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Purification and Characterization of A Calcium Binding Protein Complex in Sea Urchin Eggs

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled, "Purification and Characterization of a Calcium Binding Protein Complex from Sea Urchin Eggs" submitted by Roy M. Golsteyn in partial fulfillment of the requirements for the degree of Master of Science.

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ABSTRACT

Calcium binding proteins may mediate the calcium dependent processes involved in egg activation. Calmodulin, the sole calcium binding protein characterized in sea urchin eggs, only regulates some of these calcium dependent events. Therefore, other calcium binding proteins may also be involved in egg activation. By identifying and characterizing these proteins, one may understand how calcium can initiate the conversion of an egg into an embryo.

Analysis of the DEAE fractionated, 100,000 x g supernatant of sea urchin egg extracts by the chelex-100 calcium binding assay, revealed that calmodulin only accounted for a fraction of the total calcium binding activity. A 50 kDa and 43 kDa protein complex was purified, from the DEAE fractions that contained the highest calcium binding activity, by hydroxyapatite, gel filtration, and FPLC anion exchange chromatography. On nondenaturing gels, these proteins migrated as a single 80 kDa band that bound calcium as detected by ^{45}calcium autoradiography. The 43 kDa band was thought to be actin because it reacted with an anti-actin antibody, had a peptide digest pattern similar to rabbit actin, and bound to DNase I. The 80 kDa complex was then purified by DNase I affinity chromatography in a calcium dependent manner. A pyrene labeled actin assay revealed that the complex inhibited the rate and extent of actin polymerization.

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LIST OF ABBREVIATIONS

BSA	bovine serum albumin
DEAE	diethylaminoethyl cellulose
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetate
EGTA	ethylene glycol bis (β-aminoethyl ether)-N,N'tetraacetate
FPLC	fast protein liquid chromatography
GDP-β-S	guanosine 5'-O-(2-thiodiphosphate)
GTP-γ-S	guanosine 5'-O-(3-thiotriphosphate)
HPLC	high pressure liquid chromatography
HEPES	N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid
IP3	inositol (1,4,5) trisphosphate
IP4	inositol (1,3,4,5) tetrakisphosphate
kDa	kiloDaltons
MOPS	3-(N-morpholino)propane-sulfonic acid
NAD	nicotinamide adenine dinucleotide
SDS	sodium dodecyl sulfate
TRIS	tris(hydroxymethyl)aminomethane

1.0. INTRODUCTION

1.1. Overview

Calcium is believed to be the universal activator of all animal eggs (Jaffe, 1985). A sperm initiates an increase in intracellular free calcium when it contacts the egg surface at fertilization. The egg is then committed to divide and develop into its adult form. How a simple calcium stimulus invokes the complex processes of development is one of the fundamental questions in developmental biology.

Proteins are the most likely receptors of the intracellular calcium signal (Levine and Dalgarro, 1983). These calcium binding proteins must be present in unfertilized eggs, since the increase in intracellular calcium occurs immediately after fertilization. By identifying and characterizing the calcium binding proteins in unfertilized eggs, one may understand how calcium is involved in converting an egg into an embryo.

Calmodulin, a ubiquitous calcium binding protein, has been identified in sea urchin eggs (Head et al., 1979). It is unlikely, however, that only one calcium binding protein mediates all of the processes involved in egg activation. Therefore, we examined the 100,000 x g supernatants of unfertilized sea urchin eggs to determine if other calcium binding proteins were present. We found that calmodulin accounted for only a fraction of the total calcium binding activity in eggs. The remainder of the calcium binding activity was partly due to a novel 50 kDa actin binding protein which we have characterized. The methods used to identify this 50 kDa protein and

details of its properties are presented in this thesis. The reasons for using sea urchin eggs, the discovery and the role of calcium in eggs, and the role of actin and actin binding proteins, will also be discussed.

1.2. Sea urchin eggs as a model tissue

The sea urchin, a marine echinoderm, is an excellent organism in which to study the role of calcium in egg activation. It has been studied since the late 18th century and is the source of much of the data on egg activation, most of which is applicable to eggs from different species and phyla (Jaffe, 1983). Sea urchin eggs are particularly suited to biochemical analysis since one can acquire gram quantities. A typical harvest of eggs from a female urchin results in 2-10 mL of 100 μm diameter, mature, fertilizable eggs. It is easy to obtain sea urchin embryos that are at the same stage of development. Consequently, it is possible to detect proteins that are specific for a particular phase of the cell cycle, a feature that is not possible with an asynchronous embryo culture. Sea urchin eggs are transparent; therefore one can follow the cell cycle with a microscope. This permits the use of intracellular markers such as fluorescent dyes or antibodies in sea urchin eggs.

Eggs from other animals are not as easily studied. Mammalian eggs are extremely difficult to obtain in gram quantities. They also require a sterile and complex media for culturing, are not easily grown as synchronous cultures, and are almost too small to be

successfully microinjected. It is possible to acquire gram quantities of amphibian eggs, such as Xenopus laevis, however these are more difficult to study than sea urchin eggs. Amphibian eggs are opaque due to intracellular yolk particles. This makes microscopic observation of egg development impossible. Also, Xenopus eggs are difficult to fertilize synchronously in vitro. Therefore, the vast literature, suitability to investigative techniques, availability and ease of handling, make sea urchin eggs an ideal system in which to investigate calcium binding proteins.

1.3. An overview of egg activation

The process of fertilization is briefly outlined here to provide a framework for the biochemical events described in the following sections.

The first measurable change (see Table 1.) that occurs in sea urchin eggs after fertilization is membrane depolarization (Jaffe, 1976). This is the fast block to polyspermy (Jaffe and Gould, 1985). It ensures that only one sperm will fertilize the egg. It is followed by an immediate increase in intracellular free calcium and a later increase in intracellular pH. Eggs can be partially activated by an increase in pH alone, however, calcium is required to fully activate the eggs because it initiates a series of calcium dependent events that include the change in intracellular pH (Whitaker and Steinhardt, 1985). Once an egg is fertilized, the sperm and egg pronuclei fuse to reestablish a diploid genome and DNA and protein synthesis occur. A

TIME	EVENT
0	sperm binds to the egg
0.1 seconds	membrane depolymerization
10 "	IP3 and diacylglycerol release
25 "	intracellular calcium release
50 "	conversion of NAD to NADPH
50 "	cortical granule exocytosis
2 minutes	increase in intracellular pH
5 "	tyrosine kinase activity increases
5 "	increase in protein synthesis
8 "	microvilli elongate
25 "	pronuclear fusion
30 "	increase in DNA synthesis
110 "	mitosis and cytokinesis
24 hours	blastula
48	gastrula
72	pluteus
2-3 months	juvenile urchin

Table 1. The generalized timing of biochemical and developmental events of an sea urchin embryo; from Epel (1977), Eisen et al. (1984) and data cited in the introduction.

mitotic apparatus and contractile ring are assembled and used in mitosis and subsequent cell division. The egg continues to divide rapidly by radial cleavages until the early blastula stage. Then, the embryonic genome begins to direct further development and differentiation (Angerer and Davidson, 1984). The embryo undergoes gastrulation and a pluteus larval stage before becoming a mature urchin.

1.4. The discovery of calcium as an egg activating agent

Hermann Fol (1877) watched starfish sperm fuse with starfish eggs and thereby observed how eggs are activated (from Epel, 1977). This discovery established the role of sperm in maintaining the continuity of life through fertilization. A controversy soon arose however, when it was reported that sea urchin eggs were activated when shaken with chloroform (cited in Ishikawa, 1973). The embryologists of the time did not know how chloroform could mimic the effects of sperm. This began an examination of the mechanisms of egg activation. In 1907, Michael Guyer discovered that one could grow adult frogs from eggs activated after being pricked with a needle dipped in blood (cited in Jaffe, 1985). It was realized that eggs from many different species could be activated by being pricked with a "bloody needle". Moser identified the calcium requirement for the bloody needle activation (cited by Whitaker and Steinhardt, 1985). He realized that calcium must be present in the external medium for parthenogenetic activation to occur. A role for calcium in fertilization was suggested by D.

Mazia shortly before Moser's discovery. Mazia (1937) found that fertilized sea urchin eggs contained 15% less calcium after dialysis than unfertilized eggs. He correctly postulated that fertilized eggs contained less calcium because of a release of intracellular calcium into the cytoplasm, which was lost upon dialysis.

It was not until 1974 that further evidence was given for the calcium activation theory. Chambers et al. (1974) and Steinhardt and Epel (1974) revealed that sea urchin eggs are activated with the addition of the divalent ionophores A23187 and X537A. These compounds act as ion channels when inserted into an impermeable lipid membrane. With a sea urchin egg cultured in sea water, an ionophore will allow an influx of calcium into the egg since the free calcium concentration is 10,000 times greater in sea water than in the egg cytoplasm. Magnesium is not transported by the ionophore because its concentration is the same inside and outside of the egg (Sui and Shen, 1986). Steinhardt and Epel (1974) believe that A23187 activates eggs with an intracellular calcium increase, the same mechanism as if the egg was activated by sperm. This idea was supported by experiments in which the eggs were preloaded with ^{45}calcium and then a ^{45}calcium efflux was observed after activation. They also reported that the intracellular magnesium concentration was not affected by the ionophore treatment, which implied that the activation was due solely to calcium. A23187 can activate eggs from phylogenetically divergent species such as starfish, amphibians (Xenopus), and hamsters (Steinhardt, et al., 1974). This data suggests that all eggs are activated by a common mechanism that requires calcium.

The ability of a large variety of chemicals to activate eggs confounded the question of how are eggs activated. By 1973, over 100 methods were known to activate sea urchin eggs parthenogenetically. It was not clear how these different methods could result in egg activation until Zucker et al. (1978) microinjected aequorin, into sea urchin eggs. Aequorin is a photoprotein that undergoes a bioluminescent reaction in the presence of calcium (Shimoura and Johnson, 1970). Zucker et al. (1978) found that three representative methods of parthenogenetic activation (urea, A23187, and hypertonic sea water) caused an increase in intracellular calcium, as detected by an increase in aequorin luminescence. They postulated that many of the parthenogenetic activation methods operated through a mechanism that increases intracellular calcium.

A transient increase of intracellular free calcium was detected immediately after fertilization in aequorin loaded fish eggs (Oryzias) (Ridgeway et al., 1976; Gilkey et al., 1978). A similar calcium transient was seen in sea urchin eggs that were fertilized or treated with A23187 in the presence or absence of extracellular calcium (Steinhardt et al., 1977). This further supported the concept that egg activation at fertilization is caused by an increase in the intracellular free calcium concentration.

Aequorin allows one to quantitate the calcium flux in eggs. Although the basal calcium concentration was too low to be measured, the peak concentration was calculated to be 2.5 -4.5 μM (Steinhardt et al., 1977). A new fluorescent dye, Fura-II, was also used to quantitate the calcium concentration inside sea urchin eggs (Poenie et

al., 1985). This dye is more sensitive than aequorin, therefore they measured a basal level of calcium of 0.1 μM. The timing of the calcium transient correlated with egg activation, as determined with calcium specific dyes and photoproteins. However, direct evidence for the role of calcium was still needed.

Hamaguchi and Hiramoto (1981) microinjected calcium directly into unfertilized sea urchin eggs. They reported that 0.2 μM calcium induced egg activation, and that fertilization could be prevented by injection of a calcium chelator EGTA. EGTA loaded eggs did not fertilize even though sperm were attached to the egg surface. Microinjection of EDTA did not block egg activation since it did not bind calcium as readily as EGTA (Zucker and Steinhardt, 1978). Mammalian eggs were also activated with a calcium injection (Fulton and Whittingham, 1978).

IP3 releases calcium from intracellular stores in many different cell types (for reviews, see Berridge and Irvine, 1984; Berridge, 1984). When it was injected into sea urchin eggs, it also induced egg activation via a calcium dependent pathway (Whitaker and Irvine, 1984; Slack et al., 1986; Turner et al., 1986). IP3 activation was blocked if the eggs were first injected with a calcium chelator such as EGTA. Similar results were found with Xenopus eggs (Busa et al., 1985). The IP3 results not only substantiate the calcium hypothesis but also indicate a mechanism in which intracellular calcium is released.

There is much data to support the hypothesis that calcium is the signal that initiates egg activation and development. The requirement for calcium is suggested by the results from measuring intracellular

calcium fluxes at fertilization or activation with probes such as ^{45}calcium, photoproteins and dyes. Also one can mimic fertilization with calcium ionophores and microinjected; fertilization can be blocked with calcium chelators. All animal eggs that have been examined are activated with calcium (Jaffe, 1985).

1.5. How calcium is released from intracellular stores in sea urchin eggs

Once it was revealed that calcium may activate eggs at fertilization, the next problem was to determine the source of the calcium which contributes to the intracellular increase. As a deuterostome, sea urchin eggs have a intracellular calcium store from which calcium is released at fertilization (Jaffe, 1983). Protostome eggs do not have accessible intracellular calcium stores and cannot be activated in a calcium free medium. Therefore, the mechanism presented below may only apply to deuterostome eggs.

IP3 may be the compound that links the extracellular sperm stimulus to the intracellular calcium release. An IP3 stimulated calcium release was first suggested in sea urchin eggs when it was reported that polyphosphoinositide turnover increases at fertilization (Turner et al., 1984; Kamel et al., 1985). Since it is difficult to prelabel unfertilized sea urchin eggs with ^{32}phosphate, neither group could quantitate the polyphosphoinositide turnover. However, Kamel et al. (1985) showed a 5 fold increase in intracellular IP3 levels that occurred before the calcium increase. Whitaker and Irvine (1984),

Slack et al. (1986), and Turner et al. (1986) then showed that IP3 microinjected directly into sea urchin eggs caused the release of intracellular calcium stores.

IP3 is produced by the cleavage of polyphosphoinositides by phospholipase C, which is found in sea urchin egg membranes (Whitaker and Aitchison, 1985). Phospholipase C is thought to be activated by GTP binding proteins (G proteins) that mediate the fertilization signal between sperm and phospholipase C. The involvement of G-proteins was shown by the microinjection of GTP analogues (Turner et al., 1986; Turner et al., 1987). GTP-γ-S and cholera toxin, G_s-protein stimulators, caused the eggs to activate. The link between the G-protein and calcium release was demonstrated by blocking the GTP-γ-S activation with a subsequent EGTA injection. Conversely, GDP-β-S, a G_i-protein stimulator, prevented fertilization even though the sperm bound to the egg surface. These eggs were then rescued with an injection of IP3 or calcium.

Although the steps linking sperm attachment and calcium release have been identified, the source of the intracellular calcium is not known. Preliminary evidence suggests that the endoplasmic reticulum may be the source of the calcium transient seen at fertilization. Eisen and Reynolds (1985) used aequorin to detect the intracellular location of calcium release. They stratified the organelles within the egg with gentle centrifugation. This technique separates the lipids, endoplasmic reticulum, mitochondria, yolk platelets and the nucleus from each other while maintaining egg viability. Aequorin was injected into the stratified egg prior to fertilization. The calcium

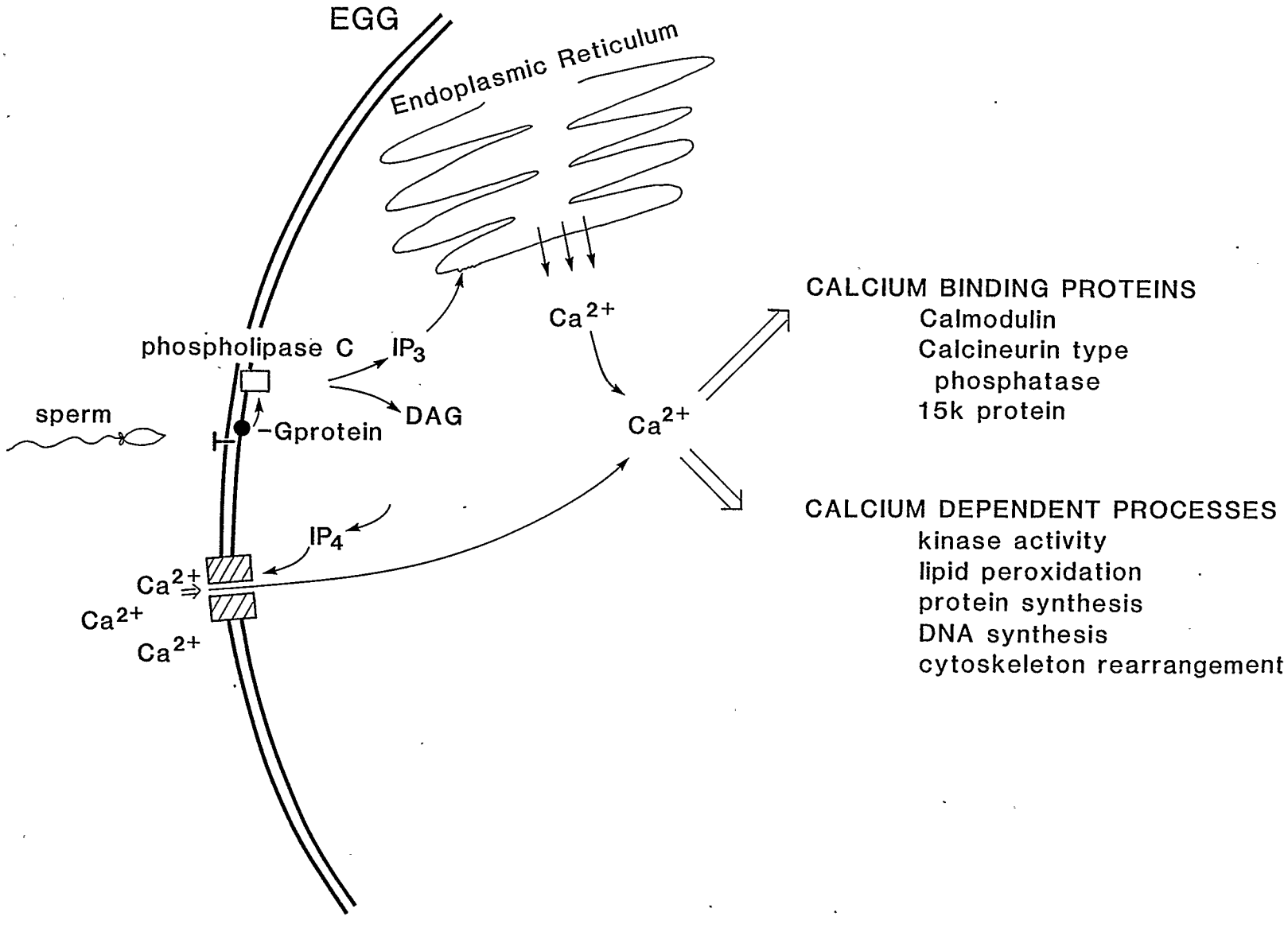
induced bioluminescence originated from a region called the clear zone. This zone is enriched with endoplasmic reticulum, suggesting that the calcium stores originate from the endoplasmic reticulum.

Clapper and Lee (1985) reported that IP3 releases calcium from a source associated with the endoplasmic reticulum. They centrifuged sea urchin egg homogenates on a percol gradient that separated the various organelles from each other. They found a nonmitochondrial fraction that sequestered calcium and then released it when stimulated with micromolar quantities of IP3. The fraction co-sedimented with the endoplasmic reticulum and also contained glucose-6-phosphatase activity; an enzyme that is specific for the endoplasmic reticulum. Although this suggested that IP3 releases calcium from the endoplasmic reticulum, evidence of IP3 binding to a receptor is still needed.

A hypothetical mechanism for calcium release in sea urchin eggs based upon the above data is shown in Fig 1. Several of the proteins must still be characterized, such as: a sperm receptor that activates the G_s-protein, the G-proteins, and an IP3 receptor. IP4 (Inositol 1,3,4,5-tetrakisphosphate) is shown as an activator of calcium channels on the plasma membrane, based upon preliminary evidence from Irvine and Moor (1986) (see also Houslay, 1987). IP4 did not release calcium from internal stores, but appeared to allow the entrance of calcium from the external medium if it was mixed with inositol 2,4,5-trisphosphate. An IP4 mechanism may be more significant to protostome eggs, which do not have intracellular calcium stores.

Fig. 1. A generalized diagram of calcium release in eggs.

A hypothetical mechanism for the increase in intracellular calcium in eggs is shown. Sperm activates phospholipase C, possibly through a sperm receptor and g-proteins. IP3 is liberated, and induces the release of calcium from the endoplasmic reticulum. Calcium activates a series of calcium dependent processes through calcium dependent proteins. IP4 may allow the entrance of calcium from the extracellular medium, although this process may only be significant to protostome eggs.


EGG
Endoplasmic Reticulum
phospholipase C
IP_3
DAG
sperm
G-protein
Ca^{2+}
IP_4
Ca^{2+}
Ca^{2+}
Ca^{2+}
Ca^{2+}
CALCIUM BINDING PROTEINS
Calmodulin
Calcineurin type phosphatase
15k protein
CALCIUM DEPENDENT PROCESSES
kinase activity
lipid peroxidation
protein synthesis
DNA synthesis
cytoskeleton rearrangement

1.6. Calcium dependent processes and calcium binding proteins in sea urchin eggs

Many biochemical processes in the egg are activated by the calcium transient that occurs at fertilization. The calcium dependent processes are: cortical granule exocytosis, hardening of the fertilization membrane, phosphorylation of NAD, increase in tyrosine, serine, and threonine kinase activity, polyphosphoinositide hydrolysis, increase in intracellular pH, increase in protein and DNA synthesis, and cytoskeletal rearrangement (Epel, 1977, 1982; Whitaker and Steinhardt, 1982, 1985). Most of these events, such as DNA and protein synthesis, are only postulated as being dependent upon calcium. The calcium binding proteins which are involved are not known.

The increase in intracellular pH that occurs several minutes after fertilization is also thought to be a calcium mediated event. It is induced by the addition of the ionophore A23187 (Whitaker and Steinhardt, 1985). A sodium/hydrogen antiporter is thought to be the enzyme responsible for the pH change (Swann and Whitaker, 1985). It is unlikely that calcium acts directly upon the antiporter because the pH change can be induced with phorbol esters (Swann and Whitaker, 1985). The calcium dependent mechanism may involve several proteins that act upon the antiporter.

Since tyrosine kinase activity is implicated with cellular transformation and differentiation (Hunter and Cooper, 1984), fertilized sea urchin eggs have been examined for the presence of

tyrosine kinases. A 5 to 10 fold increase in tyrosine kinase activity was found in membrane fractions of fertilized sea urchin eggs compared to unfertilized eggs (Dasgupta and Garbers, 1983; Ribot et al., 1984). A portion of this activity was attributed to a src related kinase as detected with a rabbit anti-src antibody (Kamel et al., 1986). Satoh and Garbers (1984) identified a calcium dependent increase in tyrosine kinase activity that occurred 5 minutes after fertilization. This increase occurred in A23187 activated eggs, fertilized eggs, eggs fertilized in 0 sodium sea water, but not in ammonia activated eggs. They believed that the tyrosine kinase activity was not detected in ammonia treated eggs because ammonia activation involves a pH increase and omits the early calcium transient. Eggs fertilized in 0 sodium sea water experience a calcium transient but not a pH increase (Shen and Steinhardt, 1979); these eggs still have tyrosine kinase activity which again suggests that only the calcium transient is required. It is not known if the tyrosine kinases require calcium directly or indirectly.

Calcium is also implicated in serine and threonine kinase activity. Eggs that were activated with A23187 have a ^{32}phosphorus phosphorylation pattern on SDS gels that was similar to fertilized eggs (Keller et al., 1980). Some of the kinases contributing to this activity are NAD kinase (Epel et al. 1981), S6 kinase (Ballinger et al., 1984) and a putative kinase C (Swann and Whitaker, 1985). The NAD kinase requires calcium and calmodulin, whereas the calcium dependent mechanism for the S6 kinase and kinase C are not known.

A calcium dependent event which is partially characterized, is an

oxygen and calcium dependent lipid peroxidizing system. Perry and Epel (1985a) showed that when calcium was added to sea urchin egg homogenates, nonmitochondrial oxygen consumption increased and a 12-L hydroxy 5,8,10,14, eicosatetraenoic acid (HETE) was formed. HETE is a product of oxidized arachidonic acid, produced by lipoxygenase. They also found that HETE was formed within 5 minutes after fertilization and disappeared by 10 minutes which correlated to the time course of the increase in intracellular calcium levels (Perry and Epel, 1985b). This calcium mediated effect was induced by A23817 but not by NH_4Cl (which activates by increasing the intracellular pH rather than a calcium flux). It is not known if lipoxygenase is a calcium binding protein or whether it is activated by another means, such as a calcium dependent kinase or by calmodulin.

A calcium dependent phospholipase activity was found in cortical granule preparations isolated from sea urchin eggs (Whitaker and Aitchison, 1985). Sea urchin eggs were attached to glass slides with polylysine and then washed with a stream of isotonic buffer (Vacquier, 1975). The buffer shears most of the egg from the slide, leaving the plasma membrane with the exposed intracellular cortical granules. When the preparation was stimulated with micromolar calcium, the products of phospholipase C activity, phosphatidylinositol 4,5-phosphate and phosphatidylinositol 4-phosphate, were released. The phospholipase C enzyme was not purified; it is not known how calcium induces the increase in phospholipase C activity.

The hardening of the fertilization membrane was attributed to a 70

kDa protein, ovoperoxidase (Turner et al., 1985). Once the fertilization membrane elevates, ovoperoxidase reacts with tyrosine residues from membrane proteins to form dityrosyl linkages. The reaction occurred extracellularly; ovoperoxidase was maximally stimulated by 10 mM calcium, which is the extracellular concentration of calcium in sea water. Ovoperoxidase was inserted into the fertilization membrane in a calcium dependent manner by a 250 kDa protein called proteoliaisin (Weidman et al., 1985). The process begins after the intracellular increase in calcium causes the cortical granules to lyse and lift the fertilization membrane off the plasma membrane. Due to the extracellular location of ovoperoxidase, and its high Ka for calcium, it is unlikely that it responds to the intracellular calcium flux.

Calmodulin regulates several functions in sea urchin eggs including cortical granule exocytosis, the conversion of NAD to NADP, kinase, and phosphatase activities. Calmodulin was the first calcium binding protein characterized in sea urchin eggs (Head et al., 1979). The 17 kDa protein was identified by comparison with bovine calmodulin on SDS and urea polyacrylamide gels, peptide mapping, amino acid analysis, and its ability to activate brain phosphodiesterase. Steinhardt and Alderton (1982) used anticalmodulin antibodies to show that calmodulin mediates cortical granule exocytosis. They first showed that 5 μM calcium causes the granules lawns to lyse. This reconfirmed the initial experiments of Baker and Whitaker (1978), who attached cortical granules to glass slides and fused the granules with physiological concentrations of ATP and calcium. Anticalmodulin

antibodies inhibited the calcium dependent exocytosis, which was reestablished with the addition of bovine calmodulin.

Calmodulin is also involved with the postfertilization activation of NAD kinase (Epel et al., 1981). Within 30 seconds after fertilization, coincident with the calcium transient, NAD was converted to NADP. The NAD kinase complexed with calmodulin, although the two proteins were separated by DEAE cellulose chromatography. If calmodulin was removed, the kinase lost its activity. The activity was restored with the addition of calcium and calmodulin.

There are several proteins that co-purify with calmodulin, as identified by a calmodulin affinity column (Iwasa and Mohri, 1983). These proteins are: the NAD kinase previously characterized by Epel, a 55 kDa + 17 kDa calcineurin-like phosphatase (Klee et al., 1979), and a 50 kDa protein with an unknown function (Iwasa and Ishiguro, 1986). The 55 kDa + 17 kDa phosphatase required calcium and calmodulin for activity. Calmodulin also associated with a myosin light chain kinase in sea urchin eggs (Chou and Rebhun, 1986). The activity increased several fold upon the addition of calmodulin and calcium. Myosin is thought to be involved with cytokinesis and the light chain kinase may regulate this process.

Other calcium binding proteins have been recently identified in sea urchin eggs. Hosoya et al. (1986) found a 15 kDa protein whose mobility shifts on SDS-polyacrylamide gels in the presence of EGTA. The function of this protein, and direct evidence of calcium binding were not reported. A 46 kDA protein was identified in sea urchin eggs with a monoclonal antibody that was originally to calcium sequestering

vesicles in HeLa cells (Petzelt and Hafner, 1986). The antibody inhibited calcium uptake in mitotic membrane systems: it is believed that it reacts with a calcium pump in mitotic asters. The pump has not yet been purified.

There are more calcium dependent processes in sea urchin eggs than there are known calcium binding proteins. Calmodulin may regulate only several of these processes; it is not known which calcium binding proteins regulate the remainder. The 15 kDa protein is the only other potential intracellular calcium binding protein identified in eggs and its function is not known. Therefore, more research is necessary to identify and characterize calcium binding proteins in eggs.

1.7. Actin and actin binding proteins

Actin is a 43 kDa intracellular polypeptide which is found in all eucaryotic cells. Along with microtubules and intermediate filaments, actin is a cytoskeletal protein, contributing to the shape and mobility of cells. It is distributed along the inside of the plasma membrane and as a mesh throughout the cytoplasm (Weber and Osborn, 1985). This intracellular network not only maintains cell shape, but may act as an anchor for glycolytic enzymes (Epel, 1982) and for factors involved with cell determination (Jeffery, 1984).

Actin regulates cell shape by its ability to self assemble. Actin monomers (g-actin) bind, end to end, into long filamentous polymers (f-actin) which may reach several micrometers in length. This reversible transition of g-actin into f-actin in cells is regulated by

actin binding proteins and intracellular ions (Maruyama and Tsukgoshi, 1984; Stossel et al., 1985). The reassembly and movement of actin in cells causes events such as muscle contraction, cell movement, and the formation of the contractile ring seen in dividing cells. The transformation of g-actin into f-actin involves at least four distinct kinetic steps: monomer activation, nucleation, filament elongation, and annealing (Pollard and Craig, 1982; Pollard and Cooper, 1986). The first step, monomer activation, involves a conformational change in g-actin upon binding a divalent cation (either magnesium or calcium) and ATP. Several actin monomers (usually 3) then bind together to form a nucleation site from which a filament will assemble. Nucleation is the rate limiting step in actin polymerization and causes the lag phase seen at the onset of polymerization. Once a nucleation site is present, actin monomers then bind and form a filament in the elongation step. An actin filament has polarity, and assembly occurs 10 times faster at the barbed end as compared to the pointed end. Barbed and pointed refer to the orientation of heavy meromyosin fragments on an actin filament. The final step, annealing, consists of the end-to-end joining of f-actin filaments observed in f-actin solutions that have been mechanically sheared; its role in cellular actin polymerization is not known. The rate of depolymerization of f-actin in the presence or absence of activating salts is much slower than the rate of polymerization. G-actin can completely polymerize in 5 minutes whereas, the same quantity of f-actin can take up to 48 hours to depolymerize (Pardee and Spudich, 1982). Other factors that affect

the rate of actin polymerization are monomer concentration, quantity and types of salts used in the buffer, and temperature.

Actin polymerization in vitro can be measured with a variety of assays. The most common methods are: measuring changes in absorbance at A_{234}, measuring changes with a rolling ball viscometer, and measuring changes in fluorescence with fluorescently labeled actin monomers. (Cooper and Pollard, 1982). There are advantages and disadvantages to each of these methods, however, the fluorescently labeled pyrene actin technique is particularly useful because it is very sensitive and noninvasive (Cooper and Pollard, 1982). Iodoacetamide-pyrene is a fluorescent label that can be ligated onto monomeric actin at a cysteine residue. Once pyrene actin polymerizes into f-actin, a 20-40 times increase in fluorescence emission occurs at 407 nm when the reaction mixture is excited at 365 nm (Kouyama and Mihashi, 1981). Unlike the rolling ball viscometry, this technique allows the actin solution to remain untouched while measurements are being taken. The properties of pyrene actin are much like native actin (Cooper et al., 1983). The time course of polymerization, elongation rate constants, and the critical concentration for assembly of pyrene actin are identical with values for native actin. Malm (1984), however, reports that profilin, an actin binding protein, did not interact with pyrene labeled actin in the same manner as native actin. This appears to be the only report of such an effect.

By itself, actin exists as either long filaments or as monomers. In nonmuscle cells however, actin exists as parallel bundles, crosslinked networks, long and short filaments, and as monomeric actin

(Weber and Osborn, 1985). Actin can also exist as a stable cellular matrix or as a dynamic structure that is the source of cell movement. Each of these forms of actin can occur at the same time, in the same cell, because of the interaction of actin binding proteins with actin.

An actin binding protein is any protein that binds to g-actin or f-actin at a physiological pH. The physiological relevance of each of these proteins is not always clear since many of the observations are made in vitro. However, the effects of these proteins upon actin usually supply an insight into how actin interacts with itself (Craig and Pollard, 1982). Actin binding proteins may organize the structure of actin filaments or regulate the rate and quantity of actin polymerization. They are classified on the basis of their interaction with g or f-actin. The classification includes g-actin sequestering proteins, capping proteins, crosslinking proteins, and proteins that bind to f-actin (Stossel et al., 1985; Pollard and Cooper, 1986).

Sequestering proteins bind to g-actin and prevent it from being taken up by actin filaments. These proteins may act as a storage reservoir for actin monomers. The salt concentration in a cell is high enough to promote all actin to polymerize, yet, only 60% of the total cellular actin exists as f-actin (Mabuchi and Spudich, 1980). Therefore, sequestering proteins are thought to maintain an accessible pool of monomeric actin. Some sequestering proteins that have been characterized include profilin, deoxyribonuclease I, depactin and a 19 kDa protein from mouse brain (Stossel et al, 1985). The deoxyribonuclease I can be used to purify actin by affinity chromatography.

Capping actin binding proteins can affect actin filaments in several ways. They can cap either end of a growing actin filament and block the addition of actin monomers to that end. In this way, the length of actin filaments may be regulated. Capping proteins also act as potent nucleation sites for filament growth. They can eliminate nucleation as the rate limiting step and increase the rate of polymerization (Tellam and Freiden, 1982). Capping proteins also sever actin filaments. They can bind to an actin molecule in the middle of a chain, excise it, and create two filaments. This may be another means in which the length of actin filaments are regulated. The interaction of several capping proteins with actin is calcium dependent. An irreversible capping protein-actin complex may form which does not dissociate even after the removal of calcium. These complexes may still act as capping proteins or nucleating proteins. Capping proteins are found in all eucaryotic cells, examples includes proteins such as villin (Glenney and Glenney, 1985), gelsolin (Yin et al., 1981) and severin (Stossel et al. 1985).

A variety of actin crosslinking proteins have also been identified in most eucaryotic cells. Proteins such as spectrin and fodrin (240 kDa), and filamin are believed to anchor actin filaments to one another in a mesh pattern and to the plasma membrane. Other proteins such as alpha-actinin, fimbrin, and villin, organize actin filaments into bundles that may project from the cell surface (Craig and Pollard, 1982; Stossel, 1984). Crosslinking proteins appear to be associated with the more permanent actin structures.

Another class of actin binding proteins bind to actin filaments but

do not affect the filament structure. Tropomyosin, an example of this class, binds to actin filaments in nonmuscle cells and confers calcium sensitivity upon the interaction of myosin and actin. The types and quantity of actin binding proteins depend upon the cell type (Glenney and Glenney, 1985). However, since as many as 10 different actin binding proteins are found in a cell, the regulation of actin may be a complicated process (Pollard and Cooper, 1986).

1.8. Actin and actin binding proteins in sea urchin eggs

Actin has several structural roles in sea urchin eggs. Aside from being a major cytoskeletal component, actin is involved in incorporating the sperm at fertilization and involved in forming two daughter cells during cytokinesis (Vacquier, 1981). This is exemplified by the addition of microfilament toxins, such as cytochalasin B, which prevent normal development of a fertilized sea urchin egg (Schatten, 1982).

Immediately after the sperm contacts the egg, a fertilization cone forms around the sperm. The cone, which is composed of f-actin, projects several micrometers from the egg surface and is thought to direct the entrance of the sperm into the egg. Cytochalasin B and E were shown to block the entrance of a sperm through the cone (Cline and Schatten, 1986). This results in an activated egg with the sperm remaining on the egg exterior (Byrd and Perry, 1980).

Microvilli, which are distributed evenly over the egg surface, elongate 8 to 10 minutes after fertilization (Cline and Schatten,

1986). The purpose of the elongation is not known, but it appears to be dependent upon the increase in intracellular calcium (Byrd and Belisle, 1985). Although the cortical granules lie immediately over the actin found at the egg cortex, no functional relationship exists between the two. Cortical granule exocytosis occurred in the presence of a variety of cytoskeletal toxins and anti-actin antibodies (Whitaker and Baker, 1983). The f-actin at the cortex may anchor the cytoskeletal actin that supports the cell (Stossel, 1984). Cytoskeletal actin may also be important in directing the development of the egg as seen in Xenopus and Ascidian embryos (Jeffery, 1984).

Actin also directs cytokinesis in sea urchin eggs. F-actin filaments were detected with labeled phalloidin along the cleavage furrow of dividing eggs (Cline and Schatten, 1986). The cytokinesis was inhibited with microfilament toxins. Each of the actin dependent processes in the egg involve the localized movement of f-actin, as seen with the fertilization cone. How actin can be modified in one region of the egg and not in an adjacent region is a conceptual problem in the study of microfilament function. The answer partly lies with actin binding proteins. Several have been characterized in sea urchin eggs.

Proteins from each class of actin binding proteins are present in sea urchin eggs. These proteins show capping activity, nucleation, and filament severing activity in vitro. Although the in vivo function and regulation of these proteins must still be determined, it is likely that they direct the actin movements seen in sea urchin eggs.

The actin binding proteins in sea urchin eggs can be classified by whether or not their activity is regulated by calcium. Four actin binding proteins - 17 kDa, 13 kDa, 58 kDa, and a 220 kDa, are not calcium dependent. The 17 kDa protein was first identified by DNase I affinity chromatography (Hosoya et al., 1982). It has similar properties to another actin binding protein called depactin, which was first characterized in starfish oocytes (Mabuchi, 1982). This protein depolymerized f-actin and inhibited the formation of f-actin. Another low molecular weight protein, which has similar activity, was also purified by DNase I affinity chromatography (Mabuchi and Hosoya, 1982). This 13 kDa protein is similar to profilin in that it reversibly bound actin monomers. Monomer sequestration may affect polymerization by altering the free monomer concentration (Stossel et al., 1985), it may also account for the high concentration of free actin in sea urchin eggs (Mabuchi and Spudich, 1980).

A 58 kDa and 220 kDa actin binding proteins were purified from actin gels that were formed by warming to 30 C and the addition of calcium (Kane, 1975). The 58 kDa protein was named fascin because it bundled actin filaments to form cross banded actin needles (Bryan and Kane, 1978). The 220 kDa protein did not interact with actin singly, rather, when it was mixed with fascin and f-actin, it caused the bundles to form a three dimensional mesh. The activity of fascin and the 220 kDa protein occurred independently of calcium even though the initial observed gelling activity was calcium dependent. This began a search for calcium dependent actin binding proteins that were responsible for the calcium dependent gelling effect.

Sedlar et al. (1983), Hosoya and Mabuchi (1984), and Wang and Spudich (1984) independently identified a 45 kDa actin binding protein whose activity was calcium dependent. The 45 kDa protein bound tightly to g-actin in the presence of calcium; the complex dissociated only after extensive dialysis with EGTA (Coluccio et al., 1986; Ohnuma and Mabuchi, 1986). Once the 45 kDa protein bound actin, it was purified by DNase I affinity chromatography (Hosoya and Mabuchi, 1984; Coluccio et al., 1986). The complex nucleated actin monomers, which increased the rate of polymerization, and it capped actin filaments, independent of calcium. The activity of the 45 kDa protein alone required calcium. The 45 kDa protein nucleated actin monomers, capped the barbed end of elongating actin filaments, and severed f-actin (Wang and Spudich, 1984; Tsukita et al., 1985; Coluccio et al., 1986). It is not known which of these functions occur in vivo.

A 100 kDa calcium dependent, actin binding protein, with similar activity as the 45 kDa protein, has also been characterized (Hosoya et al.,1986). At 1:1200 ratio of 100 kDa to actin, the 100 kDa protein reduced the rate and extent of actin polymerization. However, at 1:120 ratio, the 100 kDa protein increased the rate and extent of actin polymerization. The 100 kDa protein also severed f-actin as determined by reduced viscosity of f-actin solutions and electron micrographs. The activity of the 100 kDa protein was dependent upon the presence of micromolar calcium.

A 95 kDa actin binding protein from sea urchin eggs has a gelling activity that is inhibited by calcium (Mabuchi et al., 1985). This protein was identified as alpha actinin because of its similar

molecular weight, stokes radius, amino acid composition, and its reactivity to an anti-chicken alpha actinin antibody. Alpha actinin crosslinked f-actin into bundles as shown by viscosity measurements and electron micrographs. Rhodamine labeled alpha actinin localized in the egg cortex after fertilization. It is surprising that its activity is inhibited by calcium because actin bundling occurs during the calcium transient seen at fertilization (Vacquier, 1981).

1.9. Scope of the thesis

As discussed in the introduction, calcium activates sea urchin eggs. It is likely that calcium binding proteins participate in the conversion of an egg into an embryo. This idea is supported by the observation that many enzymatic processes are regulated by calcium. Since calmodulin only mediates a few of these processes, other calcium binding proteins must be present in eggs. By identifying the remaining calcium binding protein(s) and their function, one can then determine the biochemical events by which calcium activates eggs at fertilization.

The objective of this thesis is to determine if sea urchin eggs contain calcium binding proteins other than calmodulin. This objective will be met by:

- examining the soluble calcium binding activity of the sea urchin egg.
- identifying calmodulin in this activity
- purifying a calcium binding protein(s) responsible for the remaining calcium binding activity.
- characterizing the physical properties of this protein.
- attempting to determine the function of this protein.

2.0. Materials and Methods

The following sections describe the basic principles and methodology that were used to examine calcium binding proteins in sea urchin eggs. The buffers used in the following procedures were made with double distilled water. The chemicals used were either purchased from Sigma Chemical Company (St. Louis, Mo.) or the company listed. Also, all preparations were performed at 4°C unless otherwise stated.

2.1. Preparation of material

2.1.1. Collection of eggs

Sea urchins (Strongylocentrotus purpuratus) were collected live from Port Renfrew, British Columbia, or through the assistance of Dr. C. Glabe at the University of California at Irvine, or were purchased from Pacific Biomarine, Venice, California. The animals were induced to spawn by gently removing the mouth and draining the coelomic fluid from the exposed cavity. The cavity was filled with 0.5 M KCl and the animal was placed upside down over a beaker filled with sea water. An animal will spawn for about 10 minutes, during which 2 to 20 mL of pale to deep yellow eggs collect in the bottom of the beaker. After spawning, male and shed female animals were discarded. The eggs were washed several times by aspirating the covering sea water and resuspending them in fresh sea water. The eggs were then passed through a nylon screen (0.1 mm, Tetko Inc., N.Y.) to remove any debris such as feces or spines and to remove a translucent jelly coat that

surrounds each egg. The eggs were then either resuspended in homogenization buffer or sedimented by gentle centrifugation (1000 x g), placed into a plastic tube and stored at -80°C until required.

2.1.2. Preparation of 100,000 x g supernatants

Fresh or frozen eggs were placed into two volumes of the following buffer: 40 mM Tris (pH 7.5), 0.7 M glucose, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF),0.005 mg/mL leupeptin, 1 mM benzamidine, 25 mg/L soybean trypsin inhibitor (Hosoya et al., 1982; Waisman et al., 1985). The eggs were homogenized with either a polytron blender (30'' bursts, 10 times) for frozen eggs, or with a glass-teflon homogenizer (5 strokes over 2 minutes). Packed Chelex-100 resin (pH 7.5) (Biorad) was stirred into the mixture to make up 5% of the total volume (Waisman et al., 1985). Chelex-100 is used to remove divalent cations from the homogenate to inhibit metal activated proteases.

The homogenate was then centrifuged at 10,000 x g for 30 minutes. The supernatant was collected and stored while the pellet was gently resuspended in 1 volume of buffer and recentrifuged at 10,000 x g for 30 minutes. The two supernatants were combined and were centrifuged at 100,000 x g for 70 minutes. Occasionally, a floating lipid layer formed in the centrifugation tube after 100,000 x g centrifugation; this layer was removed and discarded. The supernatant was collected and transferred into a dialysis tubing (Spectrum, Ca.) and dialyzed against 3 x 2 L of 20 mM Tris (pH 7.5), 1mM DTT, for 18 hours. The

protein concentration of the dialysates was as high as 8 mg/mL. The dialysates were either stored at -80°C or used immediately. The colour of the 100,000 x g supernatants ranged from clear to deep orange depending upon the quantity of yolk present in the solution. Yolk was avoided because it increased the viscosity of the solution and contributed additional protein while it decreased the reproducibility of subsequent chromatographic steps. Although yolk is a major component of sea urchin eggs, it was possible to prevent it from contaminating the 100,000 x g supernatant by not freezing the eggs prior to homogenization.

2.2. Techniques used during protein purification

2.2.1. Protein determination

The protein concentration of crude and purified solutions was estimated by using the Coomassie blue dye-binding assay of Bradford (1976). 1 mL of Coomassie blue dye (Biorad) was mixed with BSA standards and egg samples followed by a brief vortex. The absorbance of each tube was measured at 595 nm against a water blank. The standard curve was constructed by plotting the absorbance versus BSA concentration. The concentration of protein in samples was then estimated by reading the abscissa from the curve.

2.2.2. Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis separates proteins on an

acrylamide matrix based upon size and, or charge of the proteins. It allows one to visualize and identify which proteins are present in a given solution. Therefore it can be used as a test for purity and also may give an indication of subunit structure and molecular weight. Three types of gels were used in the purification and analysis of sea urchin calcium binding proteins. The first and most commonly used was the discontinuous SDS gel system of Laemmli (1970). Gels were prepared from a stock solution of 30% by weight of acrylamide and 0.8% by weight of N,N'-bis-methylene acrylamide (Biorad). The final concentrations in the running gel were as follows: 0.375 M Tris-base (pH 8.6), 10 to 15% acrylamide, and 0.1% SDS. The gels were polymerized by the addition of 0.015% (v/v) tetramethylethylenediamine (TEMED) (Biorad) and 0.03% (v/v) ammonium persulfate. Stacking gels contained 0.125 M Tris-base (pH 6.8), 3% acrylamide and 0.1% SDS and were polymerized in the same way as the running gel. The electrode buffer contained 0.025 M Tris-base (pH 8.6), 0.192 M glycine, and 0.1% SDS. Protein samples were prepared in a buffer containing 0.125 M Tris-base (pH 6.8), 4% SDS, 20% glycerol, 5% 2-mercaptoethanol, and 0.004% bromophenol blue which was used as a tracking dye. The proteins were immersed in a boiling water bath for 2 to 5 minutes. If crude protein samples were being prepared, then they were centrifuged at 14,000 x g for 10 minutes prior to loading onto the gel. Electrophoresis was performed in a 0.75 mm, 12.5% slab gel at a 140 volts, constant voltage.

Nondenaturing gels were used to analyze pure protein samples in a native state. The gels were prepared in a similar manner as the SDS

gels, however, SDS and 2-mercaptoethanol were excluded from the gel and buffers. Also, the protein sample was not boiled prior to loading onto the gel. This type of gel system does not give an indication of molecular weight since the proteins migrate at a rate that is relative to a ratio of their size and charge. However, in the third gel system, 4-30% gradient, nondenaturing, one can estimate the molecular weight of a protein while maintaining the protein in a native state (Lambin and Fine, 1979). The gels were poured as a 4-30% acrylamide gradient made from a 60% acrylamide stock solution. The stock solution is 5% bis-acrylamide by weight. The running gel buffer contained 0.375 M Tris-base (pH 8.6) and 2-15% glycerol. The gel was polymerized with 0.015% TEMED and 0.03% ammonium persulfate. Although a stacking gel was not used, an additional 4% acrylamide layer was poured over the running gel so that the wells of the comb would not enter into the gradient. The samples were prepared in sample buffer made without SDS or 2-mercaptoethanol, and they were not boiled prior to loading. The running buffer contained 0.089 M Tris-base (pH 8.4), 0.08 M boric acid, and 2.5 mM EDTA. 0.75 mm gels were run for 12 to 18 hours at 375 volts, constant voltage, while the buffer was chilled with a cooling coil.

In all gel systems, proteins were visualized by staining with 0.06% Coomassie blue R-250 (Biorad) in 10% acetic acid and 25% ethanol. Destaining was performed in 10% acetic acid and 25% ethanol. If the protein concentration of the samples was below 1 μg per band, then the gels were silver stained as in instructions of Gel-Code (Pierce Chemical Co.).

2.2.3. Chelex-100 competitive calcium binding assay

The chelex assay is a competitive calcium binding assay that identifies calcium binding activity of solutions (Waisman and Rasmussen, 1983). The assay does not specifically identify calcium binding proteins, but rather, it will detect any substance that binds calcium, including anions. The principle of the assay involves the competitive binding of calcium between the sedimentable cation exchanger chelex and calcium binding proteins that remain in the supernatant. If calcium binding proteins or substances are present, then they will bind calcium and retain it in the supernatant. In the absence of calcium binding substances, the calcium binds to the chelex which is sedimented by centrifugation.

The assay was performed by mixing an aliquot (> 0.9 mL) of test solution with 0.1 μCi of $^{45}CaCl_2$ (Amersham), 0.025 mL of rapidly stirring 10% chelex slurry (Biorad), and water, to bring the volume to 1 mL in a small tube (Tokuda et al., 1987). The mixture was stirred end over end ensuring that the chelex was mixed thoroughly in the tube. After 30 minutes, the tube was centrifuged for 10 minutes at 3000 x g to sediment the chelex beads. 0.100 mL aliquots were removed from the tube and mixed with 5 mL of liquid scintillation fluid (Scintiverse, Fisher). The tubes were counted on a ^{14}C channel liquid scintillation counter for 30 seconds. Tubes which contained 5 to 10 times more counts than the water blanks were assumed to have calcium binding activity. In order to reduce calcium binding activity caused

by anions, all test substances were dialyzed on microdialysis wells against 2 L of 10 mM Tris (pH 7.5) and 0.5 mM DTT for 24 hours.

2.2.4. Phosphodiesterase assay

The phosphodiesterase assay was used to determine if calmodulin was present in a protein sample (Teo et al., 1973). Calmodulin activated phosphodiesterase converts cyclic adenosine monophosphate to 5'-adenosine monophosphate. Phosphate, which is removed by nucleotidase from 5'-adenosine monophosphate, can then be quantitated. The quantity of phosphate released can be correlated with the amount of calmodulin present in the tested sample.

The assay solutions are as follows: Assay buffer, 0.360 M Tris, 0.360 M imidazole, 45 mM $Mg(CH_3COOH)_2$ (pH 7.5); 5'nucleotidase, 10 unit/mL in 10 mM Tris and 0.5 mM $Mg(CH_3COOH)_2$ (pH 7.0); reducing agent, 24 g sodium bisulfite, 2.4 g sodium sulfite, and 500 mg 1 amino-2-naphthol-4-sulfonic acid into 200 mL water; ammonium molybdate, 0.55% in 0.5 N H_2SO_4.

The assay was performed as follows: 0.02 mL of 5 mM $CaCl_2$ was mixed with 0.100 mL of assay buffer, 0.03 mL of 5'-nucleotidase, and 0.005 mL of phosphodiesterase. Boiled test sample and water were added to bring the reaction volume up to 0.8 mL. The samples were incubated at 30°C and 0.100 mL of 10.8 mM cAMP was added to begin the reaction. After 30 minutes, 0.100 mL of 55% trichloroacetic acid was added to each tube to quench the reaction. From each tube, 0.5 mL of solution were mixed with 0.5 mL of ammonium molybdate, to which 0.05

mL of reducing agent was added. After 5 minutes, the absorbance was read at 660 nm against a water blank. High O.D. values indicate the presence of calmodulin.

2.2.5. Measuring salt concentration

The salt concentration of protein fractions was measured with each chromatographic step. Since the conductivity correlates with salt concentration, one can determine the salt concentration at which a protein fraction elutes from a column by measuring the conductivity. The conductivity of stock solutions of either 50, 100, 200, 300, 400, and 500 mM NaCl or potassium phosphate buffer were measured with a Biorad conductivity meter to derive a standard curve. The conductivity of eluted protein fractions was also measured and the corresponding salt concentration was read from the standard curve.

2.3. Calcium binding activity in 100,000 x g supernatant

Before calcium binding proteins could be purified from sea urchin eggs, it was necessary to determine if calcium binding activity exists in the soluble fraction of egg homogenates. The presence of calcium binding activity may indicate the presence of calcium binding proteins. The 100,000 x g supernatant was fractionated by DEAE ion exchange chromatography (Waisman et al., 1985). The resultant fractions represent the acidic portion of the egg soluble proteins which is the portion in which calcium binding proteins are thought to

exist (Khanna et al., 1987). The dialyzed 100,000 x g supernatant (200 mL, 4 mg/mL) was loaded onto DEAE cellulose column (Whatman DE-52, 2.5 x 20 cm) that was defined, degassed, and equilibrated with 1 L of dialysis buffer. The column was then washed with dialysis buffer until the O.D. 280 returned to the baseline. 10 mL fractions were collected as the column was eluted with a linear gradient made from 400 mL each of 20 mM Tris (pH 7.5), 1 mM DTT and 20 mM Tris (pH 7.5), 1 mM DTT, and 0.5 M NaCl. The fractions were dialyzed in microdialysis wells prior to analysis by the chelex assay. The flow through fraction from the DEAE column was also analyzed by the chelex assay. Protein concentration was measured by the Bradford assay, the salt gradient was determined by measuring the conductivity, and calmodulin was identified with the phosphodiesterase assay. Protein samples were run on 12.5% polyacrylamide gels to visualize protein separation.

2.4. Purification of proteins

The majority of the effort in this thesis was directed to the purification of a calcium binding, actin binding protein complex from sea urchin eggs. This complex was purified by two different methods, which are outlined below. Several other proteins, such as calmodulin, rabbit skeletal muscle actin, and 45 kDa-actin complex were also required for studies in this thesis; the methods used in their purification are also listed below. All purification steps were performed at 4°C and as rapidly as possible.

2.4.1. Purification of a 80 kDa protein complex

The major calcium binding activity peak, from the DEAE fractionated 100,000 x g supernatant, was pooled (fractions 62-78, Fig. 5) and dialyzed overnight against 2 x 1 L of buffer A; 10 mM potassium phosphate (pH 6.8) and 0.5 mM DTT. The dialysate (70 mL, 2 mg/mL) was applied to a 2.5 x 10 cm hydroxyapatite column (Biorad) that was previously equilibrated with 500 mL of buffer A. After the protein was loaded, the column was washed again with buffer A until the O.D. 280 returned to baseline. The column was eluted with a linear gradient of 10 to 400 mM potassium phosphate (pH 6.8) and 0.5 mM DTT over a volume of 400 mL. 10 mL fractions were collected and analyzed for calcium binding activity by the chelex assay after 48 hours of dialysis in microdialysis wells. The protein concentration was measured by the Bradford assay, and salt concentration of every 10th fraction was determined by measuring the conductivity. The peak of calcium binding activity was pooled and concentrated by ultrafiltration (Amicon, PM10) to a 2 mL volume containing 4 to 8 mgs of protein. The concentrate was then loaded onto a HPLC gel permeation column (LKB, TSK-3000SW) equilibrated with 40 mM MOPS (pH 7.1), 150 mM KCl, and 0.5 mM DTT. 3 mL fractions were collected and analyzed for calcium binding activity with the chelex assay, and protein concentration with the Bradford assay. Samples from various fractions were run onto a 12.5% polyacrylamide gel. Fractions which contained only two protein bands, one at 50 kDa and another at 43 kDa

were pooled and used in the characterization of the 80 kDa protein complex. If other protein bands were present, then the pooled fractions were loaded onto an FPLC mono-Q anion exchange column (Pharmacia). The column was equilibrated with 10 mM Tris (pH 7.5) and 0.5 mM DTT buffer which was filtered (Millipore, 0.22 μm) and degassed prior to use. After the protein was loaded (1 mg in 9 mL) and the column was washed, the protein was eluted with a 0 to 1 M NaCl gradient in 10 mM Tris (pH 7.5) and 0.5 mM DTT. Due to the low protein concentration, the chelex assay was not performed, however, the 80 kDa complex was detected and purity was established by running samples on a 12.5 % polyacrylamide gel. Occasionally, the gels were silver stained if Coomassie G250 staining was not adequate. The appropriate fractions were pooled and dialyzed in microdialysis wells for 24 hours against 10 mM Tris (pH 7.5) and 0.5 mM DTT and then stored on ice at 4°C until required. Approximately 0.5 mg of complex was purified from 100 g of eggs.

2.4.2. Purification of the 80 kDa protein complex by DNase I affinity chromatography

Since the 43 kDa component of the protein complex was thought to be actin, it was realized that DNase I affinity chromatography may be used to purify the complex (Hosoya et al. 1982). DNase I columns were either purchased from Cooper Biomedical, Pa. or synthesized according to instructions included with Pharmacia CNBr-activated Sepharose 4B. Approximately 20 mg of DNase I (grade IV Sigma or Cooper Biomedical)

was ligated to 10 mL of Sepharose 4B (Pharmacia). Before a column was used with sea urchin extracts, a small portion was tested with purified rabbit skeletal muscle actin to ensure that the column bound actin. 10 mL of 100,000 x g supernatant was brought to 50% (25 C) saturation of ammonium sulfate (Biorad) and then centrifuged for 50 minutes at 100,000 x G for 50 minutes. The pellet was dissolved into 10 mL of buffer D(10 mM HEPES (pH 7.0), 100 mM KCl, 2 mM $MgCl_2$, 20 mM 2-mercaptoethanol, 0.005% sodium azide) and dialyzed against three changes of 300 mL of buffer D over 18 hours. The dialysate was then loaded onto a 0.5 x 2 cm DNase I column and the flow through was either collected and stored or was brought to 2 mM calcium and stored. The column was then eluted with the following buffers: buffer D with 5 mM EGTA, buffer D with 1 M KCl, buffer D with 3.0 M guanidine-HCl. 0.5 to 1.0 mL fractions were collected until protein could not be detected by the Bradford assay, at which the next buffer was applied; guanidine-HCl was always the final buffer used since it removes all bound proteins. If calcium was added to the flow through, then the flow through was passed through the column again and subsequently eluted with the above buffers. Fractions containing protein were dialyzed against 1 L of 50 mM ammonium bicarbonate for 12 hours, lyophilized, and examined on a 12.5% polyacrylamide gel. The 80 kDa protein complex eluted in the EGTA fractions. If contaminants were present, the 80 kDa protein complex was purified by FPLC mono-Q anion exchange chromatography as in section 2.4.1.

2.4.3. Purification of the 45 kDa protein complex

The 45 kDa protein complex was purified from sea urchin eggs to determine if this complex was the same as the 80 kDa complex. The 45 kDa complex (Hosoya and Mabuchi, 1984) has similar properties to the 80 kDa complex. The 45 kDa complex was purified by DNase I chromatography in a manner similar to that of section 2.4.2. It elutes in the guanidine-HCl fraction. In order to minimize the amount of contaminating protein in this fraction, the complex was purified from a yolk free 100,000 x g supernatant which was first fractionated by DEAE-cellulose as described in section 2.4.1. The purity of the 45 kDa complex was determined by running the sample on a 12.5% SDS polyacrylamide gel.

2.4.4. Purification of rabbit skeletal muscle actin

Rabbit skeletal muscle actin was required for comparisons with sea urchin actin and also for pyrene labeled actin polymerization studies. Rabbit actin was purified according to the method of Pardee and Spudich (1982) and Maclean-Fletcher and Pollard (1980). Rabbit skeletal muscle (<u>latissimus dorsi</u>), excised from female New Zealand rabbits or purchased from Pel-freez (Roger, Ark.) was minced in a prechilled meat grinder. The minced muscle (200-500 g) was extracted by mixing the suspension with an overhead stirrer for 10 minutes in 5 volumes of 0.1 M KCl, 0.15 M potassium phosphate buffer (pH 6.5). The extract was filtered by squeezing it through 4 layers of preboiled and prechilled cheesecloth. The filtered muscle mince was extracted for

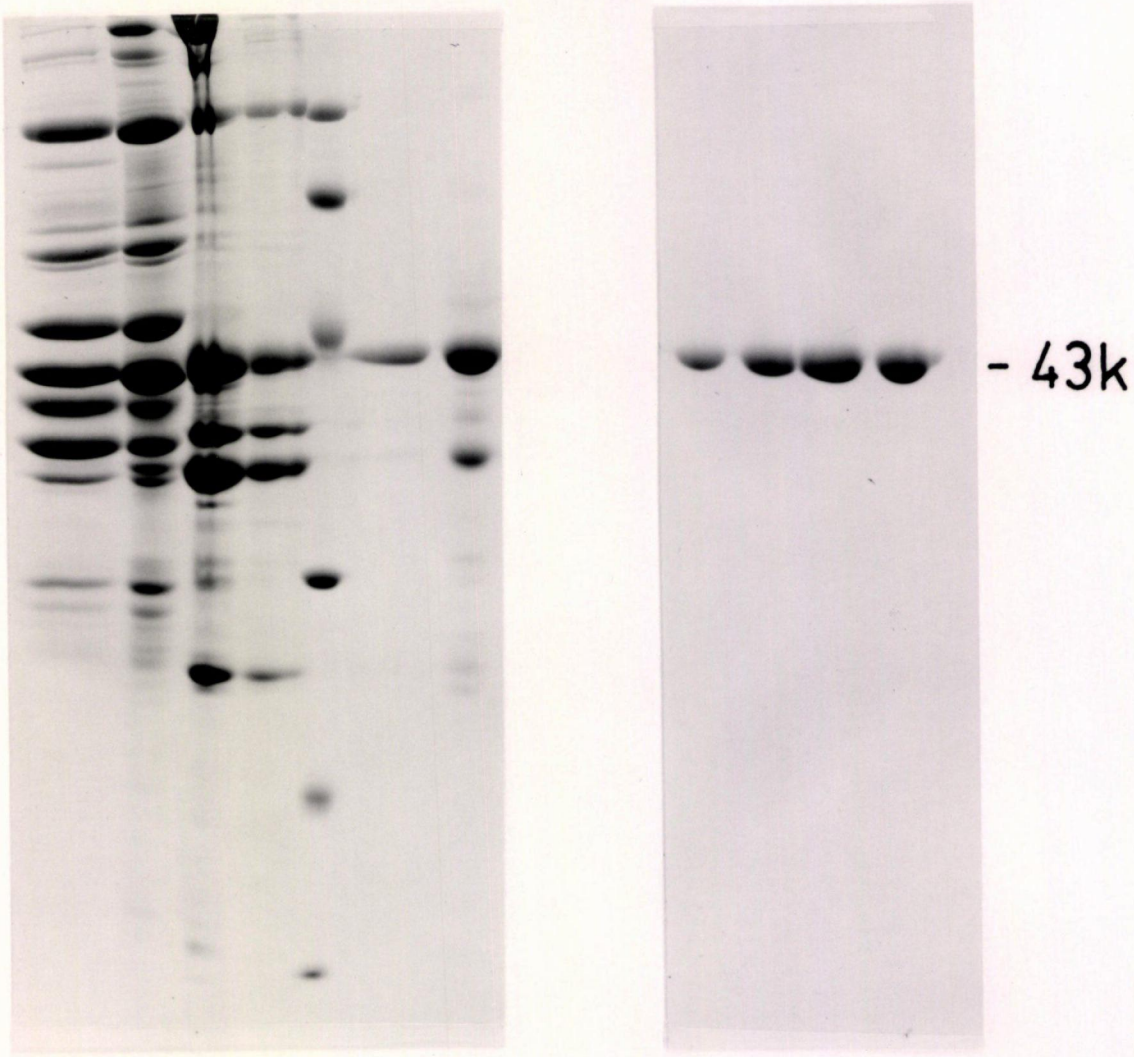
10 minutes with 10 volumes of 0.05 M $NaHCO_3$ and filtered. The filtered residue was extracted with 5 volumes of 1 mM EDTA (pH 7.0) for 10 minutes. The residue was then extracted twice with 10 volumes of water for five minutes and filtered after each extraction. The water saturated extract was quite large at this point, however, the volume was quickly reduced with the addition of 1 L of acetone and subsequent filtration through cheesecloth. The acetone extraction was repeated a total of five times for 10 minutes each. The residue was then dried overnight as acetone powder, in a fume hood. The acetone powder (10 g) was extracted with 15 volumes of buffer A (2 mM MOPS (pH 7.5), 0.1 mM $CaCl_2$, 0.33 mM ATP, 0.2 mM DTT, and 0.005% sodium azide) for 30 minutes in slushy ice. The extract was then collected through cheesecloth, followed by washing with 15 volumes of water. The filtrate was then clarified by centrifugation at 50,000 x g for 20 minutes. Solid KCl was slowly added to a final concentration of 0.6 M, and the actin in solution polymerized at room temperature for 3 hours. F-actin (filamentous) was sedimented by centrifugation at 150,000 x g for 90 minutes. The supernatant was discarded and the clear pellets were carefully washed several times with buffer A. The pellets were then homogenized with a glass teflon homogenizer in buffer A and dialyzed against 4 x 1 L of buffer A over 48 hours to form g-actin (globular). The g-actin (10 mL) was passed through a 2.5 x 30 cm Sephadex G-100 (Pharmacia) column which was equilibrated with g-actin buffer (2 mM Tris (pH 7.7), 0.33 mM ATP, 0.1 mM $CaCl_2$, 0.2 mM DTT, 0.005% sodium azide. Portions of each fraction were run on a 12.5% SDS-polyacrylamide gel to estimate purity (Fig. 2). The

Fig. 2. SDS-polyacrylamide gel analysis of rabbit skeletal muscle actin purification

The purification of rabbit skeletal muscle actin was followed by electrophoresis of the extracted proteins on a 12.5% SDS-polyacrylamide gel. The KCl wash (lane 1), $NaHCO_3$ wash (lane 2), EDTA wash (lane 3), and the water wash (lane 4) extracted many proteins including one at 43 kDa. The pelleted actin (lane 5) had several minor contaminants that remained after the high KCl salt wash (lane 6). These contaminants were removed, as seen in the peak fractions (lanes 7), by passing the actin through a G-100 Sephadex column.

ACTIN PURIFICATION

SDS-PAGE



fractions that only contained actin were pooled and polymerized by the addition of KCl to 100 mM and $MgCl_2$ to 2 mM. The f-actin was then stored on ice at 4 C. Actin concentration was determined by measuring the absorbance at 290 nm and using an extinction coefficient of 0.65 (Coluccio et al., 1986). 40 mg of actin was purified from 250 g of rabbit muscle.

2.4.5. Purification of bovine calmodulin

Calmodulin was required as a control protein for the chelex assay, the phosphodiesterase assay, and as a standard for the calcium-45 autoradiography. Fresh bovine liver was obtained from a local slaughterhouse. 1 kg of tissue was chopped and then minced in a meat grinder. The mince was mixed with 3 L of buffer containing 40 mM Tris (pH 7.5), 1 mM PMSF, 1 mM diisopropylfluorophosphate, 10 mM benzamidine, 1 mM DTT, and 20 mL of packed Chelex-100, and then homogenized in a Waring blender for several minutes (Waisman et al., 1985). The extract was centrifuged at 20,000 x g for thirty minutes and the resultant supernatant was centrifuged for 75 minutes at 100,000 x g. The supernatant was diluted into five volumes of 10 mM Tris (pH 7.5), and 1 mM DTT to which 800 mL of packed DEAE-cellulose was added (equilibrated in the same buffer). The mixture was stirred rapidly for one hour and then filtered through a scintered glass funnel. The DEAE-cellulose slurry was washed with 6 L of 10 mM Tris (pH 7.5) and poured into a 5.0 x 60 cm column. Protein was eluted with 4.4 L of 10 mM Tris (pH 7.5), 0.2 mM DTT, containing a linear

concentration gradient of 0-0.4 M NaCl. The protein fractions were dialyzed against 10 mM Tris (pH 7.5) and 1 mM DTT prior to analysis by the chelex assay and the phosphodiesterase assay. Fractions which contained calmodulin as shown by the phosphodiesterase assay, were pooled and concentrated to 2 mL by ultrafiltration (Amicon, PM10). The sample was then loaded onto a HPLC TSK-3000SW gel permeation column and eluted with 40 mM MOPS (pH 7.1), 150 mM KCl, and 0.5 mM DTT. The fractions were dialyzed against 10 mM Tris (pH 7.5) and 1 mM DTT prior to analysis by the chelex assay. The peak of calcium binding activity was pooled and dialyzed against 1 L of 20 mM Tris (pH 7.5), 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 0.5 mM DTT. The dialysate was applied to a phenyl-Sepharose column (CL-4B, Pharmacia) that was previously equilibrated with buffer C (20 mM Tris (pH 7.5), 0.1 mM $CaCl_2$. The column was washed until the A_{280} reached baseline and then washed with buffer C plus 1.0 M NaCl. Calmodulin was then eluted with 20 mM Tris (pH 7.5) and 1.0 mM EGTA. The eluted fractions were checked for purity by SDS-polyacrylamide gel electrophoresis. The pure fractions were dialyzed against 1 L of 50 mM ammonium bicarbonate for overnight and then lyophilized. The powder was then stored at -20°C until it was needed.

2.5. Characterization of the 80 kDa protein complex

Several techniques were used to examine the functional and biochemical characteristics of the purified 80 kDa protein complex. Included in the analysis were attempts to separate the complex into

component proteins, comparison with another protein complex from sea urchin eggs, and analysis of the actin portion of the complex and its role in actin polymerization.

2.5.1. ^{45}Calcium overlay autoradiography

This technique allows one to identify calcium binding proteins after they are separated on a nondenaturing or denaturing polyacrylamide gel (Maruyama, 1984). Since it was found that the 80 kDa complex was denatured in the presence of SDS, only nondenaturing gels were used for this technique. Purified complex was electrophoresed into a 4-30% polyacrylamide gel as described in section 2.2.2. One half of the gel was stained in Coomassie blue dye, while an identical half was soaked for 15 minutes in transblot buffer (0.025 M Tris-base pH 8.6, 0.129 M glycine). The unstained gel was sandwiched adjacent to an equal sized nitrocellulose sheet between two 2 mm thick, blotting papers (Biorad) (Towbin et al., 1979). It was found that MSI (Fisher) paper had the better background levels than Whatman or Biorad nitrocellulose. The sandwiched gel was inserted into a LKB transblotter such that the nitrocellulose was between the gel and the anode. Transblotting was performed at 100 volts (0.5 to 1.5 amps), with a cooling coil immersed in the buffer chamber. After 75 minutes, the nitrocellulose was removed and soaked in 100 mL of overlay buffer (10 mM imidazole (pH 6.8), 150 mM KCl, 5 mM $MgCl_2$) for 4 x 15 minute washes (Maruyama, 1984). The transblotted, polyacrylamide gel was stained in Coomassie blue dye to determine the

efficiency of the electrophoretic transfer. The nitrocellulose sheet was placed in overlay buffer which included 2 μCi/mL of ^{45}calcium. After 20 minutes, the buffer was removed and stored for reuse, and the nitrocellulose sheet was washed immediately in water and then immersed two times in water for 5 minutes. The sheet was then dried and placed under X-ray film (XAR5, Kodak) at -80°C for 18 hours and then developed. Calmodulin was used as a positive control; the high molecular weight markers (Pharmacia) were used as negative (non-calcium binding proteins) controls.

2.5.2. Comparison of 80 kDa and 45 kDa complexes

Antibodies to the 45 kDa protein (Wang and Spudich, 1984) were received from Drs. J. and A. Spudich (Stanford, Palo Alto, Ca.). The antibodies were used in an immunoblot in which the 50 kDa and 45 kDa proteins were run in adjacent lanes on a 12.5% polyacrylamide gel (Towbin et al., 1979). One half of the gel was stained with Coomassie blue dye while the identical half was transblotted onto nitrocellulose in a manner similar to that of section 2.5.1. After transblotting, the nitrocellulose sheet was incubated for 10 minutes in Tris buffered saline (TBS) (20 mM Tris-base (pH 7.5) and 0.5 M NaCl). The sheet was then incubated twice for 30 minutes in TBS with 3% gelatin in order to block remaining protein binding sites. The nitrocellulose sheet was then immersed for 1 hour in 1% gelatin-TBS containing a 1:100 dilution of the first antibody (anti-45 kDa). The sheet was then rinsed in water and washed twice in TBS plus 0.05% Tween-20 (Biorad). The

nitrocellulose sheet was then soaked in 1% gelatin-TBS plus the second antibody (anti-rabbit IgG, diluted 1:1000). After 1 hour, the sheet was rinsed with water and two, 10 minute TBS-Tween washes. The sheet was then rinsed again with water before immersing it in 100 mM phosphate buffer (pH 7.4) and 0.5 M NaCl for 10 minutes. Then 5 mg of diaminobenzene, 0.25 mL of 1% $CoCl_2$, and 0.2 mL of nickel ammonium sulfate (BDH Chemicals ltd., Poole, England) were added to the solution. The nitrocellulose sheet was developed by adding 0.009 mL of 3% H_2O_2 (Fisher) to the solution. The appearance of purple bands indicated a positive reaction with the antibody. The entire procedure was performed at room temperature and all washes were done on a rotary shaker.

2.5.3. Methods of separating the 80 kDa protein complex

Once it was realized that the calcium binding activity purified from eggs was associated with two proteins in the form of a complex, several attempts were made to separate these proteins. The purified 80 kDa complex was dialyzed against 20 mM Tris (pH 7.5), 10 mM EGTA, and 0.5 mM DTT for 48 hours and for 14 days. The samples were then electrophoresed on a 4-30% nondenaturing polyacrylamide gel which was stained and destained. A non-dialyzed sample of the complex was run as a control.

50 μg of the complex was mixed with SDS sample buffer with and without 2% 2-mercaptoethanol. The samples were run into a 12.5 SDS-polyacrylamide gel which was stained and destained.

2.5.4. Amino acid composition

The amino acid composition of the 50 kDa protein of the complex was determined so one could compare the composition with other proteins. Since the complex could only be separated by electrophoresis in SDS-polyacrylamide gels, the 50 kDa protein was electroeluted from an acrylamide gel prior to composition analysis. 0.1 mg of complex was mixed with SDS-sample buffer, boiled, and electrophoresed into a 12.5% SDS-polyacrylamide gel. The gel was briefly stained and destained, rinsed in water, and then placed in a wash buffer consisting of 100 mM Tris (pH 8.8) for 1 hour (Cleveland et al., 1977). The 50 kDa protein band was cut from the gel, and diced into 1-2 mm sections. An agarose tube gel was poured: 2 volumes of 4% agarose (Sea prep, Mandel Scientific) were mixed with 1 volume of 1.5 M Tris (pH 8.8) and 1 volume of water. The agarose solution was heated, and poured into a gel tube to 1/3 of its volume. The protein gel pieces were then inserted into the tube gel and the tube was then filled with the remaining agarose up to 1 cm from the top of the tube. After the agarose had set, the remaining space was filled with electrode buffer and sealed with a dialysis membrane. The tube was then electrophoresed at 4 milliamps per tube until the Coomassie blue dye from the polyacrylamide gel migrated into the 1 cm space. The buffer in the 1 cm space (that contains the protein) was removed with a syringe and then dialyzed overnight against water to remove the tris and SDS. The sample was then extracted with butanol to remove the Coomassie blue dye. The aqueous phase, which contained the 50 kDa

protein, was then given to the protein sequencing laboratory (Dr. D. McKay) for analysis.

2.5.5. Characterization of the 43 kDa protein

The molecular weight of the 43 kDa protein of the complex was similar to actin. To determine whether this protein was actin, several experiments were performed. A immunoblot was performed as in section 2.5.2., except that an anti-actin antibody was used (Transformation Research, Framingham, Ma.) for the primary antibody. Also rabbit skeletal muscle actin was used as a positive control.

Peptide mapping was also used to characterize the 43 kDa component of the complex (Cleveland et al., 1977). In this experiment, 0.05 mg of complex and 0.025 mg of rabbit skeletal muscle actin were electrophoresed into separate 12.5% SDS-polyacrylamide gels. The gels were briefly stained and destained and then soaked in water for one hour. The stained bands were then soaked in 5 mL of 100 mM Tris (pH 6.8) and 1% SDS twice for 30 minutes. The protein bands were then fitted into the wells of a 15% SDS-polyacrylamide gel. Each well was filled with SDS-sample buffer without 2-mercaptoethanol which contained 0.05 mg/mL V8 protease. The gel was run at 140 volts until the dye front was almost through the stacking gel, at which time the power was turned off to allow the protease time to digest the protein. After 20 minutes, the power was restored until the dye front left the gel, the gel was stained and destained. V8-protease was run alone in separate lanes as a control for band staining.

2.5.6. Polymerization of pyrene labeled actin

A method was required to determine whether the 80 kDa protein complex affects actin polymerization. Actin was labeled with a fluorescent molecule, pyrene, so one could observe the polymerization of actin with a fluorometer (Kouyama and Mihashi, 1981; Tellam and Frieden, 1982). G-actin (20 mL at 2 mg/mL) was dialyzed against 1 L of g-actin buffer without DTT or 2-mercaptoethanol for 12 hours. A 10 times molar excess of N,N-pyreneiodoacetamide (Molecular Probes, Eugene, Or.) was mixed in 0.2 mL of dimethylformamide (Fisher) and sonicated in a Braun water bath until it formed an even suspension. The mixture was then added to the rapidly stirring actin solution. KCl and $MgCl_2$ were immediately added to a final concentration of 100 mM and 2 mM respectively. The pyrene actin was then wrapped in foil to protect the solution from light, and gently rotated end over end at room temperature to allow complete mixing of the pyreneiodoacetamide. After 18 hours, the mixture was centrifuged at 150,000 x g for 90 minutes to sediment the actin. The pellet was careful washed with g-actin buffer and was then resuspended with a glass teflon homogenizer in a 5 mL volume of g-actin buffer. The pyrene labeled f-actin was then dialyzed against 4 x 1 L of g-actin buffer for 48 hours. The g-actin was clarified by centrifugation at 150,000 x g for 90 minutes. It was then passed through a 2.5 x 30 cm Sephadex G-100 column to remove unbound pyrene. If the pyrene actin concentration was below 0.5 mg/mL, it was polymerized and then depolymerized in a smaller

volume, so the concentration could be increased. The concentration of pyrene labeled actin and the molar ratio of pyrene to actin was calculated by the method of Cooper et al. (1983). Each newly synthesized pyrene labeled actin was scanned, before and after polymerization. As g-actin was transformed into f-actin, two new emission peaks were observed (Fig. 3). When not used, pyrene actin was stored as f-actin on ice.

Fluorescence studies were done in a Perkin-Elmer fluorometer in the laboratories of Dr. H. Van de Sande or Dr. D. Severson. Excitation was set at 365 nm and emission was set at 407 nm while the slit widths were set at 4 nm (Kouyama and Mihashi, 1981). Continuous recordings were made on a Perkin Elmer chart recorder set at 10 millivolt range and a chart speed of 10 mm per minute. The sensitivity of the fluorometer was set depending on the concentration of pyrene actin and actin used. Normally, the final actin concentration was 0.002 - 0.01 mM of which 5 - 20% was pyrene labeled actin. The actin was taken from stock solutions of 2 to 3 mg/mL and diluted into a final volume of 1 mL g-actin buffer in a 1x1 cm cuvette. Polymerization was induced by adding KCl and $MgCl_2$. Various concentrations of these salts were tried; 100 mM KCl and 2 mM $MgCl_2$ were chosen (Fig. 4), in agreement with Coluccio et al. (1986). Conditions were set so 50% of the maximum fluorescence would occur within 6 to 8 minutes after polymerization was initiated. The 80 kDa complex was added to the cuvette before and after polymerization was induced. For controls, either boiled complex or bovine serum albumin were added to the cuvette. Various salt concentrations were also used, including the

Fig. 3. Emission scan of pyrene labeled g-actin and f-actin

Actin was incubated for 18 hours in g-actin buffer without DTT plus 100 mM KCl, 2 mM $MgCl_2$, and 10 x molar excess (over actin) of pyrene iodoacetamide. The actin was collected by centrifugation at 150,000 x g for 90 minutes, depolymerized by dialysis in g-actin buffer, and then passed through G-100 sephadex column that removed the unreacted pyrene. The pyrene labeled g-actin was diluted to 4 μM with g-actin buffer in a 1 mL volume into a cuvette. An emission scan was taken from 250 nm to 450 nm while the solution was excited at 365 mn. The actin was then polymerized by the adjusting the buffer to 100 mM KCl and 2 mM $MgCl_2$ and the emission was read again from 250 nm to 450 nm. Two new peaks were detected in the f-actin solution; all emissions were then read at 407 nm, where one of the major peaks occurred. The structure of pyrene iodoacetamide is shown in the inset.

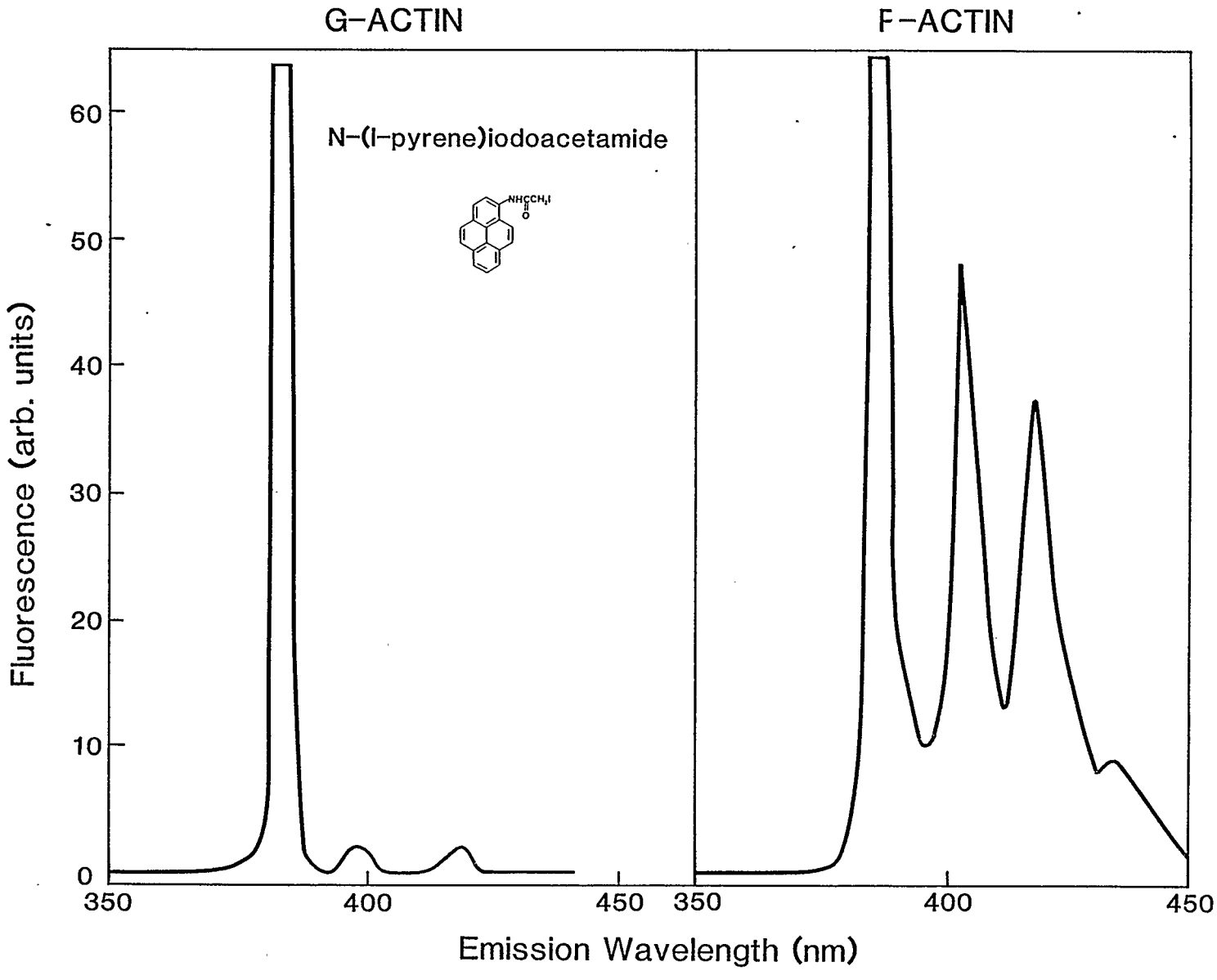
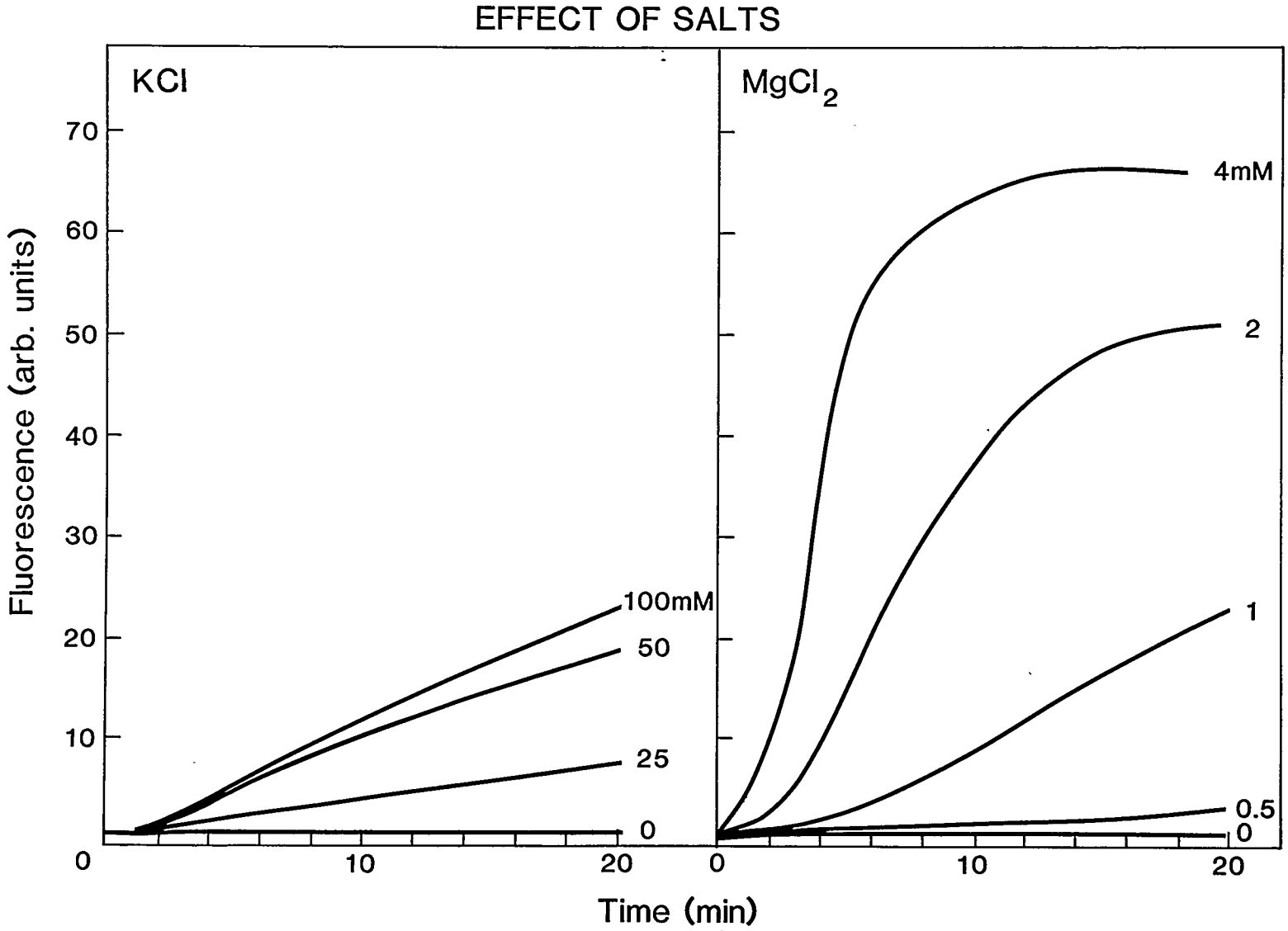

G-ACTIN
F-ACTIN
N-(1-pyrene)iodoacetamide
Fluorescence (arb. units)
0
10
20
30
40
50
60
350
400
450
Emission Wavelength (nm)

Fig. 4. The effect of KCl and $MgCl_2$ on actin polymerization

Various concentrations of KCl or MgCl2 were added to g-actin buffer that contained 2 μM g-actin, of which 10% was pyrene labeled, in a 1 mL volume. The final salt concentration is listed beside each curve. The polymerization of actin was measured in a fluorimeter with the excitation wavelength set at 365 nm and the emission wavelength set at 407 nm.


EFFECT OF SALTS
KCl
MgCl2
Fluorescence (arb. units)
70
60
50
40
30
20
10
0
100mM
50
25
0
4mM
2
1
0.5
0
10
20
10
20
Time (min)

addition of $CaCl_2$ and EGTA.

To examine the mechanism in which the complex affected polymerization, 0.2 mL of a polymerized sample with the 80 kDa complex was centrifuged at 200,000 x g for 90 minutes (Gerke and Weber, 1984). The supernatant was dialyzed against 1 L of water for 6 hours and then lyophilized. The supernatant and pellet were dissolved in equal volumes of SDS-sample buffer and electrophoresed into a 12.5% SDS polyacrylamide gel. The gel was stained with Coomassie blue dye and destained. The appearance of the 50 kDa protein in a particular lane suggests whether the complex associates with the pellet or the supernatant.

3.0. RESULTS

3.1. Overview

The results suggest that several calcium binding proteins are present in sea urchin eggs. One of these, a 50 kDa protein, binds tightly to actin and together forms a complex which inhibits the formation of filamentous actin. The purification of this complex, and the method in which its calcium binding activity and actin interactions were identified, are presented in the following sections.

3.2. Identification of calcium binding activity in 100,000 x g supernatants

A 100,000 x g egg supernatant was fractionated by DEAE anion exchange chromatography. DEAE is a suitable chromatographic step to use since most calcium binding proteins are acidic (Khanna et al. 1987) and bind to anion exchange resins such as DEAE. The DEAE flow through (proteins that did not bind to DEAE) was also tested for calcium binding activity. Occasionally, calcium binding activity was associated with this fraction. However, this was dependent upon the quantity of yolk present in the supernatant. If the 100,000 x g supernatant was yolk free, then the DEAE flow through did not possess any calcium binding activity.

The fractionated 100,000 x g supernatant from sea urchin eggs

contained a substantial amount of calcium binding activity (Fig. 5). Although there were no highly resolved peaks of activity, the profile had an initial plateau followed by a broad peak of higher calcium binding activity. The latter portion of the broad peak was partly due to the presence of calmodulin, which was identified with the phosphodiesterase assay (see Fig. 5). Since calmodulin was the only characterized calcium binding protein in sea urchin eggs (Head et al., 1979), the source of the high calcium binding activity eluting earlier than the calmodulin peak was not known. These fractions were examined for the presence of novel calcium binding proteins.

3.3. Purification of a protein complex from fractions containing the highest calcium binding activity

The fractions that contained the highest calcium binding activity from the DEAE profile were pooled and loaded onto a hydroxyapatite column. A single peak of calcium binding activity eluted at 150 mM phosphate buffer (Fig. 6). Occasionally, additional small peaks of activity were seen in subsequent preparations. If the DEAE peak was narrowly pooled, however, these smaller peaks were not observed. The flow through fractions from the hydroxyapatite column did not contain any calcium binding activity. The proteins responsible for the calcium binding activity were visible on SDS-polyacrylamide gels only after hydroxyapatite fractionation (Fig. 7). The two arrows on lane 26 (also marked by an arrow) indicate the proteins which were later shown to be the source of calcium binding activity.

Fig. 5. Chelex assay analysis after DEAE chromatography of 100,000 x g egg supernatants.

A 100,000 x g egg supernatant was loaded onto a DEAE anion-exchange resin. Proteins were eluted with a 0-500 mM NaCl linear gradient (□—□) in 20 mM Tris (pH 7.5) and 1 mM DTT. The protein concentration (o—o) was measured by the method of Bradford (1976); calmodulin (Δ—Δ) was detected by using the phosphodiesterase assay of Teo et al. (1973); and the calcium binding activity (●—●) was measured with the chelex assay (Tokuda et al., 1987), and the salt concentration was measured with a conductivity meter. Calmodulin only accounted for a fraction of the total calcium binding activity.

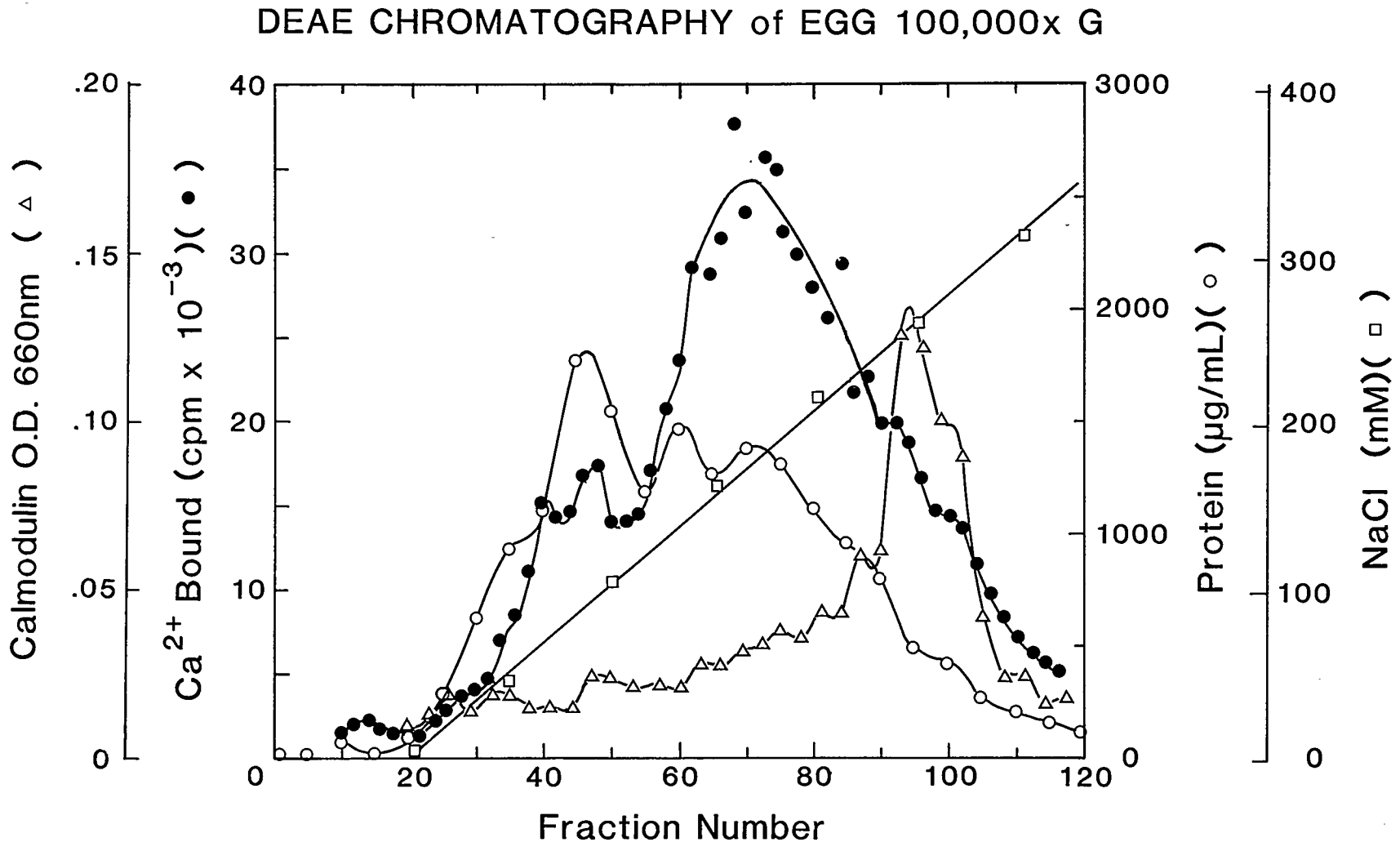

DEAE CHROMATOGRAPHY of EGG 100,000x G
Calmodulin O.D. 660nm (△)
Ca2+ Bound (cpm x 10−3)(●)
Protein (µg/mL)(○)
NaCl (mM)(□)
Fraction Number
.20
.15
.10
.05
0
40
30
20
10
0
3000
2000
1000
0
400
300
200
100
0
20
40
60
80
100
120

Fig. 6. Hydroxyapatite chromatography of DEAE fractions that contained the highest calcium binding activity.

DEAE fractions that contained the peak of calcium binding activity were pooled and loaded onto a hydroxyapatite column. The proteins were eluted with a linear gradient (□—□) of 10 to 300 mM phosphate buffer (pH 6.8) and 0.5 mM DTT. The protein concentration (○—○) was measured by the method of Bradford (1976); the calcium binding activity (●—●) was measured with the chelex assay and the salt concentration was measured with a conductivity meter. A single peak of calcium binding activity was resolved. Proteins that did not bind to the column, did not have any calcium binding activity.

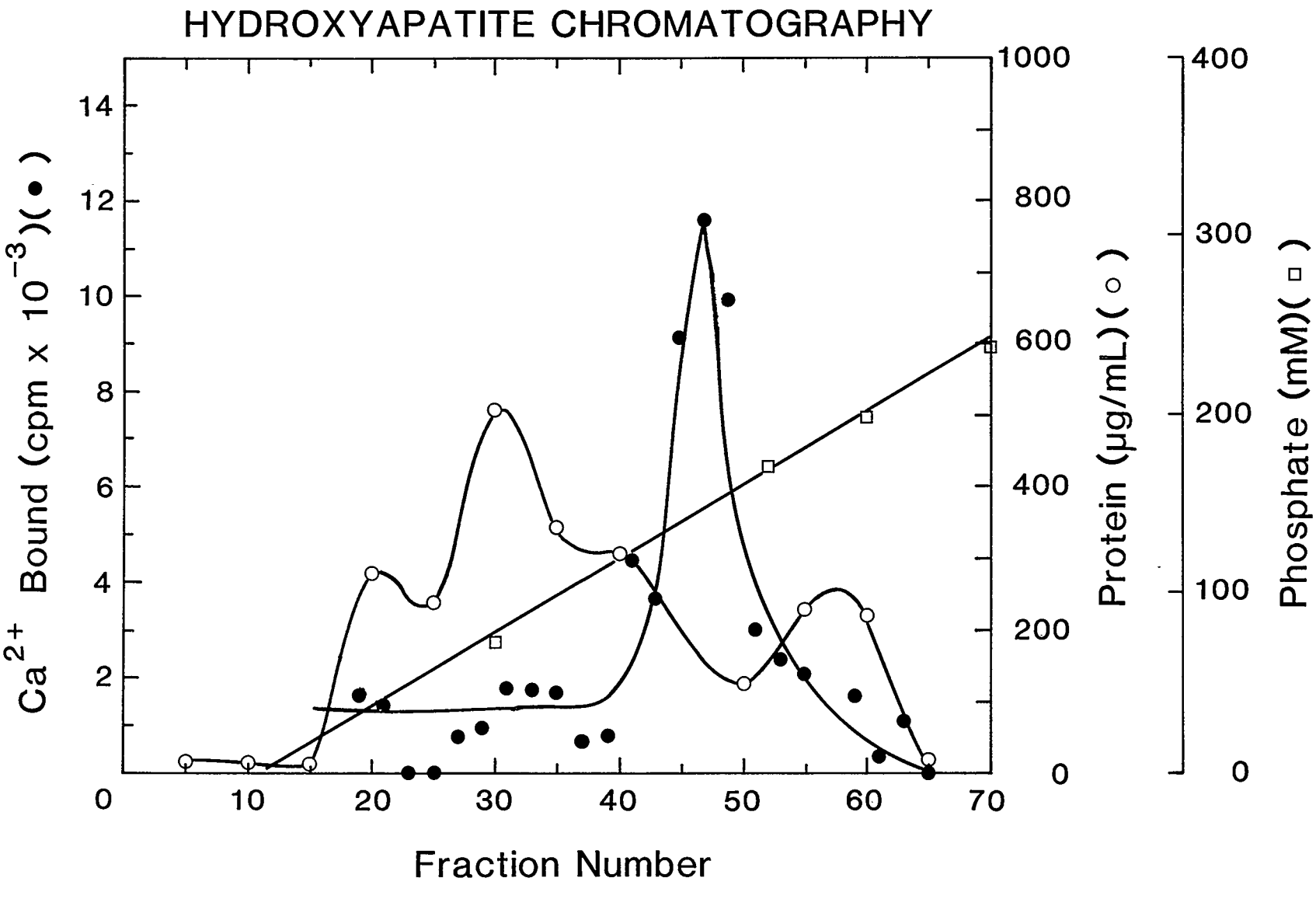
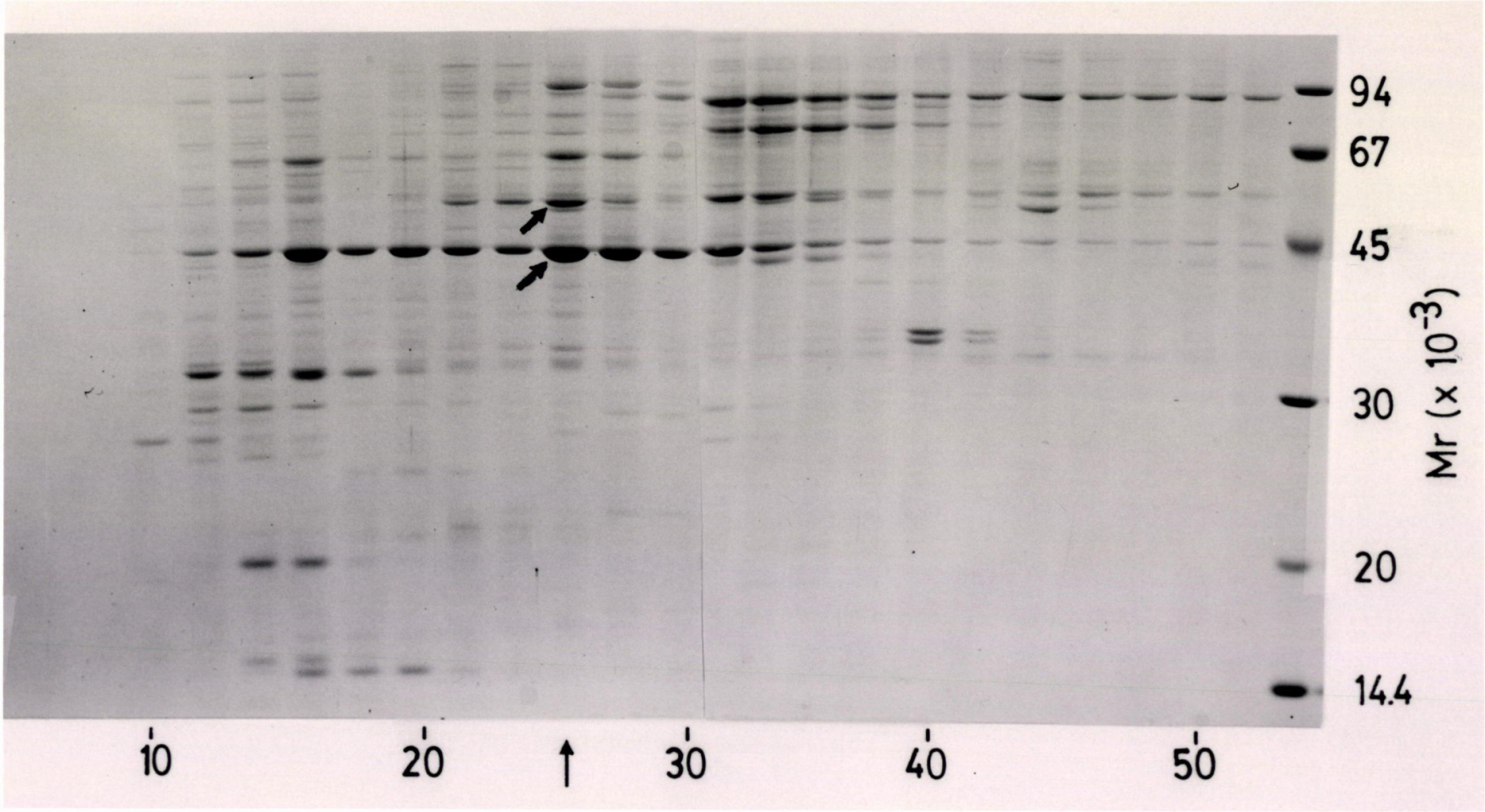

HYDROXYAPATITE CHROMATOGRAPHY
Ca^{2+} Bound (cpm x 10^{-3})(●)
Protein (μg/mL)(○)
Phosphate (mM)(□)
Fraction Number
0
2
4
6
8
10
12
14
0
200
400
600
800
1000
0
100
200
300
400
0
10
20
30
40
50
60
70

Fig. 7. An SDS-polyacrylamide gel of fractions eluted from a hydroxyapatite column.

Protein fractions from a hydroxyapatite column were run onto a 12.5% SDS polyacrylamide gel. Lane 26 (marked with an arrow) contained the two proteins that made up the 80 kDa complex (also marked with arrows) that were found to be associated with the calcium binding activity. The protein complex can be observed on gels only after this stage of purification. This gel profile is from a hydroxyapatite column that was eluted with larger fraction volumes, and with a steeper gradient than seen in Fig. 6.


94
67
45
30
20
14.4
Mr (x 10⁻³)
10
20
30
40
50

The fractions containing the calcium binding activity from the hydroxyapatite profile were pooled and loaded on a HPLC gel filtration column. One protein peak co-eluted with a single peak of calcium binding activity (Fig. 8). Samples from the peak fractions were electrophoresed onto an SDS-polyacrylamide gel that revealed two protein bands, one at 50 kDa and another at 43 kDa (Fig. 8., see inset). These two proteins could not be separated by phenyl sepharose chromatography or by FPLC mono-Q anion exchange chromatography. If other proteins were present in the calcium binding fraction from the gel filtration column, then these were removed by passing the pooled fractions through the FPLC mono-Q column. Once again, the calcium binding activity was associated with the 50 kDa and 43 kDa protein bands. The pooled fractions from each purification step were electrophoresed onto an SDS-polyacrylamide gel (Fig. 9). The 50 kDa protein was assumed to be a minor protein because it was seen only after the hydroxyapatite chromatographic step. Based upon recovery during purification, the protein complex constitutes only 0.08% of the total soluble protein in sea urchin eggs.

3.4. ^{45}Calcium overlay of 80 kDa protein complex

The 50 kDa and 43 kDa proteins migrated as a single band of approximately 80 kDa when they were electrophoresed onto a 4-30% nondenaturing polyacrylamide gel (Fig. 10). This band bound calcium in the presence of 5 mM $MgCl_2$ and 150 mM KCl as detected by ^{45}calcium overlay autoradiography (lane B), suggesting that both proteins were

Fig. 8. HPLC gel filtration of the pooled calcium binding peak from hydroxyapatite chromatography.

The peak of calcium binding activity from the hydroxyapatite column was pooled and loaded onto a HPLC gel filtration column. The fractions were tested for protein concentration and for calcium binding activity. One large protein peak (O—O) was associated with the only peak of calcium binding activity (●—●). Samples of the protein peak were electrophoresed onto 12.5% SDS-polyacrylamide gel (see inset). The peak fractions contained two proteins, one at 43 kDa and the other at 50 kDa.

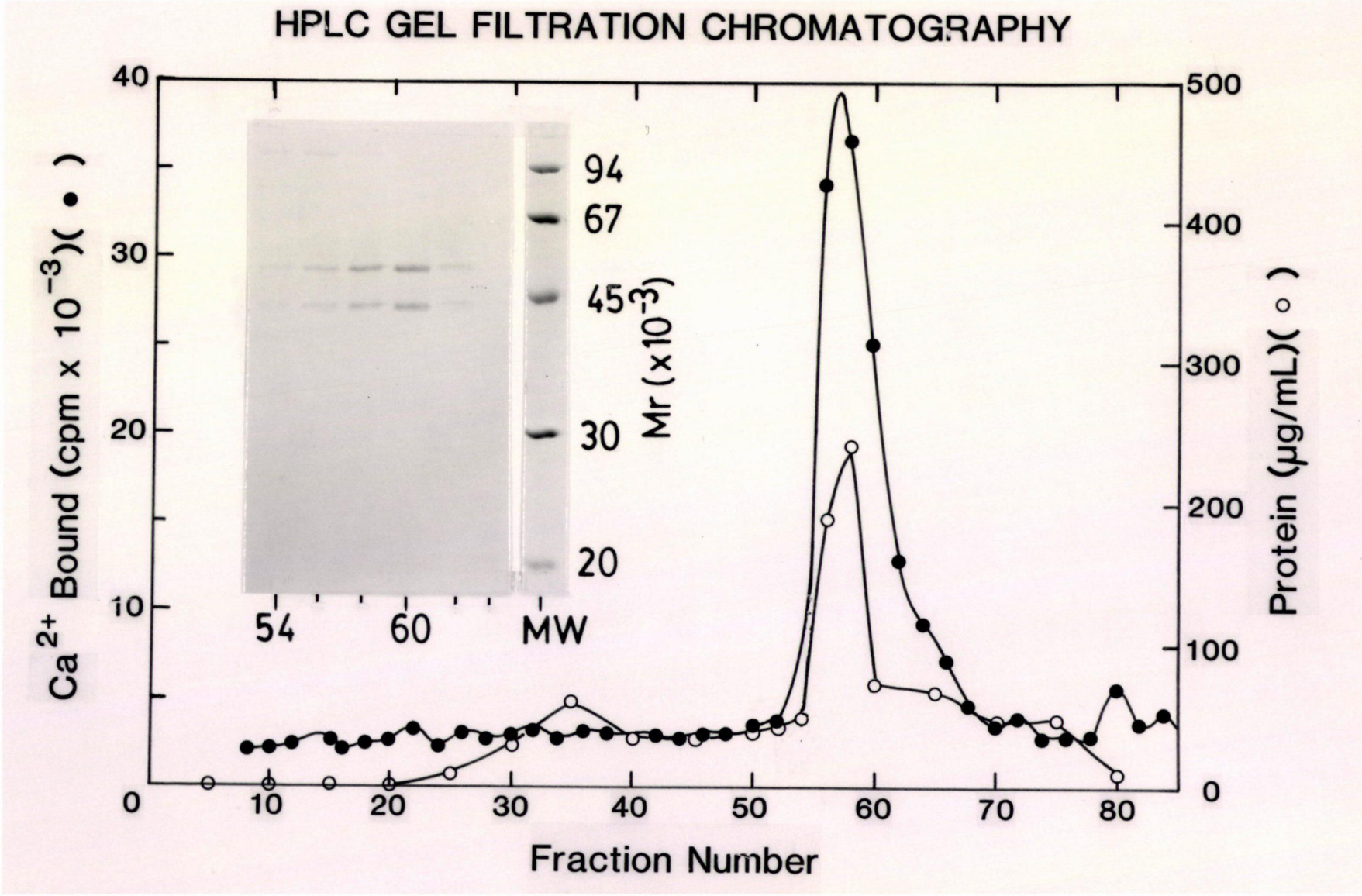

HPLC GEL FILTRATION CHROMATOGRAPHY
Ca 2+ Bound (cpm x 10−3)(•)
Protein (µg/mL)(○)
Fraction Number
Mr (x10-3)
94
67
45
30
20
54
60
MW
0
10
20
30
40
50
60
70
80
100
200
300
400
500

Fig. 9. SDS-polyacrylamide gel electrophoresis of peak fractions during purification of the 80 kDa protein complex

The peak fractions from each step of purification were electrophoresed into a 13% SDS-polyacrylamide gel in the following order: lane a, molecular weight markers; lane b, 100,000 x g; lane c, DEAE peak; lane d, hydroxyapatite peak; lane e, gel filtration peak. If necessary, contaminating proteins were removed from the gel filtration peak by passing the sample through a FPLC mono-Q anion exchange column.

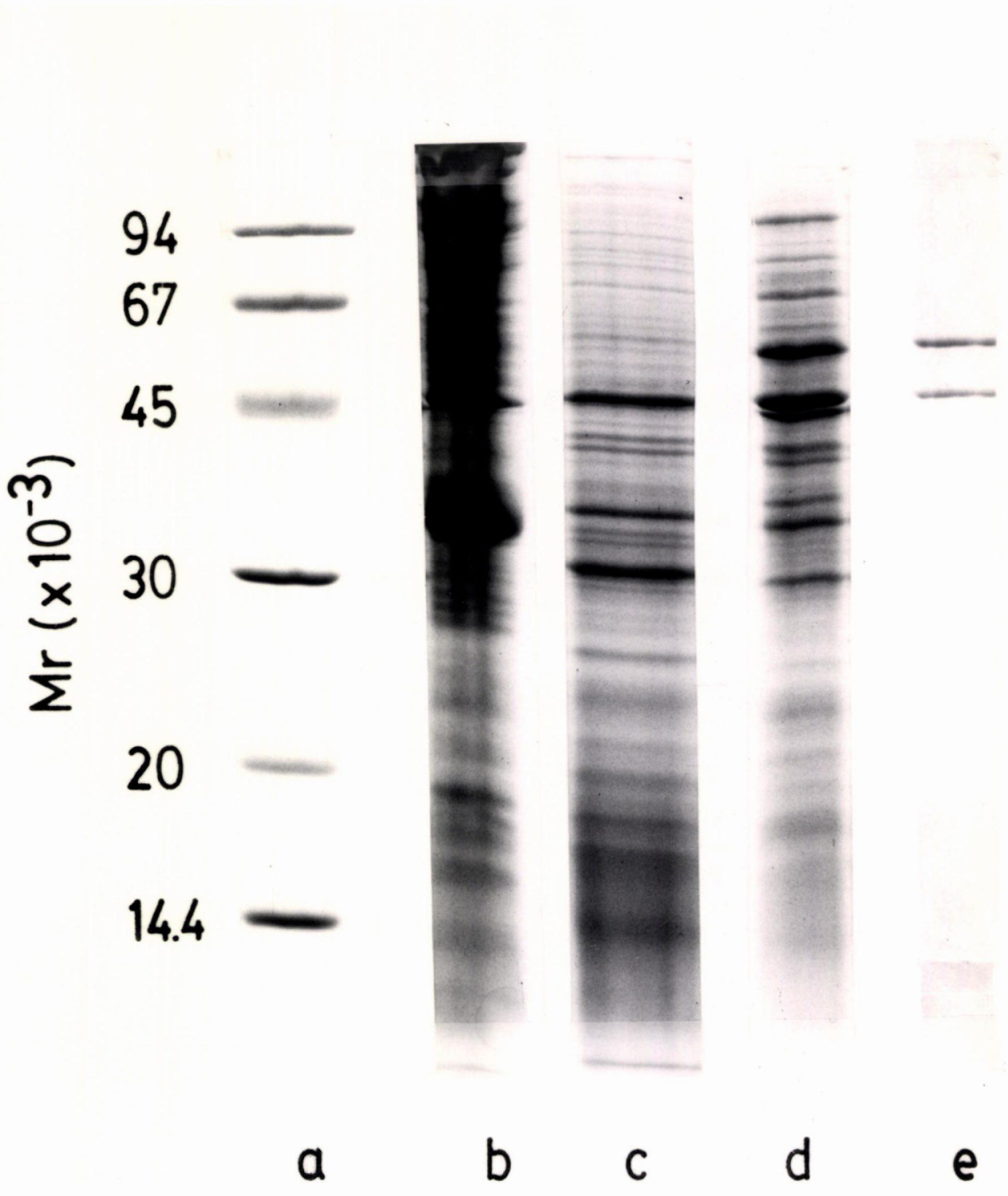
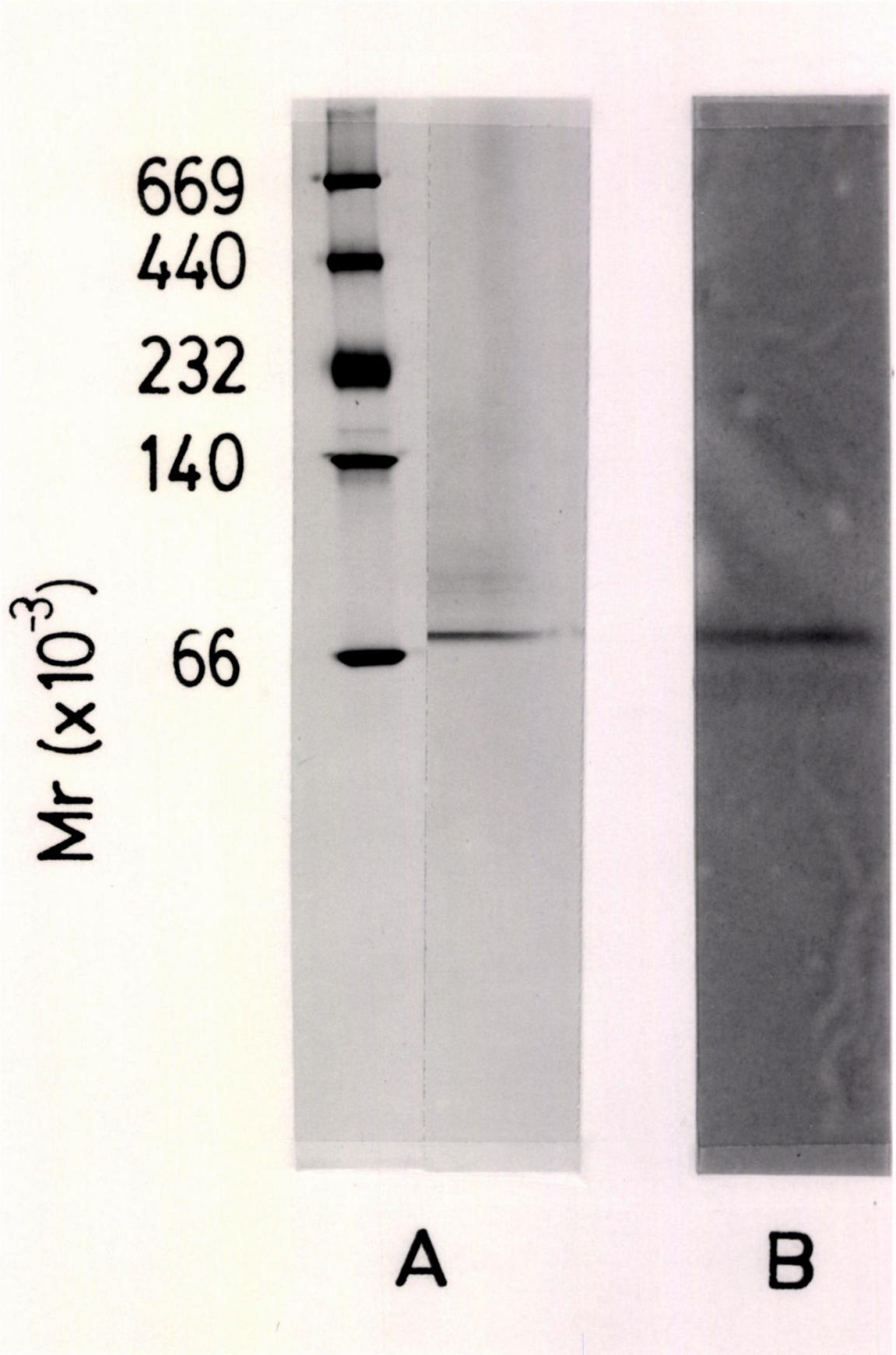

94
67
45
30
20
14.4
Mr (x10-3)
a
b
c
d
e

Fig. 10. ^{45}Calcium overlay autoradiography of the 80 kDa complex

The purified protein complex from the peak gel filtration fraction migrated as a single band on a 4-30% nondenaturing polyacrylamide gel (see A). The molecular weight was estimated to be 80 kDa by comparison with the molecular weight markers in the left lane. The proteins on the polyacrylamide gel were transblotted onto a nitrocellulose sheet that was then incubated in 10 mM imidazole (pH 7.1), 150 mM KCl, 5 mM $MgCl_2$, and 2 μCi/mL $^{45}CaCl_2$ for 15 minutes. After the sheet was dried, it was placed under high sensitivity X-ray film for 18 hours. The exposed film revealed a single band that was due to the protein complex (see B). The molecular weight markers did not bind calcium.


669
440
232
140
66
Mr (x10-3)
A
B

responsible for the calcium binding activity and that they specifically bound calcium over magnesium. Under the same conditions, the molecular weight markers (thyroglobulin, 669 kDa; ferritin, 440 kDa; catalase, 232 kDa; lactate dehydrogenase, 140 kDa; BSA, 67 kDa) did not bind calcium.

3.5. Attempts to separate the 80 kDa protein complex

Attempts were made to determine how the 50 kDa and 43 kDa proteins were bound together. In the first experiment, the complex was electrophoresed in a SDS-polyacrylamide gel, with and without 2-mercaptoethanol in the sample buffer (Fig. 11, A). The presence or absence of 2-mercaptoethanol did not affect the separation of the two proteins indicating that the proteins were not bound together by disulfide bonds. In the second experiment, the complex was dialyzed against 10 mM EGTA for 48 hours and for 14 days, then electrophoresed onto a 4-30% polyacrylamide gel (Fig. 11, B). A single band at 80 kDa revealed that the proteins remained as a complex.

3.6. Amino acid composition of the 50 kDa protein

The 50 kDa protein was isolated from the complex by electro-eluting it from an SDS-polyacrylamide gel. The amino acid composition of the protein revealed that it did not contain cysteine or tryptophan residues (Table 2). Glycine made up 20% of the residues whereas serine made up another 18%. The protein also contained more Glx and

Fig. 11. Attempts to separate the 80 kDa protein complex with 2-mercaptoethanol and EGTA

The 80 kDa protein complex was mixed with SDS-sample buffer, with and without the addition of 2-mercaptoethanol, and electrophoresed into a 12.5% SDS-polyacrylamide gel (A). The complex separated into the 43 kDa and 50 kDa components (lane 1, without 2-mercaptoethanol; lane 2, with 2% 2-mercaptoethanol; lane 3, molecular weight markers). The complex was also dialyzed against 10 mM Tris (pH 7.5), 0.5 mM DTT, and 10 mM EGTA for 2 weeks and then electrophoresed into a 4-30% nondenaturing polyacrylamide gel (B). The complex remained intact and migrated at a molecular weight equivalent to 80 kDa (lane 1) as compared to the molecular weight markers (lane 2).

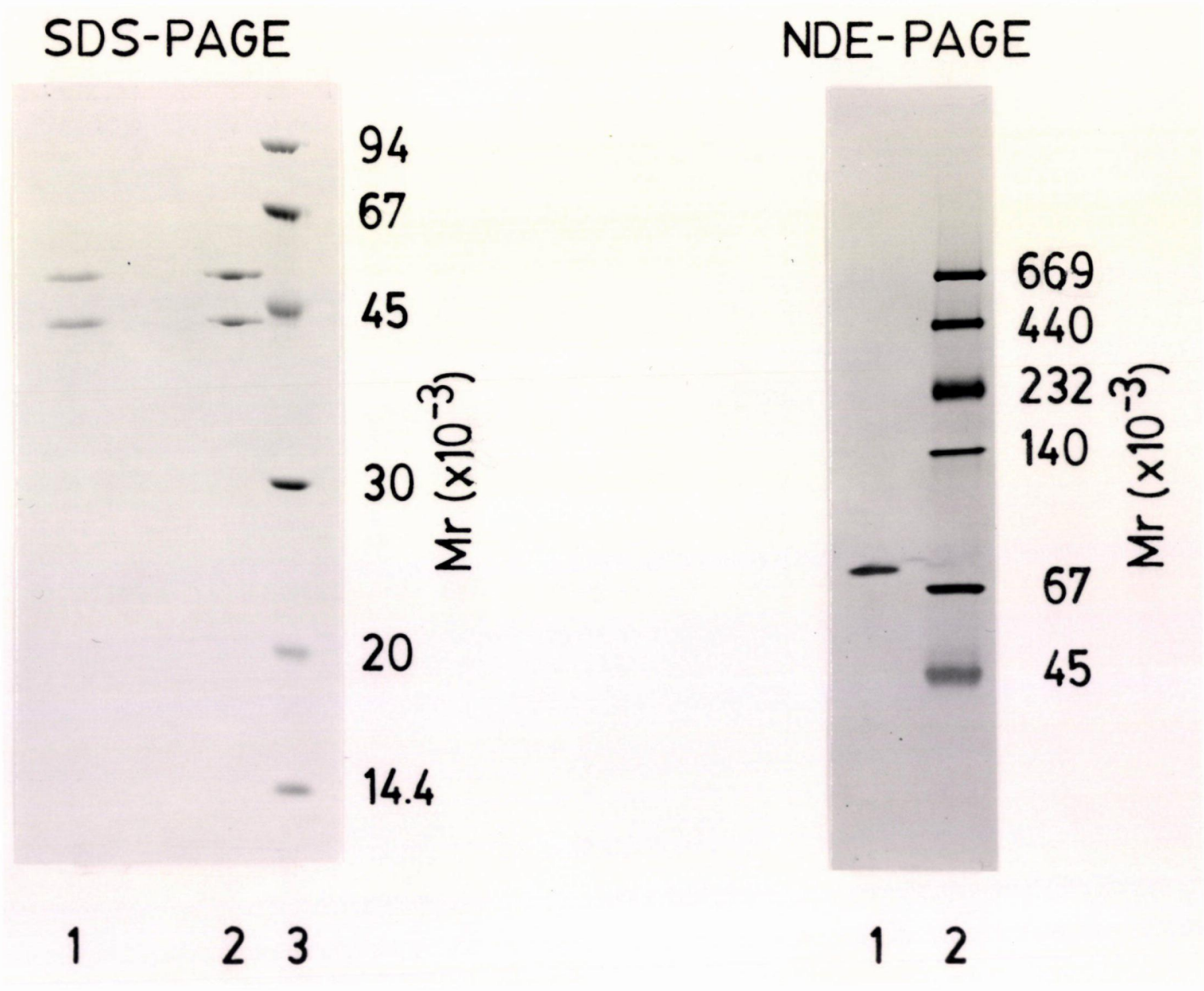

SDS-PAGE
94
67
45
30
20
14.4
Mr (x10-3)
1
2
3
NDE-PAGE
669
440
232
140
67
45
Mr (x10-3)
1
2

Table 2. Amino acid composition of the 50 kDa protein

The 50 kDa protein was eluted from a SDS-polyacrylamide gel and dialyzed against water. The protein was then dried and hydrolyzed in 6 N HCl and characterized by an automated amino acid analyzer. The protein contained 20% glycine, 18% serine and no cysteine or tryptophan.

AMINO ACID	# OF RESIDUES	%COMPOSITION
ALA	41.0	8.0
ARG	9.8	1.9
ASX	28.8	5.6
CYS	0.0	0.0
GLX	91.8	17.9
GLY	102.9	20.0
HIS	9.9	1.9
ILE	11.7	2.3
LEU	19.4	3.8
LYS	22.2	4.3
MET	4.4	0.9
PHE	10.2	2.0
PRO	18.3	3.6
SER	94.6	18.4
THR	23.6	4.6
TRP	0.0	0.0
TYR	5.1	1.0
VAL	20.2	3.9

Asx than basic amino acids.

3.7. Comparison of the 50 kDa and 45 kDa proteins

A 45 kDa protein complex was purified from sea urchin eggs in a similar manner as the 80 kDa protein complex (Wang and Spudich, 1984). Therefore it had to be determined whether or not these were the same proteins. Western blot analysis of both proteins revealed that they are not the same protein (Fig. 12, A and B). A SDS-polyacrylamide gel of the 45 kDa and 80 kDa complexes (Fig. 12 B, lanes 2 and 3) revealed the difference in molecular weight. The anti-45 kDa antibody only recognizes the 45 kDa protein (Fig. 12 A, lane 2), whereas, actin and the 50 kDa protein and the 43 kDa protein do not react with the antibody (Fig. 12 A, lanes 1 and 3). These results suggested that the 50 kDa protein was not previously characterized.

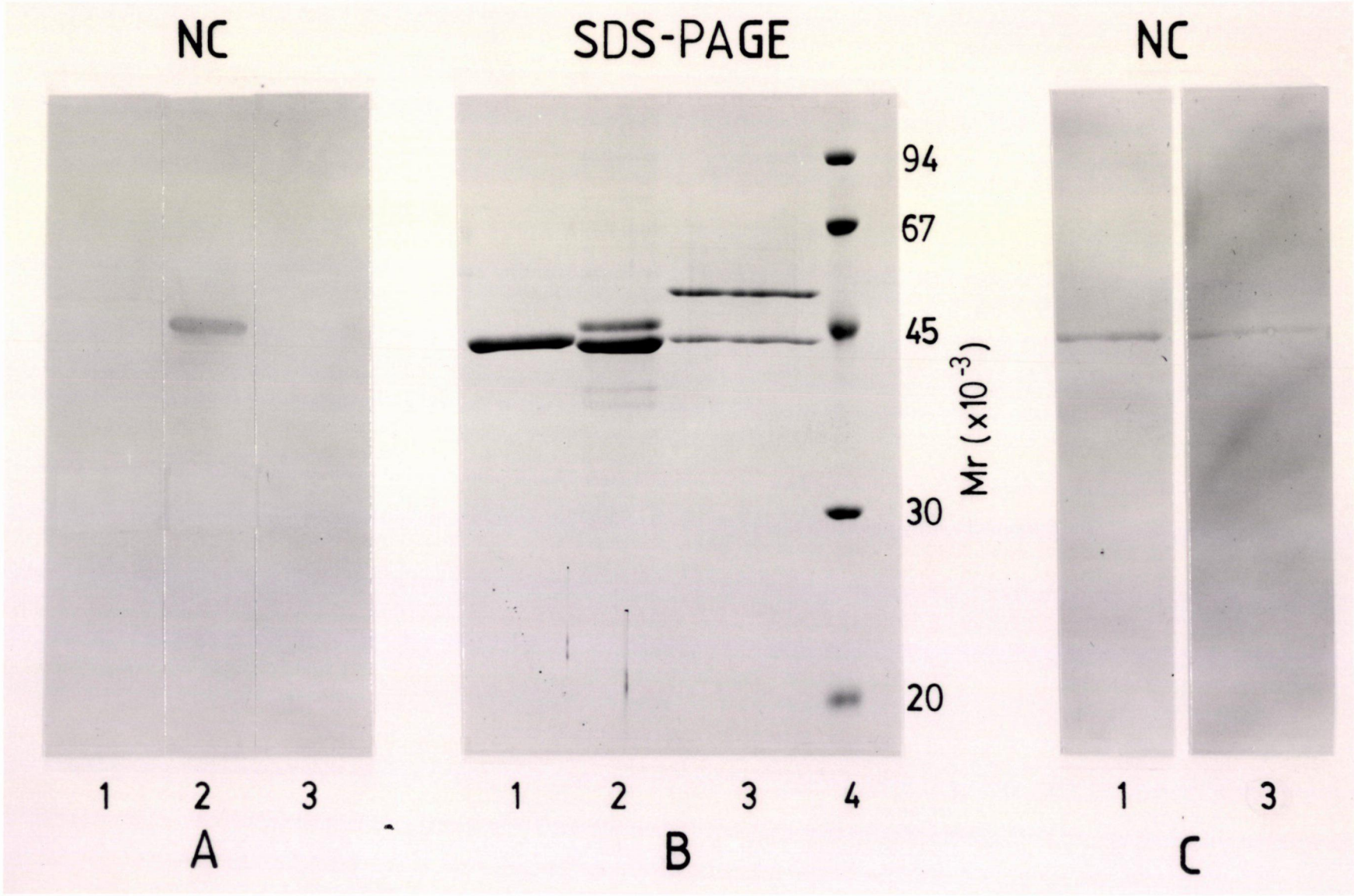
3.8. Comparison of the 43 kDa protein with actin

3.8.1. Western blot analysis

Anti-rabbit antibodies were used to determine if the 43 kDa protein of the 80 kDa complex was sea urchin actin. Anti-rabbit actin antibodies reacted with a rabbit actin control and with the 43 kDa protein band from the 80 kDa protein complex (Fig. 12, lanes 1 and 3 in B and C). The combination of antibody reactivity and the similar molecular weight to rabbit skeletal muscle actin, suggested that the 43 kDa urchin protein may be actin.

Fig. 12. Immunoblot analysis of the 45 kDa protein complex and the 80 kDa protein complex

Samples of rabbit skeletal muscle actin (B, lane 1), 45 kDa protein complex (B, lane 2), and 80 kDa protein complex (B, lane 3) were electrophoresed into a 12.5% SDS-polyacrylamide gel and then transblotted onto a nitrocellulose sheet. The sheet was incubated with anti-45 kDa protein antibody and then with an antirabbit IgG antibody that was conjugated with peroxidase. The sheet was developed with 3% H_2O_2 until the staining was complete. Only the 45 kDa protein was detected with the antibody (A, lane 2), whereas the 50 kDa protein and actin were not detected. Actin and the 80 kDa protein complex were also transblotted onto another nitrocellulose sheet and incubated with an anti-actin antibody in a similar manner as in A. The anti-actin antibody reacted with the rabbit skeletal muscle actin (C, lane 1) and with the 43 kDa protein of the 80 kDa complex (C, lane 3).


NC
SDS-PAGE
NC
94
67
45
30
20
Mr (x10-3)
1
2
3
1
2
3
4
1
3
A
B
C

3.8.2. Peptide map analysis

Rabbit skeletal muscle actin and the 43 kDa protein of the complex were digested with V8 protease by the method of Cleveland et al. (1977). There were three major bands which were common to both the rabbit and sea urchin actin (Fig. 13). Two bands appeared as doublets in rabbit actin, whereas the other band appeared at about 30 kDa. The similarities of these bands and faint minor bands suggested that the 43 kDa protein of the complex may be actin.

3.9. DNase I affinity chromatography

Since the 43 kDa protein of the complex appeared to be actin it was realized that the complex may be purified by DNase I chromatography (Hosoya et al. 1982). The 100,000 x g supernatant was brought to 2 mM calcium and loaded onto a DNase I affinity column (Fig. 14, lane b). Most proteins did not bind to the column and were collected in the flow through fraction (lane c). The arrows in lanes b and c point to two bands, a 43 kDa protein which was believed to be actin, and a 45 kDa protein which was a component of the 45 kDa protein complex as confirmed by the anti-45 kDa antibody (Fig. 12) (Wang and Spudich, 1984). These bands were reduced in intensity in the flow-through sample (lane c) and were eluted from the column in the final buffer wash of guanidine-HCl (lane f). Several unidentified actin binding proteins were removed from the column with a 1 M KCl wash (lane d).

Fig. 13. A. Peptide map of rabbit skeletal muscle actin and sea urchin actin

Complex (0.05 mg) and rabbit skeletal muscle actin (0.025 mg) were electrophoresed into separate 12.5% SDS-polyacrylamide gels. A 43 kDa band from both gels was excised and inserted into separate wells of a 15% SDS-polyacrylamide gel. The wells were filled with SDS-sample buffer without 2-mercaptoethanol but with V8 protease as in the method by Cleveland et al. (1977). The staining pattern revealed that rabbit actin (A) had several common peptides with sea urchin actin (B). Lane C contained V8 protease only, and lane D is molecular weight markers.

B. Centrifugation of 80 kDa protein complex and polymerized actin.

Complex (0.2 μM) and g-actin (8.0 μM), in 1 mL g-actin buffer, were polymerized with KCl and $MgCl_2$. After 1 hour the solution was centrifuged at 150,000 x g for 90 minutes. After the supernatant was lyophilized, the supernatant and pellet were electrophoresed into a 12.5% polyacrylamide gel which was then silver stained. A 50 kDa and 43 kDa band were visible in the supernatant (arrows, lane A), but only a 43 kDa band was visible in the pellet (lane B).

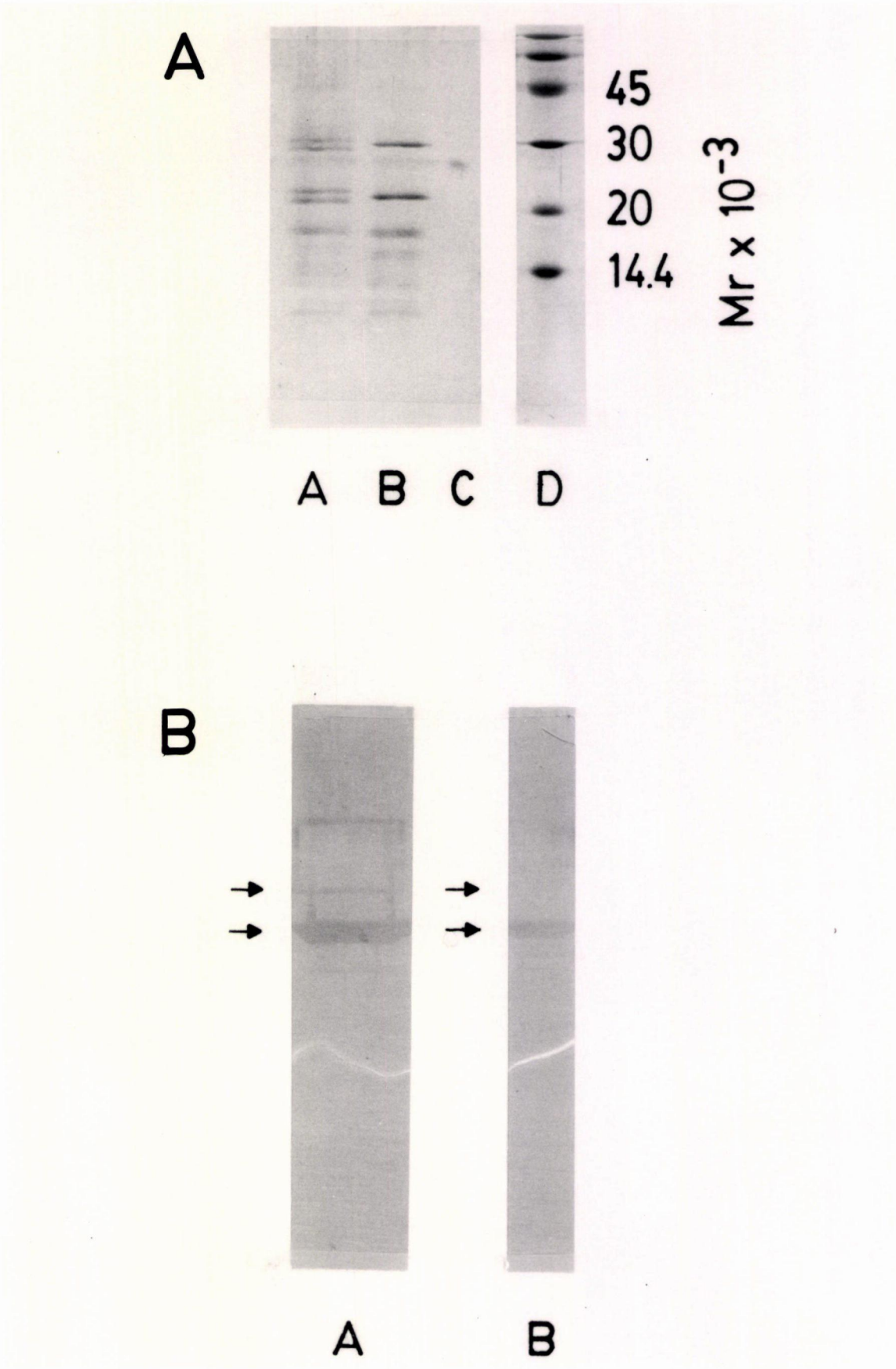
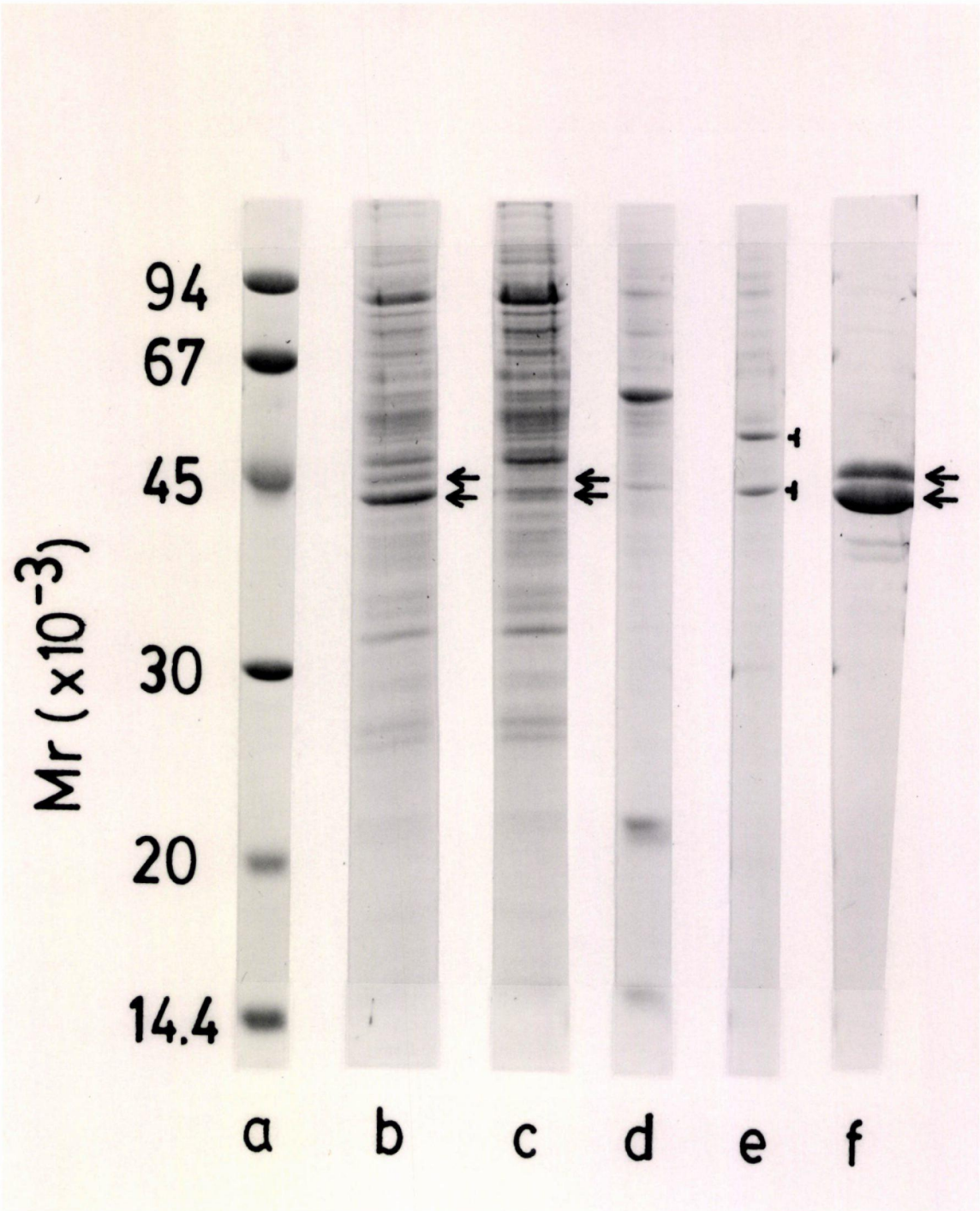

A
45
30
20
14.4
Mr x 10-3
A B C D
B
A B

Fig. 14. DNase I chromatography of 100,000 x g supernatant

Sea urchin 100,000 x g supernatant (lane b) was dialyzed against 10 mM HEPES (pH 7.0), 100 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 20 mM 2-mercaptoethanol, and 0.005% sodium azide and then loaded onto a 2 mL DNase I affinity column. A flow through fraction (lane c) was collected, as were fractions that were eluted with 1 M KCl in the same buffer (lane d), 5 mM EGTA (lane e), and 3 M guanidine-HCl (lane f). Molecular weight markers are shown in lane a. Two arrows indicate the 43 kDa and 45 kDa proteins which were present in the 100,000 x g supernatant but not in the flow through fraction. These proteins make up the 45 kDa protein complex and were eluted in the guanidine-HCl wash. The 80 kDa protein complex eluted with the EGTA wash (lane e,). If calcium was not present in the loading buffer, then the 80 kDa complex would not bind to the column. However, if calcium was added to the subsequent flow through, and the flow through was then reloaded onto the column, the complex could then be eluted with the EGTA buffer.


94
67
45
30
20
14.4
Mr (x10⁻³)
a
b
c
d
e
f

The EGTA buffer wash eluted two proteins that were the components of the 80 kDa protein complex (lane e). The interaction of the complex with the DNase I affinity column required calcium in the loading buffer. If calcium was not present, then the complex did not elute in the EGTA wash or any other wash because it did not bind to the column. If the flow through fraction was then brought to 2 mM calcium and reloaded onto the DNase I affinity column, the 80 kDa complex bound and was eluted with EGTA. This experiment implied that the 43 kDa protein of the complex was actin and that the complex binds to DNase I in a calcium dependent manner.

3.10. Purification of rabbit skeletal muscle actin

Rabbit skeletal muscle actin was required for experiments that examined the interaction of the 80 kDa protein complex with actin polymerization. The purification of actin could not be directly observed by SDS-polyacrylamide gel electrophoresis because the fraction that was purified was initially an insoluble fibrous mass. However, waste samples from each wash were run onto a SDS-polyacrylamide gel to observe which proteins were extracted (Fig. 2). The purified actin was tested for calcium binding activity by the chelex assay; it did not bind calcium, suggesting that the calcium binding activity of the 80 kDa complex was not due to the actin component.

3.11. Pyrene labeled actin assay

3.11.1. The effect of the 80 kDa protein complex upon actin polymerization

The effect of the 80 kDa protein complex upon the polymerization of actin was tested by adding the complex to a pyrene labeled actin sample and then inducing polymerization with the addition of KCl and $MgCl_2$. The complex reduced the rate of polymerization and also lowered the final concentration of f-actin in a dose dependent manner (Fig. 15). The maximum inhibition occurred at a concentration of 0.24 μM complex, which was 10% of the total actin concentration (line d). The addition of 0.16 μM BSA or denatured complex resulted in a curve which was similar to the control (line a). The complex did not have an effect if it was added (0.24 μM) to a control sample after the maximum fluorescence was reached.

3.11.2. Effect of the 80 kDa protein complex upon actin polymerization in the presence of calcium and EGTA

Since the 80 kDa protein complex bound calcium and interacted with the DNase I affinity column in a calcium dependent manner, the effect of calcium upon the interaction of the complex and actin was examined. The addition of calcium (0.2 mM) reduced the rate of actin polymerization and also slightly decreased the final fluorescence (Fig. 16, line b) as compared to the control (line a). The addition of calcium in the presence of complex did not seem to have an effect.

Fig. 15. The effect of the 80 kDa protein complex upon actin polymerization

Various amounts of the protein complex (a, 0; b, 0.05 μM; c, 0.1 μM; d, 0.2 μM) were added to a 1 mL volume containing g-actin buffer (without calcium) and 2 μM g-actin, of which 10% was pyrene labeled. Polymerization was started by adding KCl and $MgCl_2$ to a final concentration of 100 mM and 2 mM respectively. Fluorescence measurements were made at 365 nm excitation wavelength and at 407 nm emission wavelength. 0.05 μM BSA and 0.05 μM boiled 80 kDa protein complex were also added to the actin. This resulted in a curve (not shown) which was similar to the control (a).

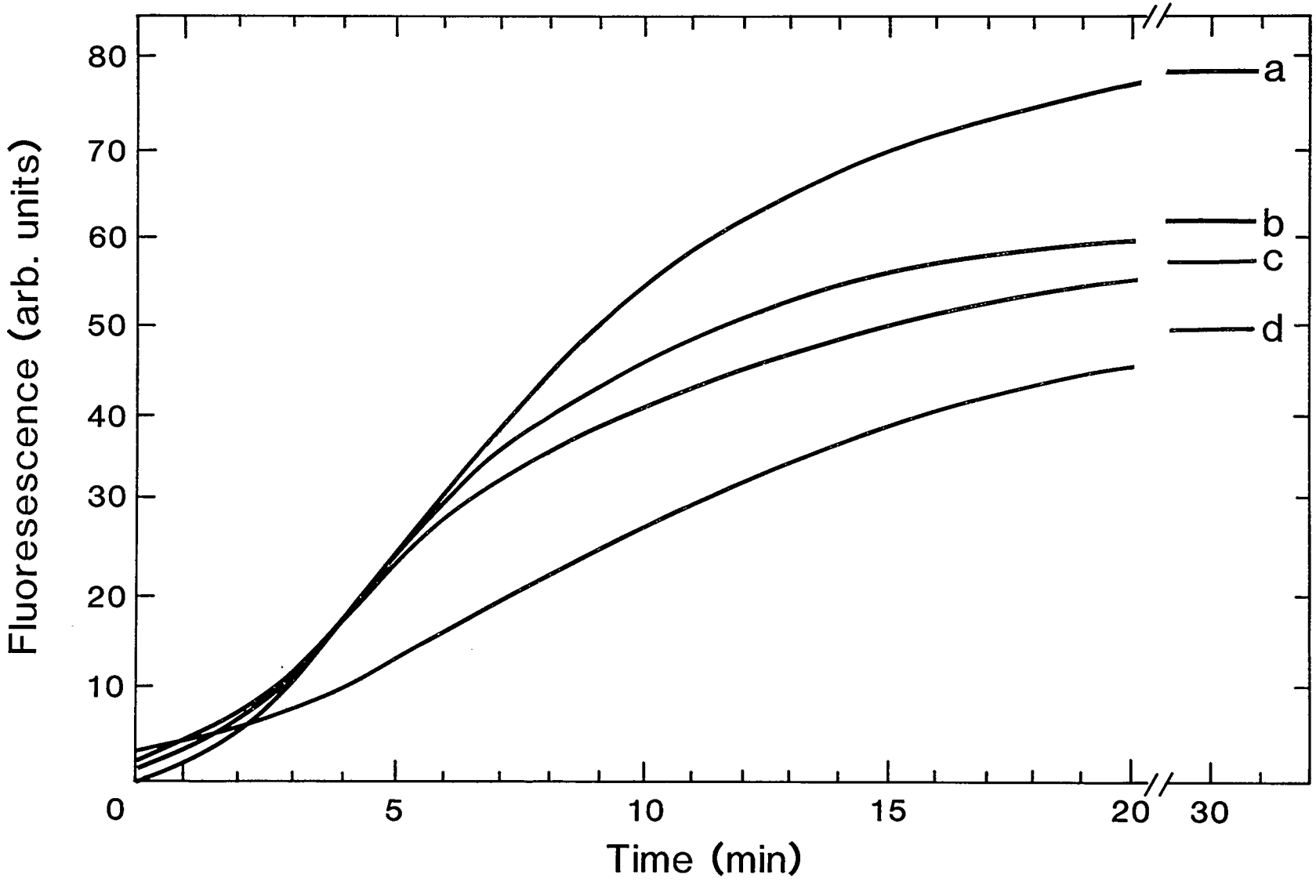
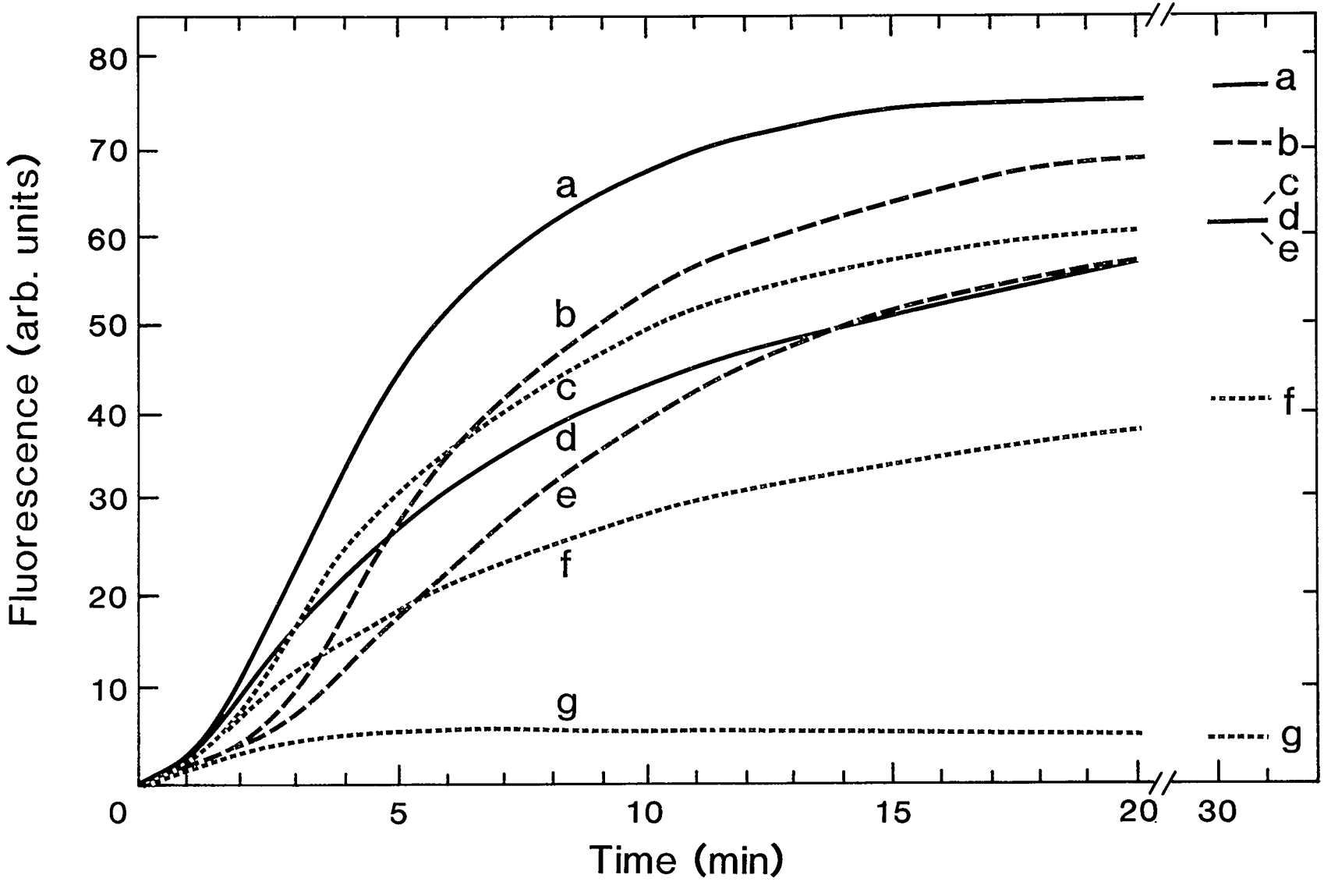

Fluoresescence (arb. units)
80
70
60
50
40
30
20
10
0
5
10
15
20
30
Time (min)
a
b
c
d

Fig. 16. The effect of calcium and EGTA upon the polymerization of actin in the presence of the 80 kDa protein complex

G-actin (8 μM total, of which 20% was pyrene labeled actin) was mixed in 1 mL of calcium free g-actin buffer and polymerized with 100 mM KCl and 4 mM $MgCl_2$ (a); 0.02 μM complex was also added (d). Calcium (0.200 mM) was added the mixture prior to polymerization (b), and in the presence of 0.02 μM complex (e). EGTA was added (0.5 mM) immediately prior to polymerization to actin alone (c), and in the presence of 0.02 μM complex (f). EGTA (2.0 mM) was also added to the g-actin solution 5 minutes prior to the addition of KCl and $MgCl_2$ (g). The addition of 0.2 mM calcium or 0.5 mM EGTA or 0.02 μM complex to the control (a), after maximum fluorescence was obtained, did not have an effect.


Fluorescence (arb. units)
80
70
60
50
40
30
20
10
0
5
10
15
20
30
Time (min)
a
b
c
d
e
f
g

The rate of actin polymerization was lower in the presence of calcium (lines d and e), however, the final fluorescence was identical. EGTA had a pronounced effect upon actin polymerization. EGTA alone (0.5 mM) inhibited the rate of actin polymerization and reduced the final fluorescence (line c); the addition of complex enhanced this effect (line f). However, if 2.0 mM EGTA was added several minutes prior to the addition of KCl and $MgCl_2$, only a slight increase in fluorescence would occur (line g). Calcium or EGTA did not have an effect if they were added to the control (line a), or to the sample treated with complex (line d), after the maximum fluorescence was reached.

3.11.3. Centrifugation of the 80 kDa complex with polymerized actin

The 80 kDa protein complex and actin were polymerized and then centrifuged to determine if the complex associates with f-actin, or remains in the supernatant. The pellet and supernatant samples were examined on a SDS-gel. The complex remained in the supernatant fraction, suggesting that it does not bind to f-actin (Fig. 13).

4.0. Discussion

Fertilization begins many biochemical processes that change an egg into a rapidly developing embryo. These processes appear to be regulated by calcium and can be initiated by any method which increases the free calcium concentration in eggs. Calmodulin was the only calcium binding protein characterized in sea urchin eggs (Head et al., 1979). It seemed unlikely that it alone regulates all of the calcium dependent events. Therefore, sea urchin eggs were examined for the presence of other calcium binding proteins. The results from this thesis indicated that calmodulin was not the only calcium binding protein in sea urchin eggs.

Analysis, by the chelex assay, of the DEAE fractionated 100,000 x g supernatant from egg extracts, suggested that a substantial amount of calcium binding activity was present in eggs. The chelex assay, however, does not identify the source of the calcium binding activity. Only after the protein has been purified, and calcium binding activity retained, can one state that a protein is responsible for calcium binding activity. Calmodulin was identified in the DEAE profile. It accounted for only a fraction of the total calcium binding activity. The source of the non-calmodulin calcium binding activity was not known; we assumed that it was due to other calcium binding protein(s). By using the chelex assay and purifying this activity by hydroxyapatite, gel filtration, and anion exchange chromatography, a 80 kDa protein complex was identified as the source of the major

calcium binding activity in egg 100,000 x g supernatants.

The presence of the 80 kDa protein complex and a 15 kDa calcium binding protein (Hosoya et al., 1986) demonstrated that calmodulin was not the only calcium binding protein in sea urchin eggs. Also, the remaining unidentified calcium binding activity from the DEAE profile (Fig. 5) may be due to other calcium binding proteins.

The calcium binding activity of the 80 kDa protein complex was instrumental to its purification by two different procedures. The complex was first purified by the chelex assay and standard chromatography. The complex was also purified by DNase I affinity chromatography since it was the only protein that bound to the column in a calcium dependent manner. Therefore, two different purification schemes were dependent upon the interaction of the complex with calcium. This implied that calcium significantly affects the complex. The ^{45}calcium overlay technique also revealed that the complex interacted with calcium (Fig. 10.).

The chelex assay suggested that the 80 kDa protein complex was the source of the highest calcium binding activity in eggs. The value for the calcium binding activity obtained by the chelex assay is a function of the following properties of a protein: protein concentration (P) and moles of calcium bound per moles of protein (n), calcium binding affinity (Kd), and the free calcium of the assay (CaF) (Waisman and Rasmussen, 1983). The ^{45}calcium bound by the test supernatant (CaB) is related to the calcium binding properties of the proteins according to the following equation:

$$CaB = \frac{n}{1 + Kd/CaF}$$

The (CaF) will be determined by the amount of chelex and the total calcium in the assay. Since the calcium binding activity is a multivariable function, one cannot characterize the calcium binding properties (Kd and n) of a protein unless two of the three variables are known. Even then, the calcium binding properties of chelex will vary with the concentration of free calcium present and therefore (CaF) in the assay will be difficult to determine. Another difficulty is that the chelex assay will respond to all calcium binding proteins in a given sample so that quantitative results can only be obtained when a single calcium binding protein is present in a test sample. The calcium binding kinetics of chelex-100 are not consistant with a simple one site model (Waisman and Rasmussen, 1983) and therefore calculation of (CaF) in the assay from known concentration and n of the chelex is difficult.

The 50 kDa protein of the complex bound tightly to a 43 kDa protein. The 43 kDa protein was thought to be actin because, its molecular weight was similar to actin, it reacted with an anti-actin antibody (Fig. 12), it bound to a DNase I affinity column (Fig. 14), and V8 protease digestion produced a peptide pattern similar to digested rabbit skeletal muscle actin (Fig. 13). The 50 kDa protein was separated from the 43 kDa protein only by SDS polyacrylamide gel electrophoresis. The two proteins did not appear to be linked by disulfide bonds (Fig. 11). Some actin binding proteins have been shown to bind tightly to actin in the presence of calcium and dissociate when the calcium is removed with EGTA. The 80 kDa protein complex did not dissociate even after two weeks of dialysis against

EGTA (Fig. 11). Therefore, the 50 kDa protein may be similar to gelsolin, in that it may irreversibly bind to actin (Kurth and Bryan, 1984).

It is not known if the 50 kDa protein exists singly in the egg or if the complex was formed during purification. Since protein actin complexes can form in the presence of calcium (Coluccio et al., 1986), calcium was not added to the buffers during purification by standard chromatography. However, EGTA was not added to chelate potentially contaminating calcium because EGTA interferes with the chelex assay. Therefore, if the complex was formed during purification, it may only require a trace quantity of calcium. If calcium causes the artifactual formation of the complex, then a method other than DNase I affinity chromatography or chelex assay is needed to purify the 50 kDa protein.

A 50 kDa calcium binding or actin binding protein has not been characterized in sea urchin eggs. A protein band at 50 kDa has been reported, however, by Hosoya and Mabuchi (1984). This protein was not described. The 80 kDa protein complex also appeared to be similar to a 45 kDa protein complex found in sea urchin eggs. (Hosoya and Mabuchi, 1984; Wang and Spudich, 1984). However, the 50 kDa protein did not react with the anti-45 kDa antibody (Fig. 12).

Proteins did not elute from the DNase I affinity column in the same manner as reported by other investigators (Hosoya and Mabuchi, 1984). The 80 kDa complex was the only protein which eluted from the column in the EGTA wash (Fig. 14). Several proteins, including 95 kDA and 17 kDa, eluted with 1 M KCl, whereas the 45 kDa complex (identified with

an anti-45 kDa antibody, Fig. 12) eluted in the guanidine-HCl wash. Hosoya et al. (1982) and Hosoya and Mabuchi (1984), found that 95 kDa, 50 kDa, some 45 kDa, 17 kDa, and 13 kDa proteins eluted in the EGTA wash, whereas most of the 45 kDa complex eluted with the 1 M KCl wash. Only a 43 kDa protein eluted with guanidine-HCl. Part of the reason for the difference in elution profiles was that we eluted the column with KCl before the EGTA wash. It is possible that proteins which elute with both KCl and EGTA may not bind in a truly calcium dependent manner. In contrast, the 80 kDa complex bound solely in a calcium dependent manner. It is not known why the 45 kDa complex eluted from our column only with guanidine-HCl.

A pyrene labeled actin assay was used to determine if the 80 kDa protein complex had an effect upon actin polymerization. The complex seemed to inhibit the rate and extent of actin polymerization (Fig. 15). The effect appeared to be independent of calcium, however, it was difficult to determine the absolute effect of the calcium and complex since calcium alone reduces the rate of polymerization (Hosoya and Mabuchi, 1984). G-actin exists a Ca-actin in the presence of calcium and as Mg-actin in the presence of magnesium. Ca-actin polymerizes slower and has a higher critical concentration for polymerization than Mg-actin (Selden et al., 1986). Ca actin must first be converted to Mg-actin when it is polymerized with the addition of magnesium. The conversion of Ca-actin to Mg-actin increases the lag phase of polymerization. Therefore the rate of polymerization will vary with calcium concentration as observed in Fig. 16. Also, 10 μM calcium was always present as carry over from

the actin buffer. EGTA was added to the assay buffer so that the effect of the complex could be observed at lower free calcium. The complex inhibited actin polymerization even more in the presence of 0.5 mM EGTA. This inhibition however, may be due to the EGTA, since 2 mM EGTA alone completely inhibited actin polymerization (Fig. 16). This effect confounds further studies of calcium and complex upon actin polymerization using this method. In subsequent experiments we have tested the effect of the 80 kDa complex on the polymerization of Mg-actin. These experiments were performed by incubating the Ca-actin with 50 μM $MgCl_2$ and 50 μM EGTA prior to the addition of complex and polymerization salts. Under these conditions the 80 kDa complex inhibited the rate and extent of polymerization in a manner similar to that reported for Ca-actin, however, the effect of the complex was unaffected by varying free calcium from 1.5×10^{-4} M to 1×10^{-10}M. Therefore under these conditions the effect of the 80 kDa complex was calcium independent (data not shown).

The ability of the 80 kDa protein complex to inhibit actin polymerization suggested that the complex may act as a capping protein (Stossel et al., 1985). The complex may bind to the growing end of a filament and prevent the addition of actin monomers. This may account for the observed substoichiometric inhibition (maximum inhibition occurred at 1:10 complex to actin); only one 50 kDa molecule may be needed to cap the actin molecules which form a filament. Conversely, in one experiment, the 80 kDa complex was added to g-actin which was then polymerized. After the maximum fluorescence was reached, the sample was then centrifuged and the supernatant and pellet were

examined by SDS polyacrylamide gel electrophoresis. The gel revealed that the complex remained in the supernatant (Fig. 13), rather than sedimenting with f-actin, which is where a capping protein would be found. However, since the complex was present in such low quantities, it is unlikely to be detected in the pellet fraction by SDS analysis. The 80 kDa protein complex did not affect actin after polymerization, therefore it did not appear to act as a severing protein. Also, even though the 50 kDa protein bound actin in a 1:1 ratio, it is not likely to be a sequestering protein since it was present in such low quantities (0.08% of total protein) relative to g-actin (4%, Mabuchi and Spudich, 1980) in the 100,000 x g supernatant. Further examination of the 80 kDa protein complex and actin filament by other techniques such as electron microscopy (Wang and Spudich, 1984) may reveal the mechanism of inhibition.

Not all actin binding proteins may actually function with actin. It is possible, therefore, that the binding of the 50 kDa protein to actin may not be relevant to the function of the protein. For example, DNase I is an actin binding protein even though DNase I and actin are found in different locations in the cell (Pollard and Cooper, 1986). However, since the 50 kDa protein was purified from an actin containing fraction, it seems likely that its function may lie with actin regulation.

There are many questions which remain to be answered about the 50 kDa protein. Does the protein exist singly in the egg, is it found in the particulate fraction, is it common to all sea urchin tissues or is it an egg specific protein? These questions could be answered after

preparation of an antibody to the 50 kDa protein. An antibody would allow one to purify the 50 kDa protein by assaying SDS polyacrylamide gels with immunoblots so that EGTA could be used in all buffers. This would eliminate the use of the chelex assay and would reveal if the 50 kDa protein binds irreversibly in the presence of calcium. Sea urchin tissues and the particulate fractions from eggs could be probed for the presence of the 50 kDa protein with an antibody. Knowing the location and quantity of the 50 kDa protein may give an indication of its function in eggs.

Questions also remain about the biochemical aspects of the 80 kDa complex. Is the 50 kDa protein, or is the interaction of the 50 kDa protein and the 43 kDa protein the source of the calcium binding activity? (The chelex assay revealed that actin alone was not responsible). How much calcium does the complex bind? With what affinity does the complex bind calcium? The answer to these questions may determine if the calcium binding properties of the complex are physiologically significant.

One of the reasons for examining calcium binding proteins was to understand how calcium can activate eggs. One needs to know more about the 80 kDa complex before its function in egg activation is known. However, there is enough data to suggest a role for the 80 kDa complex. Cytoskeletal rearrangement is one of the events which occur at fertilization (Vacquier, 1981). As actin filaments elongate in microvilli (Cline and Schatten, 1986), their growth must be regulated. The 80 kDa protein complex may form during the increase in intracellular free calcium and then limit the growth of actin

filaments. If the 80 kDa complex regulates growth without permanently binding to a filament, it may be able to regulate many filaments at the same time, and therefore, not have to be present in a high concentration. Of course, the above is only speculation.

Three intracellular calcium binding proteins are found in eggs: calmodulin, a 15 kDa with no known function, and now a 80 kDa protein complex. Since there are still more calcium dependent processes than there are proteins, it is likely that more calcium binding proteins will be identified. The chelex assay may be useful in identifying these remaining proteins.

5.0. References

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