# INVESTIGATIONS ON GLYCOSYL PHOSPHATIDYL INOSITOL-ANCHORED PROTEINS OF GOAT SPERM

**A THESIS**

*Submitted in fulfilment of the requirements for the award of the degree of*

DOCTOR OF PHILOSOPHY

*in*

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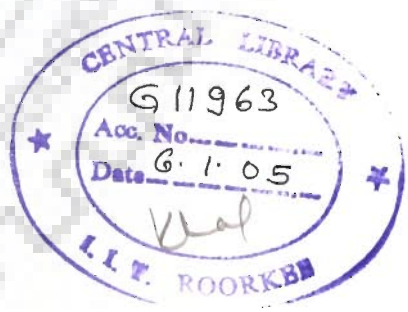
*By*

**TARUN EDGAR HUTCHINSON**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# CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled **"INVESTIGATIONS ON GLYCOSYL PHOSPHATIDYL INOSITOL-ANCHORED PROTEINS OF GOAT SPERM"** in the fulfillment of the requirement for the award of the degree of **Doctor of Philosophy** and submitted in the **Department of Biotechnology** of the **IIT Roorkee,** is an authentic record of my own work carried out during a period from **July 1999 to October 2003** under the supervision of **Dr. Ben M. J. Pereira,** Associate Professor, Department of Biotechnology.

The matter presented in this thesis has not been submitted by me for the award of any other degree of any other institute.

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This is to certify that the above statement made by the candidate is correct to the best of my knowledge.

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Dedicated
to
My Country

# Chapter - 1

# Introduction

## 1. INTRODUCTION:

The testis and ovary generate highly specialized haploid cells, sperm and egg, respectively, which come together with the sole purpose of species perpetuation. In fact, when they fail in this mission, they perish. In mammals, millions of sperm are produced and have to overcome many barriers during their journey to reach and fertilize the egg. The sperm are equipped with a strong cytoskeleton, a self-propelling mechanism, a battery of enzymes and highly distinctive molecules on their surface that make them functionally fit to undertake this stupendous task. The unique capability of sperm to fertilize the egg is not a matter of chance, but a long drawn-out process in which the germ cell is structurally and physiologically modified through successive stages of spermatogenesis in the testis (Eddy and O' Brien, 1994), maturation in the epididymis (Jones, 1989; Kirchhoff *et al.*, 1997; Tulsiani *et al.*, 1998b) and capacitation in the female genital tract (Yanagimachi, 1994; Tulsiani and Abou-Haila, 2001). At the end of all, the sperm have to compete among themselves to bind and penetrate through the many vestments of the egg to complete the process of fertilization.

It has been established that cell-cell interactions take place through molecules located on their surface membranes. The sperm plasma membrane like most other cell membranes is made up of a bi-layer lipid comprising of phospholipids and cholesterol. A change in the relative concentration in these two components influences characteristics like the permeability, fluidity,

rigidity, and stability of the sperm (Johnson, 1975; Lin and Kan, 1996). The clearest function of membrane lipid is to form amphipathic bilayer that surround cells and organelles and block leakage of hydrophilic compounds, while housing membrane proteins. However, the wide variety of lipids and proteins observed in biological membranes implies that they perform roles supplementary to simple barrier function. The general consensus today is that proteins intermingle with lipid bilayer through both covalent and non-covalent interactions (Low, 1987). Thus, proteins are distributed on either side of membrane some embedded (integral) and some attached superficially (peripheral). Mature sperm has a limited capacity for protein synthesis since they are 'stripped-down' cells lacking an endoplasmic reticulum (Kirkman-Brown *et al.*, 2000; Kobori *et al.*, 2000). This mean that sperm would have to carry the bulk of numerous molecules needed for the events leading up to fertilization. It astonishes how such a minute cell as the sperm can cope with the galaxy of molecules that would be required. Obviously, specialized mechanisms operate to meet their demands. Thus, in addition to acquisition of protein during spermatogenesis, evidence has shown that new proteins are added (Orgebin-Crist, 1987; Jones, 1989; Cooper, 1995) and at times existing proteins are modified (Tulsiani *et al.* 1998b) on the sperm surface during their passage through the male and female reproductive tracts.

The manner of protein association with lipid bilayer through covalent interactions has caught the attention of several research workers. Protein

attachment to fatty acid by amide, thioester or *o*-acyl bonds in one such arrangement (Low M.G., 1987). Glycosyl phosphatidylinositol (GPI) anchorage is yet another. Sequence analysis and biophysical measurements have shown that the glycosylated phosphatidyl inositol moiety contributes to one of the most stable and conserved class of protein and lipid bilayer interactions (McConville and Ferguson, 1993). GPI-anchored proteins are basically complex structures of phospholipids with a glycan bridge carrying proteins at one end. In a typical GPI anchored protein, the c- terminus of the protein is linked via *ethanolamine* phosphate to a glycan with the conserved backbone sequence: Man α1-2 Man α1-6 Man α1-4 GlcN-$NH_2$ which in turn is linked to the 6-position of the *myo*-inositol ring of phosphotidylinositol (McConville and Ferguson, 1993). Thus, the core structure of the GPI anchors is identical and conserved while the protein part is highly variable.

In recent times, it has been convincingly shown that the GPI anchored molecules on the surface of both sperm and egg are involved in mammalian fertilization (Coonrod *et al.*, 1999). Its presence on the outer leaflet of the lipid bilayer provides a special advantage (Low, 1987). This raises questions on how GPI-linked molecules behave and work with the exterior cell condition: Do they provide the communication system to the sperm? Do they help in egg recognition? Do they provide the site for attachment to the sperm? Or do they protect and stabilize the sperm? As of today, several GPI anchored proteins have been identified on the surface of mammalian sperm and a number of functions

have been attributed to them. The role of GPI proteins as ectoenzymes has received the most attention. Among them are the hyaluronidase (Primakoff, 1985; Thaller and Cardullo, 1995), 5' nucleotidase (Fabaini and Ronquist, 1993; Schieman *et al.*, 1994) and alpha-mannosidase (Kuno *et al.*, 2000). These molecules are believed not only to possess catalytic activity but also interact with molecules on the egg in a receptor-ligand fashion to initiate signaling. Sperm GPI proteins with exclusive receptor function has also been reported (Shetty *et al.*, 2003). In one instance, a sperm GPI-protein has been shown to act as carrier for a 'decapacitation factor' (DF) that is released from the sperm surface during the process of capacitation (Fraser, 1998). A large number of molecules, particularly the cluster of differentiation (CD) class of GPI-proteins associated with the lymphocytes, are also found to exist on the surface of sperm. They are believed to protect the sperm against complement attack (Kirchhoff and Hale, 1996). More recently, GPI-proteins such as the MAK248 that act as protease inhibitors has been reported (Yudin *et al.*, 2002).

Thus, from a survey of literature, it is abundantly clear that although the core anchor is the homologous to a large extent, the protein components have almost unrelated structure and function. Therefore, it would be highly desirable to identify as many GPI-proteins as possible from mammals and decipher their roles so that we can get a clearer picture of the mechanisms operating in the events leading to fertilization. The research trend seems to be in this direction

and accordingly the numbers of new proteins that are being identified are on the rise.

The work embodied in this thesis is also an effort to identify and characterize the functional role of GPI-anchored proteins in goat sperm. The main objectives are:

1. To separate bulk quantities goat sperm from the epididymis.
2. To isolate, purify and characterize sperm plasma membrane.
3. To check the potency of commercial source of phosphatidyl inositol specific phospholipase-C enzyme (PI-PLC from *Bacillus cereus)* using positive and negative controls.
4. To analyse sperm surface protein from the goat sperm plasma membrane by enzymatic and chemical cleavage and identify GPI proteins by SDS-PAGE.
5. To raise polyclonal antibodies and confirm GPI anchorage by western blotting.
6. To study the distribution of GPI-anchored proteins on sperm surface by immunoflourescence.
7. To determine the role of GPI anchored proteins on goat sperm surface.

Goat (*Capra indica*) was chosen as a model to investigate the GPI anchored protein since fresh testis together with epididymis was easily available throughout the year in a local abattoir where these animals are routinely

slaughtered for meat. Strict laws are in force in the country and the Institutional Animal Ethic Committee felt that the objectives of the studies could be met by using fresh tissue collected from slaughter houses and that there was no need to unnecessarily kill laboratory animals just to obtain sperm from the epididymis. Besides, not much work has been carried out on goat sperm.

# Chapter - 2

# Literature Review

# 2.0 LITERATURE REVIEW

## 2.1 History

About 50 years ago, it was observed that experimental animals, infected with anthrax exhibited amongst other chemical changes in blood chemistry, a pronounced alkaline phosphatasemia (Low, 1989). The first report of the release of alkaline phosphatase (ALP) from membranes was made during investigation of the action of anthrax toxin. A soluble factor present in toxin preparations from *Bacillus anthracis* caused a rapid and pronounced elevation of serum ALP when injected into experimental animals (Slein and Logan, 1960). This effect could also be produced by culture filtrates derived from various non-pathogenic Bacillus species and thus it was inferred that the lethal toxins of anthrax are unlikely to be factors responsible for this kind of action (Slein and Logan, 1962 a, b). Later, it was observed that a similar effect could be produced *in vitro* by incubating tissue slices with the culture filtrates and assaying the suspending media for the released ALP. The factor responsible for this effect was partially purified from *Bacillus cereus* and suggested to be a phospholipase-C with a relative specificity for phosphatidyl inositol (Slein and Logan, 1963,1965) and thus ALP came to be known as a GPI anchored protein.

The presence of GPI anchors on membrane surfaces has been widely reported in Eucarya from vertebrates to protozoa (Ikezawa, 2002) and also in species of Archaeobacteria (Kobayashi *et al.,* 1997). In an article published not too long ago, close to 100 different GPI anchored proteins that have been

identified are consolidated and presented (Low, 1989). Although the anchors have to a large extent a conserved core structure, the proteins attached to them have little similarity. The most common feature among these proteins is their attachment to the exoplasmic leaflet of the lipid bilayer of membranes through the GPI anchor. Since its first discovery in the late 1960's there has been a sudden increase in research on this ubiquitous protein modification in various cell types. The emphasis has been and is continuing to be on various aspects like the distribution, structural analysis, biosynthesis and function (Chatterjee and Mayor, 2001). Thus, today proteins as varied as enzymes, receptors, adhesion molecules, differentiation antigens and other biologically active proteins have proved to be bound to the plasma membrane by the GPI anchor.

## 2.2 Protein Arrangement On Surface Of Plasma Membrane

Proteins are arranged in varied fashion on the surface of plasma membranes. Initially, it was believed and proposed that proteins interact with the lipid bilayer only by non-covalent interactions (Singer and Nicolson, 1972). In support of this view, biophysical studies on a variety of different membrane proteins indicated that either the proteins are superficially attached to the membrane or are embedded and held by hydrophobic interaction between the fatty acid chains of phospholipid molecules. Further, among these membrane proteins two very distinctive arrangements were observed. One, as in rhodopsin, had several hydrophobic transmembrane helices connected by hydrophilic

segments protruding from the bilayer. The other type, as in glycophorin, had a less extensive arrangement with either a small proportion or none of the protein interacting with the bilayer. At that time, it was widely accepted that there is a balance of interaction between hydrophobic and polar domains of proteins with the hydrophobic core of the lipid bilayer and the surrounding medium (Singer and Nicolson, 1972). The general consensus was that these forces are not only responsible for the attachment of proteins but also assist in their correct conformation and transmembrane orientation, so that its function (that is solute transport, signal transduction, adhesion etc.) can be performed correctly. However, in the past three decades, the concept of the lone class of non-covalent interaction and attachment of biological membrane proteins has been challenged. We now know of membrane proteins covalently attached through a lipid chain (either a fatty acid chain or a prenyl group) in the cytosolic monolayer (for example NADH, Cytochrome b5 reductase, Ozols *et al.*, 1984) or via an oligosaccharide linker to phosphatidylinositol in the non cytosolic monolayer (for example, alkaline phosphatase, Ikezawa *et al.*, 1976; Low and Finean, 1977a,b; Low and Zilversmit, 1980; Low *et al.*, 1987)

**2.3 Structure of the GPI-anchor:**

According to the Ferguson (1999) GPI moiety of the glycan structures can be classified into three major categories: **type I** has a Man (α1-6) Man (α1-4) GlcN (α1-6) PI motif, **type II** has Man (α1-3) Man (α1-4) GlcN (α1-6) PI motif and **type III** is a hybrid with Man (α1-6) or (Man α1-3) Man (α1-4) GlcN

(α1-6) PI motif. The glycan structures of all GPI anchored proteins conform to type I.

In a typical GPI anchored protein, the phosphatidylinositol portion is embedded and spans the outer leaflet of the lipid bilayer of cell membranes. A glycan bridge (Man (α1-2) Man (α1-6) Man (α1-4) GlcN) connects the phosphatidylinositol to phosphoethanolamine. The phosphoethanolamine is then attached to the C-terminus of the protein via an amide linkage (see figure 1.).

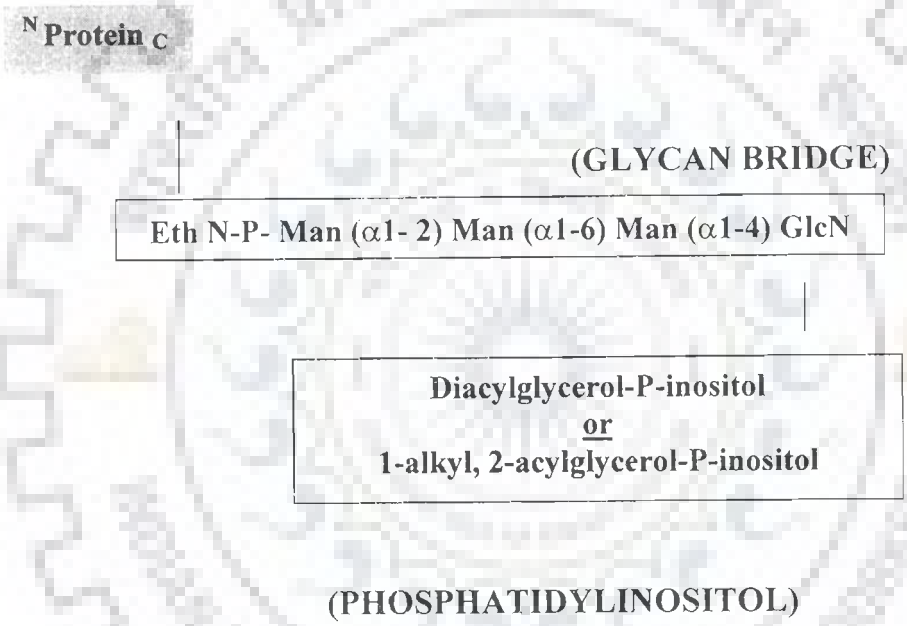



**Figure: 1. The general chemical structural organization of GPI proteins from mammalian cells**

The attachment of proteins to membranes through the GPI anchor provides several structural advantages. First, its existence in the outer leaflet of the bilayer allows lateral mobility of the molecules that is not restricted by cytoskeletal elements (Low, 1987). Second, the proteins can be released from

their anchors by the action of specific enzymes, the phospholipases C and D (Low and Finean, 1978; Low and Prasad, 1988). Finally, the amphipathic nature of the molecules makes it possible for its transfer from one cell to another through vesicles (Arienti *et al.,* 1997 a; b) and phospholipid transfer proteins (Wirtz, 1997). Thus, GPI anchors can facilitate cellular functions like intracellular sorting of proteins (Nosjean *et al.,* 1997; Muniz and Reizman, 2000), transmembrane signaling (Horejsi *et al.,* 1999) and the process of potocytosis (Anderson, 1998; Grunfelder *et al.*, 2003).

**2.4 Methods for studying GPI proteins:**

Ever since standard procedures have become available, the number of GPI proteins being reported in cells is on the increase. The identification and characterization of GPI anchored proteins is largely based on the properties of GPI structure, which is common and similar in all types of cells and membrane organizations. Various methods have been employed to study the structural and functional details of this unique class of protein with a lipid anchor and conserved glycan units. Some of the well established general and specific methods of GPI anchored protein analysis are described below. Although most protocols have been standardized and in the ready to use mode, it is always desirable to include a GPI anchored (positive) and a transmembranal protein (negative) as controls to assess the reliability of the method when actually carrying out the procedure in the laboratory.

### 2.4.1 Isolation and purification:

In order to make detailed studies of a protein it is crucial to be first able to detect, isolate and purify the molecule. It would be an added advantage if the desired protein can be obtained in adequate quantities. Although it is relatively easy to detect GPI proteins, it is rather difficult and tricky to isolate and purify these molecules in large quantities since they are membrane bound. The various strategies used in the analysis of GPI proteins are briefly outlined below.

### 2.4.2 Solubilization by detergents:

Detergents are amphipathic molecules that form micelles in water and have often been used for the solubilization of proteins from membranes. The choice of detergent is based on several characteristics like the critical micelle concentration (CMC), cloud point and hydrophilic-lipophilic balance. Triton X-100 is a non-ionic detergent that is extensively used to solubilize most transmembrane proteins that reside on membranes. Hooper and Turner (1988) examined nine kidney microvillar enzymes and found that the transmembrane proteins were all solubilized completely by detergents with low CMC, such as TX-100 and NP-40, while all of those anchored by GPI, resisted solubilization at low temperature. They also observed that detergents with a high (CMC) e.g. octyl glucoside, CHAPS, and sodium deoxycholate can solubilize substantial amount of those ectoenzymes of pig kidney microvillar membranes that are also susceptible to release by PI-PLC enzyme. While all the detergents are equally

effective in solubilizing membrane proteins not released by PI-PLC, the GPI anchored variety are resistant to triton X- 100 at low temperature. Thus, this resistance to cold solubilization and differential solubilization by detergents is considered as a characteristic feature of GPI anchorage (Hooper; 2001). In another study, several detergents have been used to solubilize GPI anchored proteins with varying degree of success (Muller *et al.*, 1994). From the data obtained, it was concluded that TX-100 poorly solubilised GPI proteins by virtue of a low CMC value, while detergents with high CMC value released substantial amount of GPI proteins. The existence of GPI proteins as complexes on membrane (Brown and Rose, 1992; Simons and Ikonen, 1997), together with the CMC of the detergents has been argued as the possible cause for the reported difference in solubilization (Hooper, 1988). The difference is so prominent that the ratio of solubilization with octyl glucoside to that of Triton X- 100 is often used as a criterion to decide if the protein of interest is transmembranal or GPI anchored (Hooper and Turner, 1988; Hooper and Bashir, 1991; Hutchinson *et al.*, 2002).

**2.4.3 Phase separation by Triton X – 114:**

Triton X-114 (TX-114) is a unique detergent that forms a clear micellar solution at low temperature but separates into two phases (i.e. detergent-enriched and detergent-depleted) at higher temperatures. This is due to the aggregation of detergent micelles when they are warmed to temperatures exceeding 20°C (Bordier, 1981). Extraction of cells or membrane with the

detergent TX-114 provides a profile (and potentially an initial purification) of amphiphilic proteins including integral membrane proteins and species bearing GPI anchors. Once a GPI linked protein is released from the component of its anchor, it will no longer partition into the detergent enriched phase. This alteration of partitioning behavior provides a rapid assay for the presence or absence of anchors. For example, in transfected cell extracts, Tamm-Horsefall glycoprotein (THP) remained primarily in the detergent phase in a TX-114 partitioning assay, indicating that it has a hydrophobic character. The TX-114 detergent associated THP was redistributed to the aqueous phase after treatment of cell extract with PI-PLC (since cleavage from the GPI anchor made it hydrophilic). The selective cleavage and differential partition between aqueous and detergent phases makes it easy to infer that the protein is GPI anchored (Rindler *et al.*, 1990).

**2.4.4 Solubilization by special agents:**

Alternative efforts have been made to device a rapid and specific method for the extraction of GPI-anchored proteins from membrane. One such method uses a derivative of taurocholate, 4'amino 7-β-benzamido taurocholic acid (BATC); it discriminates between transmembranal proteins and GPI-proteins on the basis of their physiochemical properties. Studies indicate that 8-14 fold enrichment of proteins from total membrane proteins can be achieved using concentrations between 0.1-0.3 % BATC (Muller *et al.*, 1994). It has been

further elaborated that BATC extraction has an advantage over conventional detergent extraction in the cleavage specificity and efficiency of phospholipase action, which vastly improves the release of proteins from GPI anchors.

### 2.4.5 Characterization by Chemical cleavage:

Carbohydrate units of proteoglycan and glycoprotein can be cleaved selectively by nitrous acid at glycosidic linkages involving hexosamine residues in which the amino groups are either N-sulfated or unsubstituted. In all GPI proteins, the glycosidic linkage of non-acetylated glucosamine to inositol is an unusual structure and it is also cleaved by nitrous acid deamination. During this reaction, residues of D-glucosamine are converted to 2,5 anhydro-D-mannose. Just like enzymes, the chemical method of cleavage also releases the protein in aqueous soluble forms (Ferguson, 1992). Here again, the released protein could be partitioned in TX-114 and further characterized by SDS-polyacrylamide gel electrophoresis.

### 2.4.6 Characterization by Enzymatic cleavage:

The first clue regarding the mode and site of action of Phospholipase C (PLC) stemmed from the persistent efforts of Slein and Logan, 1960; 1962; 1963; 1965. The specific nature of the enzyme came to light from the work of Ikezawa, 1976 when bacterial PLC was purified from *Bacillus cereus*. Later, Ohyabu (1978) optimized the procedure for the release of proteins specifically from phosphatidylinositol anchors. Bacterial phospholipases have been purified

from a number of sources. The enzyme preparations from *Clostridium novyi* type A (Taguchi and Ikezawa, 1978) and *Staphylococcus aureus* (Low, 1981) both showed high specificity for phosphatidylinositol (PI). These enzymes are designated as PI-PLC because they cleave the link between the diacylglycerol backbone and the phosphate group of GPI. These enzymes appear to have little or no specificity for the acyl / alkyl groups of the PI substrates (Low, 1987) but displayed marked specificity towards the inositol head group. The bacterial PI-PLC release most GPI anchored proteins in a soluble form from intact cells or membrane preparations. Similarly, Low and Finean (1977a,b) released the alkaline phosphatase by the action of PI-PLC enzyme from rabbit kidney homogenates and microsomal fractions. It is now the most common procedure used by researchers to identify the release of GPI anchored proteins (Sharom *et al.*, 1996). However, it has also come to light that several proteins with known GPI anchors are largely or completely resistant to PI-PLC. Thus, the release of 5'-nucleotidase from its anchor required hundred times more PI-PLC then alkaline phosphatase (Low, 1978). In some cases, the resistance could be due to interaction with other membrane components (Ferguson and Williams, 1988). Purified human erythrocyte (AChE) shows the same PI-PLC resistance as other cell associated enzymes. Additionally, there are numerous examples of incomplete release of other proteins by PI-PLC. For PI-PLC release experiments, membranes and cells containing the protein of interest are incubated with bacterial PI-PLC and the loss of the protein from the membrane

and/or its appearance in the supernatant is monitored following centrifugation (Hooper *et al.,* 1987; Sharom and Lehto, 2002). Another way of determining whether a protein has GPI anchor is to exploit the mobility characteristic on SDS-PAGE that occurs when the GPI anchor is removed.

Phosphatidylinositol specific phospholipase D (PI-PLD) is yet another enzyme that can release the protein from the GPI anchors, but it differs from PI-PLC in the site of cleavage. The enzyme PI-PLD cleaves the bond between inositol and the phosphate group. Experiment demonstrates that this anchor degrading activity is also abundant in mammalian plasma and human placenta (Malik and Low, 1986; Low and Prasad, 1988). Crude extracts from these sources were capable of removing the anchor from alkaline phosphatase, 5' nucleotoidase and variant surface glycoprotein (VSG). The data finally suggested that this activity is due to phospholipase-D with specificity for glycosyl phosphatidylinositol structures. Since the enzyme is inhibited by *p*-hydroxy-mercuriphenyl sulphonic acid, EGTA, and 1,10 phenanthroline, it has been concluded that serum phospholipase-D (PI-PLD) cleaves GPI structure that contain acylated inositols and is useful in their identification. However, it is not very effective on membrane bound proteins in the absence of detergent.

Lack of release of protein by phospholipase C or D does not necessarily rule out the presence of the GPI anchor. When intact cells or membrane preparations are treated with phospholipases, partial or total resistance to cleavage can also occur due to inaccessibility of the proteins to the enzymes, the

lipid environment in which it is placed or tight association of a protein with a non susceptible protein on the cell surface (Rossenberry, 1991; Sharom *et al.*, 1996; Lehto and Sharom, 1998; 2002).

**2.4.7 Detection of GPI anchored proteins by anti CRD antibody:**

Rabbit antisera raised against the soluble form of variant surface glycoprotein (VSG) contained a population of antibodies that cross-reacted with heterologous VSGs (Barbet and McGuire, 1978). The epitope involved was referred to as the 'cross-reacting determinant' (CRD). It was also found that anti-CRD antibody cross-react strongly with soluble VSGs in various assays but not with the intact membrane form that retain their dimyristyl glycerol groups (Ferguson and William, 1988; Hooper, 2001). Polyclonal antisera raised in rabbits against the PI-PLC cleaved GPI anchored protein often cross-reacted with other unrelated soluble form of GPI proteins (Hooper., 2001). Thus, it was deduced that the site of cross reactivity is cryptic in the membrane bound amphipathic form of GPI anchored proteins and is only exposed after cleavage of the GPI anchor by phospholipase-C. It is now known that the major epitope involved in this recognition is the inositol 1,2-cyclic monophosphate that is generated by phospholipase-C cleavage of the GPI anchors; other structural features may also contribute to the antigenicity (Zamze *et al.* 1988; Hooper *et*

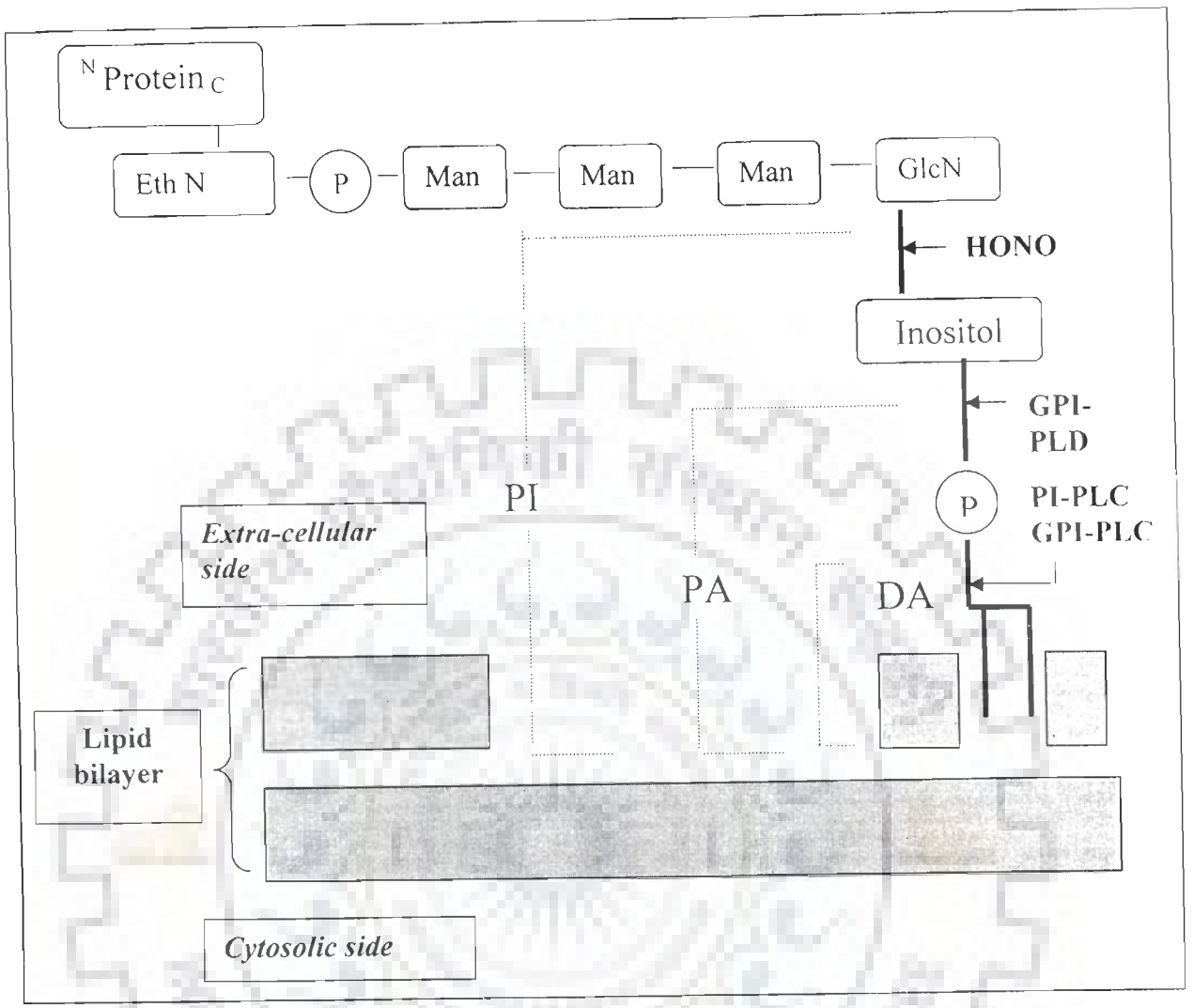



**Figure: 2. Schematic representation of the glycan core structure common to all GPI anchors.** The sites of cleavage of phospholipase C enzymes (PI-PLC, GPI-PLC), phospholipase D (GPI-PLD), and nitrous acid (HONO) are indicated as are lipid products resulting from these cleavages. PI (Phosphatidylinositol), PA (phosphatidic acid) and DAG (Diacylglycerol).

*al.,* 1991). Reactivity of protein with anti-CRD antibody after solubilization by PI-PLC strongly indicates the presence of GPI anchor and has now come to be used as a probe for the identification of this class of protein. The anti-CRD

antibody cannot react with the reaction products of either nitrous acid deamination or phospholipase D treatment because the desired epitopes for recognition are not created.

### 2.4.8 Electophoresis and immunoblotting:

Anti-CRD antibody is commercially available (Oxford Glycosystems, U.K.) and may be used for immunoprecipitating or immunoblotting proteins of interest after phospholipase C treatment. Western blot analysis and ELISA have been successfully used to identify CRD on proteins (Hooper *et al.*, 1991; Broomfield and Hooper, 1993). Alternatively, polyclonal antibodies can also be raised against the desired GPI protein and used as a probe. Several other variations are also possible. For example, the cells surface protein can be radiolabeled and proteins released by the action of PI-PLC can be identified by electrophoresis followed by autoradiography. If the protein of interest can be made to express in a cell line, then metabolic labeling with $^{3}H$ or $^{14}C$ ethanolamine can be attempted. The metabolically labeled cells can be harvested at specific time points and immunoprecipitated after subjected them to either PI-PLC treatment. The released protein can be identified by SDS-PAGE followed by fluorography (Hooper, 2001).

### 2.4.9 Localization of GPI-anchored proteins

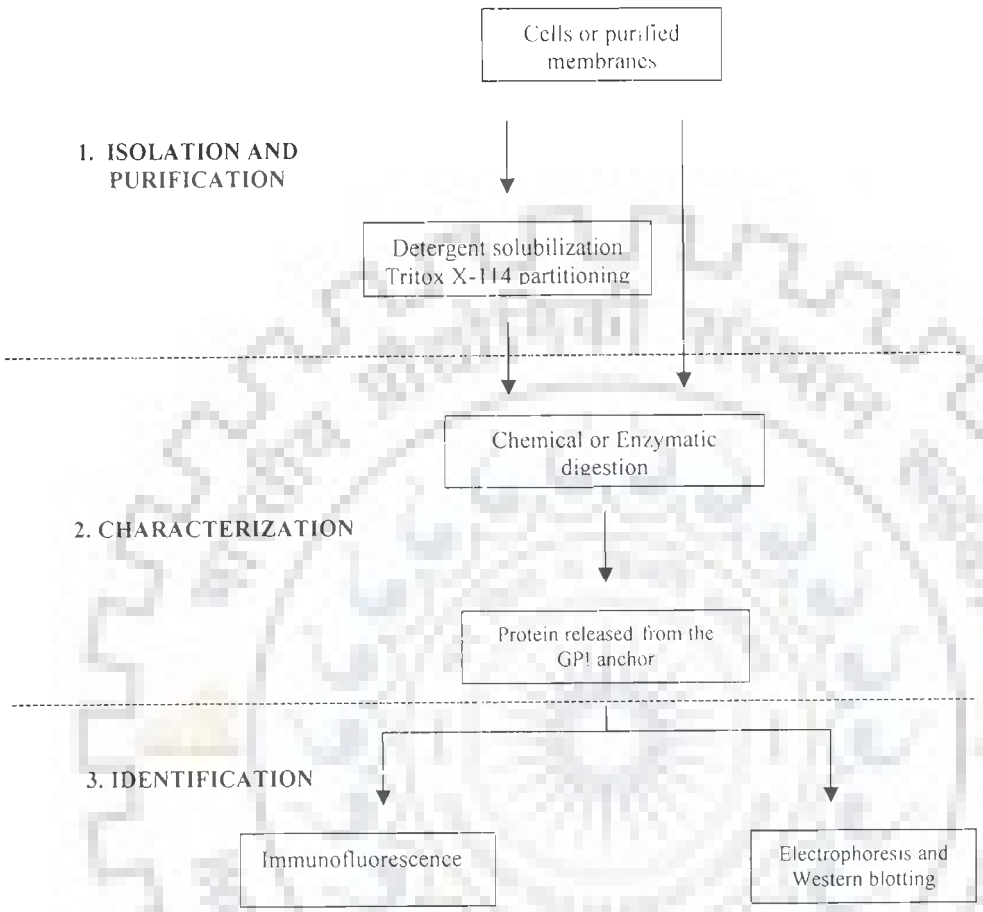
Immunofluorescence microscopy can be used to monitor the removal of GPI-anchored proteins from the cell surface following PI-PLC treatment on

whole cells both live and dead (Yudin *et al.,* 2002). In specialized cells as the sperm, this technique helps to identify the distribution of GPI proteins on specific microdomains of the membrane surface.

### 2.4.10 Predicting GPI Anchorage:

In recent times, a novel approach has been adopted for studying GPI proteins using databases created on the basis of experimental observations. The annotations take into consideration specific signal sequences, cleavage/attachment sites needed by the nascent peptides for GPI anchorage. It is claimed that even the actual site of GPI anchor attachment in a protein can be predicted. An algorithm has been developed by which the potential for a protein being GPI-anchored can be assessed (Eisenhaber *et al.,* 1999; 2000). The algorithm employs a composite prediction function: $S = S_{profile} + S_{physical\_pattern}$. $S_{profile}$ evaluates the type of amino acid preference and $S_{physical\_pattern}$ assesses the physical properties of conserved amino acid sequences. This is available free of charge to interested researchers at the public domain termed 'big-II predictor' http://mendel.imp.univie.ac.at/gpi_prediction.html. Thus, even if the complete sequence of a protein is not known, it is possible to predict whether a protein will be post-translationally modified to a GPI anchored one (Eisenhaber *et al.,* 2003). However, it must be cautioned that no matter how accurate the predictions are experimental evidence would still be required for final verification.

Figure: 3. Flow chart for the step-wise analysis of GPI-anchored proteins




## 2.5 BIOSYNTHESIS OF GPI ANCHORED PROTEINS:

Much of the knowledge that has been acquired regarding the biosynthetic pathway of GPI protein is derived from model studies involving parasites, yeast, and mammalian cell lines (Kinoshita *et al.,* 1997). It has not been possible to carry out any significant work on the biosynthesis of GPI anchors of sperm proteins since sperm are specialized cells that lack the organelles responsible for this kind of activity. Nevertheless, the steps involved are to a large extent

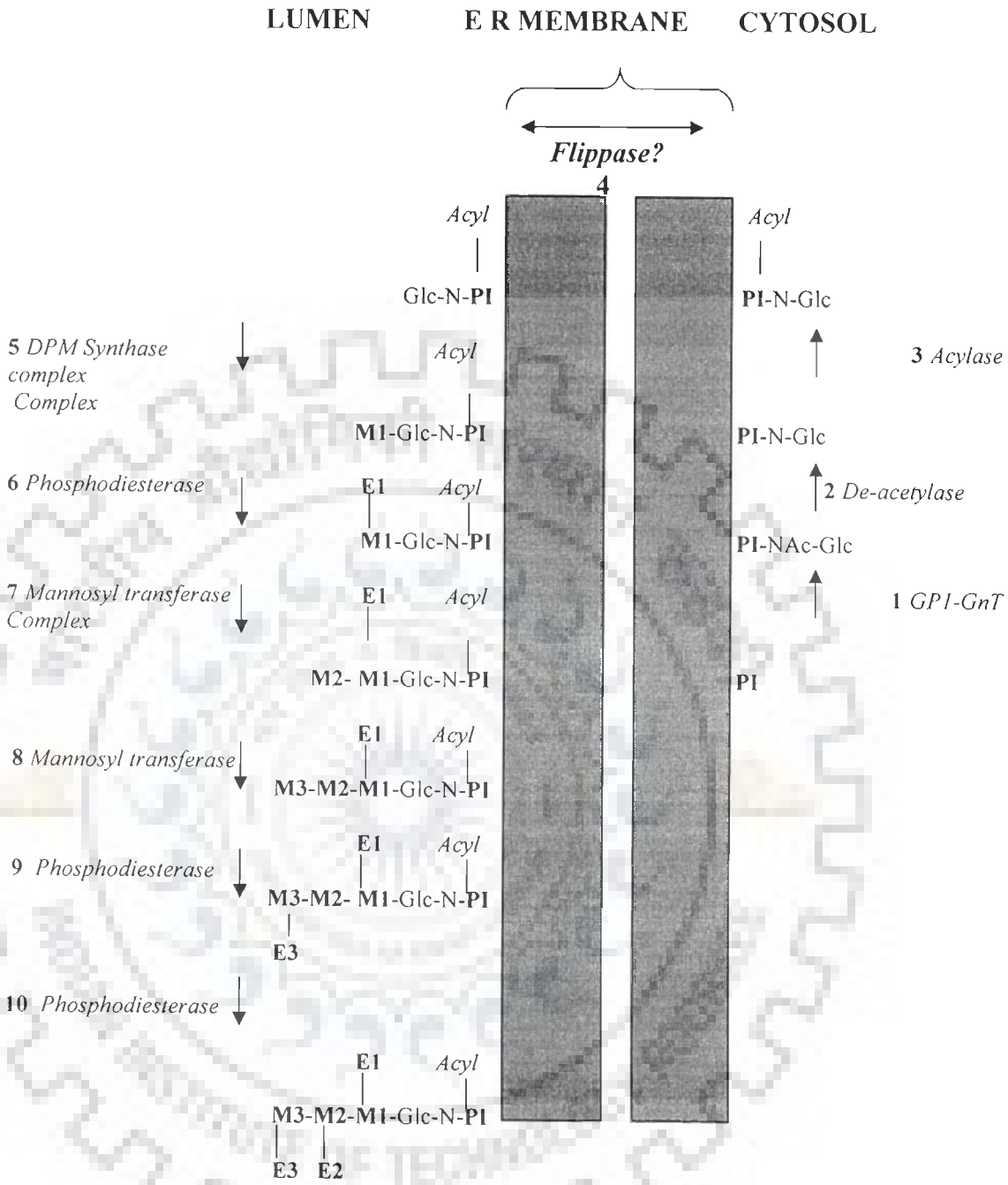



**Figure: 4. Schematic representations of GPI anchor biosynthesis in the ER.** The biosynthesis is initiated in the cytoplasmic side of the ER membrane (steps1-3). Both GlcN and the acyl group are attached at different positions of the inositol residue on PI. Tis intermediate then flips to the luminal side of the ER (step 4). The transfer of three mannosyl residues and three ethanolamine residues assembles the rest of the glycan backbone on the PI intermediate (steps 5-10). The sequence of these transfers is not same in different cells/organisms. The enzymes involved are inscribed in italics. Pi phosphatidylinositol; GlcN glucosamine; Acyl acyl group GlcNAc N-acetylglucosamine; M1, M2, M3, mannose residues; E1, E2, E3 phosphoethanolamine residues *(Reproduced with permission from the authors: Pereira et al., 2003)*

identical for all mammalian systems (Englund, 1993; McConville and Ferguson, 1993). Since several excellent comprehensive reviews have been published (Undenfriend and Kodukulu, 1995; Steven 1995; Takeda and Kinoshita, 1995) only the major steps by which GPI proteins are synthesized are recapitulated here.

### 2.5.1 The GPI anchor precursor:

The biosynthesis of the core anchor is initiated in the cytoplasmic face of the ER and completed in its lumen (Vidugiriene and Menon, 1993; 1994) Basically, sugar residues (glucosamine, mannose) and phosphoethanolamine forming a GPI anchor are sequentially added on to phosphatidylinositol forming a GPI anchor precursor (Takeda and Konishita, 1995). The construction of the GPI anchor precursor is a multi-step process requiring enzymes like glycosyl synthase, glycosyl transferase, inositol acyl transferase, deacytylase, ethanolamine phosphotransferase and mannosylases. Extensive work has been carried out on this pathway and most of the genes involved in the synthesis have already been cloned and now the effort is towards understanding the regulation of these steps (Kinoshita *et al.*, 1993).

### 2.5.2 Transfer of protein to GPI anchor:

It has been established that proteins destined for GPI anchorage are synthesized much the same way as other protein through the processes of transcription and translation. Many a time a protein attached to the GPI anchor

also exists in soluble form, which has large segments of sequence homology (Fortna *et al.,* 1999). It is believed that isoforms result from alternate splicing of the same gene (Ohta *et al.,* 1999). The proteins that are directed to be GPI anchor are synthesized as a nascent precursor with two specific signal sequences; one is the N-terminal signal peptide sequence that is responsible for its translocation into ER lumen during synthesis and the second is a small hydrophobic C-terminal stretches of 15-20 amino acids that holds the protein in the ER membrane. As soon as the protein synthesis is completed, processing take place in the ER lumen, wherein the precursor protein is cleaved, freed from the ER membrane and then attached to the pre-assembled GPI anchor. After cleavage, the C-terminal amino acid of the free protein to which the GPI anchor gets attached is termed the ω-site and always has a small side chain. The second and third amino acids referred to as ω+1 and ω+2, exhibit species-specific variations (Undenfriend and Kodukula, 1995). This is followed by a sequence of amino acids (5-7 hydrophilic and 12-20 hydrophobic residues) characteristic of an attachment signal (Micanovic *et al.,* 1990; Kodukula *et al.,* 1992; Yan *et al.,* 1998). The attachment of the protein to its anchor requires two gene product Gaalp and Pgi8p, which together form a complex having transaminase activity (Ohishi *et al.,* 2000). It has been suggested that the enzyme transaminidase binds to the attachment signal of the nascent peptide, attacks the carboxyl end of the ω-site amino acids and then guides the released peptide to the GPI anchor to complete the transamidation reaction (Chen *et al.,* 1996).

### 2.5.3 Modification in Golgi and transfer to plasma membrane:

The addition of carbohydrate moieties to the GPI protein takes place in the Golgi apparatus. This gives rise to the mature GPI anchored protein, which finally finds its way to the membrane surface. The fine details of sorting and exporting the mature GPI protein from the ER to the plasma membrane have been clearly described (Nosjean *et al.*, 1997). In a nutshell, it is suggested that protein sorting is governed by a combination of different factors, which include GPI addition, transcytosis, N- and O-glycosylation and oligomerization.

### 2.5.4. Localization in Lipid rafts:

Some GPI anchored proteins are believed to preferentially partition into specialized membrane microdomains known as DIGs (detergent insoluble glycolipids) or 'lipid rafts' (Brown and Rose, 1992; Simons and Ikonen, 1997). These membrane rafts are also enriched in cholesterol, sphingolipids and members of the Src family of tyrosine kinases (Hooper, 1999). Using simple phosphatidylcholine (PC) and cholesterol rich PC liposomes it was demonstrated that GPI anchored protein insertion, was cholesterol- dependent (Morandat *et al.*, 2002; 2003). Thus, the incorporation and clustering of GPI proteins into membrane microdomains is important for membrane trafficking and mediating cell signaling events (Stulnig *et al.*, 1997; Friedrichson and Kurzchalia, 1998; London and Brown, 2000). When the GPI anchored protein happens to be an adhesion receptor, they home into membrane rafts, form cell

adhesion complexes and are responsible for physical co-ordination of cell-cell interactions (Harris and Siu, 2002)

In the sperm of sea urchins, GPI anchored proteins and transducer proteins have been co-localized in the DIGs, implicating an organized receptor activation and signal transduction (Ohta *et al.*, 2000a,b). Immunoblot analysis, indirect and confocal immunofluorescence studies have shown the presence of membrane rafts enriched in caveolin-1 in mouse and guinea pig spermatozoa. These distinct raft domains are suggested to be involved with the biogenesis and exocytosis of the acrosome. From the data obtained it has been proposed that the caveolin-1 may be associated with the signaling pathways regulating processes such as capacitation and flagellar motility (Travis *et al.*, 2001).

**2.6 GPI-anchored proteins and Reproduction:**

The discovery that some proteins are anchored to the outer leaflet of the lipid bilayer by phosphatidylinositol provides a new approach to understand the role of sperm plasma membrane and also helps in defining the behavior and mechanism of different surface molecules in fertilization. The distribution and translocation of this class of protein to the sperm surface during maturation, storage, capacitation, acrosomal reaction and fertilization is a clear indication of their importance in reproduction (Myles, 1993). The possible modes of acquisition of GPI anchored proteins by sperm and the part played by this class of protein in the process of reproduction has been extensively reviewed (Pereira

*et al.*, 2003). In the following section, an attempt has been made to systematically survey the GPI-anchored proteins with particular emphasis on mammalian sperm.

### 2.6.1 Role of GPI anchored sperm proteins:

One of the first known GPI anchored sperm proteins detected on the posterior head region of the guinea pig was the PH-20 (Primakoff *et al.*, 1985) which has since been best-characterized (Thaler and Cardullo, 1995; Myles and Primakoff, 1997). Extensive research carried out over the years has revealed the following: First, PH-20 has hyaluronidase activity at acid and neutral pH. Second, it acts as a receptor for hyaluronic acid (HA) induced signaling. Finally, it can also recognize the zona pellucida surrounding the oocyte. The number of molecules that can be physically carried by the sperm is severely restricted by its miniscule size and weight. Thus, PH-20, also known as SPAM1, is now considered to be a multifunctional protein. Two species of PH-20 have been recognized that differ biochemically: one associated with the plasma membrane and other with the inner acrosomal membrane. The PH-20 that resides on the plasma membrane has two domains. The first domain has an enzyme active site that is responsible for the local degradation of the HA rich extracellular matrix of the cumulus at neutral pH. The second domain, the HA-binding domain leads to aggregation of PH-20 in the plasma membrane and gets linked to a 92-kDa signaling protein on the cytoplasmic leaflet of the plasma membrane (Cherr *et*

*al.*, 2001). Eventually, the intracellular calcium levels rise, triggering the acrosomal reaction. The PH-20 of the inner acrosomal membrane is the one that recognizes the zona pellucida. Thus, a rather clear picture of the PH-20 of sperm has emerged.

In the rat, the orthologue of guinea pig PH-20 is called sperm 2B1 antigen. The endoproteolytic cleavage of 2B1 in the epididymis causes a shift in the pH optimum of hyaluronidase to facilitate the penetration of spermatozoa through the cumulus oophorus (Seaton *et al.*, 2000). Since the GPI anchors span only the outer leaflet of the lipid bilayer of membranes, the migration of this protein from the tail to the acrosomal domain during capacitation of sperm is easily possible by the lateral movement of the anchor on the sperm surface free from direct cytoskeletal constraints.

Several other GPI-anchored proteins exhibiting catalytic properties have been reported. A 5'nucleotidase attached by the GPI-anchor and acquired post-testicularly was detected on human sperm membrane (Fabaini and Ronquist, 1993; Schieman *et al.*, 1994). Among the glycosidases, alpha mannosidase was released from the boar sperm plasma membrane by PI-PLC treatment, supporting a GPI-mode of anchorage (Kuno *et al.*, 2000). This sperm bound enzyme not only possesses catalytic properties but also acts as a receptor for mannose-rich residues of the egg surface (Tulsiani *et al.*, 1989). A recent study has shown that a 42-kDa sperm serine protease (TESP5) is selectively included

into lipid rafts of mouse sperm membranes presumably as a GPI-anchored protein (Honda *et al.*, 2002)

A GPI-anchored receptor that can accept a 'decapacitation factor' (DF) has been identified on the surface of mouse sperm collected from the cauda epididymis. Removal of the DF results in acceleration of capacitation and increased fertilizing ability. The DF is easily dislodged from the uncapacitated sperm surface by either centrifugation or by incubation with fucose at > 5 mM concentrations. In addition, FOTC-labelled fucosylated BSA bound specifically to the post-acrosomal region where this GPI-anchored receptor has been localized. Moreover, both excess fucose and crude DF inhibit this binding. Treatment of sperm with PI-PLC also gets rid of the DE. However, exogenous DF is unable to reassociate with PI-PLC treated sperm. Taken together, it has been concluded that DF binds via fucose residues to a GPI-anchored receptor on the sperm plasma membrane (Fraser, 1998).

Several GPI linked leukocyte antigens cluster of differentiation (CD) proteins have been detected on the surface of sperm leading one to believe that some kind of a cell-to-cell transfer occurs (Kirchhoff and Hale, 1996). Perhaps the GPI-type of anchorage facilitates the process. Decay-accelerating factor (DAF, CD55) is one such GPI-anchored membrane inhibitor of complement activation. In rats and mice, high levels of this protein have been detected on spermatids and mature sperm, perhaps protecting them from complement attack (Miwa *et al.*, 2000; 2001). In humans, a GPI-anchored gp20 sialoglycoprotein

on sperm surface has been reported with the core peptide homologous to the leukocyte peptide antigen, CD52 (Focarelli *et al.*, 1999; Della *et al.*, 2001). The same antigen is secreted by the human epididymis (HE5) and is associated with the mature of sperm. Androgen and temperature are principal factors synergistically modulating the epididymal CD52 expression (Pera *et al.*, 1996; Kirchhoff *et al.*, 1998). After the sperm in the epididymis acquires the protein, extensive refolding occurs under capacitation conditions with the exposure of sialic acid residues (Yeung *et al.*, 2001).

More recently, it has been demonstrated by microsequencing that the SAGA-1 human sperm antigen and the lymphocyte CD52 represent glycoforms (Diekman *et al.*, 2000). Unique carbohydrate epitopes of GPI anchored sperm proteins like the S19 epitope of SAGA-1 (Sperm agglutination antigen-1), which are specifically found in the epithelium of epididymis and vas deferens as well as both epididymal and ejaculated spermatozoa have been used as a contraceptive immunogen (Norton *et al.*, 2002). Monoclonal antibodies raised to S19 (S19mAb) and bound to the surface of Novasomes, a multi-lamellar liposome delivery vehicle agglutinated human spermatozoa advocating that this strategy could be employed to develop an intra-vaginal contraceptive.

Another GPI-anchored CRISP glycoprotein and a member of the CAP (CRISP, Ag 5, PR-1) family of proteins, is MAK248. The expression of this protein was originally reported to be confined to the epididymis but has now been localized to the equatorial segment and posterior head regions of

capacitated cynomolgus macaque sperm (Yudin *et al.*, 2002). It has been suggested that the protein may function as a protease inhibitor.

**Table: 1. Mammalian Sperm bound GPI-anchored proteins at a glance.**

| GPI-protein | Animal | Localization | Function | Reference |
|---|---|---|---|---|
| 2B1glycoprotein (Orthologue of PH-20) | Rat | Migrates from tail to acrosome during capacitation | Penetration of sperm through cumulus oophorus | Seaton *et al.*, 2000 |
| PH-20; SPAM-1 | Guinea pig, rat, Monkey, Human | Sperm surface/ inner acrosomal membrane | Hyaluronidase brings about local degradation of cumulus; Hyaluronic acid induced intracellular signaling; receptor for zona pellucida antigens | Myles and Primakoff, 1997; Cherr *et al.*, 2001 |
| 5'Nucleotidase (Identical to CD 73) | Human | Sperm | Ectoenzyme, Adhesion | Fabiani and Ronquist, 1993; Schiemann *et al.*, 1994 |
| α-Mannosidase | Boar | Sperm | Fertilization | Kuno *et al.*, 2000 |
| Gp20 sialoglycoprotein, HE5, SAGA-1, (identical to CD52) | Human | Ejaculated sperm | | Giovampaola *et al.*, 2001; Yeung *et al.*, 2001; Norton *et al.*, 2002; |
| Decay accelerating factor (DAF) (identical to CD55) | Mouse | Spermatids, mature sperm | Inhibitor of complement activation | Miwa *et al.*, 2001 |
| CRISP glycoprotein, MAK 248 (CAP family of protein) | Monkey | Expressed in epididymis Sperm (posterior head equatorial segment) | Protease inhibitor | Yudin *et al.*, 2002 |
| SAMP14 (ly-6/uPAR) (Super family of receptors) | Human | Testis-specific expression; Acrosomal membranes | Sperm-egg interaction | Shetty *et al.*, 2003 |

Recently, a GPI anchored human sperm protein SAMP-14 has been identified with a single functional domain similar to the Ly-6 and urokinase plasminogen activator receptor superfamily of proteins (Ly6/uPAR). Indirect immunofluorescence and immunoelectron microscopic studies of Shetty *et al.* (2003) has shown that the protein is associated with the acrosome and persists on the inner acrosomal membrane even after the acrosomal reaction. They have also suggested a role in egg-sperm interaction since antibodies against recombinant SAMP14 inhibited both the binding and fusion of human sperm to zona free hamster eggs.

Several new GPI- linked sperm proteins are being identified. Although most of these reports are preliminary in nature, a list of these molecules is tabulated for ready reference (Table 1.).

**2.6.2 Role of GPI anchored egg proteins:**

GPI anchored proteins on the surface of egg membranes are also reported to play a role in gamete fusion. Treatment of hamster oocytes with PI-PLC releases a 25-40 kDa protein cluster (PI 5-6) and inhibits human sperm-hamster egg interaction (Coonrod *et al.,* 1999). Similar results have obtained with mouse oocytes treated with PI-PLC which, releases 70 kDa (PI 5) and 35-45 kDa (pl 5.5) protein clusters from the egg surface and inhibits sperm-oolemma binding and fusion (Coonrod *et al.,* 1999). Conditional Pig-a knockout females (animals with portion of the Pig-a which encodes an enzyme involved in GPI anchor

biosynthesis in oocytes knocked out) were infertile (Alfieri *et al.*, 2003). *In vitro* fertilization assays with the eggs collected from these knockout animals were also shown to be severely deficient in their ability to fuse with sperm. However, they have cautioned that the full complement of egg GPI-anchored proteins is not essential for gamete fusion.

## 2.7 Mode of acquisition of GPI Proteins by sperm:

The general pathway of GPI-anchored protein biosynthesis is the same from yeast to higher mammals and the detailed steps involved have been extensively reviewed (Undenfriend and Kodukulu, 1995; Steven, 1995; Takeda and Kinoshita, 1995). Although, reports indicate that sperm have GPI anchored protein on their membranes, they lack endoplasmic reticulum (ER), which is crucial for the biosynthesis of this class of proteins. Moreover, in view of the large number of molecule required, the enormous number of sperm produced and the small size of this gamete, it is difficult to assume that the entire protein requirement of sperm is met with by biosynthesis in the testis alone. It is therefore likely that GPI anchor proteins are synthesized by the same metabolic pathway elsewhere and then acquired by the sperm at several sites along the reproductive tract through specialized mechanisms (Fabiani and Ronquist, 1993; Rooney and Atkinson, 1994; Kirchoff and Hale, 1996).

Sperm has limited protein synthetic capacity and it is only logical to expect that most proteins be incorporated on its surface during development in

the testis. Testicular cells are known to contain GPI anchored proteins. Ceruloplasmin (Cp), a copper binding protein with ferroxidase activity (Attieh *et al.,* 1999) is one such GPI anchored protein identified on Sertoli cells (Fortna *et al.,* 1999). Most sperm membrane protein including PH-20, a GPI anchored protein, are synthesized by developing germ cells. The origin of at least one glycosidase, the α-mannosidase found on mature sperm has been traced back to the testicular germ cells (Pereira *et al.,* 1998) and later shown to bear a GPI mode of anchorage (Kuno *et al.,* 2000). But obviously, this cannot be the only mode of acquisition of protein by sperm. Owing to the miniscule size of sperm and the requirement of a great number of molecules, continuous remodeling of the membrane surface occurs during passage through the male and female reproductive tracts. This involves loss or modification of existing proteins and also addition of new proteins to the sperm surface.

There is now evidence for GPI anchored proteins not detected on testicular germ cells making their appearance on the surface of cauda sperm (Moore *et al.,* 1989; Kirchhoff *et al.,* 1996), suggesting that the sperm acquires them during transit through the epididymis. Several regions of the male reproductive tract are known to actively support the functions of the sperm by secreting biomolecules that are incorporated into the sperm surface (Cooper, 1986; Jones 1989; Kirchhoff and Hale, 1996). Other antigens common to lymphocytes like the complement restriction factors found in the epithelial cells of the epididymis, vas deferens, seminal vesicle and prostate are also transferred

to the sperm surface (Rooney *et al.*, 1993a; Rooney and Atkinson, 1994). Even GPI anchored sperm ectoenzymes like 5' nucleotidase are transferred to spermatozoa; post testicular (Fabiani and Ronquist, 1993; Schieman *et al.*, 1994). Some of these antigens have been detected on secretory vesicles in the luminal/seminal fluid (Aumuller and seitz, 1990; Rooney *et al.*, 1993a;b; Rooney and Atkinson 1993; 1994; Fabiani and Ronquist, 1993; Fornes *et al.*, 1995; Manin *et al.*, 1995). These vesicles (prostasomes) have been shown to fuse with sperm under specified conditions (Arienti *et al.*, 1997 a; b) In addition, phospholipid transfer proteins (PL-TPs) that can act as a specific carriers for single phospholipid between membranes are known to exist in almost all eukaryotic cells (Wirtz, 1997). These facts make it possible to conceptualize a cell-to-cell transfer of GPI anchored proteins. It is likely that GPI proteins are synthesized by the epithelial cells lining the male reproductive tract (epididymis/seminal vesicles/prostate), secreted into luminal/seminal fluids, transported by secretory vesicles and ultimately incorporated into sperm by a process of fusion (Kirchhoff and Hale, 1996).

The modification of GPI anchored proteins does not stop in the male reproductive tract. Recently studies show that incubation of ejaculated human sperm in capacitation conditions opens up the HES (CD52) molecule, increasing the accessibility of some sialic acid residues and the core peptide, particularly the GPI anchor (Yeung, 2001). Therefore, as far as sperm is concerned we have evidence for GPI protein biosynthesis in testicular germ cells, acquisition /

modification of GPI proteins in the male reproductive tract and finally conformational changes in GPI proteins after ejaculation.

Another possibility is that the GPI anchor on the sperm surface remains the same but the proteins are replaced. This hypothesis stems from experiment that have demonstrated that desired proteins could be transferred to the GPI anchor on membranes, when linked to specific signal sequences (Moran and Caras, 1991 a; b; Moran *et al.,* 1991). Experiments have shown that when 29 residue of the COOH terminus of a GPI anchored protein, decay-accelerating factor, were fused to the COOH terminus of a secretory protein, the fusion protein was directed to the cell surface by means of a GPI anchor. Thus, it is possible that if proteins with these specific signal sequences are synthesized and secreted by the epithelial cells lining the reproductive tract, they could be picked up by sperm membranes with GPI anchor; provided, the appropriate enzymatic machinery for this transfer is also available in the seminal fluid.

A procedure to effect transfer of GPI linked proteins from one cell type to another in both an *in vivo* or *in vitro* system has been experimentally demonstrated under physiological conditions (Kooyman *et al.*, 1998). Based on the facts gathered from several experimental data, a consolidated hypothetical model for the replacement of the protein component in sperm GPI anchored molecules has been proposed (Pereira *et al.,* 2003). Further detailed investigations would however be necessary, to firmly establish this type of targeted delivery of proteins to the sperm surface.

## 2.8 Future scope

From the above survey, it is abundantly clear that the GPI-anchorage is structurally and functionally suited to assist the gametes to meet, fuse, fertilize and develop into embryos. In a nutshell, the GPI anchorage offers sperm special advantages that few other transmembranal proteins can give. First, sperm despite their minute size are able to manage with a limited number of proteins. This is due to the high rate of lateral mobility exhibited by GPI proteins, which ensures a better dispersion of molecules on the membrane surface. Second, the same GPI component could serve as an anchor for several proteins so that the exchange of proteins is possible on the surface of sperm membranes. In a way this feature economizes on the number of molecules sperm need to carry any given time. Third, by virtue of being GPI-anchored, proteins can also crossover from one cell to another either through secretory vesicles or phospholipid transfer proteins. The mechanism would explain how continuous remodeling of sperm membrane occurs during its sojourn in the male and female reproductive tracts.

The wide range of methods for the isolation of membranes together with the improved techniques for the identification and characterization of GPI-anchored proteins that are now available have made studies on this class of proteins far more simpler. As a result novel GPI-anchored proteins on the surface of gametes are increasingly being reported. The problem still remains because adequate amounts of these proteins are difficult to obtain. Once this is overcome and the roles of these proteins are deciphered in concert with the

mechanisms involved, our understanding of the sperm-egg interaction is bound to make great progress.

# Chapter - 3

# Materials & Methods

## 3.0 MATERIAL AND METHODS

### 3.1 Chemicals:

All chemicals used for electrophoresis, substrates used in the enzyme assays and standards were from M/s Sigma-Aldrich. Protein assay kits were obtained from Pierce Chemicals (USA). All other chemicals were of analytical grade and were obtained from Merck India, BDH (India) and SRL (India). The organic solvents were acquired from Qualigens (India) and Thomas Baker (India). Phosphatidylinositol-specific Phospholipase C (*Bacillus cereus)* and Triton X-114 were purchased from Fluka Chemie GmbH (Switzerland). Freund's adjuvant from DIFCO LABORATORIES Michigan USA, Rabbit IgG-HRP conjugate and goat antirabbit IgG-FITC conjugate were supplied by Bangalore Genei (India).

### 3.2 Tissue collection and isolation of sperm:

All procedures and experiments involving animals used in investigations on the GPI-anchored sperm proteins of Goat (*Capra indica*) have the approval of the Institutional Animal Ethics Committee. Goat testis along with the epididymis (100 ± 10 g) from mature animals was purchased from the local abattoir from time to time, collected and brought to the laboratory in ice within one hour of slaughter. The testis was perfused through the testicular artery until the epididymis was devoid of blood and became pale white. The epididymis was

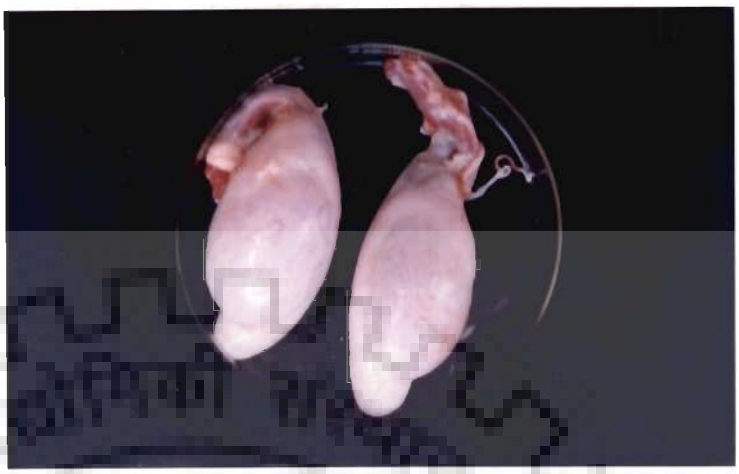
dissected out and divided into caput (head), corpus (body) and cauda (tail) as shown in Figure 5.

The segment of the epididymis belonging to 10 animals were pooled at a time and used for the recovery of sperm. Briefly, the tissue was cut (approximately, 1-2 mm thick slices) with a sharp razor blade, suspended in PBS and agitated in a shaking water bath at 37°C to release the sperm. The supernatant containing sperm cells was aspirated, passed through a nylon mesh to get rid of small tissue pieces together with other debris and then centrifuged at 600xg for 10 min. to obtain the sperm as a pellet. The pellet was washed in PBS three times, diluted to the desired cell numbers and then used for the biochemical investigations. Cell counts were made on a hemocytometer under a light microscope (Leica). The sperm prepared in the above fashion were found to be structurally intact. Identical methods have been previously used to obtain sperm from the epididymis (Tulsiani *et al.*, 1989).

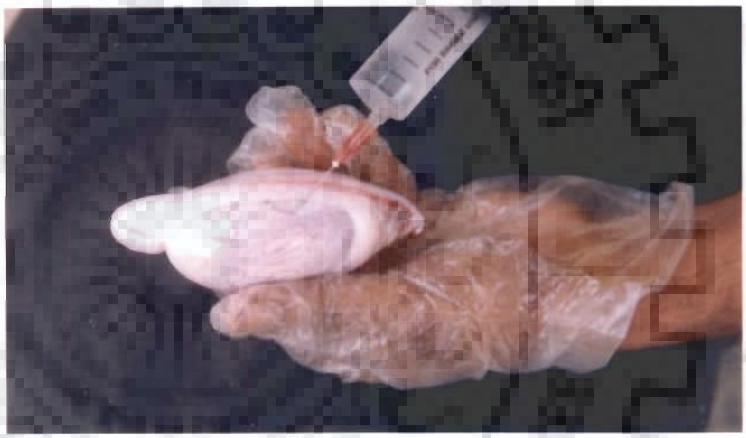
**3. 3 Ultra-structure of Goat sperm:**

Aliquots were also drawn for scanning electron microscopic (SEM) examination of the sperm. Fixation was done with 4% glutaraldehyde, and dehydrated though an alcohol series, before observation under a SEM (LEO, 435,VP). The structure of goat sperm as seen through a SEM is shown in figure 6.

(A)



(B)



(C)


Fig. 5

**Figure: 5 Steps involved in the dissection and isolation of sperm from goat testis.** (A.) Goat testis together with the epididymis ( B.) Perfusion with PBS through testicular artery (C.) Perfused epididymis showing the different segment

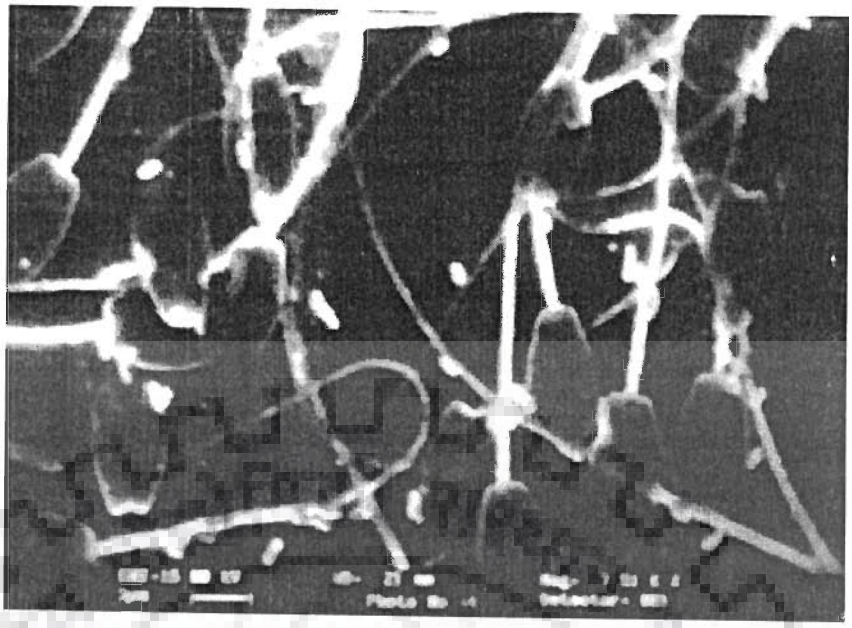


Fig. 6.

**Figure: 6 Scanning Electron micrograph of goat sperm obtained from cauda region. Bar =3μm (——)**

### 3.4 Isolation and purification of sperm plasma membrane:

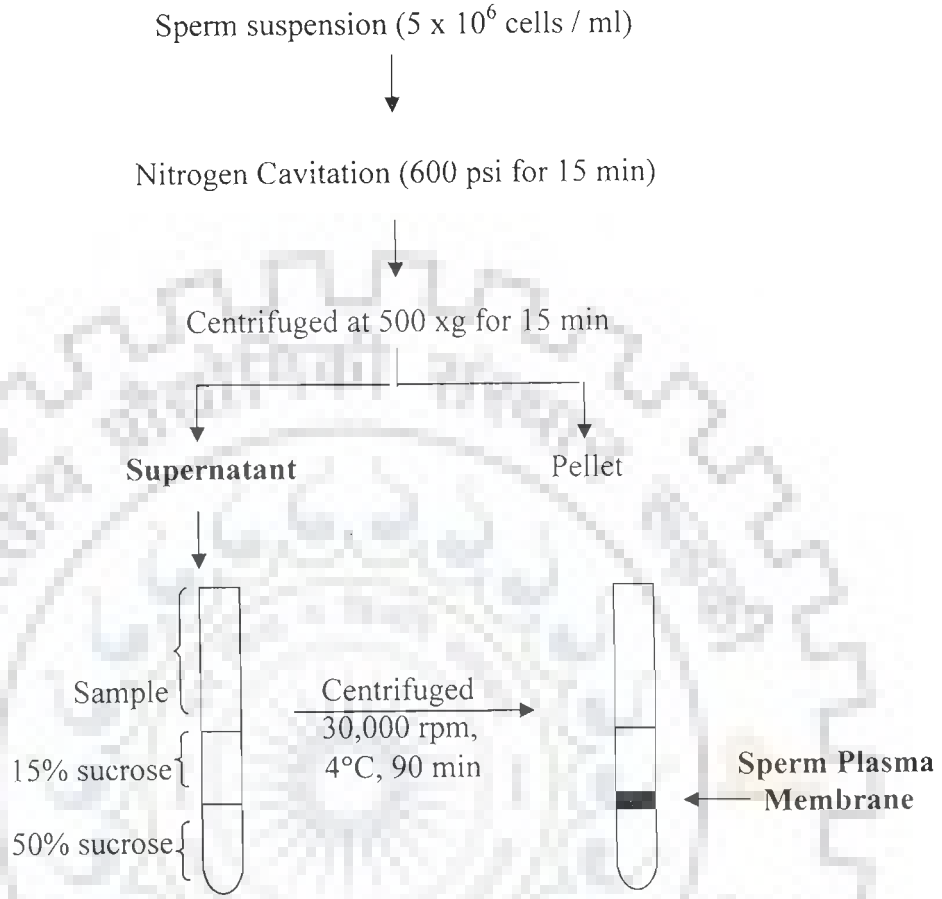
**Reagents:**

1. PBS pH 7.4: Dissolve 8gm of NaCl, 0.2g m KCl, 1.44gm of $Na_2HPO_4$ and 0.24 gm of $KH_2PO_4$ in 800ml of distilled water. Adjust the pH to 7.4 with HCl make up the volume to 1000ml, sterilize it, autoclave it for 20min at 15lbs.
2. PMSF (0.2mM): $C_7H_7FO_2S$, Mol. wt. 174.20, 0.0344gm per 1000ml of PBS.
3. Pipes buffer: (20mM pipes buffer) $C_{18}H_{18}N_2O_6O_2$. (0.604 gm in 100ml of Distilled $H_2O$).

**Procedure:**

The cauda epididymis is a storage organ for sperm and the concentration of sperm is far greater in this segment than in the caput and corpus. Therefore, spermatozoa obtained from the cauda epididymis of goat were suspended in 100 ml of ice cold PBS pH 7.4 containing 0.2mM phenylmethysulfonylfluoride (PMSF), to a final dilution of $5x10^6$ cells /ml. Plasma membrane was prepared by the original procedure of Oison *et al.* (1987) as modified by Tulsiani *et al.* (1989). The stepwise scheme of the procedure is outlined in a flow diagram (Figure 7). Briefly, 90ml of sperm suspension was transferred to a Parr Bomb cavity (S. D.HARDSON & CO. Calcutta.) for cell lysis. The spermatozoa were disrupted by nitrogen cavitation using pressure of 600 psi and an equilibration period of 15min. The cavitated sperm suspensions were centrifuged for 15min. at 500 xg and the supernatant was removed by aspiration. Aliquots (5ml) of the supernatant were layered on top of 2ml steps of a 15% (wt / vol.) and 50% (wt / vol.) sucrose solution in 20mM pipes buffer, pH 7.2. The gradient was centrifuged at 30,000 rpm for 90 min in a TST 41.14 rotor (Centrikon 2060, Kontron). The plasma membrane accumulated as a dense white band at the interphase between the 15% and 50% sucrose solutions. This band was collected by aspiration, diluted with 3 volumes of PBS and centrifuged at 105,000 xg for 30 min in Ti- 50 rotor (LS-50B, Beckman ultracentrifuge). The resulting pellet was used for biochemical and ultrastructural studies.

**Figure: 7. Flow diagram for the isolation of sperm plasma membrane.**




### 3.5 Structural characterization of sperm membrane preparation:

The firm pellet of the membrane preparation was processed for Transmission Electron Microscopy (TEM) by the procedure of David, (1973) as recommended by the Electron microscopic center, a national facility at the All India Institute of Medical Sciences, New Delhi. Briefly, the isolated sperm membrane pellet was fixed with 2.5% glutaraldehyde in 0.1M sodium cacodylated buffer, pH 7.2 at 4°C. Fixation was done for 10 –18 hours at 4°C,

after which the pellet was washed in fresh buffer, and post-fixed for two hours in 1% osmium tetraoxide in the same buffer at 4°C. After several washes in 0.1M sodium phosphate buffer, the sample was dehydrated in graded acetone solutions and embedded in CY 212 araldite. Ultra-thin sections of 60-80nm thicknesses were cut using an Ultracut-E (Reichert-Jung) ultra-microtome and the sections were stained in alcoholic uranyl acetate (10 min) and lead citrate (10 min.) before examining the grids in a transmission electron microscope (PHILIPS, CM-10), operated between 60-80 kv.

### 3.6 Biochemical Characterization of the sperm membrane preparation:

Biochemical characterization was done on the samples drawn at various stages during the preparation of sperm plasma membrane by monitoring the levels of 5' nucleotidase, acid phosphatase, RNA, DNA and protein.

#### 3.6.1 5' Nucleotidase Assay:

The enzyme 5′nucleotidase is present in abundance on the surface of plasma membranes and has been used as a marker to examine the purity of membranes during cell fractionation studies. In the present studies, aliquots of samples were suitably diluted and the 5'-nucleotidase activity was assayed according to the procedure of Touster *et al.* (1970).

**Reagents:**

1. AMP, 0.05 M
2. $MgCl_2$, 0.1 M
3. Glycine, 0.1 M
4. Stopping Reagent: 8% Trichloro acetic acid (TCA)
5. Standard Solution: 10 mM $KH_2PO_4$ diluted 1:10 to obtain 1 mM
6. Color Reagent: 6 parts of 3.6mM ammonium molybdate in 0.5M $H_2SO_4$ + 1 part 10% ascorbic acid.

**Procedure:**

The reaction mixture (total volume 0.5 ml) contained 0.005M adenosine monophosphate (AMP), 0.01M $MgCl_2$ and 0.05M glycine buffer adjusted to pH 9.1 with NaOH. The reaction was initiated by the addition of suitably diluted sample and incubated at 37°C in a water bath. The reaction was stopped after 20 minutes by the addition of 2.5 ml of 8% trichloroacetic acid. The solution was filtered through Whatman no. 42 paper and 1 ml aliquot of the filtrate was assayed for inorganic phosphate by the method of Leigh and Walker (1980b). Appropriate blanks where 8% TCA was added prior to the addition of membrane fractions were included. For the estimation of inorganic phosphate, 0.6 ml of color reagent was added to the sample as well as in blank and standard and incubation was carried out at 37°C for 45 min. After incubation, the reaction tubes were kept on ice for 5 min and the absorbance was recorded at 820 nm in a

spectrophotometer (Perkin Elmer, Lambda Bio 40). Standards (with Pi) were run in parallel concurrently.

### 3.6.2 Estimation of Deoxyribonucleic acid (DNA):

A colorimetric method using the diphenylamine reagent was employed to measure the amount of DNA in the samples (Plummer, 1990).

**Reagents:**

1. Diphenylamine reagent: 1gm of pure diphenylamine was dissolved in 100 ml of glacial acetic acid and 2.5 ml of concentrated sulphuric acid. (Freshly prepared)
2. Buffered Saline: 0.15 M NaCl, 0.015M sodium citrate, pH 7.
3. Standard Solution: Commercially available DNA, Source: herring sperm, Conc. 0.2mg/ml.

**Procedure:**

All samples were suspended, in buffered saline (0.15M NaCl, 0.015M sodium citrate pH 7) at an approximate concentration of 2 mg to 4 mg protein / ml of suspension. One ml of appropriately diluted sample, standard solution and buffered saline as a blank were added into 2 ml of freshly prepared diphenylamine reagent (1% diphenylamine w/v, 2.5% sulfuric acid v/v, dissolved in glacial acetic acid). The mixture was heated on a boiling water bath for 10 min, cooled and the extinction was recorded at 595 nm.

### 3.6.3 Estimation of Ribonucleic acid (RNA):

RNA was estimated by a colorimetric procedure employing the orcinol reagent (Plummer, 1990).

When pentoses are heated in concentrated hydrochloric acid furfurals are formed and orcinol reacts with the furfural in the presence of ferric chloride as a catalyst to give a green color. Only purine nucleotides give any significant color.

**Reagents:**

1. Orcinol Reagent: 100 mg of ferric chloride ($FeCl_3$. $6H_2O$) was dissolved in 100 ml of concentrated HCl and 3.5 ml of 6 % w/v orcinol in alcohol was added.
2. Standard: RNA commercially available.

**Procedure:**

To 2 ml of the sample, RNA standard (commercially available), and blank taken is separate test tubes, 3ml of oricinol reagent was added. The tubes were heated on a boiling water bath for 20 min. cooled and extinction measured at 665nm against an orcinol blank.

### 3.6.4 Acid Phosphatase Assay:

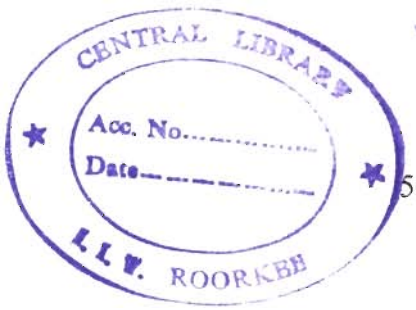
The acid phosphatase activity was measured according to the procedure outlined in Plummer, (1990) using p-nitrophenyl phosphate as substrate.

**Reagents:**

1. Sodium acetate buffer 0.2M, pH 4.5
2. Triton X-100 (10%)
3. Standard solution: 50µM p-nitrophenol standard; A 5mM stock solution, in the alkaline Tris buffer was prepared and then diluted 1 in 100 with the same buffer.
4. Substrate: 8mM p-nitrophenyl phosphate (Freshly prepared).
5. Stopping buffer: 1M Tris - HCl buffer, pH. 9.0 containing 1M $Na_2CO_3$ and 0.4M $K_2HPO_4$).

**Procedure:**

Acid Phosphatase was assayed in an incubation mixture containing (2mM p-nitrophenyl phosphate, 0.5% triton X-100 w/v, 0.12mM sodium acetate buffer, pH-4.8 and enzyme) in a final volume of 1ml. The different sub-cellular fractions were assayed for acid phosphatase, at an approximate protein concentration of 2 mg - 4 mg of protein. The reaction was carried out for the incubation period of 20 min and stopped by the addition of 1 ml of stopping buffer and the extinction was noted against water blank at 405 nm. The enzyme activity was calculated by reference to a standard curve of p- nitrophenol. The activity of acid phosphatase was expressed in terms of micromoles of p-nitrophenol released /hour.



### 3.6.5 Protein Estimation:

Protein in the samples was estimated by the Bicinchoninic acid (BCA) method using commercial kits supplied by Pierce as per manufacturers instructions. BCA reagent protein assay reagent combines the well-known Biuret reaction with the unique feature of BCA. The purple reaction formed by the interaction of two molecules of BCA with one cuprous ion ($Cu^{++}$) is water-soluble and exhibits a strong absorbance at 562nm. This allows the spectrophotometric quantification of protein in aqueous solutions.

**Reagents:**

BCA protein assay reagent was a pre-formulated reagent system that was supplied in two separate bottles.

1. Reagent A: 1000ml base reagent which contains sodium carbonate. sodium bicarbonate, BCA detection reagent and sodium tartarate in 0.2N NaOH.
2. Reagent B: 25ml of 4% copper sulfate solution.
3. The working reagent: 50 parts of reagent A with 1 parts of reagent B.
4. Standard: Bovine Serum Albumin (2mg/ml).

**Procedure**

Into appropriately labeled test tubes, 0.1 ml of each standard or unknown protein sample was pipetted. For blank, 0.1 ml diluent was used. Next, 2.0 ml of working reagent was added to each tube and mixed well. All tubes were

incubated at 37°C for 30 minutes. After incubation all tubes were cooled to room temperature and absorbance was noted at 562nm.

### 3.7 Testing the Potency of Commercial PI-PLC:

Since PI-PLC from several sources is available it becomes necessary to test the potency of the preparations and confirm their ability to cleave GPI-anchored proteins. Therefore, the ability of the PI-PLC to cleave alkaline phosphatase (ALP), a known GPI anchored protein (positive control) and adenosine triphosphatase (ATPase), a non-GPI anchored transmembranal protein (negative control) was tested. Microsomal membranes of goat kidney are known to contain both ALPase and ATPase and thus these were used for testing.

#### 3.7.1 Preparation of Microsomal membranes:

Microsomal membranes were prepared from goat kidney as per the method of Low and Finean, (1977a). Briefly, fresh goat kidney was obtained from a local abattoir with in one hour of killing the animal. The tissue was cut into thin slices and homogenized (sucrose 0.25M/L, 9ml/gm of tissue) in a glass homogenizer with a Teflon pestle (100 rpm, 12 up and down strokes). The homogenate was centrifuged at 15000 xg for 20 min in a J-21 Beckman high-speed centrifuge. The pellet was discarded and the supernatant obtained was further centrifuged at 10,5000 xg for 60 min in Beckman ultra centrifuge (LS-50B, Ti-50 rotor) to pellet the microsomal fraction.

### 3.7.2 Assay for Alkaline phosphatase (ALP):

The procedure followed was that of Low and Finean, (1977). P-Nitrophenylphosphate is used as the substrate.

**Reagents:**

1. p-Nitro-phenyl phosphate, 2mM
2. $MgCl_2$, 5mM
3. $Na_2CO_3$ / $NaHCO_3$ buffer 50mM, pH-10.1.

**Procedure:**

The microsomal fraction (obtained after cold solubilization, and PI-PLC digestion) was diluted 20 times or more in cold distilled water as desired for the reaction. The enzyme activity was assayed in an incubation mixture containing 2mM-p-nitrophenyl phosphate, 5mM $MgCl_2$ and 50mM $Na_2CO_3$ /$NaHCO_3$ buffer, pH-10.1 in a final volume of 1ml. Incubation was carried out for 30 min at 37°C and the reaction was terminated by immersing the tubes in boiling water bath for 5 minutes. The absorbance was measured at 400nm in a spectrophotometer (Perkin-Elmer Lambda Bio40). The standard curve was prepared using p-nitrophenol. The activity of alkaline phosphatase was expressed in terms of micromoles of p-nitrophenol released /hour.

### 3.7.3 Assay for Adenosine triphospahtase (ATPase):

The microsomal fractions (obtained after cold solubilization, and PI-PLC digestion) were diluted appropriately in cold distilled water as desired for the reaction. ATPase is a transmembranal protein and used as a marker to identify plasma membranes. The activity of ATPase was assayed by the method of Leigh and Walker (1980a). The activity of ATPase was estimated by measuring the amount of phosphate released by the enzyme Leigh & Walker (1980b).

**Reagents:**

1. Assay buffer: 50mM Tris-MES, pH 8.0, 3mM $MgSO_4$, 3mM ATP, 50mM KCl and 100µM Ammonium molybdate.
2. Standard solution: 10mM $KH_2PO_4$ (diluted 1:10 to obtain 1mM).
3. Stopping reagent: 10% SDS.

**Procedure:**

The ATPase was assayed by adding 100µl microsomal fraction in a 300µl assay buffer containing 50mM Tris-MES pH8.0, 3mM $MgSO_4$, 3mM ATP, 50mM KCl and with 50µl of water. The microsomal fraction was added in the last to initiate the reaction. The reaction mixture was immediately vortexed and incubated for 45 minutes at 37°C. The reaction was terminated by the addition of 10% SDS (0.5ml). Parallel standards and blanks where 10% SDS was added prior to the addition of membrane fractions were run simultaneously. As soon as the reaction was stopped, 0.6 ml color reagent was added in all tubes and

incubation was carried out for a further period of 45 min 37°C. After incubation, the reaction tubes were kept on ice for 5 minutes and the absorbance was recorded at 820nm.

### 3.7.4 Cold solubilization of membrane bound enzymes in microsomal fraction:

The use of detergents is a method of choice for solubilization of most membrane bound proteins. The solubilization depends on several characteristics of the detergents, the most important being the critical micelle concentration. Thus, it is often observed that the extent of solubilization of the same membrane bound protein from a membrane is not the same when different detergents are used. The ratio of solubilization of GPI anchored protein in triton X-100 and octylglycoside is distinct. Therefore, cold solubilization of microsomal membranes was tested at different concentrations of triton X-100 and octylglycoside.

**Reagents:**

1. Triton X-100: 0.2%, 0.4%, and 0.6% prepared in distilled water
2. Octylglycoside: 0.2%, 0.4%, and 0.6% prepared in distilled water

**Procedure:**

Equal aliquots of microsomal fraction isolated from the goat kidney were suspended into 1ml of cold distilled water (4 mg / ml of protein) and incubated

on ice for 30 min with an equal volume of detergent so that the final concentration of detergent reached 0.1%, 0.2% and 0.3%. During the period of incubation, the samples were vortexed at regular intervals and returned to the ice bath. The mixture was then centrifuged at 105000 xg for 30 min, at 4°C and the resulting supernatant was assayed for ALPase, ATPase and protein.

### 3.7.5 Cleavage by PI-PLC treatment:

Several specific phospholipases that effectively degrade only GPI species are useful for analyzing GPI anchorage of isolated proteins. A commercially available preparation of the enzyme was carefully tested before it was employed in the studies with sperm proteins.

**Reagent:**

1. Phosphatidylinositol-specific Phospholipase-C, from *Bacillus cereus*, (13.7units/mg): 5mg of enzyme was dissolved in 34.25µl of 25mMTris acetate pH 7.4, containing 50% glycerol and 0.01% sodium azide, stored at –20°C or below.

**Procedure:**

To an aliquot of microsomal fraction (1ml containing 4mg/ml of protein), 2 units of PI-PLC were added. Another equal aliquot of microsomal fraction was used as control (without PI-PLC). The reaction mixture was incubated at 37°C for 2 h to release the proteins from their GPI anchors and then centrifuged at

105000 xg for 1 h. The pellet and supernatant were collected and the activity of ALPase and ATPase was determined.

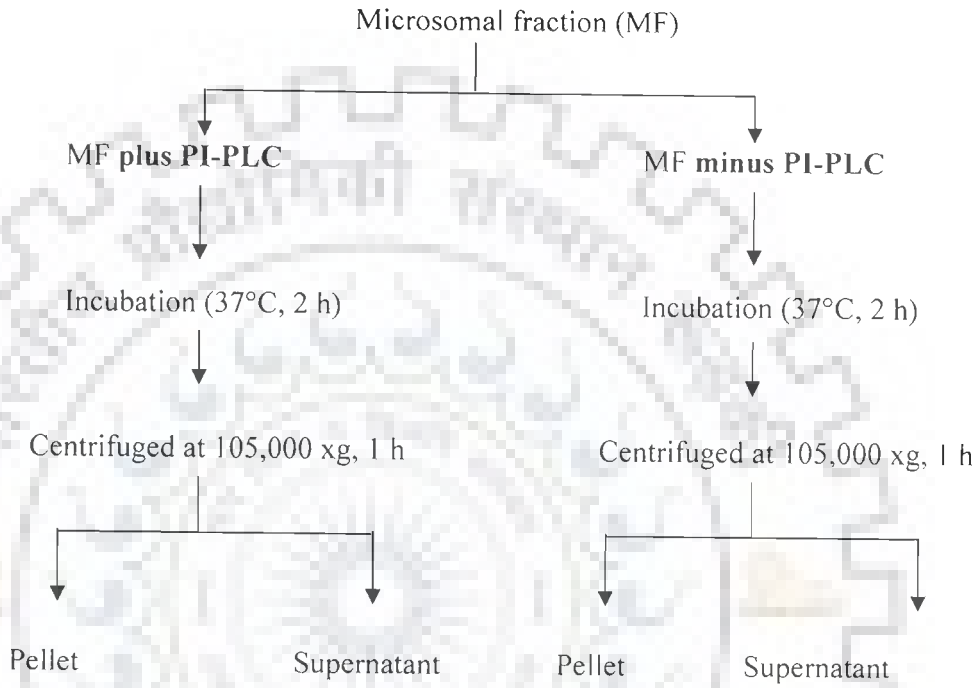



**Figure: 8. Flow chart of the protocol used for the release of GPI proteins by PI-PLC enzyme digestion**

### 3.8 Analysis of GPI anchored sperm proteins:

GPI anchored proteins are studied after cleavage by both enzymatic and chemical means. The sites of action and the reaction products are highlighted in the literature review (section 2.4.5)

### 3.8.1 Enzymatic cleavage by PI-PLC:

Once it was established using goat kidney microsomal membranes that the commercial preparation of PI-PLC was effective in releasing the proteins from their anchors, the same conditions were used to release the GPI-proteins from the sperm surface.

**Reagents:**

1. Phosphatidylinositol-specific Phospholipase-C, from *Bacillus cereus*, (13.7units/mg): 5mg of enzyme was dissolved in 34.25µl of 25mM Tris acetate pH 7.4, containing 50% glycerol and 0.01% sodium azide, stored at –20°C or below.

**Procedure:**

To equal aliquots of intact sperm/sperm plasma membrane fraction (1ml containing 4mg/ml of protein) were taken. To one aliquot 2 units of PI-PLC was added while in the other no enzyme was dispensed (control). The reaction mixture was incubated at 37°C for 2 h to release the proteins from their GPI anchors and then centrifuged at 10,5000 xg for 1 h. The pellet and supernatant were collected and the protein profiles were studied by SDS-PAGE.

### 3.8.2 Chemical cleavage by nitrous acid:

**Principle:**

An unusual property common to all GPI structures is the presence of a nonacetylated glucosamine that is glucosamine glycosidically linked to inositol. The glycosidic bond is specifically cleaved when the glucosamine undergoes nitrous acid deamination, thereby cleaving the GPI anchor. Because non-acetylated glucosamine rarely occurs outside of GPIs, nitrous acid deamination may be used to identify the GPI linkage to protein.

**Reagents:**

1. Acetate buffer (0.1M, pH 3.5): 0.82 g of sodium acetate was dissolved in 95 ml of distilled water and pH was adjusted by acetic acid and then volume was make up to 100 ml with distilled water and stored at room temperature.
2. 0.5 M $NaNO_2$, (made fresh).
3. 0.5 M NaCl.

**Procedure:**

Two equal aliquots of goat sperm ($5 \times 10^6$) were dissolved in 0.1 ml of 0.1 M acetate buffer separately. NP-40 (0.1 % v/v) detergent was added to aid solubilization and in first aliquot 0.1 ml of 0.5 M $NaNO_2$ was added, while in the second tube (control), 0.1 ml of 0.5 M NaCl was added. Incubation was

carried out for 3 hours at room temperature followed by centrifugation at 10,000 xg for 15 min. The supernatant and pellet were examined for their protein profile by SDS-PAGE.

### 3.9 Electrophoretic analysis of sperm proteins

#### 3.9.1 SDS-PAGE

SDS-PAGE was carried out according to Laemmli (1970), 10-15% gel of 1mm thickness was cast by 10-15 well comb by using Hoefer scientific instrument; SE-600 SERIES electrophoresis unit.

**Reagents:**

1. Acrylamide solution (30%): 30% w/v acrylamide solution containing 0.8% w/v N, N-methylene-bis-acrylamide: 29.2gm of acrylamide and 0.8gm of acrylamide was dissolved in 70ml of deionised water. When the acrylamide was completely dissolved, the water was added to a final volume of 100ml, solution was filtered with a Whatmann No.1 paper and stored at 4°C in a dark bottle for not more than one month.

2. Resolving buffer (1.5M Tris-Cl, pH 8.8): 18.2gm of Tris base was dissolved in 80ml of water and the pH was adjusted to 8.8 with HCl and water was added to a final volume of 100ml stored at 4°C.

3. Stacking buffer (0.5M Tris-Cl, pH 6.8): 6.1gm of Tris base was dissolved in 80ml of water and pH was adjusted to 6.8 with HCl and water was added to a final volume of 100ml, stored at 4°C.

10% sodium dodecyl sulphate (SDS, w/v): 10gm dissolved in 60ml of water and volume was leveled at 200ml by distilled water, stored at room temperature.

4. Stock sample buffer:

(0.12M Tris pH6.8, 4% SDS, 20% glycerol, 0.02% Bromophenol blue, 10% β-mercaptoethanol.

| | |
|---|---|
| Water | 1.1ml |
| 0.5M Tris-Cl, pH 6.8 | 2.4ml |
| 10% SDS | 4.0ml |
| Glycerol | 2.0ml |
| 0.5%Bromophenol blue (w/v) | 0.5ml |

Stored at room temperature, SDS-reducing was prepared by adding 100µl of β-mercaptoethanol to each 0.9ml of stock sample buffer. before use.

5. Catalyst: 10% ammonium per sulphate (APS): 100mg APS was dissolved in 1ml of water. (Freshly prepared solution was used).

6. TEMED (N, N, N, N-tetra methylethylenediamine): It was used undiluted from the bottle stored at cool dry and dark place.

7. Electrode buffer: (0.025M Tris, 0.192M glycine, 0.1%W/v SDS, pH 8.3)

0.3g Tris base, 1.4gm glycine, 1 ml 10% SDS/100ml electrode buffer.

Electrode buffer was also prepared as stock solution 5X concentration, consisting of 15gm Tris base, 72 gm glycine and 5gm SDS/liter. It was stored at room temperature in a reagent bottle of glass and was diluted to 5 times by adding 4 parts of distilled water before use.

**3.9.1.1 Casting of Gel:**

The gel plates, spacers, combs, plate holders, upper and lower buffer reservoir were thoroughly cleaned with detergent and then rinsed well in distilled water. The resolving gel was cast first then overlaid with stacking gel. The gel plates were wiped with tissue paper and the two plates taken were taken wide apart by 1mm thickness spacers, placed perpendicularly and sandwiched in between the end of the edges of glass plates. The plates and spacers sandwiched were tightened by the plate holder at the two perpendicular edges and it was fitted in a casting gel assembly, the knobs were tightened to prevent leakage. The monomer solution was prepared for 10% resolving gel by combining all of the reagents given below except the ammonium per sulfate and TEMED. The gel was cast in duplicate.

**Resolving gel recipe:**

| | |
|---|---|
| Acrylamide solution 30% | 10.0 ml |
| 0.5M Tris-Cl buffer,pH 8.8 | 7.50 ml |
| SDS 10% | 0.30 ml |
| APS 10 % | 0.30 ml |

| | |
|---|---|
| Water | 11.90 ml |
| TEMED | 15.00 µl |

**Stacking gel Recipe:**

| | |
|---|---|
| Acrylamide solution 30% | 3.40 ml |
| 0.5M Tris-Clbuffer,pH6.8 | 5ml |
| SDS10% | 0.20 ml |
| APS10% | 0.20 ml |
| Water | 11.10 ml |
| TEMED | 20.00 µl |

Gels of any other acrylamide concentration desired can be prepared by adjusting (only) the amounts of 30% monomer stock and water used in the recipes. The solution was deaerated under vacuum for at least 15 minutes. The APS and TEMED was gently mixed into the deaerated monomer solution. The solution was well mixed uniformly and poured gently in between the plated by using pipette and bulb. The resolving gel was cast up to 2/3 height on pre marked plates followed by layering of 1ml butanol. After 15 minutes the demarcation occurred between the acrylamide layer and butanol layer, indicated the complete polymerization of gel. Decant it and washed. Similarly 5-6% stacking gel was also layered on top of the resolving gel. The well was casted in a stacking gel by placing the comb in between and at the top of the two plates after 15 minutes or more the comb was removed. The gels were casted in

duplicate and plates were fixed in upper tank. The upper and lower tanks filled with electrode buffer, up to the mark as indicated on the assembly.

**3.9.1.2 Sample preparation:**

Cell/Membrane, approximate protein concentration 100-150µg/50µl were mixed with 1 volume of double strength sample treatment buffer (0.12M Tris pH 6.8, 4% SDS, 20% glycerol, 0.02% bromophenol blue, 10% β-mercaptoethanol) and heated to 95°C to boiling point for 5 minutes.

**3.9.1.3 Electrophoresis:**

The prepared samples were loaded into the wells with a protein concentration of 100-150µg and the volume was 100-200µl per well. The sample was loaded by using micro liter syringe. The gel assembly was connected to the current to allow the electrophoresis .The sample was run at 100V through stacking gel. Once the sample was concentrated at the interface of the stacking and separating gel, the voltage was increased to 120V and the electrophoresis was continued until the tracking dye reached at the bottom of the gel. After the run, the gel was removed and stained in a solution containing 0.1% Coomassie brilliant blue R-250w/v in 40% methanol, 10% Acetic acid w/w, with total volume of 200ml and kept for 1 h to overnight at room temperature. The resolution of proteins were considerably improved by destaining in acetic acid: methanol: water (10:25:65, v/v/v).

### 3.9.1.4 Determination of molecular weight:

Lambin (1978) observed that there is an excellent linear relationship between the log of molecular weight (Mr) and the log of gel concentration (%T) and the correlation between them was significantly better than the one between log (Mr) and log of relative mobility. Distances traveled by polypeptides were converted to gel concentration (%T). Log (Mr) was plotted against the log %T for the standards using double log graph paper. Standard molecular weight markers used were from Banglore Genei.

| Proteins Used | Molecular weight |
|---|---|
| Myosin, rabbit muscle | 205,000Da |
| Phosphorylase b | 97,400Da |
| Bovine serum albumin | 66,000Da |
| Ovalbumin | 43,000Da |
| Carbonic anhydrase | 29,000Da |

### 3.9.2 Preparation of Immunogen from Sperm plasma membrane:

Since GPI-anchored proteins are membrane-bound the following steps were used to prepare the antigens for the immunization of rabbits.

**Reagents:**

1. TritonX-114
2. Acetone (at -20°C)

3. Tris buffered saline (TBS) 10mM Tris-Cl, 150mM NaCl (pH 7.5)

### 3.9.2.1 Solubilization of membrane proteins and Phase partitioning by Triton X-114:

Triton X-114 is an unique type of detergent that can solubilize the majority of membrane proteins at low temperatures and when the temperature of the mixture is raised phase separation occurs. During the phase separation the hydrophilic proteins move to the aqueous phase while the hydrophobic proteins (including the GPI-anchored) are retained in the detergent phase.

### 3.9.2.2 Pre-condensation of TritonX-114 detergent

Triton X-114 often contains hydrophilic contaminants, and must be pre-condensed by several rounds of phase separation before use. To achieve this, 1.5 gm Triton X-114 was dissolved in 50 ml TBS (pH 7.5), placed in a 50 ml centrifuge tube, then chilled on ice. At this point, the solution appears transparent. The solution was warmed in a water bath to 37°C until it turns turbid and then centrifuged at 10,000 xg for 10 minutes at room temperature. The upper phase (detergent depleted) was discarded and the lower phase (detergent enriched) re-dissolved in an equal volume of ice cold TBS. (Hydrophilic contaminants partitioned in to the upper phase). The above procedure for partitioning was repeated thrice. The final detergent enriched phase contained approximate 12% detergent and was used in the studies.

### 3.9.2.3 Partitioning of Proteins:

To 5 ml of purified membrane fraction (approximate 10mg/ml protein dissolved in TBS buffer) 1 vol. of pre-condensed Triton X-114 was added. Thus, the final detergent concentration is adjusted to 2%. Incubating for 15 minutes on ice with occasional mixing solubilized the membrane proteins. At this point only a single phase appeared in the partitioning tube. The membrane mixture was centrifuged for 10 minutes at 10,000 xg in a refrigerated tabletop centrifuge at 4°C (Sigma 15K). While the pellet was discarded, the supernatant was transferred to a fresh tube and warmed to 37°C in a water bath for 15 min. The cloudy mixture was centrifuged at 10000 xg at room temperature for 10 minutes and two distinct phases are obtained. The upper (detergent depleted) phase contained soluble hydrophilic proteins while the lower (detergent enriched) phase contained protein anchored by GPI structure or by their transmembrane domains.

Antibodies against hydrophobic proteins of sperm plasma membrane, solubilized by the triton X-114 detergent (detergent enriched phase) were raised in rabbits. The detergent enriched phase was mixed with 8 volumes of acetone and kept at -20°C overnight. This was centrifuged the following day at 15,000 xg for 20 min. The pellet was washed twice to remove any traces of the detergent. The pellet was finally re-suspended in PBS and used for the immunization.

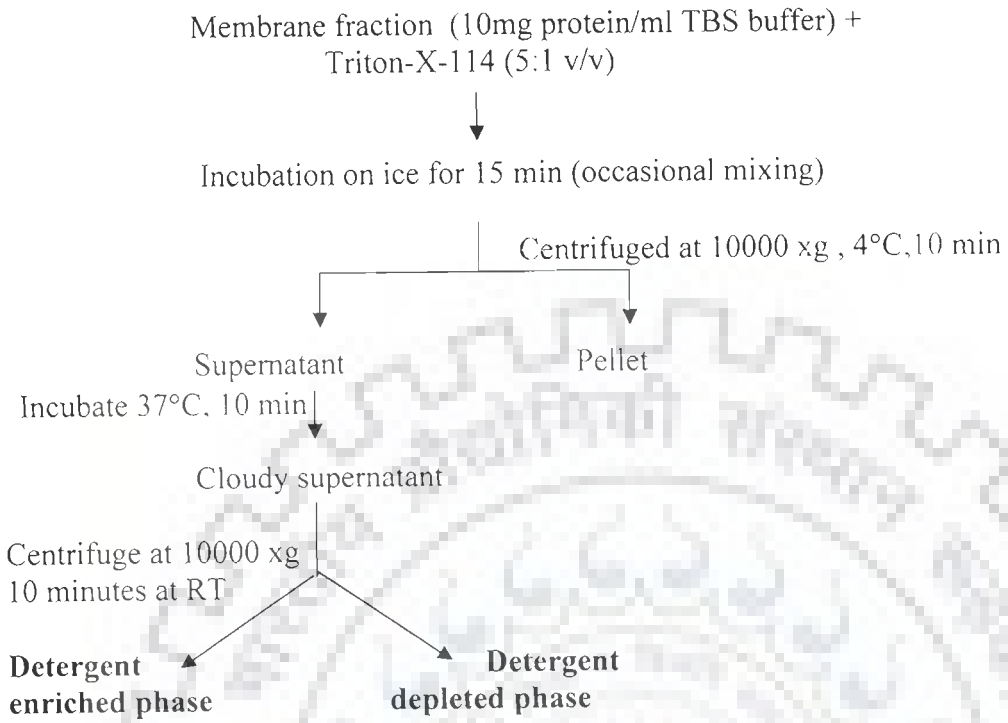


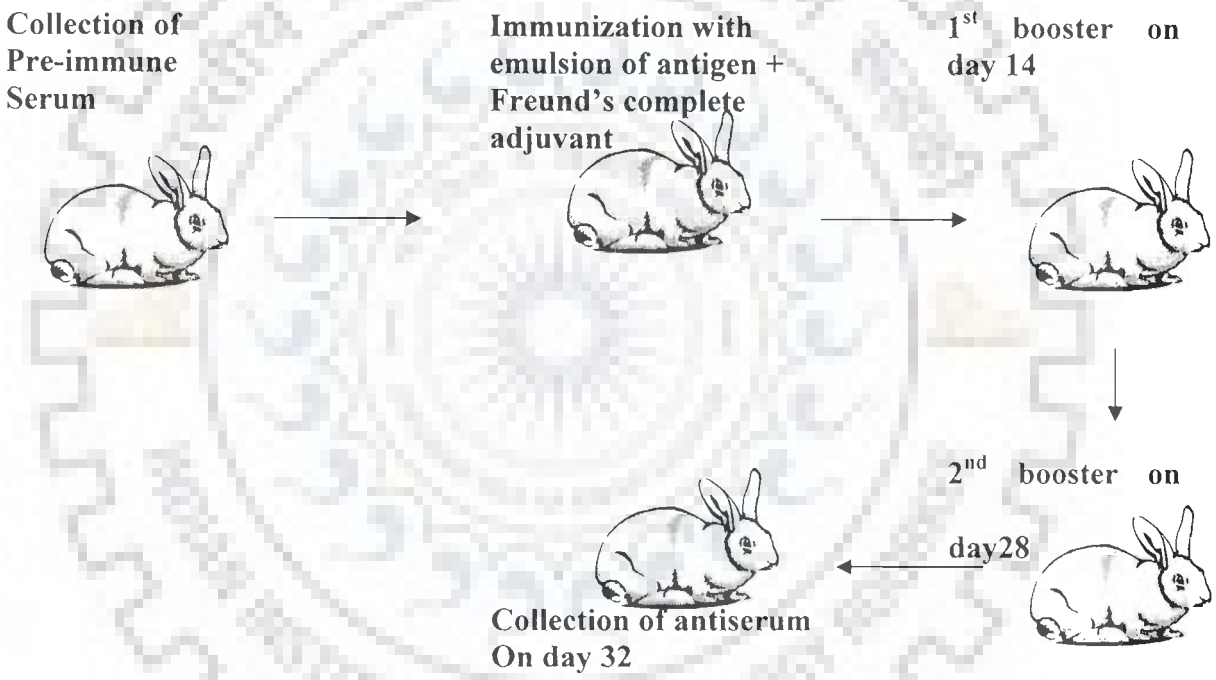

**Figure: 9. Schematic view of Triton X-114 solubilization and phase partitioning of proteins from sperm plasma membrane**

### 3.9.3 Production of polyclonal antibodies:

Two female rabbits (3 kg approx. body weight) were used for the production of polyclonal antibodies. Animals were pre-bled one week before immunization. An emulsion was prepared by mixing 2ml of Freund's complete adjuvant with equal volume of PBS containing 500µg antigen protein in a hypodermic syringe. The animals were immunized with this emulsion at multiple locations intradermally. On day 14, the first booster dose was

administered with the same antigen emulsified with incomplete Freund's adjuvant in a similar fashion. The second booster dose was given on day 28. Four days later the animals were bled through the marginal ear vein. After bleeding, blood was allowed to clot for 60 min. at room temperature. Serum from the blood was separated and stored in aliquots of 100µl at –80°C.

**Figure: 10. Immunization schedule**




### 3.9.3.1 Enzyme Linked Immuno Sorbant Assay (ELISA) for antibody titer:

**Reagents:**

1. Blocking buffer: 3% BSA in PBS.
2. Pre serum: Serum collected from the rabbit before immunization.
3. Primary Antibody: Anti serum collected from the rabbit after immunization.

4. Secondary Antibody: Commercially available tagged with horseradish peroxidase enzyme (Goat anti rabbit IgG-HRP).
5. Phosphate citrate buffer pH 5.0.
6. 0.1M Citric acid $C_6H_8O_7H_2O$.
7. 0.2M Sodium dihydrogen phosphate.
8. Both the reagents were made separately with distilled water.
9. Substrate: Orthophenylenediamine (OPD) 4 mg; Citrate buffer 0.1 M -4.8 ml, Phosphate buffer 0.2 M - 5.15ml; Hydrogen peroxide (30%) - 4µl. The substrate was prepared fresh and $H_2O_2$ was added just before the use.
10. Stopping reagent: 4N $H_2SO_4$, $H_2SO_4$-4ml: Distilled water 32 ml.

**Procedure:**

The titer of the antiserum was determined in a simple ELISA protocol. Micro titer plates were coated with 50 µl of immunizing antigen containing 5µg of protein in each well and incubated overnight at 4°C. Next day the liquid content of the wells was emptied by hand jerk. All the wells were washed three times by adding 100µl washing buffer (TBS). After washing, 100 µl blocking buffer (3% BSA in PBS) was added in each well and kept for 90 min at room temperature. Finally the contents were discarded followed by washing with TBS (thrice) as was done earlier.

The serial dilutions of pre-immune and anti-serum were done with 1% BSA in TBS. The wells were filled with 50 µl of different serial dilutions of pre-immune serum and anti-serum in duplicate and incubated for 90 min at room temperature.

After incubation, the wells were drained and washed thrice with TBS. Fifty µl of secondary antibody (Goat anti-rabbit IgG-HRP conjugate, 1:1000 dilution) were added to each well and incubated at 37°C for 90 min. followed by washing with TBS. Next, 10ml of ortho phenylene diamine reagent (4mg OPD was added in 4.8ml of 0.1M citrate buffer and 5.15 ml of 0.2M phosphate buffer, pH 5, 4µl of $H_2O_2$ was added just before the use) was added and incubated at room temperature in dark for 20min. The reaction was stopped by the addition of 50 µl of 4N $H_2SO_4$ and the absorbance was recorded with in 10min.at 492nm in an ELISA reader (Metertech Inc, model Σ960)

**3.9.4 Western blotting:**

Electrophoresis of the proteins isolated from the intact sperm/sperm plasma membrane (plus/minus PI-PLC treatment) was carried out by SDS-PAGE as outlined in section 3.9. The proteins were then transferred to nitrocellulose membranes and then immunostained as described below.

**3.9.4.1 Electro blotting:**

A semi dry transblot apparatus (Hoefer scientific system, TE 70, semiphor) was used to transfer the proteins from the polyacrylamide gels to the nitrocellulose membrane.

**Reagents:**

Transfer buffer:

| | Concentration | 1000ml |
|---|---|---|
| Tris base | 48mM | 5.80 gm |
| Glycine | 39mM | 2.90 gm |
| SDS | 0.037% | 0.37 gm |
| Methanol | 20% | 200 ml |
| Distilled water | | made up to 1000 ml |

**Procedure:**

The electrode plates were cleaned and rinsed with distilled water, eight sheets of absorbent paper (Whatman no. 1 or equivalent) and one sheet of nitro cellulose membrane to the size of the membrane were cut. The nitrocellulose sheet and the absorbent sheets were pre-soaked in transfer buffer for 10 min. The gel, nitrocellulose membrane were sandwiched between absorbent sheets as shown in the figure 11.

The bubbles were gently removed from the layers by rolling a glass rod over the sandwich. The upper electrode plate (cathode) was carefully placed over the stack without disturbing it. The electrodes were connected to the power pack to commence transfer of protein from the gel to the nitrocellulose membrane. The

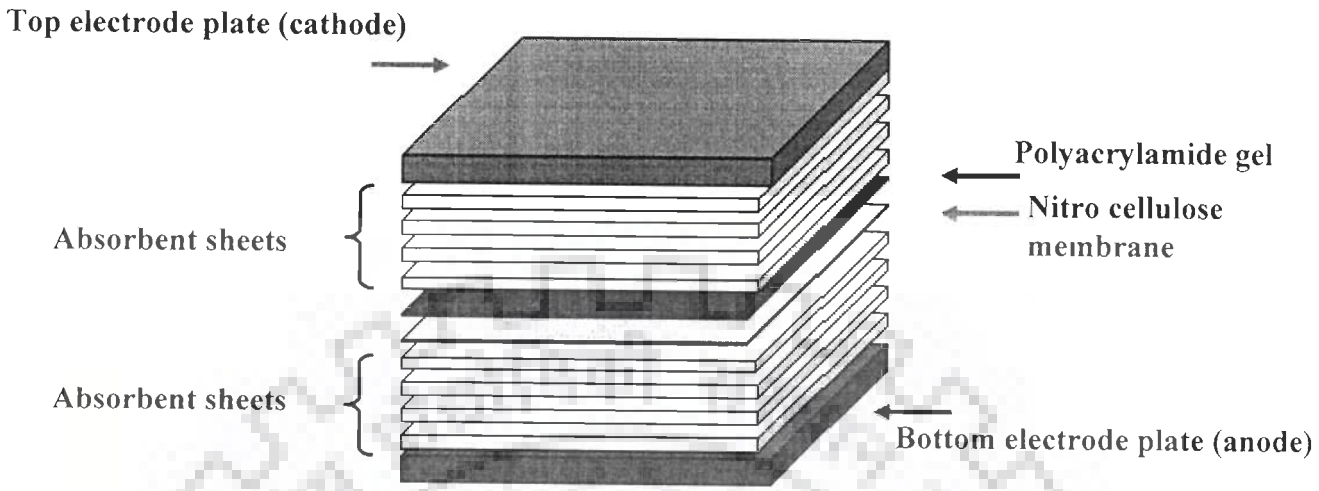



**Figure: 11 Electroblotting sandwich arrangement of absorbent sheets, polyacrylamide gel, and nitrocellulose membrane.**

transfer was carried out for 45 min at 3 mA/cm$^2$ of gel. The whole process was carried out in a cold room to avoid excess heat.

### 3.9.4.2 Immuno staining:

**Reagents:**

1. Blocking solution: 2% BSA in 0.01M PBS, it is used to block unbound sites on the membrane and prevents non-specific binding of antibodies.
2. Washing buffer: 0.01M PBS.
3. Primary antibodies: Rabbits were immunized with hydrophobic proteins which solubilised in the Triton X-114 detergent and the anti serum was diluted to 1:400 dilution in 0.2%BSA in PBS.

4. Secondary antibodies: Goat anti-rabbit IgG-HRP conjugate (Bangalore Genie).
5. Substrate: Diaminobenzidine/$H_2O_2$: (A) 5mg DAB dissolved in 5 ml PBS; (B) 30ml water + 20µl $H_2O_2$ (30%).

**Procedure:**

After electro blotting, nitrocellulose (NC) membrane was carefully placed in a blocking buffer (3% gelatin in PBS) at 37°C for 2 h. to block the non-specific binding sites. The NC membrane was washed thrice with washing buffer (PBS) and transferred into primary antibody solution (1:400 dilution in 0.5%BSA in PBS) for 1 h at 37°C. Again, the NC membrane was washed thoroughly with washing buffer (PBS) and incubated with goat anti-rabbit IgG-HRP conjugate (1:500 dilution) for 1 h at 37°C. The NC membrane was washed thrice with washing buffer and the blot was developed with developing solution (Diaminobenzidine/$H_2O_2$: (A) 5mg DAB dissolved in 5 ml PBS; (B) 30 ml water + 20µl of 30%$H_2O_2$. Developing solution was prepared by mixing by equal volume of A and B in dark). Developing was carried out in dark.

**3.10 Immunoflourescence studies:**

To map the localization of GPI anchored proteins on the surface of goat sperm a fluorochrome FITC was used.

**Reagents:**

1. PBS (0.01M, pH 7.4) containing 0.02% sodium azide.
2. Primary antibody: same as used for western blotting and ELISA.
3. Secondary antibody: Goat anti rabbit IgG-FITC.
4. Mounting media: Consists of 9 parts of glycerol and 1 part of PBS, 0.1M p-phenylene diamine (PPD). It prevents quenching of fluorescence during observation.
5. Absolute alcohol/70% acetone.

**Procedure:**

Two aliquots of sperm suspensions (1 x $10^6$) were taken in separate tubes. One was treated with PI-PLC (2units/ ml at 37°C for 90 min) while the other was used as control (untreated). Each of the samples were spread on clean special glass slides and allowed to air dry. The cells were then fixed with ethanol for 1 h. Ten µl of primary antibody (diluted 1:50) was dispensed over a glass slides and incubated for 1h at 37°C. Again the glass slides were washed thrice and 10µl secondary antibody (Goat anti-rabbit IgG-FITC, 1:50 dilution) was dispensed and incubated for 1h at 37°C. The whole process was carried out in dark room followed by washing three times with PBS. Finally the mounting medium (consisting of 9 parts of glycerol and 1 part of PBS, 0.1M p-phenylene diamine) was dispensed over a glass slide and observed under fluorescence microscope. The mounting medium prevents quenching of fluorescence during observations. The primary antibody used

was same as used for western blotting (section 3.9.4) and in the ELISA procedures (section3.9.3.2).

### 3.11 Studies on the function of GPI-anchored sperm proteins:

#### 3.11.1 Isolation and enrichment of sperm:

Goat sperm obtained from the cauda epididymis (section 3.2) was washed three times in modified Kreb's Ringer (119.4mM NaCl, 4.8 mM KCl, 1.7 mM $CaCl_2$ .$2H_20$, 1.2 mM MgS04. $7H_2O$, 25.1 mM $NaIHC0_3$, 1 mM sodium pyruvate, 25 mM sodium lactate, 5.56 mM glucose, 60 U/mL penicillin, 60 µg/mL streptomycin and 0.001 phenol red) at room temperature by centrifugation at 200 xg for 10 min. An aliquot of the sperm pellet was resuspended in small volume of MKR counted in a hemocytometer and the final volume adjusted to the desired concentration. The MKR was gassed with 5 % $CO_2$ in 95 % $O_2$ prior to use and sterilized with 0.22 µm membrane filter (Millipore India Ltd.).

#### 3.11.2 Isolation of macrophage:

The mouse peritoneal cavity was flushed with MKR using a syringe fitted with 18G needle and the peritoneal macrophages were collected. The macrophages thus obtained were washed twice in MKR by centrifugation at 120 xg for 5min at room temperature, and then used for the phagocytosis assay.

### 3.11.3 Phagocytosis assay:

Two sets of reaction were carried out for the phagocytosis studies. In one set, the sperm was treated with PI-PLC while the other was not (control). The PI-PLC treatment was carried out at 37°C for 2 h. The sperm was washed prior to addition to macrophages. Phagocytosis assays were performed in 24 well microtitre plates. Briefly, the sperm and macrophages were mixed in 100 µl of MKR at a concentration of approximately 1-10 x $10^5$ and 1-5 x $10^5$, respectively, and were co-incubated in a $CO_2$ incubator at 37° C with an atmosphere of 5% $CO_2$ in air. Co-incubation was carried out for 6 h since it has been previously reported that sperm phagocytosis reached a plateau after 6 h incubation.

### 3.11.4 Electron microscopic studies:

After co-incubation, the sample containing macrophages and sperm were pipetted several times to remove the loosely attached sperm from macrophages. The contents were transferred to a fresh tube containing 1 ml of 2.5 % glutaraldehyde in cacodylate buffer. After 1 h pre-fixation in glutaraldehyde, the samples were centrifuged at 500 xg for 10 min and washed twice in cacodylate buffer. The pellets were embedded in gelatin and fixed with 2% osmium tetroxide for 1h, dehydrated in graded ethanol series, and finally embedded in Epon 812 for making solid blocks. Thin sections were cut with an ultramicrotome (Reichert, OMU3, Austria) and the sections were stained with both uranyl acetate and lead citrate. The sections were then visualized under a transmission electron microscope (Philips, CM10, Holland).

The extent of phagocytosis was judged from the absence or presence of sperm or its components in the macrophages.

### 3.11.5 Sialic Acid Assay:

The thiobarbituric acid assay of Warren (1959) was used for the estimation of Sialic acid.

**Reagents:**

1. Sodium meta-periodate: (0.2M) in 9M phosphoric acid.
2. Sodium arsenite: (10%) in a solution of 0.5M sodium sulfate-0.1N $H_2SO_4$
3. Thiobarbituric acid: 0.6% thiobarbituric acid in 0.5M sodium sulfate.

**Procedure:**

The sperm collected from cauda region of goat epididymis was adjusted to obtain $5x10^7$ cells/ml. Two equal aliquots were made; one was treated with PI-PLC and the untreated sample was used as control. After incubation for 2h at 37°C, the samples were centrifuged and the supernatant and residual sperm pellet was assayed for sialic acid.

To sperm samples with and without PI-PLC pretreatment, 0.1ml of the periodate solution was added. The tubes were shaken and allowed to stand at room temperature for 20 minutes. Next, 1ml arsenite solution was added and the tubes were shaken until the yellowish brown color disappeared. At this time 3ml of thiobarbituric acid solution was added .The tubes were shaken, capped with a glass

bead, and then heated in a vigorously boiling water bath for 15 minutes. The tubes are then removed and placed in cold water for 5 minutes. During cooling the red color fades and the solution often become cloudy. This does not affect the final reading. The entire 4.3 ml of aqueous solution was extracted with 4.3 ml of cyclohexanone. The tubes were shaken vigorously and then centrifuged for 3 min in a tabletop centrifuge, the clear upper cyclohexanone phase was pinkish red and the color was more intense than it was in water. Optical densities of the organic phase were determined at 532 and 549 nm in a spectrophotometer (Perkin-Elmer, Lambda Bio40). Color production varied linearly with concentration of N-acetylneuraminic acid over the range usually used 0.01-0.06μM and the molecular extinction coefficient was 57,000.

**Calculation:**

The amount of N-acetylneuraminic acid present in a given sample can be determined from equation:

$$\frac{V \times O.D_{549}}{57} = \frac{4.3 \times O.D_{549}}{57} = 0.075 \times O.D_{549}$$

Where V is the final volume of test solution.

**Interfering substances:**

When tissues were subjected to the thiobarbituric acid, an absorbance maximum was found at 549 nm due to sialic acid. However, there may be a second

absorbance at 532μm due to deoxyribose for which a correction must be made since the light absorption of this interfering material at 549 nm is considerable. For the assay of sialic acid in sperm cells readings were made routinely at 549 and 532 nm and the optical density values were inserted into the equation:

μm of N-acetylneuraminic acid = $0.090 \times O.D._{549} - 0.033 \times O.D._{543}$

Equation 2 corrects for optical density at 549μm, which does not derive from N- acetylneuraminic acid. The correction is based upon the observed molecular extinction values of N-acetylneuraminic acid and 2deoxyribose at 532 and 549μm

**3.12. Statistical analysis:**

The data were subjected to statistical analysis and expressed as mean ± S.E.M. (Standard Error of mean). The S.E.M.was calculated by the following formula:

$$S.E.M. = \sqrt{\frac{\Sigma X^2 - \frac{(\Sigma X)^2}{n}}{n(n-1)}}$$

Where X = individual observations

n = number of observations

Students 't' test was used to calculate the degree of significance by the formula given below:

$$t = \frac{x_1 - x_2}{S\sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$$

Where

$$S = \sqrt{\frac{\Sigma X_1^2 - \frac{(\Sigma X_1)^2}{n_1} + \Sigma X_2^2 - \frac{(\Sigma X_2)^2}{n_2}}{n_1 + n_2 - 2}}$$

$n_1$ and $n_2$ denote the number of observations in the two classes being compared (Ostle, 1954).

Based on the degree of freedom, value of probability was obtained from the standard table given by Fischer and Yates (1948). If the calculated value was more than the table value, it is significant at the probability level.

# *Chapter - 4*

# *Results*

## 4.0 RESULTS

### 4.1 Preparation of sperm from the epididymal segments of goat:

Sperm from caput, corpus and cauda were collected as described in the section on material and methods (section 3.2). The yield and quality of sperm were examined each time by light microscopy and counting in a hemocytometer before using them for further experimental studies. A significantly high number of intact sperm were obtained as reflected from the cell counts made and representative micrographs are shown (figure 12 A, B, and C). Sperm were observed to be oriented in different directions at random in the epididymal fluid. A lot of debris together with contaminants was also present. However, after passing through a nylon mesh, centrifugation and washing, a cleaner and homogenous population of intact sperm devoid of suspended particulate matter could be recovered as shown in light micrographs (figure 13A). The morphology and structural integrity of the sperm were also confirmed by scanning electron microscopy. A typical ultra-structure of goat sperm is shown in the scanning electron micrograph (figure 13B). The good sperm count obtained is indicative of a mature and functional testis, while the high level of motility and infrequent occurrence of abnormalities in the morphology of the sperm confirm their suitability for further biochemical estimations and other studies. Occasionally, detached heads and tails of some sperm were observed in light micrograph as well as in scanning electron micrograph. This is most probably due to the reason that some of the sperm are unable to withstand the mechanical stress imposed on

them during the operation of washing. Of the three epididymal regions, the cauda segment had the best quality of sperm in terms of yield, motility and other characteristics. The yield of sperm from the corpus region of the epididymis was the lowest among the three regions. The motility of the sperm from the caput region was lower than those collected from corpus and cauda. On the whole, good quantities of intact mature sperm were obtained from the cauda region and were therefore used for most of the biochemical and other investigations.

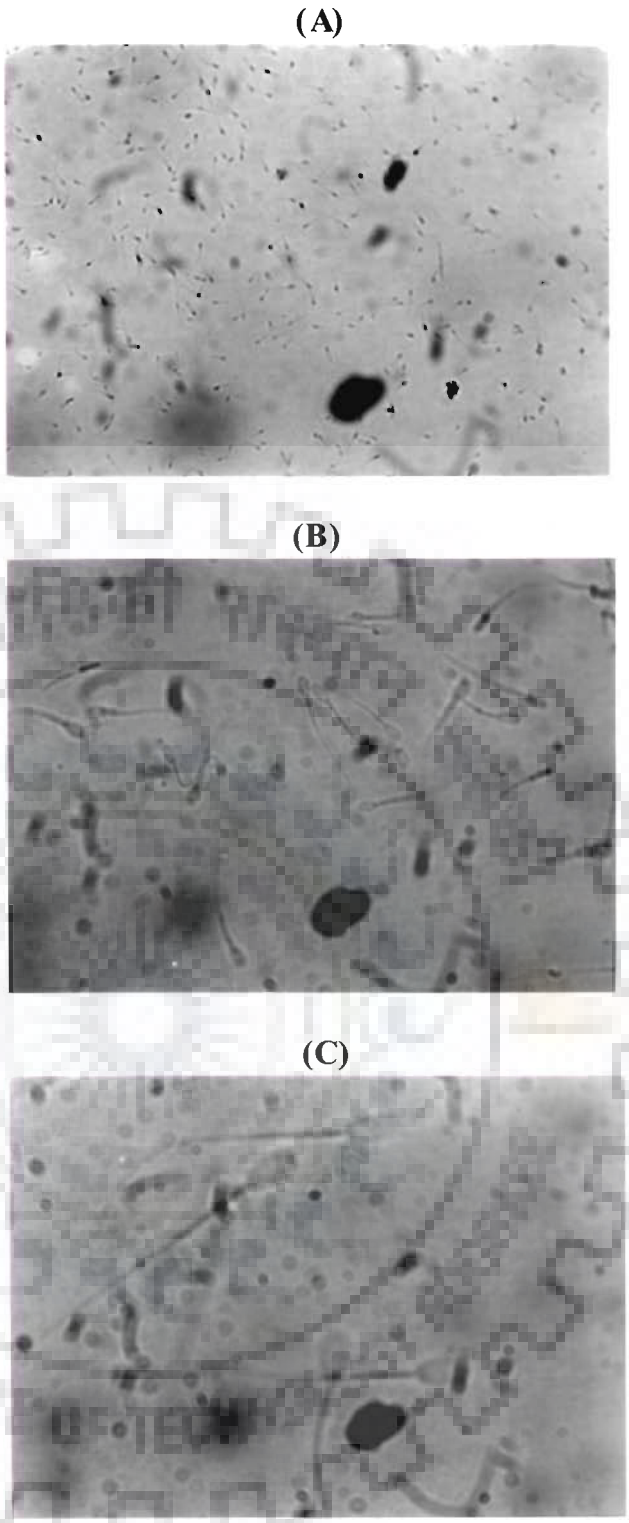
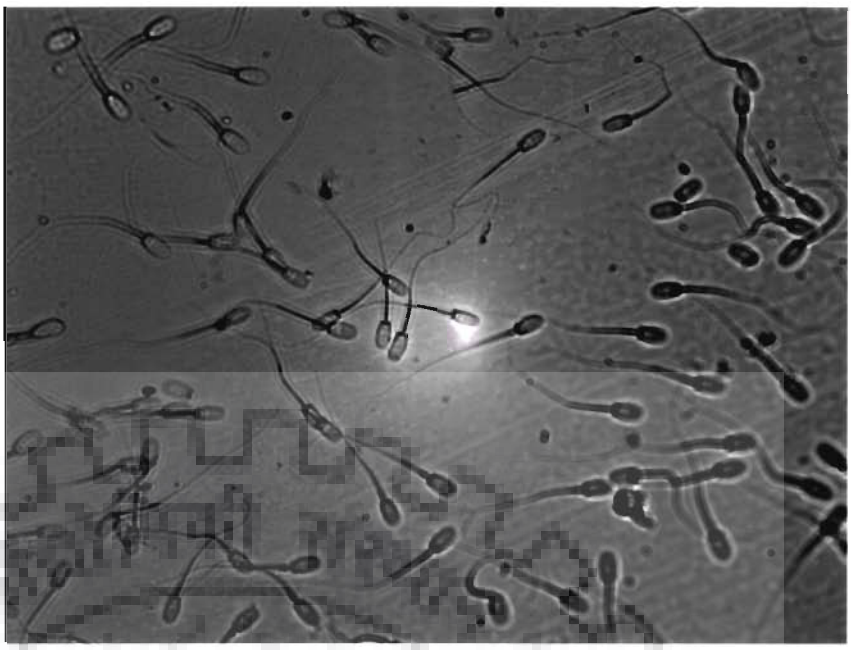



**Figure 12. Light Microscopy of spermatozoa obtained from the cauda epididymides of goat testis.** 10 µl of epididymal fluid was observed directly under microscope, (A) 10X, (B) 40X, & (C) 100X, magnification, respectively. Under lower magnification (10X) a large number of sperm can be seen suspended in the luminal fluid. Complete morphology of sperm with intact head and tail along with other luminal contents as seen at higher magnifications are shown (40X & 100X).

(A)



(B)

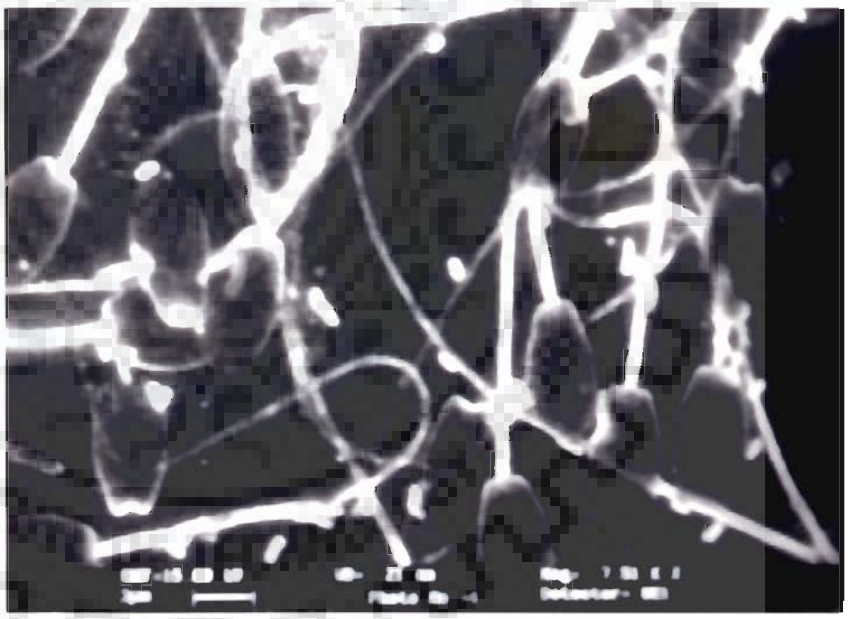


**Figure 13. Micrograph of washed goat sperm obtained from cauda region of the epididymis.**

(A) Light micrograph (40X magnification). A large number of intact sperm are seen without any contamination in background. (B) Scanning Electron Micrograph showing intact sperm with complete morphology. 3μm= ( —— ).

### 4.2 Isolation of goat sperm plasma membrane:

#### 4.2.1 Sperm disruption by nitrogen cavitation:

To isolate the membrane fraction, sperm were first disrupted by nitrogen cavitation at 600 psi for 15 min. The scanning electron micrograph (SEM) shows the extent of sperm lysis. It was observed that equilibration with nitrogen at 600 psi for 15 min led to the lysis of sperm (figure14B). SEM micrograph showed sperm homogenate with debris (containing broken heads and tails). The debris were separated from sperm homogenate by centrifugation at 600 x g for 10 min and discarded. The supernatant portion obtained after the centrifugation were collected as a crude membrane preparation.

#### 4.2.2 Preparation of sperm plasma membrane:

The crude membrane fraction was used for preparation of pure sperm plasma membrane. When the crude membrane fraction was layered on the top of the 15 and 50% sucrose step-gradient and centrifuged at 30,000 rpm for 90 min the sperm plasma membrane accumulated as a dense white band at the interphase of 15 and 50% sucrose gradient as shown in figure15. The band was recovered carefully and washed in 3 volumes of PBS. The pellet obtained was named as 'purified sperm plasma membrane' (PPM).

#### 4.2.3 Marker enzyme analysis:

Various marker enzymes and other biochemical assays were used to ascertain the identity of the sub-cellular fractions obtained at the various steps of

purification (Figure 16). Even trace amounts of DNA and RNA were not detectable in the purified sperm plasma membrane (PPM), though a significant amount of DNA and RNA was found to be associated with other fractions obtained prior to the isolation of PPM [Figure 17 (i) & (ii)]. These data indicate that isolated goat sperm plasma membrane fractions are not contaminated with any nuclear material.

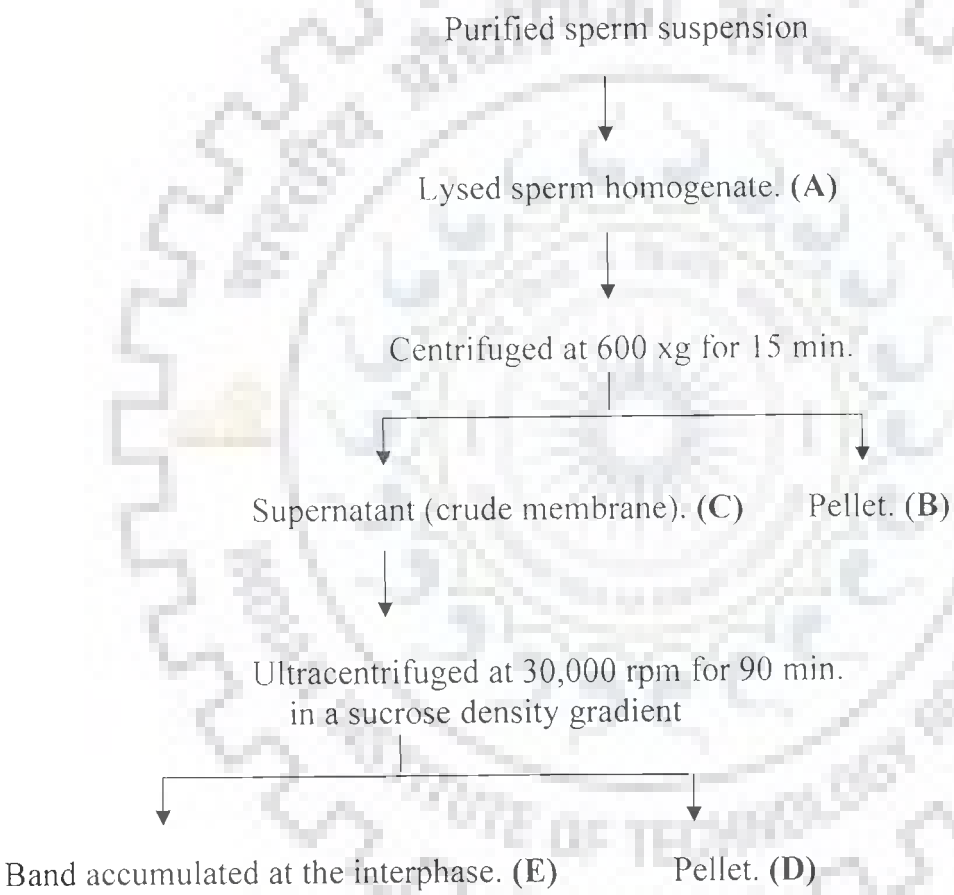



**Figure 16. Flow diagram of various sub-cellular fractions obtained during sperm plasma membrane preparation.** The pellets were washed and resuspended finally in distilled water/desired buffer. The various sub-cellular fractions obtained were named as A- Lysed sperm suspension (LSS), B -Centrifuged lysed pellet (CLP), C- Crude Plasma Membrane (CPM), D- Ultra centirifuged Pellet (UCP) and E- Purified sperm plasma membrane (PPM).

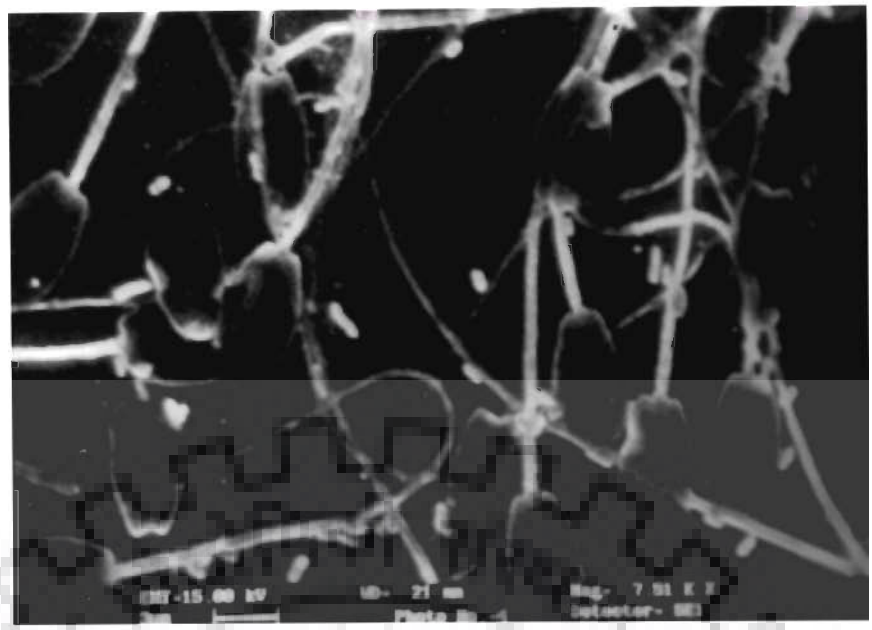
The purified sperm plasma membrane (PPM) was further checked by assays of two marker enzymes, acid phosphatase (lysosomal marker enzyme) and 5' nucleotidase enzyme (plasma membrane marker enzyme) in various sub-cellular fractions (**A, B, C, D, & E**). The result of the enzyme assay are presented in figure 17 (iii) & (iv). It is clear from the result that the sperm homogenate (A) and other crude membrane fractions (B, C, D) had much higher amounts of acid phosphatase activity. This may be due to contamination of lysosomal fraction in these crude preparations. A little acid phosphatase activity could also be seen in purified plasma membrane fraction (E) but it was significantly lower (10-fold low) compare to other crude fractions [Figure 17(iii)]. This trace activity in purified membrane fraction could be more on account of the assay procedure rather than due to lysosomal contamination. It may be pointed out that the reaction for determining phosphatase activity was carried out at acidic conditions but stopped by the addition of alkaline buffer pH 9.0. So there is a possibility that plasma membrane bound alkaline phosphatase activity was detected. On the other hand, there was high activity of 5' nucleotidase (plasma membrane bound enzyme) in purified membrane fraction compare to other crude fractions [Figure17 (iv)]. Thus, it is abundantly clear from the result that the sperm plasma membrane fraction (PPM) obtained was highly enriched with negligible contamination and therefore used for all further studies on sperm plasma membranes.

### 4.2.4 Transmission Electron Microscopy:

Transmission Electron Microscopy (TEM) was used to examine the quality of purified sperm plasma membrane (PPM). Electron microscopic analysis of the fraction isolated from the band found at the interphase between 15% and 50% sucrose gradient was performed. A large number of smooth membrane vesicles, free of debris and other contamination materials could be clearly seen (figure 18). At higher magnification, two distinct classes of membrane vesicles appear i.e. round shaped and flattened shaped as shown by arrows in figure 18D.

The enzyme marker analysis and ultra structural studies confirmed that the band that accumulated at the interphase of 15% and 50% sucrose gradient was a purified sperm plasma membrane fraction. It also ensured that the quality of plasma membrane fraction is enough good for biochemical estimations and analysis of plasma membrane proteins. As we already know that GPI anchored proteins are attached to the sperm plasma membrane through a GPI structure the use of the purified sperm plasma membrane fraction provided an easy approach to identify, characterize and analyze the surface proteins without any much interference from any other sub cellular fraction proteins.

(A)



(B)

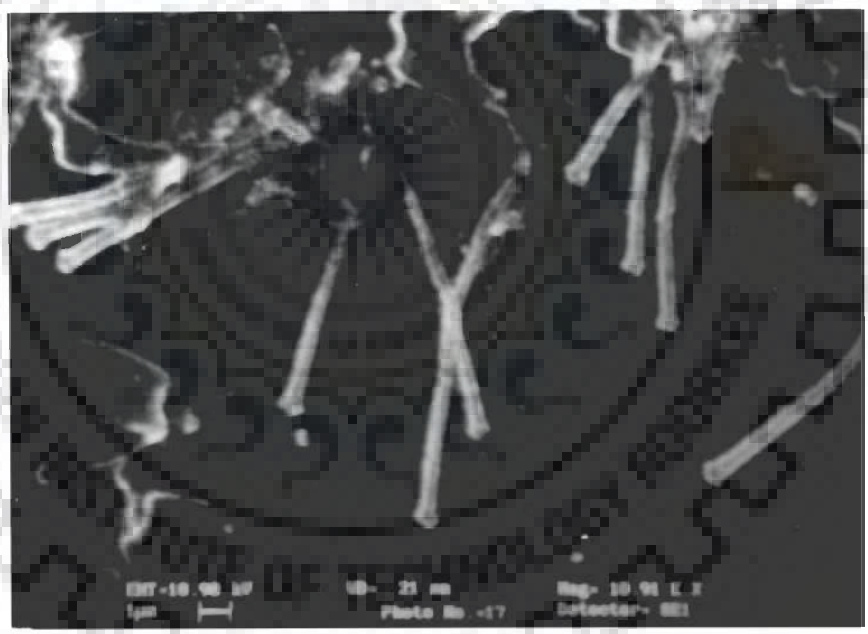


**Figure 14. Scanning Electron Micrographs of sperm before and after nitrogen cavitation.** (A) Washed intact sperm before the nitrogen cavitation process and (B) after nitrogen cavitation at 600 psi for 15 min. Broken head tail and vesicles along with debris is seen in background after lysis.

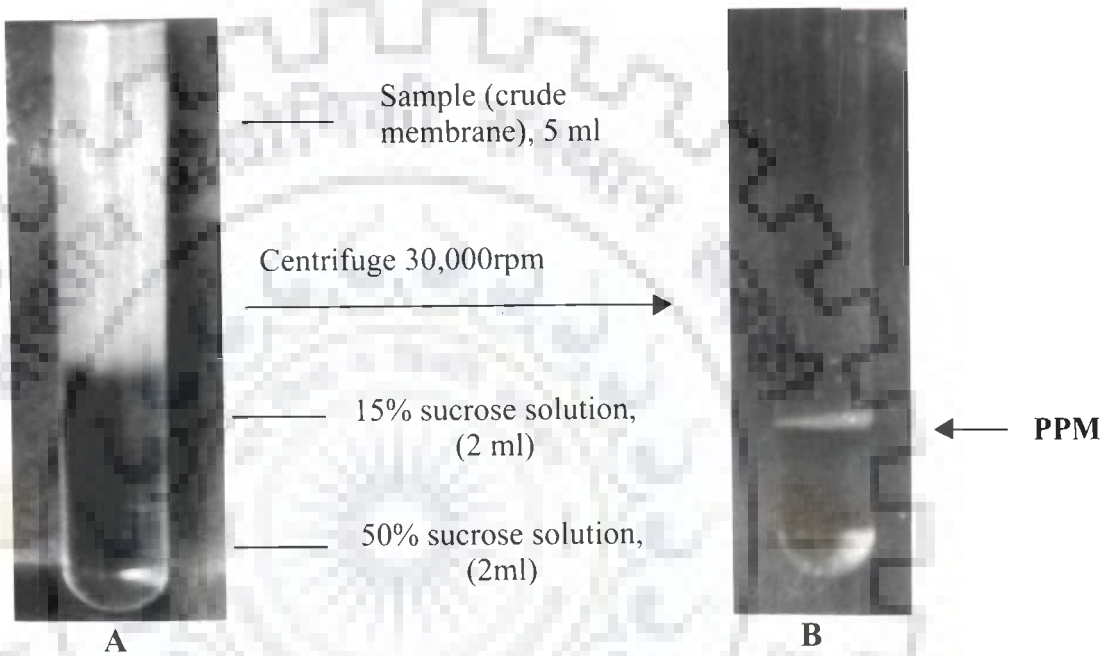



**Figure 15. Sperm plasma membrane prepared by sucrose step density gradient.** (A) 5ml crude membrane suspension was layered on the top of 2ml each 15% (w/v) and 50% (w/v) step sucrose gradient prepared in 0.2 mm pipes buffer pH 7.2. (B) After centrifugation at 30,000 rpm for 90 min, a white band of purified plasma membrane (PPM) accumulated at the interphase of 15% and 50% sucrose gradient.

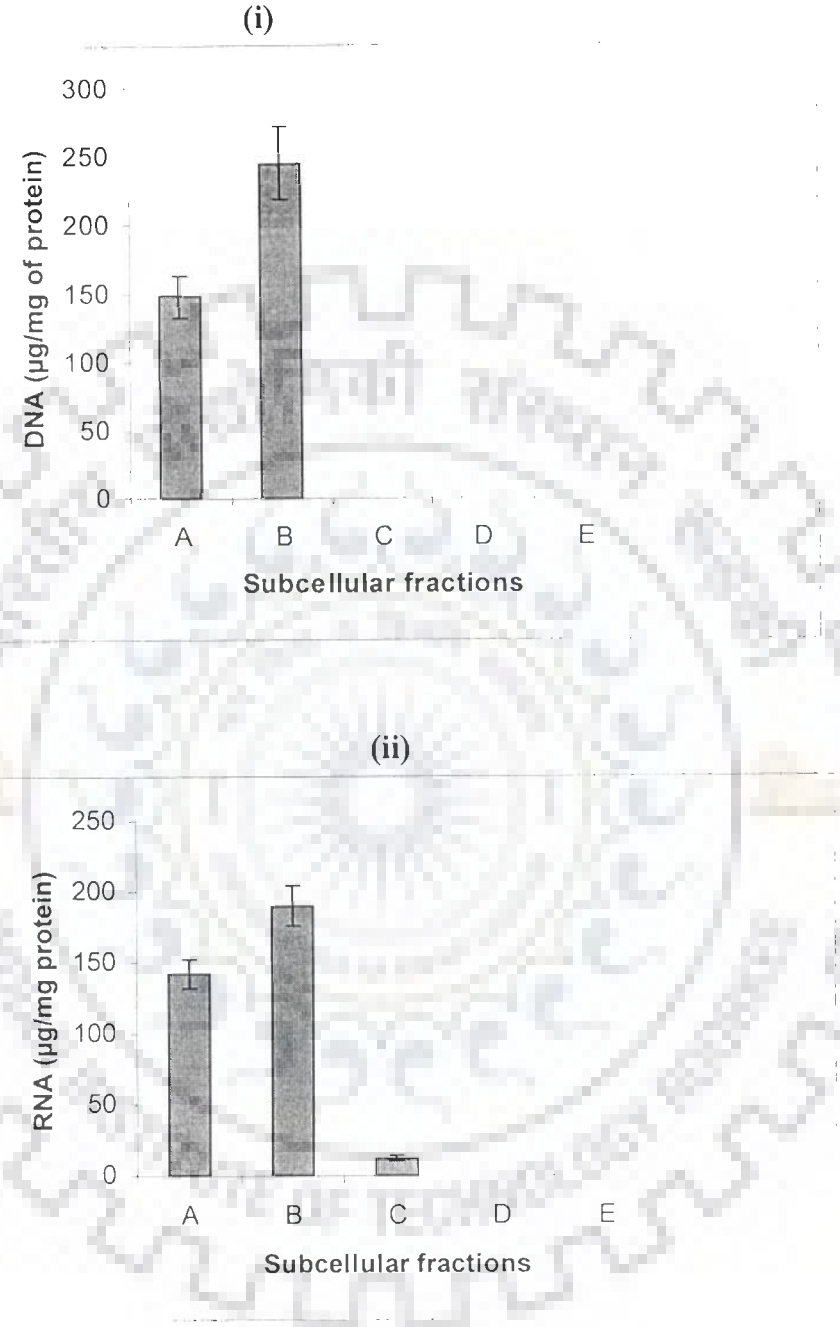



**Figure 17. DNA and RNA content of different fractions obtained during preparation of sperm plasma membrane.**

A - Lysed sperm suspension (LSS)
B - Centrifuged lysed pellet (CLP)
C - Crude Plasma Membrane (CPM)
D - Ultra centirifuged Pellet (UCP)
E - Purified sperm plasma membrane (PPM)

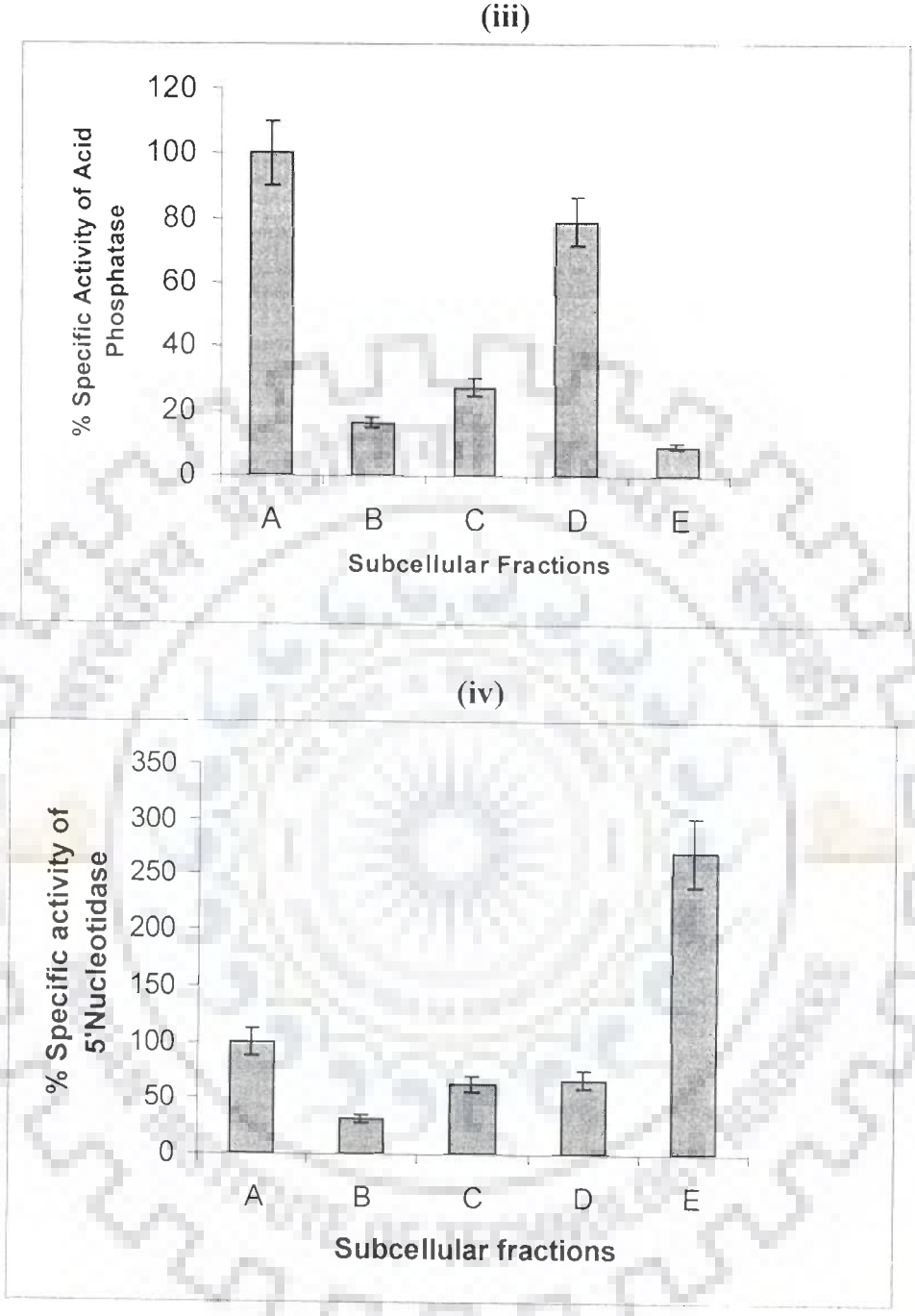



**Figure 17. The percentage specific activity of acid phosphatase and 5'-Nucleotidase in fractions obtained during preparation of sperm plasma membrane.**

A - Lysed sperm suspension (LSS)
B - Centrifuged lysed pellet (CLP)
C - Crude Plasma Membrane (CPM)
D - Ultra centirifuged Pellet (UCP)
E - Purified sperm plasma membrane (PPM)

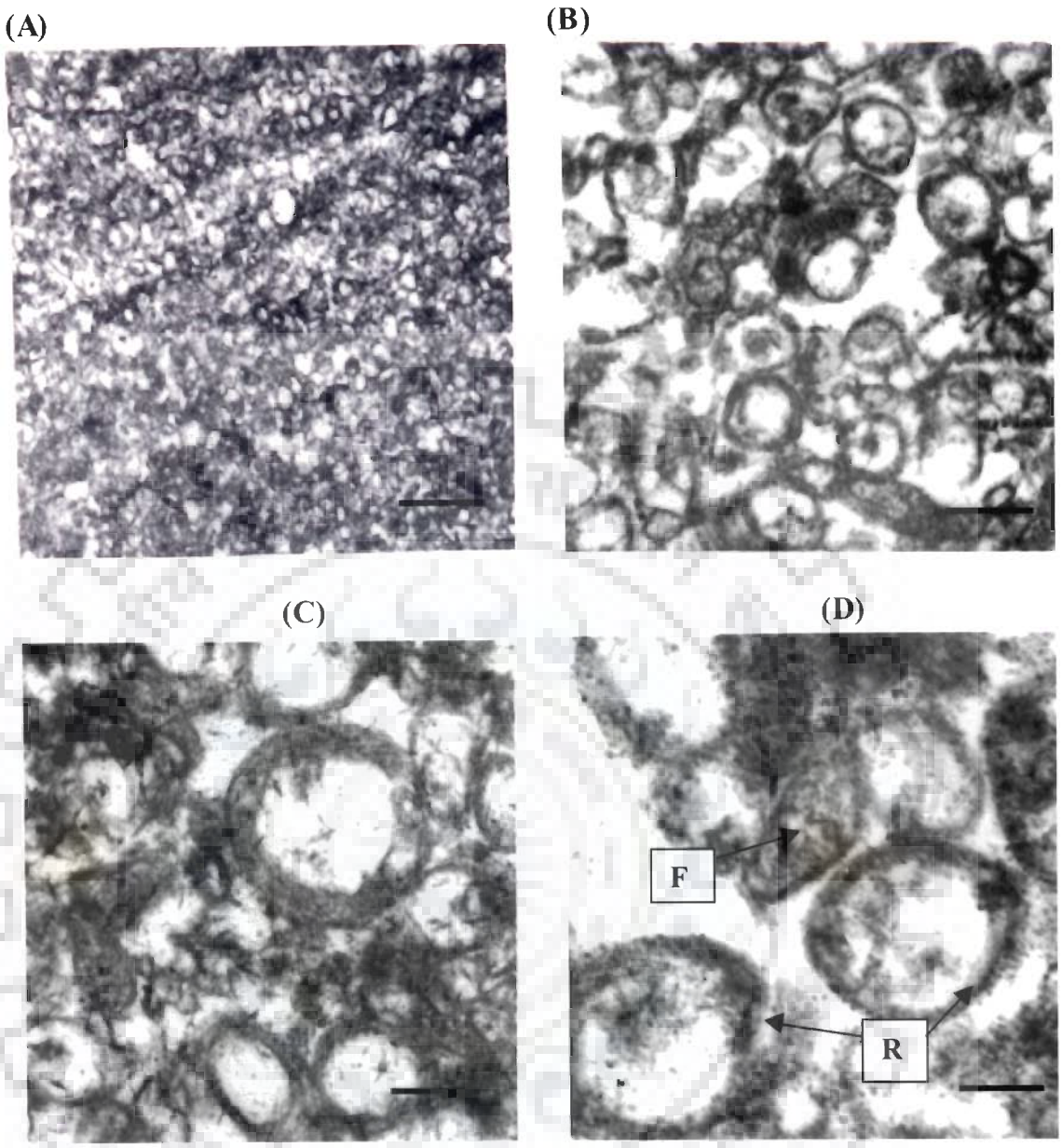



**Figure 18. Transmission electron micrographs of goat sperm plasma membrane enriched fraction.** The caudal spermatozoa were disrupted by nitrogen cavitation at 600 psi for 15 min and the membrane was purified by sucrose gradient centrifugation. The micrographs from (A) to (D) are arranged in an increasing order of magnification of the same microscopic field. The purified membrane fraction is confirmed by the presence of membranous vesicles, F indicates flattened vesicles while R indicates round vesicles. Bars (———) : (A) 0.8μm; (B) 0.44μm; (C) 0.327μm; (D) 0.164μm.

### 4.3 Identification and biochemical characterization of goat sperm and GPI anchored protein:

#### 4.3.1 Detergent solubilization of membrane bound proteins:

One of the strategies to know about the GPI anchorage of the protein is its solubilization pattern in two different detergents: triton X-100 and octyl glucoside. In case of GPI anchored proteins, the ratio of solubilization by octylglucoside to that of triton-X 100 is always more than one while in non- GPI anchored proteins it is less than one. This was confirmed by the distribution status of ALP (known GPI) and ATPase (known non-GPI) upon detergent solubilization of microsomal fraction derived from goat kidney. Not only were the activities of these enzymes estimated but the ratio of solubilization by the two detergents was also calculated. The activities of ALP and ATPase after solubilization from membrane by octyl glucoside and triton X-100 at different concentrations of detergents is given in table 2. Figure 19 depicts the ratio of the ALP and ATPase activity at different concentrations of the detergent. As expected, the value of the OG / TX-100 was found to be more than one in case of ALP, while it was less than one for ATPase. Thus, it is clear from the result that detergents solubilization ratio could be used to differentiate between the various modes of anchorage of a protein. Therefore this could be one of the general approaches to identify the GPI anchored protein.

**Table 2: Detergent solubilization of membrane bound enzymes.**

| Detergent solubilization | | MF - ATPase | MF - ALP |
|---|---|---|---|
| OG | 0.1% | 13.4 ± 0.8 | 73 ± 4 |
| | 0.2% | 8.6 ± 0.5 | 70 ± 3 |
| | 0.3% | 7.9 ± 0.4 | 69 ± 5 |
| TX-100 | 0.1% | 16.8 ± 1.0 | 62 ± 6 |
| | 0.2% | 12.8 ± 0.7 | 57 ± 4 |
| | 0.3% | 11.6 ± 0.6 | 54 ± 4 |

The specific activity of the two enzymes after cold solubilization by the respective detergent is tabulated. MF: microsomal fraction from goat kidney; ALP: alkaline phosphatase; ATPase: adenosine triphosphatase

### 4.3.2 Enzymatic cleavage:

One of the more direct approaches to identify the GPI anchored protein is its release by specific phospholipase-C enzyme (PI-PLC). Therefore this was used to identify the GPI anchored protein of goat sperm.

#### 4.3.2.1 Testing the potency of phosphatidylinositol specific phospholipase-C enzyme:

Commercial preparations of PI-PLC are available from various sources. However, besides batch-to-batch variation, these preparations differ in their potency and specificity. Therefore it would be logical to check the activity and specificity of cleavage with known GPI anchored proteins, before performing

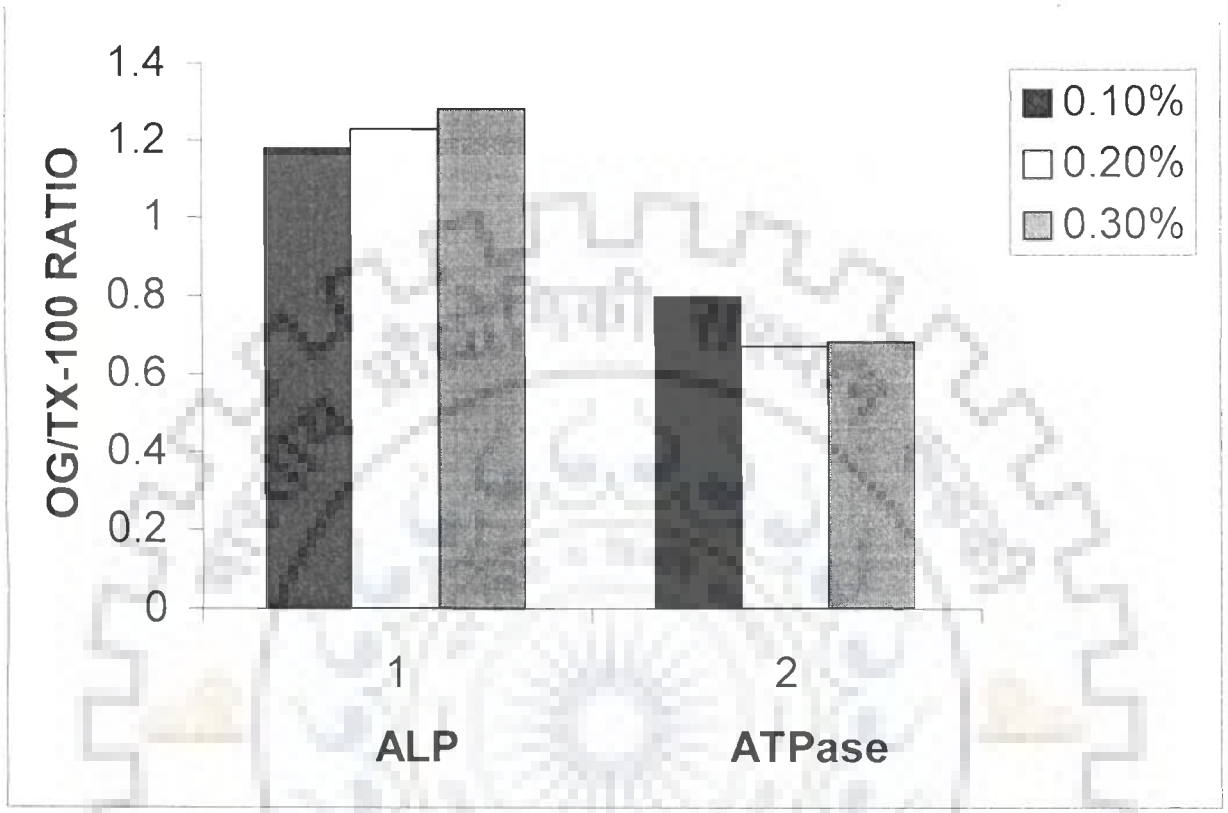



**Figure 19. OG/TX-100 solubilization ratio of membrane bound ectoenzymes.** Isolated membrane fractions were subjected to cold solubilization using detergents at 0.1, 0.2, and 0.3%. Enzymes showing values greater than 1 are supposed to be GPI-anchored. (OG, octylglucoside; TX-100, triton X-100)

the actual experiment. Hence, the potency of PI-PLC (*Bacillus cereus*) used in this study was first tested against two known plasma membrane bound proteins, ALP (known GPI anchored) and ATPase (known non-GPI anchored) both attached to microsomal membranes of goat kidney. Two equal aliquots of microsomal membranes were incubated one with and the other without PI-PLC. Measuring the activity of these ectoenzymes in the supernatant and pellet after centrifugation monitored the release of ALP and ATPase from the membrane. Centrifugation helps to pellet the microsomal membranes and supernatant contains the released protein. The results of the enzyme activities determined in both pellet as well as supernatant of PI-PLC treated and untreated samples are presented in table 3. High activity of ALP was observed in the supernatant after PI-PLC treatment compare to untreated control supernatant. On the other hand, high ATPase activity was retained in pellets of both PI-PLC treated and untreated samples. Thus, it is clear that high ALP activity in the supernatant of PI-PLC treated sample was due to release by cleavage from its GPI anchor.

**Table 3: Pattern of release of ecto-enzymes from membrane preparations after PI-PLC treatment.**

| Enzyme | Total enzyme activity in membrane | Control (-PI-PLC) | | Test (+PI-PLC) | |
|---|---|---|---|---|---|
| | | Pellet | Supernatant | Pellet | Supernatant |
| **Microsomal alkaline phosphatase** | 95.2 ±6.5 | 83.1 ±4.6 | 1.98 ±0.88 | 69.2 ±4.0* | 100.0 ±5.2* |
| **Microsomal adenosine triphosphatase** | 46.4 ±3.0 | 40.7±2.5 | 3.07 ±1.01 | 42.7±3.7 | 4.9 ±1.3 |

Microsomal Fraction was prepared as described in materials and methods. This microsomal fraction was divided into three equal aliquots. Total enzyme activity was measured in one aliquot and the remaining two were incubated in appropriate buffer with and without bacterial PI-PLC at 37°C for 2h. The enzyme released from the microsomal fraction was quantified in the pellet and supernatant after centrifuging the incubation mixture at 105,000 x g Values obtained for the pellet/supernatant fraction are compared with the corresponding controls. * $p<0.01$, Student's t-test

**4.3.2.2 Digestion of intact sperm and plasma membrane by specific PI-PLC enzyme:**

After the potency of enzymes and conditions for digestion was standardized with known GPI anchored proteins, attempt was made to identify the GPI anchored proteins of goat sperm. The intact sperm and purified goat sperm plasma membrane were treated with PI-PLC for 2 hours at 37°C and then centrifuged. Both the pellet and supernatant were recovered and analyzed for their protein profile by SDS PAGE.

### 4.4 SDS PAGE analyses of goat sperm:

#### 4.4.1 SDS PAGE profile of caput, corpus, cauda sperm and caudal sperm plasma membrane

Sperm are known to undergo extensive remodeling of their membranes during passage through the epididymis. In order to corroborate if there are any qualitative changes in the proteins during epididymal transit, the protein of sperm obtained from different sections of goat epididymis that is caput, corpus and cauda regions were separated, analyzed and compared through SDS PAGE. Protein profile of coomasie blue stained gels of sperm obtained from caput, corpus, and cauda region of epididymis are shown in figure 20. As expected a number of proteins bands were observed in each sample and the over all profile of caput, corpus and cauda was found to be similar. However, the cauda sperm figure 20, lanes 1 & 2 were found to contain some additional bands in the higher molecular weight range as compare to corpus (lanes 3, 4) and caput (lanes 5, & 6) sperm. Since the cauda sperm are believed to be mature cells, it may be inferred that the additional bands in cauda sperm may be those acquired during epididymal transit.

Since the GPI-anchored protein are associated with the membrane, the stability and proteins profile of sperm plasma membrane prepared from cauda sperm were examined. The purified cauda sperm plasma membrane fraction was resuspended in PBS buffer pH 7.4 and incubated at 37°C for different time periods that is 60, 90 and 120 min. The pellet and supernatant obtained by

centrifugation after incubation were electrophoresed. The coomasie blue stained gel profile of sperm plasma membrane is shown in figure 21. Several prominent proteins ranging from, 15-97 kDa, molecular weights were revealed on SDS-PAGE (lane 1). The sperm plasma membrane was found to be stable as no degradation or difference was observed in protein profiles after incubation at 37°C for different times (lanes 2, 3, & 4). In addition, no detectable bands were observed in supernatant fraction after incubation for 60 and 120 min (lanes 5, & 6).

**4.4.2 SDS PAGE profile of sperm and sperm plasma membrane after PI-PLC treatment:**

The sperm and purified sperm plasma membrane were treated with PI-PLC enzyme as described in material and methods, section 3.8.1. The pellet and supernatant recovered by centrifugation after enzyme treatment were analyzed for their protein profile by SDS PAGE and compared to those of untreated samples. The SDS PAGE protein profile of PI-PLC treated goat sperm is presented in figure 22. The SDS gel was analyzed by enhancing the resolution on a gel documentation system (Alpha Imager 2200 software, Alpha Innotech Corporation, San Leandro, CA., USA) and the results are represented in figure 23 (A) and (B). On comparison of protein profile of PI-PLC treated supernatant sample (figure 23 A, lane 5) with that of PI-PLC untreated supernatant (lane 4),

few new prominent protein bands (23 kDa, 27kDa, 42kDa, 57kDa, & 75kDa) were observed in supernatant of PI-PLC treated samples (Figure 23A, lane 5).

The appearance of these additional protein bands in the supernatant of PI-PLC treated samples indicates that they are most probably GPI anchored proteins since they are released from sperm after digestion with enzyme. Among them the 57 kDa protein was found to be the most prominent. The enhanced image of the same gel obtained by the 3D-aid in the Alpha Imager gel documentation system has been given for better clarity of the bands obtained (figure 23B). Examples of the released proteins after PI-PLC treatment are indicated by arrow figure 23B, lane 3. In addition, the comparison of protein profile of sperm pellet of control and PI-PLC treated samples (Figure 23A, lane 2 and 3), revealed that a 57kDa protein was depleted in PI-PLC treated sample Figure 23 (A) and (B) lane 3. This clearly indicates that the 57kDa protein is a GPI anchored protein as it is cleaved from its anchor on sperm membrane and released into supernatant fraction of PI-PLC treated samples.

Similar studies were also performed with purified goat sperm plasma membrane. The sperm plasma membrane was treated with PI-PLC under similar conditions. The pellet as well as supernatant of goat PI-PLC treated sperm membrane and control (untreated membrane), were separated on SDS PAGE and their protein profiles were compared for identification of GPI anchored protein. The results obtained are shown in figure 24. It was observed that similar to intact sperm sample a major GPI anchored protein of 57kDa also

found in sperm plasma membrane sample and it was released into the supernatant fraction after treatment with PI-PLC (figure 24, lane 4).

### 4.4.3 SDS PAGE profile of nitrous acid treated goat sperm:

Nitrous acid deamination chemically cleaves the bond between the phosphatidyl inositol and glucosamine releasing the protein from GPI anchors but leaving the phosphatidyl inositol attached with the membrane. Although this method does provide information regarding GPI anchorage, is not often used for identification GPI anchors since the conditions are not as mild as enzymatic methods. In order to verify if a similar pattern of results are obtained by chemical and enzymatic cleavage, the goat sperm suspension was treated with nitrous acid and incubated for 3 h at room temperature for chemical cleavage of GPI anchored proteins, along with control. The pellet and supernatant of both nitrous acid treated and untreated samples were collected and then electrophoresed. The SDS PAGE profile of this is represented in figure 25. As expected, a number of additional bands appeared in nitrous acid treated supernatant (Figure 25, lane 5). This was not present in the untreated supernatant lane 4. Here again, just as in the case of enzymatic treatment, the 57 kDa band was most prominent. It is thus inferred that this protein is definitely GPI anchored as it was released into the supernatant fraction from sperm after nitrous acid treatment.

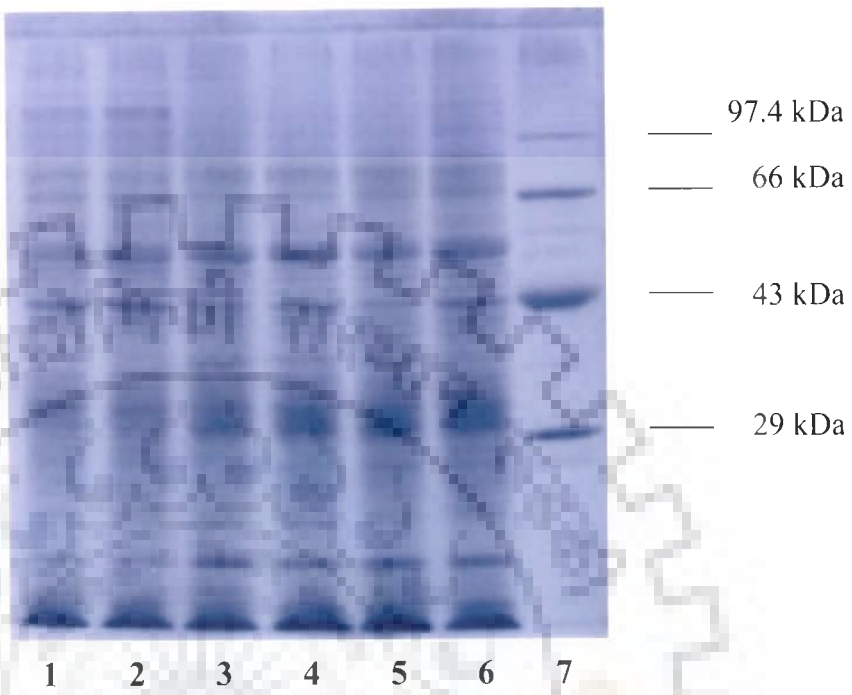



**Figure 20. SDS PAGE protein profile of sperm isolated from caput, corpus and cauda region of goat epididymis.** The samples were separated on 10% polyacrylamide gel and stained with coomasie blue R- 250. To check the reproducibility of results, two different preparations of sperm from each segment were run in parallel.

Lane 1 & 2: Sperm obtained from cauda region.
Lane 3 & 4: Sperm obtained from corpus region.
Lane 5 & 6: Sperm obtained from caput region.
Lane 7: Standard molecular weight markers.

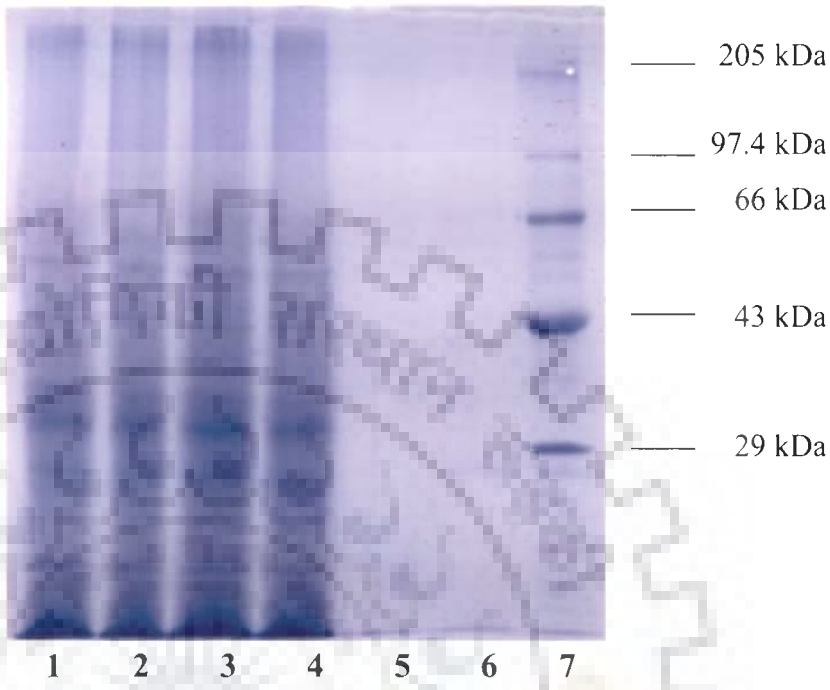



**Figure 21. SDS PAGE analysis of protein stability in plasma membrane, isolated from cauda sperm.** Lanes 1, 2, 3 & 4, shows the SDS PAGE of isolated washed plasma membrane pellet incubated for 0, 60, 90 and 120 min whereas Lanes 5 & 6, shows the SDS PAGE of aqueous portion (supernatant) obtained after incubation for 60 and 120 min and centrifugation of plasma membrane pellet. Lane 7 represents the standard molecular markers.

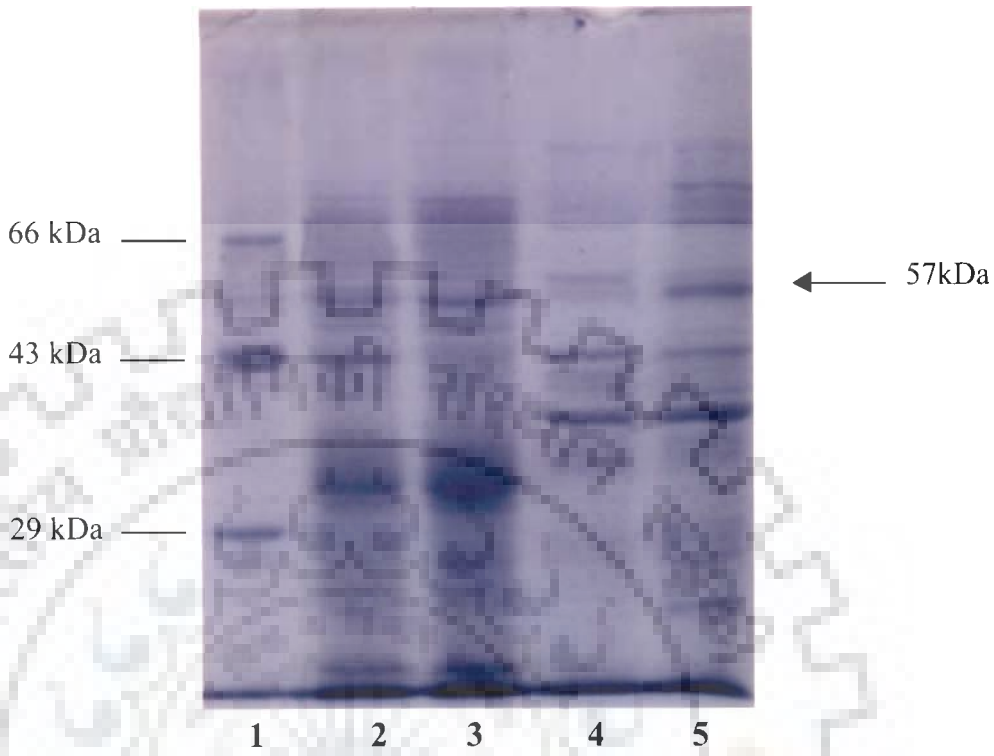



**Figure 22. The SDS PAGE protein profile of goat sperm with and without PI-PLC treatment.** Samples were incubated with and without PI-PLC enzyme for 90 min at 37C, centrifuged and pellet and supernatants were used for SDS PAGE.

Lane 1: Molecular weight markers.
Lane 2 :Sperm pellet without PI-PLC treatment.
Lane 3: Sperm pellet after PI-PLC treatment.
Lane 4: Supernatant without PI-PLC treatment.
Lane 5: Supernatant, after PI-PLC treatment.

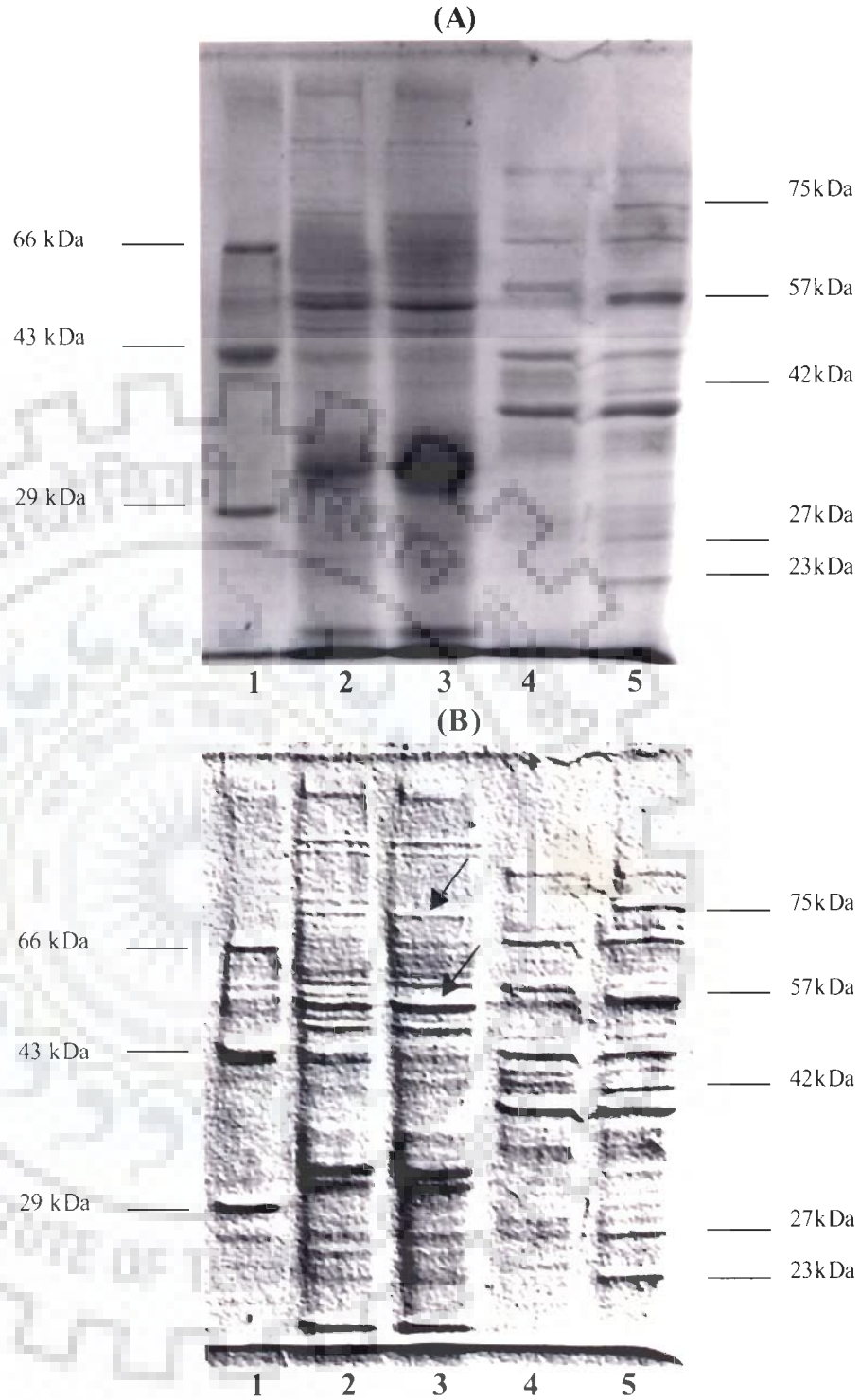



**Figure 23. Captured (A) and enhanced (B) image of SDS PAGE protein profile of goat sperm using a Gel documentation system** (The images of gels shown here is the same as in figure 22).

Lane 1: Molecular weight markers.
Lane 2 :Sperm pellet without PI-PLC treatment.
Lane 3: Sperm pellet after PI-PLC treatment.
Lane 4: Supernatant without PI-PLC treatment.
Lane 5: Supernatant after PI-PLC treatment.

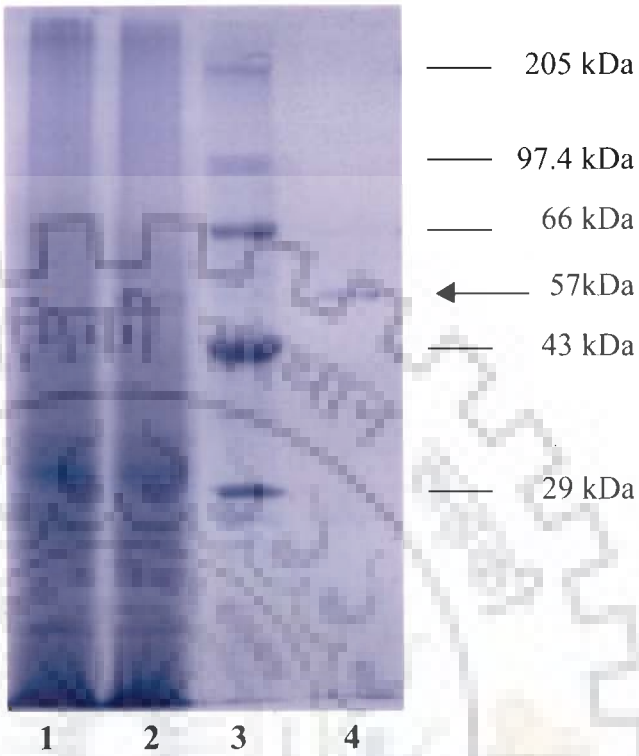



**Figure 24. The SDS PAGE protein profile of goat sperm plasma membrane with and without PI-PLC treatment.** Samples were incubated with and without PI-PLC enzyme for 90 min at 37C, centrifuged and pellet and supernatants were used for SDS PAGE.

Lane 1: Sperm plasma membrane with PI-PLC treatment.
Lane 2: Sperm plasma membrane without PI-PLC treatment.
Lane 3: Molecular weight markers.
Lane 4: Supernatant with PI-PLC treatment.

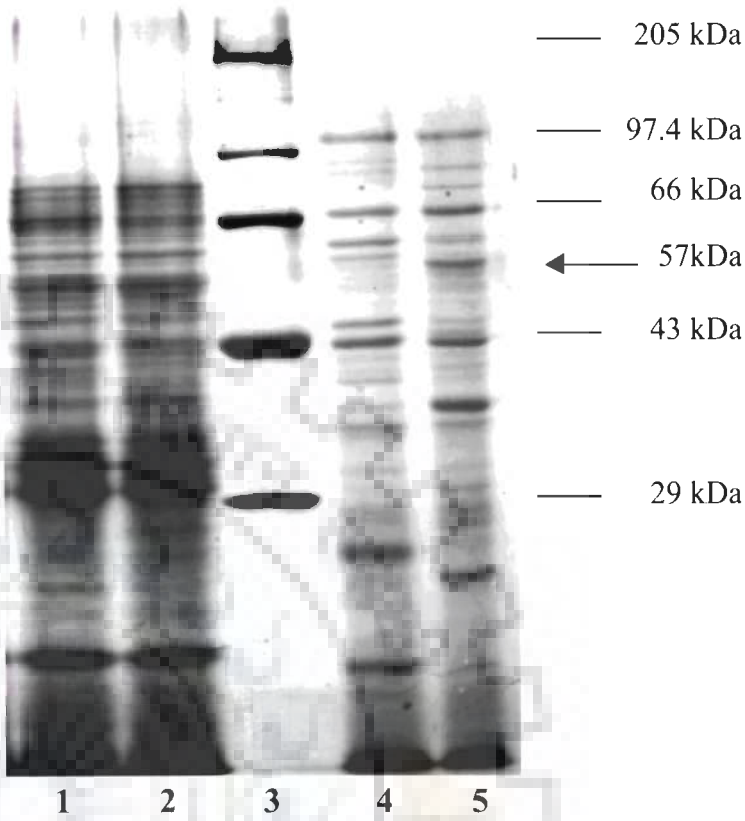



**Figure 25. SDS PAGE protein profile of goat sperm after chemical cleavage by nitrous acid.** Incubation was carried out for 3 h at room temperature for nitrous acid cleavage of GPI anchored proteins. The nitrous acid treated and control sperm were centrifuged and both pellet as well as supernatant was used for SDS PAGE.

Lane 1: Sperm pellet without nitrous acid treatment.
Lane 2: Sperm pellet after nitrous acid treatment.
Lane 3: Molecular weight markers.
Lane 4: Supernatant without nitrous acid treatment.
Lane 5: Supernatant after nitrous acid treatment.

## 4.5 Immunological studies:

### 4.5.1 Determination of titer value of polyclonal antibody:

Polyclonal antibodies were raised in rabbits against purified hydrophobic proteins of sperm plasma membrane, which were solubilized in triton X-114. The antiserum antibody titer was determined by enzyme linked immunosorbent assay (ELISA), where both sera pre-immune serum and anti-serum were compared through ELISA against the immunogen. The antiserum titer is shown in figure 26. It is clear from the results that high titer values were observed compared to pre-immune serum. The anti-serum demonstrated high values of absorbances even at dilution as low as 120,000 and showed its peak between dilutions of 400-1600. Pre-immune serum showed very little activity with antigen. Hence it is concluded that the anti-serum obtained is highly reactive to antigen (TX-114 solubilized purified hydrophobic sperm plasma membrane proteins), which also ensures specificity of polyclonal antibodies against the antigen. On the other hand, the low titer values of pre-immune serum against antigen suggest that there is less chance of non-specific binding. Thus, the polyclonal antibody obtained against the hydrophobic sperm plasma membrane proteins is suitable for other immunological applications. In this study it has been used in combination with PI-PLC treatment of membranes to identify, characterize and localize GPI proteins on the sperm.

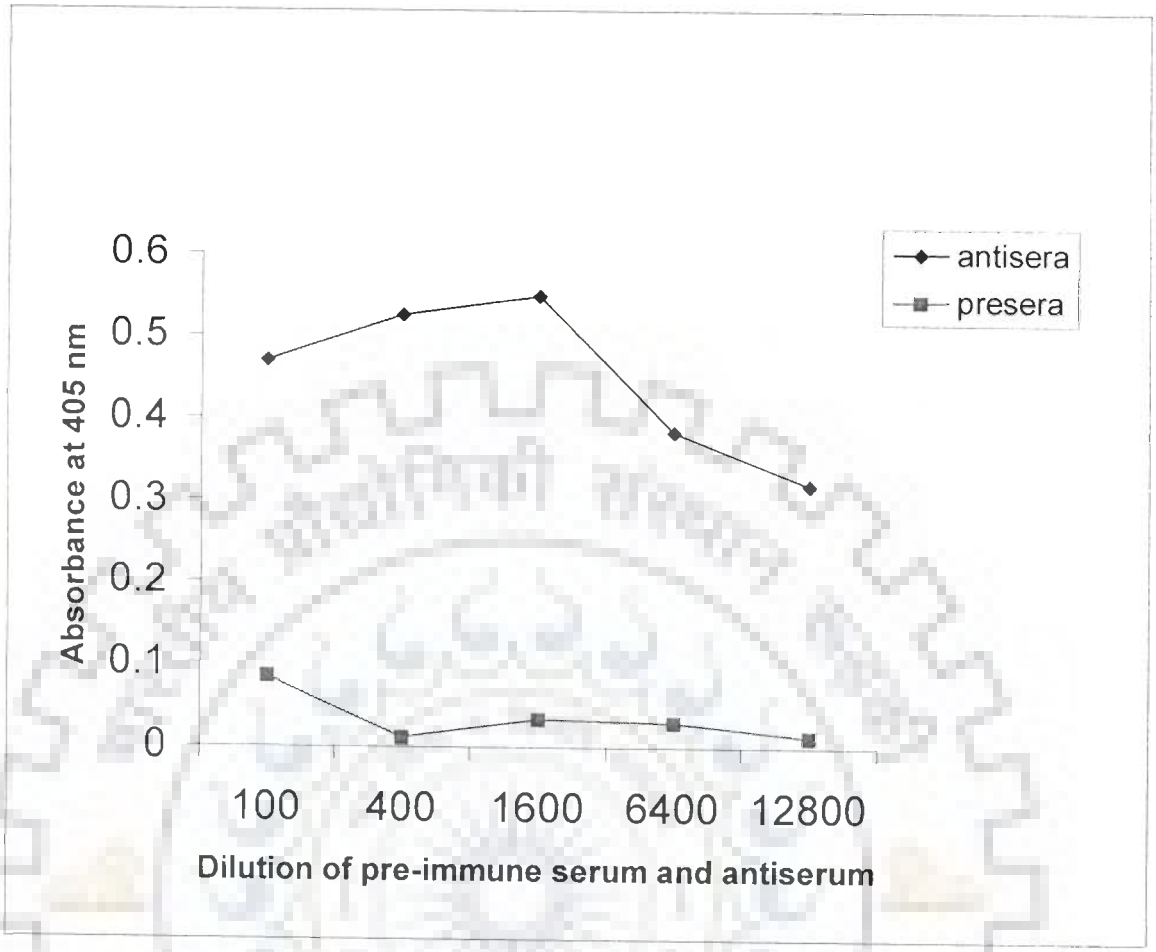



**Figure 26. Determination of antibody titer of antiserum using Enzyme Linked Immuno-sorbent Assay (ELISA).** The antiserum raised shows high values of absorbance against the immunizing antigen, while pre-immune serum shows low negligible values, confirming the specificity of antibodies raised against hydrophobic proteins of sperm plasma membrane.

### 4.5.2 Western-blot Analysis:

GPI anchored proteins are hydrophobic in its native form because they are attached to membranes by their hydrophobic domain. However, they are released from this hydrophobic domain into the aqueous phase either by enzymatic cleavage or chemical cleavage. Hence the polyclonal antibodies raised against hydrophobic proteins of sperm plasma membrane can help in detecting and localizing the GPI anchors of sperm.

Western blot using the polyclonal antibody described above was performed against sperm plasma membrane from caput, corpus and cauda sperm. For comparison purposes a purified 57kDa protein from sperm membranes (released after PI-PLC treatment and purified by gel elution, figure 27A) was also included. The result of the western blot is presented in figure 27B. The antiserum recognized two bands approximately 57kDa and 54 kDa in sperm plasma membrane from all three regions caput, corpus and cauda (figure 27B, lane 1, 2 and 3). In addition, it also recognizes the purified 57kDa protein, which was released by phospholipase-C enzyme, from hydrophobic domain of sperm plasma membrane (Figure 27B, Lane 4). The recognition of just two proteins on the blot indicates that these two proteins are immunodominant. One of these proteins was definetely GPI-anchored protein since this is the same protein (57kDa) that was released from sperm membranes by PI-PLC. The identity of the other protein could not be clearly established.

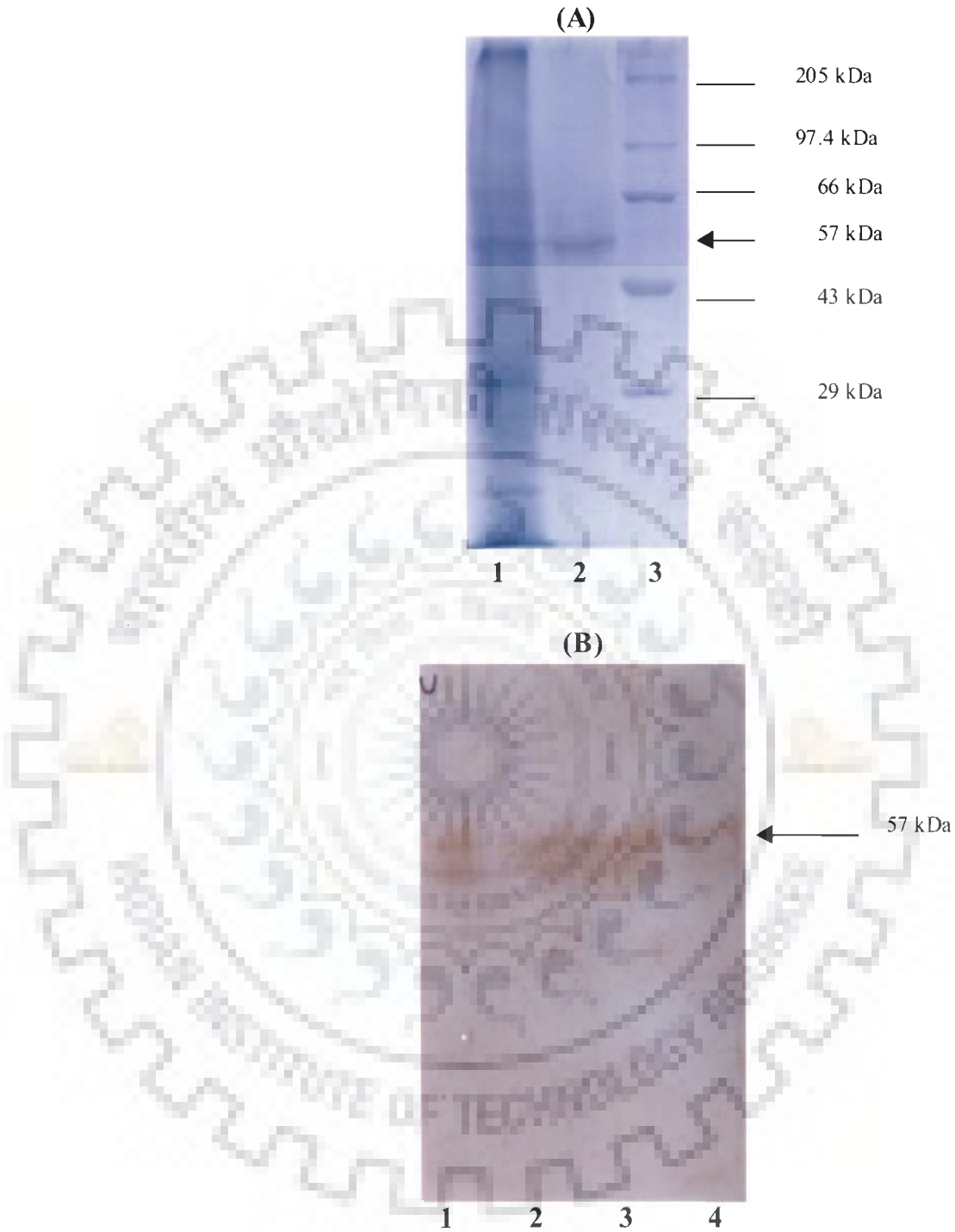



**Figure 27A. SDS PAGE of goat sperm plasma membrane proteins showing the 57-kDa GPI-protein.** Lane1, sperm plasma membrane proteins, Lane 2 represents the purified eluted 57kDa protein. Lane 3 is standard marker.

**Figure 27B. Western blot of sperm plasma membrane proteins and purified 57-kDa protein.** Lane 1, 2 & 3: plasma membrane proteins of sperm collected from caput, corpus and cauda, respectively and Lane 4: purified 57kDa GPI-protein of goat sperm.

### 4.5.3 Localization of GPI anchored proteins on goat sperm surface:

In an attempt to see the distribution and localization of identified GPI anchored proteins on the goat sperm surface immunofluorescence study was carried out using polyclonal antiserum, which was used for western blotting and ELISA.

The PI-PLC treated and control (untreated) caudal sperm were first fixed with acetone and then incubated with antiserum (primary antibody). Goat anti-rabbit IgG- FITC used as secondary antibody to localize the GPI anchors on goat sperm surface. The samples were observed under a fluorescence microscope in dark and photographed. It was assumed that PI-PLC enzyme would cleave off the GPI anchored proteins from the surface of sperm in treated sample as its specificity had been checked in the studies described earlier. On the other hand, the GPI-anchored proteins would remain attached with sperm surface in control.

The immunofluorescence micrographs of the observations are reported and represented in figures 28 and 29. It was observed that in control samples (PI-PLC untreated sperm) the whole sperm surface illuminated (produced fluorescence) including tail, mid piece as well as head region (figure 28A and 29A). On the other hand, in PI-PLC treated sperm the illumination (fluorescence) was restricted to only some parts of sperm. On closer examination of sperm surface in PI-PLC treated as well as in untreated sperm samples (figure 29 A and B), it is clear that GPI anchor proteins are present

mainly on head region of the goat sperm. The phase contrast micrographs of the same microscopic field as immunofluorescence micrograph are shown in figure 28 C and D respectively, for clarity. The magnified view of above immunofluorescence micrograph of PI-PLC untreated and PI-PLC treated samples are shown in figure 29A and B, respectively for better resolution. The hydrophobic antigens are distributed through out the sperm surface and therefore the whole sperm exhibited fluorescence in untreated sample as indicated by yellow arrows (figure 29A). While in PI-PLC treated samples only part of sperm surface was illuminated. The illuminated tail and mid piece region are indicated by blue arrow and the non--fluorescent head regions are indicated by red arrows (figure 29B). Thus, it is clear from the results that the GPI anchor proteins in goat sperm are present mainly in head region.

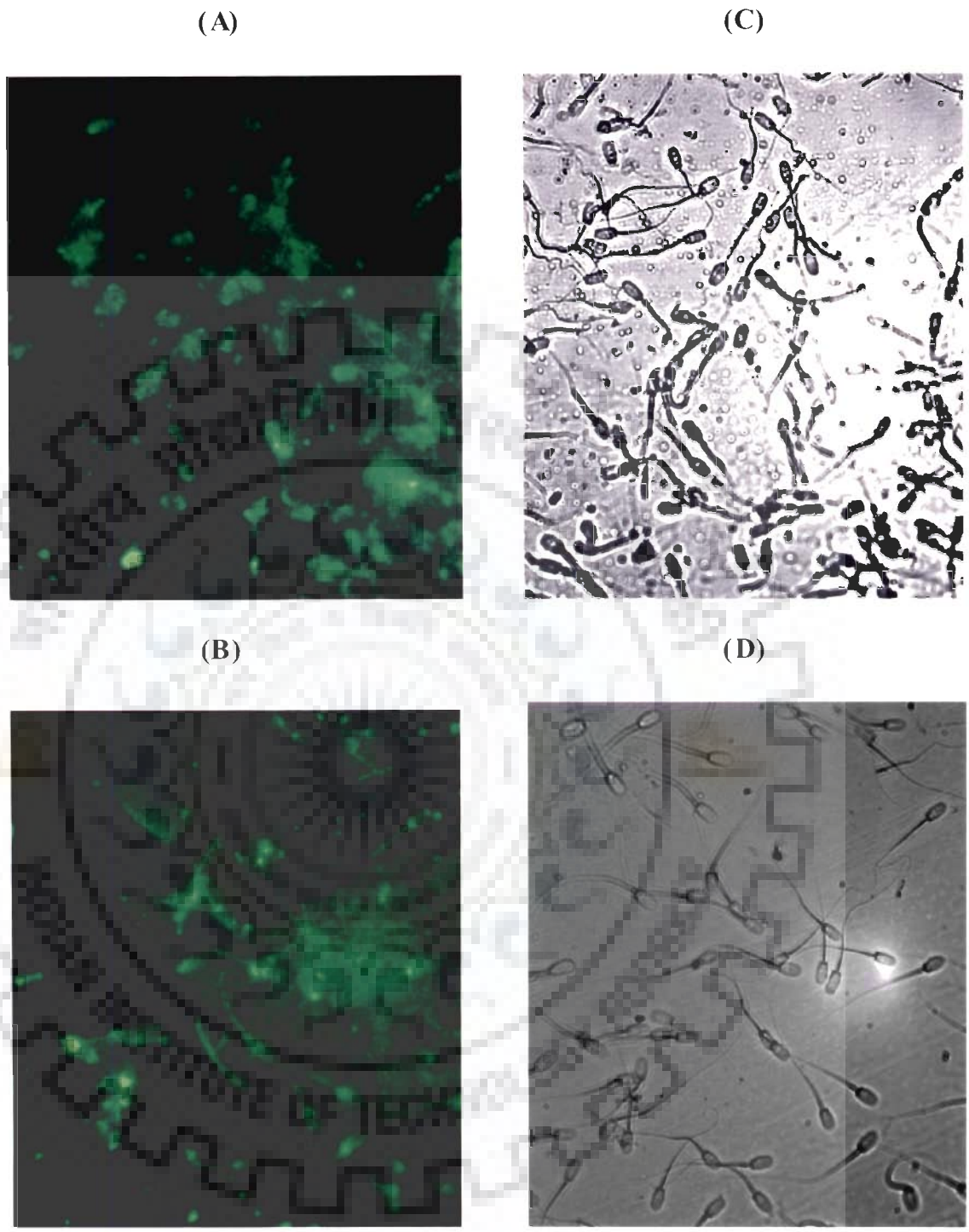



**Figure 28. Immunofluorecsence and light micrographs of sperm with and without PI-PLC treatment at low magnification** (100 X).

A: Immunofluorescence micrograph of untreated sperm.
B: Immunofluorescence micrograph of PI-PLC treated sperm.
C: Phase contrast micrograph of the same field as shown in A.
D: Phase contrast micrograph of the same field as shown in B.

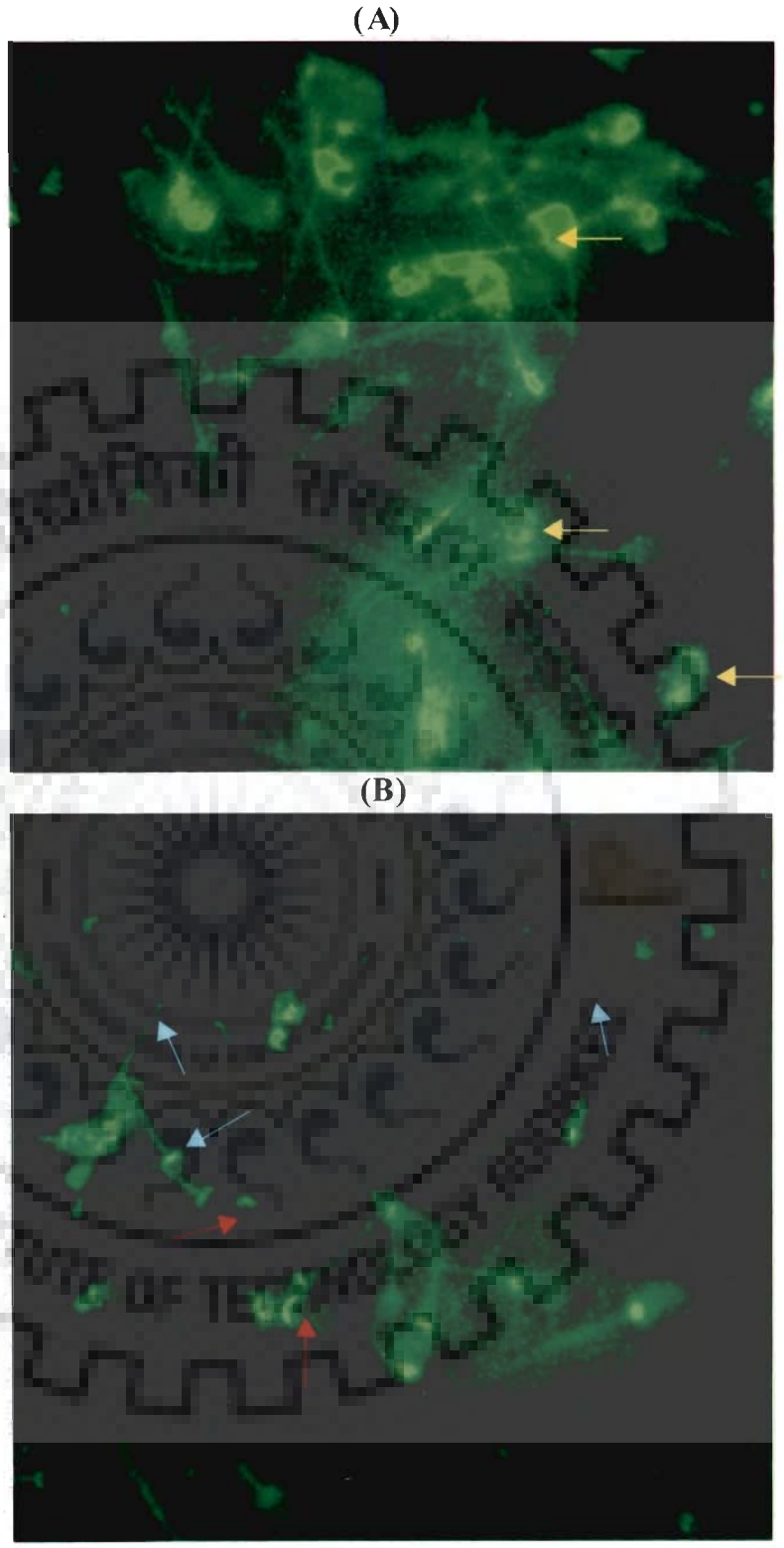



**Figure 29 Immunofluorescence micrographs of goat sperm with and without PI-PLC treatment.** (A) Goat sperm without PI-PLC treatment, where yellow arrow highlights illumination of the complete sperm (head to tail). (B) Goat sperm treated with PI-PLC, where the blue arrow indicates illuminated mid-piece and tail of sperm, while the red arrows indicate non-illuminated head region. 300X magnification.

### 4.6 Functional role of GPI-anchored Proteins:

#### 4.6.1 Assessment of survival capacity of sperm:

This experiment was conducted to assess the ability of sperm to survive phagocytosis by macrophages. Certainly and logically the key factor for its survival capacity lies at molecular level. It is suspected that some molecules present on sperm plasma membrane may be involved in protecting the sperm. In this study an attempt has been made to explore if GPI anchored proteins could be one of them.

The PI-PLC treated and untreated goat sperm were co-incubated with mice peritoneal macrophages in RPMI-1640 (section 3.11.3) and incubated for 6 h in a $CO_2$ incubator (WTB Binder, Germany) at 37°C in an atmosphere of 5% $CO_2$. The susceptibility of PI-PLC treated and PI-PLC untreated sperm to phagocytosis by macrophages was observed by transmission electron microscopy. The assay was evaluated by making comparison between electron micrographs of +PLC treated samples and -PLC treated sperm in a phagocytosis assay. The electron micrographs of macrophage incubated with untreated and treated sperm are shown in figures 30 & 31, respectively. In figure 30 a typical picture of a macrophage that is not participating in phagocytosis is shown. From figure 30 C and D, it is clear that the macrophages has no inclusions indicative of sperm phagocytosis. On the other hand cross sections of macrophages co-incubated with treated (+PLC) sperm, were seen with a large number of sperm particulates including head, mid-piece, and tail (Figure 31 A and B). The

magnified views of the same are shown in figure 31C and D, respectively. The engulfed sperm particulates are seen under both the planes longitudinal as well as transverse as indicated in figures by arrows. Thus, it is clear from the results that untreated (without PI-PLC) sperm show least susceptibility towards phagocytosis as none or negligible of the sperm particulate found to be engulfed by macrophages. On other hand, the sperm which were treated with (+PI-PLC) were found to more susceptible to phagocytosis by the macrophages. It must be emphasized that when co-incubations of intact sperm and macrophages were carried out and randomly examined (different fields of TEM) as little as 10-12% of macrophages were involved in phagocytosis. In similar experiments conducted with PI-PLC treated sperm and macrophages, this percentage of macrophages involved in phagocytosis substantially increases to 50-55%.

Therefore it can be concluded that the presence of GPI-anchored protein on sperm surface protect them from phagocytosis and may be playing an important role in survival capacity of the goat sperm in male and female genital tracts.

**4.6.2 Sialic acid concentration in proteins released by PI-PLC:**

It has been demonstrated earlier that under *in vitro* conditions, the sialoglycoprotein present on sperm surface provide protection against macrophages. It was therefore felt necessary to explore further whether the above susceptibility of sperm to macrophages was due to sialic acid or other

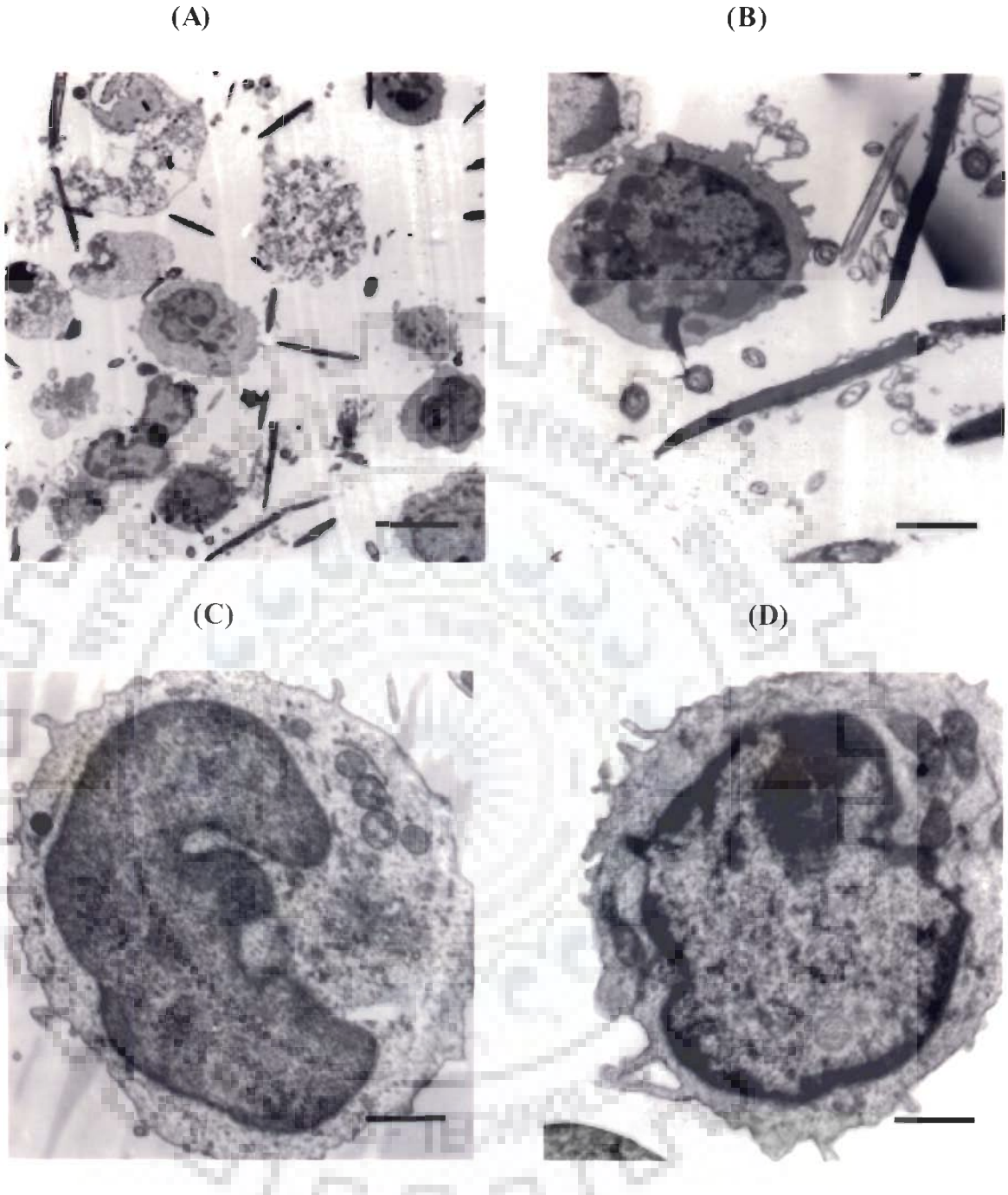



**Figure 30. Transmission electron micrographs of macrophages after co-incubation of goat sperm (without PI-PLC enzyme treatment).** Co-incubation was done for 6 h in RPMI-1640 in a $CO_2$ incubator at 37 C with an atmosphere of 5% $CO_2$. **A:** macrophages and sperm are seen scattered throughout the microscopic field. **B:** The magnified view of the same. **C** and **D:** normal macrophage; cell organelles are clearly seen but none of the sperm or sperm fragment are present in the cytoplasm of macrophage. Bars: (A) 5.6 μm; (B) 1.84 μm; (C) 1.06μm (D) 1.06 μm.

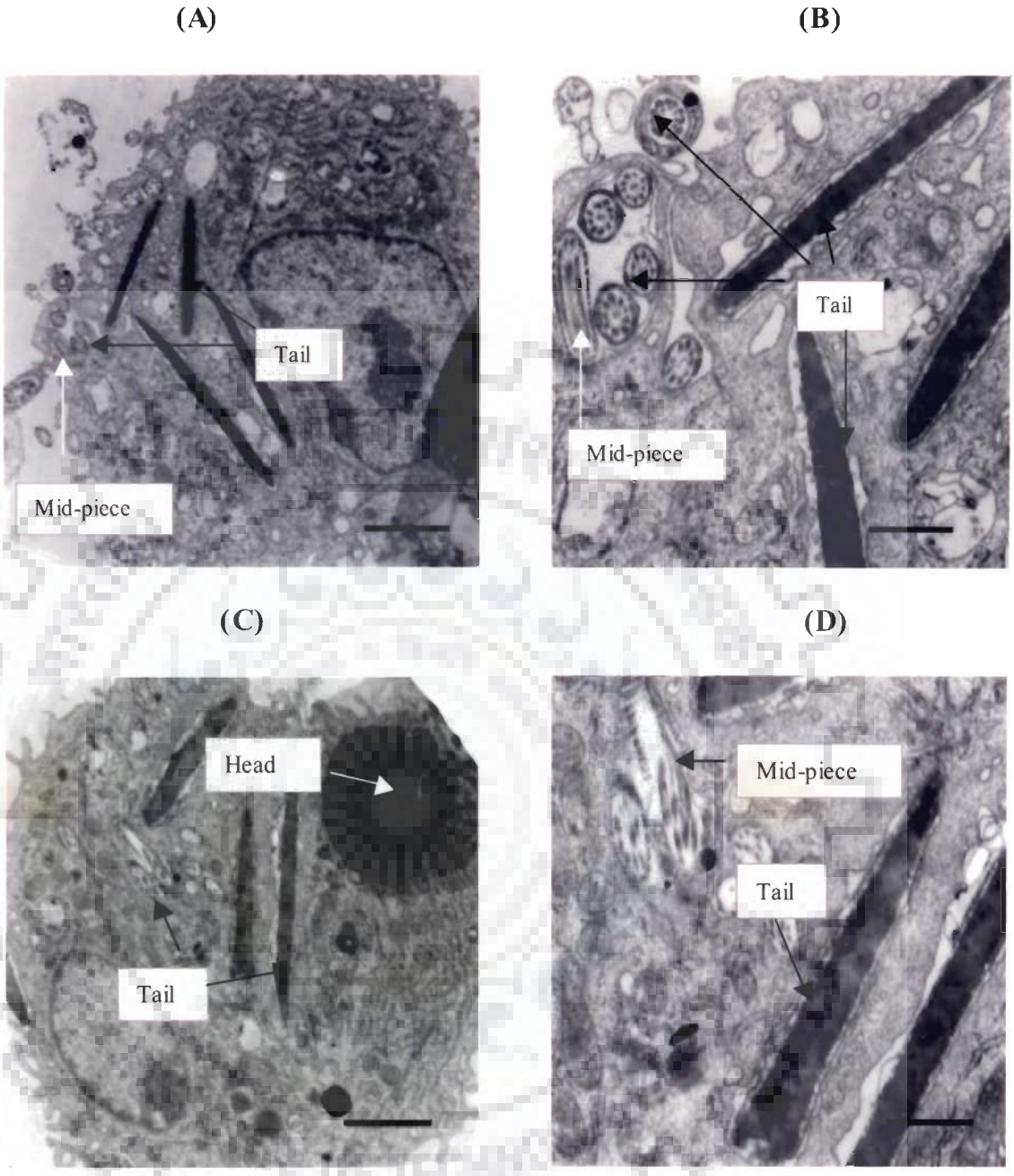



**Figure 31. Transmission electron micrographs of macrophages after co-incubation with PI-PLC treated goat sperm.** Co-incubation was done for 6 h in RPMI-1640 in a $CO_2$ incubator at 37 C with an atmosphere of 5% $CO_2$. **A:** cytoplasm of macrophage containing several sperm and sperm fragments which were engulfed by macrophage during phagocytosis, **B:** magnified view of the same macrophage in which mid-piece and tail with 9+9+2 arrangement with peripheral collar of microtubules are clearly visible. **C:** Another macrophage engaged in the phagocytosis. **D:** magnified view of the same macrophage. Bars: (A) 1.84μm; (C) 0.458μm; (B) 1.44μm; (D) 0.58μm.

reasons. Intact sperm were incubated with and without PI-PLC for 2h at 37°C, centrifuged and the amount of sialic acid was estimated in the supernatant.

However, no appreciable (almost nil) amount of sialic acid could be detected in both the supernatants. Thus, it is clear that the susceptibility towards macrophages in above experiment after PI-PLC treatment was not due to release of sialic acid. Therefore, it is unlikely that the released GPI-anchored proteins are sialylated.

# *Chapter - 5*

# *Discussion*

## 5.0 Discussion:

It is now abundantly clear that the GPI type of anchorage is one of the important modes by which proteins are tethered to the surface of sperm cells. It offers special structural advantages because unlike other transmembranal proteins, it spans only the outer leaflet of the lipid bilayer (Hooper, 2001). This trait helps in unhindered lateral mobility providing sperm the opportunity to economize on the number of molecules it needs to carry on its surface. It is a well established fact that proteins on the membrane of sperm perform vital functions to ensure that this gamete survives hostile environments and succeeds in its mission to fertilize the egg (Primakoff, 1985 ). Of late there is tremendous interest in the GPI-class of proteins and efforts have been made by several investigators not only to identify various GPI-proteins on the surface of germ cells but also to decipher their potential role. This is one such study done on the sperm of goat.

### 5.1 Purity of sperm and sperm plasma membranes:

In mammalian species, the sperm are produced in the testis, undergo maturation in the epididymis and finally are stored in a quiescent, yet viable form in the cauda region before being released when required (Graham *et al.*, 1991). Thus, the cauda epididymis is an excellent source for obtaining sperm. In fact, cauda sperm from a number of mammalian species have been used previously in a variety of research investigations. The procedures for obtaining

sperm from this storage site have also been well standardized (Tulsiani *et al.*, 1989; Hall and Killian, 1987; Skudlarek *et al.*, 1992). In the present studies, the quality of sperm obtained from the cauda epididymis of goat by using one such standard isolation protocol was tested and in terms of homogeneity, yield and viability, found to be satisfactory.

For identification of GPI-anchored proteins, it is preferable to work on isolated membranes since it helps in short-listing the number of proteins to be investigated. Thus, sperm plasma membranes were prepared by well-accepted procedures that are routinely used by other investigators to study surface molecules (Olson *et al.*, 1987; Tulsiani *et al.*, 1989; 1993). Several procedures are used for lysing sperm of which sonication ( Olson and Hamilton, 1978) and nitrogen cavitation (Olson *et al.*, 1987) are the most popular. Nitrogen cavitation has a special advantage since expansion of nitrogen gas after equilibrium at 600 psi causes cooling during sperm disruption while sonication generates heat. Therefore, since the membrane preparation was required for studying surface proteins, (which are vulnerable to heat) sperm disruption by nitrogen cavitation was adopted.

Multi-step (more than 2) gradient systems or multiple centrifugation steps have been employed for the preparation of plasma membranes (Millette, 1980). which are tedious and time consuming. In the present research investigations, centrifugation on a simple two-step sucrose gradient system was used to rapidly purify the sperm plasma membrane fraction. The identity of the membrane

preparation was checked using marker enzymes. The abundance of 5'nucleotidase and relatively poor activity of acid phosphatase were clear indications that the preparation was enriched in sperm plasma membranes. The enzyme 5'nucleotidase is considered to be one of the most specific markers for plasma membrane (Touster, 1970; Avruch, 1971; Millette, 1980). Besides plasma membrane, 5' nucleotidase has also been reported to be present in endoplasmic reticulum (Widnell and Unkeless, 1968). Fortunately, the sperm are stripped down cells and lack the endoplasmic reticulum. This fact not only reduced the possibility of contamination from these cellular organelles but also simplified the procedure of plasma membrane preparation and identification.

Transmission electron microscopic examination has been done in the past to establish the identity of membranes (O'shaughnessy and Brown 1978; Tulsiani *et al.,* 1989). The ultrastructural studies conducted in the present investigations revealed that the isolated band was mainly composed of membranous vesicles. Although two types of membranous vesicle were found, they were smooth and almost uniform in nature. Taken together, the biochemical and electron microscopic data indicate that the white band obtained at the interface of the 15-50% sucrose gradient after centrifugation is enriched in sperm plasma membranes.

**5.2 Special characteristics of GPI anchored protein:**

Unlike soluble proteins, studies pertaining to membrane proteins require unique techniques like the employment of detergents for isolation and analysis.

An important factor that influences the effectiveness of membrane protein solubilization by a detergent is its critical micellar concentration (CMC). It is well known that GPI proteins are resistant to solubilization with detergents of low CMC like triton X-100 and more readily soluble by detergents with high CMC like octyl glucoside (Hooper and Turner, 1988). This pattern of detergent solubilzation is peculiar to GPI proteins and the ratio of proteins solubilised by octyl glucoside to that solubilized by triton X-100 is used to predict the presence of GPI anchors (Muller *et al.*, 1994; Hooper, 2001). In the present studies too the solubilization pattern seen with respect to ALP (a GPI protein) and ATPase (a transmembranal protein) conformed to this rule. Therefore based on the ratio of solubilization in the two detergents, it may be inferred that ALP and not ATPase is GPI-anchored.

One possible explanation for this unique characteristic of GPI proteins is that they are targeted to specific micro-domains that do not mix freely in membranes because of acyl chain interactions (Brown and Rose, 1992; Rodgers *et al.*, 1994; Schroeder, 1998). These specialized clusters on membranes called 'rafts' are enriched in cholesterol and sphingolipids and exist in liquid ordered ($l_o$) phase or a phase with similar properties. GPI-anchored proteins become detergent-insoluble by virtue of being associated with cholesterol and sphingolipids (Hanada, *et al.*, 1995; Schroeder, *et al.*, 1998; Hooper, 1999). The importance of cholesterol has been demonstrated by treating mammalian cells with saponin, which enhances the solubilization of GPI-anchored proteins with

triton X-100 (Cerneus *et al.,* 1993). Saponin complexes with membrane cholesterol, and sequesters it, thereby preventing other interactions (Elias *et al.,* 1978). Thus, the presence of cholesterol and sphingolipids makes the entire raft complex including GPI-anchored proteins resistant to solubilization by non-ionic detergents such as triton X-100 in the cold (Brown, 2002).

There is now evidence to suggest that mouse and guinea pig sperm membranes too possess distinct raft subdomains (Travis *et al.,* 2001; Trevino *et al.,* 2001). It has been proposed that the lipid rafts on gametic cells that include GPI-proteins are functional domains coupled with signal transduction and play a role in sperm-egg interactions (Ohta, *et al.,* 2000 a,b)

**5.3 Specificity and Potency of the commercial PI-PLC preparation:**

Several methods are available to identify and study the structure of GPI-anchored proteins on cell surfaces. A distinctive characteristic of GPI proteins is its vulnerability to cleavage by phospholipases that can release proteins from their GPI-anchors. Enzymatic methods are generally preferred over chemical procedures for this purpose since they are relatively mild and have a specific site of action. Literature surveys reveal that among the GPI-degrading enzymes, phospholipase C is more extensively used than phospholipase D. Thus, phospholipase C was used in the present investigation to release proteins from their GPI-anchors. Suitable positive and negative controls were also included along side to assess the reliability of the method. This enzyme acts on GPI

proteins and converts the hydrophobic form to a soluble form, which is used as a means of separation and identification (Toutant *et al.,* 1989). The release of ALP (a known GPI anchored protein) and but not ATPase (a transmembranal protein) from membranes of goat tissues confirmed the specificity and potency of the PI-PLC used. Thus, these tests also authenticated the choice of using PI-PLC as a tool for studying proteins attached to membranes by the GPI anchor.

**5.4 A prominent 57-kDa sperm protein is GPI anchored:**

Several GPI anchored proteins have been identified on the surface of sperm from both invertebrates and vertebrates (Mendonza *et al.,* 1993; Cherr *et al.,* 2001; Kirchhoff and Hale 1996). However, there is practically no information on the GPI-anchored proteins of goat sperm. The protein profiles of caput, corpus and cauda sperm clearly demonstrate that qualitative variations occur on sperm as they transit through the epididymis. The protein profile of cauda sperm is distinct from the caput and corpus sperm. Many proteins that were not present in the caput and corpus sperm were found on cauda sperm. This is on expected lines since sperm are known to acquire antigens associated with maturation in the epididymis and the mature sperm are finally stored in the cauda before release. Thus, sperm from only the cauda epididymis was used for further investigations. SDS-PAGE analysis showed that some proteins were released from the sperm surface by PI-PLC treatment. On comparing the protein profiles of sperm with and without PI-PLC treatment, proteins with molecular weights of 75kDa, 57kDa, 42kDa, 27kDa and 23kDa were identified to be GPI-

anchored but the 57kDa protein was the most conspicuous. When the sperm plasma membrane was treated with PI-PLC and monitored by SDS-PAGE for the release of proteins only the 57kDa protein could be identified. Although all GPI-proteins are membrane anchored, differences in the GPI-anchored protein profiles of total sperm and sperm plasma membrane have been observed. Perhaps the GPI-proteins other than the 57kDa protein are associated with supplementary sperm membranes like the acrosomal membranes, which were not included in the plasma membrane fraction. Alternatively, it is possible that the initial concentrations of the other proteins are low and that they are further reduced to undetectable levels following the purification of sperm plasma membrane. Nevertheless, these results clearly demonstrated that: 1. The PI-PLC preparation used was potent and 2. Cauda sperm hold GPI-anchored proteins that are PI-PLC sensitive.

It must be pointed out that both PI-PLC-sensitive and PI-PLC-resistant species of GPI-anchored molecules have been reported in literature (Mayor *et al.*, 1990a). This was substantiated in the present experiments too since the proteins released by enzymatic cleavage were not entirely the same as those released by chemical cleavage. Although it is possible that there could be several other sperm proteins that are GPI anchored, the selective release of a limited number of proteins by PI-PLC could be due to one or more of the following reasons.

All GPI-anchored proteins share a common core structure but there are several variations arising from substitution at different sites in the glycan-bridge between the protein and lipid moiety. The details of these variations in mammals have been summarized in a recent review (Ikezawa, 2002). It is likely that such alterations in structure of GPI-anchored proteins may hinder the action of the PLC (figure 32).

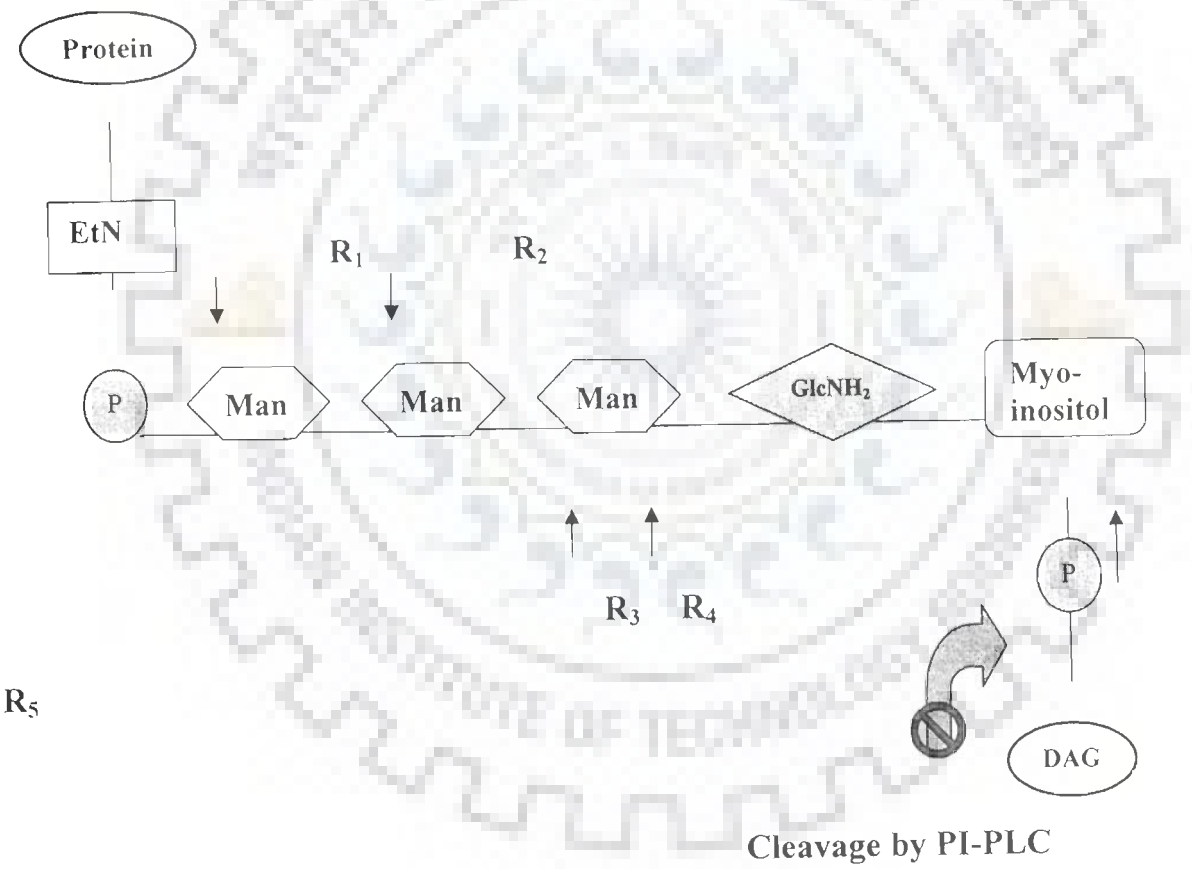



**Figure 32: Typical structure of GPI-anchor showing possible sites of substitution ($R_1$, $R_2$, $R_3$, $R_4$ and $R_5$).** Acyl substitution at R5 imparts resistance to cleavage by bacterial PI-PLC (Roberts *et al.*, 1988). EtN, ethanolamine; P, phosphate; Man, Mannose; $GlcNH_2$, glucosamine; DAG, diacylglycerol

Support to this view stems from the observation that acyl substitution in the myo-inositol moiety makes the GPI-anchor resistant to bacterial PI-PLC (Roberts *et al.,* 1988; Ferguson, 1992). In fact, it has been demonstrated that the removal of inositol-linked fatty acid by treatment with mild base or hydroxylamine treatment restores PI-PLC sensitivity (Mayor *et al.,* 1990b).

Another important factor that can influence the release of the GPI-protein by PI-PLC is the composition of the lipid environment in which they are located on lipid bilayers (Sharom and Lehto, 2002). In one study it has been convincingly shown that the efficiency of PI-PLC in releasing 5'nucleotidase reconstituted into lipid bilayer vesicles of different composition was not the same (Lehto and Sharom, 1998). It was suggested that the relative surface charge of the host plasma membrane could modulate the catalytic activity towards the GPI-anchored proteins depending on the net charge of the phospholipases (Sharom *et al.,* 1996). In addition, fluidity and packing are considered to be the important modulators of bacterial PI-PLC activity on GPI-anchors (Lehto and Sharom, 2002). It may be pointed out that the surface of the sperm has many different domains, which do not mix freely but retain their individual identity (Eddy and Koehler, 1982; Peterson *et al.,* 1987; Wolf, *et al.,* 1990; Wolf, 1995; Jones, 1998; Wolfe *et al.,* 1998; Mackie *et al.,* 2001; Baker *et al.,* 2002). Therefore, the ability of PI-PLC to release GPI-proteins may not be the same in all these microdomains. Thus, modulation of the PI-PLC action by

the lipid bilayer could be another strong reason for the release of just few GPI-proteins from sperm membranes.

It must be emphasized that GPI-anchorage is just one among several ways protein molecules are held on the membrane surface. Even among GPI proteins, there are the PI-PLC sensitive and PI-PLC resistant varieties. Besides, quantitatively the number of molecules of different GPI-proteins distributed on the surface of sperm membranes is not the same. It is quite possible that there could be other GPI-proteins released by PI-PLC from sperm membranes but their level of expression is perhaps not high enough to be clearly detected by the technique of SDS-PAGE that was used in the present study.

**5. 5 The 57kDa GPI protein is not acquired by sperm post-testicularly:**

The origin of GPI proteins of sperm has been a subject of great interest. It was originally proposed that GPI proteins are synthesized by the germ cells and introduced into sperm before gamete differentiation. It was later realized that not all proteins on sperm are acquired this way. Since sperm lack the machinery for protein biosynthesis and new proteins made their appearance on the surface of sperm at different times during their journey to meet with the egg, it was suspected that sperm acquires GPI proteins post-testicularly. Several GPI anchored proteins were found to be common between sperm and cells of the immune system. Detailed investigations revealed that proteins synthesized in other cells could be transferred on to sperm through several strategies from the

male and female reproductive tracts (Kirchhoff and Hale 1996; Pereira *et al.*, 2003). These include: incorporation through phospholipid transfer proteins (Wirtz, 1997), transfer through prostasomes (Arienti *et al.*, 1997a; b; c) and cholesterol-dependent insertion (Morandat, *et al.*, 2002). In the present studies, western blot analysis shows that the 57kDa GPI-protein was detected on the surface of sperm collected from all three regions of the epididymis: the caput, corpus and cauda. This is a clear indication that the GPI-protein was present in sperm even before it entered into the epididymis. Thus, it seems likely that sperm in the testis acquired this protein during spermatogenesis and not in the epididymis.

In the western blot analysis, in addition to the 57kDa protein, a 54kDa protein was also detected in the caput, corpus and cauda sperm. It is unlikely that this 54kDa protein is GPI-anchored protein since this was not released by either the PI-PLC treatment or chemical cleavage by nitrous acid. It may be pointed out that the first antibody used in the western blots was the antiserum raised against the protein fraction of sperm that partitioned into the detergent phase of triton X-114. This contained both transmembranal and GPI-proteins. This is perhaps the reason why the 54kDa protein was detected.

**5. 6 PI-PLC sensitive GPI-anchored proteins are predominantly localized to the sperm head:**

Immunofluorescence is a common technique used for the localization of desired molecules on the surface of sperm cells. Most of the investigators who have used immunofluorescence have either used monoclonal antibodies or polyclonal antibodies raised to purified specific antigens to look for the position of specific sperm proteins they were interested in (Tulsiani *et al.*, 1995; Yudin *et al.*, 2002). In this study, the technique of immunofluorescence is judiciously combined with PI-PLC treatment to show the localization of PI-PLC sensitive GPI proteins on the surface of goat sperm. When the intact sperm were probed with the polyclonal antibodies raised against the hydrophobic proteins fraction of sperm solubilized by triton X-114, the entire sperm exhibited fluorescence. On the other hand, when sperm were treated with PI-PLC and then probed with the same polyclonal antibody, the fluorescence was restricted to the mid-piece and tail of sperm. It must be pointed out that the fraction of proteins solubilized by TX-114, which was used as immunogen to raise polyclonal antibodies contained both transmembranal and GPI-anchored proteins. It is therefore not surprising that the entire sperm surface of intact sperm (control) showed fluorescence. However, the specific removal of the PI-PLC sensitive GPI-proteins from sperm resulted in loss of fluorescence in the head region of sperm. This implies that the PI-PLC sensitive GPI-proteins were localized predominantly to the head region of goat sperm.

### 5.7 Functional role of PI-PLC sensitive GPI anchored sperm proteins:

GPI-anchored proteins are now recognized to be useful sperm surface molecules involved in a variety of functions. As the number of GPI-proteins identified on the sperm surface is increasing, so is the curiosity in deciphering their functions. One approach to study the specific function of this class of proteins would be to strip sperm of these proteins and then examine changes in their behavior. Both chemical and enzymatic procedures are available for releasing the sperm surface GPI proteins from their anchors (Hooper, 2001). The enzymatic procedure (PI-PLC treatment) was preferred in the present set of experiments because the reaction conditions are relatively mild and the damage to the sperm structure is minimal on account of the specificity of the reaction they catalyze. To date several functions have been proposed for GPI sperm proteins. These include: adhesion properties, catalytic activity, receptor for ligands, dimerization of proteins and signal transduction (Hooper, 2001; Ikezawa, 2002). There is need to explore and identify the other novel function of this class of protein.

Vast amounts of GPI-anchored proteins exist in the cell surface of protozoa as coat proteins and protective antigens (Ikezawa, 2002). It is therefore of interest to know if GPI proteins of sperm play a protective role. A model system was therefore set up to test this hypothesis. A co-culture model has been used in the past to study the phagocytosis of residual bodies / cytoplasts from elongated spermatids by Sertoli cells (Pineau *et al.,* 1991). This procedure was

not pursued because sperm from cauda was used and their interaction with Sertoli cells is not normally seen in real life situations. The next choice was the use of epithelial cells of the reproductive tract. Spermiophagy by epithelial cells lining both the male and female reproductive tracts has been a subject of much controversy. The participation of epithelial cells lining the epididymis, vas deferens and vaginal epithelium in sperm clearance has been suggested in several species by a number of authors (Roussel *et al.*, 1967; Hoffer *et al.*, 1975; Phillips and Mahler 1977; Goyal 1982; Murakami *et al.*, 1984; Aire 2000).

Considering the enormous number of sperm produced and the small percentage of epithelial cells 'caught in action' by histological and ultrastructural investigations, it has been argued that at least the epididymal epithelial cells may not play a major role in sperm quality control by phagocytosis of defective sperm (Cooper *et al.*, 2002). Therefore epithelial cells were not employed in the present investigations.

The prevalence of phagocytosis of sperm by macrophages in the reproductive tracts has been shown since a long time (Murakami *et al.*, 1978; Ball *et al.*, 1984; Nechala and Hrudka 1984). There are even reports that macrophages play a role in mediating infertility by interacting with and destroying sperm (Muscato *et al.*, 1982; Haney *et al.*, 1983; London *et al.*, 1985; Jha *et al.*, 1996). Macrophages with phagocytic activity are also reported from human ejaculates (Blanco *et al.*, 1992). Co-incubation of sperm and peritoneal macrophages have been used to study phagocytosis *in vitro* (Toshimori *et al.*,

1991; Jha *et al.,* 1996). For evaluating sperm-macrophage interactions, sperm heads/ other parts phagocytosized in macrophages are counted after a given exposure time under standard conditions of incubation to derive at a spermiophagic index (Nechala and Hrudka 1984). The present study has therefore employed the well-accepted procedure of co-incubation of sperm and peritoneal macrophages as a model *in vitro* to assess if GPI-anchored proteins participate in the sperm disposal process.

How certain sperm are identified for elimination from the reproductive tracts is far from clear. Little information is available concerning the actual signals involved. It is well known that macrophages can recognize the Fc component of antibody molecules. Thus, in situations where auto-sperm antibodies are formed, macrophages are known to engulf sperm (London *et al.,* 1985). Macrophage-sperm interaction is also reported to be overblown in patients with accessory gland infections or subjects who have antibodies in their semen (Blanco *et al.,* 1992). But under normal circumstances there must be other mechanisms in operation for disposing sperm in the reproductive tracts. An ubiquitin-dependent mechanism for the recognition and elimination of defective spermatozoa has been recently proposed (Sutovsky *et al.,* 2001) and rebutted (Cooper *et al.,* 2002).

In the present study, the removal of GPI-anchored proteins from the sperm surface by PI-PLC treatment increased the phagocytosis of sperm by macrophages *in vitro*. From this it is obvious that the released protein but not the

anchor is involved in protecting the sperm. SDS-PAGE analysis confirmed that PI-PLC treatment of sperm released several proteins (75kDa, 57kDa, 42kDa, 27kDa and 23kDa). It is difficult to suggest which of these proteins are directly involved. Besides, the carbohydrate moieties are known to mediate signaling and communication among living cells. In particular, the sialic acid residues found at the ends of the oligosaccharide chains of many glycoproteins carry a message that determines the fate of these biomolecules. Ceruloplasmin is one such soluble sialoglycoprotein and desialylation leads to its removal from circulation. Recently, ceruloplasmin has been found to be GPI- anchored in Sertoli cells (Fortna *et al.*, 1999). gp20 Sialoglycoprotein is yet another GPI-anchored protein identified on human sperm (Focarelli *et al.*, 1999). An increase in phagocytosis by macrophages have been reported in sperm treated with the enzyme, sialidase and this action has been attributed to the removal of sialic acid from the surface (Toshimori *et al.*, 1991). Considering the above facts, it is expected that the presence or absence of sialilated GPI anchored proteins on the sperm surface may be one of the deciding factors for phagocytosis by macrophages. However, when the sialic acid of untreated and PI-PLC treated goat sperm was measured by the thiobarbuturic assay and compared, no significant changes could be observed. This implies that either the PI-PLC sensitive GPI proteins were not sialilated or the assay was not sensitive enough to detect the same.

It is not unusual for encounters between macrophages and spermatozoa even under normal physiological conditions. It may be pointed out that in the mammalian species, millions of sperm are produced but only a few are destined to reach and fertilize the egg. Sperm are competent cells that possess several functional attributes required for survival in the genital tracts until they are able to fertilize the egg. Most of the sperm perish in the reproductive tract en route to fertilizing the egg. Therefore it is logical to expect that an effective scavenging mechanism exists to get rid of not only defective and abnormal sperm but also sperm that are unable to meet the egg on time. From the experiments conducted in this study it is proposed that the absence of GPI linked proteins from their surface may be one among several signals that mark these less fortunate sperm for destruction. Under these circumstances, in may be hypothesized that one or more of these PI-PLC sensitive GPI-proteins could be a '**sperm protecting factor**' since the removal of these proteins by PI-PLC treatment enhances the susceptibility of sperm to macrophages. This mechanism may actually protect the sperm from macrophages and increase their chance of survival in the genital tracts. Further detailed investigations would be needed to pin point the GPI-protein involved.

# Chapter - 6

# Summary

## 6.0 SUMMARY:

Sperm are specialized motile male gametes, modified structurally and adapted physiologically to safely deliver their DNA to the egg with the sole intension of the propagation of species. Sperm achieve this restricted and precise function referred to as 'fertilization' through a whole sequence of events. The genesis of the sperm starts in the testis and they are made functionally fit by a process of maturation, which occurs in the epididymis. They are then stored in the distal portion (cauda) of this organ until they are ejaculated. In mammals, millions of sperm are produced which when released have to propel themselves through the female genital tract, survive for hours, recognize and bind with the egg and compete among themselves. Finally, at the end of this ordeal, only one sperm succeeds in penetrating the zona pellucida to complete the process of fertilization. The key factors that contribute to the specific nature of sperm to survive against all odds and yet fertilize the egg depend to a large extent on the molecules that adorn its surface.

The plasma membrane that envelops the sperm like most other cell membranes is composed of phospholipids and cholesterol, which not only provides a rigid, stable structure but also offers protection from the external environment. The original fluid mosaic model depicts that protein molecules are held by non-covalent interactions with the lipid bilayer, but in the last three decades, evidence point to a number of other possibilities including covalent interactions between protein and the lipid bilayer. Proteins are now known to be

functional molecules arranged in varied fashion on either side of plasma membrane, some embedded (integral) and some others loosely held (peripheral). Sperm are stripped down cells and lack endoplasmic reticulum, a recognized site of protein synthesis in eukaryotic cells. Thus, as far as the proteins of sperm surface are concerned there is little doubt that they should be acquired during their early stage of development in the testis. But taking into consideration the enormous variety of protein molecules that would be required by sperm to effectively counter the hurdles in their journey to meet with the egg and the miniscule size of the sperm, it is only logical to expect that some other special mechanisms are operational. In fact removal, redistribution or modification of existing proteins, addition of new proteins are some of the means by which extensive remodeling of sperm surface is known to occur right from the time they are produced in the testis to the time they finally fertilize the egg.

The glycosylphosphatidylinositol (GPI)- anchored protein is one of the most stable and conserved classes of arrangements that span the outer leaflet of the lipid bi-layer on the surface of membranes. The GPI structure consists of a glycan bridge between phosphatidylinositol and phosphoethanolamine; the phosphoethanolamine is in amide linkage to the C- terminus of the protein. The glycan structure [Man (α1-2) Man (α1-6) Man (α1-4) GlcN-$NH_2$] is remarkably conserved throughout evolution although it is modified in many cells and types of organisms by the addition of side chains at specific locations. Through such an anchor it is possible to link a variety of proteins to the surface of membranes.

Besides, GPI anchored proteins are of special interest since their structural organization permits free lateral movement on the membrane surface and provides an opportunity for the sperm to economize on the number of protein molecules.

In this thesis, an attempt is made to systematically identify and characterize the GPI anchored class of proteins on goat sperm and to decipher their potential functional role. Goats (*Capra indica*) were used, as the animal model since they are routinely slaughtered for meat and the epididymal tissue from which sperm were used in these studies was freely accessible from the local abattoir.

**6.1 Successful isolation of sperm and its plasma membrane:**

The testis together with the epididymis was brought to the laboratory and perfused with phosphate buffered saline through the testicular artery. The epididymis free from blood was divided into three segments: caput (head), corpus (body) and cauda (tail). The sperm from each segment was isolated and used in the studies. Since GPI anchored proteins are membrane bound, attempts were made to prepare sperm plasma membrane through nitrogen cavitation and ultracentrifugation on sucrose gradients. The protocol was the same as that used by other investigators to derive sperm plasma membranes. Nevertheless, the isolated preparation was tested for its purity, by assaying the activity of marker enzymes. In the studies it was found that 5'-nucleotidase(plasma membrane

marker) activity was high while the acid phosphatase (lysosomal marker) activity was low. Again the DNA and RNA were almost undetectable. Transmission electron microscopy of the preparation revealed membranous vesicles with characteristics akin to sperm plasma membrane. Thus, it is concluded that the preparation was an enriched fraction of plasma membrane.

**6.2 GPI-proteins have a unique detergent solubilization pattern:**

It was necessary to include a known GPI anchored and a transmembranal (TM) protein in the investigations to serve as positive and negative controls. Therefore, microsomal membranes were prepared from the goat kidney and the activity of two enzymes alkaline phosphatase (positive) and adenosine triphosphatase (negative) were used to track the fate of GPI anchored and TM proteins, respectively. Membrane bound integral proteins are generally solubilized by the use of detergents. In addition, transmembranal (TM) and GPI anchored proteins are differently solubilized in triton X-100 (TX-100) and octylglycoside (OG). The data on the ratio of enzyme activity solubilized by OG to TX-100 confirmed that alkaline phosphatase was GPI-anchored while adenosine triphosphatase was transmembranal.

**6.3 Goat sperm has several PI-PLC sensitive GPI-proteins:**

In order to specifically identify the GPI-anchored proteins, intact sperm or sperm plasma membrane fraction was co-incubated with PI-PLC *(Bacillus*

*cereus)* in Eppendorf tubes. (In separate experiments the activity of the PI-PLC was checked using alkaline phosphatase and adenosine triphosphatase as positive and negative controls as detailed earlier). After 90 min at 37°C, the tubes were centrifuged and the protein profile in the pellet and supernatant was obtained by SDS-PAGE. Control samples without PI-PLC treatment were run in parallel. On the assumption that PI-PLC treatment specifically cleaves GPI proteins from their anchors and based on the SDS-PAGE data, it is inferred that several proteins (75kDa, 57kDa, 42kDa, 27kDa and 23kDa) are GPI anchored of which the 57kDa protein is the most prominent.

**6. 4 The 57kDa GPI-Protein originates in the testis:**

Triton X-114 is a kind of detergent that can solubilize a majority of membrane proteins including GPI-anchored at low temperature (4°C) and brings about phase partitioning of hydrophobic proteins (detergent phase) and hydrophilic proteins (aqueous phase) at higher temperature (37°C). These properties were exploited to solubilize membrane bound proteins from sperm plasma membranes and separate the hydrophilic from the hydrophobic proteins. Next, antibodies were raised against the hydrophobic proteins (TM and GPI-anchored) of sperm plasma membrane, in rabbits. The antibody titer was high as determined by ELISA. Western blotting using this antiserum confirmed once again that the 57kDa protein was GPI- anchored. Since, this protein has been

found on the surface of sperm collected from all segments of the epididymis, it is inferred that this protein has a testicular origin.

### 6.5 PI-PLC sensitive GPI anchored proteins are localized on the sperm head:

The immunoflourescence studies of sperm with the polyclonal antibodies generated against the hydrophobic proteins as described above illuminated the complete sperm including head, mid-piece and tail. When the PI-PLC treated sperm was probed with the same antibody, only the mid-piece and tail showed fluorescence but not the head region. From this it is deduced that the GPI anchored proteins released from the goat sperm by the action of PI-PLC was associated with the head region.

### 6.6 PI-PLC sensitive GPI-anchored protein protect sperm:

One of the major challenges that the sperm faces is to endure hostile environment. Protection from the phagocytic activity of macrophages which they encounter both within the male and female reproductive tract is very important for the survival of sperm. In a previous study, it was demonstrated that treatment with sialidases renders sperm susceptible to phagocytosis by macrophages. Since at least one sperm surface sialoglycoprotein has been identified to be GPI anchored it was thought interesting to explore if the PI-PLC treated sperm are also susceptible to phagocytosis by macrophages. An *in vitro*

phagocytosis assay in microtiter plates was performed by co-incubating PI-PLC treated sperm with macrophages followed by fixation and electron microscopy of the macrophages. The results showed that PI-PLC treated sperm were more susceptible to phagocytosis. With a view to find out if the sialic acid residues were involved in the marking of sperm for destruction, the sialic acid level was measured in PI-PLC treated sperm and compared with untreated sperm. No significant differences were observed. It is therefore suggested that one or more of PI-PLC sensitive GPI-anchored proteins could serve as a sperm protection factor against phagocytosis by macrophages.

Thus, in a nutshell several GPI-proteins have been identified on the surface of goat sperm and a novel function of sperm protection has been attributed to the PI-PLC sensitive variety of this protein that reside predominantly on the head region.

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