—Review—

Porcine ZP4 (Zona Pellucida) as a Candidate for Contraceptive Vaccine

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The zona pellucida surrounding mammalian oocytes is a unique extracellular matrix composed of several glycoproteins. It plays an important role in sperm recognition, sperm activation and prevention of polyspermy during the fertilization process [1].

Since it was found that the zona pellucida possessed a strong tissue-specific but species-crossreactive antigenicity and that passive or active immunization with the zona antigens could induce infertility in a variety of animals, the possibility of developing a contraceptive vaccine using zona antigens have been intensively studied. In particular, porcine zona pellucida has been considered to be a potential candidate for human contraception because of its strong crossreactivity with human zona pellucida [2–4].

Recent studies on active immunization with purified porcine zona pellucida proteins demonstrated that most immunized animals became infertile along with the increase in serum antibody titers but it was always associated with the ovarian failure [5–8]. It is therefore important to segregate the antigenic epitope that inhibits the sperm-zona interactions, from other epitopes that induce ovarian failure. Recently, it has become possible to investigate the target antigens of the zona pellucida at epitope levels, since full-length cDNA sequences have been reported in various mammalian species including mice [9, 10], hamsters [11], marmosets [12], rabbits [13, 14], humans [15, 16] and pigs [17, 18]. On the basis of amino acid sequences deduced from cDNA, the immunogenicity of the synthetic peptides and the biological effects of resultant antisera have been examined [19–21]. However, it was difficult to find promising antigens using peptide synthesis because numerous peptides must be synthesized for screening of the appropriate antigens. In this regard, the strategy using monoclonal antibodies is useful for detection of effective antigen epitopes in high molecular mass proteins with numerous complicated epitopes. The aim of the present article is to review the current status for development of a contraceptive vaccine based on zona pellucida antigens.

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Immunogenicity of pZP4

Solubilized porcine zona pellucida (sPZP) was separated into four glycoprotein families (pZP1: Mr 92,000, pZP2: Mr 69,000, pZP3: Mr 55,000, pZP4: Mr 23,000) on O'Farrell's two-dimensional polyacrylaminde gel electrophoresis (PAGE) under reducing conditions, and each glycoprotein family was composed of many isomers with different charges and molecular weights (Fig. 1-a). Diagonal SDS-PAGE revealed that pZP2 and pZP4 were derived from a common parental glycoprotein pZP1. When the isolated pZP4 glycoprotein was treated with trifluoromethanesulfonic acid (TFMS) for deglycosylation, the polymorphic pattern disappeared and a single protein spot of Mr 15,000 was observed on O'Farrell's two-dimensional PAGE. This suggests that the polymorphic pattern depends on the heterogenicity of carbohydrate moieties but not on the core protein.

When mice were immunized with each of the isolated glycoprotein family, the antisera with high antibody titers were produced. All antisera except anti-pZP2 showed a strong interspecies-crossreaction with human, rabbit and hamster zona pellucida. When the antisera were added in *in vitro* fertilization system of human oocytes, three antisera to pZP1, pZP3, pZP4 exhibited a strong inhibitory effect on human sperm binding and penetration into zona pellucida of human oocytes (Fig. 2).

When female golden hamsters were immunized with sPZP or pZP4 in complete Freund's adjuvant, antisera reactive not only with porcine but also with hamster zona pellucida were produced. The animals immunized

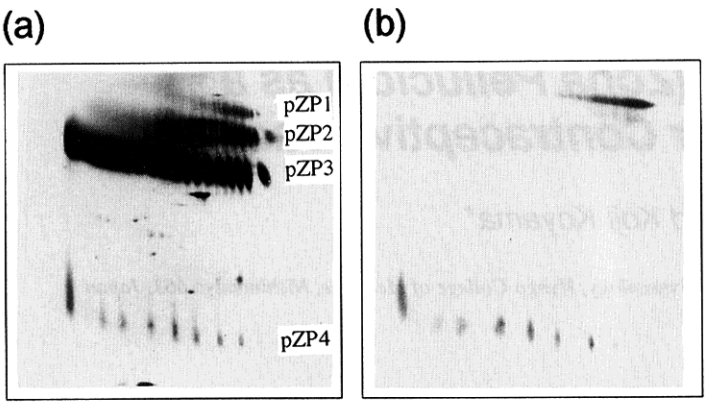

Fig. 1. Two-dimensional electrophoresis of porcine zona pellucida (a) and western-blotting with MAb-5H4 (b). Porcine zona pellucida was separated into four major glycoprotein families. MAb-5H4 reacted with pZP1 and pZP4.

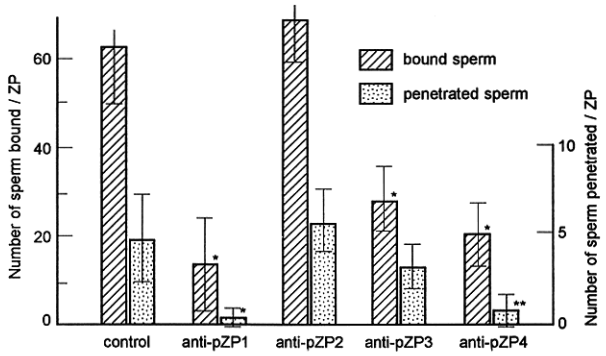

Fig. 2. Effect of antisera to four glycoprotein families on human sperm binding and penetration to zona pellucida. All antisera except anti-pZP2 showed a blocking effect on sperm binding and penetration to zona pellucida.

with sPZP became infertile permanently with remarked degenerative changes of the ovaries, while the aminals immunized with pZP4 become infertile temporarily with some ovarian degenerative changes but they recovered from infertility later [8]. The antibodies produced in the former animals were mainly reactive with pZP3, while the antibodies produced in the latter animals were mainly reacted to pZP4. These results are encouraging for further studies on the pZP4 antigens as candidates for contraceptive vaccine development.

Peptide Mapping of pZP4

The amino acid sequence of pZP4 was analyzed in order to determine the structure and possible antigen determinants. As the first step, pZP4 glycoproteins were isolated from a preparative SDS-PAGE and subjected to fragmentation by CNBr or endoproteinase LysC or AspN. The resultant fragments were fractionated by a reverse phase HPLC and then the selected peptide fragments were sequenced by automated Edman degradation method. By aligning the peptide fragments with overlapping sequences, the NH2-terminal 128-amino acid sequence was determined [22].

Cloning of cDNA Coding for pZP4

The entire amino acid sequence (133 residues) of pZP4 was deduced by cloning of cDNA coding for pZP4 [22]. cDNA cloning was carried out by PCR. mRNA was extracted from a porcine ovary and converted into single or double strand cDNA that was used as DNA templates for PCR. The primers were synthesized based on the amino acid sequence of pZP4 peptides and the nucleotide sequence of a gene (M13) in pUC18 vector. Four overlapping cDNA clones corresponding to the amino acid sequence of positions 1–77, 53–86, 70–110 and 94–198 were obtained and they were sequenced and linked together to determine the whole cDNA sequence. The molecular size deduced from the peptide comprising 198 amino acids appeared larger than that of pZP4 core peptide (Mr 15,000). Some part of the COOH-terminal sequence of the 198-amino acid peptide is considered to correspond to the NH2-terminal sequence of pZP2, because that mentioned before, pZP2 and pZP4 were derived from the common parental mol-

ecule of pZP1. By analysis of the NH2-terminal sequence of pZP2 by Edman degradation, a ten-amino acid sequence DEAVKREDSK was determined. This sequence corresponded to the amino acid sequence (positions 134–143) deduced from the constructed cDNA sequence, suggesting that pZP4 consisted of 133 amino acids and linked to the NH2-terminal portion of pZP2.

For cloning of a full length cDNA of pZP1, the 3' rapid amplification of cDNA ends (3' RACE) and RT-PCR were carried out using oligonucleotide primers. As a result, the 2.2 kbp of cDNA was isolated [18]. The pZP1 mRNA encoded a protein of 716 amino acids including a putative signal peptide of 35 residues (Fig. 3). A polyadenylation signal (AATAAA) located 13 nucleotides distal to the termination codon preceded a typical poly (A) tail of 40 nucleotides. The sequence of pZP1 coding region was 78% and 70% identical with the corresponding gene from humans, mice, respectively [16, 17].

Contraceptive Effect of Recombinant pZP1 (1–198) Proteins in Rabbits

The cloned cDNA coding for pZP1 (1–198) was inserted into a expression vector (pMAL-c2 or pET21b) to produce the recombinant fusion protein. The resultant recombinant protein (rec-pZP1) reacted with MAb-5H4 in western blotting, suggesting that the rec-pZP1 artificially produced in *E. coli* retain the antigenicity similar to that of native pZP1 and pZP4 proteins.

The rec-pZP1 (100 μg) were injected in rabbits with complete Freund's adjuvant and antibody production was monitored by ELISA and immunofluorescent staining of porcine oocytes. The rec-pZP1 produced antibodies reactive with native pZP4 and intact porcine zona pellucida and the antibody titers in ELISA ranged from 1:1,600 to 1:6,400. Immunofluorescent staining showed that the antisera reacted not only with porcine zona pellucida but also with rabbit zona pellucida, suggesting that rec-pZP1 proteins induced autoreactive autoantibodies to zona pellucida in rabbits.

When eight rabbits immunized with rec-pZP1 were caged with a fertile male, no rabbit conceived although three of them were successfully mated. In control, five of seven rabbits were successfully mated and all except one conceived and gave birth of normal size litters. Histological examination revealed that the number of growing follicles were markedly reduced in rabbits immunized with rec-pZP1 compared with control rabbits. These results indicate that rec-pZP1 can induce autoantibody reactive to self-zona antigen and significantly reduce the fertilization ability of rabbits but ovaries of the immunized rabbits are also markedly damaged. Therefore, further dissection of the antigenicity of pZP4 is necessary for development of a safer contraceptive vaccine.

Monoclonal Antibodies to pZP4

For the further characterization of the antigenicity of pZP4, monoclonal antibodies (MAbs) were produced [23] by cell fusion between mouse myeloma cells and spleen cells from a mouse immunized with pZP4. Five MAbs (2A1, 2G3, 4A2, 4E12, 5H4) produced reacted not only with intact porcine zona pellucida but also with human and rabbit zona pellucida. They also reacted with sPZP deglycosylated with TFMS treatment, suggesting that they recognize peptide antigen epitopes of porcine zona pellucida.

Three (4A2, 4E12, 5H4) of the five MAbs exhibited a significant blocking effects on sperm binding and penetration into the zona pellucida of porcine and human oocytes. MAb-5H4 specifically reacted with pZP1 and pZP4 (Fig. 1-b), but other four MAbs more strongly crossreacted with pZP3 rather than pZP4 in western blotting. Therefore, MAb-5H4 was selected for further characterization of the corresponding antigen epitope.

The reactivity of MAb-5H4 with pZP4 was preserved after deglycosylation with N-glycanase or TFMS treatments and also after treatment with trypsin or endoproteinase LysC digestion, but the reactivity was lost completely by the treatment of chymotrypsin. These results indicate that the epitope recognized by MAb-5H4 is present on a peptide portion of the pZP4 core protein.

Epitope Mapping for MAb-5H4

As the first step in identifying the epitope recognized by MAb-5H4, three cDNA segments for pZP4 (92bp coding for 1–27 residues, 346bp coding for 1–112 residues, 428bp coding for 1–133 residues) were ligated in an expression vector (pMAL-c2) and transfected to *E. coli.* The produced three recombinant proteins were examined for the reactivity with MAb-5H4 by immunoblotting. The last two transformants corresponding to pZP4 (1–112) and pZP4 (1–133) showed positive reaction (Fig. 4). This finding suggests that the MAb-5H4 epitope is present in the amino acid positions 28–112 of pZP4.

As the second step, a computer analysis [24] was carried out to identify highly flexible regions of the pZP4 peptide chain which are expected to be possible anti-

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         1                                   36                                   67
pZP1     MACRHRGDSGRPLSWLSASW   RSLLLFFPLVTSVNSIGVNQLVNTAFPGIVTCHENRMV VEFPRILGT
hZP2     ----Q--G-WS-SG-FNAG-STY--IS---A----G---D-S----P----T---D- -EIT----SSP--
mZP2     --RWQ-KA-VSSPC    GR-I--FLS-L-T----V--VSLP-SE-P----TLI-DK DEVRI--SSRFDM
                                                                                138
         KIQYTSVVDPLGLEMMNCTYVLDPENLTLKAPYEACTKRVRGHHQMTIRLIDDNAALRQEALMYHISCPVM
         -KWHA--------D-P----I----K---R-T-DN--R--H-G------VMNNS----HG-V--QFF--A-
         EKWNP----T--SEIL----A--L-RFV-KFP-ET--IK-V-GY-VN--VGDTTTDV-YKDD--HFF--AI
                                                                                205
         GAEGPDQHSGSTICMKDFMSFTFNF FPGMADENVKREDSKQR   MGWSLVVGDGEKARTLTFQEAMTQG
         QV-ETQGL-A----Q------SLPRV-S-L--       DSKGTKVQ----IE----AR-K---LP---KE-
         QA- THEI-ETVV-RR-LI--SFPQLFSRL--ENQNVSE        ---IVKI-N-TR-HI-PLKD-IVQ-
                                                                                275
         YNFLIGNQKMNIQVSFHATGVTRYSQGNSHLYMVPLKLKHVSHGQSLILASQLICVAD PVTCNATHVTLA
         FSL--D-HR-TFH-P-N-----H-V---------S---TFI-P--KV-FS--A--APD ------M--T
         FNL--DSQKVTLH-PAN---IVH-V-ES-Y--T-Q-E-LFSTT--KIVFS-HA--APDLS-A----M--T
                                                                                346
         IPEFPGKLKSVNLGSGNIAVSQLHKHGIEMETTNGLRLHFNQTLLKTNVSEKCLPHQLYLSSLKLTFHSQL
         -----------SFENQN-DVS---DN--DL-A---MK---SK-----KL-----L--F--A------LLRP
         --------E--DFGQWS-PED-W-AN--DK-A---LR-N-RKS----KP----PFY-F--SS-----YFQG
                                                                                417
         EAVSMVIYPECLCESTVSLVSEGLCTQDGFMDVKVHSHQTKPALNLDTLRVGDSSCQPTFKAPAQGLVQFR
         ETV-------------P--I-TGE---------E-Y-Y--Q---D-G-----N-----V-E-QS----R-H
         NML-T--D---H---P--I  DE--A------FE-Y-H-----------L--N-----I-KVQSV--AR-H
                                                                                487
         IPLNGCGTRHKFKNDKVIYENEIHALWAD PPSAVSRDSEFRMTVRCSYSSSNMLINTNVESLPSPEASVK
         ------Y--ED---V---------T-F---KI----------K----RND--L-I-----TP-V----
         -----Q--EG-------------ENP-SNIVF-N-------R-Y-IRDS--L-AH-KGHPS-E-F--
                                                                                558
         PGPLTLTLQTYPDNAYLQPYGDKEYPVVKYLRQPIYLEVRILNRTDPNIKLVLDDCWATSTEDPASLPQWN
         L--F--I--S----S-Q----EN---L-RF------M---V---D----------------M--D-F----
         P--LV-V--T---QS-QR--RKD---LVR-------M--KV-S-N----------------SE--A-A---Q
                                                                                629
         VVMDGCEYNLDNHRTTFHPVGSSVTYPNHHQRFDVKTFAFVSGAQGVSQLVYFHCSVFICNQLSPTFSLCS
         --V---A-D---YQ-----------H-D-Y----M-A-----E-HVL-S-------AL---R---DSP---
         I-M---E-E---YR-----A---AAHSG-Y----V-T-----E-RGL-SLI-----AL---QV-LDSP---
                                                                                672
         VTCHGPSRSRRATGTTEEEKMIVSLPGPILLLSDGSSLRD                          AVN
         ---PVS--H-----A--A---T------------D--F-GVGSSDLKASGSSGEKSRSETGEEVGSRG-MD
         ---PASL-SK-E  ANKEDT-T------------V--SKG-DP              -S--ITKDIIAK

         SKGSRTNGYVAFKTMVAMVASAGIVATLGLISYLHKKRIMMLNH
         T--HK-A-D-GS-AVA-VA-F--V-----F-Y--YE--TVS --
                 DIAS-TLG-VA-LV-SAVI--F-C--YK-RTIRF--
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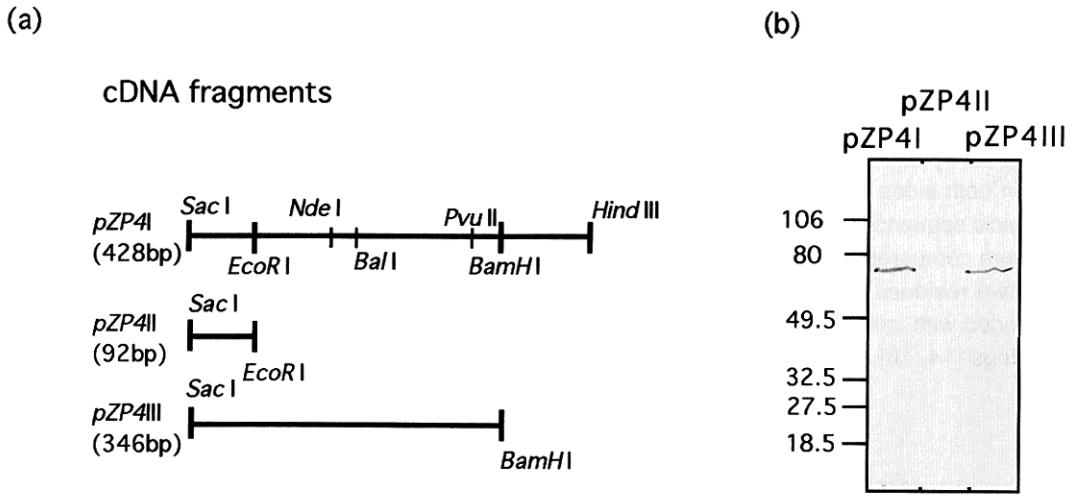

Fig. 4. Epitope mapping of MAb-5H4 by recombinant method. (a) Schematic diagram of cDNA fragments encoding for pZP4. (b) Western-blotting of bacterial cells transformed by an expression vector containing each cDNA fragment. The recombinant proteins from *pZP4*I and *pZP4*III fragments showed positive reactions with MAb-5H4. The epitope was found to be present between *EcoR*I site and *BamH*I site which corresponded to amino acid positions 28–112 of pZP4.

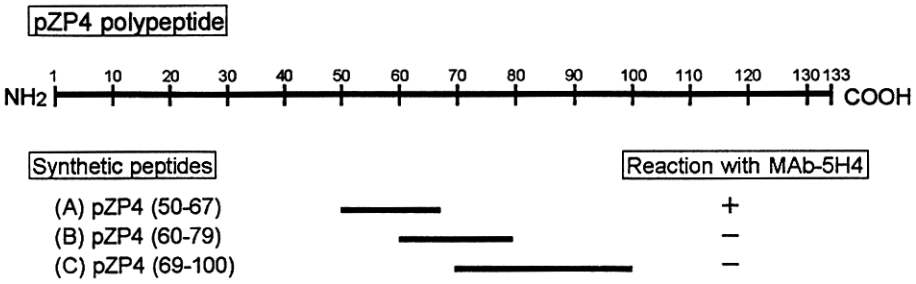

Fig. 5. Determination of the epitope for MAb-5H4. The putative peptides corresponding to the positions 50–67, 60–79, 70–100 of pZP4 were chemically synthesized for dot immunoassay with MAb-5H4. Only the peptide 50–67 reacted with MAb-5H4, suggesting that the epitope might be present on the peptide 50–59.

gen determinants. Three regions were identified to be highly flexible in the positions 28–112 residues of pZP4. On the basis of the results, three putative peptides (amino acid positions 50–67, 60–79, 70–100) that are overlapping at the NH2- and COOH-terminal ends were chemically synthesized, based on the epitope mapping by cDNA and the flexibility analysis (Fig. 5). One synthetic peptide 50–67 reacted with MAb-5H4 in dotimmunoassay, but the other two synthetic peptides positions 60–79 and 70–100 did not reacted with MAb-5H4. The specific reactivity of the synthetic peptide 50–67 was confirmed by competitive binding-inhibition assay with MAb-5H4 against immobilized native pZP4. The peptide 50-67 but not the peptides 60–79 or 70–100 dose-dependently inhibited the binding of MAb-5H4 to the native pZP4 and the degree of inhibition with the synthetic peptide 50–67 was the same degree as that with the native pZP4. Since the 60–67 amino acid se-

Fig. 3. Deduced amino acid sequence of pZP1 aligned with human and mouse ZP2. 716 putative amino acids of pZP1 are showen in line 1. The solid underline denotes a signal peptide of 35 amino acids. The arrow shows the cleavage site of pZP1 into pZP2 and pZP4. Potential N-linked glycosylation sites are marked in square branckets. Sequences of human and mouse ZP2 protein are aligned in line 2 and line 3, respectively. The positions of cysteine residues identical among three species are boxed.

quence was included in the non-reactive 60–79 peptide, the MAb-5H4 epitope seems to be present on the 50–59 amino acid sequence of (N)CTYVLDPENL(T) of the reactive peptide 50–67 of pZP4. Interestingly, a putative N-linked glycosylation site (positions 49 and 58) was present on both sides of the epitope sequence.

The amino acid sequences corresponding to the MAb-5H4 epitope were compared among six different species (Fig. 6). The two residues, valine (53) and asparagine (58) were replaced with isoleucine and lysine in rabbits, humans and dogs [14, 16], while the four residues, valine (53), proline (56), asparagine (58), leucine (59), were replaced with alanine, leucine, arginine, phenylalanine, respectively in mice [10]. Thus the fact that MAb-5H4 reacted with the zona pellucida of pigs, humans, rabbits, dogs and cats but not mice indicated that the residues of proline (56) and leucine (59) are essential for the epitope structure of the MAb-5H4 epitope. The replacement of the residues valine (53) and asparagine (58) do not affect the reactivity of the peptide 50–67 with MAb-5H4.

		50 51 52 53 54 55 56 57 58 59	Reaction with 5H4
Pig	: ZP4(ZP1)	C T Y V L D P E N L	+
Human	: ZP2	· · · I · · · · · K ·	+
Rabbit	: R75	· · · I · · · · · K ·	+
Mouse	: ZP2	· · · A · · L · R F	−
Dog	: ZPA	· · S I · · · · · K ·	+
Cat	: ZPA	· · · I · · · · · · ·	+

Fig. 6. Comparison of the sequences corresponding to MAb-5H4 epitope from various species. Positions 56 (proline) and 59 (leucine) of pZP4 seems to be important for the MAb-5H4 epitope structure.

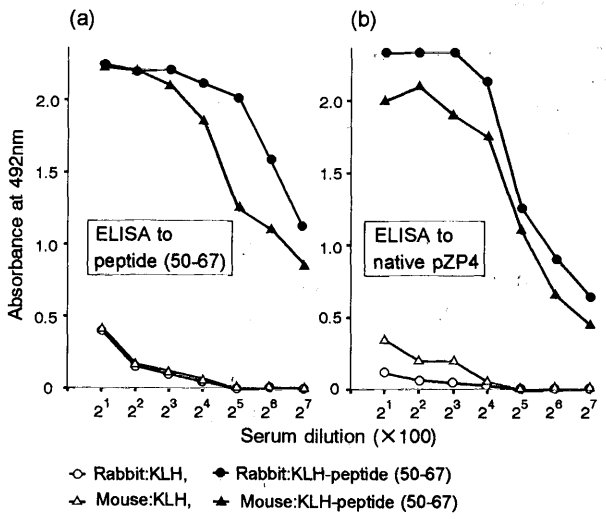

Fig. 7. Antibody production in rabbits and mice by immunization with synthetic peptide (50–67) conjugated with KLH. The antisera from both species reacted with the cognate synthetic peptide (a) and also with native pZP4 in ELISA (b).

Immunogenicity of the Synthetic Peptide 50–67

A synthetic 18mer peptide 50–67 of pZP1 was conjugated with KLH and injected into mice and rabbits with complete Freund's adjuvant. The antisera produced in both species reacted not only with the cognate synthetic peptide (Fig. 7-a) but also with native pZP4 in ELISA (Fig. 7-b). The mouse antiserum inhibited *in vitro* fertilization of porcine oocytes.

Conclusions

Heat-solubilized porcine zona pellucida separates into four major glycoprotein families (pZP1, pZP2, pZP3, pZP4). Cloning of a full-length cDNA coding for pZP1 revealed that pZP1 consisted of 681 amino acid residues, in which the NH2-terminal 133 redidues corresponded to pZP4 and the COOH-terminal 548 redidues corresponded to pZP2 and they shared a significant homology with and human mouse ZP2 [17, 25], which are considered to serve as a secondary sperm receptor in sperm-zona interaction [26]. One of the monoclonal antibody (MAb-5H4) produced against pZP4 inhibited not only boar sperm but also human sperm binding to each of the homologous oocytes. The epitope recognized by MAb-5H4 was determined to be present on a ten-amino acid sequence of pZP1 (50–59) by using epitope mapping and analysis of the flexibility of pZP4. A synthetic 18mer peptide corresponding to pZP1 (50–67) reacted with MAb-5H4, and mouse antisera raised to the synthetic 18mer peptide recognized intact porcine zona pellucida and strongly inhibited *in vitro* fertilization.

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