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Expression, Isolation and Purification of Human Zona Pellucida Protein 3

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EXPRESSION, ISOLATION AND PURIFICATION OF HUMAN ZONA PELLUCIDA PROTEIN 3

by

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ABSTRACT

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Mammalian fertilization involves interactions of sperm surface receptors with the ligands of the zona pellucida, an extracellular matrix surrounding the ovulated oocytes. In humans, the zona pellucida is composed of three major glycoproteins. One of them, ZP3, participates in the primary sperm binding and in the subsequent triggering of the spermatozoa's acrosome reaction. Studies on the role of this specific protein in the human fertilization process are hampered by the limited amount of available biologically functional proteins.

By use of a pcDNA 3.1(+) expression vector, a transfecting-vector was constructed containing a 1.3 kb histidine tagged hZP3 cDNA. This histidine tagged hZP3 cDNA containing vector was transfected to human ovarian teratocarcinoma (PA-1) cells, and a single-cell clone producing the recombinant human ZP3 glycoprotein (rhZP3) was generated. The rhZP3 glycoprotein were purified from supernatants of these cells by wheat germ agglutinin mediated glycoprotein chromatography isolation followed by the Ni-NTA mediated histidine tagged protein affinity purification. Western blotting analysis of the purified glycoproteins showed that the rhZP3 has a molecular mass ranging between

62-64 kDa which is similar to the profile of the native human ZP3. The rhZP3 triggered the acrosome reaction of spermatozoa and competitively inhibited the spermatozoa binding to the homologous zona pellucida in the hemizona assay, indicating that the recombinant glycoprotein is biologically active in both inducting the acrosome reaction and binding to its specific receptors on the surface of spermatozoa. Furthermore, the yield of the purified rhZP3 reaches the milligram level, therefore, this recombinant protein producing system can steadily supply sufficient biologically functional rhZP3 for studying the initial stages of the human fertilization process and may be adapted to clinical applications for male infatility.

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Finally, I would like to dedicate this dissertation to my parents, my dear wife, and my lovely daughter for their love and support.

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INTRODUCTION

Background

Life begins at the time of fertilization when a mature spermatozoon fuses with an ovulated oocyte to form a one-cell zygote. During fertilization, spermatozoa bind to the zona pellucida 3 (ZP3), a glycoprotein present in the oocyte extracellular glycoprotein matrix, or zona pellucida. Binding to ZP3 induces spermatozoa to undergo the acrosome reaction by which sperm cells release acrosomal lytic enzymes that promote the passage of the mobile sperm through the zona pellucida to reach the oocyte. The ZP3 plays such an important role in sperm-oocyte interaction that the study of this glycoprotein will facilitate study of the mechanism of fertilization and its disorders. Since one oocyte just contains few micrograms of zona pellucida glycoprotein depending on the species, it is very difficult to obtain sufficient amounts of ZP3 to study its biological function. Genetic engineering and molecular biology techniques are used here to circumvent this barrier and to provide a continuous source of biologically functional human ZP3 recombinant glycoprotein.

Maturation and Structure of Mammalian Spermatozoa

The spermatozoon is the end product of spermatogenesis occurring in all the seminiferous tubules. The seminiferous tubules contain large numbers of spermatogonia, located in two to three layers along the outer border of the tubular epithelium. In the first

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The model journal used for this dissertation was The Journal of *Molecular Human Reproduction.*

stage of spermatogenesis, primordial spermatogonia, called type A spermatogonia, divide four times to form 16 type B spermatogonia. At this stage, the spermatogonia migrate centrally toward and among the Sertoli cell, which forms a barrier to protect spermatogonia from being attacked by the immune system. For a period of about 24 days, each spermatogonium that crosses the barrier into the Sertoli cell layer is progressively modified to form a large primary spermatocyte. At the end of this period, each primary spermatocyte accomplishes the first meiotic division and divides into two secondary spermatocytes. Within 2 to 3 days, the second meiotic division occurs and forms four spermatids for each primary spermatocyte. During the following few weeks, each spermatid changes slowly into a spermatozoon. Unlike most invertebrates and lower vertebrates in which the spermatozoa leaving the testis already have full fertilizing capacity, the mammalian spermatozoa do not yet have the ability to fertilize oocytes while they leave the testis as highly differentiated cells. This phenomena results from the immaturity of the plasma membrane of spermatozoa and the inability of testicular spermatozoa to move progressively (Yanagimachi, 1981).

The epididymis plays a critical role in removing these functional restrictions from mammalian spermatozoa. The mammalian spermatozoa acquire their functional abilities while passing through the epididymis (Courot, 1981; Dacheus et al., 1980; Eddy et al., 1985). The deficiency of motility for testicular spermatozoa appears to be due to the immaturity of the plasma membrane and some fine metabolic adjustments such as the modification of dynein ATPase (Whit et al., 1986), the alteration in a cAMP-modulated protein kinase system (Wooten et al., 1987) and the development of the mechanism that keeps intracellular calcium ions low (Vijayaraghvan et al., 1990). The sperm plasma

membrane exposed directly to the epididymal fluid is altered step by step as the spermatozoa pass through the different regions of the epididymis (Eddy et al., 1985; Holt, 1984; Olson, 1980). The alternations of sperm plasma membrane maturation include a) changes in the distribution pattern of intramembraneous protein (Olson, 1980; Suzuki, 1981) b) cholesterol is transferred into plasma membrane of maturing spermatozoa (Suzuki, 1988; Seki et al., 1992) c) some proteins increase their negative surface charge (Yanagimachi et al., 1972; Holt, 1980) and change their lectin-binding ability dramatically while they bind to the spermatozoa (Olson et al., 1981; Magargee et al., 1988; Young et al., 1986). After fully maturating, some glycoproteins of epididymal origin or other polypeptides stabilize the plasma membrane and prevent a premature acrosome reaction, whereas others such as zona pellucida glycoprotein 2 and 3 (ZP2, ZP3) may be involved in mediating interactions between spermatozoa and the zona pellucida (Reynolds et al., 1989; Phelps et al., 1990; Phelps et al., 1987).

Mammalian spermatozoon consists of a head, a middle piece, a principal piece and an end piece. The middle piece contains the mitochondrial sheath, and the principal piece contains the fibrous sheath. The head of the mature spermatozoon is composed of the acrosome and postacrosomal regions that are separated morphologically by the equatorial segment (Figure 1). The acrosome is a membrane-bound structure covering the anterior portion of the sperm nucleus. The acrosome consists of the acrosomal cap and the equatorial segment (Yanagimachi et al., 1981). The acrosomal cap is loaded with powerful hydrolyzing enzymes such as acrosin and hyaluronidase. When sperm bind to the zona pellucida, the acrosome reaction is triggered by a series of cell-signals that induce

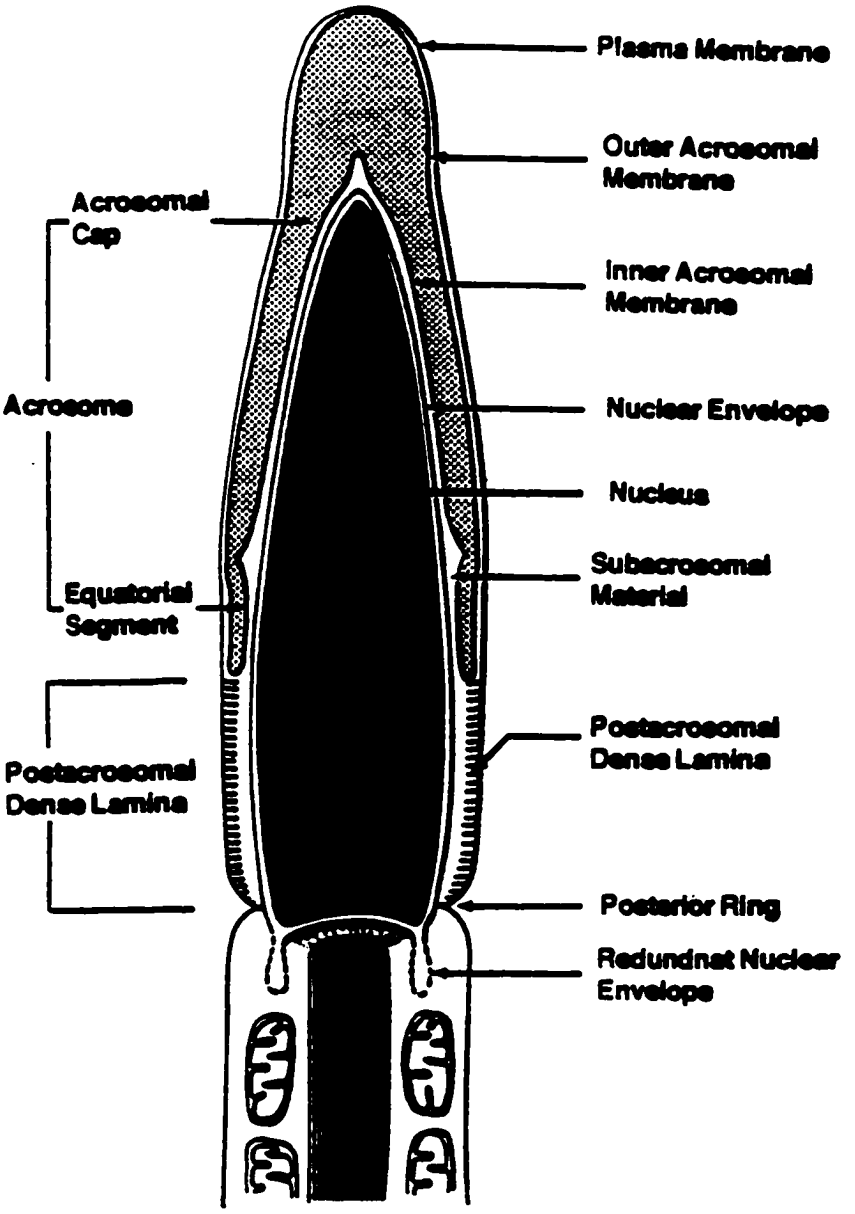

Fig. 1. The general structure of the head of a mature spermatozoon.
During the acrosome reaction, multiple fusions are formed at the outer acrosomal membrane and the overlying plasma membrane. The step occurs after specific recognition and primary binding between a putative receptor on the sperm plasma membrane and ZP3. The inner acrosomal membrane plays an important role in mediating the secondary binding between the spermatozoa and the zona pellucida.

multiple fusions between the outer acrosomal membrane and the overlying plasma membrane, which enable these hydrolyzing enzymes to be released from the acrosomal cap. These lytic enzymes help sperm to penetrate through the zona pellucida and modify the glycoproteins of the zona pellucida.

Maturation and Structure of Mammalian Oocytes

Oocytes originate from the primordial germ cells, which are found in the yolk sac endoderm and in the region of the allantois arising from the primitive streak in the early embryo. By chemotactic mechanisms, primordial germ cells migrate into the endodermal epithelium of the hindgut and then move along the dorsal mesentery of the genital ridges found in the roof of the coelom, the site of gonad development. After reaching the genital ridges, primordial germ cells proliferate for a few days. These primordial germ cells are the sole source of adult germ cells. The migration of primordial germ cells is complete while all of the cells are converted to actively dividing oogonia. A few oogonia enter the preleptotene, the interphase following the last mitotic division of oogonia, in which the final DNA replication takes place in preparation for first meiosis. This DNA synthetic activity signals the transformation of oogonia into oocytes. Oogonia progress through the meiotic process and convert themselves into nongrowing oocytes. This pool of nongrowing oocytes is the sole source of unfertilized eggs in the sexually mature adult.

At birth the ovary contains thousands of nongrowing oocytes that are hold in the prophase of first meiotic division and enclosed by a single layer of spindle-shaped

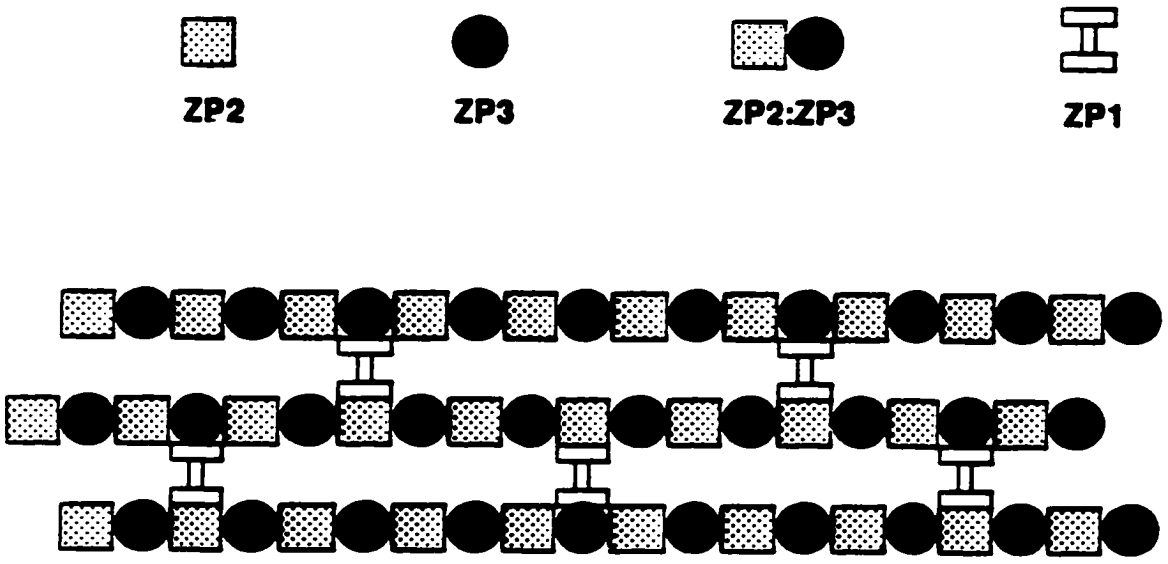

Fig. 2. Schematic representation of the arrangement of glycoproteins in mouse zona pellucida filaments. The zona pellucida is a mesh like structure composed of ZP2-ZP3 filaments which are connected by ZP1 (Wassarman, 1988).

granulosa cells. By unknown mechanisms, some of these nongrowing oocytes are pulled into the growth phase in which they synthesize and accumulate macromolecules, proliferate the surrounding granulosa cells, and lay down the extracellular zona pellucida. The zona pellucida is a mesh like structure, which is composed of ZP2-ZP3 filaments that are connected by ZP1 (Figure 2). The zona genes are not expressed in resting oocytes but are transcripted rapidly while oocytes enter the growth phase. In the latter stages of oocyte-growth, the expression of zona genes declines dramatically and there is no detectable zona pellucida synthesis in ovulated oocytes (Liang et al., 1990; Philpott et al., 1987; Bleil et al., 1980).

Fertilization

Gametogenesis results in the formation of two highly and terminally differentiated, nonproliferative haploid cells - the sperm and the oocyte. In the absence of fertilization, these cells have a relatively short life span. The interaction of these gametes at fertilization restores the ability of the fertilized oocyte to achieve full maturation and to develop into an embryo. In general, the order of initial interactions of mammalian spermatozoa and the oocyte can be divided into several steps: (i) recognition and attachment; (ii) tight binding; and (iii) secondary binding. Attachment is defined as a loose, non-specific association of the sperm head plasma membrane with the zona pellucida that follows recognition (species specific). Tight binding is a relatively strong interaction between acrosome-intact, capacitated spermatozoa and the zona pellucida. The secondary binding is mediated by ZP2 and proacrosin / acrosin located on the inner acrosomal membrane of the acrosome reacted spermatozoa (Yanagimachi, 1981).

After ejaculation, spermatozoa undergo capacitation in the female reproductive tract where their surface components acquired during epididymal transit and at ejaculation are redistributed or shed from the surface of sperm (Koehler, 1981). During capacitation, the declining of cholesterol-content of membrane changes the ratio of phospholipid / cholesterol which results in increased fluidity of the sperm membrane (Sidhu et al., 1989). At ovulation, the oocyte is surrounded by the zona pellucida which is further enveloped by the cumulus oophorus. The cumulus oophorus is composed of cumulus cells and a glycosylaminoglycan matrix which is held together by hyaluronic acid. The cumulus oophorus sets a quality control standard that only allows the capacitated spermatozoa with "intact" acrosomes to enter the cumulus (Austin, et al., 1960). Some surface enzymes of spermatozoa such as hyaluronidase, betagalactosidase, acrosin and arylsulfatase are also required for spermatozoa to penetrate the cumulus oophorus (Miyazaki, 1989; Tesarik et al., 1990). The cumulus oophorus not only serves as a barrier of spermatozoa but also provides some factors which can stimulate sperm motility and promote the acrosome reaction (Siiteri, et al., 1988; Tesarik, 1989).

Motile spermatozoa pass through the cumulus oophorus and then bind to the zona pellucida (Hartmann et al., 1972; Saling et al., 1979). Under the electron microscopy, superficial pores are found on the zona pellucida of unfertilized human oocytes. These superficial pores very similar in diameter to size of the sperm head and may be essential for sperm to penetrate the zona pellucida (Nikas et al., 1994). In mammals, binding of spermatozoa to the zona pellucida is an initial event of fertilization. Each gamete contains specific macromolecules that ensure the proper recognition and binding of sperm and oocyte as well as their subsequent fusion. In the mouse, the species best

characterized so far, the ZP3 acts as the initial sperm receptor via O-linked oligosaccharide side chain which are sterically presented by the protein backbone of the zona pellucida glycoprotein 3, ZP3 (Wassarman, 1988). The species-specific binding of sperm to ZP3, may aggregate and activate a sperm membrane protein which has tyrosine kinase property (Jones, 1990). The activated tyrosine kinase-membrane protein may trigger a signal transduction across the sperm membrane and then induces the acrosome reaction. During the acrosome reaction, the sperm plasma membrane fuses with the outer acrosomal membrane, therefore releasing the acrosomal lytic enzymes that facilitate the passage of the mobile spermatozoa through the zona pellucida. The acrosome-reacted sperm bind to zona pellucida glycoprotein 2, ZP2. This interaction is called secondary binding. Secondary binding is thought to be required for maintaining the association of the sperm with the zona pellucida while the sperm progress through it. This irreversible binding of the acrosome-reacted spermatozoa with the zona may be mediated by proacrosin/acrosin on the inner acrosomal membrane and the ZP2 in the zona pellucida (Hyne et al., 1984; Yanagimachi, 1981).

Following passage of the acrosome-reacted sperm through the zona pellucida, the spermatozoa gain access to the perivitelline space between the oocyte plasma membrane and the inner aspect of the zona pellucida. In mammals studied to date, the initial interaction of the acrosome-reacted sperm with the oocyte's plasma membrane occurs at the postacrosomal region of the sperm head (Wassarman, 1988). Since the metaphase II-arrested oocyte possess both microvillar-containing and microvillar-free domains, it was believed that the initial contact between the membranes of the respective gametes apparently occurs on a microvillar-containing domain of the oocyte (Phillips, 1991). The

microvillar-free domain overlies the metaphase spindle and may arise as a consequence of the limited release of cortical granules during oocyte maturation (Nichols et al., 1989).

The earliest detected event of mammalian oocyte activation during the fertilization is an increase in intracellular Ca^{2+} which is related to oocyte cortical granule exocytosis (Barros et al., 1988; Wassarman, 1991; Stewart-Savage et al., 1988). A consequence of cortical granule exocytosis is the releasing of cortical granule-derived glycosidases and cortical granules associated proteinases which are responsible for the loss of the sperm binding activity and the block to polyspermy (Wassarman et al., 1995). After being cleaved by these granule-derived glycosidases, the terminal carbohydrate residues of ZP3 are removed which results in losing the sperm binding ability of ZP3 (Miller et al., 1993). The ZP2 is modified by limited proteolysis which abrogates the acrosome-reacted sperm binding ability of ZP2 (Bleil et al., 1981; Ducibella et al., 1995). These modifications of zona pellucida glycoproteins respond in the zona pellucida mediated blocking of polyspermy. In the human, these functional changes are also correlated with changes in the three-dimensional structure of the zona, from one of numerous elastic ring shaped hoops with superficial pores to an amorphous mass without pores. This condensation of the outer layer of the zona pellucida may cause disorientation of sperm binding sites (Nikas et al., 1994; Familiari et al., 1988). In addition to establishing a block to polyspermy, fertilization also results in the resumption of meiosis and drives the zygote entry into the first cell cycle.

Structure of the Human Zona Pellucida Glycoproteins

The mammalian zona pellucida is composed of three secreted sulphated glycoproteins, ZP1, ZP2, and ZP3, which form heteropolymer filaments of ZP2/ZP3 that are cross-linked by ZP1 to form a mesh like structure (Wassarman et al., 1991; Wassarman, 1988). Each glycoprotein contains a signal peptide, which directs their secretion and is absent from the amino terminal sequence of the mature proteins. Human, mouse and marmoset ZP3 have a 22-amino-acid signal sequence and a 402- amino-acid secreted protein (Wassarman, 1995). All these glycoproteins possess very little α helical structure and contain a highly hydrophobic region at their carboxyl terminus which may modulate the formation of the recognizable site for spermatozoa (Liang et al., 1990). From the study of human unfertilized oocytes with silver-stained 2-D-SDS-PAGE, the human zona pellucida is composed of four major glycoproteins, termed ZP1, ZP2, and $ZP3_H$ and $ZP3_L$ (Bercegeay et al., 1995). The individual properties of the human zona pellucida components are the ZP1 (Mr =80 ~ 92 kD, pI = 4.9 ~ 5.9), the ZP2 (Mr = 58 ~ 66 kD, pI = 5.0 ~6.0), the $ZP3_H$ (Mr = 58 ~ 72 kD, pI = 3.5 ~ 5.1), the $ZP3_L$ (Mr = 54 ~ 62 kD, pI = 3.5 ~ 5.1) (Bercegeay et al., 1995).

All of these glycoproteins have relatively low isoelectric points and exhibit considerable heterogeneity on electrophoretic gels (Wassarman, 1988; Bleil et al., 1980; Wassarman, 1995). The heterogeneity of these zona glycoproteins is attributable primarily to the N-linked and O-linked oligosaccharides (Wassarman, 1991). The sialic acid at the end of the carbohydrate branches on glycoproteins may contribute to the low isoelectric point of the zona pellucida glycoproteins. The molecular weight of human ZP3 core-protein is 47,032 Da which contains 12% acidic, 8% basic, 7% aromatic, and

32% hydrophobic residues (Chamberlin and Dean, 1990). There is a very hydrophobic region which contains 26 amino acid located near the carboxyl terminal of the ZP3 core-protein. This region may play an important role in the intracellular trafficking and the interaction with the extracellular matrix (Dean, 1992).

Carbohydrate chains can be linked to proteins through the nitrogen of asparagine (N-linked), through the β-hydroxyl groups of serine, threonine, tyrosine, 5-hydroxyllysine, and 4-hydroxyproline (O-linked) or through a glycosyl-phosphatidylinositol anchor. N-linked carbohydrate chains modulate the physicochemical properties of glycoproteins by altering solubility, surface charge and adhesive properties. However, the most important role of N-linked carbohydrate is in the regulation of protein conformation. N-linked carbohydrates aid in folding of the nascent polypeptide chains and stabilization of the mature glycoproteins. Unlike the N-linked carbohydrate, the O-linked glycosylation occurs exclusively in the Golgi apparatus and the covalent attachment of N-acetylgalactosamine to an acceptor amino acid is through a α1 linkage in the O-linked glycosylation pathway.

N-linked glycans are biosynthesized by a pre-synthesized oligosaccharide moiety ($Glc_3Man_9GlcNAc_2$) which is co-translationally transferred from a lipid (dolichyldiphosphate) to the nascent polypeptide chain in the endoplasmic reticulum. These oligosaccharide moieties are further modified in endoplasmic reticulum and Golgi apparatus by trimming or adding other oligosaccharides to these carbohydrate branches. Potential N-glycosylation sites are identified as a consensus sequence Asn-Xaa-Ser/Thr in which the asparagine also has to be in an exposed position such as β-loop where it is accessible for the glycosyltransferases (Struck, et. al., 1980). N-linked glycans modulate

the physiochemical properties of the proteins to which they are attached by altering solubility, surface charge and adhesive properties. The most important role of N-linked glycan is in the regulation of protein conformation. The N-linked glycans facilitate correct disulphide bond formation, which will accelerate the folding of nascent proteins and prevent them from being degraded (Feng et. al., 1995; Rickert and Imperiali, 1995). Furthermore, the N-glycosylation is involved in the protein secretion and dimerization of zona pellucida glycoproteins (Sareneva et. al., 1994).

Mammalian ZP3 polypeptides have several potential N-linked glycosylation sites of which two are conserved. These two sites are located in relatively hydrophilic regions of polypeptide that are predicted to form reverse turns or coils (Wassarman et. al., 1995). Comparing the ZP3 of mouse, hamster, marmoset and human, each ZP3 polypeptide has 13 cysteine residues, all in identical positions, suggesting that intramolecular disulfide bonds are important in determining the glycoprotein's three-dimensional structure (Wassarman et. al., 1995). The correct folding is important for ZP3's biological function. The three-dimensional structure of ZP3 polypeptide may influence the presentation of the O-linked oligosaccharides to the receptors on spermatozoa (Wassarman, 1995).

The O-linked oligosaccharides in the C-terminal third of the ZP3 polypeptide where is quite hydrophobic domain may mediate the tight species-specific binding of free-swimming spermatozoa to unfertilized oocytes (Wassarman et. al., 1995). Unlike N-linked glycosylation, the O-linked glycosylation occurs exclusively in the Golgi apparatus. There is no lipid-coupled oligosaccharide precursor for O-linked glycosylation. Instead, the covalent attachment of N-acetylgalactosamine to an acceptor

amino acid is through an α1 linkage. Nucleotide sugars serve as substrates for all steps in O-linked glycan processing. In mouse, the O-linked oligosaccharides of ZP3 have been identified as the primary sperm-moiety in which the galactose and N-acetylglucosamine are essential for ZP3 to work as sperm receptor (Florman and Wassarman, 1985; Bleil and Wassarman, 1988).

Comparing the polypeptide primary structure of ZP3 from four different mammalian species (mouse, hamster, marmoset, and human) these four polypeptides are highly conserved in their amino acid sequence. For example, the mouse and hamster ZP3 are 82% identical; the mouse and human ZP3 are 67% identical; mouse and marmoset ZP3 are 65% identical; and the marmoset and human ZP3 are 91% identical (Wassarman et al., 1995). These highly conserved regions are proposed to play an important role in maintaining the three-dimensional structure of ZP3. The diverse regions of ZP3-polypeptide between species are believed to mediate the species-specific spermatozoa-oocyte interaction (Wassarman et al., 1995). The protease digestion study shows that the ZP3 has two domains, which are connected by a hinge region. The C-terminal domain possesses the combining site for sperm and the N-terminal domain may participate in zona pellucida filament assembly (Wassarman et al., 1995). In the mouse, the hinge region expands from amino acid 219 to 260 in which there are two proline-rich regions (residues 219-230 and residues 255-260) separated by a 24-amino-acid region. The same structure is also found in human, hamster, and marmoset (Wassarman et al., 1995).

Function of the Human Zona Pellucida Glycoproteins

The zona pellucida encases oocytes and protects the embryo from physical

damage (Edwards, 1964; Nichols et al., 1989). During the spermatozoa-oocyte interaction, these macromolecular compositions of zona pellucida not only act as specific receptors for spermatozoa but also play an important role in inducing the acrosome reaction and blocking to polyspermy (Barros et al., 1988; Wassarman, 1991). Besides, this glycoprotein-matrix also plays some important roles in regulating the normal endocrine profiles during folliculogenesis and forming a barrier to heterospecific fertilization (Stewart-Savage et al., 1988).

ZP3 is the primary receptor for spermatozoa. From studies in mice, there are four strong pieces of evidence suggesting that ZP3 use the O-linked oligosaccharide ligands on the ZP3 polypeptide backbone acting as the primary sperm receptor. First, sperm receptor activity is unaffected by destroying protein-based activity sites with a detergent (Wassarman, 1988). Second, extensive digestion with pronase also can not affect the sperm receptor activity (Leyton et al., 1989). Third, deglycosylation with trifluoromethanesulphonic acid or selective removal of O-linked oligosaccharides abrogates the ability of ZP3 to act as a sperm receptor (Florman et al., 1985). Fourth, ZP3 still keeps its sperm binding ability after being treated with endoglycosidase F which selectively removes N-linked carbohydrate (Florman et al., 1985). The interaction of ligand-receptor between the O-linked oligosaccharides of ZP3 (Wassarman et al., 1995) and a binding protein located in the sperm head plasma membrane forms multivalent interactions, which could lead to a redistribution of the binding protein, thereby converting this portion of sperm's plasma membrane to a state capable of fusing with the outer acrosomal membrane and then triggers the chain reaction leading to acrosomal reaction of spermatozoa (Florman et al., 1984; Florman et al.,1985). After fertilization,

the ZP1 may be proteolytically cleaved into small fragments whose molecular weight are similar to that of ZP2 (Bercegeay et al., 1995). This ZP1 modification can be responsible for one aspect of the zona reaction (Shabanowitz et al., 1988).

According to the concept presented by Kopf and Gerton (Kopt et al., 1991), the zona-induced acrosome reaction is mediated by a G protein pathway. The zona receptor (R_{zp}) activated by ZP3 stimulates a G protein, which in turn stimulates phospholipase C activity. Phospholipase C cleaves phosphatidylinositol (PIP_2) into diacylglycerol (DAG) and inositol triphosphate (IP_3). Inositol triphosphate increases the intracellular Ca^{2+} concentration by releasing Ca^{2+} from intracellular stores. Diacylglycerol activates Ca^{2+} dependent protein kinase C (PKC), which passes the cell signal by phosphorylating other proteins. A part of inositol triphosphate is methylated to become IP_4, which regulates the opening of voltage-dependent Ca^{2+} channels, thus allowing the influx of extracellular Ca^{2+}. Some of this Ca^{2+} acts on membrane phospholipids directly to facilitate membrane fusion. On the other pathway, the activated G protein also activates a phospholipase A_2 that cleaves phosphatidyl choline into lysophosphatidyl choline and arachidonic acid and phospholipase D that in turn cleaves phosphatidyl choline to choline and phosphatidic acid. All these products are fusogenic and may induce acrosome reaction (Flemming et al., 1981; Flemming et al., 1984). Activated G protein may trigger another chain reaction which activates adenylate cyclase, which in turn produces cAMP. The cAMP activates protein kinase A which phosphorylates proteins essential for the acrosome reaction.

The ZP2, the secondary sperm receptor, binds to proacrosin / acrosin associated with the sperm inner acrosomal membrane of acrosome-reacted spermatozoa and so bind the spermatoza more tightly to the zona pellucida (Bleil et al., 1980; Bleil et al., 1988;

Mortillo et al., 1991). After fusion of spermatozoon and oocyte, the zona pellucida triggers the zona reaction and becomes refractory both to the binding of free - swimming spermatozoa and penetration by spermatozoa that had partly penetrated the extracellular coat before fertilization. In the zona reaction, the release of the cortical granule enzyme, such as cortical granule protease and cortical granule glycosidase, into the zona pellucid may result in the partial hydrolysis or saccharide-modification of zona pellucida glycoproteins. The ZP2 which undergoes a proteolytic cleavage will associate with the block to polyspermy (Bleil, 1991; Bleil et al., 1981). After undergoing saccharide-modification, ZP3 will lose both its ability to induce the acrosome reaction and its sperm receptor activity (Lopez et al., 1985; Wassarman, 1987; Bleil et al., 1988).

Structure of the Human Zona Pellucida Protein 3 Gene

The first full - length cDNA of human ZP3 has been isolated from the human ovarian poly(A) RNA by the work of Chamberlin in 1990. This human ZP3 gene spans about 18.3 kilobase pairs and contains eight exons whose coding regions are 75% identical to those of the mouse ZP3 gene (Chamberlin et al., 1990). Both human and mouse ZP3 gene contain four short (8 - 15 bp) sequences within the first 250 bp of the 5′ flanking region. These elements may modulate oocyte-specific gene expression (Chamberlin et al., 1990). In the mouse, this region contains a cis-acting sequence located within ~ 140 bp to the gene's transcription start site which cooperate with the trans-acting factors present in growing oocytes to regulate the oocyte specific gene expression (Kinloch et al., 1989, Kinloch et al., 1989a). Both of these two genes have unusually short 5′ and 3′ untranslated regions, both contain a single open reading frame

that is 74% identical, and both code for 424 amino acid polypeptides that are 67% identical (Chamberlin et al., 1990). The similarity between the two proteins may define domains that are important in maintaining the protein structure, while the differences may play a role in mediating the species - specific events of mammalian fertilization. (Chamberlin et al., 1990; Ringuette et al., 1988).

In-Vitro Expression of Recombinant Human Zona Pellucida (rhZP3) Glycoproteins

Recombinant human ZP3 has been expressed using several different approaches, in Escherichia coli (Chapman et al., 1996), in-vitro transcription and translation (Whitmarsh et al., 1996) and in the Chinese hamster ovary (CHO) cells (Van Duin et al., 1994; Barratt et al., 1995; Brewis et al., 1996). Expression of recombinant human ZP3, using the Escherichia coli system, leads to the production of mainly insoluble protein (Chapman et al., 1996). The same situation also occurs in the baculovirus expression system using the sf9 insect cells as host cell line (Chapman et al., 1997). Insolubility is an obstacle associated with the expression of the recombinant human ZP3 in-vitro because it complicates the purification of biologically active recombinant proteins. From the study of Van Duin, the affinity - purified rhZP3 migrates between 55 - 60 kDa. The molecular size of this rhZP3 expressed in chinese hamster ovary (CHO) cells is slightly smaller than that of the native hZP3 protein (Van Duin et al., 1994). The same result was shown in a biologically active recombinant mouse ZP3 protein (Beebe et al., 1992).

The polypeptide backbone sequence of a glycoprotein is directly correlated to the sequences of DNA, but the addition and structure of carbohydrate chains is cell-, tissue-, and species-specific (Kinloch et al., 1991; Varki, 1993). These observations suggest that

the post-translational processing of these proteins during oogenesis differs to some extent from that observed in the expression system (Van Duin et al., 1994). From the previous studies, it is clear that such disparities of glycosylation do not influence the ability of these recombinant molecules to recognize complementary molecules at the sperm surface (Wassarman et al., 1995; Van Duin et al., 1994), but they may affect the capacity of these proteins to induce the acrosome reaction of sperm. Biological and functional diversities of glycoproteins are mediated by the conformational variety of their carbohydrate chains such as sequence, chain length, linkages, and covalent modification. However, the terminal sequences of carbohydrate chains are most likely to mediate specific biological function

(Varki, 1993).

Significance of the Proposed Research

Many infertility cases are due to male gamete dysfunction(s) leading to an abnormal sperm-oocyte interaction (Oehninger et. al., 1991,1997). It is very important to set up a system to identify the underlying physiopathologic mechanisms of sperm abnormalities leading to a defective sperm-oocyte interaction in patients with infertility of unknown origin. Clinical evidence indicates a high incidence of fertilization disorders in these male-factor patients, and that an abnormal sperm-zona pellucida interaction is frequently observed in these cases. Consequently, there is a fundamental need to gain a deeper understanding of human sperm-oocyte at the functional, cellular and molecular level. The molecular identity of the human sperm surface ZP3 receptor has not yet been resolved, nor is it understood what mechanisms are involved in signal transduction from

the receptor to intracellular effectors of the acrosomal exocytosis or hyperactivated motility. Among several molecules involved in the event of fertilization, ZP3 glycoprotein is believed to be the first receptor in the zona pellucida that specifically recognizes and binds with the spermatozoa. Compared to the mouse zona pellucida, less information is available for the human zona pellucida. In human, the acrosome reaction-initiating activity of intact and solubilized human zona pellucida has been reported (Shabanowitz and O'Rand, 1989; Cross, et. al., 1988). However, these experiments are performed by using very crude mixture samples with almost all of the zona pellucida components. These information can not distinguish whether hZP3 (human ZP3) alone or multi-components are involved in the induction of acrosome reaction. Therefore, a highly purified hZP3 can open a window for us to investigate the mechanism of the acrosome reaction, the pathophysiological deficiency in some cases of male infertility and the clinical diagnosis of infertility. Since ZP3 has been reported to be highly immunogenic, it may also be used for developing a long-term immunocontraceptive method (Bagavant et. al., 1997). Although the hZP3 has the potential to be such a powerful tool to answer these questions, it is very difficult to obtain sufficient amount of hZP3 from natural sources. This problem can be circumvented by using molecular cloning techniques to produce a biologically functional recombinant human ZP3 protein. Taking advantage of these techniques, we can not only produce the proteins but also we can tag the protein with a polyhistidine tail to simplify the protein purification by using a nickel chelating affinity chromatography.

The ZP proteins are glycoproteins, which support tight species-specific binding of free-swimming mammalian sperm to the unfertilized oocytes. Studies to date, suggest

that such binding is mediated in part by carbohydrate interaction (Wassarman et al., 1995). ZP3 consists of at least two different functional domains. One of the domains is included at the C-terminal third of the ZP3 polypeptide, which possess oligosaccharides that are covalently linked to serine or threonine, the O-linked oligosaccharides. From the studies in mice, results also showed that the glycosylation of one or more of five serine residues, clustered together in a polypeptide region encoded by mice ZP3 gene exon 7, is required for the sperm-binding and acrosome reaction-inducing activities (Kinloch et al., 1995). It is clear that the O-linked oligosaccharides but not N-linked oligosaccharides constitute the combining sit for biological activity of ZP3 (Florman, et al., 1985). Thus, the glycosylation is another concern in producing biological functional rhZP3 protein. Because the glycosylation is cell-, tissue- and species-specific (Varki, 1993), the host used for expressing the recombinant proteins is a critical factor for expressing rhZP3 protein preserving its biological activity. To address this concern, the PA-1 (human ovarian teratocarcinoma) cell line was chosen to express the rhZP3 protein in this research. Since the glycosylation is cell, tissue and species-specific protein modification (Varki, 1993), using PA-1 as an expression-host will match two glycosylation-criteria: tissue and species-specificity. Furthermore, PA-1 has been used as an expression host to express glycosylated native proteins such as lactosaminoglycan-carrier glycoprotein (Fukuda, et. al., 1985), heparin-binding protein (Furukawa, et. al., 1990), and recombinant fibronectin (McIlhinney and Patel, 1983) successfully. Therefore, PA-1 cell represents a valid choice as an expressing host of recombinant ZP3 glycoprotein.

The information provided by this study could have significant impact in many areas within the Reproductive Biology and Medicine field: 1) the physiology of sperm-

zona pellucida interaction in the human could be critically reviewed and expanded (i.e., temporal relationship between zona binding and penetration, acrosome reaction and motility patterns, isolation of the putative ZP3 receptor from human sperm, etc.); 2) a solid or liquid phase assay may be produced using the recombinant ZP3 thus giving access to a much simpler and universally applicable clinical diagnostic method to assess sperm-zona pellucida interaction; and 3) the generation of a naturally glycosylated recombinant ZP3 may be exploited for engineering a contraceptive vaccine against the human zona pellucida and therefore be used as a tool to drastically advance fertility regulation research.

Rationale of this Research Proposal

The binding of sperm to the zona pellucida is a crucial recognition event leading to fertilization. ZP3 plays a critical role as the primary receptor on the zona pellucida of the matured oocyte to recognize and bind the sperm (Wassarman, 1988). Moreover, it also induces the acrosome reaction of spermatozoa (Wassarman et al., 1995). Recent investigation on the composition and genetics of the zona pellucida have revealed detailed information of the structure of the zona genes, but the study of zona pellucida protein's biological function at a molecular level has been difficult. This barrier results from the difficulty in obtaining sufficient amounts of native zona pellucida proteins. This obstacle can be circumvented by using recombinant DNA technology to establish a rhZP3 protein expression system. A full-length cDNA clone of human ZP3 has been isolated and the genomic locus of human ZP3 has been well characterized (Chamberlin et al., 1990; Van Duin et al., 1992). The rhZP3 has been expressed in the CHO cell line

successfully (Van Duin et al., 1994). The reason for choosing the CHO cell as the protein expression cell line is that these cells have a good innate glycosylation quality. However the CHO cell also show some deficiency in the pattern of protein glycosylation. Van Duin et al., 1994 were not able to demonstrate binding of sperm to CHO-rhZP3. The carbohydrates of glycoproteins are not primary gene products. They are synthesized by glycosyltransferases, which are protein products of specific genes. Different cell lines may process or express different type of glycosyltransferases, which are responsible for different patterns of glycosylation. The CHO cell is a hamster overy cell line that may produce different glycosylation patterns from those of human ovary cells.

In our laboratory, total RNA was isolated from the human ovary using Chirwin's guanidium thiocyanate extraction method. This was followed by isolation of mRNA using the biotin - labeled oligo (dT) and avidin - bound polymagnetic particle method. The first single strand cDNA of hZP3 was produced by RT-PCR using a pair of oligonucleotide primers (5′ terminal or CH-1 primer and 3′ terminal or CH-2 primer) designed based upon the published sequence of the cDNA of hZP3 (Chamberlin et al., 1990). The hZP3 cDNA was subcloned into an episomal expression vector (pREP and pCEP) containing either the rous sarcoma virus promoter or the cytomegalous virus enhancer. The vectors were stablely transformed into the PA-1 cell line and the protein expression of a rhZP3 has been demonstrated (Chen, 1995). However, no pure rhZP3 protein with full functional or biological activity could originally be isolated.

The overall goal of this study is to express and purify a rhZP3 protein with functional-biological activity. Our preliminary studies showed that gel filtration chromatography and DEAE-ion-exchange purification would not effectively isolate a

pure rhZP3, because the PA-1 cell expresses many proteins possessing similar protein characteristics (such as similar molecular weight, size, and surface charge) to that of rhZP3. Besides, the rous sarcoma virus promoter and the cytomegalous virus enhancer were not efficient to obtain sufficient amounts of recombinant protein. The lower the protein expression, the more difficult the protein isolation and purification.

We therefore aimed to increase the expression of recombinant protein, to simplify the protein purification method and to enhance the specificity of protein purification as critical points to obtain a pure rhZP3. In these studies, a high-level expressing mammalian vector, the pcDNA3.1(+) vector (Invitrogen) was chosen to express a histidine tagged rhZP3, which can be purified with a nickel chelating resin. Proteins containing one or more 6xHis (six histidine) affinity tags, located at either the amino or carboxyl terminus of the protein, bind to the Ni-NTA resin(Qiagen) with a high affinity (Kd = 10^{-13} , pH 8.0), far greater than the affinity between most antibodies and antigens, or enzymes and substrates. With such high affinity, any host proteins that bind non-specifically to the Ni-NTA resin can be easily washed away under relatively stringent conditions, without affecting the binding of the 6xHis-tagged proteins. With this histidine tag, the purification of recombinant human ZP3 may be accomplished by using wheat germ agglutinin chromatography (to first isolate glycoprotein) followed by Ni-NTA affinity chromatography.

Specific Aims of This Research

Specific Aim 1: To express histidine tagged rhZP3 in the PA-1 cell line

1a: To establish a system for expression the His-tagged rhZp3 in the PA-1 cell line.

1b: To study the time course of the secretion of His-tagged rhZP3 in the PA-1 cells and to quantitate the rhZP3 production.

Because the human ZP3 gene encodes a 424 amino acid polypeptide which includes a 22-amino-acid hydrophobic putative "signal sequence" at the N terminus that is not present on the secreted glycoprotein (Wassarman et al., 1995), a tag of six histidines is fused at the C terminal of the rhZP3 glycoprotein by PCR with 5' and 3' end primers. The 3' end primer is designed to insert a set of sequence coding for 6 histidine right before the TAA stop codon of hZP3 cDNA (Wassarman, 1991). This hZP3 cDNA with 6 histidines (hZP3-6His) is introduced into the pcDNA3.1(+) vector. The pcDNA3.1(+) is a high-level mammalian expression vector which includes the human cytomegalovirus (CMV) immediate-early promoter/enhancer for high-level expression in a variety of mammalian cell lines and the bovine growth hormone (BGH) polyadenylation signal for efficient transcript stabilization and termination. For selection of stable transformants, this vector contains a neomycin resistant gene. The vectors carrying hZP3-6His (pcDNA3.1(+)-hZP3-6His) are incorporated into the PA-1 cells with the calcium phosphate-mediated transfection. The permanent transformants are selected with neomycin. A solid-phase enzyme linked immunosorbent assay (ELISA) is applied to measure the quantity of rhZP3 production by using the nondenatured purified rhZP3 as

a standard curve with which the rhZP3 concentration in collected samples can be determined.

Specific Aim 2: To isolate and purify the rhZP3

2a: To isolate the glycoprotein fraction from the conditioned culture media with wheat germ agglutinin affinity chromatography.

2b: To purify the rhZP3 from the glycoprotein fraction with the Ni-NTA nickel chelating affinity chromatography.

The glycoprotein fraction is isolated from the conditioned culture media with wheat germ agglutinin (Vector) affinity chromatography and then the histidine tagged rhZP3 is further purified from the glycoprotein fraction by Ni-NTA nickel chelating resin (Qiagen).

Specific Aim 3: Biochemical and immunological characterization of rhZP3

3a: To analyze the purified rhZP3 glycoprotein with SDS-PAGE electrophoresis and 2-D electrophoresis.

3b: To identify the rhZP3 glycoprotein with western immunoblotting.

The purity of the purified protein sample is analyzed by SDS-PAGE electrophoresis and 2-D electrophoresis. The protein-identification will be done by using western immunoblotting with use a rabbit antibody generated against a synthesized ZP3 decapeptide (D-V-T-V-G-P-L-I-F-L), the number 360 ~ 369 amino acid of human ZP3 polypeptide, as a first antibody and a goat anti-rabbit IgG conjugated with horseradish peroxidase as the second antibody.

Specific Aim 4: To evaluate the biological activity of rhZP3

4a: The acrosome reaction assay is applied to evaluate the ability of purified rhZP3 glycoprotein in inducting the acrosome reaction of spermatozoa.

4b: The hemizona assay is utilized to assess the specific binding ability of rhZP3 to spermatozoa.

Sperm-ZP interaction requires zona pellucida protein 3 (ZP3) both as sperm-oocyte binding ligand and as an acrosome reaction inducer leading to a signal transduction. An immunofluoresence technique is applied to investigate the ability of rhZP3 to induce the acrosome reaction. The hemizona assay is used to evaluate the ability of rhZP3 protein to serve as a binding ligand of sperm (Oehninger et al., 1992).

MATERIAL AND METHODS

Construction of the Recombinant hZP3-sixHis cDNA Expression Vector

Generation of Recombinant hZP3-sixHis cDNA

A mRNA extract was isolated from human ovarian tissue. Reverse Transcriptase - Polymerase Chain Reaction (RT-PCR) was performed to reverse-transcribe the hZP3 - mRNA into hZP3-cDNA. The generated hZP3-cDNA has been subcloned into the pBluescript II SK(+) plasmid (PSK) successfully by Chen, 1995.

Two pairs of primers (T3 primer/ Reverse primer and Primer B) were selected for the polymerase chain reaction (PCR) to generate the histidine tagged hZP3-cDNA from hZP3cDNA-pBluescript II SK(+) (hZP3-PSK). The T3 primer is located at the PSK from base 778 to 794 as 5'-ATA ACC CTC ACT AAAG-3' (Stratgene, La Jolla, CA). The reverse primer is located at the PSK from base 808 to 823 as 5' -AAC AGC TAT GAC CAT G- 3'(Stratgene). Primer B was designed according to the sequence of hZP3 (Chamberlin et al., 1990). Primer B is located at the 3' end of the hZP3 cDNA from base 1256 to 1282 as 5'-TT CTC GAG TTA ATG ATG ATG ATG ATG ATG TTC GGA AGC AGA CAC AGG GTG GGA GGC AGT-3'. A sequence of Xho I restriction site (CTC GAG) and a sequence coding for six histidine residues (ATG ATG ATG ATG ATG ATG) were introduced into the 5'end of primer B for the purpose of purifying the hZP3 recombinant glycoprotein from supernatants as well as for subcloning the generated histidine tagged hZP3-cDNA into the expression vector. This 3' end primer B was purchased from Gibco BRL (Grand Island, NY). These primers amplify two DNA

fragments about 1.3 kbp which contain the full length of hZP3-cDNA with a six histidine tag and a Xho I restriction site from the hZP3-PSK.

A 50 µl PCR reaction mix [5 µl Stratgene 10X reaction buffer (200 mM Tris-HCl, pH 8.0; 100 mM KCl; 60 mM $(NH_4)_2SO_4$; 20 mM $MgCl_2$; 0.1% Triton X-100; 0.25 mM dNTP (Sigma, St. Louis, MO); 0.01 µg nuclease-free bovine serum albumin (New England Biolabs, Beverly, MA); 0.5 µg T3 primer or Reverse primer; 0.5 µg primer B; 2.5U native Pfu DNA polymerase (Stratagene); 1.0 µg hZP3-PSK] was overlaid with 50 µl of mineral oil (Sigma) and run in a Perkin-Elmer Cetus DNA Thermal Cycler (Foster City, CA) for 35 cycles at 94°C, 1 min; 55°C, 1 min; and 72°C, 2min. The native Pfu DNA polymerase is isolated from *Pyrococcus furiosus*. This thermostable enzyme possesses both 5'-to- 3' DNA polymerase and 3'-to- 5' exonuclease activity. The 3'-to-5' proofreading activity results in a 12-fold increase in fidelity of DNA synthesis over Taq DNA polymerase (Flaman, et al., 1994; Cha and Tilly, 1995).

Construction of the Expression Vector

A 1.2% agarose gel electrophoresis [1.2 g Low EEO agarose (Fisher Biotech, Fair Lawn, NJ) in 100 ml TBE buffer (0.045 M Tris-Borate) (Fisher); 0.001 M EDTA (Fisher), pH 8.0; containing 20 µg Ethidium Bromide (Sigma)] was used to separate the DNA products of PCR. The 1.3 kbp hZP3-sixHis DNA band was cut out from the agarose gel and purified with a Geneclean II Kit (BLO 101, Vista, CA). The pcDNA3.1(+) plasmid (Invitrogen, Carlsbad, CA) used as a vector to generate a transfected PA-1 cell line is depicted schematically in figure 3. Briefly, the pcDNA3.1(+) vector includes the human cytomegalovirus (CMV) immediate-early

promoter/enhancer for high-level expression in a variety of mammalian cell lines, and the bovine growth hormone (BGH) polyadenylation signal for efficient transcript stabilization and termination. This vector also contains the neomycin resistance gene for selecting the stable transformants. The hZP3-sixHis-pcDNA3.1(+) was constructed by subcloning the hZP3-sixHis DNA downstream of the CMV promoter of the pcDNA3.1(+) vector.

Both hZP3-sixHis DNA and pcDNA3.1(+) were digested with BamH I / Xho I double restriction digestion. A 100 µl restriction digest mixture: 2 µg hZP3-sixHis DNA / 2µg pcDNA3.1(+); 2µl BamH I restriction enzyme (New England Biolabs, Beverly, MA); 2µl Xho I restriction enzyme (New England Biolabs); 10µl of 10X buffer (New England Biolabs); 1µl of 100X BSA (New England Biolabs). The reaction was performed at 37°C for 3 hours. Eighty microliter of H_2O and 20 µl of 3 M Sodium acetate (Sigma), pH 5.2 were added to each 100 µl restriction digested sample. Sodium acetate will neutralize the negative charge of DNA to create an ideal condition for DNA aggregation. Proteins were removed from DNA samples by phenol (Fisher) extraction which was followed by chloroform (Fisher)-2 propanol (Fisher) (24:1, v/v) extraction and then the DNA was precipitated with 100 % ethanol (Distilling Co., Inc., Pekin, IL) at -20°C overnight.

The hZP3-sixHis DNA was subcloned into pcDNA3.1(+) vector with T4 ligase (New England Biolabs). A 15 µl ligation mixture [3.5 µl of 1µg/µl hZP3-sixHis DNA; 2 µl of 1µg/µl pcDNA3.1(+); 1.5 µl T4 ligase buffer (New England Biolabs); 1 µl T4 DNA ligase (New England Biolabs); 7 µl H_2O] was used and the reaction was performed at 16°C overnight.

The DH5α E.coli. cells (BIL) were transfromed with the ligation-products. Two hundred nanogram of ligated DNA were added into 200 μl of E. coli. cells and incubated at 4°C for 1 hour. Cells were heated at 42°C for 2 minutes. After incubating at 4°C for 5 minutes, 1 ml of sterilized LB broth [10g Bacto-Tryptone (Difco, Detroit, MI); 5 g Bacto-Yeast Extract (Difco); 10 g NaCl (Fisher) in 1 L] was added and incubated at 37°C for 1 hour. E. coli cells were cultured on the ampicillin LB agar [15 g Bacto agar (Difco) in 1 L of LB broth containing 50 ~ 100 μg/ml of ampicillin (Sigma)] at 37°C overnight. The selected ampicillin resistant colonies were recultured in 20 ml of LB medium from which a number of cell containing expression vectors were amplified, and the DNA samples were purified from each selected clones with the phenol/ chloroform extraction for further identification.

Identification of the Recombinant hZP3-sixHis-pcDNA3.1(+) Expression Vectors

Confirmation of the insertion of Six Histidines Tag into the hZP3cDNA with DNA Sequencing

The Sequenase Version 2.0 DNA Sequencing Kit (Amersham) was used to perform DNA sequencing. This kit uses T7 DNA polymerase without 3'-5' exonuclease activity. The chain-termination method involves the synthesis of a DNA strand by a DNA polymerase in vitro using a single-stranded DNA template. Synthesis is initiated at only the one site where an oligonucleotide primer anneals to the template. The synthesis reaction is terminated by the incorporation of a 2', 3'-dideoxynucleoside 5'-triphosphates (ddNTPs). These nucleotides lack the 3'-OH group, which is necessary for DNA chain

elongation. When proper mixtures of dNTPs and one of the four ddNTPs are used, the enzyme-catalyzed polymerization will be terminated in a fraction of the population of chains at each site where the ddNTP is incorporated. Four separate reactions, each with ddATP, or ddTTP, or ddCTP, or ddGTP release complete sequence information by analyzing the DNA samples with 6% denature acrylamide gel. For 200 ml denature 6% Acrylamide electrophoresis gel: [30 ml 40% Acrylamide (Bio-Rad); 20 ml 10X TBE (GibcoBRL); 84 mg Urea (Fisher) in 200 ml final volume], 80 µl 20% Amoniapersulfate, and 80 µl TMEDE (Sigma). The Gel was prerun at 40 Watts for 10 minutes before loading samples.

One microliter of 2M NaOH containing 2 mM EDTA was added into 9 µl of DNA (3 ~ 5 µg DNA) and then incubated at 85°C for 5 minutes to denature the DNA. The DNA was precipitated by adding 5 µl of 1 M Sodium-Acetic Acid (Fisher), pH 5.2 and 40 µl of 100% ethanol into the denatured DNA mixture and then incubated on dry ice for 15 minutes. The DNA pellet was washed with 70% ethanol twice and then air-dried. The DNA was resuspended in 10 µl of mixture A [7 µl of H_2O; 2 µl of 5X reaction buffer (Amersham); 1 µl (10 ng DNA) of Primer]. The mixture was heated at 95°C for 5 minutes and then allowed to stand at 37°C for 15 ~ 20 minutes. The reaction mixture was prepared by adding 6 µl of mixture B [1 µl of DTT; 2 µl of label mixture (Amersham); 1 µl of ^{32}P-dATP; 2 µl of Sequenase (Amersham)] to the prepared DNA samples and then allowed to stand at room temperature for 5 minutes. Two and a half microliter of each termination mixture (ddATP, ddCTP, ddGTP, ddTTP) were added to 3.5 µl reaction mixture and then incubated at 37°C for 10 minutes. The reaction was stopped by adding 4 µl of the stop mixture and then heated up to 100°C for 5 minutes. Samples were loaded

into the gel and run at constant 25 Watts. After electrophoresis, the gel was dried with a gel dryer and then exposed to X-ray film (Fuji RX) overnight.

Confirmation of the Subcloning site of hZP3-sixHis DNA in the hZP3-sixHis-pcDNA3.1(-) Expression Vector with Restriction Endonuclease Digestion

The pcDNA3.1(+) plasmid containing hZP3-sixHis DNA [hZP3-sixHis-pcDNA3.1(+)] was isolated from the transformed E. coli clones with the alkaline lysis method (Birnboim et al., 1979; Sambrook et. al., 1989). Two milliliter of saturated growth cell were harvested by centrifugation. Pellets were resuspended in 100 µl of alkaline lysis solution I [50 mM D(+) Glucose (Sigma); 25 mM Tris-HCl (Sigma), pH 8.0; 10 mM EDTA]. Two hundred microliter of freshly prepared alkaline lysis solution II [0.2 N NaOH (Sigma); 1% SDS (Fisher)] were added into the cell suspension and incubated on ice for 5 minutes. One hundred and fifty microliter of 5 M potassium acetate [5 M potassium acetate in 30% gracial acetic acid (Fisher), pH 6] were added and incubated on ice for 5 minutes. Proteins were removed from the supernatant with phenol (Fisher) followed by chloroform (Fisher)-2 propanol (Fisher) (24:1, v/v) extraction. The DNA was precipitated with 100 % ethanol at -20°C overnight.

The purified DNA was examined by restriction endonuclease digestion to confirm the insertion of the hZP3-sixHis DNA at the downstream of the CMV promoter of the pcDNA3.1(+) vector. For Sal I restriction digestion, a 30 µl restriction digestion mixture: [1 µg hZP3-sixHis-pcDNA3.1(+); 0.5 µl Sal I restriction enzyme (New England Biolabs); 3 µl 10X buffer (New England Biolabs); 0.3 µl 100X BSA (New England Biolabs)] was used and the reaction was performed at 37°C for 3 hours. For Sma I

restriction digestion, a 30 µl restriction digestion mixture: [1 µg hZP3-sixHis-pcDNA3.1(+); 0.5 µl Sma I restriction enzyme (New England Biolabs); 3 µl 10X buffer (New England Biolabs)] was used and the reaction was performed at 25°C for 3 hours. Products of restriction endonuclease digestion were analyzed with 1 % agarose gel electrophoresis [1 g Low EEO agarose (Fisher), 100 ml TBE buffer, 20 µg Ethidium Bromide (Sigma)] at 95Volt.

Stable Transfection of PA-1 Cells with hZP3-sixHis-pcDNA3.1(+) DNA

The PA-1 cells were transfected with hZP3-sixHis-pcDNA3.1(+) DNA by using the calcium phosphate-mediated transfection method (Sambrook et al., 1989). Twenty-four hours before transfection, cells growing exponentially were harvested by trypsinization and replated at a density of $1x10^5$ to $2x10^5$ cells/ cm^2 in 60 mm tissue culture dishes (Corning) in MEM containing 10% FBS (Fetal Bovine Serum) and incubated in 5% CO_2 at 37°C. For transfecting one 60 mm monolayer of PA-1 cells: the mixture was as follows: a 50 µg/ml of DNA solution was prepared by adding 62 µl of 2M $CaCl_2$ and then sterile water was added to a final volume of 0.5 ml. The DNA solution was added drop by drop into 0.5 ml of sterile 2X HBS [280 mM NaCl; 10 mM KCl (Fisher); 1.5 mM Na_2HPO_4-2 H_2O (Fisher); 12 mM Dextrose (Sigma); 50 mM HEPES, pH 7.05 (Fisher)] with bubbling and incubated at room temperature for 30 minutes to precipitate the DNA. At the end of the incubation, the DNA precipitate was resuspended by pipetting the mixture up and down once. The culture medium was removed from the cells. The calcium phosphate-DNA suspension was transferred onto the cell monolayer and incubated at room temperature for 15 minutes. The culture medium was laid gently

onto the DNA suspension-covered cells. The cells were incubated at 37°C in a humidified incubator for 18 hours to allow the cells to incorporate the DNA by endocytosis. The cells were washed with PBS, pH 7.4 and then cultured in the culture medium for another 24 hours. The cells were replated into the selective medium [regular culture medium containing 2 mg/ml of Neomycin (Sigma)] and cultured for 2 to 3 months while changing the medium every two to three days. Since the PA-1 cell show a resistance to neomycin at the concentration of 1mg/ml, the neomycin at the concentration of 2mg/ml was used to kill the non-transfected cells. The cell replication rate will be increase even in the medium containing 2mg/ml of neomycin, if the cell density is over 70% confluence. Therefore, the cell confluence was kept at 30 to 40% confluence during the neomycin selection. After primary selection, the cells were trypsinized and a single cell was picked up with a glass needle. Selected clones were cultured in the 96-well tissue culture plate (Costa, Cambridge, MA) individually with MEM medium containing 10% FBS and 2 mg/ml of Neomycin. After reaching 80% confluency, cells were recultured into 24 well tissue culture plates (Costa) and then into 6 well tissue culture plates (Costa).

Confirmation of the Expression of the hZP3-sixHis DNA in Stably Transfected PA-1 Cells

Isolation of the Cytoplasmic RNA from Stably Transfected PA-1 Cells

One hundred-mm tissue culture plates containing 80% confluency of cells were washed with PBS. A 500 µl of Lysis buffer (10 mM Tris, pH 7.4; 1.5 mM $MgCl_2$; 0.5%

NP-40; 0.25% Sodium deoxycholate; 0.3 M Sucrose) was added onto monolayer cells which were removed from tissue culture plate with police. The cell lysate was loaded on the top of 500 µl Cushion buffer [(10 mM Tris, pH 7.4; 1.5 mM $MgCl_2$; 0.4 M Sucrose (Fisher)] and then centrifuged at 5000 x g at 4°C for 10 minutes. At this speed, the RNA will stay in the upper aqueous phase and the nuclei will sink into the cushion buffer. The upper aqueous solution was drawn into a new vial and 90 µl of Proteinase K solution [58 µl of 10X SET (100 mM Tris, pH 7.3; 10 mM EDTA; 10% SDS), 12 µl of Proteinase K (10 mg/ml) (Sigma), 17 µl of 5 M NaCl] was added. The mixture was incubated at 45°C for one hour. Proteins were removed form the solution containing RNA with Ultra Pure Phenol (GibcoBRL) extraction. One volume of iso-propanol (Fisher) was added into the RNA samples and incubated at -20°C overnight to precipitate the RNA. The concentration of RNA sample can be determined by the following equation: OD_{260} x 40 x dilution factor = RNA concentration in µg/ml.

Confirmation of the Expression of hZP3-sixHis cDNA in Stably Transfected PA-1 Cells with RT-PCR

A 30 µl RT reaction mixture: 12 µl isolated cytoplasmic RNA from the stable transfected PA-1 cell containing 11.4 µg RNA; 1 µl oligodT (GibcoBRL); 6 µl 5X M-MLV RT buffer (GibcoBRL); 2 µl nuclease free BSA 1mg/ml (GibcoBRL); 3 µl 10 mM dNTP (GibcoBRL); 2 µl M-MLV reverse transcriptase 200u/µl (GibcoBRL); 1µl RNasIn (Stratgene); 3 µl 100mM DTT (Promega). For RNA sample isolated from the PA-1 non-transfected cell, a 30 µl RT reaction mixture: 7 µl isolated cytoplasmic RNA containing 11.2µg RNA; 1 µl oligodT (GibcoBRL); 6 µl 5X M-MLV RT buffer (GibcoBRL); 2 µl

nuclease free BSA 1mg/ml (GibcoBRL); 3 μl 10 mM dNTP (GibcoBRL); 2 μl M-MLV reverse transcriptase 200u/μl (GibcoBRL); 1μl RNasIn (Stratgene); 3 μl 100mM DTT (Promega); 5 μl RNase free H_2O. The reactions were performed at 37°C for one and half hours.

The 30μl RT products were diluted to 100 μl with RNase free H_2O. A 50 μl PCR reaction mixture: [10μl of RT produce; 5μl 10X Taq reaction buffer (Promega, Madison, WI); 5μl of 2.5 M dNTP (Promega); 3.5 μl of 25 mM $MgCl_2$ (Promega); 2 μl Taq (Promega); 0.5 μl CH-1 and 0.5 μl CH-2 primer / 0.5 μl CH-1 and 0.5 μl S-1 primer / 0.5 μl CH-1 primer and 0.5 μl primer B; 23.5 μl H_2O] was overlaid with 50 μl of mineral oil and run in a Perkin-Elmer DNA thermal cycler of 35 cycles at 94°C, 1 min; 55°C, 1 min; 72°C, 2min. Products of PCR were analyzed with 1 % agarose gel electrophoresis at 95 volt.

Cell Culture and Storage of the PA-1 Cells and the Stably Transfected PA-1 Cells

Teratocarcinomas are tumors that develop spontaneously in the gonads. The PA-1 cell line (human ovarian teratocarcinoma) was originally obtained by culturing cells from the ascitic fluid of a 12-year-old girl with recurrent malignant teratoma of the ovary (Zeuthen, et. al., 1980). The PA-1 cells have a short doubling time of about 26 hours (Zeuthen, et. al., 1980). The prevalent cell type is a small, round cell that aggregates tenaciously. During serial subcultures of the cells the plating efficiency increased and the doubling time decreased (Zeuthen, et. al., 1980). Here, the PA-1 cells were cultured in Eagle's minimal essential medium (MEM medium) (Sigma) containing 5 % fetal bovine

serum (FBS) (Hyclone, Logan, Utach) and incubated at 37°C in a humidified incubator in an atmosphere of 5% CO_2 (Fukuda, et al., 1985; Furukawa, et al., 1990). After reaching 80 to 90 % confluence, cells were split from one 150-mm tissue culture dish (Corning Inc., Corning, NY) into five 150-mm tissue culture dishes. The primary selected stably transformed PA-1 cells were cultured under the same conditions as that of cultured PA-1 non-transfected cells except for supplementing with 2mg/ml of neomycin in the culture medium. After culturing for three weeks, the number of the stably transformed PA-1 cells was amplified from two 100 mm tissue culture dishes to thirty 150 mm tissue culture dishes from which the stably transformed PA-1 cells were frozen (6×10^6 cells / frozen vial) and the histidine tagged human zona pellucida glycoprotein producing PA-1 first cell stock was established.

For establishing the working cell pool, the histidine tagged human zona pellucida glycoprotein producing PA-1 cells from the cell stock were thawed and cultured in the MEM medium supplemented with 2mg/ml neomycin and 5% FBS changing the culture media every 48 hours. After culturing for three weeks, the number of the stably transformed PA-1 cells was amplified from two 100 mm tissue culture dishes to thirty 150 mm tissue culture dishes from which the stable transformed PA-1 cells were frozen (6×10^6 cells / frozen vial) and the histidine tagged human zona pellucida glycoprotein producing PA-1 working cell pool was established.

For supernatant collection, the histidine tagged human zona pellucida glycoprotein producing PA-1 cells from the working cell pool were thawed and cultured in the MEM medium supplemented with 5% FBS but without neomycin, which will inhibit the protein synthesis and reduce the protein production, with freshly changing

culture media every 48 hours. The yield of the recombinant ZP3 glycoprotein drops almost 50% after culturing the stable transformed PA-1 cell for five to seven weeks. Therefore, the stably transformed PA-1 cells are not reusable after supernatant collection.

MEM medium containing 50% FBS and 5% Dimethylsulfoxide (DMSO) (Sigma) was used as the cryogenic medium to freeze the cells -196°C in liquid nitrogen. After removing the cells from the culture-dishes with trypsin, the cells were washed with MEM without FBS and then centrifuged at 300g for 10 minutes to harvest the cells. Cell pellets were resuspended in the cryogenic medium to a final concentration of $6x10^6$ cells / ml. Each 1 ml of the cell suspension was transferred into vials and individually frozen. Vials were frozen at -80°C overnight and then transferred to liquid nitrogen for preserving at -196°C.

To thaw the cells, frozen cells in the frozen vial were incubated in a 37°C water bath for 2 minutes which was followed by washing the cells with serum free MEM medium and then centrifuged at 300g for 10 minutes. Cell pellets were resuspended in MEM medium containing 5% FBS and seeded on three 100 mm tissue culture dishes (Corning). The cells were cultured at 37°C in a humidified incubator in an atmosphere of 5% CO_2 for two to four days changing the culture medium every 48 hours.

Collection of Conditioned Cell Culture Media and Storage of Stably Transfected PA-1 Cells

Each 150 mm tissue culture plate (Corning) was seeded with 2×10^6 to 3×10^6 cells and cultured for 24 hours. The media were removed from the culture dishes and replaced with 20 ml of fresh medium. The cell culture media were collected every 48

hours until cell growth reaching 80 to 90% confluence. The collected media were centrifuged at 1000 x g for 10 minutes to remove cell debris. Protease inhibitors [100 μg/ml PMSF (Sigma), 2 μg/ml Leupeptin (Sigma), 1 μg/ml Pepstatin (Sigma), and 2 mM EDTA (Fisher)] were added to prevent protein degradation (Harlow et al., 1990). Since the glycoproteins in the supernatants of stable transformed PA-1 tend to precipitate at -20 or -80°C, the collected culture media were stored at 4°C for no longer than 48 hours before purification.

Isolation and Purification of Glycoproteins from the Cultured Media of Stably Transfected PA-1 Cells

Isolation of the Glycoproteins with Wheat Germ Agglutinin

Agarose-wheat germ agglutinin (WGA) (Vector Laboratories. Inc., Burlingame, CA) chromatography was used as a first step to isolate glycoproteins from the cultured cell media (Mintz, et. al., 1979; Osawa, et. al., 1987). Agarose-wheat germ agglutinin was equilibrated with 10 resin volume of WGA binding buffer (10 mM PBS, pH 7.4, 0.15 M NaCl). The collected supernatants were passed through the WGA resin at a flow rate of 3 resin-volume per hour in a 4°C cold room. The resin was washed with WGA binding buffer until the flow-through A_{280} was less than 0.01. Glycoproteins binding to the WGA resin were separated into two elution peaks with elution buffer A (10 mM PBS, pH 7.4, 0.15 M NaCl, 20 mM N-acetyl-D-glucosamine (Sigma)) and elution buffer B (10 mM PBS, pH 7.4, 0.15 M NaCl, 500 mM N-acetyl-D-glucosamine). After eluting glycoproteins from the resin, the resin was washed with WGA binding buffer until the

flow-through A_{280} was less than 0.01 which was followed by washing with 5 resin volume of WGA storage buffer [10 mM PBS, pH 7.4, 0.15 M NaCl, 20 mM N-acetyl-D-glucosamine, 0.08% sodium azide (Sigma)]. Eluted glycoprotein samples were dialyzed against the Ni-NTA binding buffer (50 mM PBS, pH 8.0; 300 mM NaCl) or ion-exchange binding buffer (5 mM Tris-Phosphate buffer, pH 8.5) for further purification.

Purification of hZP3 with DEAE - Cellulose Anion Exchanger

Ten grams of DEAE - Cellulose resin (Sigma) were suspended in 200 ml of 0.1N NaOH for 10 minutes at room temperature. The resin was then filtered on a glass funnel and washed with 20 volumes of deionized water. The resin was then suspended in 200 ml of 0.1N H_3PO_4 (Fisher) for 10 minutes at room temperature. The resins were then washed with D.W. until the filtrate reached neutral pH. The DEAE - Cellulose was then suspended in 200 ~ 300 ml of ion exchange binding buffer (5 mM Tris-Phosphate buffer, pH 8.5) and stored at 4° C with daily changes until pH was equilibrated. The treated DEAE - Cellulose resin was suspended in ion exchanger binding buffer and the gel was allowed settle by gravity. The column was equilibrated in the binding buffer by washing with 10 column volume of binding buffer. Thirty milliliter of glycoproteins fraction, isolated from the cultured cell media with the WGA affinity chromatography, was applied to DEAE - Cellulose column (1 x 5 cm) while collecting flow through in a sterile tube and then apply the flow through glycoprotein sample into the column again. The column was then washed with binding buffer until the A_{280} was less than 0.01. The elution was accomplished by using binding buffer at pH 6.0 and pH 4.0. The eluant which remained within the peak of 280 nm absorbance was collected. Glycerol (Fisher)

was added into the purified protein sample to a 50% final concentration and then the protein was frozen in liquid nitrogen (-196°C) and then stored at -80°C for testing of the biological activity of purified rhZP3.

Non-denaturing Purification of Histidine Tagged Glycoproteins

The histidine tagged glycoprotein (rhZP3) was purified from the glycoprotein fraction, isolated from the cultured cell media with WGA affinity chromatography, with Ni-NTA (nitrilo-tri-acetic acid) resin (Qiagen). Proteins containing one or more 6xHis affinity tags, located at either the amino or carboxyl terminus of the protein, bind to the Ni-NTA resin with an affinity ($Kd=10^{-13}$ at pH 8.0) far greater than the affinity between most antibodies and antigens, or enzymes and substrates (Hoffmann and Roeder, 1991; Janknecht et al., 1991). Two milliliter of completely resuspended 50% slurry of Ni-NTA resin were transferred into the column and then washed with 5 resin-volume of H_2O. The resin was equilibrated with 10 resin-volume of Ni-NTA binding buffer. The WGA isolated glycoprotein samples dialyzed against Ni-NTA binding buffer were passed through an equilibrated Ni-NTA column containing 1 ml resin. The flow rate was adjusted at 3 ~ 4 resin volume per hour. After passing the glycoprotein sample through the Ni-NTA column, the resin was washed with 10 resin volume of Ni-NTA binding buffer containing Tween 20 (Fisher) and 2-mercaptoethanol which was followed by washing with Ni-NTA washing buffer (50 mM PBS, pH 6.6, 300 mM NaCl) until the flow-through A_{280} was less than 0.01. His-tagged glycoproteins were eluted from the resin with Ni-NTA washing buffer containing different concentration of imidazole (Sigma). Glycerol (Fisher) was added into the purified protein sample to a 50% final

concentration and then frozen with liquid nitrogen (-196°C) and then stored at -80°C for testing the biological activity of purified rhZP3.

Denaturing Purification of Histidine Tagged Glycoproteins

Fifty milliliter of the isolated glycoprotein sample (as described in glycoprotein isolation) were dialyzed against denaturing binding buffer (6 M Guanidine hydrochloride (Fisher), 0.1 M NaH_2PO_4, 0.01 M Tris, pH 8.0) overnight with fresh changes of the denaturing binding buffer twice. Protein sample was passed through 1ml of Ni-NTA resin pre-equilibrated with the denaturing binding buffer. After passing the protein sample, the resin was washed with 10 column volumes of denaturing binding buffer which was followed by 20 column volumes denaturing washing buffer (8 M Urea, 0.1 M NaH_2PO_4, 0.01 M Tris, pH 8.0). The recombinant protein was eluted with denaturing elution buffer (8 M Urea, 0.1 M NaH_2PO_4, 0.01 M Tris, pH 4.0). The purified proteins were dialyzed against 10 mM PBS to remove salt.

Protein Assay

The BCA protein assay (Pierce, Rockford, IL) was used to measure the protein concentration of the purified protein samples. Bicinchoninic acid (BCA) in the form of its water soluble sodium salt, is a sensitive, stable and highly specific reagent for cuprous ion. BCA protein assay combines the biuret reaction (protein reacting with Cu^{2+} in an alkaline medium to produce Cu^{+}) with the unique features of BCA. The purple reaction product, formed by the interaction of two molecules of BCA with one Cu^{+}, is water-soluble and exhibits a strong absorbance at 562 nm. This allows the spectrophotometric

quantification of protein in aqueous solutions (Redinbaugh, et. al., 1986; Sorensen, et. al., 1986). The 50 μg/ml, 100 μg/ml, 150 μg/ml, 200 μg/ml, 250 μg/ml, 300 μg/ml, 350 μg/ml, 400 μg/ml, 450 μg/ml, 500 μg/ml, 550 μg/ml, 600 μg/ml, 650 μg/ml, 700 μg/ml, 750 μg/ml, 800 μg/ml, 850 μg/ml, 900 μg/ml, 950 μg/ml, 1000 μg/ml standard samples were prepared with PBS buffer and the 2 mg/ml BSA stock solution (Pierce). For 5.1 ml of working solution, 5 ml of Reagent A (Pierce) and 100 μl of Reagent B were mixed gently. Twelve and a half microliter of protein standard and unknown protein samples were added into the 96 wells microtiter plate (Costa). Two hundred and fifty microliter of working solution were added into each well and incubated at 37°C for 30 minutes. The plate was cooled down to room temperature and read with a spectrophotometer at 570 nm. The standard curve was prepared by plotting the blank corrected absorbance at 570 vs. protein concentration. Using this standard curve, the protein concentration for each unknown protein sample was determined.

SDS-PAGE Electrophoresis

A Hoefer SE 220 minigel electrophoresis apparatus (Hoefer Pharmacia Biothch Inc., San Francisco, CA) and a discontinuous SDS gel system were used to perform electrophoresis. Twenty milliliter of 8% separating gel mix [9.4 ml of H_2O, 5.3 ml of 30% Acrylamide mix (Bio-Rad), 1.5 ml of 1.5 M Tris, pH 8.8 (Fisher), 100 μl of 10% APS (Sigma), 200 μl of 10% SDS (Sigma), 12 μl of TEMED (Sigma)] were poured between the glass plates. The separating gel was overlayed with water saturated n-Butanol and after 40 minutes to 1 hour this overlay n-Butanol was removed and replaced with 8 ml of 4% stacking gel mix (5.5 ml of H_2O, 1.3 ml of 30% Acrylamide mix, 1 ml of

1M Tris, pH 6.8, 80 µl of 10% APS, 80 µl of 10% SDS, 8 µl of TEMED) (Naito, et. al., 1973). Five microgram of protein were dissolved in the denature treatment buffer (0.0625 M Tris-Cl, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, pH 6.8) and loaded into the well. Rainbow colored protein molecular weight markers (Amersham Life Science, Little Chalfont Buckinghamshire, England) were used to determine the molecular weight of protein samples. Running buffer (0.025 M Tris, 0.192 M Glycine (Fisher), 0.1% SDS) was poured into the upper and lower reservoirs. Gels were run at 25 mA constant current until the dye front reached the bottom of the gel. The gel was stained with Coomassie Blue stain solution (0.025% coomassie blue R-250 (Sigma), 40% Methanol (Fisher), 7% Acetic Acid (Fisher)) and destained with destaining solution I (50% Methanol, 10% Acetic Acid) which was followed by destaining solution II (5% Methanol, 7% Acetic Acid).

Two-Dimensional Electrophoresis

Two-dimensional electrophoresis was performed using a Tube Gel Adaptor Kit (Hoefer). Isoelectric focusing gels were made in glass tubing (7.5 cm X 1.5 mm inside diameter) sealed at the bottom with Parafilm. To prepare 5 ml of isoelectric focusing gel mixture: 2.75 g of Urea (Fisher) was added to a 100 ml side arm flask, then 665 µl of 30% Acrylamide (Bio-Rad), 1 ml of 10%(w/v) Nonidet P-40 (NP-40) (Sigma), 985 µl of H_2O + 200 µl of Ampholines, pH range 3 to 5 (Bio-Rad), + 50 µl of Ampholines, pH range 3 to 10 (Bio-Rad). The mixture of solution was swirled until the urea was completely dissolved, then 10 µl of 10% ammonium persulfate (Sigma) were added and the solution was degassed under vacuum for about 1 minute. Immediately after addition

of 7 µl of TEMED (Sigma), the solution was loaded into the gel tubes. The gel was overlayed with gel overlay solution (8 M Urea) and after 1 to 2 hours this overlay solution was removed and replaced with 5 µl sample buffer (9.5 M Urea, 2% (w/v) NP-40, 2% Ampholines (comprised of 1.6% pH range 3 to5 and 0.4% pH range 3 to 10) and 5% 2-mercapto -ethanol (Fisher)). The tubes were then loaded into the adaptor and all unused bottom ports was sealed off by inserting a cone-shaped stopper (Hoefer). The lower reservoir was filled with 0.01 M H_3PO_4 and the upper reservoir was filled with 0.02 M NaOH which should be extensively degassed to remove CO_2. The gels were then prerun at 300 volts for 30 minutes. The power was turned off, the upper reservoir was emptied, sample buffer was removed from the surface of the gels, and the samples prepared with the sample buffer were loaded. The samples were overlayed with sample overlay solution (9 M Urea, 2% (w/v) NP-40, 1% Ampholines (comprised of 0.8% pH range 3 to5 and 0.2% pH range 3 to 10)) then 0.02 M NaOH, and the chamber was refilled. After the samples were loaded, the gels were run at 400 volts for 12 hours and then at 800 volts for 1 hour. The gels were removed from the tubes and equilibrated with SDS sample buffer (10% (w/v) glycerol (Fisher), 5% 2-mecaptoethanol, 2.3% (w/v) SDS, and 62.5 mM Tris-HCl, pH 6.8, 0.1% bromphenol blue (Sigma)) at room temperature for 40 minutes with shaking. The Hoefer SE 220 minigel electrophoresis apparatus (Hoefer) and discontinuous SDS gel system were used to perform the second dimension electrophoresis. Twenty milliliter of 8% separating gel mix [9.4 ml of H_2O, 5.3 ml of 30% Acrylamide mix (Bio-Rad), 1.5 ml of 1.5 M Tris, pH 8.8 (Fisher), 100 µl of 10% APS (Sigma), 200 µl of 10% SDS (Sigma), 12 µl of TEMED (Sigma)] was poured between the glass plates. The separating gel was overlayed with water saturated n-

Butanol and after1 to 2 hours this overlay n-Butanol was removed and replaced with 8 ml of 4% stacking gel mix (5.5 ml of H_2O, 1.3 ml of 30% Acrylamide mix, 1 ml of 1M Tris, pH 6.8, 80 µl of 10% APS, 80 µl of 10% SDS, 8 µl of TEMED) (Naito, et. al., 1973). The cylindrical isoelectric focusing gel was placed on the stacking gel and fixed with 1% agarose gel which was prepared with running buffer (0.025 M Tris, 0.192 M Glycine (Fisher), 0.1% SDS). Gels were run at 25 m amp constant current until the dye front reaches the bottom of the gel. The gel was stained in Coomassie Blue stain solution (O'Farrell, 1974).

Western Blotting

The protein samples, purified by WGA affinity chromatography followed by the Ni-NTA affinity chromatography, were separated with two 8% SDS-PAGE gels using Hoefer SE 220 minigel electrophoresis apparatus (Hoefer). Rainbow colored protein molecular weight markers (Amersham) were used to determine the molecular weight of protein samples. After electrophoresis, one of the two gels was stained with Coomassie Blue stain solution.

The anti-ZP3 antiserum was purchased from Cocalico Biologicals, Inc. (Reamstown, PA). Briefly, the antiserum was produced by immunizing a rabbit with the synthetic decapeptide (D-V-T-V-G-P-L-I-F-L) which was linked to keyhole limpet hemacyanin (KLH) with the cross-linker, N-succinimidyl 3-(2-pyridyldithio) propinate (SPDP). One hundred and fifty microgram decapeptide-KLH was emulsified with an equal volume of Freund's complete adjuvant. All the emulsion was given by inject

muscle. The second boost was given one week after the first injection. The antiserum was harvested one or two weeks after the second boost.

Wet transfer of proteins from gel to hybond ECL nitrocellulose membrane (Amersham) was performed at 100 Volts for 2.5 hours at 4°C with the transfer buffer (25 mM Tris-HCl; 192 mM glysine (Fisher); 20% methanol (Fisher), pH 8.3). After transferring, the nitrocellulose membrane was blocked with blocking buffer [80% Tris-HCl buffer, pH 7.5; 15% H_2O; 5% BSA (Sigma)] at room temperature for 3 hours by gently shaking. The rabbit anti-ZP3 antiserum was used as primary antibody which was diluted at 1: 1000 in solution A (80% Tris-HCl, pH 7.5; 20% BSA). Blocked nitrocellulose membrane was incubated in the primary antibody solution at room temperature for one hour with gentle shaking. The nitrocellulose membrane was washed with washing buffer A (PBS containing 0.4% Tween 20) for 15 minutes three times with fresh changes of the washing buffer. The secondary antibody [goat anti-rabbit IgG-HRP antibody conjugate (Amersham)] was diluted by the washing buffer A at 1:2000 dilution. After washing with washing buffer, the nitrocellulose membrane was incubated in secondary antibody solution at room temperature for one hour. The membrane was washed with washing buffer B (PBS containing 0.3% Tween 20) for five minutes three times with fresh changes which was followed by washing buffer C (PBS containing 0.1% Tween 20) for five minutes three times with fresh changes of the washing buffer. The nitrocellulose membrane was exposed to the detection solution [detection reagent 1 and detection reagent 2 (1:1, v/v) (Amersham)]. The membrane was placed with protein side face to film and exposed in the film cassette for 30 to 60 seconds. The film was developed with a Konica developing machine.

ELISA of the Histidine Tagged Glycoprotein

Immunoassays are techniques for the detection and quantification of antigens or antibodies. These methods are useful for assaying proteins and the sensitivity is usually in the range of micromoles per liter (Ekins, 1981). Proteins which contain a succession of six or more histidine residues at their amino or carboxyl terminus have a strong hinding afinity to chelated nickel metal residues. Reacti-Bind Metal Chelate Plates (Pierce) takes an advantage of this affinity by coating the surface of microtiter plates with chelated nickel. Poly-histidine tagged fusion proteins will thus specifically bind to this coated surface and may be analyzed using an ELISA technique.

The rhZP3 glycoproteins purified with the Ni-NTA affinity chromatography under the non-denaturing conditions were adjusted to the following serial concentrations: 1.25 μg/ml, 2.5 μg/ml, 3.75 μg/ml, 5 μg/ml, 6.25 μg/ml, 7.5 μg/ml, 8.75 μg/ml, 10 μg/ml, 11.25 μg/ml, 12.5 μg/ml, 13.75 μg/ml, 15 μg/ml, 16.25 μg/ml, 17.5 μg/ml, 18.75 μg/ml, 20.00 μg/ml which were used as standard in the ELISA. The rhZP3 producing PA-1 cells were cultured in 150 mm tissue culture plates with 20 ml of Eagle's minimal essential medium containing 5% fetal bovine serum. A set of cell culture medium samples was collected at 24, 48 and 72 hours. At the same time of medium collecting, the total cell number in each tissue culture plates was counted by using a hemocytometer. The medium-samples from non-transfected PA-1 cells were collected with the same method as that described above. Two hundred microliters of each collected sample were used to coat the Reacti-Bind Metal Chelate Plates overnight at room temperature in a moisture chamber. After coating, microwells were blocked with 2.5% BSA in binding buffer (10mM PBS with 0.1% Tween 20, pH 8.0) and incubated in a moisture chamber

for 1 hour and then wash with washing buffer (10 mM PBS containing 0.05% Tween 20) for 6 times. Two hundred microliters of the first antibody (rabbit anti ZP3 decapeptide polyclonal antiserum) diluted at 1:2000 with dilution buffer (10mM PBS, pH 8.0) was added into each microwell and incubated in a moisture chamber at room temperature for 1 hour and then followed by washing 6 times with washing buffer. Two hundred microliter of the second antibody [Goat anti-rabbit IgG-Horseradish Peroxidase conjugates (Sigma)] diluted at 1:15,000 with dilution buffer was add into each wells and incubated under the condition describe previously. Microwells were washed with washing buffer for 6 times. The signal was developed using TMB Peroxidase ELISA Substrate System (Bio-Rad). The reaction was stopped with 2 N H_2SO_4 and then read at 450 nm.

Hemizona Assay (HZA)

In order to properly interpret the results of this sperm - zona pellucida binding test, standardized conditions have been optimized and validation studies have been extensively performed in our laboratory. An aliquot of semen (0.5ml) was diluted with 1ml of Ham's F-10 medium (Gibco) supplemented with 0.5% heat-inactivated human serum albumin (HAS). The sperm were centrifuged (5 minutes, 300 x g), then washed a second time. The final pellet was overlaid with 0.5ml of F-10 medium with HSA and incubated at 37°C, 5% CO_2 in air to achieve a separation of the motile sperm fraction. After1 hour, the sperm supernatant was removed and used for the HZA. One 100µl droplet of the control sperm suspension (500,000 motile sperm/ml) was placed in a petri dish under oil. A second dish was similarly prepared with a droplet of the sperm

suspension incubated with rhZP3 for 45 minutes. One hemizona was transferred to the control drop, while the matching hemizona was coincubated with the rhZP3-tested sperm. Gametes were incubated for 4 hours (37°C, 5% CO_2 in air). After 4 hours of coincubation, each hemizona was removed and pipetted five times to dislodge loosely attached sperm

(Hodgen et. al., 1988; Oehninger et al., 1991). The number of sperm tightly bound to the outer surface was counted. For each hemizona pair, the HZI was calculated as follows: (number of test sperm bound to the hemizona / number of fertile sperm bound to hemizona) X 100 (Burkman, et al.,1988).

Acrosome Reaction

The acrosomal status of spermatozoa was evaluated using the fluorescent probe fluorescein isothiocyanate (FITC) - labeled Pisum Sativum agglutinin (PSA; Vector) following previously described techniques (Mahony, 1991, 1993, Cross, 1986). Assay slides were read using an epifluorescent microscope at 400X magnification; duplicate were evaluated for each treatment and time analyzed, assessed blindly by two different experienced observers and results averaged. At least 200 cells were evaluated per slide. Acrosome reacted sperm are diagnosed when a total loss of the acrosomal cap is observed (bar pattern), or no immunofluorescence is seen at all. Acrosome-intact sperm typically show staining of the entire acrosomal cap. The acrosomal status of control and test (rhZP3-treated) sperm was examined under basal conditions (spontaneous acrosome reaction). Different rZP3 concentrations, different times of exposure to rZP3 (1 min through 60 min), and different sperm capacitation conditions (immediately post - swim -

up, 1, 4, 8 and 24 hours after swim - up) were also assessed. Chi-square analyses was used to statistically examine differences within simple comparisons, whereas ANOVA will be used when multiple comparisons were performed. All biological experiments were performed under the approval of IRB of Eastern Virginia Medical School.

RESULTS

Construction of hZP3-sixHis-pcDNA3.1(+) Expression Vector

Generation of hZP3-sixHis cDNA

A mRNA extract was isolated from human ovarian tissue. The hZP3-cDNA generated by RT-PCR was subcloned into the pBluescript II SK(+) plasmid (PSK) by Chen, 1995.

In order to improve the purification of the rhZP3, six histidines were fused to the hZP3 glycoprotein. Since the 22 amino acid signal peptide located at N terminal not exists in the mature hZP3 glycoprotein, the histidine tag was fused to the hZP3 glycoprotein at the C terminal. To generate a histidine tagged hZP3 DNA, primer B was designed to introduce six histidine-codons before the TAA stop codon of hZP3 cDNA. In addition, primer B also introduce a Xho I restriction site to hZP3 DNA after the TAA stop codon. The Xho I restriction site was used for subcloning the hZP3-sixHis DNA into the pcDNA3.1(+) vector. The reverse primer of PSK and the primer B specifically amplified an expected 1406 bp DNA fragment which contains a 110 bp up stream DNA fragment, hZP3 cDNA (1272 bp), six histidine-codon (18 bp) and Xho I restriction site (6 bp) (figure 3). The T3 primer and primer B specifically amplified an expected 1363 bp DNA fragment which contains a 67 bp up stream DNA fragment, hZP3 cDNA (1272 bp), six histidine-codon (18 bp) and Xho I restriction site (6 bp)(figure 3).

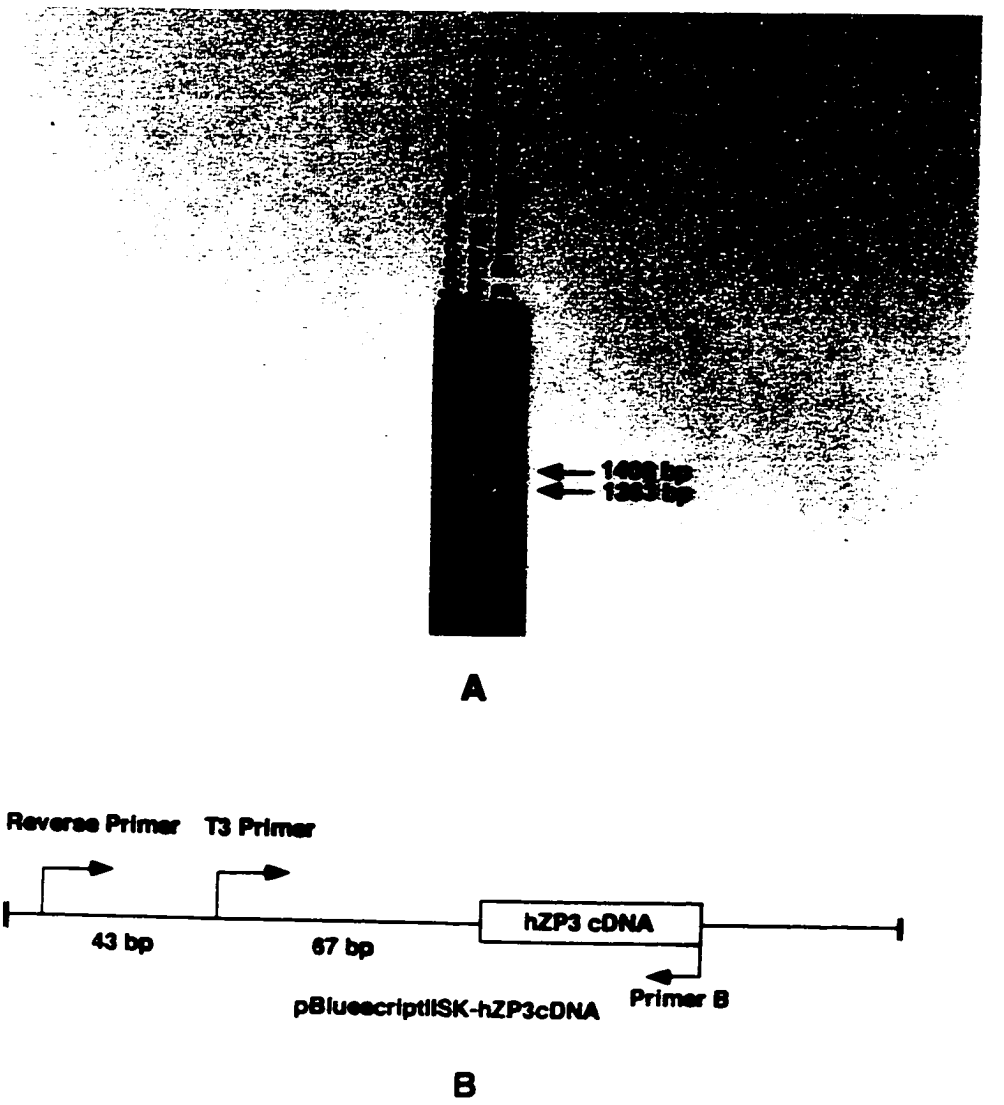

Fig. 3. Introduction of a six-histidine tag into the hZP3 cDNA with PCR.
(A) The reverse primer and primer B amplified a 1406 bp DNA fragment which contains 110 bp of upstream sequence, 1272 bp of hZP3cDNA, 18 bp of sequence coding for six histidines, and 6 bp of sequence coding for Xho I restriction site from the vector pBluescriptIISK-hZP3cDNA. A 1363 bp DNA fragment which contains 67 bp of upstream sequence, 1272 bp of hZP3cDNA, 18 bp of sequence coding for six histidines, and 6 bp of sequence coding for Xho I restriction site was amplified using the T3 primer and primer B.
(B) The relative location of the reverse primer and T3 primer in the pBluescriptII-hZP3cDNA is shown. The T3 primer and reverse primer introduced a 67 bp and 110 bpDNA fragment at the upstream of the hZP3cDNA respectively.

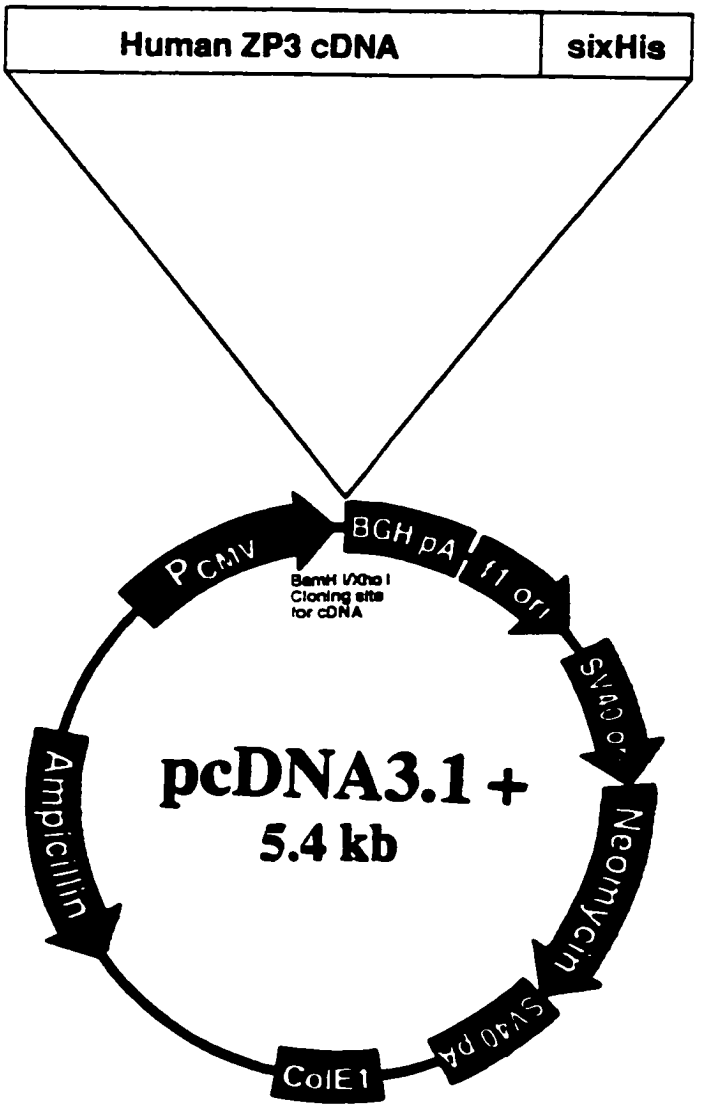

Fig. 4. Construction of the hZP3-sixHis expression vector. The hZP3-sixHis DNA was cloned into the pcDNA3.1(+) right at the downstream of the CMV promoter. pcDNA3.1(+) vector includes the human cytomegalovirus (CMV) immediate-early promoter/enhancer for high-level expression in a variety of mammalian cell lines, and the bovine growth hormone (BGH) polyadenylation signal for efficient transcript stabilization and termination. This vector also contains the neomycin resistance gene for selecting the stable transformants.

CGT AAC CGC AGG CAT GTG ACA GAA GAA GCA GAT GTC ACC GTG GGG

CCA CTG ATC TTC CTG GAC AGG AGG GGT GAC CAT GAA GTA GAG CAG

TGG GCT TTG CCT TCT GAC ACC TCA GTG GTG CTG CTG GGC GTA GGC

CTG GCT GTG GTG GTG TCC CTG ACT CTG ACT GCT GTT ATC CTG GTT

CTC ACC AGG AGG TGT CGC ACT GCC TCC CAC CCT GTG TCT GCT TCC

GAA **CAT CAT CAT CAT CAT CAT** TAA **CTC GAG** AA

six histidines XhoI restriction site

Fig. 5. DNA sequencing for confirming the insertion of six-histidine tag in rhZP3 DNA. The DNA sequencing shows that a set of the DNA sequence (5'-CAT, CAT, CAT, CAT, CAT, CAT-3') coding for six histidines and a set of DNA sequence (5'-CTC,GAG-3') coding for XhoI restriction site were introduced into the hZP3 cDNA. The TAA, the stop codon of the hZP3 cDNA, was inserted between the six histidine codons and XhoI restriction site codon.

Subcloning the hZP3-sixHis into the pcDNA3.1(-)Vector

The 1.3 kbp hZP3-sixHis DNA generated by PCR was separated from other DNA fragments by 1.2% agarose gel electrophoresis and the 1.3 kbp DNA fragments were purified with the Geneclean II Kit. Both hZP3-sixHis DNA and pcDNA3.1(+) vector were double digested with BamH I / Xho I restriction enzymes. The 1.3 kbp hZP3-sixHis was subcloned in pcDNA3.1(+) downstream of the human cytomegalovirus (CMV) promoter with the T4 ligase (figure 4). Two hundred nanograms of ligation–products, hZP3-sixHis-pcDNA3.1(+), were used to transform 200 μl of lag phase DH5α E.coli cells. The transformed E.coli colonies were selected with ampicillin LB agar plates. Fourteen out of two hundred clones were selected. The DNA samples isolated from the selected E.coli. colonies were examined by PCR using CH1 primer (located at hZP3 cDNA from base -3 to 18) and primer B to confirm the insertion of hZP3-sixHis DNA in the pcDNA3.1(+) vector. The PCR reaction amplified a 1296 bp DNA fragment from the hZP3-sixHis-pcDNA3.1(+). Three out of the fourteen selected clones show an expected PCR-product. The hZP3-sixHis-pcDNA3.1(+) DNA was isolated from one of the three selected transformed E.coli. clones with the alkaline lysis method. The insertion site of six histidines in the hZP3-sixHis DNA was confirmed with the partially DNA sequencing analysis (figure 5). The insertion site of hZP3-sixHis DNA in the pcDNA3.1(+) was confirmed with Sal I and Sma I restriction digestion analysis (figure 6).

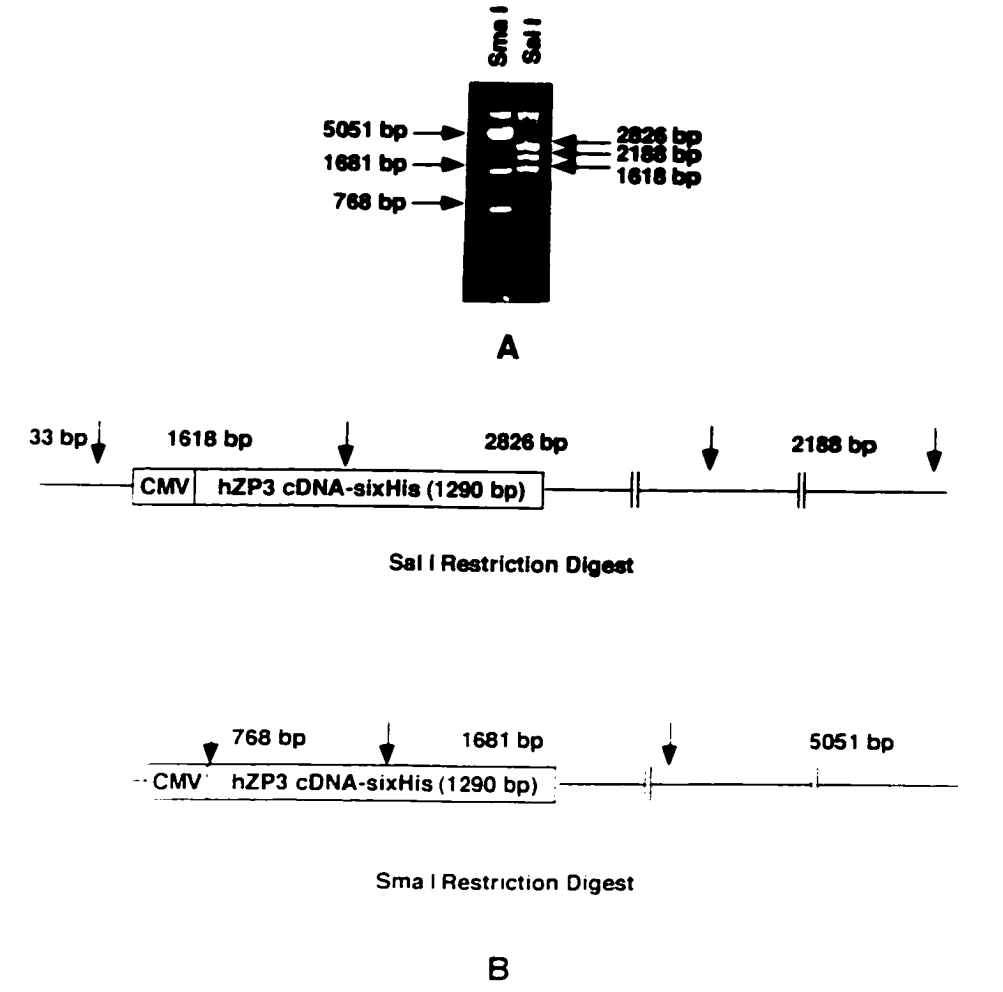

Fig. 6. Restriction map of hZP3-sixHis-pcDNA3.1(+). The pattern of the restriction digestion of Sma I and Sal I confirms the hZP3-sixHis DNA being inserted right at the downstream of the CMV promoter of the pcDNA3.1(+) vector (panel A). The restriction sites of Sal I and Sma I are presented on the hZP3-sixHis-pcDNA3.1(+) DNA (panel B).

Transfection and Selection of ZP3-sixHis-Expressing Clones

The calcium phosphate-mediated transfection method was used to incorporate the hZP3-sixHis-pcDNA3.1(+) into the PA-1 host cells. In order to establish the stably transformed PA-1 cell line, the transfected PA-1 cells were selected with neomycin. Since the PA-1 cell is resistant to neomycin at the concentration of 500μg/ml (suggested by Invitrogen) and 1mg/ml, the concentration of neomycin was increased to 2mg/ml to kill the non-transfected cells. However, the PA-1 cell still exhibited some resistant to 2mg/ml of neomycin when the cell density is over 70% confluence. Therefore, the cell confluence was kept at 30 to 40% during the neomycin selection. When the cell confluence decreased below 30%, the cells from each plate were trypsinized and recultured on new plates at 40% confluence. After selecting for 3 months, two stably transformed clones were obtained.

The RNA samples purified from the PA-1 non-transfected and the selected stably transfected PA-1 cells were applied to RT-PCR to verify the expression of the hZP3-sixHis recombinant DNA in the stably transfected PA-1 host cells. In figure 7, the CH1 / CH2 primer and CH1 / Primer B amplify an expected 1272 bp and 1296 bp hZP3 DNA fragment respectively from the cDNA template which was reverse-transcribed from the RNA isolated from the stably transformed PA-1 cells. For the RNA samples from non-transfected PA-1 cells, no amplified signals are detected. This data supports the expression of the hZP3-sixHis recombinant DNA in the stably transfected PA-1 cells.

The western blot analysis was applied to confirm the secretion of rhZP3 glycoproteins into the culture medium. A 48-hour conditional culture media was collected from one 100 mm tissue culture plate. The proteins in the media were

precipitated with 5% trichloroacetic acid and then centrifuged at 10,000 x g (4°C) for 30 minutes. The protein pellets were washed with 70% ethanol twice. The protein samples were analyzed with SDS-PAGE followed by the western blot analysis. A protein band with a molecular weight of approximately 60 kDa was recognized by the rabbit anti-ZP3-decapeptide antiserum. Therefore, it can be verified that the rhZP3 glycoproteins produced by stably transformed PA-1 cells are secreted into culture medium.

Ion Exchange Purification of rhZP3

Each 150 mm tissue culture plate (Corning) was seeded with 2 x 10^6 to 3 x 10^6 cells. At this cell density, the PA-1 cell will reach 40% confluence within 24 hours. After 24 hours, the media was removed from the culture plates and replaced with 20 ml of fresh medium without neomycin. Neomycin will not be used during the media collection because it inhibits protein synthesis. The cell culture media were collected every 48 hours until cells reached 80 to 90% confluency. The wheat germ agglutinin (WGA) affinity chromatography was used for affinity-isolating the glycoproteins directly from the culture media of the selected stably transfected PA-1 cells. The glycoproteins binding to the WGA affinity column were eluted with WGA elution buffer (10 mM PBS, 0.15 M NaCl, 500mM N-acetyl-D-glucosamine, pH 7.4). The isolated glycoproteins were dialyzed against the ion exchange binding buffer and then purified with the DEAE anion exchange chromatography. The rhZP3 glycoproteins were eluted with 5 mM Tris-phosphate at pH 6.0 and pH 4.0. With the ion exchange purification, 3 to 4 mg of glycoproteins can be recovered from one liter culture media.

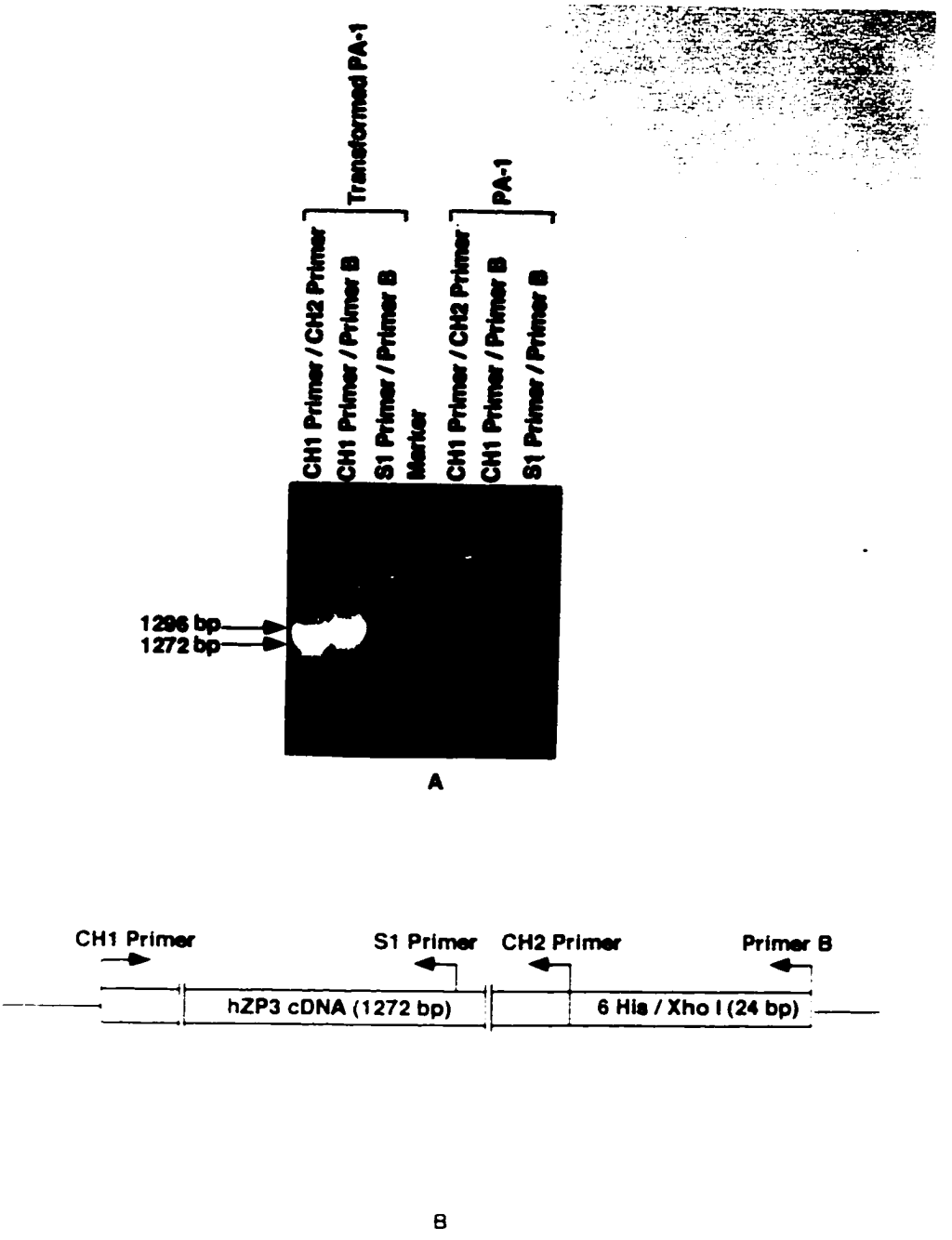

Fig. 7. Confirm the expression of the hZP3-sixHis DNA in the stably transfected PA-1 cells with RT-PCR. The RNA samples were isolated from the stably transformed PA-1 cells and non-transfected PA-1 cell. Both RNA samples were reverse transcribed into DNA samples that were used as template in the PCR reaction. The CH1 / CH2 primer and CH1 / Primer B amplify a 1272 bp of hZP3 DNA and a 1296 bp DNA which contains the hZP3 DNA, six histidine tag codons and Xho I restriction site codon respectively.

In figure 8, Coomassie brilliant blue staining and western blot analysis are shown after SDS-PAGE electrophoresis of the proteins purified by the WGA and DEAE anion exchange chromatography. The results show that about 20% purity can be reached. The western blotting shows that the 62~66 kDa rhZP3 can be eluted under elution condition of either pH 6.0 or pH 4.0. This phenomena indicates that the rhZP3 expressed in the PA-1 cell may display a heterogeneous surface charge, which may be due to different amounts of sialic acid on the end of the carbohydrate chains, on the rhZP3 glycoproteins.

Under the pH 4.0 elution condition, a 140 kDa protein band was eluted. This protein band also was recognized by the rabbit anti-ZP3 decapeptide antiserum in the western blot analysis. In figure 9, a stronger washing condition with pH 6.0 was applied to wash the DEAE anion exchange resin, the 140 kDa proteins can be separated from the 62~66 kDa rhZP3 glycoproteins. This 140 kDa glycoprotein may be a dimer of rhZP3 glycoproteins.

Ni-NTA Affinity Purification of Histidine Tagged rhZP3

The Ni-NTA nickle-chelating resins were used for affinity purification of histidine tagged rhZP3 glycoproteins. The culture media were collected as the same method as described in the ion exchange purification. In figure 10, the purity and yield between the non-denatured and denatured protein purification are compared. The purification under denaturing condition did not show significant improvement in the purity of the rhZP3 glycoproteins. This phenomenon may result from other glycoproteins, which contain neighboring histidine residues. The glycoproteins, which express different amounts of neighbor histidine residues will exhibit different levels of

Ni-NTA binding ability. The yield of the denatured purification is about 30% lower than that of non-denatured purification. The biological activity of the rhZP3 is another concern in using the denaturing purification. Since the rhZP3 purified under denaturing conditions may not go back to their native structure during the renature procedure. Therefore, the denature-purification is not a good choice for rhZP3 purification.

To improve the purity of the rhZP3, glycoproteins bound to the WGA column were separated into two glycoprotein fractions with different elution condition: 10 mM PBS, 0.15 M NaCl, 500mM N-acetyl-D-glucosamine, pH 7.4 and 10 mM PBS, 0.15 M NaCl, 20 mM N-acetyl-D- glucosamine, pH 7.4. About 80 to 90% of the histidine tagged rhZP3 glycoproteins stay in the glycoprotein fraction eluted by elution buffer containing 20 mM N-acetyl-D- glucosamine (figure 11). In the Ni-NTA affinity chromatography, about 80% of the histidine tagged rhZP3 are eluted from the Ni-NTA resins with the elution buffer containing 40mM imidazole (figure 11).

To break through the barriers of purity and yield, the washing and eluting conditions were modified. The Ni-NTA binding buffer containing 0.4% Tween 20 and 10 mM 2-mercaptoethanol was used to wash the Ni-NTA resin to reduce the background proteins which were co-purified with the rhZP3. The elution buffer containing 20mM imidazole was used to elute the rhZP3 glycoprotein. Under this elution condition, fewer background proteins were co-purified with the rhZP3. The partially embedded histidine tag may account for the weak interaction between the histidine tagged recombinant protein and Ni-NTA resins. This phenomenon allows the histidine-tagged rhZP3 to be eluted from Ni-NTA resins with low imidazole concentration.

In figure 12, the purified histidine tagged rhZP3 (Mr = 62 ~ 66 kDa) shows a purity above 80%. The western blot analysis shows two signals for the solubilized native human ZP3 glycoproteins. One has the molecular weight about 54 kDa ($ZP3_L$) and the other has the molecular weight about 58 kDa ($ZP3_H$). The rhZP3 glycoproteins expressed by PA-1 cells show a slightly larger molecular weight than these native human ZP3 glycoproteins.

The effect of aggregation and insolubility property of rhZP3 causes serious problems in handling this recombinant glycoprotein. The gradient elution had been used to elute the rhZP3 from the affinity column previously. Under the elution conditions, the rhZP3 glycoproteins will distribute in 50 to 100 ml of elution buffer. After concentrating with a centriprep10, the recovery will drop to few microgram levels because most of the glycoproteins lose during the concentration procedure. This low protein recovering may due to the aggregation of the rhZP3 or the adhesion of rhZP3 to the filter membrane of centriprep during the concentration. Precipitation is also not a proper way for handing the rhZP3 since rhZP3 is very difficult to return to the solution after this procedure.

Because of aggregation and insolubility, above 80% of the rhZP3 will be lost during concentrating or dialyzing the purified proteins. Therefore, to obtain a highly purified rhZP3 glycoprotein we should prevent any concentration or dialysis procedures after being eluted from the Ni-NTA affinity column. To circumvent these obstacles, the elution step becomes the most critical step during the purification procedure. The rhZP3 was eluted from the Ni-NTA affinity column by incubating the resin with two resin bed volumes of elution buffer for two minutes and then blowing the solution out of the

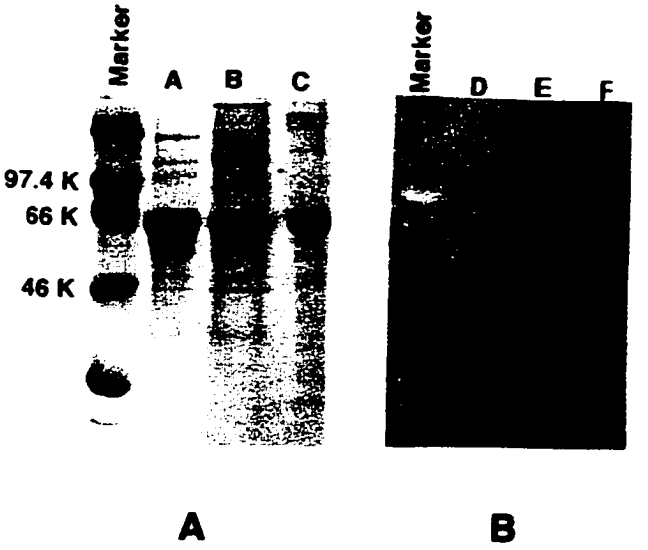

Fig. 8. Purification of rhZP3 with the wheat germ agglutinin mediated glycoprotein isolation followed by DEAE anion exchange separation. Coomassie brilliant blue staining (panel A): lane A, glycoproteins bind in the DEAE anion exchange resins were eluted with the 5mM Tris-Phosphate buffer, pH 6.0; lane B, glycoproteins were eluted from the DEAE resins with the 5mM Tris-Phosphate buffer, pH 4.0; lane C, the proteins from non-transfected PA-1 cells were purified with the same method as that used in purifying the rhZP3 and eluted with the 5mM Tris-Phosphate buffer, pH 4.0. Western blot analysis stained with rabbit anti-synthetic decapeptide of hZP3 antiserum, 1:1000 dilution. Panel B: lane D, a 62 to 66 kDa rhZP3 was detected by the antiserum; lane E, both the 62 ~ 66 kDa rhZP3 glycoprotein band and a glycoprotein band with the molecular weight about 140 kDa was detected by the antiserum; lane F, no proteins from the non-transfected PA-1 cells were recognized by the antiserum.

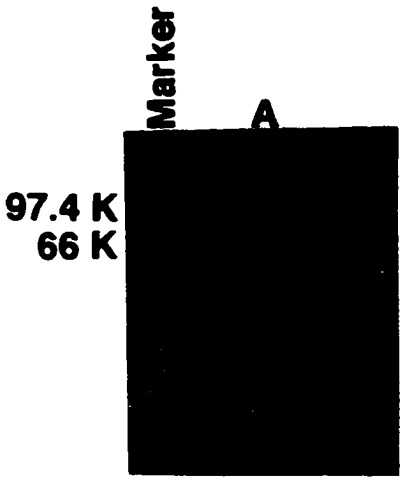

Fig. 9. Purification of 140 kDa ZP3 like glycoprotein with WGA and DEAE anion exchange. The glycoproteins isolated from the culture media of the stably transfected PA-1 cells with the wheat germ agglutinin affinity chromatography followed by the DEAE anion exchange separation were analyzed by western blot. After purified glycoproteins bind to the DEAE ion exchange resins, the resins were washed with 5mM Tris-Phosphate buffer, pH 6.0 and then the glycoproteins were eluted from the ion exchange resin with 5mM Tris-Phosphate buffer, pH 4.0. Western blot analysis stained with rabbit anti-synthetic decapeptide of hZP3 antiserum, 1:1000 dilution. Lane A, the 140 kDa glycoprotein band can be separated from the 62 ~ 66 kDa rhZP3.

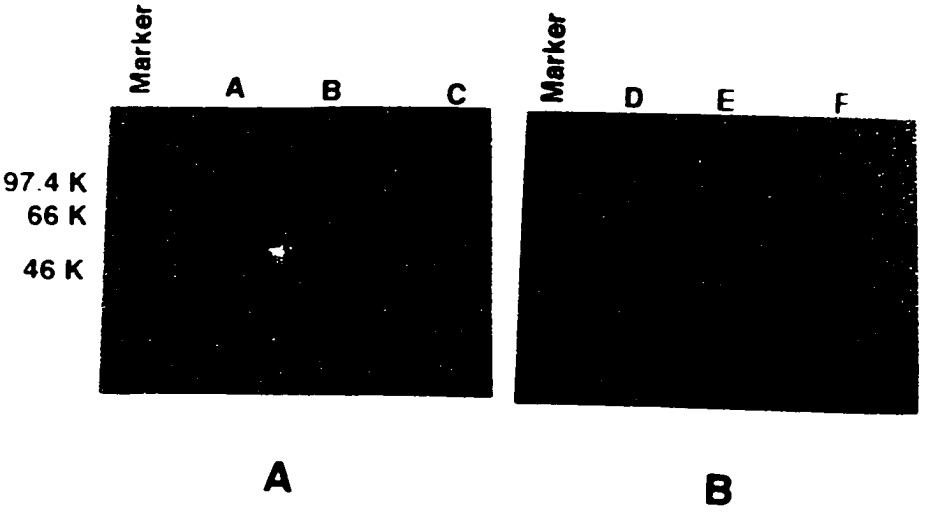

Fig. 10. Purification of rhZP3 with WGA and Ni-NTA affinity chromatography. The rhZP3 glycoproteins purified with the wheat germ agglutinin affinity chromatography followed by Ni-NTA affinity chromatography under the non-denaturing or denaturing purification conditions. Coomassie brilliant blue staining (panel A): lane A, rhZP3 glycoproteins were purified under the non-denaturing condition; lane B, rhZP3 glycoproteins were purified under the denaturing condition; lane C, the culture media of non-transfected PA-1 cells was purified with WGA affinity glycoproteins isolation followed by the Ni-NTA affinity chromatography under the non-denaturing condition. Western blot analysis stained with rabbit anti-hZP3 decapeptide antiserum (panel B): lane D, E, the Ni-NTA non-denaturing purification condition show a stronger rhZP3 glycoprotein signal than that of denaturing purification; lane F, no proteins from the non-transfected PA-1 cells were recognized by the antiserum.

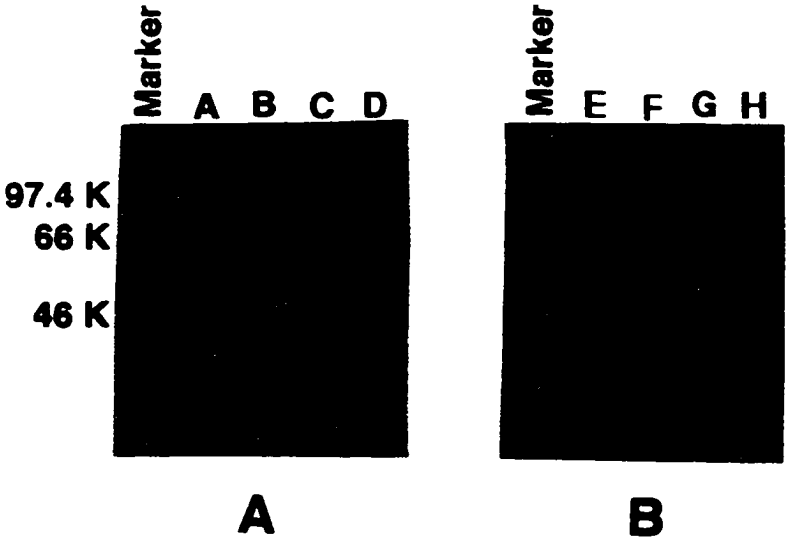

Fig. 11. Study of different purification condition of rhZP3 with WGA and Ni-NTA affinity chromatography. The glycoproteins in the stable transfected PA-1 cells were isolated by the WGA affinity column from which the glycoproteins were eluted with WGA elution buffer A (10 mM PBS, pH 7.4, 0.15 M NaCl, 20 mM N-acetyl-D-glucosamin) and WGA elution buffer B (10 mM PBS, pH 7.4, 0.15 M NaCl, 500 mM N-acetyl-D-glucosamin). The glycoprotein fractions eluted by different elution condition were further purified with the Ni-NTA affinity chromatography under the non-denature conditions. Coomassie brilliant blue staining (panel A): lane A, Glycoproteins eluted with WGA elution buffer B were further purified with the Ni-NTA affinity chromatography from which the rhZP3 glycoproteins were eluted with Ni-NTA elution buffer (50 mM PBS, 300 mM NaCl, 80 mM imidazole, pH 6.6); lane B, rhZP3 glycoproteins were purified under the same purification procedure as that used in lane A but the rhZP3 glycoproteins were eluted from Ni-NTA affinity column with the elution buffer (50 mM PBS, 300 mM NaCl, 40 mM imidazole, pH 6.6); lane C, Glycoproteins eluted with elution buffer A were further purified with the Ni-NTA affinity chromatography from which the rhZP3 glycoproteins were eluted elution buffer (50 mM PBS, 300 mM NaCl, 80 mM imidazole, pH 6.6); lane D, rhZP3 glycoproteins were purified under the same purification procedure as that used in lane C but the rhZP3 glycoproteins were eluted from Ni-NTA affinity column with the elution buffer (50 mM PBS, 300 mM NaCl, 40 mM imidazole, pH 6.6). Western blot analysis stained with rabbit anti-synthesized hZP3 decapeptide antiserum (panel B): lane E and F, From the glycoproteins eluted with the WGA elution buffer B, a trace amount of rhZP3 glycoproteins were detected; (panel B) lane G and H, From the glycoproteins eluted with the WGA elution buffer A, about 80% rhZP3 glycoproteins binding to the Ni-NTA resins were eluted from Ni-NTA affinity column with the 40 mM imidazole (lane H).

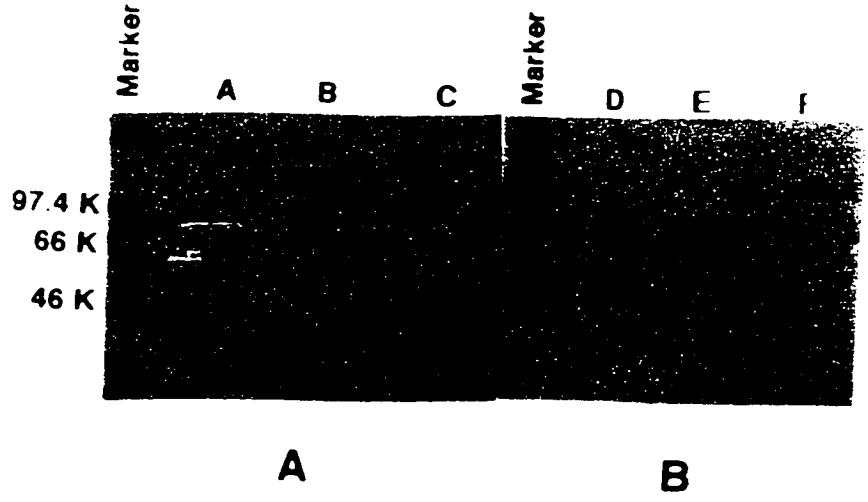

Fig. 12. The comparison of rhZP3 and solubilized native human ZP3. Coomassie brilliant blue staining (panel A) and western blot (panel B) are shown after SDS-PAGE of the protein fraction eluting from the Ni-NTA affinity column with the elution buffer: 50 mM PBS, 300 mM NaCl, 20 mM imidazole, pH 6.6 (lanes A and D), of a batch of 20 heat-solubilized human zona used as a positive reference (lanes B and E). As a negative control, the glycoproteins purified from non-transfected PA-1 cells using the same method as that used to purify rhZP3 was concentrated 15 times and loaded into SDS-PAGE gel. (lanes C and F).

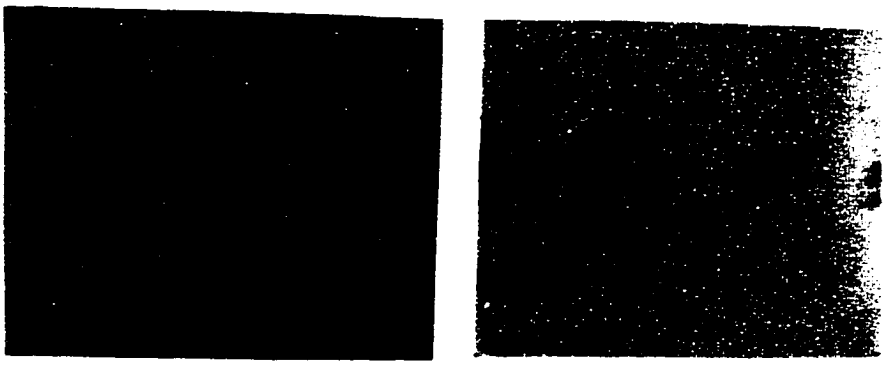

Fig. 13. Two-dimensional electrophoresis of rhZP3. The Coomassie brilliant blue staining (panel A) and western blot (panel B) are shown after two-dimensional electrophoresis which was applied to separate the glycoproteins of 62 ~ 66 kDa into two glycoprotein bands which have different isoelectric point (pI = 3.4 ~5.0, pI = 5.8 ~ 6.7). The glycoprotein band of the lower pI value which shows a strong signal in the western blot analysis stained with rabbit anti-hZP3 decapeptide antiserum accounts for 70 to 80% of the 62 ~ 66 kDa glycoproteins.

column. Under this elution condition, the purified rhZP3 can be eluted at the small volume with the concentration about 1mg/ml.

In figure 13, the protein and western blot are shown after two-dimensional electrophoresis was applied to separate the glycoproteins of 62 ~ 66 kDa into two glycoprotein bands which have different isoelectric points (pI = 3.4 ~5.0, pI = 5.8 ~ 6.7). The glycoprotein band of the lower pI value accounts for 70 to 80% of the 62 ~ 66 kDa glycoproteins. On the basis of these experiments, it can be inferred that a combination of affinity chromatography with the wheat germ agglutinin affinity column followed by the Ni-NTA affinity column can purify the rhZP3 glycoproteins at 50 to 60% purity.

The ELISA Study of rhZP3 Glycoprotein

The hZP3 glycoprotein secretion time course of stable transformed PA-1 cell was analyzed with the ELISA in which a nickel chelate plates were used to bind the histidine tagged proteins in the culture media specifically. The culture media was collected at 24, 48, and 72 hours. At the same time of medium collecting, the total cell number in each tissue culture plates was counted. The WGA/Ni-NTA affinity purified rhZP3 were used as the standard to determine the concentration of the samples. The samples collected at 24, 48, and 72 hours were centrifuged with Centrifuge 5415C at 14000rpm (4°C) to remove cell debris. Two hundred microliter media from each samples were analyzed with ELISA to determine the rhZP3 concentration. In figure 14, the hZP3 glycoprotein producing PA-1 cell shows an inhibition of hZP3 glycoprotein secretion. For 24 hours culturing after seeding, one million stable transfected PA-1 cells can produce 1.83 μg of the rhZP3 glycoproteins. At 48 hours, one million cells produce about 1.7 μg of the

rhZP3 glycoproteins. However, at the 72 hours, the rhZP3 glycoprotein productions show a significant decrease to about 1.51 μg/million cells. This phenomenon of protein secretion inhibition may result from the feedback inhibition of the rhZP3 glycoproteins in the culture medium. The degradation and aggregation of recombinant glycoproteins may also account for this phenomenon.

The Biological Activity of rhZp3 Glycoprotein

The ability of the rhZP3 to induce the sperm acrosome reaction and to competitively inhibit the binding between spermatozoa and zona pellucida were assessed with the acrosome reaction assay and hemizona assay. The analysis of the acrosome reaction observed in response to purified rhZP3 (500 ng/ml) and a Ca^{2+} ionophore revealed that both of these reagents were capable of inducing a significant increase (about 1.9 fold) in acrosome reaction compared with the control incubation (Ham's F-10 with HSA, Protein purified from non-transfected PA-1 cells).

In the hemizona assay, the purified rhZP3 glycoproteins show the ability to inhibit about 70% of the binding between spermatozoa and zona pellucida at the concentration of 500 ng/ml.

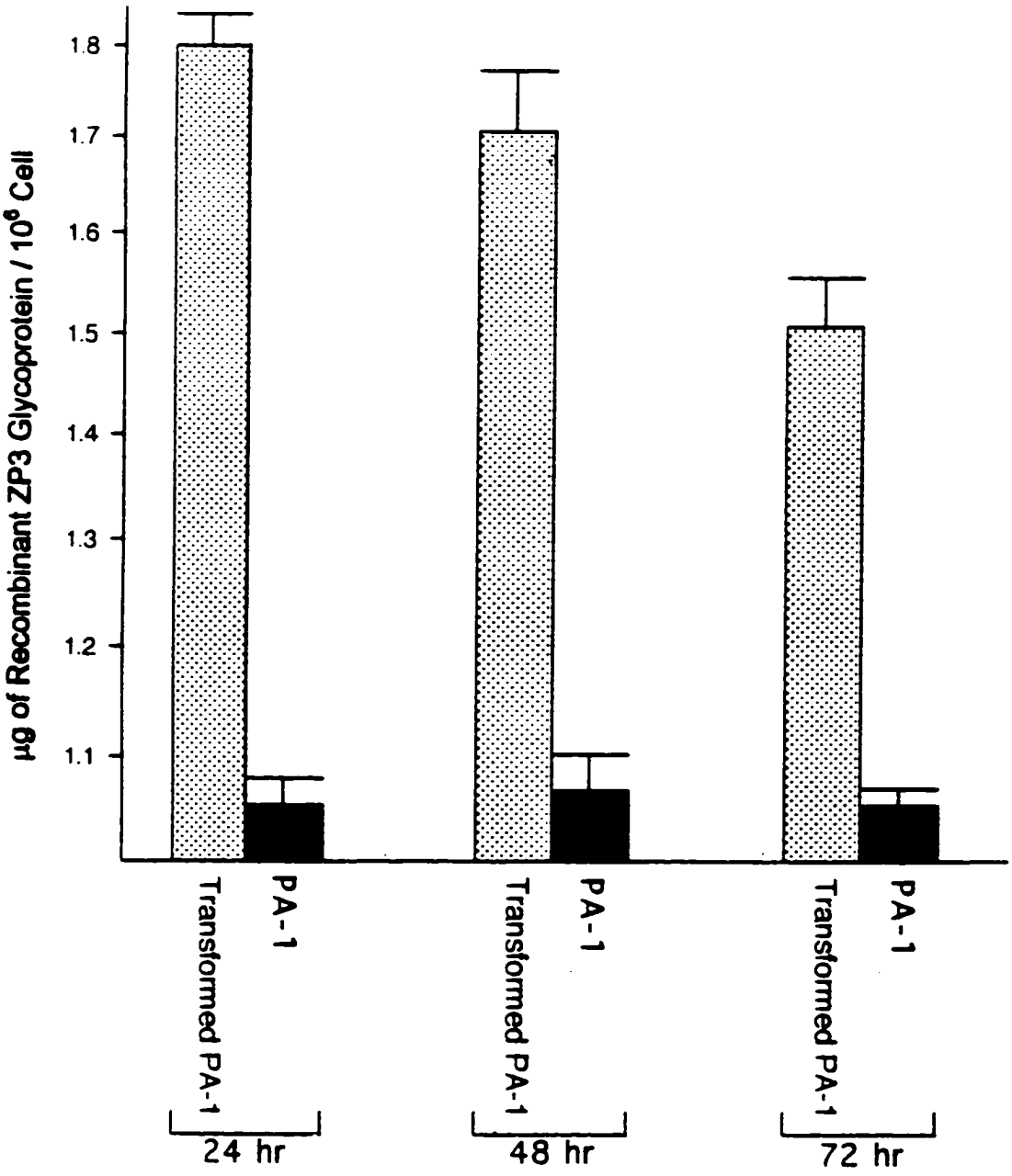

Fig. 14. ELISA study of recombinant protein-secretion. The feedback inhibition of the secretion of histidine tagged recombinant hZP3 glycoprotein in stably transformed PA-1 cells. The ELISA study reveals that the secretion of the rhZP3 glycoproteins at 24, 48 and 72 hours are 1.83 µg/10^6 cells, 1.7 µg/10^6 cells and 1.51 µg/10^6 cells respectively. It shows a significant decreasing of rhZP3 glycoprotein secretion at 72 hours of culturing time. Therefore, the supernatants were collected every 48 hours for rhZP3 purification.

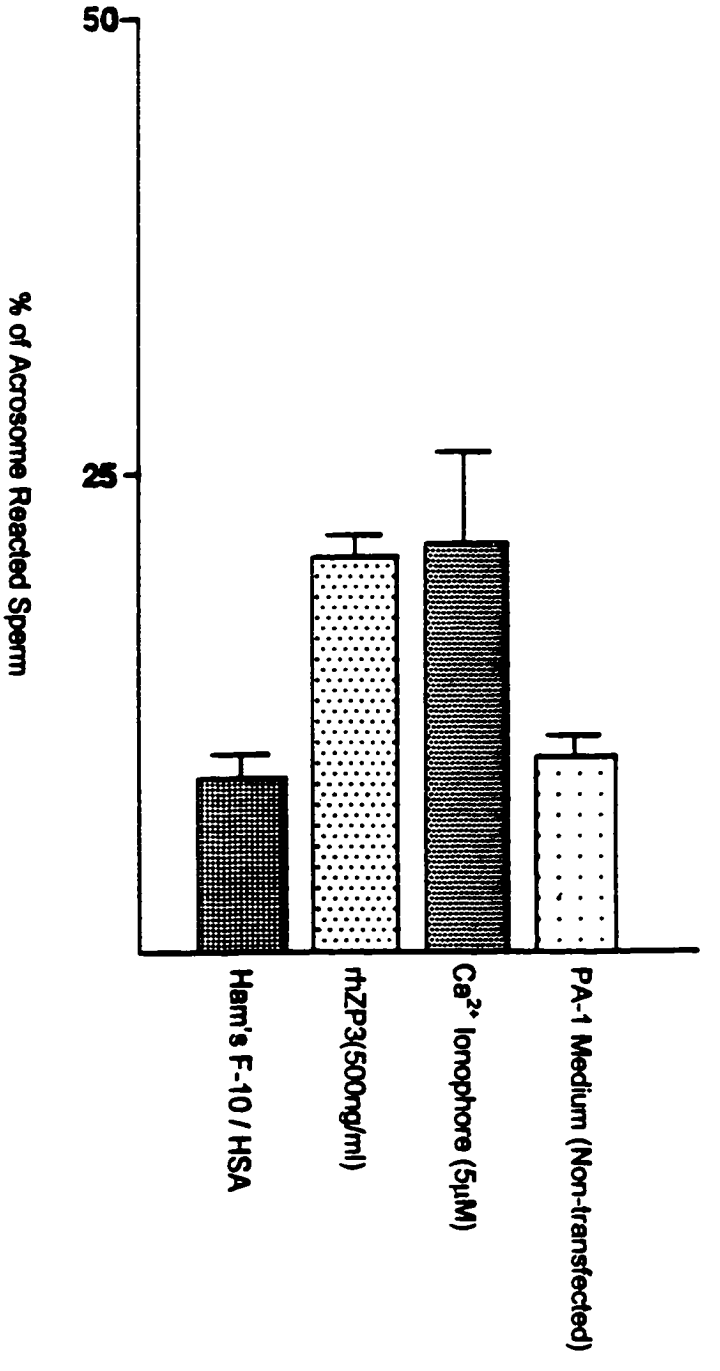

Fig. 15. The effect of Ni-NTA affinity-purified rhZP3 glycoproteins in increasing the acrosome reaction of human spermatozoa. Capacitated human spermatozoa were preincubated in the presence of rhZP3 glycoproteins at concentration of 30 ng/ml, Ca^{2-} ionophore, Ham's F-10 and purified glycoproteins from non-transfected PA-1 for 30 minutes. The purified rhZP3 and Ca^{2+} ionophore show the ability to increase about 1.9 fold of the acrosome reaction of spermatozoa.

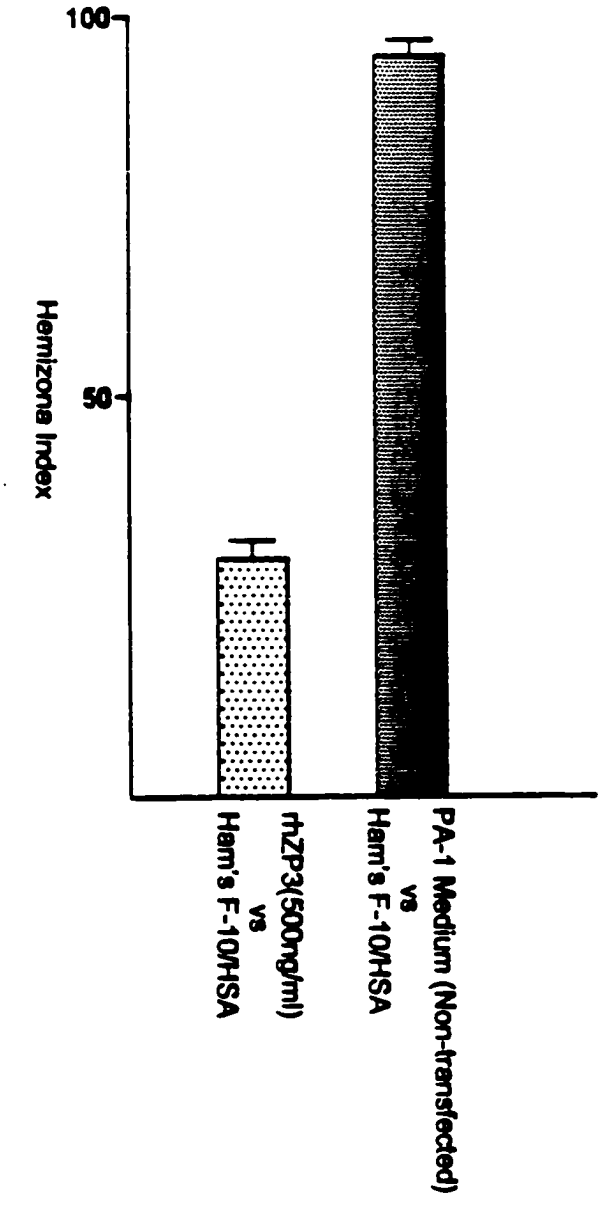

Fig. 16. The effect of Ni-NTA affinity-purified rhZP3 glycoproteins in inhibiting the binding between spermatozoa and zona pellucida in the hemizona assay. The purified rhZP3 glycoproteins show the ability to inhibit 70% spermatozoa-zona pellucida binding under the concentration of 500 ng/ml.

DISCUSSION

The expression of recombinant zona pellucida protein 3(rZP3) in the mouse and rabbit and human demonstrated that it is possible to use genetic engineering and molecular biology techniques to express recombinant ZP3 glycoproteins with the ability to induce acrosomal reaction of spermatozoa (Kinlock et al., 1991; Prasad et al., 1996; Beebe et al., 1992; Van Duin et al., 1994). With these technologies, scientists not only can express a recombinant ZP3 glycoprotein but also can reveal the mechanisms involved in gamete interaction at the molecular level. For example, exon swapping and mutagenesis with mouse ZP3 gene demonstrated that the C-terminus of ZP3 protein is responsible for inducing the acrosome reaction (Kinloch et al., 1995).

The recombinant human ZP3 has been expressed using several different approaches, e.g. Escherichia coli (Chapman et al., 1996), *in vitro* transcription and translation (Whitmarsh et al., 1996) and in Chinese hamster ovary (CHO) cells (Van Duin et al., 1994). However, none of these systems can produce a rhZP3 with strong and full biological activities (the ability to induce the acrosome reaction and inhibit the binding between the oocyte and spermatozoa) and have a recovery of the rhZP3 at milligram levels. All these approaches face the technical difficulties of producing highly purified biologically functional glycosylated zona pellucida proteins (Van Duin et al., 1994; Barratt et al., 1995). The insolubility and adequate glycosylation are the main problems associated with the expression of recombinant zona pellucida proteins (Chapman and Barratt, 1996).

In the first approach, we tried to express the rhZP3 with the pBlueBacHis vector (Invitrogen, San Diego, CA), a polyhedrin-directed recombinant protein expression

system of baculovirus, in the sf9 and H5 insect cell lines. This protein expression system has been proved having the ability to produce the recombinant proteins at milligrams level (Takeuchi et al.,1992; Chen et al., 1993). However, the glycosylation and the insolubility of the protein are two main disadvantages of this baculovirus expression system. Since the glycosylation is species-specific and tissue-specific (Varki, 1993), the sf9 and H5 insect cell lines may not produce a similar glycosylation to that of oocyte. The insolubility is the major disadvantage of the baculovirus expression system in expressing the rhZP3. The insolubility property of rhZP3 expressed in baculovirus expression system is worse than that observed in other protein expression systems using the mammalian cell lines. Using the wheat germ agglutinin combining with ProBond (Invitrogen) affinity chromatography, the total recovery of the purified recombinant protein from one liter supernatant is about 30 to 50 μg of rhZP3 at 20 to 30 % purity. Therefore, an effective mammalian expression vector, the pcDNA3.1(+) vector, was selected to express this rhZP3 glycoproteins in PA-1 cell line, a human ovarian teratocarcinoma cell.

The pcDNA3.1(+) vector contains two important elements: the human cytomegalovirus (CMV) immediate-early promoter/enhancer for high-level expression in a variety of mammalian cell lines and the bovine growth hormone (BGH) polyadenylation signal for efficient transcript stabilization and termination. The RT-PCR result shows that the pcDNA3.1(+) has strong capacity to express the recombinant ZP3 glycoproteins in the PA-1 cells. This result supports the high yield of recombinant ZP3 protein (about 7 mg of rhZP3/L of cell culture media)

According to the natural characteristic of ZP3, this glycoprotein tends to form a polymer like structure (Bleil and Wassarman, 1980; Yanagimachi, 1988). This character implies that there are distinct molecular structures in the ZP3-polypeptide backbone which will facilitate protein-protein polymerization, or some specific metabolic pathway, or some intracellular signal transduction mechanism that conducts the polymerization (Wassarman, 1990). This polymerization character of ZP3 leads to aggregate and precipitate out of the solution. The insolubility of rhZP3 glycoproteins not only complicates the protein purification but also causes some problems in preserving the collected culture media. The protein-precipitates were observed in the collected culture media when these media were stored at -20°C or -80°C for two to three weeks.

The rhZP3 glycoproteins such as expressed in CHO cells partition into insoluble fraction in which they may aggregate with other ZP3-proteins or non-ZP3-proteins thus decreasing the concentration of the rhZP3 in the collected culture media (Chapman et al., 1997). The lower rhZP3 in the soluble phase, the more difficulty in purifying pure rhZP3. The aggregation situation also causes problem during the dialysis. After Ni-NTA affinity purification, about 80 to 90% of the purified glycoproteins in the soluble phase are aggregated into a glycoprotein-sheet during the dialysis. Furthermore, the aggregated proteins are very difficult to get back to the soluble phase without using any detergents. Therefore, the dialysis procedure should not be used in processing the highly purified rhZP3 glycoprotein samples.

The ZP3 is difficult to purify since it is a mixture of heterogeneous glycoproteins. The affinity purification is a good choice to purify such kind of heterogeneous proteins. The efficiency of antibody-antigen affinity chromatography depends on the specificity of

antibody and the interaction between antigen and antibody. If the antibodies have high affinity to their antigens, they have the ability to recognize antigen specifically and immobilized antibodies may bind the antigen very tightly on the resins. It may need an extreme condition (such as low pH) to elute the antigen from affinity-resins. Under this situation, the biological activities of eluted proteins may be lost during exposure to such elution conditions. The Ni-NTA affinity chromatography does not have these kinds of situations. The dissociate constant (Kd) between the Ni-NTA and the histidine tag is 10^{-13} at pH 8.0 (Hoffmann and Roeder, 1991) and is far greater than the affinity between most antibodies and antigens, or enzymes and substrates. Moreover, most of six histidines -tagged-recombinant proteins can be eluted from Ni-NTA under a mild condition with 80mM (Hoffmann and Roeder, 1991) or lower concentrations of imidazole which can be dialyzed away easily. Therefore the Ni-NTA affinity chromatography can circumvent the disadvantages of antibody-antigen affinity chromatography.

Eluting the rhZP3 glycoproteins from the affinity column is a critical step during the rhZP3 protein purification. The gradient elution is a common procedure used in the affinity chromatography but it is not suitable for purifying rhZP3 glycoproteins. Since the rhZP3 is a mixture of heterogeneous glycoproteins, which have different molecular weights and different isoelectric points. The gradient elution method will elute the rhZP3 glycoproteins in a wide gradient-range. Therefore, the rhZP3 will be eluted in a big elution volume which will cause a serious situation, because above 80% of purified rhZP3 glycoproteins will be lost during the concentrating procedure which is the major contribution to the low recovery of the rhZP3 glycoprotein purification. For example, the

purification of recombinant mouse ZP3 glycoproteins in which a Centricon 30 is applied to concentrate the eluted protein sample (Beebe et al., 1992). This procedure results in a low recovery of rmZP3 (20 ~ 100μg from 1 liter culture medium) (Beebe et al., 1992). The rmZP3 glycoproteins may stick to the membrane or wall of tube of the Centricon 30 during the concentration. Compared to our recovery of about 2μg of rhZP3/ml of cell culture media, this concentration procedure results in a relatively low recovery at 20 to 100 ng of rZP3/ml of cell culture media (Beebe et al., 1992).

Many studies indicate that both the three-dimensional structure of ZP3 glycoprotein and carbohydrates attached to it are required for mediating its biological activity. (Florman and Wassarman, 1985). The carbohydrates are not primary gene products. They are synthesized by glycosyltransferases, which are protein products of the gene. Different cell lines may express different glycosyltransferases, which produce different pattern of glycosylation on proteins. The four mammalian ZP3 genes that have been cloned thus far, i.e., mouse, human, hamster, and marmoset ZP3, all encodes homologous proteins of 422-424 amino acids. Post-translational processing of the protein in the case of mouse recombinant ZP3 produced in Chinese hamster ovary (CHO) cell lead to glycosylated protein of about 83 kDa (Bebee, 1992), whereas in the other species, ZP3 has a molecular weight of approximately 55 kDa (Wassarman and Litscher, 1995). All these recombinant ZP3 glycoprotein have smaller size than that of native ZP3. The explanation for this difference is still unclear, but it is conceivable that the variation in the extent and/or patterns of glycosylation accounts for the disparate molecular weight of rhZP3 glycoproteins. Since the glycosylation is species-specific and tissue-specific (Varki, 1993), the PA-1 cell, derived from human ovarian tissue, was used as a host cell

line to express the rhZP3 glycoproteins. In addition, the PA-1 cell has been proved to have the ability to synthesize a wide variety of oligosaccharides (Fukuda et al., 1985; Furukawa et al., 1990).

Despite of these difficulties, we have demonstrated a new system to express a biologically functional recombinant human ZP3 glycoproteins and a purification system, which can circumvent these obstacles as described above. Purification of the recombinant human ZP3 from the cell culture media of histidine-tagged rhZP3-producing PA-1 cells has been accomplished by a protein purification system composed of wheat germ agglutinin affinity chromatography glycoprotein isolation and Ni-NTA mediated histidine tagged recombinant protein affinity purification. To achieve a high degree of purity of rhZP3, the rhZP3 is eluted from Ni-NTA affinity column into two pools by different elution conditions. This strategy produced a lower overall yield, but it provided a 50 to 60% purity of rhZP3 glycoproteins that have molecular weight between 62 to 66 kDa right in the range of the native ZP3 ($ZP3_H$, Mr = 58 ~ 72 kDa, pI = 3.5 ~ 5.1; $ZP3_L$, Mr = 54 ~ 62 kDa, pI = 3.5 ~ 5.1) reported by Bercegeay at 1995. Analyzing the yield indicates that about 2 mg quantities of high purity rhZP3 have been purified from 1 liter cell culture media of histidine-tagged rhZP3-producing PA-1 cells. Compared to the 7mg total expressed recombinant ZP3 glycoproteins in one liter cultured cell media, there is about 30% of recombinant proteins being recovered. It leaves some room for us to enhance the recovery rate. If a set of eight to ten histidines is introduced into the recombinant protein at C-terminal or at N-terminal right after the signal peptides, these longer histidine tag may provide an stronger interaction between the recombinant protein and the Ni-NTA resin which allows a stronger washing condition to be applied to wash

off these contaminate proteins. Therefore, both the purity and the recovery of the rhZP3 may be enhanced.

Characterizing the purified glycoproteins with the SDS-PAGE electrophoresis, two-dimensional electrophoresis and western blotting analysis shows that the rhZP3 expressed in the stable-transfected PA-1 is heterogeneous. This rhZP3 glycoprotein has a molecular weight range between 62 ~ 66 kDa which is similar to their native molecular weight profile. Purified rhZP3 was subjected to the isoelectric focusing followed by SDS-PAGE and western blotting analysis. The results indicated that the rhZP3 glycoproteins was isoelectric focused predominantly between 3.4 and 5.0.

Compared to the 55 ~ 60 kDa rhZP3 expressed in CHO cells (Van Duin et al., 1994), the rhZP3 expressed in PA-1 cell has a higher molecular weight indicating that the PA-1 cell could perform a better glycosylation on the rhZP3. The *in vitro* translation experiment demonstrated that our hZP3-cDNA was able to produce a 47 kDa nonglycosylated core protein which exhibited full-length protein backbone of the hZP3 (Chen, 1995). There is 15to19 kDa different between the glycosylated and non-glycosylated rhZP3 protein. Typically, a complex type N-linked or O-linked oligosaccharide is about 3 - 4 kDa. It indicates that the rhZP3 core protein might have four to six oligosaccharide side chains being attached to it and at least one of them is O-linked oligosaccharide, because the biological activity has been shown to be absolutely dependent on correct O-linked glycosylation (Wassarman, 1989,1991). Carbohydrate side chains are important for stabilizing proteins (Parekh et al., 1989; Rickert et al., 1995; Sareneva et al., 1994). These oligosaccharide side chain may provide a long half-life (up to 10 days) to this highly glycosylated rhZP3 (Dean, 1992; Chamberlin et al., 1990).

Under our storage condition (50% glycerol freeze with liquid nitrogen and then stored at -80°C), the purified rhZP3 glycoproteins can preserve their biological activity about 30 days.

All these results indicate that rhZP3 expressed in PA-1 cell is characteristically and immunologically related to the native hZP3. In the biological function study, the rhZP3 expressed in PA-1 cell can increase the acrosome reaction by 1.9 fold at the concentration of 30 ng/ml, which is about 1/1000 of the concentration of the rhZP3 expressed in CHO cells used to induce a similar acrosome reaction (Van Duin et al., 1994). The hemizona assay shows that the rhZP3 can competitively inhibit the binding between spermatozoa and zona by 70%. Furthermore, the rhZP3 expressed in the PA-1 cells can induce the acrosome reaction within 30 minutes after spermatozoa exposed to rhZP3. This is almost 12 times faster than that of rhZP3 expressed in CHO cells (Van Duin et al., 1994). The stronger biological activity of recombinant hZP3 glycoproteins expressed in PA-1 cell line may result from the more similar glycosylation pattern to that of native human ZP3.

With this high-level rhZP3 expression system in hand, the highly purified rhZP3 glycoprotein may provide an easier way to identify the complementary receptors on spermatozoa. Studies using native zona pellucida have identified several candidate receptors on the plasma membrane of spermatozoa such as zona receptor kinase (Burks et al., 1995). This rhZP3 expression and purification system may provide the necessary techniques to express recombinant zona receptor kinase (rZRK) and then the kinetics of interaction between the rhZP3 and rZRK can be examined. If the rhZP3 can interacts with the recombinant zona receptor kinase in a similar manner to native zona pellucida

then the primary cell signalling mechanisms associated with the zona receptor kinase can be studied.

This highly purified rhZP3 can be used to reveal the crystal structure of rhZP3 that allows significant advances in the understanding of how molecules interact. Such information provides the baseline for more detail studies. The site-directed mutagenesis studies, for example, can reveal the information about the biologically active site of the ZP3 (Kinloch et al., 1994). These informations may provide a foundation with which to gain additional knowledge of those protein and carbohydrate domains that are critical for species-specific fertilization. The information about the structure of rhZP3 also highlights the way for designing the drugs/molecules that can inhibit/activate the proteins involved in the fertilization.

The other goal in the production of rhZP3 is to explore its use for contraceptive research. Recombinant zona pellucida glycoproteins have been used as antigens to stimulate an immune response, however, the initial enthusiasm for this approach shows a long-term side effect due to the subsequent ovarian dysfunction following immunization in several species (Paterson et al., 1996; Bagavant et al., 1997). A possible mechanism is autoimmune ovarian disease induced by ZP3-specific autoreactive T cells, demonstrated in mice immunized with a murine ZP3 peptide (Bagavant et al., 1997). The selected mouse ZP3 epitopes have allowed induction of limited supression of fertility without accompanying ovarian pathology (Lou et al., 1995). However, the possible presence of ZP3 on primordial follicles may contribute to ovarian pathology and thus complicate the choice of peptide antigens (Grootenhuis et al., 1996). Production of rhZP3 glycoprotein can be used for epitope mapping studies. For example, Gupta et al. (1996) used such an

approach to identify amino acid residues 133-144 and 205-216 of procine ZP3α which are important in the sperm-zona recognition process and thus may be important candidate epitopes for contraceptive design.

CONCLUSIONS

The conclusions of these studies are summarized as follows:

1. The PA-1 cell line has been stable transfected with the hZP3-sixHis-pcDNA3.1(+) vector. The RT-PCR analysis proves that the histidine tagged hZP3 cDNA has been successfully expressed in the PA-1 cells. This stably transfected PA-1 cell line is capable of producing and secreting the biologically active rhZP3 at a high expression level (7mg/litter cultured cell media).
2. The rhZP3 glycoproteins purified by wheat germ agglutinin chromatography followed by Ni-NTA affinity chromatography are heterogeneous. The two dimensional electrophoresis, SDS- PAGE electrophoresis and western immunoblotting analysis show that the rhZP3 glycoproteins have a range of molecular weights between 62 ~ 66 kDa. and an isoelectric point between 3.4 ~ 5.0.
3. Two-dimensional electrophoresis followed by the western immunoblotting analysis shows that the purified rhZP3 has a purity about 60% at a recovery of 2μg of rhZP3/ ml of collected culture media.
4. The ELISA analysis reveals that the rhZP3 producing PA-1 cells show a feedback inhibition on the recombinant glycoprotein secretion. At the culture time of 72 hours, the rhZP3 producing PA-1 cells show a significant decrease in rhZP3 production.
5. The protein purification system composed of a wheat germ agglutinin glycoprotein isolation followed by DEAE anion exchange can isolate both a 140 kDa glycoprotein and 62~66 kDa rhZP3 but the purity is less than 20%.
6. The purified rhZP3 shows the ability to increase the acrosome reaction of spermatozoa 1.9 fold at the concentration of 30 ng/ml. In the hemizona assay, the

purified rhZP3 competitively inhibits about 70% of the normal level of spermatozoa-zona pellucida binding at the concentration of 500 ng/ml.

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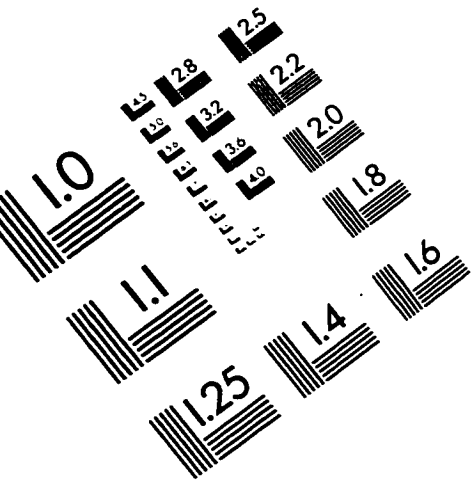
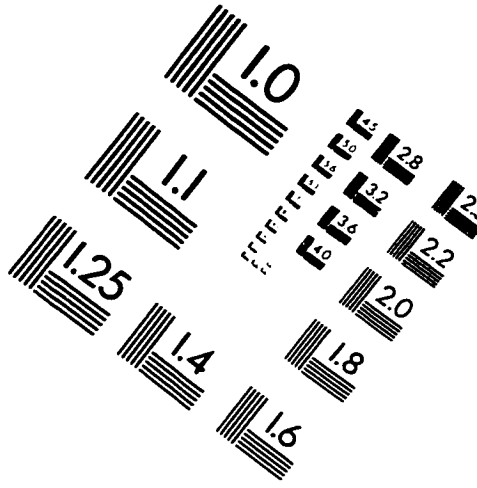
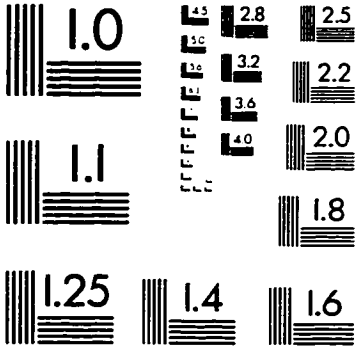
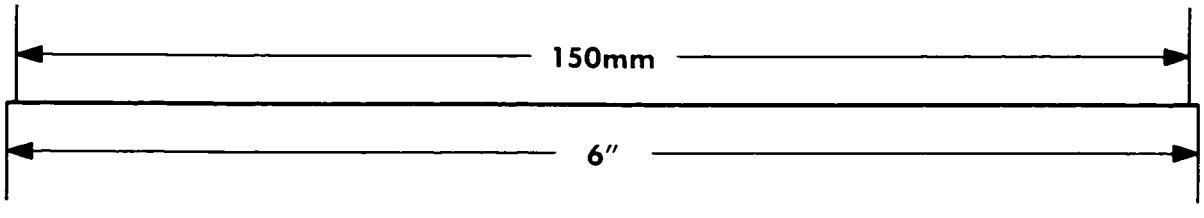
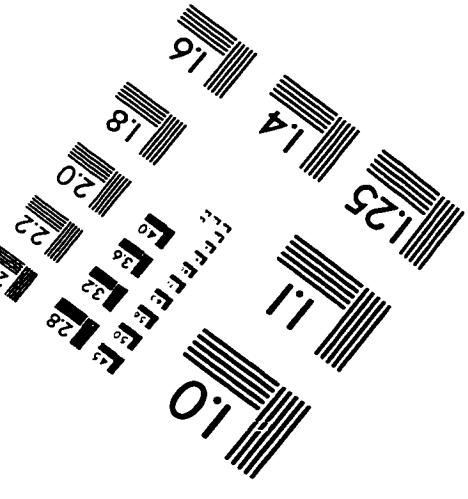
IMAGE EVALUATION TEST TARGET (QA-3)

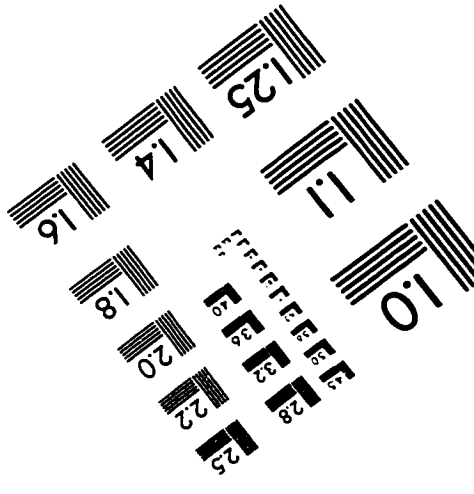












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