PURIFICATION AND BIOCHEMICAL CHARACTERIZATION OF A PROTEIN COMPLEX FROM THE SEA URCHIN, *Strongylocentrotus purpuratus*: POSSIBLE FUNCTIONAL ROLE FOR THE YOLK GRANULE ORGANELLE DURING EMBRYONIC DEVELOPMENT

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PURIFICATION AND BIOCHEMICAL CHARACTERIZATION OF A PROTEIN COMPLEX FROM THE SEA URCHIN, *Strongylocentrotus purpuratus*: POSSIBLE FUNCTIONAL ROLE FOR THE YOLK GRANULE ORGANELLE DURING EMBRYONIC DEVELOPMENT

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ABSTRACT

The yolk granule is the most abundant membrane-bound organelle present in the eggs and embryos of many animals including avians, amphibians, insects, mollusks and echinoderms. The sea urchin yolk granules comprise about one-third of the volume of the egg. The classical view of the yolk granule is that it provides nutrition to the growing embryo, but the finding that the composition of the sea urchin yolk granule does not change until the later stages of larval development changed this view, suggesting that the yolk granules are not just benign storage organelles, but might be involved in some other cellular events occurring during embryonic development.

Several studies have demonstrated that sea urchin yolk granules harbor several components destined for export. Therefore, we hypothesized that the yolk granules might be involved in transportation and fusion events. We have isolated a protein of 240 kDa present in the yolk granules of eggs from the sea urchin, *Strongylocentrotus purpuratus,* by ion exchange chromatography using the anion exchange resin, Q-Sepharose Fast Flow. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of this protein under non-reducing conditions revealed that this was a complex composed of three polypeptides of 160 kDa, 120 kDa and 90 kDa. Western blots performed using the anti-toposome antibody demonstrated that the 240 kDa protein complex was the toposome which was proteolytically processed, while the 160 kDa polypeptide was the major yolk granule protein derived from it. Peptide mapping confirmed that the 240 kDa toposome was the precursor for the 160 kDa major yolk granule protein.

We have biochemically characterized the calcium-dependent phospholipid binding and vesicular aggregating activity of the 240 kDa protein complex. As revealed

by liposome binding assays, the 240 kDa protein complex bound phospholipids in a calcium-dependent manner. Liposome aggregation assays demonstrated that this 240 kDa protein complex was capable of driving vesicular aggregation, which was also found to be a calcium-dependent process. The yolk granule aggregation assays allowed us to demonstrate that this aggregating activity was a physiologically relevant process. The anti-toposome antibody could specifically inhibit the calcium dependent phospholipid binding, liposome aggregation and the yolk granule aggregating activity of the 240 kDa protein complex. The exposure of yolk granules to trypsin inhibited aggregation suggesting that this process was driven by protein present in the outer surface of the yolk granule membranes. Analysis of the yolk granule membranes isolated by density gradient ultracentrifugation suggested that the membranes were rich in high molecular weight polypeptides of 160 k, 130 k, 120 k and 90 k. Collectively, these data suggest that the 240 kDa protein or the polypeptides derived from it could be the factor/s involved in vesicular aggregation and transportation events involving the yolk granule.

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BCIP	5-bromo-4-chloro-3-indolyl phosphate
BPB	Bromophenol blue
BSA	Bovine serum albumin
CBB	Coomassie brilliant blue
CFSW	Calcium-free sea water
CMFSW	Calcium- magnesium free sea water
DTT	Dithiothreitol
EDTA	Ethylenediamine tetraacetic acid
EGTA	ethylene glycol-bis (β-aminoethyl ether) N, N, N', N'-tetraacetic acid
HPF	Hours post fertilization
IgG	Immunoglobulin G
MFSW	Millipore-filtered sea water
NBT	Nitro blue tetrazolium chloride
OD	Optical density
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TCA	Trichloroacetic acid

Chapter 1: Introduction

1.1 Sea urchin as a model system for developmental studies

The sea urchin is an echinoderm that has been widely used to study developmental processes. The ease of obtaining large numbers of gametes made it an ideal model system for developmental studies. The fact that these can be fertilized *in vitro* and embryos can be grown makes it an attractive animal system to study. The embryos have a relatively small number of cells in comparison to more complex organisms. The pluteus stage embryos of *Strongylocentrotus purpuratus* consist of approximately 1,500 cells (Angrerer and Davidson, 1984). Cell lineage mapping has been performed and a number of molecular markers for specific genes and gene products are available to follow the differentiation of cells during development. For example, the cytoskeletal genes CyIII and Spec (*Strongylocentrotus purpuratus* ectoderm specific) are expressed by aboral ectoderm cells (Angrerer and Davidson, 1984). As well, the utility of the embryo has been enhanced by the ability to produce and culture transgenic embryos (Flytzanis *et al.*, 1985; Cameron and Davidson, 1991).

The sea urchin is also a widely used animal model to study the function of yolk granules since it is a very abundant organelle in the eggs and embryos as well as early larvae of the sea urchin. Therefore it is possible to obtain a large amount of material for the biochemical studies of yolk granule function. This has made the sea urchin an ideal system to study the function of yolk granules during embryonic and larval development.

1.2 The yolk granule

The yolk granule is a relatively large organelle, which is spherical or oval in shape having a diameter of 1-2 μm. It is the most abundant membrane-bound organelle

present in the eggs, embryos and early larvae of the sea urchin, comprising approximately one-third of cytoplasmic volume. The membrane covering the yolk granule is approximately 9 nm in thickness (Armant *et al.*, 1986). Yolk granules consist of substructures of small particles, some of which resemble membrane-bound vesicles (Armant *et al.*, 1986; Ii *et al.*, 1978, Yokota et al, 1993). As revealed by negative staining, these vary in diameter from about 10 to 50 nm (Ii *et al.*, 1978). These vesicular yolk subparticles are commonly found throughout development and are considered to be lipoproteins present in the yolk granules. Most of the water soluble lipoprotein components in the egg are of yolk granule origin (Ii *et al.*, 1978). These constitute about 40% of the total yolk material suggesting that yolk contains a large amount of lipid combined with lipoproteins. The sea urchin yolk lipoproteins resemble VLDL from human serum or hen yolk more than serum LDL because they contain a high triglyceride content (Ii *et al.*, 1978).

Yolk is also found in several other invertebrates (e.g. insects, mollusks and crustaceans) as well as vertebrates (e.g. fish, amphibians and avians). In echinoderms, the yolk granules are found spread evenly throughout the cytoplasm of the eggs and embryonic cells while in most of the other animals the yolk material forms a separate compartment known as the yolk sac in which the yolk granules are housed. A single layer of serosal cells lines the yolk sac, separating the yolk material from the rest of the cytoplasm.

1.2.1 Yolk granules can be separated into two fractions of differing buoyant densities

Armant *et al.* (1986) have developed a method for isolating yolk granules using sucrose density gradient centrifugation. When a crude yolk granule preparation was fractionated by sucrose density gradient centrifugation, the procedure revealed the presence of two major density classes of yolk granules. The more buoyant fraction of yolk granules was designated the "low-density yolk granule fraction" while the less buoyant class of yolk granules were designated the "high-density yolk granule fraction". The low-density yolk granule fraction was homogenous. The high-density yolk granule fraction was slightly contaminated with mitochondria. Similar results could be obtained by sucrose density gradient fractionation of the crude yolk granules isolated from various stage embryos including blastula, gastrula and pluteus. Electron microscopic analysis revealed that these two groups of yolk granules bear the same morphological structures. There is no indication of a change in the relative proportion of these two density classes of yolk granule during embryonic development. The reasons for the existence of two different density groups of yolk granules are not known.

1.2.2 Morphology of the yolk granules changes during embryogenesis

Sea urchin yolk granules can be classified into four types according to their morphology when viewed under the electron microscope: dense, intermediate, sparse and lysosomal yolk granules (Yokota *et al.*, 1993). Dense yolk granules have densely packed substructures consisting of coarse granules or micelles while the sparse yolk granules had loosely packed substructures. Yolk granules in sea urchins undergo morphological

changes during embryonic development as shown by the gradual replacement of the prevalent dense granules by the less dense structures (Yokota *et al.*, 1993). In unfertilized eggs, most of the yolk granules are dense granules whereas they are rarely observed in gastrulae. It is suggested that the yolk granules change from the dense state to the sparse state via the intermediate state, as embryonic development proceeds.

Recent data suggest that the yolk granule is a dynamic organelle even during the early developmental stages. The biochemical changes occurring in the yolk granules may be responsible for the morphological changes occurring in the yolk granules. The glycoproteins present in the yolk granules of sea urchins are initially present as a very high molecular weight precursor, which is proteolytically processed as embryonic development proceeds (Yokota and Kato, 1988; Scott and Lennarz, 1988; Scott *et al.*, 1990; Gratwohl *et al.*, 1990; Reimer and Crawford, 1995). A study performed on lipid components present in the yolk granules of sea urchin embryos suggests that the lipid composition of the yolk granule undergoes dynamic changes (unpublished observations). These compositional changes of the yolk lipids are observed starting from 10 minutes post fertilization. Also, there is evidence suggesting that the yolk granule proteins are utilized for the membrane assembly during the embryonic development (Gratwohl *et al.*, 1990). These as well as other biochemical changes occurring during the embryonic development may account for the morphological changes occurring in the yolk granules observed through the course of embryonic development. Still, the exact reason for these morphological changes remain to be elucidated.

1.2.3 Acidification of yolk granules during embryogenesis

Studies done on yolk granules of sea urchin as well as insects (e.g. the stick insect *Carausius morosus*) suggest that the yolk granules become slightly acidified during the early stages of embryonic development. The time taken for the acidification of yolk granules varies from species to species. In *Strongylocentrotus purpuratus,* the acidification is completed by 6 hours post fertilization while in sea urchin, *Lytechinus pictus* it is completed by 48 hours post fertilization (Table 1 in appendix) (Mallya *et al.*, 1992). In *Strongylocentrotus purpuratus,* the observed decrease in the pH is 0.7 units, where it drops from about pH 6.8 to pH 6.1. In *Carausius morosus* it drops down to pH 5.5 - 5.8 following fertilization (Fausto *et al.*, 2001a). The pH drop seems to be a transient event.

According to the studies done with the stick insect *Carausius morosus*, not all the yolk granules in the embryo become acidified at the same time (Fausto *et al.*, 2001). Acidified yolk granules are rather scarce and randomly distributed in vitellophages of early embryos. They tend to increase gradually in number as development proceeds to completion.

How the yolk granule of sea urchin is acidified remains unclear (Yokota *et al.*, 1993). It is possible that a Na^+/H^+ ATPase is activated, which is already present in the yolk granule membrane. Fusion of lysosomes or other endosomal vesicles with the yolk granules is another possibility (Schuel *et al.*, 1975). The presence of lysosomal-like hydrolases in the yolk granules with acidic pH optima supports the latter hypothesis. Yolk granule membrane fusion with the lysosomes to acquire the acidic environment is seen in other animals (Fausto *et al.*, 2001a). In these animals, lysosomes play a major

role in yolk utilization. In the sea urchin system, there is little evidence to support the fact that the lysosomes play a major role in yolk degradation. Therefore it was believed that the proton pump is responsible for the sea urchin yolk acidification.

Chloroquine is shown to be effective in dissipating proton gradients in a variety of biological systems by specifically interfering with the proton pump. Interestingly, the level of 160kDa protein was unchanged when chloroquine was added to *Strongylocentrotus purpuratus* embryos 6 hours after fertilization (Mallya *et al.*, 1992). According to this observation, the yolk acidification of sea urchin might be caused by Na^+/H^+ ATPase system. This result also provides strong indirect support for the idea that acidification of yolk granules is important for the proteolysis of the major yolk granule protein. A study done with *Carausius morosus* has shown that chloroquine could interfere with yolk acidification suggesting that the proton pump is the main machinery involved in the process of yolk acidification.

1.3 Yolk proteins

Yolk protein comprises about 10-15% of total egg protein (Ichio *et al.*, 1978; Kari and Rottmann, 1985; Ozaki, 1980; Harrington and Easton, 1982). The majority of the yolk proteins are glycoproteins. They range in molecular weight from about 35 kDa to over 300 kDa. The major protein component present in the sea urchin yolk granule is vitellogenin, a high molecular weight protein of 220k –270k (Malkin *et al.*, 1965). This protein represents approximately 8% of the total egg protein (Kari and Rottmann, 1985). In several sea urchin species (*Strongylocentrotus purpuratus*, *Lytechnius pictus* and *Strongylocentrotus droebachiensis*), the predominant protein ranges from about 200 kDa

to 300 kDa. (Harrington and Easton, 1980). Analysis of the protein fraction of yolk granules by SDS-PAGE has revealed that this major protein component stains positive for carbohydrates using periodic acid-Schiff (PAS) method suggesting that this is also a glycoprotein.

The glycoproteins in the yolk granule are predominantly of the polymannose, N-linked type. The predominance of polymannose-type glycoprotein in yolk granules was further demonstrated by their staining with concanavalin A-colloidal gold in sectioned embryos. Comparison of the physical and chemical properties of these glycoproteins from different sea urchin species revealed striking similarities in amino acid and monosaccharide composition (Table 2 in appendix) and PI values (Table 3 in appendix) (Scott and Lennarz, 1988). Yolk glycoproteins in echinoderms contain a considerable amount of carbohydrate. The 160-90 kDa proteins from all sea urchin species showed decrease of 30 kDa upon deglycosylation (Scott and Lennarz, 1988). The major yolk glycoprotein in all three sea urchin species studied contained approximately 10 nmol of glucosamine and 40 nmol of mannose per nanomole of protein. Fucose is either absent (*Arbacia punctulata*) or present at low levels (*Strongylocentrotus purpuratus* and *Lytechinus pictus*). The amount of glucose present is variable (Table 2 in appendix) (Scott and Lennarz, 1988). Isoelectric point determination by two dimensional gel electrophoresis reveal that the PI of all the yolk glycoproteins were in the neutral to basic range (Table 3 in appendix) (Scott and Lennarz, 1988).

1.3.1 Deposition of yolk proteins in the oocytes

In many animals, the main yolk glycoproteins have been shown to be maternally derived. The synthesis of yolk proteins in various other tissues and their accumulation in oocytes is known as vitellogenesis. Vitellogenesis has been extensively studied in vertebrate and invertebrate animals. In vertebrates it is synthesized in the liver of avians (Bergink *et al.*, 1974; Tata, 1976) and amphibians (Wahli *et al.*, 1981). In invertebrates, vitellogenin is produced in the intestine of nematodes (Kimble and Sharrock, 1983) and echinoderms (Shyu *et al.*, 1986) and in the fat body of the female insects (Wojchowski *et al.*, 1986).

In sea urchins, yolk glycoproteins are deposited both in the testes and the ovary (Ozaki *et al.*, 1980; Unuma *et al.*, 1998). It is synthesized by the intestine and secreted into the coelomic fluid of the adult sea urchin where it is the major protein of the coelomic fluid (Harrington and Easton, 1982; Cervello *et al.*, 1994). From the coelom, this protein is absorbed by accessory cells (nutritive phagocytes) of the gonad. Before gametogenesis, the protein is stored in the accessory cells, which is gradually translocated to the gametes by a process of endocytosis during their formation (Tsukahara, 1970).

Studies done with the stick insect *Carausius morosus* have shown that vitellogenin is localized in the Golgi complex and the secretory granules in the fat body tropocytes prior to its deposition in the oocytes (Fausto *et al.*, 2001b; Snigirevskaya *et al.*, 1997). The lysosomal system plays an important role in the termination of vitellogenesis in tropocytes by degrading biosynthetic organelles and secretory granules. At this point vitellogenin is found in tropocyte autophagolysosomes. The yolk protein precursors are then internalized by oocytes, which are found in the microvillus membrane, coated

vesicles, early endosomes and late endosomes from which it then goes to transitional yolk bodies.

The oocytes are endowed with the capability of sequestering vitellogenin from the maternal blood-stream through a process of receptor-mediated endocytosis (Stifani *et al.*, 1990, Schonbaum *et al.*, 1995). Following the selective uptake by the receptors, the vitellogenins are deposited in the developing oocytes. In mature yolk granules vitellogenin is found as vitellin, which is the crystallized storage form of vitellogenin.

Although the key component of the uptake of vitellogenin is the receptor and although vitellogenins from a variety of organisms have been characterized, much less is known about their receptors. In vertebrates, one of the best studied receptor is the chicken oocytes 95kDa receptor which is shown to bind two components of chicken yolk, vitellogenin and very low density lipoproteins (VLDL) (Stifani *et al.*, 1990). Recently, Bujo *et al.* (1994) have shown that the 95 kDa receptor is closely related to the mammalian VLDL receptor (VLDLR) (Schonbaum *et al.*, 1995). Molecular characterization of mosquito vitellogenin receptor revealed that it has a high homology to the *Drosophila* yolk protein receptor (Sppington *et al.*, 1996).

1.3.1.1 Proteolytic processing of vitellins during yolk deposition

Studies performed on vitellins from ovarian follicles and newly laid eggs of the insect *Carausinus morosus* suggest that the vitellins present as different variant forms and are comprised of polypeptides of various sizes (Giorgi *et al.*, 1997; Fausto *et al.*, 2001b). This is thought to be due to the post-endocytotic processing during yolk deposition. The ovarian follicles progressively more advanced in development exhibit a more complex

vitellin profile, showing a unique set of polypeptides for different stages of development (Fausto *et al.*, 2001b).

Also in *Xenopus laevis,* the vitellogenin is enzymatically split into a number of lipovitellins and phosvitin polypeptides upon entering the oocytes (Willey & Wallace, 1981; Wallace, 1985, Wallace and Selman, 1985). The proteolytic processing of the newly endocytosed vitellogenin may be a precondition for the resulting polypeptides to be transferred to the yolk granules in a crystalline form. This model can still be applied to all oviparous species including most fishes in which yolk is stored in a fluid form.

1.3.2 Processing of sea urchin yolk glycoproteins

1.3.2.1 Toposome is the precursor for the major yolk granule protein

Toposome, which is a form of vitellogenin, is the major glycoprotein present in unfertilized sea urchin eggs. It is known that this is the precursor for the major yolk granule protein, a high molecular mass protein of 160k-180k that is abundant in the eggs (Yokota *et al.*, 1993; Scott and Lennarz, 1988; Scott *et al.*, 1990; Yokota and Kato, 1987). Major yolk granule protein is also present in the coelomic fluid of both sexes of sea urchins at slightly higher molecular weight (195-200 kDa) than it is in the yolk granules of the eggs. It has been estimated to comprise greater than 50% of the total protein in the coelomic fluid (Harrington and Easton, 1982; Cervello *et al.*, 1994).

The toposome, as well as the major yolk protein, is further proteolytically processed by a protease present in the yolk granules. These are the precursors for the majority of the glycoproteins present in the sea urchin yolk granules.

1.3.2.2 Proteolytic processing of major yolk glycoproteins

As mentioned earlier, the toposome is proteolytically cleaved giving rise to the major yolk granule protein, which is the major glycoprotein present in the sea urchin yolk granules of eggs and embryos in early stages. There are also less abundant proteins derived from the toposome, which are known as minor yolk granule proteins. As embryonic and larval development proceeds, these major and minor yolk granule proteins are processed by limited proteolysis, giving rise to smaller molecular weight cleavage products (Kari and Rotman, 1985; Armant *et al.*, 1986; Scott and Lennarz, 1989; Lee *et al.*, 1989). This results in changing the protein profile of the yolk granule as embryonic development proceeds (Scott *et al.*, 1990; Yokota *et al.*, 1993). The changes in the protein profile can be revealed by reducing SDS-PAGE analysis of the yolk proteins from different developmental stages. The protein profile is stage-specific. Electrophoretic analysis under non-reducing conditions has shown high molecular weight bands derived from toposome and the major yolk granule protein throughout embryonic development. Despite the proteolytic cleavage, the total mass of the toposome and the major yolk granule protein remain constant throughout embryonic development thereby maintaining its overall physico-chemical properties. Apparently, the polypeptide products remain covalently bound to each other by intermolecular disulfide bonds even after proteolytic cleavage (Armant *et al.*, 1986; Scott and Lennarz, 1988).

Studies done with different species of sea urchins revealed that the protein profile of the major yolk granule protein of each embryonic stage is species-specific. In *Strongylocentrotus purpuratus*, the major vitellogenin, the toposome is about 243 kDa while the major yolk granule protein, which is derived from this toposome, is about 160

kDa (Scott *et al.*, 1990; Scott and Lennarz, 1988). The major yolk granule protein gives rise to relatively smaller molecular weight species of 115 kDa, 108 kDa, 90 kDa, 83 kDa and 68 kDa as development proceeds. In *Hemicentrotus pulcherrimus*, the major yolk granule protein is a 178kDa species, which is processed to give rise to 114 kDa, 94 kDa, 72 kDa and 61 kDa polypeptides (Yokota and Kato, 1987; Yokota *et al.*, 1993). *Anthocidaris crassispina* bears a major yolk granule protein of 180 kda, which is processed to give rise to 112 kDa, 92 kDa, 70 kDa and 56 kDa polypeptides (Yokota and Kato, 1987). When embryonic development proceeds, higher molecular mass species derived from the major yolk granule protein gradually disappear while the lower molecular mass species appear.

Studies done with insect models have also shown that the yolk polypeptides undergo limited proteolysis to yield a number of cleavage products of lower molecular mass (Fausto *et al.*, 2001b; Cecchettini *et al.*, 2001). The yolk proteins are not exhaustively degraded within the yolk sack, but the yolk sac gradually decreases in size as a result of translocation of yolk polypeptides to the perivitelline fluid. Immunolocalization studies have shown that the yolk polypeptides are initially associated with the vitellophages as well as the yolk granules contained in the vitellophages. By subsequent stages of development, the yolk granules appear to be present in the serosal cells lining the yolk sac and just underneath the plasma membrane of these cells suggesting that the yolk polypeptides are shuttled by serosal cells transcytotically from the yolk sac to the perivitelline fluid. These polypeptides are also associated with the vesicles budding from the Golgi apparatus suggesting an involvement of the trans-Golgi

network in the translocation of yolk cleavage products. The yolk cleavage products possibly undergo post-translational modification(s) in the Golgi apparatus.

1.3.2.3 The enzyme responsible for yolk glycoprotein processing is present as an inactive pro-protease

The mild acidification of the yolk granules occurring after fertilization has been shown to be the major factor influencing the activation of a dormant protease, which is involved in yolk glycoprotein processing (Medina *et al.*, 1988; Mallya *et al.*, 1992; Fausto *et al.*, 2001a). The time taken for the acidification process and the activation of this dormant protease differ from species to species within the class *Echinoidea.* For example, in *Strongylocentrotus purpuratus* the yolk processing starts by 6 hours post-fertilization (HPF) while in *Lytechinus pictus* it starts by 48 hours post-fertilization (Table 1in appendix) (Mallya *et al.*, 1992). In *Strongylocentrotus purpuratus* the proteolysis is essentially complete by 18 hours.

Studies done on insects (Fausto *et al.*, 2001a) as well as echinoids (Mallya *et al.*, 1992) have shown that the enzyme responsible for proteolytic processing of vitellins and the major yolk granule protein is a cathepsin-B-like protease. Studies performed using the stick insect *Carausius morosus* have shown that the protease is initially present as a pro-protease (Fausto *et al.*, 2001a). In this study the pro-protease was identified by immunoblotting in yolk extracts of progressively more advanced embryos. A specific monoclonal antibody raised against this pro-protease was used to demonstrate that it was gradually processed to yield a lower molecular weight polypeptide as development proceeds to completion. This latter polypeptide was identified as a protease using

electrophoresis in polyacrylamide gels containing yolk extracts. Pro-protease labeling is gradually lost as the yolk granules are progressively more acidified as embryonic development proceeds. The yolk granules probed simultaneously for acidification and latent pro-protease yielded labeling patterns partially superimposed. These observations suggest that the enzyme responsible for the processing of yolk proteins is a protease, which is proteolytically processed in an acidic environment.

In the sea urchin, the yolk protease was studied *in vitro* to identify the effect of pH (Mallya *et al.*, 1992). The pH activity profile revealed that the enzyme is inactive at pH 6.8 and that maximal activity was observed at pH 6.1, which is the pH of the yolk granules at blastula stage. At 4.2, the pH at which most lysosomal enzymes are optimally active, this protease shows only 30% of maximal activity. Thus the drop of pH by 0.7U observed *in vivo* would be expected to afford optimum conditions for yolk protease activity (Table 1 in appendix). This study confirmed that the acidification of the yolk granules is shown to be the controlling step in the activation of this protease.

As revealed by the studies performed using the stick insect *Carausius morosus*, the pro-protease was shown to be a maternally derived gene product since there was no evidence to show that it was synthesized in the egg or the embryos (Fausto *et al.*, 2001a). The molecular weight of the precursor was determined to be 57 kDa, which finally gives rise to the active protease of 40 kDa (Fausto *et al.*, 2001a). When a monoclonal antibody was specifically raised against the 57 kDa protein and tested by immunoblotting on different developmental stages, the 57 kDa protein was shown to exhibit a precursor product relationship with the 40 kDa protease. Appearance of this latter polypeptide could also be mimicked *in vitro* by partially digesting 57 kDa polypeptide with papain.

This indicated that pro-protease conversion may be triggered by an enzyme member of the papain superfamily. When newly laid eggs of the stick insect were incubated *in vitro* at 37°C for timed intervals ranging from 0 to 72 hours and the resulting extracts resolved by reducing SDS-PAGE, the results showed that vitellin polypeptides processed under these conditions reproduce the electrophoretic patterns yielded by yolk sacs during development (Fausto *et al.*, 2001a). This suggests that the newly laid eggs are enzymatically autonomous, requiring no external supply to sustain vitellin proteolysis. Proteolysis seen in the embryos was clearly associated with the appearance of the 40 kDa polypeptide, which is the active form of the protease.

The enzyme activity of the yolk protease was tested in the presence of several inhibitors to identify which class of protease it is belonged to (Table 4 in appendix) (Mallya *et al.*, 1992). Inhibition by Z-Phe-Ala-CH_2F confirmed that it is a cathepsin-B like protease. Leupeptin, which is a competitive inhibitor of thiol proteases, completely inhibited the yolk protease. It seems likely that the enzyme responsible for yolk glycoprotein proteolysis cleaves at peptide linkages involving the carboxyl groups of lysine or arginine or both. The inhibition of the protease activity by serine protease inhibitors might be due to the inhibition of the enzymes responsible for the activation of the dormant pro-protease.

1.3.3 Posttranslational modification of yolk proteins

There is evidence showing that the yolk proteins are posttranslationally modified. Studies performed with insects suggest that the vesicles in the serosal cells carrying vitellogenin cleavage products are first targeted to the Golgi apparatus and only

subsequently delivered to the apical cell pole for exocytotic release (Fausto *et al.*, 2001b; Snigirevskaya *et al.*, 1997). The actual involvement of trans Golgi network in the vesicular translocation of vitellin cleavage products through the serosal cells suggest that they may have been posttranslationally modified either by proteolytic processing (Friederich *et al*, 1988; Steiner, 1998; Molloy *et al.*, 1999) or by glycosylation (Dunphy *et al.*, 1985; Varki, 1998). There is also evidence to show that the yolk polypeptides are covalently modified by the addition of sulfate or phosphate groups (Dhadialla and Raikhel, 1990; Niimi *et al.*, 1994).

Interestingly, the vitellin cleavage products acquire a neutral isoelectric point upon transfer to the perivitelline fluid (Cecchettini *et al.*, 2001), whereas the vitelline polypeptides are highly electronegative as long as they are confined within the yolk sac (Giorgi *et al.*, 1999). These observations suggest that the vitellins are being posttranslationally modified in the Golgi apparatus leading to changes in their chemical properties.

1.4 Functions of the yolk granule

1.4.1 Yolk provides nutrition for the embryonic development of many animals

The yolk is believed to be a nutrient store of lipid and protein materials. The classical view of the yolk material is that it provides nutrition for the embryonic and larval development. Generally, lipid components of the yolk granules are known to be the major energy source while proteins, mainly the vitellogenin serve as an amino acid supply (Reimer and Crawford, 1995). This is true for most living systems, which harbor yolk granules or yolk sacs. For example, in insects, the yolk provides storage of raw

material to sustain embryonic development (Mc-Gregor and Loughton 1977; Yamashita and Indrasith, 1988). In amphibians and avians the yolk has been shown to provide a reservoir of raw materials used during embryogenesis (Willey and Wallace, 1981; Wallace, 1985).

Because the general characteristics of yolk granules of sea urchin embryos are similar to those of the other species (Williams, 1967), a similar function has been assumed for the sea urchin yolk granules. Armant *et al.* (1986) suggested that in sea urchins, the yolk glycoproteins could be utilized during larval development if food was scarce. Functions for sea urchin yolk granules as a site of catabolism are suggested by the presence of several acid hydrolases, including acid phosphatase (Schuel *et al.*, 1975). It is not clear whether these are originally of yolk granule or lysosomal origin. Such catabolic enzymes could be used to generate amino acids, fatty acids or carbohydrates from the stored macromolecular forms. However, there is little evidence supporting this view in the sea urchin system given that the composition of the yolk granule remains unchanged throughout development (Armant *et al.*, 1986; Scott *et al.*, 1990). Analysis of the low density yolk granules isolated throughout embryonic development revealed little or no change in the mass of a variety of constituents including protein, lipid (phospholipid and triglyceride), carbohydrate (hexose and sialic acid) and nucleic acid (RNA) until the pluteus stage (Table. 1 in appendix) (Armant *et al.*, 1986). Studies have shown that the rate of the yolk utilization is not related to the nutritional status of the embryo and early larvae, suggesting that it is not a ready-to-use food source in an event of starvation (Armant *et al.*, 1986; Kari and Rotman, 1985). In *Strongylocentrotus purpuratus*, the yolk granules and associated glycoproteins usually disappear on the 7th

day of the feeding larval stage. This process does not speed up in starvation, suggesting that providing nutrition to the growing embryo and larvae is not the main role of yolk (Armant *et al.*, 1986, Scott *et al.*, 1990).

Large quantities of major yolk granule protein are produced in both male and female sea urchins, whereas vitellogenins are specific to females in other animal classes such as nematodes, insects and vertebrates, negating the possibility that major yolk granule protein serves as a vitellogenic store of amino acids (Shyu *et al.*, 1986). These findings support the view that the yolk granules are not just benign storage organelles that feed the embryonic and larval stages of sea urchin development. At least in the echinoderm system, yolk proteins might be involved in biochemical processes other than those providing nutrition; however the functions of the yolk granule proteins in the sea urchin system are still not well identified. Different studies on sea urchin yolk granules suggest different functions for it, complicating its picture.

1.4.2 The sea urchin yolk granule is not a site of synthesis

The vesicular structure of the yolk granules suggested the possibility that these organelles function in either lipogenesis or membrane biogenesis. No enzyme activities required for lipid synthesis, such as fatty acyl coenzyme A lyase, glycerol phophate acyl transferase, choline phosphotransferase or ethanolamine phosphotransferase were detected, however (Armant *et al.*, 1986). Therefore, it is unlikely that the yolk granule is a site of lipid synthesis.

To determine any *de novo* synthesis of the yolk proteins, the sea urchin embryos were labeled with (^{35}S)-methionine and the yolk granules were isolated and analyzed by

SDS-PAGE followed by autoradiography (Armant *et al.*, 1986). Major yolk associated glycoproteins were not labeled indicating that they were not synthesized *de novo* during embryogenesis. Surprisingly, the sea urchin yolk granules harbor a considerable amount of RNA, which accounts for approximately 12% of its weight (Armant *et al.*, 1986). Studies identified unique classes of 9S and 12S RNA in yolk granules of *Strongylocentrotus purpuratus*. Another important study was performed with RNA present in the yolk granules to investigate whether the yolk granule RNAs are translationally active. No translatable RNA was identified, since no translation products were detected in an *in vitro* translation system (Armant *et al.*, 1986). Overall, this evidence strongly suggests that the yolk granule is not a site of anabolism of proteins or lipids.

1.4.3 Major yolk granule protein is a nutrient store for sea urchin spermatogenesis

During sea urchin gametogenesis (spermatogenesis in males and oogenesis in females), the vitellogenin is deposited in both types of gametes (Shyu *et al.*, 1986). In females, the major yolk granule protein is retained during the vitellogenic phase of oogenesis from nutritive phagocytes to the growing oocytes where it is packaged into yolk granules and stored in mature eggs (Ozaki *et al.*, 1986; Harrington and Ozaki, 1986; Unuma *et al.*, 1998).

It has been suggested that, in males, the major yolk granule protein serves as a nutrient store for spermatogenesis (Unuma *et al.*, 1998). As immunohistochemical studies reveal, the major yolk granule protein depletes from the degenerating nutritive phagocytes in the follicular lumen during spermatogenesis. The protein is not detected in

the stored spermatozoa. The disappearance of the vitellogenins during spermatogenesis suggests that it provides nutrients for gametogenesis, at least for spermatogenesis. Although vitellogenin is deposited in both male and female gametes, since all the vitellins disappear by the stage of sperm maturation, almost all the vitellin in the embryos is maternally derived.

1.4.4 Yolk granule proteins are utilized for the assembly of new membranes

The important change resulting from cell division during development from egg to blastula is the formation of new membranes. The original membrane-bound toposomes are not diluted out during cell division, but rather replenished from an internal reservoir (Gratwohl *et al.,* 1990). The origin of many proteins present in sea urchin embryonic cells is shown to be the yolk granule (Gratwohl *et al.,* 1990; McClay and Fink, 1982; Wessel *et al.*, 2000; Alliegro and McClay, 1988; Fuhrman *et al.*, 1992; Brennan and Robinson, 1994).

The toposome is shown to have two different types of storage vesicles, the cortical granules and the yolk granules. In the yolk granules, these are present in the membrane as well as stored free in the interior compartment (Gratwohl *et al.,* 1990). Toposome, which is present in the cortical granules, is concised to the electron dense lammellar compartment (Gratwohl *et al.,* 1990). It is suggested that the toposomes in the two compartments are destined for different structures and functions (Gratwohl *et al.,* 1990). As visualized by immunogold labeling, fertilization results in a double layer of labeling. The glycoprotein in the lammellar compartment of the cortical granule is exocytosed at fertilization during the cortical reaction and found to become a part of the

double layer enveloping the embryo on the outside of the hyaline layer. These toposomes in the cortical granules are incorporated in to the hyaline layer as unmodified 160 kDa polypeptides. On the other hand, as explained earlier, the toposomes present in the yolk granules are proteolytically modified. The toposomes, which are processed in this way, are secreted in this form to all the external surfaces of newly formed cells. An immunogold labeling experiment, which was performed to trace these modified toposomes in the sea urchin embryo, demonstrated that it is transported and deposited in all cell surfaces, apical, lateral and basal (Noll *et al.*, 1985). These observations suggest that the yolk granules act as the reservoir of toposomes present in the newly formed membranes. About 25% of the toposomes from hatched blastula embryos were associated with purified membranes and 75% were associated with the cytoplasm. Both these fractions exhibited a similar degree of processing when analyzed by reducing SDS-PAGE (Matranga *et al.*, 1986). The clusters of labeling identified in the cytoplasm not associated with the membranes or the yolk granules might be such toposomes in transit. These were often located over what appeared to be remnants of disintegrating granules, particularly near membranes or Golgi stacks near the apical surface. It was suggested that some of these structures might represent vesicles in the process of fusion with membranes. The labeling seen near the Golgi stacks support the hypothesis that the toposome and/or major yolk granule protein are being post-translationally modified in the Golgi network.

1.4.5 Role of the toposome as morphogenic cell adhesion particles

Cell-cell adhesion is a highly specific interaction, which regulates differentiation, morphogenesis and embryonic development. Cervello and Matranga (1988) describe a function for the sea urchin, *Paracentrotus lividus,* toposome in mediating cell adhesion in the embryonic system.

Dissociated cells from the sea urchin embryo have a remarkable ability to re-associate into a developing embryo (Herbst, 1900; Giudice, 1962). The biological significance of the 200 kDa and 180 kDa polypeptides isolated from the coelomic fluid of the blastula stage embryo were tested using a morphogenetic cell aggregation assay on dissociated blastula stage cells (Cervello and Matranga, 1988). The number and size of the aggregates determined the significance of the aggregation activity. Toposome precursors proved to be active in promoting cell adhesion even in its unprocessed form (Cervello and Matranga, 1988). This occurred even when cells were rendered aggregation-incompetent by non-cytolytic n-butanol extraction. These observations suggest that precursors of the toposomes contain all the epitopes of the contact site involved in cell aggregation. The antibodies raised against the toposome obtained from mesenchyme blastula embryos inhibited the aggregation activity, further supporting the finding that toposome or toposome-derived peptides are involved in aggregation.

Since the precursors retain cell-adhesion promoting activity, processing may serve to generate positional diversities among the cells during embryogenesis. In immunohistochemical studies the toposome-specific monoclonal antibodies stained cell surface structures in a pattern consistent with a code specifying the position of a cell in the embryo (Matranga and Cervello, 1984; Noll *et al.*, 1985; Matranga *et al.*, 1987). It is

suggested that the extracellular toposomes rivet the apical lamina to the surface and underlying cytoskeleton of the microvilli while the modified toposomes from the yolk granules are responsible for position-specific cellular adhesion as they are released to the newly formed cells. The vitellogenins and the major yolk granule protein, which they found to be 200 kDa and 180 kDa polypeptides respectively in sea urchin *Paracentrotus lividus,* were shown to be a storage form of cell adhesion molecules. It was concluded that the toposome and the major yolk granule protein are probably involved in the important events related to positioning of the embryo.

1.4.6 Storage and transportation function

There is a possibility that the major yolk granule protein serves as a carrier of substances present in the cells. Yolk proteins are generally known to be important in regulating embryogenesis by sequestering diverse molecules for subsequent timed release. While the vitellogenins can be covalently modified with carbohydrates, phosphates and sulfates, they can also non-covalently bind lipids, hormones, vitamins and minerals (Lagueux *et al.*, 1981; Kunkel and Nordin, 1985; Byrne *et al.*, 1989; Dhadialla and Raikhel, 1990; Niimi *et al.*, 1994). Yolk proteins of *Drosophila melanogaster* have been known to bind steroids and it has been proposed that breakdown of the major yolk granule protein leads to timed release of these hormones (Bownes *et al.*, 1988). The major yolk granule protein of *Drosophila malanogaster* is found to be distinct from vitellogenins.

1.4.6.1 Storage and transportation of metal ions

Higher concentrations of free iron and calcium in the living systems can cause harmful effects; therefore, these should be finely regulated to maintain the ideal free levels for the proper function of the organisms. The major yolk granule protein has been shown to act as a metal ion chelator, which also maintains a reservoir of these metal ions. The metal ions could be slowly released according to the cells needs, reducing the risk of having high concentrations of metal ions in the living cells.

1.4.6.1.1 Storage and transportation of iron

In vertebrates, transferrins are glycoproteins involved in reversible binding and transportation of iron. Vertebrate transferrins are monomeric glycoproteins (~80 kDa) that consist of two domains with similar amino acid sequence, each with a single iron binding site (Baker and Lindley, 1992). Crystallographic studies have shown that the iron binding sites are stabilized by many intrachain disulfides that coordinate iron binding (Baker and Lindley, 1992).

As mentioned previously, the major yolk granule protein has historically been classified as a vitellogenin based on its abundance in the yolk granules. Although the physiological function of the major yolk protein in the sea urchin has been analyzed for over two decades, this classification as a vitellogenin has yielded conflicting results. The fact that major yolk granule protein is found in both sexes of sea urchins suggests that it should play a physiological role in embryogenesis, as well as in gametogenesis.

Brooks and Wessel (2002) recently presented the primary structure of the major yolk granule protein as predicted from cDNAs of sea urchin species and demonstrated

that instead of resembling vitellogenin, the major yolk granule protein contained transferring-like iron binding domains. It is suggested that major yolk granule protein transports iron to the ovary and testes to meet the proliferative demands of gametogenesis and embryogenesis. When the full-length deduced amino acid sequence of *Strongylocentrotus purpuratus* and the partial amino acid sequence of *Lytechinus variegates* were aligned to one another, 57.6% identity was shown in a 742 amino acid overlap. When these sequences were compared with the other proteins in the NCBI database, surprisingly no similarity to reported vitellogenins was found. It was noted, however, that the major yolk granule protein contained two transferrin-like motifs. Both species of sea urchin had a sequence identity between 24-28% to both vertebrate and invertebrate transferrins (sequence identity between vertebrate and insect transferrin is only 25-30%). It was therefore proposed that the major yolk granule protein is a member of the transferrin superfamily. The consensus sequence includes serum transferrin, melanotransferrin, ovotransferrin (from egg white) and lactotransferrin (from milk, white blood cells and other secretary fluids) from vertebrates and four transferrin domains from insects.

The major yolk granule protein was able to bind iron as determined by iron overlay assay followed by phosphoimaging (Brooks and Wessel, 2002). Coelomic fluid showed distinct iron binding with bands that increased in intensity in accordance with an increase in protein concentration. Coelomic fluid of both sexes of sea urchin contained proteins capable of binding iron. Immunoprecipitation studies revealed that the major yolk granule protein in the coelomic fluid could bind iron. It was suggested that its function of major yolk granule protein is to transport iron ions in coelomic fluid.

It is worthwhile to recall that the major yolk granule protein occurs as a single species of high molecular weight in coelomic fluid that is processed into several distinct smaller fragments when embryonic development proceeds, and this processing is shown to be concomitant with the acidification of yolk granules that occurs during embryogenesis. Since many transferrins release iron at low pH, the major yolk granule protein processing maybe linked to iron release from this protein.

These results partially help to resolve the mysteries of major yolk granule protein utilization in embryonic development and its occurrence in the male gonad. The packaging of major yolk granule protein into yolk granules may serve as a mechanism of iron delivery during gametogenesis and embryogenesis. Further, they suggest that major yolk granule protein packaging in coelomyocytes could have a dual function of providing bacteriostatic function in coelomic fluid as well as delivery of major yolk granule protein-bound iron to the gonads. The authors of this paper argue that the assignment of the major yolk granule protein as a vitellogenin was previously based on its abundancy in yolk granules, coelomic fluid and intestines but not on any protein sequence data.

1.4.6.1.2 Storage and transportation of calcium

Calcium ions are common messengers in intracellular signaling and transportation events and play a significant role in regulation of meiosis in mammalian oocytes. A study performed with pig oocytes (Petr *et al.*, 2001) revealed that the yolk granule may function as a site of calcium storage. It was suggested that these deposits probably serve as a source of calcium for calcium-dependent events.

The ability of yolk proteins to bind calcium is supported by Cervello and Matranga (1988), who showed that the cell aggregation activity caused by the toposome is calcium-dependent. This study provides the first evidence that sea urchin vitellogenin binds calcium. Therefore the calcium deposits observed by Petr *et al.* (2001) might be due to calcium binding by the vitellogenins and/or its derivatives.

1.4.7 Function of yolk granules in the protein export pathway

Several studies have shown that the yolk granules act as a storage compartment for many proteins destined for export. HLC-32, a major protein component of the hyaline layer and the basal lamina extracellular matrices of the sea urchin embryo, is present in the yolk granules of unfertilized eggs (Mayne and Robinson, 1998). Following fertilization, there is a coincidental loss of HLC-32 from the yolk granules and its appearance in the extracellular matrices. Studies done with *Xenopus leavis* suggest that a lectin is localized to the yolk granules and some other vesicles in the egg, prior to its appearance on the cell surface (Outenreath *et al.*, 1988). Echinonectin, a hyaline layer protein, has been localized to membrane-bound vesicles in the unfertilized egg (Fuhrman *et al.*, 1992). It is believed that these proteins are exported from the yolk granules by a transport pathway involving yolk granules and/or associated vesicles. The sea urchin yolk granules also house RNA, which has been shown to be translationally inert (Armant *et al.*, 1986). Additionally, there is evidence for the localization of RNAse to yolk granules in bullfrog oocytes, suggesting that these organelles act as a cellular compartment for some cytoplasmic enzymes (Liao and Wang, 1994; Wang *et al.*, 1995). Therefore, some structural protein components as well as functional protein components

destined for export have been shown to be associated with the yolk granules. Collectively, these findings suggest that the yolk granules, and perhaps additional membrane-bound vesicles, are involved in an export pathway to the embryonic cell surface. In this context, Cruetz *et al.* (1996) has demonstrated the association of a nematode annexin with yolk granules in *Caenorhabditis elegans* oocytes. A study done with *Xenopus laevis* identified the association of synexin (annexin VII) with the yolk granules of the oocytes (Srivastava *et al.*, 1996). Synexin is a calcium-dependent phospholipid-binding and membrane-fusion protein in the annexin gene family. It is capable of forming calcium channels and plays a role in exocytotic secretion.

In the sea urchin embryo, immunogold labeling studies have shown that toposome is transported to the outer membrane of the embryonic cells (Cervello and Matranga, 1988). This observation further supports the view that the yolk proteins are involved in an export pathway. Further work will be required to determine if the yolk granules and associated proteins play a crucial role in transportation events.

1.5 Research focus

Although the yolk proteins have been investigated for decades, the precise function of the yolk granules in the sea urchin embryo is not very clear (Kane, 1965; Infante and Nemer, 1968; Kondo, 1972; Kondo and Koshihara, 1972; Ii *et al.*, 1978; Harrington and Easton, 1982; Kari and Rotman, 1985; Shyu *et al.*, 1986; Armant *et al.*, 1986; Gratwohl *et al.*, 1990). Being abundant in eggs and embryos, yolk protein should be involved in important cellular events occurring during fertilization and embryonic development. As many studies reveal, the yolk granule houses several proteins destined

for export. On the other hand, the vitellins have been shown to be an important component of yolk granule membranes as well as newly formed membranal structures including plasma membranes of embryonic cells. Based on these data, we hypothesize that the yolk proteins, mainly the toposome and the major yolk granule protein, are involved in transportation and membrane fusion events.

In mammalian systems, proteins involved in transportation and membrane fusion events are calcium-dependent, phospholipid-binding proteins (e.g. annexin VII). In this study, we investigated the capacity of yolk granule proteins to engage in membrane binding and aggregation reactions. A radiolabeled, calcium binding assay was used to investigate the calcium-binding capacity of yolk proteins. A multilamellar liposome-binding assay was employed to investigate the calcium-dependent phospholipid-binding activity of the proteins, while a unilamellar liposome-aggregation assay was used to investigate the vesicular-aggregation activity of these proteins. Western blots and peptide maps were created to identify precursor product relationship between the yolk proteins.

Chapter 2. Materials and Methods

2.1 Materials

All reagents were of the highest grade available. Acetone, chloroform, methanol, glacial acetic acid, potassium chloride (KCl), sodium chloride (NaCl), magnesium chloride ($MgCl_2$), sodium bicarbonate ($NaHCO_3$), imidazole, urea, NP-40, sodium iodide (NaI) and ethylenediaminetetraacetic acid (EDTA) were purchased from Fisher Scientific (Fair Lawn, New Jersey, USA) and calcium chloride ($CaCl_2$), sodium azide (NaN_3), cobalt chloride ($CoCl_2.6H_2O$), tris (hydroxymethyl) aminomethane (Tris), glycerol, glycine, dithiothreitol (DTT), ethylene glycol- bis (β-aminoethyl ether) N, N, N',N'-tetraacetic acid (EGTA), bromophenol blue (BPB), tween-20, bovine serum albumin (BSA), brain lipid extract, phosphatidyl inositol (PI), phosphatidyl serine (PS), phosphatidyl ethanolamine (PE) and phosphatidyl choline (PC) were obtained from Sigma-Aldrich Canada (Oakville, ON, Canada). Sucrose, acrylamide, bisacrylamide, sodium dodecyl sulfate (SDS), Coomassie brilliant blue (CBB) and anti-mouse IgG were purchased from Bio-Rad (Hercules, Canada), Q- Sepharose Fast Flow, Chelating Sepharose Fast Flow, silver staining kit and $^{45}CaCl_2$ were purchased from Amersham Biosciences (Uppsala, Sweden), 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitro blue tetrazolium chloride (NBT) were obtained from Boehringer Mannheim corp. (Indianapolis, IN, USA) and benzamidine was purchased from Eastman Kodak Company (Rochester, NY, USA).

Strongylocentrotus purpuratus was purchased from Seacology, Vancouver, British Columbia. Anti-toposome antibody was a kind gift from Dr. V. Matranga, 'Instituto di Biologia dello Sviluppo', Palermo, Italy. Troponin-C was a gift from Dr. D. Heeley, Department of Biochemistry, Memorial University of Newfoundland.

2.2 Methods

2.2.1 Growth of embryos

Gametes were obtained from *Strongylocentrotus purpuratus* by intracoelomic injection of 0.5 M KCl. Eggs were washed three times in ice-cold Millipore-filtered sea water (MFSW; 0.45 μm filter size) and fertilized with a 100-fold numerical excess of sperm. Embryos were cultured with constant aeration at 12°C in cylindrical chambers containing paddles rotating at 40 rpm. Samples were harvested at different times after fertilization. Eggs and embryos were stored frozen at –70°C.

2.2.2 Preparation of yolk granules by differential centrifugation

Yolk granules were isolated from unfertilized eggs as described by Yokota and Kato (1998). Eggs were washed in ice-cold Millipore filtered sea water (MFSW), followed by calcium-, magnesium-free sea water (CMFSW). Yolk granules were prepared both in the presence and absence of EDTA. The eggs were resuspended in a solution of either 0.5 M KCl (pH 7.0) or 0.5 M KCl containing 1 mM EDTA (pH 7.0) and homogenized in a hand-held Dounce homogenizer at 0°C. The homogenate was centrifuged at 400xg for 4 min at 4°C and the pellet was discarded. The supernatant was centrifuged at 2,400xg for 10 min at 4°C. The pellet was resuspended in the same starting solution and centrifuged at 400xg for 4 min at 4°C. The supernatant was again centrifuged at 2,400xg for 10 min at 4°C. The final pellet contained yolk granules and was used for the preparation of yolk granule protein extracts and yolk granule membranes and for the yolk granule aggregation assays.

2.2.3 Preparation of protein

2.2.3.1 Preparation of yolk granule protein extracts in the presence and absence of EGTA

Yolk granules were suspended in 0.5 M KCl containing 1mM $CaCl_2$ for 30 minutes at 0°C and centrifuged at 2,400xg for 10 min at 4°C. The resulting supernatant was centrifuged at 50,000xg for 1 hr at 4°C and the supernatant was termed the "yolk granule calcium extract". Yolk granules, pelleted at 2,400xg were extracted with 0.5 M KCl containing 5 mM EGTA and centrifuged at 2,400xg for 10 min at 4°C. The resulting supernatant was centrifuged at 50,000xg for 1 hr at 4 °C and the supernatant was termed the "yolk granule EGTA extract".

2.2.3.2 Preparation of yolk granule membrane proteins

2.2.3.2.1 Sucrose density gradient ultracentrifugation

Sucrose density gradient ultracentrifugation was performed to isolate the yolk granule membranes using the method of Vater and Jackson (1989). Hypotonic lysis of the yolk granules was performed by suspending the granules in 20 mM Tris, pH 8.0 at 0 °C for 45 min. The yolk granules, which were not lysed, were pelleted out by spinning at 2,400xg for 10 min and discarded. The supernatant, containing the contents of lysed granules (4.1 mL), was made 50% (w/v) sucrose using a 78% (w/v) sucrose stock prepared in 90 mM KCl, 10 mM benzamidine, 0.2% NaN_3, 50 mM Tris-HCl (pH 8.0). This was layered under a discontinuous gradient of 40% (w/v) (9.5 mL), 25% (w/v) (5.5 mL) and 0% (0.8 mL) sucrose in the same buffer. The gradient was centrifuged at 87,000xg for 16 hrs at 4 °C. Fractions (0.5 mL) were collected starting from the bottom

of the tube. The 25/40% and 0/25% interfaces contain the yolk granule membranes. The fractions at these interfaces were pooled separately and diluted with 10 volumes of 10 mM Tris-HCl, containing 0.05% NaN_3 (pH 7.0) and centrifuged at 17,000xg for 1 hr at 4 °C to pellet out the yolk granule membranes. The pellets were then resuspended in a small volume of 10mM Tris-HCl, containing 0.05% NaN_3 (pH 7.0).

2.2.3.2.2 Extraction of yolk granule membrane proteins

a. Extraction with EDTA

The membrane fraction was made 10 mM EDTA, incubated at room temperature for 30 min and centrifuged for 30 min at 30 psi in an air driven, bench top ultracentrifuge (Beckman airfuge). The supernatant was termed the "yolk granule membrane EDTA extract".

b. Extraction with salt

Salt extraction was used to release proteins externally bound to the membranes. The membrane suspension was made 0.5 M in NaCl, incubated at room temperature for 30 min and centrifuged for 30 min at 30 psi in the airfuge. The supernatant was termed the "yolk granule membrane salt extract".

2.2.3.3 Extraction of surface proteins from eggs and embryonic cells

Dissociation of *Strongylocentrotus purpuratus* embryos into single cells was achieved by the method of Matranga *et al.* (1986). Embryos (1 mL) prepared in MFSW were pelleted and resuspended in dissociation medium of CMFSW containing 5 mM EDTA (3 mL) at 0°C. The embryos were dissociated by gently pipetting in and out. The

intact embryonic cells were isolated by differential centrifugation as follows: The intact embryos were pelleted by centrifuging at 150 xg for 10 min at 4°C. The pellet was discarded. The supernatant was centrifuged at 800xg for 10 min at 4°C to pellet the embryonic cells. The embryonic cells were washed 3 times with CMFSW at 0°C by resuspending and centrifuging at 800xg. The embryonic cells were then extracted with CMFSW containing 10 mM EDTA. The EDTA extract was centrifuged at 50,000xg for 1 hr at 4 °C and the supernatant was named the embryonic cell EDTA extract. The eggs prepared in MFSW were washed 3 times with CMFSW by resuspending and centrifuging at 150 xg at 4°C. The eggs were then extracted with CMFSW containing 10 mM EDTA. The EDTA extract was centrifuged at 50,000xg for 1 hr at 4 °C and the supernatant was termed the "egg EDTA extract".

2.2.4 Separation of proteins

2.2.4.1 Anion exchange chromatography

Q-Sepharose Fast Flow resin (Fast-Q) was equilibrated with 10 column volumes of 10 mM Tris (pH 8.0). Protein samples, which were dialyzed against equilibration buffer, were loaded onto the column. Unbound proteins were washed off the column with 5 column volumes of equilibration buffer and bound proteins were eluted using a step gradient of NaCl, ranging from 0.1 M to 1 M (step size- 0.1 M) in the equilibration buffer.

2.2.4.2. Immobilized metal ion affinity chromatography (IMAC)

Chelating Sepharose Fast Flow resin was washed with 5 column volumes of distilled water, added 5 column volumes of 0.2 M $CoCl_2$ and again washed with 5 column volumes of distilled water and equilibrated with 10 column volumes of 50 mM Imidazole containing 6 M urea (pH 7.0). The sample was extensively dialyzed against the equilibration buffer and loaded onto the column. Unbound proteins were washed off the column with 5 column volumes of equilibration buffer and the bound proteins were eluted with 1 M NaCl followed by a step gradient of guanidine thiocyanate ranging from 1 M to 5 M (step size- 1 M) in the equilibration buffer.

2.2.5 Determination of protein concentration

Protein samples were precipitated with an equal volume of 20% (w/v) trichloro acetic acid (TCA; AnalaR) on ice for 20 min, centrifuged in an eppendorf centrifuge at 16,000xg for 10 min and the supernatants were discarded. The protein concentrations were determined by the method of Lowry *et al.* (1951) using BSA as a standard. Optical density (OD) was read at 750 nm in a spectrophotometer (Spectronic 601, Milton Roy)

2.2.6 Identification of proteins

2.2.6.1 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Electrophoresis was carried out using a mini-gel electrophoretic apparatus (ThermoEC). SDS-PAGE was performed according to the method of Laemmli (1970), in 1.5 mm thick 10% (w/v) polyacrylamide [30% (w/v) acrylamide, 0.8% (w/v) bisacrylamide] slab gels. Protein samples were precipitated with an equal volume of 20%

(w/v) TCA on ice for 20 min, centrifuged in an Eppendorf centrifuge at 16,000xg for 10 min and the supernatants were discarded. Pellets were resuspended in 30 µl of Laemmli solubilizing solution [0.1 M DTT, 0.2 M Tris, 2% (w/v) SDS, 32% (v/v) glycerol and 0.1% (w/v) BPB] with an additional 5 µl of 10% (w/v) SDS. Samples were boiled for 3 min and electrophoresed at a constant current of 20 mA. Electrophoresis buffer contained 25 mM Tris, 200 mM glycine and 0.1% (w/v) SDS. Following electrophoresis the gels were stained at room temperature using a silver staining kit. Alternatively some gels including the gels used in quantitative assays were stained with 0.25% (w/v) CBB in 45% (v/v) methanol and 10% (v/v) acetic acid for 30 min at 37 °C and destained in a solution containing 10% (v/v) acetic acid and 7% (v/v) methanol at 37 °C. The gels were photographed and analyzed (molecular weight analysis and densitometric analysis) in the Gel Documentation System (Alpha Innotech Corporation) using the ChemiImager software program.

2.2.6.2 Western blots

Protein samples were fractionated by SDS-PAGE on a 3%-12% (w/v) polyacrylamide gradient gel (Laemmli, 1970). The proteins were transferred on to a nitrocellulose membrane (Gelman Sciences) by electroblotting at 60V for 2 hrs at room temperature in a transfer buffer containing 25 mM Tris, 0.2 M glycine and 20% (v/v) methanol. Proteins of known molecular mass were excised and stained separately with 0.1% (w/v) amido black in 45% (v/v) methanol, 7% (v/v) acetic acid and destained in a solution containing 45% (v/v) methanol, 7% (v/v) acetic acid. The membrane was blocked by overnight incubation in 50 mM Tris-HCl (pH 7.4) containing 150 mM NaCl,

3% (w/v) BSA at room temperature. The nitrocellulose membrane was probed with the anti-toposome antibody at 1: 500 dilution in wash buffer of 50 mM Tris-HCl (pH 7.4) containing 0.05% (v/v) NP-40, 0.1% (w/v) SDS, 20 mM NaI and 2% (w/v) BSA, for 6 hrs at room temperature. The nitrocellulose membrane was washed overnight at room temperature in wash buffer, then 3 x 30 min in TTBS [20 mM Tris containing 0.5 M NaCl and 0.05% (w/v) tween-20 (pH 7.4)]. It was incubated for 1 hr in TTBS containing goat anti-mouse IgG conjugated with alkaline phosphatase at a dilution of 1: 3000, washed 2x 15 min in TTBS and then 2x 15 min in TBS [20 mM Tris-HCl containing 0.5 M NaCl (pH 7.4)]. The antigen-antibody complex was visualized by treating the membrane in 100 mM $NaHCO_3$, 1 mM $MgCl_2$ (pH 9.8) containing 0.03% (w/v) NBT and 0.015% (w/v) BCIP in the dark. The color reaction was stopped by transferring the membrane into distilled water.

2.2.7 Peptide mapping

Partial peptide maps were generated using the method of Cleveland *et al.* (1976). Proteins (10 μg) were electrophoresed on a 10% (w/v) polyacrylamide gel (Laemmli, 1970), the gel was stained with CBB for 30 min at room temperature and destained for less than 1 hr at room temperature to visualize stained proteins with minimum acid hydrolysis. The 240 kDa, 160 kDa and 120 kDa species were excised. The gel slices were soaked 30 min at room temperature with occasional swirling in 10 mL of a solution containing 0.125 M Tris-HCl, 0.1% (w/v) SDS and 1 mM EDTA (pH 6.8) and were placed at the bottom of the sample wells of a 12% (w/v) polyacrylamide gel containing a 4 % (w/v) polyacrylamide stacking gel, which was 1cm in height. The gel slices were

then overlaid with 10 μg/mL *Staphylococcus aureus-V8* proteinase (Calbiochem) in 0.125 M Tris–HCl containing 0.1% (w/v) SDS, 1mM EDTA and 20% (v/v) glycerol (pH 6.8). The amount of enzyme used for digestion varied depending on the molecular weight of the polypeptide substrates. The amounts of enzyme used to digest the polypeptides were:

i. 240 kDa-10 μl (0.1 μg)

ii. 160 kDa-5 μl (0.05 μg)

iii. 120 kDa-4 μl (0.04 μg)

Proteins were electrophoresed into the stacking gel for 20 min at a constant current of 10 mA to bring the enzyme and the polypeptide substrate into contact. The current was turned off for 30 min to allow the digestion to take place. The resulting peptides were resolved in a 12% (w/v) separating gel by electrophoresing at a constant current of 20 mA and the gel was silver stained.

2.2.8 Biochemical characterization of proteins

2.2.8.1 Calcium binding assay

A dot blot assay was performed on the 0.4 M fraction (5 μg) eluted from the Fast-Q resin. Troponin-C (1 μg) and BSA (2 μg) were used as positive and negative controls respectively. The nitrocellulose membrane was equilibrated in the calcium-binding buffer containing 10 mM Imidazole and 60 mM KCl for 1 hr at room temperature and then was incubated in the same buffer containing 7.5 μM $CaCl_2$ and 1 mCi/L of $^{45}CaCl_2$ for 15 min. The membrane was washed for 5 min in 1mM Tris-HCl, pH 7.5, air-dried

and calcium binding was detected using the phosphoimager (Alpha Innotech Corporation).

2.2.8.2 Characterization of membrane binding and vesicular aggregation activities

2.2.8.2.1 Preparation of liposomes

Multilamellar liposomes were prepared using brain lipid extract containing 10% (w/w) PI, 50% (w/w) PS and several other brain lipids. Brain lipid extract was solubilized in 2:1 chloroform: methanol by vortex mixing for 4 min, the solvent was evaporated to dryness under nitrogen gas and dried under vacuum for 1 hr. The resulting residue was resuspended in the liposome binding buffer containing 50 mM Imidazole, 150 mM NaCl and 0.1 mM EGTA (pH 7.4) at a concentration of 20 mg/mL, by vortex mixing for 4 min.

Additionally, multilamellar liposomes were prepared containing the same phospholipid composition as yolk granule membranes of unfertilized sea urchin eggs (Table 5 in appendix). Phospholipid composition of yolk granules was determined using standard assays; phospholipids were separated using the method of Rouser *et al.* (1967) and individual classes were quantitated by the method of Bartlett *et al.* (1959). Cholesterol was not used as a component for the liposome preparation. Phosphatidyl serine, phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl inositol and sphingomyelin were mixed together at the ratio of 3.9: 6.3: 12.4: 3.1: 3.6 and dissolved in 2:1 Chloroform: methanol by vortex mixing for 4 min. The solvent was evaporated to dryness under nitrogen gas and dried under vacuum for 1 hr. The resulting residue was resuspended in the liposome binding buffer; 50 mM Imidazole, 150 mM NaCl and 0.1 mM EGTA (pH 7.4) at a concentration of 20 mg/mL, by vortex mixing for 4 min.

Unilamellar PS liposomes were prepared by the method of Lee *et al.* (1997). Phosphatidyl serine stock solution was prepared in acetone at a concentration of 20 mg/mL by vortex mixing for 4 min. The solvent was evaporated to dryness, the resulting residue was freeze-dried for 1 hr and solubilized in 8 mL of extrusion buffer of 10 mM Hepes, 100 mM NaCl and 100 μM EDTA (pH 7.2) by vortex mixing for 4 min to make multilamellar vesicles. Multilamellar vesicles were extruded once through two 0.2 μm millipore filters (nuclepore, Whatman) and then ten times through two 0.1 μm millipore filters using an extrusion apparatus (Lipex Biomembranes Inc.) under nitrogen gas to make unilamellar PS liposomes. The phosphate concentration of the unilamellar PS liposome preparations was determined by the method of Bartlet *et al.* (1959) and the molarity of the phosphate was calculated accordingly.

2.2.8.2.2 Liposome binding assays

Liposome binding assays were performed following the method of Spenneberg *et al.* (1998) with some modifications. Multilamellar or unilamellar liposomes (2 mg/mL) were incubated with the proteins in the binding buffer containing 50 mM Imidazole, 150 mM NaCl and 0.1 mM EGTA (pH 7.4) in the presence of 5 mM free calcium for 30 min at room temperature. The MAXChelator software program was used to determine the free calcium concentrations. Inhibition of liposome binding by the anti-toposome antibody was assayed by incubating the protein with 5 mM free calcium in the presence of the antibody at a concentration of 1 μg/mL. Liposomes were pelleted out in the airfuge by spinning at 30 psi for 30 min. Supernatants were saved and the pellets were resuspended and washed with binding buffer containing 5 mM $CaCl_2$ (Calcium wash

buffer). The unbound fraction and the residual pellet were analyzed by reducing SDS-PAGE.

2.2.8.2.3 Liposome aggregation assays

Liposome aggregation assays were carried out by the method of Lee *et al.* (1997). Proteins were incubated with 1.25 mM $CaCl_2$ in the aggregation buffer containing 40 mM histidine, 300 mM sucrose and 0.5 mM $MgCl_2$, (pH 6.0) for 12 min at room temperature. Unilamellar PS liposomes were added to the mixture to obtain a final concentration of 95 μM and the OD was recorded at 350 nm in 2 min intervals for a total of 20 min. Inhibition of liposome aggregation by anti-toposome antibody was determined by pre-incubating the proteins with the antibody for 20 min at a concentration of 1 μg/mL. In some experiments, the unilamellar vesicles were harvested by spinning in the airfuge for 20 min at 30 psi, the pellet washed once with the aggregation buffer containing 1.25 mM calcium and the supernatant and the final pellet analyzed by SDS-PAGE to identify the proteins bound to the unilamellar PS liposomes.

2.2.8.2.4 Yolk granule aggregation assay

Crude yolk granule preparations were resuspended in a solution containing 40 mM histidine, 300 mM sucrose, 0.5 mM $MgCl_2$ and 0.5 M KCl (pH 6.0). Aggregation assays were performed at room temperature in the presence of 1.25 mM calcium. Aggregation of yolk granules was monitored by measuring the OD_{350} at 2 min intervals.

Chapter 3. Results

3.1 Analysis of yolk granule protein extracts

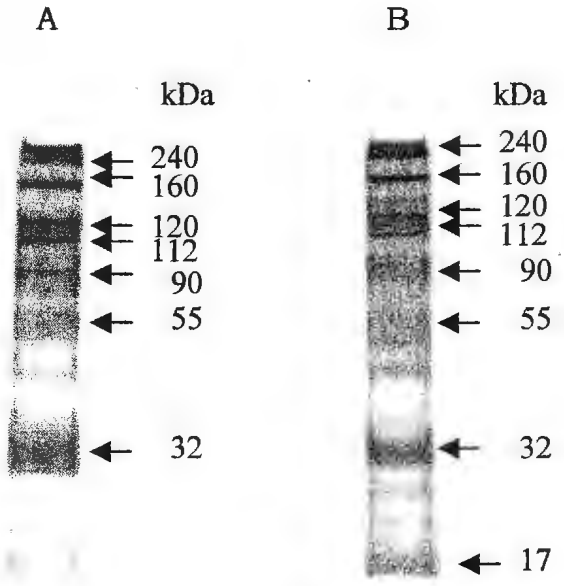
All the solutions used in yolk granule protein preparations contained 0.5 M KCl to create an isotonic environment to reduce the osmotic lysis of granules. The yolk granules prepared in 0.5 M KCl were first washed with 0.5 M KCl containing 1mM $CaCl_2$ (yolk granule calcium extract) to extract any proteins non-specifically bound to the yolk granule membrane. The second extraction was performed with 0.5 M KCl containing 1 mM EGTA (yolk granule EGTA extract), which is a specific chelator of calcium. Any proteins bound to the yolk granule membranes in a calcium-dependent manner should appear in the yolk granule EGTA extract; therefore, theoretically, the protein profiles of the two extracts should be different. Instead, as revealed by SDS-PAGE analysis, both the yolk granule extracts, yolk granule calcium extract and yolk granule EGTA extract, had almost the same protein profile (Fig.3.1). Despite using isotonic solutions, some yolk granules may have ruptured releasing their contents. The apparent molecular weights of the polypeptides in the extracts ranged between 17 k and 240 k as determined by SDS-PAGE (Laemmli, 1970) under reducing conditions. The apparent molecular weights of the polypeptides present in the yolk granule extracts are indicated in Fig. 3.1.

3.2 Separation of proteins present in the yolk granule calcium extract

Ion exchange chromatography was performed using an anion exchange resin, Q-Sepharose Fast Flow (fast-Q) to achieve a partial separation of the proteins present in the yolk granule calcium extract. After loading the column, the bound proteins were eluted with a step gradient of NaCl ranging from 0.1 M to 1 M. Sodium dodecyl sulfate

Fig. 3.1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of yolk granule extracts.

The yolk granules prepared in 0.5 M KCl were first washed with 0.5 M KCl containing 1mM $CaCl_2$ (yolk granule calcium extract) and then with 0.5 M KCl containing 1 mM EGTA (yolk granule EGTA extract). Aliquots (5 μg) of yolk calcium extract (Panel A) and yolk granule EGTA extract (Panel B) were fractionated in a 10% (w/v) polyacrylamide gel (Laemmli, 1970). The gel was silver-stained. The apparent molecular masses of the polypeptides are shown.


A
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120
112
90
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B
kDa
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120
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55
32
17

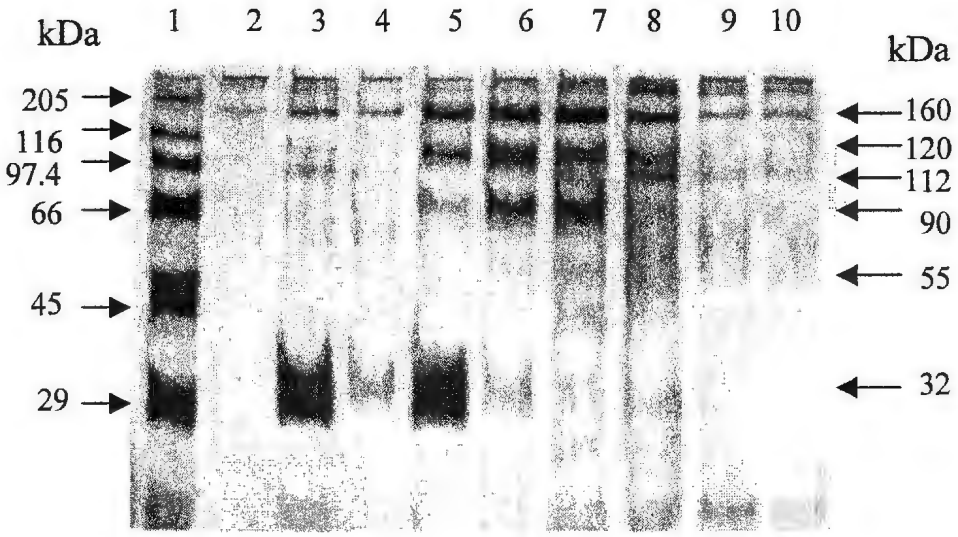
polyacrylamide gel electrophoresis (Laemmli, 1970), under reducing conditions, revealed that each fraction had a different protein profile (Fig. 3.2).

3.3 Proteins present in the 0.3 M and 0.4 M fractions eluted from the fast-Q resin are components of a high molecular weight protein complex of 240k

The liposome aggregation assays performed on the 0.3 M and 0.4 M fractions eluted from the fast-Q resin demonstrated that these fractions had the liposome aggregating activity (see section 3.8, Results). A wide range of chromatographic methods were attempted in order to further separate the proteins present in the 0.3 M and 0.4 M fractions eluted from the fast-Q resin. However, none of the methods were successful. These results led us to suspect that the proteins were present as a high molecular weight complex in the native state. To investigate this possibility, the 0.3 M and 0.4 M fractions eluted from the fast-Q resin were run in a 10% (w/v) polyacrylamide gel, following solubilization in Laemmli solubilizing solution (Laemmli, 1970) in the presence and absence of DTT (Fig. 3.3). Dithiothreitol is a reducing agent, which is capable of cleaving intramolecular and intermolecular disulfide bonds. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (Laemmli, 1970) under reducing conditions showed three major bands at 160 kDa, 120 kDa and 90 kDa in the 0.3 M and 0.4 M fractions (Fig. 3.3) A different protein profile was seen in SDS-PAGE (Laemmli, 1970) under non-reducing conditions, where a major band was observed having a molecular weight of 240 k (Fig. 3.3). This 240 kDa protein was largely absent when the 0.3 M and 0.4 M fractions eluted from the fast-Q resin were electrophoresed under reducing conditions.

Fig. 3.2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of fractions eluted from the anion exchange resin.

Anion exchange resin, Q-Sepharose Fast Flow (fast-Q) was equilibrated with 10 mM Tris, pH 8.0, at 4°C and protein present in the yolk granule calcium extract (1mg) was loaded onto the column. The column was washed with equilibration buffer and the bound proteins were eluted with a step gradient of NaCl ranging from 0.1 M to 1 M (step size-0.1M) in the same buffer. Aliquots (5μg) of the unbound fraction (lane 2), first wash (lane 3), and elutions from 0.1 M to 0.7 M NaCl (lane 4 to lane 10 respectively) were fractionated in a 10% (w/v) polyacrylamide gel (Laemmli, 1970). The molecular mass markers were run in lane 1. The gel was silver-stained.


kDa
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10
kDa
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97.4
66
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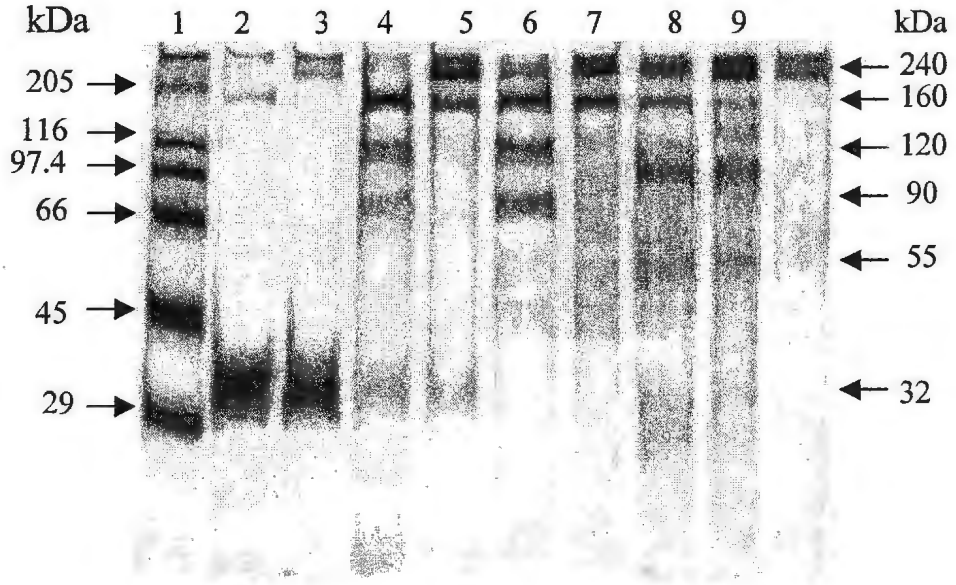
160
120
112
90
55
32

Fig. 3.3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of fractions eluted from fast-Q resin, under reducing and non-reducing conditions.

Aliquots (5 μg) of the 0.2 M fraction (lanes 2 and 3), the 0.3 M fraction (lanes 4 and 5), the 0.4 M fraction (lanes 6 and 7) and the 0.5 M fraction (lanes 8 and 9) were boiled in Laemmli solubilizing solution containing DTT (lanes 2, 4, 6 and 8) and in Laemmli solubilizing solution without DTT (lanes 3, 5, 7, and 9) and fractionated in a 10% (w/v) polyacrylamide gel (Laemmli, 1970). The molecular mass markers were run in lane 1. The gel was silver-stained.


kDa
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kDa
205
116
97.4
66
45
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240
160
120
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32

This result suggested that the 160 k, 120 k and 90 k polypeptides exist as a high molecular weight complex of 240 k associated through intermolecular disulfide bonds.

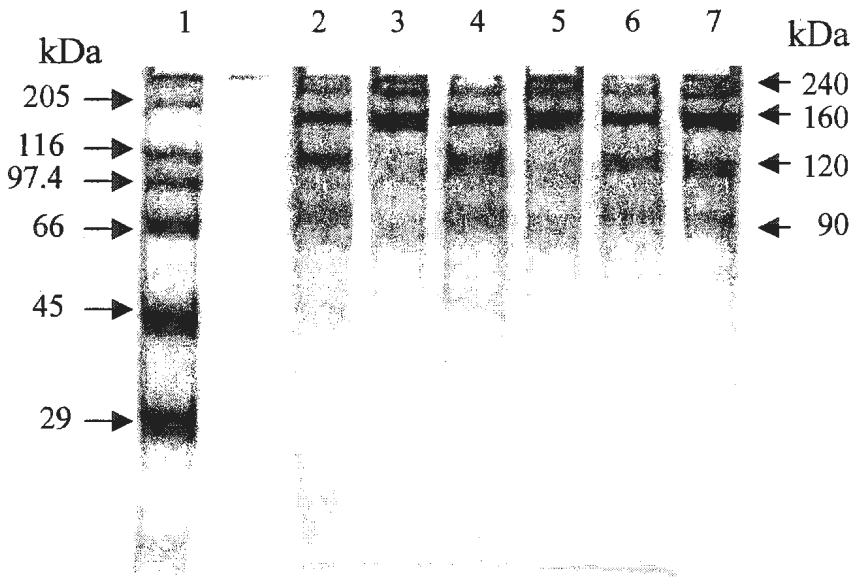
An experiment was performed to determine milder reducing conditions that can be used to separate the polypeptides present in the 240k high molecular weight protein complex. The protein present in the 0.4 M fraction eluted from the fast-Q resin was incubated for 1 hr under different reducing conditions as follows: i) at room temperature in the presence of 10 mM DTT; ii) at room temperature in the presence of 100 mM DTT; iii) at 37°C in the presence of 100 mM DTT. The incubations were performed in duplicate. Following incubation, the protein present in each aliquot was precipitated using an equal volume of 20 % (w/v) TCA. The precipitates were fractionated in a 10% (w/v) polyacrylamide gel (Laemmli, 1970), side by side after treating with different solubilizing conditions: one aliquot was boiled in the Laemmli solubilizing solution containing DTT while the other fraction was boiled in the Laemmli solubilizing solution without DTT (Fig. 3.4). Pretreatment of proteins with 100 mM DTT at 37°C for 1 hr could generate the same protein profile that was observed when the sample was treated with Laemmli solubilizing solution containing DTT. This demonstrated that when treated under these milder conditions, the 240 kDa protein could be reduced to give rise to the free polypeptides of which it was composed.

3.4 The 240 kDa protein is the toposome of *Strongylocentrotus purpuratus*

A Western blot experiment was performed using the yolk granule calcium extract and the 0.4 M fraction eluted from the fast-Q resin (Fig. 3.5). The protein (10 μg) present in the yolk granule calcium extract and the 0.4 M fraction eluted from the fast-Q resin

Fig. 3.4. Determination of minimum reducing conditions required to separate the polypeptides present in the 0.4 M fraction eluted from the fast-Q resin.

Aliquots (5 μg) of 0.4 M fraction eluted from the fast-Q resin were treated with 10 mM DTT (lanes 2 and 3) at room temperature for 1 hr, 100mM DTT (lanes 4 and 5) at room temperature for 1 hr and 100 mM DTT at 37°C for 1 hr. The protein present in the samples were precipitated by adding an equal volume of 20% (w/v) TCA. The precipitates were boiled in Laemmli solubilizing solution containing DTT (lanes 2, 4, 6 and 8) and in Laemmli solubilizing solution without DTT (lanes 3, 5, 7, and 9) and fractionated in a 10% (w/v) polyacrylamide gel (Laemmli, 1970). The molecular mass markers were run in lane 1. The gel was silver-stained.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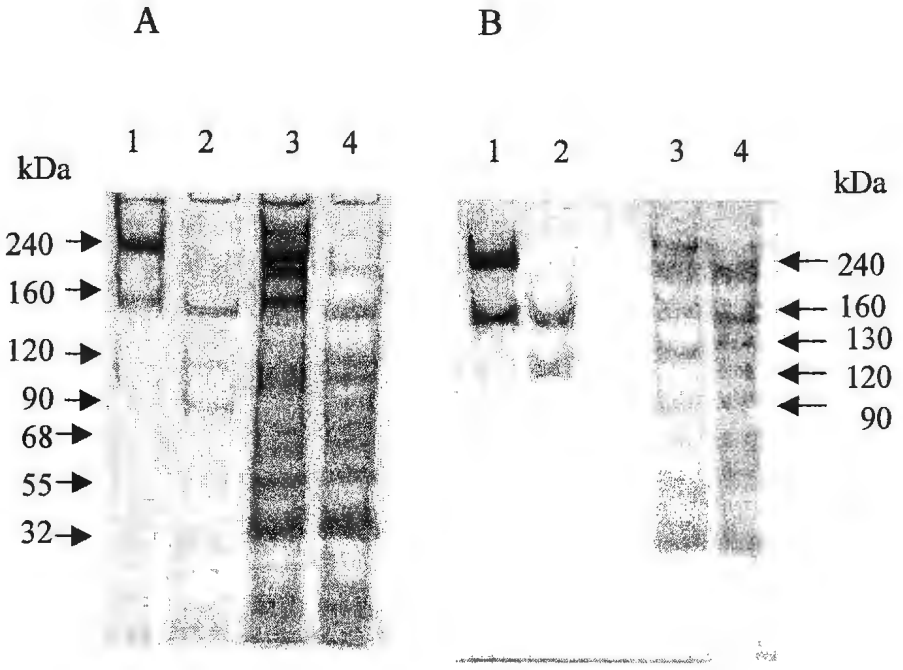
29
kDa
240
160
120
90

Fig. 3.5. Western blot experiment on 0.4 M fraction eluted from the fast-Q resin.

Panel A. Aliquots (5 μg) of yolk granule calcium extract (lanes 3 and 4) and 0.4 M fraction eluted from the fast-Q resin (lanes 1 and 2) were boiled in Laemmli solubilizing solution containing DTT (lanes 2 and 4) and in Laemmli solubilizing solution, without DTT (lanes 1 and 3) and fractionated in a 3-12% (w/v) polyacrylamide gel (Laemmli, 1970). The gel was silver stained.

Panel B. Aliquots (10 μg) of yolk granule calcium extract (lanes 3 and 4) and 0.4 M fraction eluted from the fast-Q resin (lanes 1 and 2) were boiled in Laemmli solubilizing solution containing DTT (lane 2 and 4) and in Laemmli solubilizing solution without DTT (lane 1 and 3) and fractionated in a 3- 12% (w/v) polyacrylamide gel (Laemmli, 1970) and transferred onto nitrocellulose. The nitrocellulose was probed with the anti-toposome antibody at a dilution of 1: 500 (v/v). It was then incubated for 1 hr in TTBS containing goat anti-mouse IgG conjugated with alkaline phosphatase at a dilution of 1: 3000 and the antibody was visualized by treating the membrane in 100 mM $NaHCO_3$, 1mM $MgCl_2$, pH 9.8 containing 0.03% (w/v) NBT and 0.015% (w/v) BCIP in the dark. The apparent molecular masses of the polypeptides are shown.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130
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90

were electrophoresed in a 3- 12% (w/v) polyacrylamide gel (Laemmli, 1970) following solubilizing the samples under reducing and non-reducing conditions and then electroblotted onto nitrocellulose. The antibody used in the Western blots was raised against the toposome of the sea urchin, *Paracentrotus lividus* and was a gift from Dr. V. Matranga, 'Instituto di Biologia dello Sviluppo', Palermo, Italy. We expected that this anti-toposome antibody would cross react with the toposome of the sea urchin, *Strongylocentrotus purpuratus*. The 240 kDa and 160 kDa polypeptide bands in both the yolk granule calcium extract and the 0.4 M fraction eluted from fast-Q resin were prominent in the Western blot demonstrating that these two polypeptides cross reacted with the antibody (Fig. 3.5). These results suggest that the 240 kDa polypeptide is the toposome of the *Strongylocentrotus purpuratus*. The SDS-PAGE experiments performed under reducing and non-reducing conditions suggest that the 160 kDa polypeptide was covalently bound to the 240 kDa complex (Fig. 3.3). On the other hand, the 160 kDa polypeptide was abundant in our yolk granule protein extracts. Therefore, we suggest that the 160 kDa polypeptide is the major yolk granule protein derived from the 240 kDa toposome.

3.5 Precursor product relationship of the 240 kDa polypeptide with the 160 kDa and 120 kDa polypeptides

A partial peptide mapping experiment was carried out following the method of Cleveland *et al.* (1976) with some modifications to determine if the 240 kDa polypeptide was the precursor for the 160 kDa and 120 kDa polypeptides. Protein present in the 0.4 M fraction eluted from the fast-Q resin was electrophoresed in a 10% (w/v)

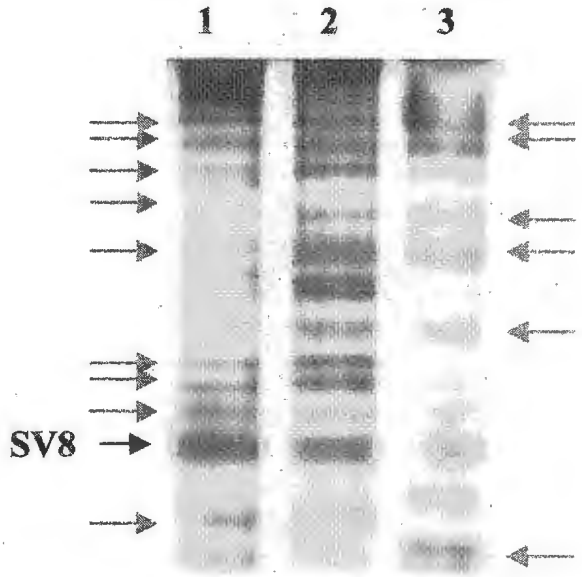
polyacrylamide gel following solubilizing under reducing and non-reducing conditions (Laemmli, 1970). The 240 kDa, 160 kDa and 120 kDa polypeptides were excised from the gel following brief staining with CBB. The proteins were digested with *Staphylococcus aureus V8* (SV8) protease as described in section 2.2.7, Materials and Methods. The resulting peptides were resolved in a 12% (w/v) polyacrylamide gel. The peptide map was visualized by silver staining. The resulting peptide maps (Fig. 3.6) showed that the 240 kDa polypeptide shared common peptides with the 160 kDa and 120 kDa polypeptides. Some peptide fragments were shared by only 240 kDa and 120 kDa polypeptides (shown by red arrows), while some peptide fragments were shared by only 240 kDa and 160 kDa polypeptides (shown by blue arrows). The other peptide fragments were common to all three proteins. This result provides strong evidence to suggest that the 160 kDa and 120 kDa polypeptides are derived from the 240 kDa protein.

3.6 Identification of calcium binding proteins present in the 0.4 M fraction eluted from the fast-Q resin

Calcium binding experiments were carried out to determine if the polypeptides present in the 0.4 M fraction eluted from the fast-Q resin could bind calcium. This experiment was an approach to identify the proteins involved in calcium-dependent liposome aggregation, since the polypeptides which bind calcium should be involved in this aggregation process. The proteins were incubated with ^{45}Ca and the binding was detected by phosphoimaging. No labeling was detected when the calcium binding assay was performed using the 0.4 M fraction eluted from the fast-Q resin following fractionation by SDS-PAGE (Laemmli, 1970) under reducing conditions. The

Fig. 3.6. Partial peptide mapping of polypeptides present in the 0.4 M fraction eluted from the fast-Q resin.

The 240 kDa, 160 kDa and 120 kDa polypeptides were excised from a 10% (w/v) SDS-PAGE gel following brief staining with CBB. The proteins were digested with *Staphylococcus aureus V8* (SV8) protease as described in section 2.2.7, Materials and Methods. The resulting peptides were resolved in a 12% (w/v) polyacrylamide gel and silver-stained. Peptide patterns arising from the 240 kDa (lane 1), 160 kDa (lane 2) 120 kDa (lane 3) polypeptides are shown and the position of the SV8 in the gel is indicated. The red arrows indicate the peptide fragments shared between 240 kDa and 120 kDa polypeptides while the blue arrows indicate the peptide fragments shared between 240 kDa and 160 kDa polypeptides.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SV8

denaturation occurring during SDS-PAGE may have accounted for the loss of calcium binding ability of the proteins present in the 0.4 M fraction. Therefore, a dot blot assay was performed in which the 0.4 M fraction eluted from the fast-Q resin was directly applied to nitrocellulose, followed by incubation with ^{45}Ca to determine whether any proteins present in the 0.4 M fraction bound calcium. Labeling was seen using the 0.4 M fraction in a dot blot (Fig 3.7). This result demonstrated that one or more polypeptides present in the 240 kDa protein complex was capable of binding calcium. The dot blot assay was performed by Dr. John Robinson.

3.7 Lipososome binding of yolk granule proteins

Many proteins involved in membrane fusion and transportation events have the capacity to bind phospholipids in a calcium-dependent manner (Boustead *et al.*, 1993). We were therefore interested in determining whether the yolk granule proteins exhibit calcium-dependent phospholipid binding. Liposome binding assays were performed to determine whether the proteins present in the yolk granule calcium extract and the 0.4 M fraction eluted from the fast-Q resin bind phospholipids in a calcium-dependent manner. The assays were performed using multilamellar liposomes prepared using a brain lipid extract. The brain lipid extract was composed of 10% (w/w) PI, 50% (w/w) PS and several other phospholipids. When a liposome binding assay was carried out on the yolk granule calcium extract, a range of proteins were shown to bind phospholipids in the presence of 5 mM calcium (Fig. 3.8). The apparent molecular masses of these proteins were detected as 160 k, 120 k, 112 k, 90 k, 68 k, 55 k and 32 k. The liposome binding assay performed on the proteins present in the 0.4 M fraction eluted from the fast-Q resin

Fig. 3.7. Calcium-binding assay on proteins present in the 0.4 M fraction eluted from the fast-Q resin.

Calcium-binding assay was performed in a dot blot of the 0.4 M fraction eluted from the fast-Q resin. An aliquot (5 μg) of the 0.4 M fraction was placed on nitrocellulose (2) and incubated with 7.5 μM $CaCl_2$ containing 1 mCi/L ^{45}Ca in calcium binding buffer. Troponin C (1 μg) was used as the positive control (1) and BSA (2 μg) was used as the negative control (3). Calcium binding was detected by PhosphoImaging in the gel documentation system (Alpha Innotech Corporation). Troponin C used in the assay was a gift from Dr. David Heeley. The calcium binding assay was performed by Dr. John Robinson.

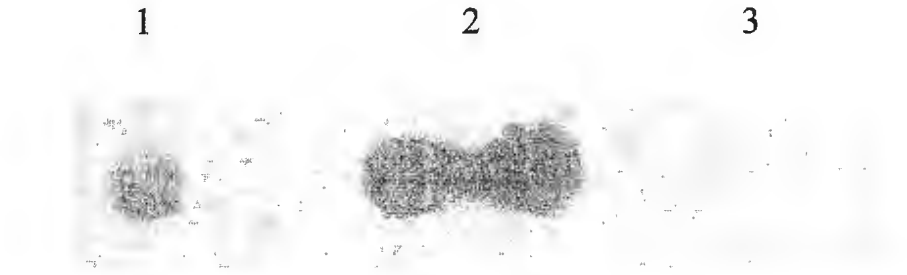
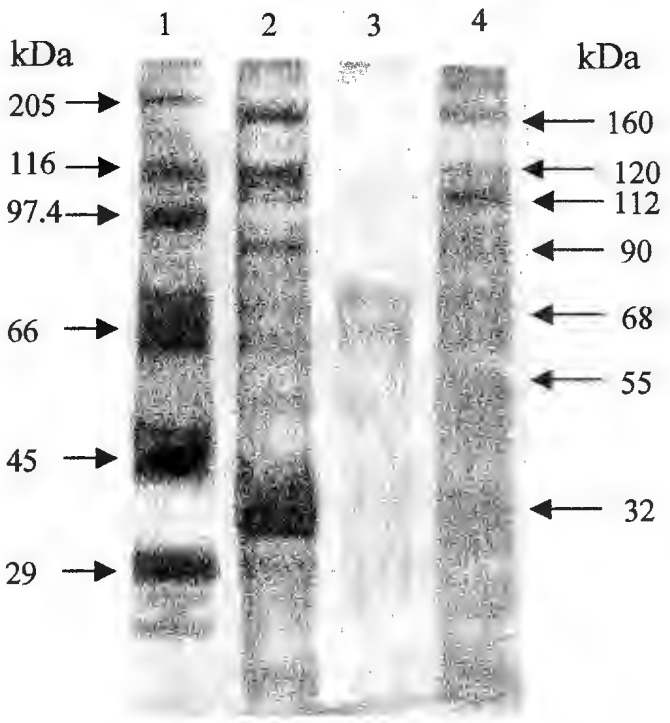

1
2
3

Fig. 3.8. Liposome binding assay on proteins present in the yolk granule calcium extract. An aliquot of yolk granule calcium extract (10 μg) was incubated with multilamellar brain lipid liposomes for 30 min at room temperature in the presence of 5 mM calcium, the liposomes were pelleted in an airfuge and washed once with calcium wash buffer. The supernatant (lane 2), calcium wash (lane 3) and the bound fraction (pellet, lane 4) were fractionated in a 10% (w/v) polyacrylamide gel (Laemmli, 1970). Molecular mass markers were run in lane 1. The gel was silver-stained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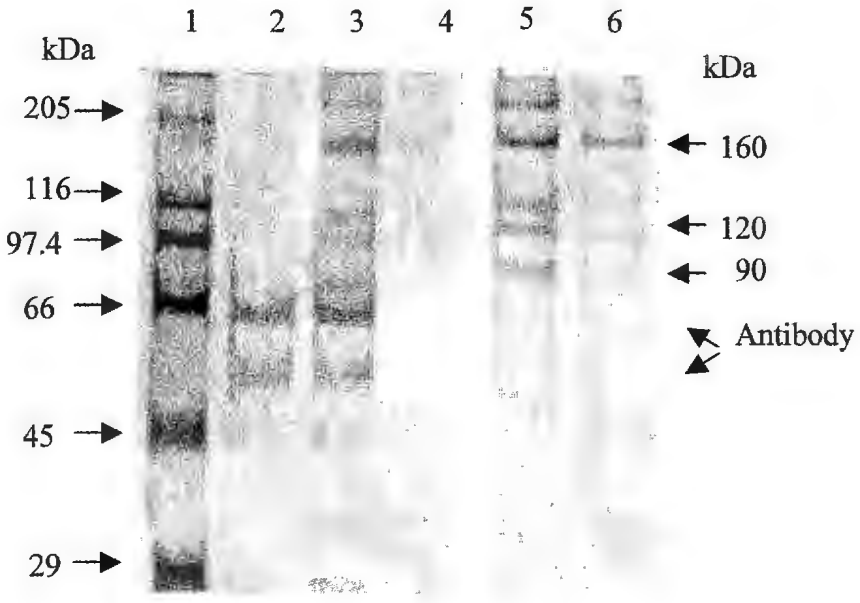
revealed that polypeptides present in this fraction, mainly 160 kDa and 120 kDa, bind phospholipid vesicles in the presence of 5 mM calcium (fig. 3.9). Liposome binding assays were also performed with the multilamellar liposomes having the phospholipid composition of the yolk granule membranes (Refer to Table. 5 in appendix) (Fig. 3.10). The 160 kDa and 120 kDa polypeptides present in the 0.4 M fraction bound to these liposomes. Collectively, these data suggested that polypeptides present in the 0.4 M fraction eluted from the fast-Q resin were capable of binding to phospholipid vesicles in the presence of 5 mM calcium.

Liposome binding assays were performed with different concentrations of calcium to determine the relationship between calcium concentration and the amount of protein binding. The unbound (supernatant) and bound (pellet) fractions were electrophoresed in an 8% (w/v) polyacrylamide gel (Laemmli, 1970) and the gel was stained with CBB. The 160 kDa bands were quantified by densitometry, the percentage bound was determined and then plotted against calcium concentration (Fig 3.11). The percentage bound showed a positive correlation with the calcium concentration in the medium and the maximum percentage binding of 25% was obtained at a free calcium concentration of 200 μM. The apparent Kd for binding was approximately 25 μM as calculated from the graph (Fig. 3.11).

The binding assays described above were also performed in the presence of the anti-toposome antibody to determine if this antibody inhibits protein binding to liposomes (Fig. 3.9 and 3.10). The presence of this anti toposome antibody at a concentration of 1 μg/mL inhibited the protein present in the 0.4 M fraction eluted from the fast-Q resin, binding to liposomes.

Fig. 3.9. Liposome binding assay on proteins present in the 0.4 M fraction eluted from the fast-Q resin with multilamellar brain lipid vesicles.

An aliquot (5 μg) of 0.4 M fraction eluted from the fast-Q resin was incubated with multilamellar brain lipid liposomes for 30 min at room temperature in the presence of 5 mM calcium. The liposomes were pelleted in an airfuge and both the unbound fraction (supernatant, lane 5) and the bound fraction (pellet, lane 6) were fractionated in an 8% (w/v) polyacrylamide gel (Laemmli, 1970). In parallel, a second liposome binding assay was carried out in the presence of anti-toposome antibody. An aliquot (5 μg) of 0.4 M fraction eluted from the fast-Q resin was pre-incubated with anti-toposome antibody for 20 min and incubated with multilamellar brain lipid liposomes for 30 minutes at room temperature in the presence of 5 mM calcium. The unbound fraction (lane 3) and the bound fraction (lane 4) were fractionated in an 8% (w/v) polyacrylamide gel and then silver-stained. Lanes 1 and 2 contain molecular mass markers and anti-toposome antibody respectively. The anti-toposome antibody was a gift from Dr. V. Matranga, 'Instituto di Biologia dello Sviluppo', Palermo, Italy.


1
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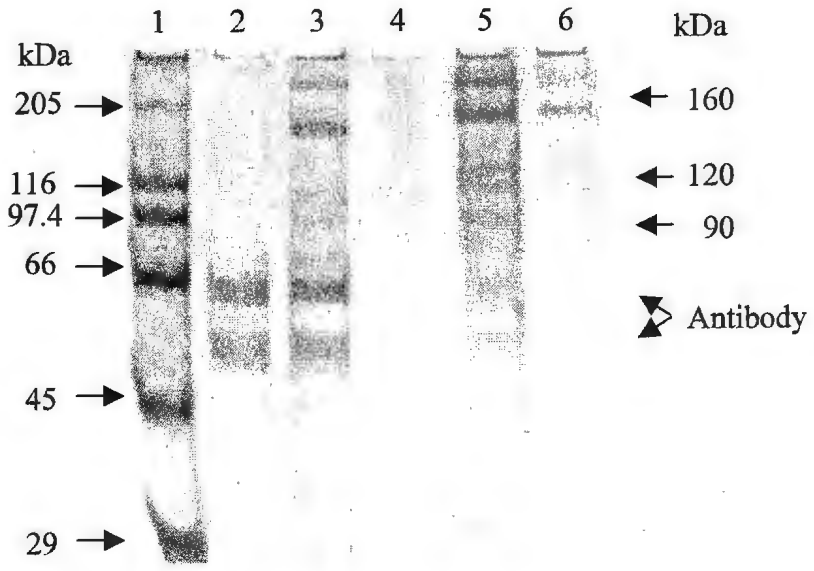
4
5
6
kDa
205
116
97.4
66
45
29
kDa
160
120
90
Antibody

Fig. 3.10. Liposome binding assay using proteins present in the 0.4 M fraction eluted from the fast-Q resin and multilamellar liposomes having the same phospholipid composition as that in the yolk granule membrane.

The yolk granule membranes were analyzed for the phospholipid composition using standard assays (refer to Table. 5 in appendix). The phospholipid analysis was performed by Scott Pelley in Dr. Philip Davis's Lab. An aliquot (5 μg) of the 0.4 M fraction eluted from the fast-Q resin was incubated for 30 min at room temperature in the presence of 5 mM calcium and multilamellar liposomes with the same phospholipid composition as the yolk granule membrane. The liposomes were pelleted in an airfuge and the unbound fraction (supernatant, lane 5) and the bound fraction (pellet, lane 6) were fractionated in an 8% (w/v) polyacrylamide gel (Laemmli, 1970). In parallel, a second liposome binding assay was carried out in the presence of anti-toposome antibody. An aliquot (5 μg) of 0.4 M fraction eluted from the fast-Q resin was pre-incubated with anti-toposome antibody for 20 min and incubated with multilamellar liposomes for 30 min at room temperature in the presence of 5 mM calcium. The unbound fraction (lane 3) and the bound fraction (lane 4) were fractionated in an 8% (w/v) polyacrylamide gel. The gel was silver stained. Lane 1 and lane 2 contain molecular mass markers and anti-toposome antibody respectively. The anti-toposome antibody was a gift from Dr. V. Matranga, 'Instituto di Biologia dello Sviluppo', Palermo, Italy.


1
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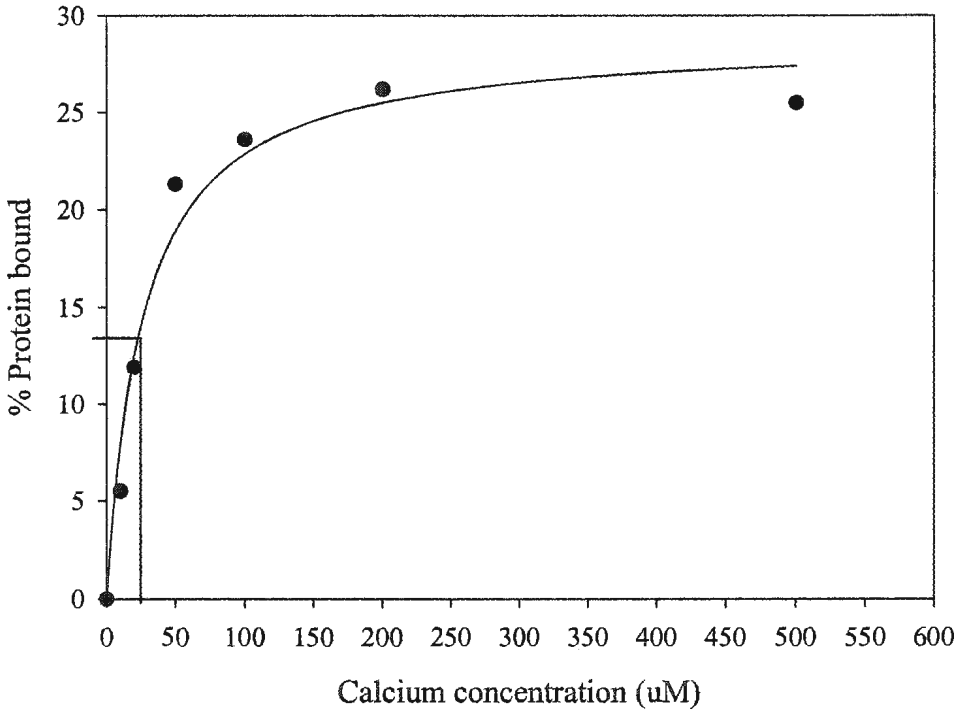
205
116
97.4
66
45
29
kDa
160
120
90
Antibody

Fig. 3.11. Effect of calcium concentration on protein binding to liposomes.

Aliquots (5 μg) of the 0.4 M fraction eluted from the fast-Q resin were incubated with multilamellar brain lipid liposomes in the presence of different concentrations of calcium for 30 min. Liposomes were pelleted and the pellets and supernatants were fractionated in an 8% (w/v) polyacrylamide gel (Laemmli, 1970), stained with CBB and the 160 kDa band in the pellets and supernatants were quantified by densitometry, in the gel documentation system using the ChemiImager software program (Alpha Innotech Corporation) . The percentages of bound protein were calculated and plotted against calcium concentration. Apparent Kd for binding was approximately 25 μM. The experiment was performed only once.


30
25
20
15
10
5
0
% Protein bound
0
50
100
150
200
250
300
350
400
450
500
550
600
Calcium concentration (uM)

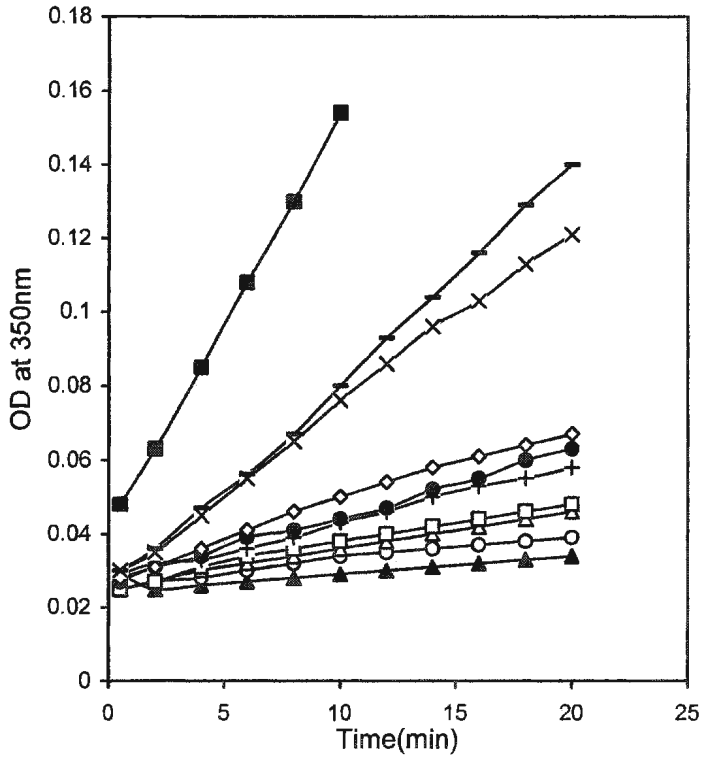
3.8 Liposome aggregation by yolk granule proteins

Liposome aggregation assays were performed to investigate whether the proteins were capable of driving aggregation of vesicular structures in a calcium-dependent manner. The proteins of the yolk granule calcium extract demonstrated aggregating activity in the presence of 1.25 mM calcium (data not shown). Each time a liposome aggregation assay was performed, two control experiments were carried out; one with calcium in the absence of proteins to demonstrate that calcium alone was not able to drive liposome aggregation, and the other with protein in the absence of calcium to demonstrate that the aggregation driven by proteins was calcium-dependent.

The aggregation assay was then performed on fractions eluted from the fast-Q resin (Fig. 3.12); in all assays, 5 μg of protein was used. The fractions, eluted from the fast-Q resin in 0.3 M, 0.4 M and 0.5 M NaCl demonstrated liposome aggregating activity while the other fractions had little or no aggregating activity. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (Laemmli, 1970) of these fractions under reducing conditions showed three major polypeptides in each fraction (Fig. 3.3). Polypeptides of 160 kDa and 120 kDa were common to all three fractions suggesting that one or both these proteins might be involved in liposome aggregation. Both the 0.3 M and 0.4 M fractions eluted from the fast-Q resin had a polypeptide of 90 kDa, which suggested that this also might be involved in liposome aggregation.

The polypeptides involved in aggregation may bind to the liposomes. Following liposome aggregation by the 0.4 M fraction eluted from the fast-Q resin, the vesicles were pelleted by centrifugation in the airfuge. The supernatant and the pellet were analyzed by SDS-PAGE (Laemmli, 1970) under reducing conditions. The 160 kDa, 120 kDa and

Fig. 3.12. Liposome aggregation assays on fractions eluted from the fast-Q resin. Aliquots (5 μg) of fractions eluted from the fast-Q resin, i.e. the unbound (□), wash (▲), and the 0.1 M (◇), 0.2 M (+), 0.3 M (×), 0.4 M (■), 0.5 M (−), 0.6 M (△), 0.8 M (●) NaCl eluates were incubated with 1.25 mM calcium in the aggregation buffer for 12 min. Unilamellar PS liposomes were then added and the OD_{350} was recorded every 2 min for a total of 20 min. A control experiment was carried out with calcium alone, omitting the protein, to demonstrate that calcium alone could not drive liposome aggregation at a concentration of 1.25 mM (O). This experiment was performed four times and the data presented in the figure are representative of the data obtained.


0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
OD at 350nm
0
5
10
15
20
25
Time(min)

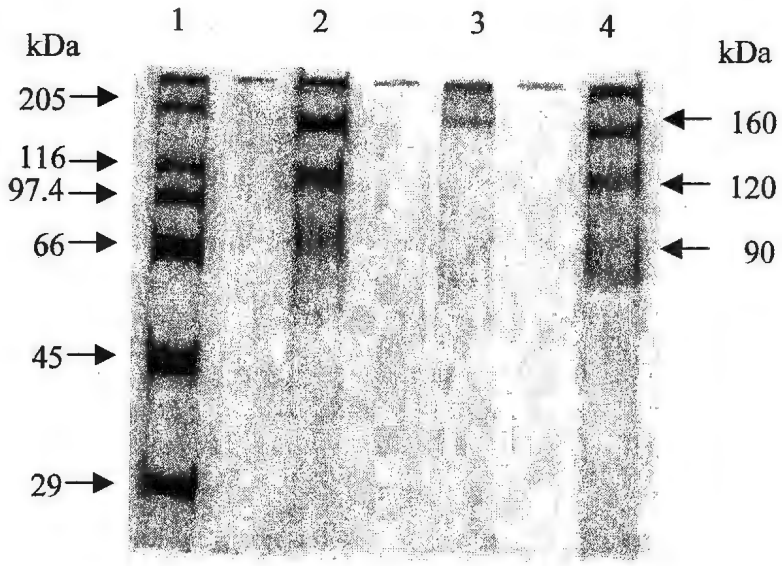
90 kDa polypeptides appeared in the liposome pellet suggesting that the 240 kDa complex was bound to the liposomes (Fig. 3.13).

A liposome aggregation assay was performed with the 0.4 M fraction eluted from the fast-Q resin upon treating it with 100 mM DTT at 37 °C for 1 hr, to determine if the reduced form of the 240 kDa protein complex possesses liposome aggregating activity (Fig. 3.14). A good level of liposome aggregating activity was observed in the reduced state as well (Fig. 3.14), suggesting that one or more polypeptides present in the 240 kDa complex were involved in liposome aggregation even in the reduced form. Analysis of the unilamellar liposome pellet by SDS-PAGE (Laemmli, 1970) revealed that all three polypeptides were bound to the liposomes (Fig 3.15). It is possible that all three polypeptides have the capacity to bind to liposomes and drive liposome aggregation. On the other hand, the polypeptides might remain associated by the help of other intermolecular bonds, which are not affected by DTT treatment (e.g. ionic, hydrophobic and hydrogen bonds).

Liposome aggregation assays were performed to determine the effect of protein concentration on the rate of aggregation. The assay was performed with the yolk granule calcium extract and with the 0.4 M fraction eluted from the fast-Q resin. In both cases, the rate of aggregation was dependent on the concentration of protein. The initial rates of aggregation ($\triangle OD_{350}$/ min) were calculated and plotted against the protein concentration for both the yolk granule calcium extract (Fig. 3.16. panel A) and the 0.4 M fraction eluted from the fast-Q resin (Fig 3.16, panel B). The maximum initial rate of liposome aggregation was attained with ~50 μg/mL of protein present in the yolk granule calcium extract. Only ~25 μg protein from the 0.4 M fraction eluted from the fast-Q

Fig. 3.13. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the liposome pellet following the unilamellar PS liposome aggregation assay.

An aliquot (5 μg) of the 0.4 M fraction eluted from the fast-Q resin was incubated with 1.25 mM calcium for 12 min. Unilamellar PS liposomes were then added and the aggregation was monitored by measuring the OD at 350 nm. After 20 min, the liposomes were pelleted by spinning in an airfuge and the supernatant (lane 3), and the pellet (lane 4) were fractionated in a 10% (w/v) polyacrylamide gel (Laemmli, 1970) under reducing conditions along with the 0.4 M fraction eluted from the fast-Q resin (lane 2) and the high molecular weight markers (lane 1). The gel was silver-stained.


1
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kDa
205
116
97.4
66
45
29
kDa
160
120
90

Fig. 3.14. Liposome aggregation by the 0.4 M fraction eluted from the fast-Q resin, in its reduced form.

An aliquot (5 μg) of the 0.4 M fraction eluted from the fast-Q resin was pre-incubated with 100 mM DTT at 37 °C for 1 hour and was then incubated with 1.25 mM calcium in the aggregation buffer. Unilamellar PS liposomes were then added and the aggregation was monitored by measuring the OD_{350} at 2 min intervals for 14 min (♦). Control assays were performed with the 0.4 M fraction eluted from the fast-Q resin, without pre-incubating with DTT (▲), and the 0.4 M fraction eluted from the fast-Q resin, in the absence of calcium (●). This experiment was performed two times and the data presented in the figure are representative of the data obtained.

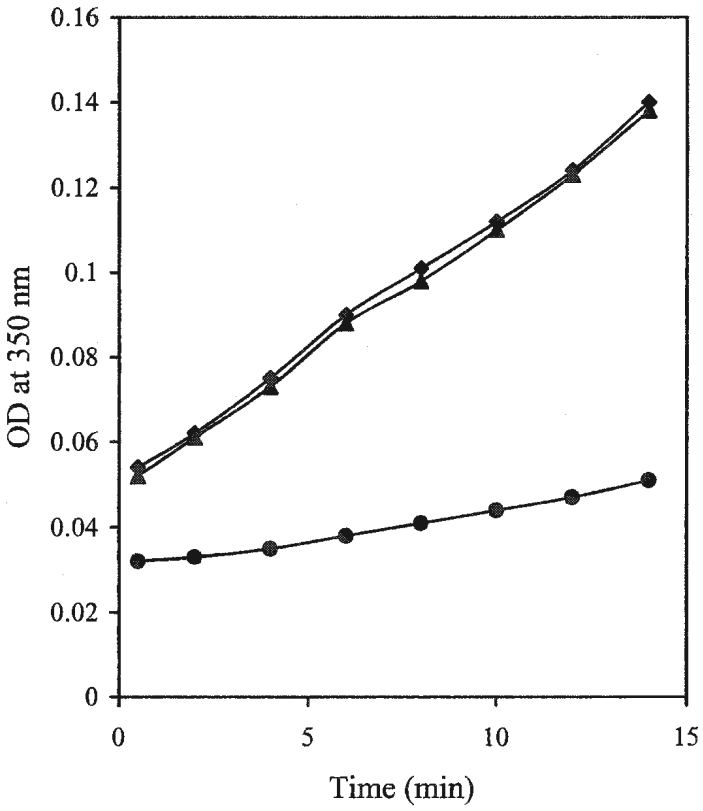
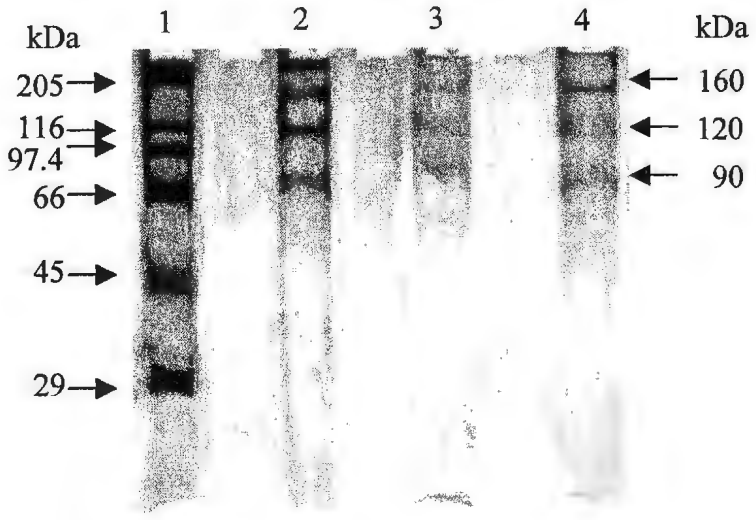

0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
OD at 350 nm
0
5
10
15
Time (min)

Fig. 3.15. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the liposome pellet following unilamellar PS liposome aggregation by proteins present in the 0.4 M fraction eluted from the fast-Q resin, in its reduced state.

An aliquot (5 μg) of the 0.4 M fraction eluted from the fast-Q resin, was pre-incubated with 100 mM DTT at 37 °C for 1 hr and then was incubated with 1.25 mM calcium in the aggregation buffer. Unilamellar PS liposomes were then added and the aggregation was monitored by measuring the OD at 350 nm. After 20 min, the liposomes were pelleted by spinning in an airfuge and the supernatant (lane 3) and the pellet (lane 4) were fractionated in a 10% (w/v) polyacrylamide gel (Laemmli, 1970) along with reduced 0.4 M fraction eluted from the fast-Q resin (lane 2) and the molecular mass markers (lane 1). The gel was silver-stained.


kDa
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kDa
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66
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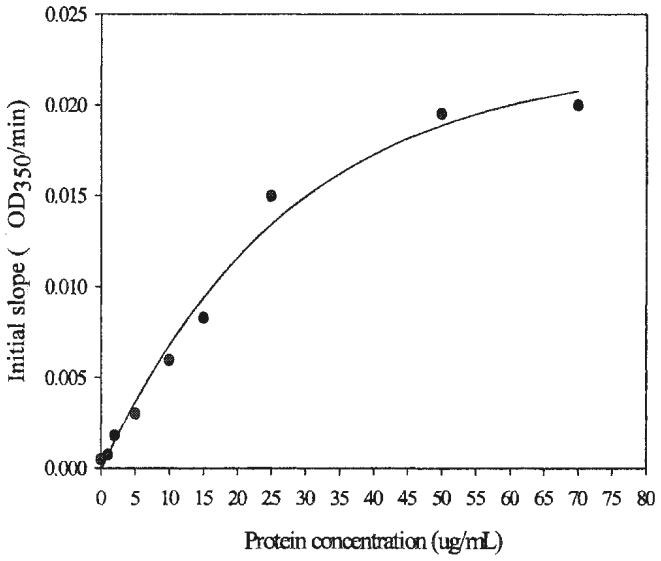
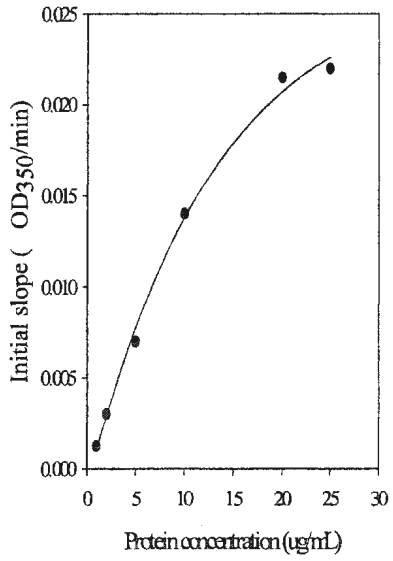
160
120
90

Fig. 3.16. Determination of the effect of protein concentration on liposome aggregation. Liposome aggregation assays were performed with the yolk granule calcium extract and the 0.4 M fraction eluted from the fast-Q resin, at different protein concentrations. The initial rates ($\triangle OD_{350}$/min) of aggregation by the yolk granule calcium extract and the 0.4 M fraction eluted from the fast-Q resin were calculated and plotted against the protein concentration (panel A and panel B respectively). This experiment was performed two times and the data presented in the figure are representative of the data obtained.

A


0.025
0.020
0.015
0.010
0.005
0.000
Initial slope (OD350/min)
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80
Protein concentration (ug/mL)

B


0.025
0.020
0.015
0.010
0.005
0.000
Initial slope (OD350/min)
0 5 10 15 20 25 30
Protein concentration (ug/mL)

resin, was required to reach this maximum initial velocity, suggesting that the aggregating activity might be relatively more concentrated in this fraction.

Calcium is known to aggregate liposomes on its own at higher concentrations. Two sets of liposome aggregation assays were carried out to determine the effect of calcium ion on aggregation: one with different concentrations of calcium in the absence of protein to determine the effect of calcium alone on aggregation, and the other with different concentrations of calcium in the presence of a fixed amount (5 μg) of protein present in the 0.4 M fraction eluted from the fast-Q resin, to determine the effect of calcium concentration on protein driven aggregation. In each case, the initial rates of aggregation ($\triangle OD_{350}$/min) were determined and plotted against the calcium concentration (Fig. 3.17). Calcium alone could drive liposome aggregation above 1.5 mM, but was unable to cause liposome aggregation below this concentration. The protein present in the 0.4 M fraction eluted from the fast-Q resin was capable of driving liposome aggregation below 1.5 mM calcium, a concentration at which calcium alone could not drive liposome aggregation. In the presence of protein, the rate of aggregation was dependent on the calcium concentration used in the assay. The rate of protein driven aggregation was fast, and the effect of calcium itself was minimal at 1.25 mM calcium, which was the concentration of calcium used in our assays.

Liposome aggregation assays were performed to investigate the effect of other metal ions on the aggregating activity of the proteins present in the 0.4 M fraction (Fig. 3.18). Proteins present in the 0.4 M fraction could drive liposome aggregation in the presence of 1.25 mM barium while no aggregation was observed in the presence of 1.25 mM magnesium.

Fig. 3.17. Determination of the effect of calcium concentration on aggregation.

Two sets of liposome aggregation assays were performed, one with a fixed amount (5 μg) of protein present in the 0.4 M fraction eluted from the fast-Q resin and different concentrations of calcium and the other with different concentrations of calcium in the absence of protein. The initial rates of aggregation ($\triangle OD_{350}$/min) with calcium alone (●) and protein in the presence of calcium (O) were calculated and plotted against the calcium concentration.

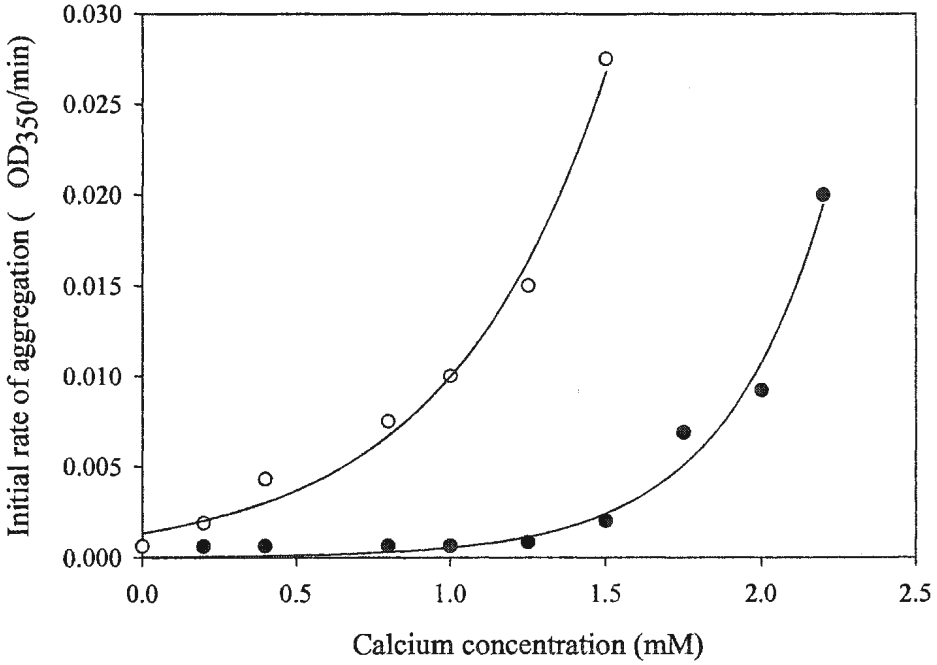
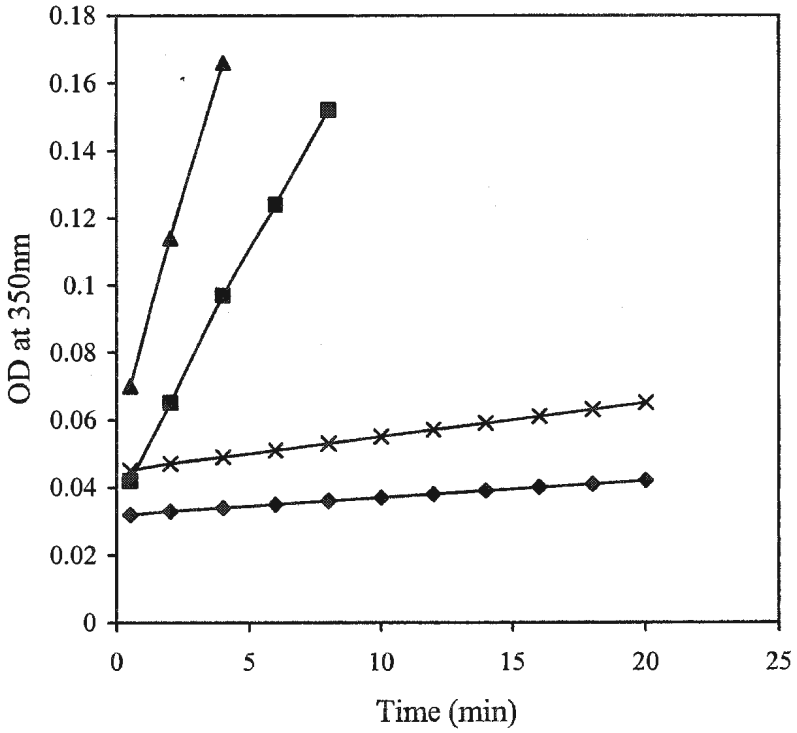

0.030
0.025
0.020
0.015
0.010
0.005
0.000
Initial rate of aggregation (OD350/min)
0.0
0.5
1.0
1.5
2.0
2.5
Calcium concentration (mM)

Fig. 18. Determination of the effect of the other metal ions on liposome aggregation by proteins present in the 0.4 M fraction eluted from the fast-Q resin.

Aliquots (5 μg) of 0.4 M fraction eluted from the fast-Q resin were incubated with 1.25 mM calcium (■) 1.25 mM barium (▲) or 1.25 mM magnesium (×) for 12 min. Upon adding unilamellar PS liposomes, the aggregation was monitored by measuring the OD_{350} at 2 min intervals for 20 min. Control (♦) did not contain any metal ions. The experiment was performed only once.


0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
OD at 350nm
0
5
10
15
20
25
Time (min)

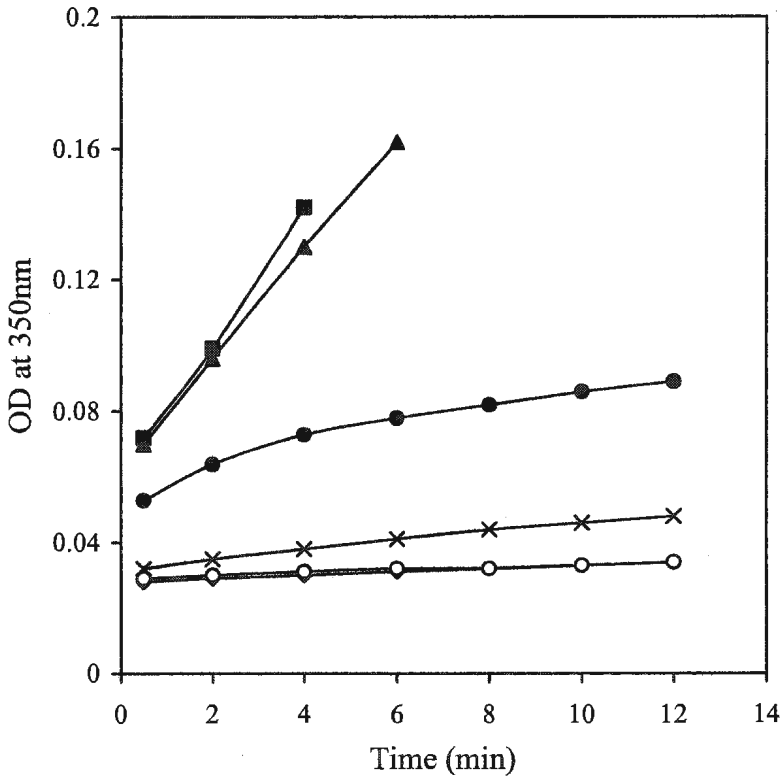
The liposome aggregation assays were also performed in the presence of the anti-toposome antibody to determine the effect of this antibody on the aggregation driven by the proteins present in the 0.4 M fraction eluted from the fast-Q resin (Fig. 3.19). Anti-toposome antibody could specifically inhibit the aggregating activity of the proteins present in the 0.4 M fraction eluted from the fast-Q resin. The aggregation was completely inhibited when the proteins were pre-incubated with the antibody at room temperature for 20 min, at a concentration of 1 μg/mL. The inhibition of the binding of the protein present in the 0.4 M fraction eluted from the fast-Q resin to the liposomes could be attributed to the inhibitory effect of the antibody on aggregation (Figs 3.9 and 3.10).

3.9 The major yolk granule protein in *Strongylocentrotus purpuratus* is capable of aggregating liposomes

Immobilized metal ion affinity chromatography (IMAC) was employed to isolate the 160 kDa polypeptide. Copper was first bound to the Chelating Sepharose Fast Flow resin. After loading the 0.4 M fraction eluted from the fast-Q resin onto the column, the bound proteins were eluted with a step gradient of guanidine thiocyanate. The 160 kDa polypeptide was bound to the Chelating Sepharose Fast Flow resin and eluted at 3 M guanidine thiocyanate (Fig. 3.20). The 3 M guanidine thiocyanate fraction was dialyzed extensively to remove the guanidine thiocyanate and a liposome aggregation assay was performed (Fig. 3.21). The protein demonstrated liposome aggregating activity in the presence of calcium, providing strong evidence to suggest that the major yolk granule

Fig. 3.19. Inhibition of the liposome aggregation caused by the protein present in the 0.4 M fraction eluted from the fast-Q resin, by the anti-toposome antibody.

An aliquot (5 μg) of 0.4 M fraction eluted from the fast-Q resin was incubated with 1.25 mM calcium for 12 min. Unilamellar PS liposomes were then added and the aggregation was monitored by measuring the OD_{350} at 2 min intervals for 12 min (▲). The effect of the anti-toposome antibody on the aggregation was tested by pre-incubating the protein present in the 0.4 M fraction with the anti-toposome antibody (1 μg/ mL) for 20 min before commencing the assay (×) and with the protein present in the 0.4 M fraction eluted from the fast-Q resin, in the presence of the anti-toposome antibody (1 μg/ mL), but without preincubating (●). An assay was performed with the 0.4 M fraction eluted from the fast-Q resin, pre-incubated with pre-immune serum (1 μg/ mL) for 20 min (1 μg) to demonstrate that the pre-immune serum did not have an effect on the liposome aggregation caused by the protein present in this fraction (■). Two control assays were performed with the anti-toposome antibody (1 μg/ mL) alone (♦) and pre-immune serum alone (O) omitting the protein present in the 0.4 M fraction eluted from the fast-Q resin, to demonstrate that the proteins contained in these fractions did not exhibit liposome aggregating activity. The curves resulted from these latter two control experiments are superimposed and indistinguishable from one another. This experiment was performed two times and the data presented in the figure are representative of the data obtained.


0.2
0.16
0.12
0.08
0.04
0
OD at 350nm
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2
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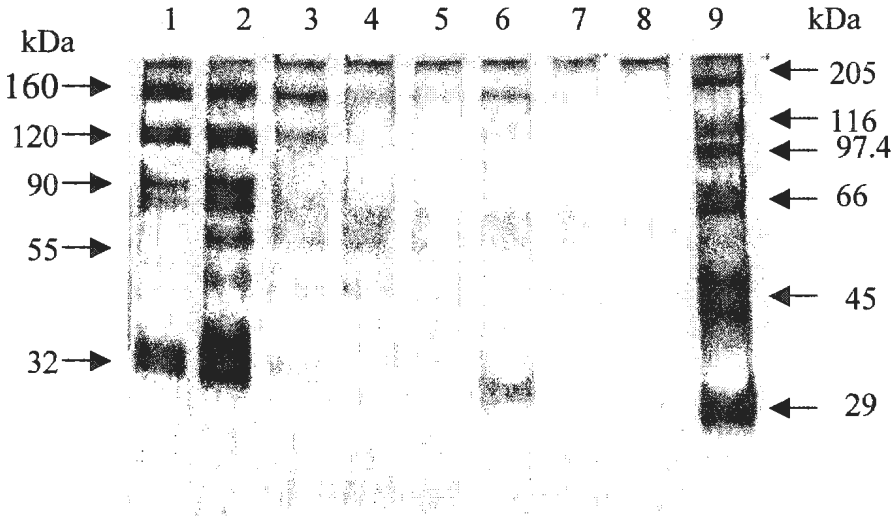
8
10
12
14
Time (min)

Fig. 3.20. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the fractions eluted from the Chelating Sepharose Fast Flow resin.

Copper was bound to the Chelating Sepharose Fast Flow resin and was equilibrated with 10 column volumes of 50 mM Imidazole containing 6 M urea (pH 7.0). An aliquot (100 μg) of the 0.4 M fraction eluted from the fast-Q resin, which was extensively dialyzed against the equilibration buffer was loaded onto the column. The unbound proteins were washed off the column using 5 column volumes of equilibration buffer and the bound proteins were eluted using 1 M NaCl and then using a step gradient of guanidine thiocyanate ranging from 1 M to 5 M (step size-1 M). Aliquots (5 μg) of the unbound fraction (lane 1), wash (lane 2), 1 M NaCl eluate (lane 3) and guanidine thiocyanate eluates (lanes 4 to 8) were boiled in Laemmli solubilizing solution and fractionated in a 10% (w/v) polyacrylamide gel (Laemmli, 1970) and silver stained. The molecular mass markers were run in lane 9.


1
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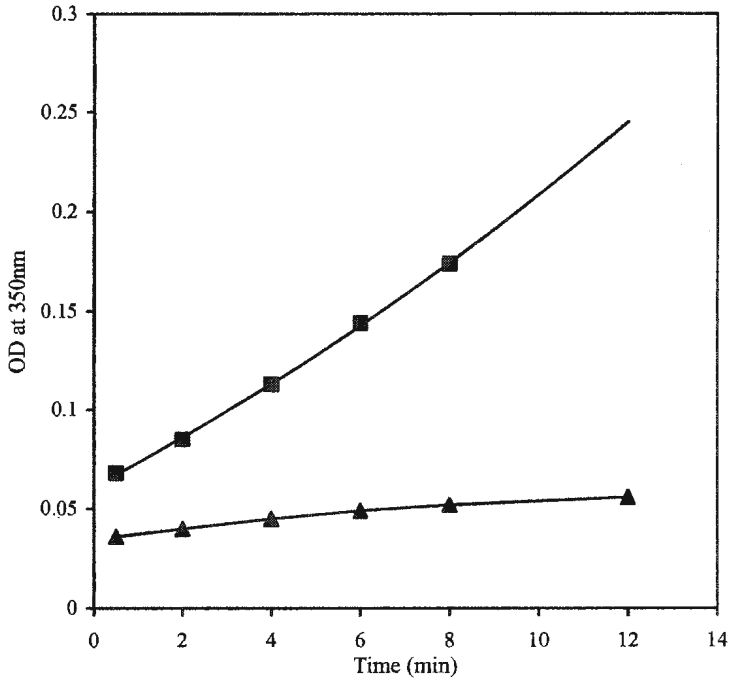
3
4
5
6
7
8
9
kDa
160
120
90
55
32
kDa
205
116
97.4
66
45
29

Fig. 3.21. Liposome aggregation assay on proteins present in the 3 M guanidine thiocyanate fraction eluted from the Chelating Sepharose Fast Flow resin.

An aliquot (0.5 μg) of the 3 M guanidine thiocyanate fraction, eluted from the Chelating Sepharose Fast Flow resin was incubated with 1.25 mM calcium for 12 min. Unilamellar PS liposomes were then added and the aggregation was monitored by measuring the OD_{350} at 2 min intervals for a total of 20 min (■). A control experiment was performed with calcium alone omitting the protein (▲). The experiment was performed only once.


0.3
0.25
0.2
0.15
0.1
0.05
0
OD at 350nm
0
2
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8
10
12
14
Time (min)

protein in the sea urchin was involved in calcium-dependent vesicular aggregation. The low molecular weight 29 k polypeptide appearing in the 3 M guanidine thiocyanate eluate (Fig. 3.20) was probably not involved in liposome aggregation, as it was not present in any of the fractions which demonstrated aggregating activity. Since the 29 kDa polypeptide was not present in the starting material, we suggest that it is a peptide derived from a protein present in the starting material during the chromatographic procedure.

3.10 Yolk granule aggregation

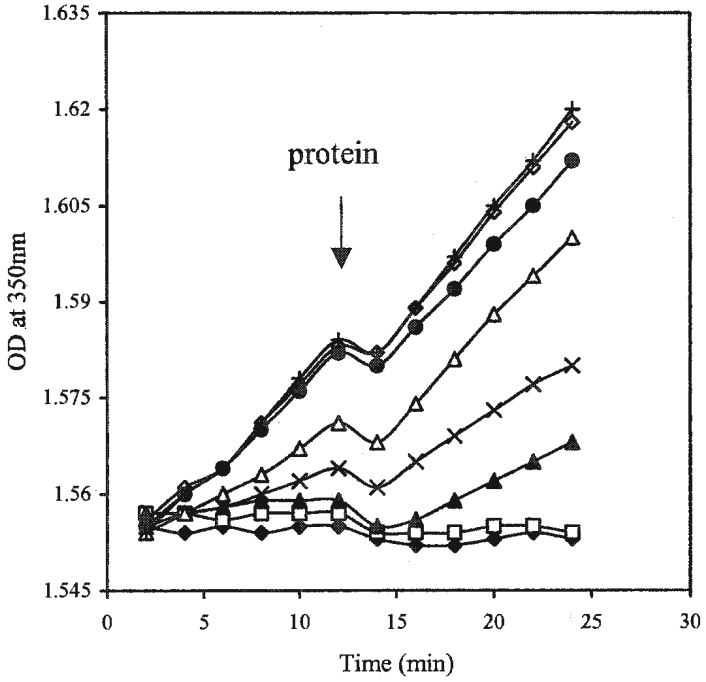
3.10.1 Proteins present in the 0.4 M fraction eluted from the fast-Q resin can drive yolk granule aggregation

We have investigated the ability of the protein present in the 0.4 M fraction eluted from the fast-Q resin, to mediate aggregation of vesicular structures, which are naturally associated with the cells in order to determine the physiological relevance of the aggregating activity. Yolk granule aggregation assays were employed to reach this goal. The assays were performed using the yolk granules prepared in 0.5 M KCl in the presence (Fig. 3.22, panel B) and absence (Fig. 3.22, panel A) of EDTA. Since EDTA can chelate calcium, the yolk granules prepared in the presence of EDTA should be deficient of any membrane proteins, bound to them in a calcium dependent manner. The aggregation assay was performed in two steps as follows: Initially only calcium was added to the yolk granule suspension and aggregation was monitored by measuring the OD_{350} at 2 min intervals for 12 min. After 12 min, the protein present in the 0.4 M fraction eluted from the fast-Q resin was added to the yolk granule suspension and the aggregation was monitored for another 12 min. The experiment was performed with

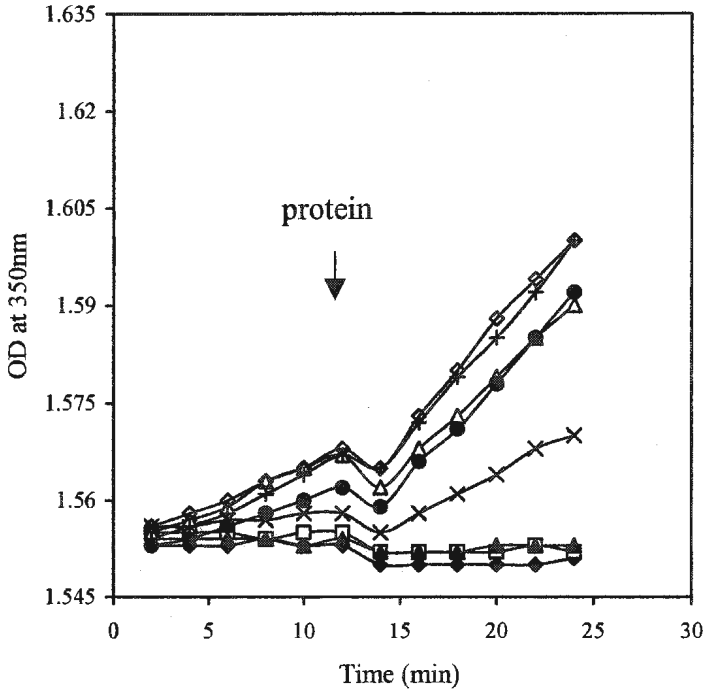
Fig. 3.22. Yolk granule aggregation assays.

Aggregation assays were performed using yolk granules prepared in 0.5 M KCl (panel A) and yolk granules prepared in 0.5 M KCl containing 1 mM EDTA (panel B). Aggregation was monitored by measuring the OD at 350 nm. At zero time, calcium was added to the yolk granule suspension and the OD was recorded at 2 min intervals for a total of 12 min. At 12 min, the protein (5 μg) present in the 0.4 M fraction eluted from the fast-Q resin was added to the yolk granule suspension and the OD monitored for another 12 min. The experiments were performed with a series of calcium concentrations, i.e. 1 μM (□), 10 μM (▲), 100 μM (×), 500 μM (△), 1mM (●), 1.25 mM (+), 2 mM (◇). A control assay was performed in the absence of calcium (♦). The dip in absorbance observed upon the addition of protein was caused by the dilution effect. This experiment was performed two times and the data presented in the figure are representative of the data obtained.

A


1.635
1.62
1.605
1.59
1.575
1.56
1.545
OD at 350nm
protein
0
5
10
15
20
25
30
Time (min)

B


1.635
1.62
1.605
1.59
1.575
1.56
1.545
OD at 350nm
protein
0
5
10
15
20
25
30
Time (min)

different calcium concentrations to determine the effect of calcium concentration on yolk granule aggregation (Fig. 3.22). The yolk granules prepared in 0.5 M KCl were able to aggregate in the presence of calcium, suggesting that the yolk granules harbor proteins, which can drive aggregation in the presence of calcium. The rate of yolk granule aggregation was positively correlated with the concentration of calcium used in the assay (Fig. 3.23). Interestingly, the yolk granules prepared in the presence of EDTA were not able to aggregate at the same rate suggesting that the EDTA treatment strips off the proteins, which mediate the yolk granule aggregation (Fig. 3.23). Collectively these observations suggest that the proteins, which drive the yolk granule aggregation, were bound to the yolk granule membrane in a calcium-dependent manner. The aggregating activity of the protein as well, was shown to be calcium-dependent.

Alternatively, another yolk granule aggregation assay was performed with the yolk granules prepared in 0.5 M KCl after extracting the granules with 0.5 M KCl containing 10 mM EGTA (Fig. 3.24). The yolk granules lost the aggregating activity upon the EGTA extraction. Supplementation of the yolk granules with the protein present in the EGTA extract could restore the lost aggregating activity of the yolk granules. A Western blot was performed on the EGTA extract using the anti-toposome antibody to identify the proteins present. The polypeptides of 240 kDa, 160 kDa, 120 kDa and 32 kDa appeared in the Western blot. The pattern of the Western blot of the EGTA extract was similar to that of the 0.4 M fraction eluted from the fast-Q resin (Fig. 3.25).

Fig. 3.23. The effect of calcium concentration on yolk granule aggregation. Aggregation assays were performed using the yolk granules prepared in 0.5 M KCl (Fig. 22, panel A) and the yolk granules prepared in 0.5 M KCl containing 1 mM EDTA (Fig. 22. panel B) in the presence of a series of calcium concentrations. The initial rates ($\triangle OD_{350}$/min) of the calcium-driven yolk granule aggregation by the yolk granules prepared in 0.5 M KCl (●) and the yolk granules prepared in 0.5 M KCl containing 1 mM EDTA (O) were calculated using the curves before protein was added and these values were plotted against the calcium concentration. This experiment was performed two times and the data presented in the figure are representative of the data obtained.

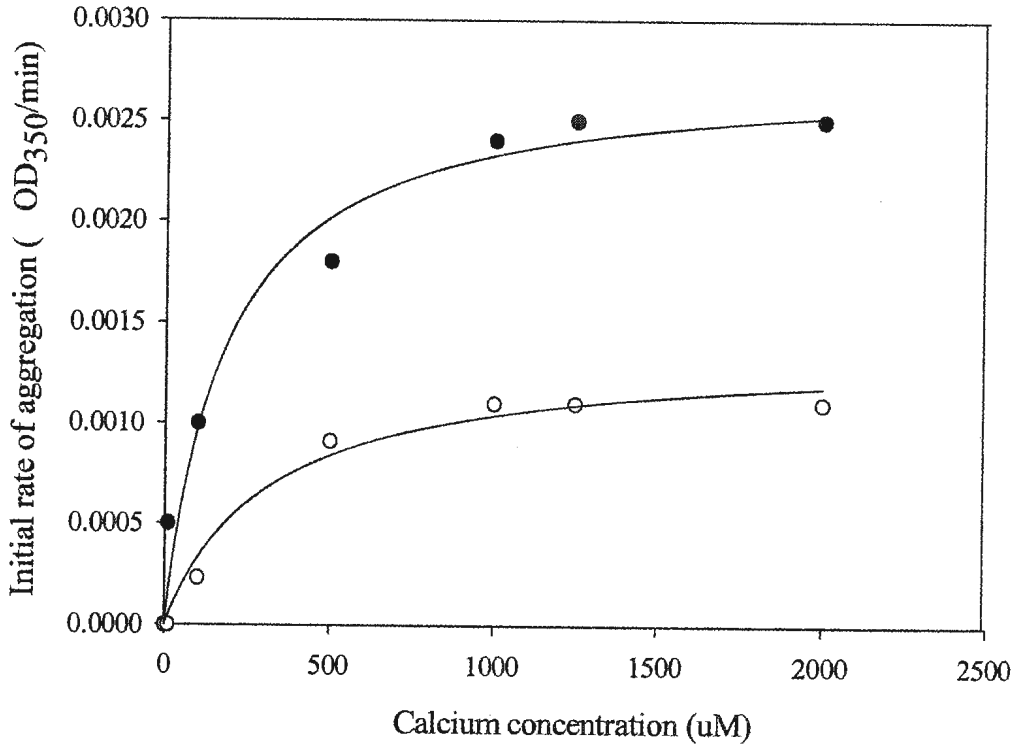

0.0030
0.0025
0.0020
0.0015
0.0010
0.0005
0.0000
Initial rate of aggregation (OD350/min)
0
500
1000
1500
2000
2500
Calcium concentration (uM)

Fig. 3.24. The effect of EGTA extraction of the granules on yolk granule aggregation. Yolk granules prepared in 0.5 M KCl were resuspended in aggregation buffer containing 0.5 M KCl. The yolk granule suspension was made 1.25 mM calcium and the aggregation was monitored by measuring the OD_{350} at 2 min intervals for a total of 20 min (▲). The effect of EGTA extraction of yolk granules was examined as follows. The yolk granules prepared in 0.5 M KCl were extracted with EGTA. Yolk granules were pelleted and the aggregation was performed with these yolk granules as explained above (■). In another assay, the protein present in the EGTA extract (10 μg) was added to the yolk granules which were extracted with EGTA before commencing the assay, to determine if the proteins present in this extract could to restore the calcium driven yolk granule aggregation (♦). A control assay was performed with yolk granules prepared in 0.5 M KCl in the absence of calcium (●). The experiment was performed only once.

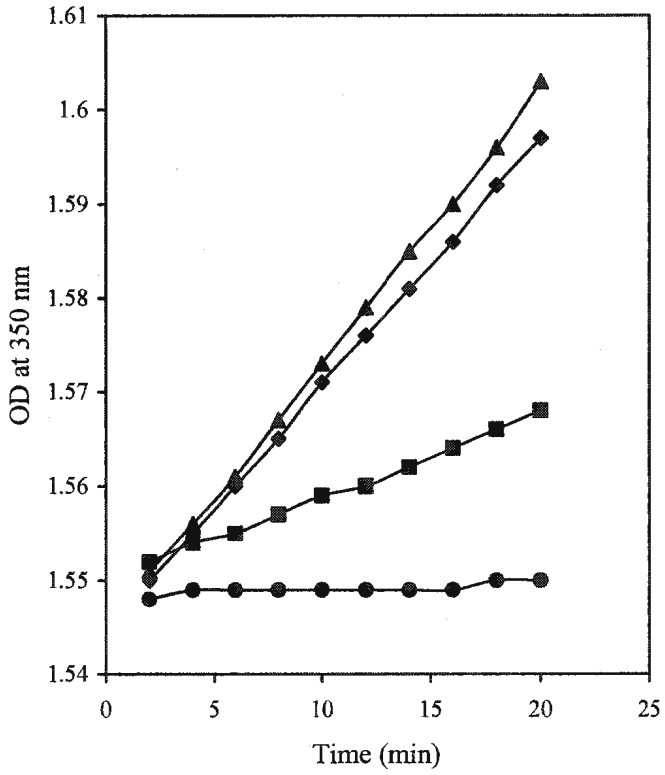
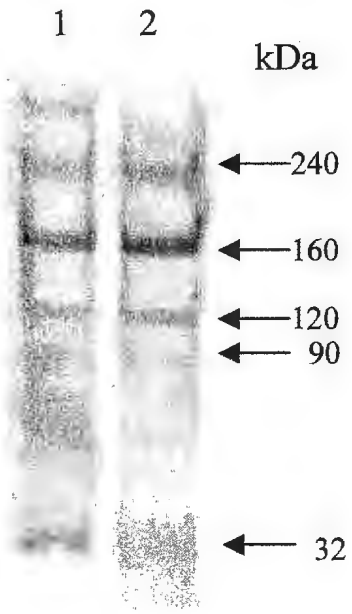

1.61
1.6
1.59
1.58
1.57
1.56
1.55
1.54
OD at 350 nm
0
5
10
15
20
25
Time (min)

Fig. 3.25. Western blot analysis of the yolk granule EGTA extract.

Aliquots (10 μg) of the yolk granule EGTA extract (lane 1) and the 0.4 M fraction eluted from the fast-Q resin (lane 2) were boiled in Laemmli solubilizing solution containing DTT, fractionated in a 3-12% (w/v) polyacrylamide gel (Laemmli, 1970) and transferred onto nitrocellulose. The nitrocellulose was probed with the anti-toposome antibody at a dilution of 1: 500 (v/v). It was then incubated for 1 hour in TTBS containing goat anti-mouse IgG conjugated with alkaline phosphatase at a dilution of 1: 3000 and the antibody was visualized by treating the membrane in 100 mM $NaHCO_3$, 1mM $MgCl_2$, pH 9.8 containing 0.03% (w/v) NBT and 0.015% (w/v) BCIP in the dark. Molecular masses of the proteins are shown.


1
2
kDa
240
160
120
90
32

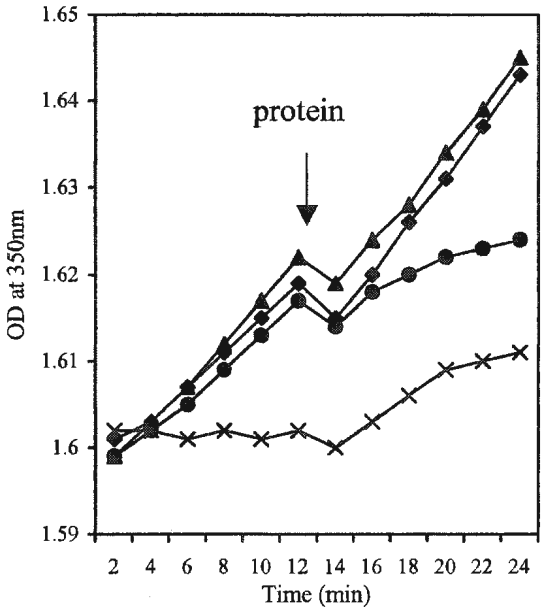
3.10.2 The anti-toposome antibody inhibits the yolk granule aggregation

The effect of the anti-toposome antibody on yolk granule aggregation was determined by pre-incubating the yolk granules with the antibody as well as by pre-incubating protein present in the 0.4 M fraction eluted from the fast-Q resin, with the antibody. Pre-incubation of the yolk granules with the anti-toposome antibody inhibited the calcium-driven aggregation of yolk granules prepared in 0.5 M KCl (Fig. 3.26, panel A). This result suggests that the proteins responsible for aggregation present in the yolk granule membranes are exposed to the periphery of the yolk granules. This allows the antibody to react directly with the proteins, thereby inhibiting their aggregating activity. Although the protein present in the 0.4 M fraction eluted from the fast-Q resin could restore the lost aggregating activity of the yolk granules prepared in the presence of EDTA, pre-incubation of the 0.4 M fraction eluted from the fast-Q resin with the anti-toposome antibody abolished this action (Fig. 3.26, panel B).

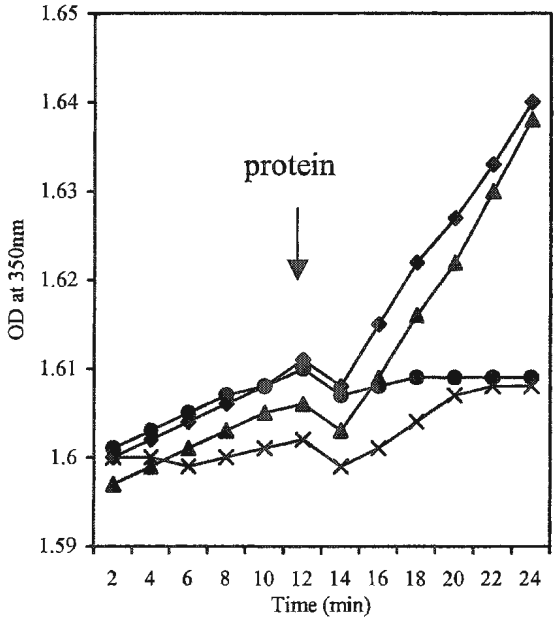
Collectively these data suggest that the aggregating activity of 240 kDa protein complex and/or one or more polypeptides contained in this protein complex is a physiologically relevant function. This result also suggests that the proteins responsible for the aggregation are originally present on the surface of the yolk granules. Both the membrane binding, as well as the aggregating activity, was shown to be mediated by calcium. Most importantly, we have demonstrated that the proteins present in the 0.4 M fraction eluted from the fast-Q resin are involved in yolk granule aggregation.

Fig. 3.26. Inhibition of yolk granule aggregation by the anti-toposome antibody. Yolk granule aggregation assays were performed using the granules prepared in 0.5 M KCl (panel A) or in 0.5 M KCl containing 1mM EDTA (panel B). At zero time, 1.25 mM calcium was added to the yolk granule suspension and the aggregation was monitored by measuring the OD_{350} at 2 min intervals for a total of 12 min. At 12 min, the protein (5 μg) present in the 0.4 M fraction eluted from the fast-Q resin was added to the yolk granule suspension and the aggregation was monitored for another 12 min (♦). The effect of anti-toposome antibody on yolk granule aggregation was tested in two ways; i.) Yolk granules were pre-incubated with the anti-toposome antibody for 20 min before commencement of the assay (×) ii.) The protein present in the 0.4 M fraction was pre-incubated with anti-toposome antibody for 20 min before adding to the yolk granule suspension (●). A control experiment was performed by pre-incubating the yolk granules with pre-immune serum to demonstrate that the pre-immune serum did not inhibit the yolk granule aggregation (▲). The dip in absorbance observed upon the addition of protein was caused by the dilution effect. The experiment was performed only once.

A


1.65
1.64
1.63
1.62
1.61
1.6
1.59
OD at 350nm
protein
2 4 6 8 10 12 14 16 18 20 22 24
Time (min)

B


1.65
1.64
1.63
1.62
1.61
1.6
1.59
OD at 350nm
protein
2 4 6 8 10 12 14 16 18 20 22 24
Time (min)

3.10.3 Exposure of yolk granules to trypsin abolishes their calcium-driven aggregation

Yolk granule aggregation assays were performed following exposure of the granules to trypsin. Trypsin, being membrane-impermeable, cleaves only the peripherally located membrane proteins. Yolk granule aggregation assays were performed using the granules prepared in 0.5 M KCl with different concentrations of trypsin (Fig. 3.27). Inhibition was not observed at a trypsin concentration of 0.2 μg/mL but a partial inhibition was observed at a trypsin concentration of 0.5 μg/mL, while a complete inhibition was seen with 1 μg/mL trypsin (Fig 3.27). These observations proved that the yolk granule aggregation was driven by proteins, which were susceptible to tryptic digestion and located at the periphery of the yolk granules. When yolk granules previously treated with trypsin, were supplemented with protein present in the 0.4 M fraction eluted from the fast-Q resin, the lost aggregation activity was restored successfully (Fig. 3.28).

3.11 Analysis of yolk granule membrane proteins

We were interested in finding whether the 240 kDa and 160 kDa polypeptides were components of the yolk granule membranes. Density gradient ultracentrifugation was employed to isolate yolk granule membranes. The fractions spanning the whole gradient were analyzed by SDS-PAGE (Laemmli, 1970) under reducing conditions to determine the protein profiles (Fig. 3.29). The 50% (w/v) and 40% (w/v) sucrose fractions (lanes from 3 to 25 and lanes from 27 to 35 respectively) had relatively higher concentration of low molecular weight and medium molecular weight proteins. The 30 k polypeptide,

Fig. 3.27. Determination of the effect of exposing yolk granules to trypsin on yolk granule aggregation.

Yolk granules prepared in 0.5 M KCl were resuspended in the aggregation buffer containing 0.5 M KCl. To find the effect of exposing yolk granules to trypsin on yolk granule aggregation, the granules were pre-incubated with trypsin at 0.1 μg/mL (◇), 0.2 μg/mL (▲), 0.5 μg/mL (×), 1 μg/mL (□) and 2 μg/mL (●) before commencing the aggregation assay. The yolk granule suspension was then made 1.25 mM calcium and the aggregation was monitored by measuring the OD_{350} at 2 min intervals for a total of 20 min. A control assay was performed in the absence of trypsin (■). The experiment was performed only once.

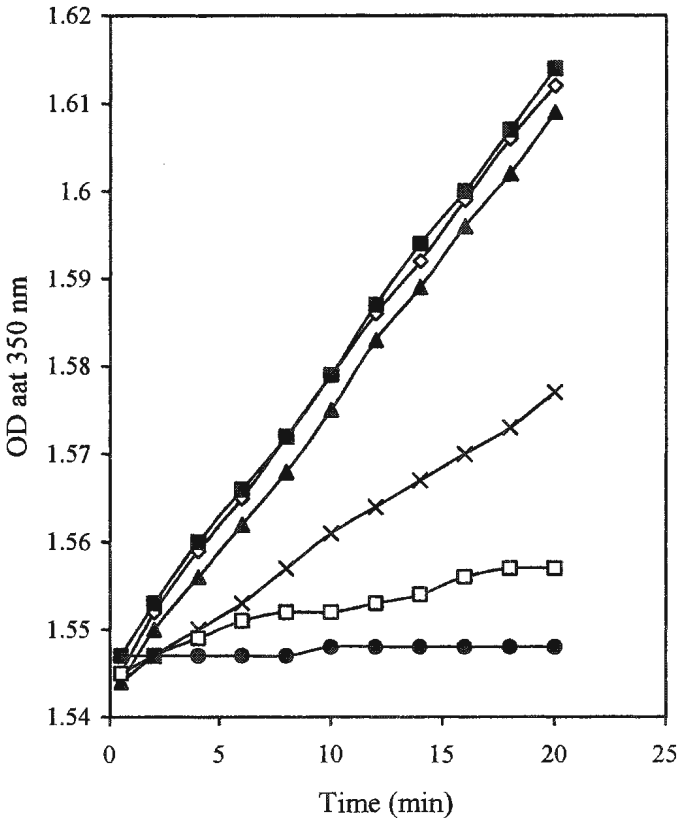

1.62
1.61
1.6
1.59
1.58
1.57
1.56
1.55
1.54
OD aat 350 nm
0
5
10
15
20
25
Time (min)

Fig 3.28. Ability of the protein present in the 0.4 M fraction eluted from the fast-Q resin to restore the lost aggregating activity of yolk granules exposed to trypsin.

The yolk granules prepared in 0.5 M KCl was resuspended in the aggregation buffer containing 0.5 M KCl. The effect of exposing yolk granules to trypsin on yolk granule aggregation was determined by two methods as following. i.) The yolk granules were pre-incubated with trypsin (1 μg) for 2 min at room temperature and the aggregation assay was performed as described below (▲). ii) Trypsin (1 μg) was added at 10 min after initiation of the yolk granule aggregation assay (♦). The ability of the protein present in the 0.4 M fraction eluted from the fast-Q resin to restore the lost aggregating activity of yolk granules exposed to trypsin was determined as following. Yolk granules exposed to trypsin (1 μg/mL) were pelleted by centrifuging in a bench top eppendorf centrifuge for few seconds and resuspended in the aggregation buffer containing 0.5 M KCl and the aggregation assay was performed. For the aggregation assays, the yolk granule suspension was made 1.25 mM calcium, the protein (5 μg) present in the 0.4 M fraction was then added and the aggregation was monitored by measuring the OD_{350} at 2 min intervals for a total of 20 min (●). Control assays were performed with the yolk granules prepared in 0.5 M KCl in the presence of 1.25 mM calcium (■) and absence of calcium to demonstrate that the yolk granule aggregation was calcium-dependent (X). The experiment was performed only once.

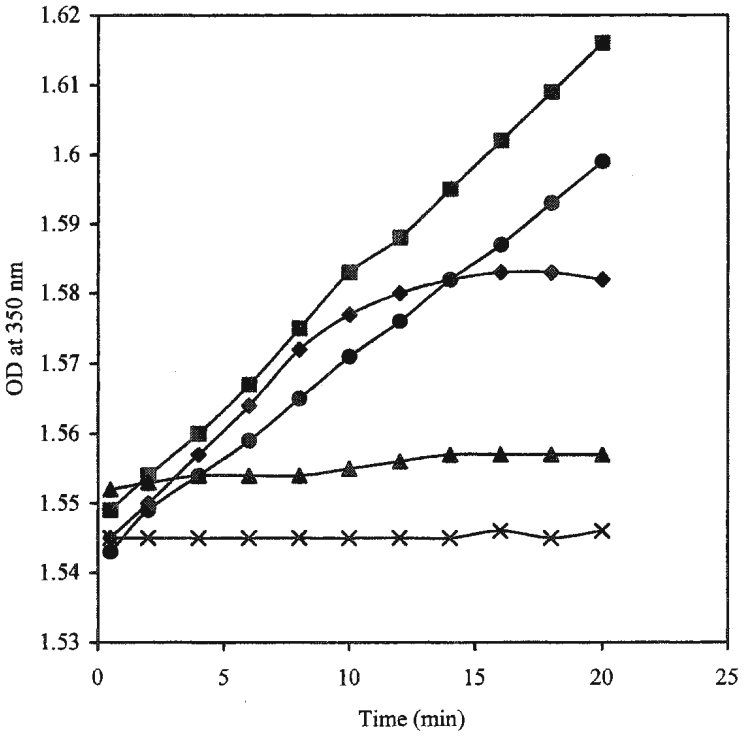
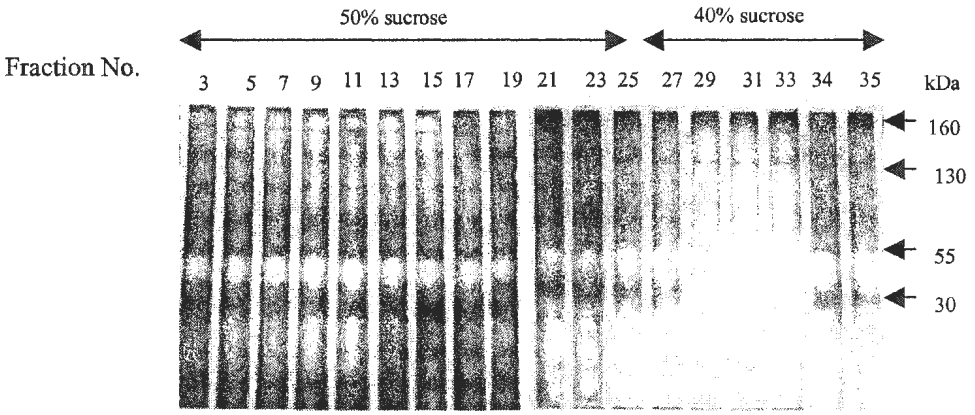
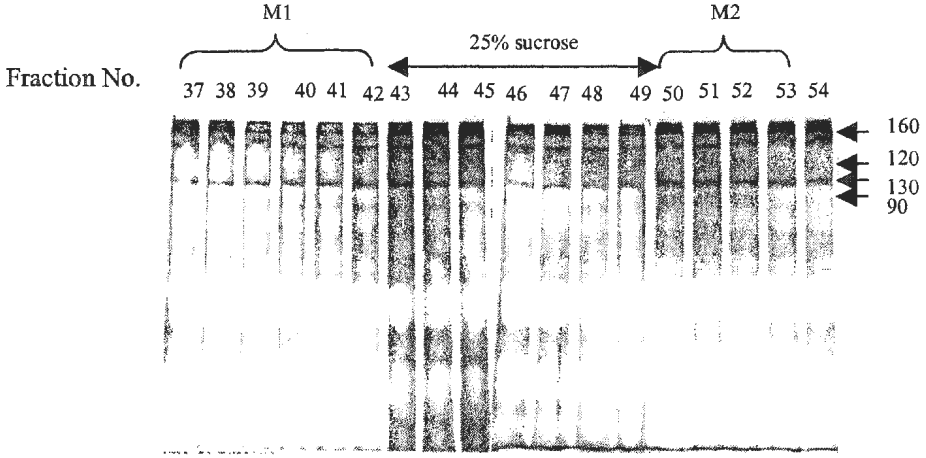

1.62
1.61
1.6
1.59
1.58
1.57
1.56
1.55
1.54
1.53
OD at 350 nm
0
5
10
15
20
25
Time (min)

Fig. 3.29. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of fractions collected from the sucrose density gradient.

Yolk granules were lysed by suspending in a hypotonic solution of 20 mM Tris (pH 7.0). The lysate was made 50% (w/v) sucrose using a 78% (w/v) sucrose stock and was layered under a discontinuous gradient of 40% (w/v) (9.5 mL), 25% (w/v) (5.5 mL and 0% (0.8 mL) sucrose. The gradient was centrifuged at 87,000xg for 16 hours and fractions of 0.5 mL were collected starting from bottom of the tube. Aliquots (5 μg) of fractions spanning the gradient were fractionated in a 10% (w/v) polyacrylamide gel (Laemmli, 1970). The gel was silver-stained. The 25/40% interface (fraction No. 37 to 42) and 0/25% interface (fraction No. 50 to 53) contained the yolk granule membranes. The fraction numbers and the corresponding sucrose densities are indicated in the figure.


50% sucrose
40% sucrose
Fraction No.
3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 34 35
kDa
160
130
55
30


M1
M2
25% sucrose
Fraction No.
37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54
160
120
130
90

which is an abundant low molecular weight species present in the yolk granules of the sea urchins, was present in larger quantities in these fractions. The latter fractions from the gradient contained relatively larger amounts of higher molecular weight species, including 160 k and 120 k polypeptides. According to Vater and Jackson (1989), as well as our observations, the membrane fraction was confined to both the 25/40% and 0/25% interfaces. Interestingly, the fractions from these interfaces mainly had high molecular weight proteins. The two sets of membrane fractions from the 25/40% and 0/25% interfaces were pooled separately (designated M1 and M2 respectively), diluted and centrifuged at high speed to pellet the membrane particles. When the final membrane preparation was analyzed by SDS-PAGE (Laemmli, 1970) under non-reducing conditions, both the M1 and M2 fractions were shown to contain 160 kDa, 130 kDa, 120 kDa, 90 kDa and 55 kDa as major polypeptides (Fig. 3.30). These results suggested that the yolk granule membrane was composed of mainly high molecular weight polypeptides of 160k, 130k, 120k and 90k. When electrophoresed under non-reducing conditions, these preparations showed a totally different protein profile (Fig. 3.31). The high molecular weight polypeptides of 240k and 160k were prevalent in the membrane preparations with some other high molecular weight polypeptides of 140k, 110k, 90k medium molecular weight polypeptides of 76 and 62 k. These results demonstrated that the 240k high molecular weight protein complex and the 160 kDa major yolk granule protein were present in the yolk granule membrane. It is possible that the yolk granule membrane is composed of proteins generated from the toposome including the 160 kDa major yolk granule protein.

Fig. 3.30. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the yolk granule membrane fractions under reducing conditions.

Yolk granule membranes were prepared by sucrose density gradient ultracentrifugation by the method of Vater and Jackson (1989). The 25/40% interface (fractions 37 to 42) and the 0/25% interface (fractions 50 to 53) containing the yolk granule membranes were pooled (M1 and M2 respectively) and centrifuged at 170,000xg to pellet the yolk granule membranes. The pellet was then resuspended in a small volume of 10 mM Tris-HCl (pH 7.0) and aliquots (5 μg) of the M1 (lane 1) and the M2 (lane 2) were fractionated in a 10% (w/v) polyacrylamide gel (Laemmli, 1970) and silver-stained. Lane 3 has molecular mass markers.

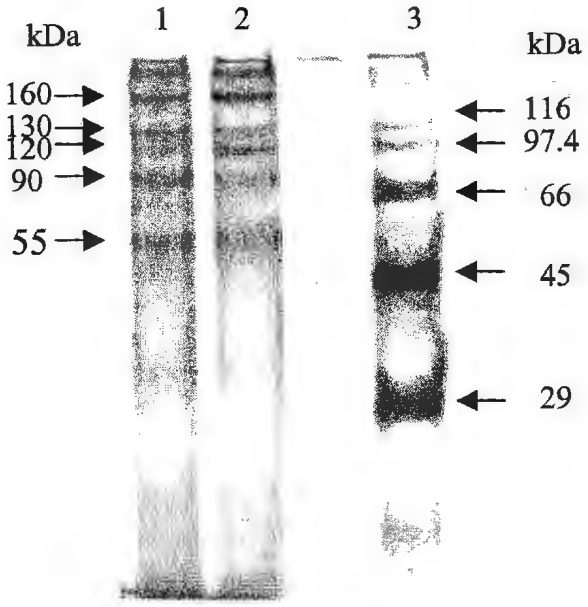
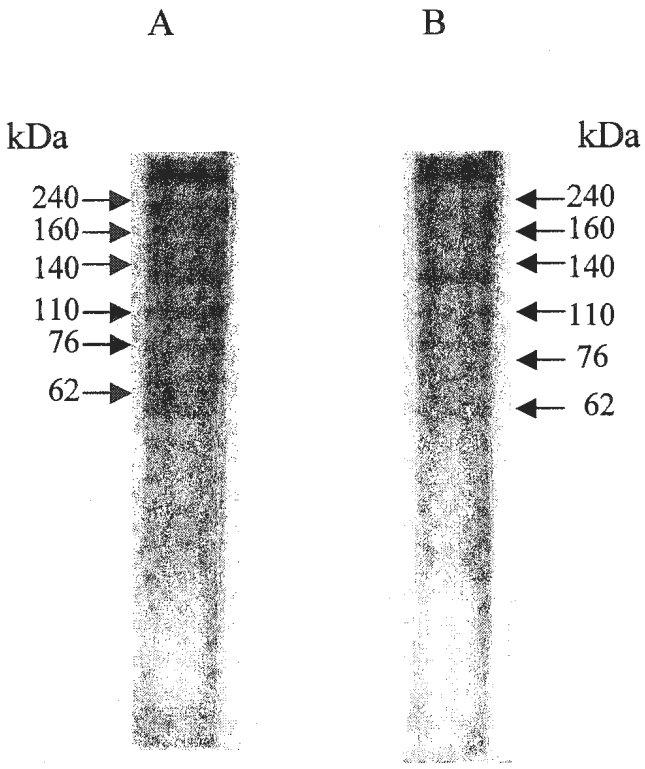

kDa
1
2
3
kDa
160
130
120
90
55
116
97.4
66
45
29

Fig. 3.31. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of yolk granule membrane preparations under non-reducing conditions.

Aliquots (5 μg) of the M1 (Panel A) and M2 (Panel B) membrane fractions were fractionated in a 10% (w/v) polyacrylamide gel (Laemmli, 1970) after boiling in Laemmli solubilizing solution without DTT. The gel was silver-stained. The molecular masses of the proteins are shown.


A
B
kDa
240
160
140
110
76
62
kDa
240
160
140
110
76
62

The M1 membrane fraction was extracted with a buffer containing EDTA to determine if the proteins were bound to membranes in a calcium-dependent manner. The membranes were pelleted, and the supernatant (EDTA extract) and the pellet were analyzed by SDS-PAGE (Laemmli, 1970) under reducing conditions (Fig. 3.32). The 160 kDa, 130 kDa, 120 kDa and 90 kDa polypeptides appeared in the supernatant suggesting that the membrane binding of these proteins was mediated by calcium. When a liposome aggregation assay was performed on the EDTA extract, an excellent liposome aggregation activity was observed, suggesting that one or more proteins present in the EDTA extract of the yolk granule membrane might be physiologically involved in aggregation of yolk granules (Fig. 3.33). When the M1 membrane fraction was washed with 0.5 M NaCl, the polypeptides of 160 kDa, 130 kDa, 120 kDa and 90 kDa were shown to be in the supernatant suggesting that these proteins could be eluted by high salt washes (Fig. 3.34). The high salt washes should release mainly the peripherally located proteins from the membranes.

3.12 Analysis of the surface proteins in eggs and embryonic cells

Immunocytochemical studies on sea urchin embryos have demonstrated that the yolk proteins are transported and present in the plasma membranes of the eggs and embryonic cells (Gratwohl *et al.*, 1990). The embryos from different stages (1 hours post fertilization (HPF), 2 HPF, 7 HPF, 25 HPF, 44 HPF and 70 HPF) were dissociated by the method of Matranga *et al.* (1986) in order to separate the cells. The embryonic cells were isolated by differential centrifugation. The eggs and the embryonic cells were extracted with EDTA. We expected to see calcium-dependent proteins present on the surface of

Fig. 3.32. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of EDTA extract of high density yolk granule membrane fraction.

The M1 membrane fraction was made 10 mM EDTA, incubated at room temperature for 30 min and was centrifuged for 30 min at 30 psi in an airfuge. Aliquots (5 μg) of the EDTA extract (supernatant, lane 1) and the membrane pellet (lane 2) were fractionated in a 10% (w/v) polyacrylamide gel (Laemmli, 1970). A control assay was performed in parallel in the absence of EDTA (supernatant, lane 3 and pellet, lane 4). The gel was silver-stained. Lane 5 has molecular mass markers.

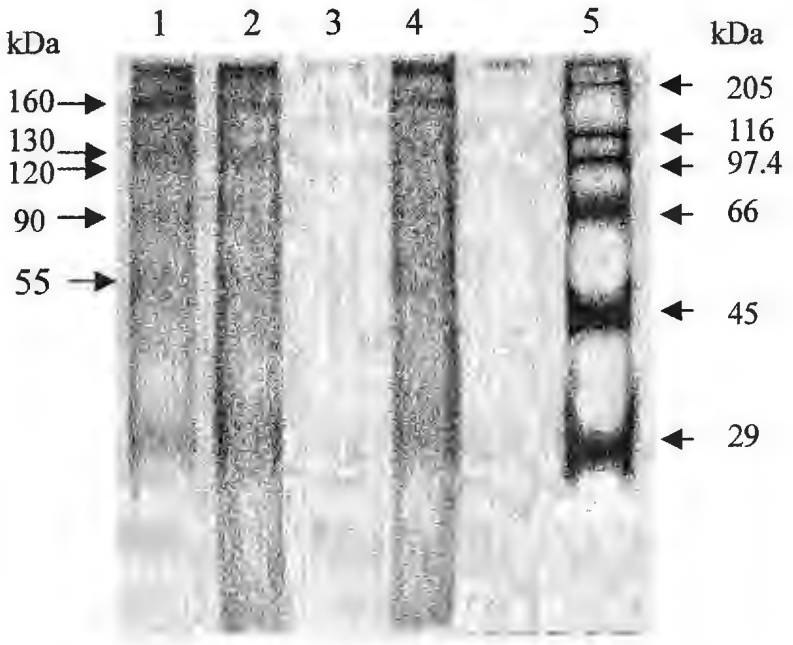

kDa
1
2
3
4
5
kDa
160
130
120
90
55
205
116
97.4
66
45
29

Fig. 3.33. Liposome aggregation assay on EDTA extracts of the yolk granule membrane fractions.

Aliquots (5 μg) of the M1 (×) and the M2 (♦) membrane fractions were pre-incubated with 1.25 mM calcium for 12 min. Unilamellar PS liposomes were then added and the aggregation was monitored by measuring the OD_{350} at 2 min intervals for 14 -20 min. Control assays were performed with protein present in the EDTA extract of M1, in the absence of calcium (■), protein present in the EDTA extract of M2 in the absence of calcium (O), and with 1.25 mM calcium in the absence of protein (▲). The experiment was performed only once.

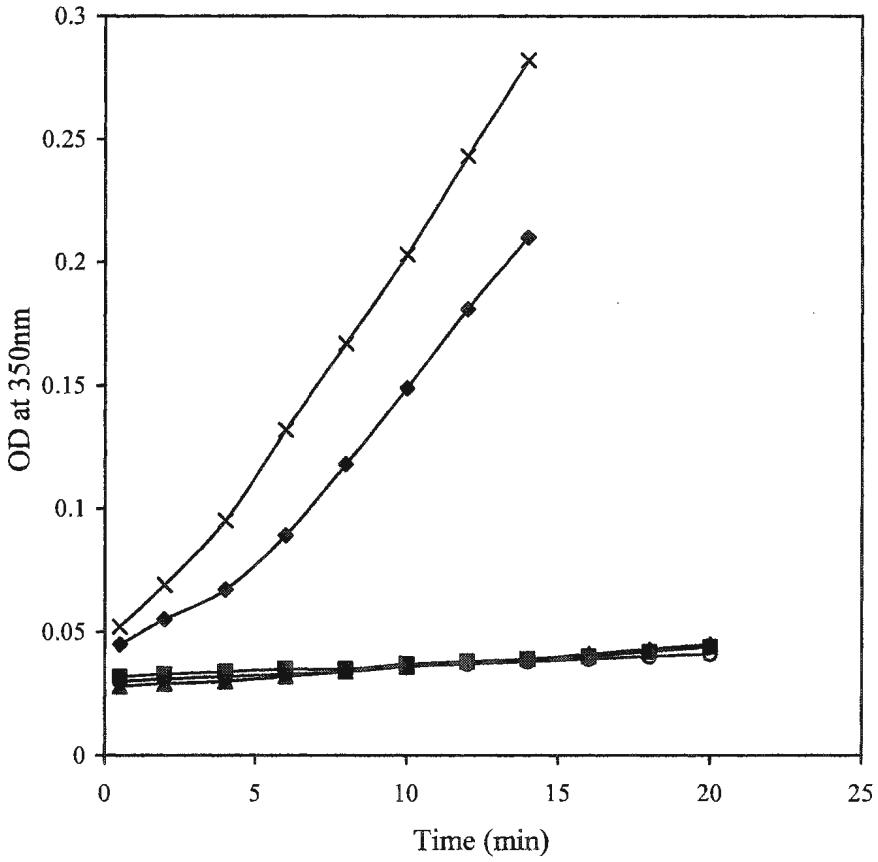
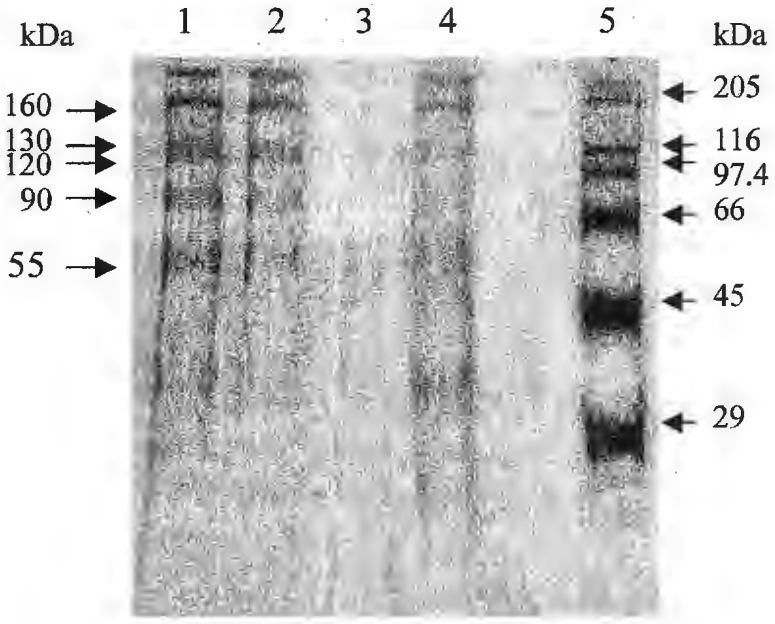

0.3
0.25
0.2
0.15
0.1
0.05
0
OD at 350nm
0
5
10
15
20
25
Time (min)

Fig. 3.34. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of salt extract from yolk granule membrane fractions.

The M1 membrane fraction was made 0.5 M NaCl, incubated at room temperature for 30 min and was centrifuged for 30 min at 30 psi in an airfuge. Aliquots (5 μg) of the salt extract (lane 1) and the membrane pellet (lane 2) were fractionated in a 10% (w/v) polyacrylamide gel (Laemmli, 1970). A control assay was performed in parallel in the absence of NaCl (supernatant, lane 3 and pellet, lane 4). The gel was silver-stained. Lane 5 has molecular mass markers.


kDa
1
2
3
4
5
kDa
160
130
120
90
55
205
116
97.4
66
45
29

these cells in these EDTA extracts. Sodium dodecyl sulfate gel electrophoresis (Laemmli, 1970) under reducing conditions demonstrated that these extracts were enriched with high molecular weight polypeptides including 240k, 160k (Fig. 3.35). As development proceeds, these high molecular weight proteins decrease while the low molecular weight proteins start to appear. The most striking change was the gradual disappearance of the 160 kDa polypeptide by 7 HPF, with the appearance of relatively lower molecular weight polypeptides ranging from 90 k to 44 k.

A Western blot was performed on the EDTA extract of eggs and cells from 25 HPF embryos using the anti-toposome antibody (Fig. 3.36). The anti-toposome antibody cross-reacted with 240 kDa, 160 kDa, 120 kDa and 90 kDa polypeptides present in the egg EDTA extract (Fig. 3.36, lane 1), while it cross-reacted with some lower molecular weight species of 55 k, 44 k and 32 k in the EDTA extracts of the cells from 25 HPF stage (Fig. 3.36, lane 2). This suggested that the lower molecular weight polypeptides present in the EDTA extract of the cells from the 25 HPF stage were derived from the toposome. Obviously the 240 kDa toposome and the 160 kDa major yolk granule protein present in the surface of the embryos are not been proteolytically processed while they are sitting on the cell surface, since the enzyme involved in yolk protein processing is located in the yolk granules. Therefore, this observation suggests that the yolk proteins, which are enzymatically modified, are continuously transported to the plasma membrane throughout the embryonic development.

Fig. 3.35. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of egg and embryonic cell extracts.

Embryos were dissociated using the method explained by Matranga *et al.* (1986). Embryonic cells were isolated by differential centrifugation. Eggs and the embryonic cells were washed with CMFSW and extracted with CMFSW containing 10 mM EDTA. Extracts were spun at 87,000xg to obtain the supernatants. Aliquots (5 μg) of extracts from the eggs (lane 1) and the embryos of 1 HPF (lane 2), 2 HPF (lane 3), 7 HPF (lane 4), 25 HPF (lane 5) and 70 HPF (lane 6) were fractionated in a 3-12% (w/v) polyacrylamide gel (Laemmli, 1970) and silver-stained. Lane 7 has molecular mass markers.

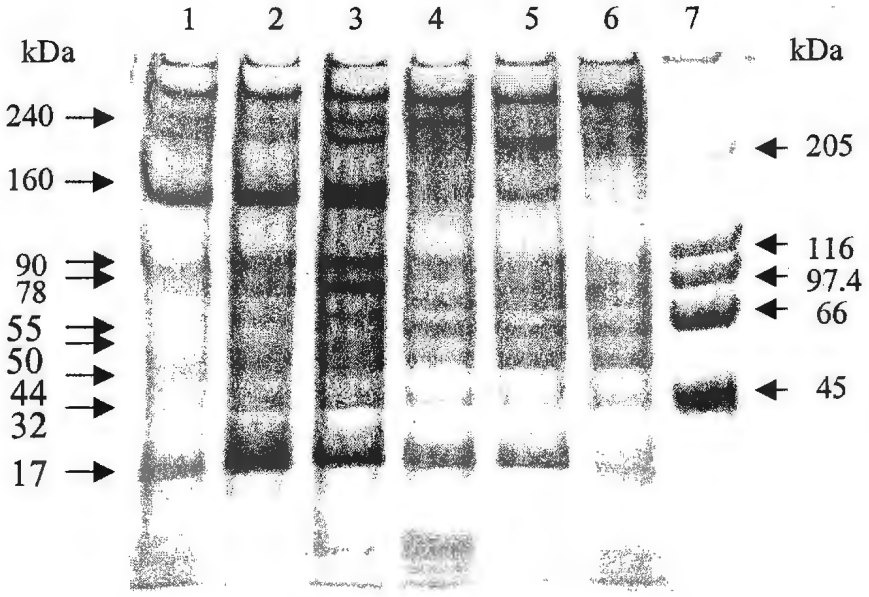
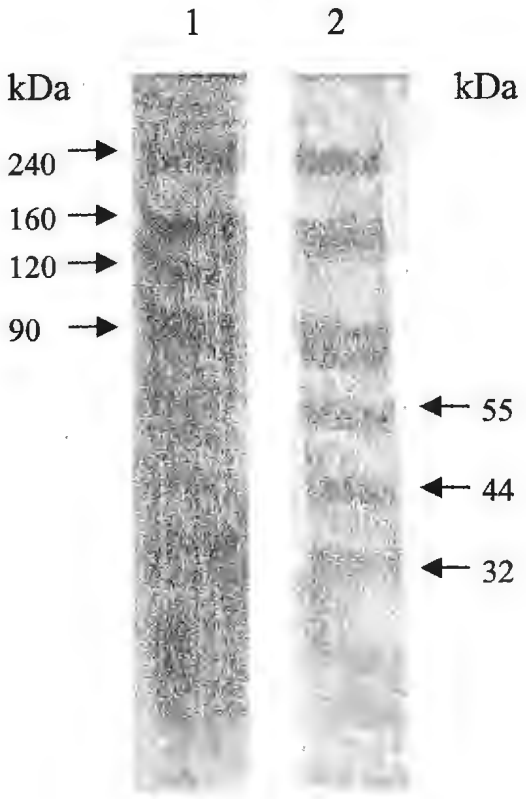

1
2
3
4
5
6
7
kDa
240
160
90
78
55
50
44
32
17
kDa
205
116
97.4
66
45

Fig. 3.36. Western blot analysis of EDTA extracts of eggs and cells from 25 HPF embryos.

Aliquots (5 μg) of the EDTA extracts of eggs (lane 1) and cells from 25 HPF embryos (lane 2) were fractionated in a 3-12% (w/v) polyacrylamide gel (Laemmli, 1970) and transferred onto nitrocellulose. The nitrocellulose membrane was probed with the anti-toposome antibody at a dilution of 1: 500 (v/v). It was then incubated for 1 hour in TTBS containing goat anti-mouse IgG conjugated with alkaline phosphatase at a dilution of 1: 3000 and the antibody was visualized by treating the membrane in 100 mM $NaHCO_3$, 1mM $MgCl_2$, pH 9.8 containing 0.03% (w/v) NBT and 0.015% (w/v) BCIP in the dark. Molecular masses of the proteins are shown.

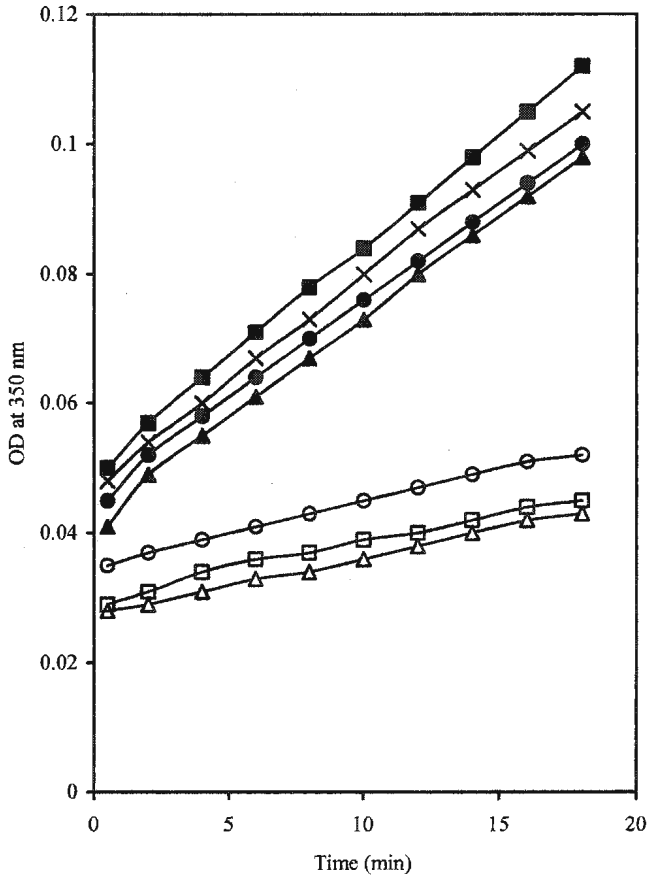

1
2
kDa
kDa
240
160
120
90
55
44
32

A liposome binding assay was performed with the protein present in the 25 HPF embryonic cell extract. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the liposome pellet under reducing conditions demonstrated that the polypeptides of 90 kDa, 55 kDa, 50 kDa, 44 kDa, and 32 kDa were present in this fraction. When the liposome pellet was analyzed by SDS-PAGE under non reducing conditions, a single band at 240 kDa was observed (results not shown). This result suggested that the 240 kDa protein complex was capable of binding to the liposomes even after further proteolytic processing. Unlike the 240 kDa protein complex present in the eggs, this protein complex contains relatively lower molecular weight polypeptides of 90 k, 55 k, 50 k, 44 k, and 32 k. The 160 kDa and the 120 kDa polypeptides were reduced in quantity in this protein complex suggesting that these polypeptides were proteolytically processed to give rise to the lower molecular weight polypeptides.

A liposome aggregation assay was performed with the EDTA extracts of embryonic cells from different developmental stages to investigate whether the proteolytic processing of the yolk proteins affects the liposome aggregating activity (Fig. 3.37). Protein (10 μg) was used in the assays after extensively dialyzing the extracts to remove EDTA. All the protein extracts exhibited liposome aggregating activity. The rate of aggregation was not significantly affected by the proteolytic processing of the 240 kDa protein. The liposome aggregation driven by the proteins present in the 25 HPF embryonic cell extract was inhibited when the protein present in this extract was pre-incubated with the anti-toposome antibody for 20 min prior to the aggregation assay (fig. 3.37). These results suggest that the aggregation was driven by the 240 kDa complex or the polypeptides derived from it.

Fig. 3.37. The effect of proteolytic processing of the 240 kDa toposome on liposome aggregating activity.

Aliquots (10 μg) of the EDTA extract of eggs (■) and EDTA extracts of cells from 1 HPF embryos (×), 7 HPF embryos (●) and 25 HPF embryos (▲) were incubated with 1.25 mM calcium in the aggregation buffer for 12 min. Unilamellar PS liposomes were then added and the aggregation was monitored by measuring the OD_{350} at 2 min intervals for 18 min. Another aggregation assay was performed with the 25 HPF embryonic cell extract after pre-incubating the protein present in this extract with the anti-toposome antibody for 20 min (O). Control assays were performed with EDTA extracts of egg (□) and EDTA extracts of cells from 25 HPF embryos (△) in the absence of calcium. The experiment was performed only once.


0.12
0.1
0.08
0.06
0.04
0.02
0
OD at 350 nm
0
5
10
15
20
Time (min)

Chapter 4: Discussion

4.1 Role of the yolk granule in embryonic development

The yolk granule is the most abundant organelle present in the eggs and early embryos of many animals, including avians, amphibians, molluscs and echinoderms. The echinoderm yolk granules comprise about one third of the volume of the egg (Armant *et al.*, 1986). Although the yolk granule has been studied for several decades, little is known about the function of this organelle in embryonic development. The classical view of the yolk granule is that it provides nutrition to the growing embryos. Of course, this fact is true for most of the other animals whose eggs house yolk granules. For example, the yolk granule of avians, insects and amphibians has been widely studied and is believed to serve as a storage site for raw material needed to sustain embryonic development (Willey and Wallace, 1981; Wallace, 1985, Mc-Gregor and Laughton, 1977). Based on its basic structure and composition, it is believed that the sea urchin yolk granules have the same function in embryonic development.

Armant *et al.* (1986) observed that the composition of the yolk granules and yolk-associated contents, including protein, lipid, carbohydrate and nucleic acid remain constant throughout development until the 7^{th} day of larval development in the sea urchin, *Arbacia punctulata*. Starvation of the larvae did not speed up the process of yolk utilization suggesting that the yolk components were not to be used when food was scarce. The finding that the sea urchin yolk granules did not change in composition until later stages of larval development, even during starvation raised the question of the function of the yolk granule.

The sea urchin yolk granule is not involved in anabolic processes, as it does not contain anabolic enzymes (Armant *et al.*, 1986). The RNA present in the yolk granules is

translationally inert, suggesting that the yolk granule is not a site of *de novo* synthesis of proteins (Armant *et a.l*, 1986). Other reports examining yolk granule have proposed several functions for it, but whether these can be applied to all the animal systems is questionable. For example, in the sea urchin, vitellogenin is found to be present in gametes from both sexes (Shyu *et al.*, 1986) and the reason for its occurrence in male gametes was unknown, until Unuma *et al.* (1998) found that it is involved in providing nutrition to the growing spermatozoa. Yolk proteins have been shown to bind lipids, hormones, vitamins and metal ions, thereby participating in storage and transportation of these materials (Laueux *et al.*, 1981; Kunkel and Nordin, 1985; Byrne *et al.*, 1989; Dhadialla and Raikhel, 1990 and Niimi *et al.*, 1994). The finding that the major yolk granule protein is involved in iron binding suggests that the sea urchin yolk proteins are engaged in some important functions both in male and female gametes (Brooks and Wessel, 2002).

Several studies have shown that the yolk granules house components destined to be exported. Mayne and Robinson (1998) have observed that HLC-32, which is a major component present in the extracellular matrices (both the hyaline layer and basal lamina) of the sea urchin, is initially present in the yolk granules. As revealed by immunohistochemical studies, this component gradually disappears from the yolk granules as development proceeds. This disappearance was shown to be concomitant with the appearance of HLC-32 in the extracellular matrices, suggesting that it is exported from the yolk granules as development proceeds. In addition, echinonectin, which is a component of the hyaline layer is found to be associated with yolk granules (Fuhrman *et al.*, 1992). The RNA found in yolk granules, which is translationally inert (Armant *et al.*,

1986), might be transported to and activated in the cytoplasmic compartment of the cell. These findings support the hypothesis that the yolk granule is involved in storage and transportation events in the embryo. In this context, Srivastava *et al.* (1996) identified synexin (annexin VII) in yolk granules of *Xenopus laevis* embryos. Synexin is a calcium-dependent phospholipid binding protein, which forms calcium channels and is involved in membrane fusion events. It is thought to play a significant role in exocytotic secretion.

Based on these data, we hypothesized that the yolk granule might be involved in intracellular transportation and membrane fusion and the yolk proteins might play a key role in these events. Findings that the yolk proteins are associated with yolk granule membrane as well as other membranal structures, mainly the newly formed cytoplasmic membranes of embryonic cells, partially supported our hypothesis. In further support of this hypothesis, Cervello and Matranga (1989) demonstrated that the toposome has the capacity to mediate embryonic cell aggregation, which is a calcium-dependent process. Our study was mainly focused on biochemical characterization of yolk proteins and especially, their role in membrane binding and vesicular aggregation.

4.2 Biochemical characterization of yolk proteins

4.2.1 Toposome in *Strongylocentrotus purpuratus* eggs is composed of three polypeptides

The toposome is the precursor for the majority of the yolk proteins (Kari and Rotman, 1985; Armant *et al.*, 1986; Scott and Lennarz, 1988; Lee *et al.*, 1989; Yokota *et al.*, 1993). It is found to be processed proteolytically, giving rise to lower molecular weight yolk proteins (Kari and Rotman, 1985; Armant *et al.*, 1986; Scott and Lennarz,

1988; 1989; Yokota *et al.*, 1993). A maternally-derived cathepsin-B like protease is found to be engaged in this limited proteolysis of the yolk protein precursors (Fausto *et al.*, 2001; Mallya *et al.*, 1992). Despite this proteolytic processing, the molecular mass of the toposome remains constant throughout embryonic development since the polypeptides derived remain bound to each other by disulfide links (Armant *et al.*, 1986; Scott and Lennarz, 1988; 1989; Yokota *et al.*, 1993).

According to Scott and Lennarz (1988), the toposome of *Strongylocentrotus purpuratus* is a protein of 243 kDa. We have isolated a 240 kDa species from the yolk granule protein extracts using anion exchange chromatography. The 240 kDa polypeptide was bound to the resin and was eluted mainly in the 0.3 M and 0.4 M salt fractions. Sodium dodecyl sulfate gel electrophoresis of this protein under reducing and non-reducing conditions demonstrated that it was composed of three polypeptides of 160 kDa, 120 kDa and 90 kDa linked together by disulfide bonds. This observation is consistent with the finding of Scott and Lennarz (1988) who demonstrated that the 243 toposome, which they have isolated from the sea urchin egg, was composed of three polypeptides of 160 kDa, 115 kDa and 90 kDa. It is not surprising that chromatographic procedures could not be successfully employed to separate these polypeptides present in the 0.4 M NaCl eluate since they remain covalently linked by disulfide bridges even after proteolytic processing.

Anti-toposome antibody raised against the toposome of *Paracentrotus lividus* cross reacted with the 240 kDa, 160 kDa and 120 kDa polypeptides in an immunoblot, suggesting that the 240 kDa polypeptide was the toposome in *Strongylocentrotus purpuratus* while the 160 kDa polypeptide was the major yolk granule protein derived

from the toposome. In addition, the 160kDa polypeptide was abundant in the yolk granule protein extracts, further suggesting that it is the major yolk granule protein present in *Strongylocentrotus purpuratus.* Peptide mapping confirmed that the 240 kDa polypeptide was the precursor for the 160 kDa and 120 kDa polypeptides since they shared common peptides with the 240 kDa polypeptide. According to our observations, as well as the findings of Scott and Lennarz (1988), the 160 kDa polypeptide is the major yolk granule protein derived from the 240k high molecular weight toposome of *Strongylocentrotus purpuratus.*

4.2.2 Calcium-dependent phospholipid binding and vesicular-aggregating activity of the toposome

We were interested in defining a biological role for the yolk granule in the sea urchin embryonic system, mainly by investigating whether the yolk granule-associated proteins are involved in membrane binding and vesicular aggregation events. Many proteins involved in membrane aggregation, fusion and intracellular transportation belong to the family of annexins. These proteins, which are involved in membrane fusion events, bind acidic phospholipids in a calcium-dependent manner (Grewal *et al.*, 2000; Filipenko and Waisman, 2000; Mailliard *et al.*, 1995; Stradal and Gimona, 1999, Boustead *et al.*, 1993; Spenneberg *et al.*, 1998; Creutz *et al*, 1996; Maekawa *et al.*, 1994; Koster *et al.*, 1993). Calcium binding provides positive charges for the proteins, which can mediate acidic phospholipid binding (Lee *et al.*, 1997; Spenneberg *et al.*, 1998; Boustead *et al.*, 1993; Filipenko and Waisman, 2000).

To achieve our goal, a liposome-binding assay (Spenneberg *et al.*, 1998) was employed to characterize the calcium-dependent binding of yolk granule protein to multilamellar liposomes. In addition, a unilamellar phosphatidyl serine aggregation assay (Lee and Pollard, 1997) was utilized to investigate the ability of yolk proteins to drive vesicular aggregation. The aggregation assays performed with the individual fractions from the fast-Q separation demonstrated that the 0.3 M, 0.4 M and 0.5 M salt-elutions contained liposome aggregating activity (Fig. 3.12). All of these fractions had three major polypeptides, as revealed by reducing SDS-PAGE analysis (Fig. 3.2). The 0.3 M and 0.4 M fractions had the same protein profile comprising 160 kDa, 120 kDa and 90 kDa polypeptides (fig. 3.2). On the other hand, the 0.5 M fraction had a slightly different protein profile having polypeptides of 160 kDa, 120 kDa, 112 kDa and also a minor polypeptide of 55 kDa (Fig. 3.2). Non-reducing SDS-PAGE analysis demonstrated that all the 0.3 M, 0.4 M and 0.5 M fractions contained a high molecular weight polypeptide of 240 k (Fig. 3.3). Studies done on the insect, *Carausius morosus* have demonstrated that the vitellogenin can occur in different variant forms, which are composed of different sets of polypeptides (Giorgi *et al.*, 1997). It is possible that, in *Strongylocentrotus purpuratus*, there are two variant forms of the toposome which give rise to the 160 kDa major yolk granule protein. Observation of the 160 kDa major yolk granule protein and the 120 kDa polypeptide in all the three fractions suggest that one or both of these proteins could be involved in liposome aggregation. Possibly, the 90 kDa polypeptide appearing in the 0.3 M and 0.4 M fractions and 112 kDa and 55 kDa polypeptides appearing in the 0.5 M fraction are also involved in liposome aggregation.

We were then interested in biochemically characterizing the phospholipid binding and vesicular aggregating activities of the 240 kDa polypeptide, which is the toposome of *Strongylocentrotus purpuratus*. Liposome binding assays performed on the proteins present in the 0.4 M fraction eluted from the fast-Q resin demonstrated that the 240 kDa complex could bind liposomes in a calcium-dependent manner (Figs. 3.9 and 3.10). One can clearly see that the extent of binding is dependent on the concentration of free calcium present in the medium (Fig. 3.11). Maximum binding of 25 % was observed at a calcium concentration of 200 μM. Above the concentration of 200 μM, the percentage bound remained constant at 25% possibly because the binding sites on the liposomes were limiting (Fig. 3.11).

The calcium concentration in the unfertilized sea urchin egg is 0.1 μM (Torok *et al.*, 1998). Even during calcium influx that occurs in a few milliseconds after fertilization, the intracellular calcium level rises only to 2 μM (Torok *et al.*, 1998). We have observed that the calcium concentrations required for the vesicular binding as well as aggregation assays were higher than the physiological intracellular calcium concentration. This is consistent with the work from other laboratories (Lee and Pollard, 1997; Koster *et al.*, 1993). This is not surprising since *in vitro* systems are incomplete systems, which are deficient in important factors responsible for mediating phospholipid binding and vesicular aggregation. On the other hand *in vivo* systems most probably utilize efficient mechanisms to bind phospholipids even at lower calcium concentrations.

The 240 kDa protein complex was also capable of driving calcium-dependent unilamellar liposome aggregation (Fig. 3.12). The rate of unilamellar liposome aggregation was clearly dependent on the protein concentration (Fig. 3.16). Analysis of a

unilamellar liposome pellet by SDS-PAGE revealed that the whole 240 kDa protein complex was associated with the liposomes (Fig. 3.13). The percentage binding reached approximately 90%. In contrast, the liposomes prepared using brain lipids bound only about 25% of protein present in the 0.4 M fraction eluted from the fast-Q resin. This result suggests that, the liposomes prepared using phosphatidyl serine strongly interact with the protein. Brain lipids on the other hand are composed of only 50 % of phosphatidyl serine (Refer to 2.2.8.2.1). It must be noted that the proteins involved in membrane fusion events (especially annexins) have a high affinity for acidic phospholipids (Lee *et al.*, 1997; Spenneberg *et al.*, 1998; Boustead *et al.*, 1993; Filipenko and Waisman, 2000).

Calcium, which is a positively charged ion, has been shown to mediate binding of annexin like proteins to the acidic phospholipids. The 240 kDa protein complex, in its non reduced state, was capable of binding calcium as shown by a radiolabeled calcium binding assay (Fig. 3.7). We then examined the effect of the other metal ions on liposome aggregation driven by the 0.4 M fraction eluted from the fast-Q resin. According to Lee and Pollard (1997), liposome aggregation is a divalent cation-dependent process. It was demonstrated that barium, but not magnesium, supports annexin driven liposome aggregation (Lee and Pollard, 1997). We have demonstrated that the liposome aggregation driven by protein present in the 0.4 M fraction eluted from the fast-Q resin was dependent on calcium as well as barium, but not magnesium, supporting the view that the protein present in this fraction has annexin like properties (Fig. 3.18).

The liposome binding as well as the aggregating activity may be mediated by only one or two polypeptides present in the 240 kDa protein complex. There is evidence to

show that the polypeptides derived from the toposome remain bound to each other by disulfide bonds even after the proteolytic processing (Yokota *et al.*, 1993; Scott and Lennarz, 1988; Scott *et al.*, 1990). We suggest that the disulfide linkages between the polypeptides present in the 240 kDa protein complex could be reduced by treating the protein complex with 100 mM DTT at 37°C for 1 hour (Fig. 3.4). When the 0.4 M fraction eluted from the fast-Q resin treated as above was used in the liposome aggregation assay, a good level of liposome aggregating activity was observed (Fig. 3.14). As revealed by SDS-PAGE analysis under reducing conditions (Laemmli, 1970), all three polypeptides were found to be associated with the liposome pellet, despite using the 0.4 M fraction eluted from the fast-Q resin, treated with DTT (Fig. 3.15). It is possible that all three polypeptides have the capacity to bind to liposomes and drive liposome aggregation. On the other hand, the polypeptides might remain associated by the help of ionic, hydrophobic and/or hydrogen bonds.

The yolk granule aggregation assay allowed us to directly observe the physiological relevance of the aggregating activity of the 240 kDa protein complex. The yolk granules used in the assay were prepared in 0.5 M KCl in the presence and absence of EDTA. The observation that the yolk granules prepared in 0.5 M KCl, preserving its membrane bound protein, aggregated in the presence of calcium suggested that the protein originally contained in the yolk granule membrane was involved in granule aggregation (Fig. 3.22). In contrast, the yolk granule aggregation could be decreased by extracting granules with EDTA or EGTA. This suggested that the proteins responsible for granule aggregation were bound to yolk granule membranes in a calcium-dependent manner and washed off by EDTA or EGTA treatment (Fig. 3.22 and Fig. 3.24

respectively). One can see that the rate of yolk granule aggregation is dependent on the calcium concentration in the medium (Fig. 3.22). Supplementation of the yolk granules prepared in the presence of EDTA with the proteins present in the 0.4 M fraction eluted from the fast-Q resin, restored the aggregating activity of the granules (Fig. 3.22). Based on these observations, we suggest that, the 240 kDa protein complex, which is the toposome of *Strongylocentrotus purpuratus,* plays a major role in yolk granule aggregation. This action could be physiologically relevant since the 240 kDa protein complex is found to be a component present in the yolk granule membrane (Figs. 3.30 and 3.31). By mediating yolk granule aggregation and possibly fusion, the 240 kDa protein complex may play a role in an export pathway involving these granules.

Exposure of yolk granules to trypsin completely abolished the calcium-driven yolk granule aggregation, demonstrating that the granule aggregation was a protein-dependent process (Figs. 3 27 and 3.28). It is worth noting that trypsin, which is membrane impermeable, could only cleave peripherally located membrane proteins. Therefore, these observations suggest that the proteins responsible for yolk granule aggregation are located on the outer surface of the yolk granule membranes.

The anti-toposome antibody blocked the calcium-dependent phospholipid binding activity of the 240 kDa protein complex, suggesting that the antibody can specifically interact with the complex to reduce the binding of the protein to the multilamellar liposomes (Figs. 3.9 and 3.10). The liposome aggregation driven by the 240 kDa protein complex was decreased in the presence of anti-toposome antibody (Fig. 3.19). We have also observed that yolk granule aggregation could be reduced by pre-incubating yolk granules with the anti-toposome antibody (Fig. 3.26). The inhibition may have resulted

from antibody blocking the binding sites of phospholipids or calcium in the 240 kDa protein complex.

4.3 Yolk granule membrane is rich in high molecular weight yolk proteins

The yolk granule proteins are thought to be utilized for the assembly of new membranes. (Gratwohl *et al.*, 1990). It has been also noted that the yolk proteins are constituents of yolk granule membranes (Gratwohl *et al.*, 1990). We have isolated yolk granule membranes by sucrose density gradient ultracentrifugation following the method of Vater and Jackson (1989). According to Vater and Jackson (1989), as well as our observations, the yolk granule membranes could be separated into two fractions of differing buoyant densities. This may be a consequence of the occurrence of two buoyant classes of yolk granules as observed by Armant *et al.* (1985). Sodium dodecyl polyacrylamide gel electrophoresis of the two different classes of yolk granule membranes suggested that the protein present in both the yolk granule membrane classes were qualitatively similar. Both the yolk granule membrane classes contained high molecular weight proteins where we mainly observed polypeptides of 160 kDa, 130 kDa, 120 kDa and 90 kDa (Fig. 3.30). Sodium dodecyl sulfate gel electrophoresis under non-reducing conditions revealed that the both the yolk granule membrane classes contained polypeptides of 240 kDa, 160 kDa, 140 kDa, 110 kDa, 90 kDa, 76 kDa and 62 kDa (Fig. 3.31). According to these observations we propose that the yolk granule membrane contains the proteolytically processed 240 kDa toposome. Two different classes of yolk granule membranes may have resulted from quantitative compositional differences of the protein present in the yolk granule membrane.

The proteins present in the membrane particles could be washed off the yolk granule membranes using EDTA, suggesting that the proteins were bound to the membrane in a calcium-dependent manner (Fig. 3.32). The majority of the proteins present in the yolk granule membranes could be eluted using high salt washes, leading us to believe that these are peripherally attached membrane proteins (Fig. 3.33). The protein present in the EDTA washes was capable of driving liposome aggregation in a calcium-dependent manner suggesting that one or more proteins attached to the yolk granule membranes might be involved in yolk granule aggregation (Fig. 3.34).

4.4 Plasma membranes of eggs and embryonic cells contain yolk proteins

Immunocytochemical localization of the toposome in hatched blastula embryos of the sea urchin has shown that the toposome is present in the plasma membranes of embryonic cells (Gratwohl *et al.*, 1990). The method of Matranga *et al.* (1986) was used to dissociate the embryonic cells from various stage embryos. The intact cells were isolated by differential centrifugation and extracted with EDTA. We expected to find the calcium dependent membrane binding proteins in these extracts. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (Laemmli, 1970), under reducing conditions, demonstrated that the protein profile of the EDTA extracts changes as embryonic development proceeds (Fig. 3.35). A Western blot performed using anti-toposome antibody demonstrated that these extracts had the 240 kDa polypeptide which was proteolytically processed as development proceeds (Fig. 3.36). Relatively lower molecular weight polypeptides cross reacted with the anti-toposome antibody at the 25 HPF stage.

Several studies have revealed that the protein profile of the yolk granule changes as development proceeds (Scott *et al.*, 1990; Yokota *et al.*, 1993). This occurs due to the proteolytic processing of the yolk protein precursors (Medina *et al.,* 1988; Mallya *et al.*, 1992; Fausto *et al.*, 2001a). As we have observed, the protein profile of the embryonic cell EDTA extracts changes as embryonic development proceeds (Fig. 3.35). Since the protease responsible for the yolk protein processing is localized to the yolk granules (Medina *et al.,* 1988; Mallya *et al.*, 1992; Fausto *et al.*, 2001), we propose that the toposome, which is proteolytically modified while it is present in the yolk granules, is continuously transported and deposited in the plasma membranes.

Liposome binding assay demonstrated that the 240 kDa protein complex present in the 25 HPF embryonic cell extract was bound to the liposomes in the presence of calcium (results not shown). Proteins that are present in the EDTA extracts of eggs and those from cells of embryos at different stages demonstrated calcium-dependent liposome aggregation (Fig. 3.37). The liposome aggregation driven by the proteins present in these embryonic cell extracts was inhibited in the presence of the anti-toposome antibody suggesting that the aggregation was driven by the 240 kDa complex or the polypeptides derived from it (Fig. 3.37). This result demonstrates that the yolk protein proteolytic processing has no effect on its aggregating activity (Fig. 3.37). The toposome, which was highly processed in the later stages of development, demonstrated the same aggregating activity, as the toposome that was partially processed in the egg extract (Fig. 3.37). Therefore, we suggest that, no matter what the degree of proteolytic processing, the toposome may play a role in vesicular aggregation and possibly fusion events throughout embryonic development.

4.5 General conclusions

As mentioned earlier, the yolk granules have been shown to harbor many components destined for export. Therefore, we hypothesized that the yolk granule is involved in the export pathway. The yolk proteins may play a role in transportation and membrane fusion events. Therefore, the focus of this study was the biochemical characterization of the phospholipid binding and vesicular aggregating activity of the yolk proteins.

We have identified, and isolated, the partially processed toposome in *Strongylocentrotus purpuratus* eggs, and biochemically characterized the calcium-dependent phospholipid binding and vesicular aggregating activity of this abundant high molecular weight yolk protein, which is the precursor for the majority of yolk proteins. The 240 kDa protein complex has the ability to bind calcium in its non-reduced state. It also binds to liposomes in a calcium-dependent manner. The protein complex was capable of aggregating liposomes in a calcium-dependent manner, which is a property of annexins. The toposome was shown to be actively participating in vesicular aggregating events regardless of its continuous proteolytic processing occurring in the yolk granule during embryonic development. The yolk granule may be involved in a continuous budding and fusion process with the membranal structures, including the plasma membranes, leading to the release of yolk granule contents. The 240 kDa protein complex may play a role in the export pathway involving yolk granules.

4.6 Future directions

According to our observations, the high molecular weight yolk granule protein, toposome, may play a role in an export pathway involving the yolk granules by mediating vesicular aggregation and perhaps fusion events. The toposome isolated from sea urchin eggs was composed of three major polypeptides of 160 kDa, 120 kDa and 90 kDa. These polypeptides should be isolated and characterized individually to identify which polypeptide/s is/are involved in phospholipid binding and vesicular aggregation. The actual molecular weight of the polypeptides derived from the toposome and the sequences of these polypeptides may be determined by mass spectroscopic analysis. Mass spectroscopy can also be utilized to generate the peptide maps of purified polypeptides and identification of peptide fragments. Once the amino acid sequences of the polypeptides are determined, the genes coding these polypeptides can be isolated from the sea urchin genome. These genes can then be analyzed to identify the consensus sequences responsible for phospholipid binding and calcium binding. This will help to determine if the polypeptides present in the 240 kDa protein complex are homologous to the annexin family of proteins.

Nuclear magnetic resonance studies can be used to determine the structural properties of the protein domains. These data will be important in understanding the 3D structure of these membrane binding proteins. In addition, circular dichroism spectroscopy can be performed to determine the conformational changes occurring in the proteins upon calcium binding and phospholipid binding.

We have demonstrated that the anti-toposome antibody could inhibit the phospholipid binding (Figs. 3.9) and vesicular aggregating activity (Fig. 3.19) of the

toposome. Most importantly, we have observed that the anti-toposome antibody could decrease the yolk granule aggregation, when the yolk granules were exposed to this antibody (Fig. 3.26). Therefore, we suggest that the anti-toposome antibody could be used for blocking the physiological action of the toposome in *in vivo* assays utilizing live embryos. This would allow us to investigate the physiological function of this abundant yolk protein in embryonic development.

Appendix

Time of harvest	*Strongylocentrotus purpuratus*		*Lytechinus pictus*	
	pH of YG	pH change	pH of YG	pH change
Egg	6.8	-	7.0	-
12 h (blastula)	6.1	-0.7	-	-
24 h (hatched blastula)	6.1	-0.7	7.2	+0.2
42 h (mid gastrula)	-	-	7.2	+0.2
48 h (late gastrula)	-	-	6.2	-0.8
72 h (prism)	-	-	6.5	-0.5

Table 1. Changes in pH during embryonic development of *Strongylocentrotus purpuratus* and *Lytechinus pictus.*

(Adapted from Mallya *et al.*, 1992)

Monosaccharide	nmol CHO/nmol protein			Normalized ratio*		
	SP	LP	AP	SP	LP	AP
Fucose	0.27	0.72	0.00	0.10	0.17	0.00
Galactosamine	0.51	1.25	0.65	0.19	0.30	0.09
Glucosamine	5.47	8.26	14.55	2.00	2.00	2.00
Galactose	0.62	0.69	0.84	0.23	0.17	0.11
Glucose	4.54	3.90	4.34	1.66	0.94	0.60
Mannose	22.81	31.24	67.01	8.34	7.56	9.21

Table 2. Comparison of carbohydrate compositions of 160 to 170kDa yolk glycoproteins. CHO = carbohydrate, SP = *Strongylocentrotus purpuratus,* LP = *Lytechinus pictus,* AP = *Arbacia puntulata*

*Values normalized assuming that the oligosaccharide chains contained two N-acetylglucosamine residues.

(Scott and Lennarz, 1988)

Species	Molecular mass (kDa)	pI range
Strongylocentrotus purpuratus	160	7.2-7.8
	115	7.6-8.0
	108	7.2-8.0
	90	6.7-8.0
Lytechinus pictus	160	6.9-7.8
	115	7.2-7.8
	108	6.3-7.6
	90	6.9-7.6
Arbacia punctulata	170	6.8-7.6
	112	6.2-6.8
	90	6.2-6.9

Table 3. Comparison of molecular masses and isoelectric points of yolk glycoproteins. (Scott and Lennarz, 1988)

Inhibitor	YP-160 proteolysis inhibited	Class of enzyme inhibited
Antipain	Yes	Thiol proteinase
Leupeptin	Yes	Thiol/Serine proteinases
Z-Phe-Ala-CH_2F	Yes	Thiol proteinase (cathepsin-B)
Pepstatin	No	Aspartic proteinase (pepsin)
Chymostatin	No	Serine proteinase (chymotrypsin)
Elastatinal	No	Serine proteinase (elastase)
TLCK	Yes	Serine proteinase (trypsin)
Banzamidine	Yes	Serine proteinase (trypsin-like)

Table 4. Effect of enzyme inhibitors on YP-160 proteolysis *in vivo* and the class of proteinase they inhibit.

YP- yolk protein, TLCK- Tosyl Lysyl Chloromethylketone

(Mallya *et al.*, 1992)

	mole %
Phosphatidyl choline	29.5
Phosphatidyl ethanolamine	11.5
Phosphatidyl serine	18.3
Phosphatidyl inositol	14.6
Sphingomyelin	20.5
Cholesterol	6.0

Table 5. The lipid composition of the yolk granule membrane in unfertilized sea urchin egg.

The phospholipid analysis was performed by Scott Pelley in Dr. Philip Davis's lab.

References

Alliegro, M. C. and McClay, D. R. (1988). Storage and mobilization of extracellular matrix proteins during sea urchin development. *Developmental Biology.* **125:** 208-216.

Angrerer, R. C. and Davidson, E. H. (1984). Molecular indices of cell lineage specification in sea urchin embryos. *Science.* **226:** 1153-1160.

Armant, D. R., Carson D. D., Decker, G. L., Welply, J. K. and Lennarz, W. J. (1986). Characterization of yolk platelets isolated from developing embryos of *Arbacia punctulata. Developmental Biology.* **113:** 342-355.

Baker, E. N. and Lindley, P. F. (1992). New perspectives on the structure and function of transferrins. *Journal of Inorganic Biochemistry.* **47:** 342-355.

Bartlett, G. R. (1959). Phosphorus assay in column chromatography. *Journal of Biological Chemistry.* **234:** 466-468.

Bergink, E. W. and Wallace R. A. (1974). Precursor-product relationship between amphibian vitellogenin and the yolk proteins, lipovitellin and phosvitin. *Journal of Biological Chemistry.* **249:** 2897-2903.

Boustead, C. M., Brown, R. and Walker, J. H. (1993). Isolation, characterization and localization of annexin V from chicken liver. *Biochemistry Journal.* **291:** 601-608.

Bownes, M., Shirras, A., Blair, M., Collins, J. and Coulson A (1988). Evidence that insect embryogenesis is regulated by ecdysteroids released from yolk proteins. *Proceedings. National Academy of Sciences. USA.* **85:** 1554-1557.

Brennan, C. and Robinson, J. J. (1994). Cloning and characterization of HLC-32, a 32 kDa protein component of the sea urchin extraembryonic matrix, the hyaline layer. *Developmental Biology*. **165:** 556-565.

Brooks, J. M. and Wessel, G. M. (2002). The major yolk protein in sea urchin is a transferrin-like iron binding protein. *Developmental Biology*. **245:** 1-12.

Byrne, B. M. Gruber, M and Ab, G. (1989). The evolution of egg yolk proteins. *Progress in Biophysics and Molecular Biology*. **53:** 33-69.

Cameron, R. A. and Holland, N. D. (1985). Demonstration of the granular layer and the fate of the hyaline layer during the developments of a sea urchin (*Lytechinus variegates*). *Cell and Tissue Research*. **239:** 455-458.

Cecchettini, A., Falleni, A., Gremigni, V., Locci, M. T., Masetti, M., Bradley, J. T. and Giorgi, F. (2001). Yolk utilization in stick insects entails the release of vitelline polypeptides into the perivitelline fluid. *European Journal of Cell Biology*. **80:** 458-465.

Cervello, M., Arizza, V., Lattuca, G., Parrinello, N. and Matranga, V. (1994). Detection of vitellogenin in a subpopulation of sea urchin coelomocytes. *European Journal of Cell Biology*. **64:** 314-319.

Cervello, M. and Matranga, V. (1989). Evidence of a precursor-product relationship between vitellogenin and toposome, a glycoprotein complex mediating cell adhesion. *Cell Differentiation and Development*. **26**: 67-76.

Cleveland, D. W., Fischer, S. G., Kirschner, M. W. and Laemmli, U. K. (1977). Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. *The Journal of Biological Chemistry*. **252:** 1102-1106.

Creutz, C. E., Snyder, S. L., Daigle, S. N. and Redick, J. (1996). Identification, localization and functional implications of an abundant nematode annexin. *Journal of Cell Biology.* **132:** 1079-1092.

Dhadialla, T. S. and Raikhel, A. S. (1990). Biosynthesis of mosquito vitellogenin. *Journal of Biological Chemistry.* **265:** 9924-9933.

Dunphy, W. G., Brands, R. and Rothman, J. E. (1985). Attachment of terminal N-acetylglucosamine to asparagines-linked oligosaccharides occurs in the central cisternae of the Golgi stack. *Cell.* **40:** 463-472.

Fausto, A. M., Gambellini, G, Mazzini, M., Cecchettini, A., Masetti, M. and Giorgi, F. (2001a). Yolk granules are differentially acidified during embryo development in the stick insect *Carausius morosus. Cell and Tissue Research.* **305:** 433-443.

Fausto, A. M., Gambellini, G, Mazzini, M., Cecchettini, A., Masetti, M., Locci, M. T., and Giorgi, F. (2001b). Serosa membrane plays a key role in transferring vitellin polypeptides to the perivitelline fluid in insect embryos. *Development, Growth and Differentiation.* **43:** 725-733.

Filipenko, N. R. and Waisman, D. M. (2000). Characterization of the Ca^{2+} binding sites of annexin II tetramer. *The Journal of Biological Chemistry.* **275 (49):** 38877-38884.

Flytzanis, C. N., McMahon, A. P., Hough-Evans, Katula, B. R., Britten, K. S. and Davidson, E. H. (1985). Persistence and integration of cloned DNA in post embryonic sea urchins. *Developmental Biology.* **108:** 431-442.

Friederich, E., Fritz, H. J. and Huttner, W. B. (1988). Inhibition of tyrosine sulfation in the trans-Golgi retards the transport of a constitively secreted protein to the cell surface. *Journal of Cell Biology.* **107:** 1655-1667.

Fuhrman, M. H., Suhan, J. P. and Ettensohn, C. A. (1992). Developmental expression of echinonectin, an endogenous lectin of the sea urchin embryo. *Development, Growth and Differentiation*. **34:** 137-150.

Giorgi, F., Bradley, J. T. and Nordin, J. H. (1999). Differential vitellin polypeptide processing in insect embryos. *Micron*. **30:** 579-596.

Giorgi, F., Cecchettini, A., Locci, M. T., Masetti, M. and Peccatori, M. (1997). Native vitellins are modified during ovarian development in the stick insect, *Carausius morosus* (Br.) *Archives of Insect Biochemistry and Physiology*. **36:** 335-348.

Giudice, G. (1962). Restitution of whole larvae from disaggregated cells of sea urchin embryos. *Developmental Biology*. **5:** 402-411.

Gratwohl, E. K. M., Kellenberger, E., Lorand, L. and Noll, H. (1991). Storage, ultrastructural targeting and function of toposomes and hyaline in sea urchin embryogenesis. *Mechanisms of Development*. **33:** 127-138.

Grewal, T., Heeren, J., Mewawala, D., Schnitgerhans, T., Wendt, D., Salomon, G., Enrich, C, Beisiegel, U. and Jackle, S. (2000). Annexin VI stimulates endocytosis and is involved in the trafficking of low density lipoprotein to the pre-lysosomal compartment. *The Journal of Biological Chemistry*. **275 (43) :** 33806-33813.

Harrington, F. E. and Easton, D. P. (1980). A putative precursor to the major yolk protein of the sea urchin. *Developmental Biology*. **94:** 505-508.

Harrington, F. E. and Ozaki (1986). The major glycoprotein precursor in echinoids is secreted by coelomocytes into the coelomic plasma. *Cell Differentiation*. **19:** 51-57.

Ichio, I., Deguchi, K., Kawashima, s., Endo, S. and Ueta, N. (1978). Water soluble lipoproteins from yolk granules in sea urchin eggs. I. Isolation and general properties. *Journal of Biochemistry (Tokyo).* **84:** 737-749.

Ii, I., Deguchi, K., Kawashima, S., Endo, S. and Ueta, N. (1978). Water soluble lipoproteins from yolk granules in sea urchin eggs. *Journal of Biochemistry.* **84:** 737-749.

Infante, A. A. and Nemer, M. (1968). Heterogeneous ribonucleoprotein particles in the cytoplasm of sea urchin embryos. *Journal of Molecular Biology.* **32:** 543-565.

Kane, R. E. (1965). Identification and isolation of the mitotic apparatus protein. *Biological Bulletin.* **129:** 396.

Kari, B. E. and Rottmann, W. L. (1985). Analysis of changes in yolk glycoprotein complex in developing sea urchin embryo. *Developmental Biology.* **108:** 18-25.

Kimble, J and Sharrock, W. J. (1983). Tissue specific synthesis of yolk proteins in *Caenorhabditis elegans. Developmental Biology.* **96:** 189-196.

Kondo, H. (1972). Properties of glycoprotein particles and their changes during development of sea urchin eggs and embryos. *Experimental Cell Research.* **72:** 519-532.

Kondo, H. and Koshihara, H. (1972). Immunological studies on the 26S particles of the sea urchin eggs. *Experimental Cell Research.* **75:** 385-396.

Koster, J. J., Boustead, C. M., Middleton, C. A. and Walker, J. H. (1993). The sub-cellular localization of annexin V in cultured chick-embryo fibroblasts. *Biochemistry Journal.* **291:** 595-600.

Kunkel, J. G. Nordin, J. H. (1985). Yolk proteins. In "Comprehensive insect physiology; Biochemistry and Pharmacology" (G. A. Kerkuto and L. J. Gilbert, Eds.), pp. 83-111. Pergamon Press, Oxford.

Laemmli, U.K. (1970). Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature (London).* **272:** 680-685.

Lagueux, M., Harry, P. and Hoffmann, J. A. (1981). Ecdysteroids are bound to vitellin in newly laid eggs of *Locusta. Molecular and Cellular Endocrinology.* **24:** 325-338.

Lee, G. F., Fanning, E. W., Small M. P. and Hille, M. B. (1989). Developmentally regulated proteolytic processing of a yolk glycoprotein complex in the embryos of the sea urchin, Strongylocentrotus purpuratus. *Cell Differentiation and Development.* **26:** 5-18.

Lee, G., and Pollard, H. B. (1997). Highly sensitive and stable phosphatidyl serine liposome aggregation assay for annexins. *Analytical Biochemistry.* **252:** 160-164.

Liao, Y. D. and Wang, J. J. (1994). Yolk granules are the major compartment for bullfrog (*Rana catesbeiana*) oocyte specific ribonuclease. *European Journal of Biochemistry.* **222:** 215-220.

Maekawa, S., Toriyama, M. and Sakai, H. (1994). Purification of a sea urchin calpactin like protein which is excluded from the mitotic apparatus region. *Biochemistry and Molecular Biology International.* **3:** 155-163.

Mailliard, W. S., Haigler, H. T. and Schlaepfer, D. D. (1996). Calcium-dependent binding of S100C to the N-terminal domain of annexin I. *The Journal of Biological Chemistry.* **271 (2) :** 719-725.

Malkin, L. I., Mangan, J. and Gross, P. R. (1965). A crystalline protein of high molecular weight from cytoplasmic granules in sea urchin eggs and embryos. *Developmental Biology*. **12:** 520-542.

Mallya, S. K., Partin, J. S., Valdizan, M. C. and Lennarsz, W. J. (1992). Proteolysis of the major yolk glycoprotein is regulated by acidification of the yolk platelets in sea urchin embryos. *The Journal of Cell Biology*. **117:** 1211-1220.

Matranga, V. and Cervello, M. (1984). Monoclonal antibodies raised against cell surface components of sea urchin embryos show the expression of different antigenic patterns. *Journal of Embryology and Experimental Morphology*. **82:** (Suppl.1) p.89.

Matranga, V., Kuwasaki, B. and Noll, H. (1986). Functional characterization of toposomes from sea urchin blastula embryos by a morphogenic cell aggregation assay. *EMBO Journal*. **5:** 3125-3132.

Matranga, V., Zito, F. and Cervello, M. (1987). A monoclonal antibody recognizes a subclass of toposomes specifying for mesodermal structures. *European Journal of Cell Biology*. **44:** (Suppl. 21) p.45.

Mayne, J. and Robinson, J. J. (1998). The sea urchin egg yolk granule is a storage compartment for HLC-32, an extracellular matrix protein. *Biochemistry and Cell Biology*. **76:** 83-88.

McClay, D. R. and Fink, R. D. (1982). Sea urchin hyalin: Appearance function in development. *Developmental Biology*. **92:** 285-293.

McGregor, D. A. and Laughton, B. (1977). Amino acid composition, degradation and utilization of locust vitellogenin during embryogenesis. *Wilhelm Roux's Archives*. **181:** 113-122.

Medina, M., Leon, P. and Vallejo, C. G. (1988). Drosophila cathepsin B-like protease: A suggested role in yolk degradation. *Development, Growth and Differentiation.* **31:** 241-247.

Molloy, S. S., Anderson, E. D., Jean, F. and Thomas, G. (1999). Bio-cycling the furin pathway: From TGN localization to pathogen activation and embryogenesis. *Trends in Cell Biology.* **9:** 28-35.

Niimi, T., Yoshimi, T. and Yamashita, O. (1993). Vitellin and egg-specific protein as metal binding proteins of the silkworm, *Bombyx mori. Journal of Sericultural Science. Japan.* **62:** 310-318.

Noll, H., Matranga, V., Cervello, M., Humphreys, T., Kuwasaki, B. and Adelson, D. (1985). Characterization of toposomes from sea urchin blastula cells: A cell organelle mediating cell adhesion and expressing positional information. *Proceedings. National Academy of Sciences. USA.* **82:** 8062-8066.

Outreath, R. L., Roberson, M. M. and Barondes, S. H. (1988). Endogenous lectin secretion into the extracellular matrix of early embryos of *Xenopus laevis. Developmental Biology.* **125:** 187-194.

Ozaki, H. (1980). Yolk proteins of the sand dollar *Dendraster excentricus. Development, Growth and Differentiation.* **22:** 365-372.

Parrish, C. C. (1995). Seasonal studies of seston lipids in relation to microplankton species composition and scallop growth in South Broad Cove, Newfoundland. *Marine Ecology Progress Series.* **129:** 151.

Petr, J., Rozinek, J., Hruban, V., Jilek, F., Sedmikova, M., Vanourkova, Z. and Nemecek, Z. (2001). Ultrastructural localization of calcium deposits during *in vitro* culture of pig oocytes. *Molecular Reproduction and Development.* **58:** 196-204.

Reimer, C. L. and Crawford B. J. (1995). Identification and partial characterization of yolk and cortical granule proteins in eggs and embryos of the starfish, *Pisaster ochracens*. *Developmental Biology*. **167:** 439-457.

Rouser, G. (1967). Column chromatographic and associated procedures in separation and determination of phosphatides and glycolipids. In "*Lipid chromatographic analysis*" (Edited by Margiveti 6.V.). Vol. 1, pp. 99-162. Marcel Dekker, New York.

Sappington, T. W., Kokoza, V. A., Cho, W. L. and Raikhel, AA. S. (1996). Molecular characterization of the mosquito vitellogenin receptor reveals unexpected high homology to the *Drosophila* yolk protein receptor. **93:** 8934-8939.

Schonbaum, C. P., Lee, S. and Mahowald, A. P. (1995). The *Drosophila* yolkless gene encodes a vitellogenin receptor belonging to the low density lipoprotein receptor superfamily. *Proceedings. National Academy of Sciences. USA*. **92:** 1485-1489.

Schuel, H.., Wilson, W. L., Wilson, J. R. and Bressler, R. S. (1975). Heterogeneous distribution of "lysosomal" hydrolases in yolk platelets isolated from unfertilized sea urchin eggs by zonal centrifugation. *Developmental Biology*. **46:** 404-412

Scott, L. B. and Lennarz, W. J. (1988). Structure of a major yolk glycoprotein and its processing pathway by limited proteolysis are conserved in echinoids. *Developmental Biology*. **132:** 91-102.

Scott, L. B., Leahy, P. S., Decker, G. L. and Lennarz, W. J. (1990). Loss of yolk platelets and yolk glycoproteins during larval development of the sea urchin embryo. *Developmental Biology*. **137:** 368-377.

Shyu, A. B., Raff, R. A. and Blumental, T. (1986). Expression of the vitellogenin gene in female and male sea urchin. *Proceedings. National Academy of Sciences. USA*, **83:** 3865-3869.

Snigirevskaya, E. S., Hays, A. R. and Raikhel, A. S. (1997). Secretory and internalization pathways of mosquito yolk protein precursors. *Cell and Tissue Research.* **290:** 129-142.

Spenneberg, R., Osterloh, D and Gerke, V. (1998). Phospholipid vesicle binding and aggregation by four novel fish annexins are differentially regulated by Ca^{2+}. *Biochimica et Biophysica Acta.* **1448:** 311-319.

Srivastava, M., Goping, G., Caohuy, H., McPhie, P. and Pollard, H. B. (1996). Detection and localization of synexin (annexin VII) in *Xenopus* adult and embryonic tissues using an antibody to the *Xenopus* N-terminal PGQM repeat domain. *Experimental Cell Research.* **229:** 14-19.

Steiner, D. F. (1998). The proprotein convertases. *Current Opinions in Chemical Biology.* **2:** 31-39.

Stifani, S., Barber, D. L., Nimpf, J. and Schneider, J. (1990). A single chicken oocytes plasma membrane protein mediates uptake of very low density lipoprotein and vitellogenin. *Proceedings. National Academy of Sciences. USA.* **87:** 1955-1959.

Stradal, T. B. and Gimona, M. (1999). Ca^{2+} Dependent association of S100A6 (calcyclin) with the plasma membrane and the nuclear envelope. *The Journal of Biological Chemistry.* **274 (44):** 31593-31596.

Tata, J. R. (1976). The expression of the vitellogenin gene. *Cell.* **9:** 1-14.

Torok, K., Wilding, M., Groigno, L., Patel, R. and Whitaker, M. (1998). Imaging the special dynamics of calmodulin activation during mitosis. *Current Biology.* **8:** 692-699.

Tsukahara, J. (1970). Formation and behavior of pinosomes in the sea urchin oocytes during oogenesis. *Development, Growth and Differentiation.* **12:** 53-64.

Unuma, T, Suzuki, T., Kurokawa, T., Yamamoto, T. and Akiyama, T. (1998). A protein identical to the yolk protein is stored in the testes in male red sea urchin, *Reudocentrotus depressus*. *Biological Bulletin.* **194:** 92-97.

Varki, A. (1998). Factors controlling the glycosylation potential of the Golgi apparatus . *Trends in Cell Biology.* **8:** 34-40.

Wahli, W. Dawid, I. B., Pyffel, G. U. and Weber, R. (1981). Vitellogenesis and the vitellogenin gene family. *Science.* **212:** 298-304.

Wallace, R. A. (1985). Vitellogenins and oocytes growth in nonmammalian vertebrates. *Developmental Biology.* **1:** 127-177.

Wallace, R. A. and Selman, K (1985). Major protein changes during vitellogenesis and maturation of *Fundulus* oocytes, *Developmental Biology.* **110:** 492-498.

Wang, J. J., Tang, P. C., Chao, S. H., Cheng, C. H., Ma, H. J. and Liao, Y. D. (1995). Immunocytochemical localization of ribonuclease in *Rana catesteiana* oocytes. *Cell and Tissue Research.* **280:** 259-265.

Wessel, G. M. Zaydfudim, V. Yuin-tsee, Hsu, J., Laidlaw, M. and Brooks, J. M. (2000). Direct molecular interaction of a conserved yolk granule protein in sea urchins. *Development, Growth and Differentiation.* **42:** 507-517.

Wiley, H. S. and Wallace, R. A. (1981). The structure of vitellogenin . Multiple vitellogenins in *Xenopus laevis* give rise to multiple forms of yolk proteins. *Journal of Biochemistry*. **256:** 8626-8634.

Williams, J. (1967). Yolk utilization. In " *The biochemistry of animal development*". (R. Weber, Ed.),vol. II. Pp.343-378. Academic Press, New York/ London.

Wojchowski, D. M., Parson, P., Nordin, J. H. and Kunkel. (1986). Processing of pro-vitellogenin in insect fat body: A role for high mannose oligosaccharide. *Developmental Biology*. **116:** 422-430.

Yamashita, O. and Indrasith, L. S (1988). Metabolic fates of yolk proteins during embryogenesis in Arthropods. *Development, Growth and differentiation*. **30:** 337-346.

Yokota, Y. and Kato, K. H. (1988). Degradation of yolk proteins in sea urchin eggs and embryos. *Cell Differentiation*. **23:** 191-200.

Yokota, Y., Kato, K. H. and Mita, M. (1993). Morphological and biochemical studies on yolk degradation in the sea urchin, *Hemicentrotus pulcherrimus. Zoological Science*. **10:** 661-670.