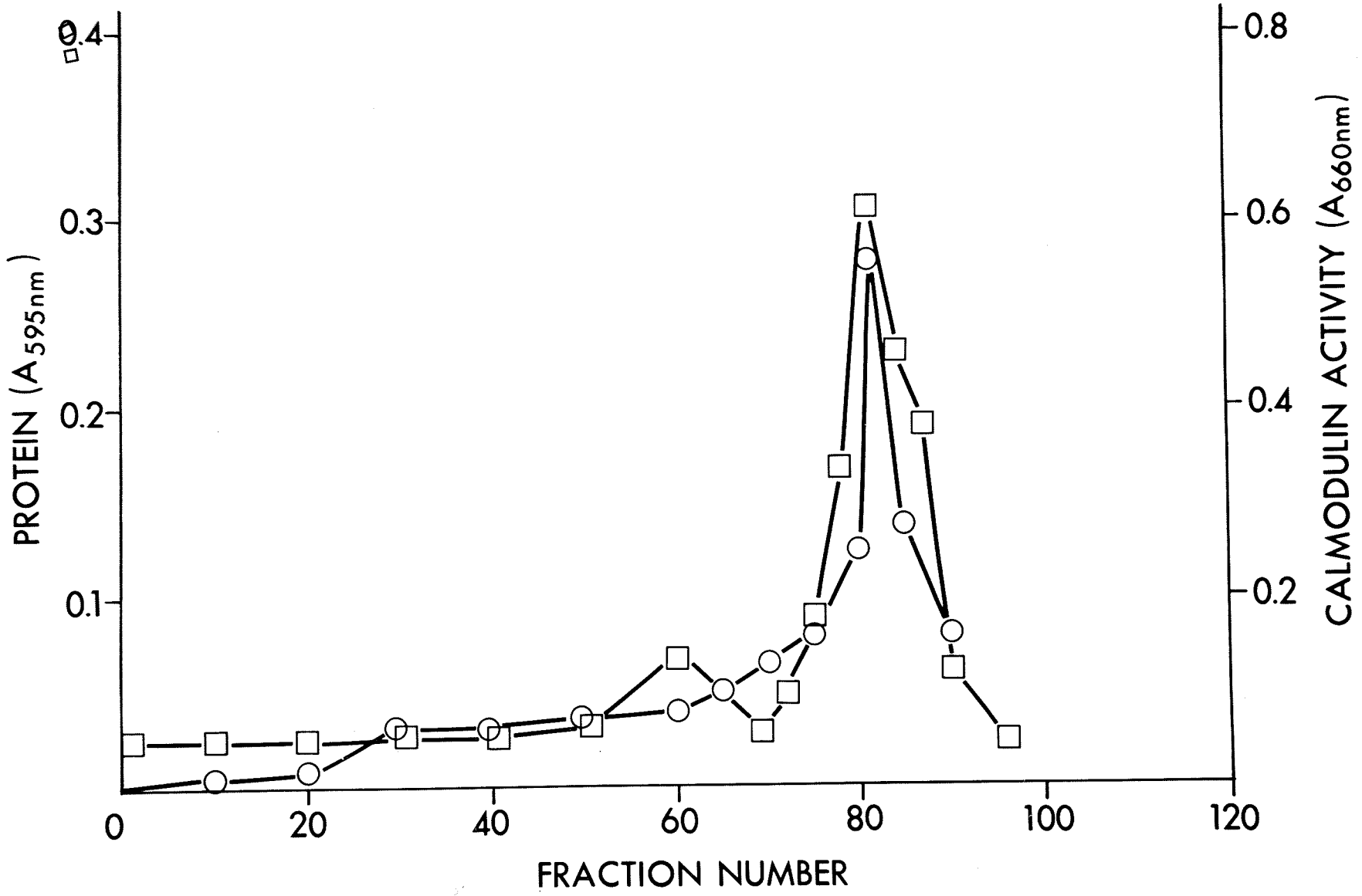

PROTEIN (A_{595nm})
CALMODULIN ACTIVITY (A_{660nm})
FRACTION NUMBER
0.4
0.3
0.2
0.1
0.8
0.6
0.4
0.2
0
20
40
60
80
100
120

FIGURE 4

Elution profile for protein (-O- -) and calmodulin (- - -) from Sephadex G-100 column. The calmodulin active fractions from DEAE-cellulose were dialyzed, lyophilized and dissolved in 3.1 ml of buffer C (section 2.4.1). This extract was applied to the Sephadex G-100 column. Calmodulin was eluted with buffer C containing 500 μM Ca^{2+}. Fractions 77 to 88 were pooled, dialyzed, lyophilized and dissolved in 1.3 ml of Tris buffer with 50 μM Ca^{2+}.

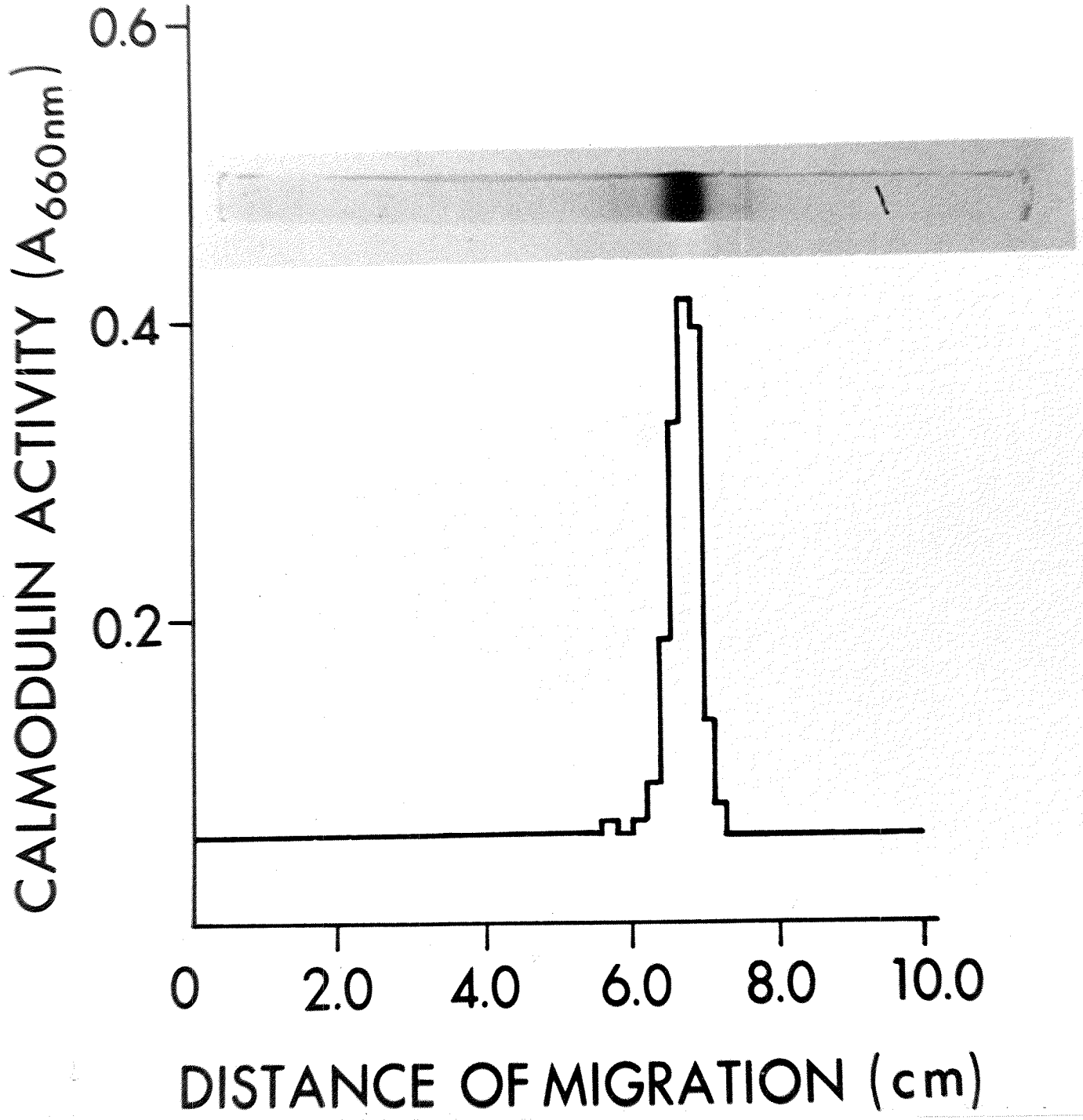

0.6
0.4
0.2
CALMODULIN ACTIVITY (A_{660nm})
0
2.0
4.0
6.0
8.0
10.0
DISTANCE OF MIGRATION (cm)

FIGURE 5

Titration curves for purified cabbage calmodulin (-o-o-) and bovine brain calmodulin (-●-●-) using calmodulin dependent bovine brain cyclic nucleotide phosphodiesterase.

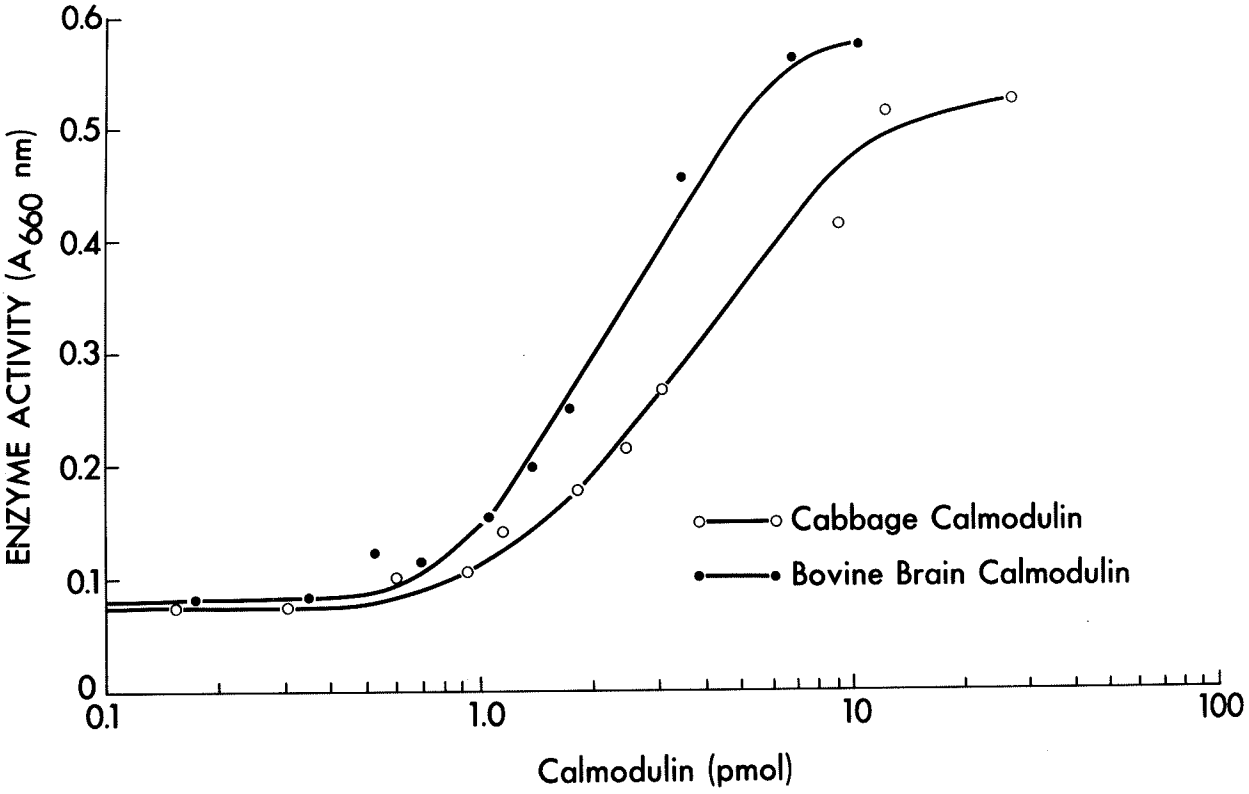


FIGURE 6

Two 5 µl aliquots of post-G-100 cabbage calmodulin each containing 20 µg of protein were subjected to disc gel electrophoresis on 12% polyacrylamide gels. One of the gels was stained for protein (upper section) while the other was sliced into segments, extracted with 100 µl of buffer C (section 2.4.1) and then assayed for calmodulin activity (lower section).

polyacrylamide SDS gel electrophoresis again single major protein bands were obtained with R_f values identical to the bovine brain calmodulin (R_f=0.70 for 10% gels, and R_f=0.51 for 15% gels). A densitometric scan of the 10% electrophoretic gel performed at 595 nm (Fig 7) showed that the calmodulin peak comprised approximately 97% of the total protein. Comparatively, SDS gel electrophoresis (using 15% polyacrylamide gels) of the calmodulin purified by fluphenazine-Sepharose chromatography produced a single major protein band comigrating with bovine brain calmodulin.

Additional evidence for the purity of the Sephadex G-100 cabbage calmodulin was demonstrated by the interaction of cabbage calmodulin with calmodulin binding protein 1 and the subsequent gel filtration of the complex (Fig. 8). When the Sephadex G-100 cabbage calmodulin sample was applied alone to the G-100 column, there was calmodulin activity and a substantial amount of protein in fraction numbers 46 to 58. However, when an equivalent volume of sample was mixed with an excess of calmodulin binding protein 1 and then applied to the same column, all the calmodulin activity appeared in the earlier fractions and only a minimal amount of protein remained in fractions 46 to 58. Thus, there was very little ccntaminating protein within the Sephadex G-100 cabbage calmodulin sample.

The UV absorption spectrum for the cabbage calmodulin was similar to the bovine brain calmodulin with absorption peaks occurring at 267, 263, 259, 253 and 246 nm (Fig. 9). The amino acid composition for the plant calmodulins (Table II)

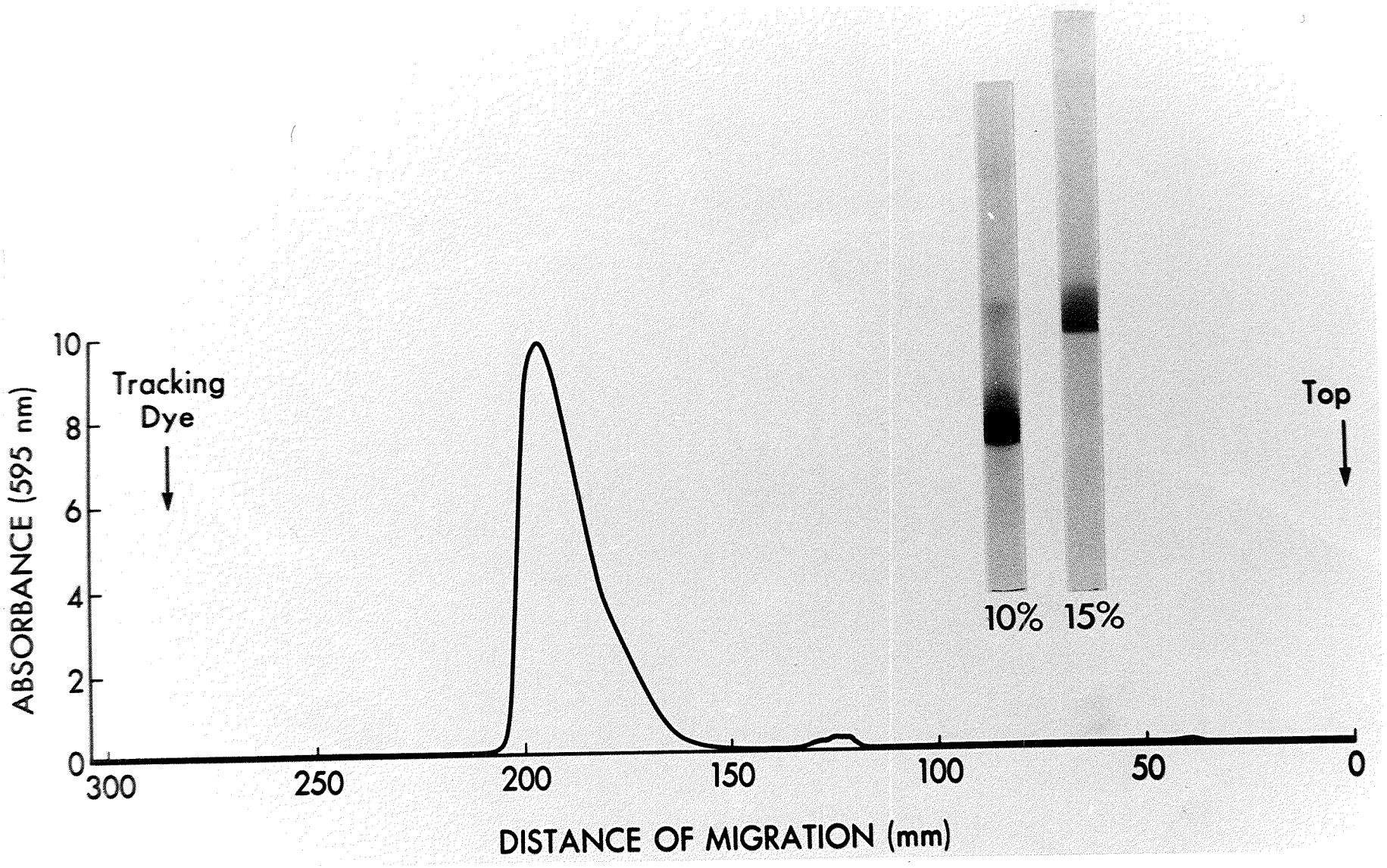

ABSORBANCE (595 nm)
10
8
6
4
2
0
Tracking Dye
300
250
200
150
100
50
0
DISTANCE OF MIGRATION (mm)
Top
10%
15%

FIGURE 7

Twenty microgram samples of the Sephadex G-100 cabbage calmodulin were subjected to 10% and 15% SDS polyacrylamide gel electrophoresis. The 10% gel was scanned using a Flur-Vis Autoscanner (Helena Laboratories), at a wavelength of 595 nm.

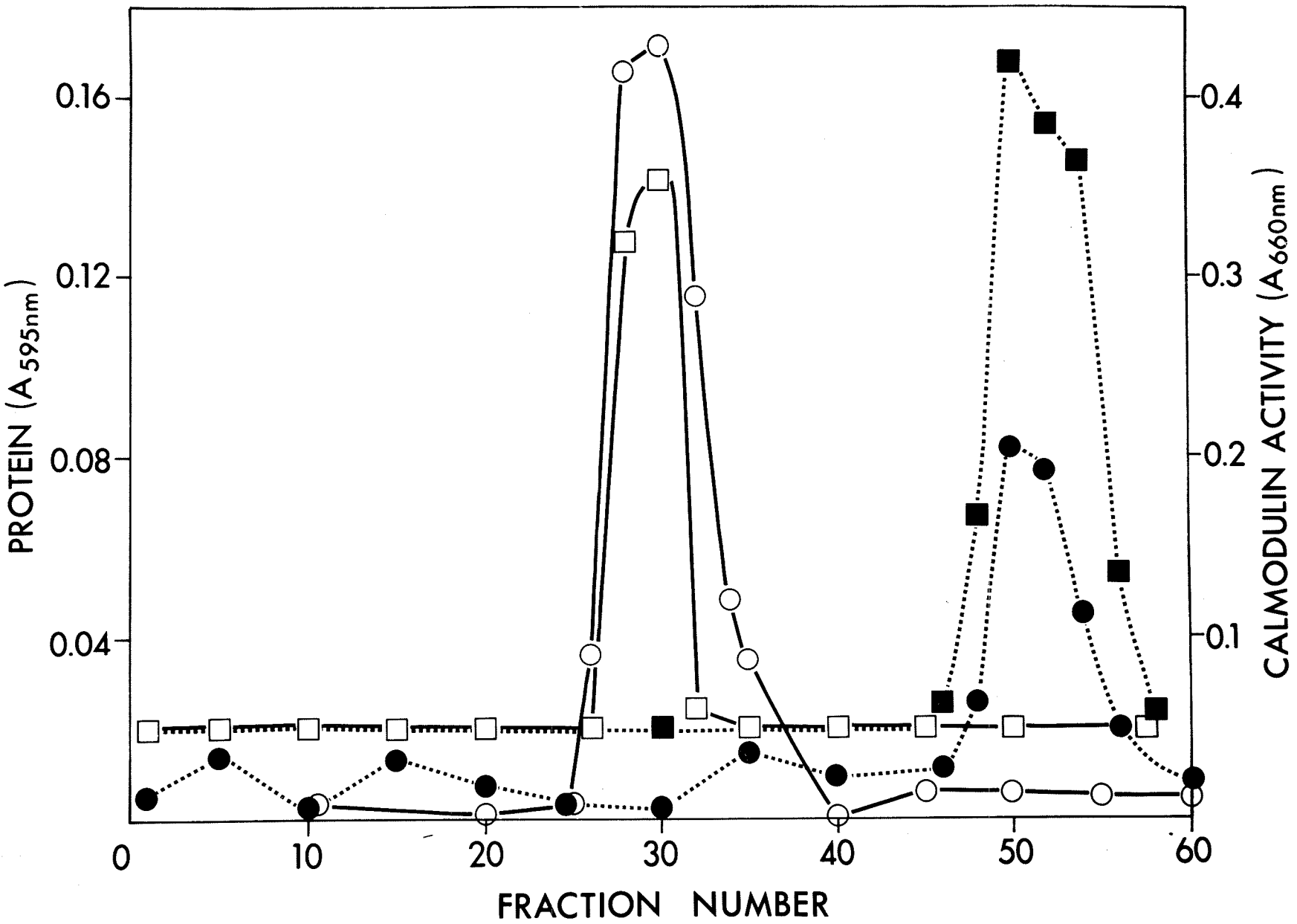

0.16
0.12
0.08
0.04
PROTEIN (A_{595nm})
0.4
0.3
0.2
0.1
CALMODULIN ACTIVITY (A_{660nm})
0
10
20
30
40
50
60
FRACTION NUMBER

FIGURE 8

Gel filtration analysis of cabbage calmodulin in the presence and absence of calmodulin binding protein I. A purified calmodulin sample (200 µg/50 µl) was chromatographed on a Sephadex G-100 column (90 x 1.5 cm) and the fractions were analyzed for protein concentration (-●-●-) and calmodulin activity (-■-■-). In a separate experiment, a pure sample of cabbage calmodulin was mixed with 2.6 mg of calmodulin binding protein I and then chromatographed on the same Sephadex G-100 column. Fractions were analyzed for protein concentration (-○-○-) and calmodulin activity (-□-□-).

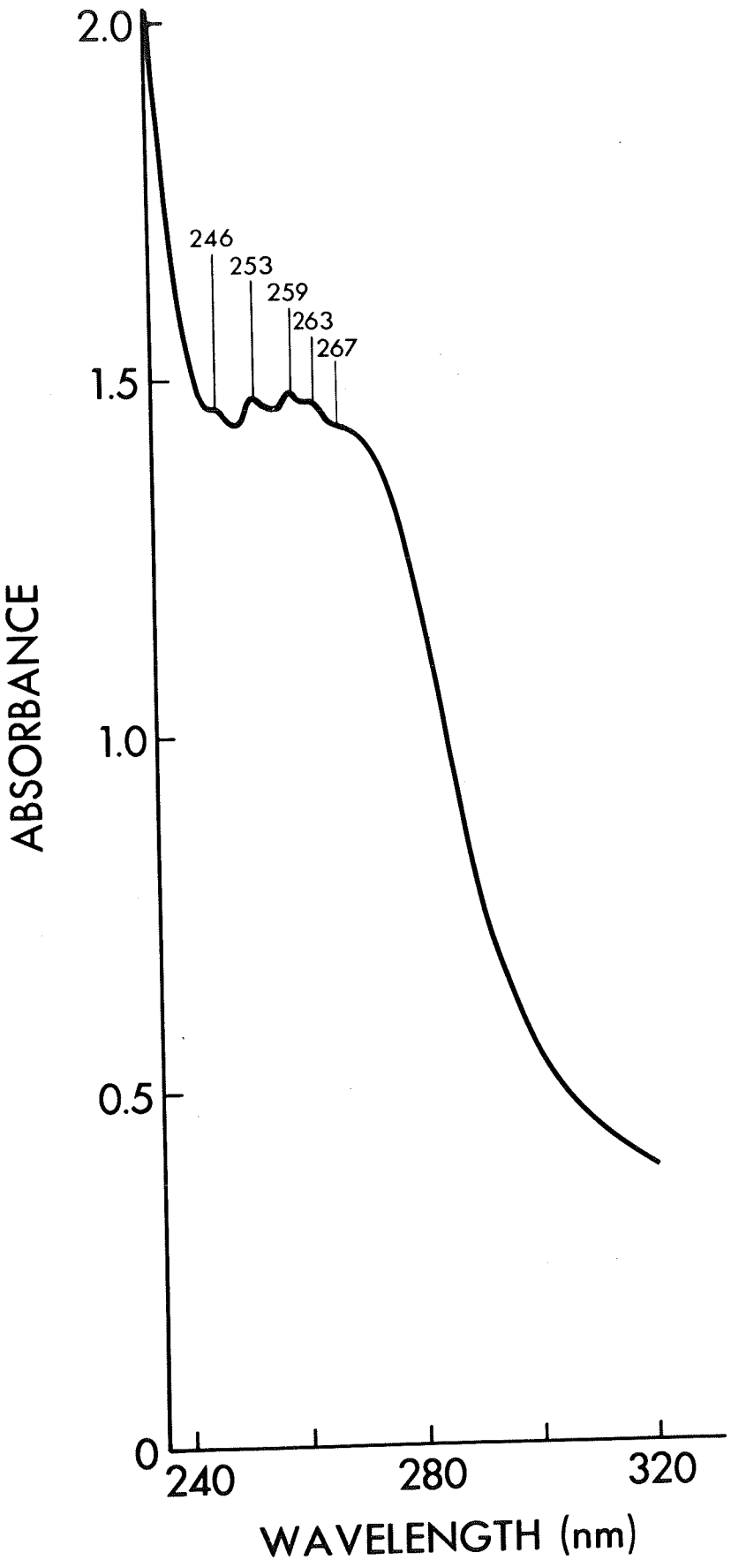

2.0
1.5
1.0
0.5
0
ABSORBANCE
246
253
259
263
267
240
280
320
WAVELENGTH (nm)

FIGURE 9

UV absorption spectrum of the post G-75 cabbage calmodulin extract. A 750 µl sample containing 2.3 mg of protein/ml was examined for absorbance of UV light through the range of 320 to 240 nm. Absorbance peaks occurred at 267, 263, 254, 253 and 246 nm.

TABLE II

AMINO ACID ANALYSIS

(Mole of amino acid per mole of calmodulin)

AMINO ACID	BRAIN	CABBAGE	CUCUMBER	ALFALFA	SPINACH
Lysine	7	9	9	8	10
Histidine	1	2	2	1	1
Arginine	6	5	5	6	5
Aspartate	22	23	26	25	27
Threonine	12	8	11	9	10
Serine	3	6	6	6	7
Glutamate	25	27	33	31	31
Proline	2	4	3	3	3
Glycine	11	11	12	12	12
Alanine	11	11	7	7	8
Cysteine*					
Valine	7	8	8	8	8
Methionine	8	6	9	7	8
Isoleucine	7	6	7	7	7
Leucine	9	11	12	11	12
Tyrosine	2	1	1	2	1
Phenylalanine	8	8	9	8	9
Trimethyllysine	1	1	1	1	1

*Not Determined

also resembles that of the earthworm calmodulin (22), and bovine brain calmodulin (94,95). Characteristically, these proteins contain a high number of acidic residues, a high phenylalanine to tyrosine ratio, a low content of histidine, and one residue of trimethyllysine. However, the plant calmodulins contain a higher proportion of the acidic residues, aspartate and glutamate, than do the brain and earthworm calmodulins.

3.5 Additional Characteristics of the Purified Plant Calmodulins

The slab gel electrophoresis show slight impurities for cucumber and spinach calmodulins although, in the presence of 0.1 mM Ca^{2+}, the R_f values are identical to that of the bovine brain calmodulin (Fig. 10). In the presence of the EGTA the calmodulins undergo a mobility shift, although the plant calmodulins exhibit less of a shift than the brain calmodulins (Fig. 11)

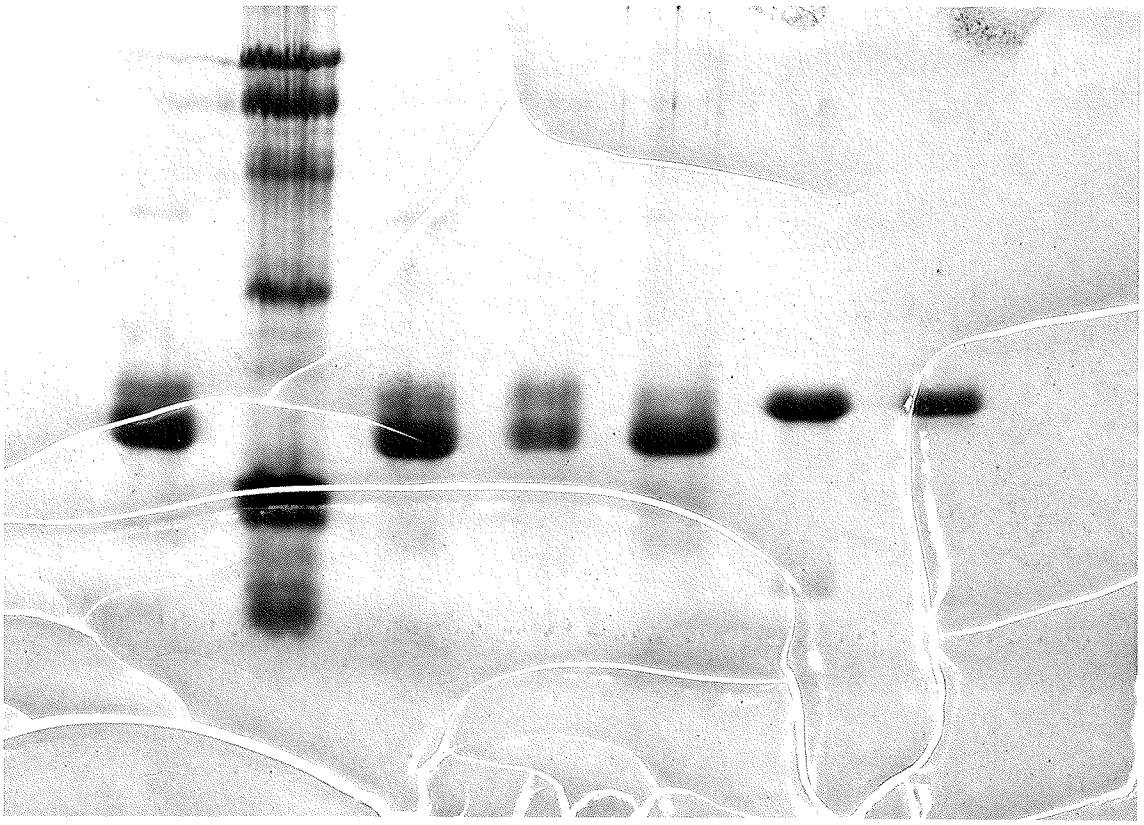
The cyanogen bromide cleavage maps as produced by high pressure liquid chromatography are identical for the spinach and cucumber calmodulins, which in turn show a slightly different pattern to that of the bovine brain calmodulin (Fig. 12).

The phosphodiesterase activation curve is similar for the brain and plant calmodulins (Fig. 13). The amounts required for half maximal activation are 2.6 pmoles for brain calmodulin, 3.2 pmoles for spinach calmodulin and 5 pmoles

FIGURE 10

Sodium dodecyl sulfate polyacrylamide gel electrophoresis of purified plant and brain calmodulins. Identify from left to right as follows: i) standards, including egg albumin, myoglobin and cytochrome, 5 µg each, ii) cucumber calmodulin, 5 µg, iii) spinach calmodulin, 5 µg, iv) bovine brain calmodulin, 5 µg tracks, v) to viii) include the same sequence of proteins at 10 µg each. Samples were applied at the cathodic terminal.

CATHODE



ANODE

FIGURE 11

Sodium dodecyl sulfate polyacrylamide gel electrophoresis of purified plant and brain calmodulins in the presence of 1mM EGTA (the gel buffer contained 0.1 mM EGTA). Identity from left to right is as follows: tracks i) and iii) cucumber calmodulin, 5 µg, ii) standards, including phosphorylase b, bovine serum albumin, ovalbumin, chymotrypsinogen, myoglobin, cytochrome C, and bacitracin, 5 µg each, iv) alfalfa calmodulin 5 µg, v) spinach calmodulin, 5 µg, vi) and vii) bovine brain calmodulin, 5 µg. Samples were applied at the cathodic terminal.

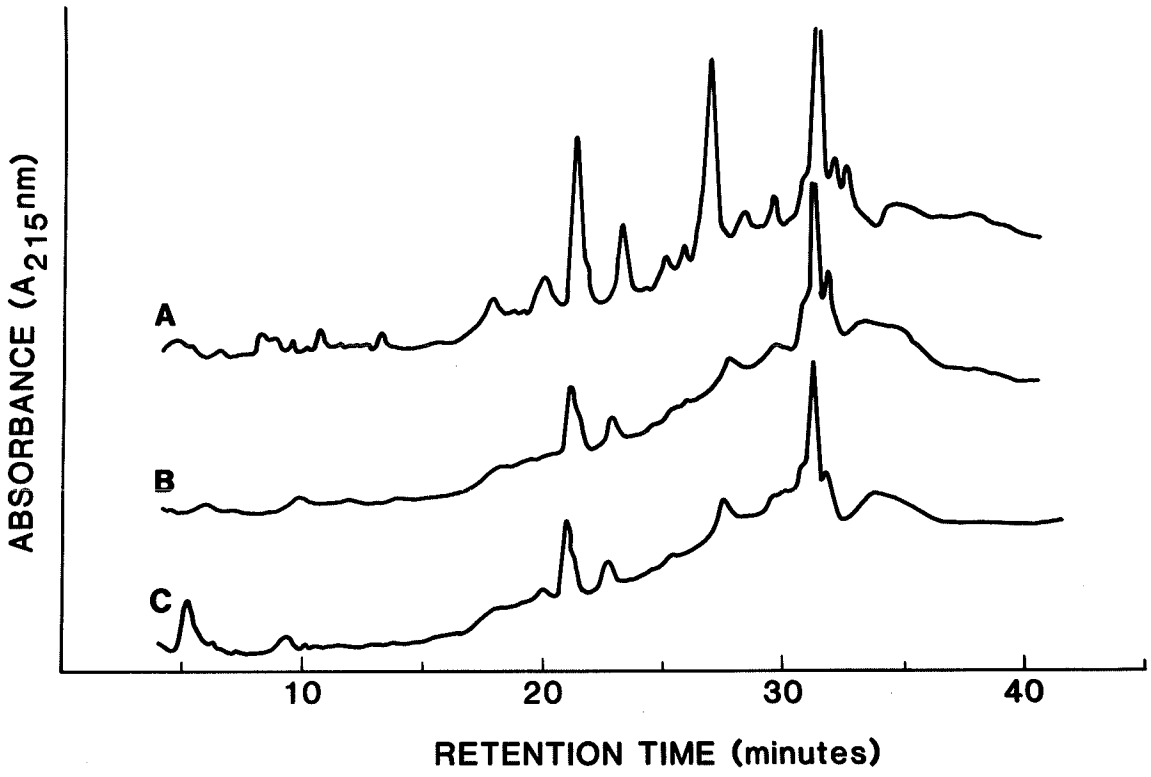
FIGURE 12

Elution pattern of cyanogen bromide treated calmodulins following HPLC (see Methods section).

A. CNBr treated bovine brain calmodulin

B. CNBr treated cucumber calmodulin

C. CNBr treated spinach calmodulin A 0 to 70% gradient of acetonitrile in 0.1% H_3PO_4 was employed for the separation. The flow rate was 1 ml/min.



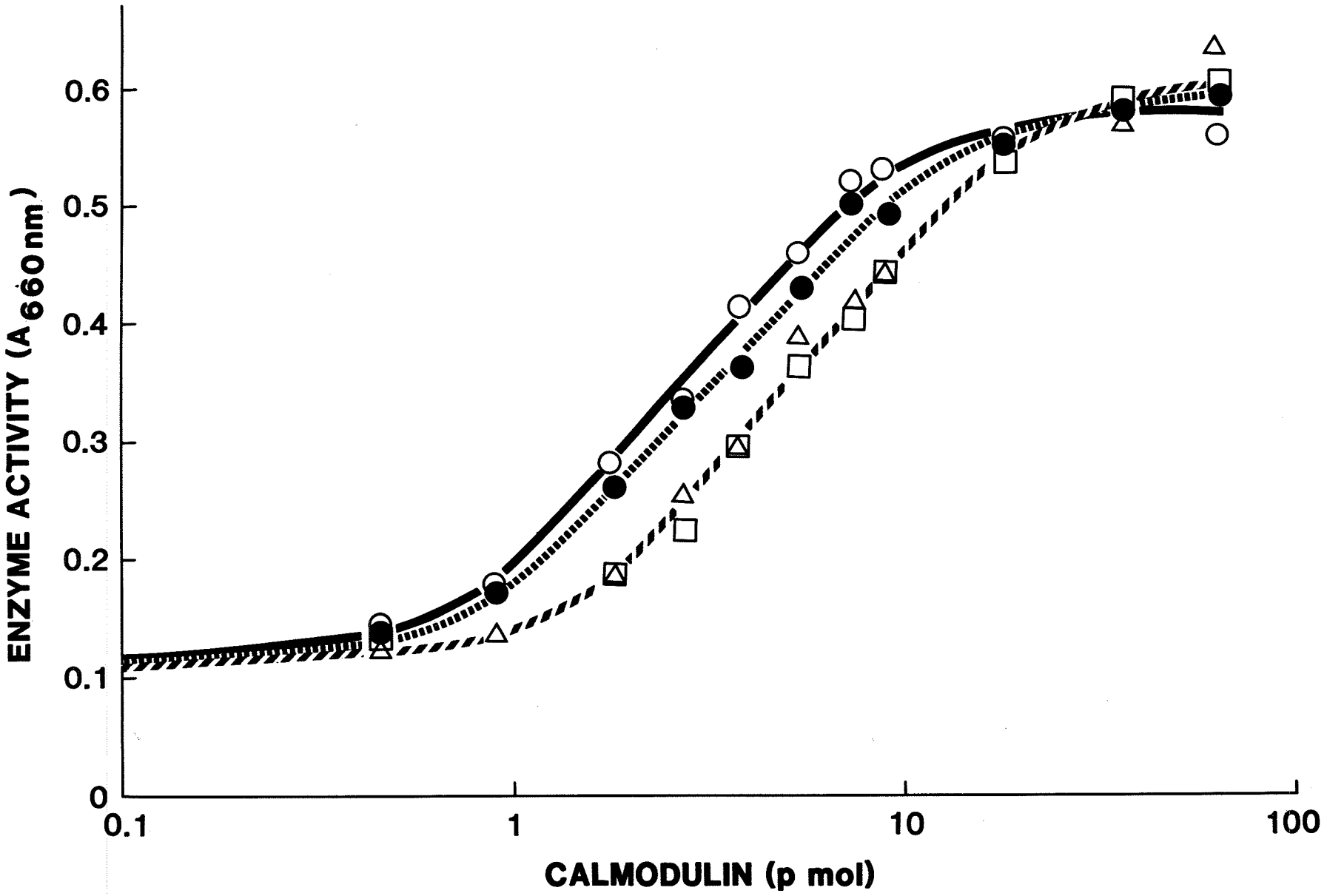

ENZYME ACTIVITY (A_{660nm})
0.6
0.5
0.4
0.3
0.2
0.1
0
0.1
1
10
100
CALMODULIN (p mol)

FIGURE 13

Calmodulin dependent cyclic nucleotide phosphodiesterase activation curve by the purified calmodulins. Brain calmodulin, o-o; spinach calmodulin, ●---●; alfalfa calmodulin, □---□; cucumber calmodulin, △---△.

for both alfalfa and cucumber calmodulin.

When performing the radioimmunoassay, it was discovered that there was considerable non-specific binding of the ^{3}H-labeled calmodulin to the Pansorbin; i.e., labeled calmodulin would bind to the Pansorbin even in the absence of antibody to calmodulin. Usually this value was 45 to 50% of the total radioactivity. Therefore, for each assay a control was employed which included incubating antisera to mouse IgG with the labeled calmodulin. The value obtained for the percent bound radioactivity for this control was subtracted from those values obtained from the incubations with the antibody to calmodulin.

It was also found that the percent of radioactivity bound was much greater in the presence of EGTA. Therefore, all radioimmunoassays included 1mM EGTA.

For the competition assay with unlabeled purified calmodulins, the dilution of 1:20 was chosen from the antiserum dilution curve (Fig. 14) as this dilution factor gives the highest value for the percent of bound radioactivity within the linear region of the curve.

The radioimmunoassay for the purified calmodulins shows a linear region from approximately 5 to 1,000 ng for both brain and plant calmodulins (Fig. 15). With this antiserum therefore, the immunological properties of the calmodulins are very similar.

It has been demonstrated (90) that the inhibition of calmodulin-dependent cyclic nucleotide phosphodiesterase occurs by direct calmodulin-ophiobolin A interaction. Here,

FIGURE 14

Antiserum dilution curve. Fifteen nanograms of ^{3}H-labeled calmodulin (9,200 cpm) in 50 ml phosphate buffered saline with 1mM EGTA (PBS-EGTA) was incubated with 50ml of PBS-EGTA and 50 ml of the following dilutions of antiserum: 1:10, 1:20, 1:40, 1:60, 1:80, 1:100, 1:500 and 1:1,000. The immunologic reaction was followed as described in the methods section. Pansorbin was employed to separate the antibody-linked calmodulin from the free antigen, and the percent radioactivity bound to pansorbin was determined. The final value was that obtained after subtracting percent bound radioactivity from the control, which consisted of labeled calmodulin and antiserum to mouse IgG.

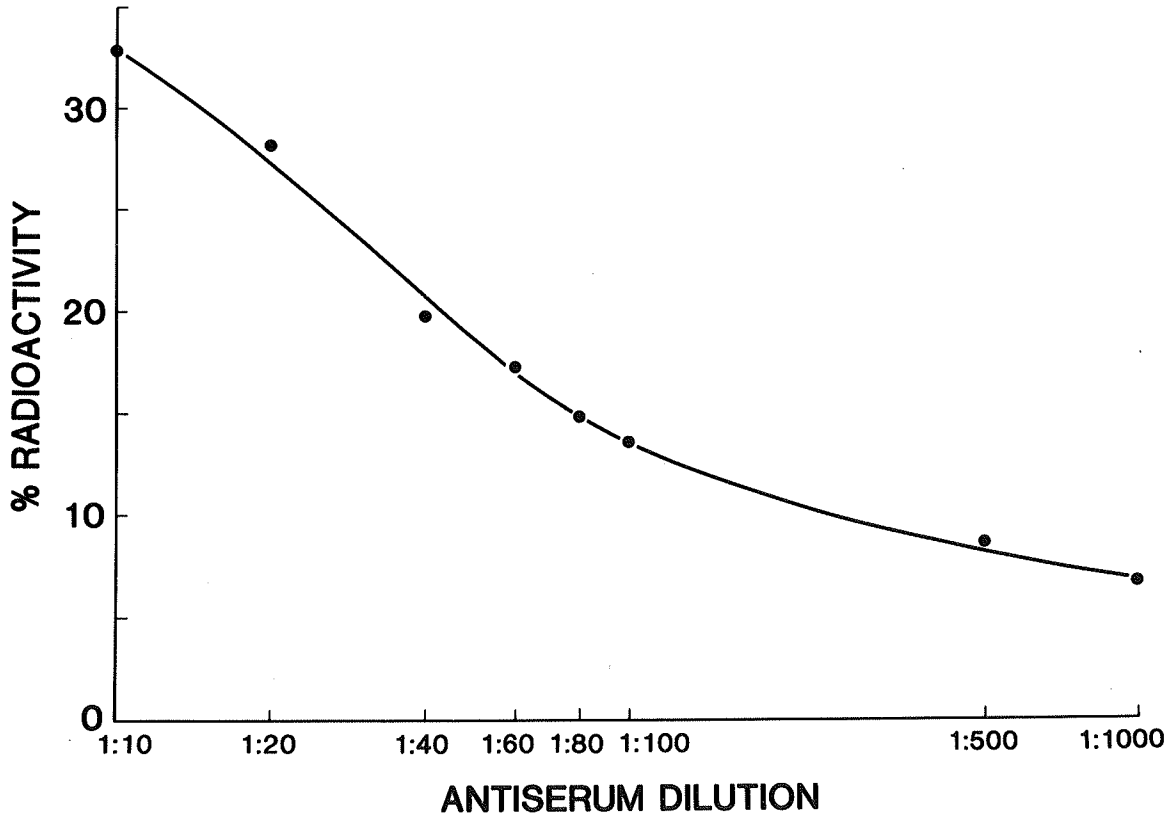
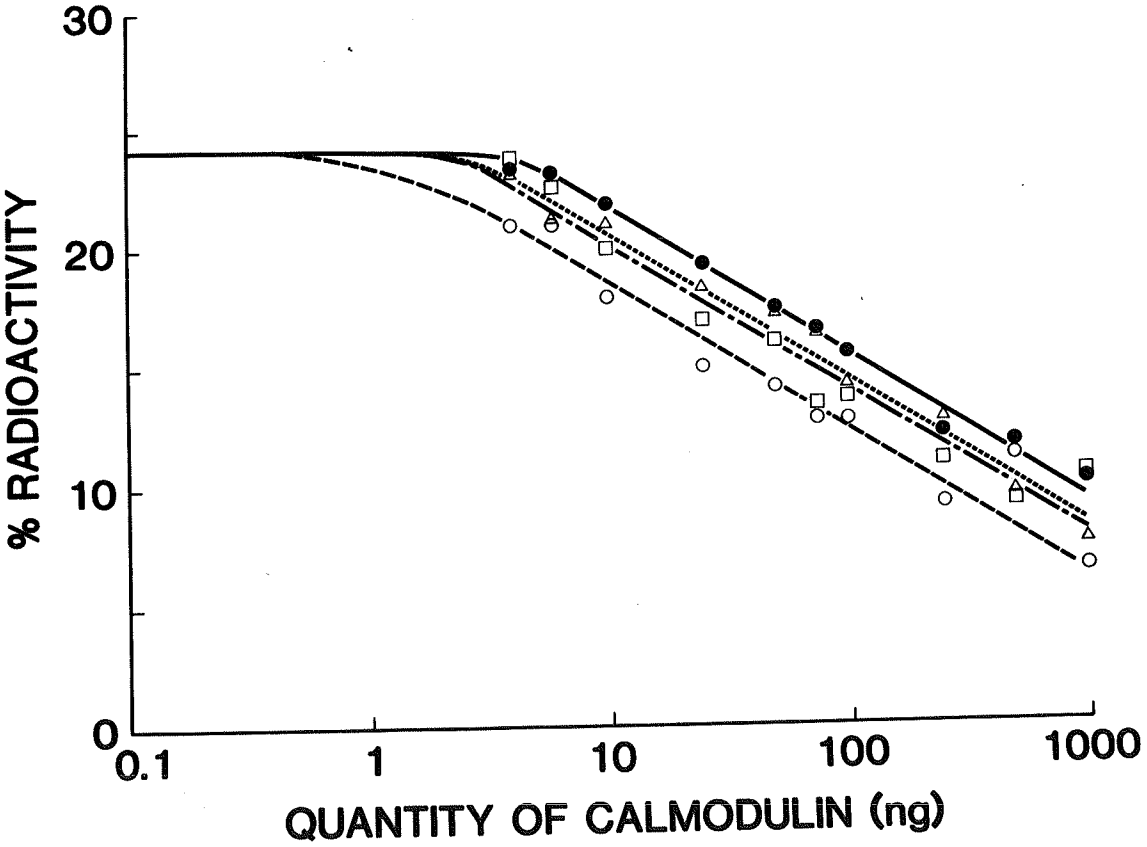


FIGURE 15

Radioimmunoassay of purified brain and plant calmodulins. Purified brain and plant calmodulins with a final quantity ranging from 4 to 1,000 ng (in 50 ml of PBS-EGTA) were mixed with 50 ml of ^{3}H-labeled calmodulin (15 ng, 9,200 cpm) and 50 ml of antiserum (1:20 dilution in PBS-EGTA), and incubated for a further 30 minutes as described (see methods section). A control was also performed in which antiserum to mouse IgG was incubated with ^{3}H-labeled calmodulin and 50 ml of PBS-EGTA. This value was subtracted from those obtained with the incubations using purified calmodulins and antibody to calmodulin.



it is shown that the inhibition kinetics of the ophiobolin A are similar for the brain and spinach calmodulins with half maximal inhibition occurring at approximately 10μM (Fig. 16).

3.6 Isolation of Cyclic Nucleotide Phosphodiesterase Inhibitor

In the presence of bovine brain calmodulin, the dialyzed P. areolatum crude homogenate exhibited potent inhibition of the enzyme (Fig. 17). Similar inhibition kinetics were observed with purified spinach calmodulin (results not shown).

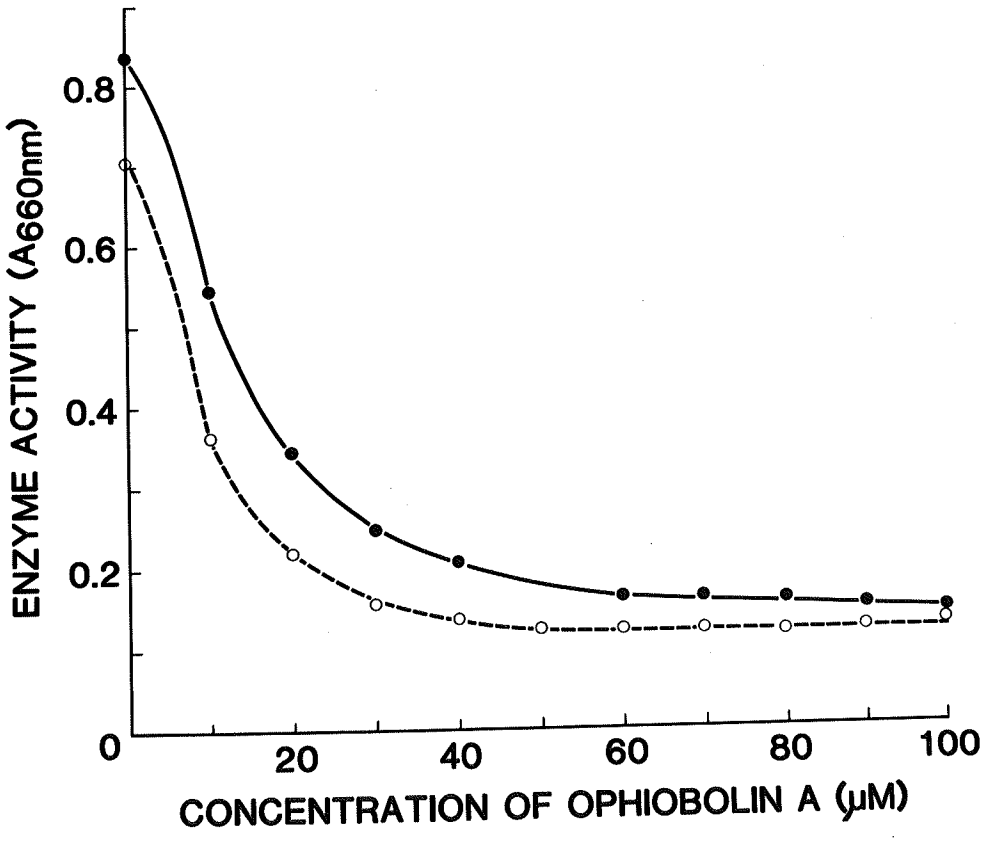
3.7 Purification of the Inhibitor

The fern crude homogenate was applied to a DEAE-cellulose and a CM-cellulose column. The inhibitor bound very strongly to the DEAE-cellulose and most of the inhibitor activity could be released very slowly by washing the column with buffer H containing 3 M urea and 1 M NaCl. The inhibitor did not bind to the CM-cellulose.

The inhibitor bound very strongly to the calmodulin-Sepharose 4B complex and could be released with buffer F containing 6 M urea and 1 M NaCl. The inhibitor also bound strongly although with lower capacity to glycine-Sepharose 4B. However, the recovery of the inhibitor from the glycine-Sepharose 4B exceeds that from the calmodulin-Sepharose 4B (85% and 53% respectively).

FIGURE 16

Inhibition of calmodulin-dependent cyclic nucleotide phosphodiesterase reacttion with ophiobolin A. Calmodulin-dependent cyclic nucleotide phosphodiesterase was performed as described in the Experimental Section using approximately 4 units of brain calmodulin in one series and approximately 4 units of spinach calmodulin in another series. The activity of the phosphodiesterase was determined in the presence of ophiobolin A at concentrations ranging from 10 to 100 μM.



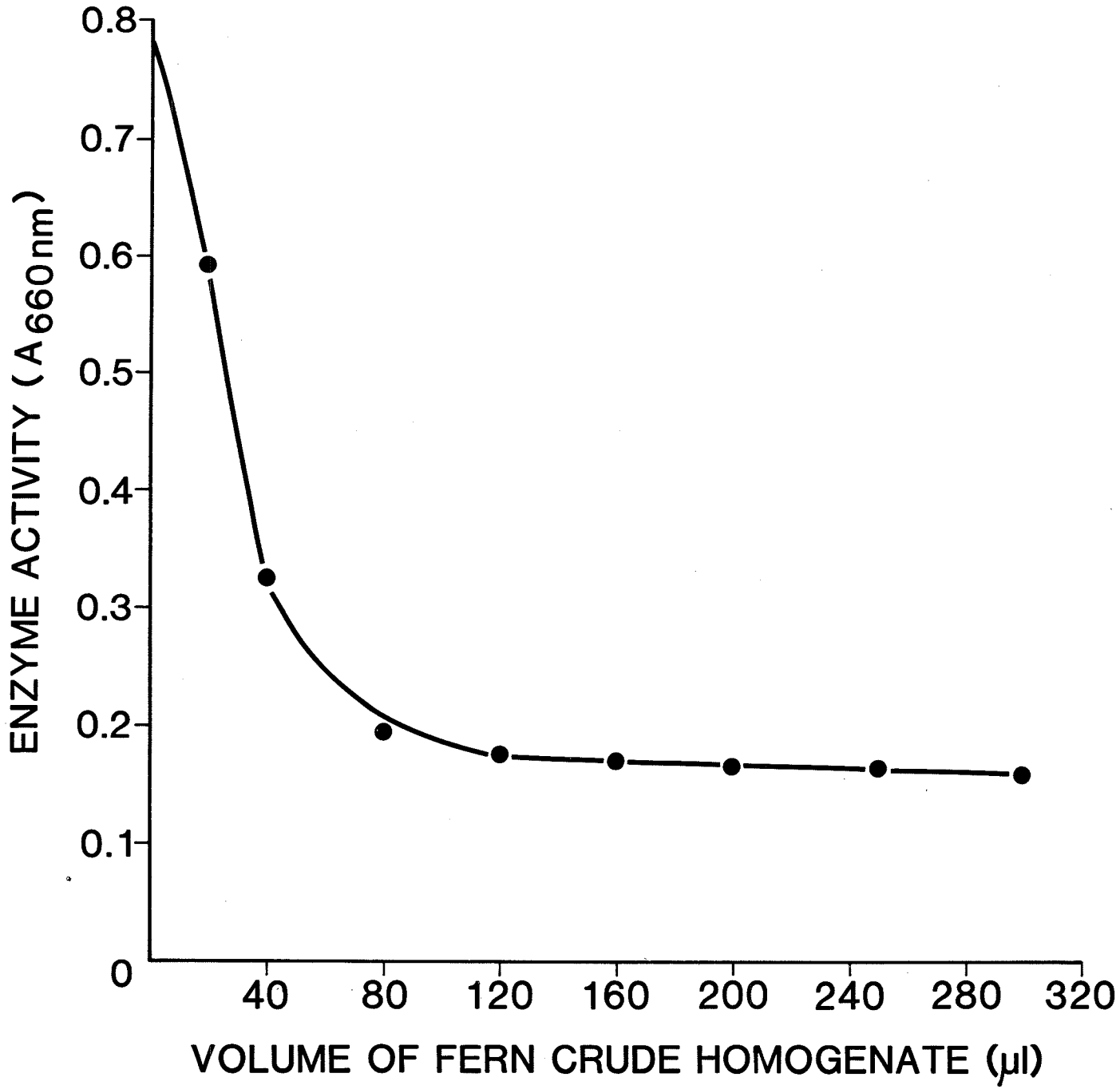

0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
ENZYME ACTIVITY (A660nm)
40
80
120
160
200
240
280
320
VOLUME OF FERN CRUDE HOMOGENATE (µl)

FIGURE 17

Calmodulin-dependent cyclic nucleotide phosphodiesterase inhibitory activity in fern crude homogenate. Phosphodiesterase activity was assayed as described in the presence of increasing volumes of the fern crude homogenate.

Therefore, the former was chosen for routine inhibitor purification.

3.8 Physical and Chemical Properties of the Inhibitor

The inhibiting factor(s) was non-dialyzable. The very strong binding to DEAE-cellulose and lack of binding to CM-cellulose indicates that the inhibitor is negatively charged. Furthermore, the inhibitor was shown to be stable to a variety of harsh treatments including the combination of acid or base treatment with high temperature (Table III). A slight loss in activity was observed following base treatment. The inhibitor was also stable to urea and guanidine-HCl denaturants (Table III).

The partially purified fern inhibitor was stable to the ethanol, 2-propanol and chloroform treatments (Table III). Further, protease digestion did not alter the inhibitor activity (Table III), nor did RNase or DNase treatment.

3.9 Enzyme Inhibition Kinetics

The inhibitor exhibits more pronounced inhibition on the calmodulin activated phosphodiesterase than on the basal and calmodulin independent forms of the enzyme (Fig. 18). Half maximal inhibition of enzyme activity was achieved with 55 ul of the inhibitor. At the same level of inhibitor there is a slight activation of the basal form of the enzyme and a negligible effect on the independent form.

TABLE III

INHIBITOR ACTIVITY FOLLOWING CHEMICAL TREATMENT

Treatment of Crude Homogenate	Volume required for 1/2 maximal inhibition (µl)
Control	57
Base (1N NaOH)	128
Acid (1N HCl)	73
Urea (3M) + NaCl (0.5M)	57
Guanidine-HCl	57
Treatment of Partially Purified Inhibitor	
Control	57
Trypsin	57
Pronase	55
Ethanol	57
2-Propanol	44
Chloroform	52

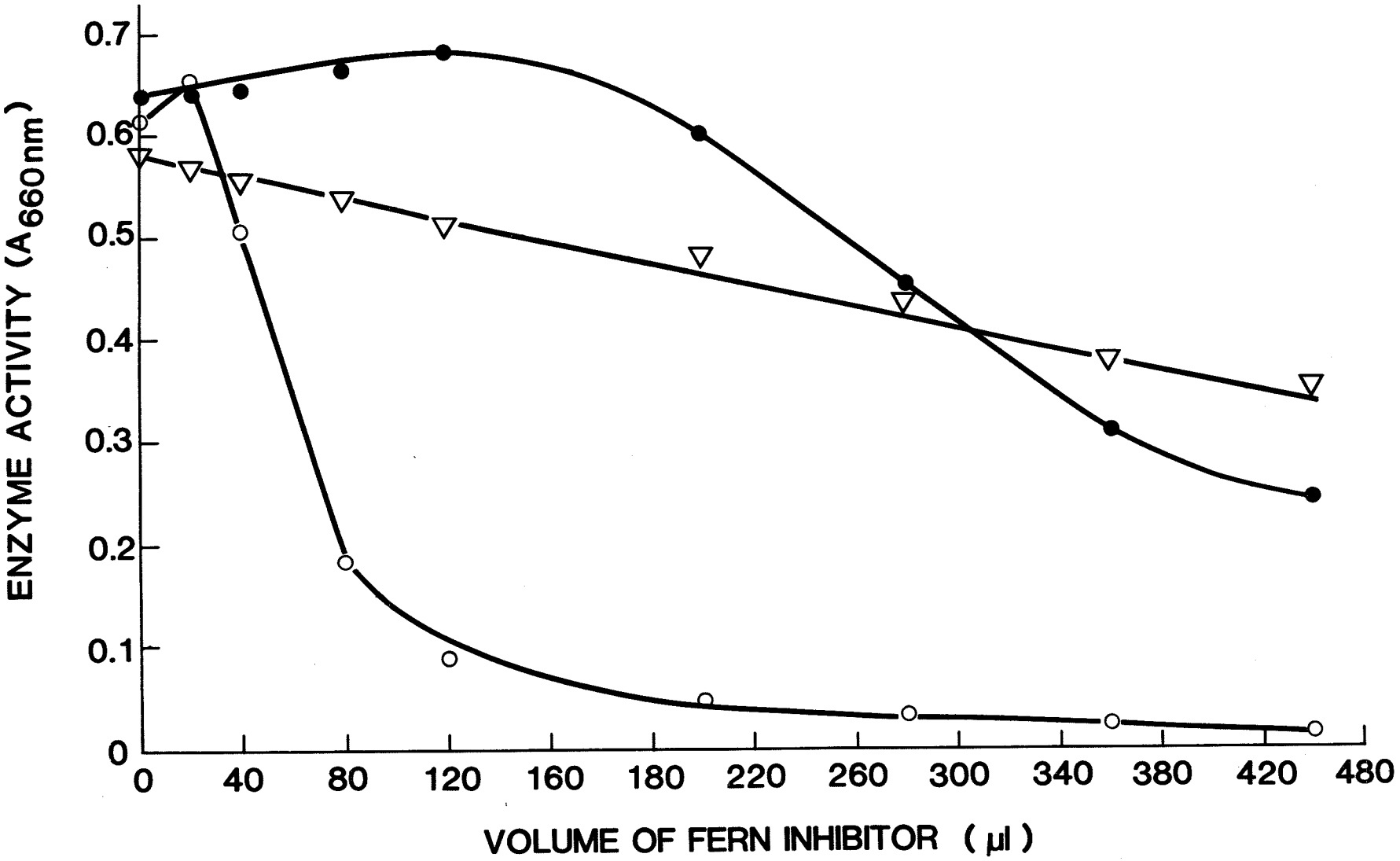

ENZYME ACTIVITY (A_{660nm})
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
40
80
120
160
180
220
260
300
340
380
420
480
VOLUME OF FERN INHIBITOR (µl)

FIGURE 18

Preferential inhibition of calmodulin-dependent phosphodiesterase. Phosphodiesterase (calmodulin-dependent, basal and calmodulin-independent forms) was titrated with increasing volumes of the glycine-Sepharose purified fern inhibitor: (-○-○-), 0.012 units of the enzyme with 4 units of calmodulin; (- ● - ● -), 0.012 units of the enzyme, no calmodulin; (-▽-▽-) 0.011 units of the calmodulin-independent form of the enzyme.

The inhibition on the enzyme is not attributed to chelation of Ca^{2+}. The inhibition kinetics were identical regardless of an increase in Ca^{2+} concentration in the enzyme assay from 0.1 to 1.0 mM Ca^{2+} (Fig. 19).

The inhibition kinetics of the inhibitor have been compared to that by calcineurin by monitoring the rate of inhibition. Calcineurin inhibits the enzyme activation by competing with the enzyme for calmodulin (71-74). The rate of inactivation of the enzyme by calcineurin was dependent upon the release of free calmodulin from the calmodulin-enzyme complex, a slow process. Comparatively, the inhibitor, at the same quantity as calcineurin (5 units) had a much more rapid rate of inhibition (Fig. 20). Therefore, the inhibitor can bind to the calmodulin-enzyme complex.

The inhibitor was not competitive with calmodulin. Further addition of calmodulin from 4 to 40 units to the enzyme assay mixture in the presence of 2 units of the inhibitor does not reverse the inhibition (Fig. 21).

The activation of the enzyme by lysophosphatidylcholine (LPC) is reversible and is not dependent on Ca^{2+} (3). Maximal activation is achieved at 90 μM LPC (Fig. 22). In the presence of 3 units of the inhibitor there was an additional 2-fold stimulation of the enzyme. At low concentrations of LPC (20 μM), the stimulation by the inhibitor was not observed (Fig. 23). In fact, the inhibition kinetics for the lipid activated enzyme at low LPC concentration resemble that for the calmodulin activated form

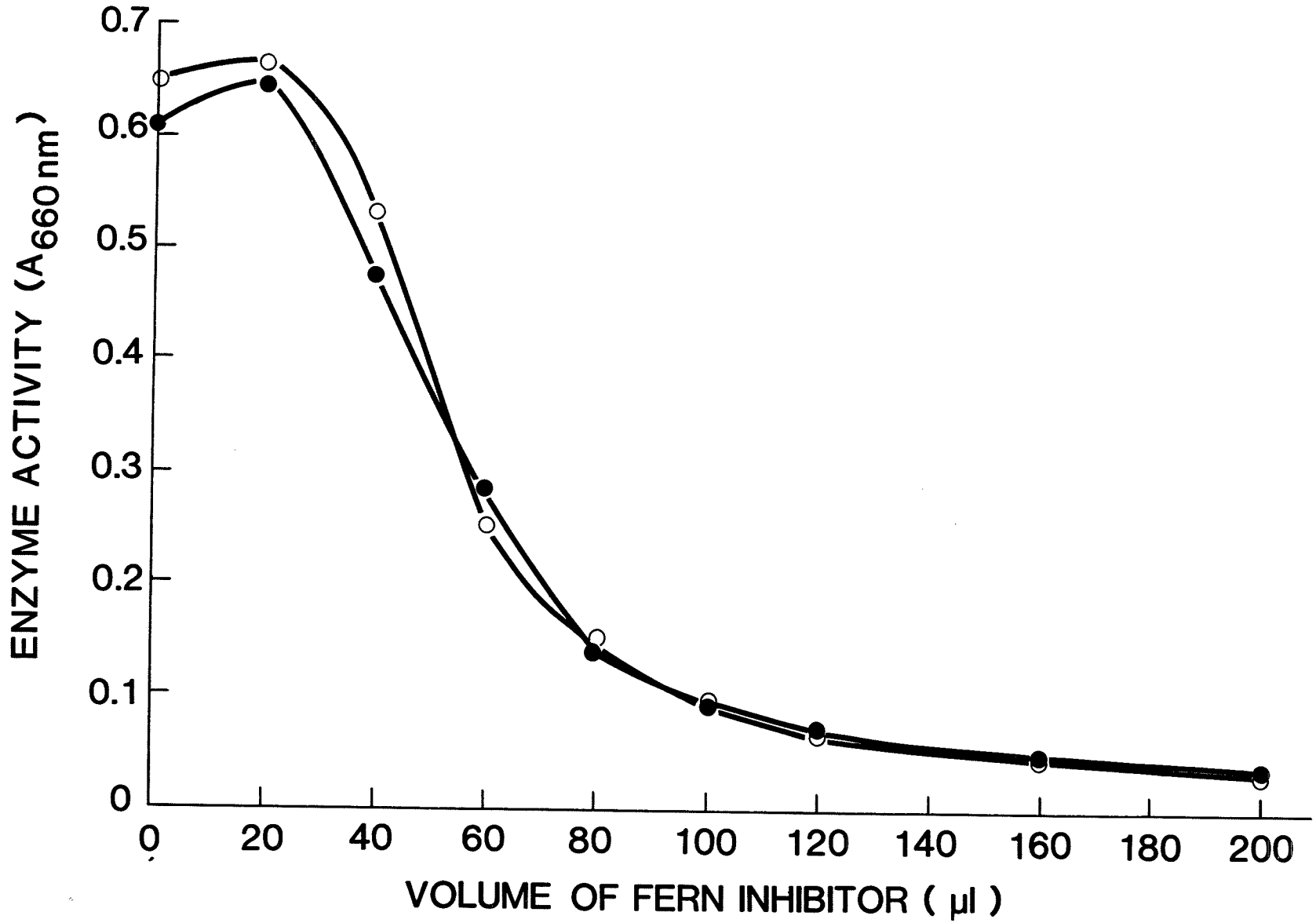

ENZYME ACTIVITY ($A_{660\,nm}$)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
20
40
60
80
100
120
140
160
180
200
VOLUME OF FERN INHIBITOR (µl)

FIGURE 19

Effect of increasing volumes of inhibitor on the activity of 0.10 (-●-●-) and 1.0 (-○-○-) mM concentrations of Ca^{2+}.

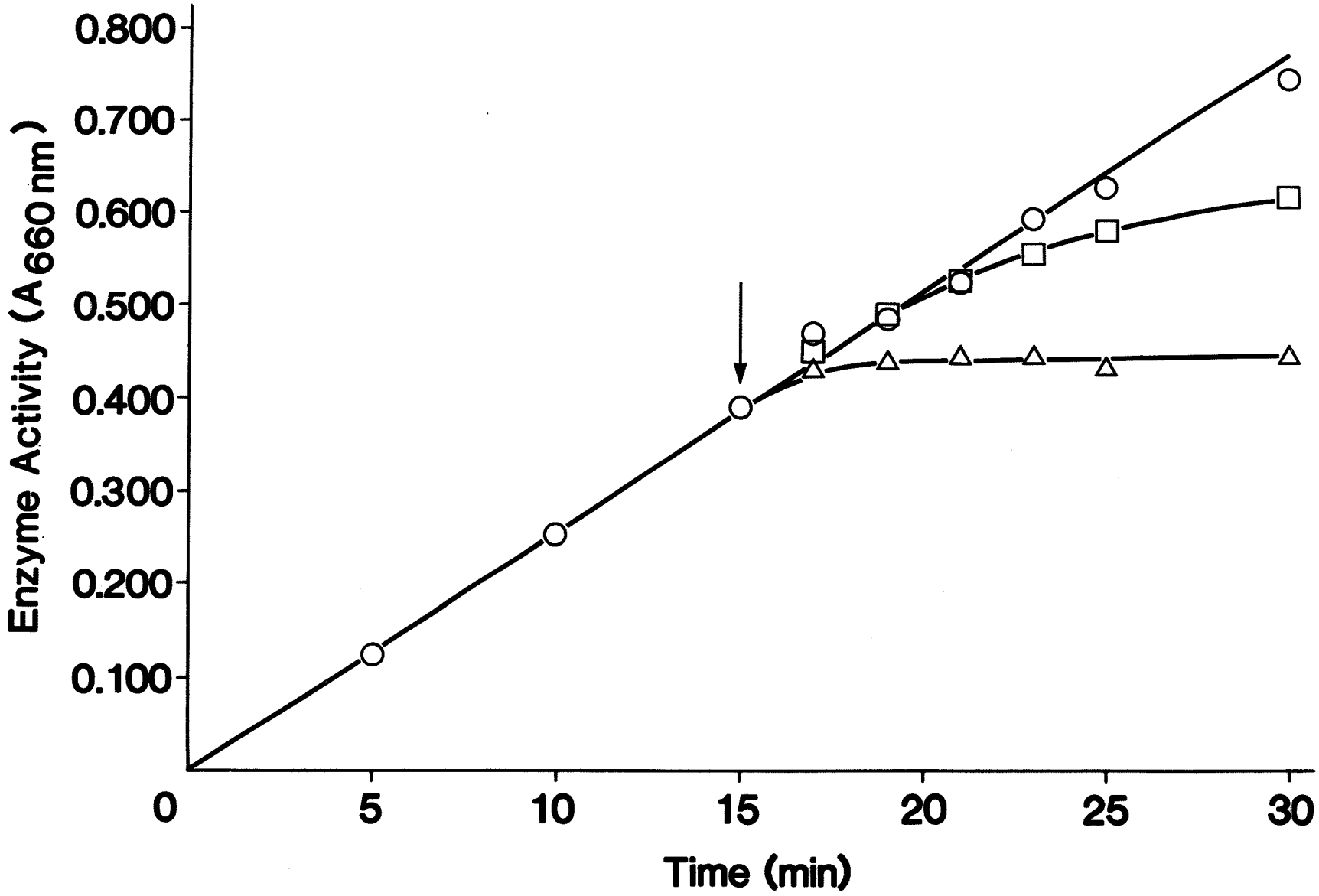

0.800
0.700
0.600
0.500
0.400
0.300
0.200
0.100
Enzyme Activity (A660 nm)
0
5
10
15
20
25
30
Time (min)

FIGURE 20

Inhibition kinetics of the inhibitor and calcineurin. The enzyme activity was determined at specific time intervals during the reaction. At the 15 minute time interval, 200 µl of either the fern inhibitor or calcineurin (5 units of each) was added to separate reaction vessels. The enzymes reaction was terminated after incubation intervals of 2, 4, 6, 8, 10 and 15 minutes and the enzyme activity was determined as described in the text: (-○-○-), no addition of inhibitor; (-□-□-), 5 units of calcineurin; (-△-△-), 5 units of the inhibitor.

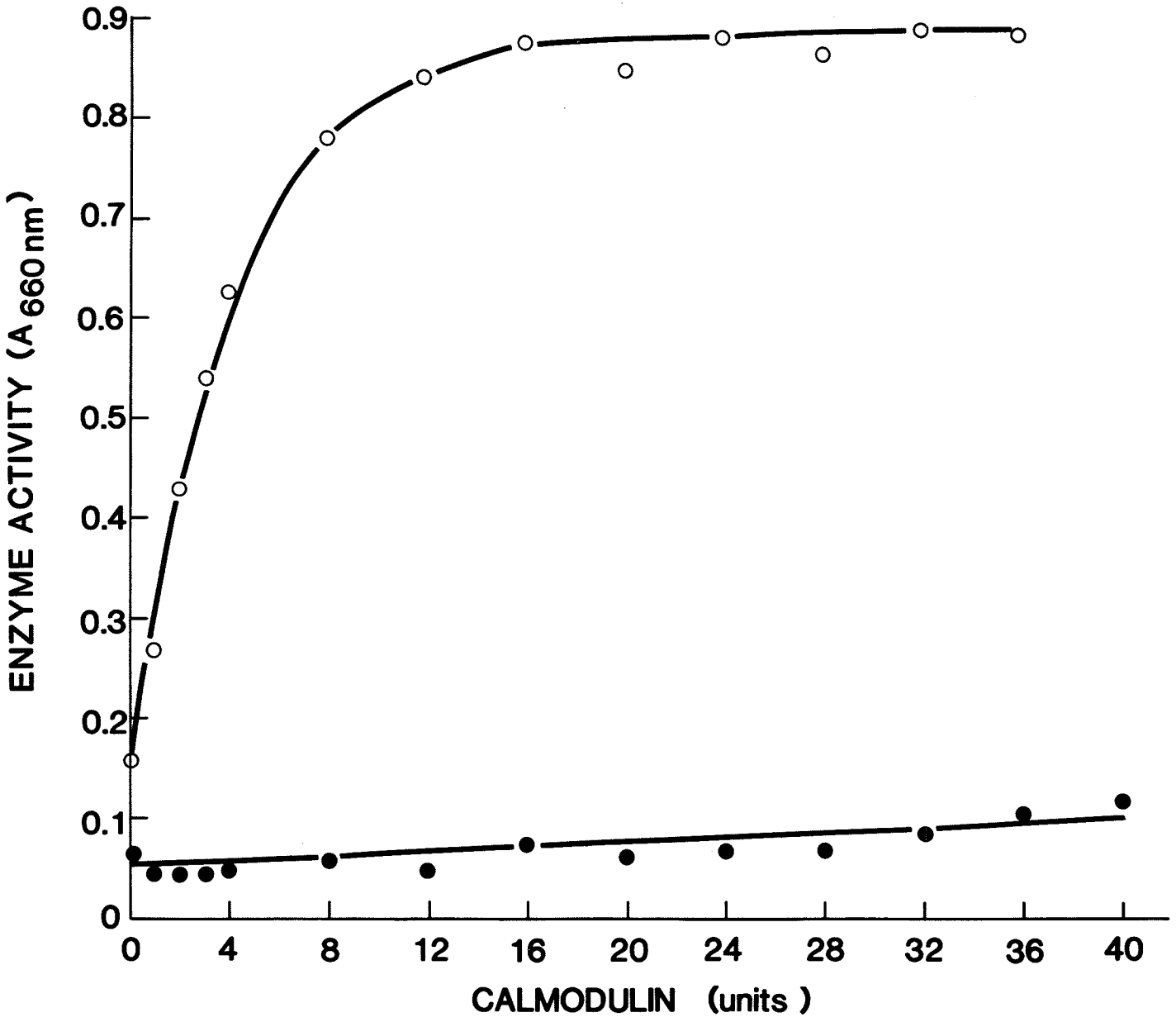

ENZYME ACTIVITY ($A_{660\,nm}$)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
4
8
12
16
20
24
28
32
36
40
CALMODULIN (units)

FIGURE 21

Titration of the enzyme (0.012 units) with calmodulin in the presence (-●-●-) and absence (-o-o-) of 2 units of the inhibitor.

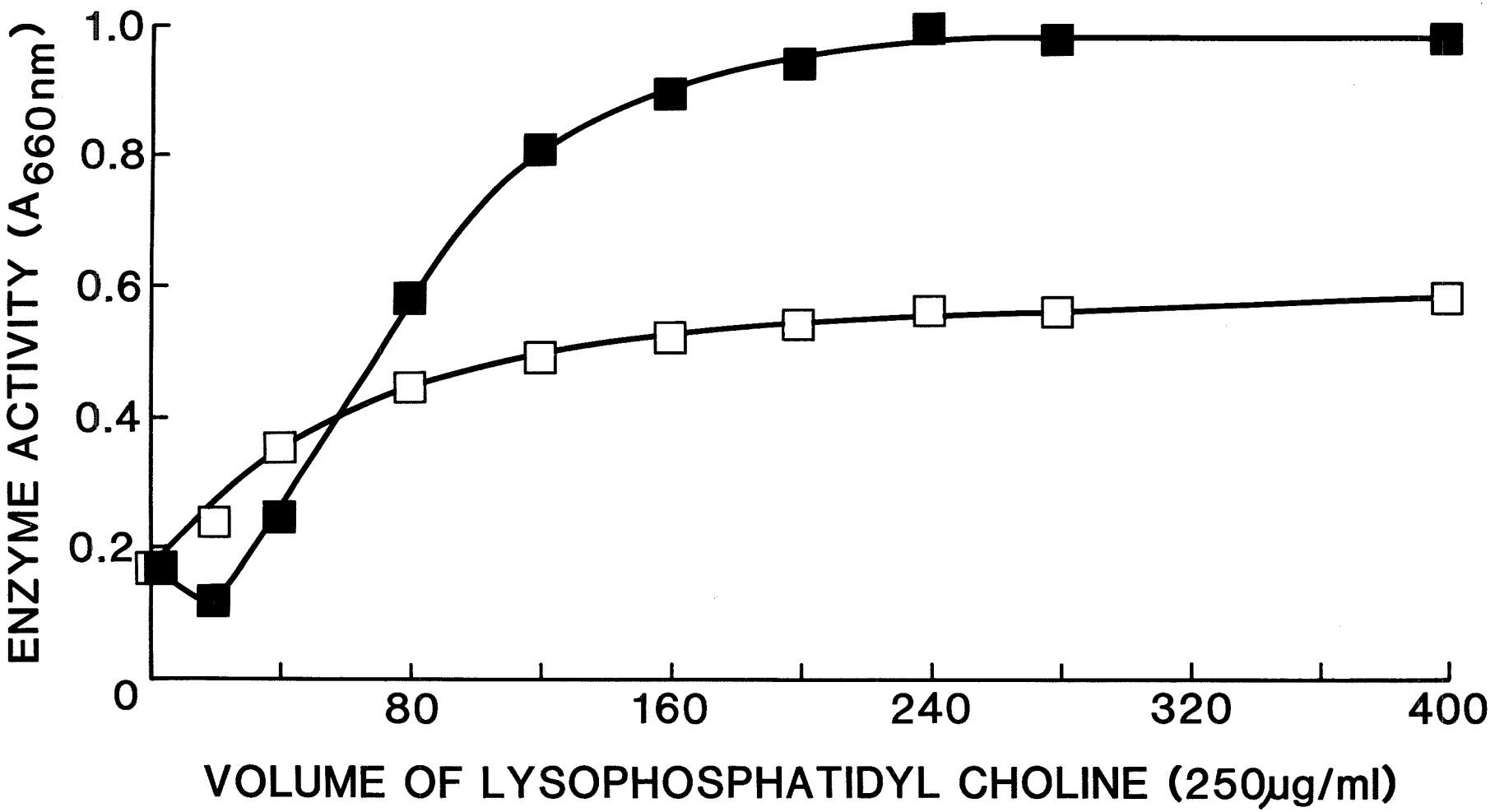

ENZYME ACTIVITY (A_{660nm})
1.0
0.8
0.6
0.4
0.2
0
80
160
240
320
400
VOLUME OF LYSOPHOSPHATIDYL CHOLINE (250μg/ml)

FIGURE 22

Activation of the enzyme with lysophosphatidylcholine (LPC) and the effect of the inhibitor. The enzyme was titrated with increasing volumes of LPC (.25mg/ml) in the presence (-■-■-) and absence (-□-□-) of 3 units of the inhibitor.

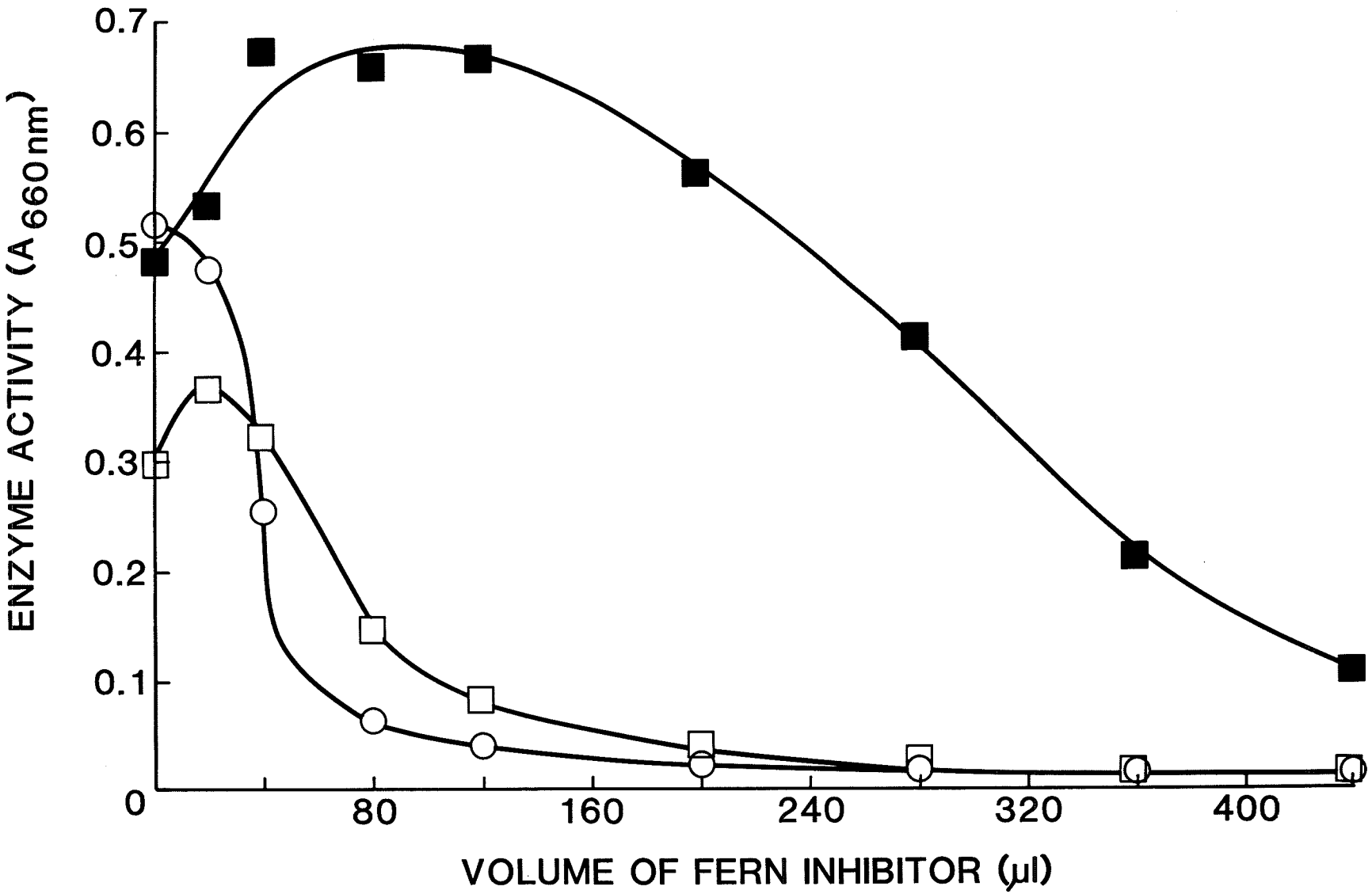

ENZYME ACTIVITY (A_{660nm})
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
80
160
240
320
400
VOLUME OF FERN INHIBITOR (µl)

FIGURE 23

Titration of calmodulin-activated and LPC-activated phosphodiesterase with the inhibitor. Phosphodiesterase which was activated by 15 pmoles calmodulin (-o-o-), 30 µl LPC (0.25 mg/ml) (-□-□-), and 60 µl LPC (1.0 mg/ml) (-■-■-) was titrated with increasing volumes of the inhibitor.

(Fig. 23). The difference observed at low and high concentration of LPC may indicate the presence of two LPC binding regions on the enzyme.

The enzyme has been activated through limited proteolysis by digestion with trypsin for 2 minutes at 30°C. The trypsin activated enzyme was not stimulated by calmodulin (results not shown), nor with LPC (Fig. 24). However, the trypsin activated enzyme bears similar inhibition kinetics to the calmodulin activated enzyme (Fig. 25). Furthermore, when trypsin activated enzyme is inhibited with five units of the inhibitor, high concentration of LPC can reverse the inhibition (Fig. 24). The trypsin digested enzyme retains the binding site for high concentrations of LPC.

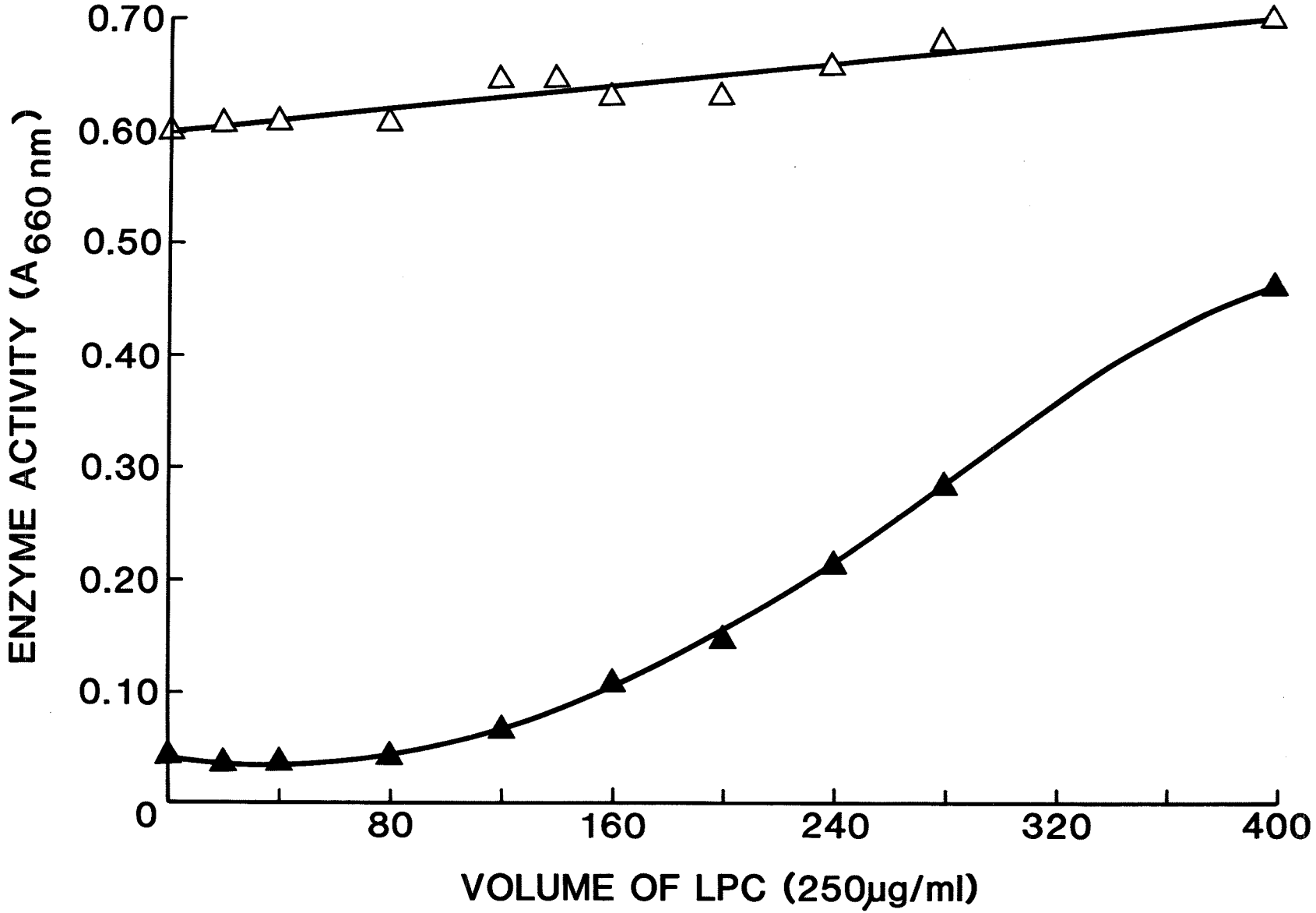

0.70
0.60
0.50
0.40
0.30
0.20
0.10
0
ENZYME ACTIVITY ($A_{660\,nm}$)
80
160
240
320
400
VOLUME OF LPC (250µg/ml)

FIGURE 24

Titration of trypsin-activated phosphodiesterase with LPC in the presence (-▲-▲-) and absence (-△-△-) of 5 units of the inhibitor.

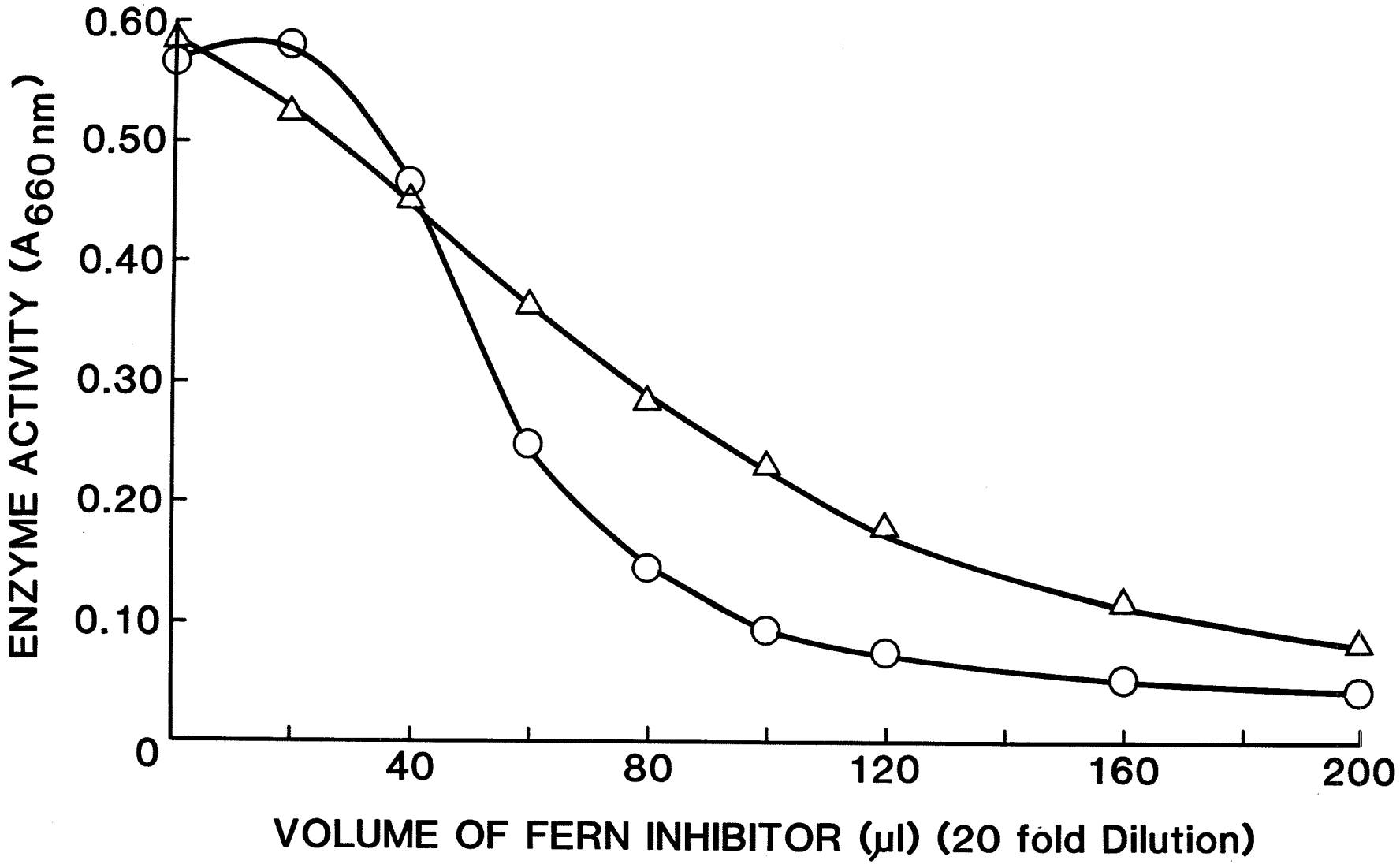

ENZYME ACTIVITY ($A_{660\,nm}$)
0.60
0.50
0.40
0.30
0.20
0.10
0
40
80
120
160
200
VOLUME OF FERN INHIBITOR (µl) (20 fold Dilution)

FIGURE 25

Titration of calmodulin-activated and trypsin-activated phosphodiesterase with the inhibitor. Calmodulin-activated enzyme (-o-o-) and trypsin-activated enzyme (-Δ-Δ-) were incubated as described in the presence of increasing volumes of the inhibitor.

DISCUSSION

Cheung (5) originally demonstrated that mammalian calmodulins lack tissue specificity. Calmodulins from various mammalian cells display identical specific activity toward bovine brain phosphodiesterase. Subsequently, it was found that calmodulins from many invertebrates could also activate the phosphodiesterase (21). The suggestion that calmodulin lacks tissue and species specificity is further substantiated in the study by Waisman (75) which demonstrated the activation of the phosphodiesterase by plant extracts. More extensive characterization on the purified plant calmodulins has verified the similarities between the mammalian and plant calmodulins on the basis of chemical, physical, biological and immunological properties. The results suggest that the structure required for the interaction between calmodulin and the calmodulin-dependent phosphodiesterase is conserved in the plant proteins. Cyclic nucleotide phosphodiesterases are present in plants (52), although the regulation of plant phosphodiesterase by calmodulin has not been demonstrated as yet.

In addition to the activation of bovine brain cyclic nucleotide phosphodiesterase, plant calmodulins are also capable of interacting with bovine brain calmodulin binding protein I and of activating rabbit skeletal muscle myosin light chain kinase. These observations are consistent with the idea that the interaction of calmodulin with various calmodulin-regulated enzymes involves the same "binding site"

on calmodulin. Presumably, this "binding site" is required for the function of calmodulin in plants.

In mammalian tissues,, calmoldulin can regulate the activities of many enzymes and therefore, is implicated in the regulation of several cellular processes (for review, see refs 16-19). This phenomenon suggests that calmodulin is a general Ca^{2+}-mediatory protein. It is possible that calmodulin plays a similar general Ca^{2+}-mediatory role in plants and in lower forms of animals. However, the specific cellular processes regulated by calmodulin in plants are in the process of delineation. Thus far, the only plant enzymes which have been shown to be calmodulin dependent are NAD kinase (53,96,97) and Ca^{2+}-Mg^{2+} ATPase (98). In addition, Grand et al (59) have presented preliminary evidence for calmodulin binding proteins in barley and fungi. Furthermore, at least seven proteins are observed on gel electrophoresis from a fraction exhibiting Ca^{2+} dependent binding to Calmodulin-Sepharose (96). Further studies are required in order to establish the physiological functions of the protein in plants..

From amino acid sequence studies, calmodulin has been demonstrated to belong to a family of homologous proteins which also includes parvalbumins, myosin light chains, troponin-C, and mammalian intestinal Ca^{2+} binding proteins (for review, see ref 99). On the basis of sequence information, Barker et al (100) have constructed a family tree for these proteins which shows that calmodulin is the most slowly changing protein in the family. The results

which illustrate that plant calmodulins possess similar molecular properties to the mammalian calmodulins are consistent with the idea that this protein is highly conserved in structure. Similar results were obtained from researchers working on calmodulins from a variety of plant sources (59-61,101) from fungi (44,53,59), and from protozoans (35,102).

Important diferences do exist however, within the plant calmodulins. They have a higher proportion of lysyl, aspartyl, glutamyl and proline residues than do the mammalian calmodulins. The cyanogen bromide peptide map shows a very similar pattern for the plant calmodulins i.e. the spinach and cucumber, while some differences occur when compared with brain calmodulin. These results corroborate those by Jarrett et al (97) who have shown significant difference in primary structure between peanut seed and brain calmodulin by examination of the CNBr peptides by gel electrophoresis.

The chemical and physical properties of the inhibitor show a highly stable, water soluble, negatively charged, organopolymer which is neither a protein nor a nucleic acid. The lack of identity has permitted quantitation on the basis of enzymatic inhibitory activity rather than on a per mol basis.

The inhibitor exhibited a much higher potency of inhibition on the calmodulin-activated activity than on the basal activity of the calmodulin-dependent cyclic nucleotide phosphodiesterase. Since high concentrations of calmodulin did not reverse or prevent the inhibition, it is suggested

that the inhibition is not due to the competition between calmodulin and the inhibitor for binding to the enzyme, nor to the competition of the enzyme and the inhibitor for calmodulin binding. The possibility that the inhibitor antagonizes the calmodulin activation by Ca^{2+} binding is also ruled out since excessively high concentrations of Ca^{2+} had no effect on the enzyme inhibition.

It is plausible therefore, to suggest that the inhibitor binds to the activated enzyme at a site distinct from the calmodulin binding site to induce enzyme inhibition. Several other observations also support such a suggestion. The time course of the enzyme inhibition by the inhibitor is clearly different from that by calcineurin. Since calcineurin inhibits the calmodulin activation of the enzyme reaction by binding to calmodulin, the time course of its inhibition represents that of the dissociation of calmodulin from the enzyme. The observation that the inhibition of the phosphodiesterase by the inhibitor was instantaneous strongly supports the view that the inhibitor binds to the activated enzyme to cause the enzyme inhibition. The observation that the inhibitor showed potent inhibitory activity against the trypsin-activated and lysophophotidylcholine-activated enzyme is also in agreement with such a suggestion. The trypsin-activated phosphodiesterase does not require calmodulin for activation suggesting that all or part of the calmodulin binding domain has been removed from the enzyme. The inhibition of the enzyme by the inhibitor, therefore, indicates that the binding of the inhibitor is independent of

the presence of the calmodulin-binding domain.

The effect of the inhibitor on the LPC-activated enzyme is complex and cannot be explained by a simple one site effect. At low LPC concentration, the inhibition kinetics resemble the calmodulin-activated enzyme. At high LPC concentration, the inhibitor stimulates enzyme activity. The integrity of the binding site at high LPC concentration is maintained during proteolysis because analogous to the intact enzyme, the inhibition of the trypsin-activated form of the enzyme cn be reversed with high LPC concentration.

The study has described a complex organic molecule with unique chemical and physical characteristics, the properties of which have been employed in kinetic analysis of the calmodulin-dependent cyclic nucleotide phosphodiesterase. A knowledge of the inhibitor structure will help in elucidating further the structure and the regulatory properties of the enzyme.

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