Comparison and characterisation of specific fertilisation proteins in human (*Homo sapiens*) and baboon (*Papio anubis*) spermatozoa

by

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I, RODI OMONDI OJOO HEREBY DECLARE THAT THE WORK CONTAINED IN THIS THESIS IS MY ORIGINAL WORK AND HAS NOT BEEN PRESENTED FOR A DEGREE IN ANY OTHER UNIVERSITY

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SUMMARY

Previous studies have shown presence of shared antigens on baboon and human spermatozoa and significant homology between some of the testicular and spermatozoal antigens in the baboon and man. The baboon therefore, has been considered a possible non-human primate model for the study of human male reproduction.

The present studies have investigated fertilisation proteins in the two species. These fertilisation proteins were localized on spermatozoa by immunofluorescence using a commercial monoclonal antibody (mAb) and monoclonal antibodies previously raised against specific antigens on the heads of rodent or human spermatozoa. Among the monoclonal antibodies used were mAb M1 (Noor and Moore, 1999), mAb 18.6, (Moore *et al.*, 1990), mAb IAM-1 and mAb ES-1 (Al Eisa *et al.*, 2001) and mAb 4G10 that was a commercial antibody. Biochemical characterisation was done using the same monoclonal antibodies and involved separation and identification of sperm proteins using one-dimensional gels and immunoblotting. The study then focused on characterisation of one of the fertilisation proteins, inner acrosomal membrane –1 (IAM-1). This entailed the use of two-dimensional gel electrophoresis, immunoblotting, end terminal sequencing, peptide mass spectrometry and partial amino acid sequencing to characterise the protein.

This study identified shared determinants on baboon and human spermatozoa using mAbs 18.6, 4G10, IAM-1 and ES-1. These were

immunolocalised to similar domains on spermatozoa in the two species. One dimensional immunoblots corroborated the immunolocalisation studies demonstrating similarity of determinants recognised by the same mAbs in the two species. Some determinants like M1 were found in rodents but not in the two primates. ES-1 was localized by immunofluorescence to the equatorial segment and tail in both human and baboon spermatozoa. One-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis of human sperm protein extracts and immunoblotting using mAb ES-1, revealed immunoreactive bands of apparent molecular weights, 24, 28, 40, 47 and 50 kDa, while in baboon sperm extracts immunoreactive bands were recognised at 34, 38, 47, and 50 kDa.

Characterisation of IAM-1 using one dimensional immunoblots revealed it was a protein with four immunoreactive bands of apparent molecular weights 28, 32, 39 and 45 kDa in man while in the baboon, 3 immunoreactive bands of apparent molecular weights, 32, 38 and 44kDa were identified. Two-dimensional gel electrophoresis and immunoblotting revealed IAM-1 has native pI's of between 3.86 – 4.0. Immunofluorescence localized this antigen to the anterior acrosome and equatorial region of both human and baboon spermatozoa.

Treatment with different extraction solutions revealed that this protein was an integral membrane protein or a lipid-anchored protein intimately attached to the inner acrosomal membrane and equatorial segment of human spermatozoa. Mass spectrometry and amino acid sequencing isolated several peptides and the overall picture obtained suggested IAM-1 was end-terminally blocked, non-

glycosylated and a trypsin-like protease. Taken together, immunological and biochemical data obtained suggested that the protein IAM-1 could be a membrane anchored trypsin-like protease most likely involved in sperm-zona penetration

The ultimate goal of this study was identification of suitable gamete-specific proteins integrally involved in the fertilisation process, which can be used for immunocontraception. Further work remains to be done to confirm the suitability of the IAM-1 as a possible candidate protein for testing. This work provides additional evidence for use of the baboon as a model for study of human reproduction.

TABLE OF CONTENTS

Chapter 1

GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1. Gametogenesis

Gametogenesis or the formation of gametes occurs in the male and female gonads resulting in haploid gametes in the two sexes. In the male the process is referred to as spermatogenesis. This is a well orchestrated process of cellular proliferation, meiotic division and differentiation that occurs when sexual maturity is attained and results in the formation of spermatozoa. It involves a series of mitotic divisions by spermatogonial cells that result in the formation of other spermatogonia, a subset of which enter meiosis to become primary spermatocytes. The resultant primary spermatocytes undergo first meiotic division giving rise to secondary spermatocytes which then progress through second meiotic division to give rise to haploid round spermatids. The last mitotic and subsequent meiotic divisions during spermatogenesis are characterised by incomplete cytokinesis, a feature that ensures that the resultant daughter cells remain connected via cytoplasmic bridges and develop synchronously until spermiation. Each round spermatid undergoes a differentiation process termed spermiogenesis during which it is transformed into a polarised spermatozoon with a distinct head and tail. The spermatozoon is structurally and functionally adapted to deliver the genetic material in its nucleus to the female egg (Eddy and O'Brien, 1994; de Krester and Kerr, 1994; Johnson and Everitt, 1995).

In the female gonad, the oogonia undergo oogenesis. In most mammals during foetal life there is an initial phase of oogonial mitosis followed by a period when all the oogonia enter first meiotic division to become primary oocytes. These oocytes then develop through the stages of meiotic prophase I to diplotene (or dictyate) stage before birth where they remain arrested until the period preceding ovulation at sexual maturity. At this time, the oocyte resumes first meiotic division and undergoes an asymmetrical cell division. In this process most cytoplasm, half of the chromosomes and practically all the organelles are retained in the oocyte while the rest is extruded in the smaller polar body. In most mammals meiotic division in the ovulated oocyte is then arrested again in metaphase II. Therefore at ovulation there is a metaphase II oocyte delimited by the oolemma, the first polar body in the perivitelline space, the zona pellucida which is an acellular matrix enclosing both the oocyte and first polar body, all invested by the outermost cumulus layer. The oocyte has the substrates and mRNAs needed for synthesis of bio-molecules required for initial embryonic development. Completion of meiosis marked by the extrusion of a second polar body occurs after the sperm fuses with the egg (Johnson and Everitt, 1995).

1.2. Gamete morphology

1.2.1. Sperm morphology

The sperm has a head and tail or flagellum, enveloped by the plasma membrane. The head is attached to the tail at the neck. The head comprises an

acrosome, the nucleus, cytoskeletal components and the cytoplasm. Most mammalian sperm have spatulate shaped heads that are flattened in the anterior-posterior axis. However, in most rodents sperm have a falciform-shaped head with the acrosome overhanging the convex margin of the nucleus.

The tail is composed of a connecting piece, middle piece, principal piece and the end or terminal piece. There are species differences in the shapes and sizes of the heads of mammalian spermatozoa and the lengths and relative sizes of the flagella components. (Fouquet and Kann, 1994; Eddy and O'Brien, 1994; Breed, 1997)

1.2.1.1. ***Plasma membrane structure***

This is the external envelope of both the head and tail components of sperm. The sperm plasma membrane has been subdivided into several domains (Fig.1) based on the evidence that different regions of the sperm surface have different organization and structure of membranes (Primakoff and Myles, 1983; Holt, 1984). The sperm head plasma membrane is subdivided into, acrosomal (anterior head) and post-acrosomal (posterior head) regions (Fig.1). The acrosomal region is further subdivided from the anterior aspect caudally into, marginal segment (or peripheral rim, anterior band, apical segment) domain found on the anterior acrosomal margin, the principal segment (acrosomal segment) domain that covers the major part of the acrosome and the equatorial segment (posterior acrosome) domain that forms the posterior part of the acrosome (Fig.1). The marginal together with the principal segment domains are often also termed the anterior acrosome or acrosomal cap (Eddy and O'Brien, 1994). The post-acrosomal region (posterior head, post-

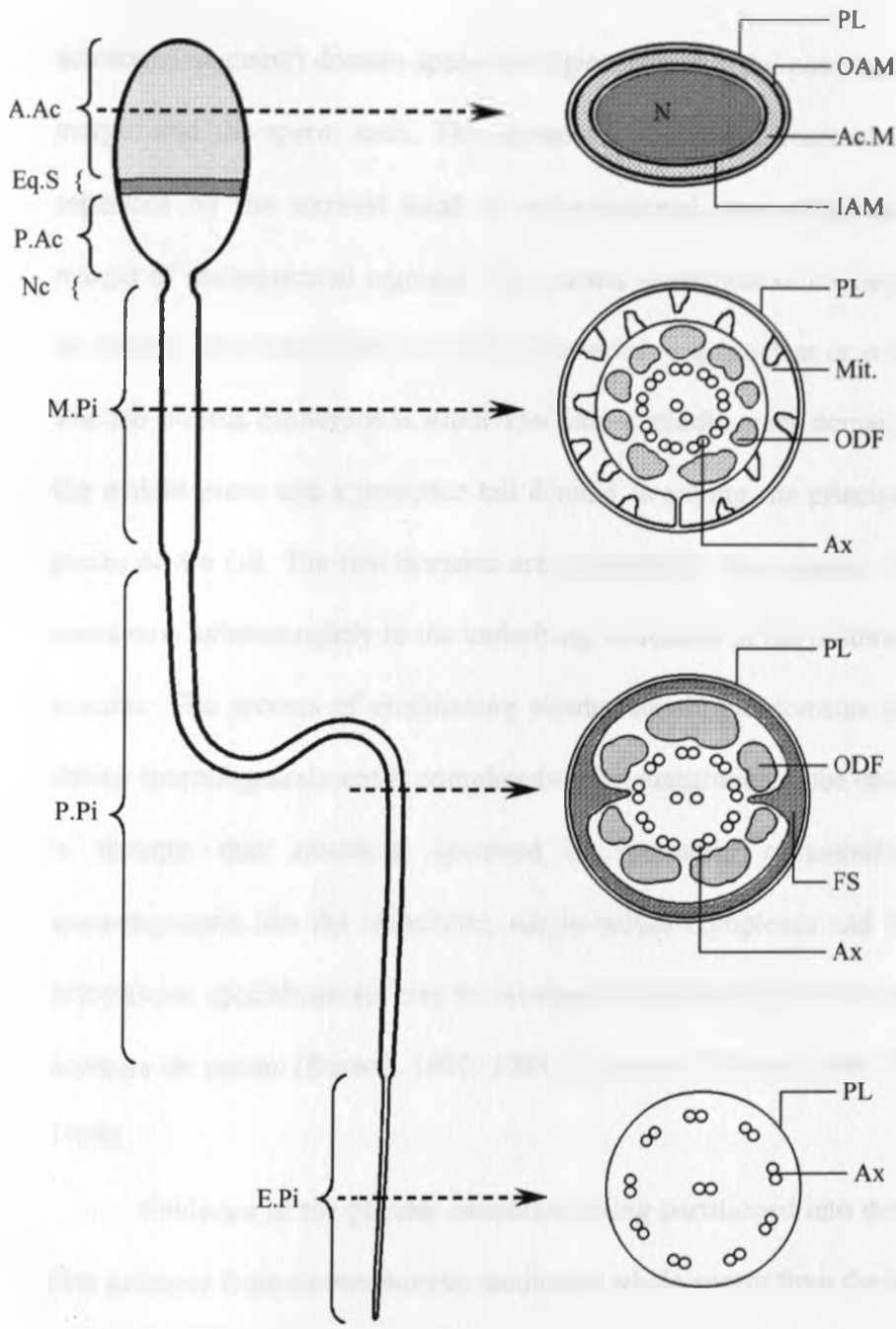


Fig.1. Structure of the human spermatozoon showing the different domains on the head and tail. The dotted-bold lines with arrows indicate transverse sections made at that level.
A.Ac: anterior acrosome, Eq.S: equatorial segment, P.Ac: post acrosomal region, M.Pi:middle piece, P.Pi: principal piece, E.Pi: end piece.
PL: plasma membrane, Ax: microtubules of axoneme, FS: Fibrous sheath, ODF: Outer dense fiber, Mit: mitochondria, OAM: Outer acrosomal membrane.
Ac.M:acrosomal matrix, IAM: inner acrosomal membrane, N:nucleus, Nc:Neck
(After Fawcett, 1975; Eddy and O'Brien, 1994)

acrosomal segment) domain spans the region between the posterior acrosomal margin and the sperm neck. The acrosomal and post-acrosomal regions are separated by the serrated band or sub-acrosomal ring along the posterior margin of the equatorial segment. The plasma membrane of the head region is de-limited from that of the tail by the posterior ring (nuclear or striated ring). The tail plasma membrane is subdivided into a middle piece domain overlying the middle piece and a posterior tail domain overlying the principal and end pieces of the tail. The two domains are separated by the annulus. The plasma membrane adheres tightly to the underlying structures at the posterior ring and annulus. The process of establishing plasma membrane domains commences during spermiogenesis and is completed during maturation in the epididymis. It is thought that structures involved in spermatid re-modelling during spermiogenesis like the manchette, tubulo-bulbar complexes and Sertoli cell ectoplasmic specializations may be involved in formation of plasma membrane domains on sperm. (Russell, 1980; 1984; Eddy and O'Brien, 1994; Toshimori, 1998).

Evidence of the plasma membrane being partitioned into domains was first gathered from electrophoretic studies on whole sperm from the rabbit, bull and ram. In these experiments sperm migrated towards the anode tail-first (Bangham, 1961; Nevo *et al.*, 1961). These results suggested presence of a negative charge on the sperm surface that was more concentrated on the sperm tails. This has been confirmed using positively charged colloidal iron hydroxide that bound more to the flagellum of ram and stallion sperm (Yanagimachi *et al.*, 1972; Lopez *et al.*, 1987).

Studies using different lectins as probes for polysaccharide chains had variable results with regard to their distribution on the sperm surface. Although there were more binding sites on the heads, there was more tail to tail aggregation observed as a result of the overall greater surface area of the tails (Koehler, 1981; Lee and Ahuja, 1987). Freeze-fracture, freeze-etch and surface replica studies have shown differences in the plasma membrane of different regions corresponding to separate domains. The intra-membranous particles visualized using these techniques vary in number, size and distribution in different plasma membrane domains (Friend and Fawcett, 1974).

Within species there are altered patterns, sizes and distribution of intra-membranous particles during maturation, capacitation and acrosome reaction (Eddy and O'Brien, 1994). Regional specializations in the plasma membrane of the sperm head are seen at the equatorial and marginal segments of the acrosome, post-acrosomal region and posterior ring. The plasma membrane specializations at the posterior ring, especially the tight apposition with the nuclear envelope, may effectively separate the sperm into two compartments with differences in ionic balance and metabolism (Eddy and O'Brien, 1994).

Along the tail there are modifications seen in the plasma membrane of the middle piece in association with the mitochondria. The plasma membrane adheres tightly to the annulus, possibly separating the middle piece as a distinct domain from the caudal parts of the tail (Friend and Fawcett, 1974). Other studies using filipin to demonstrate sterol presence and distribution, and polymixin to demonstrate presence of anionic lipids, confirm regional

differences of surface components (Bearer and Friend, 1980; Lopez and de Souza, 1991).

Antibodies have been used to localise the surface antigens on sperm and to define their roles using specific bioassays. Antisera against germ cells or spermatozoa components have been used but tend to lack specificity. To overcome this, monoclonal antibodies against specific epitopes of surface antigens have been raised to further enhance the study of specific sperm surface antigens. These have been useful in localisation and characterisation of several sperm surface antigens in different mammals (see Eddy and O'Brien, 1994 for review). However, they also have limitations as they may recognise similar epitopes shared by different proteins (Villaroya and Scholler, 1986).

The sperm plasma membrane proteins are modified along the epididymis by: addition of new components, unmasking or modification of pre-existing sperm moieties or loss of sperm-surface components. Most modifications could involve alterations in the glycoproteins and the glycolipids and this takes place in specific regions of the epididymis (Eddy *et al.*, 1985).

1.2.1.2. ***Sperm nucleus***

This contains the haploid paternal set of chromosomes in the form of highly condensed chromatin associated with protamines. The latter are low molecular weight proteins that are very basic and rich in arginine and cysteine. They are synthesized in the spermatids and due to abundant cysteine content, form covalent disulphide bonds stabilizing the DNA by cross-linkages. Other nucleoproteins could be involved in the stabilization of chromatin too (Warrent and Kim, 1978).

1.2.1.2.1. *Nuclear envelope*

This encloses the sperm nucleus. Unlike most somatic cell nuclear membranes that have 40-60 nm separation of the membrane bilayer, the sperm nuclear envelope has a 7-10 nm separation only. Most of the envelope lacks nuclear pores except for the area caudal to the posterior ring or redundant nuclear envelope where there are abundant hexagonally arranged pores (Fawcett, 1975). The inner surface of the nuclear membrane has a protein meshwork lining called the nuclear lamina that forms the nuclear skeletal framework anchoring the chromatin. It is generally made up of four related proteins namely, lamin A, B_1, B_2 and C in mammals (Mckeon *et al.*, 1986; Eddy and O'Brien, 1994).

1.2.1.2.2. *Sperm head cytoskeleton*

This comprises sub-acrosomal, post-acrosomal and para-acrosomal components. The sub-acrosomal component lies between the inner acrosomal membrane and the nuclear envelope and is equivalent to the perforatorium of rodent sperm, while the post-acrosomal cytoskeleton is delimited by the nuclear envelope, the post-acrosomal plasma membrane and the posterior ring. The two parts form the perinuclear theca (Bellvé *et al.*, 1992). The third component, the para-acrosomal cytoskeleton lies between the acrosome and the plasma membrane. It has been described in the hamster but could be present in other species too (Olson and Winfrey, 1985; Eddy and O'Brien, 1994). The sperm cytoskeleton has proteins ranging from 8 – 80 kDa. Though some of these proteins could be structural in function others are now known to be cytosolic proteins, basic proteins and nuclear-like histones (Oko *et al.*, 2001).

Some of these proteins could be involved in spermatid differentiation events like, nuclear and acrosomal shaping, acrosomal-nuclear attachment and later on, sperm- egg interaction (Eddy and O' Brien 1994; Oko *et al*, 2001).

1.2.1.2.3. *Acrosome*

This overlies the anterior aspect of the sperm nucleus and is therefore indented on the caudal aspect where it is attached to the nucleus. The acrosome comprises an inner acrosomal membrane that immediately invests the anterior portion of the outer layer of the nuclear envelope, an outer acrosomal membrane which is continuous with the inner acrosomal membrane at the equatorial region and the acrosomal contents enclosed by the two membranes (Fig.1). The outer acrosomal membrane is in turn invested by the plasma membrane. The acrosome is subdivided into an acrosomal cap or anterior acrosome and the posterior acrosome or equatorial segment. These regions are further subdivided into segments corresponding to those found on the plasma membrane namely the apical, principal and equatorial segments of the acrosome. Generally, the marginal segment is the acrosomal portion extending beyond the nucleus, the principal segment overlies the nucleus and the equatorial segment overlies the equator of spatulate sperm or lateral surfaces of falciform-shaped sperm. There are a few exceptions to these general divisions as seen in some species where the sperm head is neither spatulate nor falciform shaped, like the woolly opossum. In the latter species, there is no identifiable equatorial segment (Phillips, 1970). There is species variation in size, shape and relative proportions of the acrosomal cap to the equatorial segment.

Acrosomal contents have ordered arrangements seen in different

species varying from lamellar to crystalline to cobblestone-like (Flechon, 1974; Friend and Fawcett, 1974). The acrosomal membrane has an ordered arrangement of intra-membranous particles peculiar to different segments. The outer acrosomal membrane is composed of 12 - 290 kDa proteins including glycoproteins, calmodulin binding proteins and phosphorylated proteins (Olson *et al.*, 1985). The inner acrosomal membrane is connected to the outer membrane by bridges in boar spermatozoa (Russell *et al.*, 1979). Acrosomal contents include proteases such as proacrosin, acrosinin, acrosin inhibitors, β-galactosidase and hyaluronidase among other proteins. It is notable that acrosomal hyaluronidase and proacrosin are spermatogenic cell-specific isozymes. Using monoclonal and polyclonal antibodies, many antigens of unknown function have been localised in the acrosome (Goldberg, 1977; Eddy and O'Brien, 1994).

1.2.1.3. ***Sperm tail***

The components of the tail or flagellum include the neck (connecting piece), middle piece, principal and end piece (Fig.1). The base of the tail is attached to the nucleus by the connecting piece. The tail contains a centrally located axoneme (Fig.1). The axoneme extends from the connecting piece to the end piece of the tail. It is composed of a central pair of microtubules surrounded by nine peripheral doublets (Fawcett, 1981). Each doublet has a complete microtubule A, and a C-shaped microtubule B. Each A microtubule has two dynein arms extending to the next B microtubule of the adjacent doublet in a clockwise manner, viewed from the tail base to the end piece. There are spokes that radiate from the central microtubules to the outer

peripheral pairs. The peripheral doublets are named 1 to 9 from the microtubule pair on the plane bisecting the central pair. The axoneme is encircled by nine outer dense fibres which have a wrapping of helically arranged mitochondria in the middle piece while in the principal piece the axoneme is surrounded by the outer dense fibres that are then covered by the fibrous sheath (Fig.1). The end piece lacks any peri-axonemal components. The outer dense fibres and the fibrous sheath form the cytoskeleton of the sperm tail (Fawcett, 1975; Eddy and O'Brien, 1994; Fouquet and Kann, 1994).

1.2.1.3.1. *Connecting piece*

This is formed by the capitulum or the neck portion that fits into the implantation fossa on the sperm head and the segmental columns. The outer part of the nuclear envelope is adherent to the basal plate at the implantation fossa. The capitulum fits caudal to the basal plate. From the capitulum, two major and five minor segmental columns arise. The major columns split into two each and together with the five minor columns are continuous with the nine outer dense fibres. The distal centriole gives rise to the axoneme of the sperm tail. The two centrioles may regress during spermiogenesis, or the proximal one may be retained in some species. The connecting piece terminates at the beginning of the middle piece (Fawcett and Phillips, 1969; Fawcett, 1975).

1.2.1.3.2. *Middle piece*

This is the part of the tail between the connecting piece and the beginning of the principal piece at the annulus. It has the axoneme centrally ringed by nine outer dense fibres giving the 9+9+2 arrangement found in

mammalian sperm (Fig.1). Outer dense fibres differ between species in size and shape but also among themselves. They are named according to the corresponding microtubule doublet. Generally numbers 1, 5, 6 and 9 are bigger than the rest. They taper from the middle piece to the distal principal piece. The smaller fibres terminate before the larger ones in the principal piece (Telkka *et al.*, 1961; Eddy and O'Brien, 1994). Outer dense fibers are thought to be involved in providing elastic recoil during flagellar motion, due to abundant disulfide linkages in their protein composition. The mitochondrial sheath surrounds the outer dense fibres of the middle piece. These are helically wrapped end to end along it and invested externally by plasma membrane. There are species differences in numbers of mitochondria and lengths of the middle piece. The caudal limit of the mitochondria along the middle piece is marked by the annulus (Fawcett, 1975).

1.2.1.3.3. *Principal piece*

It is the longest segment of the flagellum. It is characterised by the fibrous sheath, formed by two longitudinal columns attached together by semi-circular ribs, in mammalian and some avian spermatozoa. The fibrous sheath surrounds the axoneme and the outer dense fibers except numbers 3 and 8. It is invested by the plasma membrane externally. The longitudinal columns run peripheral to microtubules 3 and 8 and are attached to the outer dense fibers 3 and 8 in the proximal part of the principal piece and microtubule doublets 3 and 8 distally. The longitudinal columns are in the dorso-ventral axis of the sperm tail defined by the plane perpendicular to the central microtubule doublets of the axoneme. The ribs attach to the longitudinal columns and

branch to attach to each other. The fibrous sheath is thought to modulate flagellar movement (Fawcett, 1975; Eddy and O'Brien, 1994).

1.2.1.3.4. *End piece*

This forms the terminal part of the flagellum. The beginning of the end piece is marked by the termination of the fibrous sheath of the principal piece. It is formed by the axoneme and the overlying plasma membrane. It lacks peri-axonemal structures. The microtubules of the axoneme terminate at different levels caudally within the end piece (Fawcett, 1975; Fouquet and Kann, 1994).

1.2.2. Oocyte/Egg morphology

At ovulation in most mammals, the oocyte/egg comprises ooplasm containing the maternal chromosomes arrested in metaphase II enveloped in an oolemma. Surrounding the oolemma is the perivitelline space where the 1st polar body is found. Enclosing the perivitelline space is the zona pellucida which is invested by the cumulus layer. The egg plasmalemma (oolemma) has microvilli along its surface except for the area overlying the mitotic spindle (Phillips and Shalgi, 1980). Underlying the area with microvilli, are the cortical secretory granules (Wassarman and Albertini, 1994).

1.2.2.1. *Cumulus Layer*

This comprises cells that are remnants of the follicular granulosa cells in an extracellular matrix secreted by these cells, rich in hyaluronic acid (Camaioni *et al.*, 1996). This layer is shed off in some species like the bovine and ovine, prior to mammalian gamete interaction. It may also be modified during oviductal transport by interaction with the oviductal epithelium.

Cumulus cells may have paracrine influence on the fallopian tubes by secreting steroids, peptides and cytokines (Yanagimachi, 1994; Hunter, 2002; Talbot *et al.*, 2003).

1.2.2.2. *The Zona Pellucida (ZP)*

This is the egg extracellular matrix that envelopes the ovum together with the perivitelline space around it, in all mammalian oocytes. It is thought to be secreted solely by the developing oocyte in the course of oogenesis (Yanagimachi, 1994; Eberspaecher *et al*, 2001) or both the oocyte and the surrounding granulosa cells of the corona radiata (Lee and Dunbar, 1993). It varies in thickness from, less than 2μm in some marsupials to 27μms in the bovine (Dunbar and Wolgemuth, 1984). It is composed of 3 sulphated glycoproteins ZP1, ZP2 and ZP3 in most species, (Wassarman, 1988; Harris *et al.*, 1994). This nomenclature was based on the migration of these glycoproteins on SDS gels. There is another nomenclature based on length of the coding region of genes for zona glycoproteins, ZPA, ZPB and ZPC with ZP1 corresponding to ZPB, ZP2 to ZPA and ZP3 to ZPC of mice and humans (Harris *et al.*, 1994). Homologues of ZP proteins have been isolated in lower vertebrates like fish (Lyons *et al.*, 1993; Chang *et al.*, 1996). The zona pellucida functions in mediation of species specificity in gamete interaction (O'Rand, 1988), blocking polyspermy (Barros and Yanagimachi, 1972) and embryo protection prior to implantation (Mcleskey *et al.*, 1998). Studies using knock-out mice without ZP have indicated it is essential for fertilisation (Rankin *et al.*, 1996).

1.3. Overview of mammalian fertilisation

Mammalian fertilisation involves several sequential steps including, deposition of sperm in the female tract, transport or motility during which there is capacitation, cumulus penetration, sperm-egg recognition and primary ZP binding, acrosome reaction, secondary binding and sperm penetration of the ZP, sperm-oolemmal binding then fusion and egg activation (Yanagimachi, 1994).

1.3.1. Capacitational Changes on Sperm

Following spermiation in the testis, there is progressive alteration in the biochemical composition of the sperm plasma membrane along the epididymis. This involves changes of the lipid composition and membrane protein components of the sperm plasma membrane. The membrane proteins may be integral or surface adsorbed proteins and may be replaced or modified as the sperm traverse the epididymis (Yanagimachi, 1994). In the course of sperm maturation in the epididymis, there is overall increase in the net surface negative charge and glycosylation of sperm membrane proteins, changes thought to be associated with stabilization of the plasma membrane preventing premature acrosomal reaction and hypermotility.

Ejaculated mammalian spermatozoa are initially unable to fertilize and require a period of conditioning in the female tract during which they attain functional competence for fertilisation as a result of physiological changes that occur on and within them. This process is known as capacitation

(Yanagimachi, 1994; Töpfer-Petersen *et al.*, 2000) and was first described independently by Austin (1951) and Chang (1951). Capacitation is required for acrosome reaction that is induced by physiological stimuli like zona glycoproteins (Florman and First, 1988). Furthermore, capacitation primes sperm receptors to respond to the oocyte vestments and initiates signal transduction pathways leading to acrosome reaction (Töpfer-Petersen, 2000). Capacitation is accompanied by membrane changes like, increased membrane fluidity, sperm surface antigen expression and modification, protein phosphorylation, membrane hyperpolarization and intracellular changes like Ca^{2+} concentration and pH (Storey, 1995; Brewis and Moore, 1997). The exact relationship between these modifications is not clear and neither is there synchronous capacitation of all spermatozoa in a given semen sample *in vitro* (Baldi *et al.*, 2000). It is also known that capacitation is modulated by molecules of seminal plasma origin such that spermatozoa undergo capacitational changes while at the same time there is inhibition of spontaneous acrosome reaction (Fraser *et al.*, 2003)

1.3.1.1. ***Sperm plasma membrane changes***

The sperm plasma membrane is directly exposed to the capacitating environment within the female reproductive tract and undergoes changes that alter the stability of the membrane, making it more sensitive to the egg and its vestments (Yanagimachi, 1994). Two groups of decapacitation proteins have been identified:

a) those of seminal plasma origin with low molecular weights, 5-23 kDa

b) and those of epididymal origin with high molecular weights, 129-259 kDa.

The decapacitation proteins are either adsorbed on the sperm surface during epididymal transit or from seminal plasma during ejaculation. They are either structurally modified or removed during capacitation of sperm within the female reproductive tract (Yanagimachi, 1994). Addition of decapacitation proteins found in seminal fluid is associated with the inhibition of capacitation in human sperm (Luconi *et al.*, 2000). Some of these proteins could act by activating a Ca^{2+} Atpase that maintains low intracellular Ca^{2+} concentration in epididymal sperm (Adeoya-Osiguwa and Fraser, 1996). Other antigens are masked or unmasked on the sperm plasma membrane and yet others redistributed on the plasma membrane, during capacitation (Yanagimachi, 1994).

Other changes during capacitation are in plasma membrane glycoproteins and peripheral membrane proteins. These may include removal of the terminal sialic acid in glycoproteins. The removal of sialoglycoconjugates from spermatozoa occurs during capacitation *in vivo* under the influence of sialic acid binding protein which is secreted by the uterus (Yanagimachi, 1994; De Jonge, 2005). Involvement of glycolipids like seminolipid of seminal vesicle origin has also been documented. Seminolipid is initially sulphated and localised over the anterior acrosomal surface, soon after ejaculation where it is believed to be involved in stabilizing the plasma membrane. With capacitation, it migrates to equatorial segment where it is in

de-sulphated form and could take part in the sperm-egg fusion later in fertilisation (Flesch and Gadella, 2000).

Freeze fracture studies of acrosomal membranes in the hamster, human and guinea pig sperm demonstrated capacitation associated alteration in distribution of integral membrane proteins, seen as intra-membranous particles (IMP). The IMP–free areas were found to have few or no sterols and anionic lipids. The guinea pig middle piece plasma membrane also showed alteration in IMP distribution following capacitation (Bearer and Friend, 1990).

Fluorescence photobleaching technique has been used on mouse sperm to demonstrate fluidity alterations of the plasma membrane. While the overall content of phospholipids in the plasma membrane may not change during capacitation, their distribution within the lipid bilayer is altered and there is also increased phospholipid methylation and synthesis of phosphatidylcholine from phosphatidylethanolamine (Yanagimachi, 1994; Baldi *et al.*, 2000).

Altered fluidity of the plasma membrane improves aggregation of sperm surface proteins, increasing avidity for zona ligands (O'Rand *et al.*, 1988). Cholesterol content of mammalian sperm in most species studied affects capacitation *in vitro* (Cross, 1998). It has been suggested that loss of cholesterol from sperm membranes during capacitation increases fusogenicity of the acrosomal and plasma membranes. However, experiments testing this hypothesis indicate that fusogenicity changes may not be directly attributable to efflux of cholesterol from the plasma membrane.

Loss of cholesterol from the sperm membranes is however, associated with overall increase in the intracellular pH and concomitant increased acrosomal responsiveness *in vitro* (Cross, 1998). Cholesterol efflux from the plasma membrane *in vitro* has also been found to be correlated with transmembrane signalling events involving cAMP and protein tyrosine kinases resulting in protein tyrosine phosphorylation during capacitation (Visconti *et al.*, 1999). It is noteworthy that species with low cholesterol content of the mature sperm plasma membrane, like boar and ram have shorter capacitation times (1-2 hours) while those with higher content like human and horse sperm have relatively longer times (6-8 hours) (Flesch and Gadella, 2000). Efflux of cholesterol *in vitro* has been mediated by albumin or other compounds like cyclodextrin with high affinity for cholesterol; however *in vivo* correlates of these compounds have not been clarified (Flesch and Gadella, 2000). These authors have also proposed involvement of intracellular lipid transport proteins in movement of sterols or lipids from plasma membrane to the acrosomal membranes, as an alternative hypothesis to that of cholesterol efflux.

1.3.1.2. ***Intracellular Changes of Sperm***

Among the changes that occur within sperm during capacitation is the increase in intracellular concentrations of Ca^{2+}. This has been observed in sperm of several mammals including human (Baldi *et al.*, 2000). Concentration of Ca^{2+} in sperm is regulated by ionic pumps in the plasma membrane, a Ca^{2+}-ATPase, a Ca^{2+}/H^{+} exchanger system and a Na^{+}/Ca^{2-} antiporter. Although these ionic pumps are found in sperm, how they regulate intracellular Ca^{2+} has not been elucidated. Ca^{2+} are believed to be sequestered intracellularly, though

their precise location is unclear as mature sperm lack an endoplasmic reticulum while the acrosome that was thought to be a possible store does not retain significant amounts of Ca^{2+} (Kirkman-Brown *et al.*, 2000; Baldi *et al.*, 2000). Recently, it has been suggested that prostasomes of seminal fluid origin could have a role in Ca^{2-} availability to spermatozoa during capacitation *in vivo* (Arienti *et al.*, 2004; De Jonge, 2005). The other internal changes during capacitation include increase in intracellular Na^{+} and decrease in zinc concentrations. Bicarbonate ions have been shown to regulate adenyl cyclase activity and cAMP metabolism in hamster sperm and are also required for protein tyrosine phosphorylation during capacitation. Furthermore, the involvement of bicarbonate ions in capacitation is protein kinase A dependent and alters fluidity of the sperm plasma membrane (Visconti *et al.*, 1990; Baldi *et al.*, 2000; De Vries *et al.*, 2003).

1.3.1.3. ***Changes in Sperm Motility***

Hyperactivated motility has been defined as the kind of swimming pattern exhibited by most sperm in the ampulla of the oviduct at fertilisation, while activated motility is that shown by cauda epididymal sperm when released into physiological medium, where the progress is in a straight trajectory (Yanagimachi, 1994). Hyperactivated motility on the other hand, is characterised by pronounced flagellar movements, marked lateral excursion of the sperm head and a non linear trajectory.

Hyperactivated movement of sperm was first reported by Yanagimachi, (1969) who observed that it was related to acquisition of fertilizing ability by spermatozoa, and also proposed its role in zona penetration. Other possible

benefits of this motility include, release of fertilizing sperm from the oviductal isthmus epithelial cells (Demott and Suarez, 1992), penetration of mucous found in the oviductal isthmus of various species (Jansen, 1980; Suarez, *et al*, 1992; 1997) and traversing the cumulus mass (Ho and Suarez, 2001).

Various factors including progesterone, follicular fluid and hormonal changes in blood supply have been proposed as triggers to hypermotility *in vivo* (Ho and Suarez, 2001). Calcium ions are required for axonemal function and together with other factors like bicarbonate ions and cAMP may be crucial for initiation and maintenance of hyperactivated motility (Lindemann *et al*, 1991; Suarez and Ho, 2003; Ho and Suarez, 2003). However, the precise details of their involvement are not yet elucidated.

1.3.2. Sperm – egg interaction

Sperm – egg interactions occur at various levels on the egg surface. Each level involves different receptors on the sperm and egg surface. The sperm has to pass consecutively through the egg vestments to penetrate into the egg. These are successively, the cumulus oophorus, the zona pellucida and the oolemma (Fig. 2).

1.3.2.1. *Sperm-Cumulus interaction*

Sperm-cumulus interaction is poorly understood despite this being the first egg vestment to be penetrated by the fertilizing spermatozoon in most mammals (Kirkman-Brown *et al.*, 2002). The cumulus layer comprises outer cumulus cells and inner corona radiata cells in a hyaluronic acid matrix. The corona cells send processes to each other and through the zona to the oocyte. These processes form gap junctions that are thought to be involved in cell-cell

communication particularly during oocyte growth. Passage through the cumulus possibly primes sperm for acrosome reaction (Tesarik *et al.*, 1988, Kirkman-Brown *et al.*, 2002). Sperm passage through this vestment is physical involving hyperactivated motility and possibly enzymatic activity due to sperm surface hyaluronidases like PH-20 (Hunnicutt *et al.*, 1996; Primakoff and Myles, 2002). The cumulus may also act as a selective barrier excluding uncapacitated and acrosome-reacted sperm (Saling, 1991). Another function attributed to this layer is selection of abnormal and some normal sperm that are then phagocytised by leukocytes found among the cumulus cells (Nottola *et al.*, 1998).

1.3.2.2. ***Sperm – zona interaction***

Mammalian sperm-zona interaction is thought to be species specific mediated by the glycan-recognising receptors on the sperm surface and their complementary ligands on the zona pellucida. This interaction occurs in two steps namely, primary sperm-zona binding and secondary sperm-zona binding. Briefly, it involves primary binding between acrosome-intact sperm and the ZP, an interaction that is mediated by ZP3 which is one of the zona glycoproteins of the unfertilised egg and specific ZP3 receptors on the sperm plasma membrane. The binding of sperm surface receptors to ZP3 triggers acrosomal exocytosis, an event that is followed by secondary sperm-ZP interaction between molecules on the acrosome-reacted sperm and another zona glycoprotein, ZP2 (Mcleskey *et al.*, 1998; Tulsiani and Abou-Haila, 2001; Wassarman, 2005).

1.3.2.2.1. *Zona Proteins*

ZP3

ZP3 is responsible for species-specific binding of spermatozoa to the zona pellucida i.e. sperm-egg recognition and primary binding resulting in adhesion of the sperm to the zona pellucida and induction of acrosome reaction, a pre-requisite for fertilisation (Bleil and Wassarman, 1980; Wassarman, 1990; Saling, 1991). Sperm binding to mouse ZP3 has been investigated and found to be determined by O-linked oligosaccharides specifically linked to serine residues 332 and 334 on the C-terminal half of the ZP3 molecule (Florman and Wassarman, 1985; Chen *et al.*, 1998; Rosiere and Wassarman, 1992). The ZP3 polypeptide backbone may either have a role in supporting and orienting the oligosaccharide chains for interaction with the sperm receptors or have a direct role in the actual sperm binding (Bagavant *et al*, 1993 a, b). Following primary binding, sperm undergo acrosomal reaction characterised by multiple point fusions between the outer acrosomal membranes and overlying plasma membrane, formation of hybrid vesicles and exposure of the acrosomal contents and inner acrosomal membrane (Yanagimachi, 1994; Brewis and Moore, 1997). Following fertilization, glycosidases of oocyte cortical granule origin cleave the oligosaccharides on ZP3 responsible for sperm-zona recognition (Dean, 2004).

ZP2

Following acrosome reaction, the inner acrosomal membrane is exposed to the ZP matrix. At this stage sperm binding to the egg is mediated by ZP2 which binds sperm molecules in the acrosomal matrix or inner acrosomal

membrane: it is responsible for maintenance of sperm binding to the egg, functioning thus as a secondary receptor (Bleil *et al.*, 1988). Recent work however, suggests ZP2 may be an important component of a three dimensional lattice formed by ZP glycoproteins, required for sperm binding to the ZP.

Following fertilization, ZP2 is cleaved by enzymes of cortical granule origin altering the structure of this lattice. This modification alters the binding affinity of the zona matrix to other spermatozoa. (Moller and Wassarman, 1989; Dean, 2004).

ZP1

In murine eggs it is thought to have a structural role, cross-linking ZP2-ZP3 heterodimers to form a three dimensional ZP matrix (Greve and Wassarman, 1985). Porcine and rabbit ZP1 however, show sperm binding properties similar to murine ZP3 (Yonezawa, *et al.*, 1995; Prasad *et al.*, 1996) In porcine, ZP1 binds to sperm membrane receptors via N-linked oligosaccharides (Yonezawa *et al.*, 1995). A combination of ZP1 and ZP3 is still required for optimal sperm binding (Yurewicz *et al.*, 1993).

1.3.2.2.2. *Primary Sperm-ZP interactions*

Among the important sperm proteins implicated in primary zona binding are β 1-4 galactosyltransferase, sp56, a receptor tyrosine kinase known as Zona Receptor Kinase (ZRK) and spermadhesins. While most of the other putative ZP3 receptors have been isolated in non-primate mammals, it is only ZRK that has been isolated in human sperm too.

β 1-4 Galactosyltransferase (Galtase)

β 1-4 galactosyltransferase was first reported as a primary zona binding candidate by Shur and Hall, (1982). It is found intracellularly as a glycosyltransferase in a number of cell types though it is a cell surface protein in others including mouse sperm (Evans *et al.*, 1995; Shaper *et al.*, 1990). The surface antigen acts as a lectin, binding N-acetylglucosamine sugar residues. Use of galactosylated ZP proteins indicated interaction of Galtase with ZP3 (Miller *et al.*, 1992a) and transgenic mice over-expressing surface Galtase on sperm bound more ZP3 compared to controls (Youakim *et al.*, 1994). Sperm from Galtase null mice are unable to undergo acrosome reaction in presence of mouse ZP3 or anti-Galtase antibodies, unlike normal mouse sperm, indicating involvement of this protein in acrosome reaction induction in this species (Lu and Shur, 1997; Talbot *et al.*, 2003).

Sp56

This is a 56 kDa peripheral membrane protein found on the dorsal surface of the mouse sperm head. It is also found on hamster sperm. It covalently associates with purified mouse ZP3 (Bleil and Wassarman, 1990) and ^{125}I labelled ZP3 glycopeptides (Cheng *et al.*, 1994). Purified Sp56 binds ZP of unfertilized mouse eggs but not fertilized ones and inhibits sperm-egg binding *in vitro* (Bookbinder *et al.*, 1995). Sp56 has been cloned and sequenced; the sequence obtained confirming it could be a peripheral membrane protein. It is however not found in guinea pig or human sperm (Bookbinder *et al*, 1995).

Receptor Tyrosine Kinases

Several sperm proteins that bind to zona glycoproteins and undergo autophosphorylation on exposure to zona pellucida glycoproteins have been identified (Naz and Ahmad, 1994). These proteins have molecular weights of 95 kDa, 63 kDa, 51 kDa and 14 - 18 kDa (Saling, 1991; Naz and Ahmad, 1994). The importance of tyrosine phosphorylation in sperm-zona interactions was demonstrated by experiments in which inhibition of protein tyrosine kinase prevented acrosomal exocytosis, effectively blocking fertilisation (Leyton *et al.*, 1992).

A 95kDa mouse sperm surface protein was identified as the major phosphotyrosine protein. It had tyrosine kinase activity, was implicated in ZP3 binding, and was involved in events leading to acrosomal exocytosis in sperm (Saling, 1991; Leyton *et al.*, 1992). This mouse 95 kDa protein designated Zona Receptor Kinase (ZRK) was also recognised on human sperm by a monoclonal antibody (mAb) 97.25 and shown to be involved in human sperm-zona interaction (Moore *et al.*, 1987). The gene coding for the human homologue of this protein was cloned and its sequence determined (Burks *et al.*, 1995). Recombinant human ZP3 induced phosphorylation of ZRK on human spermatozoa, a process that is linked to a signal transduction pathway which culminates in acrosomal exocytosis (Brewis *et al.*, 1995; Mcleskey *et al*, 1998). Furthermore, acrosomal exocytosis was inhibited by specific antibodies targeting ZRK (Moore *et al.*, 1995). The precise details of the signal transduction pathway are however not yet fully elucidated.

Spermadhesins

These are 12-16 kDa proteins some of which bind ZP glycoproteins. They include AWN-1, AQN-1 and AQN-3. The ones that bind to the ZP glycoproteins are similar in not being N-glycosylated at the 50th Asparagine amino acid, a feature that has been related to primary ZP binding (Calvete *et al.*, 1993 a, b; 1994). These antigens are localised on the acrosomal cap and have been identified on pig, equine and dog sperm (Töpfer-Petersen *et al.*, 1995).

Work done in different species indicates involvement of more than one specific sperm receptor in primary sperm-zona binding. It is possible that alternate pathways or receptors have evolved to ensure fertilisation in the absence of a particular one (Barber and Fayrer-Hosken, 2000) or the receptors act together or independently in the process to enhance chances of successful sperm-zona binding (Chapman and Barratt, 1996). Furthermore, it has been suggested that ZP3 has multivalent low and high affinity interactions with several sperm based receptors during primary sperm-zona interaction forming a fertilisation complex required for this phase of gamete interaction (Thaler and Cardullo, 1996).

1.3.2.2.3. *Secondary Sperm-ZP binding*

Secondary binding occurs following acrosome reaction and the resultant exposure of the acrosomal contents and inner acrosomal membrane. It has been suggested that acrosome reaction may facilitate secondary binding in three ways; by externalization of ligand proteins, by promoting protein migration across the fluid membrane to access the binding sites such as for PH-

20, or by alterations on pre-existing membrane proteins possibly by the acrosomal contents (Fénichel and Durand-Clément, 1998). Among the sperm molecules identified as possible receptors for ZP2 binding are PH-20, Proacrosin, Rabbit sperm autoantigens (RSAs) and Sp17.

PH-20

This was initially identified as a membrane protein, anchored by glycosyl phosphatidylinositol to the sperm plasma membrane of the post-acrosomal region in guinea pig sperm (Phelps *et al.*, 1988). It has since been localised within the acrosome in the same species. This protein has both ZP binding and hyaluronidase domains, with the latter possibly involved in cumulus matrix dispersal at fertilisation (Primakoff *et al.*, 1988; Lin *et al.*, 1994). A monoclonal antibody against the ZP binding domain inhibited adhesion of acrosome reacted sperm to the ZP (Primakoff *et al.*, 1985). PH-20 has been cloned in other species like mouse (Lathrop *et al.*, 1990), humans and cynomolgous monkeys (Lin *et al.*, 1993).

Proacrosin

This is a zymogen found intra-acrosomally in all mammalian sperm. During acrosome reaction it is cleaved to yield the active form, acrosin. Localization within the acrosome, ZP binding properties and proteolytic activities are suggestive of a role in ZP secondary binding and penetration (Töpfer-Petersen, 1996). Molecular analysis has identified residues on proacrosin that could be involved in ZP binding (Mcleskey *et al.*, 1998) which was supported by findings of Tsubamoto *et al.*, (1996) showing binding of porcine ZP2 to proacrosin. However proacrosin-null mice are fertile indicating

that the role played by acrosin during fertilisation may not be essential (Baba *et al.*, 1994).

Rabbit Sperm Autoantigens (RSAs)

These are low molecular weight proteins found on sperm and spermatogenic cells identified by ZP binding properties. Antibodies against these proteins inhibit sperm-egg interaction *in vitro* and *in vivo* (O'Rand, 1981; O'Rand *et al.*, 1984). Two clones that were isolated from a rabbit testis cDNA library and sequenced, yielded a predicted 17kDa protein named Sp17. The latter is specific to the testis and has also been isolated in human and mouse testis (Kong *et al*, 1995; Lea *et al*, 1996). It was also isolated and sequenced in the baboon testis (Adoyo *et al.*, 1997). Sequencing of the cDNA clones obtained for the three species revealed a high degree of conservation of Sp17 between the species. Antisera against Sp17 localised the protein on the acrosome and it has further been suggested that the protein remains on the remnants of acrosomal and plasma membranes at the equatorial region following acrosomal exocytosis to be involved in secondary binding to the ZP (Richardson *et al.*, 1994).

1.3.2.3. ***Sperm-Oolemmal Interactions***

The later stages of mammalian gamete interaction prior to fertilisation, involve adhesion and eventual fusion of membranes of the gametes. The precise details of mechanism of membrane fusion in eukaryotes remain undetermined. There is however growing evidence that specific sperm ligands and corresponding oolemmal receptors could be involved in sperm-oolemmal adhesion and fusion. It has been reported that only acrosome-reacted sperm

naturally reach the perivitelline space to interact with the egg oolemma (Moore and Bedford, 1983) and that the sperm plasma membrane which persists at the equatorial region following acrosome reaction, is the initial point of sperm-egg fusion (Moore and Bedford, 1978; Bedford *et al*, 1979). The membranous portion of sperm acrosome persisting after acrosome reaction, the equatorial segment, due to its role in initial fusion, has been the focus of investigations regarding sperm-oolemmal interaction (see illustration in Fig.2).

1.3.2.3.1. *Sperm fusion proteins*

A number of putative spermatozoal fusion proteins of testicular and epididymal origins, have been reported by various investigators. Some of these proteins are localised at the equatorial segment following acrosome reaction like equatorin/MN9, GII/M13, DE, while others like fertilin are located on the post-acrosomal plasma membrane of the sperm head (Primakoff *et al*, 1987, Allen and Green, 1995; Toshimori *et al.*, 1998; Noor and Moore, 1999).

Equatorin (MN9)

This is a 38-48 kDa protein complex in mice and in rat sperm. It is also found in human sperm where it is predominantly localised at the equatorial segment, between the inner and outer acrosomal membranes. It persists at the equatorial segment even following acrosome reaction (Toshimori *et al*, 1992; Toshimori, 2001). Antibodies against equatorin do not affect sperm-egg binding but significantly inhibit the fusion step *in vitro* (Toshimori *et al*, 1998; Toshimori, 2001). Intra-oviductal administration of mouse mAb MN9 also significantly reduced fertilisation *in vivo* (Yoshinaga *et al.*, 2001).

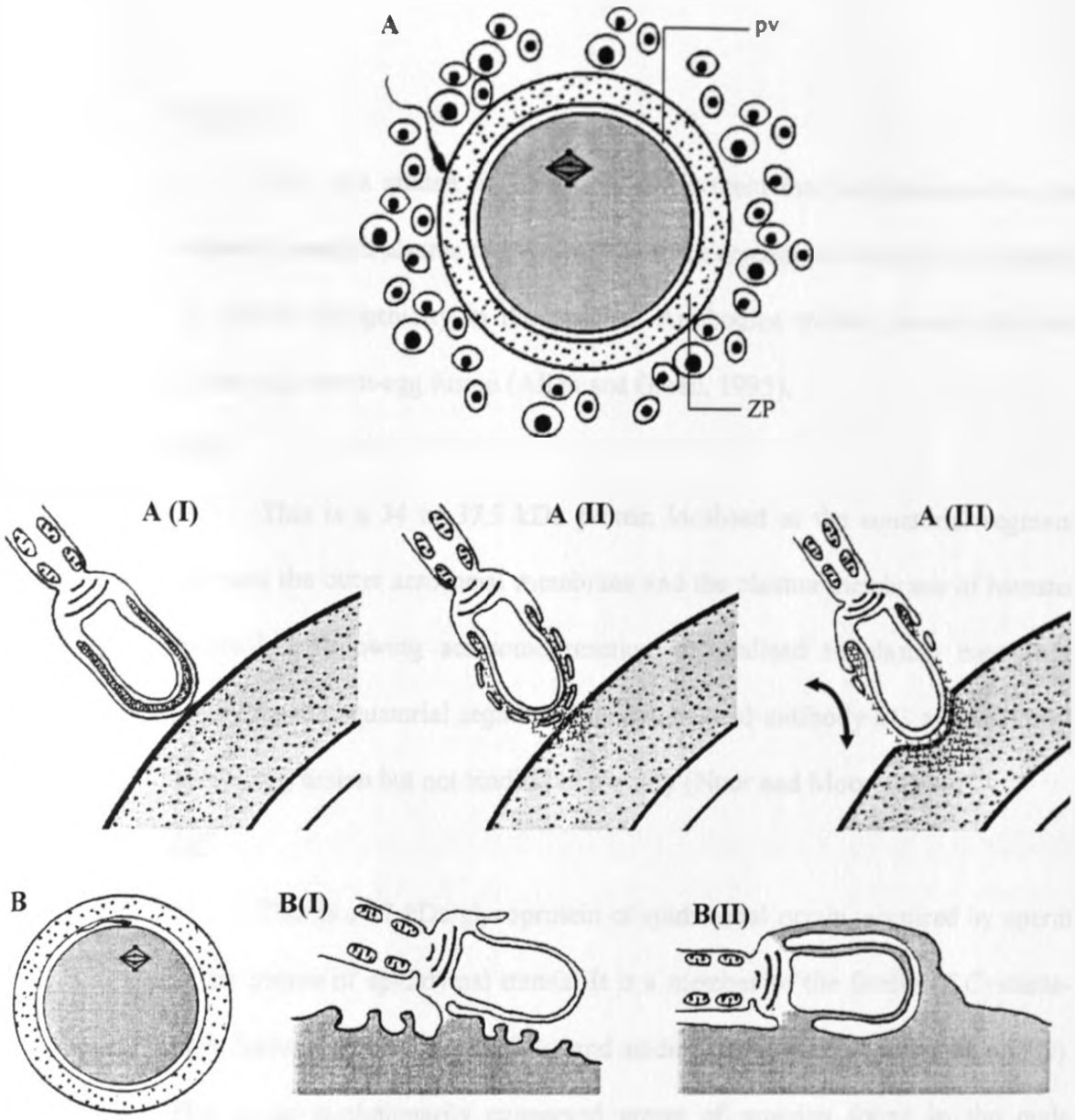


Fig. 2 Schematic diagrams of successive stages in mammalian sperm-egg interactions. (A) The fertilizing sperm penetrates the cumulus layer to reach the surface of the zona pellucida (ZP). At the zona surface, the acrosome intact sperm (AI) undergoes primary binding that induces acrosome reaction involving multiple fusion sites along the plasma and outer acrosomal membranes leading to vesiculation and release of acrosomal matrix contents (AII). The matrix contents and inner acrosomal membrane proteins could participate in secondary sperm-zona binding and alignment of the head leading to zona penetration. The actual penetration of the zona pellucida (AIII) could be mechanical (arrows) and may also involve hydrolytic enzymes in the matrix and membrane proteases. Once in the perivitelline space (B) the sperm adheres to the oolemma, motility ceases and fusion commences at the plasma membrane overlying the equatorial segment (BI) and spreads to involve the rest of the inner acrosomal membrane and sperm head (BII). ZP: zona pellucida, pv: perivitelline space. (After Brewis & Moore, 1997).

G11/M13

This is a guinea pig sperm 34kDa protein that is translocated to the equatorial region plasma membrane following acrosome reaction. It is thought to acquire fusogenicity in the process. Antibodies to this protein inhibited guinea pig sperm-egg fusion (Allen and Green, 1995).

M1

This is a 34 or 37.5 kDa protein localised at the equatorial segment between the outer acrosomal membrane and the plasma membrane of hamster sperm but following acrosome reaction is localised to plasma membrane overlying the equatorial segment. The monoclonal antibody M1 also inhibited sperm-egg fusion but not binding or motility (Noor and Moore, 1999).

DE

This is a 37 kDa glycoprotein of epididymal origin, acquired by sperm in the course of epididymal transit. It is a member of the family of *C*ysteine-*R*ich *S*ecretory *P*roteins (CRISPs) and acidic epididymal glycoprotein (AEG). This is an evolutionarily conserved group of proteins found in the male reproductive tract among other tissues (Xu and Hamilton, 1996). DE has been identified in mouse, rat and human spermatozoa using molecular cloning techniques. The human cDNA form of this protein is also referred to as the acidic epididymal glycoprotein related protein (ARP) (Hayashi *et al.*, 1996).

Monoclonal antibody against DE inhibits fertilisation *in vivo*, and sperm-oolemma fusion but not binding. (Cohen *et al*, 2000). Though initially located on the dorsal sperm head, it is later translocated to the equatorial segment during capacitation (Toshimori, 2001). Additionally, specific DE

binding sites were localised on the mouse oolemmal surface implying possible involvement of DE in sperm-oolemmal fusion despite having no disintegrin domain or fusion motif within its structure (Cohen *et al*, 2000). More recent findings have indicated that DE-mediated gamete interactions are dependent on the native conformation of the molecule which is in turn influenced by formation of disulfide bonds (Ellerman *et al.*, 2002).

The ADAM family

The ADAM (*a d*isintegrin *a*nd *m*etalloprotease) family comprises cell membrane-associated multidomain zinc dependent proteases that also have adhesive properties. At least 29 members of this family of proteins have been identified and have high sequence homology and domain organization. Apart from the key structural domains namely the disintegrin and metalloproteinase domains, ADAMs also have the following domains within their structure, a pro-domain, a cysteine-rich domain, epidermal growth factor-like domain, transmembrane domain and cytoplasmic tail domains (Cho *et al*, 1998; Kaushal and Shah, 2000). Five of the ADAMs, ADAM2 (fertilin β), 3 (Cyritestin), 5, 16, 18 are expressed as proteins in male germ cells and spermatozoa.

Fertilin

Fertilin (formerly PH-30) is among the putative fusion proteins that have been investigated intensively. It is a heterodimeric protein that belongs to the ADAM family made of two subunits α (ADAM 1) and β (ADAM 2). Fertilin α and β which are initially in precursor form (with all domains found in ADAMs) during spermatogenesis. They undergo proteolytic processing in

the course of testicular and epididymal transit, removing the pro and metalloprotease domains, leaving the disintegrin domain as the N-terminal domain in both subunits (Cho *et al*, 1998).

The Fertilin β disintegrin domain has an ECD (glu-cys-asp) tri-peptide motif instead of the RGD (arg-gly-asp) motif found at the same position, in closely related Snake Venom Metalloproteinases (SVMPs). It is this tri-peptide motif that is associated with integrin binding, especially the presence of the terminal aspartic acid (Zhu *et al*., 2000; Mclaughlin *et al.*, 2001).

Fertilin was first identified on guinea pig sperm where it is located on the post-acrosomal plasma membrane (Primakoff *et al.*, 1987). It has subsequently been cloned and sequenced in the mouse, rat, rabbit, bovine, macaque and human (Frayne and Hall, 1999). The monoclonal antibody against fertilin β inhibited sperm–egg fusion (Primakoff *et al.*, 1987). The disintegrin domain of fertilin β is thought to bind to a complementary integrin on the egg oolemma that are associated with specific tetraspanin proteins, to effect sperm-egg adhesion (Chen *et al.*, 1999). However, there are indications that fertilin β may be involved in sperm-zona interaction too (Cho *et al*, 1998). CD9 the tetraspanin associated with the integrin α6β1, in murine eggs, mediates binding of a 57kDa protein (possibly fertilin β) or recombinant peptides of fertilin β to the egg (Chen *et al*, 1999). Recombinant peptides to the binding region of the fertilin β disintegrin domain, inhibit sperm-egg adhesion and fusion (Primakoff and Myles, 2000).

Fertilin α, by virtue of having both a disintegrin and fusion peptide is thought to have a role in sperm-oolemma adhesion and fusion respectively. The fusion component is in the form of a hydrophobic cysteine rich sequence within the structure of fertilin α that has similarities with a virus fusion peptide and probably behaves like one during sperm oolemma fusion (Martin *et al*, 1998; Wolfe *et al*, 1999). Functional fertilin α is however not expressed on human sperm implying that it may not be essential for human sperm-oolemmal interactions (Jury *et al*, 1998).

Cyritestin (ADAM 3)

This is a 55kDa protein with a disintegrin domain. The protein is located at the equatorial segment and inner acrosomal membrane of sperm following acrosome reaction. Anti-cyritestin antibodies strongly inhibit sperm-oolemmal binding in the mouse (Yuan *et al*, 1997). Peptide analogues of disintegrin domain of mouse ADAM3 also inhibit gamete binding and fusion (Linder and Heinlein, 1997; Yuan *et al.*, 1997). It has been proposed that Fertilin β and ADAM3 may act sequentially or concurrently in sperm-egg adhesion (Cho *et al.*, 1998). The antibody inhibition assays and location of this protein at the equatorial segment of sperm, suggest possible involvement in the binding and fusion process of egg and sperm.

1.3.2.3.2. *Egg fusion proteins*

Sperm bind to the egg oolemma at the region with microvilli (Shalgi and Phillips, 1980). However, microvilli are not necessarily associated with sperm receptors on the egg oolemma as growing hamster oocytes have fusion ability before microvilli development and changes in pH affecting microvilli do

not alter sperm-oolemma binding (Yanagimachi *et al.*, 1980; Zuccotti *et al.*, 1991).

Oolemmal fusibility is transient and is lost after fertilisation occurs. This is almost immediate in the rabbits, mice and humans and gradual in others like the hamster (Austin, 1961; Wolf, 1978; Zuccotti *et al.*, 1991; Sengoku *et al.*, 1995). Among the egg receptors that are being investigated in relation to sperm-oolemmal fusion are the integrins. These are associated with tetraspanin proteins.

Integrins

These are proteins found on the surface of different cell types that function as receptors for cell-cell and cell-matrix interactions. Over 24 heterodimeric integrins have been identified so far. An integrin comprises α and β subunits. The α subunit is composed of 3-4 divalent cation binding domains, a single transmembrane portion, and a short cytoplasmic region (Ruoslahti, 1996 a, b; Evans, 2001). The β subunit comprises relatively large extracellular domains of 48-56 cysteine residues that are often clustered in four repeated motifs (Hogervorst *et al.*, 1990). Integrins bind ligands like collagen, laminin, vitronectin, fibronectin and RGD (Arg-Gly-Asp)-containing peptides. It has been noted that for binding most integrins require an acidic residue like the aspartate in the RGD peptides of cognate ligands (Evans, 2001). However some integrins bind more than one ligand whilst others are associated with dual functions like secretion and other cellular functions (Ruoslahti, 1996 a,b). Their activation triggers phosphorylation of intracellular proteins, increased

gene transcription, changes in cytoskeletal arrangement among other changes in cells (Mcleskey *et al.*, 1998).

Integrins are involved in the sperm-oolemma interaction. Evidence for this was obtained from observations that RGD peptides, which are known integrin ligands, inhibit fusion of zona free hamster eggs to human and hamster sperm (Bronson and Fusi, 1990; Fusi *et al.*, 1993). Secondly, integrins have also been identified distributed on microvilli at the fusogenic area of the egg oolemma and lastly though indirectly, is the fact that there has been identification of an integrin binding disintegrin domain in fertilin, one of the putative sperm fusion proteins (Blobel *et al*, 1992).

One of the integrins, α6β1 had been implicated in the murine sperm-oolemmal fusion process by experiments that showed that:

a) antibodies against the integrin inhibit sperm-egg binding; b) peptides that bind other integrins do not inhibit sperm-egg binding or fusion and; c) sperm binds transfected cells expressing α6β1 and such binding is inhibited by peptides containing the disintegrin domain of fertilin (Almeida *et al.*, 1995). Recent experimental evidence however contradict these earlier findings suggesting that the integrins α6β1 may not be crucial in the sperm-oolemmal interaction (Miller *et al.*, 2000).

Tetraspanins

These comprise a family of integral plasma membrane proteins with four transmembrane domains, two extracellular loops and three short cytoplasmic segments. This group of proteins are associated with integrins, MHC class II glycoproteins and with each other (Chen *et al*, 1999). They have

been implicated in cellular processes like, growth control, intracellular signalling, migration and adhesion though their precise mode of action is unclear (Hemler, 1998; Chen *et al.*, 1999).

CD9

CD9 is a member of the transmembrane tetraspan superfamily that interacts with the integrins, especially β1 integrins and other membrane proteins (Miller *et al.*, 2000; Kaji *et al*, 2000). It is expressed on egg microvilli at the fusogenic area of the egg oolemma (Kaji *et al.*, 2000). It is also expressed in other somatic cells such as the muscle cells (Tachibana and Hemler, 1999). The role of this tetraspan has been investigated using knock-out mice. CD9 null female mice were infertile when mated with normal males. This was attributed to the marked reduction in sperm-egg fusion ability as a result of the absence of this protein. The sperm–egg binding was however, unaffected (Kaji *et al*, 2000). Antibodies against CD9 inhibited sperm-egg binding and fusion and additionally, the antibodies also inhibited the binding of the disintegrin domain of fertilin β to murine eggs (Miller *et al.*, 2000; Chen *et al*, 1999).

Other Oolemmal Tetraspanins

Other tetraspanins that are also associated with β1 integrins and CD9 have also been identified on the mouse egg surface. These are CD81 and CD98 (Takahashi *et al.*, 2001). Antibodies to CD98 significantly inhibited sperm-egg binding and binding of ADAM3 disintegrin domain. It is thought that tetraspanins interact with each other, with integrins and with other integrin-associated proteins forming a tetraspan web that defines and maintains the site

of sperm fusion in murine fertilisation (Takahashi *et al.*, 2001). The tetraspan web has been hypothesized to be involved in cell signalling at raft-like micro domains of the plasma membrane (Rubinstein *et al*, 1996).

1.3.2.3.3. *Fusion mechanisms*

Fusion results in the plasma membranes of the sperm and egg becoming continuous. It has been hypothesized from viral fusion experiments that fusion proteins share common fusion peptides that are short sequences of hydrophobic amino acid residues of α helical structure. Peptides of this structure, due to their hydrophobic nature, are able to enter the lipid bilayer of cell membranes causing destabilization and eventual fusion (Snell and White, 1996; Pecheur *et al.*, 1999). Among the putative sperm fusion proteins is fertilin α that has a hydrophobic motif with a possible fusion role. However, sperm-egg fusion still occurs in knockout mice lacking detectable levels of fertilin α (Talbot *et al.*, 2003).

1.3.2.3.4. *Egg activation*

Egg activation is triggered off by sperm-oolemmal fusion. The actual fusion occurs in the region of the egg oolemma with microvilli and sub-plasmalemmal cortical secretory granules (Mcleskey *et al.*, 1998). Consequently there is cytosolic mobilisation of Ca^{2+} from the point of fusion in oscillating waves to the rest of the egg, exocytosis of Ca^{2+} dependent cortical granules into the perivitelline space to prevent polyspermy, resumption of meiosis, pronuclei formation, syngamy, initiation of DNA replication and cleavage (Yanagimachi, 1994; Töpfer-Petersen, 1999).

It has been proposed that the trigger for the events that take place in the course of egg activation could be due to a soluble sperm factor introduced in the egg following fusion of the gametes (Parrington, 1996; Swann and Parrington, 1999). Nitric oxide synthase has been identified as a possible sperm factor introduced into the egg at fertilization. Moreover, nitric oxide produced by activity of this enzyme was shown to trigger egg activation events (Kuo *et al.*, 2000). However more recently, another possible factor, a gamete specific form of phospholipase C called PLCζ (zeta) has been implicated in egg activation. Its introduction into the egg by sperm is associated with calcium oscillations which are in turn associated with egg activation (Swann *et al*, 2006). It remains to be determined conclusively, which of the factors is responsible for egg activation.

1.4. Contraception possibilities and use of non-human primates

At the present growth rates, the world population is estimated to reach 28 billion people by the year 2050 (Aitken, 2001). This presents a problem of overpopulation with grave implications for the future. Population growth rates are at present highest in the developing countries where there is no corresponding increase in resources like health facilities or food production to cater for the numbers. These great numbers could also lead to environmental degradation and a myriad of social and economic problems as many compete for limited resources available (Aitken, 2001). Effective, affordable and acceptable contraception may be one of the solutions to the global problem of overpopulation.

A number of methods of contraception are currently available but they have varying acceptability due to, having undesirable side effects, offering inadequate protection against pregnancy, unsuitability or unavailability for a proportion of the population due to cultural, economic or personal reasons (Frayne and Hall, 1999). One of the possible methods of contraception that is receiving increasing attention is immunocontraception. This method makes use of the body's immune system to block an indispensable step in the process of gamete interaction during fertilisation. Events in the fertilisation sequence including, sperm capacitation, sperm-cumulus interaction, sperm-zona binding, the acrosome reaction, zona penetration and gamete fusion present potential targets for immunological interception (Bedford, 1994). The feasibility of immunocontraception as a potentially viable alternative method of fertility regulation is emphasized by the presence of large numbers of overtly healthy men and women that are infertile due to presence of anti-sperm antibodies (Diekman *et al.*, 2000).

1.4.1. Qualities of an ideal immunocontraceptive

The suitability of an antigen for use in human immunocontraception is determined by its tissue specificity, involvement in human fertility and immunogenicity. A good contraceptive vaccine would therefore have to be safe, reliable, affordable, easy to administer, capable of evoking a homologous response in most or all individuals and acceptable to a wide range of the population. Further, it should have a long lasting but reversible contraceptive effect that can be assessed by simple assays (Frayne and Hall, 1999).

1.4.2. Immunocontraception antigens

Immunisation of women (Baskin, 1932), female rabbits (Menge, 1971) and mice (Tung *et al.*, 1979) with isologous sperm or testicular extracts resulted in reduced fertility levels. Similarly, immunisation of females using porcine ZP extracts reduced fertility in a number of animal species including rodents (Hasegawa *et al.*, 1992), equidae (Liu *et al*, 1989), non-human primates (Sacco *et al.*, 1983; Dunbar *et al.*, 1989; Bagavant *et al.*, 1994) and elephants (Fayrer-Hosken *et al.*, 1999). It is also known that antisperm antibodies (ASAs) may be responsible for up to 30% of male or female cases of infertility (Naz and Menge, 1994). Additionally, 70% of vasectomies are associated with formation of ASAs (Liskin *et al.*, 1983) and vasovasostomy done to restore fertility is often associated with persisting infertility largely implicating ASAs (Goldberg *et al.*, 2001). These findings together, indicate the potential for using isologous or heterologous sperm or zona antigens for immunocontraception. A number of gamete antigens have been tested or are being tested as immunocontraceptives. They include zona-derived and sperm antigens.

1.4.2.1. *Zona pellucida antigens*

Autoantibodies against zona antigens were proposed to be a cause of infertility in women (Shivers and Dunbar, 1977). Antisera against ZP inhibited sperm binding to homologous eggs in hamsters, mice and rats (Gwatkin *et al.*, 1977; Tsunoda and Chang, 1976). These observations triggered interest in use of ZP antigens for immunocontraception. Subsequently, immunisation of female squirrel monkeys (*Saimiri sciureus*) and cynomolgous monkeys (*Macaca fascicularis)* with solubilized porcine ZP glycoproteins, resulted in

reduced fertility of the animals and depletion of ovarian follicles by the immune response that was generated (Sacco *et al.*, 1983; Gulyas *et al.*, 1983). Similar observations were made on baboons immunised with porcine ZP (Dunbar *et al.*, 1989).

The induction of ovarian pathology could not be attributed to the sugars on the ZP glycoproteins, as deglycosylated ZP antigens had similar effects on the ovary. It was then realised that the pathology arose due to presence of T-cell epitopes in the ZP antigens and these were responsible for T-cell mediated immunity, resulting in oocyte destruction in the ovaries (Mahi-Brown, 1996). Distinction of B-cell epitopes responsible for humoral immune response from T-cell epitopes on the ZP antigens was achieved, offering the possibility of using specific peptides for immunocontraception (Millar *et al.*, 1989). Immunisation of female cynomolgous monkeys (*Macaca fascicularis*) with synthetic peptides of a human ZP3 epitope and a macaque homologue of the same ZP3 epitope, resulted in induction of a humoral response to the epitope and infertility. There was no ovarian pathology seen in the study (Mahi-Brown, 1996). The duration of infertility and reversibility of it were not determined conclusively in the latter experiment. However, the findings supported possible use of zona glycoprotein peptides in female immunocontraception.

It has been suggested that immunisation of female animals with ZP antigens may alter surface carbohydrate moieties of the zona proteins, affecting sperm-zona recognition and binding (Barber and Fayrer-Hosken, 2000). Alternatively, IgG the major class of immunoglobulin involved in the humoral immune response generated, may sterically hinder sperm receptor(s) from

binding to the zona glycans. IgG may also bind to the zona glycoproteins achieving the same effect, especially when serum and oviductal levels of the antibodies are high (Paterson and Aitken, 1990). However, *in vitro* penetration of canine zonae by homologous sperm is inhibited by anti-porcine ZP polyclonal and monoclonal antibodies whereas the sperm-binding is not (Mahi-Brown *et al.*, 1985; Bamezai *et al.*, 1988). Monoclonal antibodies against mouse ZP2 and ZP3 also inhibit penetration but not binding of mouse sperm to the zonae (East *et al.*, 1985). These findings suggest that there are additional mechanisms responsible for immunocontraception using ZP antigens other than those proposed above including possible premature conformational changes of ZP ligands akin to the zona block that occurs after egg activation (Barber and Fayrer-Hosken, 2000).

1.4.2.2. *Sperm Antigens*

Various sperm specific antigens with immunocontraceptive potential have been identified using antibodies generated following immunisation of rodents with extracts of whole sperm or purified sperm components. A number of the antigens of immunocontraceptive interest have been shown to be involved in sperm–zona recognition like, SP10, SP17, FA-1 and PH-20 and sperm-oolemmal interactions like, fertilin α, fertilin β and cyritestin.

SP10

This protein was first identified in the human sperm localised within the acrosome (Herr *et al.*, 1990a). Later it was identified in other species including mouse, fox, bovine, porcine and non –human primates like the baboon and

rhesus monkey using immunological and molecular biology techniques (Herr *et al.*, 1990b, Freemerman *et al.*, 1993; Beaton *et al.*, 1995; Reddi *et al*, 1995, Coonrod *et al.*, 1996). Antibodies against SP-10 inhibit, *in vitro* mouse fertilisation, bovine sperm-zona binding and human sperm penetration of zona-free hamster eggs (Coonrod *et al.*, 1996; Liu *et al.*, 1989; Anderson *et al.*, 1987). More recently, a monoclonal antibody pep-SP10 against a peptide of human SP-10 localised SP-10 to the equatorial region of human sperm after acrosome reaction and inhibited human sperm to zona free hamster egg binding but not human sperm-zona binding (Hamatani *et al.*, 2000). The findings that SP-10 is specifically localised in the maturing germ cells of the testis and on sperm coupled with the observation that it remains associated with the equatorial segment of the sperm head following acrosomal reaction, make SP-10 a prime vaccine candidate. Furthermore, immunisation of mice and macaques with human SP-10 causes antibody secretion in the female reproductive tract (Srinivasan *et al.*, 1995; Kurth *et al.*, 1997a). Similarly, immunisation of macaques with recombinant macaque SP-10 generated oviductal antibodies that cross-reacted with human sperm SP-10 (Kurth *et al.*, 1997b).

Sp17

This is a member of the rabbit sperm autoantigens (RSAs) (see RSAs in section 1.3.2.2.3). Immunisation of female cynomolgous monkeys with human Sp17, that shares >96% homology with the macaques, induced an immune response with IgG class antibodies expressed in the oviductal fluid that recognised macaque Sp17 on sperm (Lea *et al.*, 1998a). However, in mice,

immunisation with a chimeric synthetic Sp17 peptide reduced fertility in one strain that was not correlated to serum or vaginal antibody levels. In another strain of mice, it induced no immune response (Lea *et al*, 1998b). Recent work has localised Sp17 in the fibrous sheath of sperm tail and in other body tissues in mice and humans (Lea *et al.*, 2004). Presence of Sp17 in other body tissues renders it unsuitable as an immunocontraceptive agent.

FA-1

Fertilisation antigen 1 (FA-1), a sperm specific glycoprotein, has been isolated in murine, rabbit, bull, macaque and human sperm. The murine FA-1 has been cloned and sequenced (Naz, 1996a). In all species mentioned, the antibodies to FA-1 inhibit fertilisation in vitro at the level of sperm-zona interaction (Naz *et al.*, 1992). Furthermore, recombinant FA-1 caused reduced fertility of mice immunised with the protein. This contraceptive effect was reversible (Naz and Zhu, 1998). These findings are indications of the potential of this protein for use as a possible immunocontraceptive.

PH-20

This sperm antigen is involved in secondary sperm – ZP binding. PH-20 has been discussed briefly earlier, in section 1.3.2.2.3. Antibodies against PH-20 inhibit sperm – zona binding in vitro (Primakoff *et al.*, 1985) and there is significant infertility induced following immunisation with PH-20, in both male and female guinea pigs (Primakoff *et al.*, 1988). In males, the infertility is accompanied by autoimmune orchitis (Tung *et al.*, 1997). There are no further investigations of female immunocontraception using the molecule though it was promising.

SP56

This has so far been identified in the mouse spermatids and sperm (see section 1.3.2.2.2). In the latter it has high affinity for mouse ZP3 glycoprotein and is thought to be involved in sperm –zona binding (Bookbinder *et al.*, 1995). It was earlier on thought to be a peripheral membrane protein but recent work has demonstrated that it is an intracrosomal protein (Kim *et al.*, 2001). The limitation of Sp56 is that it has been isolated only in the mouse sperm (Bookbinder *et al.*, 1995).

β 1-4 galactosyl transferase

Like SP56 this sperm protein is found in mouse sperm and is involved in sperm –zona binding. It has been discussed in section 1.3.2.2.2 in more detail. It is however an unlikely immunocontraceptive agent as there is a related somatic cell isoenzyme that has a high degree of homology with the germ cell protein (Frayne and Hall, 1999).

Lactate Dehydrogenase (LDH- C_4)

Sperm specific LDH-C_4 is an isozyme that shares antigenic epitopes with a somatic cell form lactate dehydrogenase (Gupta and Chaturvedi, 2000). Synthetic B-cell epitopes of the enzyme have been used in immunisation of female baboons with significant reduction in fertility (O'Hern *et al.*, 1995). However, the mechanism of immunocontraception is unclear. In male baboons, immunisation with a B-cell epitope of the synthetic peptide successfully reduced fertility without autoimmune orchitis. The infertility involved reduced sperm – zona binding though it is not clear whether this protein is normally involved in sperm –zona interactions (Goldberg *et al.*, 2001). This antigen has

been tested in animal models and the results are promising. The only constraints may be that, the precise function of LDH-C4 in the fertilisation process is unclear and the presence of a somatic cell isoenzyme with close sequence homology.

The ADAM Family

Among the members of the ADAM family that have been considered for immunocontraceptive use due to their possible involvement in sperm – oolemmal interaction are fertilin α, fertilin β and cyritestin (see also section 1.3.2.3.1).

In man, macaque and rodents fertilin α and β have been cloned and sequenced. The tissue distribution of fertilin and cyritestin differs between the rodents and the macaque, with both fertilin β and cyritestin being spermatogenic cell specific in the rodents while in the macaque fertilin β is sperm specific but cyritestin is found in other somatic cells. Fertilin α was however found distributed widely in other somatic tissues in both primates and the rodents. Furthermore, though both fertilin α and cyritestin genes exist in man, the proteins are not expressed on germ cells (Jury *et al*, 1997; Frayne and Hall, 1998). Taken together, these findings imply that only fertilin β may be considered as a candidate for primate immunocontraception. Although *in vitro* trials have indicated its involvement in sperm – oolemmal binding, *in vivo* immunisation trials of female guinea pigs with fertilin β were not promising (Ramarao *et al.*, 1996)

Other proteins have been considered like those of epididymal origin that possibly function in sperm-egg interaction such as protein DE (see section 1.3.2.3.1). It is known that antibodies against DE inhibit sperm penetration of zona-free eggs *in vitro* (Cuasnicu *et al.*, 1990) and in vivo insemination with sperm exposed to DE reduces fertilisation rates in female rats (Hall and Tubbs, 1997). Male rats immunised with purified DE have reduced fertility and no apparent autoimmune reaction along the reproductive tract (Ellerman *et al.*, 1998). These findings suggest that proteins acquired in the epididymis participate in fertilisation and could be important for immunocontraception.

As evidenced in the number of proteins considered in relation to immunocontraception using sperm antigens, there is no definite single protein that has been identified to be essential in the fertilisation process. Furthermore, there are species variations in expression or non-expression of some of the proteins considered. It is also not clear to date, whether sperm proteins function in concert or sequentially or whether some are redundant in different species. It is likely that they have concerted action to maximise fertilising ability and therefore loss of individual proteins may not significantly affect sperm fertilising ability (Frayne and Hall, 1999). This could explain the limited effect some knock–out experiments may have on fertilisation. On the other hand, it could also make interpretation of some of the results from these experiments difficult.

1.4.3. Non- human primates

Laboratory rodents have been used extensively to study various aspects of reproduction. A lot of the foregoing literature review on important and basic aspects of fertilisation is based on results of studies done on mice, rats, guinea pigs and hamsters. However, the findings from these experimental systems may not necessarily be representative of that in humans. Since the eventual target of immunocontraception benefits is humans, it therefore would be ideal if information regarding safety, efficacy, and nature of immune response evoked by a particular candidate protein could be as representative as possible of the situation in humans. Use of human subjects for experimentation is however restricted due to ethical and legal implications and as pointed out by Wango (1990), experimental results obtained from laboratory and other non-primate animals need to be validated in non-human primates before extension of the same studies to human subjects.

Non-human primates are closer to man with regard to structure and functions of several biological systems. Evolutionarily, the apes like gorillas, chimpanzees and orang-utans are closer to man than the Simian primates like baboons and macaques. However, the apes are endangered species therefore, generally unavailable for research endeavours (Graham, 1981; Mahi-Brown, 1996). The focus of non-human primate studies in reproduction has been on the simian primates particularly the baboon and macaques.

Various studies in reproduction have shown the suitability of the baboon as a model for studying human reproduction. Morphological findings show that spermatogenesis in the olive baboons (*Papio cynocephalus*) is

intermediate between the pattern in the rodents and man (Chowdhury and Steinberger, 1976). Several sperm antigens have now been identified in the baboon using monoclonal antibodies (Isahakia, 1988; 1989; Isahakia and Bambra, 1990). A number of these monoclonal antibodies against baboon sperm antigens were shown to cross react with antigens on human spermatozoa (Isahakia and Bambra, 1990). Molecular biological techniques used in characterization of sperm or testicular antigens for use as immunogens, have demonstrated significant homology between some of these in the baboon and human, like LDH-(C4) with 99.3% homology (O'Hern *et al.*, 1995). Baboon SP17 predicted protein, was found to have 97% similarity to the human, 74% homology to mouse and 77% similarity to that of the rabbit (Adoyo *et al.*, 1997). Sequencing of SP-10 in different species revealed 85% homology between baboon and human SP-10 and 60% homology between the mouse and the human form (Freemerman *et al.*, 1993). These data are strong indicators of the suitability of the baboon as a model for studies in human male reproduction. The studies on the baboon highlighted above indicated

i) significant homology of isolated sperm proteins in this species with those of humans

ii) existence of cross-reactivity between human antisera and baboon sperm antigens

and

iii) presence of significant morphological correlates in human and baboon spermatogenesis

Based on this background this particular study was carried out. It is hoped that results obtained from this study would strengthen the case for use of the baboon as suitable non-human primate model for study of various immunocontraceptive agents (particularly sperm antigen based agents), prior to use in humans.

1.5. Aims of Study

1. To identify fertilisation antigens common to the baboon and human sperm.

2. To characterise some of the common sperm antigens.

3. To specifically characterise Inner Acrosomal Membrane –1 (IAM-1), one of the putative fertilisation antigens, on human spermatozoa.

CHAPTER 2

IMMUNOLOCALISATION, 1-D PAGE AND IMMUNOBLOTTING OF FERTILISATION ANTIGENS

2.0. Introduction

2.1. Sperm-egg interactions

Mammalian fertilisation is a complicated process involving a number of cell-matrix and cell-cell interactions. The fertilising spermatozoon has to penetrate the egg vestments successively from the cumulus mass externally, to the zona pellucida and finally the egg oolemma (Yanagimachi, 1994; Brewis and Moore, 1997). The sperm has proteins complementary to egg receptors that are of importance in gamete interaction during fertilisation. These are found on its plasma membrane, outer acrosomal membrane, acrosomal matrix, inner acrosomal membrane and equatorial segment.

For sperm to penetrate the egg *in vivo*, they undergo a series of poorly defined events within the female reproductive tract collectively called capacitation.In most species, capacitation is associated with hyperactivated motility of sperm, protein tyrosine phosphorylation, plasma membrane destabilization, modification and expression of various sperm surface antigens (Brewis and Moore,

1997; de Lamirande *et al.*, 1997). Only capacitated spermatozoa can undergo zona pellucida induced acrosome reaction and fertilise zona-intact eggs (Yanagimachi, 1994; De Jonge, 2005).

Sperm penetration of the cumulus is thought to be mechanical due to the hyperactivated motility and possibly enzymatic too due to at least one sperm surface hyaluronidase, PH-20 (Bedford, 1998; Primakoff and Myles, 2002). The zona pellucida presents the next barrier to sperm. Acrosome-intact sperm-zona interaction is species specific due to presence of specific ligands on sperm and complementary receptors on the zona (Wassarman, 1999). It involves initial loose binding, specific tight binding, induction of a signal transduction cascade in sperm that results in acrosomal exocytosis, then secondary binding leading to zona penetration (Brewis and Wong, 1999). The oolemma of the egg presents the final barrier to sperm. Its penetration involves sperm antigens at the equatorial segment and those on the inner acrosomal membrane interacting with the oolemmal receptors leading to sperm-egg fusion.

2.1.1. Sperm – Oolemmal Interactions

Sperm-oolemmal fusion is a key event during fertilisation that involves close approximation of the gamete membranes and their subsequent fusion. It is now known from mammalian gametes studied, that fusion at the surface of the oolemma is initiated at the region of the sperm head called the equatorial segment (Moore

and Bedford, 1978; Santhananthan *et al.*, 1986; Taggart *et al.*, 1993). This region acquires fusion competence following acrosome reaction (Yanagimachi, 1988; Arts *et al.*, 1993) hence is of importance in the study of sperm-egg fusion.

Recently, attempts have been made to identify specific molecular components involved in the fusion process. So far among the possible sperm molecules identified include fertilin α and β, Cyritestin and a protein of epididymal origin, DE (see reviews by Primakoff and Myles, 2002 and Cuasnicu *et al.*, 2001). Other putative fusogenic proteins localised at the equatorial segment of mammalian sperm have been identified and include M29 (Saling *et al.*, 1985), equatorin (MN9) (Toshimori *et al.*, 1992), G11 and G13 (Allen and Green, 1995) and M1 (Noor and Moore, 1999). Fertilin α, β and cyritestin have all been well characterised and belong to the ADAM (*A Di*sintegrin *A*nd *M*etalloproteinase) protein family. Initial findings that fertilin α had a fusion peptide in its structure were suggestive of a role in sperm-oolemmal fusion (Blobel *et al*, 1992). However, doubts were cast on its significance in the fusion process by the recent findings indicating that fertilin α may not be expressed on human and gorilla sperm (Jury *et al.*, 1998). Furthermore, even though this family of proteins may be involved in sperm-egg interaction, recent evidence from knock-out experiments in mice indicate that they may not be involved at the oolemmal binding and fusion step which in turn suggests the likelihood of other more significant molecules determining this step (Yamagata *et al.*, 2002; Primakoff and Myles, 2002).

DE is a 32 kDa rat epididymal protein associates with the sperm surface in the epididymis and is detectable to the point of fertilisation. It becomes localised to the equatorial segment during capacitation of sperm hence could play a part in sperm-egg fusion. It has also been found to have 40% homology to human ARP an epididymal protein with similar localization on human sperm. Both of these proteins are classified under the CRISP (*C*ysteine *R*ich *S*ecretory *P*roteins) family. Antibodies against the human ARP protein significantly inhibit sperm to zona free hamster egg penetration. Furthermore, complementary binding sites for DE and ARP have been localised on the egg plasmalemma of mice and humans respectively. Work done on these two proteins as reviewed recently by Cuasnicu *et al.*, (2001), indicates they could be promising leads. However, further experiments like the use of knock-out mice for these proteins could contribute to better understanding of their specific roles in the gamete fusion process.

Another molecule that is being considered is equatorin, an antigen of 38-48 kDa recognised by a mAb MN9 that is localised on inner matrix of the equatorial segment in rodent and human sperm (Toshimori *et al.*, 1998). In mice, mAb MN9 blocks *in vitro* fertilisation at the level of sperm-egg fusion and not binding. Following gamete fusion in the same species, equatorin was still detectable in the egg up to the commencement of cleavage of the embryo (Manandhar and Toshimori, 2001). M1 and M29 resemble equatorin in terms of the molecular weights obtained in the different studies and the ability of antibodies to block

sperm-egg fusion without affecting the binding process (Saling *et al.*, 1985; Noor and Moore, 1999). While it appears that there may be differences in ultrastructural localization of equatorin compared to M1 the methods used for ultrastructural studies in each case were different and there is still the possibility that M1 and equatorin could still be the same molecule. Further characterisation including isolation and peptide sequencing could resolve these doubts. From the foregoing it is evident that the equatorial segment may have a number of putative fusogenic proteins and is still open to investigations in the different species.

2.2. Monoclonal Antibodies in Fertilisation Studies

Ever since this technology was first developed by Kohler and Milstein (1975), it has found wide applicability in various fields of scientific research including reproduction. The mammalian spermatozoon has functionally important sperm specific proteins that are also potent autoantigens (Caron and Saling, 1991). Antisperm antibodies are known to impair sperm function at various levels including, maturation, transport, acrosome reaction and fertilisation (Diekman and Goldberg, 1995). It is with the aim of developing methods of disrupting normal gamete function that a lot of effort has been directed towards identifying various sperm specific antigens. Monoclonal antibodies have been used extensively in the selective process of identifying and characterising various sperm antigens (Anderson *et al.*, 1987).

Our laboratory has over the recent past, raised various monoclonal antibodies against determinants found on sperm in different mammals. Among these were mAb 18.6 that recognises a determinant that so far appears conserved in the anterior acrosome of most mammals (Moore *et al*, 1990), mAb M1 whose cognate antigen is possibly involved is sperm-egg fusion (Noor and Moore, 1999). More recently, Al Eisa *et al*, (2001) raised monoclonal antibodies against purified human sperm heads. Some of these mAbs recognised antigens associated with the equatorial segment of acrosome reacted human sperm indicating, that they could possibly have a role in sperm-egg interactions in humans. Among mAbs raised were *I*nner *A*crosomal *M*embrane-1 (IAM-1), that recognises an antigen located on the inner acrosomal membrane and equatorial region of human sperm. It is testis and sperm specific and has so far been identified in human but not rodent sperm. It inhibited *in vitro* human sperm – zona free hamster egg binding but not fusion (Al-Eisa *et al.*, 2001). The other mAb was equatorial segment-1 (ES-1). The cognate ES-1 antigen was also detected at the equatorial segment of human sperm. ES-1 mAb inhibited both human sperm-egg binding and human sperm- zona free hamster egg fusion (Al Eisa *et al.*, 2001).

In the present study, there was further biochemical characterisation of the cognate antigens to the two mAbs IAM-1 and ES-1 on human and baboon sperm and baboon testis. Both of these antigens are thought to be involved in sperm-egg interaction particularly the adhesion and fusion process. The expression patterns of

antigens recognised by some of the monoclonal antibodies previously raised against determinants in other species, was also examined in the baboon testis and sperm.

2.3. Specific aims of this chapter

1. Immunolocalisation of the cognate antigens to anti-phosphotyrosine antibodies, mAbs IAM-1, ES-1, M1 and 18.6 on human and baboon spermatozoa and baboon testis, using indirect immunofluorescence technique (IIF).
2. Biochemical characterisation of the fertilisation antigens mentioned in 1 above using one dimensional polyacrylamide gel electrophoresis.

2.4. Materials and Methods

2.4.1. Sperm Preparations

2.4.1.1. *Human sperm preparation*

Fresh semen samples were obtained from proven healthy donors. Following liquefaction for a minimum of 30 minutes at 37°C, some of the semen was used for "swim-up" (see 2.4.1.4 below) while the rest was processed further. Of the latter, 1.5 ml samples were aliquoted into micro-centrifuge tubes and centrifuged at 960 g for 8 minutes. The supernatants were discarded and the pellets resuspended in 1.5 mls of phosphate buffered saline (PBS) pH = 7.4 (prepared as detailed in Appendix). These were centrifuged at 960g for 8 minutes, the supernatants discarded and pellets resuspended in PBS. The washing with PBS was then repeated twice. The resultant pellet was well resuspended in 200µls of PBS. The sperm concentration was determined with a Neubauer Haemocytometer (see 2.4.1.2) and the sample either used immediately or stored at -20°C until required.

2.4.1.2. *Sperm count determination*

The concentration of spermatozoa was determined using a Neubauer haemocytometer according to the protocol provided in the WHO Laboratory Manual for Examination of Human Semen and Sperm-Cervical Mucus Interaction (1999). Briefly this was determined as follows:

Sperm counts were made in 5 smaller squares in the central part of the Neubauer chamber giving a total count N. To obtain the sperm concentration the following formula was applied:

$N \times 5 \times 10^4$ = Number of sperms /1ml.

Where:

N is the sum of the sperm counts determined in the 5 central squares in the chamber.

The factor 5 is used as the central chamber of the haemocytometer has 25 small squares.

10^4 is the volume of the central chamber.

2.4.1.3. *Baboon sperm preparation*

Male Olive baboons (*Papio anubis*) were anaesthetised with a mixture of Ketamine hydrochloride (10% Ketaset®, Bristol laboratories) and Xylazine hycrochloride (2% Rompun®, Bayer, Leverkeusen) mixed in a ratio of 100:50mg respectively (the actual dosage rate used was 8mg/kg body weight of Ketamine hydrochloride and 0.4mg/kg of Xylazine hydrochloride in the mixture). Semen was collected from the anaesthetised baboons by rectal electro-ejaculation using a standard rectal probe (Standard Precision Electronics Inc., Littleton, Co., USA). The semen was allowed to liquefy at 37°C for at least 30 minutes. Some of the semen was used for "swim-up" that was performed as detailed in section 2.4.1.4.

The rest was washed, concentration determined and stored as detailed for human spermatozoa in section 2.4.1.1 above.

2.4.1.4. *Sperm swim-up technique*

1 ml Biggers, Whitten and Whittingham (BWW) medium (see Appendix) with added 0.3% BSA was pre-warmed to 37°C in a 15 mls falcon tube. 1ml of semen was carefully pipetted under the BWW. The tube was incubated at 37°C, 5% CO_2 tilted at an angle of 45°. After 1½ hours swim-up, the top 0.75 mls of medium was collected and washed with PBS three times, each time pelleting the spermatozoa at 960g. The sperm pellet was then well resuspended in 200µls PBS and the concentration determined. The spermatozoa were stored frozen at -20°C until required for further experiments. For some of the washed spermatozoa, concentration was adjusted with PBS to 5 million per ml. 10µls of the resuspended sperm was placed on a multispot slide and air dried in preparation for subsequent Indirect Immunofluorescence staining.

2.4.2. Baboon Testis Preparation

Baboon (*Papio anubis*) testes were collected following orchiectomy on anaesthetised (as detailed in 2.4.1.3 above) healthy adult male animals that were being culled at the Institute of Primate Research, Karen, Kenya. The testes were immediately stored in liquid nitrogen and later at –70 °C until required.

Table 1. Reagents used in preparation of stacking and different percentages of resolving gels for SDS polyacrylamide gels.

	Stacking Gel	Resolving Gels	
	4%	**7.5%**	**10%**
40% Bis Acrylamide	1.0ml	1.88mls	2.5mls
0.5M Tris-HCl pH=6.8	2.52mls	-	-
1.5M Tris-HCl pH=8.8	-	2.5mls	2.5mls
10% SDS	100µls	100µls	100µls
Distilled Water	6.36mls	5.47mls	4.8mls
10% APS	50µls	50µls	50µls
TEMED	10µls	5µls	5µls

APS and TEMED were the last two reagents added during gel preparation. APS - Ammonium persulphate (BDH), TEMED - N N N' N' - Tetramethylethylene diamine (Sigma), SDS - Sodium dodecyl sulphate (BDH), Bis Acrylamide - N N'-methylene-bisacrylamide (Promega).

2.4.3. One dimensional SDS Polyacrylamide Gel Electrophoresis (1D SDS-PAGE)

One dimensional SDS – PAGE was done according to the method of Laemmli (1970). Briefly, electrophoresis reagents were prepared according to the protocol given in Table 1. The gels were either 7.5% resolving gels for the separation of high molecular weight proteins or 10% gels for separation of the medium to low molecular weight proteins. The reagents were mixed carefully, taking care not to form air bubbles and poured to set in a prepared Mini Protean™ II electrophoresis system (BIO-RAD). Immediately, the top of the gel was layered with 70% industrial methylated spirit (IMS) and the gel was allowed to set. After the resolving gel had set, the 4% stacking gel ingredients (Table 1) were mixed together, the IMS poured off, and the stacking gel layered on top of the resolving gel. A comb was immediately inserted in the stacking gel to create loading wells. As soon as the stacking gel had set, the comb was removed, the mini protean apparatus assembled for electrophoresis, and electrode running buffer (25mM Tris, 192mM Glycine , 0.5% SDS) added to fill the inner and outer chambers of the apparatus. Molecular weight standards and samples (see 2.4.3.1. below) were loaded in the wells and electrophoresis carried out (at 150V and 40mA) for 60 minutes or till dye front migrated to the bottom of the resolving gel.

2.4.3.1. *Sample loading*

Samples loaded onto the wells were prepared as follows. Each well was loaded with sperm proteins extracted from at least 250,000 sperm. This required amount of spermatozoa in PBS was mixed with reducing or non-reducing loading buffer (Table 2) at a ratio of 1: 4, sample:loading buffer. The sample-buffer mixture was boiled for 5 minutes. The mixture was centrifuged at 10,600g for 10 minutes.

Table 2. Reagents used in preparation of non-reducing and reducing sample loading buffers for One Dimensional Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

	Loading buffers	
Reagents	**Reducing**	**Non-reducing**
Distilled Water	4.0mls	4.4mls
0.5mls Tris HCl pH=6.8	1.0mls	1.0mls
Glycerol	800µls	800µls
10% w/v SDS	1.6mls	1.6mls
2-β mercaptoethanol	400µls	-
0.05% w/v Bromophenol blue	200µls	200µls

About 25µls of the supernatant was loaded into each of the wells of the prepared gel. Molecular weight markers were diluted 1:3 with reducing sample

loading buffer, boiled for 1 minute then loaded alongside the samples onto the prepared wells on the gel. For ES-1 and anti-phosphotyrosine cognate antigen determinations, reducing loading buffer (Table 2) was used to treat the sperm sample while for IAM-1 non-reducing buffer (Table 2) was used for protein extractions.

2.4.4. Western Blotting

This was carried out according to a modified version of the method by Towbin *et al.*, (1979).

2.4.4.1. *Semi-Dry Protein Transfer*

This mode of protein transfer involved the use of the Trans-blot® semi-dry transfer cell (BIO-RAD). Protein transfer was done onto Immobilon-P (Millipore) polyvinylidene fluoride (PVDF) or Trans Blot (BIO-RAD), nitrocellulose membrane.

The PVDF membrane was prepared as follows:

A piece of the membrane cut to the size of the 1-D gel that was run was immersed in absolute methanol for 15 seconds, then 2 minutes in distilled water and then equilibrated in Towbins transfer buffer (25 mM Tris, 19 mM glycine, 20% methanol) for at least 5 minutes before use.

Alternatively, nitrocellulose membrane cut to the size of the gel was prepared by equilibration in Towbins transfer buffer for 5 minutes.

Following electrophoresis, the gel was briefly equilibrated in Towbins transfer buffer for 5 minutes. Five Whatman® filter papers cut to the size of the gel were soaked in transfer buffer and placed on the anodic plate of the semi-dry transfer unit. The piece of nitrocellulose or PVDF membrane, previously cut to the size of the gel and prepared for protein transfer as detailed above, was then placed on the filter papers. Immediately, the gel was placed carefully on the membrane and its orientation marked on the same. Another set of five Whatman® filter papers, previously cut to gel size and soaked in transfer buffer were placed on the gel while taking care to expel any air bubbles trapped between them. The cathodic plate of the Trans-blot transfer unit was placed on top of the filter papers completing the assembly for protein transfer. Protein transfer was done at a constant voltage of 10V for 60 minutes.

2.4.4.2. ***Tank Transfer (Wet Protein Transfer)***

In this mode of protein transfer following electrophoresis, the gel was equilibrated in Towbins transfer buffer for 5 minutes. A protein transfer cassette had been prepared as follows:

A clean scotch brite pad larger in size than the gel was placed on the anodic plate (light coloured). Then five Whatman® filter papers cut to the size of the gel (that had been soaked in transfer buffer) were placed on it, taking care to expel any air bubbles trapped between them with a clean glass rod. A piece of nitrocellulose or PVDF membrane cut to the gel size prepared and pre-equilibrated in Towbins

transfer buffer (as in 2.4.4.1. above) was then placed on the filter papers. Immediately, the gel was placed carefully on the membrane and its orientation marked on the same. The sandwich was completed by placing five other filter papers, pre-wetted in the transfer buffer, on the gel, and finally covering these with the scotch brite pad and then the cathodic plate (black colour). The transfer sandwich unit was then immersed in the Towbins transfer buffer within the Trans-Blot Electrophoretic Transfer Cell (BIO-RAD). Cooling of the apparatus was achieved by running circulating water and use of a stirrer in the transfer cell. The transfer was done at 200V, 400mA and 200W for 3 hours.

2.4.5. Immuno-blotting

2.4.5.1. *Protocol I*

Following protein transfer to PVDF or nitrocellulose membranes, the membranes were carefully removed and the lanes with the samples cut out and placed in blocking solution (blocking done to prevent non-specific antibody binding to the membrane) of 3% w/v Bovine Serum Albumin (BSA) (Sigma) in PBST (0.05% v/v Tween-20® (Sigma) in Phosphate buffered saline), on a rocking platform (Biometra) at room temperature for 2 hours. Alternatively, the membrane was placed in blocking solution made up of 5% w/v non-fat dried milk in PBST, at $4^{O}C$ overnight (12 hours). All subsequent steps were done at room temperature

with the membranes agitated on the rocking platform. After blocking, the membranes were washed three times in PBST. Each wash was performed for 5 minutes. This was followed by incubation of the membranes in primary antibody for 1½ hours. Primary antibodies were monoclonal antibodies prepared using hybridoma technology following immunisation of mice against specific sperm components. Negative controls not treated with primary antibody but treated with secondary antibody, were incorporated in the experiments. After incubation in primary antibody, the membranes were washed three times (10 minutes for each wash) in PBST. They were then incubated for 2 hours in secondary antibody (diluted 1:1500 with 3% BSA in PBST). The secondary antibodies used were either Anti-Mouse IgG Alkaline Phosphatase Conjugate (Sigma) or Anti-Mouse IgG Horseradish peroxidase conjugate (Amersham Lifescience). For the primary antibody ES-1, the secondary antibody used was Goat Anti-mouse IgM (Mu chain) (ICN Biomedicals Inc.) diluted 1:1500 (with 3% BSA in PBST). Secondary antibody incubation was followed by five consecutive washes of 10 minutes each in PBST. Lanes with the molecular weight markers were stained separately for total protein as detailed in section 2.4.7.

2.4.5.2. *Protocol II*

Total protein staining and immunodetection was combined according to the protocol of Chevallet *et al.*, (1997). All the subsequent steps were performed on a rocking table (Biometra). This method involved the removal of the PVDF membrane following protein transfer; washing 3 times for 5 minutes (3 x 5) each in 30% ethanol, followed by washing 3 x 5 in 0.05% v/v Tween20 in distilled water. Staining for total protein was carried out overnight in colloidal gold total protein stain (Bio-rad) diluted 1:3 with 0.05% Tween20 in distilled water. The membrane was then rinsed 3 x 5 minutes in PBS containing 0.05% Tween20 and 1% w/v, polyvinylpyrrolidone (Sigma). Blocking was then done in the same solution for 2 hours. Then primary antibody incubation was performed with IAM-1 supernatant for 2 hours. The membrane was then rinsed 6 x 5 minutes in PBS with 0.05% Tween20. This was followed by 1 hour incubation with secondary antibody that was Anti-mouse IgG (raised in Sheep) conjugated to horse radish peroxidase (Amersham Pharmacia Biotech) and diluted 1:1,500 with PBS containing 0.05% Tween20. Finally there were 6 x 5 minutes washes in PBS containing 0.05% Tween20. Visualization of Immuno-reactions was done using Enhanced Chemiluminescence (ECL+Plus) Western blotting detection kit (Amersham Pharmacia Biotech) according to the manufacturers protocol described below.

2.4.5.3. ***Immuno-detection***

2.4.5.3.1. *Alkaline-phosphatase detection*

When the secondary antibody used was Anti-Mouse IgG Alkaline Phosphatase Conjugate, the substrate buffer (0.1M Tris-HCl pH = 9.5, 0.2M NaCl and 0.001M $MgCl_2$) was used to wash the membranes once after the last wash with PBS, following incubation with secondary antibody. The membranes were then incubated in a substrate solution made of 5 mls of substrate buffer, 33 µl of NBT (50 mg/ml Nitro blue tetrazolium) and 16.5 µl of BCIP (50mg/ml of 5-bromo-4-chloro-3-indolyl-phosphate) for 15 minutes to allow for colour development. The immunoblots were scanned into a Computer that had Image master 1-D Software (Pharmacia Biotech) that enabled detection and calculation of apparent molecular weights of the immunoreactive bands.

2.4.5.3.2. *Enhanced Chemiluminescence detection*

Where the secondary antibody was Anti-Mouse IgG Horseradish peroxidase conjugate (Amersham Lifescience), detection involved use of the Enhanced Chemiluminescence+Plus (ECL+Plus) Western blotting detection kit (Amersham Pharmacia Biotech) according to the manufacturers protocol. This involved mixing the two solutions provided in the kit: ECL+Plus substrate solution in Tris buffer (Solution A) and Acridan solution in dioxane and ethanol (Solution B) in the ratio of 40:1 respectively in a container covered with aluminium foil. In the dark room, about 4 mls of the solution was spread on the membrane that was placed on clean

cling film and covered by the same to prevent drying. The reaction was given 5 minutes to develop and excess mixture blotted off; the membrane was immediately wrapped in clean cling film and exposed to Kodak XAR -5 film (Anachem Ltd) that was carefully positioned on the blot and marked in pencil (for orientation purposes), at 20, 40, 60 seconds then at 3, 10 or 15 minutes to determine the optimal exposure. The films were developed for 2 - 5 minutes in Kodak Industrex manual developer (Kodak Industrie, France), diluted 1: 2.33 in distilled water. The films were then fixed in Kodak industrex manual fixer (Kodak Industrie, France) diluted 1:2.33 (in distilled water), for 2 - 5 minutes. The films were washed in running water for 5 minutes and air -dried. Images were scanned into a computer and analysed with the help of the Image master 1D software (Pharmacia Biotech).

2.4.5.3.3. *Enhanced Chemiluminescence Glycoprotein Detection*

This was done using the ECL™ glycoprotein detection system (RPN 2190, Amersham Pharmacia Biotech), according to the manufacturers instructions. Briefly, it involved the preparation of human sperm sample for SDS-PAGE gel electrophoresis as outlined in section 2.4.1.1 above, using SDS non-reducing loading buffer for protein extraction and solubilisation. For sample preparation, at least 250,000 human spermatozoa were used for protein extraction per well on a 1-D gel prepared as detailed in section 2.4.3.1 above. A positive protein control, transferrin (at least 500 ng/lane) was run in one of the lanes on the gel. A negative control was included which had sperm protein that did not undergo the periodate

oxidation step but was treated with non-reducing loading buffer. The gel was run as detailed above and proteins transferred to Immobilon – P (Millipore) PVDF membrane using the Tank transfer unit (BIO-RAD). The membrane was removed following successful protein transfer and dried as follows: it was placed in 100% methanol for 15 seconds then placed to air dry on a clean filter paper at room temperature for 15 minutes. It was then stored in a fridge at 4^0C till the glycoprotein detection was done. Solutions required were provided in the glycoprotein detection kit. Dilutions of these solutions were made according to the manufacturer's protocol. All subsequent incubations were performed on a rocking table (Biometra). The detection for glycoprotein was preceded by rehydration of the PVDF membranes by placing in 100% methanol for 15 seconds then double distilled water for 2 minutes and then PBS for 10 minutes. Thereafter, they were incubated for 20 minutes (in the dark) in a solution of 10mM sodium metaperiodate (this step oxidized the carbohydrate moieties) dissolved in 100mM acetate buffer, pH = 5.5. This was followed by two rinses in PBS then 3 washes of 10 minutes each in PBS. The membranes were then incubated for 60 minutes in a solution made of 4 µl of 0.125mM biotin hydrazide (this reagent biotinylated any carbohydrates present) dissolved in 20mls of 100mM acetate buffer, pH =5.5. This was followed by two brief rinses and 3 washes of 10 minutes each in PBS. Subsequently there was blocking by incubation for 60 minutes in 20 mls of 5% blocking agent in PBS. Washing was done as had been outlined in the previous step

with PBS. Thereafter the membranes were incubated for 30 minutes in streptavidin horseradish peroxidase conjugate (diluted 1:6000 in PBS) and then a washing step carried out as outlined above in PBS. Detection was done with the ECL+Plus kit according to the protocol in section 2.4.5.3.2 above.

2.4.6. Total Protein Staining for Gels

2.4.6.1. *Silver Staining Protocol*

In this protocol the Silver Staining Kit (Amersham Pharmacia Biotech) was used according to the manufacturer's instructions. All the incubations and washings were done on a rocking table (Biometra) at room temperature. Briefly, following 1D SDS-PAGE, the gel was placed in fixing solution (40% v/v ethanol, 10% glacial acetic acid and 50% distilled water) for 30 minutes. This was followed by a five minutes wash in distilled water, repeated twice. The gel was then incubated in sensitizing solution (30% v/v ethanol, 0.125% w/v glutaraldehyde, 0.83M sodium acetate, 0.2% w/v sodium thiosulphate) for 30 minutes. It was then washed with distilled water for five minutes, this repeated twice. The gel was then placed in silver solution (in 0.25% w/v silver nitrate, 0.014% formaldehyde and 90% v/v distilled water) for 20 minutes. It was washed twice in distilled water, each time for a minute, then placed in developing solution (0.24M sodium carbonate and 0.0074 w/v formaldehyde) for 2-15 minutes. When there was visually sufficient development of the protein spots, the gel was transferred to stop solution (40mM

EDTA in distilled water) for 10 minutes then washed three times in distilled water for 5 minutes each. The images of the gels were scanned into a computer.

2.4.6.2. ***Coomassie Blue staining***

Following SDS-PAGE, gels were covered well with excess Coomassie blue stain (0.1% w/v Coomassie Blue R-250 (Sigma) in 40% methanol, 10% acetic acid and 50% distilled water) and placed on a rocking platform for 1 hour. The Coomassie stain was decanted off and the gel destained in several changes of destaining solution (40% methanol, 10% acetic acid in distilled water) until the protein bands were well defined against minimal background. A digital image of the gel was acquired using a scanner connected to a computer.

2.4.7. Total protein staining for Western blots

2.4.7.1. ***Colloidal Gold total protein stain***

Colloidal Gold Total Protein Stain (BIO-RAD) was used according to manufacturer's instructions for the purpose of detection of protein bands following western blotting. Briefly, the blot was treated as follows: three washes of 10 minutes each in PBST then three rinses of 2 minutes each, in distilled water. The stain was diluted 1:4 in distilled water and staining done until the protein bands were clearly visible. The blot was washed 3 times for 10 minutes each in distilled water. Images were scanned into a computer.

2.4.8. Differential extraction of human sperm proteins

This was done to determine the optimal extraction solution(s) for IAM-1. Human sperm was prepared as detailed in section 2.4.1.1. Approximately 85 million sperm were diluted in 3.25mls of PBS in a sterile bijou bottle that was placed on ice and sonicated at an amplitude of 15μ for 2 minutes. The suspension was vortex mixed and equal amounts aliquoted into four microfuge tubes. These were each centrifuged at 17,000g for 30 minutes. The supernatant was aspirated and discarded. 200μl of different extraction solutions (Table 3 under results) were added to each microfuge tube and the sperm suspension mixed well. Extraction was done for 12 hours at room temperature whilst shaking the tubes. Sperm proteins were extracted from approximately 20 million spermatozoa per microfuge tube.

After 12 hours of extraction, the tubes were centrifuged at 13,000g for 10 minutes, the supernatants aspirated and aliquoted into clean sterile labeled microfuge tubes. 25μls of the urea containing protein extracts were aliquoted into two separate tubes then 10% glycerol and a grain of bromophenol blue added. The different solutions were mixed well and each loaded on a separate well on a 4% stacking, 10% resolving gel (prepared as outlined previously in section 2.4.3).

The 1M NaCl sperm supernatant (after centrifugation) was desalted using a Micron® YM-3 Centrifugal filter device (Millipore) according to the manufacturer's instructions. The resultant protein solution was diluted with non-reducing loading buffer and treated as outlined in section 2.4.3.1 above before loading onto the

prepared resolving gel. Low molecular weight markers were loaded then 1D SDS-PAGE run and then semi-dry protein transfer onto PVDF membranes carried out as outlined previously (see 2.4.4.1). The subsequent combination of colloidal gold staining and immunoblotting using mAb IAM-1 as the primary antibody and a secondary anti-mouse IgG antibody was done as detailed previously in section 2.4.5.2.

2.4.9. Indirect Immunofluorescence Technique (IIF)

Comparative IIF staining was done on human and baboon spermatozoa and baboon testis using a set of monoclonal antibodies (mAbs) as primary antibodies. These were either commercially obtained or had been raised in mice against specific epitopes on mammalian sperm. The secondary antibodies used were anti-mouse IgG or anti-mouse IgM (for mAb ES-1 only) conjugated to fluorescein isothiocyanate (FITC).

2.4.9.1. *Human sperm slide preparations*

Swim-up sperm washed as detailed in section 2.4.1 were used for IIF. The sperm concentration was determined with a Neubauer haemocytometer as detailed in 2.4.1.2 above then adjusted to 5-10 million cells/ml using PBS.

A 100µl of adjusted sperm suspension was then used to make a thin smear that was spread evenly on a slide and air dried. For multispot slides, 10 µl per spot was

spread evenly on each spot and air dried. The slides were fixed in methanol for 15 seconds at room temperature, air dried then stored at 4 °C until used.

2.4.9.2. ***Antibody labeling of sperm slides***

The slides with sperm smears were covered with supernatant of mAbs (primary antibody). A cover slip was placed carefully on top of the supernatant and the slides were incubated for 1½ hours at 37°C in a humidified chamber. Negative controls (incubated with PBS instead of supernatant) were included.

Following incubation all the slides were gently washed 3 times with PBS to remove unbound primary antibody. They were then incubated for 1 hour in a humidified chamber at 37°C, with FITC conjugated Anti-mouse IgG (whole molecule) (Sigma) diluted 1:200 with 3% BSA in PBST. At the end of the incubation all slides were rinsed gently 5 times with PBS and prepared for microscopy as detailed in 2.4.9.4. below.

2.4.9.3. ***Baboon testicular slide preparations***

Baboon testicular pieces were removed from storage at –70°C and immersed in liquid nitrogen. The tissues were mounted in OCT compound (Tissue-Tek, Miles, IN) and 6 µm sections were cut in a cryomicrotome. The cut sections were mounted on poly-L-lysine coated slides and were placed briefly at room temperature for the sections to adhere to the slides. They were then fixed in 100% methanol for 1 minute followed by 100% Acetone for 15 seconds, air-dried and stored at –20 °C till processed.

2.4.9.3.1. *Labeling of baboon testicular slides*

Testicular slides prepared as detailed above, were removed from –20 °C and allowed to equilibrate at room temperature for 5 minutes. The slides were then flooded with supernatants of mAbs 18.6 and IAM-1. Negative control slides (without primary antibody) were flooded with PBS. The slides were incubated for 1 hour in a humidified chamber kept at 37 °C. After 1 hour the slides were gently rinsed 3 times with PBS, then flooded with secondary antibody that was FITC conjugated Anti-mouse IgG (whole molecule, Sigma) diluted 1:1500 in 3% BSA in PBST. These were then incubated at 37 °C in a humidified chamber for 1 hour and then rinsed with PBS and prepared for microsocopy as detailed in 2.4.9.4 below.

2.4.9.4. ***Microscopy***

After 3 washes in PBS (following incubation with secondary antibody) the slides were mounted in Mowiol-Dabco anti-fade preparation, and sealed with a coverslip. The pattern and degree of immunofluorescence was examined with an Olympus BH2-RFCA phase-contrast microscope fitted with a UV lamp and filter. The Images were captured with the aid of a CCD camera linked to a computer with Scion software (Scion Corp.,USA).

For Confocal microscopy, following secondary antibody incubation the slides were gently washed with PBS and briefly air-dried. They were then mounted in Vectashield® Mounting Medium with Propidium Iodide (Vector Laboratories Incorporation) and sealed with a coverslip. The slides were viewed in a Leica TCS

4D Confocal microscope using a 400 mW laser, with a range from 457- 675nm (visible light), Krypton – Argon and Images captured with Scanware software v5.1a (Leica).

2.5. Results

2.5.1. *Indirect Immunofluorescence (IIF)*

2.5.1.1. Phosphotyrosine Proteins

2.5.1.1.1. *Human Spermatozoa*

Similar patterns of fluorescent labeling were observed using the two antiphosphotyrosine monoclonal antibodies (mAbs), PY20 and 4G10. On both human and baboon sperm there appeared to be significantly more intense fluorescence with mAb 4G10 than with mAb PY20, hence the former was preferred for further experiments. Depending on the time-lapse during capacitation, human sperm probed with mAb 4G10 as the primary antibody showed regional differences in patterns of immunofluorescence.

Freshly ejaculated human sperm (Fig. 3a) had fluorescence of the annulus, and the caudal parts of the principal piece. Following swim-up, most sperm had flourescence of the annulus and entire principal piece (Fig.3b). In some spermatozoa, there was additional fluorescence of the equatorial band and anterior acrosome. Following about 3 hours of capacitation *in vitro*, there was significant increase in the intensity of fluorescence of the principal piece and a portion at the neck - sperm head junction (Fig. 3c), most likely, the centrioles or segmental columns of the neck. At this stage the annulus could not be readily distinguished from the rest of the principal piece.

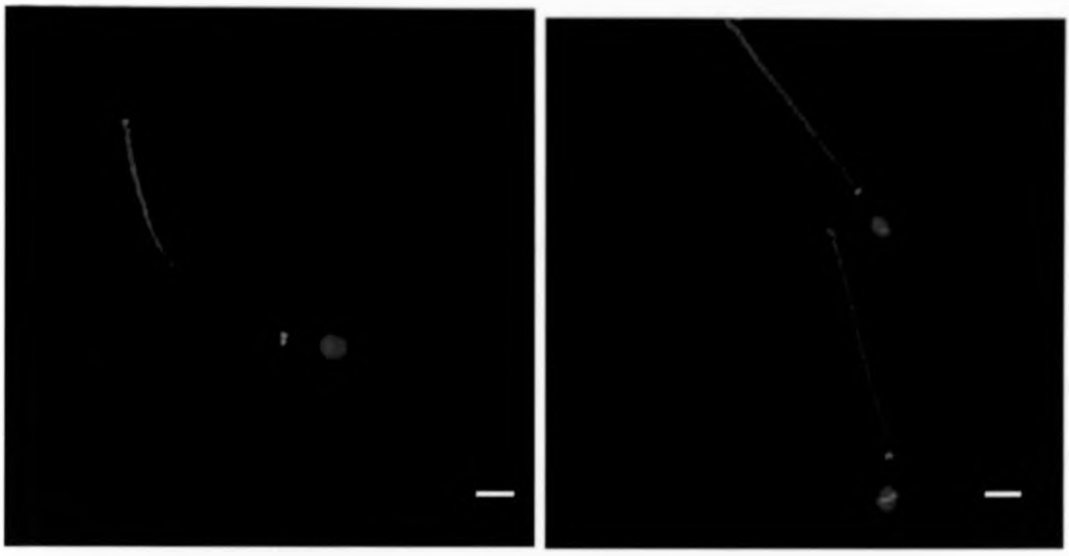


Fig. 3a, 3b Confocal pictures of human sperm showing initial anti-phosphotyrosine labelling of the annulus region and caudal principal piece of the tail in freshly ejaculated sperm that progresses to involve the entire principal piece in swim-up sperm . In some sperm there was also labeling of the anterior acrosome and equatorial segment. Each scale bar = 5μm

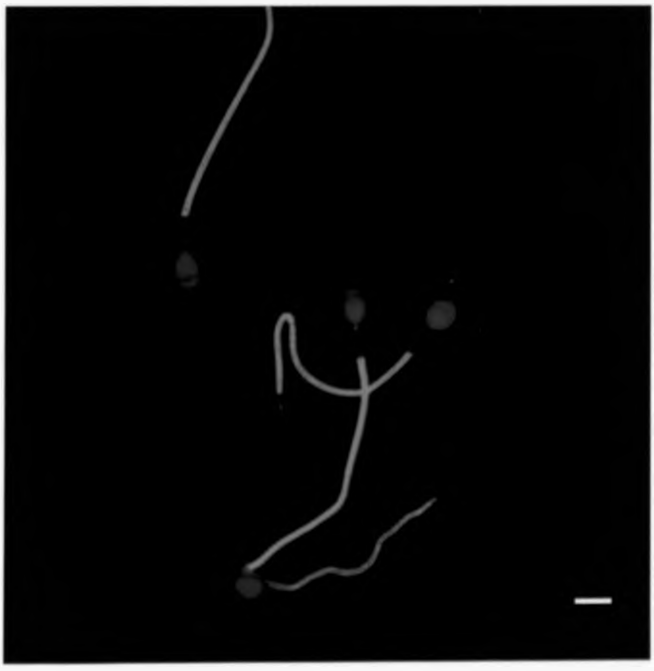


Fig. 3c Confocal image of human sperm capacitated for 3 hours showing intense epifluorescence of the principal piece of sperm tails. Scale bar = 5μm

2.5.1.1.2. *Baboon Sperm*

Baboon sperm used for IIF were swim-up sperm. Under these conditions, baboon sperm probed with mAb 4G10 had fluorescence of the equatorial band, anterior acrosome and post acrosomal regions of the head and parts of the middle piece (Fig. 4a). Other sperm had fluorescence of the entire principal and end pieces of the tail (Fig. 4b).

2.5.1.2. ***Monoclonal Antibody 18.6***

2.5.1.2.1. *Human and Baboon Sperm*

Human sperm probed with mAb 18.6 had intense fluorescence of the entire anterior acrosome (Figs. 5a, 5b). Baboon sperm probed with the same mAb had similar results, with epifluorescence of the anterior acrosome (Figs. 6a, 6b).

2.5.1.2.2. *Baboon Testis*

Indirect immunofluorescence of baboon testis was carried out using mAb 18.6. The cognate antigen was localised only on the acrosomes of luminal spermatozoa (Figs. 7a, 7b) within the seminiferous tubules.

2.5.1.3. ***Monoclonal Antibody IAM-1***

2.5.1.3.1. *Human Sperm*

Human sperm probed with IAM-1 as the primary antibody had epifluorescence of the anterior acrosome and the equatorial band (Figs. 8a, 8b). These findings were similar in both capacitated and non-capacitated human sperm.

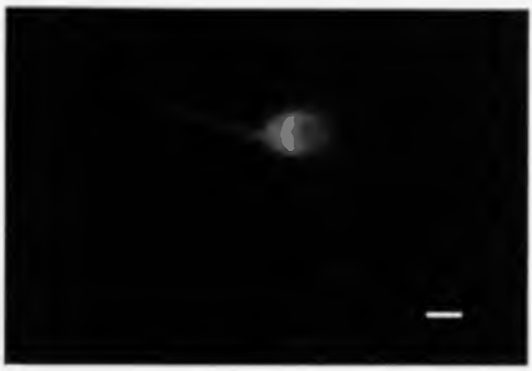


Fig. 4a Baboon sperm probed with anti-phosphotyrosine 4G10 showing epifluorescence of the equatorial band , anterior acrosomal and post acrosomal region of the head and the middle piece. Scale bar = 2.8 μm

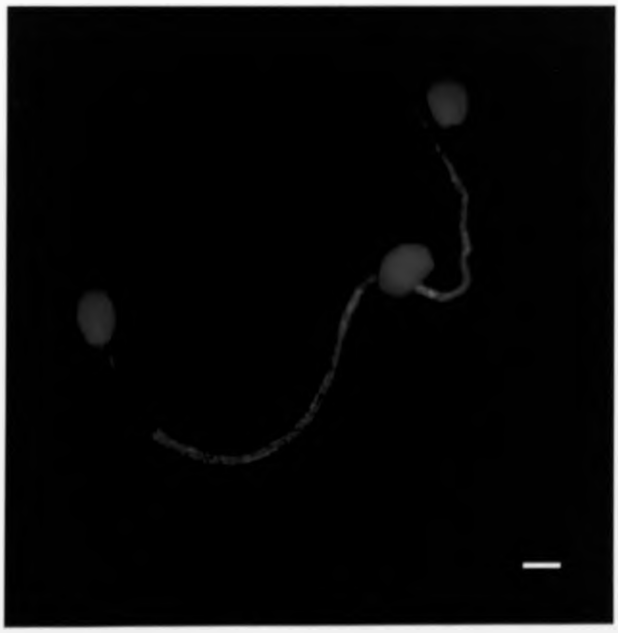


Fig. 4b Confocal picture of swim–up Baboon sperm probed with anti-phosphotyrosine 4G10, showing epifluorescence of the principal and end pieces of the tail. Scale bar = 2.8μm

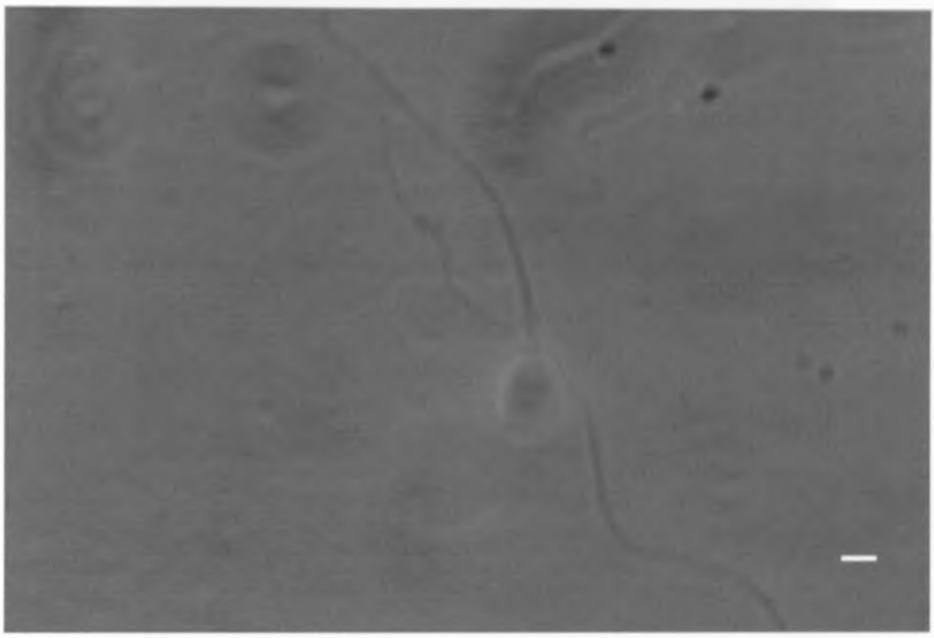


Fig. 5a Phase contrast micrograph of human sperm. Scale bar = 1.9μm

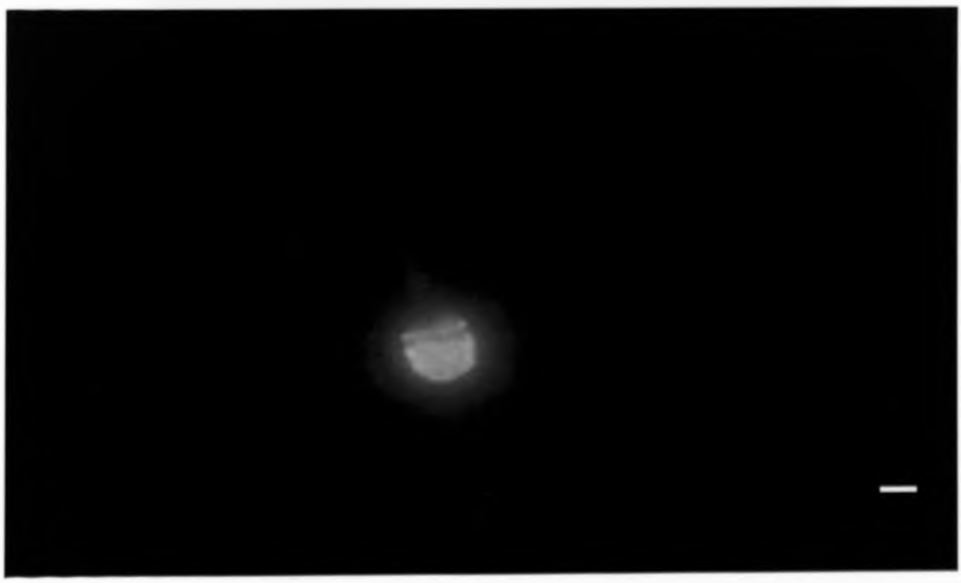


Fig. 5b Epifluorescence of the anterior acrosome of human sperm probed with mAb 18.6. Scale bar = 1.9μm

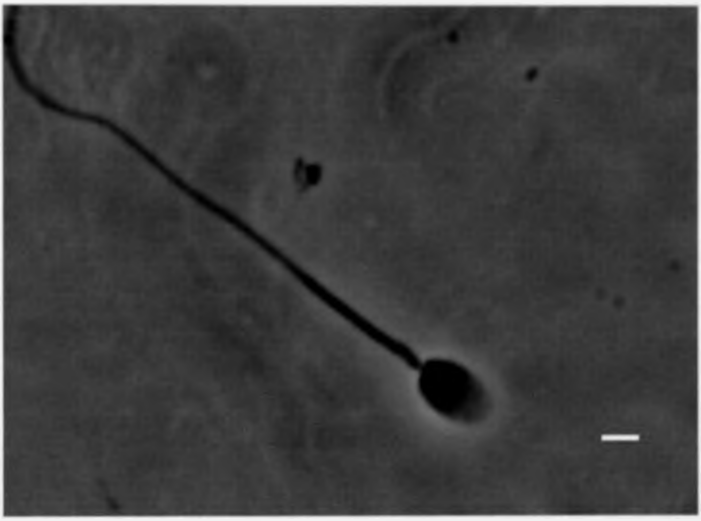


Fig. 6a. Phase contrast micrograph of baboon sperm. Scale bar = 2.2μm

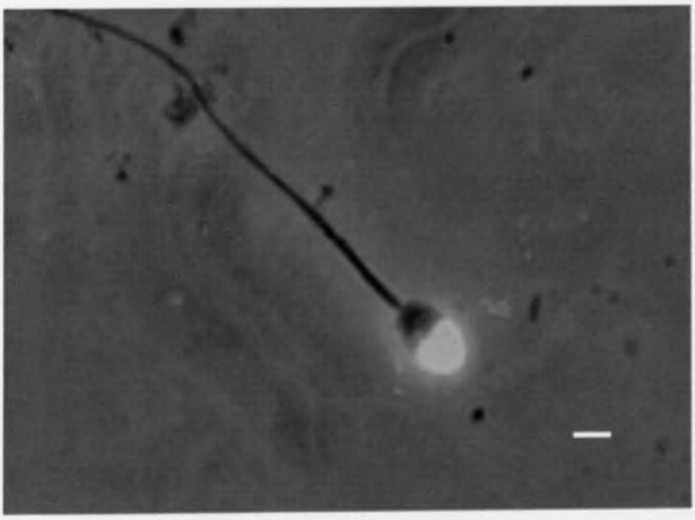


Fig. 6b Baboon sperm probed with mAb 18.6 showing epifluorescence of the anterior acrosome. Scale bar = 2.2μm

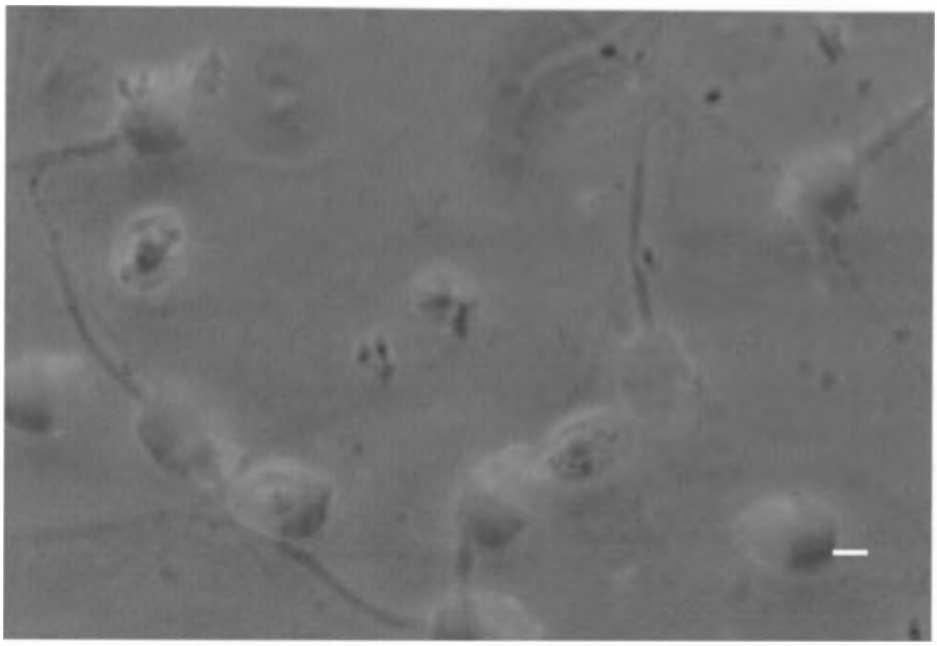


Fig.7a Phase contrast micrograph of luminal spermatozoa in baboon seminiferous tubules. Scale bar = 1.6μm

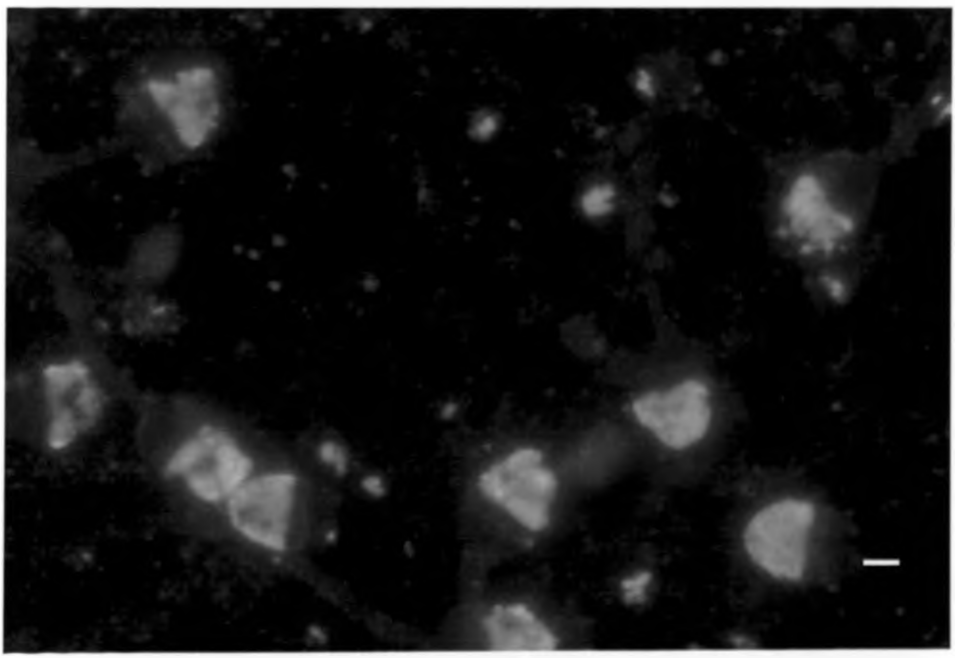


Fig.7b Luminal spermatozoa of the baboon seminiferous tubules probed with mAb 18.6. There is fluorescence of the anterior acrosomes of the spermatozoa. Scale bar = 1.6μm

2.5.1.3.2. *Baboon Sperm*

There were two patterns of staining observed, one where there was epifluorescence of the equatorial band together with patchy staining of the anterior acrosome (Fig. 9a) and a second pattern where there was intense fluorescence along the equatorial band only (Fig. 9b). Predominantly more of the swim-up baboon sperm had second pattern of epifluorescence with only the equatorial band stained.

2.5.1.3.3. *Baboon Testis*

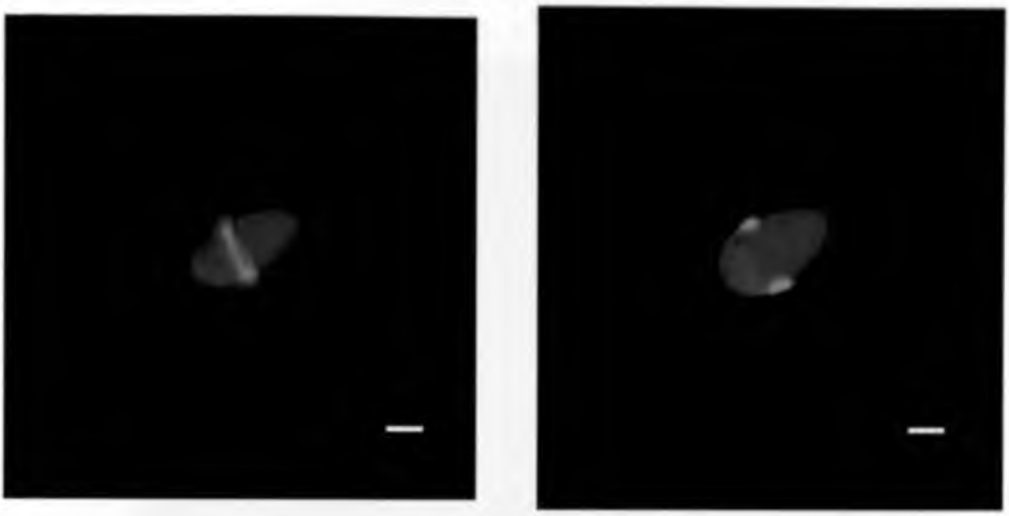
Baboon testis probed with IAM-1 had fluorescence concentrated toward the luminal aspect of the seminiferous epithelium around the maturing spermatids and on luminal spermatozoa (Figs. 10a, 10b).

2.5.1.4 ***Monoclonal antibody ES-1***

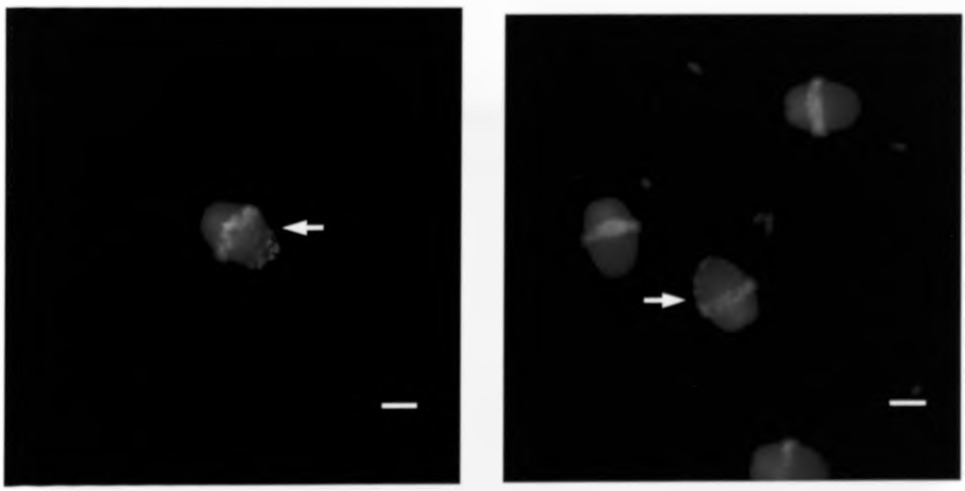
Baboon sperm probed with mAb ES-1 had fluorescence of the equatorial band, part of the post-acrosomal region of the head and the tail especially the middle piece (Fig. 11a). Human sperm on the other hand probed with the same antibody had almost similar pattern of staining with fluorescence of the equatorial band, post-acrosomal region and most of the tail (Fig.11b).

2.5.1.5. ***Monoclonal antibody M1***

Baboon and human sperm probed with mAb M1 had no fluorescence (Figs. 12a, 12b) implying lack of this particular antigen on sperm in both species.



Figs. 8a, 8b Confocal images of human sperm at different planes, demonstrating equatorial segment and anterior acrosome localisation of the cognate antigen of mAb IAM-1. Each scale bar = 1.5μm



Figs. 9a, 9b
Confocal Images of swim-up baboon sperm probed with mAb IAM-1. The cognate antigen was localized predominantly at the equatorial segment of the spermatozoa but some (arrows) had both anterior acrosome and equatorial segment localisation. Each scale bar = 2μm

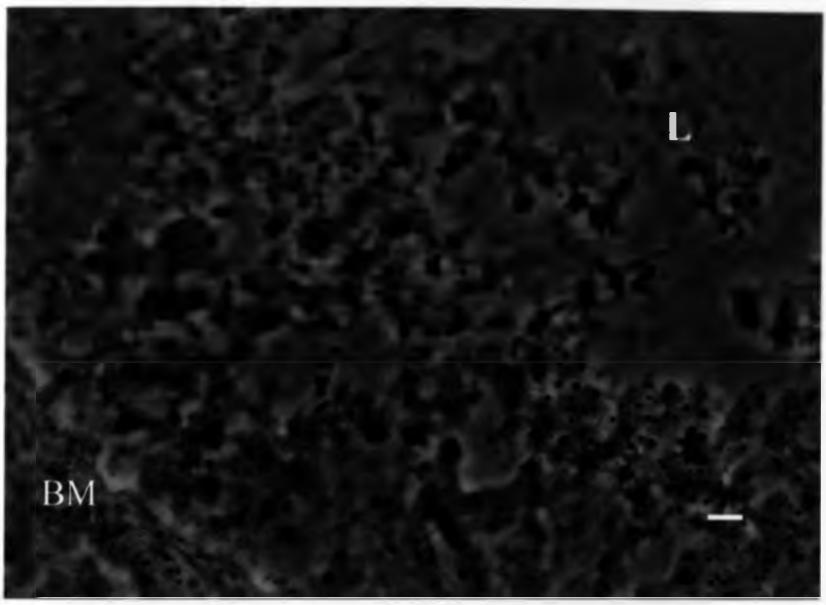


Fig. 10a Phase contrast photomicrograph of baboon seminiferous epithelium. BM: Basement membrane, L:Lumen. Scale bar = 5μm

Fig. 10b Baboon seminiferous epithelium showing immunolocalisation of the cognate antigen to IAM-1 in the part of the epithelium adjacent to the lumen (L) of the tubule. BM:Basement membrane, L:Lumen. Scale bar = 5μm

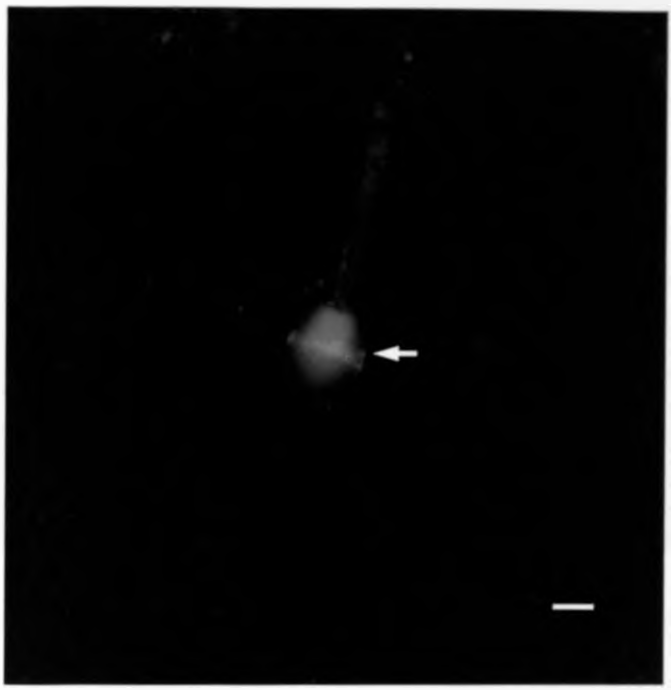


Fig. 11a Confocal image of baboon sperm probed with mAb ES-1. The cognate antigen is mainly localised at the equatorial segment (arrow). Scale bar = 1.9μm

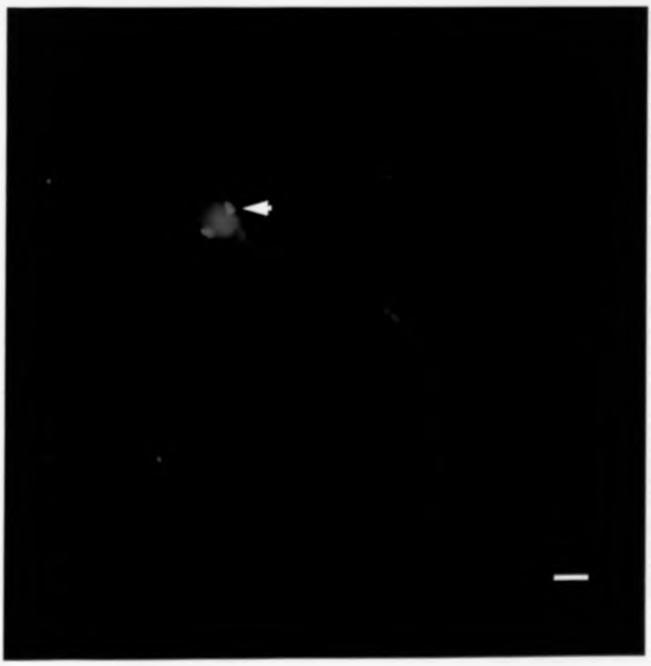


Fig. 11b Confocal Image of human sperm that has been probed with mAb ES-1. The antigen is localised mainly at the equatorial segment (arrow) and along the sperm tail. Scale bar = 2.5μm

Fig. 12a Phase contrast micrograph of baboon sperm. Scale bar

Fig. 12b Micrograph of the baboon sperm in the figure above pr
with monoclonal antibody M1. There is no epiflouresence of the h
or the tail of the sperm. Scale bar = 3.6μm

2.5.2. Immunoblots

2.5.2.1. *IAM-1*

Monoclonal antibody IAM-1 (mAb IAM-1) recognised four bands of apparent molecular weights 28, 32, 39 and 45 kDa on immunoblots of human sperm proteins (Figs.13 and 15), under non-reducing conditions. On baboon sperm immunoblots, mAb IAM-1 recognised 3 protein bands at apparent molecular weights, 32, 38 and 44 kDa under non-reducing conditions (Fig.15). The three immunoreactive bands recognised by mAb IAM-1 on baboon sperm immunoblots appeared similar to three bands of approximately the same apparent molecular weights found on the human sperm immunoblots (Fig.15).

2.5.2.2. *ES-1*

Monoclonal antibody ES-1 (mAb ES-1) was immunoreactive with several bands of protein on both human and baboon sperm immunoblots (Fig. 15). In baboon sperm, mAb ES-1 recognised bands of apparent molecular weights, 24, 28, 40, 47, 50 and 71 kDa. In human sperm, immunoreactive bands were at 34, 38, 47 and 50 kDa. The mAb ES-1 recognised similar protein bands of apparent molecular weights 47 and 50 kDa in both human and baboon sperm (Fig.15).

2.5.2.3. *Phosphotyrosine Proteins*

Immunoblots of baboon testis and sperm probed with anti-phosphotyrosine monoclonal antibodies PY20 and 4G10 revealed presence of several phosphotyrosine containing proteins of low, medium and high apparent molecular

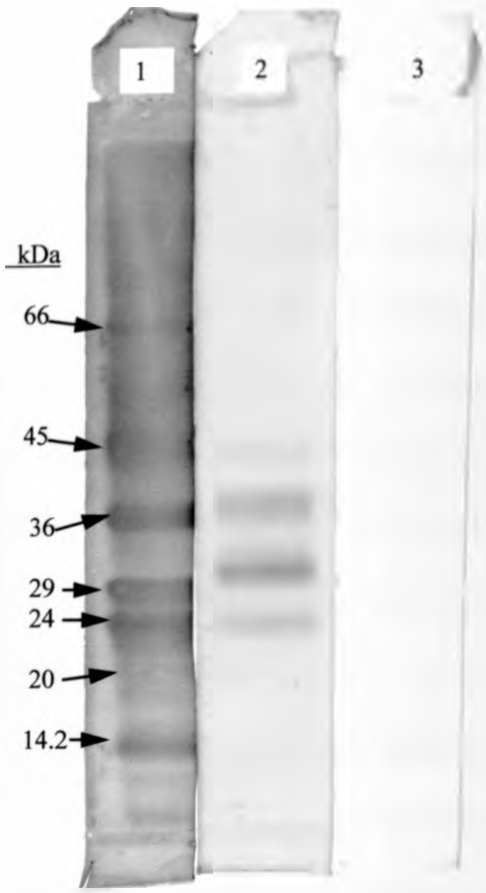


Fig. 13 Immunoblot of human sperm probed with mAb IAM-1 and detected with alkaline phosphatase. Four immunoreactive bands were evident between ~ 26 and 44 kDa under non-reducing conditions.Lane 1– molecular weight marker, lane 2– human sperm extract and lane 3– control.

weights. In baboon testis immunoblots, bands of apparent molecular weights; 32, 41, 45, 55, 62, 90, 107 and 129 kDa were observed (Fig.14a). Baboon sperm immunoblots had phosphotyrosine containing proteins of apparent molecular weights 29, 37, 44, 47, 57, 66, 82, 105, 118 and 145 kDa (Fig.14b).

2.5.2.4. ***Differential extraction of human sperm proteins***

Different protein bands were resolved on Western blots of extracts of human sperm proteins obtained using various solutions (Table 3). A strongly ionic solution of 1M NaCl extracted several proteins ranging from 16 – 70 kDa. However, the cognate protein bands recognised by mAb IAM-1 that would have fallen within this range were not represented in this group (Table 3, Figs 16a, 16b). Solutions with high concentration of Chaotropes, 9M Urea and 2M Thiourea and detergents Triton X-100 and ASB-14 extracted the IAM-1 protein that was resolved on immunoblots as 2 bands of apparent molecular weights 24 and 28 kDa. Using the 7M Urea lysis solution, it appeared as if too little of the IAM-1 protein was extracted to be detectable by ECL on the blot though other protein bands were detectable at 28 and 29 kDa in the same sample (Table 3, Figs. 16a,16b). Indirect Immunofluorescence performed on sperm following extraction of sperm proteins in the various solutions, indicated presence of the IAM-1 protein at the inner acrosomal membrane and equatorial band of sperm heads that had been sonicated then treated with 1M NaCl solution and no epifluorescence on sperm treated with the other extraction solutions (Table 3).

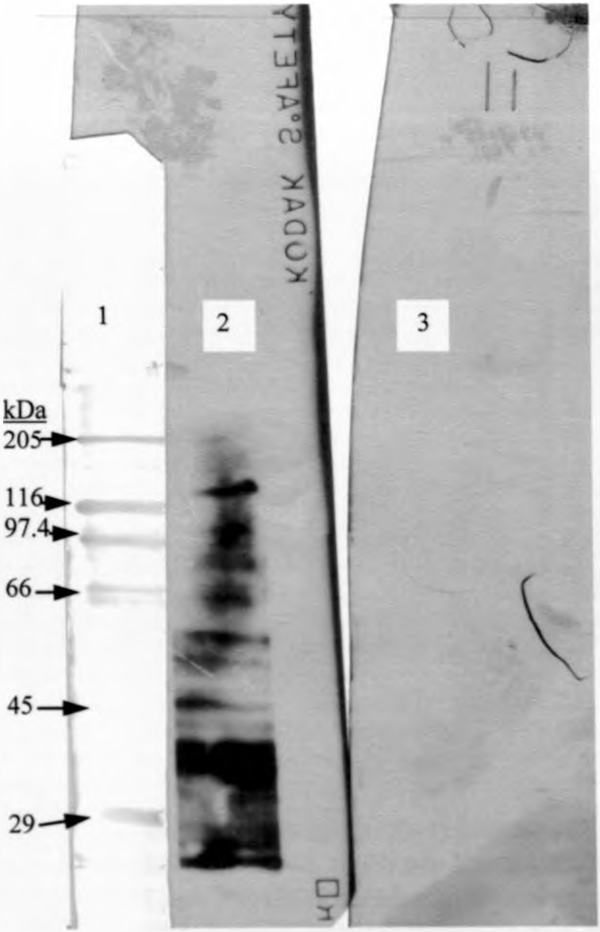


Fig. 14a Baboon testis immunoblot probed with the antiphosphotyrosine mAb PY-20 and detection done using ECL. Several bands ranging from ~ 32kDa to 128kDa were recognized.Lane 1 - molecular weight markers ,Lane 2 - ECL overlay of baboon testis extract, Lane 3 - ECL overlay of control lane.

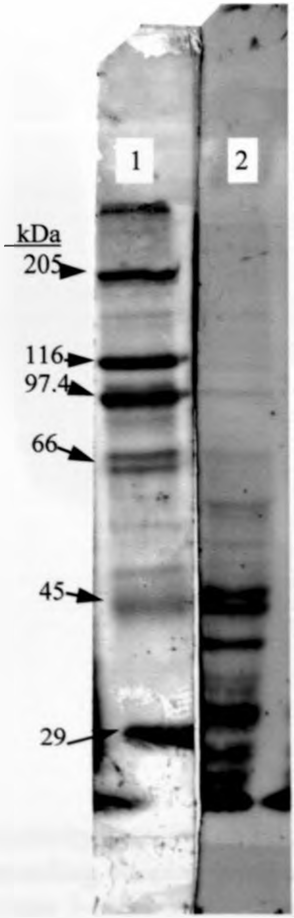


Fig. 14b Immunoblot of Baboon sperm probed with antiphosphotyrosine mAb 4G10 and detection done using alkaline phosphatase.Several immunoreactive bands were evident between ~ 28 and 145 kDa. Lane 1– molecular weight markers and lane 2– the baboon sperm lane.

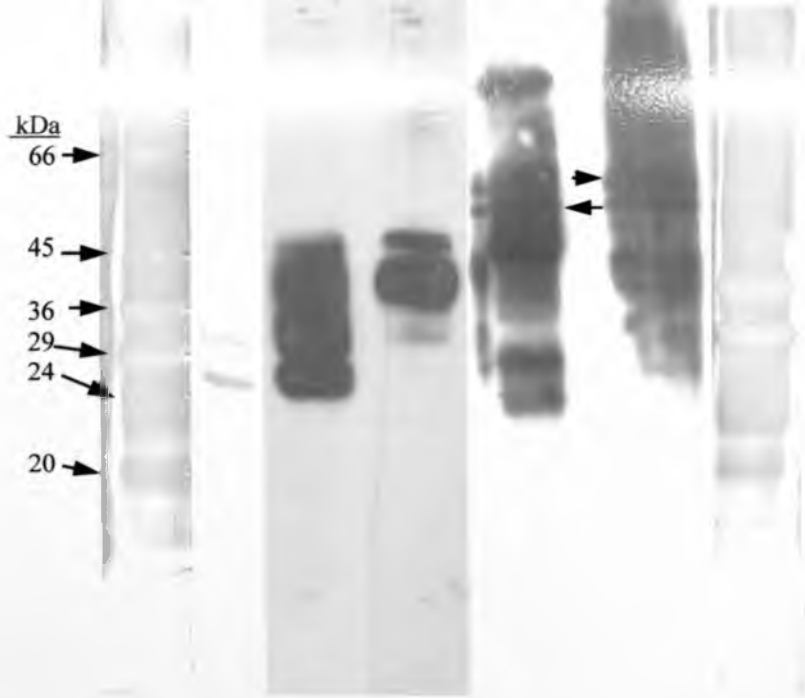


Fig. 15 Immunoblots of human and baboon sperm showing immunoreactivity to two monoclonal antibodies, IAM-1 and ES-1. Lanes 1 and 7 are low molecular weight markers with the corresponding weights in kilodaltons adjacent to Lane 1. Lane 2-IAM-1 on human sperm using alkaline phosphatase detection, Lane 3-IAM-1 on human sperm using ECL detection, Lane 4-baboon sperm IAM-1 using ECL detection, Lane 5-ES-1 on baboon sperm using ECL detection and Lane6–ES-1 on human sperm using ECL detection. Arrows on lanes 5 and 6 show common bands.

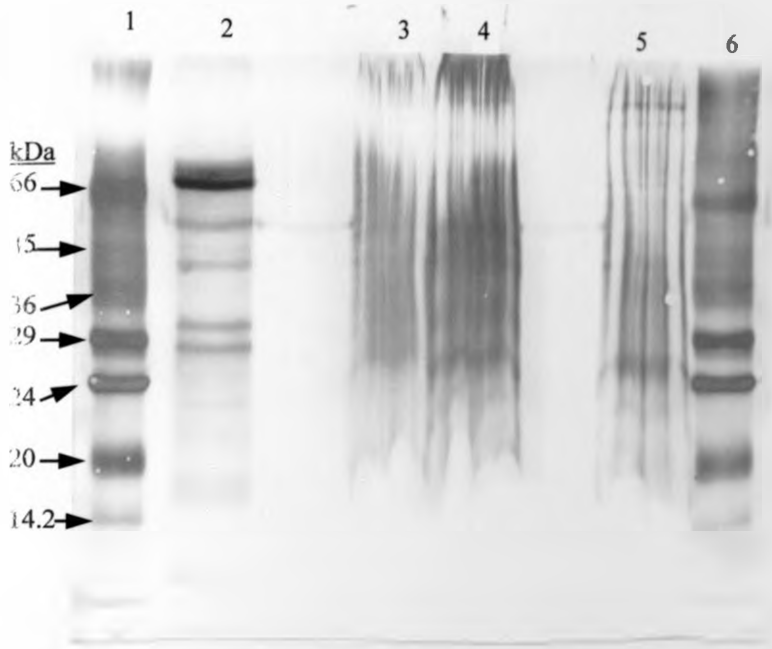


Fig. 16a Colloidal gold total protein staining of a western blot following extraction of whole human sperm proteins using different solubilizing agents. Lanes 1 & 6 are low molecular weight markers, Lanes 2-5 are sperm extracts using different treatments, 2-1M NaCl, 3 - 7M Urea lysis solution, 4 - 9M Urea lysis solution, 5 - 9M Urea and Thiourea lysis solution.

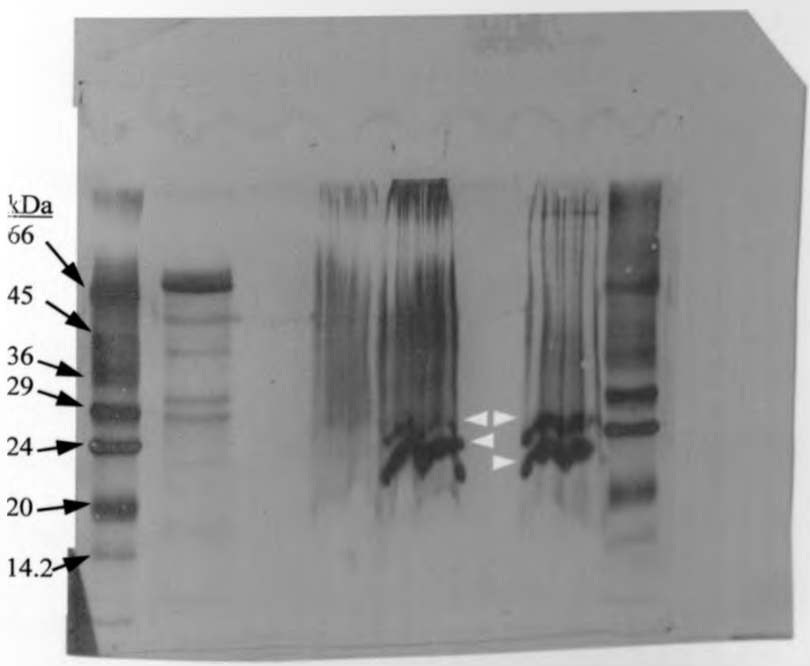


Fig. 16b ECL Kodak film overlay of the above western blot of human sperm proteins showing detection of bands immunoreactive to mAb IAM-1 (white arrowheads) in only lanes 4 and 5 at ~ 23kDa and 27 kDa

Table 3. Summary of total protein staining, immunoblot and IIF results on human sperm following protein extraction using different solutions.

Extraction Solutions	Bands of Protein extracted (kilodaltons)	Bands Immunoreactive to IAM-1	Sperm Indirect Immunofluorescence (IIF) after protein extraction
1M NaCl	16, 23, 29, 32, 42, 45, 49,70	-	+
7M Urea, 2% ASB14, 8mM PMSF, 0.5% CA, 1% Triton X-100, 2M Thiourea	28, 49	-	-
9M Urea, 2% ASB14, 8mM PMSF, 0.5%CA, 1% Triton X-100	24, 26, 28, 48	24, 28	-
9M Urea, 2% ASB14, 8mM PMSF, 0.5% CA, 1% Triton X-100, 2M Thiourea	24, 28, 38, 43	24, 28	-

1-D SDS-PAGE was performed and resultant Western Blots stained with Colloidal Gold total protein stain then subsequent immunoblotting done on the same blots using mAb IAM-1 with ECL detection. IIF was done on the sperm following protein extraction. ASB14 - Amidosulfobetaine detergent , PMSF -

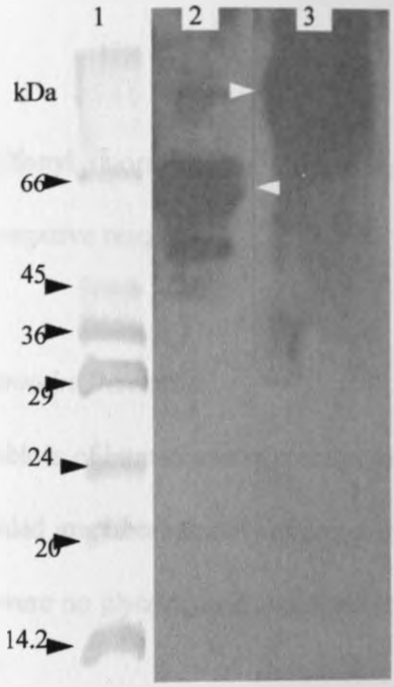


Fig. 17 Total carbohydrate detection on human sperm protein extract using the ECL glycoprotein detection system (Amersham). Lane 1– low molecular weight markers, lane 2 - positive control ECL overlay for transferrin, lane 3 – ECL overlay of lysis buffer extract of human sperm. The white arrowheads show bands positive for glycoproteins

Phenylmethylsulfonyl fluoride, CA- Carrier Ampholyte pH 4 – 7., + indicates positive IIF. – negative results.

2.5.2.5. ***Glycoprotein Detection***

Immunoblots of human sperm proteins extracted under non reducing conditions revealed immunoreactivity of proteins of molecular weights greater than 66 kDa. There were no glycoproteins detected at the level of the bands representing IAM-1 (Fig.17).

2.6. Discussion

There is a lot of similarity in results between human and baboon sperm immunoblots and Indirect Immunofluorescence (IIF) labeling, using different primary monoclonal antibodies.

2.6.1. IAM-1

2.6.1.1. *Immunoblotting and Immunolocalisation*

mAb IAM-1 recognises epitopes of 4 bands of proteins of apparent molecular weights, 28, 32, 39, and 45 kDa on human sperm immunoblots. Similar results were observed on human sperm by Al-Eisa *et al.*, (2001) with a ladder of 3 to 4, 32-42 kDa proteins on immunoblots. The bands immunoreactive to this antibody in baboon sperm immunoblots, were of similar molecular weights to those of human sperm. However, the band with the lowest molecular weight (28kDa) was not evident in baboon sperm immunoblots. Therefore, based on similarity in apparent molecular weight of immunoreactive proteins, there was similarity in the proteins recognised by mAb IAM-1 in baboon and human spermatozoa.

Presence of heterogenous peptides immunoreactive to the same antibody had been observed in SP-10, an intra-acrosomal protein in human sperm and sperm agglutination antigen-1 (SAGA-1) or CD52 that is found on the sperm plasma membrane (Herr *et al.*, 1992; Diekman *et al.*, 1997; Diekman, 2003). These researchers attributed this phenomenon to either the hydrolysis of the protein by

acrosomal proteases or to alternative splicing mechanisms occurring during synthesis of the protein. However, despite the inclusion of protease inhibitors in the extraction solutions, there was the presence of multiple mAb IAM-1 immunoreactive bands as a consistent feature in different human sperm samples tested, suggestive of the existence of different isoforms of the cognate protein, most likely due to alternative splicing.

Indirect Immunofluorescence of baboon and human spermatozoa using the same mAb demonstrated similarity in the pattern of labeling in sperm from the two species, with epifluorescence of the anterior acrosome and equatorial band. However in the baboon spermatozoa used in this study, there was predominantly epifluorescence of the equatorial band unlike in human spermatozoa where there was intense labeling of both the anterior acrosome and equatorial segment. Confocal microscopy revealed greater intensity of FITC labeling at the equatorial segment in human sperm compared to the anterior acrosome. It is not clear whether the pattern of staining in baboon sperm is as a result of changes resulting from commencement of the capacitation process during the swim-up process or whether in this species this antigen is actually predominantly localised in the equatorial segment of sperm. IIF of baboon testis also revealed presence of IAM-1 in the germinal epithelium especially within the seminiferous epithelium around maturing spermatids and on the luminal spermatozoa. This is consistent with findings in human testicular sections by Al-Eisa *et al.*, (2001) who observed that this antigen

confirmed presence of IAM-1 labeling on sperm heads that had been subjected to NaCl treatment and no labeling on sperm that had been subjected to the detergent - chaotrope combination. These results suggest that IAM-1 is a protein with relatively hydrophobic amino acid residues, possibly intimately related to the lipid portion of the sperm acrosomal membranes as an integral membrane protein or well-anchored to the membrane, hence the inability of strongly ionic solutions to extract it (Santoni *et al.*, 2000). Detergent-chaotrope lysis solution however, overcame this due to the amphipathic nature of detergent molecules that disrupt the arrangement of the bilipid layer of the sperm membranes and concomitantly the bonding of this protein to the lipid layer (Rabilloud, 1996).

Glycoprotein detection done on human sperm protein extract under non-reducing conditions revealed presence of glycoproteins of molecular weights greater than 66 kDa. IAM-1 under these conditions, had polypeptides of molecular weights ranging from 26 – 44 kDa. This was observed on parallel immunoblots of the same samples probed with the mAb IAM-1. These results suggest that IAM-1 is not a glycoprotein. Work done by Al-Eisa *et al.*, (2001) on IAM-1 demonstrated its location on the inner acrosomal membrane of acrosome reacted human sperm. Sperm glycoproteins are particularly associated with its plasma membrane as peripheral and integral membrane proteins although some are found in the acrosomal matrix. They are important in the interactions of sperm and their immediate environment during; the maturation process in the epididymis,

capacitation within the female tract and finally sperm-zona interactions (Yanagimachi, 1994; Diekman, 2003). The finding that IAM-1 is non-glycosylated would be consistent with its location on the inner acrosomal membrane and inner side of the equatorial segment, parts of the sperm that are not in direct contact with its immediate environment until after acrosome reaction. It therefore appears from our data, that IAM-1 is a polymorphic, non-glycosylated protein that is an integral membrane protein or a protein well-anchored to the lipid component of the inner acrosomal membrane and equatorial segment.

2.6.2. ES-1

mAb ES-1 recognised several bands on immunoblots of both baboon and human spermatozoa under reducing conditions. Among the bands that appeared common to both species were those at molecular weights 38 – 40, 47 and 50 kDa. Earlier findings (Al-Eisa *et al.*, 2001) indicated this antibody recognised a single band of an apparent molecular weight of 40kDa in human sperm. In our experiments there appear to be at least four bands on human sperm and about six in baboon. IIF of baboon spermatozoa revealed labeling predominantly at the equatorial segment. This pattern was also observed in human spermatozoa. However, the middle piece was also labeled in baboon spermatozoa while in human sperm the entire tail was also labeled. The proteins responsible for the tail labeling could possibly account for the other bands observed in immunoblots of sperm in the

two species. It should however be noted that in both baboon and human spermatozoa the predominant labeling was that of the equatorial band. The tail epifluorescence was not as intense as that of the equatorial band. Though Al-Eisa *et al.*, (2001) had observed a single band of ~ 40kDa our data indicated more than one band on immunoblots in both species. This could have arisen due to use of different batches of supernatant of the mAb ES-1 for our experiments. It is therefore possible that our supernatant was not entirely monospecific to the particular epitope localised at the equatorial segment but additionally, recognised other epitopes of tail proteins in both human and baboon spermatozoa. It is however noteworthy that there was close similarity in the molecular weights of immunoreactive bands recognised by the ES-1 supernatant in man and baboon sperm immunoblots and there was definite labeling for this antigen at the equatorial segment of spermatozoa in both species. ES-1 has been implicated in sperm-oocyte interaction, specifically sperm-oolemmal interaction with mAb ES-1 significantly inhibiting human sperm-zona free hamster oocyte interaction (Al-Eisa *et al.*, 2001). The antigen ES-1 is localised on the surface of the outer acrosomal membrane in human spermatozoa although it migrates to the plasma membrane surface following acrosome reaction (Al-Eisa *et al.*, 2001). This antigen could therefore be an important antigen in human and possibly baboon sperm-oolemmal binding and fusion process.

2.6.3. Phosphotyrosine Proteins

Protein tyrosine phosphorylation is one of the key intracellular events that take place during capacitation and acrosome reaction of mammalian spermatozoa affecting subsequent sperm-egg interactions (Naz, 1996b). The present study demonstrated presence of phosphotyrosine containing proteins in baboon spermatozoa and testis through immunoblots and also through indirect immunofluorescence of baboon sperm. Indirect immunofluoresence localised tyrosine phosphorylated proteins on human sperm too. Anti-phosphotyrosine monoclonal antibodies 4G10 and PY20 used were specific for phosphotyrosine residues in protein.

There was similarity in patterns of IIF labeling of human and baboon spermatozoa using anti-phosphotyrosine monoclonal antibodies implying similarity of proteins involved in the two species. In human spermatozoa there were progressive changes in the anti-phosphotyrosine labeling of sperm right from freshly ejaculated state through swim-up to capacitated state after 3 hours incubation. In non-capacitated spermatozoa of both species, the annulus and caudal parts of the principal pieces were labeled, with some additionally having labeling of the anterior acrosome and equatorial segment.

In a number of swim-up baboon spermatozoa, the principal and end pieces of the flagellum were labeled. It was also noted that capacitation in human spermatozoa is associated with certain changes including, increased intensity of

labeling of the principal piece and labeling in the region of the centrioles. Carrera *et al.*, (1996) also observed labeling in the neck region of capacitated human sperm though they did not expound on its significance. The centriolar apparatus of spermatozoa is known to be structurally related to the axoneme and possibly the latter is also involved in hypermotility observed in capacitated sperm. It has been reported that some phosphotyrosine containing proteins could be related to the axoneme in the boar (Berruti and Martegani, 1989). It is therefore possible that some phosphotyrosine proteins could be related to the centrioles too and are involved in capacitational changes in the sperm flagellum. The other possible alternative is labeling of the segmental columns in the neck region or connecting piece. These columns are structurally continuous caudally as the outer dense fibres that are also related to the fibrous sheath of the principal piece of the tail. It would be expected therefore that the same proteins contribute to the formation of these structures and hence the presence of phosphotyrosine containing proteins in the neck segment. However, during capacitation, the middle piece did not show labeling by IIF. The reason for this is unclear if the foregoing is true. It is possible that presence of a helical mitochondrial sheath around the outer dense fibres in the middle piece reduces accessibility of the antibodies to the phosphorylated proteins of the outer dense fibres. This has been corroborated by findings of other experiments in the past which indicated that the mitochondria of the middle piece could form a barrier reducing access of chemicals to the outer dense fibers here

(Kim *et al.*,1997). It has also been suggested that there could be actual differences in the chemical composition of the outer dense fibres of the middle piece and those of the principal piece, a possibility that could also explain the different responses observed in chemical treatments on these different segments of the sperm tails (Kim *et al.*, 1997). Labeling at the neck region of capacitated human spermatozoa therefore needs to be clarified using immuno-electron microscopy to elucidate the actual structures involved. The annulus demarcates the middle piece from the principal piece of the tail. The labeling of the annulus in non-capacitated sperm could possibly be due to initial phosphorylation of tyrosine containing proteins commencing at this point and then spreading to involve the entire principal piece in the course of capacitation. It has been observed that capacitation is associated with increased intensity of phosphotyrosine labeling on human spermatozoa particularly the labeling of the acrosome (Naz *et al*, 1991; Naz, 1996b). Furthermore, it is also known that in humans, at least three sperm flagellum proteins undergo tyrosine phosphorylation during capacitation. Two of these, 105 and 81 kDa, were localised in the fibrous sheath of human spermatozoa (Leclerc *et al*, 1997) while the third, a 95 kDa protein, was specifically localised on the ribs of the fibrous sheath of human sperm tail (Mandal *et al.*, 1999). In our study, there was significantly increased phosphotyrosine labeling of the principle piece of human sperm after 3 hours incubation. These sperm at this point demonstrated hypermotility. These labeling changes could be due to the previously identified fibrous sheath proteins

undergoing phosphorylation that is associated with hypermotility. Indeed the hyperactivated motility of sperm is now accepted as a concomitant feature of capacitation (Mortimer *et al.*, 1998) Phosphorylation of tyrosine containing proteins of the sperm flagellum associated with hypermotility, has also been observed in the mouse (Urner *et al.*, 2001) and hamster (Si and Okuno, 1999). We did not however, observe any significant increase in phosphotyrosine labeling of the acrosome of capacitated sperm. There appear to be different observations on patterns of tyrosine phosphorylation in human sperm:

i) Some authors have observed changes of IIF phosphotyrosine labeling in human sperm from tail regions in non-capacitated sperm, to the acrosomal regions in capacitated or zona exposed sperm (Naz *et al.*, 1991; Naz, 1996b).

ii) Others however, have found that under capacitating conditions human sperm show predominant labeling of the principal piece and neck region (Carrera *et al.*, 1996).

It has also been observed by other investigators that even in the same batch of spermatozoa there is heterogeneity of labeling for phosphotyrosine containing proteins after capacitation (Carrera *et al.*, 1996; Urner *et al.*, 2001), an observation attributed to presence of relatively high percentages of abnormal spermatozoa in human ejaculate (Carrera *et al.*, 1996) and also possibly due to the fact that ejaculated human spermatozoa are not all homogenously mature. This is supported

by recent data that has shown existence of substantially different human sperm subpopulations in each normal ejaculate, each with varied rates of tyrosine phosphorylation in response to *in vitro* capacitating conditions (Buffone *et al.*, 2004). However, under the capacitation conditions used in our study there were predominant changes in labeling of the sperm flagellum in human spermatozoa.

Immunoblots of baboon testis and spermatozoa identified low, medium and high molecular weight phosphotyrosine containing proteins. Some of the proteins had almost similar apparent molecular weights in baboon testis and spermatozoa and possibly were the same proteins. Naz and Ahmad, (1994) reported presence of 14-18 kDa, 51kDa, 63kDa and 95kDa phosphotyrosine containing proteins. Our studies did not however include demonstration of changes in degree of tyrosine phosphorylation of these protein bands following capacitation of baboon sperm. This, coupled with possible effects of zona proteins on the baboon sperm protein tyrosine phosphorylation, would be a possible area for further study in an endeavour to understand capacitational changes in spermatozoa of this species.

2.6.4. M1

mAb M1 was tested by indirect immunofluorescence on human and baboon sperm where it did not recognise any determinant in both species. This particular antibody had been raised against purified hamster sperm heads on which it recognised an epitope at the equatorial segment of hamster sperm (Noor and

Moore, 1999). This antibody cross-reacted with spermatozoa from a number of mammals (Noor and Moore, 1999). However, in our experiments there was no evidence of cross-reactivity with human and baboon spermatozoa implying that the particular determinant was not conserved in these primates.

2.6.5. 18.6

Monoclonal antibody 18.6 labeled the anterior acrosome in both baboon and human spermatozoa. Baboon testis probed with the same antibody had FITC labeling on luminal spermatozoa and none on the germinal epithelium of the seminiferous tubules. The mAb 18.6 has been used as a specific marker of the acrosome (Moore *et al.*, 1990). The cognate antigen is localised to the acrosomal membranes and contents of human spermatozoa. This antigen appears to be one that is conserved in spermatozoa of eutherian mammals as it has been observed in a number of species including rodents. However, a precise role of this antigen in the fertilisation process is still unclear as the antibody does not affect sperm-zona free hamster and sperm-zona (intact) binding interactions in hamster and humans (Moore *et al*, 1990). Nevertheless its conservation in mammalian sperm and localization on acrosomal membranes and acrosomal matrix is suggestive of a role in the fertilisation process. Our results confirm earlier observations on localization of the antigen at the anterior acrosome of human spermatozoa and additionally demonstrate presence of the same antigen at a similar locus in baboon spermatozoa.

Presence of the antigen on luminal spermatozoa and not the seminiferous epithelium could imply that the epitope for mAb 18.6 becomes "unmasked" just at the time of spermiation or the final modifications to the antigen resulting in the immunoreactive epitope occurs almost at spermiation.

2.6.6. Conclusions

The studies demonstrate presence of shared antigens like IAM-1, ES-1, Phosphotyrosine proteins and 18.6 in man and the baboon spermatozoa using both indirect immunofluorescence (IIF) and immunoblots of 1-D gels. Some antigens found in rodents like M1 appear not to be conserved in primates. IAM-1 is a non-glycosylated integral membrane protein or a protein well anchored to the inner acrosomal and equatorial segment membranes.

CHAPTER 3

2-D PAGE AND BIOCHEMICAL ANALYSIS OF IAM-1 BY MASS SPECTROMETRY

3.0. Introduction

3.1. One vs. Two-dimensional gel electrophoresis

One dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) offers a means of separating proteins on the basis of their molecular weight. The drawback with this mode of separation is the limited resolution it offers making it impossible to distinguish between proteins of similar molecular weight and post-translationally modified forms of the same protein (Graves and Haystead, 2002). Two-dimensional (2-D) SDS-PAGE offers a reproducible method of resolving individual proteins from a complex mixture on the basis of both their charges and molecular weights (Dove, 1999) thus providing a solution to some of the problems inherent in the one-dimensional separation. Furthermore, 2-D gels enable separation of isoforms and post-translationally modified proteins on the basis of their charge differences (Brewis, 1999).

3.2. 2-D gel electrophoresis

3.2.1. Principles of 2-D gel electrophoresis

Two-dimensional (2-D) gel electrophoresis is a technique for separation of proteins involving a combination of a first dimension where proteins are separated along a pH gradient on the basis of differences in their charges (isoelectric focusing) and a second dimension where focused proteins are further separated on an SDS polyacrylamide gel on the basis of their molecular weights (O'Farrel, 1975; Fichmann and Westermeier, 1999).

Isoelectric focusing (IEF) is possible as amino acids are amphoteric molecules that either possess a net negative, positive or zero charge depending on the pH of the solution they are contained in (Berkelman *et al.*, 1998). The net charge a protein possesses is contributed by its amino acid side chains, its carboxyl and amino termini and groups contributed as a result of post-translational modifications. At pH values below their isoelectric points (pI) amino acids have a net positive charge and at pH values above their pI they have a net negative charge. At their pI, they have no net charge. In IEF, proteins migrate along a pH gradient under the influence of an electric field. Therefore, a protein with a net positive charge migrates towards the cathode and as the pH increases along the gradient it becomes progressively less positive until it reaches its pI where it stops moving. Likewise, a protein with net negative charge migrates towards the anode until it reaches its pI, where it becomes stationery in the electric field as it has no net charge (Berkelman *et al.*, 1998). Thus proteins can be separated on the basis of very small charge

differences using IEF. The individual proteins are concentrated at their different isoelectric points under the influence of the electric field, hence the term "isoelectric focusing".

The concept of 2-D gel electrophoresis was first documented by Macko and Stegemann (1969). However, the actual 2-D methods were run successfully only after Klose (1975) and O'Farell (1975) separately developed effective sample preparation protocols. The basic method initially involved use of a tube gel rod that had mixtures of carrier ampholytes (small soluble amphoteric molecules) that had high buffering capacity close to their pI. When an electric field was applied across the rod the ampholytes established the pH gradient when they migrated to their pIs. The sample was focused in this gradient and then transferred to a vertical slab gel for the second dimension. This method became the 'IsoDalt' system when it was improved by Anderson and Anderson (1978), who optimized running conditions for replicate samples.

The technique of Immobilized pH gradients (IPG's) was introduced by Bjellquist *et al.* (1982). The IPG's differed from the earlier pH gradients in that the acrylamido buffers (these comprise a single basic or acidic group linked to an acrylamide monomer) that form the pH gradient are covalently incorporated into a polyacrylamide gel when it is cast (Berkelman *et al.*, 1998). The proportion of acidic or basic acrylamido buffer content defines the resultant pH gradient. The acrylamide monomers of the buffers link to the bisacrylamide and acrylamide monomers of the gel and undergo simultaneous polymerization and incorporation into the gel matrix as it sets. The gel is supported by casting on a

plastic backing. Görg *et al.*, (1988) pioneered application of IPG technology to 2-D electrophoresis. Use of IPG's in the first dimension of 2-D electrophoresis (also called IPG-Dalt) resulted in enhanced reproducibility and resolution, improved ease of handling of the gel and stability of the pH gradient (Berkelman *et al.*, 1998; Fichmann and Westermeier, 1999). These features coupled with the high loading capacity of IPGs have led to rapid spot identification enabling microsequencing, amino acid analysis and mass spectrometry (Görg and Weiss, 1999).

3.2.2. 2-D gel electrophoresis of sperm proteins

Sperm proteins from various species have been separated using 2-D gel electrophoresis with different objectives in mind:

2-D gel electrophoresis has been applied to general studies of sperm proteins such as in the determination of electrophoretic maps of plasma membrane proteins in boar sperm (Russell *et al.*, 1983) and human sperm (Naaby-Hansen, 1990). It has also been applied to more specific studies like those investigating phosphorylated sperm plasma membrane proteins in bovine epididymal sperm (Noland *et al.*, 1984), identifying specific proteins such as actin in rabbit sperm (Welch and O'Rand, 1985) or in the isolation of cognate proteins for antisperm, auto and iso-antibodies (Shetty *et al.*, 1999). Recently, post-vasectomy autoantibodies that were predominantly against outer dense fibre proteins of rat sperm were characterised by 2-D electrophoresis (Flickinger *et al.*, 2001).

When plasma membranes from human sperm were analysed, up to 260 proteins between pIs 4.5 - 7.8 were identified (Naaby-Hansen, 1990). The limitations of 2-D techniques using human sperm include the small protein recovery per ejaculate, staining variability of extracted proteins and the limited samples available per individual. Kritsas *et al.*, (1992), described an improved technique using mini-gels, for limited sperm sample protein analysis. The preponderance of acidic proteins in boar and bovine sperm plasma membrane extracts was observed (Peterson *et al.*, 1983; Fenner *et al.*, 1992).

Several specific sperm proteins have been characterised by 2-D SDS-PAGE. Among the proteins characterised are:

(i) P26h, which is a sperm protein of epididymal origin involved in gamete interaction in the hamster. This protein was shown to be relatively hydrophilic with a basic pI of about 8.3 (Coutu *et al.*, 1996).

(ii) SP-10, a polymorphic intra-acrosomal protein of molecular weights 18-45kDa has been analyzed using 2-D gel electrophoresis (Freemerman *et al.*, 1994). The protein has been recognised in several species including, humans, baboons, macaques, pigs, foxes, mice and rats. The SP-10 peptides in human sperm were found to have pIs clustered around 4.9 (Freemerman *et al.*, 1994).

(iii) MSA-63, an antigen of molecular weight 24-84 kDa and pI range of 4-6 has been characterised in the mouse using 2-D (Liu *et al.*, 1992).

3.2.3. Membrane protein isolation

Molloy, (2000) reviews various techniques used by different investigators for 2-D membrane protein analysis. It has been noted that 2-D electrophoretic separations of proteins in both eukaryotes and prokaryotes is characterised by poor representation of the membrane and other hydrophobic proteins (Santoni *et al.*, 2000; Molloy, 2000). In prokaryote cell preparations using carrier ampholyte tube gels and IPG's for 2-D runs, there was poor representation of the hydrophobic membrane proteins compared to those predicted in genomes (Wilkins *et al*, 1998; Molloy, 2000). This was attributed to poor initial solubilisation of the proteins using conventional solubilising solutions, reduced solubility, and loss of protein at pI due to precipitation and adsorption (Rabilloud, 1998; Molloy, 2000). In spite of using the current range of detergents, incorporation of thiourea and reducing agents in the solubilisation buffer, resolution of certain more hydrophobic membrane proteins is still not possible (Molloy *et al*, 2000). There is so far no universal protocol that works effectively in extraction of both eukaryotic and prokaryotic membrane proteins. Moreover, almost all the protocols are best suited to isolating denatured or reduced proteins. There is therefore, need to test and adapt specific protocols depending on the nature of the particular membrane protein of interest.

3.3. Mass spectrometric identification of proteins

Mass spectrometry is becoming increasingly applied in identification of purified proteins due to its sensitivity of analysis and high sample throughput (Griffin *et al.*, 1992; Aebersold and Mann, 2003). It can be used in concert with other techniques of protein purification like 2-D gel electrophoresis that can separate specific proteins in a complex mixture of proteins (Aebersold and Mann, 2003). Separated or purified proteins are digested and then subjected to mass spectrometry. Mass spectrometers can analyse peptide masses providing a peptide mass fingerprint or peptides to give an amino acid sequence by tandem mass spectrometry (MS/MS) (Graves and Haystead, 2002). There are different types of mass spectrometers (MS) but they all essentially have three components:

(i) an ion source that can be Electrospray ionization (ESI) or *M*atrix *a*ssisted *l*aser *d*esorption/*i*onization (MALDI),

(ii) a mass analyser which could be an ion trap, time of flight (TOF), quadrupole and Fourier transform ion cyclotron analysers,

(iii) a detector that records the number of ions at each mass/charge (m/z) value.

Therefore based on different combinations of these basic components, there different mass spectrometers like the Triple quadrupole MS, Quadrupole time of flight MS, Fourier transform MS and MALDI-TOF MS (Aebersold and Mann, 2003). Peptides are analyzed in gas phase where they are ionized and charged. Using MALDI-TOF (time of flight) mass spectrometer the result is a mass spectrum depicting mass/charge (m/z) ratios of the peptides in the digest. The

m/z data obtained are used to search the protein databases using peptide-database searching algorithms (peptide matching/peptide mass fingerprinting) to identify the specific protein or family of related proteins (Figeys *et al.*, 2001; Aebersold and Mann, 2003; Steen and Mann, 2004). It is also possible to obtain the internal peptide sequence of the digested protein using tandem mass spectrometry in Triple Quadrupole MS and Quadrupole TOF MS and other MS. In these MS a specific peptide can be selected and its peptide bonds sequentially broken giving the partial sequence of amino acids making up the protein (Graves and Haystead, 2002). There are different algorithms available for searching sequence databases such as Peptide search, Sequest, Protein prospector and Mascot. These make use of the partial sequence data to identify matching proteins from predicted and known sequences within databases (Steen and Mann, 2004).

3.4. Specific aims of this chapter

1. To identify the specific protein recognised by mAb IAM-1 on human sperm using 2-D gel electrophoresis techniques.
2. To obtain sufficient quantities of the protein for characterisation, using peptide mass fingerprinting and/or N-terminal sequencing and/or internal peptide sequencing.

3.5. Materials and Methods

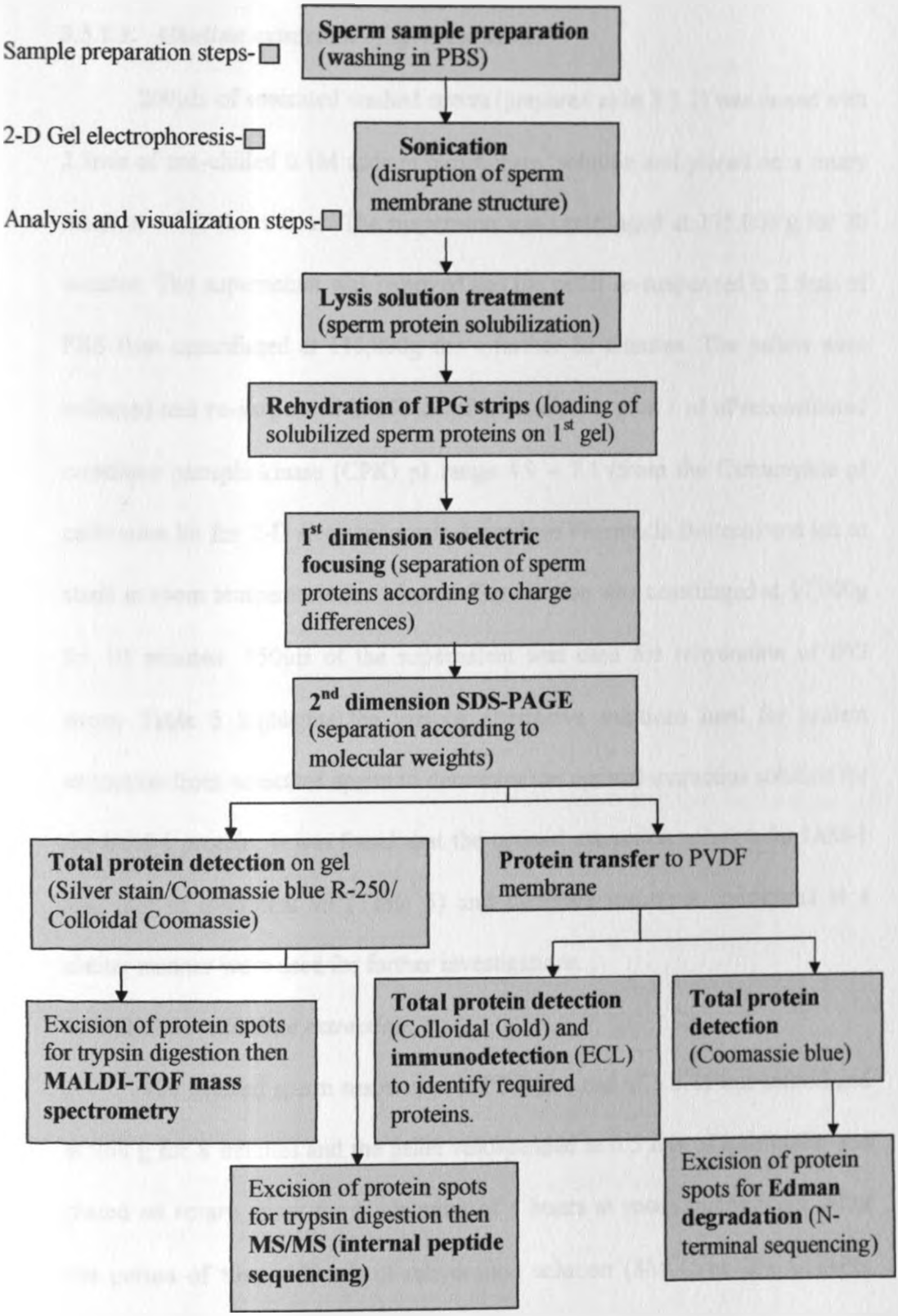
2-D electrophoresis was performed according to a modified version of the protocol by Berkelman *et al.*, (1998) to optimize conditions for isolation of non-reducing proteins. Briefly, it involved the following steps depicted in the flow chart (Table 4):

- Sample preparation
- Rehydration of IPG strips
- Isoelectric Focusing
- 2nd Dimension SDS PAGE
- Visualization and analysis of results

3.5.1. Sample preparation

Human sperm was prepared according to the protocol given earlier in Chapter 2 (2.4.1.1). Following this initial treatment, 50 - 150 million sperm were used for protein extraction from sperm for 2-D gels. The sperm were resuspended in 3.25 mls of Phosphate buffered saline (PBS) pH=7.4, placed on ice and sonicated in a Soniprep 150 Ultrasonic disintegrator (Sanyo Gallenkamp PLC) at an amplitude of 10.5 μ for 2 minutes. The resultant solution was centrifuged at 10,600g for 10 minutes and the pellet resuspended in 200μls of PBS.

Table 4. Flow chart of protocol used in isolation and characterization of IAM-1 from human sperm.



3.5.1.1. ***Alkaline extraction of sperm proteins***

200µls of sonicated washed sperm (prepared as in 3.5.1) was mixed with 2.5mls of pre-chilled 0.1M sodium bicarbonate solution and placed on a rotary mixer at 4 °C. for 1 hour. The suspension was centrifuged at 115,000 g for 20 minutes. The supernatant was removed and the pellet re-suspended in 2.5mls of PBS then centrifuged at 115,000g for a further 20 minutes. The pellets were collected and re-suspended in 350µls of lysis solution plus 1 µl of reconstituted creatinine phosphokinase (CPK) pI range 4.9 – 7.1 (from the Carbamylate pI calibration kit for 2-D electrophoresis Amersham Pharmacia Biotech) and left to stand at room temperature for 1 hour. The solution was centrifuged at 17,000g for 10 minutes. 350µls of the supernatant was used for rehydration of IPG strips. Table 5 highlights the various alternative solutions used for protein extraction from sonicated sperm to determine the optimal extraction solution for the IAM-1 protein. It was found that the optimal extraction solution for IAM-1 was that in treatment vii (Table 5) and therefore solutions constituted in a similar manner were used for further investigations.

3.5.1.2. ***Non- alkaline extraction***

The washed sperm suspension in PBS (see end of 3.5.1) was centrifuged at 960 g for 8 minutes and the pellet resuspended in 0.5 mls of lysis buffer and placed on rotary mixer for a minimum of 4 hours at room temperature. After this period of time, 250 µls of rehydration solution (8M Urea, 2% CHAPS, 0.5% v/v IPG buffer and few grains of bromophenol blue in distilled water) was added to the 100 µls of lysis solution (treatment (iv) in Table 5) -sperm

Table 5. Various solutions used for extraction of sperm proteins for 1-D gel electrophoresis and observed efficacy in IAM-1 extraction of each as assessed using intensity of ECL on immunoblots.

Treatment solution used	**Immunodetection using ECL**
i) SDS reducing loading buffer	---
ii) SDS non-reducing loading buffer	++++
iii) 1M Sodium Chloride extraction then SDS (non-reducing) loading buffer to supernatant	---
iv) Lysis solution with 9.5M Urea, 4% Chaps, 0.5%IPG pH4 – 7, PMSF, Distilled Water.	+++
v) Lysis solution with 9.5M Urea, 4% Chaps, 0.5%IPG pH4-7, PMSF, DTT, Distilled Water,	---
vi) Lysis solution with 9.5M Urea, 0.5-2% Triton X-100, 0.5% IPG, PMSF, Distilled water	+
vii) Lysis solution with 9M Urea, 2M Thiourea, 2% ASB-14, 1%Triton X-100, 0.5% IPG pH4-7, PMSF, Distilled water	++++

- Indicates nothing detected using ECL

+ Indicates weakly positive response

+++ Strong positive response

++++ Very strong positive response

CHAPS (3-(3-cholamidopropyl)-dimethylammonio)-1-propanesulfonate)

PMSF- Phenylmethane sulfonyl fluoride(Sigma)

DTT - Dithiothreitol

suspension plus 1 µl of reconstituted creatinine phosphokinase and kept standing for a further 1 hour. The sample was then centrifuged at 10,600 g and the supernatant removed and loaded on to a reswelling tray to rehydrate an immobilised pH gradient (IPG) strip. For the treatment vii in Table 5, having ASB-I4 as the detergent, the same lysis extract was used for rehydration of the IPG strip with no additional rehydration solution.

3.5.2. 2-D electrophoresis

3.5.2.1. *Rehydration of Immobilized pH Gradient Strips (IPGs)*

Rehydration and sample loading of IPG strips was done at the same time. 350µls or 300µls of rehydration solution (containing the sample) was aliquoted onto the slots on the Immobiline DryStrip Reswelling Tray (Amersham Pharmacia Biotech UK Limited). The 350µls was for 18 cm IPG strips (Amersham Pharmacia Biotech.) while 300µls was for 17cm IPG strips (Bio-rad). The IPG strips were carefully placed on the rehydration solution gel face downwards and overlain with 2 mls of IPG cover fluid (Amersham Pharmacia Biotech). The strip was allowed to rehydrate for a minimum of 12 hours at room temperature.

3.5.2.2. *Isoelectric Focusing (IEF)*

This step required preparation of the Immobiline Dry Strip Kit (Amersham Pharmacia Biotech) comprising an Immobiline DryStrip Tray, DryStrip aligner, electrodes, electrode strips, sample cup bar and sample cups. The kit was set up on a Multiphor II electrophoresis unit (Amersham Pharmacia

Biotech.) cooling plate. The MultiTemp III thermostatic circulator (Amersham Pharmacia Biotech.) connected to the electrophoresis unit, was set to 20 °C. Rehydrated IPG strips were removed from the reswelling tray, rinsed with distilled water briefly and excess moisture blotted off. They were placed on the Immobiline drystrip aligner, gel face upwards within the tray, with the anodic side facing the positive side of the cooling plate. Two IEF electrode strips, each 110 mm long, were cut and soaked in 500 µls of distilled water. The IEF strips were aligned in contact with the ends of the gels on either ends of the IPG strips. The appropriate electrodes were placed on top of the electrode strips at the ends of the IPG strips.

Where additional sample solution was loaded to the IPG strips, sample cups were placed on the sample bar, positioned on the Immobiline drystrip tray in contact with the cathodic electrode. The sample cups were carefully adjusted in contact with the IPG strips. IPG cover fluid was poured to cover the IPG strips and sample cups. 100µls of sample was loaded onto the sample cups and it gravitated into the sample cups.

IEF was done for a total of 75,000 - 80,000 Volt Hours in gradient mode as in Table 6.

Table 6. Programme used for Isoelectric focusing for 18 cm IPG strips

Phase	Voltage(V)	Current(mA)	Power(W)	VH
1	500	1	5	1
2	500	1	5	3500
3	3,500	1	5	10,000
4	3,500	1	5	72,500

The electrode strips were changed twice during IEF. At the end of IEF, the IPG strip was either immediately stored at -20 °C or equilibrated (see Appendix for equilibration solution) for 30 minutes before the second dimension SDS PAGE.

3.5.2.3. ***2nd dimension SDS PAGE***

The thermostatic circulator was set to 15 °C. A precast ExcelGel® SDS, gradient 8-18 polyacrylamide gel (Amersham Pharmacia Biotech), 110 x 245 x 0.5 mm, was opened and air-dried for 8 minutes then carefully placed on about 4 mls of cover fluid on the cooling plate. A Multiphor buffer strip positioner (Amersham Pharmacia Biotech.) was set in place with the cathodic and anodic buffer strips positioned on either side, in contact with the gel. The IPG strip was removed from the equilibration solution and excess solution blotted off the ends. It was then positioned, gel face downwards, on the SDS gel parallel to the cathodic buffer strip. Sample application pieces were placed at either end of the IPG strip. 3µls of low molecular weight markers (Dalton Mark VII-L TM, SIGMA) , 14,200 – 66,000 kDa, was diluted with 7µls of equilibration solution and 5µls of the resulting solution loaded on two sample application pieces. The two pieces were placed one on either side of the IPG strip, at the same level, on the SDS gel.

The Multiphor II electrode holder was aligned with the buffer strips and placed on the strips. Electrophoresis was then run as shown in Table 7.

Table 7. Schedule used for electrophoresis in the 2nd dimension SDS-PAGE .

Step	Voltage(V)	Current (mA)	Power (W)	Duration(h:min)
1	600	20	30	0.30
2	600	50	30	1.14

After step 1 or when the dye front was 0.2 cm from the IPG strip, the system was paused and application pieces and the IPG strip removed. The cathodic buffer strip was moved to the IPG strip position and Step 2 of electrophoresis commenced. At the end of the run, the gel was removed from the Multiphor II apparatus and excess oil blotted off the plastic backing of the gel.

3.5.3. Visualization of results

3.5.3.1. *Total protein detection on gels*

This was done according to the protocols described earlier in Chapter 2 (2.4.6.).

The gels were stained for total protein using Silver stain (see protocol on 2.4.6.1) or Colloidal Coomassie as detailed below.

3.5.3.2. *Colloidal Coomassie gel staining*

This was done using Brilliant Blue G-Colloidal Concentrate (Sigma) or Colloidal Coomassie according to the following protocol. Following second dimension SDS-PAGE, the gel was fixed (in 45% Methanol, 1% Acetic acid) for 30 minutes, then the fixative solution decanted off and the Colloidal

Coomassie stain, prepared according to the manufacturers instructions (diluted first 1:4 with distilled water then 4:1 in absolute methanol), added. The gel was placed on a rocking platform at room temperature, for 18 – 24 hours. The stain was decanted off and the gel destained in distilled water until suitable contrast of protein spots was obtained.

3.5.3.3. ***2-D Semi-dry protein transfer***

Protein Transfer involved use of the Multiphor II Novablot Unit (Amersham Pharmacia Biotech). Protein transfer was done to Immobilon-P (Millipore), polyvinylidene fluoride (PVDF) or Trans Blot (Bio-rad), nitrocellulose membrane. Both the anodic and cathodic novablot graphite electrodes were saturated with distilled water then the excess blotted off. The anodic electrode was fitted onto the multiphor II buffer tank. Two sets of 9 filter papers (Whatmans) were cut to the dimensions 110 x 240 mm, and wetted in CAPS transfer buffer (prepared as described in the Appendix). The transfer buffer used was CAPS buffer where there was subsequent protein sequencing of the spots obtained on the blots. A similar size of PVDF membrane was cut and pre-wetted in 100% methanol for 15 seconds. It was then hydrated in distilled water for 2 minutes. This was followed by equilibration in the transfer buffer for 5 minutes. One set of 9 pre-wetted filter papers was placed on the anodic electrode. The gels were separated from the plastic backing using a novablot unit. The PVDF membrane was carefully placed on the gel and the plastic backing with gel and membrane placed on the anodic electrode. The plastic backing was carefully removed and the gel overlain by the other set of 9 pre-

wetted filter papers. The cathode electrode was then fitted over the sandwich, the safety lid placed and the unit connected. The transfer was done at 150mA (about 0.8mA /cm^2 per hour) for 2 hours.

3.5.3.4. ***Total protein detection on blots***

Total protein detection and immunodetection was combined according to the protocol of Chevallet *et al.*, (1997) to facilitate accurate localisation of immunoreactive spots. Briefly this involved: removal of the PVDF membrane from the Novablot unit following protein transfer. All the subsequent steps were performed on a rocking table; washing, 3 times for 5 minutes (3 x 5) each in 30% ethanol, followed by washing 3 x 5 in 0.05% v/v, Tween20 in distilled water; staining overnight in colloidal gold total protein stain (Bio-rad) diluted 1:3 with 0.05% Tween20 in distilled water for total protein; The membrane was then rinsed 3 x 5 minutes in PBS containing 0.05% Tween20 and 1% w/v, polyvinylpyrrolidone (Sigma); blocking was then done in the same solution for 2 hours followed by primary antibody incubation with IAM-1 supernatant for 2 hours. The membrane was then rinsed 6 x 5 minutes in PBS with 0.05% Tween20. This was followed by a 1 hour incubation with Anti-mouse IgG (raised in Sheep) conjugated to horse radish peroxidase (Amersham Pharmacia Biotech), diluted 1:1,500 with PBS containing 0.05% Tween20. Finally there was a 6 x 5 minutes wash in PBS containing 0.05% Tween20.

Visualization of Immunoreactions was done using Enhanced Chemiluminescence (ECL+Plus) Western blotting detection kit (Amersham

Pharmacia Biotech) according to the manufacturers protocol described earlier in Chapter 2 (see 2.4.5.3.2).

3.5.3.5. ***Excision of protein bands or spots***

3.5.3.5.1. *Gel proteins*

This was done following Colloidal Coomassie staining of the gel, for MALDI–TOF (*M*atrix *A*ssisted *L*aser *D*esorption *I*onisation *T*ime *O*f *F*light mass spectrometry) and for Q-TOF (Quadrupole/orthogonal time of flight) tandem mass spectrometry. The bands of interest were identified, carefully excised with sterile scapel blades and placed in separate sterile microfuge tubes. A part of the gel without protein (a control spot) was cut and placed in a microfuge. The samples were then kept at 4 ^{O}C until analyzed. Peptide mass fingerprinting was performed using a Voyager-DE PRO MALDI-TOF mass spectrometer at the Microchemical Facility of Babraham Institute in Cambridge UK.

For tandem mass spectrometry, peptides resulting following trypsin digestion were separated by reverse phase HPLC in a 15 cm PepMap C18 column then internal peptide sequencing was performed using a Quadrupole/orthogonal acceleration time of flight (Q-TOF) mass spectrometer (Micromass).

3.5.3.5.2. *Blot proteins*

Western blots were performed on human sperm 2-D gels and these were stained for total protein using Coomassie blue stain (protocol similar to 2.4.6.2). Spots corresponding to IAM-1 were carefully excised and placed in sterile microfuge tubes. They were stored at 4 ^{0}C until analyzed. N-terminal sequencing was performed by Edman degradation at the Babraham Institute in Cambridge, UK.

3.6. Results

3.6.1 IAM-1 extraction

Extraction of human sperm proteins using various lysis solutions shown in Table 5 (treatments i – vii) provided further information regarding the physico-chemical properties of IAM-1 and its interactions with mAb IAM-1. Inclusion of reducing agents whether β-mercaptoethanol as in treatment (i) or dithiothreitol as in (v) resulted in non-recognition of the antigen by the mAb IAM-1. Extraction solutions containing the anionic detergent SDS and the zwitterionic amido sulfobetaine detergent ASB-14 realized the most efficient extraction of IAM-1 from the sperm membranes. Furthermore, when the detergent ASB-14 was used, efficiency of extraction was enhanced at high urea concentrations (9M) and by inclusion of the additional chaotrope thiourea as in treatment (vii). Extraction solutions with the non-ionic detergent Tritron X-100 as the only detergent resulted in poor recovery of IAM-1 (treatment vi). Use of strongly ionic solutions of sodium chloride alone did not extract IAM-1 from the sperm membranes (treatment iii).

It was also noted that despite strong immunoreactivity using ECL detection on 2-D blots, the protein IAM-1 was barely discernible on 2-D blots stained with colloidal gold total protein stain (Figs. 19, 20, 21). In some of these blots other sperm proteins were intensely stained indicating that a good amount of other sperm proteins were actually transferred onto the membranes (Fig. 19).

3.6.2. 2-D electrophoresis

3.6.2.1. *2-D gels*

Several human sperm proteins were resolved following 2-D electrophoresis. These proteins were mainly clustered between pI's 4.2 and 8.7. They were relatively low molecular weight proteins of between 10 – 80 kDa (Fig. 18).

3.6.2.2. *2-D immunoblots*

3.6.2.2.1. *Non–alkaline protein immunoblots*

Immunoreactive spots were accurately assigned on Western blots according to the method of Chevallet *et al.*, (1997). Immunoprobing with mAb IAM-1 on 2-D blots of the human sperm proteins (extracted without prior alkaline incubation with 100mM sodium carbonate) revealed immunoreactive spots of apparent pI's 3.86 – 4.0. These spots had apparent molecular weights of 23, 27 and 32 kDa (Fig 19).

3.6.2.2.2. *Alkaline protein immunoblots*

When human sperm proteins were extracted following incubation under alkaline conditions (with 100mM sodium carbonate) and 2-D separation performed, immunoblots of the resolved proteins probed with mAb IAM-1 had immunoreactive spots with pI's of 5.8 and 6.2 and apparent molecular weights of about 25 kDa (Fig. 20).

When narrower range Immobilised pH gradient strips of pH 4-7 were used for the 2-D separation of the same proteins, the resolved IAM-1

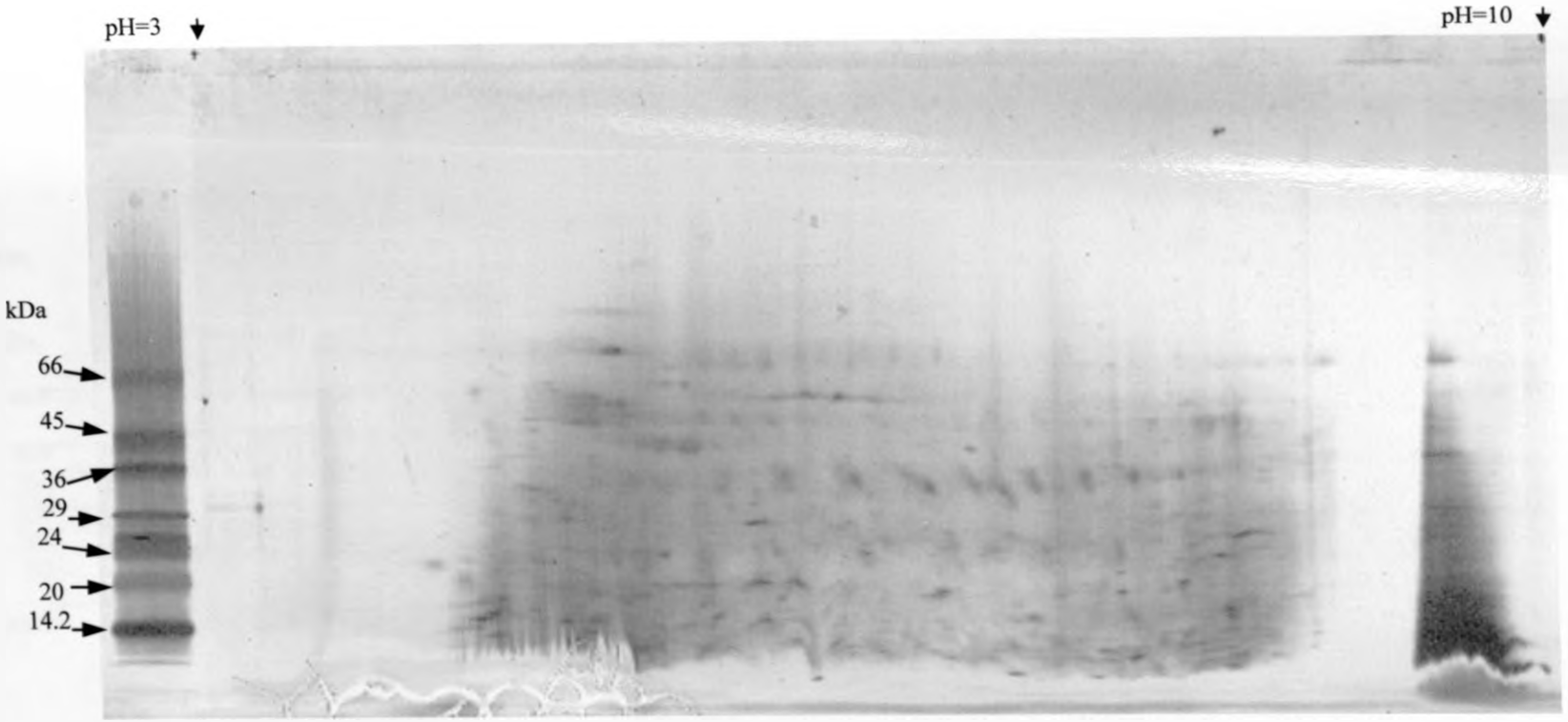


Fig. 18 Silver stained SDS polyacrylamide gel of human sperm proteins following 2-dimensional electrophoresis. A linear immobilized pH gradient strip of pH range 3-10 was used for first dimension isoelectric focusing. The molecular weights in kilodaltons are indicated on the left.

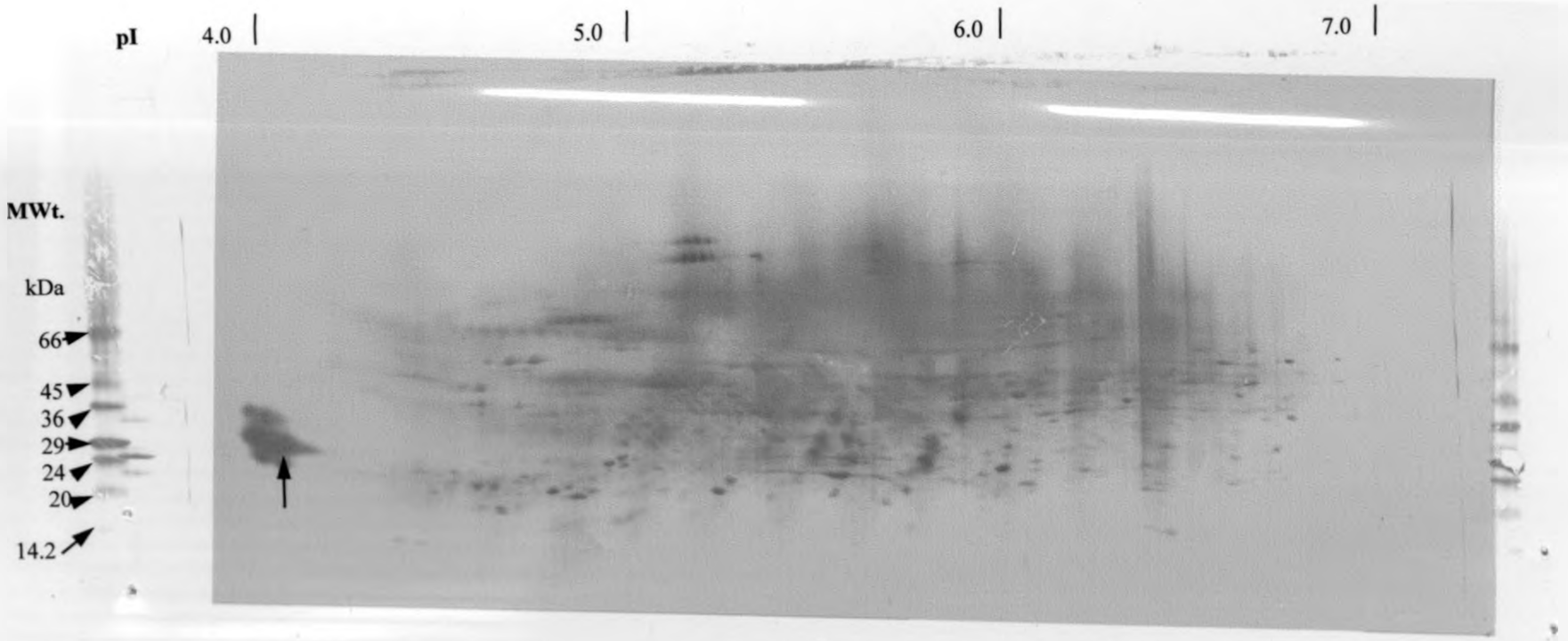


Fig. 19 Immunoblot of whole human sperm, lysis buffer protein extract (without alkaline treatment), stained with colloidal gold total protein stain. The blot was overlain with ECL Kodak film (see overlay) and the arrow indicates the position of protein spots immunoreactive to mAb IAM-I of apparent pI ~3.86– 4.0 and apparent molecular weights of ~ 23, 27 and 32 kDa.

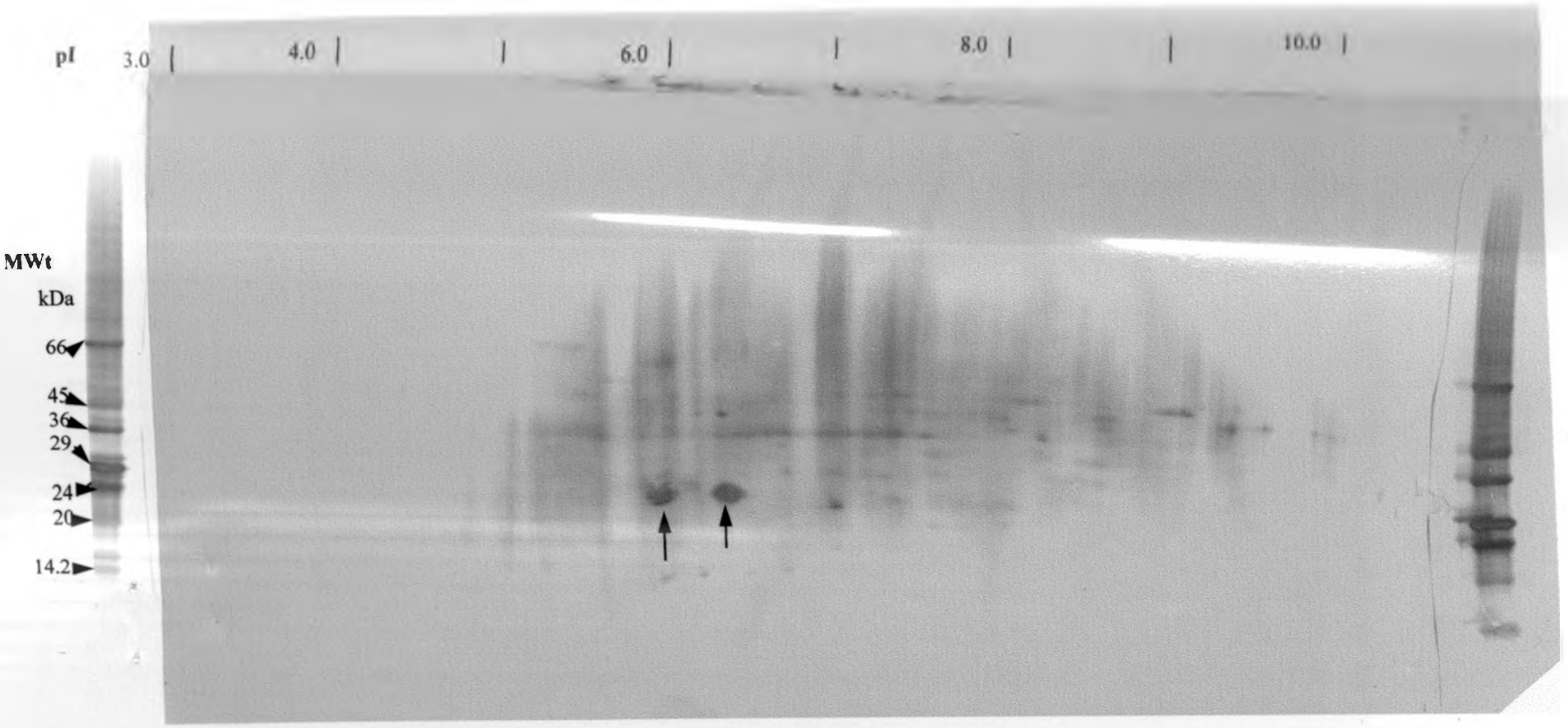


Fig. 20 Immunoblot of whole human sperm lysis buffer (under alkaline conditions) protein extract, stained with colloidal gold total protein stain . The overlay of ECL Kodak film shows spots immunoreactive to mAb IAM-I (arrows on immunoblot) of pI~ 5.8 and 6.2 and apparent molecular weight of ~25kDa.

Fig. 21 Immunoblot of whole human sperm lysis buffer protein extract, following alkaline treatment and 2-dimensional gel electrophoresis, stained with colloidal gold total protein stain. Arrows show position of the proteins immunoreactive to mAb IAM-1, of mean apparent pIs ~5.32, 5.61 and 6.07 and mean apparent molecular weights of ~ 24kDa.

immunoreactive spots had pI's of 5.27 - 5.37, 5.5 - 5.72 and 5.98 - 6.16 and corresponding apparent molecular weights of 23.5, 24.5 and 23.3 kDa respectively (Fig. 21). Therefore it was apparent that alkaline incubation prior to extraction shifted the pI of IAM-1 to make it less acidic.

3.6.3. N-terminal sequencing

An N-terminal amino acid sequence was obtained from protein spots on human sperm western blots (Table 8). The selected spots corresponded to immunoreactive IAM-1 protein spots. The sequence of 10 amino acids obtained was not complete as there were no amino acids detected at positions 4, 6 and 8. Furthermore, in some cases there were changes in the amino acid residues with each cycle performed as in 2, 3 and 9 (Table 8). A search of the protein databases using the possible combinations of the amino acids did not yield any significant match.

Table 8. N-terminal sequence of IAM-1

1	2	3	4	5	6	7	8	9	10
S	L	G	X	A	X	I	X	G	R
	V	L						L	
	G								

3.6.4. Peptide mass fingerprinting

Following trypsin digestion and MALDI-TOF mass spectrometry, mass spectra were obtained for two spots corresponding to IAM-1 immunoreactive

proteins excised from the western blots (Figs 22a, 22b). The resultant masses representative of peptides from trypsin digestion of the two spots are listed in Table 9. Various protein databases were interrogated using these masses, in an attempt to identify the proteins in the two spots. The search engine used was the MS-Fit (http://www-fbsc.ncifcrf.gov/ucsfhtml3.2/msfit.htm) while the protein databases interrogated included SWISS-PROT (Tables 10 and 11) and NCBInr databases. However for both of the spots there was low percentage of peptide masses matched to actual proteins in the databases with the highest for Spot 1 being 23% (Table 10) and for Spot 2 being 33% (Table 11) in the SWISS-PROT database.

3.6.5. Internal peptide sequencing

Internal peptide sequencing of two immunoreactive spots yielded a number of peptides. These were matched in the protein databases to Trypsin like proteins, Keratin, Glutathione transferase mu 3, alpha 3/7 tubulin, Ras related protein and others (Table 12). It was noteworthy that keratin, trypsin-like proteins and alpha tubulin were detected in both of the spots. Keratin was matched to that of human and sheep. The trypsin however was matched to trypsin found in three different species namely, human, porcine and canine.

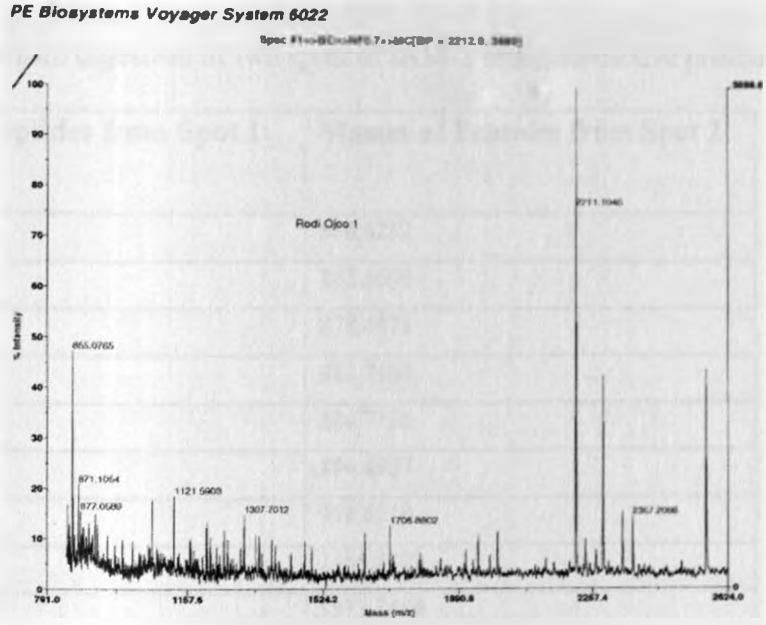


Fig.22a. MALDI mass spectrum of one of the spots immunoreactive to IAM-1 of apparent molecular weight 23,419 Da and pI~ 6.0

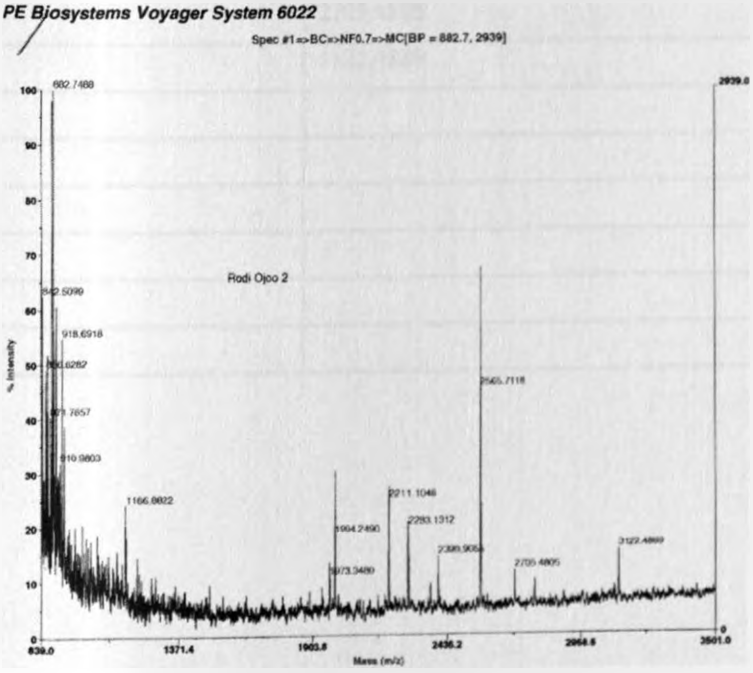


Fig.22b. MALDI mass spectrum of one of the protein spots immunoreactive to IAM-1of apparent molecular weight 25,941 Da and pI~ 5.6

Table 9. Masses of Peptides obtained from MALDI-TOF mass spectrometer following trypsin digestion of two spots of IAM-1 immunoreactive proteins.

Masses of Peptides from Spot 1	Masses of Peptides from Spot 2
846.3955	856.6282
855.0765	862.5609
871.1054	878.4478
872.3176	882.7488
877.0589	884.7136
883.0702	894.4957
886.5982	918.6918
900.1572	1166.8822
917.3644	1973.3489
923.5960	1994.2490
1066.1256	2283.1312
1121.5903	2399.9055
1207.5269	2565.7118
1209.6414	2705.4805
1253.6869	3122.4889
1307.7012	
1708.8802	
2283.1831	
2338.1567	
2367.2086	
2566.2191	

Table 10. Data obtained for Spot 1 masses using MS-Fit search engine on the SwissProt database. The top 5 matches are shown in the lower part of table.

Sample ID (comment): **Magic Bullet digest**
Database searched: **SwissProt.11.02.2001**
Molecular weight search (**1000 - 50000 Da**) selects **73280** entries.
Full pI range: **100225** entries.
Species search (**MAMMALS**) selects **18164** entries.
Combined molecular weight, pI and species searches select **11425** entries.
MS-Fit search selects **74** entries (results displayed for top **5** matches).

Considered modifications: | **Peptide N-terminal Gln to pyroGlu** | **Oxidation of M** | **Protein N-terminus Acetylated** |

Min. # Peptides to Match 4	Peptide Mass Tolerance (+/-) 500.000 ppm	Peptide Masses are monoisotopic	Digest Used Trypsin	Max. # Missed Cleavages 1	Cysteines Modified by acrylamide	Peptide N terminus Hydrogen (H)	Peptide C terminus Free Acid (OH)	Input # Peptide Masses 21

Result Summary

Rank	MOWSE Score	# (%) Masses Matched	Protein MW (Da)/pI	Species	SwissProt. 11.02.2001 Accession #	Protein Name
1	1.12e+03	5/21 (23%)	46664.3 / 6.42	MOUSE	P43883	ADIPOPHILIN (ADIPOSE DIFFERENTIATION-RELATED PROTEIN) (ADRP)
2	479	4/21 (19%)	47911.9 / 8.92	RAT	O35167	ACETYLCHOLINESTERASE COLLAGENIC TAIL PEPTIDE PRECURSOR (ACHE Q SUBUNIT) (ACETYLCHOLINESTERASE-ASSOCIATED COLLAGEN)
3	414	4/21 (19%)	36118.7 / 7.60	MOUSE	O35459	DELTA3,5-DELTA2,4-DIENOYL-COA ISOMERASE, MITOCHONDRIAL PRECURSOR
4	261	4/21 (19%)	35071.5 / 8.72	SHEEP	P05028	SODIUM/POTASSIUM-TRANSPORTING ATPASE BETA-1 CHAIN (SODIUM/POTASSIUM-DEPENDENT ATPASE BETA-1 SUBUNIT)
5	244	4/21 (19%)	43294.3 / 5.69	PIG	O46409	APOLIPOPROTEIN A-IV PRECURSOR (APO-AIV)

Table 11. Data obtained from masses derived from Spot 2 using MS-Fit search engine on the SwissProt. database. The top 5 matches are listed in the lower part of the table.

Sample ID (comment): **Magic Bullet digest**
Database searched: **SwissProt.11.02.2001**
Molecular weight search (**1000 - 50000 Da**) selects **73280** entries.
Full pI range: **100225** entries.
Species search (**MAMMALS**) selects **18164** entries.
Combined molecular weight, pI and species searches select **11425** entries.
MS-Fit search selects **20** entries (results displayed for top **5** matches).

Considered modifications: | **Peptide N-terminal Gln to pyroGlu** | **Oxidation of M** | **Protein N-terminus Acetylated** |

Min. # Peptides to Match **4**	Peptide Mass Tolerance (+/-) **500.000 ppm**	Peptide Masses are **monoisotopic**	Digest Used **Trypsin**	Max. # Missed Cleavages **1**	Cysteines Modified by **acrylamide**	Peptide N terminus **Hydrogen (H)**	Peptide C terminus **Free Acid (OH)**	Input # Peptide Masses **15**

Result Summary

Rank	MOWSE Score	# (%) Masses Matched	Protein MW (Da)/pI	Species	SwissProt. 11.02.2001 Accession #	Protein Name
1	480	4/15 (26%)	36720.4 / 7.13	BOVIN	P05980	PROSTAGLANDIN-F SYNTHASE 1 (PGF SYNTHASE 1) (PGF 1) (PROSTAGLANDIN-D2 11 REDUCTASE 1) (PGFSI)
2	411	4/15 (26%)	26908.9 / 6.75	BOVIN	P09611	PLACENTAL LACTOGEN I PRECURSOR (BPLP-I)
3	353	5/15 (33%)	47578.9 / 9.17	HUMAN	P09543	2',3'-CYCLIC NUCLEOTIDE 3'-PHOSPHODIESTERASE (CNP) (CNPASE)
4	182	4/15 (26%)	41834.7 / 8.91	HUMAN	O00532	SERINE/THREONINE-PROTEIN KINASE KKIALRE (CYCLIN-DEPENDENT KINASE-LIKE 1)
5	177	4/15 (26%)	43135.8 / 6.28	HUMAN	P27361	MITOGEN-ACTIVATED PROTEIN KINASE 3 (EXTRACELLULAR SIGNAL-REGULATED KINASE 1) (ERK-1) (INSULIN-STIMULATED MAP2 KINASE) (MAP KINASE 1) (MAPK 1) (P44-ERK1) (ERT2) (P44-MAPK) (MICROTUBULE-ASSOCIATED PROTEIN-2 KINASE)

Table 12. Internal peptide sequence of various proteins obtained from tandem mass spectrometry of 2 presumptive IAM-1 protein spots and corresponding matches derived from the protein database searches

Spot 1						
Peptide Sequences	***Database Match***	***Score***	***Peptides Matched***	***Molecular weight (Da)***	***pI***	***Accession***
LGEHNIDVLEGNEQFINAAK LSSPATLNSR VATVSLPR	Trypsin precursor (Porcine) (EC 3.4.21.4)	428.6	3	24,391	7.4	P00761
QLVESDINGLR	Keratin, type I cuticular HA5 (Hair keratin)	135.4	1	47,578	4.8	Q92764
VDIIENQVMDFR LKPQYLEELPGQLK	Glutathione S-transferase mu 3	119.2	2	26,670	5.4	P21266
EIVDLVLDR	Tubulin alpha-3/alpha7 chain	72.6	1	49,941	5.0	P05214
LFDQAFGLPR	Heat shock 27 kDa protein (HSP27)	71.3	1	22,920	6.6	P42929
VGEYSLYIGR	Serum amyloid p-component precursor	53.3	1	25,369	6.5	P3743
VVLIGDSGVGK	Ras related protein Rab-11a (RAB-11)	42.9	1	24,375	6.5	P24410
FQELESETLK	Prostatic acid phosphatase precursor	39.4	1	44,548	6.2	P15309
NKATSFHLGQLK	Hypothetical 40.7 kDa protein in Pyk1-S	33.4	1	40,683	8.7	P39729
Spot 2						
LGEHNIDVLEGNEQFINAAK LSSPATLNSR IITHPNFNGNTLDNDIMLIK VATVSLPR	Trypsin precursor (Porcine) (EC3.4.21.4)	438.9	4	24,391	7.4	P00761
QLVESDINGLR DSLENTLTETEAR	Keratin, type I microfibrillar 48 kDa	234.6	2	46,656	4.8	P02534
TQNPMVTGTSVLGVK	Proteasome beta chain precursor	83.4	1	29,174	5.9	P28373
VLEGNEQFINAAK	Trypsin III precursor (Human) (EC 3.4.21.4)	61.1	1	26,758	6.3	P15951
TLLDIDNTR	Keratin, type I cytoskeletal 9	53.0	1	61,969	5.2	P35527
ILDELTLCR	Keratin, type I cuticular HA3-I	31.6	1	45,917	4.9	O76009
VATISLPR	Trypsin, anionic precursor (Canine) (EC3.4.21.4)	31.2	1	26,404	4.7	P06872
IIATASVLGVK	6-phosphofructokinase beta subunit	26.3	1	10,4970	6.9	Q03216
EIVDLVLDR	Tubulin alpha chain, testis-specific	24.0	1	49,975	5.0	P18288

3.7. Discussion

Two-dimensional gel electrophoresis (2-D) is a powerful tool that enables separation of complex mixtures of proteins into purified constituent polypeptides. 2-D reference maps of human spermatozoal proteins have recently been established (Naaby-Hansen, 1990; Xu *et al.*, 1994; Naaby-Hansen *et al.*, 1997). However, despite availability of this data it is just a few of the proteins that have actually been identified on the maps and of these, fewer still have undergone molecular characterisation. Among the subset of sperm proteins so far identified using 2-D are a number of sperm surface proteins including those of seminal plasma origin (Naaby-Hansen *et al.*, 1997) and those found on the sperm membranes (Xu *et al.*, 1994). Sperm surface proteins overlying the inner acrosomal membrane and equatorial segment such as IAM-1 would be of particular interest as they could be important in gamete interaction at fertilisation

3.7.1. IAM-1 extraction

Three-dimensional conformation of proteins is important functionally and is dependent on non-covalent interactions between regions along the polypeptide chain. It is also known that some antibodies are directed against the resultant conformational epitopes on the protein molecules (Rabilloud, 1996). Our experimental results using reducing agents in extraction solutions indicate that there is the presence of at least one conformational epitope on the IAM-1

protein as treatment of sperm proteins with these agents resulted in non-recognition of the cognate protein by mAb IAM-1. For solubilisation of proteins to take place, there has to be disruption of its non-covalent interactions (Rabilloud, 1996). The combination of chaotropes like urea and thiourea with reducing agents completely unfold proteins, with chaotropes breaking intermolecular hydrogen and hydrophobic bonds while reducing agents break any disulfide bonds.

In our study, inclusion of reducing agents like the alkyl thiol, mercaptoethanol or cyclizable thiols like dithiothreital (DTT) in the extraction solution irreversibly affected the subsequent binding of the mAb IAM-1 to the cognate antigen. This was most likely due to presence of a conformational epitope whose integrity was maintained by the presence of one or more disulfide bonds. It is therefore hypothesized here that inclusion of the reducing agents reduced the disulfide bond(s) leading to the loss of native conformation of the protein and with it, the epitope for mAb IAM-1, resulting in non-recognition by the antibody.

Use of a combination of colloidal gold total protein stain which is a relatively sensitive protein stain (detects as low as 1ng of protein), coupled with ECL detection methods demonstrated that the mAb IAM-1 had a very strong affinity for the cognate protein as it immunoreacted with quantities of the antigen that were barely discernable on western blots of human sperm. The intense staining of other sperm proteins on the same 2-D blots where IAM-1

was barely visible indicated that insufficient amounts of IAM-1, unlike many other sperm proteins, were transferred and bound to the blots.

This could have arisen due to:

i) low amounts of the protein being initially available on sperm membranes or

ii) poor extraction of IAM-1 from the sperm membranes or

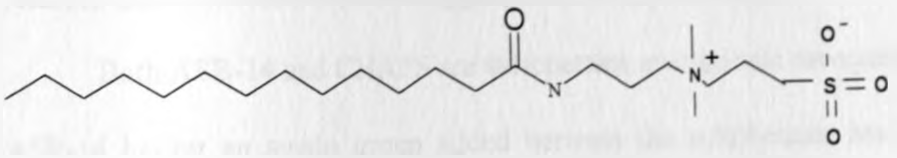
iii) loss of the protein during the 2-D electrophoresis

iv) loss of the protein during the processing of the blots.

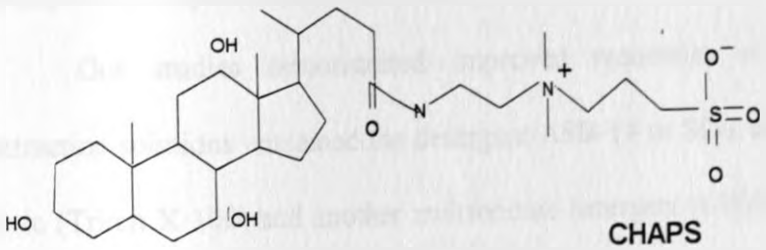
Loss of membrane and other poorly soluble proteins during isoelectric focusing (IEF) occurs during sample entry into the IEF gel due to stacking of proteins and aggregation resulting in precipitation and more so during IEF when proteins near their isoelectric point. At this crucial stage loss of proteins can either arise from adsorption of the proteins to the relatively hydrophobic matrix of the Immobilized pH gradient strip or aggregation due to reduced repulsive charges on the individual proteins close to isoelectric point leading to precipitation (Rabilloud, 1996; Rabilloud, 1998). Attempts were made to optimize extraction of this membrane protein and reduce loss of the same during IEF as follows:

i) Addition of thiourea to solubilisation buffer as was done enhances protein solubility even close to isoelectric point and improves the overall protein loading capacity of the gel (Rabilloud, 1998).

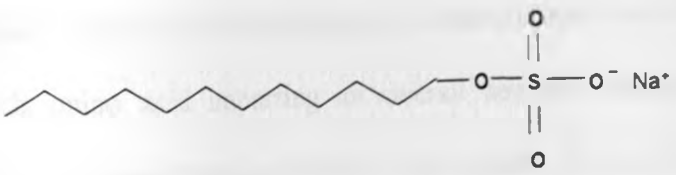
ii) Urea denatures proteins including relatively hydrophobic ones, in aqueous solutions exposing hydrophobic residues to the solvent.



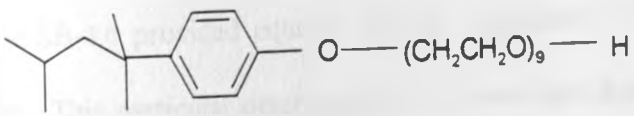
ASB14



CHAPS



SDS



Triton X-100

Fig. 23. Structural formulae of the detergents used in extraction of IAM-1 from human sperm. ASBI4 and CHAPS are zwitterionic detergents while SDS is an anionic detergent and Triton X-100 is a non-ionic detergent

iii) Detergents are used to prevent hydrophobic interactions of the exposed residues that could lead to protein aggregation and precipitation.

Both ASB-14 and CHAPS are sulfobetaine zwitterionic detergents, with ASB-14 having an amido group added between the sulfobetaine head and a linear alkyl hydrophobic tail while CHAPS has the amido group between the polycyclic hydrophobic tail and the sulfobetaine head (Fig. 23).

Our studies demonstrated improved recoveries of IAM-1 when extraction solutions contained the detergent ASB-14 or SDS compared to non-ionic (Triton X-100) and another zwitterionic detergent (CHAPS). It is known that SDS is a particularly effective anionic detergent useful in solubilising a wide range of proteins. In the solubilisation process at least two SDS molecules bind to each amino acid imparting an overall negative charge to the denatured protein molecules (Molloy, 2000). This curtails the use of this detergent in Isoelectric focusing as this separation technique exploits differences in the intrinsic surface charge of protein molecules (Santoni *et al.*, 2000).

ASB-14 provided equally efficient extraction of IAM-1 in solubilising solutions. This particular detergent has also been used successfully in extraction of relatively hydrophobic bacterial membrane proteins (Molloy *et al.*, 2000). Its greater efficacy as a sulfobetaine detergent has been attributed to the presence of its long alkyl hydrophobic tail unlike the polycyclic hydrophobic tail in CHAPS (see Fig.23). Indeed the findings that ASB-14 and SDS provide relatively more efficient extraction of IAM-1 compared to Tritron X-100 and CHAPS would be consistent with the hypothesis that IAM-1 is a relatively

hydrophobic integral membrane protein or a membrane protein well anchored to the inner acrosomal membrane.

Strong solutions of NaCl disrupt bonding due to ionic or electrostatic interactions between peripheral membrane proteins and other membrane proteins or with the phospholipids of the membranes (Rabilloud, 1996; Santoni *et al.*, 2000). The same occurs when solutions with high pH are used as was the case during sodium carbonate treatment of sperm in our extractions. In fact Fujiki *et al.*, (1982) state that carbonate treatment discriminates between peripheral and integral proteins with the latter remaining attached to cell membranes after carbonate treatment at pH=11. Therefore failure of carbonate treatment coupled with concentrated NaCl solutions to extract IAM-1 confirmed that the protein was not a peripheral membrane protein but one intimately associated with the acrosomal membranes possibly as an integral membrane protein or protein well anchored to the lipid component of the membranes.

3.7.2. 2-D gels and immunoblots

Earlier 2-D work on sperm proteins using carrier ampholyte gel rods for the first dimension had preponderance of neutral and acidic proteins between pI's 5.6–6.9. These were relatively low molecular weight proteins of less than 100kDa (Naaby-Hansen, 1990; Naaby-Hansen *et al.*,1997). Similar findings were obtained using immobilised pH gradient gels for the first dimension in 2-D separation of human sperm proteins (Xu *et al.*, 1994). It was also noted in the

latter study that membrane proteins constituted less than 15% of proteins isolated by 2-D electrophoresis of human sperm. However, so far there are no studies reporting separation of human sperm proteins under non-reducing conditions as were used in our experiments. Our findings on 2-D gels of human sperm proteins were in agreement with the earlier findings where there was more representation of the lower molecular weight proteins (<80kDa) and proteins with pI's of less than 9 (Naaby-Hansen, 1990).

Other techniques such as use of *N*on *E*quilibrium *pH* gradient electrophoresis (NEPHGE) have improved the resolution of more basic sperm proteins (Naaby-Hansen *et al.*, 1997). However, for the purposes of our study, use of conventional immobilized pH gradients were adequate to localize the protein of interest, IAM-1, on 2-D gels.

Other earlier 2-D SDS PAGE studies have also recorded the presence of proteins that formed "charge trains" or different isoforms (Naaby-Hansen, 1990; Xu *et al.*, 1994; Diekman *et al.*, 1997; Naaby-Hansen *et al.*, 1997). These were a series of spots that were isoforms of the same protein on the gels with almost similar apparent molecular weights but differing pIs. They are thought to arise as a result of variations in the extent of post-translational modifications on the particular proteins (Naaby-Hansen *et al.*, 1997). From our study, IAM-1 is an acidic protein that in native form has a pI of about 4.0. Treatment of this protein with an alkaline solution of sodium carbonate (pH=11) altered its pI to between 5.3 and 6.0. It is likely that the acidic nature of the native protein could be partly contributed to by post–translational groups linked to the protein.

Post-translational modifications of proteins are important in determining their eventual location within the cell, lifespan within the cell like acetylation and regulating activity of cellular proteins as in phosphorylation. Post-translational modification of proteins like phosphorylation, are also known to alter migration patterns of proteins on gels (Hao *et al.*, 2002). It is therefore conceivable that addition of alkali to IAM-1 hydrolyzes the post-translationally added groups responsible for acidity of the protein, with the resultant isoforms of the protein having higher or more basic pI values. Predominance of negatively charged amino acid residues like aspartic acid and glutamic acid within the structure of IAM-1 could also contribute to its acidic pI.

It is unlikely that alteration of the pI of IAM-1 was due to dissociation of subunits of the protein as there were no significant changes in the apparent molecular weights of the isoforms before and after alkaline treatment. Therefore, the proposed mechanism involving hydrolysis of post-translational groups by the alkaline treatment is the more likely mechanism by which there was alteration in the pI value of IAM-1.

3.7.3. N-terminal sequencing

N-terminal sequencing was performed on protein spots excised from human sperm western blots. The sequence obtained for 10 cycles was incomplete as no amino acids were detected in some cycles. In other cycles the precise identification of the amino acid obtained was uncertain as more than one type of amino acids was obtained at the same position. Inconsistency in the

amino acids obtained at certain positions during sequencing was attributed to low quantity of sample protein that necessitated use of higher sensitivity settings for detection. The higher settings could have contributed to variations due to background interference.

Apparent lack of amino acids at certain positions on the other hand could have arisen as a result of blocked N-terminal amino acids. It is known that many eukaryotic proteins have blocked N-terminal amino acids due to post-translational modification and as a result are refractory to Edman degradation particularly the initial coupling step of the process (Brown and Roberts, 1976; Brown, 1979). Therefore blockage of N-termini by glycosylation, acetylation, formylation, fatty acid acylation and cyclization or other modifications could have contributed to the missing amino acids at some of the positions in the protein. Under such circumstances internal peptide sequencing can be carried out to get a partial sequence for identification or the protein can be processed further to remove the groups blocking the N-termini (LeGendre *et al.*, 1993). A search of the protein data bases using the various permutations of the sequence data we obtained was inconclusive as there were no significant matches. Therefore, peptide mass fingerprinting and internal peptide sequencing of the protein was carried out to characterise IAM-1.

3.7.4. Peptide mass fingerprinting

Database searches using mass spectra obtained from two spots corresponding to IAM-1 were inconclusive. The percentage of peptides matched to particular proteins was generally less than 45% for each of the spots. Identification of a protein in peptide mass fingerprinting is dependent on availability of matches within the protein or translated genomic databases (Aebersold and Mann, 2003). Furthermore, the more the peptides that match (higher % matching) the more likely a protein can be related to a particular family of proteins or to a specific protein from the database (Figeys *et al.*, 2001). Despite obtaining a large number of peptides in digests for both spots, the percentage of peptides matched per protein spot was relatively low possibly indicating that IAM-1 could be a novel sperm membrane protein hitherto undefined in the protein databases.

3.7.5. Internal peptide sequencing

Data obtained from internal peptide sequencing of 2 spots immunoreactive to mAb IAM-1 revealed a number of peptide sequences with homology to trypsin-like proteins in both spots. The other types of protein common to both spots were the keratin related proteins and alpha tubulin proteins. Other proteins were identified in only one of the two spots that were sequenced.

The sequencing of multiple peptides of different proteins in each of the spots could also be attributed to the non-reducing conditions used in our

experiments that resulted in horizontal smears rather than distinct spots in the 2-D gels. It is known that the presence of reducing agents during isoelectric focusing improves resolution of protein spots in the second dimension of separation (Görg *et al.*, 2000). Due to the constraints of the non-reducing conditions required for IAM-1 isolation it was possible that other adjacent proteins with close pIs', were also excised for analysis from the resolving gel, contributing to the peptides of several proteins obtained in each spot sequenced. Some of the proteins that had high scores, appeared in both protein spots and had a few similar biochemical characteristics to IAM-1 are discussed below.

3.7.5.1. *Keratin*

There were a number of peptides isolated that were matched to various keratin types in humans and sheep. Keratins are proteins expressed in body cells particularly epidermal cells as cytokeratins or soft alpha keratins while in hair and other keratinized structures they are expressed as hair or hard alpha keratin. Both cytokeratins and hair keratin exist as heterodimers of type I (acidic) and type II (basic to neutral) proteins forming intermediate filaments (Rogers *et al.*, 1998). Cytokeratins have also been identified in germ cells, particularly the sperm flagellum (Hinsch *et al.*, 2003).

In our experiment four keratin types were isolated with three being of human origin and one of sheep wool origin. It is likely that the two human cuticular keratins (Q92764 and O76009) and one sheep wool keratin (P02534) were contaminants introduced during the processing of the samples. The other

keratin (P35527) isolated could have also been a contaminant from skin cells or could have been a sperm protein.

Recent work involving antibodies raised against purified recombinant peptides of epidermal K9 recognised testicular K9 found associated with manchette microtubules. Furthermore, significant homology in cDNA and amino acid sequences of testicular and epidermal K9 was observed when the gene was cloned in the two tissues (Mochida *et al.*, 2000). However, following spermatid remodelling and disappearance of the manchette the fate of K9 is unclear. Although keratins have been isolated as components of the mature sperm cytoskeleton, they are found in the outer dense fibres and the fibrous sheath of the flagellum (Kierszenbaum, 2001; Hinsch *et al.*, 2003). Furthermore the spermatid K9 has an apparent molecular weight of 62-64 kDa. (Mochida *et al.*, 2000). Therefore while sperm cytokeratins are found in the flagellum of spermatozoa, IAM-1 is found associated with the acrosomal membranes and equatorial region of the spermatozoon. The biochemical characteristics further suggest that IAM-1 is not K9 and therefore the latter could have been either a contaminant or a sperm protein.

3.7.5.2. ***Trypsin-like Proteins***

It is noteworthy that there were different peptides obtained during the sequencing of both spots, with a number corresponding to trypsin-like proteins in database searches. The entries with the best matches for both spots were however, homologous to a trypsin-like serine protease of porcine origin (accession number: P00761). This could imply that the porcine trypsin used for

digestion prior to Q-TOF MS/MS, underwent autolysis and the resultant peptides were sequenced.

On the other hand, the matching of certain peptides obtained to human and canine trypsin-like proteins complicates the picture further. While the peptide that was matched to the human trypsin III precursor (P35030) is similar to a segment of one of the peptides that was matched to porcine trypsin (P00761), the short peptide that was matched to canine trypsin anionic precursor differed from either human or porcine trypsin by one amino acid isoleucine substituting for valine in the canine trypsin. However, it is known that partial sequence data cannot guarantee a 100% identity as some proteins differ by a small number of amino acids that may not be in the segment sequenced (Charbonneau, 1993). The import of these findings would be that there could be a trypsin-like protein in human sperm that has certain structural characteristics similar to canine and porcine trypsins.

Expression of trypsin-like proteins in the testis especially on haploid germ cells and epididymal spermatozoa has been observed in the mouse and pig (Akama *et al.*, 1994; Ohmura *et al.*, 1999). Trypsin–like proteases are serine proteases with Arg/Lys-Xaa cleavage specificity. Among the serine proteases with such catalytic function found in the mammalian sperm acrosome are proacrosin/acrosin (Crosby *et al.*, 1998), trypsin-like proteases (Akama *et al.*, 1994) and some of the *te*sticular *s*erine *p*roteases 1-5 (TESP 1-5) identified in mouse sperm (Honda *et al.*, 2002b) among others. In both the mouse and human sperm, serine protease activity during sperm-zona interaction appears to

have a crucial function as inhibitors of these proteases actually prevent sperm-zona penetration in vitro (Fraser, 1982; Liu and Baker, 1993).

It is thought that trypsin-like proteases in mammalian sperm may be involved in enzymatic dispersal of acrosomal contents during acrosome reaction, limited hydrolytic activity on the zona pellucida during sperm penetration, cleavage and subsequent activation of certain acrosomal matrix constituents and lectin-like carbohydrate binding properties and hence involvement in sperm-zona pellucida secondary binding (Ohmura *et al.*, 1999; Honda *et al.*, 2002a). It has indeed been suggested that among the proteases activated following acrosome reaction are a group of serine proteases that are associated with the acrosomal membranes and this latter group may subsequently be involved in sperm-zona pellucida interactions (Honda *et al.*, 2002b).

An inner acrosomal membrane-associated trypsin-like protein in human sperm would therefore be an attractive prospect as IAM-1 is also associated with the inner acrosomal membrane. Furthermore, following preparation of human sperm under alkaline conditions in our experiments, we resolved proteins of pI's between 5.3 – 6.2 and apparent molecular weights between 23-25kDa. The theoretical calculated molecular weights for trypsins range between 24 – 27kDa in the different species with pI's of 4.7 – 7.4. It would therefore be in keeping with these expected results and with the known functions of trypsin-like proteases in acrosomes of spermatozoa to have such a protein in the human sperm acrosome. Therefore, on the basis of the biochemical parameters of

trypsin-like proteins, the functional characteristics of serine proteases that form acrosomal contents in other species, and the relatively high scores obtained in matches for trypsin-like proteins in our experiments and the proposed functions of trypsin-like serine proteases in the acrosome, it appears that IAM-1 is a membrane-anchored trypsin-like protein.

3.7.5.3. *Glutathione S-transferase*

Two of the peptides sequenced following the trypsin digestion were matched to human glutathione S-transferase mu3. Glutathione S-transferases exist in several forms and are expressed in many body tissues. They are primarily cytosolic detoxification enzymes that offer protection against reactive oxygen species of exogenous or endogenous origin (Hayes and Pulford, 1995; Kidd *et al.*, 2003). Reactive oxygen species include the superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\cdot}OH$) and hydroxide anion (^{-}OH) which are produced during metabolic redox reactions in body cells (Loscalzo, 2004). Though there are three classes of this enzyme in the body, it is only the μ class that is expressed in brain and testicular tissue (Campbell *et al.*, 1990). The testicular Glutathione S-transferase μ class isoenzyme has a pI of about 5.2, similar to the brain one and both are thought to be associated with maintaining the integrity of the blood-testicular and blood-brain barriers in the respective organs (Campbell *et al.*, 1990).

Catalytically active μ class Glutathione S-transferases have also been identified on goat sperm plasma membrane where they also appear to be involved in sperm-egg interaction particularly sperm-zona binding (Hemachand

et al., 2002). Furthermore, introduction of solubilised zona pellucida to spermatozoa causes aggregation of Glutathione S-transferases and induces acrosome reaction. The sperm membrane associated Glutathione S-transferase had molecular weights of 23 –26 kDa but were removed by treatment with strongly ionic solutions and immunolocalised to the sperm surface indicating they were peripheral membrane proteins (Hemachand *et al.*, 2002). Our findings of peptides matching to μ class Glutathione S-transferase in our 2-D gels is in agreement with the earlier findings regarding presence of this enzyme on the membranes associated with sperm. While earlier investigations pointed to its presence in the human testis-blood barrier (Campbell *et al.*, 1990), this study demonstrates that it is one of the protein constituents of the ejaculated human sperm. Further immunolocalisation work needs to be done to determine its specific location on human sperm. It is however unlikely that Glutathione S-transferase mu3 is IAM-1, judging from its biochemical characteristics (Hemachand *et al.*, 2002) that identify it as a peripheral protein on the plasma membrane.

3.7.5.4. *Tubulin alpha-3/7*

This was first identified in mice where it is exclusively expressed in the testis (Villasante *et al.*, 1986). Genes encoding for the same protein were found in humans. The alpha-3 and alpha-7 tubulins are identical though coded for by different genes (Villasante *et al.*, 1986). It is also known that alpha and beta tubulins form dimers (Fanarraga *et al.*, 1999). Subsequently other studies have

shown that alpha-3/7 tubulins are testis and sperm specific in humans. In the sperm they are components of the axoneme while in the testis they have been localised to the manchette, a structure associated with morphogenesis of sperm during spermiogenesis (Mochida *et al.*, 1998; 1999). Therefore, Tubulin alpha-3/7 are cytoskeletal proteins found in germ cells.

The expected molecular weight of these proteins is about 49kDa with a pI of about 5.0. From our experiment it had an apparent molecular weight of 25kDa. It could be that the hydrolysis of the tubulin dimers by the alkaline conditions used in the experiment resulted in much smaller molecules of close to 25kDa. IAM-1 on the other hand is not a cytoskeletal protein and is localised to the acrosome and equatorial segment and not the tail of sperm. Inclusion of tubulin alpha3/7 among the digested peptides in both protein spots may be due to the constraints in our study where reducing agents were not used in the 2-D separation of proteins.

3.7.5.5. *Heat shock 27kDa protein*

Heat shock proteins are produced in various mammalian cells in response to elevated temperature or exposure to stressful environments (Sarge, 1995). In these cells they act as molecular chaperones involved in protein folding and transport and mediate development of tolerance to the physico-chemical stress (Bohring *et al.*, 2001). Among the heat shock proteins that have been localised on the surface of spermatozoa are 95kDa and 70 kDa proteins (Miller *et al.*, 1992b). More recently analysis of the human genome has revealed

that one of the outer dense fiber proteins HspB10, has a lot of similarity to the 27kDa human heat shock protein (Fontaine *et al.*, 2003). Our finding of a 27 kDa heat shock protein is therefore in agreement with earlier work that heat shock proteins are found on sperm despite the inactive state of the sperm genome. The precise function of these proteins however is still undetermined as it is unlikely that they have similar functions in germ cells to that in somatic cells.

3.7.5.6. *Proteasomes*

One of the peptides sequenced was matched to proteasome beta chain precursor. Proteasomes are multicatalytic complexes of cellular proteases involved in ATP dependent degradation of ubiquinated proteins (Tipler *et al.*, 1997). Proteasomes have been found in all eukaryotic cells so far studied (Wojcik *et al.*, 2000). Work done on human and mouse spermatozoa demonstrated presence of 26S proteasomes in the gametes (Tipler *et al.*, 1997). Furthermore, proteasomes have been localised on the acrosome, post-acrosomal region, nuclear vacuoles of neck region, mid-piece and cytoplasmic droplet of normal human spermatozoa (Ziemba *et al.*, 2002). Other work has localised proteasomes particularly at the centriolar region in the neck and acrosome of human sperm (Wojcik *et al.*, 2000). It has been suggested that these enzymes could be involved in degradation of histones leading to nuclear condensation during spermiogenesis (Wojcik *et al.*, 2000).

With regard to mature spermatozoa, proteasomes are thought to be involved in degradation of paternal mitochondria during mammalian fertilisation and in sperm-zona interaction (Sutovsky *et al.*, 2003; 2004). While IAM-1 is testis and sperm specific, proteasomes are generally found in many somatic cells other than sperm. It is therefore unlikely that IAM-1 could be a proteasome. However, the finding of proteasomes in our 2-D studies on human sperm is consistent with other studies that have localised these enzymes on mammalian sperm (Tipler *et al.*, 1997; Ziemba *et al.*, 2002; Sutovsky *et al.*, 2004)

The Ras related protein Rab-11a (RAB-11) like proteasomes is also found in a number of somatic cells in human and also mouse tissues (Gromov *et al.*, 1998). On the same basis of tissue and species specificity it is unlikely that IAM-1 is Rab-11a.

3.7.6. Conclusion

Investigations to characterise IAM-1 were inconclusive with regard to the precise identity of the protein. Examination of data obtained from the internal peptide sequencing however, pointed to IAM-1 being a trypsin-like protein more than any of the other proteins that were isolated. Biochemical data supported its association with the acrosomal membranes as a membrane anchored protein or integral membrane protein which in its native state has a pI of 3.86-4.0 and apparent molecular weight of between 23-25 kDa.

Chapter 4

GENERAL DISCUSSION AND CONCLUSION

4.1. Fertilisation Antigens

A number of sperm antigens conserved among several mammalian species have been studied (Feuchter *et al.*, 1981; Isahakia and Alexander, 1984; Eddy and O'Brien, 1994; Naz, 1999). The initial observations were made using antisera against whole sperm preparations (Hansen, 1972; D'Almeida and Voisin, 1977). Later on, with the development of the hybridoma technique by Kohler and Milstein (1975), monoclonal antibodies raised against specific epitopes of sperm antigens confirmed the presence of common antigens on mammalian spermatozoa (Feuchter *et al.*, 1981; Isahakia and Alexander, 1984; Eddy and O'Brien, 1994; Naz, 1999).

The present study on various fertilisation antigens in human and baboon spermatozoa provided insight into the nature of some of the determinants found in the two species. The primary antibodies used were monoclonal antibodies (mAbs) previously raised against specific components of rodent or human spermatozoa. Some of the mAbs like M1 that was raised against a determinant on hamster spermatozoa (Noor and Moore, 1999), failed to recognise any determinant on both human and baboon sperm. Others like mAbs ES-1 and IAM-1 raised against determinants on purified human sperm heads (Al-Eisa *et al.*, 2001), recognised determinants at identical domains on baboon and human spermatozoa.

Furthermore, there was homology in the corresponding proteins on immunoblots of sperm in the two species. Phosphotyrosine proteins were not restricted to particular domains on spermatozoa in both species but were found in both the head and tail of sperm in the two species. Furthermore, the patterns of phosphotyrosine staining of baboon and human spermatozoa were similar. mAb 18.6 that recognises an antigen found on the anterior acrosome of spermatozoa in a number of mammals (Moore *et al.*, 1990) localised similar determinants on the anterior acrosome on both human and baboon sperm.

The import of all these findings taken together is that there is similarity in a number of fertilisation antigens localised on baboon and human sperm. Earlier work using rabbit antisera against testicular tissue demonstrated the presence of shared testicular antigens among human, chimpanzee, rhesus monkey and the baboon (Menge and Fuller, 1975). Moreover, further work using monoclonal antibodies confirmed presence of similar epitopes on sperm from human, monkey, dogs, rabbits, rats and mice as shown by cross-reacting antibodies in these species (Isahakia and Alexander, 1984). Other studies have also confirmed presence of shared antigens of testicular origin on baboon and human sperm (Isahakia, 1988). Therefore, on one hand, our results particularly with mAb 18.6 and anti-phosphotyrosine mAbs demonstrate presence of antigens, which conserved in a number of mammalian species, in human and baboon sperm. On the other hand, failure of mAb M1 to recognise any antigen on baboon and human sperm is indicative that this particular determinant is not conserved in these species and

therefore may not be of importance with regard to fertilisation in these and possibly other primates.

4.1.1. *ES-1*

ES-1 was one of the antigens that was localised on both baboon and human sperm. Our studies demonstrated identical localisation of the ES-1 antigen on the equatorial segment of both baboon and human sperm. Furthermore, immunoblots of baboon and human sperm revealed cross-reacting antigens of similar molecular weights in the two species. Electron microscopic immunolocalisation had shown ES-1 on the plasma membrane overlying the equatorial segment of acrosome-reacted human sperm (Al-Eisa *et al.*, 2001). Moreover, *in vitro* fertilisation inhibition studies using human sperm and zona-free hamster oocytes indicated that the mAb ES-1 significantly inhibited sperm-oolemmal fusion process (Al-Eisa *et al.*, 2001). Taken together, our present findings and those of earlier studies (Al Eisa *et al.*, 2001) indicate that the determinant ES-1 may be involved in sperm-oolemmal fusion in the two primates and suggests homology of the protein in the two species. The extent of homology would need to be determined by sequencing of the isolated purified proteins as the monoclonal antibody recognises a specific epitope found on the protein in the two species.

In mammals, the plasma membrane overlying the apical equatorial segment is the part of the acrosome-reacted sperm that initially fuses with the oolemma (Moore and Bedford, 1978; Bedford *et al.*, 1979; Sathananthan *et al.*,

1986). As in sperm-zona pellucida interactions it is thought that there maybe initial recognition followed by adhesion events likely to involve molecules of the plasma membrane at the equatorial segment and corresponding ligands on the oolemma (Brewis and Moore, 1997; Evans *et al.*, 2001). Furthermore, fusogenicity of the equatorial segment unlike other domains of acrosome-reacted sperm has been shown using liposomes (Arts *et al.*, 1993). Though poorly understood, the acquisition of fusion competence by the plasma membrane overlying the equatorial segment is thought to be the result of action by some of the acrosomal contents during acrosome reaction (Takano *et al.*, 1993; Yanagimachi, 1994). The presence of ES-1 on the plasma membrane of the equatorial segment of acrosome-reacted sperm makes it a suitable candidate for further investigation regarding sperm-oolemmal interactions.

Among the other sperm equatorial segment associated proteins that have been isolated in mammals, are the 40kDa mouse protein recognised by M29 (Saling *et al.*, 1986), the 30-51kDa proteins for G11 and M13 (Allen and Green, 1995), the 37.8 and 34 kDa hamster protein, M1 (Noor and Moore, 1999), a 36 kDa protein of pI~5.5 that was identified on human sperm (Auer *et al.*, 2000), a 38 - 48 kDa protein called equatorin recognised by mAb MN9 that has been isolated from sperm in rodents and man (Manandhar and Toshimori, 2001) and equatorial segment protein of 34-38kDa and pI of 5.1-5.4 (Wolkowicz *et al.*, 2003). One common finding for these protein constituents of the equatorial segment is that

specific antibodies against certain epitopes on these proteins inhibit sperm-oolemmal fusion (Wolkowicz *et al.*, 2003).

Another interesting observation is that the molecular weights of the proteins associated with the equatorial segment as reported by the different investigators, tend to be clustered around 38-40kDa. However, most of them have not been fully characterised hence it is difficult to know whether some could just be the same phylogenetically conserved proteins isolated in different species. ES-1 has five bands including a 38kDa band and 40 kDa band in baboon and humans respectively. Isolation and sequencing of the protein may be necessary to characterise it further and determine whether the immunoreactive bands seen on 1-D SDS gels are isoforms of the same protein and whether they could be related to any of the other equatorial segment proteins that have been isolated in other species and in humans.

4.1.2. *IAM-1*

This protein was localised on both human and baboon sperm. mAb IAM-1 localised the determinants to the equatorial segment and the anterior acrosome in both species. Specific isolation and characterisation of this particular protein through the protocols used in our experiments were not entirely unequivocal. This was attributed to a number of inherent constraints due to the physical characteristics of the cognate protein that have been discussed in the previous

chapter. Nevertheless, the following properties of the protein became evident from our experimental data:

i) The protein was either an integral membrane protein or a protein that is well anchored to the acrosomal membranes by a lipid anchor (Fig.24)

ii) The protein first appears during spermiogenesis in the seminiferous epithelium associated with developing round spermatids

iii) The IAM-1 protein had a number of immunoreactive bands of molecular weights of 28, 32, 39 and 45 kDa in human while it was 32, 38 and 44 kDa in baboon sperm 1-D immunoblots.

iv) The IAM-1 protein from human sperm separated by 2-D electrophoresis had a pI of 3.86 - 4.0 and molecular weight 23-32 kDa while under alkaline treatment it had a pI of 5.3-6.2 and molecular weight of 23-25 kDa. This data suggests that IAM-1 could be post-translationally modified making it more acidic in its native state.

v) IAM-1 had at least one conformational epitope that was (or were) irreversibly altered on introduction of reducing agents to the extraction solutions.

vi) IAM-1 is a non-glycosylated membrane protein.

vii) IAM-1 could be a trypsin-like protein.

Other than transmembrane proteins, eukaryotic proteins are attached to the membranes by lipid anchors (Fig. 24), the most abundant being those anchored by glycosylphosphatidylinositol (GPI) (Udenfriend and Kodukula, 1995). There are reports in literature of lipid anchored sperm membrane proteins in the acrosomal

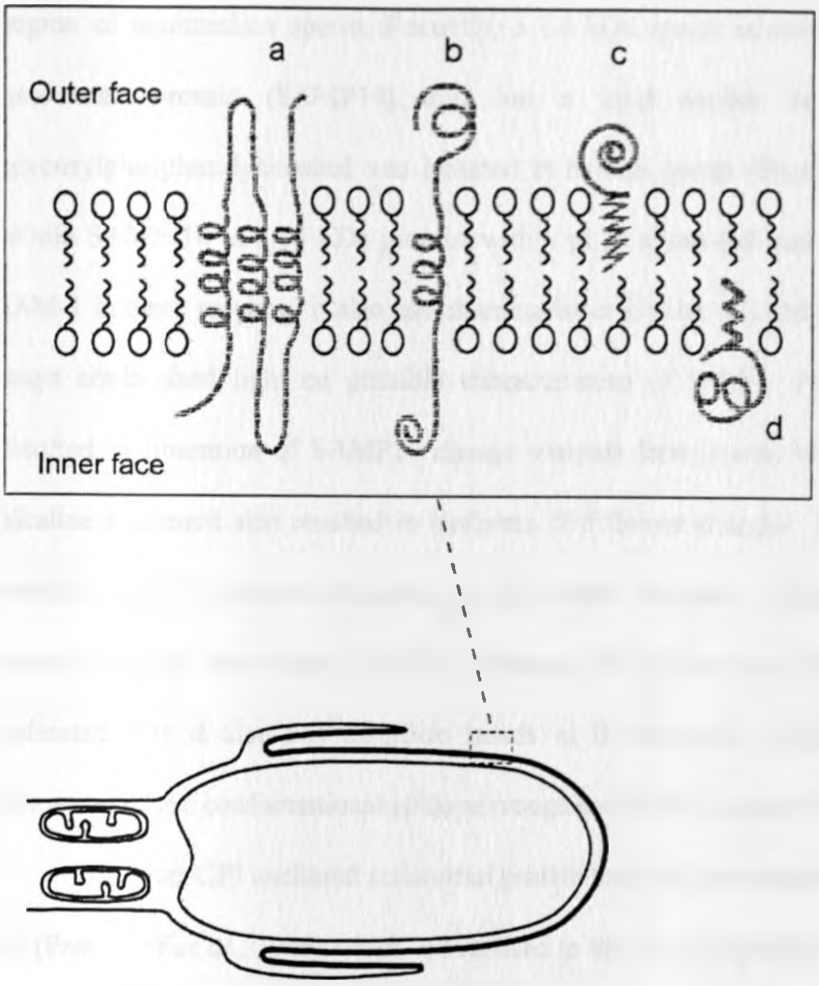


Fig. 24. Acrosome-reacted human sperm showing distribution of IAM-1(bold line) on the inner acrosomal membrane and equatorial segment. Hypothesized ways in which IAM-1 is anchored to the inner acrosomal membrane and equatorial segment is shown in the enlarged box. a- is an integral membrane protein with multiple transmembrane segments, b- a single pass integral membrane protein, c- is a glycosylphosphatidyl inositol anchored membrane protein, d- is a prenyl or fatty acid anchored protein.

region of mammalian sperm. Recently, a 14 kDa *s*perm *a*crosomal *m*embrane-associated *p*rotein (SAMP14) that has a lipid anchor in the form of glycosylphosphatidylinositol was isolated in human sperm (Shetty *et al.*, 2003). While SAMP 14 is a 14 kDa protein with a pI of about 4.8 and is dissimilar to IAM-1 in these respects, it also has characteristics similar to IAM-1 and in certain ways could shed light on possible characteristics of IAM-1. PIPLC treatment resulted in liberation of SAMP14 charge variants from sperm while for IAM-1 alkaline treatment also resulted in isoforms of different charges. SAMP14 has a domain with 10 cysteine residues on the amino terminus. Although it is not presently clear how many cysteine residues are present on IAM-1 our data indicated that it also has disulfide bonds in its structure contributing to the formation of the conformational epitope recognised by the cognate antibody.

Another GPI anchored acrosomal protein that has been characterised is PH-20 (Primakoff *et al.*, 1985) which is localised to the inner acrosomal membrane of human sperm following acrosome reaction (Sabeur *et al.*, 1997). However, its localization on the entire human sperm head plasma membrane before acrosome reaction and molecular weight differences are basic differences PH-20 has when compared to IAM-1 (Sabeur *et al.*, 1997).

IAM-1 bears considerable similarity to SP-10 another acrosomal protein that has been identified in human sperm. SP-10 is reported as being first seen in the human testis where it is associated with the round and maturing spermatids of the adluminal compartment (Herr *et al.*, 1990a). In the present study, IAM-1 was

observed on the adluminal spermatids undergoing spermiogenesis within the seminiferous tubules of the baboon. A similar pattern of IAM-1 distribution had been observed in the human testis. On human spermatozoa, IAM-1 was localised on the inner acrosomal membrane and equatorial segment following ionophore induced acrosomal reaction (Al-Eisa *et al.*, 2001). This distribution is identical to that of SP-10 after ionophore induced acrosome reaction (Herr *et al.*, 1990a). It has been reported that there is a pool of SP-10 that is anchored to the acrosomal membranes by TX-114 resistant anchors and that this pool is extracted only by extreme pH conditions and strong detergents (Foster and Herr, 1992). This compares favourably with the present data on IAM-1 which is not extracted by strong ionic solutions, very poorly extracted in TX-100 another non-ionic detergent and requires extreme pH, chaotropes and strong zwitterionic detergents for effective extraction. Based on these similarities, this could suggest lipid anchorage of IAM-1 too as in the SP-10 case.

The present study has demonstrated IAM-1 on both baboon and human sperm while SP-10 had also been identified earlier on sperm of both species (Herr *et al.*, 1990b). From the foregoing it is tempting to suggest that IAM-1 could be SP-10; however, there are fundamentally significant differences in the biochemical data obtained from each of these two human sperm proteins. While SP-10 appears on SDS-PAGE gels as a series of polymorphic peptides that have a molecular weight range between 18 -32 kDa in epididymal and ejaculated human sperm

(Herr *et al*, 1990a; Foster *et al.*, 1994), IAM-1 had about 4 bands between 28-45 kDa in ejaculated human sperm.

Presence of multiple immunoreactive peptides of different molecular weights has been attributed to endoproteolytic processing of intra-acrosomal proteins or alternative splicing during transcription of the gene (Wright *et al.*, 1990; Herr *et al.*, 1992). Furthermore, it was noted that proteolytic processing of SP-10 could begin during spermiogenesis within the testis and proceed to the epididymal maturation stage (Foster *et al.*, 1994). It is possible that IAM-1 protein undergoes activation in the form of proteolytic hydrolysis in the course of acrosome reaction hence the multiple immunoreactive bands seen. The pI of the native protein is also clustered at 4.9 for SP-10 (Herr *et al.*, 1990a) while that of IAM-1 is between 3.8-4.0. These biochemical parameters confirm that though both are acidic proteins, they are distinctly different on the basis of their molecular weights and isoelectric points.

Another protein that has been localised to the equatorial segment and anterior acrosome of sperm in a hamster, rat, rabbit, bull and human sperm is a 22kDa protein referred to as SP22 (Klinefelter *et al.*, 2002). The human homologue is DJ-1. However, despite similar immunolocalisation of SP-22 and IAM-1 on human sperm and despite evidence of synthesis of SP-22 in spermatids within the testis, SP-22 has been found in other tissues including the brain (Klinefelter *et al*, 2002). It belongs to a family of conserved proteins found even in prokaryotes (Welch *et al.*, 1998). SP22/DJ-1 appears to be involved in zona

penetration and sperm-oolemmal fusion (Klinefelter *et al.*, 2002; Okada *et al.*, 2002). IAM-1 on the other hand appears to be testis and sperm specific (Al-Eisa *et al.*, 2001), and so far appears to be found in primates and man and not rodents.

IAM-I is localised on the inner acrosomal membrane and at the equatorial segment of human sperm following acrosome reaction indicating possible involvement in secondary sperm-zona interactions and the fusion process of gametes (Al-Eisa *et al.*, 2001). Following acrosome reaction and loss of the acrosomal ghost as the sperm penetrates the zona pellucida, the plasma membrane overlying the equatorial segment and inner acrosomal membrane become the surface components on the anterior part of the sperm head. IAM-1 has been immunolocalised under electron microscopy to the inner acrosomal membrane and equatorial segment of acrosome-reacted human sperm (Al-Eisa *et al.*, 2001). Our studies have also shown similar immunolocalisation on capacitated and non-capacitated human sperm permeabilised by methanol fixation. Moreover, there was greater intensity of immunofluorescence at equatorial segment of human sperm compared to anterior acrosome under confocal microscopy. Our studies using non-capacitated baboon sperm also revealed predominant localization on the equatorial segment in most of the methanol permeabilised sperm. On the basis of equatorial segment localization it appears that IAM-1 could have a role in sperm-oolemmal interaction particularly the fusion process.

There are possible hypotheses related to the localization of IAM-1 on the inner acrosomal membrane. Evidence from the present study on internal peptide

sequence determination though equivocal, suggests this membrane protein is a trypsin-like protease. Other observations we made were that it is intimately bound or anchored to the inner acrosomal membrane and equatorial segment. This was evident from:

i) the findings that following disruption of the acrosome by sonication there was mAb IAM-1 epifluorescence on the anterior 2/3 of human sperm, likely to be the inner acrosomal membrane and equatorial segment

ii) the extraction experiments with differing extraction solutions where despite sonicating human sperm and treating the resultant sperm heads with strongly ionic 1M NaCl solution, there was still mAb IAM-1 epifluorescence evident on the sperm heads.

A membrane-anchored serine-protease would be particularly useful as the sperm penetrates the zona pellucida. Being associated with the inner acrosomal membrane, IAM-1 would not participate in initial sperm-zona recognition events. However, it could participate in the secondary sperm-zona interaction. Secondary sperm-zona binding involves more persistent adhesion than the initial contact that induces acrosomal exocytosis (Brewis and Moore, 1997). Though poorly studied in man and other primates, it has been studied in more detail in the mouse where the receptor on the zona pellucida has been identified as ZP2 (Bleil *et al.*, 1988).

ZP2 is implicated as it binds acrosome-reacted sperm and not acrosome-intact sperm (Mortillo and Wassarman, 1991). There is no consensus so far on the corresponding sperm ligand for ZP2. However, proacrosin/acrosin has been

proposed as a possible ligand for ZP2. While acrosin is a serine protease with other hydrolytic functions in the acrosomal matrix, it was also shown to have lectin-like zona binding activity possibly involving strong ionic interactions of the carbohydrate moieties of ZP2 and basic residues on proacrosin/acrosin (Richardson and O'Rand, 1996; Howes and Jones, 2002). The case for acrosin or trypsin-like protease involvement in zona penetration by human sperm was further strengthened by the work of Liu and Baker (1993) that demonstrated lack of zona penetration when trypsin inhibitor was used *in vitro*. This was complicated somewhat by the observations that knock-out mice lacking the acrosin gene were fully fertile in natural mating experiments, suggesting acrosin activity was not requisite for zona penetration by sperm (Baba *et al.*, 1994). Taken together these findings imply that there could be other trypsin-like proteases essential in zona penetration by sperm.

In mice a family of other acrosomal testicular serine proteases (TESP) have been identified that could be involved in zona penetration (Ohmura *et al.*, 1999). Indeed it has been suggested that rather than having direct effects on the ZP during penetration of sperm, acrosin may act indirectly by activating other acrosomal proteases and dispersing acrosomal matrix components (Honda *et al.*, 2002a). While there have been differing opinions on the importance of acrosomal hydrolytic enzymes in sperm penetration of the eutherian zona pellucida (Yanagimachi, 1994; Bedford, 1998) it is unlikely that this event could be wholly

physical particularly in light of earlier work showing effects of trypsin inhibitors on sperm penetration of the zona pellucida (Liu and Baker, 1993).

It is therefore more likely that both the motility associated physical penetration and acrosomal hydrolytic action is important in zona penetration by eutherian sperm. The slit like passage of these sperm through the zona may more likely be due to interplay of limited hydrolysis particularly by acrosomal membrane proteases that are exposed to the zona pellucida following acrosome reaction coupled with the shearing action of the motile sperm head (Bedford, 1998; Primakoff and Myles, 2002). While the acrosomal matrix proteases like proacrosin/acrosin may serve important functions like dispersal of acrosomal contents and activating other enzymes proteolytically and transient retention of acrosome-reacted sperm on the zona surface (Honda *et al.*, 2002a; Howes and Jones, 2002), it is those proteases well anchored to the inner acrosomal membrane that would be better placed particularly when it comes to providing the limited hydrolysis required as the sperm penetrates the zona pellucida.

Work on human sperm by Al-Eisa *et al.*, (2001) suggested involvement of IAM-1 in sperm-zona interaction as mAb IAM-1 significantly inhibited sperm-zona interaction and not sperm-oolemmal interaction. It is therefore our hypothesis that among the trypsin-like membrane proteases involved in sperm-zona pellucida interaction in human sperm is IAM-1. This is by virtue of its intimate association with the inner acrosomal membrane even following acrosome reaction. It would

therefore not be affected by dispersion of acrosomal contents but can interact with the ZP directly as the sperm penetrates this layer.

These studies have demonstrated presence of cross-reacting antibodies for shared determinants in baboon and human sperm. Some of the determinants like IAM-1 and ES-1 have been localised in humans but not in rodents. It also demonstrated that these antigens could be conserved in primates as they are localised at similar domains and have almost similar biochemical parameters in the two species. Other determinants are shared among many mammals hence appear to be generally conserved. The results of these studies are in agreement with earlier studies that have demonstrated similar determinants on baboon and human sperm (Isahakia and Alexander, 1984; Isahakia, 1988). They add additional evidence for use of the baboon as a model for studying some of the human fertilisation proteins. Further work focused on characterising IAM-1. Our study developed a protocol for isolation of this protein from human sperm under non-reducing conditions. It was found to be an integral membrane protein or membrane anchored protein of testicular origin. IAM-1 could be a trypsin-like protease involved in secondary sperm-zona interaction.

4.2. Conclusion

This study has localised a number of sperm fertilisation antigens common to human and baboon sperm using different monoclonal antibodies. The monoclonal antibodies were mAb 18.6, M1, anti-phosphotyrosine, ES-1 and IAM-

l raised against components of mammalian sperm in different species. The techniques involved indirect immunofluorescence of sperm and testicular sections and immunoblotting.

It was found that other than mAbM1 that was negative, the other monoclonal antibodies recognised similar determinants on human and baboon sperm. The study then focused on Inner acrosomal membrane protein (IAM-1) for which 2-D gels of human sperm protein extract were run to identify and characterise the protein. In the process, a protocol for extraction of sperm membrane proteins under non-reducing conditions was developed.

IAM-1 was found to be a 28-45kDa protein with a pI of 3.8-4.0 for human sperm. It was found to be an integral membrane protein or lipid anchored membrane protein of testicular origin. While peptide mass fingerprinting indicated IAM-1 could be a novel protein, internal peptide sequencing suggested it could be a trypsin-like protein. It seems likely that IAM-1 could be involved in sperm penetration of the zona pellucida.

4.2.1. Future directions

In the course of the present studies alternative lines of possible investigations were opened up particularly in regard to IAM-1 and ES-1:

IAM-1

1) Phosphatidlyinositol specific phospholipase C can be used to cleave phosphate-glycerol bonds in phospholipids and glycosylphosphatidylinositol (GPI) anchors

(Udenfriend and Kodukula, 1995). Treatment of the sonicated sperm with this enzyme should liberate the protein IAM-1 if it is indeed anchored to the inner acrosomal and the equatorial segment membranes by means of a GPI anchor.

2) Confirmation of the gene sequence of IAM-1 will confirm our findings regarding the possibility of it being a Trypsin-like membrane protein. In this regard it would be necessary to screen a human or baboon testicular cDNA expression library with the cognate monoclonal antibody and sequence the clones that are identified. This could be possible as the present studies identified the cognate antigen in the seminiferous epithelium of the testis. The sequence of the entire open reading frame of this protein would provide useful data on the function of the protein depending on the motifs identifiable from the sequence. Furthermore, synthetic peptides could then be synthesized to determine the effect of antibodies against these on fertilisation in the baboon or human and whether mAb IAM-1 would recognise them. However, based on the fact that mAb IAM-1 recognises a conformational epitope, screening an expression library using this antibody is limiting as it would depend entirely on similarity of the tertiary structure of bacteria-expressed protein to native IAM-1.

Immunoprecipitation may not also be feasible for this protein as the antibody recognises a conformational epitope. This epitope is affected by the reducing agents that would be used during immunoprecipitation.

3) In our experiments, it was possible that presence of other more soluble sperm proteins reduced efficiency of 2-D separation of IAM-1. In this regard pre-

fractionation may be necessary step before 2-D isolation of IAM-1. This would involve extraction using high salt concentrations like 1M NaCl to strip off peripheral membrane proteins before subsequent extraction using detergent-chaotrope mixture.

ES-1

This protein was isolated under reducing conditions and therefore is amenable to studies incorporating use of reducing agents. Further work isolating the protein from 2-D gels and sequencing the product should be possible using either baboon or human sperm. Homologous proteins or the protein itself can be identified from the databases and its function derived too based on its amino acid sequence. Synthetic peptides could be generated to test and confirm its function.

Phosphotyrosine proteins

These can be investigated particularly in the baboon on the basis of similarity in labeling patterns of human and baboon spermatozoa observed in the present studies. Investigations could cover determination of the specific proteins phosphorylated on baboon sperm during capacitation and on exposure to zona glycoproteins. There is increasing data on phosphorylated proteins in human sperm during capacitation and acrosome reaction with minimal comparative data in other primates. Therefore, these studies would provide useful comparative information.

Appendix

(1) *BWW Medium* (Biggers, Whitten, Whittingham Solution)

This medium was prepared as detailed below. To 400mls of double distilled water (ddH_2O), the following salts were added while stirring the solution to dissolve,

94.59mM NaCl	2.77g
4.78mM KCl	0.178g
1.71mM $CaCl_2.2H_2O$	0.127g
1.19mM KH_2PO_4	0.081g
1.19mM $MgSO_4.7H_2O$	0.147g
25.07mM $NaHCO_3$	1.053g
5.56mM D-glucose	0.5g
0.25mM Pyruvic acid (Sodium pyruvate)	0.014g
Penicillin/Streptomycin	0.5ml/400ml

2.00g of Lactic acid Sodium salt (or equivalent sodium lactate syrup) were weighed and carefully transferred into a 50ml sterile universal bottle. 50mls of ddH_2O was added to the universal bottle and mixed well to dissolve the salt. The sodium lactate solution was then added to 400mls solution that had been prepared, and the resultant solution made up to 500ml with ddH_2O.The pH was corrected to 7.8 using NaOH or HCl, while the osmolarity was approximately 299-308 milli osmols. The solution was stored at 4°C.

Night before use: 200mls of BWW medium was filtered with a 0.45µm filter(Millipore) for use. 20ml of the filtered BWW was aliquoted in a universal bottle and labeled as a Control. A further 20mls filtered BWW was aliquoted in another universal bottle. 20 mls of filtered BWW was mixed well in a universal bottle with 20mls of paraffin oil and left to equilibrate. A 5mls aliquot of BWW was also made in another universal bottle. All the media was incubated overnight at 37°C and 5% CO_2

Morning of use: Three drops of phenol red were added to the Control bottle. This was then kept in the incubator as a measure of the BWW acidity (pink indicated a pH 7.2-7.4, and goes yellow as acidity increases). The actual pH of the BWW was measured and recorded using the 5 ml aliquot. 0.3% BSA was added to one of the 20ml aliquots and it was used for Swim-up of sperm.

(2) *Phosphate buffered saline (PBS)*

Chemical	Concentration	Amount
NaCl	137mM	80g
KCl	2.7mM	2g
Na_2HPO_4	10mM	14.4g
KH_2PO_4	1.76mM	2.4g
Double distilled H_2O		1 litre

A X10 stock solution of Phosphate buffered saline was prepared as above. The solution was stirred at room temperature until all the salts dissolved. To make a

working solution, a 1:10 dilution was made using double distilled water. This was aliquoted in 500ml bottles and sterilized by autoclaving.

(3) *SDS Equilibration Solution/Buffer*

This was constituted as follows while stirring the solution,

Chemical Ingredients	Amount	Final Concentration
1.5M Tris-HCl pH 8.8	3.35mls	50mM
Urea	36.035g	6M
87%v/v Glycerol	34.5mls	30% v/v
SDS	2.0g	2%w/v
Double distilled	100mls	

25ml aliquots of the solution were made and frozen at –20°C.

Prior to use the aliquots were thawed to room temperature and a few grains of bromophenol blue added before equilibrating Immobilized pH gradient strips (IPG's) for the second dimension SDS PAGE.

(4) *CAPS 3-[cyclohexylamino]-1-propanesulfonic acid buffer for protein transfer*

(a) Preparation of Stock Solution of CAPS buffer {10X CAPS (100mM, pH=11): 22.13 g. of CAPS was dissolved in 900mls of distilled water. Titration with 2M NaOH (about 20mls) made the pH 11. Distilled water was added to make a total volume of 1 liter. The stock solution was stored at 4°C.

(b) Electroblotting buffer (1X stock buffer in 10% methanol):

This was prepared by making a 1:1:8 dilution of 10X CAPS stock buffer: methanol: distilled water respectively.

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