reports in the murine model, questioning the role of the consensus furin cleavage site (CFCS) in secretion of the zona proteins and incorporation in the matrix prompted us to express the baculovirus-expressed hZP3 without the C-terminal TD ($hZP3_{(1-348)}$), that lies immediately after the CFCS (Williams and Wassarman, 2001; Qi *et al.*, 2002; Zhao *et al.*, 2002). However, this protein also did not get secreted into the culture supernatant. It can be speculated that the secretory signals present in the *Sf21* cells may not be recognized by the foreign human zona proteins.

The expression of hZP2 in *E. coli* yielded several low molecular weight proteins in addition to the ~90 kDa main protein band as observed in the Western blot of induced host cells harboring the recombinant hZP2 plasmid. Various reasons that can be attributed to the presence of lower molecular weight fragments are, i) multiple initiation sites in mRNA, ii) premature translation termination iii) specific or non-specific proteolysis of the full-length protein, and iv) a combination of the above.

On SDS-PAGE, the *E. coli*- as well as baculovirus-expressed hZP2, hZP3 and hZP4 fusion proteins showed a slightly retarded mobility as is evident from their apparent molecular weights as compared to the theoretical values, which is sometimes the case with polyhistidine tagged fusion proteins. In case of the baculovirus-expressed recombinant proteins, the apparent molecular weights show greater deviation than the expected values and this may be attributed to glycosylation of these recombinant proteins in the insect cells. The characterization of native ZP glycoproteins from human oocytes by various groups has revealed heterogeneity and variability in their mobility in SDS-PAGE (Shabanowitz and O'Rand, 1988; Bercegeay *et al.*, 1995; Gupta *et al.*, 1998; Bauskin *et al.*, 1999). Some of the discrepancies in the apparent molecular weights assigned to various ZP glycoproteins may be attributed to the different nomenclature used by various investigators. Using antibodies against synthetic peptides, it has been documented that hZP2 is comprised of 90-110 kDa and hZP3 as 53-60 kDa (Bauskin *et al.*, 1999). The baculovirus-expressed recombinant hZP2 revealed a band of ~105 kDa and $hZP3_{(1-424)}$ ~65 kDa (Figure 13), which were comparable to the native proteins. Lectin binding analysis indeed revealed the presence of both N- and O-linked glycosylation in the baculovirus-expressed recombinant proteins, which were absent in the *E. coli*-expressed proteins (Figures 17 and 18). The predominant glycosylations in the four baculovirus-expressed recombinant ZP glycoproteins were characterized by the binding of lectins ConA (specific for mannose α 1-3/1-6 residues, N-linked) and

राष्ट्रीय प्रतिरक्षाविज्ञान संस्थान
NATIONAL INSTITUTE OF IMMUNOLOGY

CERTIFICATE

This is to certify that the thesis entitled, "**Molecular and Functional Analysis of Recombinant Human Zona Pellucida Proteins**" submitted by Ms. Sanchita Chakravarty, in partial fulfillment of **Ph.D.** degree of Jawaharlal Nehru University, embodies the work done by the candidate under my guidance at the **National Institute of Immunology, New Delhi**. This work is original and has not been submitted in part or in full for any other degree or diploma of any university.

SKGupta

Satish K. Gupta, Ph.D., FNA, FNASc, FASc, FAMS
Staff Scientist-VII
National Institute of Immunology
[Supervisor]

अरूणा आसफ अली मार्ग, नई दिल्ली - 110067 फैक्स : 91-11-26162125, 26177626 तार : IMMUNOLOGY
Aruna Asaf Ali Marg, New Delhi - 110067 Fax : 91-11-26162125, 26177626 Grams : IMMUNOLOGY
दूरभाष (Telephones) EPABX : 26717121 - 45 & 26717010 - 19

Contents	Page No

Acknowledgements

I take this opportunity to express my deep sense of gratitude to my supervisor, Dr. Satish K. Gupta, whose constant support, guidance, encouragement and motivation has enabled me to complete my thesis work successfully. He has been instrumental in inculcating discipline, confidence and a rational scientific approach in me that has helped me throughout the course of my thesis. I wish to thank Dr. Sandip K. Basu and Prof. Avadhesha Surolia, the Directors, NII, for their kind help and encouragement. I also thank my Doctoral Committee members, Dr. K. Muralidhar, Dr. Chandrima Shaha, Dr. Subeer Majumdar and Dr. Madhulika Srivastava for their help and valuable suggestions.

I sincerely thank Mr. Akhilesh K. Aggarwal and Ms. Sanju for their help in the academic matters including CSIR correspondences. Sh. Babulal and Sh Satish Sharma are thanked for their help in bringing out this thesis.

It has been a wonderful experience working in this lab, both professionally and personally. I extend my sincere thanks to Abhishek, Anasua, Anurag, Archana, Beena, Deepika, Gagan, Manish, Neela, Neelu, Pankaj, Pankaj Jr., Sangeeta, Sonika and Suraj for their help in all frontiers and making my stay in the lab a memorable experience. Gagan is especially thanked for being the ideal senior one can look up to, by going out of her way umpteen times to lend a helping hand both with my experiments and personal problems. Rajat and Raghav are thanked for their technical help.

Staying at RSH has been a wonderful experience especially with friends like Divya, Neetha, Paroma and Pooja around.

I am obliged to the National Institute of Immunology and Council of Scientific and Industrial Research, Government of India for providing me a fellowship during the course of my thesis.

Needless to say, I am most indebted to my beloved parents for their blessings, constant support and confidence that made me tide through the difficult times. This list would be incomplete without acknowledging Shilpi, my sister and Archana and Rohin, my unforgettable friends. Last but not the least, I wish to acknowledge Raj, who has been a pillar of strength for me, both on the professional and personal front, especially at times when I had almost given up. Had it not been for his faith in me, this thesis would not have seen the light of the day.

Sanchita

ABBREVIATIONS

µg	microgram
aa	amino acid
Ab	antibody
AcNPV	*Autographa californica* nuclear polyhedrosis virus
BCA	bicinchoninic acid
bm	bonnet monkey
bp	base pairs
BSA	bovine serum albumin
BWW	Biggers Whitten Whittingham
cDNA	complimentary deoxyribonucleic acid
CFA	complete Freund's adjuvant
ConA	Concanavalin A
DBA	*Dolichos biflorus* agglutinin
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotide triphosphates
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbant assay
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
GalNAc	N-acetylgalactosamine
GlNAc	N-acetylglucosamine
h	hour
HRPO	horseradish peroxidase
IFA	incomplete Freund's adjuvant
i.p.	intraperitoneally
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilobase
kDa	kilodalton

LB	Luria Bertani
LMP	low melting point
mA	milliamperes
MAbs	monoclonal antibodies
MCS	multiple cloning site
mg	milligram
min	minutes
ml	millilitre
mQ	milliQ
mRNA	messenger ribonucleic acid
mZP	mouse zona pellucida
Ni-NTA	nickel nitrilotricaetic acid
nt	nucleotide
O/N	overnight
OPD	orthophenylenediamine
PBS	phosphate buffered saline
PBST	PBS containing Tween-20
PCR	polymerase chain reaction
PEG	polyethylene glycol
PSA	*Pisum sativum* agglutinin
RBITC	rhodamine-B isothiocyanate
rpm	revolutions per minute
RPMI	Rosweli Park Memorial Institute
RT	room temperature
SBA	Soybean agglutinin
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SE	standard error
sec	seconds
Sf	*Spodoptera frugiperda*
SS	signal sequence
TAE	Tris-acetate-EDTA
TD	transmembrane-like domain
TE	Tris-EDTA

TEMED	N,N,N',N'-tetramethylenediamine
TRITC	Tetramethylrhodamine isothiocyanate
U	units
WGA	wheat germ agglutinin
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
ZP	zona pellucida
ZP1	zona pellucida glycoprotein 1
ZP2	zona pellucida glycoprotein 2
ZP3	zona pellucida glycoprotein 3
ZP4	zona pellucida glycoprotein 4

Introduction

Procreation in mammals takes place when the spermatozoon and the egg unite by an extraordinary cell fusion event that gives rise to an original individual and triggers a very sophisticated developmental program in the newly formed zygote. Initial contact between the two gametes occurs when the sperm attach to the zona pellucida (ZP), an extracellular translucent matrix surrounding the mammalian egg. The attachment of sperm strengthens into a tenacious binding with the ZP, a prerequisite for zona penetration. This binding is mediated by specific interaction between ZP glycoproteins and egg-binding proteins on the sperm surface, and induces the acrosome reaction which involves fusion and vesiculation of outer acrosomal membrane and sperm plasma membrane at many sites, release of the vesicles, and exposure of the inner acrosomal membrane. Then, the acrosome-reacted sperm penetrate the ZP, reach the perivitelline space, and fuse with the egg. To block polyspermy, transient depolarization of the egg plasma membrane and release of cortical granules including various enzymes to the perivitelline space are induced immediately after sperm-egg fusion, resulting in physical and chemical alterations of the ZP and inactivation of its sperm-binding ability. The ZP further plays an important role in protection of the blastocyst prior to implantation.

In various species, ZP is primarily composed of three glycoproteins (Harris *et al.*, 1994). These are designated as zona pellucida glycoprotein-1 (ZP1), -2 (ZP2) and -3 (ZP3) based on their mobility in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Recent studies, however, revealed that human ZP is composed of four glycoproteins classified as ZP1, ZP2, ZP3 and ZP4 (Lefievre *et al.*, 2004; Conner *et al.*, 2005) suggesting that the number of ZP glycoproteins is not conserved across the species.

Various studies suggest that in mouse, ZP3 serves as the putative primary sperm receptor and is also responsible for inducing acrosomal exocytosis (Bleil and Wassarman, 1980a; Beebe *et al.*, 1992). In this process, O-linked oligosaccharides play a critical role (Florman and Wassarman, 1985; Bleil and Wassarman, 1988). In the porcine system, however, both O-linked oligosaccharides (Yurewicz *et al.*, 1991) and the tri- and tetra-antennary neutral complex type of N-linked oligosaccharides (Nakano *et al.*, 1996; Yonezawa *et al.*, 1999) of ZP3 have been implicated for its sperm receptor activity. The critical appraisal of the role of ZP3 and other ZP glycoproteins during fertilization in humans was hampered due to their non-availability in highly purified

form from native source. Subsequently, employing recombinant human ZP3 (hZP3) expressed in Chinese hamster ovary (CHO) cells, it was demonstrated that incubation of capacitated human spermatozoa with hZP3 leads to induction of acrosomal exocytosis (van Duin *et al.*, 1994). Further, recombinant hZP3 expressed in *Escherichia coli* and presumably lacking glycosylation also induced acrosomal exocytosis, suggesting that the presence of carbohydrates on ZP3 polypeptide backbone may not be an absolute requirement for ZP3 to induce acrosome reaction (Chapman *et al.*, 1998).

The ZP2, in mouse model has been shown to serve as the secondary receptor that maintains the binding of the acrosome-reacted spermatozoa to the ZP, while the ZP1 acts as a cross-linker of the ZP2-ZP3 heterodimeric filaments (Greve and Wassarman, 1985; Bleil *et al.*, 1988). However, in the rabbit model, rec55 (homologue of ZP4) binds to the spermatozoa in a dose-dependent manner (Prasad *et al.*, 1996). Rabbit ZP4 also binds to recombinant Sp17 (a family of sperm autoantigens), further reiterating its importance during sperm-oocyte interaction (Yamasaki *et al.*, 1995). The porcine ZP3β (homologue of ZP3) fails to bind to the sperm receptors whereas ZP3α (homologue of ZP4)-ZP3β heterocomplexes bind with high affinity to boar sperm membrane vesicles, suggesting involvement of more than one ZP protein in sperm recognition (Yurewicz *et al.*, 1998). Additional studies have also demonstrated that recombinant bonnet monkey (*Macaca radiata*) ZP4 (bmZP4) expressed in *E. coli* binds to the head region of the capacitated spermatozoa and the binding shifts to the equatorial segment, post-acrosomal domain and mid-piece of the acrosome-reacted spermatozoa, indicating a role for ZP4 in the sperm binding (Govind *et al.*, 2001).

The above described observations suggest that the functional role of the individual ZP glycoproteins during the complex process of fertilization as delineated in mouse model may not be tenable in other species. Hence, in order to delineate the functions of ZP glycoproteins in a particular species, independent investigations should be carried out and without any bias based on the findings in murine model. This prompted us to revisit the role of ZP glycoproteins during fertilization in humans and to investigate the importance of glycosylation in this process. In the present study, attempts have been made to obtain the human zona proteins expressed in prokaryotic as well as eukaryotic expression systems in the absence of chaotropic agents and evaluate their functional attributes with respect to interaction with human spermatozoa. The *E. coli* expression system would express non-glycosylated zona proteins whereas eukaryotic expression

system would produce glycosylated recombinant zona proteins. For this purpose, hZP2, hZP3 and hZP4, devoid of N-terminal signal sequence (SS) and C-terminal transmembrane-like domain (TD) have been cloned and expressed in *E. coli*, while in baculovirus, full-length hZP2, hZP3 and hZP4 have been expressed. Additionally, hZP3, including SS but excluding TD has also been expressed in baculovirus. The conditions have been optimized for the purification of the recombinant proteins to obtain them in renatured form. Full-length hZP3 has also been expressed in a mammalian expression vector and the recombinant protein transiently expressed in Chinese hamster ovary-K1 (CHO-K1) cells. The *E. coli*- and baculovirus-expressed purified renatured proteins, along with cell lysate of CHO-K1 cells transiently transfected with hZP3 harboring expression vector, have been evaluated for their ability to bind to capacitated and/or acrosome-reacted human spermatozoa *in vitro* by direct binding assay using fluorochrome labeled zona proteins and/or by indirect immunofluorescence assay using specific antibody probes. The above have also been analyzed for their sperm receptor function by assessing their ability to induce acrosomal exocytosis in capacitated spermatozoa. This led to the finding that baculovirus-expressed hZP3 and hZP4 expressed in both the eukaryotic expression systems could induce acrosome reaction in capacitated human sperm. In order to understand the role of carbohydrate residues present on ZP glycoproteins in induction of the acrosome reaction, baculovirus-expressed hZP3 and hZP4 have been obtained by growing infected *Sf21* insect cells in the presence of tunicamycin. Alternatively, baculovirus-expressed purified recombinant proteins have been chemically deglycosylated. Both glycosylated recombinant human ZP glycoproteins and those deficient in N- or O-linked glycosylation have subsequently been assessed for their ability to induce acrosomal exocytosis in human sperm.

Several candidate ligands have been characterized on spermatozoa that are involved in binding to ZP glycoproteins (Wassarman, 1999). Recently, the proteasome complex present on sperm has been shown to play an important role during fertilization (Morales *et al.*, 2003; Pizzaro *et al.*, 2004). In the present thesis, attempts have been made to understand the role of sperm proteasome by employing recombinant human zona proteins during binding and induction of acrosome reaction.

Thus, in the present thesis, efforts have been made to elucidate the functional attributes of the individual human ZP glycoproteins. These studies will further our understanding

of the critical steps of sperm-egg interaction during human fertilization which, in turn, may provide new insights into dealing with the global problem of infertility on one hand, and development of novel contraceptives on the other.

Review of Literature

Zona Pellucida

Zona pellucida (ZP) is an acellular translucent sulfated glycoproteinaceous matrix that surrounds the mammalian oocyte. The ZP matrix is synthesized and secreted by the oocyte during follicular development and plays a very important role in mediating critical steps during fertilization. It acts as a species-specific "docking site" (Yanagimachi, 1977; Gwatkin and Williams, 1977) for the binding of sperm to the oocyte, induces acrosomal exocytosis in the zona bound spermatozoa (Bleil and Wassarman, 1983), prevents polyspermy (Gulyas and Yuan, 1985) and plays an important role in the protection of pre-implanted blastocyst. The murine ZP is composed of three biochemically and immunologically distinct glycoproteins designated as ZP1, ZP2 and ZP3 based on their mobility on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). These glycoproteins have also been classified on the basis of the size of their mRNA transcripts as ZPA, ZPB and ZPC, ZPA being the longest and ZPC, the shortest (Harris *et al.*, 1994), leading to a dual nomenclature (Table 1). Recent studies have shown that human ZP is composed of four ZP glycoproteins designated as ZP1, ZP2, ZP3 and ZP4 (Lefievre *et al.*, 2004; Conner *et al.*, 2005). In the present thesis, the nomenclature pertaining to the recent human ZP glycoproteins classification is used and ZP1/B (by earlier classification) in humans is referred to as ZP4 (Table 1).

Ultrastructure and Development

Formation of the ZP starts in the growing oocyte and increases in width as the oocyte increases in diameter (Wassarman and Mortillo, 1991). The ZP matrix appears to be fibrillogranular in nature, and its thickness, rigidity and size varies from species to species (Baranska *et al.*, 1975, Phillips and Shalgi, 1980a; b). The thickness of the ZP varies from <2 μm in marsupials to about 27 μm in cows (Dunbar and Wolgemuth, 1984). Scanning electron micrographs (SEM) of zonae from several species reveal that the ZP surface ultrastructure consists of an extensive network of filaments interspersed with numerous pores of varying sizes. High resolution SEM studies with mouse as well as human oocytes show that ZP appears as a delicate meshwork of thin interconnected filaments in a regular alternating pattern of wide and tight meshes/pores (Familiari *et al.*, 2006). These pores appear larger at the outer surface of the zona than inner surface. Ultrastructural cytochemical findings also suggest that the porous region of the human

Table 1: Classification of ZP glycoproteins in mouse and humans

	Zona protein			
Mouse				
Original classification	ZP1 (623 aa)	ZP2 (713 aa)	ZP3 (424 aa)	
Alternate classification	ZPB	ZPA	ZPC	
Human				
Earlier classification	ZP1 (540 aa)	ZP2 (745 aa)	ZP3 (424 aa)	
Earlier alternate classification	ZPB (540 aa)	ZPA (745 aa)	ZPC (424 aa)	
Recent classification; used in present thesis	ZP4 (540 aa)	ZP2 (745 aa)	ZP3 (424 aa)	ZP1 (638 aa)

ZP is limited to 25% of the external region of the human ZP, while the compact region constitutes ~75% of the total ZP (Jimenez-Movilla *et al.*, 2004). The amorphous spongy outer surface of zona with larger pores may facilitate sperm penetrability as human ZP with a more compact and smoother outer surface has been shown to be less penetrable (Familiari *et al.*, 1988; 1992).

A mature mouse oocyte has about 5-7 μm thick ZP that contains about 3-4 ng of protein (Bleil and Wassarman, 1980b; Wassarman and Mortillo, 1991). Electron microscopy has revealed that mouse ZP is a matrix comprised of 2-3 μm long interconnected filaments each possessing a structural repeat of 14-15 nm made up of a ZP2-ZP3 heterodimer formed via non-covalent bonds and cross-linked by ZP1 homodimers (Greve and Wassarman, 1985). In the mouse ZP, the ratio of ZP2:ZP3 is close to 1:1, whereas ZP1 is approximately 9% of the combined molar amounts of ZP2 and ZP3. The small amount of ZP1 relative to ZP2 and ZP3 may have important implications for the distribution of ZP1 cross-links, since the number of cross-linking sites potentially exceeds the number of ZP1 dimer molecules by a considerable margin (Green, 1997). Using double and triple immunolocalization experiments, it was ascertained that the spacing of ZP1 dimers as cross-linking sites of the ZP2-ZP3 heterodimers may be much closer than previously proposed (El-Mestrah *et al.*, 2002). Though it is known that ZP2-ZP3 dimers may be formed in oocytes prior to secretion as the secretory vesicles in growing oocyte contain both newly synthesized ZP2 and ZP3, the site of ZP2-ZP3 dimer formation is unknown (Wassarman and Mortillo, 1991). Perhaps, a certain degree of ZP pre-assembly occurs within the growing mouse oocyte as formation of ZP filaments was observed when purified mouse ZP2 and ZP3 were co-incubated under proper conditions *in vitro* (Wassarman *et al.*, 1996). However, the extent of this pre-assembly remains undetermined.

The development of mouse ZP begins with the non-uniform secretion of nascent ZP into localized pockets between the oocyte surface and surrounding follicular cells. In the early stages of oocyte growth, the ZP filaments appear to be organized into hexagonal arrays with uniform pore size between the oocyte and the follicle cells. As the oocyte grows and increases in size, the pockets of ZP filaments coalesce and form a uniform coat around the cell. During the growth of the oocyte, the microvilli increase in number and size, and along with the cellular projections from the cumulus cells, establish junctions with the oocyte. This increases the surface area of contact and forms

channels through ZP to provide nourishment to the growing oocyte (Wassarman and Mortillo, 1991).

Biochemical Characterization of the ZP Glycoproteins

Identification and analysis of the ZP glycoproteins from various species have been done using one as well as 2-dimensional (2-D) SDS-PAGE.

Murine and porcine models

In the most extensively studied model, mouse (m), the ZP is comprised of three families of glycoproteins, mZP1 (180-200 kDa), mZP2 (120-140 kDa) and mZP3 (83 kDa), based on their mobility on a SDS-PAGE run under non-reducing conditions (Bleil and Wassarman, 1980b). Under non-reducing conditions, porcine (p) ZP resolved into 2 separate glycoproteins designated as pZP1 (80-90 kDa) and pZP3 (55 kDa) (Yurewicz *et al.*, 1987) whereas, under reducing conditions, it resolved into 4 different families of glycoproteins classified as pZP1 (82 kDa), pZP2 (61 kDa), pZP3 (55 kDa) and pZP4 (21 kDa). The pZP2 and pZP4 have been shown to be derived from pZP1 by proteolysis and are cross-linked by intermolecular disulfide bonds (Hedrick and Wardrip, 1987; Yurewicz *et al.*, 1987) while pZP1 has been shown to be homologous to mZP2 (Taya *et al.*, 1995). Enzymatic deglycosylation with endo-β-galactosidase revealed that pZP3 is composed of two biochemically and immunologically distinct glycoproteins, pZP3α and pZP3β (Hedrick and Wardrip, 1987; Yurewicz *et al.*, 1987).

Human ZP glycoproteins

Under reducing conditions, zonae from human (h) unfertilized eggs separated into three acidic proteins with molecular weight ranging from 64-78 kDa (hZP2), 57-73 kDa (hZP3) and 90-110 kDa (hZP4; previously classified as ZP1/ZPB) (Shabanowitz and O'Rand, 1988). However, hZP2 and hZP4 co-migrated at 92-120 kDa under non-reducing conditions. Silver stained 2-D SDS-PAGE analysis of ZP components from unfertilized oocytes revealed that the hZP3 family shows marked charge heterogeneity (Bercegeay *et al.*, 1995). Polyclonal antibodies generated in rabbits against recombinant bonnet monkey (bm) ZP proteins were used as specific probes to characterize human ZP glycoproteins in Western blot of heat solubilized human ZP (hSIZP) resolved by SDS-PAGE (Gupta *et al.*, 1998). Under non-reduced conditions, hZP2, hZP3 and hZP4 resolved as 100, 53 and 60 kDa polypeptides respectively whereas under reduced conditions, dominant reactivity of hZP2, hZP3 and hZP4 was

localized to 65, 58 and 63 kDa and faint reactivity to 96, 138 and 53 kDa bands respectively. In 2-D SDS-PAGE, hZP4 was shown to be comprised of two chains at 63-58 and 55-45 kDa, each consisting of multiple isomers. The hZP2 was less acidic when compared with hZP3 and hZP4 and comprised a major component of 65 kDa and a minor component of approximately 96 kDa. The 65 kDa component displayed a higher degree of charged isomers in comparison with the 96 kDa component. The hZP3 comprised a broad band in the range 68-58 kDa (Gupta *et al.*, 1998). These studies show conclusively that the hZP4 overlaps with hZP3 and that in previous studies, hZP2 was likely to have been misinterpreted as being hZP4.

In spite of having very similar polypeptide backbone, the ZP proteins from various species have differences in their relative order of migration on SDS-PAGE. It is observed because of the differential post-translational modifications including glycosylation, which alter the effective molecular weight of the ZP proteins, and hence their migration (Wassarman, 1999). Variations in glycosylation also provide extensive charge heterogeneity to the ZP proteins and they exist as several isoelectric species. For instance, the pZP3 family has been shown to consist of 20 charged isomers with apparent pI values in the range of 3.5-6.0.

Molecular and Structural Characterization of ZP Glycoproteins

Genomic organization

Investigations pertaining to the genomic organization of the ZP proteins of different species have shown a similar intron/exon structure for each gene family. The *Zp2* family has 18 exons (human and cynomolgus monkey have an extra exon at C-terminus), *Zp3* family consists of 8 exons and *Zp4* family has 12 exons. However, the length of the chromosomal region comprising the locus of ZP genes varies from as small as 6.5 kilobases (kb) in m*Zp1* (Epifano *et al.*, 1995a) to as big as 18.3 kb in h*Zp3* (Chamberlin and Dean, 1990). In mouse, *Zp1*, *Zp2* and *Zp3* are single copy genes located on chromosomes 19, 7 and 5 respectively (Lunsford *et al.*, 1990; Epifano *et al.*, 1995b). The m*Zp1* has 12 exons ranging in size from 82-364 base pairs (bp) and encodes for a 623 aa long polypeptide. The exon size in m*Zp2* ranges from 45-190 bp, with a mRNA transcript of 2201 nucleotides (nt) encoding for a polypeptide of 713 aa while the m*Zp3* gene consists of 8 exons spanning 92-338 bp transcribed into 1302 nt mRNA encoding for a 424 aa polypeptide (Kinloch *et al.*, 1988; Liang and Dean,

1993). The exon map for h*Zp4* is similar to m*Zp1* and spans 11 kb. The transcript encodes for a 540 aa long polypeptide with just 39% similarity at the aa level with mZP1, which is due to the fact that m*Zp1* has an elongated exon 3 (Harris *et al.*, 1994). The newly documented h*Zp1* gene is located on chromosome 11 and encodes a polypeptide of 638 aa (Lefievre *et al.*, 2004). It is the true orthologue of mZP1 and shares a sequence identity of 67% at the aa level with mZP1 (Hughes and Barratt, 1999). Comparison of the human *Zp1* and murine *Zp1* genes indicates significant conservation of intron-exon size and organization, and of regulatory sequences. In addition, the m*Zp1* and h*Zp1* genes are in a region of conserved synteny between human chromosome 11 and mouse chromosome 19. The h*Zp2* has 19 exons (one more than that in mouse) transcribed into a 2235 nt long mRNA transcript, which encodes a 745 aa long polypeptide. The mZP2 and hZP2 share a sequence identity of 57% at aa level (Liang and Dean, 1993). The h*Zp3* gene contains 8 exons spanning 18.3 kb, the transcript of which encodes for a 424 aa polypeptide. The aa sequence identity shared between hZP3 and mZP3 is 67% (Chamberlin and Dean, 1990).

In addition to the genomic clones, the cDNA clones have also been characterized for mZP1 (Epifano *et al.*, 1995a), mZP2 (Liang and Dean, 1993), mZP3 (Ringuette *et al.*, 1986), hamster ZP3 (Kinloch *et al.*, 1990), rabbit 55 kDa protein (rec55; homologue of hZP4; Schwoebel *et al.*, 1991), rabbit 75 kDa protein (rec75; homologue of mZP2; Lee *et al.*, 1993), rabbit 45 kDa protein (rec45; homologue of mZP3; Harris *et al.*, 1994), pZP3α (homologue of hZP4; Yurewicz *et al.*, 1993a), pZP4 (homologue of mZP2; Taya *et al.*, 1995), pZP3β (homologue of mZP3; Harris *et al.*, 1994), marmoset ZP3 (Thillai-Koothan *et al.*, 1993), hZP1 (Anasua Ganguly, Gamete Antigen Laboratory, National Institute of Immunology, New Delhi, personal communication), hZP2 (Liang and Dean, 1993), hZP3 (Chamberlin and Dean, 1990), hZP4 (Harris *et al.*, 1994), dog and cat ZP1, ZP2, ZP3 (Harris *et al.*, 1994), bmZP2 (Jethanandani *et al.*, 1998), bmZP3 (Kolluri *et al.*, 1995) and bmZP4 (Gupta *et al.*, 1997).

Common structural attributes

There are a number of common features that are shared between various ZP glycoproteins. These are i) short 5' and 3' untranslated regions; ii) N-terminal hydrophobic signal peptide sequence to direct them into a secretory pathway and which ultimately gets cleaved off from the mature protein; iii) potential N- and O- linked glycosylation sites; iv) a C-terminal hydrophobic transmembrane-like domain (TD) that

plays a role in the intracellular trafficking of the proteins; v) a potential consensus proprotein convertase (furin) cleavage site (CFCS) upstream of the transmembrane domain, RXR/KR (altered to SRRR in hZP4 and rabbit ZP4 and SRNN in cow ZP4); and vi) a ZP signature domain. The position of the characteristic structural features of the polypeptide backbone of human ZP glycoproteins is tabulated in Table 2.

a) ***Significance of CFCS***: The role of CFCS in the secretion of ZP proteins and their assembly in ZP matrix has been extensively investigated. Enzymatic deglycosylation and 2-D thin-layer chromatographic analyses of mouse ZP glycoproteins suggested that mZP2 and mZP3 are processed at their CFCS prior to secretion and incorporation into ZP (Litscher *et al.*, 1999). Using site-directed mutagenesis studies and a specific inhibitor of furin-like enzymes, it was demonstrated that secretion of nascent mZP3 from transfected cells is dependent on its cleavage at CFCS (Williams and Wassarman, 2001). This observation was further substantiated by microinjecting epitope-tagged (Myc and Flag) cDNAs for mZP2 and mZP3 into the germinal vesicle (nucleus) of growing oocytes isolated from juvenile mice, wherein excision of the C-terminal region of the ZP glycoproteins was found to be an essential requirement for assembly into the oocyte ZP (Qi *et al.*, 2002). However, another group investigating the significance of CFCS in secretion of mZP3 in mouse embryonic fibroblast cells and its incorporation in the ZP of mouse oocyte demonstrated that cleavage of mZP3 at its CFCS is not required for its secretion, intracellular trafficking or its incorporation into the zona matrix (Zhao *et al.*, 2002). The growing oocytes of transgenic mice expressing mZP3 with a mutated CFCS had normal appearing zonae pellucidae and incorporation of mZP3 in their ZP matrix was equivalent to that of normal mice.

b) ***'ZP domain'***: All ZP glycoproteins (except for cat ZP3 and mZP1) share a sequence designated as the 'ZP domain' along with many eukaryotic extracellular proteins from a wide variety of organisms, from nematodes to mammals. The 'ZP domain' consists of approximately 260 amino acids including 8 conserved cysteine (Cys) residues and additional conservation of hydrophobicity, polarity and turn forming tendency at a number of other positions, which are probably essential for the three dimensional structure of the proteins in which the domain is present (Bork and Sander, 1992). This domain is also found in other proteins like the transforming growth factor (TGF)-βR III, uromodulin, pancreatic secretory granule protein GP2, α- and β-tectorins, DMBT-1 (deleted in brain tumor-1), Nomp A (no-mechanoreceptor-potential-A), Dumpy and

Table 2: Characteristic features of human ZP glycoproteins

ZP Protein	Length of polypeptide (aa)	Signal sequence (aa)	ZP domain (aa)	Transmembrane -like domain (aa)
ZP1	638	1-25	279-548	602-622
ZP2	745	1-38	372-637	717-736
ZP3	424	1-22	45-304	387-409
ZP4	540	1-21	188-460	506-526

Cuticulin-1, *Drosophila* genes *miniature* and *dusky* etc (Wassarman *et al.*, 2001; Roch *et al.*, 2003). Identification of 'ZP domain' within various proteins involved in the formation of filaments or matrices led to the proposal that this sequence motif might be responsible for their ability to assemble (Killick *et al.*, 1995; Legan *et al.*, 1997). Mutations in 'ZP domain' of α-tectorin resulted in defective tectoral membrane assembly, underlying human hearing disorder demonstrating that 'ZP domain' plays a role in polymerization (Jovine *et al.*, 2002). These evidences suggest that filaments of different 'ZP domain' proteins may share a common three-dimensional architecture and the membrane-anchoring segments of their precursors are crucial in assembly, but they have no role to play in the secretion of the proteins.

Corollary to the above observations, replacement of the 'ZP domain' with enhanced green fluorescent protein (EGFP) did not prevent secretion of mZP3, suggesting the presence of other trafficking signals. Analysis of linker-scanning mutations of a mZP3-EGFP fusion protein in transient expression assays and in transgenic mice identified an 8 aa hydrophobic region required for its secretion and incorporation into the ZP matrix (Zhao *et al.*, 2003). This patch is conserved among mouse zona proteins and lies between the CFCS and the TD. Cleavage of mZP3 released the ectodomain from the TD and mass spectrometry analysis of native ZP located the cleavage site N-terminal to the CFCS and distinct from the hydrophobic patch (Zhao *et al.*, 2003). In order to study a general mechanism for assembly of 'ZP domain' proteins, using microinjection of mutated DNA constructs into growing oocytes and mammalian cells, a conserved duplicated motif comprising of two hydrophobic sequences, an internal hydrophobic patch (IHP) within the 'ZP domain' and an external hydrophobic patch (EHP) within the C-terminal pro-peptide regulating the assembly of mouse ZP proteins has been identified (Jovine *et al.*, 2004). Cleavage of ZP precursors results in loss of the EHP, thereby activating secreted polypeptides to assemble by using the IHP within the ZP domain (Jovine *et al.*, 2004). Recent identification of a placenta-specific 1 (PLAC1) protein sharing sequence homology with the N-terminal half of the 'ZP domain' (ZP-N), but not with its C-terminal half (ZP-C) provides a function for the 2 termini of the 'ZP domain' in ZP proteins (Jovine *et al.*, 2006). Studying the assembly of PLAC1 into filaments by electron microscopy showed the ability of ZP-N to be self-sufficient as an actively folding unit, prompting a re-evaluation of the architecture of the 'ZP domain'

and its polymers. Furthermore, it is suggested that ZP-C might play a regulatory role in the assembly of 'ZP domain' protein complexes.

c) ***Trefoil motif***: In addition to the 'ZP domain', a Trefoil motif or P-domain has also been recognized in ZP1 and ZP4, and is absent in ZP2 and ZP3. The Trefoil domain is a 42 amino acid, Cys rich region found in a family of small polypeptides called the Trefoil family (Thim, 1989). This module has been described in several proteins that have diverse biological activities like spasmolytic polypeptide (SP), intestinal trefoil factor (ITF), pS2 etc (Bork, 1993). The domain consists of 6 Cys residues, linked by three disulfide bonds with a 1-5, 2-4, 3-6 Cys pairing, and usually occurs in 1-6 copies. The Trefoil family members are mucin-associated and largely found in epithelia of gastrointestinal tissues. The domain may have a role in renewal and pathology of mucous epithelia (Hoffmann and Hauser, 1993). Most of the proteins containing the Trefoil domain are thought to be growth factors. This domain has also been found in two intracellular enzymes (Tomasetto *et al.*, 1990). All the members of ZP1 and ZP4 families conform to this domain except mZP1, which deviates from this signature motif at aa residues 235 and 240. The presence of such a module in the heavily glycosylated ZP glycoproteins suggests a more general role, such as specific binding to carbohydrates.

Conservation during evolution

There is evidence indicating an overall conserved backbone structure of the ZP proteins from different species because of the conserved nature of the Cys residues present in the ZP glycoproteins of various species. The ZP1, ZP2, ZP3 and ZP4 families contain 11, 18, 12 and 19 conserved Cys residues respectively. Out of these conserved Cys residues, 10 are present in the 'ZP domain' of ZP1 and ZP4 while 8 in ZP2 and ZP3. In addition to the homology observed within a given ZP protein from different species, a considerable amount of homology is also seen between the aa sequence of ZP1 and ZP2 indicating the involvement of a common ancestral gene. A 348 aa domain (aa residues 268-623) in mZP1 is found to have 47% similarity (32% identity) with mZP2 (aa residues 363-713) and is encoded by 8 exons in both cases (Epifano *et al.*, 1995a). Probably, mZP1 and mZP2 have originated from the same ancestral gene that has been duplicated and reutilized by exon shuffling (Harris *et al.*, 1994). The mature polypeptide chains of hZP1 and hZP4 share an identity of 47% at the aa level, which is the highest between any two ZP proteins in humans. The sequence identity at the

deduced aa level of the four ZP glycoproteins from various mammalian species with their respective human homologues is listed in Table 3.

The ZP glycoproteins have been conserved throughout the course of vertebrate evolution. The vitelline envelope (VE) from *Xenopus laevis* eggs is composed of glycoproteins gp69, gp41 and gp37 that are homologous to the three mammalian ZP glycoproteins, ZP2, ZP3 and ZP4 respectively (Hedrick, 1996). Comparison of the deduced aa sequence of gp69 revealed that it shared a sequence identity of 28.5%, 27.6% and 26.9% with mouse, pig and human ZP2 while the gp41 showed a sequence identity of 40.9%, 40.0% and 40.8% with human, pig and mouse ZP3 respectively. The gp37 aa sequence, when compared with the ZP4 sequence from human, pig and mouse revealed a sequence identity of 41.6%, 41.7% and 36.8% respectively. Since the VE around *Xenopus* eggs contains three glycoproteins structurally related (39-48% aa similarity) to the three mouse zona proteins, investigations pertaining to incorporation of the mouse zona proteins into *Xenopus* VE using synthetic mRNAs encoding ZP1, ZP2, and ZP3 proteins injected into the cytoplasm of *Xenopus* oocytes were carried out (Doren *et al.*, 1999). After 20 h of incubation, localization of post-translationally modified zona proteins was detected with monoclonal antibodies (MAbs) specific to mZP1, mZP2, and mZP3. Thus, the mouse zona proteins appear to have been sufficiently conserved through 350 million years of evolution to be incorporated into the extracellular envelope surrounding *Xenopus* eggs.

Developmental Regulation of ZP Glycoproteins' Expression

During embryo development in mouse, the expression of the three ZP glycoproteins has been shown to be restricted only to the oocytes (Ringuette *et al.*, 1986; Liang *et al.*, 1990; Epifano *et al.*, 1995b). The mZP1 and mZP3 transcripts were detected only in the growing oocytes (Philpott *et al.*, 1987; Epifano *et al.*, 1995b) while the mZP2 transcripts could be observed in oocytes before the growth phase of oogenesis and even prior to birth, as early as 16 days of gestation (Millar *et al.*, 1993). As the mouse oocyte growth takes place, all three zona transcripts coordinately accumulate and represent approximately 1.5% of the total mRNA in 50-60 μm oocytes. In the later stages of oogenesis, their abundance declines and each zona transcript is present in ovulated eggs at less than 5% of its maximal level (Millar *et al.*, 1993). Using immunolabeling technique, association of the mouse ZP proteins was observed with the golgi apparatus,

Table 3: Sequence identity at the deduced amino acid level of the four ZP glycoproteins from various species with their respective human homologues

ZP Family	Species	% Identity to Human
ZP1	Rat	66.0
	Chicken	55.0
	Quail	52.0
	Mouse	64.0
ZP2	Mouse	57.0
	Rabbit	72.0
	Pig	64.0
	Bonnet monkey	94.2
ZP3	Mouse	67.0
	Rabbit	69.0
	Pig	74.0
	Bonnet monkey	93.9
ZP4	Rabbit	71.0
	Pig	68.0
	Bonnet monkey	92.0

secretory granules, and a complex structure called vesicular aggregate, in mouse ovarian follicles and is suggestive of the active involvement of these subcellular organelles in processing of the three glycoproteins before their secretion to form the ZP (El-Mestrah *et al.*, 2002). An asymmetric spatial distribution of the three ZP glycoproteins in the mouse zona matrix was also revealed at various stages of follicular development. By inhibiting *de novo* biosynthesis of specific zona proteins with antisense oligonucleotides, it was ascertained that mZP2 and mZP3 are independent of each other in their biosynthesis but are dependent upon each other for their incorporation into the zona pellucida matrix (Tong *et al.*, 1995).

The expression of porcine ZP2 (Taya *et al.*, 1995), rabbit ZP2 (rec75; Lee *et al.*, 1993), marmoset ZP3 (Thillai-Koothan *et al.*, 1993) and gp41 from *Xenopus* (Kubo *et al.*, 1997) has been observed to be oocyte-specific. However, immunohistochemical analysis of rabbit ovaries revealed that rec55 was localized in the oocytes of primordial follicles and the granulosa cells (GCs) of primary follicles but was undetectable in the GCs of large antral follicles (Lee and Dunbar, 1993). But later investigations by the same group using *in situ* hybridization failed to detect the presence of rec55 in the GCs (Dunbar *et al.*, 1994). A plausible explanation for the paradoxical detection of rec55 in GCs grown *in vitro* but not by *in situ* hybridization of ovarian sections is that rec55 gene might be de-repressed in GCs cultured in the absence of oocytes. *In situ* hybridization of cynomolgus monkey ovarian sections using digoxigenin-labeled cDNA probes specific for the mRNA encoding ZP2, ZP3 or ZP4 demonstrated the presence of ZP2 in growing follicles at all stages and in the GCs of mature preovulatory follicles (Martinez *et al.*, 1996). The ZP3 was detected in oocytes at all stages of folliculogenesis as well as in GCs, while ZP4 was present in secondary follicles and to a lesser extent in tertiary follicles, but was not found in primordial, primary or antral follicles or GCs. In brushtail possum, *in situ* hybridization revealed that expression of ZP4 was restricted to oocytes of primordial and primary follicles while no expression was detected in the surrounding GCs (Haines *et al.*, 1999). In variance with the data from the mouse model, using hZP3 specific antibodies, the hZP3 has been shown to be present in the oocyte as well as in the GCs of primordial, primary and secondary follicles of the human ovary (Grootenhuis *et al.*, 1996).

In mice, the expression of the ZP genes in a coordinate, oocyte-specific manner during the growth phase of oogenesis suggested that the transcription of the zona genes may be

controlled by shared regulatory element(s) (Epifano *et al.*, 1995b). The first report in this direction came from observations pertaining to the regulation of oocyte-specific expression of the sperm receptor gene during mouse development by *cis*-acting elements present in the m*Zp3* gene 5'-flanking region (Lira *et al.*, 1990). Analysis of the upstream sequences of mouse and human *Zp2* and *Zp3* genes revealed the presence of 5 short conserved DNA sequences (4-12 bp), that are 60-100% identical and upstream of the TATAA box (I, IIA, IIB, III and IV; Millar *et al.*, 1991). Mutation analysis established that the 12 bp element IV, present approximately 200 bp upstream of the TATAA box, was both necessary and sufficient for high level expression of a reporter gene under ZP promoter in mouse oocytes. Oligonucleotides corresponding to the conserved upstream regulatory elements from either ZP2 or ZP3 form DNA-protein complexes of identical mobility in gel retardation assays indicating the involvement in binding of common regulatory factors to conserved element IV responsible for coordinated expression of the oocyte-specific *Zp2* and *Zp3* genes (Millar *et al.*, 1991). A putative transcription factor, the zona activating protein (ZAP) has also been implicated in the regulation of expression of mouse as well as human *Zp2* and *Zp3* genes in mouse and human models (Millar *et al.*, 1993). The onset of m*Zp2* transcription and the profile of its subsequent accumulation correlate with the ZAP-1 DNA binding activity. DNA binding activity similar to that of ZAP-1 has also been detected in the ovarian extracts from rat, human and possum suggesting the conservation of ZAP-1 protein in mammals. Another oocyte-specific 60 kDa protein (OSP-1), binding to nucleotides -99 to -86 of the m*Zp3* promoter, has also been identified and proposed as a mammalian oocyte-specific transcription factor (Schickler *et al.*, 1992).

Generation of ZP Proteins by Recombinant DNA Technology

The critical appraisal of the role of individual ZP glycoproteins during fertilization has been hampered due to their non-availability in highly purified form, free from other ovarian contaminants from the native source. In order to circumvent this limitation, over the years, several groups have successfully expressed and purified recombinant ZP proteins in different heterologous expression systems (Kinloch *et al.*, 1991; Beebe *et al.*, 1992; van Duin *et al.*, 1994; Barratt and Hornby, 1995; Gupta *et al.*, 1997; Kaul *et al.*, 1997; Chapman *et al.*, 1998; Jethanandani *et al.*, 1998; Patra *et al.*, 2000; Yonezawa

et al., 2005). The mouse L-929 cell line stably transformed with a recombinant plasmid encoding mZP3 and green monkey CV-1 cells infected with a recombinant vaccinia virus containing mZP3 expressed a 60-70 kDa mZP3 protein, which differed in molecular weight from native mZP3 (~83 kDa). However, the recombinant mZP3 obtained was biologically active, inhibited sperm-zona binding with a potency equivalent to that of native ZP and triggered acrosomal exocytosis in capacitated mouse sperm (Beebe *et al.*, 1992). The mZP3 has also been expressed in mouse embryonal carcinoma (EC) cells and the recombinant mZP3 inhibited binding of the sperm to ovulated eggs and induced acrosome reaction *in vitro* (Kinloch *et al.*, 1991). On the other hand, hamster ZP3, expressed in EC cells, failed to bind to the spermatozoa or induce acrosome reaction in hamster sperm (Kinloch *et al.*, 1991). However, hamster ZP3 expressed under mZP3 promoter, purified from the oocytes of transgenic mice was found to contain both sperm binding activity as well as the ability to induce acrosome reaction (Kinloch *et al.*, 1992).

The genes corresponding to bmZP1 and bmZP2 have been cloned and expressed in the *E. coli* expression system (Gupta *et al.*, 1997; Jethanandani *et al.*, 1998) while bmZP3 has been expressed both in *E. coli* as well as baculovirus-expression systems (Kaul *et al.*, 1997; Gahlay and Gupta, 2003). In the porcine model, pZP2 has been cloned and expressed in *E. coli* as well as in mammalian cells such as CHO, CHO-K1, 239T and LLC-PK1 cells (Tsubamoto *et al.*, 1996; Yamasaki *et al.*, 1996; Tsubamoto *et al.*, 1999a). A recent study has demonstrated that recombinant pZP4 expressed in insect cells has the ability to bind bovine, but not porcine sperm (Yonezawa *et al.*, 2005). The baculovirus-expressed rabbit rec55 bound to acrosome-intact spermatozoa in a dose dependent manner and an alteration in rec55 localization was observed in the acrosome-reacted sperm (Prasad *et al.*, 1996).

The hZP3 has been expressed in bacteria (Barratt and Hornby, 1995), yeast (Harris *et al.*, 1999), baculovirus (Harris *et al.*, 1999) and CHO cells (van Duin *et al.*, 1994; Harris *et al.*, 1999). Both the *E. coli* expressed as well as the mammalian recombinant hZP3 exhibited the ability to induce acrosomal exocytosis in capacitated human spermatozoa (van Duin *et al.*, 1994; Chapman *et al.*, 1998). The recombinant hZP2 expressed in *E. coli* showed binding to acrosome-reacted human spermatozoa (Tsubamoto *et al.*, 1999b).

Mammalian Fertilization

The ZP proteins play a key role in mediating the initial attachment and binding of the spermatozoa through complementary receptor-ligand recognition on the gamete surface. These interactions initiate a complex cascade of events culminating in fertilization and formation of an embryo (Snell and White, 1996; Wassarman *et al.*, 2001).

Penetration through follicular cells

The capacitated spermatozoa with intact acrosome, penetrate cumulus oophorus, which consists of several layers of ovarian follicular GCs embedded in an ovarian extracellular matrix composed of hyaluronic acid surrounding the ovulated egg (Yanagimachi, 1994). The hyaluronidase activity of a sperm membrane protein PH-20 enables sperm to penetrate the layer of cumulus cells surrounding the oocyte (Myles and Primakoff, 1997). However, sperm from PH-20 null mice can penetrate cumulus cells and fertilize the egg, suggesting that PH-20 is not an absolute requirement for fertilization and other hyaluronidase(s), in conjunction with PH-20 may play an important role in sperm penetration (Baba *et al.*, 2002). One such protein, a 55-kDa hyaluronan-hydrolyzing protein was found to be abundant in wild-type and PH-20-deficient mouse sperm (Kim *et al.*, 2005). This protein was identified as a single-chain hyaluronidase, named Hyal5, present on the plasma and acrosomal membranes of sperm presumably as a GPI-anchored protein. Hyal5 protein purified from mouse epididymal sperm as well as PH-20-deficient mouse sperm were capable of dispersing cumulus cells from the cumulus mass, suggesting that Hyal5 may function principally as a "cumulus matrix depolymerase" in the mouse sperm penetration through the cumulus (Kim *et al.*, 2005).

Functional attributes of ZP glycoproteins during fertilization

a) *Sperm-ZP binding: primary sperm receptor function*

The species-specificity of sperm-ZP binding follows the process of initial penetration through cumulus layer. The p95 (Leyton and Saling, 1989), β-galactosyl transferase (GalTase; Miller *et al.*, 1992) and sperm protein-56 (sp56; Bleil and Wassarman, 1990) are a few sperm surface molecules that have been implicated as cognate ligands for zona proteins in various mammalian species. In mouse, the amino terminal of ZP3 was suggested to have dimerization activity with ZP2 while the sperm receptor activity was

attributed to the C terminal (Wassarman and Litscher, 1995). The initial adhesion event between mouse sperm and the ZP is a high affinity event involving about 30,000 binding sites (300 molecules/μm^2) ascribed to ZP3 which are sufficient to tether a sperm to the extracellular matrix prior to the induction of acrosomal exocytosis (Thaler and Cardullo, 1996). The contact becomes more tenacious and the bound sperm undergoes acrosome reaction (Bleil and Wassarman, 1983; Wassarman, 1999). Of the three glycoproteins that constitute the mouse ZP, only purified mZP3 binds exclusively to the head of acrosome-intact sperm and induces them to undergo acrosomal exocytosis (Bleil and Wassarman, 1983, 1986). Even at nanomolar concentrations, purified, unfertilized egg mZP3 is a very effective inhibitor of sperm-egg binding whereas, at similar concentrations, mZP3 from fertilized eggs or early embryos has no effect on binding of sperm to eggs *in vitro* (Wassarman, 1999). It results as a consequence of the 'zona reaction', wherein limited modification of the O-linked oligosaccharides of mZP3 takes place generating an altered form of ZP3 termed as $ZP3_f$ that can no longer recognize and bind to sperm (Bleil and Wassarman, 1983). The importance of mZP3 in mediating sperm receptor activity was further reinforced by observations that ZP3 null mice were infertile and had ZP-less oocytes (Liu *et al.*, 1996; Rankin *et al.*, 1996). In hamster and humans also, ZP3 performs the primary sperm receptor function (Moller *et al.*, 1990; van Duin *et al.*, 1994).

Going a step further, to delineate the functional domain of mZP3, using exon-swapping, hamster ZP3 exon-6, -7, and -8 were individually replaced with the corresponding exons of mZP3 (Williams *et al.*, 2006). While EC-expressed hamster ZP3 was unable to inhibit binding of mouse sperm to eggs *in vitro*, the substitution of mZP3 exon-7 for hamster ZP3 exon-7, but not mZP3 exon-6 or -8, can impart inhibitory activity to EC-expressed hamster ZP3, signifying that mZP3 exon-7 may be the critical 'sperm-combining site' on the ZP.

Porcine ZP3α (homologous to human ZP4) was demonstrated to bind to isolated boar sperm membrane vesicles whereas ZP3β (homologous to mZP3) did not show sperm binding ability (Sacco *et al.*, 1989; Yurewicz *et al.*, 1993b). However, on subsequent investigations, it was ascertained that heterocomplexes of ZP3α and ZP3β, and not individual proteins are responsible for high affinity binding to the boar sperm

membrane vesicles, as the ZP3α preparation in the previous study was found to contain a minor contaminant of ZP3β (Yurewicz *et al.*, 1998).

In the rabbit model, baculovirus-expressed rec55 (homologue of hZP4) showed a dose-dependent binding to rabbit spermatozoa (Prasad *et al.*, 1996). Both the rabbit ZP proteins, rec45 (homologue of mZP3) and rec55 bind to recombinant Sp17, a sperm specific protein, suggesting the involvement of more than one receptor on ZP in mediating sperm-oocyte interaction during fertilization, a molecular mechanism similar to the porcine system (Yamasaki *et al.*, 1995).

In the non-human primates, using indirect immunofluorescence as well as direct binding assay, it was observed that *E. coli*-expressed bmZP4 binds to the principal segment of the acrosomal cap of capacitated bonnet monkey spermatozoa (Govind *et al.*, 2001). The binding of bmZP4 in acrosome-reacted spermatozoa shifted to the equatorial segment, postacrosomal domain, and midpiece region. This binding event indicates that ZP4, in non-human primates, may have a functional role during fertilization (Govind *et al.*, 2001).

In *Xenopus laevis*, both the envelope glycoproteins ZP3 and ZP4 possessed independent ligand activity, but ZP3 was the major ligand for sperm binding (75%; Vo and Hedrick, 2000). Mixing of isolated ZP2, ZP3 and ZP4 in a ratio of 1:4:4 (equal to that in the *Xenopus* egg envelope) resulted in a synergistic enhancement in sperm binding. Thus, ZP3 possessed both independent and hetero-oligomeric-dependent ligand activities for sperm binding.

b) *Secondary sperm receptor function*

Following induction of acrosome reaction in the ZP bound sperm, the acrosome-reacted sperm must remain bound to eggs, despite loss of plasma membrane from the anterior region of the head and exposure of inner acrosomal membrane. The continued binding and penetration of the sperm has been attributed to ZP2 in mouse, thus called the secondary sperm receptor (Bleil *et al.*, 1988). The exclusive binding of ZP2 and inability of ZP3 to bind to acrosome-reacted sperm suggests that redistribution of sperm surface molecules takes place during acrosome reaction and while some ligands get exposed, others may get hidden. The guinea pig PH-20 (Sperm Adhesion Molecule-1 or SPAM-1; Myles and Primakoff, 1984) and pig acrosin (Yonezawa *et al.*, 1995) are the putative cognate ligands present on the sperm that bind to ZP2.

c) ***Role of supramolecular structures***

In mouse, ZP1 has been implicated in maintenance of the structural integrity of ZP matrix by cross-linking the filaments of ZP2-ZP3 heterodimers (Greve and Wassarman, 1985). Discovery of the fourth protein in human ZP, ZP1, raised a possibility that the presence of four zona proteins may support species-specific sperm binding in humans. However, the presence of 4 ZP glycoproteins in rat too, is not sufficient to support human sperm binding to rodent eggs (Hoodbhoy *et al.*, 2005). Hence, additional determinants must be responsible for taxon-specific fertilization among mammals. As it has been difficult to ascribe sperm binding to a single ZP glycoprotein, there is a possibility that supramolecular structures play an important role in sperm-egg recognition. The 3-D structure(s) involving one or more egg proteins, to which the sperm bind have not been determined and need further elucidation.

d) ***Gamete fusion and block to polyspermy***

Subsequent to sperm penetration, adherence and fusion of the gamete plasma membranes take place. Numerous studies have implicated several molecules on both the sperm and egg as being involved in fusion. The integrin family members present on the oolemma have been shown to play an essential role in sperm-egg binding and fusion (Almeida *et al.*, 1995; Chen *et al.*, 1999). Recent studies, however, suggest that additional molecules on the oocyte may also have a role to play in the sperm-egg fusion (He *et al.*, 2003). Post fusion, the contents of cortical granules (CG), a special organelle in the oocyte, are released into the perivitelline space, a phenomenon called 'cortical reaction', causing the ZP to become refractory to subsequent sperm binding and penetration ('zona reaction'). The CG exudates cause sperm receptor modification and zona hardening that imparts ZP resistance against proteases, low pH and reducing agents and thus, blocks polyspermic penetration. In mouse, the modification of mZP3 to $mZP3_f$ results in the loss of sperm receptor sites and limited proteolysis of ZP2 produces small molecular weight glycopeptides of ~23 kDa that remain non-covalently bound to ZP2 by intramolecular disulfide bonds (Moller and Wassarman, 1989; Wassarman and Mortillo, 1991). In *Xenopus laevis*, a mechanism for egg envelope hardening involving ZP2 proteolysis by an egg metalloprotease as a triggering event has been implicated (Lindsay and Hedrick, 2004). These phenomena collectively bring about the block to polyspermy.

e) *Delineation of functional attributes of ZP glycoproteins using transgenic mice*

The function of individual ZP glycoproteins in the process of fertilization has been elucidated by targeted mutagenesis of individual ZP genes in embryonic stem cells to generate null mice defective for one of the zona proteins. In *Zp1* null mice, the ZP was composed of only ZP2 and ZP3 and the matrix was more loosely organized than zonae around normal oocytes (Rankin *et al.*, 1999). These mice had perturbed folliculogenesis and after mating with males, fewer two-cell embryos were recovered from *Zp1* null females, and their litters were significantly smaller than those produced by normal mice. Hence, although mZP1 is not essential for sperm binding or fertilization, it is required for the structural integrity of the ZP to minimize precocious hatching and reduced fecundity (Rankin *et al.*, 1999). In *Zp2* null mice, ZP1 and ZP3 were synthesized and formed a thin zona matrix in early follicles that was not sustained in pre-ovulatory follicles (Rankin *et al.*, 2001). The abnormal zona matrix did not affect initial folliculogenesis, but there was a significant reduction in the number of antral stage follicles in ovaries isolated from mice lacking a ZP. Few eggs were detected in the oviduct after stimulation with gonadotropins, and no two-cell embryos were recovered after mating *Zp2* null females with normal male mice. Thus, ZP2 has a crucial role during fertilization and early embryo development (Rankin *et al.*, 2001). The *Zp3* null mice had follicles with germinal vesicle intact oocytes but completely lacked a ZP matrix and had a disorganized corona radiata (Liu *et al.*, 1996; Rankin *et al.*, 1996). The females were sterile and the developmental potential of the oocytes was highly compromised. On hormone-induced ovulation, cumulus masses were present in the oviducts of homozygous mutant mice, but zona-free eggs were observed in only half of the females and, in these, less than 10% of the normal number of eggs were detected. No zona-free two-cell embryos were recovered from homozygous mutant female mice after mating with fertile males, and none became pregnant or produced offspring (Rankin *et al.*, 1996). The portion of the oocyte facing the follicular antrum was naked and thus, the extent of follicle-oocyte interaction was also found to be compromised in the $mZP3^{-/-}$ mice although the oocytes seem to grow normally and the follicles reach the antral stage of development in the absence of ZP assembly (Liu *et al.*, 1996).

Sperm Adhesion Proteins Involved in Sperm-Oocyte Interaction

The species-specific interaction between the male and female gametes during fertilization is mediated by complementary receptors present on the surface of the sperm and the egg. Using various biochemical, immunohistochemical and molecular biology techniques, a number of putative ligands present on the sperm surface and possibly involved in interaction with the ZP, have been elucidated and are listed in Table 4. However, only few are described in detail below.

Primary binding to the oocyte

One of the candidate adhesion molecules discovered in mouse is a protein of 95 kDa, p95, that binds to oligosaccharides on mZP3 and also reacts with anti-phosphotyrosine antibodies in a ZP concentration-dependent manner, supporting the idea that the same 95 kDa sperm protein serves as a ZP3 receptor and as a tyrosine kinase substrate (Leyton and Saling, 1989). Further evidence exists that p95 is aggregated by ZP3 on sperm plasma membrane, which stimulates tyrosine kinase activity leading to acrosomal exocytosis (Saling, 1991). Thus, p95 acts as an autophosphorylating tyrosine kinase, like those serving as receptors for various growth factors (Leyton and Saling, 1989). Treatment with tyrosine kinase inhibitors completely abrogates the stimulation of protein tyrosine phosphorylation by ZP, which in turn inhibits the sperm acrosome reaction. Sequence analysis of p95 at aa level revealed identity to a ubiquitous mouse hepatoma hexokinase. Interestingly, Western blots with anti-phosphotyrosine antibodies showed that only the germ cell component of the testis possessed a tyrosine-phosphorylated form of hexokinase, suggesting the unique role of p95 in fertilization (Kalab *et al.*, 1994). However, LL95, a p95-specific MAb failed to recognize any known hexokinase, suggesting that p95 may not be a hexokinase (Leyton *et al.*, 1995). The homologue of p95 in humans is called zona receptor kinase (ZRK), whose kinase activity is stimulated by hZP3 (Burks *et al.*, 1995). The sequence of p95 is identical with *c-mer*, a proto-oncogene, questioning its potential role in sperm-egg binding (Bork, 1996; Tsai and Silver, 1996).

Another putative ligand on sperm for primary interaction is known as sp56, a peripheral membrane protein, present on the outer surface of the mouse sperm head plasma membrane as demonstrated by immunohistochemical and immunoblotting studies, using MAbs (Bleil and Wassarman, 1990; Cheng *et al.*, 1994). Results of immunoprecipitation of sperm extracts incubated with mZP3, by either a polyclonal

Table 4: Candidate sperm proteins involved in sperm-egg interaction

Sperm protein	Species	References
Primary Binding		
β-galactosyltransferase (GalTase)	Mouse, guinea pig, rat, cow, pig, rabbit	Miller *et al.*, 1992; Gong *et al.*, 1995
Zona receptor kinase (ZRK)/p95	Mouse, human	Leyton and Saling, 1989; Burks *et al.*, 1995
Sperm protein-56 (sp56)	Mouse, guinea pig	Bleil and Wassarman, 1990; Cheng *et al.*, 1994; Bookbinder *et al.*, 1995
Spermadhesins	Pig	Sanz *et al.*, 1991; 1993
Mannose-binding protein (MBP)/α-D-mannosidase	Rat, human	Tulsiani *et al.*, 1989; Cornwall *et al.*, 1991; Yoshida-Komiya *et al.*, 1999
Secreted protein containing a cleavable signal sequence, N-terminal Notch-like type II EGF repeats and C-terminal Discoidin/F5/8 Complement domains (SED1)	Pig, mouse	Ensslin *et al.*, 1998; Ensslin and Shur, 2003; Shur *et al.*, 2006
Sperad	Guinea pig	Quill and Garbers, 1996
Galactose binding protein	Rat	Abdullah and Kierszenbaum, 1989
Sperm agglutination antigen-1	Human	Diekman *et al.*, 1997
APz	Pig	Topfer-Peterson *et al.*, 1985; Peterson and Hunt, 1989
Fertilization antigen-1 (FA-1)	Human, mouse	Naz and Ahmad, 1994; Kadam *et al.*, 1995
NZ-1, NZ-2, NZ-3	Human	Naz and Zhu, 1997; 2001; Zhu and Naz, 1998
Secondary Binding		
PH-20/SPAM1	Guinea pig, mouse, rat, macaque, human	Lathtrop *et al.*, 1990; Lin *et al.*, 1993; Hou *et al.*, 1996
Proacrosin	Pig, mouse	Fock-Nuzel *et al.*, 1984; Jones, 1991; Urch and Patel, 1991; Yonezawa et al., 1995
Rabbit sperm antigens (RSA) e.g. Sp 17	Rabbit, mouse, human, baboon	O'Rand and Porter, 1982; O'Rand *et al.*, 1988; Lea *et al.*, 1996
Zonadhesin	Pig, human, mouse	Hardy and Garbers, 1994; 1995; Lea *et al.*, 2001
SP10	Human, mouse, cow, pig, fox, baboon, macaque	Herr *et al.*, 1990a; b; Coonrod *et al.*, 1996
SP38	Pig	Mori *et al.*, 1993; 1995
P36	Human	Auer *et al.*, 2004

antibody directed against mZP3 or a MAb directed against sp56, suggest that mZP3 is specifically associated with sp56 (Cohen and Wassarman, 2001). The sp56 binds to ZP3 and ZP3 oligosaccharides but does not bind to ZP3 from fertilized mouse eggs. It contains multiple consensus repeats of 60 aa, termed as Sushi domains, as well as a C4b-binding domain which is present in a protein family that includes several proteins involved in protein-protein interactions (Cheng *et al.*, 1994; Bookbinder *et al.*, 1995). Contrary to the initial localization of sp56 on the plasma membrane, later investigations showed that it is present in the acrosomal matrix of acrosome-intact sperm, suggesting its function in acrosomal matrix-zona interactions during and immediately following acrosome reaction in mouse (Foster *et al.*, 1997). AM67, a secretory component of the guinea pig sperm acrosomal matrix, is orthologous to mouse sp56 (Foster *et al.*, 1997). A highly related homologue of sp56 is present in hamster and rat, but not in human.

A third primary sperm receptor for ZP3 is sperm membrane β1,4-galactosyltransferase (GalTase), which recognizes terminal O-linked GlcNAc residues on mZP3 and plays a significant role in sperm-egg binding (Miller *et al.*, 1992; Gong *et al.*, 1995). Inhibition of GalTase or GalTase binding site on mZP3 inhibits its binding ability to sperm (Shur and Hall, 1982; Miller *et al.*, 1992). Aggregation of GalTase by multivalent oligosaccharide ligands on ZP3 and by synthetic polymers terminating in GlcNAc elicited G protein activation, culminating in induction of acrosome reaction (Gong *et al.*, 1995; Shi *et al.*, 2001). The cytoplasmic domain of cell surface GalTase appears to enable it to function as a signal-transducing receptor for extracellular oligosaccharide ligands. After induction of acrosome reaction, GalTase gets relocalized to a new membrane domain where it cannot bind ZP3. Upon activation of the egg, modification of the GalTase binding site on ZP3 by the release of N-acetylglucosaminidase from the egg cortical granules leads to loss in the sperm receptor activity of ZP3 in fertilized egg and 2-cell embryos (Miller *et al.*, 1993). Sperm from transgenic mice over-expressing GalTase exhibited higher rates of G protein activation than wild-type sperm, which rendered transgenic sperm hypersensitive to their ZP3 ligand and led to precocious acrosomal exocytosis (Youakim *et al.*, 1994). Further, sperm from GalTase null mice were refractory to ZP3 and failed to undergo acrosome reaction in response to zona glycoproteins (Shi *et al.*, 2001). However, other studies have shown that GalTase null sperm bind to ZP but have lower fertilization ability *in vitro*. This implies that other receptor-ligand interactions may also play a role in mediating sperm-oocyte binding.

The most recent model suggests that GalTase functions as the ZP3 receptor that is responsible for inducing the acrosome reaction, whereas other sperm surface receptors dictate initial sperm-zona binding (Shi *et al.*, 2001; Nixon *et al.*, 2001). GalTase is present in guinea pig, mouse, rat, bovine, porcine and rabbit sperm, with guinea pig, mouse and rat having higher expression levels of GalTase than the others (Larson and Miller, 1997).

Other sperm specific proteins that have been found to be involved in sperm-egg interactions include spermadhesins, a family of 12-16 kDa secretory proteins expressed as the products of boar seminal plasma (Sanz *et al.*, 1991; 1993). At ejaculation, the spermadhesins form a protective coat around the sensitive acrosomal region of the sperm head, thus possibly preventing premature acrosome reaction. Isolated porcine spermadhesins bind the ZP glycoproteins in a cation-dependent manner using a carbohydrate recognition mechanism (Topfer-Petersen, 1999).

Secondary binding to the oocyte

After initial binding of the sperm to the ZP, sperm surface PH-20 is one of the ligands implicated in secondary sperm receptor function and has been studied in a number of species including the guinea pig and mouse (Lathtrop *et al.*, 1990), rat (Hou *et al.*, 1996), macaque and human (Lin et al., 1993). Antibodies generated against PH-20 significantly reduce sperm-zona pellucida binding *in vitro* (Primakoff *et al.*, 1985). It is a bi-functional glycosylphosphatidyl inositol (GPI) anchored integral membrane glycoprotein with separate domains, one of which acts as a hyaluronidase enabling the sperm to penetrate through the cumulus cells surrounding the oocyte, while the other acts as a ZP2 binding domain (Myles and Primakoff, 1997; Hunnicutt *et al.*, 1996; Cherr *et al.*, 2001). Two forms of PH-20, one in capacitated spermatozoa (64 kDa) and the other in acrosome-reacted spermatozoa (53 kDa) are known to be present (Li *et al.*, 1997; Sabeur *et al.*, 1997). The PH-20 is located exclusively in the posterior head region in acrosome-intact boar sperm but following acrosome reaction, it is re-localized on the inner acrosomal membrane and its concentration increases three fold (Myles and Primakoff, 1984; Cowan *et al.*, 1986; Yudin *et al.*, 1998).

Another sperm protein, acrosin, a serine endoprotease with a trypsin-like cleavage activity is localized in the acrosomal matrix as an enzymatically inactive zymogen, proacrosin (~53 kDa; Honda *et al.*, 2002). Proacrosin had initially been identified as a mZP2 binding protein present on the acrosome-reacted sperm (Jones, 1991).

Subsequently, it was also shown to be the cognate ligand for ZP2 on sperm in porcine system (Yonezawa *et al.*, 1995). At an alkaline pH, the autoactivation of proacrosin leads to its conversion into α-acrosin by an internal cleavage at N terminus (Arg22-Val24) to create a 22 aa light chain and a 376 aa heavy chain which remains attached by two disulfide bonds to form a heterodimer (Fock-Nuzel *et al.*, 1984). Further activation is brought about by three sequential endoproteolytic cleavages at the C-terminal end, to remove 70 aa residues, including a characteristic polyproline motif that yields a highly active β-acrosin with a molecular weight of ~35-38 kDa (Cechova *et al.*, 1988; Baba *et al.*, 1989a; b). While α-acrosin acquires protease activity helping the spermatozoa to penetrate the ZP (Planchenault *et al.*, 1991), β-acrosin has a role as a secondary binding protein (Topfer-Petersen and Henschen, 1987; Urch and Patel, 1991). Addition of suramin (polysulfonate anti-cancer drug), inhibited the retention of the acrosome-reacted spermatozoa with the egg surface during fertilization (Howes *et al.*, 2001). However, acrosin null mice were still capable of penetrating the zona, though a delay in the penetration due to dispersal of acrosomal proteins from the acrosome during exocytosis was observed (Adham *et al.*, 1997; Yamagata *et al.*, 1998). The interaction is mediated through ionic bonds between polysulphate groups on ZP oligosaccharides and basic residues on the surface of proacrosin/acrosin (Jansen *et al.*, 1995; Moreno *et al.*, 1998).

A family of carbohydrate mediated zona binding membrane glycoproteins, rabbit sperm antigens (RSAs) has also been advocated in the binding of rabbit sperm to the ZP. Three low molecular weight proteins (13 ± 2 kDa) designated as RSA-1, RSA-2 and RSA-3 have been isolated and characterized from the rabbits (O'Rand and Porter, 1982). The Sp17 is a member of the RSA family and is localized in human, rabbit, mouse and baboon testes (O'Rand *et al.*, 1988; Kong *et al.*, 1995; Lea *et al.*, 1996; Adoyo *et al.*, 1997). The expression of Sp17 is seen on acrosomal ghost and equatorial region of acrosome-reacted sperm. The N-terminal region of the protein, which is highly conserved, contains an A-kinase anchoring protein (AKAP) binding motif. Recombinant rabbit Sp17 expressed in COS cells binds to recombinant ZP3 and ZP4 from rabbits via two conserved heparin binding motifs (Yamasaki *et al.*, 1995). Sp17 is conserved throughout all the mammalian species and its gene is also expressed in metastatic cells and during mucosal immune responses (Dong *et al.*, 1997; Tatlow *et*

al., 2000) suggesting its important role in cell migration and/or adhesion as well as fertilization (Lacy and Sanderson, 2001).

Of late, emerging evidence for the participation of ubiquitin-proteasome pathway in the process of sperm-ZP penetration has been generated in invertebrates, ascidians and mammals (Sutovsky *et al.*, 2004). The 26S proteasome is a multi-subunit protease that selectively recognizes and degrades egg coat substrate proteins tagged by covalent ligation of a small, multimeric protein ubiquitin. Structurally, the 26S proteasome is a holoenzyme composed of a barrel-shaped 20S core capped with two 19S regulatory complexes, on each side of the 20S barrel. The 19S regulatory complex is composed of at least 17 subunits of a total mass of ~1 MDa. It recognizes the polyubiquitin tail on the ubiquitinated substrate protein by virtue of its subunit, Rpn10/S5a. Other 19S subunits are implicated in the binding and removal of polyubiquitin chain from the ubiquitinated substrate before it is translocated to the 20S core (Glickman and Ciechanover, 2002). The 20S proteasomal core is composed of four concentric rings containing seven proteasomal subunits of the α-type (two outer rings) and seven subunits of the β-type (two inner rings). The α-subunits form the accessory structures while the β-subunits harbor the protease activities responsible for the hydrolysis of the linearized polypeptide chains (Tanaka, 1998). In porcine system, one or more of the ZP glycoproteins are ubiquitinated and 26S proteasomes associated with the inner acrosomal membrane are exposed as a result of acrosomal exocytosis bringing about proteasome mediated proteolysis of the ZP (Sutovsky *et al.*, 2003; 2004). Sperm-ZP penetration may involve deubiquitination of ZP, with several proteasomal subunits undergoing phosphorylation. The location of proteasome in mature human sperm was restricted to the neck, acrosomal and post-acrosomal regions (Wojcik *et al.*, 2000, Bialy *et al.*, 2001; Morales *et al.*, 2004). Use of proteasome-specific inhibitors led to a block in ZP-induced acrosome reaction, documenting a role for proteasomes in human fertilization (Morales *et al.*, 2003). Mouse sperm proteasome has also been found to participate in ZP binding and induction of acrosome reaction (Pasten *et al.*, 2005). Investigations in other mammalian species like hamster, rat, bovine and rabbit have also suggested a role for sperm proteasomes in gamete interaction (Pizzaro *et al.*, 2004).

Fusion with oolemma

Following penetration into the zona matrix, several other proteins mediating the fusion of the sperm and egg plasma membranes come into play. These include sperm fertilin and DE. Fertilin (PH-30) is a heterodimer of α (guinea pig ~60 kDa) and β (guinea pig ~40 kDa) N-glycosylated subunits, both of which are members of the ADAM family (Wolfsberg and White, 1996; Evans, 1999). Both subunits are synthesized as precursors (~100 kDa) by spermatogenic cells and are proteolytically processed to form the mature protein. Since peptides with sequences pertaining to the α-subunit as well as disintegrin domain of the β-subunit can prevent the binding of sperm to ZP denuded eggs (Myles *et al.*, 1994; Evans *et al.*, 1995, 1997), it was proposed that the binding of acrosome-reacted spermatozoa to egg plasma membrane is supported by interactions between fertilin's disintegrin domain and integrin ($\alpha_6\beta_1$) receptors on the oolemma while fertilin-α mediates fusion via its viral fusion peptide domain (Hynes, 1992; Almeida *et al.*, 1995; Graves, 1995).

DE is another candidate molecule belonging to CRISP family, which mediates gamete fusion in rat, mouse and human (Cameo and Blaquier, 1976; Cohen *et al.*, 2000a, Cohen and Wassarman, 2001). Expressed in an androgen dependent manner and originally located on the sperm head, it migrates to the equatorial region concomitantly with the occurrence of acrosome reaction (Rochwerger and Cuasnicu, 1992). Two populations of DE are associated with spermatozoa, a major population, which is loosely arranged with the cells and is released from the spermatozoa during capacitation, and a tightly associated protein that stays bound after capacitation and migrates to the equatorial segment subsequent to acrosomal exocytosis (Cohen *et al.*, 2000b). The relocation of DE after acrosome reaction to the equatorial region suggests its role in sperm-egg fusion.

Role of Oligosaccharide Moieties in Sperm-ZP Interaction

Pioneering studies have suggested that sperm-egg interactions are mediated by carbohydrate moieties present on the opposing gamete surfaces (Macek and Shur, 1988; Wassarman and Litscher, 1995).

Murine model

There is considerable evidence implicating the important role of oligosaccharides in the sperm receptor function of mZP3. Extensive enzymatic digestion of purified mZP3 led

to the generation of small ZP3 glycopeptides (~1.5-6 kDa) that retained the ability to bind to mouse sperm, but failed to induce acrosomal exocytosis (Florman *et al.*, 1984). These results suggest that the sperm receptor activity of mZP3 is dependent only on its carbohydrate components, whereas acrosome reaction-inducing activity is dependent on its polypeptide chain as well. Chemical or enzymatic removal of all mZP3 oligosaccharides resulted in complete inactivation of the sperm receptor function. However, selective removal of O-linked oligosaccharides from mZP3 by trifluoromethanesulfonic acid destroyed its sperm receptor activity, whereas no such effect was observed on the removal of N-linked oligosaccharides by endo-β-N-acetyl-D-glucosaminidase (Florman and Wassarman, 1985). Further, the enzymatic removal of N-linked oligosaccharides from mZP3 indicated that neither N-linked oligosaccharides nor sialic acid is an essential element of the mZP3 combining site for sperm (Litscher and Wassarman, 1996). In another report, the presence of a fucosyl residue on an oligosaccharide at micromolar concentrations inhibited binding of sperm to eggs *in vitro* (Johnston *et al.*, 1998). Interestingly, lewis-X containing glycans, when clustered on a neoglycoprotein, bind mZP3 receptors on sperm and induce sperm to undergo the acrosome reaction in a dose-dependent and capacitation-dependent manner (Hanna *et al.*, 2004).

Initial studies implicated galactose in α- or β-linkage at the non-reducing terminus of O-linked oligosaccharides and N-acetylglucosamine (GlcNAc) in β-linkage as the sugar determinants on mZP3 responsible for binding of sperm to the ZP (Bleil and Wassarman, 1988; Miller *et al.*, 1992). However, mice deficient in glycosyl transferase, which amends terminal galactose in α-linkage, were found to be fully fertile (Thall *et al.*, 1995; Liu *et al.*, 1997) implicating galactose in β-linkage or GlcNAc or both as critical residues (Wassarman, 1999). Mannose has also been implicated to play an important role in the murine sperm receptor activity. Incubation of mouse spermatozoa in the presence of increasing concentrations of D-mannose resulted in a dose-dependent decrease in the number of spermatozoa bound per egg and a dose-dependent inhibition of the sperm mannosidase activity (Cornwall *et al.*, 1991). Another view is that murine sperm-ZP binding is mediated by two independent oligosaccharide-binding sites on sperm. The first (apparently high affinity) site binds both the α-linked galactosyl and

fucosylated moieties whereas the second (apparently low affinity) site binds galactose residues in β-linkage (Johnston *et al.*, 1998).

Using exon-swapping and site-directed mutagenesis studies, it was ascertained that sperm-binding and acrosome reaction inducing activity of mZP3 resides within exon-7 at its C-terminus, which also harbors 5 conserved Ser residues, glycosylation of one or more of which, is critical for sperm receptor activity (Kinloch *et al.*, 1995). The 5 Ser residues were individually mutated by site-directed mutagenesis and the recombinant mutant mZP3 proteins thus obtained were analyzed for their sperm receptor activity *in vitro* (Chen *et al.*, 1998). As compared with wild-type mZP3, mutations of Ser-329, Ser-331, or Ser-333 had no effect on sperm receptor activity. On the other hand, mutation of either Ser-332 or Ser-334 resulted in complete inactivation of mZP3 as the sperm receptor suggesting that these aa residues may carry oligosaccharides that are recognized by the ligands present on mouse spermatozoa. However, mutation of either Ser-332 or Ser-334 to Thr residues did not affect the sperm receptor activity of mZP3, suggesting that Thr residues can replace the 2 evolutionarily conserved Ser residues without hampering the sperm binding ability of mZP3 (Williams *et al.*, 2003).

Porcine model

Both N- and O-linked oligosaccharides have been thought to play a role in sperm-oocyte interaction in pigs. Preliminary studies showed that preincubation of boar sperm with ZP3 or purified O-glycans, but not N-glycans, inhibited subsequent attachment to zona-encased oocytes (Yurewicz *et al.*, 1991). However, the inhibition by O-glycans was 2 to 3 orders less effective than native ZP3 as competitive ligands. Contradicting reports suggest that di-, tri- or tetra-antennary, neutral, complex-type N-linked carbohydrate chain(s) localized in the N-terminal region of pZP3α mediate sperm-egg binding in pigs (Nakano *et al.*, 1996). An *in vitro* competition assay revealed that tri- and tetra-antennary glycan chains of pZP3α possess a sperm binding activity stronger than that of di-antennary chains located on Asn-220 of pZP3α (Kudo *et al.*, 1998). In support of the above information, the N-terminal peptide of ZP3α (aa residues 137-247; including Asn-220) also exhibited sperm binding function (Yonezawa *et al.*, 1997). Later studies demonstrated the presence of a lactosaminoglycan type carbohydrate moiety in all three ZP proteins of pig and rabbit but absent in the mouse and rat, which appears to be involved in sperm recognition and structural organization (Yurewicz *et al.*, 1987; Dunbar *et al.*, 2001).

Humans

Using lectins as histochemical markers for carbohydrates on ZP, it has been shown that the most pronounced differences between the ZP of humans and other mammals is the presence of very high concentration of D-mannose residues in human ZP, reflecting a high content of asparagine-linked oligosaccharides (Maymon *et al.*, 1994). Additionally, in sugar competition assays, mannose has been implicated in human fertilization (Mori *et al.*, 1989). Several oligosaccharide moieties along with complex glycoconjugates bearing selectin-like ligands have also been shown to be involved in the human sperm-egg binding (Patankar *et al.*, 1993; Miranda *et al.*, 1997; Oehninger *et al.*, 1998). In a hemizona binding assay, a significant decrease in the number of human spermatozoa bound to the hemizona was observed when spermatozoa were pre-incubated with sugars like GlcNAc, mannose, fucose and galactose implicating these residues in human sperm-ZP binding *in vitro* (Miranda *et al.*, 1997).

Sugar residues and species-specificity

Apart from their involvement in sperm-egg binding, the carbohydrate residues on the ZP glycoproteins also render species specificity to sperm-zona interaction. Recombinant hamster ZP3 expressed in mouse EC cells failed to bind to hamster sperm or induce acrosome reaction. However, mZP3 expressed under similar experimental conditions could bind to mouse sperm and also induce acrosomal exocytosis when incubated with capacitated sperm (Kinloch *et al.*, 1991). It may be attributed to the improper glycosylation of hamster ZP3 in mouse cell line making it unrecognizable by hamster sperm. In another study, transgenic mice with oocytes expressing chimeric zona composed of mZP1, mZP2 and hZP3 (gene encoding mZP3 replaced with gene encoding hZP3) have been developed. Oocytes from these transgenic mice failed to bind human sperm. Interestingly, these oocytes still recognized and bound mouse sperm resulting in conception (Rankin *et al.*, 1998). The oligosaccharides present on hZP3 in these transgenic mice were identical to mZP3 from normal mice, which may be responsible for recognition and binding of mouse sperm rather than human sperm (Dell *et al.*, 2003). In a recent study, sperm binding assays with recombinant porcine ZP glycoproteins, expressed using the baculovirus-*Sf9* insect cell system, demonstrated that recombinant pZP4 bound bovine sperm weakly but did not bind porcine sperm (Yonezawa *et al.*, 2005). Further, in the presence of recombinant pZP3, bovine sperm binding activity was greatly increased, but porcine sperm still failed to bind. The major

sugar chains added to pZP4 in insect cells were similar in structure to the major neutral N-linked chains of the bovine zona and are possibly responsible for bovine specific sperm binding activity (Yonezawa *et al.*, 2005). Perhaps, variations in the distribution of sugar residues in the ZP of different species, but not the polypeptide backbone are responsible for species-specific determination of sperm-egg interaction (Skutelsky *et al.*, 1994; Parillo *et al.*, 2000).

Contrary to the above observations, recombinant hZP3 expressed in *E. coli* has shown the ability to induce acrosome reaction in human sperm to levels similar to that obtained using purified glycosylated recombinant hZP3 expressed in CHO cells, suggesting a significant role of the polypeptide backbone in sperm recognition (van Duin *et al.*, 1994; Chapman *et al.*, 1998). Even in bonnet monkey, *E. coli* expressed ZP2, ZP3 as well as ZP4 have been shown to exhibit binding to bonnet monkey spermatozoa (Govind *et al.*, 2001; Gahlay *et al.*, 2002). Additionally, glycosylation of bmZP3 is required for inducing acrosomal exocytosis in the bonnet monkey spermatozoa (Gahlay and Gupta, 2003).

Induction of Acrosomal Exocytosis

Mammalian sperm acrosome is a Golgi-derived organelle that forms a cap over the anterior two-thirds of the sperm nucleus. Exocytosis of the acrosome (acrosome reaction) causes the acrosomal contents to be released and the inner acrosomal membrane to become the limiting membrane of the anterior sperm head. The physiological agents that have been implicated in induction of acrosome reaction in different mammalian species include progesterone, serum albumin, hydrolytic enzymes (particularly proteases), hormones including biogenic amines, estradiol, and arachidonic acid metabolites, sulfur-containing β-amino acids, glycosaminoglycans such as hyaluronic acid and one/more ZP glycoproteins (Meizel, 1985; Osman *et al.*, 1989; Roldan *et al.*, 1994). The ZP glycoproteins from various species have been studied in great detail to delineate the component that induces acrosome reaction in sperm and the pathway involved in acrosomal exocytosis (Beebe *et al.*, 1992; van Duin *et al.*, 1994; Chapman *et al.*, 1998; Gahlay and Gupta, 2003). Among the various physiological and pharmacological inducers of acrosome reaction, ZP3 has been accepted as the natural agonist (except in guinea pig) that initiates the acrosome reaction upon binding of acrosome-intact mammalian spermatozoa to ZP (Bleil and

Wassarman, 1983; Ward and Kopf, 1993). Though purified mZP3 and large mZP3 glycopeptides induce sperm to acrosome-react *in vitro*, purified mZP3 O-linked oligosaccharides and small mZP3 glycopeptides bind to mouse sperm but do not induce acrosomal exocytosis. However, cross-linking of proteolytic mZP3 fragments bound to sperm by anti-mZP3 antibodies led to aggregation of sperm receptors and induced sperm to undergo acrosome reaction (Leyton and Saling, 1989). In humans, initial studies revealed that intact as well as acid disaggregated ZP induces acrosomal exocytosis in human sperm (Cross *et al.*, 1988). In a later study, employing solubilized human zona, a dose-dependent increase in induction of acrosome reaction in human spermatozoa was observed (Franken *et al.*, 1996). In the quest to investigate the fertilizing potential of human sperm, various researchers have ascertained that solubilized human zona induces acrosome reaction in human sperm and inhibits human sperm-egg interaction *in vitro* (Lee *et al.*, 1992; Franken *et al.*, 1996). These findings indicate that aggregation of sperm surface receptors via multivalent interactions with a variety of chemically dissimilar zona ligands trigger the activation of second messenger pathways leading to the acrosome reaction.

Signaling Events during Acrosomal Exocytosis

The activation of the ligand(s) on the sperm surface by binding of ZP3 is the prerequisite in the signaling pathway that brings about the induction of acrosome reaction in ZP3-bound sperm. This leads to the activation of a cation channel through a pertussis toxin insensitive and voltage-insensitive mechanism on one hand and activation of a pH regulator, resulting in a transient alkalinization of internal pH causing an activation of calcium channels on the other. Sperm maintain an inwardly negative membrane potential, and conductance through cation channels produces a depolarizing current. Sperm membrane depolarization from ~-60 mV to ~-30 mV by binding of mZP3 to the sperm receptor(s) activates a cation channel (impermeable to anions) that conducts monovalent and divalent cations. Inward depolarizing currents thus generated, depolarize the sperm membrane potential and open up the voltage sensitive T-type low voltage Ca^{2+} channels. However, the voltage dependent inactivation of T currents occurs within 50-100 msec during depolarization (Arnoult *et al.*, 1996; Lievano *et al.*, 1996; Santi *et al.*, 1996), thereby terminating the ZP3-induced Ca^{2+} influx. But a sustained release of Ca^{2+} is an absolute requirement for induction of

acrosome reaction. Exposure of sperm to ZP glycoproteins also generates a second wave of sustained elevated Ca^{2+} levels by activation of L-type voltage-dependent Ca^{2+} channels (Florman and First, 1988; Florman *et al.*, 1992; Florman, 1994). The T-type channels may also be modulated by their state of tyrosine phosphorylation during capacitation and ZP3 stimulation. Other intracellular effectors of ZP action such as elevated pH and inositol phosphate-3 (IP_3) may also contribute to Ca^{2+} mobilization. In fact, internal alkanization is initiated by ZP3 through a pertussis toxin-sensitive pathway that signifies mediation of sperm guanine nucleotide-binding regulatory (G) proteins of the G_i type. Pertussis toxin strongly inhibited ZP-induced sustained elevations in intracellular Ca^{2+} levels but failed to block progesterone-induced acrosomal exocytosis in human sperm (Endo *et al.*, 1987; Lee *et al.*, 1992; Tesarik *et al.*, 1993a). In addition, interaction of ZP/ZP3 with its sperm receptor(s) stimulates a G_i protein-independent Ca^{2+} channel responsible for membrane depolarization and a G_i protein-sensitive alkalinization of the sperm cytoplasm which activates an L-type voltage-sensitive Ca^{2+} channel which, in turn, causes massive Ca^{2+} influx necessary for the acrosomal exocytosis (Florman *et al.*, 1998). It involves the heterotrimeric G proteins, G_i1 and G_i2, which activate voltage-dependent Ca^{2+} channels in other systems (Ward *et al.*, 1994). Participation of a second G protein, $G_{\alpha q/11}$ has also been suggested (Walensky and Snyder, 1995). Receptor activation of $G_{\alpha q}$ (a pertussis toxin-insensitive process) activates phospholipase Cβ1 with subsequent hydrolysis of phosphatidylinositol 4, 5-bisphosphate and the generation of IP_3 and diacylglycerol (DAG). Subsequently, IP_3 binds to the IP_3 receptors and induces the release of Ca^{2+} (Walensky and Snyder, 1995). The high intracellular free Ca^{2+} concentration together with DAG leads to membrane fusion and finally, acrosomal exocytosis (Harrison and Roldan, 1990; Roldan and Murase, 1994). However, the receptors that activate the sperm G proteins as well as the second messengers activated downstream during ZP3 stimulation of the sperm have still not been elucidated.

Another pathway resulting in activation of sustained Ca^{2+} influx in sperm by ZP3 involves the *Drosophila melanogaster Trp2* (transient receptor potential-2) gene that encodes a light activated cation channel in photoreceptor cells (Jungnickel *et al.*, 2001). Although the 7 members of the classical mammalian TRP (TRPC) family are homologues of the dipteran gene, encode "special assignment" channels, recruited to diverse signaling pathways and are candidate subunits of phospholipase-C (PLC)-

dependent Ca^{2+} entry channels, the mechanism is not yet properly understood (Minke and Cook, 2002). Alternative models have been proposed, either based on lipid products or PLC hydrolysis or generation of IP_3 and activation of calcium store depletion operated entry pathway (Minke and Cook, 2002). Members of soluble N-ethylmaleimide-senstive factor-attachment protein receptor (SNARE) proteins present in the acrosomal region of sea urchin and mammalian sperm may link Ca^{2+} entry to exocytosis (Schulz *et al.*, 1997; Michaut *et al.*, 2000; Tomes *et al.*, 2002).

The regulation of acrosomal exocytosis is also mediated by human follicular fluid (Tesarik, 1985), progesterone (Roldan *et al.*, 1994) and the presence of intracellular proteases with a trypsin- or a chymotrypsin-like activity (Morales *et al.*, 1994). There seems to be existence of two progesterone receptors on human sperm plasma membrane, one responsible for Ca^{2+} influx and modulated by protein-kinase C (PKC) and the other, selectively permeable to sodium that is not under PKC control. Alternatively, PKC inhibition might change ion selectivity of a single progesterone-activated channel, thus decreasing Ca^{2+} permeability, while leaving sodium permeability unchanged (Blackmore *et al.*, 1990; Foresta *et al.*, 1995). The signaling by progesterone may also lead to the opening of a plasma membrane Cl^- channel, which appears to be a part of a receptor resembling neuronal γ-aminobutyric acid receptor type A ($GABA_A$), to induce Cl^- efflux (Wistrom and Meizel, 1993), in addition to the activation of a protein tyrosine kinase (Tesarik *et al.*, 1993b). A sperm trypsin-like enzyme has been shown to play a direct or indirect role in the membrane events of hamster sperm acrosome reaction either by being involved in the dispersal of the acrosomal matrix or in the membrane events associated with acrosome reaction or both (Dravland *et al.*, 1984). Supporting evidence in humans showed that trypsin as well as chymotrypsin inhibitors blocked the acrosome reaction induced by the human follicular fluid and ZP (Pillai and Meizel, 1991; Llanos *et al.*, 1995).

Thus, the pathway leading to acrosomal exocytosis can be divided into 3 stages: (i) receptor aggregation, (ii) activation of intracellular signaling pathways, and (iii) sustained Ca^{2+} influx through voltage dependent Ca^{2+} channels. However, although it is clear that multiple second messenger systems regulate this process, yet the relationships between these systems and their relative order of activation has not been conclusively determined in studies employing native ZP glycoproteins.

Future Directions

Keeping in view all the information pertaining to ZP glycoproteins in various species available till date, a lot of unexplained questions still seem to exist with respect to the role of individual ZP glycoproteins in sperm-egg interaction as well as the exact composition of the ZP matrix. Recombinant DNA technology and other advanced techniques may prove to be a good handle in elucidation of the different steps in the process of fertilization that still remain elusive. There is a need to critically understand the role of oligosaccharide moieties associated with the zona proteins in fertilization. Though a host of candidate sperm surface molecules have been implicated in binding to ZP proteins, the actual identity and mechanism of receptor-ligand interactions and the downstream signaling events that follow still needs further clarification. Perhaps species-specific multiple ligand interactions between the male and female gametes lead to successful fertilization. Particularly, in humans, based on the relevant immunogenic epitopes from both sperm and egg proteins, development of effective and safer contraceptive vaccines may become more feasible. On the other hand, a lucid understanding of gamete interactions may aid in dealing with the serious global concern of infertility.

Materials and Methods

REAGENTS

Chemicals

Urea, tris, glycine, ethanol, acrylamide, percoll, N-[2-hydroxyethyl]piperazine-N'-[2-thanesulfonic acid] (HEPES), N,N'-methylenebisacrylamide, trypsin, sodium dodecyl sulfate (SDS), N,N,N',N'-tetramethylenediamine (TEMED), β-mercaptoethanol, lysozyme, dithiothreitol (DTT), L-glutamine, Pristane, mineral oil, phenol, ethidium bromide, bromophenol blue, xylene cyanol, Coomassie brilliant blue-R250, calcium chloride, Ribonuclease A (RNase), rhodamine-B isothiocyanate (RBITC), sodium acetate, glucose, chloroform, polyethylene glycol-400 (PEG-400), calcium ionophore (A23187), trypan blue, sodium azide, dimethyl sulfoxide (DMSO), sodium deoxycholate, 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal), progesterone, pertussis toxin, tunicamycin, sodium bicarbonate ($NaHCO_3$) were procured from Sigma Chemical Co., St. Louis, MO, USA. Agarose, low melting point (LMP) agarose, ethylenediaminetetraacetic acid (EDTA), oxidized glutathione, reduced glutathione, 4-chloro-1-naphthol, were purchased from Amresco, Solon, Ohio, USA. Glycerol, isopropyl-β-D-thiogalactopyranoside (IPTG), ammonium persulfate, guanidine hydrochloride were obtained from USB Corporation, Cleveland, Ohio, USA. NHS-LC-biotin, fluorescein isothiocyanate isomer-I (FITC) were procured from Pierce, Rockford, IL, USA. Lipofectamine-2000 was obtained from Invitrogen Corporation, Carlsbad, CA, USA. Methanol, acetic acid, formaldehyde, diethyl ether, isopropyl alcohol, isoamylalcohol were purchased from BDH, Merck Limited, Worli, Mumbai, India.

Reagents for Enzyme Immunoassay

Bovine serum albumin (BSA) and Tween-20 (polyoxyethylene-20-sorbitan monolaurate) were procured from Amresco. Orthophenylenediamine (OPD) was obtained from Sigma whereas hydrogen peroxide was purchased from BDH.

Media and Antibiotics

Tryptone, yeast extract and agar were obtained from Pronadisa, Laboratorios Conda, S. A., Madrid, Spain. Dulbecco's Modified Eagle's Medium (DMEM), Rosweli Park Memorial Institute (RPMI)-1640 medium, Ham's F-12 Nutrient Mixture Kaighn's Modification (F-12K) medium and HAT mixture were procured from Sigma. Grace's Insect Cell Culture medium, lactalbumin hydrolysate and yeastolate ultrafiltrate were purchased from GIBCO^{TM}, Invitrogen Corp., Carlsbad, USA. Antibiotics ampicillin

(sodium salt) and gentamycin sulfate were obtained from Sigma and the antibiotic antimycotic solution was procured from GIBCO. Heat inactivated fetal calf serum was obtained from Biological Industries, Hibbutz Beit, Haemek, Israel. The tissue culture grade chemicals used for making Biggers Whitten Whittingham (BWW) medium and embryo-tested quality of albumin (Fraction V powder) used to supplement the BWW medium were purchased from Sigma. Agarplaque-Plus^{TM} agarose was obtained from PharMingen, San Diego, CA, USA.

Bacterial Strains and Plasmids

BL21[DE3]pLysS and DH5α strains of *E. coli* were purchased from Stratagene, La Jolla, CA, USA. The *E. coli* expression vector pRSET-A and mammalian expression vector pcDNA6/V5-His-B were procured from Invitrogen. Baculovirus transfer vector pAcHLT-A was obtained from PharMingen.

Kits

PCR-Script^{TM} Amp Cloning kit was procured from Stratagene. Plasmid DNA purification midi kit was purchased from QIAGEN GmbH, Hilden, Germany. The BaculoGold Transfection Kit was obtained from PharMingen. Lectin kit-I, II and III were purchased from Vector Laboratories Inc., Burlingame, CA, USA. Bicinchoninic Acid (BCA) Protein Assay Kit was obtained from Pierce.

Primers and Enzymes

Different oligonucleotide primers were custom made either by Sigma Genosys Ltd., Bangalore, India, Microsynth GmbH, Hilden, Germany or Bio Basic Inc., Ontario, Canada.

Restriction enzymes *BamH I, Bgl II, EcoRI, Kpn I* and *Sac I*, Vent DNA polymerase and T4 DNA ligase were obtained from New England Biolabs (NEB), Beverly, MA, USA. Taq DNA polymerase was purchased from Promega, Madison, WI, USA while Shrimp Alkaline Phosphatase was procured from USB.

Molecular Weight Markers

The λ DNA-*Hind* III digest DNA and 1 kb DNA ladder were purchased from NEB. Prestained broad range SDS-PAGE standards were obtained from Bio-Rad, Hercules, CA, USA.

Antibodies and Conjugates

Goat anti-mouse IgG (H+L) conjugated to horseradish peroxidase (HRPO), goat anti-rabbit IgG (H+L) HRPO, rabbit anti-goat IgG (H+L) HRPO, goat anti-mouse IgG

(H+L) FITC conjugate and goat anti-rabbit IgG (H+L) FITC were procured from Pierce. Streptavidin-HRPO, avidin conjugated to FITC and mouse monoclonal antibody isotyping kit were purchased from Sigma. Tetramethylrhodamine isothiocyanate (TRITC) conjugated to *Pisum sativum* agglutinin (PSA) was obtained from Vector Laboratories.

Others

All other chemicals employed were of AR grade and procured from S.D. Fine Chemicals Ltd., Mumbai, India or Sisco Research Laboratories, Mumbai, India. Nickel-nitrilotriacetic acid (Ni-NTA) resin was purchased from QIAGEN. Poly-prep slides and dialysis tubings were obtained from Sigma. Ultrafiltration assembly, Centricon centrifugal filter devices and YM30 membranes were purchased from Amicon Corp., Lexington, MA, USA. Filtration membranes of 0.22 and 0.45 microns were procured from Millipore, Bedford, MA, USA. Complete and incomplete Freund's adjuvants were obtained from Difco laboratories, Detroit, MI, USA. Complete protease inhibitor cocktail tablets were purchased from Roche Applied Science, Mannheim, Germany. Tissue culture flasks (T-25, T-75 and T-175), 6 well, 24 well and 96 well tissue culture plates and 96 well microtitration plates were procured from Nunc a/s, Rosakilde, Denmark. The spinner bottles were obtained from Thermolyne, Barnstead International, Iowa, USA. Glass micro-slides and coverslips were purchased from Blue Star, Polar Industrial Corporation, Mumbai, India.

METHODS

I. CLONING AND EXPRESSION OF RECOMBINANT HUMAN ZONA PROTEINS IN PROKARYOTIC EXPRESSION SYSTEM

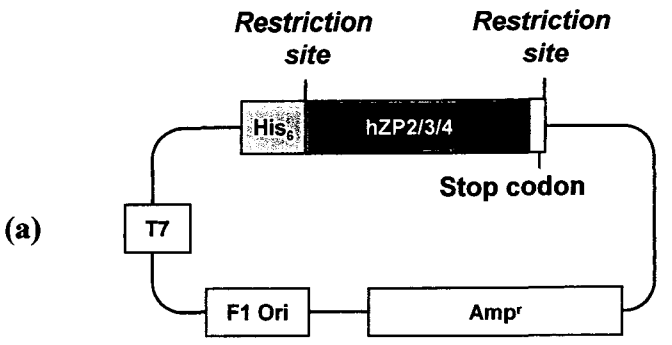
a) Cloning of hZP2, hZP3 and hZP4 in *E. coli*

The human zona proteins were cloned in the prokaryotic expression vector, pRSET-A (Figure 1a) and the constructs used for the expression of the respective proteins in *E. coli*.

i) PCR amplification

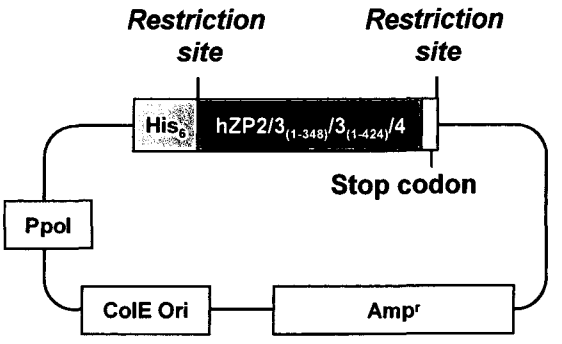
The cloning of recombinant hZP3 in *E. coli* was initiated from its pBluescript clone (kindly made available by Dr. Jurrien Dean, Laboratory of Cellular and Developmental Biology, NIDDK, National Institutes of Health, Bethesda, Maryland, USA) as a template. An internal cDNA fragment of hZP3 (67-1044 bp; 23-348 aa), devoid of the N-terminal signal sequence (SS) and C-terminal transmembrane-like domain (TD), was amplified by polymerase chain reaction (PCR). The above amplicon was cloned and expressed as a fusion protein with polyhistidine tag at the C-terminus in pRSET-A expression vector (Invitrogen) under the T7 promoter *lac* operator control (Figure 1a). In the hZP3 construct, the forward primer had an *EcoR I* restriction enzyme site and the reverse primer contained a *BamH I* restriction enzyme site. The primer sequences pertaining to the construct are specified in Table 5. The PCR amplification was performed in a 50 µl reaction volume (50 mM KCl, 10 mM Tris-HCl pH 9.0, 0.1% Triton X-100) using 10-20 ng of the template deoxyribonucleic acid (DNA), 50 pmole of the forward and the reverse primers, 25 mM $MgCl_2$, 10 mM deoxyribonucleotide triphosphates (dNTPs) and 5 units (U) of Taq DNA polymerase (Promega) for extension. The template was initially denatured at 94°C for 10 min followed by amplification, which was carried out for 25 cycles of denaturation at 94°C for 60 sec, primer annealing at 53°C for 90 sec and extension at 72°C for 90 min followed by a final extension at 72°C for 15 minutes.

For cloning of hZP2 in pRSET-A, a hZP2 cDNA internal fragment (-SS, -TD; 115-1935 bp; 38-645 aa) was amplified by PCR from its parent pBluescript-hZP2 clone (a kind gift from Dr. Jurrien Dean) harboring the full-length hZP2 cDNA. A similar strategy was adopted for PCR amplification as for hZP3 except that primer annealing



(a)

pRSET-A-ZP2/3/4
BL21[DE3]pLysS



(b)

pAcHLT-A-ZP2/3$_{(1-348)}$/3$_{(1-424)}$/4
***Sf21* cells**

Figure 1: Schematic representation of the constructs pertaining to the expression of human zona proteins in *E. coli* and baculovirus expression vectors. (a) The cDNA corresponding to hZP2/3/4 (devoid of SS and TD) was cloned in the *E. coli* expression vector pRSET-A. The vector had a T7 promoter (PT7) and a 6X histidine tag (His_6) at the N-terminus. It also had a F1 origin of replication (F1 Ori) and an ampicillin resistance gene (Amp^r). (b) Full length hZP2/3/4 and hZP3 (without TD) were cloned in the baculovirus transfer vector pAcHLT-A which harbors in the N-terminus, a late polyhedrin promoter (Ppol) and a His_6 tag. It also contains a ColE origin of replication (ColE Ori) and an Amp^r gene.

Table 5: Salient features of various constructs designed for expression of human ZP proteins in *E. coli* and baculovirus

Construct	Expression system	Forward Primer*	Reverse Primer*[a]
hZP3	*E. coli*	5'-CG***GGATCC***CA ACCCCTCT-3'	5'-CG***GAATTC***TTA GGAAGCA-3'
$hZP3_{(1-348)}$	Baculovirus	5'-CG***GAATTC***ATGG AGCTGAGCTAT-3'	5'-GA***AGATCT***TCAGG AAGCAGACCTGGA-3'
$hZP3_{(1-424)}$	Baculovirus	Same as for $hZP3_{(1-348)}$	5'-CG***GAATTC***TCATT CGGAAGCAGA-3'
hZP2	*E. coli*	5'-GA***AGATCT***TCATA GATGTTTCTCAGTTG-3'	5'-CG***GAATTC***TTAAGA GGACACAGGGCA-3'
hZP2	Baculovirus	5'CG***GAATTC***ATGGCGT GCAGGCAGAGAGGAG GCTCTTGGAGTCCC-3'	5'-GA***AGATCT***TCAGTG ATTTGACACAGT-3'
hZP4	*E. coli*	5'-CG***AGCTCA***AGCCTG AGGCACCA-3'	5'-GG***GGTACC***TTAACT GAGATCAGGACA-3'
hZP4	Baculovirus	5'-CG***AGCTCA***TGTGGC TGCGG-3'	5'-GG***GGTACC***TCATTG ACACATTTG-3'

*Restriction enzyme site is denoted in bold italics

[a]Stop codon in the reverse primer is underlined

was done at 48°C. The primers used, listed in Table 5, had an *EcoR I* restriction site in the forward primer and a *Bgl II* site in the reverse primer.

Amplification of an internal fragment of hZP4 cDNA (-SS, -TD; 64-1389 nt; 22-463 aa) in pRSET-A vector was also performed from its parent pBluescript clone (kindly provided by Dr. S. V. Prasad, Baylor College of Medicine, Houston, Texas, USA) under similar conditions as that of hZP3 construct except that the PCR was carried out in a reaction buffer containing 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl pH 8.8, 2 mM $MgSO_4$ and 0.1% Triton X-100 by 2 U of Vent DNA polymerase (NEB). The restriction sites incorporated were *Sac I* in the forward primer and *Kpn I* in the reverse primer. The primer sequences are given in Table 5.

ii) Agarose gel electrophoresis and ligation of PCR amplified products in pPCR-Script Amp SK (+) cloning vector

Following PCR amplification, the amplicons were analyzed for confirmation of the expected fragment sizes by electrophoresis on a 1% agarose gel containing 0.5 μg/ml ethidium bromide at 10 V/cm using 1X Tris-Acetate-EDTA (TAE) buffer (40 mM Tris pH 8.0, 20 mM acetic acid and 1 mM EDTA). DNA was visualized at a wavelength of 312 nm on UV transilluminator. Subsequently, the correctly amplified products were resolved on a 1% low melting point (LMP) agarose gel and purified from the gel. For gel purification, the band of interest was excised and melted at 65°C for 20 min. One-tenth the volume of 5 M NaCl was added to the melted agarose and incubated at 65°C for another 10 min. DNA was then extracted twice by adding an equal volume of phenol equilibrated with TE (Tris-EDTA; 10 mM Tris pH 8.0 and 1 mM EDTA) to the melted contents of the tube followed by extraction with phenol : chloroform : isoamyl alcohol (25:24:1) and then with chloroform : isoamyl alcohol (24:1). DNA was precipitated by addition of 2 volumes of chilled 100% ethanol to the aqueous phase and incubating the contents at -70°C for 1 h. The DNA pellet was collected by centrifugation at 12,000 X g for 20 min and washed with 70% ethanol. The pellet was air-dried, resuspended in 25 μl TE and quantitated.

The gel purified PCR products were polished for making them blunt-ended before being cloned into the *Srf I* restricted pPCR-Script Amp SK(+) cloning vector. The blunting reaction was carried out at 72°C for 30 min using 0.5 U of cloned *Pfu* DNA polymerase, 10 mM dNTPs and 10X polishing buffer (Stratagene). The blunt-ended

PCR products were then ligated into the pre-digested pPCR-Script Amp SK(+) cloning vector, using a vector to insert ratio of 1:40 in a 10 µl reaction volume for 1 h at room temperature (RT). The reaction mixture comprised of 10 ng of pPCR-Script Amp SK(+) cloning vector, 4 U of T4 DNA ligase, 0.5 µl of 10 mM rATP, 1 µl of 10X reaction buffer and 5 U of *Srf I* restriction enzyme. The buffers and enzymes used for blunting as well as for ligation were supplied along with the PCR-Script™ Amp cloning kit (Stratagene).

iii) Media composition and bacterial culture

The Luria Bertani (LB; pH 7.4) medium was prepared in double distilled water by adding 1% NaCl, 0.5% yeast extract and 1% tryptone, and sterilized by autoclaving under pressure (15 lbs/inch^2) for 20 min. Solid growth medium was prepared by adding 1.5% agar to LB prior to autoclaving. Appropriate antibiotics were added after cooling the medium to approximately 50-60°C. Bacterial cultures were grown in LB medium at 37°C in an orbital shaker set at 200 revolutions per minute (rpm) unless otherwise stated. Ampicillin was used at a concentration of 100 µg/ml and chloramphenicol at 37 µg/ml. DH5α strain of *E. coli* was grown in plain LB medium without any antibiotic selection while BL21[DE3]pLysS strain of *E. coli* were selected on chloramphenicol.

iv) Preparation of competent cells and transformation

The DH5α strain of *E. coli* was grown overnight (O/N) in LB at 37°C and subcultured (1:100) in 100 ml of fresh LB medium. The culture was grown until it reached an absorbance of 0.4 at 600 nm (A_{600}). The culture was centrifuged at 2500 X g for 10 min at 4°C. The cell pellet was resuspended in 10 ml of freshly prepared, sterile, ice cold $CaCl_2$ (100 mM) solution and incubated for 30 min on ice. Cells were centrifuged at 2500 X g and the pellet was very gently resuspended in 2 ml of chilled $CaCl_2$ (100 mM) containing 15% glycerol. Aliquots of 100 µl were dispensed into sterile, chilled 1.5 ml microcentrifuge tubes and stored at -70°C until further use. Competent cells of other strains of *E. coli* were also prepared in a similar manner after growing them in the appropriate selection medium.

For transformation, each ligation mix from the above reactions was separately added to a vial of DH5α competent cells thawed on ice. The contents were gently mixed and incubated on ice for 30 min. The cells were then exposed to a heat shock at 42°C for 90 sec and further incubated on ice for another 5 min. The transformed cells were then

grown in 1 ml of LB medium for 1 h at 37°C with constant shaking for the expression of the ampicillin resistance marker gene (β-lactamase). Aliquots from each transformation were plated separately on LB plates containing 100 μg/ml ampicillin (LB_{amp}), 80 μg/ml of X-gal (Sigma) and 20 mM of IPTG (Amresco). The plates were incubated at 37°C for 12-14 h for growth of ampicillin-resistant colonies along with blue-white selection, following which white colonies were picked up for further analysis.

v) Small scale plasmid DNA isolation and restriction digestion

White transformants picked up following blue-white selection were inoculated in 3 ml of LB_{amp} medium and grown O/N. The next day, 1.5 ml aliquots of O/N culture were harvested by centrifugation at 10,000 X g for 1 min in a microfuge. The supernatant was discarded and the pellet was resuspended in 100 μl of chilled TEG (25 mM Tris-HCl pH 8.0, 10 mM EDTA and 50 mM glucose) and incubated on ice for 5 min. After incubation, 200 μl of freshly prepared alkaline-SDS (0.2 N NaOH, 1% SDS) was added and the contents were mixed gently by inversion. This was followed by incubation for 7 min at RT. Post-incubation, 150 μl of ice-cold sodium acetate solution (3 M, pH 5.2) was added to the mixture, mixed gently by inversion and incubated on ice for 15 min. After incubation, the contents were centrifuged at 12,000 X g for 20 min at 4°C and the supernatant was carefully transferred to a fresh tube. DNA was precipitated with 0.6 volumes of isopropanol and incubated at RT for 20 min. The DNA pellet was obtained by centrifugation at 12,000 X g at RT for 20 min, air-dried and dissolved in 200 μl of TE. To remove RNA contamination, 50 μg of DNase free RNase was added and incubated for 1 h at 37°C. Plasmid DNA was then extracted once with an equal volume of TE equilibrated phenol followed by extraction with phenol : chloroform : isoamyl alcohol (25:24:1) and then with chloroform : isoamyl alcohol (24:1). DNA was precipitated by addition of 2 volumes of chilled 100% ethanol to the aqueous phase and incubating the contents at -70°C for 1 h. The DNA pellet was obtained by centrifugation at 12,000 X g for 15 min, washed with 70% ethanol, air-dried and resuspended in 25 μl TE. The clones were checked for the presence of insert by restriction digestion analysis. The plasmid DNA isolated from the transformants were subjected to *EcoR I-Bgl II, EcoR I-BamH I* and *Sac I-Kpn I* restriction digestion for hZP2, hZP3 and hZP4 respectively according to the manufacturer's instructions. The

digested products were checked for the release of the respective insert on a 1% agarose gel. One positive clone was selected from each set of transformations and the plasmid DNA was purified in large amount for insert preparation.

vi) Large scale plasmid DNA isolation

Selected transformants were grown O/N in 250 ml of LB_{amp}. The bacterial cells from the O/N cultures were harvested by centrifugation at 4000 X g for 10 min at 4°C. The cell pellet was resuspended in 5 ml of TEG solution containing lysozyme (2.0 mg/ml in 10 mM Tris-HCl pH 8.0) and incubated at RT for 15 min. Alkaline-SDS (10 ml) was added to the mixture and again incubated at RT for 10 min after mixing the contents gently by inverting the tube. Post-incubation, chilled sodium acetate solution (7.5 ml) was added and the contents were incubated on ice for 15 min. After incubation, the mixture was centrifuged at 10,000 X g at 4°C and processed in a similar fashion as described above up to addition of isopropanol. The DNA pellet was resuspended in 500 µl TE containing 20 µg/ml RNase and incubated for 1 h at 37°C. Plasmid DNA was then extracted as described above. The DNA pellet was air-dried and finally dissolved in 200 µl of TE.

vii) Cloning of the hZP2, hZP3 and hZP4 constructs into pRSET-A expression vector

The pRSET-A vector plasmid DNA was digested with *EcoR I-Bgl II, EcoR I-BamH I* and *Sac I-Kpn I* restriction enzymes respectively for the hZP2, hZP3 and hZP4 constructs and purified after resolution on a 1% LMP agarose gel. Similarly, the inserts from the hZP2, hZP3 and hZP4 constructs were also digested and gel purified. Each of the pre-digested inserts was ligated with the linearized vector at a ratio of 10:1 in a 10 µl reaction volume for 16 h at 16°C using 4 U of T4 DNA ligase (NEB) in 1X Ligase buffer [50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 1 mM ATP, 25 µg/ml BSA]. Subsequently, 5 µl of the ligated mix was transformed into BL21[DE3]pLysS competent cells for the expression of various constructs of human zona proteins in *E. coli.*

b) Expression of recombinant *E. coli*-expressed hZP2, hZP3 and hZP4

i) Checking the Expression of recombinant hZP proteins

In order to check the expression of recombinant proteins, the transformants with respect to the three constructs were inoculated in 1 ml of LB containing 100 µg/ml of ampicillin and 37 µg/ml of chloramphenicol and grown O/N at 37°C. Following day,

the cells were subcultured (1:100 dilution) in 1 ml of LB_{amp} and grown at 37°C until the A_{600} reached a value of 0.5-0.6. The cultures were then induced with 1 mM IPTG for 2.5 h at 37°C to induce expression of the fusion protein under the control of T7 promoter. Non-transformed host cells were grown in parallel as control cultures and processed identically. Simultaneously, uninduced transformed cells were also grown as a control. The cells were centrifuged at 10,000 X g in a microfuge for 1 min and the resulting pellet was stored at -70°C until used.

ii) SDS-PAGE and Western blot analysis of the expressed recombinant proteins

The cell pellet obtained from each of the 1 ml cultures was resuspended in 50 µl each of milli Q (MQ) water and 2X sample buffer (0.0625 M Tris pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol and 0.01% bromophenol blue). The samples were boiled for 10 min and 15 µl of each sample was resolved on a 0.1% SDS-10% PAGE (Laemmli, 1970). The expression of the recombinant protein was checked by Western blot analysis for which the SDS-PAGE resolved proteins were electrophoretically transferred O/N to a 0.45 µm nitrocellulose membrane (Bio-Rad) at a constant current of 50 milliampere (mA) in Tris-Glycine buffer (25 mM of Tris-HCl and 200 mM glycine) containing 20% methanol (Towbin *et al.*, 1979). The membrane was then washed once with phosphate buffered saline (PBS; 50 mM Phosphate and 150 mM NaCl, pH 7.4) and non-specific sites were blocked with 3% BSA in PBS for 60 min at RT. All the subsequent incubations were carried out for 1 h at RT and each incubation was followed by three washings with PBS containing 0.1% Tween-20 [PBST (0.1%)]. Post-blocking, the membrane was incubated with 1:1000 dilution of the primary antibody. The bound antibody was revealed using HRPO conjugated secondary antibody at a dilution of 1:2000. The blot was developed with 0.6% (w/v) 4-chloro-1-naphthol (Amresco) in 50 mM PBS containing 25% methanol and 0.06% H_2O_2. The reaction was stopped by washing the membrane extensively with MQ water. The expression of recombinant hZP3 expressed in *E. coli* was detected by employing ascites of murine MAb, MA-451 (diluted 1:1000) generated against porcine pZP3β (homologue of hZP3), and cross-reactive with hZP3 (Kaul *et al.*, 1997). The expression of *E. coli*-expressed hZP2 and hZP4 was detected by using 1:1000 dilution of ascites of murine MAbs, MA-925 and MA-813 generated against *E. coli*-expressed recombinant bonnet monkey (*Macaca radiata*) ZP2 (bmZP2; unpublished observation) and bonnet

monkey ZP4 (bmZP4; Govind *et al.*, 2000) respectively. For detection of the bound monoclonal antibody, goat anti-mouse immunoglobulin conjugated to HRPO (Pierce) was used at a dilution of 1:2000.

iii) Localization of the expression of recombinant human zona proteins

For the localization experiments, a 100 ml culture of the respective pRSET-A clone was grown and induced with 1 mM IPTG as described above for expression. After induction for 2.5 h, the culture was divided into two aliquots of 50 ml each and cells were harvested by centrifugation at 4000 X g for 30 min at 4°C. For cytosolic localization, one aliquot was resuspended in 5 ml of sonication buffer (50 mM phosphate pH 7.8, 300 mM NaCl). The sample was frozen in liquid nitrogen and then thawed in chilled water. The cells were lysed by brief sonication using a Branson Sonifier-450 for 6 cycles of 90 sec each (30 Watt output; Branson Ultrasonic Corp., Danbury, CT, USA) and centrifuged at 10,000 X g for 20 min at 4°C. The supernatant and the pellet represent the soluble and insoluble components of the cell pellet respectively. In order to check for periplasmic localization of the recombinant proteins, each of the second aliquots of induced cells was resuspended separately in 10 ml each of hypertonic solution (30 mM Tris pH 8.0, 20% sucrose, 1 mM EDTA) and incubated at RT for 10 min with shaking. Cells were centrifuged at 10,000 X g for 10 min. The pellet was subjected to an osmotic shock in 10 ml of 5 mM $MgSO_4$. Cells were stirred for 10 min in an ice water bath and centrifuged at 10,000 X g at 4°C for 10 min. The supernatant collected represents the periplasmic fraction. The various fractions were analyzed by SDS-PAGE and Western blotting as described above.

II. PURIFICATION OF E. coli EXPRESSED RECOMBINANT PROTEINS

a) Under denaturing conditions

For purification of recombinant hZP2, hZP3 and hZP4 expressed in *E. coli*, clones with respect to these constructs were grown at a shake-flask level (250 ml culture/flask; total volume 1 L) as described above. The cells were induced with 1 mM IPTG for 2.5 h at 37°C, centrifuged at 4000 X g for 30 min at 4°C and stored at -70°C until used. The recombinant protein was purified by nickel affinity chromatography. The cell pellet (~1g) of each clone was solubilized in 5 ml of buffer A (6 M guanidine hydrochloride, 0.1 M NaH_2PO_4, 0.01 M Tris pH 8.0). The suspension was centrifuged at 8000 X g for 15 min at 4°C and the supernatant containing the recombinant fusion protein was mixed

with buffer A equilibrated Nickel-Nitrilotriacetic acid resin (Ni-NTA resin; QIAGEN) and kept for gentle end-to-end shaking for 1 h at RT. The resin was loaded onto a column and washed with 10 bed-volumes of buffer A. The column was subsequently washed with 5 bed-volumes each of buffers B and C, which contained 8 M Urea, 0.1 M NaH_2PO_4 and 0.01 M Tris and had successively reducing pH values of 8 and 6.3 respectively. The recombinant protein was eluted with buffers D and E (composition identical to that of buffers B and C), in which the pH was further reduced to 5.9 and 4.5 respectively. The eluted protein, after analysis on SDS-PAGE, was concentrated in an Amicon concentrator using YM30 membrane (Amicon) for all the three recombinant human zona proteins. The concentration of purified proteins was estimated by bicinchoninic acid assay (BCA; Pierce) using BSA as the standard.

b) Renaturation of *E. coli*-expressed recombinant proteins

In order to obtain the purified protein in soluble form without urea, the Ni-NTA purified recombinant protein was extensively dialyzed against renaturation buffer (50 mM Tris-HCl pH 8.5, 1 mM EDTA, 0.1 mM reduced glutathione, 0.01 mM oxidized glutathione and 10% sucrose) for 96 h with 5 changes of the dialysis buffer to assist in removal of urea and refolding of the protein. The refolded protein was further dialyzed against 20 mM Tris pH 7.4. The protein concentration of the purified and dialyzed recombinant protein was estimated using BCA.

c) Fluorescein isothiocyanate (FITC) labeling of the recombinant proteins

In order to prepare recombinant protein-FITC conjugate, an aliquot of the recombinant protein was dialyzed against 0.5 M carbonate buffer (pH 9.0) and incubated with FITC Isomer I (Pierce) at a molar ratio of 1:24 for 1.5 h at RT with end-to-end mixing. Post-incubation, unbound FITC was removed from the labeled recombinant protein by extensive dialysis against PBS pH 7.4. All the above treatments were performed under light-protected conditions. The fluorescein/recombinant protein molar ratio (F/P) of the respective FITC-labeled recombinant protein was determined by spectrophotometric analysis and calculated using the following formula:

$$\text{Molar F/P} = \frac{\text{Molecular weight of the protein}}{389} \times \frac{A_{495}/195}{A_{280}-[(0.35 \times A_{495})]/E^{0.1\%}}$$

where: 389 is the molecular weight of FITC in Da

195 is the absorption $E^{0.1\%}$ of bound FITC at 490 nm at pH 13.0

$(0.35 \times A_{495})$ is the correction factor due to the absorbance of FITC at 490 nm

$E^{0.1\%}$ is the absorption at 280 nm of a protein at 1.0 mg/ml

III. CLONING AND EXPRESSION OF ZONA PROTEINS IN BACULOVIRUS EXPRESSION SYSTEM

a) Cloning of hZP2, hZP3 and hZP4

The cDNAs corresponding to hZP2, hZP3 and hZP4 were also cloned in baculovirus-transfer vector, pAcHLT-A and expressed in baculovirus expression system (Figure 1b).

i) PCR amplification

In pAcHLT-A, the baculovirus transfer vector (PharMingen), two constructs of hZP3, both harboring the SS, but one excluding the TD [+SS, -TD; 1-1044 bp; 1-348 aa; $hZP3_{(1-348)}$] while the other, including the TD [+SS, +TD; 1-1272 bp; 1-424 aa; $hZP3_{(1-424)}$] were made. The cDNA corresponding to the above two constructs were PCR amplified by Taq DNA polymerase using the template DNA as described above for cloning in *E. coli*. The primers for $hZP3_{(1-348)}$ consisted of an *EcoR I* restriction enzyme site in the forward primer and a *Bgl II* restriction enzyme site in the reverse primer while both the primers for $hZP3_{(1-424)}$ had *EcoR I* restriction sites (Table 5). The PCR conditions were identical to the ones used during amplification of the hZP3 insert for its cloning in the pRSET-A prokaryotic expression vector. However, the annealing of primers to the template DNA was performed at 48°C and 47°C for $hZP3_{(1-348)}$ and $hZP3_{(1-424)}$ respectively.

The cDNAs corresponding to hZP2 (+SS, +TD; 1-2235 nt; 1-745 aa) and hZP4 (+SS, +TD; 1-1620 bp; 1-540 aa), as depicted in figure 1, were also similarly PCR amplified using identical PCR conditions as described above except that primer annealing was done at 46°C and 45°C for hZP2 and hZP4 respectively. The primers employed for amplification of hZP2 had an *EcoR I* restriction site in the forward and a *Bgl II* restriction enzyme site in the reverse primer, whereas those for hZP4 had *Sac I* and *Kpn I* restriction sites in forward and reverse primers respectively (Table 5).

ii) Ligation and cloning of the PCR amplified products into pPCR-Script Amp SK(+) vector

Pertaining to each of the above constructs, the PCR amplified insert was gel purified, blunt-ended, and ligated into the pPCR-Script Amp SK(+) vector as described above. After transformation into DH5α cells, plasmid DNA was purified from the white transformants and restriction analysis performed with appropriate enzymes for

confirmation of the release of insert of right size. The inserts were purified from their respective positive clones as described above and used for ligation in the pAcHLT-A baculovirus transfer vector.

iii) Cloning into pAcHLT-A transfer vector

The pAcHLT-A transfer vector was digested with the respective enzymes, as used for preparation of the insert, and ligated to the insert using T4 DNA ligase as described before. In case of preparation of digested vector for cloning of hZP4 in baculovirus-expression sytem, the linearized vector was first dephosphorylated using Shrimp Alkaline Phosphatase (USB) in order to reduce re-ligation of vector. The dephosphorylation reaction was carried out in a reaction buffer containing 20 mM Tris-HCl pH 8.0 and 10 mM $MgCl_2$ at 37°C for 1 h using 2 U of the enzyme. The enzyme was completely and irreversibly inactivated by heating the reaction mix at 65°C for 15 min before being used for ligation. Five µl of the ligation mix was transformed into DH5α competent cells and plated on a LB_{amp} (100 µg/ml) plate. Colonies thus obtained were screened for the presence of the insert by isolating plasmid DNA from them and analyzing them for the release of insert by restriction digestion. One of the positive clones containing the insert for each of the three constructs, was used for large-scale purification of DNA using the midi DNA purification column (QIAGEN). A single colony of the respective clone was picked up from a freshly streaked LB_{amp} plate, inoculated into 3 ml of LB_{amp} medium and incubated for 8 h at 37°C with vigorous shaking (~250 rpm). Subsequently, 100 µl of this primary culture was inoculated into 100 ml of LB_{amp} medium and grown at 37°C for 16 h. The culture was centrifuged at 6000 X g for 15 min at 4°C. Plasmid DNA was purified from the pellet using QIAGEN DNA purification kit according to the manufacturer's instructions. Since the DNA being purified had to be used for transfection into the baculovirus genome, all steps after centrifugation at the isopropanol step were carried out under sterile conditions to prevent contamination during transfection.

b) Co-transfection of recombinant baculovirus transfer vector into insect cells

i) Cell culture techniques

Wild type *Autographa californica* nuclear polyhedrosis virus (AcNPV) and the recombinant AcNPV viruses were grown in confluent monolayers of an insect cell line obtained from Fall armyworm, *Spodoptera frugiperda* (*Sf*) *21*, maintained in TNM-FH

medium composed of Grace's insect cell culture medium supplemented with 3.33 g/L lactalbumin hydrolysate (GIBCO), 3.33 g/L yeastolate ultrafiltrate (GIBCO), 10% Fetal Calf Serum (FCS) and an antibiotic-antimycotic solution containing 100 U/ml penicillin G sodium, 100 µg/ml streptomycin sulfate and 250 ng/ml of amphotericin B as fungizone (GIBCO).

ii) Generation of recombinant baculoviruses by co-transfection

For *in vivo* homologous recombination of the genes of interest into AcNPV genome at the polyhedrin locus, the different recombinant baculovirus transfer vectors were separately co-transfected along with the BaculoGold™ linearized baculovirus DNA into *Sf21* insect cells using the BaculoGold Transfection Kit (PharMingen). *Sf21* cells were seeded at a density of 0.8 X 10^6 cells/well (~80% confluency) into two wells (control and experimental) of a 6-well tissue culture plate (Nunclon) for each construct. The recombinant baculovirus transfer vector DNA (5 µg) containing the insert of interest was mixed gently with 0.5 µg of linearized BaculoGold™ DNA in a microcentrifuge tube and incubated at RT for 5 min, following which 1 ml of Transfection Buffer B (25 mM HEPES pH 7.1, 125 mM $CaCl_2$ and 140 mM NaCl) was added to the reaction mix. The old medium from the experimental well was replaced with 1 ml of Transfection Buffer A (Grace's Medium supplemented with 10% FCS pH 6.2) while medium of the control well was replaced with 3 ml of fresh TNM-FH. After 5 min, the Transfection Buffer B/DNA solution was added dropwise, with gentle rocking of the plate after every 3-5 drops, to the experimental well. The plate for each of the constructs was incubated at 27°C for 5 h to allow *in vivo* recombination of the respective insert into the baculovirus genome. After 5 h, medium from both the wells of the plates was replaced with fresh TNM-FH medium and the plates were incubated at 27°C for 5 days. After 5 days, supernatant from the respective experimental wells (co-transfection supernatant) was collected and used for setting up a plaque assay to purify the recombinant virus expressing the protein of interest.

iv) Plaque assay for isolating recombinant viruses

For each construct, *Sf21* cells seeded in a 6-well tissue culture plate at a concentration of 0.8 X 10^6 cells/well (~70% confluency) were incubated with 100 µl of serial dilutions (from 10^0-10^4) of the co-transfection supernatant from the experimental well for 1 h at RT to allow infection of *Sf21* cells by the viruses. Simultaneously, one of the

wells was infected with the wild type AcNPV virus as a control. The viral inoculum was aspirated after 1 h and 2 ml of cooled agarose (1.5% Agarplaque-Plus™ Agarose; PharMingen) was added to each well and allowed to set. TNM-FH medium (2 ml) was added to each well and the plate was incubated at 27°C for 96 h after which the medium was removed and cells were stained with staining solution (0.03% neutral red in TNM-FH; 2 ml/well). Thereafter, the staining solution was aspirated and the plates were inverted and incubated at 27°C for 5 h. Plaques appearing as clear zones, were marked and picked up by removing an agar plug directly above the plaque using a sterile 200 µl tip and viruses were allowed to diffuse out in 200 µl of TNM-FH medium O/N at 4°C. This served as the initial viral stock for the individual plaques obtained.

c) Expression of recombinant proteins in baculovirus

i) Screening of the recombinant viruses

To check for the expression of the recombinant proteins, 0.8 X 10^6 *Sf21* cells/well (~70% confluent) were seeded in a 6-well tissue culture plate and infected with 100 µl of the viral supernatant from 6 individual, randomly picked plaques corresponding to each construct for 1 h at RT, following which the viral inoculum was replaced by 2 ml of fresh TNM-FH medium. One of the wells was infected with the wild type AcNPV virus as a negative control. After 96 h of infection, the cells were washed twice with PBS, the pellet resuspended in 50 µl each of MQ water and 2X SDS-PAGE sample buffer, 30 µl of which was resolved on SDS-PAGE and analyzed by Western blot analysis for the expression of recombinant protein as described above. In addition, to 30 µl of the culture supernatant from each plaque, 2X SDS-PAGE sample buffer was added, boiled for 10 min and analyzed by Western blot along with the cell pellet for the expression of the respective recombinant protein. The supernatant obtained from the positive plaque(s) for each set of co-transfection, which served as a virus stock, was harvested and filter sterilized through a 0.22 µm filter. The virus titer of the stock was determined by a plaque assay and the virus was stored either at 4°C (for short term storage) or at -70°C (for long term storage) and used for amplification of virus as described below.

ii) Amplification of recombinant viruses

For amplification of recombinant virus from the positive plaques of each construct, *Sf21* insect cells were infected with the viral stock from the positive plaque at a low

multiplicity of infection (MOI<1) and the supernatant was harvested 7 days post infection (p.i.). The titer of the amplified virus was determined by a plaque assay.

iii) Time kinetics analysis of recombinant protein expression post-infection

To determine the time-course of recombinant protein expression, *Sf21* cells were seeded at a density of 0.4 $X10^6$/well in a 6-well tissue culture plate and infected with the recombinant virus at a MOI of 3 for 5 different time intervals (24, 48, 72, 84 and 96 h). The infected cells from each time interval were pelleted, washed with PBS and analyzed for the expression of recombinant protein by Western blot, as described above.

IV. PURIFICATION OF BACULOVIRUS-EXPRESSED RECOMBINANT PROTEINS

For the production of recombinant proteins expressed in baculovirus, 2 X 10^8 *Sf21* cells were infected with the recombinant viruses at a MOI of 3 and cultured at 27°C for optimum time intervals pertaining to maximum expression of the respective recombinant protein either as a monolayer culture or in suspension. For a suspension culture, the insect cells were grown in Spinner bottles (Thermolyne) at 27°C on a biological stirrer (Thermolyne cellgro stirrer) at a speed of 42 rpm. Cells were harvested from culture by centrifugation at 1000 X g for 5 min and washed twice with PBS. The cell pellets were stored at -70°C until used.

a) Purification of the recombinant proteins under denaturing conditions

The baculovirus expressed recombinant proteins were also purified under denaturing conditions using a Ni-NTA affinity column essentially as described for *E. coli*-expressed recombinant human zona proteins. The eluted fractions of purified baculovirus-expressed recombinant hZP2, $hZP3_{(1-348)}$, $hZP3_{(1-424)}$ and hZP4 were concentrated in an Amicon concentrator using YM30 membrane.

b) Renaturation of the purified proteins

The Ni-NTA purified recombinant proteins were extensively dialyzed against renaturation buffer as described above. The refolded protein was further dialyzed against 20 mM Tris pH 7.4. The protein concentrations were estimated by BCA.

iii) Fluorochrome Labeling of the Recombinant Proteins

For preparing recombinant protein-FITC conjugate, the respective recombinant proteins were processed as described above for the *E. coli* expressed recombinant proteins. In addition, recombinant $hZP3_{(1-424)}$ and hZP4 expressed in baculovirus were also

conjugated to rhodamine B isothiocyanate (RBITC; 536 Da; excitation wavelength = 544 nm; Sigma), as described for FITC conjugation. The FITC as well as RBITC-labeled recombinant proteins were analyzed by 0.1% SDS-10% PAGE as described later followed by Coomassie staining.

V. CHARACTERIZATION OF CARBOHYDRATE RESIDUES OF THE PURIFIED RECOMBINANT PROTEINS

In order to determine the nature of sugar residues present, if any, in *E. coli* and baculovirus-expressed recombinant proteins, a lectin-binding assay in an Enzyme Linked Immunosorbent Assay (ELISA) was performed. Microtitration plates (Nunclon) were coated with the recombinant protein at a concentration of 500 ng/well in PBS for 1 h at 37°C followed by O/N at 4°C. All subsequent washings were done three times in 0.05% PBST. The plate was blocked with 1% BSA in PBS (200 µl/well) for 90 min at 37°C followed by incubation with the respective biotinylated lectin (1 µg/ml; 100 µl/well). The bound biotinylated lectins were revealed by incubating with 100 µl/well of HRPO conjugated streptavidin (1:3000; Pierce) at 37°C for 1 h. The enzyme activity was detected by adding 100 µl/well of 0.05% orthophenylenediamine (OPD) and 0.06% H_2O_2 in 50 mM citrate phosphate buffer pH 5.0 following which the reaction was stopped by adding 50 µl/well of 5 N H_2SO_4. The absorbance was read at 490 nm with 630 nm as the reference filter. Twenty one lectins available in the Lectin kit-I, II and III (Vector Laboratories) were used for determination of the type of glycosylation. The assay was repeated twice for each set of recombinant proteins.

To confirm the specificity of the lectins binding to the recombinant proteins, lectin blots were also performed. *E. coli* and baculovirus-expressed recombinant human zona proteins (1 µg/lane) were resolved on SDS-PAGE and processed for immunoblotting as described above except that post-blocking, the membrane was incubated with 20 µg/ml of the respective biotinylated lectins and their binding was subsequently revealed using HRPO-conjugated streptavidin (1:2000; Pierce).

VI. REMOVAL OF SUGAR RESIDUES FROM BACULOVIRUS-EXPRESSED RECOMBINANT HUMAN ZP PROTEINS

a) Removal of N-linked carbohydrate residues from $hZP3_{(1-424)}$ and hZP4

In order to produce baculovirus-expressed recombinant $hZP3_{(1-424)}$ and hZP4 devoid of Asn-linked glycans, *Sf21* cells were infected with the respective recombinant viruses along with addition of different concentrations (5, 10, 20 or 40 μg/ml of culture medium) of tunicamycin (from a stock solution of 1 mg/ml in 0.1 N NaOH; Sigma), cultured and processed for protein purification as described above. The modified, purified recombinant $hZP3_{(1-424)}$ and hZP4 will be referred to as tunica-$hZP3_{(1-424)}$ and tunica-hZP4 respectively.

b) Chemical O-linked deglycosylation of $hZP3_{(1-424)}$ and hZP4

To cleave the O-glycosydic linkages, 0.5 mg each of purified baculovirus-expressed $hZP3_{(1-424)}$ and hZP4 (0.5 mg/ml in 20 mM Tris pH 7.4) was added to an equal volume of 0.02 N NaOH and the mixture was incubated at 50°C for 24 h. Post-incubation, the alkali treated recombinant proteins were dialyzed extensively against PBS pH 7.4 and stored at -20°C until use. The alkali treated recombinant $hZP3_{(1-424)}$ and hZP4 will henceforth be referred to as alkali-$hZP3_{(1-424)}$ and alkali-hZP4 respectively.

Both the N- and O-linked deglycosylated baculovirus-expressed recombinant $hZP3_{(1-424)}$ and hZP4 were analyzed for their carbohydrate content in a lectin-binding assay in ELISA as described above.

VII. CLONING AND EXPRESSION OF hZP3 IN MAMMALIAN EXPRESSION SYSTEM

a) Cloning of hZP3 in pcDNA6/V5-His-B expression vector

i) PCR Amplification

To enable cloning of full-length hZP3 (+SS, +TD; 1-1272 bp; 1-424 aa) in the pcDNA6/V5-His-B mammalian expression vector, the cDNA corresponding to hZP3 was PCR amplified by Taq DNA polymerase from its parent pBluescript clone (mentioned before) as the template DNA using 5'-CG***GGATCC***ACCATGGAGCTGAGCTAT–3' having a *BamH I* restriction enzyme site as the forward primer and 5'-CG***GAATTC***TTCGGAAGCAGACAC-3' with *EcoR I* restriction site as the reverse primer. The restriction enzyme sites are denoted in bold italics. In the forward primer, a Kozak translation initiation sequence shown underlined

with initiation codon depicted in bold was added to ensure proper initiation of translation. The PCR conditions were identical to that used earlier for its cloning in the pRSET-A prokaryotic expression vector. However, the annealing of primers to the template DNA was performed at 46°C.

ii) Ligation and cloning of the PCR amplified hZP3 into pPCR-Script Amp SK(+) vector

The PCR amplified hZP3 insert was purified from gel, blunt-ended, and ligated into the pPCR-Script Amp SK(+) vector as described before. Following transformation into DH5α cells, plasmid DNA was purified from the white transformants and release of insert of right size, confirmed by restriction analysis, performed with appropriate enzymes. The hZP3 insert was purified from one of the positive clones as described previously and used for ligation in the pcDNA6/V5-His-B expression vector.

iii) Cloning into pcDNA6/V5-His-B expression vector

The pcDNA6/V5-His-B vector was restricted with the appropriate enzymes, as used for preparation of the insert, and ligated to the insert using T4 DNA ligase as described previously. The transformation of the ligation mix, screening of the colonies thus obtained for insert fallout of the correct size and large scale purification of plasmid DNA from one of the positive clones was performed as described above. Since the DNA being purified had to be used for transfection into the mammalian cells, all steps following centrifugation at the isopropanol step were carried out under sterile conditions to prevent contamination during subsequent transfection. The recombinant hZP3 construct will henceforth be referred to as pcDNA-hZP3.

b) Expression of hZP3 in CHO-KI cells

i) Cell Culture Techniques

The CHO-KI cells were propagated in T-25 culture flasks using Ham's F-12K medium (Sigma) supplemented with 10% FCS at 37°C in a humidified chamber with 5% CO_2 in air. For subculturing, the spent medium from the cells was discarded and the cells were trypsinized in Ham's F-12K medium supplemented with 0.5% trypsin and 0.2% EDTA. Cells were pelleted by centrifugation at 250 X g for 10 min followed by their resuspension in Ham's F-12K medium supplemented with 10% FCS and distribution in fresh T-25 flasks.

ii) Transient transfection of CHO-K1 cells with pcDNA-hZP3

For transient transfection, cells were seeded on coverslips in a 24-well tissue culture plate at a density of 5 X 10^4 cells/well a day prior to transfection. To standardize *in vitro* transfection conditions for optimum transient expression of human ZP3, varying amounts of plasmid DNA were mixed with Lipofectamine 2000 in a ratio of DNA : Lipofectamine 2000 of 1:2 in plain Ham's F-12K medium (devoid of FCS; total volume of 100 µl). This mixture was incubated for 25 min at RT and the seeded CHO-K1 cells on coverslips were washed twice with plain Ham's F-12K medium followed by the addition of 400 µl of plain medium/well. Subsequently, the DNA-Lipofectamine 2000 complex was added dropwise to the cells and incubated at 37°C in humidified atmosphere of 5% CO_2 for 5 h. Thereafter, the transfection mix was removed from the cells, 500 µl/well of Ham's F-12K medium supplemented with 10% FCS was added and the cells were incubated for a further period of 48 h. Untransfected cells in one well of the 24-well plate served as the negative control in the experiment. Post-incubation, the cells were processed for visualization of the expression of human ZP3 by indirect immunofluorescence. After standardization of the plasmid DNA concentration in a 24-well plate, large scale transfection was done in T-25 culture flasks by appropriately scaling up the cell density, medium volume and amount of DNA as well as Lipofectamine 2000.

iii) Indirect Immunofluorescence Assay

For detection of hZP3 expression by transfected CHO-K1 cells, an indirect immunofluorescence assay was performed. The transiently transfected CHO-K1 cells were washed once with PBS, fixed in chilled methanol for 5 min and blocked with PBS supplemented with 3% BSA at 4°C for 1.5 h. All subsequent washings of the cells were done with PBS. The cells were incubated with 1:20 dilution of either preimmune rabbit serum or rabbit polyclonal antibodies against $hZP3_{(1-424)}$ (kindly provided by Dr. Suraj K., Gamete Antigen Laboratory, National Institute of Immunology, New Delhi, India) for 1 h at 4°C. The bound antibodies were revealed by incubating the cells with FITC-conjugated goat anti-rabbit immunoglobulins (diluted 1:800; Pierce). Finally, the coverslips with cells were mounted in PBS : glycerol (1:9) and examined under a fluorescent microscope.

iv) Analysis of expressed hZP3 by immunoblot

Subsequent to large scale transient transfection, approximately 2 X 10^6 CHO-K1 cells were trypsinized and the cell pellet obtained was resuspended in 100 µl of PBS pH 7.4. The cells were lysed with 5-6 consecutive freeze thaw cycles (freezing in chilled ethanol bath followed by immediate thawing in a 37°C water bath) followed by brief centrifugation to separate the lysate from the cell debris. The cell lysate was mixed with 2X SDS-PAGE sample buffer and processed for SDS-PAGE followed by immunoblot analysis as described above. Murine monoclonal antibody against baculovirus-expressed recombinant $hZP3_{(1-424)}$ (MA-1552; culture supernatant diluted 1:5), made available by Mr. Pankaj Bansal, Gamete Antigen Laboratory, National Institute of Immunology, New Delhi, was used as a probe for hZP3 expression, followed by its detection using HRPO conjugated goat anti-mouse immunoglobulins. Untransfected CHO-K1 cells taken as control were also processed identically.

VIII. GENERATION OF MONOCLONAL ANTIBODIES

a) Immunization and fusion

After due approval from the Institutional Animal Ethical Committee, male BALB/c mice (8-10 week old, Small Experimental Animal Facility, National Institute of Immunology, New Delhi, India) were immunized subcutaneously (*s.c.*) with recombinant baculovirus-expressed hZP4 (50 µg/animal) emulsified with complete Freund's adjuvant (CFA; Difco). Animals were boosted intraperitoneally (*i.p.*) two times at 4 week intervals by the same amount of recombinant protein but emulsified with incomplete Freund's adjuvant (IFA; Difco). After 8-10 weeks, mouse showing the highest antibody titers against baculovirus-expressed recombinant hZP4 (as determined by ELISA; described later) was selected, and administered a total of 500 µg of recombinant hZP4 intravenously through tail vein over a period of three consecutive days. The day after the last booster injection, the mouse was euthanised by ether anesthesia and the spleen was removed aseptically in RPMI-1640 medium. The spleen was teased with serrated-toothed forceps to obtain single cell suspension of splenocytes. The clumps and membrane fragments were allowed to settle down and splenocytes were collected by centrifugation of the supernatant at 500 X g for 10 min. Before fusion, SP2/O-Ag 1.4 mouse myeloma cells (maintained at logarithmic phase of growth) were harvested by centrifugation at 500 X g for 10 min and counted in a

haemocytometer. The splenocytes were mixed with the myeloma cells (9 X 10^6) in a 2:1 ratio, and collected by centrifugation at 500 X g for 10 min and washed twice with RPMI-1640 medium. To the pellet, 0.5 ml of 50% PEG-400 solution was added drop by drop with constant shaking. The pellet was rocked continuously for 1 min and 4 ml of RPMI-1640 was added dropwise over a span of 5 min and then the volume was made to 25 ml with RPMI-1640 medium. The cells were centrifuged at 300 X g for 10 min and the pellet was resuspended in 30 ml of RPMI-1640 containing 20% FCS and 0.1 mM sodium hypoxanthine, 0.4 μM aminopterin, and 0.016 mM thymidine (HAT; Sigma). The cells (200 μl/well) were overlaid on the six 24 well culture plates pre-seeded with 10^5 feeder cells (peritoneal macrophages from BALB/c mice) per well. The used medium was replaced with fresh medium at 48 and 96 h.

b) Screening of the hybrid cells

After 10 days of fusion, supernatants from all the wells were checked for the production of antibodies against baculovirus-expressed hZP4 by ELISA (described later). The hybrids from positive wells (positive for the production of antibodies against recombinant hZP4) were cloned by limited dilution to obtain stable hybrid single cell clones secreting antibodies against recombinant hZP4. The positive clones were frozen in 10% dimethyl sulfoxide (DMSO) in FCS at a concentration of 1-2 million cells/ml in liquid nitrogen.

c) Production of MAbs

Selected hybridomas were grown in large scale either in tissue culture flasks or as ascites to produce sufficient amounts of MAbs. Hybrid cells were grown in T-75 tissue culture flasks containing RPMI-1640 medium with 10% FCS. Cells were subcultured 1:10 in new flasks and culture supernatants were collected after growing the cells for 2-3 days. For production of ascites, the cells were grown in peritoneal cavity of Pristane primed BALB/c mice. The mice were sensitized with 0.5 ml of Pristane (Sigma) and after 10-12 days, 10 X 10^6 healthy hybrid cells were injected *i.p.* using a 22G needle. The ascites developed after 1-2 weeks was collected using an 18G needle and the cells were removed by centrifugation at 4000 X g. The supernatant containing the antibodies was separated, aliquoted and stored at -20°C till further use.

IX. CHARACTERIZATION OF MAbs

a) Enzyme Linked Immunosorbant Assay (ELISA)

i) Reactivity with recombinant human ZP glycoproteins

The culture supernatants and ascites of the MAbs generated against baculovirus-expressed recombinant hZP4 were tested for their reactivity with it and the other zona proteins in an ELISA. Microtitration plates were coated at an optimized concentration of 200 ng/well of the respective recombinant protein for 1 h at 37°C and O/N at 4°C. The coated plates were washed once and blocked with 1% BSA for 2 h at 37°C. All subsequent incubations were carried out at 37°C and were followed by three washings with PBST (0.05%). Post-blocking, the plates were washed and incubated with either 100 µl of MAbs culture supernatant, ascites fluid (diluted 1:1000 with PBS) or culture supernatant from SP2/O myeloma cells. Bound antibodies were revealed by rabbit anti-mouse immunoglobulins conjugated to HRPO (Pierce) at an optimized dilution of 1:2000 for 1 h and color was developed with OPD as described before.

ii) Isotyping

The isotypes of the MAbs were determined using mouse monoclonal antibody isotyping reagents (Sigma) by indirect ELISA. The microtitration plates coated with baculovirus-expressed hZP4 and blocked with BSA as described above, were incubated with 100 µl of respective MAbs culture supernatant for 2 h at 37°C. The incubation with MAb was followed by the addition of goat anti-mouse isotype specific antibodies at 1:1000 dilution and the plate was furthe incubated for 90 min at 37°C. The binding was revealed by rabbit anti-goat IgG-HRP conjugate (Pierce) at an optimized dilution of 1:25,000 for 1 h at 37°C and processed for color development with OPD as described earlier.

b) Western Blot

The specificity of MAbs was also analyzed in a Western blot. For each MAb, two µg each of the baculovirus-expressed recombinant hZP2, $hZP3_{(1-424)}$ and hZP4 were resolved by SDS-PAGE and processed for Western blot wherein post-blocking, the nitrocellulose membrane was incubated with respective MAb culture supernatant diluted 1:5 with PBS for 90 min at RT. The bound antibodies were revealed by incubation with goat anti-mouse IgG-HRPO conjugate (Pierce) at an optimized dilution of 1:2000 and the enzyme reaction estimated as described earlier.

c) Indirect Immunofluorescence with Bonnet Monkey Ovarian Sections

Owing to the paucity of availability of human ovarian tissue, bonnet monkey ovarian sections were used for evaluation of the MAbs for their ability to recognize native ZP. The pre-fixed ovarian sections were washed with PBS and blocked at 37°C for 1 h with 1% normal goat serum in PBS. The sections were then incubated with respective MAbs (1:5 dilution of culture supernatants) for 90 min. Negative controls included ovarian sections incubated with culture supernatant obtained by growing SP2/O myeloma cells. The binding was revealed by goat anti-mouse immunoglobulins conjugated to FITC (1:800; Sigma) and processed for mounting and examination as described before.

X. EVALUATION OF THE BINDING OF RECOMBINANT ZONA PROTEINS WITH HUMAN SPERMATOZOA AND INDUCTION OF ACROSOME REACTION

a) Evaluation of binding of recombinant human zona proteins with sperm

i) Medium preparation

The medium used for all the sperm experiments, Biggers-Whitten-Whittingham medium (pH 7.4; BWW; Biggers *et al.*, 1971) was prepared by adding 94.8 mM NaCl, 4.78 mM KCl, 1.7 mM $CaCl_2.2H_2O$, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 0.25 mM sodium pyruvate, 43.6 mM sodium lactate, 5.5 mM glucose, 10 mM HEPES and 20 μg/ml gentamycin in double distilled water. Phenol red was added as a pH indicator and the medium was filter sterilized through a 0.45 μm filter before use. The medium was freshly prepared every time before an experiment. While BWW medium supplemented with 2.6% BSA (Sigma; embryo tested) was used as a medium during capacitation, BWW supplemented with 0.3% BSA was used for washing of the sperm, in binding and induction of acrosome reaction of human spermatozoa.

ii) Semen collection and capacitation of human spermatozoa

All experiments using human spermatozoa were carried out under informed consent and following the clearance from the Institutional Bio-safety and Ethical Committee. Semen samples were collected from healthy donors and subjected to liquefaction at RT for 30 min. Aliquots (0.5 ml) of semen were layered over a two-step Percoll density gradient (Suarez *et al.*, 1986). The lower fraction of the gradient (95% Percoll) consisted of 475 μl of sterile Percoll and 25 μl of 18% NaCl-200 mM HEPES buffer pH 7.4, which made the Percoll solution approximately isotonic to spermatozoa. The

upper fraction of the gradient (47.5% Percoll) comprised of 250 µl Percoll, 12.5 µl of NaCl-HEPES buffer and 250 µl of BWW medium with 0.3% BSA. The gradients layered with semen were centrifuged at 500 X g for 30 min at RT. The pellets comprising of >90% motile spermatozoa were pooled and washed with 5 ml of BWW medium supplemented with 0.3% BSA thrice by centrifugation at 500 X g for 10 min each. The sperm pellet was resuspended in BWW medium supplemented with 2.6% BSA at a concentration of 10 X 10^6 sperm/ml and incubated in aliquots of 1 ml for 16 h at 37°C in a humidified chamber with 5% CO_2 in air for capacitation.

iii) Induction of acrosome reaction by calcium ionophore-A23187

For the preparation of acrosome-reacted spermatozoa used in binding studies, the capacitated and motile sperm were washed once with BWW medium and incubated with BWW medium containing calcium ionophore-A23187 (10 µM; Sigma) for 20 min. The sperm suspension was washed with BWW medium and the sperm pellet was gently resuspended in BWW medium containing 0.3% BSA.

iv) Evaluation of sperm binding in a direct binding assay

The binding of recombinant *E. coli* and baculovirus-expressed proteins to capacitated as well as acrosome-reacted spermatozoa was further evaluated by direct binding assay using FITC-labeled recombinant proteins. In addition, recombinant $hZP3_{(1-424)}$ induced acrosome-reacted sperm obtained as described later, were also employed to study the binding characteristics of FITC conjugated baculovirus-expressed hZP2 and hZP4. Post capacitation or after induction of acrosome reaction, 5 X 10^6 sperm were incubated in a total reaction volume of 50 µl with 2.5 µg of the respective recombinant protein-FITC conjugate for 15 min at 37°C with 5% CO_2 in humidified air followed by incubation at 4°C for 30 min. Sperm were pelleted at 500 X g for 5 min, washed thoroughly to remove any unbound protein and subsequently, fixed in 4% paraformaldehyde for 30 min at RT. Post-fixation, the sperm were washed once with 50 mM PBS pH 7.4 and 20 µl aliquots were spotted onto poly-L-lysine coated slides (Sigma). The spots were air-dried, washed once with PBS and stained with 5 µg/ml *Pisum sativum* agglutinin (PSA) conjugated to tetramethylrhodamine isothiocyanate (TRITC-PSA, Vector Laboratories) for 30 min at RT for analysis of the acrosomal status of the sperm. Any spermatozoa that demonstrated complete loss of PSA staining in the acrosome or revealed staining at the equatorial region was classified as acrosome-reacted. Sperm showing PSA staining

in the acrosome were scored as capacitated (acrosome-intact). Slides were then washed thrice with PBS and observed for double staining (fluorescence) under a LSM 510 confocal microscope (Zeiss, Baden-Wuerttenberg, Germany) or Eclipse 80*i* fluorescence microscope (Nikon, Chiyoda-ku, Tokyo, Japan) using an oil immersion objective. In all the binding experiments, at least 100 sperm were scored for each reaction and the experiment was repeated thrice with semen sample from two different males to rule out any ambiguity.

iv) Evaluation of sperm binding by indirect immunofluorescence assay

The binding reaction was carried out in a similar manner as described above except that unlabeled recombinant zona proteins were used instead of the FITC-labeled proteins. In addition, 30 µl of the cell lysate of CHO-K1 cells transfected with pcDNA-hZP3 plasmid, prepared as described above, was also assessed for its binding to capacitated human sperm. The binding of the recombinant protein to sperm was evaluated by indirect immunofluorescence assay in the following manner. The slides were blocked with 3% BSA at 37°C for 1 h. Post-blocking, the slides were washed thrice with PBS. All subsequent incubations were carried out at 37°C for 1 h, followed by three washings with PBS. All the antibody dilutions were made in PBS + 0.1% BSA and filter sterilized through a 0.45 µm filter. The slides were incubated with 1:20 dilution (containing 0.1% BSA) of either rabbit pre-immune serum or rabbit polyclonal antibodies against the respective recombinant baculovirus-expressed protein. The bound antibodies were detected with goat anti-rabbit immunoglobulin-FITC used at an optimized dilution of 1:800 and simultaneously stained with PSA-TRITC (5 µg/ml) to study the acrosomal status.

v) Co-localization of the binding of baculovirus-expressed recombinant hZP3$_{(1-424)}$ and hZP4 on capacitated sperm

To investigate, if recombinant hZP3$_{(1-424)}$ and hZP4 expressed in baculovirus can simultaneously bind to human spermatozoa, RBITC conjugated hZP3$_{(1-424)}$ and FITC labeled hZP4 or vice versa were co-incubated at a molar ratio of 1:1 with capacitated human sperm as described above and assessed for RBITC as well as FITC fluorescence under the fluorescence microscope.

vi) Competitive displacement studies

In order to determine the specificity of binding of recombinant hZP2, hZP3 and hZP4 expressed in *E. coli* and baculovirus, 5 X 10^6 capacitated or acrosome-reacted sperm

were incubated with 2.5 μg FITC conjugated recombinant protein alone or in the presence of 1:5 and 1:10 fold molar excess of unlabeled recombinant human zona proteins in a reaction volume of 50 μl at 37°C and 5% CO_2 in humidified air for 15 min, followed by incubation at 4°C for 30 min. Further processing was done as described above for the direct binding experiments. The results are expressed as percentage of capacitated and/or acrosome-reacted sperm exhibiting the binding of FITC conjugated respective recombinant protein, in the absence or presence of competing unlabeled recombinant zona proteins.

In addition, specificity of the binding of baculovirus-expressed $hZP3_{(1-424)}$ and hZP4 to capacitated sperm was ascertained by employing MAbs generated against the respective recombinant protein. To 50 μl of culture supernatant obtained by growing hybrid cell clones secreting MAbs against $hZP3_{(1-424)}$ (made available by Mr. Pankaj Bansal, Gamete Antigen Laboratory, National Institute of Immunology, New Delhi) /hZP4, 2.5 μg of FITC conjugated $hZP3_{(1-424)}$/hZP4 (2.5 μl) was added and the reaction mixture incubated for 1 h at RT. Subsequently, to the incubating mixture, 5 X 10^6 capacitated sperm (50 μl of BWW with 0.3% BSA) were added and further incubated at 37°C and 5% CO_2 in humidified air for 15 min followed by incubation at 4°C for 30 min. The sperm were then fixed, processed and analyzed as described above for the evaluation of binding characteristics. The experiments were done in duplicates and repeated thrice and the values are represented as mean ± SEM.

b) Induction of acrosome reaction by recombinant human zona proteins

The ability of recombinant zona proteins to induce acrosome reaction in capacitated human spermatozoa was studied with respect to the *E. coli*, baculovirus as well as mammalian expressed human zona proteins. Human sperm (1 X 10^6), capacitated in BWW with 2.6% BSA for 16 h at 37°C with 5% CO_2 in air as described above, were incubated with individual recombinant protein or in combinations in a total reaction volume of 100 μl under similar conditions but in BWW supplemented with 0.3% BSA. For assessing the acrosome reaction inducing ability of mammalian expressed hZP3, the cell lysate (30 μl) of pcDNA-hZP3 transfected CHO-K1 cells was incubated with the capacitated human sperm. In order to account for the spontaneous induction of acrosome reaction, sperm were also incubated with BWW + 0.3% BSA alone. To understand the mechanism of action of recombinant proteins, capacitated spermatozoa

were treated with pertussis toxin (1 μg/ml; Sigma), an inhibitor of G_i protein, for 30 min at 37°C with 5% CO_2 in humidified air prior to the addition of recombinant protein for the induction of acrosome reaction. The acrosomal status of the sperm was evaluated using PSA staining. All the experiments were done "blind" with coded samples. In all the cases, at least 100 sperm were scored and the experiment was repeated at least thrice with 2 different semen samples.

c) Statistical Analysis

The results pertaining to the binding of recombinant human ZP glycoproteins to the capacitated as well as acrosome-reacted spermatozoa are presented as mean ± SEM of at least three independent experiments. In the competitive displacement experiments, the statistical differences between the means of the binding percentage of sperm to FITC conjugated recombinant protein alone and with the competitor or within an experimental group treated with two or more different competitors were compared by using one way ANOVA followed by Newmans-Keuls Multiple Comparison Test. In the experiments pertaining to the induction of acrosome reaction, the statistical analysis was done by comparing the means of the medium control (BWW + 0.3% BSA) and experimental sets or within two experimental groups as described above. A p value of <0.05 was considered to be statistically significant in all cases.

XI. EFFECT OF PROTEASOME INHIBITORS ON RECOMBINANT ZP PROTEIN- SPERM INTERACTION

Employing proteasome-specific inhibitors, lactacystin (LC), *clasto*-lactacystin β-lactone (CLBL) and Z-Leu-Leu-Leu-CHO (MG-132) kindly provided by Dr. Peter Sutovsky, University of Missouri-Columbia, Columbia, MO, USA, the role of sperm proteasome in sperm-egg interaction was studied. Binding as well as induction of acrosome reaction experiments with the capacitated and/or acrosome-reacted human sperm were carried out as described above except that during incubation, along with the recombinant protein, 100 μM of either of the above inhibitors was added to the reaction mixture. Appropriate control reactions were also set up wherein water in case of LC, DMSO for CLBL and ethanol for MG132 replaced the respective inhibitor in the reaction mixture. Post-incubation, the binding as well as acrosomal status of the spermatozoa was assessed by fluorescence microscopy as explained earlier.

Results

I. PURIFICATION AND CHARACTERIZATION OF RECOMBINANT HUMAN ZONA PELLUCIDA GLYCOPROTEINS

A critical delineation of the role of individual ZP glycoproteins during mammalian fertilization is pertinent for a comprehensive understanding of the complex cascade of events that are involved in its successful accomplishment. These studies become more relevant since the role of individual ZP glycoprotein in sperm-egg interaction varies from one species to another. Thus, in the present study, the three human zona proteins have been cloned and expressed in the prokaryotic as well as eukaryotic expression systems in order to address these issues. These studies would also provide us with an opportunity to elucidate the role played by the polypeptide backbone and the carbohydrate moieties in the bioactivity of the individual zona proteins.

a) Cloning, expression and purification of recombinant human zona proteins in *E. coli*

The cloning and expression of human zona proteins in *E. coli* was undertaken to obtain the recombinant human zona proteins in non-glycosylated form. The constructs corresponding to internal cDNA fragments of hZP2, hZP3 and hZP4, devoid of the respective SS and TD were PCR amplified and cloned under a T7 promoter downstream of a polyhistidine tag in pRSET-A, an *E. coli* expression vector as described in *Materials and Methods*. Before initiating expression studies, the pRSET-A clones encompassing the cDNA encoding hZP2, hZP3 and hZP4 were analyzed to determine the nt sequence for ascertaining that the insert was in frame and no mutations had occurred during PCR amplification that might lead to changes in the aa sequence of the encoded proteins. The schematic representation of the constructs has been shown in Figure 1a as described earlier.

i) *hZP2*

The hZP2 cDNA was PCR amplified from its parent clone as described in *Materials and Methods* to obtain a 1.8 kb amplicon as shown in Figure 2a. Thereafter, it was cloned in pPCR-Script Amp SK(+) cloning vector. The restriction digestion of the positive clones with *EcoR I* and *Bgl II* revealed the release of 1.8 kb hZP2 insert (Figure 2b). The released insert was purified and subsequently cloned in pRSET-A expression vector, wherein the positive clone was again identified by restriction analysis (Figure 2c). The expression of hZP2 in its pRSET-A clone was assessed as described in *Materials and Methods* and the recombinant hZP2 was expressed as

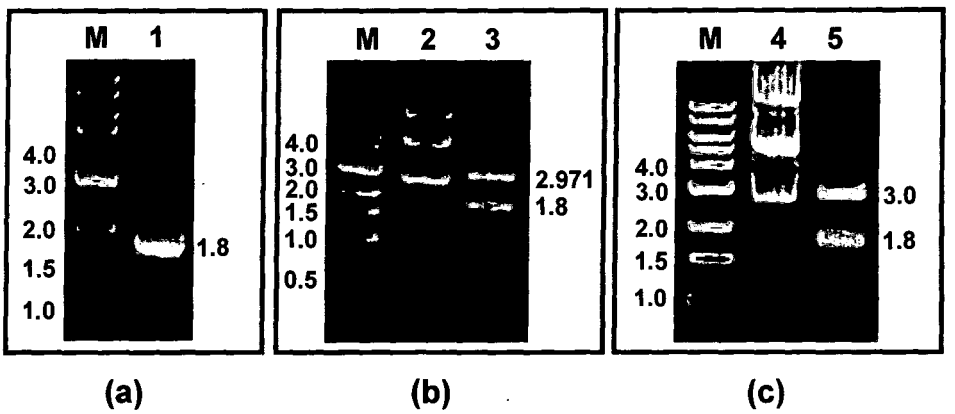


Figure 2: Cloning of hZP2 in *E. coli* expression vector. An internal fragment of hZP2 cDNA (115-1935 nt; 38-645 aa), excluding the SS and TD, was PCR amplified and finally cloned in pRSET-A expression vector as described in *Materials and Methods*. Panel (a) represents the 1.8 kb PCR amplified hZP2 cDNA as analyzed by agarose gel electrophoresis. Panel (b) represents the restriction pattern of hZP2 clone in pPCR-Script Amp SK(+) plasmid digested with *EcoR I* and *Bgl II* restriction enzymes. Panel (c) represents the restriction profile of pRSET-A plasmid harboring hZP2 cDNA with the enzymes *EcoR I* and *Bgl II*. Lanes are represented as M: 1 kb DNA ladder; lane 1: PCR amplified hZP2 cDNA; lanes 2 and 3: undigested and digested hZP2-pPCR-Script Amp SK(+) clone plasmid DNA, respectively; lanes 4 and 5: undigested and digested hZP2-pRSET-A clone plasmid DNA, respectively. The molecular weights depicted are in kb.

polyhistidine tagged fusion protein. A typical expression profile by Western blot, of the cell lysate from the BL21[DE3]pLysS host cells transformed with the hZP2 construct is shown in Figure 3a. The expression of hZP2 in *E. coli* was found to be leaky as some amount of the recombinant protein (~90 kDa) was detected even in uninduced cells harboring the recombinant plasmid. However, in the induced cells, in addition to the main protein band at ~90 kDa, several low molecular weight bands were also observed. These bands might represent premature translation termination or specific/non-specific degradation products/proteins.

Further, analysis of the localization of hZP2 expression in *E. coli* revealed that its expression was restricted to insoluble intracellular fractions of host cells and not in the soluble or periplasmic fractions (Figure 3b). The subtle differences in the relative mobility of the recombinant protein in relation to the molecular weight marker in the immunoblot analyses may be due to the fact that different batches of pre-stained molecular weight markers have been used during the course of this study. Though the molecular weight markers used are from the same company, the same protein standards of different batches have different apparent molecular weights.

The recombinant hZP2 was purified using Ni-NTA affinity chromatography under denaturing conditions in the presence of 8 M urea as described in *Materials and Methods*. The polyhistidine tag at the N-terminus of the recombinant hZP2 facilitated its purification by the Ni-NTA column. The purified hZP2 was renatured by dialysis for gradual removal of urea in the presence of oxidized and reduced glutathione with sucrose as a stabilizing agent. The Coomassie-stained SDS-PAGE as well as the Western blot analysis of the purified *E. coli*-expressed recombinant hZP2 revealed a single band corresponding to ~90 kDa (Figure 4).

ii) *hZP3*

The hZP3 cDNA devoid of its SS and TD was PCR amplified to obtain a 978 bp amplicon that was finally cloned in the pRSET-A expression vector as described above for hZP2 (Figure 5). The positive clone was induced with IPTG and checked for the expression of the recombinant hZP3. The immunoblot profile of the expression of hZP3 in *E. coli* is shown in Figure 6a. The recombinant hZP3 was expressed as a single band of molecular weight ~50 kDa and its expression was observed to be tightly regulated as the recombinant protein was detected only in the induced cells harboring the recombinant plasmid. By immunoblot analysis, the recombinant hZP3 was found to be

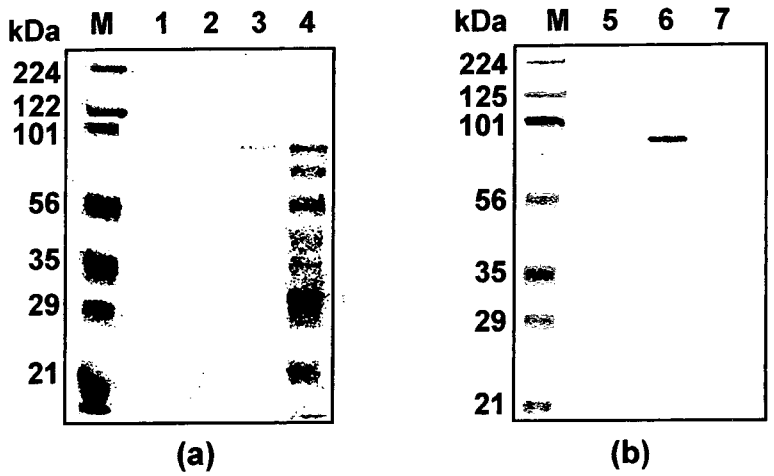


Figure 3: Immunoblot analysis of the expression and cellular localization of hZP2 in *E. coli*. BL21[DE3]pLysS cells transformed with hZP2 construct in pRSET-A were grown in LB medium and induced with 1mM IPTG for 2.5 h. Pellet of 1 ml of uninduced and induced culture was subjected to boiling (a) or fractionated into soluble, insoluble and periplasmic components followed by boiling (b) in 2X SDS-PAGE sample buffer (0.0625 M Tris pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol and 0.01% bromophenol blue), electrophoresed and processed for Western blot as described in *Materials and Methods*. The *E. coli*-expressed hZP2 was detected by using murine monoclonal antibody against bonnet monkey ZP2 (bmZP2), MA-925 which cross-reacts with hZP2 as described in *Materials and Methods*. Various lanes are represented as M: Molecular weight markers; lanes 1 and 2: uninduced and induced wild type host cells respectively; lanes 3 and 4: uninduced and induced cells harboring hZP2-pRSET-A plasmid respectively; lanes 5-7: soluble, insoluble and periplasmic cellular compartments respectively of host cells harboring hZP2-pRSET-A plasmid.

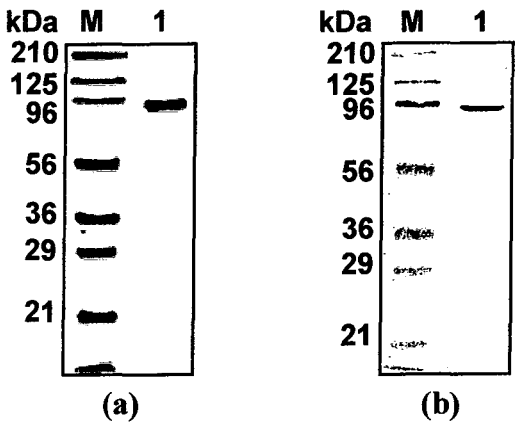


Figure 4: Electrophoretic and immunoblot analysis of purified recombinant hZP2 expressed in *E. coli*. The recombinant hZP2 was purified under denaturing conditions using Ni-NTA affinity column, followed by renaturation and subsequently resolved on 0.1% SDS-10% PAGE under reducing conditions as described in *Materials and Methods*. Panel (a) represents the Coomassie stained SDS-PAGE profile of *E. coli*-expressed purified recombinant hZP2 (lane 1, 5μg/lane) while panel (b) depicts the immunoblot profile (lane 1, 2 μg/lane). The recombinant hZP2 was revealed in the Western blot using antibodies as described in the legend to Figure 3. Lane M represents molecular weight markers.

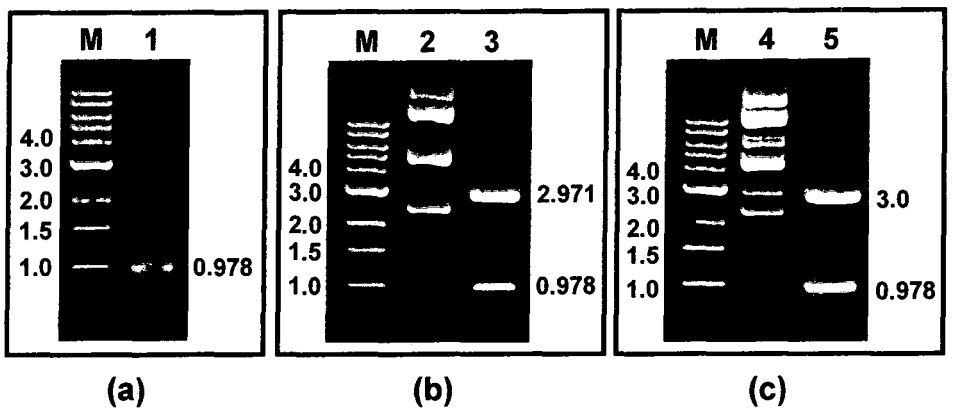


Figure 5: Cloning of hZP3 in *E. coli* expression vector. An internal fragment of hZP3 cDNA (67-1044 nt; 23-348 aa), excluding the SS and TD, was PCR amplified and finally cloned in pRSET-A expression vector as described in *Materials and Methods*. Panel (a) represents the 978 bp PCR amplified hZP3 cDNA as analyzed by agarose gel electrophoresis. Panel (b) represents the restriction pattern of hZP3 clone in pPCR-Script Amp SK(+) plasmid digested with *EcoR I* and *BamH I* restriction enzymes. Panel (c) represents the restriction profile of pRSET-A plasmid harboring hZP3 cDNA with the enzymes *EcoR I* and *BamH I*. Lanes are represented as M: 1 kb DNA ladder; lane 1: PCR amplified hZP3 cDNA fragment; lanes 2 and 3: undigested and digested hZP3-pPCR-Script Amp SK(+) clone plasmid DNA, respectively; lanes 4 and 5: undigested and digested hZP3-pRSET-A clone plasmid DNA, respectively. The molecular weights depicted are in kb.

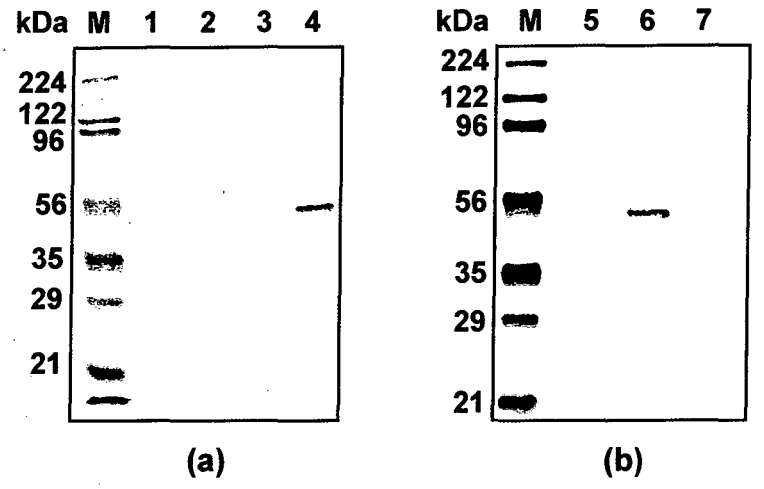


Figure 6: Western blot analysis of the expression and cellular localization of hZP3 in *E. coli*. BL21[DE3]pLysS cells transformed with hZP3 construct in pRSET-A were grown in LB medium and induced with 1mM IPTG for 2.5 h. Pellet of 1 ml of uninduced and induced culture was subjected to boiling (a) or fractionated into soluble, insoluble and periplasmic components followed by boiling (b) in 2X SDS-PAGE sample buffer, electrophoresed and processed for Western blot as described in *Materials and Methods*. The *E. coli*-expressed hZP3 was detected by using murine monoclonal antibody against porcine ZP3β (homologue of hZP3), MA-451 that is cross-reactive with hZP3 as described in *Materials and Methods*. Various lanes are represented as M: Molecular weight markers; lanes 1 and 2: uninduced and induced wild type host cells respectively; lanes 3 and 4: uninduced and induced cells harboring hZP3-pRSET-A plasmid respectively; lanes 5-7: soluble, insoluble and periplasmic cellular compartments respectively of host cells harboring hZP3-pRSET-A plasmid.

localized only in the intracellular insoluble fraction of the host cells (Figure 6b). Purification of the recombinant hZP3 was done using Ni-NTA affinity column under denaturing conditions followed by its renaturation as described in *Materials and Methods*. The purified recombinant hZP3 as assessed in Coomassie stained SDS-PAGE and Western blot, revealed a single band of molecular weight of ~50 kDa (Figure 7).

iii) *hZP4*

A 1.326 kb internal cDNA fragment of hZP4, excluding the SS and TD was PCR amplified (Figure 8a), cloned in the pPCR-Script Amp SK(+) cloning vector and subsequently in pRSET-A expression vector as described in *Materials and Methods*. The positive clones were identified in the both steps by restriction analysis using *Sac I* and *Kpn I* restriction enzymes. The restriction digestion profiles of one of the positive clones in both the cloning and expression vectors are depicted in Figures 8b and c.

The recombinant hZP4 expression in *E. coli* was assessed as described in *Materials and Methods*. The profile of hZP4 expression as analyzed by Western blot showed that it is present as a single band of ~65 kDa in the host cells containing the recombinant plasmid (Figure 9a). The localization of recombinant hZP4 was restricted to the insoluble cellular fraction of host cells (Figure 9b). The purification of the *E. coli*-expressed recombinant hZP4 and its subsequent renaturation were executed as described above for *E. coli*-expressed hZP2 and hZP3 and the profile of purified recombinant hZP4 is depicted in Figure 10.

From 1 litre culture of BL21[DE3]pLysS cells grown at shake flask level and induced with IPTG, an approximate yield of 1 mg of each of the recombinant hZP2, hZP3 and hZP4 individually was obtained.

b) Cloning and expression of recombinant human ZP glycoproteins in baculovirus expression system

To study the importance of carbohydrate residues in the bioactivity of human zona proteins, the human zona proteins were cloned and expressed in the baculovirus expression system in order to obtain these proteins in the glycosylated form. The cDNAs corresponding to the full length hZP2, hZP3 ($hZP3_{(1-424)}$), hZP4 as well as hZP3 devoid of TD ($hZP3_{(1-348)}$) were PCR amplified from their respective parent clones as described in *Materials and Methods* (Figure 11). The amplified products obtained were independently cloned in the pPCR-Script Amp SK(+) cloning vector as described in *Materials and Methods* and restriction analyses with appropriate enzymes

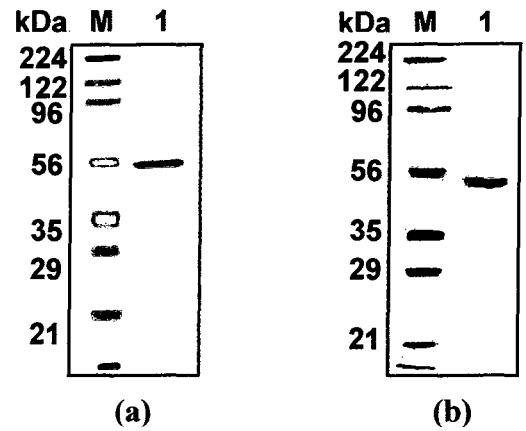


Figure 7: Electrophoretic and immunoblot analysis of purified recombinant hZP3 expressed in *E. coli*. The recombinant hZP3 was purified under denaturing conditions using Ni-NTA affinity column, followed by renaturation and subsequently resolved on 0.1% SDS-10% PAGE under reducing conditions as described in *Materials and Methods*. Panel (a) represents the Coomassie stained SDS-PAGE profile of *E. coli*-expressed purified recombinant hZP3 (lane 1, 5 μg/lane) while panel (b) depicts the immunoblot profile (lane 1, 2μg/lane). The recombinant protein was detected in the immunoblot using antibodies as described in legend to Figure 6. Lane M represents molecular weight markers.

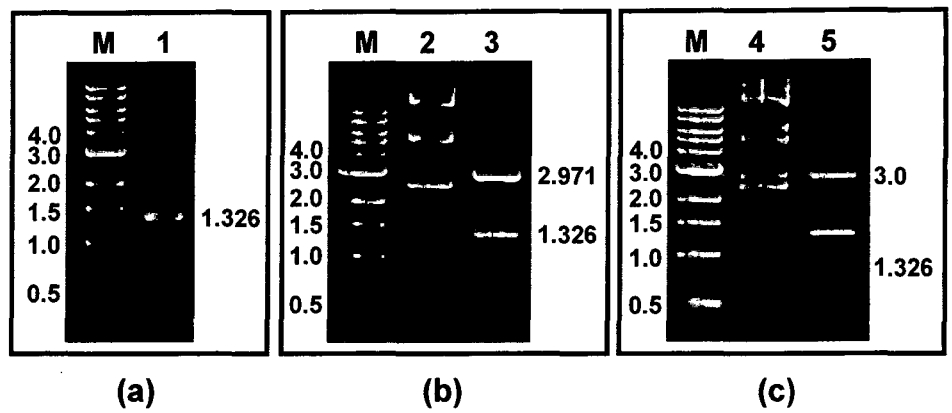


Figure 8: Cloning of hZP4 in *E. coli* expression vector. An internal fragment of hZP4 cDNA (64-1389 nt; 22-463 aa), excluding the SS and TD, was PCR amplified and finally cloned in pRSET-A expression vector as described in *Materials and Methods*. Panel (a) represents the 1.326 kb PCR amplified hZP4 cDNA as analyzed by agarose gel electrophoresis. Panel (b) represents the restriction pattern of hZP4 clone in pPCR-Script Amp SK(+) plasmid digested with *Sac I* and *Kpn I* restriction enzymes. Panel (c) represents the restriction profile of pRSET-A plasmid harboring hZP4 cDNA with the enzymes *Sac I* and *Kpn I*. Lanes are represented as M: 1 kb DNA ladder; lane 1: PCR amplified hZP4 cDNA fragment; lanes 2 and 3: undigested and digested hZP4-pPCR-Script Amp SK(+) clone plasmid DNA, respectively; lanes 4 and 5: undigested and digested hZP4-pRSET-A clone plasmid DNA, respectively. The molecular weights depicted are in kb.

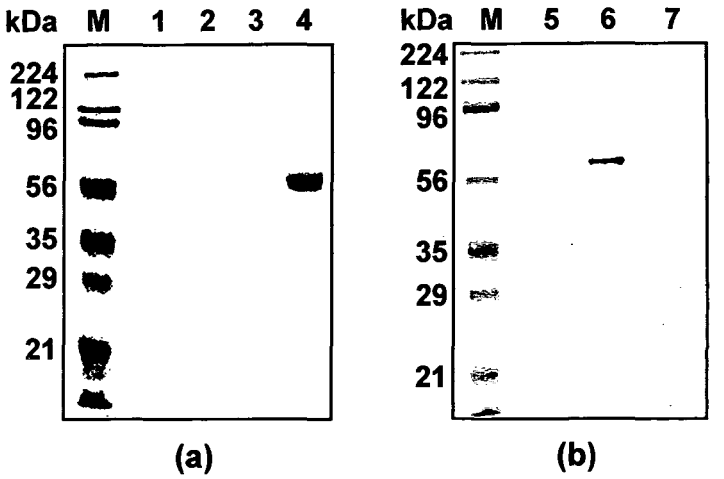


Figure 9: Immunoblot analysis of the expression and cellular localization of hZP4 in *E. coli.* BL21[DE3]pLysS cells transformed with hZP4 construct in pRSET-A were grown in LB medium and induced with 1mM IPTG for 2.5 h. Pellet of 1 ml of uninduced and induced culture was subjected to boiling (a) or fractionated into soluble, insoluble and periplasmic components followed by boiling (b) in 2X SDS-PAGE sample buffer, electrophoresed and processed for Western blot as described in *Materials and Methods*. The *E. coli*-expressed hZP4 was detected by using murine monoclonal antibody against bmZP4, MA-813 that cross-reacts with hZP4 as described in *Materials and Methods*. Various lanes are represented as M: Molecular weight markers; lanes 1 and 2: uninduced and induced wild type host cells respectively; lanes 3 and 4: uninduced and induced cells harboring hZP4-pRSET-A plasmid respectively; lanes 5-7: soluble, insoluble and periplasmic compartments respectively of host cells harboring hZP4-pRSET-A plasmid.

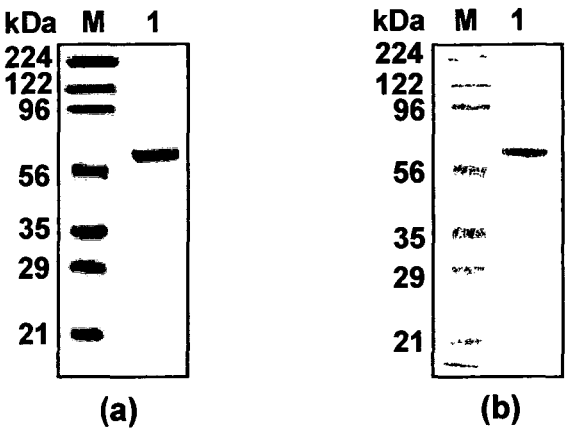


Figure 10: Electrophoretic and immunoblot analysis of purified recombinant hZP4 expressed in *E. coli*. The recombinant hZP4 was purified under denaturing conditions using Ni-NTA affinity column, renatured and subsequently resolved on 0.1% SDS-10% PAGE under reducing conditions and processed for Coomassie staining or immunoblotting as described in *Materials and Methods*. Panel (a) represents the Coomassie stained SDS-PAGE profile of *E. coli*-expressed purified recombinant hZP4 (lane 1, 5μg/lane) while panel (b) depicts the immunoblot profile (lane 1, 2 μg/lane). The recombinant hZP4 was revealed in the Western blot using antibodies as described in the legend to Figure 9. Lanes are represented as M: Molecular weight markers; lane 1: *E. coli*-expressed recombinant hZP4.

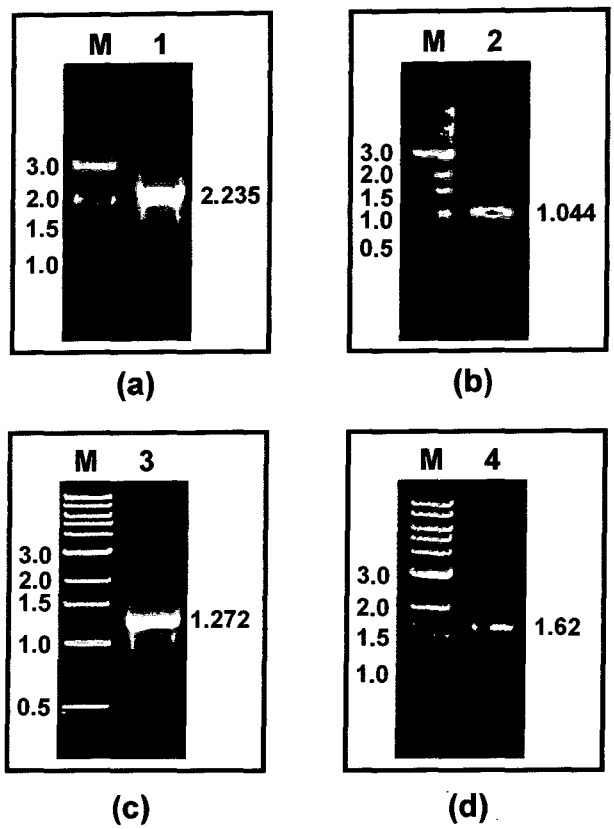


Figure 11: PCR amplification of the cDNAs corresponding to human zona proteins for cloning in baculovirus-expression system. The internal fragments of hZP2 cDNA (1-2235 nt; 1-745 aa; panel a), hZP3 cDNA (1-1272 nt, 1-424 aa; panel c), hZP4 cDNA (1-1620 nt, 1-540 aa; panel d), including the SS and TD and hZP3 cDNA (1-1044 nt, 1-348 aa; panel b), excluding the TD were PCR amplified from their respective parent clones as described in *Materials and Methods*. Lanes are represented as M: 1 kb DNA ladder; lanes 1-4: PCR amplified cDNA products of hZP2, $hZP3_{(1-348)}$, $hZP3_{(1-424)}$ and hZP4 respectively. The molecular weights depicted are in kb.

were performed to identify the positive clones for each of the four constructs. A representative positive clone for each of the constructs along with its restriction profile is shown in Figure 12, left panel. The insert purified from the recombinant cloning vector was subsequently cloned downstream to a polyhistidine tag under the control of late polyhedrin promoter in the baculovirus transfer vector, pAcHLT-A as mentioned in *Materials and Methods*. Positive clones for all the constructs, as identified by restriction analysis, are shown in Figure 12, right panel.

The recombinant proteins were expressed in *Sf21* insect cells following generation of recombinant baculoviruses for the individual ZP constructs as described in *Materials and Methods*. Immunoblot analysis of the total cell lysate from *Sf21* cells infected with the recombinant virus specific for hZP2, $hZP3_{(1-348)}$, $hZP3_{(1-424)}$ and hZP4 revealed single bands corresponding to ~105, ~55, ~65 and ~75 kDa respectively (Figure 13). The expression of all the four recombinant proteins was specific, as no expression was detected in *Sf21* cells infected with the wild type AcNPV virus (Figure 13). Analysis of the expression of the four recombinant proteins in baculovirus revealed that these are absent in the supernatant and are present only in the cell lysate of virus infected *Sf21* cells.

c) Time kinetics of expression for baculovirus-expressed recombinant human zona proteins

To investigate the optimum time required for the maximum expression of the individual recombinant human zona protein by *Sf21* cells, the *Sf21* cells were infected with the recombinant baculovirus for various time periods as described in *Materials and Methods*. The time kinetics experiments with the various constructs pertaining to the expression of recombinant proteins in *Sf21* cells revealed that the expression of $hZP3_{(1-424)}$ and hZP4 could be detected as early as 24 h pi while hZP2 and $hZP3_{(1-348)}$ showed detectable amounts of expression only at 48 h pi (Figure 14). The maximum expression of baculovirus-expressed hZP2 as well as $hZP3_{(1-424)}$ was observed at 84 h pi and a decrease in the amount of protein expressed was observed at 96 h pi in both the cases. On the other hand, $hZP3_{(1-348)}$ and hZP4 were maximally expressed only when the *Sf21* cells were incubated with the respective recombinant viruses for 96 h (Figure 14).

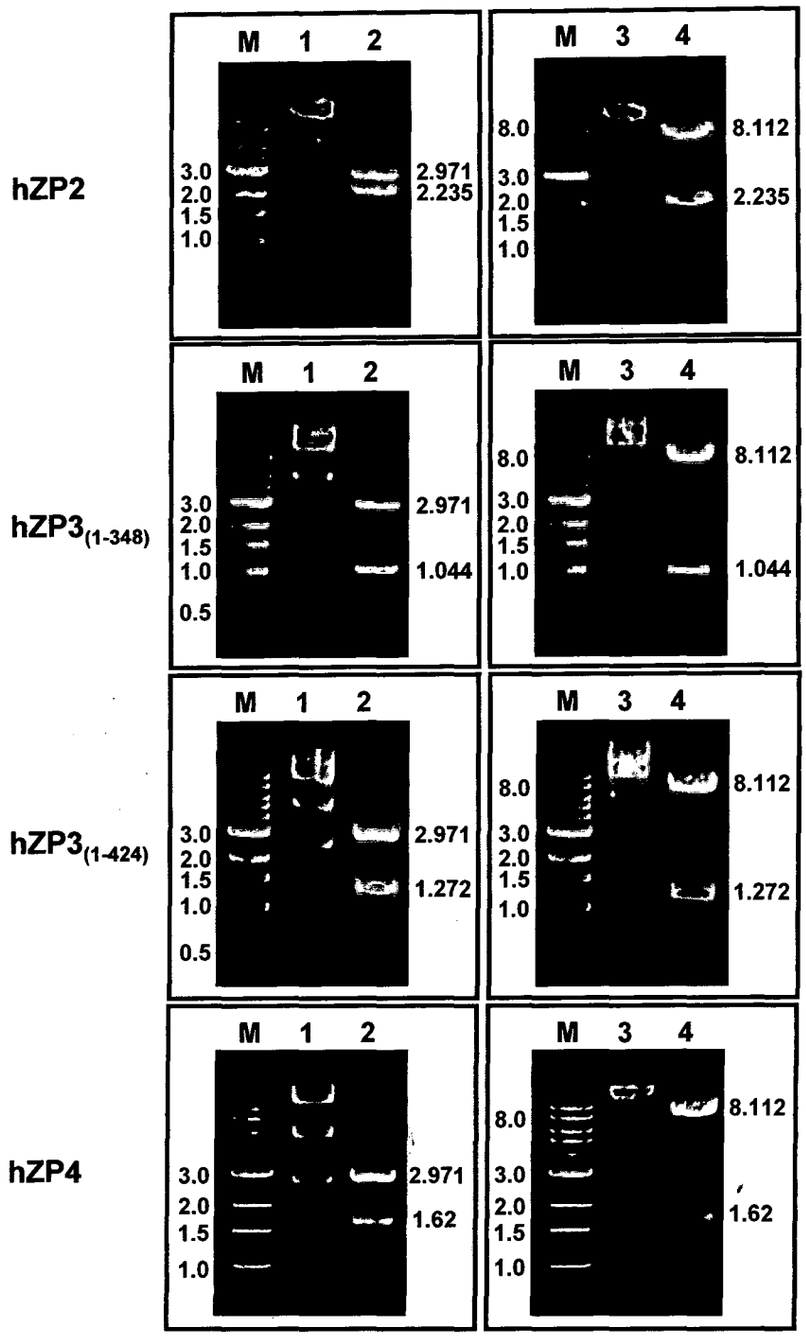


Figure 12: Cloning of cDNA encoding human zona proteins in baculovirus transfer vector. The PCR amplified cDNA products pertaining to hZP2, $hZP3_{(1-348)}$, $hZP3_{(1-424)}$ and hZP4 were independently cloned in pPCR-Script Amp SK(+) cloning vector and positive clones identified by restriction analysis as described in *Materials and Methods*. The insert purified from the positive clone from each construct was subsequently cloned in pAcHLT-A transfer vector as described in *Materials and Methods*. The left panel represents the restriction patterns of positive clones in pPCR-Script Amp SK(+) plasmid digested with the respective restriction enzymes. The right panel represents the restriction profile of pAcHLT-A plasmid harboring the respective cDNA with the respective enzymes. Lanes are represented as M: 1 kb DNA ladder; lanes 1 and 2: undigested and digested pPCR-Script Amp SK(+) clone plasmid DNA, respectively; lanes 3 and 4: undigested and digested pAcHLT-A clone plasmid DNA, respectively. The molecular weights depicted are in kb.

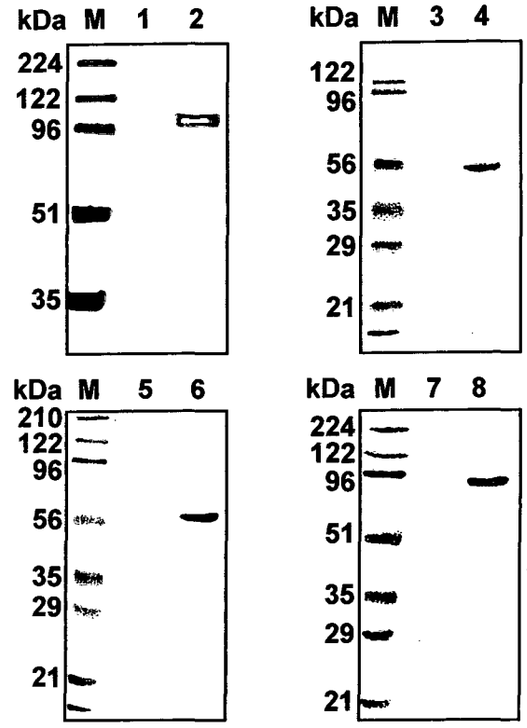


Figure 13: Western blot analysis of the expression of recombinant human ZP glycoproteins in baculovirus. Recombinant viruses from 6 randomly picked plaques corresponding to hZP2, $hZP3_{(1-348)}$, $hZP3_{(1-424)}$ and hZP4 were used to infect *Sf21* cells (0.8 X 10^6) for 96 h. As a negative control, *Sf21* cells were also infected with the wild type AcNPV virus. The infected cells were processed for Western blot as described in *Materials and Methods*. A representative immunoblot pattern of one of the positive plaques showing the expression of the respective recombinant protein is depicted. Lanes are represented as M: Molecular weight markers; lanes 1, 3, 5 and 7: *Sf21* cells infected with wild type AcNPV virus; lanes 2, 4, 6 and 8: *Sf21* cells infected with recombinant virus expressing hZP2, $hZP3_{(1-348)}$, $hZP3_{(1-424)}$ and hZP4 respectively.

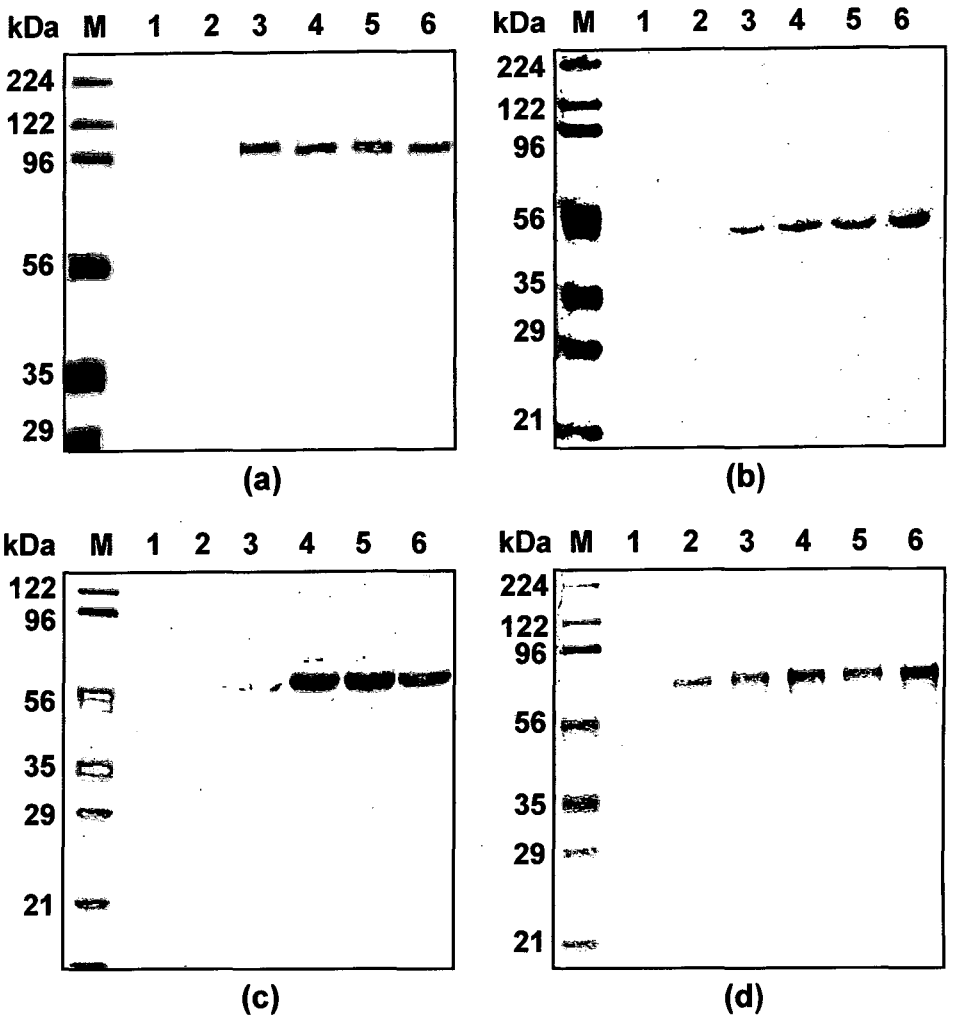


Figure 14: Time course analysis of the expression of recombinant human ZP glycoproteins in baculovirus expression system. *Sf21* cells were infected independently with recombinant virus harboring constructs encoding (a) hZP2, (b) $hZP3_{(1-348)}$, (c) $hZP3_{(1-424)}$ and (d) hZP4 at a MOI of 3.0. Post-infection, the infected *Sf21* cells were harvested at different time intervals and their respective pellets solubilized in 2X SDS-PAGE sample buffer, electrophoresed and processed for immunoblot as described in *Materials and Methods*. Lanes are represented as M: Molecular weight markers; lane 1: AcNPV infected *Sf21* cells harvested at 96 h pi; lanes 2-6: respective recombinant virus infected *Sf21* cells harvested at 24, 48, 72, 84 and 96 h pi respectively.

d) Purification of baculovirus-expressed recombinant human zona proteins and their renaturation

The recombinant baculovirus-expressed human ZP glycoproteins, hZP2, $hZP3_{(1-348)}$, $hZP3_{(1-424)}$ and hZP4 were purified under denaturing conditions using Ni-NTA resin and subsequently renatured as described in *Materials and Methods*. The Coomassie stained SDS-PAGE and immunoblot profiles of these proteins are shown in Figures 15 and 16. These analyses revealed that the purified baculovirus-expressed recombinant hZP2, $hZP3_{(1-348)}$, $hZP3_{(1-424)}$ and hZP4 migrated as single bands corresponding to ~105, ~55, ~65 and ~75 kDa respectively (Figure 15, 16). An average yield of ~250-500 μg of each of the purified baculovirus-expressed recombinant proteins was obtained from *Sf21* cells grown in one spinner flask (50 X 10^6 cells/flask) and transfected with the respective recombinant baculovirus.

e) Characterization of glycosylation of recombinant human zona proteins

Since the expression of the human zona proteins was undertaken in *E. coli* and baculovirus-expression systems to obtain them in non-glycosylated and glycosylated forms respectively, it was prudent to determine the carbohydrate moieties, if any, present in these recombinant proteins. With this in view, the *E. coli*- and baculovirus-expressed recombinant human zona proteins were analyzed by an *in vitro* lectin binding assay as well as by lectin blots as described in *Materials and Methods*. The baculovirus-expressed recombinant hZP2 exhibited strong reactivity with Concanavalin A (ConA) and Jacalin, and weak reactivity with *Pisum sativum* agglutinin (PSA), *Dolichos biflorus* agglutinin (DBA) and Soybean agglutinin (SBA; Figure 17). Both the baculovirus-expressed recombinant $hZP3_{(1-348)}$ and $hZP3_{(1-424)}$ showed identical profile of glycosylation and bound strongly to ConA and Jacalin, and weakly to Wheat germ agglutinin (WGA) and PSA (Figure 18a). The recombinant hZP4 expressed in baculovirus exhibited strong binding with ConA, Jacalin and weak binding with DBA and PSA (Figure 18b). No binding with any of the biotinylated lectins was observed with the *E. coli*-expressed recombinant hZP2, hZP3 and hZP4. While ConA has oligosaccharide specificity towards mannose α 1-3 or mannose α 1-6 residues, Jacalin binds to α-O glycosides of Gal or GalNac moieties. Oligosaccharide specificity of PSA is α-linked mannose containing oligosaccharides, with an N-acetylchitobiose-linked α-fucose residue included in the receptor sequence and SBA recognizes GalNAc residues in α or β-linkages. DBA binds to GalNAc residues in α-linkage and WGA has

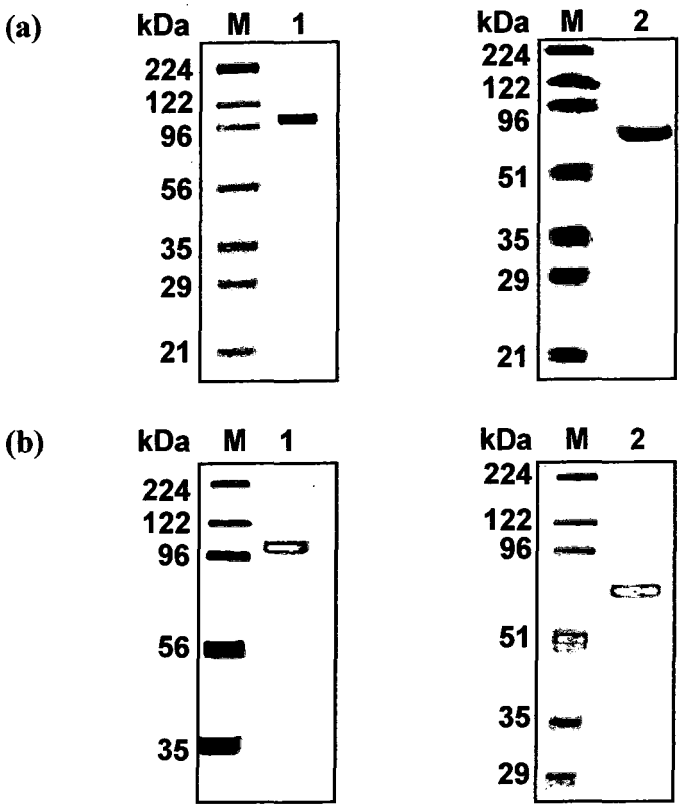


Figure 15: Electrophoretic and immunoblot analysis of purified baculovirus-expressed recombinant hZP2 and hZP4. The recombinant hZP2 and hZP4 expressed in baculovirus were purified under denaturing conditions using Ni-NTA affinity column, renatured and resolved on 0.1% SDS-10% PAGE under reducing conditions as described in *Materials and Methods*. Panel (a) represents the Coomassie stained SDS-PAGE profile of baculovirus-expressed recombinant hZP2 (lane 1, 5 μg/lane) and hZP4 (lane 2, 5 μg/lane) while panel (b) depicts the immunoblot profile of baculovirus-expressed recombinant hZP2 (lane 1, 2 μg/lane) and hZP4 (lane 2, 2 μg/lane) . Lane M represents molecular weight markers.

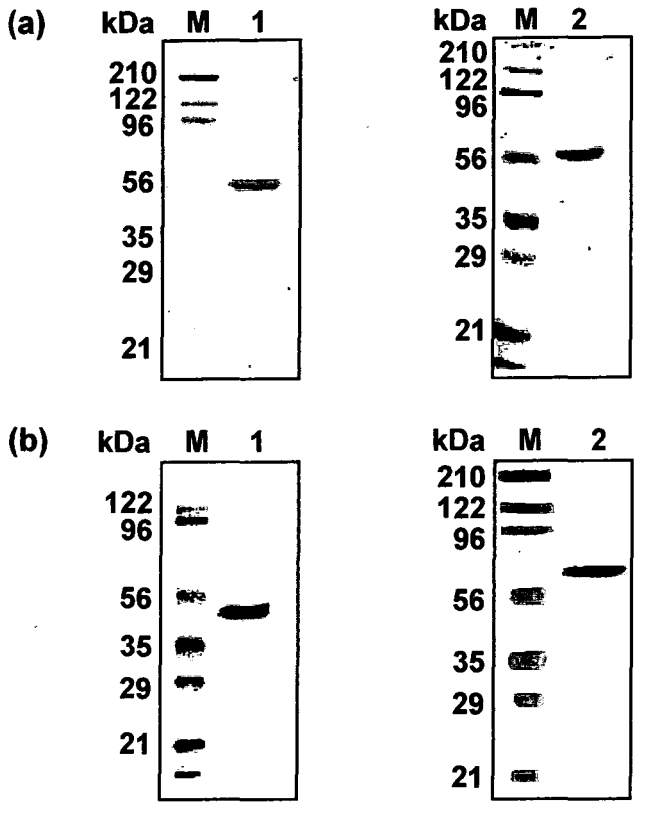


Figure 16: Electrophoretic and immunoblot analysis of purified baculovirus-expressed recombinant $hZP3_{(1-348)}$ and $hZP3_{(1-424)}$. The baculovirus-expressed recombinant $hZP3_{(1-348)}$ and $hZP3_{(1-424)}$ were purified under denaturing conditions using Ni-NTA affinity column, renatured and resolved on 0.1% SDS-10% PAGE under reducing conditions as described in *Materials and Methods*. Panel (a) represents the Coomassie stained SDS-PAGE profile of baculovirus-expressed recombinant $hZP3_{(1-348)}$ (lane 1, 5 μg/lane) and $hZP3_{(1-424)}$ (lane 2, 5 μg/lane) while panel (b) depicts the immunoblot profile of baculovirus-expressed recombinant $hZP3_{(1-348)}$ (lane 1, 5 μg/lane) and $hZP3_{(1-424)}$ (lane 2, 5 μg/lane). Lane M represents molecular weight markers.

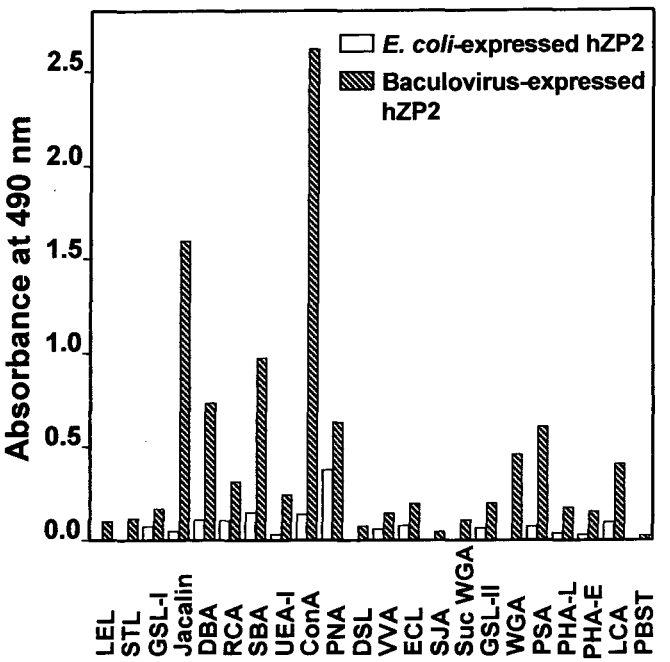


Figure 17: Profile of lectins binding to *E. coli*- and baculovirus-expressed human ZP2 in ELISA. Microtitration plates were coated with the baculovirus-expressed recombinant hZP2 (500 ng/well) and processed for evaluation of binding to 21 different biotinylated lectins in an ELISA as described in *Materials and Methods*. Values are expressed as absorbance obtained with various lectins binding to the respective recombinant protein, after deducting the non-specific binding of the lectins to the uncoated wells. Each bar represents a mean of duplicate experiments. The lectins tested were LEL: *Lycopersicon esculentum* lectin, STL: *Solanum tuberosum* lectin, GSL-I: *Griffonia simplicifolia* lectin I, Jacalin, DBA: *Dolichos biflorus* agglutinin, RCA: *Ricinus communis* agglutinin, SBA: Soybean agglutinin, UEA-I: *Ulex europaeus* agglutinin I, ConA: Concanavalin A, PNA: Peanut agglutinin, DSL: *Datura stramonium* lectin, VVA: *Vicia villosa* agglutinin, ECL: *Erythrina cristagalli* lectin, SJA: *Sophora japonica* agglutinin, Suc WGA: Succinylated Wheat germ agglutinin, GSL II: *Griffonia simplicifolia* lectin II, WGA: Wheat Germ agglutinin, PSA: *Pisum sativum* agglutinin, PHA-L: *Phaseolus vulgaris* leucoagglutinin, PHA-E: *Phaseolus vulgaris* erythroagglutinin, and LCA: *lens culinaris* agglutinin.

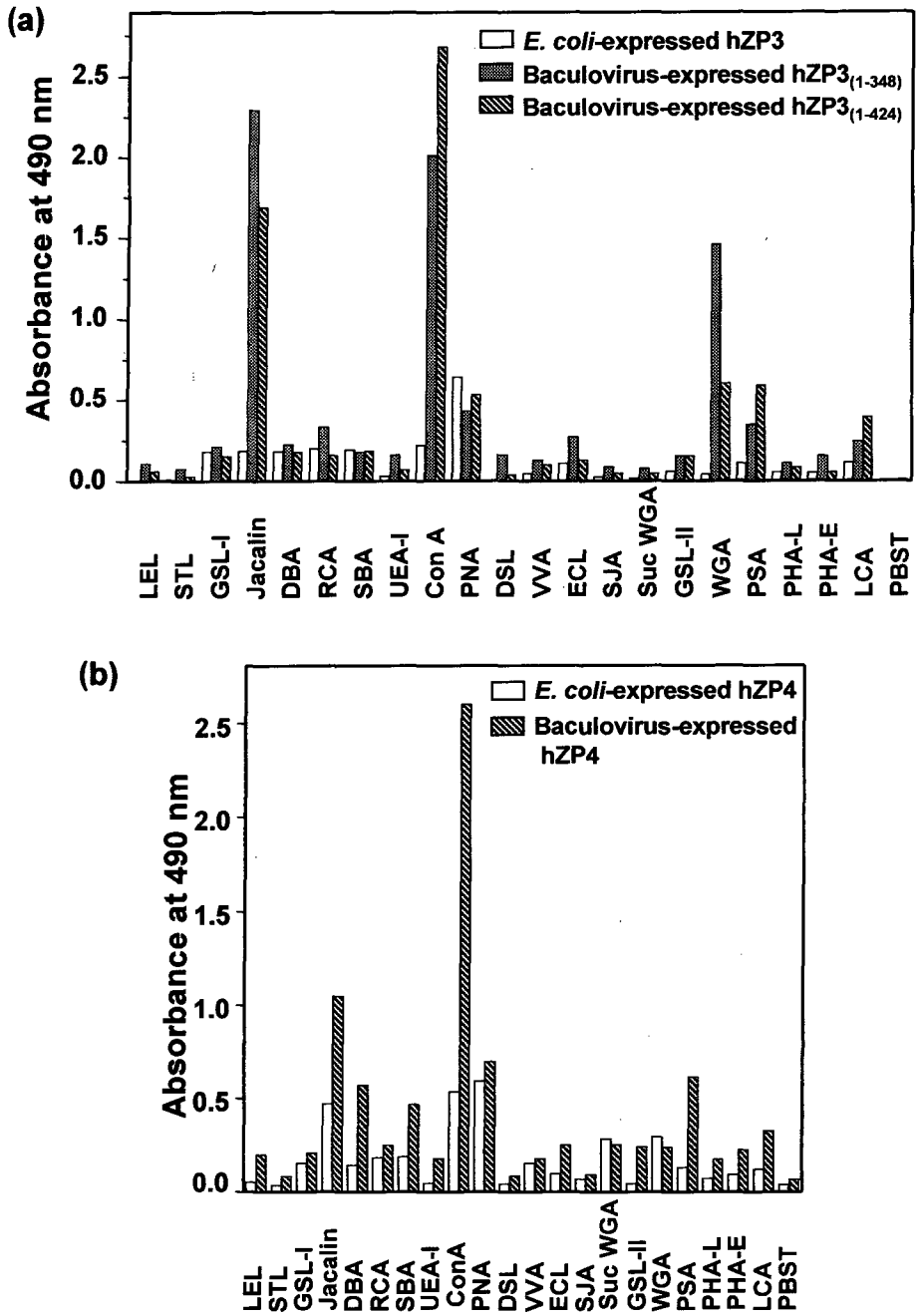


Figure 18: Profile of lectins binding to *E. coli*- and baculovirus-expressed human ZP3 and ZP4 in ELISA. Microtitration plates were coated with the respective recombinant protein (500 ng/well) and processed for evaluation of binding to 21 different biotinylated lectins in an ELISA as described in *Materials and Methods*. Values are expressed as absorbance obtained with various lectins binding to the respective recombinant protein, after deducting the non-specific binding of the lectins to the uncoated wells. Each bar represents a mean of duplicate experiments. The lectins tested are mentioned in legend to Figure 17.

oligosaccharide specificity towards GlcNAc and neuraminic acid residues. While ConA, PSA and WGA have oligosaccharide specificity towards N-linked sugar residues, Jacalin, DBA and SBA detect the presence of O-linked carbohydrate moieties. The lectin blots pertaining to the selected lectins exhibiting reactivity with the recombinant proteins reconfirmed the specificity of the above lectins to bind to the respective recombinant baculovirus-expressed proteins. Since ConA and Jacalin bound to all the baculovirus-expressed recombinant proteins, the representative lectin blots of the *E. coli* as well as baculovirus-expressed recombinant proteins with ConA and Jacalin are shown in Figure 19. These results confirm that the baculovirus-expressed recombinant human zona proteins obtained are glycosylated and have both N- and O-linked sugar residues while the corresponding *E. coli*-expressed recombinant proteins are non-glycosylated.

f) Conjugation recombinant human zona proteins with fluorochromes

To obtain a handle for direct detection of the recombinant human zona proteins in the *in vitro* sperm binding assays (described later), the Ni-NTA affinity purified *E. coli*- and baculovirus-expressed recombinant zona proteins were conjugated to FITC and/or RBITC as described in *Materials and Methods*. The molar F/P ratios of the FITC conjugated recombinant proteins were found to be 0.97 and 1.24 for *E. coli*- and baculovirus-expressed hZP2 respectively; 1.09 for *E. coli*-expressed hZP3, 1.60 for baculovirus-expressed $hZP3_{(1-424)}$; 1.22 and 1.06 for *E. coli*- and baculovirus-expressed hZP4 respectively. The RBITC labeled baculovirus-expressed $hZP3_{(1-424)}$ and hZP4 were found to have molar F/P ratios of 1.32 and 0.99 respectively. The profile of the FITC and RBITC labeled human ZP glycoproteins expressed in baculovirus as analyzed on SDS-PAGE is shown in Figure 20.

g) Expression of hZP3 in mammalian expression system

The carbohydrate moieties of the ZP glycoproteins have been suggested to play an important role in species-specific sperm-egg recognition as well as their ability to induce acrosomal exocytosis in different species including humans (Florman *et al.*, 1984; Florman and Wassarman, 1985; Mori *et al.*, 1989; Yurewicz *et al.*, 1991; Nakano *et al.*, 1996; Miranda *et al.*, 1997; Oehninger *et al.*, 1998; Gahlay and Gupta, 2003). Owing to the differential glycosylation of the recombinant proteins expressed in insect cells and mammalian cells (Yonezawa *et al.*, 2005), it was prudent to express these proteins in mammalian expression system. The cDNA encoding hZP3 (1-1272 nt; 1-

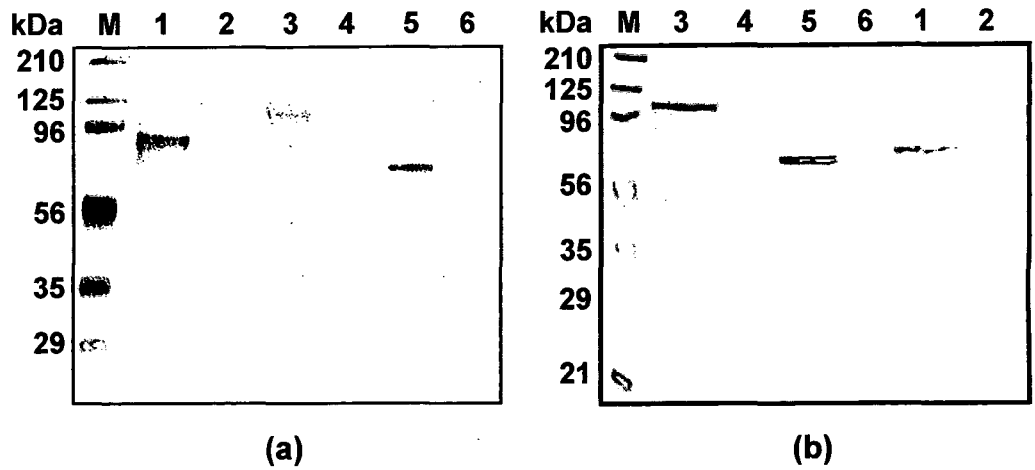


Figure 19: Immunoblot profile of lectins binding to *E. coli* and baculovirus-expressed human zona proteins. The recombinant proteins (1 μg/lane) were resolved on SDS-PAGE and processed for immunoblotting with 20 μg/ml each of (a) ConA and (b) Jacalin as described in *Materials and Methods*. Lanes are represented as M: Molecular weight markers; lanes 1, 3 and 5 represent baculovirus-expressed hZP2, $hZP3_{(1-424)}$ and hZP4 respectively; lanes 2, 4 and 6 represent *E. coli*-expressed hZP2, hZP3 and hZP4 respectively.

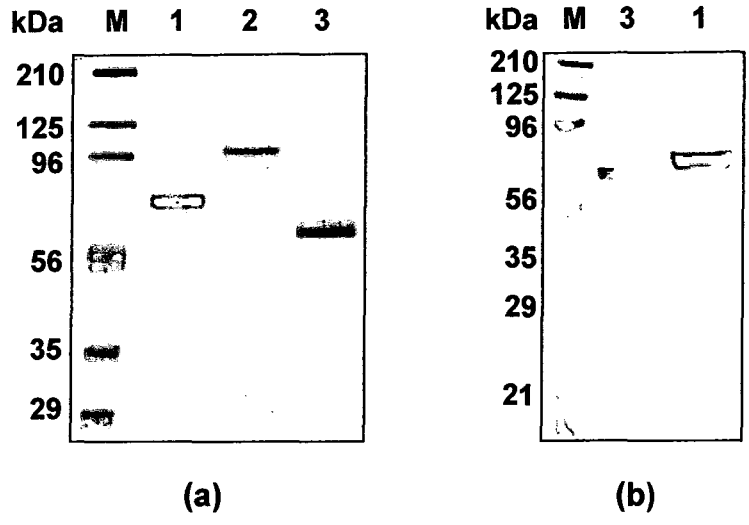


Figure 20: Analysis of purified recombinant human zona proteins conjugated to FITC/RBITC by SDS-PAGE. The purified baculovirus-expressed recombinant hZP2, $hZP3_{(1-424)}$ and hZP4 were conjugated to FITC (panel a)/RBITC (panel b) as described in *Materials and Methods* and resolved by 0.1% SDS-10% PAGE under reducing conditions and analyzed by Coomassie blue staining. Lanes are represented as M: Molecular weight markers; lanes 1-3: FITC/RBITC conjugated baculovirus-expressed recombinant hZP4, hZP2 and $hZP3_{(1-424)}$ respectively (2 μg/lane) .

424 aa) was PCR amplified with *BamH I* restriction site in the forward primer and *EcoR I* restriction site in the reverse primer (Figure 21a). The PCR amplified cDNA fragment was purified, cloned in pPCR-Script Amp SK(+) cloning vector followed by final cloning in the pcDNA6/V5-His-B vector under CMV promoter. The positive clone was confirmed by restriction digestion (Figure 21) and nt sequencing. The pcDNA-hZP3 plasmid DNA was purified by alkaline lysis using plasmid DNA purification kit (QIAGEN) and used to study the expression of hZP3 in a transient *in vitro* expression system employing CHO-K1 cells as described in *Materials and Methods*. To standardize the optimum transfection conditions for the expression of hZP3, CHO-K1 cells, in a 24-well tissue culture plate, were transfected with 0.5-16 μg of pcDNA-hZP3 plasmid DNA (plasmid DNA : Lipofectamine 2000 :: 1:2). Transfected cells were processed for indirect immunofluorescence employing murine MAb, MA-1552 generated against baculovirus-expressed $hZP3_{(1-424)}$ as described in *Materials and Methods*. Maximum expression of hZP3 was observed at 2 μg DNA concentration (~30% transfection efficiency; Figure 22; Table 6). Further, to optimize the Lipofectamine 2000 concentration for transfection, pcDNA-hZP3 plasmid DNA at 2 μg was used with varying amounts of Lipofectamine 2000 (1-8 μg) and it was observed that a ratio of DNA : Lipofectamine 2000 :: 1:2 gave the best transfection efficiency (Table 7). The expression of hZP3 was also assessed in the cellular lysates of CHO-K1 cells transiently transfected with pcDNA-hZP3 by Western blot using murine monoclonal antibody, MA-1552 against baculovirus-expressed $hZP3_{(1-424)}$ (made available by Mr. Pankaj Bansal, Gamete Antigen Laboratory, National Institute of Immunology, New Delhi) as described in *Materials and Methods*. The recombinant hZP3 expressed in CHO-K1 cells migrated as a doublet with molecular weight between ~60-70 kDa (Figure 23). The untransfected CHO-K1 cells did not show the expression of the recombinant protein.

II. GENERATION AND CHARACTERIZATION OF MURINE MONOCLONAL ANTIBODIES (MAbs) AGAINST BACULOVIRUS-EXPRESSED hZP4

In order to investigate the functional characterization of human ZP glycoproteins, in particular, hZP4, it was prudent to use highly specific MAbs developed against hZP4. Hence, attempts were made to generate MAbs against baculovirus-expressed recombinant hZP4. A panel of 10 MAbs (MA-1650, -1654, -1657, -1660, -1662, -1665,

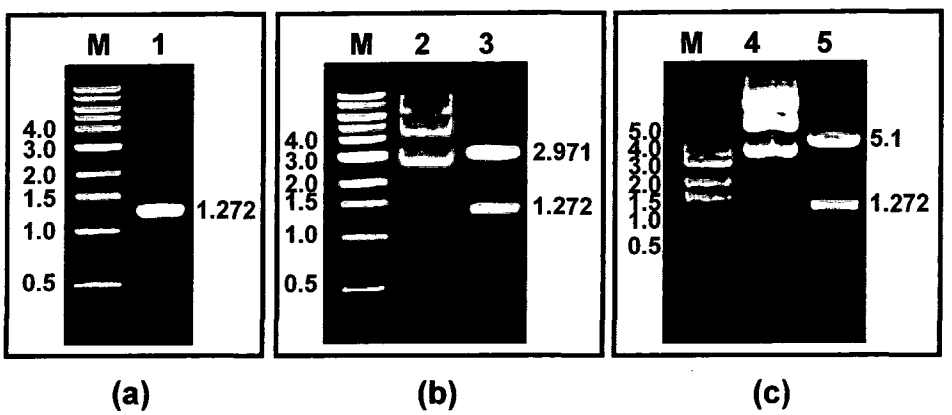


Figure 21: Cloning of hZP3 in mammalian expression vector. An internal fragment of hZP3 cDNA (1-1272 nt; 1-424 aa), excluding the SS and TD, was PCR amplified and finally cloned in pcDNA6/V5-His-B expression vector as described in *Materials and Methods*. Panel (a) represents the 1.272 kb PCR amplified hZP3 cDNA as analyzed by agarose gel electrophoresis. Panel (b) represents the restriction pattern of hZP3 clone in pPCR-Script Amp SK(+) plasmid digested with *BamH I* and *EcoR I* restriction enzymes. Panel (c) represents the restriction profile of pcDNA6/V5-His-B plasmid harboring hZP3 cDNA with the enzymes *EcoR I* and *BamH I*. Lanes are represented as M: 1 kb DNA ladder; lane 1: PCR amplified hZP3 cDNA fragment; lanes 2 and 3: undigested and digested hZP3-pPCR-Script Amp SK(+) clone plasmid DNA, respectively; lanes 4 and 5: undigested and digested pcDNA-hZP3 clone plasmid DNA, respectively. The molecular weights depicted are in kb.

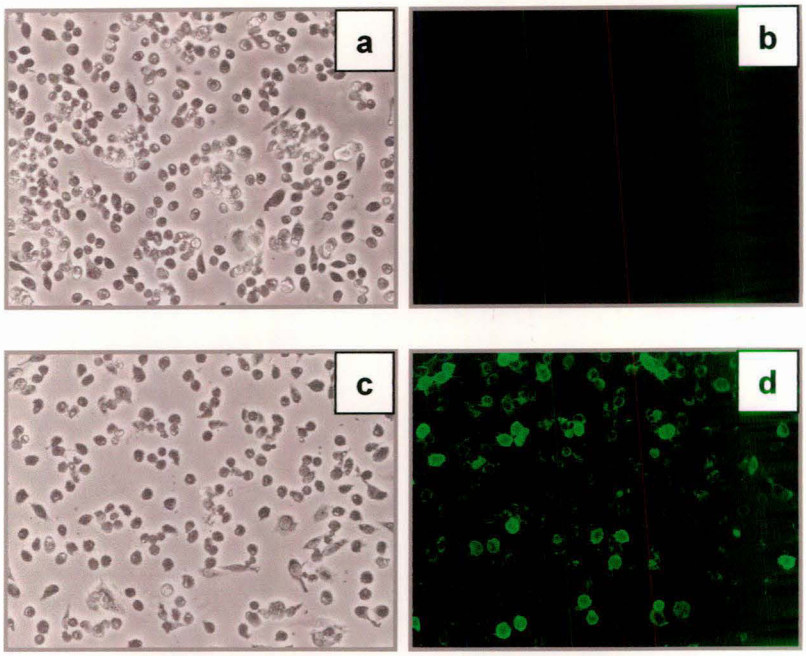


Figure 22: Expression of recombinant hZP3 in CHO-K1 cells. In a 24-well tissue culture plate, CHO-K1 cells (5 X 10^4/well) were transfected with pcDNA-hZP3 plasmid DNA as described in *Materials and Methods*. After 48 h, transfected cells were processed for detection of expression of hZP3 in an indirect immunofluorescence assay using either preimmune rabbit serum (a and b) or rabbit polyclonal antibodies against $hZP3_{(1-424)}$ (c and d) as described in *Materials and Methods*. The left panel represents the phase contrast image of CHO-K1 cells while the corresponding right panel shows the fluorescent frame of the same (X 200).

Table 6: Optimization of pcDNA-hZP3 plasmid DNA concentration for *in vitro* transient expression of hZP3 in CHO-K1 cells

Amount of DNA (μg)*	Amount of Lipofectamine 2000 (μg)	Expression levels of hZP3
0.5	1.0	+
1.0	2.0	+++
2.0	4.0	++++
4.0	8.0	++
8.0	16.0	+
16.0	32.0	Cell death

*The amount of plasmid DNA used to transfect 5 X 10^4 cells/well in a 24-well tissue culture plate

(+) positive for expression; number of '+' indicates degree of expression

Table 7: Optimization of ratio between concentration of pcDNA-hZP3 plasmid DNA and Lipofectamine 2000 for *in vitro* transient expression of hZP3 in CHO-K1 cells

Amount of DNA (μg)*	Amount of Lipofectamine 2000 (μg)	Expression levels of hZP3
2.0	1.0	+
2.0	2.0	+
2.0	4.0	++++
2.0	8.0	+

*The amount of plasmid DNA used to transfect 5 X 10^4 cells/well in a 24-well tissue culture plate

(+) positive for expression; number of '+' indicates degree of expression

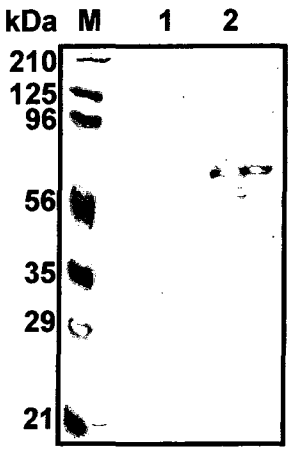


Figure 23: Immunoblot of expression of recombinant hZP3 in CHO-K1 cells. CHO-K1 cells (2 X 10^6), transiently transfected with pcDNA-hZP3 were lysed by consecutive freeze-thaw cycles as described in *Materials and Methods*. The cell lysate obtained was resolved on SDS-PAGE and processed for Western blot to study the expression of recombinant hZP3 as described in *Materials and Methods*. The hZP3 expression was revealed by using murine monoclonal antibody, MA-1552 against baculovirus-expressed $hZP3_{(1-424)}$. Lanes are represented as M: Molecular weight markers; lanes 1 and 2: cell lysates corresponding to untransfected and pcDNA-hZP3 transfected CHO-K1 cells respectively.

-1667, -1671, -1673 and -1677) was raised against baculovirus-expressed recombinant hZP4 (Table 8). All the 10 MAbs showed strong reactivity with the recombinant hZP4 in an ELISA. The isotype analysis revealed 7 MAbs to be of IgG1, and one each of IgG2a, IgG2b and IgM isotypes (Table 8). Further, immunoreactivity analysis by ELISA against baculovirus-expressed recombinant hZP2, $hZP3_{(1-424)}$ and hZP4 revealed that all the MAbs recognized specifically baculovirus-expressed hZP4 and did not cross-react with the other zona proteins (Figure 24a). The specificity of these MAbs was further characterized in Western blot where all the MAbs were found to specifically recognize baculovirus-expressed hZP4. Representative immunoblots pertaining to MA-1660 and MA-1671 are shown in Figure 24b. One of the above two MAbs, MA-1660 was also evaluated for its ability to recognize native zona. Due to non-availability of human oocytes, it was investigated by employing bonnet monkey ovarian cryosections. The rationale for using bonnet monkey ZP was that bmZP4 has 92% sequence identity with hZP4 at aa level. MA-1660 exhibited intense fluorescence with the native zona but did not react with any other ovarian cell types (Figure 25). The culture supernatant obtained by growing SP2/O myeloma cells failed to show any fluorescence with native ZP suggesting the specific reactivity of MA-1660 to native ZP.

III. BINDING CHARACTERISTICS OF RECOMBINANT HUMAN ZONA PROTEINS WITH HUMAN SPERM

Various studies have demonstrated that in mouse, ZP3 is responsible for the initial binding of the sperm to the oocyte and induction of acrosomal exocytosis, thereby assigning it the primary sperm receptor function (Bleil and Wassarman, 1980a; 1983; Mortillo and Wassarman, 1991; Beebe *et al.*, 1992). The binding sites of the zona proteins on the sperm have been studied in great detail in the murine and porcine models (Bleil and Wassarman, 1986; Mortillo and Wassarman, 1991; Thaler and Cardullo, 1996; Burkin and Miller, 2000; Kerr *et al.*, 2002). In mouse, it has been shown that about 60% of the ZP3 binding sites are present on the acrosomal cap region whereas 40% sites reside in the post-acrosomal region (Bleil and Wassarman, 1986; Mortillo and Wassarman, 1991). However, recent evidence suggests that only 20% of the ZP3 binding sites are located on the acrosomal cap and the remaining sites are present on the post-acrosomal region (Kerr *et al.*, 2002). The presence of both high and

Table 8: Characterization of murine monoclonal antibodies generated against recombinant baculovirus-expressed hZP4

Monoclonal antibody	Reactivity in ELISA with recombinant hZP4* (Absorbance at 490 nm)	Antibody isotype
MA-1650	1.42	IgM
MA-1654	1.40	IgG1
MA-1657	1.40	IgG1
MA-1660	1.51	IgG2b
MA-1662	1.48	IgG2a
MA-1665	1.49	IgG1
MA-1667	1.40	IgG1
MA-1671	1.48	IgG1
MA-1673	1.53	IgG1
MA-1677	1.34	IgG1

*Culture supernatant used at a dilution of 1:5

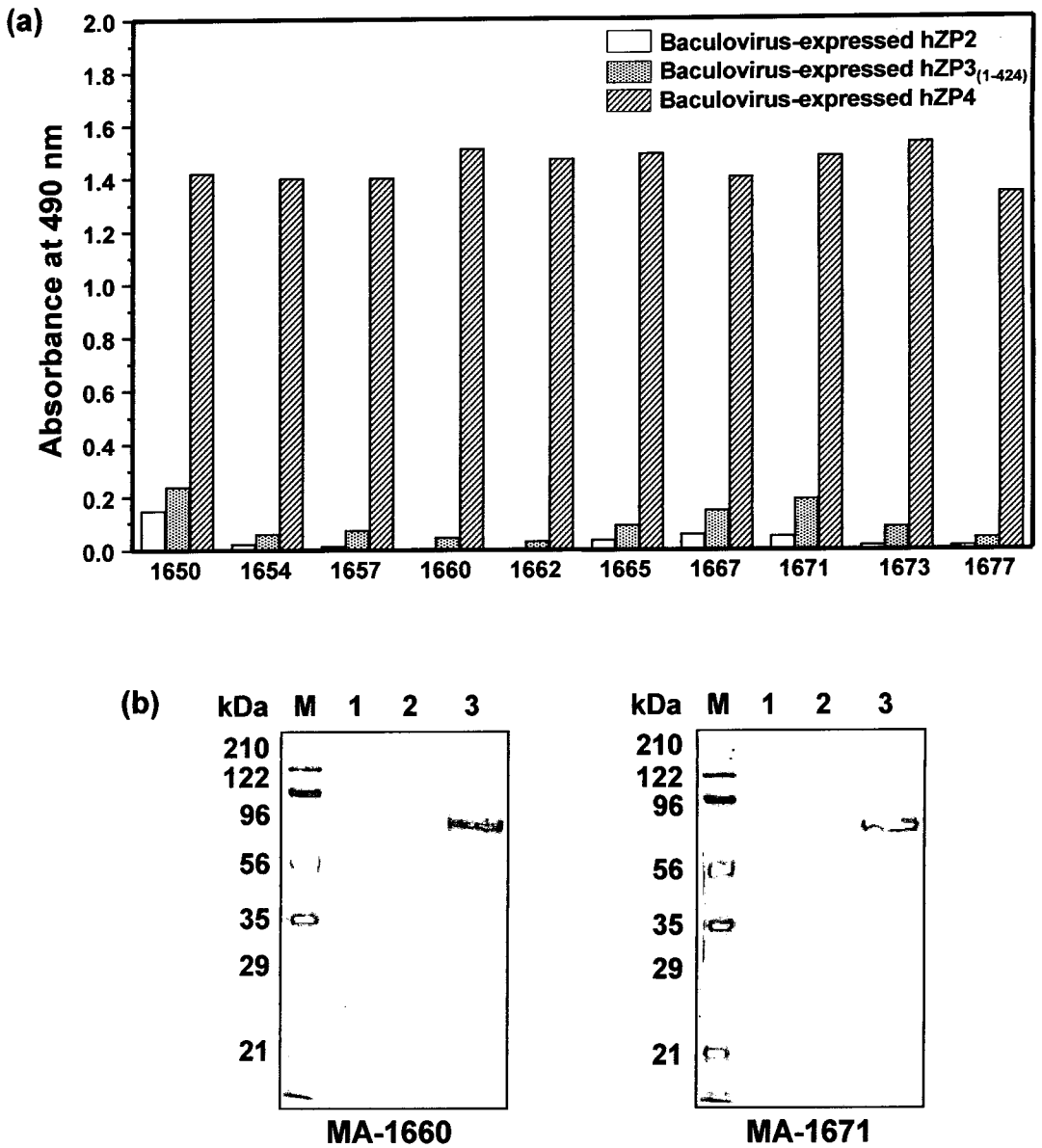
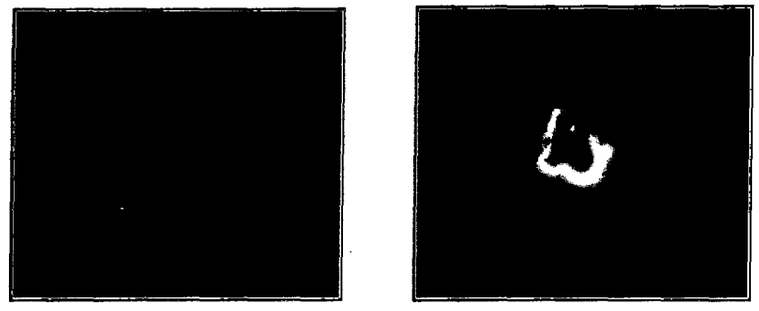


Figure 24: Specificity of monoclonal antibodies generated against baculovirus-expressed recombinant hZP4 in (a) ELISA and (b) Western blot. (a) Microtitration plates were coated with 200 ng/well of the recombinant human zona proteins and processed for evaluation of binding of various MAbs as described in *Materials and Methods*. In the present experiment, neat culture supernatants of the respective MAbs were used. Each bar represents a mean of duplicate experiments. (b) Respective baculovirus-expressed recombinant human zona proteins (2 μg/lane) were resolved on SDS-PAGE followed by processing for immunoblot with 1:5 dilution of culture supernatant of the hybridoma cell clones as described in *Materials and Methods*. Lanes are represented as M: Molecular weight markers; lanes 1-3: baculovirus-expressed recombinant hZP2, $hZP3_{(1-424)}$ and hZP4 respectively.



(a) (b)

Figure 25: Reactivity of murine monoclonal antibody generated against baculovirus-expressed recombinant hZP4 with bonnet monkey ZP by indirect immunofluorescence. Bonnet monkey ovarian cryosections were incubated with 1:5 dilution of the culture supernatants obtained by growing either SP2/O myeloma cells or MA-1660 hybridoma cell clone and processed for indirect immunofluorescence as described in *Materials and Methods*. Representative immunofluoresence patterns are shown as (a) SP2/O culture supernatant, (b) MA-1660 (X 100).

low affinity ZP binding sites on the sperm surface has also been documented in mouse (Thaler and Cardullo, 1996). In humans, the information regarding the localization of the binding sites of zona glycoproteins on the sperm as well as the kinetics of binding is scanty. To seek information pertaining to some of these unanswered questions, the purified recombinant human zona proteins expressed in *E. coli* and baculovirus as well as the cell lysate of CHO-K1 cells transfected with pcDNA-hZP3 plasmid were assessed for their binding characteristics with human spermatozoa in a direct and/or indirect binding assay as described in *Materials and Methods*.

a) Binding studies with *E. coli*-expressed recombinant human zona proteins

The FITC conjugated recombinant proteins were used in the direct binding assay whereas unlabeled purified proteins were employed for indirect binding assay. The acrosomal status of the spermatozoa showing binding to the recombinant proteins was simultaneously assessed using PSA-TRITC staining as described in *Materials and Methods*. The results obtained for both the direct as well as indirect binding assays were identical. For ease of interpretation and to avoid duplication, data pertaining to only direct binding assay is presented below. Binding studies revealed that 16.08 ± 2.75% (Mean ± SEM) of capacitated sperm exhibited binding of *E. coli*-expressed hZP3 (Table 9). Two distinct binding patterns were observed (Table 9, Figure 26). Binding to the acrosomal cap was observed in 32.32% of sperm among those showing binding of *E. coli*-expressed hZP3, whereas 67.68% sperm showed binding of recombinant hZP3 to the equatorial region (Table 9, Figure 26A, B). On the other hand, acrosome-reacted sperm (by calcium ionophore) did not show any binding of *E. coli*-expressed hZP3 to the acrosomal cap. However, 25.65 ± 2.55% acrosome-reacted sperm showed the binding of recombinant hZP3 on the equatorial region (Table 9, Figure 26C). Interestingly, 14.96 ± 1.37% capacitated sperm also exhibited the binding of *E. coli*-expressed recombinant hZP4 (Table 9, Figure 26). In contrast to recombinant hZP3, the major binding profile of recombinant hZP4, under similar experimental conditions, was observed on the acrosomal cap (79.50% of those positive for binding of recombinant hZP4; Table 9, Figure 26D). Only 20.50% sperm showed binding of *E. coli*-expressed hZP4 to the equatorial region (Table 9, Figure 26E). Acrosome-reacted sperm did not show any binding of recombinant hZP4 to the acrosomal cap and only 15.15 ± 1.90% sperm showed binding of recombinant hZP4 to the equatorial region (Table 9, Figure 26F). The *E. coli*-expressed recombinant hZP2 failed to bind to

Table 9: Binding characteristics of *E. coli*-expressed recombinant human zona proteins with human spermatozoa in direct binding assay

Recombinant protein	Percent binding to spermatozoa	Binding patterns	
		Acrosomal cap	Equatorial region
Capacitated (acrosome-intact) sperm			
hZP2	0.00		
hZP3	16.08 ± 2.75	32.32%	67.68%
hZP4	14.96 ± 1.37	79.50%	20.50%
Acrosome-reacted sperm (calcium ionophore mediated)			
hZP2	18.75 ± 1.45	0.00%	100.00%
hZP3	25.65 ± 2.55	0.00%	100.00%
hZP4	15.15 ± 1.90	0.00%	100.00%

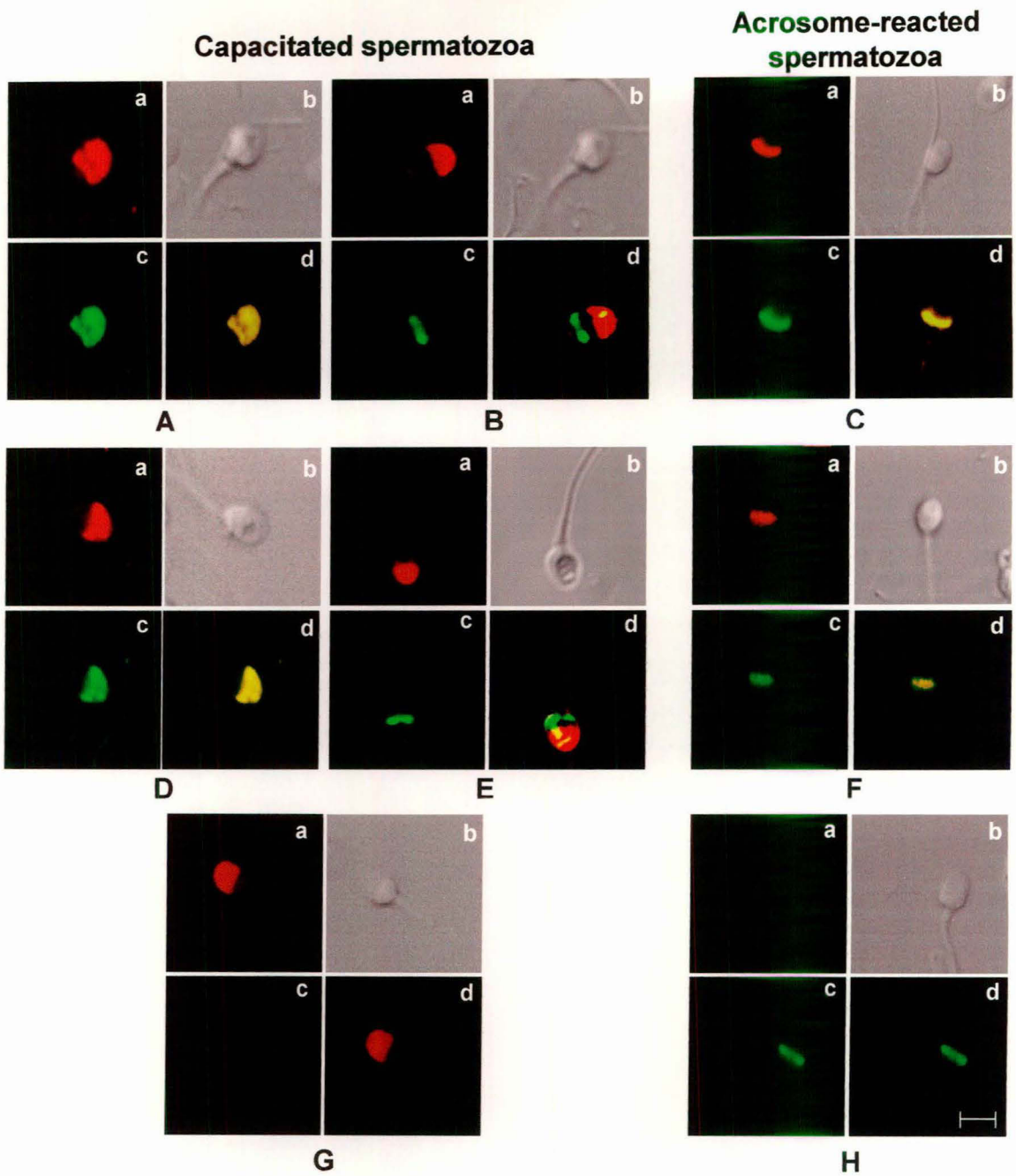


Figure 26: Binding profile of *E. coli*-expressed recombinant human zona proteins with human spermatozoa. Capacitated (left panel) or acrosome-reacted sperm (right panel; 5 X 10^6/50 µl) were incubated with 2.5 µg of FITC conjugated *E. coli*-expressed recombinant hZP2 (G and H), hZP3 (A-C) or hZP4 (D-F) and processed as described in *Materials and Methods*. The acrosomal status was determined by labeling the sperm with TRITC-PSA. The images were captured using Eclipse 80*i* fluorescence microscope (Nikon). In each panel, the sub-panels are represented as a: PSA-TRITC fluorescence; b: phase contrast; c: FITC-ZP protein fluorescence; and d: overlap of fluorescent frames. The scale bar represents 2.5 µm.

capacitated sperm but showed binding to the equatorial region in 18.75 ± 1.45% of the acrosome-reacted sperm (Figure 26G, H).

b) Evaluation of binding characteristics of baculovirus-expressed recombinant human ZP glycoproteins with spermatozoa

The results obtained with respect to the binding of recombinant baculovirus-expressed human ZP glycoproteins were similar to those observed with the corresponding *E. coli*-expressed proteins. The percentage of spermatozoa showing the binding as well as the distribution of binding patterns of baculovirus-expressed FITC conjugated hZP2, $hZP3_{(1-424)}$ and hZP4 are presented in Table 10. The binding profiles of the above proteins with capacitated and acrosome-reacted spermatozoa as studied by the direct binding assay are shown in Figure 27. Since, calcium ionophore, a chemical agonist for acrosome reaction was used to obtain acrosome-reacted spermatozoa to study the binding characteristics of zona proteins, the results were reconfirmed using spermatozoa that were induced to undergo acrosome reaction with recombinant $hZP3_{(1-424)}$, the physiological agonist, and then the binding characteristics analyzed as described in *Materials and Methods*. The binding profile of baculovirus-expressed recombinant hZP2 and hZP4 to $hZP3_{(1-424)}$ induced acrosome-reacted spermatozoa was similar to that observed with calcium ionophore mediated acrosome-reacted sperm (Table 10). Since baculovirus-expressed $hZP3_{(1-424)}$ and hZP4 exhibited a similar binding profile to capacitated spermatozoa, $hZP3_{(1-424)}$ and hZP4 labeled with FITC and RBITC respectively or vice versa were co-incubated with capacitated sperm to observe whether the two proteins can bind simultaneously to capacitated sperm. Interestingly, both the RBITC labeled hZP4 and FITC conjugated $hZP3_{(1-424)}$ showed simultaneous binding to the acrosomal cap of capacitated sperm (Figure 28, left panel). A similar pattern was observed when the FITC labeled hZP4 and RBITC conjugated $hZP3_{(1-424)}$ were used to analyze their binding to capacitated spermatozoa (Figure 28, right panel).

c) Specificity of binding of recombinant human ZP proteins to spermatozoa

The specificity of the binding of recombinant human zona proteins expressed in *E. coli* and baculovirus to spermatozoa was investigated in a competitive displacement binding assay using 5 and 10 fold molar excess of unlabeled recombinant human zona proteins in presence of FITC conjugated recombinant human zona proteins as described in *Materials and Methods*. The binding of FITC conjugated baculovirus-expressed $hZP3_{(1-424)}$ to capacitated spermatozoa was reduced from 20.0% to 3.5 and 1.6% when co-

Table 10: Binding characteristics of baculovirus-expressed recombinant human zona proteins with human spermatozoa in direct binding assay

Recombinant protein	Percent binding to spermatozoa	Binding patterns	
		Acrosomal cap	Equatorial region
Capacitated (acrosome-intact) sperm			
hZP2	0.00		
$hZP3_{(1-424)}$	19.08 ± 2.05	36.10%	63.90%
hZP4	17.95 ± 2.37	87.90%	12.10%
Acrosome-reacted sperm (calcium ionophore mediated)			
hZP2	18.75 ± 1.45	0.00%	100.00%
$hZP3_{(1-424)}$	25.65 ± 2.55	0.00%	100.00%
hZP4	15.15 ± 1.90	0.00%	100.00%
Acrosome-reacted sperm ($hZP3_{(1-424)}$ mediated)			
hZP2	18.50 ± 2.75	0.00%	100.00%
hZP4	14.85 ± 1.60	0.00%	100.00%

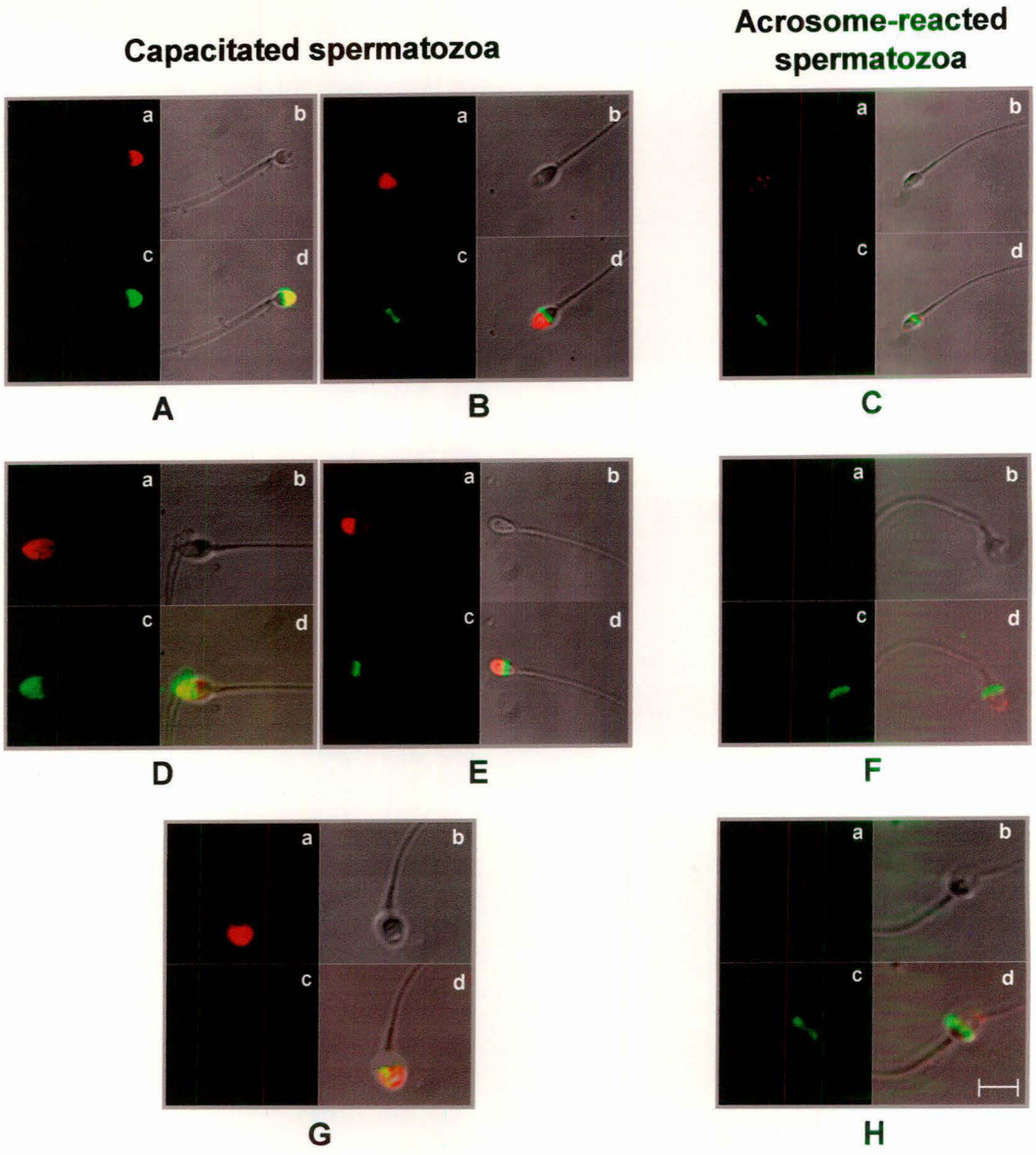


Figure 27: Profile of binding of baculovirus-expressed recombinant human zona proteins to human spermatozoa. Capacitated (left panel) or acrosome-reacted sperm (right panel; 5 X 10^6/50 µl) were incubated with 2.5 µg of FITC conjugated baculovirus-expressed recombinant hZP2 (G and H), $hZP3_{(1-424)}$ (A-C) or hZP4 (D-F) and processed as described in *Materials and Methods*. The acrosomal status was determined by labeling the sperm with TRITC-PSA. The images were captured using LSM 510 confocal microscope (Zeiss). In each panel, the sub-panels are represented as a: PSA-TRITC fluorescence; b: phase contrast; c: FITC-ZP protein fluorescence; and d: overlap of phase contrast and fluorescent frames. The scale bar represents 5 µm.

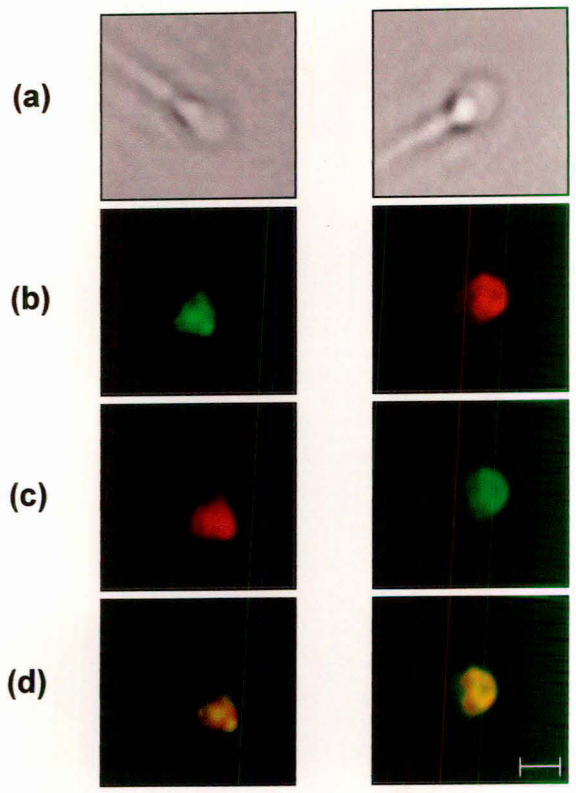


Figure 28: Co-localization of the binding of baculovirus-expressed recombinant hZP3$_{(1-424)}$ and hZP4 to capacitated sperm. Capacitated human sperm (5 X 10^6/50 µl) were co-incubated with FITC labeled baculovirus-expressed hZP3$_{(1-424)}$/hZP4 and RBITC labeled hZP4/hZP3$_{(1-424)}$ at a molar ratio of 1:1 as described in *Materials and Methods*. The left panel represents the binding of FITC labeled hZP3$_{(1-424)}$ and RBITC conjugated hZP4 while the right panel shows the binding of RBITC labeled hZP3$_{(1-424)}$ and FITC conjugated hZP4 to capacitated human spermatozoa. The images were captured using Eclipse 80*i* fluorescence microscope (Nikon). The panels are represented as a: phase contrast; b: binding of baculovirus-expressed recombinant hZP3$_{(1-424)}$; c: binding of baculovirus-expressed hZP4; and d: overlap of fluoresecent frames. The scale bar represents 2.5 µm.

incubated with 5 and 10 fold molar excess of unlabeled ZP3 respectively ($p<0.01$) (Figure 29a). However, in presence of baculovirus-expressed hZP2 and hZP4, no significant decrease ($p>0.05$) in the binding of $hZP3_{(1-424)}$ was observed. Similarly, unlabeled baculovirus-expressed hZP4 and hZP2, when used at 5 and 10 fold molar excess, significantly inhibited the binding of the respective FITC conjugated recombinant proteins to 2.6 and 2.0% respectively from 15.2% ($p<0.01$) for baculovirus-expressed hZP4, and 5.5 and 3.0% respectively from 21.2% for baculovirus-expressed hZP2 ($p<0.01$; Figure 29b, c). Lesser and non-significant inhibition in the binding of FITC labeled hZP4 to capacitated spermatozoa was observed when hZP2 ($p>0.05$) and hZP3 ($p>0.05$) were used at 10 fold excess as competitors (Figure 29b). The binding of FITC labeled baculovirus-expressed hZP2 to the acrosome-reacted sperm was reduced, which was not however, statistically significant, in the presence of 10 fold excess of baculovirus-expressed $hZP3_{(1-424)}$ ($p>0.05$) and hZP4 ($p>0.05$) respectively (Figure 29c).

Similar analysis of binding of the *E. coli*-expressed recombinant human zona proteins revealed that the binding of these proteins was also highly specific for each individual protein (Figure 30). The binding of each of the three FITC conjugated *E. coli*-expressed recombinant zona proteins to capacitated and/or acrosome-reacted sperm was specifically inhibited only in the presence of the respective unlabeled protein and not by the other *E. coli*-expressed zona proteins (Figure 30). The highly specific binding of the *E. coli*- and baculovirus-expressed human zona proteins prompted us to investigate if both the non-glycosylated and glycosylated recombinant zona proteins had similar binding sites and affinity to bind to human sperm. These competitive binding assays performed as described in *Materials and Methods* revealed that the unlabeled *E. coli*-expressed recombinant zona proteins, used at 5 and 10 fold molar excess, were competent to inhibit binding of the corresponding FITC labeled baculovirus-expressed recombinant proteins (Figure 31). Similarly, the binding of FITC labeled *E. coli*-expressed proteins was also inhibited in the presence of 5 and 10 fold molar excess of the corresponding unlabeled baculovirus-expressed recombinant proteins (Figure 31). These studies suggest that the binding of the recombinant zona proteins to sperm may not require the presence of carbohydrate residues and can be mediated by the polypeptide backbone only.

Figure 25: Competitive inhibition studies to determine the specificity of binding to paclovirus-expressed ZA glycoproteins with mutant spermatozoa. Capacitated or calcium ionophore induced acrosome-reacted mutant sperm (2 X 10^6\20 µl) were incubated with 2.5 µg to 5 µg conjugated recombinant paclovirus-expressed (a) pZA3$^{(1-434)}$ (b) pZA4 and (c) pZA2 in the absence or presence of 2-10 fold molar excess of multiepitope paclovirus-expressed pZA3$^{(1-434)}$ and pZA4 as described in Materials and Methods. The Y-axis represents the percent of sperm showing binding to the TTC conjugated recombinant protein either in presence or absence of multiepitope competing protein. Data is represented as mean ± SEM of three independent experiments. The statistical significance is calculated using one way ANOVA followed by Newmans-Keuls Multiple Comparison Test. Bars labeled with different letters identify means that are statistically significant.

Figure 30: Competitive inhibition studies to determine the specificity of binding of *E. coli*-expressed ZP glycoproteins with human spermatozoa. Capacitated or calcium ionophore induced acrosome-reacted human sperm (2 X 10^6\20 µl) were incubated with 2.5 µg of FITC conjugated recombinant *E. coli*-expressed (a) hZP3, (b) hZP4 and (c) hZP2 in the absence or presence of 2-10 fold molar excess of unlabeled *E. coli*-expressed hZP2, hZP3 and hZP4 as described in Materials and Methods. The Y-axis represents the percent of sperm showing binding of the FITC conjugated recombinant protein either in presence or absence of unlabeled competing protein. Data is represented as mean ± SEM of three independent experiments. The statistical significance is calculated using one way ANOVA followed by Newmans-Keuls Multiple Comparison Test. Bars labeled with different letters identify means that are statistically significant.

The specificity of binding of recombinant baculovirus-expressed $hZP3_{(1-424)}$ and hZP4 to capacitated spermatozoa was further ascertained using murine MAbs generated against the recombinant human zona proteins expressed in baculovirus. A significant inhibition in the binding of the FITC conjugated $hZP3_{(1-424)}$ pre-incubated with MAbs against $hZP3_{(1-424)}$, MA-1552 and MA-1567, to the capacitated spermatozoa was observed ($p<0.01$; Figure 32a). However, no inhibition in the binding of FITC labeled $hZP3_{(1-424)}$ to the sperm was observed in the presence of MA-1660, MAb specific for hZP4 ($p>0.05$). Similarly, the binding of FITC labeled hZP4 to the capacitated sperm was inhibited by pre-incubation of recombinant protein with MAbs against hZP4 (MA-1660 and MA-1662; $p<0.02$) but not with MA-1567, generated against $hZP3_{(1-424)}$ ($p>0.05$; Figure 32b).

d) Ability of hZP3 expressed in mammalian cells to bind to human spermatozoa

As discussed above, it was pertinent to investigate the binding characteristics of the hZP3 expressed in mammalian expression system. The cell lysate of CHO-K1 cells expressing recombinant hZP3 was also analyzed by an indirect binding assay for its ability to bind to human sperm as described in *Materials and Methods*. These studies revealed that although the binding profile of hZP3 as revealed by MA-1552, a MAb specific for $hZP3_{(1-424)}$ was similar to that observed with the *E. coli*- and baculovirus-expressed hZP3, the number of sperm showing binding of mammalian-expressed hZP3 was 9.3 ± 2.05%. Hence, lesser number of capacitated sperm exhibited the binding of mammalian expressed hZP3 than *E. coli*-/baculovirus-expressed recombinant hZP3.

IV. INDUCTION OF ACROSOMAL EXOCYTOSIS BY RECOMBINANT HUMAN ZONA PROTEINS

At the pre-fertilization stage, the binding of zona proteins to the sperm leads to induction of acrosomal exocytosis in zona bound sperm. While ZP has been shown to be the physiological agonist of acrosome reaction *in vivo* in different species including human (Cross *et al.*, 1988), progesterone and follicular fluid have also been shown to induce acrosome reaction (Osman *et al.*, 1989; Tesarik, 1985). Amongst the pharmacological agents, calcium ionophore brings about acrosomal exocytosis in spermatozoa (Tesarik, 1985). The ZP glycoproteins from various species have been studied in great detail to delineate the component that serves as the inducer of acrosomal exocytosis in the sperm after the initial recognition between the sperm and

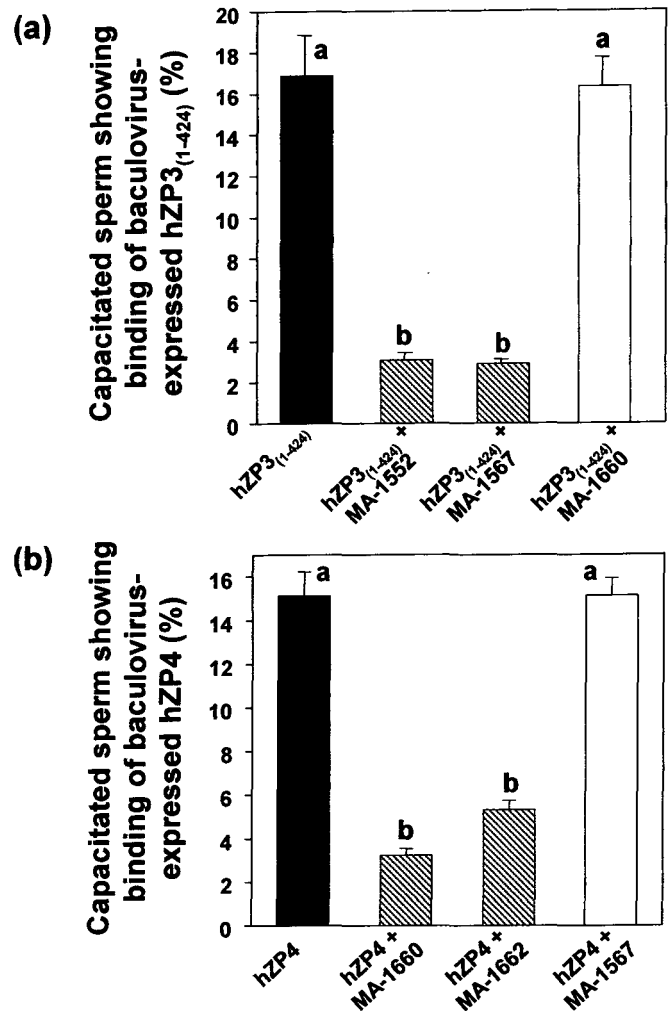


Figure 32: Monoclonal antibody mediated inhibition in the binding of baculovirus-expressed recombinant $hZP3_{(1-424)}$ and hZP4 to capacitated human sperm. The FITC conjugated baculovirus-expressed recombinant $hZP3_{(1-424)}$ and hZP4 were pre-incubated with the MAbs generated against either $hZP3_{(1-424)}$ (MA-1552, MA-1567) or hZP4 (MA-1660, MA-1662) and subsequently analyzed for their binding to capacitated human sperm as described in *Materials and Methods*. Panel (a) represents the binding of $hZP3_{(1-424)}$ whereas panel (b) depicts the binding of hZP4 to capacitated sperm. The Y axis shows the percentage of sperm exhibiting binding of the recombinant protein to capacitated human sperm in the absence (black bars), presence of specific MAbs (striped bars) and non-specific MAbs (open bars). Data is presented as Mean ± SEM of three independent experiments. Bars labeled with different letters identify means that are statistically significant.

the egg (Beebe *et al.*, 1992; van Duin *et al.*, 1994; Chapman *et al.*, 1998; Gahlay and Gupta, 2003). To assess whether the individual recombinant zona proteins are able to bring about acrosome reaction in human sperm, the *E. coli*- and baculovirus-expressed recombinant hZP2, hZP3 and hZP4 were evaluated for their ability to induce acrosomal exocytosis in the human capacitated spermatozoa in an *in vitro* assay followed by analysis of the acrosomal status of the sperm by TRITC-PSA staining as described in *Materials and Methods*. Any spermatozoa that demonstrated complete loss of PSA staining in the acrosome or revealed staining at the equatorial region was classified as acrosome-reacted. Sperm showing PSA staining in the acrosome were scored as capacitated (acrosome-intact).

a) Dose response and time kinetics of ZP glycoprotein mediated acrosome reaction

Dose response studies performed with baculovirus-expressed recombinant $hZP3_{(1-424)}$ and hZP4 used at various concentrations (1, 2, 5, 10, 20 and 100 µg/ml) revealed that as low as 1 µg/ml (100 ng/reaction) of both the recombinant proteins induced a significant increase in acrosome reaction (Figure 33a). The increase in number of capacitated sperm undergoing acrosomal exocytosis reached saturation at concentration of 20 µg/ml for both the recombinant proteins and did not increase with further increase in the protein concentration. Time-kinetics studies performed with 20 µg/ml of the recombinant proteins revealed that significant induction of acrosomal exocytosis by $hZP3_{(1-424)}$ and hZP4 can be seen as early as 15 min after exposure of the capacitated spermatozoa to the recombinant protein (Figure 33b). However, the maximum acrosomal exocytosis was observed at 60 min, which shows a decline at 120 and 240 min.

b) Percent induction of acrosomal exocytosis by recombinant zona proteins

After optimization of the dose of the recombinant proteins and time of their incubation with the capacitated sperm for induction of acrosomal exocytosis, all the recombinant human zona proteins were analyzed for their ability to induce acrosome reaction in human sperm. The results obtained in the presence of 20 µg/ml of the respective recombinant protein, when incubated with capacitated sperm for 60 min, are shown in Table 11. Incubation of the capacitated sperm with *E. coli*-expressed recombinant hZP3 did not induce significant increase ($p>0.05$) in acrosomal exocytosis (16.01 ± 0.39%) as compared to the respective medium control (12.50 ± 1.70%). However, when capacitated sperm were incubated with baculovirus-expressed $hZP3_{(1-424)}$, a significant

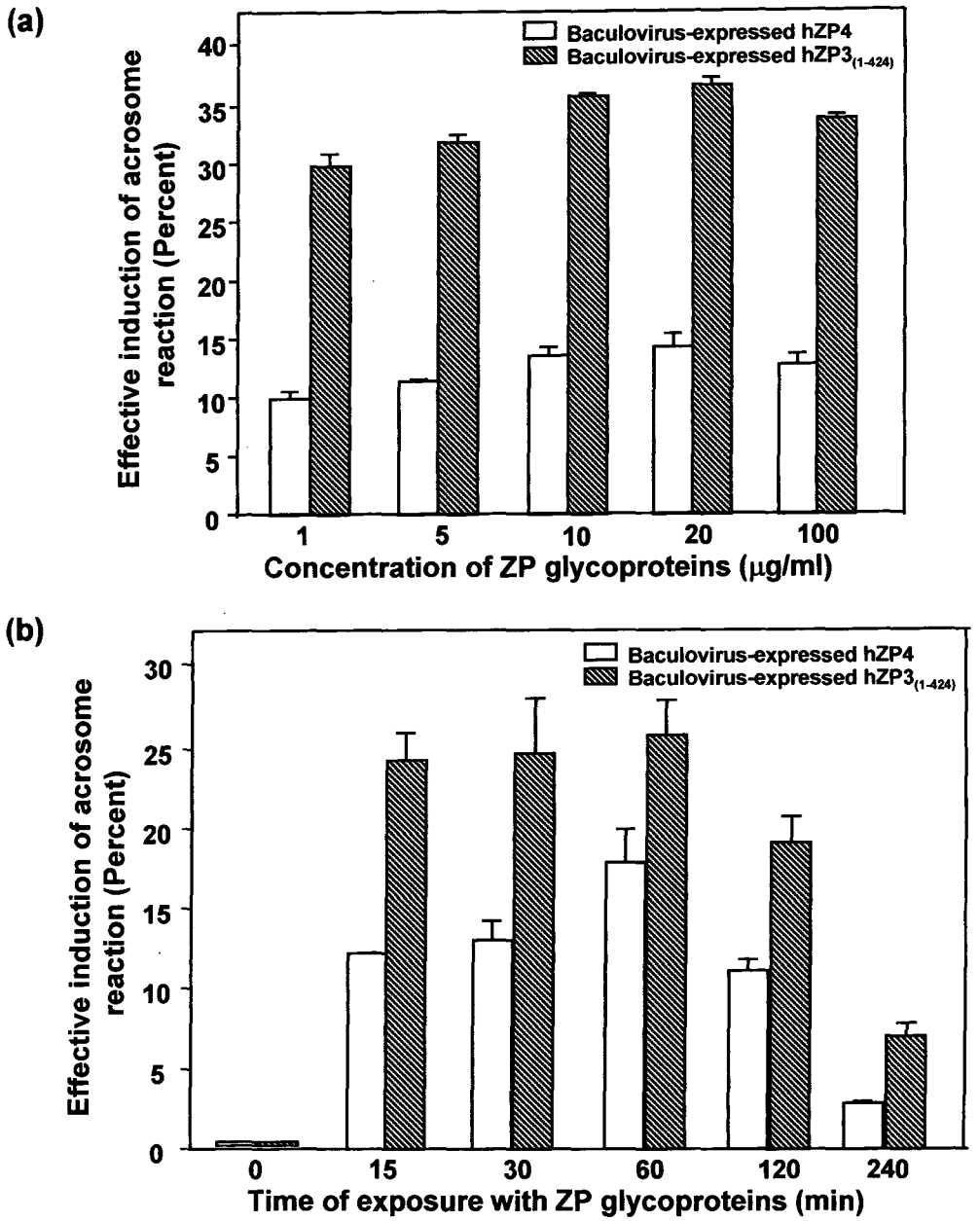


Figure 33: Induction of acrosome reaction in capacitated human spermatozoa: dose response and time kinetics of baculovirus-expressed $hZP3_{(1-424)}$ and hZP4. Capacitated sperm (1 X 10^6/100 μl) were incubated with the respective recombinant protein at varying concentrations (Panel a) for 60 min, and subsequently analyzed for acrosomal status by TRITC-PSA staining as described in *Materials and Methods*. To optimize the time required for induction of acrosomal exocytosis, capacitated sperm (1 X 10^6/100 μl) were incubated with or without 2 μg/100 μl of baculovirus-expressed $hZP3_{(1-424)}$ or hZP4 for varying time points (Panel b). Y-axis represents effective induction of acrosome reaction which is the percent induction of acrosome reaction in presence of the respective recombinant protein minus the percent of acrosome reaction observed in the presence of medium alone. Values are Mean ± SEM of 3 different experiments using semen samples from at least two different male donors.

Table 11: Induction of acrosome reaction in capacitated human sperm by recombinant human ZP proteins

Treatment[a]	Percent Induction of Acrosomal Exocytosis[b] (Mean ± SEM)	Statistical Significance[c]
Group I		
Control (BWW + 0.3% BSA)	12.50 ± 1.70	
E. coli-expressed hZP3	16.01 ± 0.39	p=0.1145
Baculovirus-expressed $hZP3_{(1-424)}$	38.99 ± 4.54	p=0.0055*
Calcium Ionophore	52.11 ± 2.75	p<0.0001*
Group II		
Control (BWW + 0.3% BSA)	7.81 ± 0.83	
E. coli-expressed hZP4	10.26 ± 0.79	p=0.0883
Baculovirus-expressed hZP4	19.08 ± 1.70	p=0.0005*
Group III		
Control (BWW + 0.3% BSA)	5.42 ± 0.26	
E. coli-expressed hZP2	5.98 ± 1.54	p>0.05
Baculovirus-expressed hZP2	6.47 ± 0.27	p=0.1502
Group IV		
Control (BWW + 0.3% BSA)	9.04 ± 1.55	
Baculovirus-expressed $hZP3_{(1-424)}$	34.73 ± 4.14	p=0.0005*
Baculovirus-expressed hZP4	22.06 ± 2.67	p=0.0019*
Baculovirus-expressed $hZP3_{(1-424)}$ + hZP4	29.25 ± 4.42	p=0.0017*

[a]Capacitated sperm were incubated with the recombinant proteins at a concentration of 2 μg/100μl for 60 min. The baculovirus-expressed recombinant $hZP3_{(1-424)}$ and hZP4 were used in the co-incubation experiments at a molar ratio of 1:1. Calcium ionophore was used at a concentration of 10 μM.

[b]Percent induction of acrosomal exocytosis was calculated by dividing the number of acrosome reacted sperm by total number of sperm counted and multiplying by 100. Values represent mean ± SEM of at least three independent experiments.

[c]Statistical significance with respect to the medium control was calculated by one way ANOVA followed by Newmans-Keuls Multiple Comparison Test.

*Values are statistically significant.

increase (38.99 ± 4.54%; p=0.0055) in the acrosomal exocytosis was observed. Calcium ionophore, a chemical agonist of acrosomal exocytosis, used as a positive control, also showed a significant increase in the percentage of sperm undergoing acrosome reaction (52.11 ± 2.75%; p<0.0001). The baculovirus-expressed recombinant hZP4 also induced a significant increase in the acrosome reaction (19.08 ± 1.70%; p=0.0005) as compared to the medium control (7.81 ± 0.83%; Table 11). The *E. coli*-expressed hZP4 as well as *E. coli* and baculovirus-expressed hZP2 failed to induce any significant increase in the acrosomal exocytosis in capacitated spermatozoa (Table 11).

The observed induction of acrosome reaction mediated by baculovirus-expressed $hZP3_{(1-424)}$ and hZP4 independently prompted us to study whether the two recombinant proteins had a synergistic effect on the induction of acrosome reaction in human sperm. When the capacitated spermatozoa were incubated with a combination of baculovirus-expressed recombinant $hZP3_{(1-424)}$ and hZP4 at a molar ratio of 1:1, the induction of acrosome reaction was found to be 29.25 ± 4.42%, which is not statistically significant as compared to 34.73 ± 4.14% with $hZP3_{(1-424)}$ alone (p=0.20) or 22.06 ± 2.67% with hZP4 alone (p=0.12; Table 11). However, induction of acrosome reaction with the cocktail was statistically significant as compared to the medium control (p=0.0017).

Since, baculovirus-expressed $hZP3_{(1-424)}$ was found to have the ability to induce acrosome reaction in capacitated human sperm, attempts were made to investigate the function of the TD, if any, in inducing acrosomal exocytosis using two proteins, $hZP3_{(1-348)}$, devoid of the TD and $hZP4_{(1-424)}$, including the TD. It was found that both the proteins were competent to induce acrosome reaction in capacitated sperm (Figure 34). Though there was marginally higher increase in percent induction of acrosome reaction with recombinant $hZP3_{(1-424)}$ than with $hZP3_{(1-348)}$, the difference was not statistically significant (p>0.05).

c) Evaluation of acrosome reaction inducing ability of mammalian-expressed hZP3

The cell lysate prepared from CHO-K1 cells transiently transfected with pcDNA-hZP3 and expressing the recombinant protein as shown above (Figure 23), was also assessed for its ability to induce acrosomal exocytosis in capacitated sperm as described in *Materials and Methods*. The cell lysate of CHO-K1 cells expressing hZP3 was competent to bring about acrosomal exocytosis in 38.91 ± 1.51% of capacitated human sperm which was significantly higher than untransfected CHO-K1 cell lysate mediated

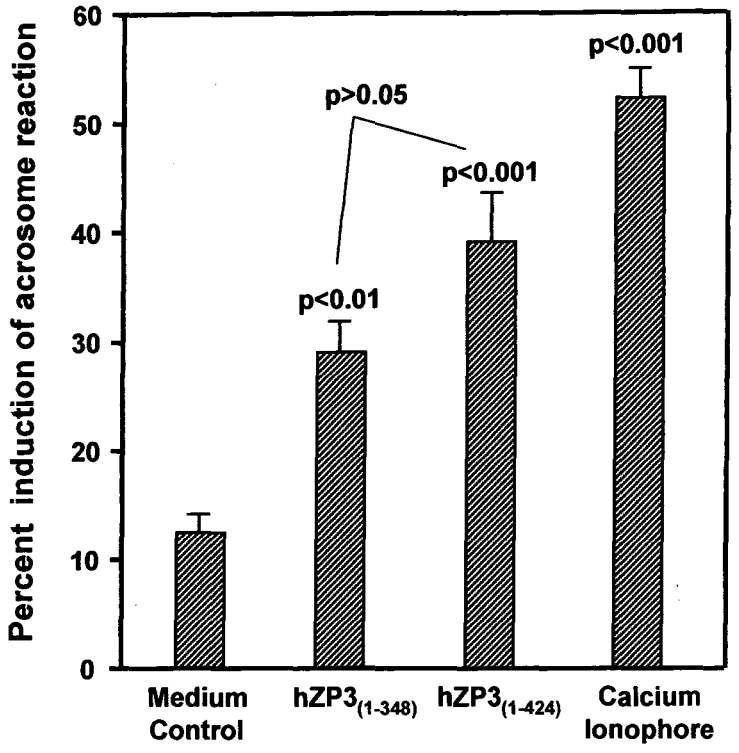


Figure 34: Analysis of the ability of baculovirus-expressed recombinant $hZP3_{(1-348)}$ and $hZP3_{(1-424)}$ to induce acrosome reaction in capacitated human spermatozoa. Capacitated sperm (1 X 10^6/100 µl) were incubated with the respective recombinant protein (2 µg/100 µl) for 1 h and subsequently analyzed for acrosomal status by TRITC-PSA staining as described in *Materials and Methods*. Values are expressed as Mean ± SEM of three different experiments using semen samples from three male donors. The p values show the statistical significance of the treated group as compared to the medium control.

induction of acrosome reaction, taken as negative control (11.97 ± 1.07%; $p<0.005$; Figure 35).

V. IMPORTANCE OF CARBOHYDRATE MOIETIES ON ZP GLYCOPROTEINS

It is known that glycosylation of zona proteins plays a crucial role in the initial binding event that takes place between the sperm and the ZP (Yonezawa *et al.*, 2005). In different mammalian species, different carbohydrate moieties have been implicated for the sperm receptor function. In mouse, O-linked oligosaccharides of ZP3 play a critical role (Florman and Wassarman, 1985; Bleil and Wassarman, 1988) while in the porcine system, both O-linked oligosaccharides (Yurewicz *et al.*, 1991) and the tri- and tetra-antennary neutral complex type of N-linked oligosaccharides (Nakano *et al.*, 1996; Yonezawa *et al.*, 1999) of ZP3 have been implicated for its sperm receptor activity. In human zona proteins, both N- as well as O-linked glycans are known to be present (Maymon *et al.*, 1994; Miranda *et al.*, 1997). However, the role of the glycosylation of human zona proteins critical for mediating sperm-ZP interactions is yet to be clearly elucidated.

a) Removal of selective carbohydrate moieties from baculovirus-expressed $hZP3_{(1-424)}$ and hZP4

In an attempt to understand the relevance of N- and O-linked glycans of zona proteins for mediating acrosomal exocytosis in capacitated human sperm, the N- and O-linked sugar residues were selectively removed from the recombinant human zona proteins expressed in baculovirus. The addition of N-linked sugar residues on proteins during expression is known to be inhibited by tunicamycin, which has been used for deglycosylation of a variety of glycoproteins including zona proteins. Chemical deglycosylation by mild alkali treatment leads to removal of O-linked carbohydrate moieties from glycoproteins with no adverse effect on the polypeptide backbone. Using tunicamycin and mild alkali treatment for removal of N- and O-linked glycans respectively, selectively deglycosylated baculovirus-expressed recombinant $hZP3_{(1-424)}$ and hZP4 were obtained as described in *Materials and Methods*.

The obtained deglycosylated recombinant proteins were analyzed for their carbohydrate content by lectin binding in ELISA as described in *Materials and Methods*. Only those lectins showing binding to the glycosylated baculovirus-expressed recombinant proteins as assessed earlier were tested in this assay. The purified recombinant tunica-

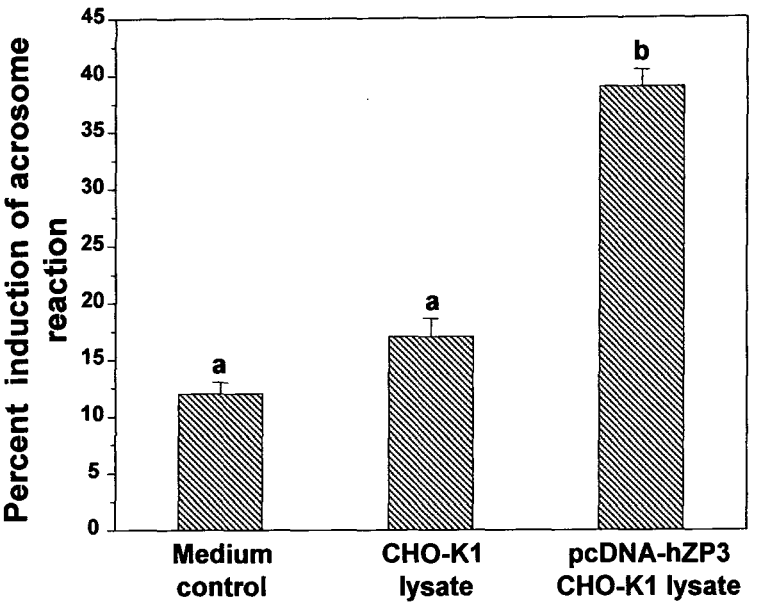


Figure 35: Analysis of the ability of recombinant hZP3 expressed in CHO-K1 cells to induce acrosome reaction in capacitated human spermatozoa. Capacitated sperm (1 X 10^6/100 µl) were incubated with the cellular lysates of untransfected and pcDNA-hZP3 transfected CHO-K1 cells for 1 h and subsequently analyzed for acrosomal status by TRITC-PSA staining as described in *Materials and Methods*. Values are expressed as Mean ± SEM of three different experiments using semen samples from at least two male donors. Bars labeled with different letters identify means that are statistically significant.

$hZP3_{(1-424)}$ and tunica-hZP4 showed reduced binding of ConA as compared to the respective wild type recombinant proteins in ELISA (Table 12a). The reduced binding to ConA, however, did not vary with the increase in concentration of tunicamycin from 5-20 μg/ml. Incubation of *Sf21* cells with 40 μg/ml tunicamycin led to a drastic decrease in the recombinant protein expression and hence, was not used for further analysis. Tunicamycin had no effect on the binding of Jacalin, a lectin recognizing O-linked oligosaccharides. On the contrary, assessment of carbohydrate content of recombinant alkali-$hZP3_{(1-424)}$ and alkali-hZP4 revealed reduced reactivity with Jacalin while the reactivity with ConA remained unaffected (Table 12b).

b) Ability of deglycosylated $hZP3_{(1-424)}$ and hZP4 to induce acrosome reaction

Following deglycosylation, it was imperative to study whether removal of N-/O-linked glycosylation had any effect on the ability of recombinant $hZP3_{(1-424)}$ and/or hZP4 to induce acrosome reaction in human spermatozoa. To achieve this, different recombinant modified proteins having reduced N-/O-linked sugar residues were analyzed for their ability to induce acrosomal exocytosis in capacitated human spermatozoa as described in *Materials and Methods*. The percentage of spermatozoa undergoing acrosomal exocytosis, when incubated with tunica-$hZP3_{(1-424)}$ as well as tunica-hZP4 expressed in the presence of all the concentrations of tunicamycin, was reduced significantly in comparison to the respective wild type recombinant proteins (Figure 36). However, no significant differences in the reduction of acrosome reaction were observed when sperm were treated with $hZP3_{(1-424)}$ or hZP4 purified from *Sf21* cells grown in presence of 5, 10 and 20 μg/ml of tunicamycin. The acrosome reaction inducing ability of these proteins was not completely abolished and was significantly higher than the percent of sperm showing spontaneous acrosomal exocytosis (Figure 36).

Interestingly, the alkali-$hZP3_{(1-424)}$ and alkali-hZP4, partially devoid of O-linked glycans, showed the ability to induce acrosome reaction in capacitated sperm equivalent to baculovirus-expressed $hZP3_{(1-424)}$ and hZP4 respectively, with no significant differences (Figure 37).

Table 12: Characterization of deglycosylated baculovirus-expressed recombinant $hZP3_{(1-424)}$ and hZP4 for binding to lectins in ELISA

(a)

Recombinant protein	Concentration of tunicamycin (µg/ml)	Absorbance at 490 nm with lectins	
		ConA	Jacalin
$hZP3_{(1-424)}$	0	2.78	1.38
	5	1.75	1.36
	10	1.58	1.35
	20	1.60	1.32
hZP4	0	2.93	0.88
	5	1.32	0.75
	10	1.30	0.78
	20	1.30	0.80

(b)

Recombinant protein	Absorbance at 490 nm with lectins	
	ConA	Jacalin
$hZP3_{(1-424)}$	2.57	1.40
alkali-$hZP3_{(1-424)}$	2.48	0.67
hZP4	2.82	0.96
alkali-hZP4	2.69	0.48

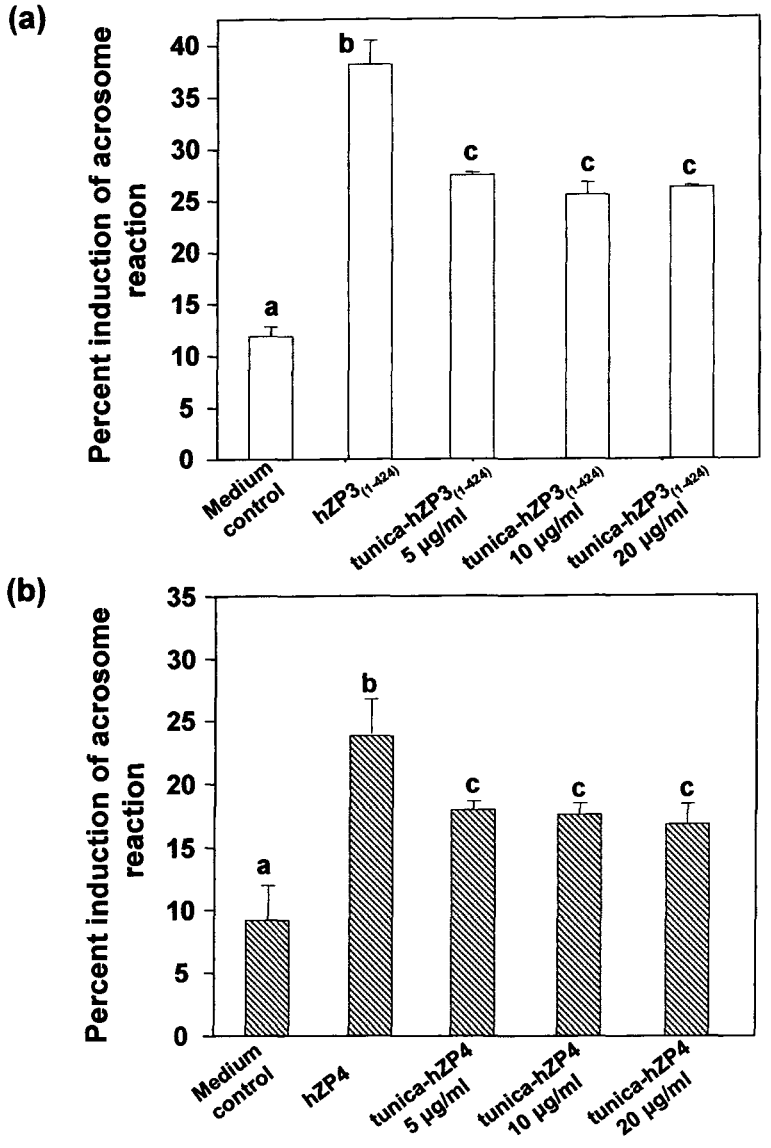


Figure 36: Analysis of the ability of baculovirus-expressed recombinant tunica-hZP3$_{(1-424)}$ and tunica-hZP4 to induce acrosome reaction in capacitated human spermatozoa. Capacitated sperm (1 X 10^6/100 µl) were incubated with the respective recombinant protein (2 µg/100 µl) for 1 h and subsequently analyzed for acrosomal status by TRITC-PSA staining as described in *Materials and Methods*. Values are expressed as Mean ± SEM of three different experiments using semen samples from at least two male donors. Bars labeled with different letters identify means that are statistically significant.

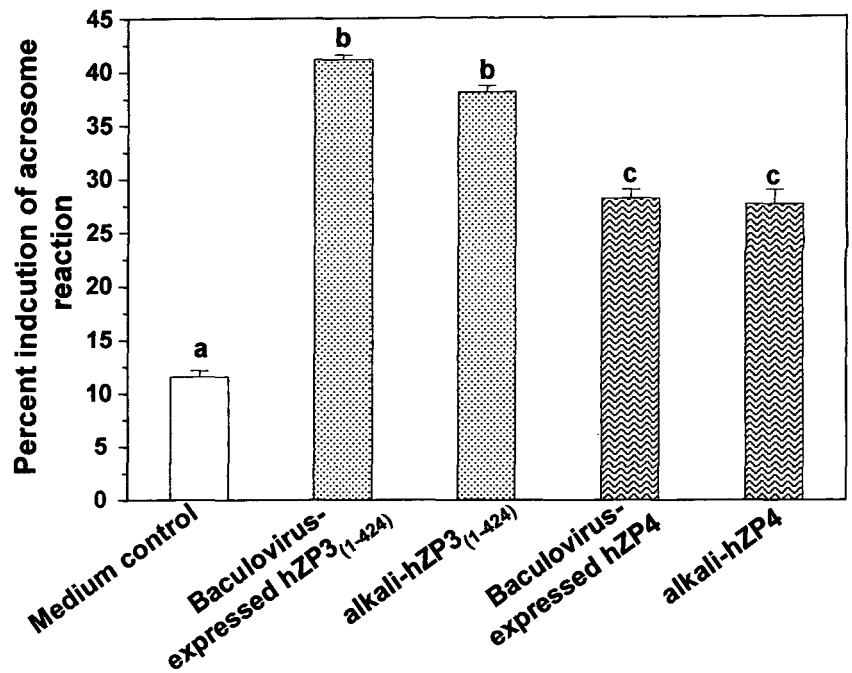


Figure 37: Analysis of the ability of baculovirus-expressed recombinant alkali-hZP3$_{(1-424)}$ and alkali-hZP4 to induce acrosome reaction in capacitated human spermatozoa. Capacitated sperm (1 X 10^6/100 µl) were incubated with the respective untreated or alkali-treated recombinant protein (2 µg/100 µl) for 1 h and subsequently analyzed for acrosomal status by TRITC-PSA staining as described in *Materials and Methods*. Values are expressed as Mean ± SEM of three different experiments using semen samples from at least two male donors. Bars labeled with different letters identify means that are statistically significant.

VI. ROLE OF SPERM PROTEASOME IN INTERACTION WITH ZP GLYCOPROTEINS

Sperm proteasome has been recently advocated to be involved at different steps in the interaction between sperm and zona in various mammalian species, especially porcine model (Sutovsky *et al.*, 2004). In the present study, proteasome-specific inhibitors were employed to investigate the function of sperm proteasome, if any, in binding and induction of acrosomal exocytosis in human spermatozoa mediated by recombinant human zona proteins as described in *Materials and Methods*. In the presence of the inhibitors LC, CLBL and MG132, each used individually at a concentration of 100 μM, there was no effect on the binding of recombinant baculovirus-expressed human ZP glycoproteins with spermatozoa. The results of these experiments are presented in Table 13. The binding profiles of recombinant hZP2, $hZP3_{(1-424)}$ and hZP4 with capacitated and/or acrosome-reacted sperm in the presence of the inhibitors were identical to the results obtained in absence of either of the inhibitors.

Since the binding of the recombinant human zona proteins to sperm was not inhibited in the presence of proteasome-specific inhibitors, it was prudent to investigate if the induction of acrosome reaction mediated by recombinant human zona proteins was affected in the presence of the proteasome-specific inhibitors. These studies performed as described in *Materials and Methods*, revealed that presence of either of the proteasome-specific inhibitors individually at a concentration of 100 μM, abrogated the induction of acrosomal exocytosis in capacitated human sperm by baculovirus-expressed recombinant $hZP3_{(1-424)}$ as well as hZP4 in a statistically significant manner (Figure 38a, b). The calcium ionophore induced acrosome reaction, however, remained unaffected in the presence of all the inhibitors used independently (Figure 38c).

Table 13: Binding characteristics of baculovirus-expressed recombinant human zona proteins with human spermatozoa

Treatment[a]	Percent binding to spermatozoa	Binding patterns	
		Acrosomal cap	Equatorial region
Capacitated (acrosome-intact) sperm			
Baculovirus-expressed hZP2	0.00		
Baculovirus-expressed hZP2 + LC	0.00		
Baculovirus-expressed hZP2 + CLBL	0.00		
Baculovirus-expressed hZP2 + MG132	0.00		
Baculovirus-expressed $hZP3_{(1-424)}$	19.08 ± 2.05	36.10%	63.90%
Baculovirus-expressed $hZP3_{(1-424)}$ + LC	17.86 ± 1.60	35.00%	65.00%
Baculovirus-expressed $hZP3_{(1-424)}$ + CLBL	19.50 ± 2.10	31.79%	68.21%
Baculovirus-expressed $hZP3_{(1-424)}$ + MG132	18.00 ± 1.50	38.15%	61.85%
Baculovirus-expressed hZP4	17.95 ± 2.37	87.90%	12.10%
Baculovirus-expressed hZP4 + LC	16.45 ± 2.85	80.50%	19.50%
Baculovirus-expressed hZP4 + CLBL	15.98 ± 3.22	85.65%	14.35%
Baculovirus-expressed hZP4 + MG132	17.23 ± 1.04	77.60%	22.0%
Acrosome-reacted sperm (calcium ionophore mediated[b])			
Baculovirus-expressed hZP2	18.75 ± 1.45	0.00%	100.00%
Baculovirus-expressed hZP2 + LC	19.20 ± 2.32	0.00%	100.00%
Baculovirus-expressed hZP2 + CLBL	17.33 ± 1.00	0.00%	100.00%
Baculovirus-expressed hZP2 + MG132	17.50 ± 3.11	0.00%	100.00%
Baculovirus-expressed $hZP3_{(1-424)}$	25.65 ± 2.55	0.00%	100.00%
Baculovirus-expressed $hZP3_{(1-424)}$ + LC	23.12 ± 1.50	0.00%	100.00%
Baculovirus-expressed $hZP3_{(1-424)}$ + CLBL	26.00 ± 0.22	0.00%	100.00%
Baculovirus-expressed $hZP3_{(1-424)}$ + MG132	24.75 ± 2.00	0.00%	100.00%
Baculovirus-expressed hZP4	15.15 ± 1.90	0.00%	100.00%
Baculovirus-expressed hZP4 + LC	16.10 ± 1.75	0.00%	100.00%
Baculovirus-expressed hZP4 + CLBL	14.55 ± 2.07	0.00%	100.00%
Baculovirus-expressed hZP4 + MG132	15.00 ± 1.90	0.00%	100.00%

[a]Capacitated/acrosome-reacted sperm were incubated with the FITC labeled recombinant proteins at a concentration of 2.5 μg/50 μl in the absence/presence of 100 μM of proteasome-specific inhibitors, lactacystin (LC)/*clasto*-lactacystin β-lactone (CLBL)/MG132 and processed for analysis of binding as described in *Materials and Methods*.

[b]Calcium ionophore was used at a concentration of 10 μM.

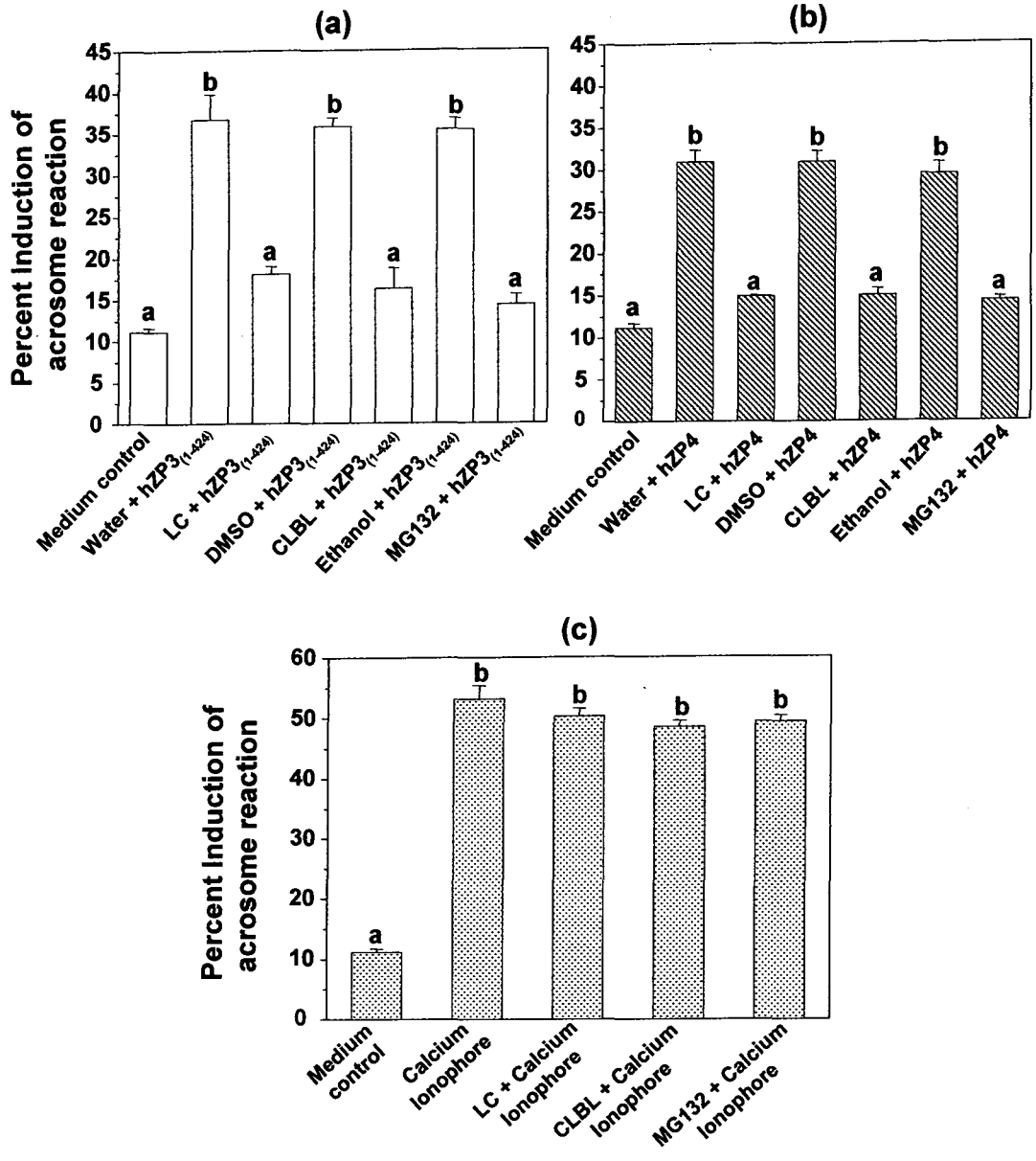


Figure 38: Analysis of the ability of baculovirus-expressed recombinant $hZP3_{(1-424)}$ and hZP4 to induce acrosome reaction in the presence of proteasome-specific inhibitors in capacitated human spermatozoa. Capacitated sperm (1 X 10^6/100 µl) were incubated with recombinant (a) $hZP3_{(1-424)}$/(b) hZP4-bac (2 µg/100 µl) or (c) calcium ionophore in presence of 100 µM of the proteasome-specific inhibitors, LC, CLBL and MG132 independently or their respective control solvents for 1 h and subsequently analyzed for acrosomal status by TRITC-PSA staining as described in *Materials and Methods*. Values are expressed as Mean ± SEM of three different experiments using semen samples from at least two male donors. Bars labeled with different letters identify means that are statistically significant.

Discussion

The sequence of events leading from the interaction of ZP with sperm and culminating in fertilization is a very complex cascade and has been studied in great detail in different vertebrate species. A critical appraisal of the role of individual ZP glycoproteins is essential to gain insight into the various steps involved in the pre-fertilization stage. Various studies have demonstrated that in mouse, ZP3 is responsible for initial binding of the sperm to the oocyte and induction of acrosomal exocytosis, thereby assigning it the primary sperm receptor function (Bleil and Wassarman, 1980b; 1983; Beebe *et al.*, 1992). Mouse ZP2 plays a role as the secondary sperm receptor and maintains the binding of acrosome-reacted spermatozoa to the oocyte (Bleil *et al.*, 1988). The mZP1 dimers maintain the structural integrity of the ZP matrix by cross-linking the mZP2-mZP3 heterodimer filaments (Greve and Wassarman, 1985). In the porcine system, however, the heterocomplexes of pZP3 and pZP4 bind to the boar sperm membrane vesicles with high affinity whereas the individual glycoproteins fail to bind (Yurewicz *et al.*, 1998). In the rabbit, ZP3 as well as ZP4 are known to bind to the recombinant Sp17, a sperm protein (Yamasaki *et al.*, 1995). In humans, the information regarding the localization of the binding sites of zona glycoproteins on the sperm as well as the kinetics of binding is scanty owing to the non-availability of zona proteins due to ethical restrictions. However, recombinant hZP3 expressed both in prokaryotic or eukaryotic expression systems, have shown the ability to induce acrosome reaction in the capacitated human sperm (van Duin *et al.*, 1994; Chapman *et al.*, 1998). Looking at all these findings, it becomes evident that the delineation of the role of ZP glycoproteins in one mammalian species can not be extrapolated to the other species.

With these in mind, an attempt has been made in the present thesis to generate human ZP glycoproteins by recombinant DNA technology and employ the recombinant proteins thus generated, to understand their functional attributes at the pre-fertilization stage in humans.

To achieve the above objectives, the cDNAs corresponding to the hZP2, hZP3 and hZP4 (without SS and TD) have been cloned and expressed in *E. coli*, while the full length hZP2, hZP3 ($hZP3_{(1-424)}$) and hZP4, in addition to a construct of hZP3 without TD ($hZP3_{(1-348)}$), have been expressed in baculovirus expression system. The hZP3 has also been expressed transiently in CHO-K1 mammalian cells. The availability of the recombinant human zona proteins in both non-glycosylated as well as glycosylated forms would enable us to delineate the role of polypeptide backbone and the glycosyl

moieties that are advocated to play a crucial role in species-specific sperm-egg recognition.

The recombinant zona proteins have been expressed as polyhistidine-tagged fusion proteins in *E. coli* as well as baculovirus expression system. In *E. coli*, their expression in the *lon* and *ompT* proteases deficient BL21[DE3]pLysS host strain would aid in minimizing the lower molecular weight fragments which may arise due to degradation. Expression of the recombinant zona proteins as polyhistidine-tagged fusion proteins allowed their convenient purification by Ni-NTA affinity column. All the recombinant human zona proteins expressed in *E. coli* were present in the insoluble cell fractions in the form of inclusion bodies, as observed in the localization studies (Figure 3b, 6b, 9b). It is well documented that expression in *E. coli* leads to over-expression of the recombinant protein resulting in the formation of inclusion bodies (Kaul *et al.*, 1997; Harris *et al.*, 1999). Over-expression of the recombinant protein in the cell creates a situation where folding intermediates are present in high concentration. These intermediates, owing to their exposed hydrophobic regions, therefore, have a propensity to form aggregates. Additionally, the lack of post-translational modifications in *E. coli* has also been suggested to contribute to aggregation. In previous studies from our laboratory, recombinant zona proteins expressed in *E. coli* could be purified only under denaturing conditions in the presence of urea to maintain it in a soluble form (Kaul *et al.*, 1997). However, for investigating these proteins for their biological function, the presence of urea becomes a major limiting factor. Hence, the recombinant protein was purified under denaturing conditions in the presence of 8 M urea, which was gradually removed by dialysis against a renaturation buffer containing oxidized and reduced glutathione and sucrose. The use of oxidized and reduced glutathione would help in disulfide bond formation between the Cys residues present in the polypeptide backbone and has been used by many researchers to successfully refold the recombinant proteins expressed as inclusion bodies in *E. coli* (Patra *et al.*, 2000; Govind *et al.*, 2001). The presence of sucrose in the renaturation buffer acts as a stabilizing agent.

In contrast to mammalian cells, failure to observe the secretion of baculovirus-expressed human zona proteins in the medium is in agreement with that observed by other investigators (Harris *et al.*, 1999). The plausible reason for this phenomenon is not clear. It may be possible that *Sf21* cells lack furin-like enzymes and fail to cleave the TD and hence, failure to secrete the expressed ZP glycoproteins. Contradicting

reports in the murine model, questioning the role of the consensus furin cleavage site (CFCS) in secretion of the zona proteins and incorporation in the matrix prompted us to express the baculovirus-expressed hZP3 without the C-terminal TD ($hZP3_{(1-348)}$), that lies immediately after the CFCS (Williams and Wassarman, 2001; Qi *et al.*, 2002; Zhao *et al.*, 2002). However, this protein also did not get secreted into the culture supernatant. It can be speculated that the secretory signals present in the *Sf21* cells may not be recognized by the foreign human zona proteins.

The expression of hZP2 in *E. coli* yielded several low molecular weight proteins in addition to the ~90 kDa main protein band as observed in the Western blot of induced host cells harboring the recombinant hZP2 plasmid. Various reasons that can be attributed to the presence of lower molecular weight fragments are, i) multiple initiation sites in mRNA, ii) premature translation termination iii) specific or non-specific proteolysis of the full-length protein, and iv) a combination of the above.

On SDS-PAGE, the *E. coli*- as well as baculovirus-expressed hZP2, hZP3 and hZP4 fusion proteins showed a slightly retarded mobility as is evident from their apparent molecular weights as compared to the theoretical values, which is sometimes the case with polyhistidine tagged fusion proteins. In case of the baculovirus-expressed recombinant proteins, the apparent molecular weights show greater deviation than the expected values and this may be attributed to glycosylation of these recombinant proteins in the insect cells. The characterization of native ZP glycoproteins from human oocytes by various groups has revealed heterogeneity and variability in their mobility in SDS-PAGE (Shabanowitz and O'Rand, 1988; Bercegeay *et al.*, 1995; Gupta *et al.*, 1998; Bauskin *et al.*, 1999). Some of the discrepancies in the apparent molecular weights assigned to various ZP glycoproteins may be attributed to the different nomenclature used by various investigators. Using antibodies against synthetic peptides, it has been documented that hZP2 is comprised of 90-110 kDa and hZP3 as 53-60 kDa (Bauskin *et al.*, 1999). The baculovirus-expressed recombinant hZP2 revealed a band of ~105 kDa and $hZP3_{(1-424)}$ ~65 kDa (Figure 13), which were comparable to the native proteins. Lectin binding analysis indeed revealed the presence of both N- and O-linked glycosylation in the baculovirus-expressed recombinant proteins, which were absent in the *E. coli*-expressed proteins (Figures 17 and 18). The predominant glycosylations in the four baculovirus-expressed recombinant ZP glycoproteins were characterized by the binding of lectins ConA (specific for mannose α 1-3/1-6 residues, N-linked) and

Jacalin (specific for α-O glycosides of Gal or GalNAc moieties, O-linked) as shown in Figures 17 and 18. Using immunocytochemistry, a recent study has also shown the presence of both ConA and Jacalin binding to the native human ZP (Jimenez-Movilla *et al.*, 2004). The presence of very high concentration of D-mannose residues in human ZP has earlier also been documented, reflecting a high content of asparagine-linked oligosaccharides (Maymon *et al.*, 1994).

After the purification of the glycosylated recombinant proteins, a panel of 10 MAbs, (MA-1650, -1654, -1657, -1660, -1662, -1665, -1667, -1671, -1673 and -1677) was raised against baculovirus-expressed recombinant hZP4 to develop a probe to recognize hZP4, and use them as a tool in functional assays with spermatozoa. These were characterized for their specificity and isotypes. All the MAbs were highly specific for the recombinant hZP4 and one of the MAbs, MA-1660 was tested and found positive for its reactivity with native bonnet monkey zona in an indirect immunofluorescence assay.

The availability of both non-glycosylated and glycosylated recombinant human zona proteins provided us with a handle to further our understanding pertaining to the interaction between the sperm and the individual zona proteins at the pre-fertilization stage. Conditions were optimized for analyzing the binding characteristics of the recombinant zona proteins to capacitated and acrosome-reacted spermatozoa *in vitro* by a direct binding assay and by indirect immunofluoresence using specific antibodies. Binding experiments revealed that baculovirus-expressed recombinant $hZP3_{(1-424)}$ as well as hZP4 were competent to bind to the capacitated spermatozoa (Figure 27). Various studies have demonstrated that in mouse, ZP3 is responsible for the initial binding of the sperm to the oocyte and induction of acrosomal exocytosis, thereby assigning it the primary sperm receptor function (Bleil and Wassarman, 1980b; 1983; Mortillo and Wassarman, 1991; Beebe *et al.*, 1992). However, in rabbit, porcine and bonnet monkey, ZP4 has also been shown to bind to homologous spermatozoa (Prasad *et al.*, 1996; Yurewicz *et al.*, 1998; Govind *et al.*, 2001). The ZP4 is not, however, present in the mouse ZP. In the present study, for the first time, it has been demonstrated that hZP4 is also competent to bind to the capacitated (acrosome-intact) spermatozoa.

However, the distribution of the binding patterns differed between the baculovirus-expressed $hZP3_{(1-424)}$ and hZP4 (Table 10). The hZP4, as compared to hZP3, elicited

higher binding percentage on the acrosomal cap of the acrosome-intact capacitated sperm, whereas, majority of the hZP3 bound sperm showed its localization in the equatorial region. The binding sites of the zona proteins on the sperm have been studied in great detail in the murine and porcine models (Bleil and Wassarman, 1986; Mortillo and Wassarman, 1991; Thaler and Cardullo, 1996; Burkin and Miller, 2000; Kerr *et al.*, 2002). In mouse, it has been shown that about 60% of the ZP3 binding sites are present on the acrosomal cap region whereas 40% sites reside in the post-acrosomal region (Bleil and Wassarman, 1986; Mortillo and Wassarman, 1991). However, our findings are supported by recent evidence in the mouse model suggesting that only 20% of the mZP3 binding sites are located on the acrosomal cap and the remaining sites are present on the post-acrosomal region (Kerr *et al.*, 2002).

The lesser binding percentages of baculovirus-expressed $hZP3_{(1-424)}$ and hZP4 to capacitated human spermatozoa, of the order of ~15-20%, obtained in the present study may be corroborated by a report where more than 75% of motile sperm from fertile men have been shown to be incapable of binding to native ZP (Liu *et al.*, 2003). It is also possible that the observed binding percentages in the present study may not conform with the physiological values due to the phenomenon of induction of acrosomal exocytosis, subsequent to the binding of baculovirus-expressed $hZP3_{(1-424)}$ or hZP4 to the capacitated sperm. Indeed, analysis of acrosomal status of the capacitated sperm after incubation with the baculovirus-expressed $hZP3_{(1-424)}$ and hZP4 revealed an increase in the number of sperm having undergone acrosome-reaction (data not shown). An alternate plausible explanation for the observed lower binding percentages of hZP3 and hZP4 to capacitated spermatozoa may also be due to the possibility that the sperm population employed in these studies from different healthy donors may represent sperm at different stages of maturation.

In the calcium ionophore induced acrosome-reacted spermatozoa, binding of both the baculovirus-expressed recombinant $hZP3_{(1-424)}$ and hZP4 was restricted to the equatorial region of the sperm, since the binding sites on the acrosomal cap are lost with the loss of acrosomal membrane. Despite having similar glycosylation as baculovirus-expressed $hZP3_{(1-424)}$ and hZP4, the baculovirus-expressed recombinant hZP2 failed to bind to capacitated acrosome-intact spermatozoa and bound to the acrosome-reacted spermatozoa, emphasizing the significance of studying individual zona proteins with respect to their function in sperm-zona interaction. It has also been

shown earlier that *E. coli*-expressed recombinant hZP2 binds to acrosome-reacted, but not to capacitated human spermatozoa (Tsubamoto *et al.*, 1999b). The hZP2 binding sites are restricted to only acrosome-reacted spermatozoa which conforms to the notion of ZP2 performing the secondary sperm receptor function in the mouse model, where it maintains the binding of acrosome-reacted sperm to eggs after the primary receptor recognition on the sperm surface has been established by ZP3 (Bleil *et al.*, 1988).

The binding patterns of baculovirus-expressed recombinant ZP glycoproteins to the spontaneously induced acrosome-reacted sperm obtained during the capacitation process were comparable to the profile obtained in the calcium ionophore-induced acrosome-reacted sperm (data not shown). Additionally, binding of recombinant hZP2 and hZP4 in baculovirus-expressed $hZP3_{(1-424)}$ mediated acrosome-reacted sperm also exhibited an identical binding profile to that observed in calcium ionophore induced acrosome-reacted sperm. These observations suggest that binding profile of the recombinant ZP glycoproteins to calcium ionophore induced acrosome-reacted sperm may not be an artifact.

Interestingly, the *E. coli*-expressed recombinant zona proteins were also found to be equally competent as their baculovirus-expressed counterparts to bind to human sperm (Table 9). The non-glycosylated recombinant proteins also exhibited identical binding profiles as observed with baculovirus-expressed recombinant human zona proteins to capacitated and acrosome-reacted human sperm (Figure 26). These findings suggest that the polypeptide backbone of the human zona proteins may be sufficient to act as the docking site for sperm on the ZP matrix and carbohydrate residues present on the recombinant zona proteins may be dispensible for the binding event *per se*. These results are corroborated by similar findings wherein the polypeptide backbone of bonnet monkey ZP3 and ZP4 have been independently shown to be sufficient to bind to homologous spermatozoa (Govind *et al.*, 2001; Gahlay *et al.*, 2002). However, in the mouse system, presence of O-linked oligosaccharides on mZP3 is critical for its binding to the capacitated spermatozoa (Florman and Wassarman, 1985; Wassarman and Litscher, 1995).

The binding of the recombinant human zona proteins was confirmed to be highly specific by significantly competing out FITC conjugated recombinant proteins by 5 and 10 fold excess of the corresponding non-glycosylated or glycosylated unlabeled proteins (Figures 29-31). In addition, binding to the sperm was also shown to be

glycoprotein-specific as the binding of FITC conjugated recombinant proteins could not be inhibited significantly by a 10 fold molar excess of the other competing unlabeled recombinant zona proteins. The specificity of the binding of baculovirus-expressed $hZP3_{(1-424)}$ and hZP4 to the capacitated spermatozoa was further reiterated by observations that their binding was obliterated by the MAbs against the respective recombinant protein (Figure 32). The competitive inhibition studies suggest that the hZP3 and hZP4 have distinct binding sites on the sperm. Demonstration of the simultaneous binding of baculovirus-expressed $hZP3_{(1-424)}$ and hZP4 on the acrosomal cap of capacitated human sperm further strengthens the notion that hZP3 and hZP4 indeed bind to different ligands on sperm surface.

In mammals, acrosome reaction of the capacitated spermatozoa is critical for successful fertilization. Acrosome reaction refers to the sequential process of fusion and fenestration of the outer acrosomal membrane and its overlying plasma membrane, followed by release of the acrosomal contents that facilitate penetration of the sperm through the ZP (Morales and Llanos, 1996). Both physiological and pharmacological agents have been implicated as inducers of acrosome reaction. While ZP has been shown to be the physiological agonist of acrosome reaction *in vivo* in different species including human (Cross *et al.*, 1988), progesterone and follicular fluid have also been shown to induce acrosome reaction (Osman *et al.*, 1989; Tesarik, 1985). Amongst the pharmacological agents, calcium ionophore brings about acrosomal exocytosis in spermatozoa (Tesarik, 1985). In the present study, induction of acrosome reaction by baculovirus-expressed recombinant $hZP3_{(1-424)}$ and its failure by *E. coli*-expressed hZP3 (Table 11), reiterate that glycosylation of the hZP3 is essential for induction of acrosome reaction. The recombinant $hZP3_{(1-424)}$ employed in the present study has been shown to have high mannose sugar moieties added to the polypeptide backbone (Figure 18). These findings correlate perfectly with earlier observations where mannose has been implicated to play an important role in the sperm receptor activity (Mori *et al.*, 1989; Cornwall *et al.*, 1991; McLeskey *et al.*, 1998). However, this is in contrary to an earlier report where hZP3 expressed in *E. coli* has been shown to induce acrosome reaction in capacitated spermatozoa but only after 18 h of exposure of sperm to the recombinant protein (Chapman *et al.*, 1998), in contrast to 1 h in our studies. To understand whether the CFCS of glycosylated hZP3 can modulate its ability to induce acrosome reaction in human sperm, $hZP3_{(1-348)}$ was also analyzed along with $hZP3_{(1-424)}$

in the *in vitro* assay for induction of acrosome reaction. The results revealed no significant differences between the ability of the two proteins to induce acrosomal exocytosis in capacitated spermatozoa (Figure 34), suggesting that the acrosome reaction inducing ability of the baculovirus-expressed hZP3 does not lie within the TD. Interestingly, baculovirus-expressed hZP4 also showed the ability to induce acrosomal exocytosis in capacitated human spermatozoa whereas the *E. coli*-expressed protein failed to do so. This protein has been shown to be involved in binding to homologous spermatozoa in rabbit, porcine and bonnet monkey in addition to ZP3 (Prasad *et al.*, 1996; Yurewicz *et al.*, 1998; Govind *et al.*, 2001). However, no information pertaining to the ability of ZP4 to induce acrosomal exocytosis in homologous sperm is available in any of the above mammalian species. The results of this study show that ZP4 may also have a functional role in acrosomal exocytosis in human.

The dose-response results indicate that as less as 1 μg/ml of baculovirus-expressed recombinant $hZP3_{(1-424)}$ as well as hZP4 are sufficient to induce a significant acrosome reaction in capacitated human sperm, though the maximum induction of acrosome reaction was observed at 20 μg/ml (Figure 33a). Earlier reports using recombinant hZP3 have also demonstrated that 5-20 μg/ml of the recombinant protein is required to induce significant acrosomal exocytosis in human sperm (van Duin *et al.*, 1994; Chapman *et al.*, 1998). The amount of baculovirus-expressed recombinant hZP3 needed for inducing acrosome reaction in human sperm in an *in vitro* system far exceeds the amount that is present *in vivo* where the ZP surrounding a single human oocyte consists of about 5 ng of hZP3 (van Duin *et al.*, 1994). The reason for this difference may be the presence of other factors in the milieu of the female reproductive tract that act in synergy with the zona proteins to bring about acrosomal exocytosis in the sperm. The observed ability of the baculovirus-expressed hZP3 and hZP4 to induce acrosome reaction is not due to post mortem acrosomal loss as no change either in the sperm motility or in the sperm viability was observed when the sperm were incubated with the recombinant proteins (data not shown).

The time-kinetics studies revealed that the acrosome reaction in human sperm can be observed as early as 15 min post exposure to baculovirus-expressed $hZP3_{(1-424)}$ and hZP4 and reaches a maximum at about 60 min (Figure 33b). Subsequently, an increase in the spontaneous acrosome reaction in the presence of medium alone, was responsible for decrease in the effective induction of acrosome reaction observed at 120 and 240

min in the presence of $hZP3_{(1-424)}$ as well as hZP4. The tendency of spermatozoa to undergo increased spontaneous acrosome reaction, when they have been incubated for prolonged periods *in vitro* has been documented earlier (van Duin *et al.*, 1994).

The baculovirus-expressed hZP2 failed to induce acrosomal exocytosis in capacitated spermatozoa in spite of sharing the nature of glycosylation with baculovirus-expressed hZP3 and hZP4. This observation suggests that the polypeptide backbone of the protein may not be completely dispensable for induction of acrosome reaction. It may be possible that the polypeptide backbone of hZP3 or hZP4 facilitates appropriate disposition of critical sugar residues that are important for induction of acrosome reaction. The observation that baculovirus-expressed hZP4 does not act synergistically with $hZP3_{(1-424)}$ to bring about enhanced induction of acrosome reaction in sperm may be due to the possibility that both the proteins either recognize same receptor on the spermatozoa or soluble glycosylated hZP3 and hZP4 cannot bind simultaneously to spermatozoa due to stearic hindrance. However, present findings showing simultaneous localization of both the recombinant proteins on the sperm surface (Figure 28) rules out the possibility of their binding to the same receptor on sperm.

Present findings that Pertussis toxin, an inhibitor of G_i protein mediated signaling pathway, completely abolished baculovirus-expressed $hZP3_{(1-424)}$ induced acrosome reaction are in agreement with earlier studies where mouse ZP induced acrosome reaction was shown to be blocked by Pertussis toxin (Tesarik *et al.*, 1993a), However, inability of Pertussis toxin to inhibit baculovirus-expressed hZP4 mediated acrosomal exocytosis suggests that induction of acrosome reaction mediated by recombinant hZP4 follows a similar mechanism as that of progesterone, which has a G_i protein independent pathway (Tesarik *et al.*, 1993a). These observations indicate that hZP3 and hZP4 may follow different downstream signaling mechanisms to bring about the induction of acrosome reaction. Although the downstream signals following the induction of acrosome reaction by solubilized zona have been studied by various groups, additional studies need to be carried out to investigate the precise mechanism involved in mediation of acrosomal exocytosis in human spermatozoa by hZP3 and hZP4 independently. The present finding that glycosylated hZP4 can also induce capacitated spermatozoa to undergo acrosome reaction opens new avenues with respect to the functions of the ZP glycoproteins at different stages of sperm-oocyte interaction, and of the interplay between the various glycoproteins present in the zona matrix.

Since, different carbohydrate residues are critical for mediating sperm-egg interaction in different species, there is a need to delineate the relevance of N- as well as O-linked oligosaccharide moieties present on zona proteins in human sperm-ZP interaction. To achieve this, the baculovirus-expressed recombinant $hZP3_{(1-424)}$ and hZP4 were selectively deglycosylated in the presence of tunicamycin and alkali treatment respectively for removal of N- and O-linked glycosides respectively. However, only partial reduction in the N- and O-linked glycosylation of the respective recombinant $hZP3_{(1-424)}$ and hZP4 was observed (Table 12). Partial abrogation of the acrosomal exocytosis induction ability of the recombinant tunica-$hZP3_{(1-424)}$ and tunica-hZP4, deficient in N-linked glycans (Figure 36), suggests that N-linked glycosylation of zona proteins in humans may be involved in the induction of acrosome reaction. In contrast, failure of alkali-$hZP3_{(1-424)}$ and alkali-hZP4, having reduced O-linked glycans to have any significant decrease in the induction of acrosomal exocytosis (Figure 37) suggests that O-linked glycans of hZP3 and hZP4 may not be critical for mediation of induction of acrosome reaction in human sperm. These results are in agreement with earlier reports emphasizing the importance of mannose binding receptors on human sperm surface that have been directly correlated with fertilization outcome following conventional IVF insemination (Tesarik *et al.*, 1991; Benoff, 1997). In mouse, small ZP3 glycopeptides (~1.5-6 kDa) generated by extensive enzymatic digestion of purified mZP3 retained the ability to bind to mouse sperm, but failed to induce acrosomal exocytosis (Florman *et al.*, 1984). These results suggest that the sperm receptor activity of mZP3 is dependent only on its carbohydrate components, whereas acrosome reaction-inducing activity is dependent on its polypeptide chain as well. Further, selective removal of O-linked oligosaccharides from mZP3 destroyed its sperm receptor activity, whereas no such effect was observed on the removal of N-linked oligosaccharides (Florman and Wassarman, 1985). In addition, deglycosylation studies have also indicated that neither N-linked oligosaccharides nor sialic acid is an essential element of the mZP3 combining site for sperm (Litscher and Wassarman, 1996). The mechanism of sperm-ZP interaction in humans, thus, seems to be more like the porcine system, where the N-linked oligosaccharides have been implicated (Nakano *et al.*, 1996; Kudo *et al.*, 1998) and unlike the murine model, where O-linked glycans play a major role in the sperm receptor function of zona proteins (Florman *et al.*, 1984; Florman and Wassarman, 1985). However, the exact mechanism and characterization

of the critical sugar residues that play a key role in gamete interaction are yet to be clearly elucidated.

Though, the baculovirus-expressed recombinant human zona proteins obtained in the present study are functionally bioactive, they may not have identical conformation and post-translational modifications, mainly glycosylation as the native proteins. The investigations pertaining to the binding as well as acrosome reaction inducing ability of the native human zona proteins to human sperm, in addition to the critical appraisal of the importance of specific carbohydrate residues in the function of these proteins are crucial to understand the complex physiological events that take place during interaction of the sperm and the egg. However, non-availability of purified native zona proteins in substantial amounts and the ethical considerations have led to the use of recombinant DNA technology to obtain the recombinant zona proteins in large amounts. The disadvantage in employing recombinant zona proteins for functional studies is the differential glycosylation of these proteins in different expression systems, which may or may not conform to the glycosylation status of the native proteins. In a recent study, porcine ZP3 expressed in baculovirus has been shown to bind to bovine but not porcine sperm (Yonezawa *et al.*, 2005). The recombinant proteins expressed in baculovirus expression system show preferential addition of N-linked carbohydrate moieties that mainly consist of high mannose type carbohydrate chains that may change the specificity of their interactions with other proteins. The addition of high mannose type carbohydrate residues recognized by ConA in the recombinant proteins employed in the present study may be advantageous, as in humans, high mannose type carbohydrate chains indeed have been shown to be present on native zona proteins (Jimenez-Movilla *et al.*, 2004). Also, human sperm have been reported to harbor mannose binding sites on the surface that have been implicated to play a crucial role in sperm-egg interaction (Youssef *et al.*, 1996). The purification of native proteins, if not performed to homogeneity, may also lead to incorrect deductions, as in the porcine system, in an earlier report, ZP4 was demonstrated to bind to the isolated boar sperm membrane vesicles (Yurewicz *et al.*, 1993b). However, on subsequent investigations, it was ascertained that heterocomplexes of ZP3 and ZP4, and not individual proteins are responsible for high affinity binding to the boar sperm membrane vesicles, as the ZP4 preparation in the previous study contained a minor contaminant of ZP3 (Yurewicz *et al.*, 1998). Nevertheless, it may still be critical to study the functional attributes of the

zona proteins from the native source, in addition to the employment of recombinant proteins for various investigations, in order to get a better insight into the physiological role of zona proteins in the process of fertilization.

Keeping these in mind, hZP3 was cloned and expressed in mammalian expression system (CHO-K1 cells) to obtain the recombinant protein in a form that may be more acceptable and close to the native protein than the previously generated baculovirus-expressed protein. The ability of cell lysate of CHO-K1 cells transiently expressing hZP3 to bind to and induce acrosome reaction in capacitated sperm suggests that the mammalian-expressed hZP3 is functionally active (Figure 35). However, further optimization needs to be done to enable the secretion of the recombinant protein in the culture supernatant in order to purify it in the native conformation. Moreover, the purification of the recombinant hZP3 from transfected CHO-K1 cells and its characterization needs further careful execution for its acceptable extrapolation to the *in vivo* conditions.

Fertilization is a very well-coordinated and tightly regulated interaction between the sperm and the egg. A proper understanding of the signals between the sperm receptor i.e. zona and the cognate ligands on the sperm surface is mandatory to dissect out the entire process that takes place before the sperm penetrates the oolemma. In the present study, though the functions of the zona proteins have been characterized with respect to their binding ability and competence to induce acrosome reaction in human sperm, the function of the sperm counterparts have not been investigated. In fact, definitive information pertaining to the identification and characterization of sperm ligands specific for human zona proteins is still lacking in literature. Taking a cue from recent studies where sperm 26S proteasome has been implicated in human as well as porcine fertilization (Morales *et al.*, 2003; Sutovsky *et al.*, 2004), the baculovirus-expressed human zona proteins were assessed for their ability to bind to and induce acrosome reaction in human sperm in the presence of proteasome-specific inhibitors. Though the binding characteristics of ZP glycoproteins remained unaffected, the presence of proteasome inhibitors drastically reduced the $hZP3_{(1-424)}$ and hZP4 mediated acrosomal exocytosis in spermatozoa. Acrosome reaction by calcium ionophore, however, was not blocked by the presence of the proteasome inhibitors. These results suggest that the sperm proteasome does not come into play during the initial recognition event but may be crucial for ZP mediated induction of acrosome reaction in human spermatozoa.

With an overview of the available information and in light of the current findings, it becomes apparent that in addition to the role of individual zona proteins, the interplay between different zona proteins that constitute the ZP matrix also has a crucial role during fertilization. In mouse, ZP1 maintains the structural integrity of ZP matrix by cross-linking the filaments of ZP2-ZP3 heterodimers (Greve and Wassarman, 1985). Discovery of the fourth protein in human ZP, ZP1, suggested that the presence of four zona proteins may support species-specific sperm binding in humans (Lefievre *et al.*, 2004; Conner *et al.*, 2005). However, the presence of fourth ZP glycoproteins in rat too, is not sufficient to support human sperm binding to rodent eggs (Hoodbhoy *et al.*, 2005). Hence, additional determinants must be responsible for taxon-specific fertilization among mammals. As it has been difficult to ascribe sperm binding to a single protein or carbohydrate determinant in the ZP, there is a possibility that supramolecular structures play an important role in sperm-egg recognition. However, the exact mechanism that mediates species-specific interaction between the sperm and the ZP needs further elucidation.

Summary

Over the years, the ZP glycoproteins, constituting the mammalian ZP matrix have been a subject of intense investigations for gaining insight into the interplay between the sperm and the egg during fertilization. These glycoproteins, either alone, in conjunction with each other or with other factors in the physiological milieu, play an important role in different stages of fertilization. Four glycoproteins, hZP1, hZP2, hZP3 and hZP4 comprise the human ZP matrix as advocated recently. Keeping in view the elucidated functions of the different zona proteins in various mammalian species, in the present thesis, attempts have been made to understand the role of human zona proteins in their interaction with spermatozoa at the pre-fertilization stage. To achieve this, the three human zona proteins, hZP2, hZP3 and hZP4 have been expressed in *E. coli* and baculovirus-expression systems to obtain them in non-glycosylated and glycosylated forms respectively. It has helped in delineating the role of the polypeptide backbone vis-à-vis glycans in mediating different functions. For expression in *E. coli*, the hZP2, hZP3 and hZP4, excluding their respective N-terminal SS and C-terminal TD, were cloned downstream of T7 promoter in pRSET-A expression vector and expressed as polyhistidine-tagged fusion proteins in BL21[DE3]pLysS host strain of *E. coli* deficient in the *lon* and *ompT* proteases to aid in minimizing the lower molecular weight fragments which may arise due to proteolytic degradation. The expression of all the above recombinant proteins was localized exclusively in the insoluble intracellular fraction of the host cells. Hence, they were purified under denaturing conditions in the presence of 8 M urea, by Ni-NTA affinity chromatography. Subsequently, the purified recombinant proteins were renatured by extensive dialysis in buffer containing oxidized and reduced glutathione to aid in the formation of disulfide bonds. The SDS-PAGE analysis of the purified recombinant hZP2, hZP3 and hZP4 revealed bands of ~90, ~50 and ~65 kDa respectively. The approximate yield of each recombinant protein from an induced 1 litre culture (at shake flask level) of BL21[DE3]pLysS cells was 1 mg.

In addition, the hZP2, hZP3 ($hZP3_{(1-424)}$) and hZP4 including their SS and TD, along with an additional construct of hZP3, including SS but devoid of TD ($hZP3_{(1-348)}$), were cloned in baculovirus transfer vector, pAcHLT-A and expressed in *Sf21* insect cells. The baculovirus-expressed purified recombinant hZP2, $hZP3_{(1-348)}$, $hZP3_{(1-424)}$ and hZP4 had apparent molecular weights corresponding to ~105, ~55, ~65 and ~75 kDa respectively. An average yield of ~250-500 μg of each of the purified baculovirus-expressed recombinant proteins was obtained from transfected *Sf21* cells grown in one

spinner flask (50 X 10^6 cells/flask). Characterization of the carbohydrate moieties by lectin binding analysis revealed that all the baculovirus-expressed proteins contained both N-linked glycosides mainly having mannose α 1-3 and/or mannose α 1-6 residues (ConA) and O-linked glycosylation having α-O glycosides of Gal or GalNAc moieties (Jacalin). Some other oligosaccharide residues were also found to be present on these glycoproteins in lesser amounts as demonstrated by weak reactivity with other lectins. The *E. coli*-expressed zona proteins did not react with any of the lectins. These results confirmed that the recombinant proteins expressed in *E. coli* were non-glycosylated while the baculovirus-expressed proteins were glycosylated.

In order to develop a probe to recognize hZP4 and employ in functional assays, a panel of 10 MAbs, MA-1650, -1654, -1657, -1660, -1662, -1665, -1667, -1671, -1673 and -1677 was generated against baculovirus-expressed hZP4. Analysis of reactivity of the MAbs in ELISA and Western blot revealed that the above MAbs reacted only with hZP4 and not with hZP2 and hZP3. The isotype analysis revealed 7 MAbs to be of IgG1, and one each of IgG2a, IgG2b and IgM isotypes. Further analysis by indirect immunofluorescence revealed that MA-1660 reacted with native bonnet monkey ZP (bonnet monkey ZP4 and hZP4 share 92% sequence identity at aa level).

Evaluation of binding characteristics of the *E. coli*- as well as baculovirus-expressed recombinant zona proteins both by direct and indirect binding assays revealed similar results. Both the glycosylated and non-glycosylated hZP3 bound to the capacitated (acrosome-intact) spermatozoa showing two different binding patterns. Majority of the capacitated sperm showed binding of recombinant hZP3 to the equatorial region while a lesser percentage of spermatozoa exhibited the binding of the recombinant protein to the acrosomal cap. The *E. coli*- and baculovirus-expressed recombinant hZP4 also showed similar binding patterns as hZP3 but their distribution was different. In case of hZP4, a higher percentage of spermatozoa showed the binding of the recombinant protein to the acrosomal cap than to the equatorial region. Co-localization of baculovirus-expressed $hZP3_{(1\text{-}424)}$ and hZP4, labeled with different fluorochromes, on the sperm, revealed that although the two proteins may have similar localization sites on sperm, they may bind to different ligands on the sperm surface. Competitive inhibition binding studies, employing 5 and 10 molar excess of the competing zona proteins, further reconfirmed the specificity of binding of these proteins to the human sperm. For example, binding of FITC labeled hZP3 to the capacitated spermatozoa was

inhibited by the unlabeled hZP3, but not hZP2 and hZP4. The binding of the non-glycosylated and glycosylated recombinant hZP3 and hZP4 was however, restricted only to the equatorial region of the acrosome-reacted sperm. Under similar experimental conditions, the recombinant hZP2 expressed both in *E. coli* and baculovirus, failed to bind to capacitated (acrosome-intact) spermatozoa, but bound to acrosome-reacted sperm at the equatorial region. Since the non-glycosylated and glycosylated recombinant ZP proteins bind with similar binding profiles to capacitated and acrosome-reacted human spermatozoa, it might be speculated that the carbohydrate moieties of the ZP glycoproteins may not be critical in mediating the initial binding event *per se* between the sperm and the zona, and that the polypeptide backbone of the zona proteins may be sufficient for this event.

When analyzed for their ability to induce acrosome reaction in capacitated human spermatozoa, the baculovirus-expressed recombinant $hZP3_{(1-424)}$ and hZP4 were able to induce significant induction of acrosome reaction in human sperm whereas their corresponding *E. coli*-expressed counterparts failed to do so. In addition, baculovirus-expressed $hZP3_{(1-348)}$ was found to be equally competent as $hZP3_{(1-424)}$ in inducing acrosome reaction in capacitated sperm, suggesting that the TD may not have a role to play in the process of acrosomal exocytosis mediated by hZP3. The recombinant hZP2 expressed either in *E. coli* or in baculovirus did not induce acrosomal exocytosis in capacitated sperm. These results reiterate earlier findings attributing hZP3 the primary sperm receptor function and hZP2, the secondary receptor role once the sperm undergoes acrosomal exocytosis. The present investigations also envisage an important role for hZP4 in the sperm receptor function and induction of acrosomal exocytosis. Moreover, these findings suggest that though, the binding of recombinant zona proteins to the sperm may be mediated by their polypeptide backbone alone, the ability to induce acrosome reaction by hZP3 and hZP4 needs the involvement of oligosaccharide residues. In an effort to delineate the signaling pathway by which baculovirus-expressed $hZP3_{(1-424)}$ and hZP4 induce acrosome reaction in human sperm, Pertussis toxin, an inhibitor of G_i protein was employed. The results demonstrated that while $hZP3_{(1-424)}$ follows a G_i protein dependent pathway for bringing about acrosome reaction in human sperm, the mechanism followed by hZP4 is G_i protein independent.

The expression of hZP3 in mammalian expression system was also undertaken to further understand the influence of glycosylation that might be taking place in insect

cells as compared to mammals. Transient transfection studies with pcDNA6/V5-His-B mammalian expression vector harboring cDNA encoding hZP4 in CHO-K1 cells demonstrated its expression in the cell lysate as a doublet corresponding to 60-70 kDa. Further studies are needed to investigate, if hZP3 expressed by CHO-K1 cells is secreted in the culture medium. In an indirect binding assay, using MAbs generated against baculovirus-expressed $hZP3_{(1-424)}$, the cell lysate from CHO-K1 cells transfected with pcDNA-hZP3 showed binding of hZP3 to the human spermatozoa. The hZP3 present in the cell lysate of CHO-K1 cells was also competent to induce significant induction of acrosome reaction when compared with the cell lysate of untransfected CHO-K1 cells.

To ascertain the importance of specific oligosaccharide residues of zona proteins in the interaction with the spermatozoa, recombinant $hZP3_{(1-424)}$ and hZP4 were obtained by growing transfected *Sf21* cells in the presence of varying concentrations of tunicamycin to make these proteins deficient in N-linked glycosylation. Further, purified baculovirus-expressed recombinant $hZP3_{(1-424)}$ and hZP4 were treated with mild alkali to selectively remove O-linked glycosylation. Assessment of these partially deglycosylated proteins for their ability to induce acrosome reaction revealed that removal of N-linked glycosylation led to a significant reduction in the acrosome reaction inducing ability of baculovirus-expressed $hZP3_{(1-424)}$ and hZP4. On the other hand, removal of O-linked sugar residues had no significant effect on their ability to induce acrosome reaction, suggesting that in humans, N-linked carbohydrate residues of ZP glycoproteins may be more relevant in mediating acrosomal exocytosis in spermatozoa.

After analysis of the sperm-binding characteristics and acrosome reaction inducing ability of the recombinant human zona proteins, an attempt was made to understand, if the recently described sperm proteasome has a function in mediating binding and/or induction of acrosome reaction in human sperm by the zona proteins. The presence of proteasome-specific inhibitors did not influence the binding of the recombinant baculovirus-expressed hZP2, $hZP3_{(1-424)}$ and hZP4 to the spermatozoa. However, the ability of baculovirus-expressed $hZP3_{(1-424)}$ and hZP4 to induce acrosome reaction in human sperm was significantly inhibited in presence of these inhibitors. This implies that the sperm proteasome may not have a direct role in sperm-egg binding but may influence subsequent events such as zona mediated induction of acrosome reaction.

These studies will facilitate in furthering our understanding pertaining to the role of zona proteins during the process of human fertilization.

Bibliography

Abdullah M, Kierszenbaum AL. (1989) Identification of rat testis galactosyl receptor using antibodies to liver asialoglycoprotein receptor: purification and localization on surfaces of spermatogenic cells and sperm. J Cell Biol **108** 367-375.

Adham IM, Nayernia K, Engel W. (1997) Spermatozoa lacking acrosin protein show delayed fertilization. Mol Reprod Dev **46** 370-376.

Adoyo PA, Lea IA, Richardson RT, Widgren EE, O'Rand MG. (1997) Sequence and characterization of the sperm protein Sp17 from the baboon. Mol Reprod Dev **47** 66-71.

Almeida EA, Huovila AP, Sutherland AE, Stephens LE, Calarco PG, Shaw LM, Mercurio AM, Sonnenberg A, Primakoff P, Myles DG, White JM. (1995) Mouse egg integrin alpha 6 beta 1 functions as a sperm receptor. Cell **81** 1095-1104.

Arnoult C, Cardullo RA, Lemos JR, Florman HM. (1996) Activation of mouse sperm T-type Ca^{2+} channels by adhesion to the egg zona pellucida. Proc Natl Acad Sci USA **93** 13004-13009.

Auer J, Camoin L, Courtot AM, Hotellier F, De Almeida M. (2004) Evidence that P36, a human sperm acrosomal antigen involved in the fertilization process is triosephosphate isomerase. Mol Reprod Dev **68** 515-523.

Baba D, Kashiwabara S, Honda A, Yamagata K, Wu Q, Ikawa M, Okabe M, Baba T. (2002) Mouse sperm lacking cell surface hyaluronidase PH-20 can pass through the layer of cumulus cells and fertilize the egg. J Biol Chem **277** 30310-30314.

Baba T, Kashiwabara S, Watanabe K, Itoh H, Michikawa Y, Kimura K, Takada M, Fukamizu A, Arai Y. (1989a) Activation and maturation mechanisms of boar acrosin zymogen based on the deduced primary structure. J Biol Chem **264** 11920-11927.

Baba T, Michikawa Y, Kawakura K, Arai Y. (1989b) Activation of boar proacrosin is effected by processing at both N- and C-terminal portions of the zymogen molecule. FEBS Lett **244** 132-136.

Baranska W, Konwinski M, Kujawa M. (1975) Fine structure of the zona pellucida of unfertilized egg cells and embryos. J Exp Zool **192** 193-202.

Barratt CLR, Hornby DP. (1995) Induction of the acrosome reaction by rhuZP3. In: *Human sperm acrosome reaction*, Ed: Fenichel P and Parinaud J, Colloque INSERM/John Libbey Eurotext, Montrague, France, Vol 326, pp 105-122.

Bauskin AR, Franken DR, Eberspaecher U, Donner P. (1999) Characterization of human zona pellucida glycoproteins. Mol Hum Reprod **5** 534-540.

Beebe SJ, Leyton L, Burks D, Ishikawa M, Fuerst T, Dean J, Saling P. (1992) Recombinant mouse ZP3 inhibits sperm binding and induces the acrosome reaction. Dev Biol **151** 48-54.

Benoff S. (1997) Carbohydrates and fertilization: an overview. Mol Hum Reprod **3** 599-637.

Bercegeay S, Jean M, Lucas H, Barriere P. (1995) Composition of human zona pellucida as revealed by SDS-PAGE after silver staining. Mol Reprod Dev **41** 355-359.

Bialy LP, Ziemba HT, Marianowski P, Fracki S, Bury M, Wojcik C. (2001) Localization of a proteasomal antigen in human spermatozoa: immunohistochemical electron microscopic study. Folia Histochem Cytobiol **39** 129-130.

Biggers JD, Whitten WK, Whittingham DG. (1971) The culture of mouse embryos *in vitro*. In Daniel JC Jr (eds), Methods in Mammalian Embryology. WH Freeman and Co., San Francisco, USA, pp. 86-116.

Blackmore PF, Beebe SJ, Danforth DR, Alexander N. (1990) Progesterone and 17 alpha-hydroxyprogesterone. Novel stimulators of calcium influx in human sperm. J Biol Chem **265** 1376-1380.

Bleil JD, Greve JM, Wassarman PM. (1988) Identification of a secondary sperm receptor in the mouse egg zona pellucida: role in maintenance of binding of acrosome-reacted sperm to eggs. Dev Biol **128** 376-385.

Bleil JD, Wassarman PM. (1980a) Mammalian sperm-egg interaction: identification of a glycoprotein in mouse egg zonae pellucidae possessing receptor activity for sperm. Cell **20** 873-882.

Bleil JD, Wassarman PM. (1980b) Structure and function of the zona pellucida: identification and characterization of the proteins of the mouse oocyte's zona pellucida. Dev Biol **76** 185-202.

Bleil JD, Wassarman PM. (1983) Sperm-egg interactions in the mouse: sequence of events and induction of the acrosome reaction by a zona pellucida glycoprotein. Dev Biol **95** 317-324.

Bleil JD, Wassarman PM. (1986) Autoradiographic visualization of the mouse egg's sperm receptor bound to sperm. J Cell Biol **102** 1363-1371.

Bleil JD, Wassarman PM. (1988) Galactose at the nonreducing terminus of O-linked oligosaccharides of mouse egg zona pellucida glycoprotein ZP3 is essential for the glycoprotein's sperm receptor activity. Proc Natl Acad Sci USA **85** 6778-6782.

Bleil JD, Wassarman PM. (1990) Identification of a ZP3-binding protein on acrosome-intact mouse sperm by photoaffinity crosslinking. Proc Natl Acad Sci USA **87** 5563-5567.

Bookbinder LH, Cheng A, Bleil JD. (1995) Tissue- and species-specific expression of sp56, a mouse sperm fertilization protein. Science **269** 86-89.

Bork P, Sander C. (1992) A large domain common to sperm receptors (Zp2 and Zp3) and TGF-beta type III receptor. FEBS Lett **300** 237-240.

Bork P. (1993) A trefoil domain in the major rabbit zona pellucida protein. Protein Sci **2** 669-670.

Bork P. (1996) Sperm-egg binding protein or proto-oncogene? Science **271** 1431-1432.

Burkin HR, Miller DJ. (2000) Zona pellucida protein binding ability of porcine sperm during epididymal maturation and the acrosome reaction. Dev Biol **222** 99-109.

Burks DJ, Carballada R, Moore HD, Saling PM. (1995) Interaction of a tyrosine kinase from human sperm with the zona pellucida at fertilization. Science **269** 83-86.

Cameo MS, Blaquier JA. (1976) Androgen-controlled specific proteins in rat epididymis. J Endocrinol **69** 47-55.

Cechova AK, Topfer-Petersen E, Henschen A. (1988) Boar proacrosin is a single-chain molecule which has the N-terminus of the acrosin α-chain (light chain). FEBS Lett **241** 136-140.

Chamberlin ME, Dean J. (1990) Human homolog of the mouse sperm receptor. Proc Natl Acad Sci USA **87** 6014-6018.

Chapman NR, Kessopoulou E, Andrews P, Hornby D, Barratt CR. (1998) The polypeptide backbone of recombinant human zona pellucida glycoprotein-3 initiates acrosomal exocytosis in human spermatozoa *in vitro*. Biochem J **330** 839-845.

Chen J, Litscher ES, Wassarman PM. (1998) Inactivation of the mouse sperm receptor, mZP3, by site-directed mutagenesis of individual serine residues located at the combining site for sperm. Proc Natl Acad Sci USA **95** 6193-6197.

Chen MS, Tung KS, Coonrod SA, Takahashi Y, Bigler D, Chang A, Yamashita Y, Kincade PW, Herr JC, White JM. (1999) Role of the integrin-associated protein CD9 in binding between sperm ADAM 2 and the egg integrin alpha6beta1: implications for murine fertilization. Proc Natl Acad Sci USA **96** 11830-11835.

Cheng A, Le T, Palacios M, Bookbinder LH, Wassarman PM, Suzuki F, Bleil JD. (1994) Sperm-egg recognition in the mouse: characterization of sp56, a sperm protein having specific affinity for ZP3. J Cell Biol **125** 867-878.

Cherr GN, Yudin AI, Overstreet JW. (2001) The dual functions of GPI-anchored PH-20: hyaluronidase and intracellular signaling. Matrix Biol **20** 515-525.

Cohen DJ, Ellerman DA, Cuasnicu PS. (2000a) Mammalian sperm-egg fusion: evidence that epididymal protein DE plays a role in mouse gamete fusion. Biol Reprod **63** 462-468.

Cohen DJ, Rochwerger L, Ellerman DA, Morgenfeld MM, Busso D, Cuasnicu PS. (2000b) Relationship between the association of rat epididymal protein "DE" with spermatozoa and the behavior and function of the protein. Mol Reprod Dev **56** 180-188.

Cohen N, Wassarman PM. (2001) Association of egg zona pellucida glycoprotein mZP3 with sperm protein sp56 during fertilization in mice. Int J Dev Biol **45** 569-576.

Conner SJ, Lefievre L, Hughes DC, Barratt CL. (2005) Cracking the egg: increased complexity in the zona pellucida. Hum Reprod **20** 1148-1152.

Coonrod SA, Herr JC, Westhusin ME. (1996) Inhibition of bovine fertilization *in vitro* by antibodies to SP-10. J Reprod Fertil **107** 287-297.

Cornwall GA, Tulsiani DR, Orgebin-Crist MC. (1991) Inhibition of the mouse sperm surface alpha-D-mannosidase inhibits sperm-egg binding *in vitro*. Biol Reprod **44** 913-921.

Cowan AE, Primakoff P, Myles DG. (1986) Sperm exocytosis increases the amount of PH-20 antigen on the surface of guinea pig sperm. J Cell Biol **103** 1289-1297.

Cross NL, Morales P, Overstreet JW, Hanson FW. (1988) Induction of acrosome reactions by the human zona pellucida. Biol Reprod **38** 235-244.

Dell A, Chalabi S, Easton RL, Haslam SM, Sutton-Smith M, Patankar MS, Lattanzio F, Panico M, Morris HR, Clark GF. (2003) Murine and human zona pellucida 3 derived from mouse eggs express identical O-glycans. Proc Natl Acad Sci USA **100** 15631-15636.

Diekman AB, Westbrook-Case VA, Naaby-Hansen S, Klotz KL, Flickinger CJ, Herr JC. (1997) Biochemical characterization of sperm agglutination antigen-1, a human sperm surface antigen implicated in gamete interactions. Biol Reprod **57** 1136-1144.

Dong G, Loukinova E, Smith CW, Chen Z, Van Waes C. (1997) Genes differentially expressed with malignant transformation and metastatic tumor progression of murine squamous cell carcinoma. J Cell Biochem Suppl **28-29** 90-100.

Doren S, Landsberger N, Dwyer N, Gold L, Blanchette-Mackie J, Dean J. (1999) Incorporation of mouse zona pellucida proteins into the envelope of *Xenopus laevis* oocytes. Dev Genes Evol **209** 330-339.

Dravland JE, Llanos MN, Munn RJ, Meizel S. (1984) Evidence for the involvement of a sperm trypsinlike enzyme in the membrane events of the hamster sperm acrosome reaction. J Exp Zool **232** 117-128.

Dunbar BS, Avery S, Lee V, Prasad S, Schwahn D, Schwoebel E, Skinner S, Wilkins B. (1994) The mammalian zona pellucida: its biochemistry, immunochemistry, molecular biology and developmental expression. Reprod Fertil Dev **6** 331-347.

Dunbar BS, Timmons TM, Skinner SM, Prasad SV. (2001) Molecular analysis of a carbohydrate antigen involved in the structure and function of zona pellucida glycoproteins. Biol Reprod **65** 951-960.

Dunbar BS, Wolgemuth DJ. (1984) Structure and function of the mammalian zona pellucida, a unique extracellular matrix. In: *Modern Cell Biology*, Ed: Satri B, Alan R. Liss, New York, Vol 3, pp 77-111.

El-Mestrah M, Castle PE, Borossa G, Kan FW. (2002) Subcellular distribution of ZP1, ZP2, and ZP3 glycoproteins during folliculogenesis and demonstration of their topographical disposition within the zona matrix of mouse ovarian oocytes. Biol Reprod **66** 866-876.

Endo Y, Lee MA, Kopf GS. (1987) Evidence for the role of a guanine nucleotide-binding regulatory protein in the zona pellucida-induced mouse sperm acrosome reaction. Dev Biol **119** 210-216.

Ensslin MA, Shur BD. (2003) Identification of mouse sperm SED1, a bimotif EGF repeat and discoidin domain protein involved in sperm–egg binding. Cell **114** 405–417.

Ensslin M, Vogel T, Calvete JJ, Thole HH, Schidtke J, Matsuda T, Toepfer-Petersen E. (1998) Molecular cloning and characterization of P47, a novel boar sperm-associated zona pellucida-binding protein homologous to a family of mammalian secretory proteins. Biol Reprod **58** 1057–1064.

Epifano O, Liang LF, Dean J. (1995a) Mouse Zp1 encodes a zona pellucida protein homologous to egg envelope proteins in mammals and fish. J Biol Chem **270** 27254-27258.

Epifano O, Liang LF, Familari M, Moos MC Jr, Dean J. (1995b) Coordinate expression of the three zona pellucida genes during mouse oogenesis. Development **121** 1947-1956.

Evans JP, Schultz RM, Kopf GS. (1995) Mouse sperm-egg plasma membrane interactions: analysis of roles of egg integrins and the mouse sperm homologue of PH-30 (fertilin) beta. J Cell Sci **108** 3267-3278.

Evans JP, Schultz RM, Kopf GS. (1997) Characterization of the binding of recombinant mouse sperm fertilin alpha subunit to mouse eggs: evidence for function as a cell adhesion molecule in sperm-egg binding. Dev Biol **187** 94-106.

Evans JP. (1999) Sperm disintegrins, egg integrins, and other cell adhesion molecules of mammalian gamete plasma membrane interactions. Front Biosci **4** D114-131.

Familiari G, Nottola SA, Macchiarelli G, Micara G, Aragona C, Motta PM. (1992) Human zona pellucida during *in vitro* fertilization: an ultrastructural study using saponin, ruthenium red, and osmium-thiocarbohydrazide. Mol Reprod Dev **32** 51-61.

Familiari G, Nottola SA, Micara G, Aragona C, Motta PM. (1988) Is the sperm-binding capability of the zona pellucida linked to its surface structure? A scanning electron microscopic study of human *in vitro* fertilization. J In Vitro Fert Embryo Transf **5** 134-143.

Familiari G, Relucenti M, Heyn R, Micara G, Correr S. (2006) Three-dimensional structure of the zona pellucida at ovulation. Microsc Res Tech **69** 415-426.

Florman HM, Arnoult C, Kazam IG, Li C, O'Toole CM. (1998) A perspective on the control of mammalian fertilization by egg-activated ion channels in sperm: a tale of two channels. Biol Reprod **59** 12-16.

Florman HM, Bechtol KB, Wassarman PM. (1984) Enzymatic dissection of the functions of the mouse egg's receptor for sperm. Dev Biol **106** 243-255.

Florman HM, Corron ME, Kim TD, Babcock DF. (1992) Activation of voltage-dependent calcium channels of mammalian sperm is required for zona pellucida-induced acrosomal exocytosis. Dev Biol **152** 304-314.

Florman HM, First NL. (1988) Regulation of acrosomal exocytosis. II. The zona pellucida-induced acrosome reaction of bovine spermatozoa is controlled by extrinsic positive regulatory elements. Dev Biol **128** 464-473.

Florman HM, Wassarman PM. (1985) O-linked oligosaccharides of mouse egg ZP3 account for its sperm receptor activity. Cell **41** 313-324.

Florman HM. (1994) Sequential focal and global elevations of sperm intracellular Ca^{2+} are initiated by the zona pellucida during acrosomal exocytosis. Dev Biol 165 152-164.

Fock-Nuzel R, Lottspeich F, Henschen A, Muller-Esterl W. (1984) Boar acrosin is a two-chain molecule. Isolation and primary structure of the light chain; homology with the pro-part of other serine proteinases. Eur J Biochem **141** 441-446.

Foresta C, Rossato M, Di Virgilio F. (1995) Differential modulation by protein kinase C of progesterone-activated responses in human sperm. Biochem Biophys Res Commun **206** 408-413.

Foster JA, Friday BB, Maulit MT, Blobel C, Winfrey VP, Olson GE, Kim KS, Gerton GL. (1997) AM67, a secretory component of the guinea pig sperm acrosomal matrix, is related to mouse sperm protein sp56 and the complement component 4-binding proteins. J Biol Chem **272** 12714-12722.

Franken DR, Henkel R, Kaskar K, Habenicht UF. (1996) Defining bioassay conditions to evaluate sperm/zona interaction: inhibition of zona binding mediated by solubilized human zona pellucida. J Assist Reprod Genet **13** 329-332.

Gahlay GK, Gupta SK. (2003) Glycosylation of zona pellucida glycoprotein-3 is required for inducing acrosomal exocytosis in the bonnet monkey. Cell Mol Biol (Noisy-le-grand) **49** 389-397.

Gahlay GK, Srivastava N, Govind CK, Gupta SK. (2002) Primate recombinant zona pellucida proteins expressed in *Escherichia coli* bind to spermatozoa. J Reprod Immunol **53** 67-77.

Glickman MH, Ciechanover A. (2002) The ubiquitin-proteasome proteolytic pathway: destruction for the sake of construction. Physiol Rev **82** 373-428.

Gong X, Dubois DH, Miller DJ, Shur BD. (1995) Activation of a G protein complex by aggregation of beta-1,4-galactosyltransferase on the surface of sperm. Science **269** 1718-1721.

Govind CK, Gahlay GK, Choudhury S, Gupta SK. (2001) Purified and refolded recombinant bonnet monkey (*Macaca radiata*) zona pellucida glycoprotein-B expressed in *Escherichia coli* binds to spermatozoa. Biol Reprod **64** 1147-1152.

Govind CK, Hasegawa A, Koyama K, Gupta SK. (2000) Delineation of conserved B cell epitope on bonnet monkey (*Macaca radiata*) and human zona pellucida glycoprotein-B by monoclonal antibodies demonstrated inhibition of sperm-egg binding. Biol Reprod **62** 67-75.

Graves BJ. (1995) Integrin binding revealed. Nat Struct Biol **2** 181-183.

Green DP. (1997) Three-dimensional structure of the zona pellucida. Rev Reprod **2** 147-156.

Greve JM, Wassarman PM. (1985) Mouse egg extracellular coat is a matrix of interconnected filaments possessing a structural repeat. J Mol Biol **181** 253-264.

Grootenhuis AJ, Philipsen HL, de Breet-Grijsbach JT, van Duin M. (1996) Immunocytochemical localization of ZP3 in primordial follicles of rabbit, marmoset, rhesus monkey and human ovaries using antibodies against human ZP3. J Reprod Fertil Suppl **50** 43-54.

Gulyas BJ, Yuan LC. (1985) Cortical reaction and zona hardening in mouse oocytes following exposure to ethanol. J Exp Zool **233** 269-276.

Gupta SK, Sharma M, Behera AK, Bisht R, Kaul R. (1997) Sequence of complementary deoxyribonucleic acid encoding bonnet monkey (*Macaca radiata*) zona pellucida glycoprotein-ZP1 and its high-level expression in *Escherichia coli.* Biol Reprod **57** 532-538.

Gupta SK, Yurewicz EC, Sacco AC, Kaul R, Jethanandani P, Govind CK. (1998) Human zona pellucida glycoproteins: characterization using antibodies against recombinant non-human primate ZP1, ZP2 and ZP3. Mol Hum Reprod **11** 1058-1064.

Gwatkin RB, Williams DT. (1977) Receptor activity of the hamster and mouse solubilized zona pellucida before and after the zona reaction. J Reprod Fertil **49** 55-59.

Haines BP, Rathjen PD, Hope RM, Whyatt LM, Holland MK, Breed WG. (1999) Isolation and characterization of a cDNA encoding a zona pellucida protein (ZPB) from the marsupial *Trichosurus vulpecula* (Brushtail possum). Mol Reprod Dev **52** 174-182.

Hanna WF, Kerr CL, Shaper JH, Wright WW. (2004) Lewis X-containing neoglycoproteins mimic the intrinsic ability of zona pellucida glycoprotein ZP3 to induce the acrosome reaction in capacitated mouse sperm. Biol Reprod **71** 778-789.

Hardy DM, Garbers DL. (1994) Species-specific binding of sperm proteins to the extracellular matrix (zona pellucida) of the egg. J Biol Chem **269** 19000-19004.

Hardy DM, Garbers DL. (1995) A sperm membrane protein that binds in a species-specific manner to the egg extracellular matrix is homologous to von Willebrand factor. J Biol Chem **270** 26025-26028.

Harris JD, Hibler DW, Fontenot GK, Hsu KT, Yurewicz EC, Sacco AG. (1994) Cloning and characterization of zona pellucida genes and cDNAs from a variety of mammalian species: the ZPA, ZPB and ZPC gene families. DNA Seq **4** 361-393.

Harris JD, Seid CA, Fontenot GK, Liu HF. (1999) Expression and purification of recombinant human zona pellucida proteins. Protein Expr Purif **16** 298-307.

Harrison RA, Roldan ER. (1990) Phosphoinositides and their products in the mammalian sperm acrosome reaction. J Reprod Fertil Suppl **42** 51-67.

He ZY, Brakebusch C, Fassler R, Kreidberg JA, Primakoff P, Myles DG. (2003) None of the integrins known to be present on the mouse egg or to be ADAM receptors are essential for sperm-egg binding and fusion. Dev Biol **254** 226-237.

Hedrick JL, Wardrip NJ. (1987) On the macromolecular composition of the zona pellucida from porcine oocytes. Dev Biol **121** 478-488.

Hedrick JL. (1996) Comparative structural and antigenic properties of zona pellucida glycoproteins. J Reprod Fertil Suppl **50** 9-17.

Herr JC, Flickinger CJ, Homyk M, Klotz K, John E. (1990a) Biochemical and morphological characterization of the intra-acrosomal antigen SP-10 from human sperm. Biol Reprod **42** 181-193.

Herr JC, Wright RM, John E, Foster J, Kays T, Flickinger CJ. (1990b) Identification of human acrosomal antigen SP-10 in primates and pigs. Biol Reprod **42** 377-382.

Hoffmann W, Hauser F. (1993) The P-domain or trefoil motif: a role in renewal and pathology of mucous epithelia? Trends Biochem Sci **18** 239-243.

Honda A, Siruntawineti J, Baba T. (2002) Role of acrosomal matrix proteases in sperm-zona pellucida interactions. Hum Reprod Update **8** 405-412.

Hoodbhoy T, Joshi S, Boja ES, Williams SA, Stanley P, Dean J. (2005) Human sperm do not bind to rat zonae pellucidae despite the presence of four homologous glycoproteins. J Biol Chem **280** 12721-12731.

Hou S-T, Ma A, Jones R, Hall L. (1996) Molecular cloning and characterization of rat sperm surface antigen 2B1, a glycoprotein implicated in sperm-zona binding. Mol Reprod Devel **45** 193-203.

Howes E, Pascall JC, Engel W, Jones R. (2001) Interactions between mouse ZP2 glycoprotein and proacrosin; a mechanism for secondary binding of sperm to the zona pellucida during fertilization. J Cell Sci **114** 4127-4136.

Hughes DC, Barratt CL. (1999) Identification of the true human orthologue of the mouse *Zp1* gene: evidence for greater complexity in the mammalian zona pellucida? Biochim Biophys Acta **1447** 303-306.

Hunnicutt GR, Primakoff P, Myles DG. (1996) Sperm surface protein PH-20 is bifunctional: one activity is a hyaluronidase and a second, distinct activity is required in secondary sperm-zona binding. Biol Reprod **55** 80-86.

Hynes RO. (1992) Integrins: versatility, modulation, and signaling in cell adhesion. Cell **69** 11-25.

Jansen S, Quigley M, Reik W, Jones R. (1995) Analysis of polysulfate-binding domains in porcine proacrosin, a putative zona adhesion protein from mammalian spermatozoa. Int J Dev Biol **39** 501-510.

Jethanandani P, Santhanam R, Gupta SK. (1998) Molecular cloning and expression in *Escherichia coli* of cDNA encoding bonnet monkey (*Macaca radiata*) zona pellucida glycoprotein-ZP2. Mol Reprod Dev **50** 229-239.

Jimenez-Movilla M, Aviles M, Gomez-Torres MJ, Fernandez-Colom PJ, Castells MT, de Juan J, Romeu A, Ballesta J. (2004) Carbohydrate analysis of the zona pellucida and cortical granules of human oocytes by means of ultrastructural cytochemistry. Hum Reprod **19** 1842-1855.

Johnston DS, Wright WW, Shaper JH, Hokke CH, Van den Eijnden DH, Joziasse DH. (1998) Murine sperm-zona binding, a fucosyl residue is required for a high affinity sperm-binding ligand. A second site on sperm binds a nonfucosylated, beta-galactosyl-capped oligosaccharide. J Biol Chem **273** 1888-1895.

Jones R. (1991) Interaction of zona pellucida glycoproteins, sulphated carbohydrates and synthetic polymers with proacrosin, the putative egg-binding protein from mammalian spermatozoa. Development **111** 1155-1163.

Jovine L, Janssen WG, Litscher ES, Wassarman PM. (2006) The PLAC1-homology region of the ZP domain is sufficient for protein polymerisation. BMC Biochem **7** 11.

Jovine L, Qi H, Williams Z, Litscher E, Wassarman PM. (2002) The ZP domain is a conserved module for polymerization of extracellular proteins. Nat Cell Biol **4** 457-461.

Jovine L, Qi H, Williams Z, Litscher ES, Wassarman PM. (2004) A duplicated motif controls assembly of zona pellucida domain proteins. Proc Natl Acad Sci USA **101** 5922-5927.

Jungnickel MK, Marrero H, Birnbaumer L, Lemos JR, Florman HM. (2001) Trp2 regulates entry of Ca^{2+} into mouse sperm triggered by egg ZP3. Nat Cell Biol **3** 499-502.

Kadam AL, Fateh M, Naz RK. (1995) Fertilization antigen (FA-1) completely blocks human sperm binding to human zona pellucida: FA-1 antigen may be a sperm receptor for zona pellucida in humans. J Reprod Immunol **29** 19-30.

Kalab P, Visconti P, Leclerc P, Kopf GS. (1994) p95, the major phosphotyrosine-containing protein in mouse spermatozoa, is a hexokinase with unique properties. J Biol Chem **269** 3810-3817.

Kaul R, Afzalpurkar A, Gupta SK. (1997) Expression of bonnet monkey (*Macaca radiata*) zona pellucida-3 (ZP3) in a prokaryotic system and its immunogenicity. Mol Reprod Dev **47** 140-147.

Kerr CL, Hanna WF, Shaper JH, Wright WW. (2002) Characterization of zona pellucida glycoprotein 3 (ZP3) and ZP2 binding sites on acrosome-intact mouse sperm. Biol Reprod **66** 1585-1595.

Killick R, Legan PK, Malenczak C, Richardson GP. (1995) Molecular cloning of chick beta-tectorin, an extracellular matrix molecule of the inner ear. J Cell Biol **129** 535-547.

Kim E, Baba D, Kimura M, Yamashita M, Kashiwabara S, Baba T. (2005) Identification of a hyaluronidase, Hyal5, involved in penetration of mouse sperm through cumulus mass. Proc Natl Acad Sci USA **102** 18028-18033.

Kinloch RA, Mortillo S, Stewart CL, Wassarman PM. (1991) Embryonal carcinoma cells transfected with ZP3 genes differentially glycosylated similar polypeptides and secrete active mouse sperm receptor. J Cell Biol **115** 655-664.

Kinloch RA, Mortillo S, Wassarman PM. (1992) Transgenic mouse eggs with functional hamster sperm receptors in their zona pellucida. Development **115** 937-946.

Kinloch RA, Roller RJ, Fimiani CM, Wassarman DA, Wassarman PM. (1988) Primary structure of the mouse sperm receptor polypeptide determined by genomic cloning. Proc Natl Acad Sci USA **85** 6409-6413.

Kinloch RA, Ruiz-Seiler B, Wassarman PM. (1990) Genomic organization and polypeptide primary structure of zona pellucida glycoprotein hZP3, the hamster sperm receptor. Dev Biol **142** 414-421.

Kinloch RA, Sakai Y, Wassarman PM. (1995) Mapping the mouse ZP3 combining site for sperm by exon swapping and site-directed mutagenesis. Proc Natl Acad Sci USA **92** 263-267.

Kolluri SK, Kaul R, Banerjee K, Gupta SK. (1995) Nucleotide sequence of cDNA encoding bonnet monkey (*Macaca radiata*) zona pellucida glycoprotein-ZP3. Reprod Fertil Dev 7 1209-1212.

Kong M, Richardson RT, Widgren EE, O'Rand MG. (1995) Sequence and localization of the mouse sperm autoantigenic protein, Sp17. Biol Reprod **53** 579-590.

Kubo H, Kawano T, Tsubuki S, Kawashima S, Katagiri C, Suzuki A. (1997) A major glycoprotein of *Xenopus* egg vitelline envelope, gp41, is a frog homolog of mammalian ZP3. Dev Growth Differ **39** 405-417.

Kudo K, Yonezawa N, Katsumata T, Aoki H, Nakano M. (1998) Localization of carbohydrate chains of pig sperm ligand in the glycoprotein ZPB of egg zona pellucida. Eur J Biochem **252** 492-499.

Lacy HM, Sanderson RD. (2001) Sperm protein 17 is expressed on normal and malignant lymphocytes and promotes heparan sulfate-mediated cell-cell adhesion. Blood **98** 2160-2165.

Laemmli UK. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227** 680-685.

Larson JL, Miller DJ. (1997) Sperm from a variety of mammalian species express beta1,4-galactosyltransferase on their surface. Biol Reprod **57** 442-453.

Lathrop WF, Carmichael EP, Myles DG, Primakoff P. (1990) cDNA cloning reveals the molecular structure of a sperm surface protein, PH-20, involved in sperm-egg adhesion and the wide distribution of its gene among mammals. J Cell Biol **111** 2939-2949.

Lea IA, Richardson RT, Widgren EE, O'Rand MG. (1996) Cloning and sequencing of cDNAs encoding the human sperm protein, Sp17. Biochim Biophys Acta **1307** 263-266.

Lea IA, Sivashanmugam P, O'Rand MG. (2001) Zonadhesin: characterization, localization, and zona pellucida binding. Biol Reprod **65** 1691-1700.

Lee MA, Check JH, Kopf GS. (1992) A guanine nucleotide-binding regulatory protein in human sperm mediates acrosomal exocytosis induced by the human zona pellucida. Mol Reprod Dev **31** 78-86.

Lee VH, Dunbar BS. (1993) Developmental expression of the rabbit 55-kDa zona pellucida protein and messenger RNA in ovarian follicles. Dev Biol **155** 371-382.

Lee VH, Schwoebel E, Prasad S, Cheung P, Timmons TM, Cook R, Dunbar BS. (1993) Identification and structural characterization of the 75-kDa rabbit zona pellucida protein. J Biol Chem **268** 12412-12417.

Lefievre L, Conner SJ, Salpekar A, Olufowobi O, Ashton P, Pavlovic B, Lenton W, Afnan M, Brewis IA, Monk M, Hughes DC, Barratt CL. (2004) Four zona pellucida glycoproteins are expressed in the human. Human Reprod **19** 1580-1586.

Legan PK, Rau A, Keen JN, Richardson GP. (1997) The mouse tectorins. Modular matrix proteins of the inner ear homologous to components of the sperm-egg adhesion system. J Biol Chem **272** 8791-8801.

Leyton L, Saling P. (1989) Evidence that aggregation of mouse sperm receptors by ZP3 triggers the acrosome reaction. J Cell Biol **108** 2163-2168.

Leyton L, Tomes C, Saling P. (1995) LL95 monoclonal antibody mimics functional effects of ZP3 on mouse sperm: evidence that the antigen recognized is not hexokinase. Mol Reprod Dev **42** 347-358.

Li MW, Cherr GN, Yudin AI, Overstreet JW. (1997) Biochemical characterization of the PH-20 protein on the plasma membrane and inner acrosomal membrane of cynomolgus macaque spermatozoa. Mol Reprod Dev **48** 356-366.

Liang LF, Chamow SM, Dean J. (1990) Oocyte-specific expression of mouse *Zp-2*: developmental regulation of the zona pellucida genes. Mol Cell Biol **10** 1507-1515.

Liang LF, Dean J. (1993) Conservation of mammalian secondary sperm receptor genes enables the promoter of the human gene to function in mouse oocytes. Dev Biol **156** 399-408.

Lievano A, Santi CM, Serrano CJ, Trevino CL, Bellve AR, Hernandez-Cruz A, Darszon A. (1996) T-type Ca^{2+} channels and alpha1E expression in spermatogenic cells, and their possible relevance to the sperm acrosome reaction. FEBS Lett **388** 150-154.

Lin Y, Kimmel LH, Myles DG, Primakoff P. (1993) Molecular cloning of the human and monkey sperm surface protein PH-20. Proc Natl Acad Sci USA **90** 10071-10075.

Lindsay LL, Hedrick JL. (2004) Proteolysis of *Xenopus laevis* egg envelope ZPA triggers envelope hardening. Biochem Biophys Res Commun **324** 648-654.

Lira SA, Kinloch RA, Mortillo S, Wassarman PM. (1990) An upstream region of the mouse ZP3 gene directs expression of firefly luciferase specifically to growing oocytes in transgenic mice. Proc Natl Acad Sci USA **87** 7215-7219.

Litscher ES, Qi H, Wassarman PM. (1999) Mouse zona pellucida glycoproteins mZP2 and mZP3 undergo carboxy-terminal proteolytic processing in growing oocytes. Biochemistry **38** 12280-12287.

Litscher ES, Wassarman PM. (1996) Characterization of mouse ZP3-derived glycopeptide, gp55, that exhibits sperm receptor and acrosome reaction-inducing activity *in vitro*. Biochemistry **35** 3980-3985.

Liu C, Litscher ES, Mortillo S, Sakai Y, Kinloch RA, Stewart CL, Wassarman PM. (1996) Targeted disruption of the m*Zp3* gene results in production of eggs lacking a zona pellucida and infertility in female mice. Proc Natl Acad Sci USA **93** 5431-5436.

Liu DY, Baker HW, Pearse MJ, d'Apice AJ. (1997) Normal sperm-zona pellucida interaction and fertilization *in vitro* in alpha-1,3-galactosyltransferase gene knockout mice. Mol Hum Reprod **3** 1015-1016.

Liu DY, Garrett C, Baker HW. (2003) Low proportions of sperm can bind to the zona pellucida of human oocytes. Hum Reprod **18** 2382-2389.

Llanos M, Morales P and Meizel S. (1995) Evidences for a role of a chymotrypsin-like activity in the increase in intracellular calcium required for the human sperm acrosome reaction. Noticiero de Biologia **3** 58P.

Lunsford RD, Jenkins NA, Kozak CA, Liang LF, Silan CM, Copeland NG, Dean J. (1990) Genomic mapping of murine *Zp2* and *Zp3*, two oocyte-specific loci encoding zona pellucida proteins. Genomics **6** 184-187.

Macek MB, Shur BD. (1988) Protein-carbohydrate complementarity in mammalian gamete recognition. Gamete Res **20** 93-109.

Martinez ML, Fontenot GK, Harris JD. (1996) The expression and localization of zona pellucida glycoproteins and mRNA in cynomolgus monkeys (*Macaca fascicularis*). J Reprod Fertil Suppl **50** 35-41.

Maymon BB, Maymon R, Ben-Nun I, Ghetler Y, Shalgi R, Skutelsky E. (1994) Distribution of carbohydrates in the zona pellucida of human oocytes. J Reprod Fertil **102** 81-86.

McLeskey SB, Dowds C, Carballada R, White RR, Saling PM. (1998) Molecules involved in mammalian sperm-egg interaction. Int Rev Cytol **177** 57-113.

Meizel S. (1985) Molecules that initiate or help stimulate the acrosome reaction by their interaction with the mammalian sperm surface. Am J Anat **174** 285-302.

Michaut M, Tomes CN, De Blas G, Yunes R, Mayorga LS. (2000) Calcium-triggered acrosomal exocytosis in human spermatozoa requires the coordinated activation of Rab3A and N-ethylmaleimide-sensitive factor. Proc Natl Acad Sci USA **97** 9996-10001.

Millar SE, Lader E, Liang LF, Dean J. (1991) Oocyte-specific factors bind a conserved upstream sequence required for mouse zona pellucida promoter activity. Mol Cell Biol **11** 6197-6204.

Millar SE, Lader ES, Dean J. (1993) ZAP-1 DNA binding activity is first detected at the onset of zona pellucida gene expression in embryonic mouse oocytes. Dev Biol **158** 410-413.

Miller DJ, Gong X, Decker G, Shur BD. (1993) Egg cortical granule N-acetylglucosaminidase is required for the mouse zona block to polyspermy. J Cell Biol **123** 1431-1440.

Miller DJ, Macek MB, Shur BD. (1992) Complementarity between sperm surface beta-1,4-galactosyltransferase and egg-coat ZP3 mediates sperm-egg binding. Nature **357** 589-593.

Minke B, Cook B. (2002) TRP channel proteins and signal transduction. Physiol Rev **82** 429-472.

Miranda PV, Gonzalez-Echeverria F, Marin-Briggiler CI, Brandelli A, Blaquier JA, Tezon JG. (1997) Glycosidic residues involved in human sperm-zona pellucida binding *in vitro*. Mol Hum Reprod **3** 399-404.

Moller CC, Bleil JD, Kinloch RA, Wassarman PM. (1990) Structural and functional relationships between mouse and hamster zona pellucida glycoproteins. Dev Biol **137** 276-286.

Moller CC, Wassarman PM. (1989) Characterization of a proteinase that cleaves zona pellucida glycoprotein ZP2 following activation of mouse eggs. Dev Biol **132** 103-112.

Morales P, Kong M, Pizarro E, Pasten C. (2003) Participation of the sperm proteasome in human fertilization. Hum Reprod **18** 1010-1017.

Morales P, Llanos M. (1996) Interaction of human spermatozoa with the zona pellucida of oocyte: Development of the acrosome reaction. Front Biosci **1** 146-160.

Morales P, Pizarro E, Kong M, Jara M. (2004) Extracellular localization of proteasomes in human sperm. Mol Reprod Dev **68** 115-124.

Morales P, Socias T, Cortez J, Llanos MN. (1994) Evidences for the presence of chymotrypsin-like activity in human spermatozoa with a role in the acrosome reaction. Mol Reprod Dev **38** 222-230.

Moreno RD, Sepulveda MS, de Ioannes A, Barros C. (1998) The polysulphate binding domain of human proacrosin/acrosin is involved in both the enzyme activation and spermatozoa-zona pellucida interaction. Zygote **6** 75-83.

Mori E, Baba T, Iwamatsu A, Mori T. (1993) Purification and characterization of a 38-kDa protein, sp38, with zona pellucida-binding property from porcine epididymal sperm. Biochem Biophys Res Commun **196** 196-202.

Mori E, Kashiwabara S, Baba T, Inagaki Y, Mori T. (1995) Amino acid sequences of porcine Sp38 and proacrosin required for binding to the zona pellucida. Dev Biol **168** 575-583.

Mori K, Daitoh T, Irahara M, Kamada M, Aono T. (1989) Significance of D-mannose as a sperm receptor site on the zona pellucida in human fertilization. Am J Obstet Gynecol **161** 207-211.

Mortillo S, Wassarman PM. (1991) Differential binding of gold-labelled zona pellucida glycoproteins mZP2 and mZP3 to mouse sperm membrane compartments. Development **113** 141-149.

Myles DG, Kimmel LH, Blobel CP, White JM, Primakoff P. (1994) Identification of a binding site in the disintegrin domain of fertilin required for sperm-egg fusion. Proc Natl Acad Sci USA **91** 4195-4198.

Myles DG, Primakoff P. (1984) Localized surface antigens of guinea pig sperm migrate to new regions prior to fertilization. J Cell Biol **99** 1634-1641.

Myles DG, Primakoff P. (1997) Why did the sperm cross the cumulus? To get to the oocyte. Functions of the sperm surface proteins PH-20 and fertilin in arriving at, and fusing with, the egg. Biol Reprod **56** 320-327.

Nakano M, Yonezawa N, Hatanaka Y, Noguchi S. (1996) Structure and function of the N-linked carbohydrate chains of pig zona pellucida glycoproteins. J Reprod Fertil Suppl **50** 25-34.

Naz RK, Ahmad K. (1994) Molecular identities of human sperm proteins that bind human zona pellucida: nature of sperm-zona interaction, tyrosine kinase activity, and involvement of FA-1. Mol Reprod Dev **39** 397-408.

Naz RK, Zhu X. (1997) Molecular cloning and sequencing of cDNA encoding for a novel testis-specific antigen. Mol Reprod Dev **48** 449-457.

Naz RK, Zhu X. (2001) Molecular cloning and sequencing of a novel cDNA encoding for a protein involved in human sperm function. Biochem Biophys Res Commun **284** 911-917.

Nixon B, Lu Q, Wassler MJ, Foote CI, Ensslin MA, Shur BD. (2001) Galactosyltransferase function during mammalian fertilization. Cells Tissues Organs **168** 46-57.

O'Rand MG, Porter JP. (1982) Purification of rabbit sperm autoantigens by preparative SDS gel electrophoresis: amino acid and carbohydrate content of RSA-1. Biol Reprod **27** 713-721.

O'Rand MG, Widgren EE, Fisher SJ. (1988) Characterization of the rabbit sperm membrane autoantigen, RSA, as a lectin-like zona binding protein. Dev Biol **129** 231-40.

Oehninger S, Patankar M, Seppala M, Clark GF. (1998) Involvement of selectin-like carbohydrate binding specificity in human gamete interaction. Andrologia **30** 269-274.

Osman RA, Andria ML, Jones AD, Meizel S. (1989) Steroid induced exocytosis: the human sperm acrosome reaction. Biochem Biophys Res Commun **160** 828-833.

Parillo F, Fagioli O, Dall'Aglio C, Verini-Supplizi A. (2000) Lectin histochemical detection of sulfoglycans in the zona pellucida of mammalian antral oocytes. Acta Histochem **102** 193-202.

Pasten C, Morales P, Kong M. (2005) Role of the sperm proteasome during fertilization and gamete interaction in the mouse. Mol Reprod Dev **71** 209-219.

Patankar MS, Oehninger S, Barnett T, Williams RL, Clark GF. (1993) A revised structure for fucoidan may explain some of its biological activities. J Biol Chem **268** 21770-21776.

Patra AK, Gahlay GK, Reddy BV, Gupta SK, Panda AK. (2000) Refolding, structural transition and spermatozoa-binding of recombinant bonnet monkey (*Macaca radiata*) zona pellucida glycoprotein-C expressed in *Escherichia coli.* Eur J Biochem **267** 7075-7081.

Peterson RN, Hunt WP. (1989) Identification, isolation, and properties of a plasma membrane protein involved in the adhesion of boar sperm to the porcine zona pellucida. Gamete Res **23** 103-118.

Phillips DM, Shalgi R. (1980a) Surface architecture of the mouse and hamster zona pellucida and oocyte. J Ultrastruct Res **72**, 1-12.

Phillips DM, Shalgi RM. (1980b) Surface properties of the zona pellucida. J Exp Zool **213**, 1-8.

Philpott CC, Ringuette MJ, Dean J. (1987) Oocyte-specific expression and developmental regulation of ZP3, the sperm receptor of the mouse zona pellucida. Dev Biol **121** 568-575.

Pillai MC, Meizel S. (1991) Trypsin inhibitors prevent the progesterone-initiated increase in intracellular calcium required for the human sperm acrosome reaction. J Exp Zool **258** 384-393.

Pizarro E, Pasten C, Kong M, Morales P. (2004) Proteasomal activity in mammalian spermatozoa. Mol Reprod Dev **69** 87-93.

Planchenault T, Cechova D, Keil-Dlouha V. (1991) Matrix degrading properties of sperm serine proteinase, acrosin. FEBS Lett **294** 279-281.

Prasad SV, Wilkins B, Skinner SM, Dunbar BS. (1996) Evaluating zona pellucida structure and function using antibodies to rabbit 55 kDa ZP protein expressed in baculovirus expression system. Mol Reprod Dev **43** 519-529.

Primakoff P, Hyatt H, Myles DG. (1985) A role for the migrating sperm surface antigen PH-20 in guinea pig sperm binding to the egg zona pellucida. J Cell Biol **101** 2239-2244.

Qi H, Williams Z, Wassarman PM. (2002) Secretion and assembly of zona pellucida glycoproteins by growing mouse oocytes microinjected with epitope-tagged cDNAs for mZP2 and mZP3. Mol Biol Cell **13** 530-541.

Quill TA, Garbers DL. (1996) Sperad is a novel sperm-specific plasma membrane protein homologous to a family of cell adhesion proteins. J Biol Chem **271** 33509-33514.

Rankin T, Familari M, Lee E, Ginsberg A, Dwyer N, Blanchette-Mackie J, Drago J, Westphal H, Dean J. (1996) Mice homozygous for an insertional mutation in the *Zp3* gene lack a zona pellucida and are infertile. Development **122** 2903-2910.

Rankin T, Talbot P, Lee E, Dean J. (1999) Abnormal zonae pellucidae in mice lacking ZP1 result in early embryonic loss. Development **126** 3847-3855.

Rankin TL, O'Brien M, Lee E, Wigglesworth K, Eppig J, Dean J. (2001) Defective zonae pellucidae in *Zp2*-null mice disrupt folliculogenesis, fertility and development. Development **128** 1119-1126.

Rankin TL, Tong ZB, Castle PE, Lee E, Gore-Langton R, Nelson LM, Dean J. (1998) Human ZP3 restores fertility in Zp3 null mice without affecting order-specific sperm binding. Development **125** 2415-2424.

Ringuette MJ, Sobieski DA, Chamow SM, Dean J. (1986) Oocyte-specific gene expression: molecular characterization of a cDNA coding for ZP-3, the sperm receptor of the mouse zona pellucida. Proc Natl Acad Sci USA **83** 4341-4345.

Roch F, Alonso CR, Akam M. (2003) Drosophila *miniature* and *dusky* encode ZP proteins required for cytoskeletal reorganisation during wing morphogenesis. J Cell Sci **116** 1199-1207.

Rochwerger L, Cuasnicu PS. (1992) Redistribution of a rat sperm epididymal glycoprotein after *in vitro* and *in vivo* capacitation. Mol Reprod Dev **31** 34-41.

Roldan ER, Murase T, Shi QX. (1994) Exocytosis in spermatozoa in response to progesterone and zona pellucida. Science **266** 1578-1581.

Roldan ER, Murase T. (1994) Polyphosphoinositide-derived diacylglycerol stimulates the hydrolysis of phosphatidylcholine by phospholipase C during exocytosis of the ram sperm acrosome. Effect is not mediated by protein kinase C. J Biol Chem **269** 23583-23589.

Sabeur K, Cherr GN, Yudin AI, Primakoff P, Li MW, Overstreet JW. (1997) The PH-20 protein in human spermatozoa. J Androl **18** 151-158.

Sacco AG, Yurewicz EC, Subramanian MG, Matzat PD. (1989) Porcine zona pellucida: association of sperm receptor activity with the alpha-glycoprotein component of the Mr = 55,000 family. Biol Reprod **41** 523-532.

Saling PM. (1991) How the egg regulates sperm function during gamete interaction: facts and fantasies. Biol Reprod **44** 246-251.

Santi CM, Darszon A, Hernandez-Cruz A. (1996) A dihydropyridine-sensitive T-type Ca^{2+} current is the main Ca^{2+} current carrier in mouse primary spermatocytes. Am J Physiol **271** C1583-1593.

Sanz L, Calvete JJ, Mann K, Schafer W, Schmid ER, Topfer-Petersen E. (1991) The amino acid sequence of AQN-3, a carbohydrate-binding protein isolated from boar sperm. Location of disulphide bridges. FEBS Lett **291** 33-36.

Sanz L, Calvete JJ, Mann K, Gabius HJ, Topfer-Petersen E. (1993) Isolation and biochemical characterization of heparin-binding proteins from boar seminal plasma: a dual role for spermadhesins in fertilization. Mol Reprod Dev **35** 37-43.

Schickler M, Lira SA, Kinloch RA, Wassarman PM. (1992) A mouse oocyte-specific protein that binds to a region of mZP3 promoter responsible for oocyte specific mZP3 gene expression. Mol Cell Biol **12** 120-127.

Schulz JR, Wessel GM, Vacquier VD. (1997) The exocytosis regulatory proteins syntaxin and VAMP are shed from sea urchin sperm during the acrosome reaction. Dev Biol **191** 80-87.

Schwoebel E, Prasad S, Timmons TM, Cook R, Kimura H, Niu EM, Cheung P, Skinner S, Avery SE, Wilkins B, Dunbar BS. (1991) Isolation and characterization of a full length cDNA encoding the 55 kDa rabbit zona pellucida protein. J Biol Chem **266** 7214-7219.

Shabanowitz RB, O'Rand MG. (1988) Characterization of the human zona pellucida from fertilized and unfertilized eggs. J Reprod Fertil **82** 151-161.

Shi X, Amindari S, Paruchuru K, Skalla D, Burkin H, Shur BD, Miller DJ. (2001) Cell surface beta-1,4-galactosyltransferase-I activates G protein-dependent exocytotic signaling. Development **128** 645-654.

Shur BD, Hall NG. (1982) A role for mouse sperm surface galactosyltransferase in sperm binding to the egg zona pellucida. J Cell Biol **95** 574-579.

Shur BD, Rodeheffer C, Ensslin MA, Lyng R, Raymond A. (2006) Identification of novel gamete receptors that mediate sperm adhesion to the egg coat. Mol Cell Endocrinol **250** 137-148.

Skutelsky E, Ranen E, Shalgi R. (1994) Variations in the distribution of sugar residues in the zona pellucida as possible species-specific determinants of mammalian oocytes. J Reprod Fertil **100** 35-41.

Snell WJ, White JM. (1996) The molecules of mammalian fertilization. Cell **85** 629-637.

Suarez SS, Wolf DP, Meizel S. (1986) Induction of the acrosome reaction in human spermatozoa by a fraction of human follicular fluid. Gamete Res **14** 107-121.

Sutovsky P, Manandhar G, McCauley TC, Caamano JN, Sutovsky M, Thompson WE, Day BN. (2004) Proteasomal interference prevents zona pellucida penetration and fertilization in mammals. Biol Reprod **71** 1625-1637.

Sutovsky P, McCauley TC, Sutovsky M, Day BN. (2003) Early degradation of paternal mitochondria in domestic pig *(Sus scrofa)* is prevented by selective proteasomal inhibitors lactacystin and MG132. Biol Reprod **68** 1793-1800.

Tanaka K. (1998) Molecular biology of the proteasome. Biochem Biophys Res Commun **247** 537-541.

Tatlow D, Brownlie R, Babiuk LA, Griebel P. (2000) Differential display analysis of gene expression during the induction of mucosal immunity. Immunogenetics **52** 73-80.

Taya T, Yamasaki N, Tsubamoto H, Hasegawa A, Koyama K. (1995) Cloning of a cDNA coding for porcine zona pellucida glycoprotein ZP1 and its genomic organization. Biochem Biophys Res Commun **207** 790-799.

Tesarik J, Carreras A, Mendoza C. (1993a) Differential sensitivity of progesterone- and zona pellucida-induced acrosome reactions to pertussis toxin. Mol Reprod Dev **34** 183-189.

Tesarik J, Moos J, Mendoza C. (1993b) Stimulation of protein tyrosine phosphorylation by a progesterone receptor on the cell surface of human sperm. Endocrinology **133** 328-335.

Tesarik J. (1985) Comparison of acrosome reaction-inducing activities of human cumulus oophorus, follicular fluid and ionophore A23187 in human sperm populations of proven fertilizing ability *in vitro*. J Reprod Fertil **74** 383-388.

Thaler CD, Cardullo RA. (1996) The initial molecular interaction between mouse sperm and the zona pellucida is a complex binding event. J Biol Chem **271** 23289-23297.

Thall AD, Maly P, Lowe JB. (1995) Oocyte Gal α1,3Gal epitopes implicated in sperm adhesion to the zona pellucida glycoprotein ZP3 are not required for fertilization in the mouse. J Biol Chem **270** 21437-21440.

Thillai-Koothan P, van Duin M, Aitken RJ. (1993) Cloning, sequencing and oocyte-specific expression of the marmoset sperm receptor protein, ZP3. Zygote **1** 93-101.

Thim L. (1989) A new family of growth factor-like peptides. 'Trefoil' disulphide loop structures as a common feature in breast cancer associated peptide (pS2), pancreatic spasmolytic polypeptide (PSP), and frog skin peptides (spasmolysins). FEBS Lett **250** 85-90.

Tomasetto C, Rio MC, Gautier C, Wolf C, Hareuveni M, Chambon P, Lathe R. (1990) hSP, the domain-duplicated homolog of pS2 protein, is co-expressed with pS2 in stomach but not in breast carcinoma. EMBO J **9** 407-414.

Tomes CN, Michaut M, De Blas G, Visconti P, Matti U, Mayorga LS. (2002) SNARE complex assembly is required for human sperm acrosome reaction. Dev Biol **243** 326-338.

Tong ZB, Nelson LM, Dean J. (1995) Inhibition of zona pellucida gene expression by antisense oligonucleotides injected into mouse oocytes. J Biol Chem **270** 849-853.

Topfer-Petersen E, Friess AE, Nguyen H, Schill WB. (1985) Evidence for a fucose-binding protein in boar spermatozoa. Histochemistry **83** 139-145.

Topfer-Petersen E, Henschen A. (1987) Acrosin shows zona and fucose binding, novel properties for a serine proteinase. FEBS Lett **226** 38-42.

Topfer-Petersen E. (1999) Carbohydrate-based interactions on the route of a spermatozoon to fertilization. Hum Reprod Update **5** 314-29.

Towbin H, Staehelin T, Gordon J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci USA **76** 4350-4354.

Tsai JY, Silver LM. (1996) Sperm-egg binding protein or proto-oncogene? Science **271** 1432-1434.

Tsubamoto H, Hasegawa A, Inoue M, Yamasaki N, Koyama K. (1996) Binding of recombinant pig zona pellucida protein 1 (ZP1) to acrosome-reacted spermatozoa. J Reprod Fertil Suppl **50** 63-67.

Tsubamoto H, Yamasaki N, Hasegewa A, Koyama K. (1999a) Expression of a recombinant porcine zona pellucida glycoprotein ZP1 in mammalian cells. Protein Expr Purif **17** 8-15.

Tsubamoto H, Hasegawa A, Nakata Y, Naito S, Yamasaki N, Koyama K. (1999b) Expression of recombinant human zona pellucida protein 2 and its binding capacity to spermatozoa. Biol Reprod **61** 1649-1654.

Tulsiani DR, Skudlarek MD, Orgebin-Crist MC. (1989) Novel alpha-D-mannosidase of rat sperm plasma membranes: characterization and potential role in sperm-egg interactions. J Cell Biol **109** 1257-1267.

Urch UA, Patel H. (1991) The interaction of boar sperm proacrosin with its natural substrate, the zona pellucida, and with polysulfated polysaccharides. Development **111** 1165-1172.

van Duin M, Polman J, De Breet IT, van Ginneken K, Bunschoten H, Grootenhuis A, Brindle J, Aitken RJ. (1994) Recombinant human zona pellucida protein ZP3 produced by Chinese Hamster Ovary cells induces the human sperm acrosome reaction and promotes sperm-egg fusion. Biol Reprod **51** 607-617.

Vo LH, Hedrick JL. (2000) Independent and hetero-oligomeric-dependent sperm binding to egg envelope glycoprotein ZPC in *Xenopus laevis*. Biol Reprod **62** 766-774.

Walensky LD, Snyder SH. (1995) Inositol 1,4,5-trisphosphate receptors selectively localized to the acrosomes of mammalian sperm. J Cell Biol **130** 857-869.

Ward CR, Kopf GS. (1993) Molecular events mediating sperm activation. Dev Biol **158** 9-34.

Ward CR, Storey BT, Kopf GS. (1994) Selective activation of G_i1 and G_i2 in mouse sperm by the zona pellucida, the egg's extracellular matrix. J Biol Chem **269** 13254-13258.

Wassarman PM, Jovine L, Litscher ES. (2001) A profile of fertilization in mammals. Nat Cell Biol **3** E59-64.

Wassarman PM, Litscher ES. (1995) Sperm-egg recognition mechanisms in mammals. Curr Top Dev Biol **30** 1-19.

Wassarman PM, Liu C, Litscher ES. (1996) Constructing the mammalian egg zona pellucida: some new pieces of an old puzzle. J Cell Sci **109** 2001-2004.

Wassarman PM, Mortillo S. (1991) Structure of the mouse egg extracellular coat, the zona pellucida. Int Rev Cytol **130** 85-110.

Wassarman PM. (1999) Mammalian fertilization: molecular aspects of gamete adhesion, exocytosis, and fusion. Cell **96** 175-183.

Williams Z, Litscher ES, Jovine L, Wassarman PM. (2006) Polypeptide encoded by mouse ZP3 exon-7 is necessary and sufficient for binding of mouse sperm *in vitro*. J Cell Physiol **207** 30-39.

Williams Z, Litscher ES, Wassarman PM. (2003) Conversion of Ser to Thr residues at the sperm combining-site of mZP3 does not affect sperm receptor activity. Biochem Biophys Res Commun **301** 813-818.

Williams Z, Wassarman PM. (2001) Secretion of mouse ZP3, the sperm receptor, requires cleavage of its polypeptide at a consensus furin cleavage-site. Biochemistry **40** 929-937.

Wistrom CA, Meizel S. (1993) Evidence suggesting involvement of a unique human sperm steroid receptor/Cl- channel complex in the progesterone-initiated acrosome reaction. Dev Biol **159** 679-690.

Wojcik C, Benchaib M, Lornage J, Czyba JC, Guerin JF. (2000) Proteasomes in human spermatozoa. Int J Androl **23** 169-177.

Wolfsberg TG, White JM. (1996) ADAMs in fertilization and development. Dev Biol **180** 389-401.

Yamagata K, Murayama K, Okabe M, Toshimori K, Nakanishi T, Kashiwabara S, Baba T. (1998) Acrosin accelerates the dispersal of sperm acrosomal proteins during acrosome reaction. J Biol Chem **273** 10470-10474.

Yamasaki N, Richardson RT, O'Rand MG. (1995) Expression of the rabbit sperm protein Sp17 in COS cells and interaction of recombinant Sp17 with the rabbit zona pellucida. Mol Reprod Dev **40** 48-55.

Yamasaki N, Tsubamoto H, Hasegawa A, Inoue M, Koyama K. (1996) Genomic organization of the gene for pig zona pellucida glycoprotein ZP1 and its expression in mammalian cells. J Reprod Fertil Suppl **50** 19-23.

Yanagimachi R. (1977) Specificity of sperm-egg interaction. In: *Immunobiology of Gametes*, Ed: Edidin M and Johnson M, Cambridge University Press, Cambridge, pp 225-295.

Yanagimachi R. (1994) Mammalian fertilization. In: *The Physiology of Reproduction*, Ed: Knobil E and Neill JD, Raven Press, New York, pp 189-317.

Yonezawa N, Fukui N, Kudo K, Nakano M. (1999) Localization of neutral N-linked carbohydrate chains in pig zona pellucida glycoprotein ZPC. Eur J Biochem **260** 57-63.

Yonezawa N, Hatanaka Y, Takeyama H, Nakano M. (1995) Binding of pig sperm receptor in the zona pellucida to the boar sperm acrosome. J Reprod Fertil **103** 1-8.

Yonezawa N, Kudo K, Terauchi H, Kanai S, Yoda N, Tanokura M, Ito K, Miura K, Katsumata T, Nakano M. (2005) Recombinant porcine zona pellucida glycoproteins expressed in *Sf9* cells bind to bovine sperm but not to porcine sperm. J Biol Chem **280** 20189-20196.

Yonezawa N, Mitsui S, Kudo K, Nakano M. (1997) Identification of an N-glycosylated region of pig zona pellucida glycoprotein ZPB that is involved in sperm binding. Eur J Biochem **248** 86-92.

Yoshida-Komiya H, Tulsiani DR, Hirayama T, Araki Y. (1999) Mannose-binding molecules of rat spermatozoa and sperm-egg interaction. Zygote **7** 335-346.

Youakim A, Hathaway HJ, Miller DJ, Gong X, Shur BD. (1994) Overexpressing sperm surface beta 1,4-galactosyltransferase in transgenic mice affects multiple aspects of sperm-egg interactions. J Cell Biol **126** 1573-1583.

Youssef HM, Doncel GF, Bassiouni BA, Acosta AA. (1996) Mannose-binding sites on human spermatozoa and sperm morphology. Fertil Steril **66** 640-645.

Yudin AI, Cherr GN, Vandevoort CA, Overstreet JW. (1998) Rearrangement of the PH-20 protein on the surface of macaque spermatozoa following exposure to anti-PH-20 antibodies or binding to zona pellucida. Mol Reprod Dev **50** 207-220.

Yurewicz EC, Hibler D, Fontenot GK, Sacco AG, Harris J. (1993a) Nucleotide sequence of cDNA encoding ZP3 alpha, a sperm-binding glycoprotein from zona pellucida of pig oocyte. Biochim Biophys Acta **1174** 211-214.

Yurewicz EC, Pack BA, Armant DR, Sacco AG. (1993b) Porcine zona pellucida ZP3 alpha glycoprotein mediates binding of the biotin-labeled M(r) 55,000 family (ZP3) to boar sperm membrane vesicles. Mol Reprod Dev **36** 382-389.

Yurewicz EC, Pack BA, Sacco AG. (1991) Isolation, composition and biological activity of sugar chains of porcine oocyte zona pellucida 55K glycoproteins. Mol Reprod Dev **30** 126-134.

Yurewicz EC, Sacco AG, Gupta SK, Xu N, Gage DA. (1998) Hetero-oligomerization-dependent binding of pig oocyte zona pellucida glycoproteins ZPB and ZPC to boar sperm membrane vesicles. J Biol Chem **273** 7488-7494.

Yurewicz EC, Sacco AG, Subramanian MG. (1987) Structural characterization of the Mr = 55,000 antigen (ZP3) of porcine oocyte zona pellucida. Purification and characterization of alpha- and beta-glycoproteins following digestion of lactosaminoglycan with endo-beta-galactosidase. J Biol Chem **262** 564-571.

Zhao M, Gold L, Dorward H, Liang LF, Hoodbhoy T, Boja E, Fales HM, Dean J. (2003) Mutation of a conserved hydrophobic patch prevents incorporation of ZP3 into the zona pellucida surrounding mouse eggs. Mol Cell Biol **23** 8982-8991.

Zhao M, Gold L, Ginsberg AM, Liang LF, Dean J. (2002) Conserved furin cleavage site not essential for secretion and integration of ZP3 into the extracellular egg coat of transgenic mice. Mol Cell Biol **22** 3111-3120.

Zhu X, Naz RK. (1998) Cloning and sequencing of cDNA encoding for a human sperm antigen involved in fertilization. Mol Reprod Dev **51** 176-183.