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# Amino acid sequence of a porcine zona pellucida glycoprotein ZP4 determined by peptide mapping and cDNA cloning

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Mammalian zona pellucida is an attractive target for developing a contraceptive vaccine. In this study, the amino acid sequence of a core protein of porcine zona pellucida glycoprotein ZP4 was determined by peptide mapping and cDNA cloning. Two kinds of ZP4 peptides were identified: one consisted of 128 amino acid residues and the other of 133 amino acid residues with an additional five amino acid sequence at the carboxy-terminal end of the 128 amino acid peptide. Both peptides had two potential *N*-linked glycosylation sites. The 128 amino acid peptide showed 39.1% similarity to the amino-terminal region of mouse ZP2 polypeptide. The positions of five cysteine residues were the same for porcine ZP4 and mouse ZP2. The cloned cDNA possessed an additional 195 nucleotides at the 3′ end of the sequence corresponding to the 133 amino acid peptide. This additional sequence was found to encode the amino-terminal 10 amino acid sequence of porcine ZP2 polypeptide. These results suggest that porcine ZP4 and ZP2 are derived from a common parent polypeptide by proteolytic cleavage at the position between 133 and 134 residues.

## Introduction

The zona pellucida is an extracellular matrix surrounding mammalian oocytes and has several functions essential for fertilization, including sperm recognition, induction of acrosome reaction and prevention of polyspermy (Wassarman, 1990). The zona pellucida has a strong immunogenicity against heterogeneous species of animals, and the antibodies produced strongly inhibit fertilization *in vivo* and *in vitro* (Tsunoda and Chang, 1978; Sacco, 1979; Tsunoda *et al.*, 1981). Immunological analyses have revealed that the zona pellucida possesses species-specific and interspecies crossreactive antigens (Sacco, 1977; Isojima *et al.*, 1984; Drell and Dunbar, 1984; Koyama *et al.*, 1985). The species-specific antigens may be related to the species-specific recognition of spermatozoa by the zona pellucida, while the crossreactive antigens may be associated with other zona pellucida functions necessary for fertilization.

It has been shown, by DNA cloning, that a mouse zona pellucida component, mZP3, possesses a polypeptide sequence conserved among different species (Ringuette *et al.*, 1988; Margaret and Dean, 1990; Kinloch *et al.*, 1990). This finding suggests that the gene encoding mZP3 is derived from a common ancestor gene. In general, it is believed that the interspecies crossreactive antigens are less likely to produce antibodies because of the T-cell tolerance to common antigens. However, animals immunized with zona pellucida from different species can produce self-reactive antibodies to the zona pellucida of immunized animals (Gwatkin *et al.*, 1977; Sacco *et al.*, 1983; Mahi-Brown *et al.*, 1985; Hasegawa *et al.*, 1992). It has been reported that canine zona pellucida not only produces self-reactive anti-zona antibodies, but also induces infertility in immunized dogs (Mahi-Brown *et al.*, 1985). Although it has not been confirmed, several clinical reports have shown that anti-zona antibodies can be detected in the sera of some infertile women (Shivers and Dunbar, 1977; Caudle *et al.*, 1987). These results indicate that self-reactive antibodies can be produced in animals by active immunization with interspecies crossreactive antigens. On the basis of species crossreactivity, many researchers have engaged in studies of contraceptive vaccines by using porcine zona pellucida as an antigen source in several animal species including non-human primates (Sacco *et al.*, 1983, 1987; Gulyas *et al.*, 1983; Skinner *et al.*, 1984; Mahi-Brown *et al.*, 1985; Dunbar *et al.*, 1989; Liu *et al.*, 1989; Upadhya *et al.*, 1989; Paterson *et al.*, 1992). Most animals immunized with porcine zona pellucida or its components became infertile; however, this infertility was always associated with ovarian dysfunction or failure. It is therefore necessary to isolate an antigen component that induces antibodies that inhibit only spermatozoa–zona interactions but are without any undesirable side effects. We attempted to obtain such an antigen from porcine zona using monoclonal antibodies (Isojima *et al.*, 1984; Koyama *et al.*, 1985, 1991, 1992; Hasegawa *et al.*, 1988).

Biochemical analyses indicated that porcine zona pellucida is composed of three or four glycoprotein families with unusually high degrees of charge heterogeneity (Dunbar *et al.*, 1981; Yurewicz *et al.*, 1983; Hedrick and Wardrip, 1986).

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Koyama *et al.* (1991) showed using two-dimensional PAGE under reducing conditions that heat-solubilized porcine zona pellucida separated into four glycoprotein families with average $M_r$ values of 92 000, 69 000, 55 000 and 23 000. These were designated pZP1, pZP2, pZP3 and pZP4, respectively. A monoclonal antibody (5H4) produced to pZP4 inhibited the binding of human spermatozoa to human zona pellucida *in vitro* (Koyama *et al.*, 1992), and the antigen recognized by the monoclonal antibody was found to be a peptide portion of the pZP4 glycoprotein. In addition, active immunization with pZP4 induced temporary infertility in hamsters (Hasegawa *et al.*, 1992). These results encouraged us to study the molecular structure of pZP4 further, with the aim of developing a contraceptive vaccine. The purpose of this study was to determine the amino acid sequence of pZP4 core protein using the technique of peptide mapping, and to confirm this by cDNA cloning.

## Materials and Methods

### *Preparation of pZP4*

Porcine zonae pellucidae were collected from minced pig ovaries by sieving them through nylon screens as described by Isojima *et al.* (1984) and Hedrick and Wardrip (1986). The zonae pellucidae were solubilized in 50 mmol carbonate buffer $l^{-1}$ (pH 9.6) by heating at 70°C for 30 min. Two different pZP4 preparations were used for determination of the amino-terminal amino acid sequence. For the first preparation, solubilized porcine zonae (4 mg) were fractionated by one-dimensional SDS–PAGE (Laemmli, 1970) and the pZP4 glycoproteins corresponding to the average $M_r$ of 23 000 were electroeluted from the gel in 2.5 mmol Tris–glycine buffer $l^{-1}$ (pH 8.3) containing 0.01% SDS for 3 h at 4°C. The collected material was dialysed extensively against 5 mmol $NH_4HCO_3$ $l^{-1}$ and was lyophilized. For the second preparation, solubilized porcine zonae (1 mg) were subjected to two-dimensional electrophoresis according to a modification of the O'Farrell method (O'Farrell, 1974; Hedrick and Wardrip, 1986) and the separated proteins were transblotted onto Immobilon P membrane (polyvinylidene difluoride membrane Code No. 00010, Millipore Corp., Bedford, MA) in 10 mmol 3-cyclohexylaminopropane-sulfonic acid (CAPS) blotting buffer $l^{-1}$ (pH 11.0) containing 10% methanol, by electrophoresis at 10 V at 4°C overnight. The membrane was stained with 0.1% Coomassie brilliant blue-R in methanol: $H_2O$ (1:1, v:v) for 10 min at room temperature and was destained with methanol:acetic acid:water (5:1:4, v:v:v) for 30 min (Matsudaira, 1987). One single spot (pI 5.2, $M_r$ 21 000) was excised from the membrane and used for the sequence analysis. The two-dimensional electrophoresis was carried out six times to obtain enough material for sequencing.

### *Pyridylethylation of pZP4*

To protect cysteine residues from Edman degradation, pZP4 material was subjected to reduction and alkylation before fragmentation. Lyophilized pZP4 (1 mg) was dissolved in 300 µl of 0.5 mol Tris–HCl buffer $l^{-1}$ (pH 8.5) containing 7 mol guanidine $l^{-1}$ and 10 mmol EDTA $l^{-1}$, and 1 µl of pyridylethylene and 2 µl of tri-*n*-butyl-phosphin were added to the solution. After incubation under nitrogen gas for 4 h at room temperature, the mixture was dialysed extensively against 5 mmol $NH_4HCO_3$ $l^{-1}$.

### *Cleavage of pZP4 by CNBr*

A sample of 200 µg pyridylethylated pZP4 was added to 200 µl 70% formic acid containing CNBr (10 mg $ml^{-1}$) and incubated overnight at room temperature. The CNBr-treated material was dried by flushing with nitrogen gas. The dried material was dissolved in 200 µl of 70% formic acid and then subjected again to flushing with nitrogen gas. This procedure was repeated twice, and the final material was dissolved in 200 µl of $H_2O$ before application to a reverse-phase HPLC column.

### *Proteolytic digestion of pZP4*

Two enzymes (endoproteinases LysC and AspN) with different cleavage specificity were used to prepare pZP4 peptide fragments. For the endoproteinase LysC digestion, 200 µg of pyridylethylated pZP4 was dissolved in 200 µl of 25 mmol Tris–HCl buffer $l^{-1}$ (pH 8.5) containing 1 mmol EDTA $l^{-1}$, and was mixed with 200 µl of endoproteinase LysC solution (25 µg $ml^{-1}$, Boehringer–Mannheim Yamanouchi). The digestion was performed at a ratio of enzyme to substrate of 1:40 for 5 h at 37°C, according to the manufacturer's directions. The treatment with endoproteinase AspN (Boehringer–Mannheim) was carried out at an enzyme to substrate ratio of 1:100 for 8 h at 37°C in 10 mmol Tris–HCl $l^{-1}$, (pH 7.2), according to the manufacturer's directions.

### *Separation of pZP4 peptide fragments*

Peptide fragments derived from pZP4 glycoproteins by treatment with CNBr or proteolytic enzymes were separated in a reverse phase HPLC (600E Waters, Milford, MA). A test sample was applied to a µ Bondapak $C_{18}$ (3.9 mm × 30 cm, Waters) equilibrated with 5% acetonitrile in water containing 0.1% trifluoroacetic acid (TFA). The column was eluted with a linear gradient of acetonitrile (5–50%) at a flow rate of 0.85 ml $min^{-1}$. The eluate was monitored by absorbance at 220 nm.

### *Analysis of amino-terminal amino acid sequence of pZP4*

Amino-terminal amino acid sequences were determined using an automated gas phase protein sequencer (Model 477A Applied Biosystems Japan, Tokyo). The Immobilon P membranes transblotted from pZP4 were directly applied to the glass filter, while the soluble pZP4 peptide samples obtained from HPLC were concentrated to 100 µl and applied on polybrene-treated glass filter for fixation of peptide fragments.

### *Analysis of carboxy-terminal amino acid sequence of pZP4*

The carboxy-amino acid sequence of pZP4 was estimated by successive digestion of the carboxy-terminal amino acid as reported by Hayashi *et al.* (1973). pZP4, 1 nmol $l^{-1}$, was

treated with 0.01 nmol of carboxypeptidase Y $l^{-1}$ (Takara Biochemicals, Kyoto) in 50 µl of 0.1 mol pyridine–acetate buffer $l^{-1}$ (pH 5.6) containing 0.5% SDS at 20°C. Aliquots (5 µl) of the reaction mixture were taken at 0 min, 1 min, 2 min, 5 min, 10 min, 20 min, 30 min and 60 min after the reaction, and 1 µl of acetic acid was added to each sample to stop the reaction. Released amino acids in each sample were analysed by an amino acid analyser (L-8500 Hitachi, Tokyo).

*Analysis of amino-terminal amino acid sequence of pZP2*

pZP2 was isolated by treating solubilized porcine ZP (1 mg) with *Escherichia freundii* endo-β-galactosidase (0.17 mU, Seikagaku-Kogyo Corp., Tokyo) in 1 ml of 0.1 mol $CH_3COONH_4$ $l^{-1}$ (pH 5.6) at 37°C overnight. The digested material was subjected to SDS–PAGE, followed by transblotting to Immobilon P. After staining with Coomassie brilliant blue-R, the protein band corresponding to pZP2 was excised and applied to the amino acid sequencer, as described above.

*cDNA cloning for pZP4*

cDNA cloning for pZP4 was carried out by the polymerase chain reaction using a PCR reagent kit (Gene Amp DNA Amplification Reagent kit, Perkin Elmer Cetus, Norwalk, CT) and an automatic PCR apparatus (DNA Thermal Cycler, Perkin Elmer Cetus). The reaction was carried out as one cycle under the following conditions: (1) thermal denaturation: 94°C for 1 min; (2) primer annealing: 60°C for 2 min; and (3) primer extension: 72°C for 3 min. The same cycle was repeated thirty times, and then the final reaction was carried out at 72°C for 7 min to complete one round of polymerase chain reaction. DNA primers were synthesized on an automated DNA synthesizer (Cyclone plus DNA Synthesizer, Milligen/Biosearch, Bedford, MA) based on the amino acid sequence of pZP4, or the nucleotide sequence of the cloned DNA segments, or the sequence of M13 gene in vector pUC18. Amplified DNA segments were cloned into a pUC19 vector and sequenced by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977). For preparation of a DNA template for PCR, mRNA was extracted from a porcine ovary using an mRNA extraction kit (Fast Track mRNA isolation kit, Invitrogen, San Diego, CA) and converted into single or double stranded cDNAs which were then used as DNA templates for PCR. For cloning of a DNA segment coding for the amino acid sequence between positions 70 and 110 of pZP4, a cDNA synthesis kit (Oligo dT, Pharmacia, Stockholm) was used to synthesize a single-stranded cDNA. In the cloning of 3′ and 5′ regions of the gene, two cDNA synthesis kits (Oligo dT, from Pharmacia and from Promega, Madison, WI) were used to synthesize double-stranded cDNAs. Each of the resulting cDNAs was linked to an *Eco* RI/*Not* I adaptor and then inserted into pUC18 vector at *Eco* RI site. For cloning of a DNA segment coding for the amino acid sequence between positions 1 and 61 of the pZP4, a cDNA synthesis kit (Amersham Japan, Tokyo) was used with the primer 5′-GGTGATGGCCACGCACTC-3′ [(-)strand DNA sequence coding for the amino acid between positions 70 and 77 of pZP4] to synthesize double-stranded cDNA, which was used as a DNA template.

*Computer-aided sequence analyses*

Comparison of amino acid sequences was performed with a computer program using the database of Kyushu University. Hydropathicity of the amino acid sequence of pZP4 was determined by the Kyte and Doolittle algorithm (1982) and the secondary structure was determined in accordance with Chou and Fasman (1978).

## Results

*Amino acid sequence of pZP4 by peptide mapping*

Solubilized porcine zona pellucida was separated into four major glycoprotein families (pZP1, pZP2, pZP3, pZP4) with marked heterogeneity in $M_r$ and pI values (Fig. 1a). The pZP4 glycoprotein isolated by electroelution from one-dimensional SDS–PAGE gel revealed the same heterogeneity (Fig. 1b). When this pZP4 preparation was subjected to analysis of amino-terminal amino acid sequence, it was found to be X-X-X-Asn-Gln-Leu-Val-Asn-Thr-Ala-Phe-Pro-Gly-Ile-Val-Thr-Cys-His-Glu-Asn-Arg-Met-Val-Val-Glu-Phe (X is the unidentified cycle in Edman degradation method). The first three amino acid residues could not be determined owing to the large amount of contaminating glycine that could not be removed from the sequencing material even after extensive dialysis. The sequence of the first three amino acids was determined by isolating a member of the pZP4 glycoprotein family ($M_r$ 21 000 and pI 5.2) (marked with a circle in Fig. 1a) from transblotted Immobilon P. The amino-terminal amino acid sequence was determined as Ile-Gly-Val-Asn-Gln-Leu-Val-Asn-Thr-Ala. From these results, 26 residues of the amino-terminal sequence of pZP4 were determined as follows: (Ile)-Gly-Val-Asn-Gln-Leu-Val-Asn-Thr-Ala-Phe-Pro-Gly-Ile-Val-Thr-Cys-His-Glu-Asn-Arg-Met-Val-Val-Glu-Phe. Occasionally, the first amino-terminal amino acid Ile was missing.

The internal amino acid sequence of pZP4 was determined by aligning the peptide sequence of the products fragmented with CNBr, endoproteinase LysC and AspN. Reverse-phase HPLC patterns of the fragments of pZP4 were obtained by these treatments (Fig. 2). By aligning the peptide fragments with overlapping sequences, an amino-terminal 110-amino acid sequence and another ten-amino acid sequence were determined (Fig. 3a, b). However, no sequence connecting these two peptides could be detected. The same results were obtained in repeated analyses.

*Cloning of cDNA coding for pZP4*

The entire amino acid sequence of pZP4 was deduced through cloning of the following four DNA segments: (a) cloning of a DNA segment coding for the amino acid sequence between positions 70 and 110 of pZP4; (b) cloning of a 3′ region of the pZP4 gene, that is, a DNA segment coding for the amino acid sequence between positions 94 and 198 of pZP4; (c) cloning of a 5′ region of the pZP4 gene, that is, a DNA segment coding for the amino acid sequence between positions 53 and 86 of pZP4 and (d) cloning of a DNA segment coding for the amino acid sequence between positions

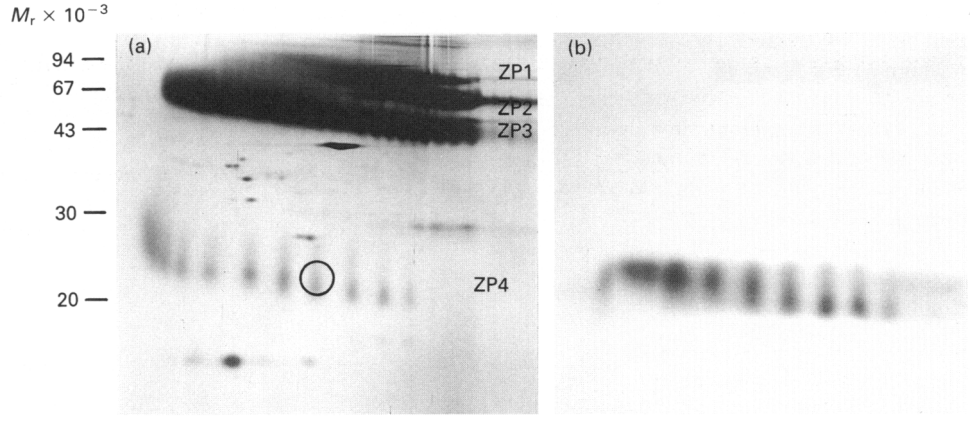


**Fig. 1.** Two-dimensional electrophoresis of solubilized porcine zona pellucida and porcine ZP4 isolated from preparative SDS–PAGE. (a) Solubilized porcine zona pellucida (100 μg) was separated into four major glycoprotein families designated as pZP1, pZP2, pZP3 and pZP4. One family member in pZP4 (marked with a circle) was used for the analysis of amino-terminal amino acid sequence after transblotting to Immobilon P membrane. (b) pZP4 (10 μg) prepared by one-dimensional SDS–PAGE from solubilized porcine zona pellucida was applied to two-dimensional electrophoresis. No other component was present in the pZP4 preparation and 100 μg of this preparation was used for analysis of amino-terminal amino acid sequence.

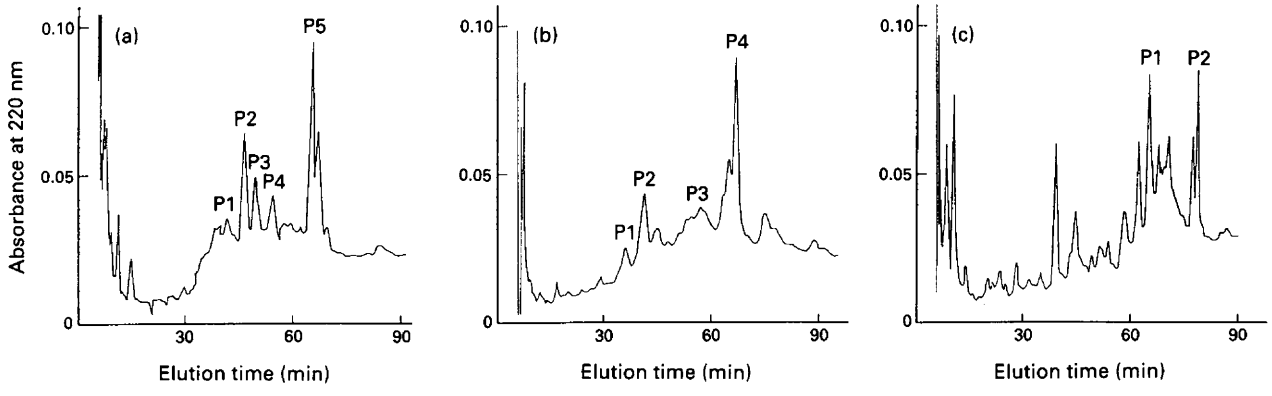


**Fig. 2.** Reverse-phase HPLC of fragmented porcine zona pellucida polypeptide ZP4. Porcine ZP4 was cleaved with (a) CNBr, (b) endoproteinase LysC and (c) endoproteinase AspN. The numbered peptide peaks were subjected to the amino-terminal amino acid sequencing.

1 and 77 of pZP4 (see Fig. 4). The four overlapping cDNA clones corresponding to the amino acid sequences of positions 1–77, 53–86, 70–110 and 94–198 were sequenced and linked together to determine the DNA sequence (Fig. 5). The amino acid sequence deduced from the constructed cDNA was identical to the sequence determined by the peptide analysis of pZP4. The deduced amino acid sequence also contained a sequence consisting of eight amino acids (His-Ser-Gly-Ser-Thr-Ile-Lys-Met) in positions 111–118 which had not been determined by the Edman degradation of pZP4, which corresponded to the amino acid sequence connecting the two peptides (Fig. 3a, b). These results suggested that the pZP4 polypeptide consisted of at least 128 amino acid residues. The sequence contained two potential *N*-linked glycosylation sites (positions 49 and 58) and 15 possible *O*-glycosylation sites. The amino acid residue (49) that could not be determined by the Edman degradation method was found to be Asn, which had probably been concealed by glycosylation of pZP4 molecules. Although no other amino acid sequence other than the sequence of the 128 residues with Phe128 as the carboxy-terminal amino acid was detected by peptide mapping of pZP4, the cloned cDNA did not contain the stop codon after the codon corresponding to the carboxy-terminal Phe128. In addition, the cloned cDNA contained 594 nucleotides, which is 210 nucleotides longer than the 384 nucleotides expected from the 128 amino acid residues of the sequenced pZP4 peptide.

### *Amino-terminal amino acid sequence of pZP2*

Previously we suggested that pZP2 and pZP4 were derived from pZP1 by proteolytic cleavage (Koyama *et al.*,

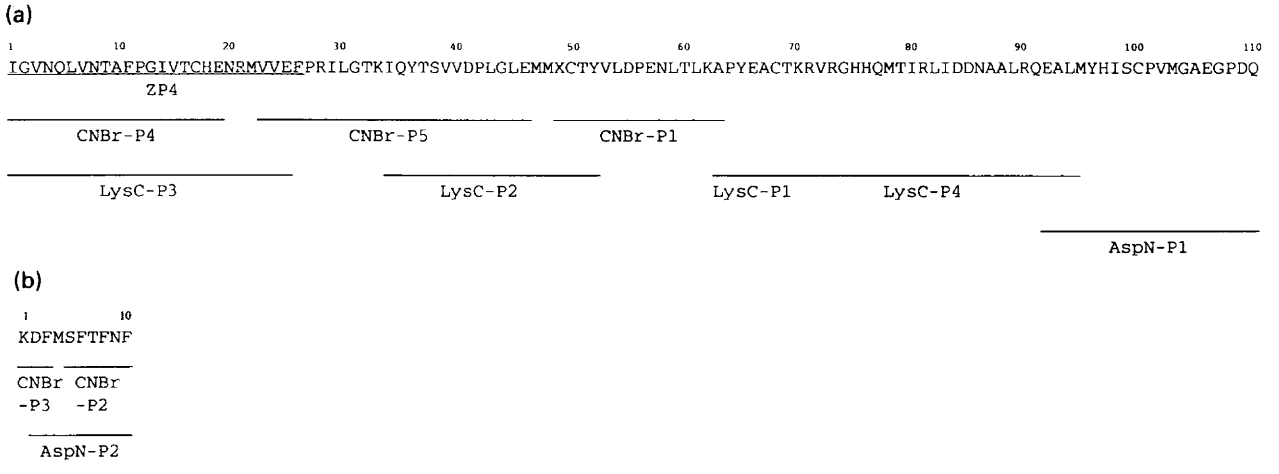


**Fig. 3.** Alignment of the amino acid sequence of porcine zona pellucida ZP4 peptides. Two peptide sequences (a), (b) were determined by aligning the peptide fragments with overlapping sequences. The amino acids are shown in the single-letter amino acid code. No amino acid was identified in position 49 (X). No sequence connecting peptides (a), (b) could be detected in the Edman degradation of porcine ZP4.

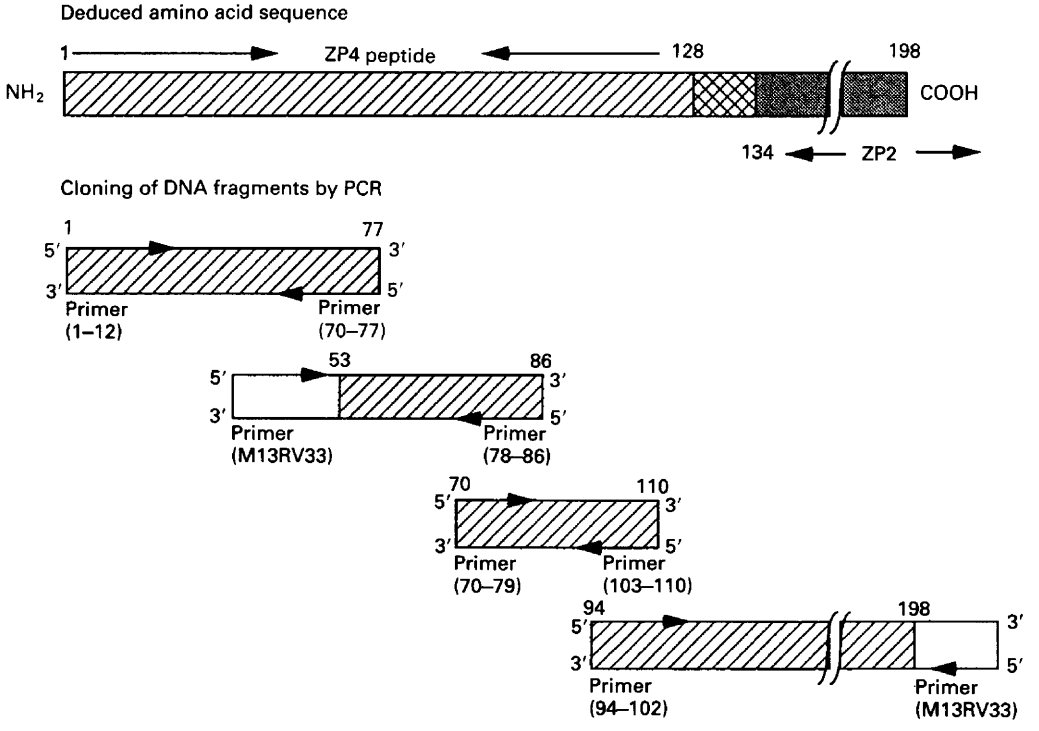


**Fig. 4.** Strategy for determining the sequence of porcine zona pellucida polypeptide ZP4 gene. The complete nucleotide sequence of the porcine ZP4 gene was determined independently by cloning the four DNA segments in the following order: (1) a DNA segment coding for the amino acid sequence from position 70 to 110, (2) a DNA segment coding for the amino acid sequence from position 53 to 86, (3) a DNA segment coding for the amino acid sequence from position 94 to 198, and (4) a DNA segment coding for the amino acid sequence from position 1 to 77.

1991). It was therefore necessary to determine whether the additional cDNA sequence in this study corresponds to the amino acid sequence of the pZP2 polypeptide. The amino-terminal amino acid sequence of pZP2 was therefore analysed and a ten-amino acid sequence (Asp-Glu-Asn-Val-Lys-Arg-Glu-Asp-Ser-Lys) determined. This sequence corresponded to the amino acid sequence (134–143) deduced from the 5′-region (30 nucleotides) of the additional cDNA sequence (210 nucleotides).

### *Carboxy-terminal amino acid sequence of pZP4*

A sample of purified pZP4 was treated with carboxypeptidase Y and the released amino acids were analysed on the basis

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1                                                  11
ATC GGC GTT AAT CAA CTC GTT AAT ACA GCA TTT CCA GGT ATT GTC ACT TGC CAT GAA AAT
Ile Gly Val Asn Gln Leu Val Asn Thr Ala Phe Pro Gly Ile Val Thr Cys His Glu Asn

21                                                 31
AGA ATG GTA GTG GAA TTT CCA AGA ATT CTT GGC ACT AAG ATA CAG TAC ACC TCT GTG GTG
Arg Met Val Val Glu Phe Pro Arg Ile Leu Gly Thr Lys Ile Gln Tyr Thr Ser Val Val

41                                                 51
GAC CCT CTT GGT CTT GAA ATG ATG AAC TGT ACC TAT GTT CTG GAC CCA GAA AAC CTC ACC
Asp Pro Leu Gly Leu Glu Met Met Asn Cys Thr Tyr Val Leu Asp Pro Glu Asn Leu Thr

61                                                 71
CTG AAG GCC CCA TAT GAA GCC TGT ACC AAA AGA GTG CGT GGC CAT CAC CAA ATG ACC ATC
Leu Lys Ala Pro Tyr Glu Ala Cys Thr Lys Arg Val Arg Gly His His Gln Met Thr Ile

81                                                 91
AGA CTC ATA GAT GAC AAT GCT GCT TTA AGA CAA GAG GCT CTC ATG TAT CAC ATC AGC TGT
Arg Leu Ile Asp Asp Asn Ala Ala Leu Arg Gln Glu Ala Leu Met Tyr His Ile Ser Cys

101                                               111
CCT GTT ATG GGA GCA GAA GGC CCT GAT CAG CAT TCG GGA TCC ACA ATC TGC ATG AAA GAT
Pro Val Met Gly Ala Glu Gly Pro Asp Gln His Ser Gly Ser Thr Ile Cys Met Lys Asp

121                                               131
TTC ATG TCT TTT ACC TTT AAC TTT TTT CCT GGG ATG GCT GAC GAA AAT GTG AAA CGT GAG
Phe Met Ser Phe Thr Phe Asn Phe Phe Pro Gly Met Ala Asp Glu Asn Val Lys Arg Glu

141                                               151
GAT TCG AAG CAG CGC ATG GGA TGG AGC CTT GTA GTT GGT GAC GGT GAA AGA GCC CGA ACT
Asp Ser Lys Gln Arg Met Gly Try Ser Leu Val Val Gly Asp Gly Glu Arg Ala Arg Thr

161                                               171
CTG ACC TTT CAG GAG GCC ATG ACC CAA GGA TAT AAT TTC CTG ATA GGG AAC CAG AAG ATG
Leu Thr Phe Gln Glu Ala Met Thr Gln Gly Tyr Asn Phe Leu Ile Gly Asn Gln Lys Met

181                                               191                        198
AAC ATC CAA GTG TCA TTC CAT GCC ACT GGA GTG ACT CGC TAC TCG CAA GGT AAC ···
Asn Ile Gln Val Ser Phe His Ala Thr Gly Val Thr Arg Tyr Ser Gln Gly Asn ···
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**Fig. 5.** The primary structure of cloned cDNA and deduced amino acid sequence for porcine zona pellucida polypeptide ZP4. The locations of potential *N*-linked glycosylation sites (Asn-X-Thr) are underlined. The amino acid sequence (1–133) corresponds to porcine ZP4 and the additional sequence (134–198) corresponds to the amino-terminal region of porcine ZP2. The arrow indicates the cleavage site between porcine ZP4 and porcine ZP2.

**Table 1.** Amino acids (pmol) released from the carboxy-terminal region of the porcine zona pellucida peptide ZP4 by carboxypeptidase Y

| Reaction time (min) | Thr | Asn | Phe | Pro | Gly | Met | Ala |
|---|---|---|---|---|---|---|---|
| 1 | * | 69 | 111 | * | 10 | * | 125 |
| 2 | 29 | 94 | 204 | * | 40 | 100 | 178 |
| 5 | 112 | 145 | 443 | * | 79 | 216 | 281 |
| 10 | 231 | 296 | 770 | 294 | 227 | 351 | 420 |
| 20 | 362 | 351 | 1053 | 338 | 293 | 468 | 510 |
| 30 | 372 | 366 | 1272 | 543 | 422 | 529 | 536 |
| 60 | 499 | 515 | 1655 | 614 | 419 | 537 | 615 |

*Significant yields were not detected.

of reaction time to determine whether the five-amino acid sequence (129–133) belongs to the carboxy-terminal portion of pZP4 or whether the sequence is deleted from both polypeptides of pZP4 and pZP2 when they were generated by the proteolytic cleavage. This enzyme released seven amino acids from the carboxy-terminal region of pZP4 (Table 1). Five (Phe, Pro, Gly, Met, Ala) of the seven amino acids were residues included in the 129–133 sequence of pZP4 (Fig. 5). Ala seems to be the carboxy-terminal amino acid of the pZP4 polypeptide, as this amino acid was released in the highest yield during the first 1 min treatment. This suggests that the pZP4 polypeptide consists of 133 amino acid residues. However, Phe was also detected in almost the same yield to Ala at 1 min treatment before the time when Met and Gly were released. This suggests that the pZP4 preparation used for this experiment also contained a polypeptide the carboxy-terminal amino acid of which is Phe128. As the reaction proceeded, not only Met132, Gly131 and Pro130, but also Asn127 and Thr125

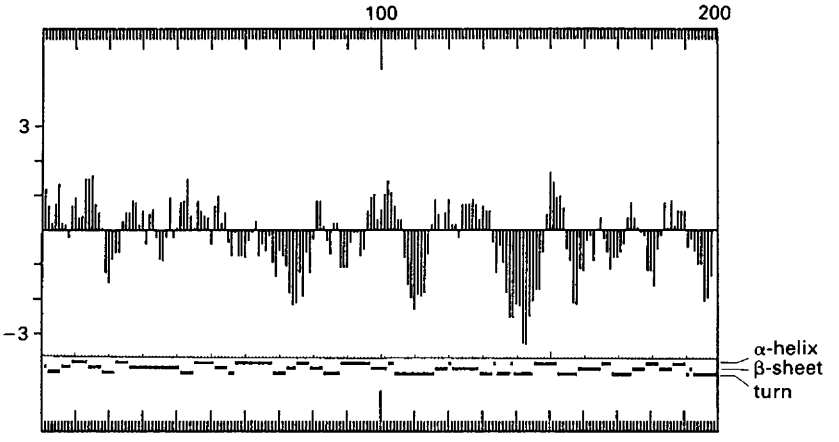


**Fig. 6.** Hydropathicity plot and secondary structure of 198 amino acid sequence deduced from the cloned DNA for porcine zona pellucida polypeptide ZP4. Hydropathicity was determined by the Kyte and Doolittle algorithm (1982). Secondary structures were determined in accordance with Chou and Fasman (1978).

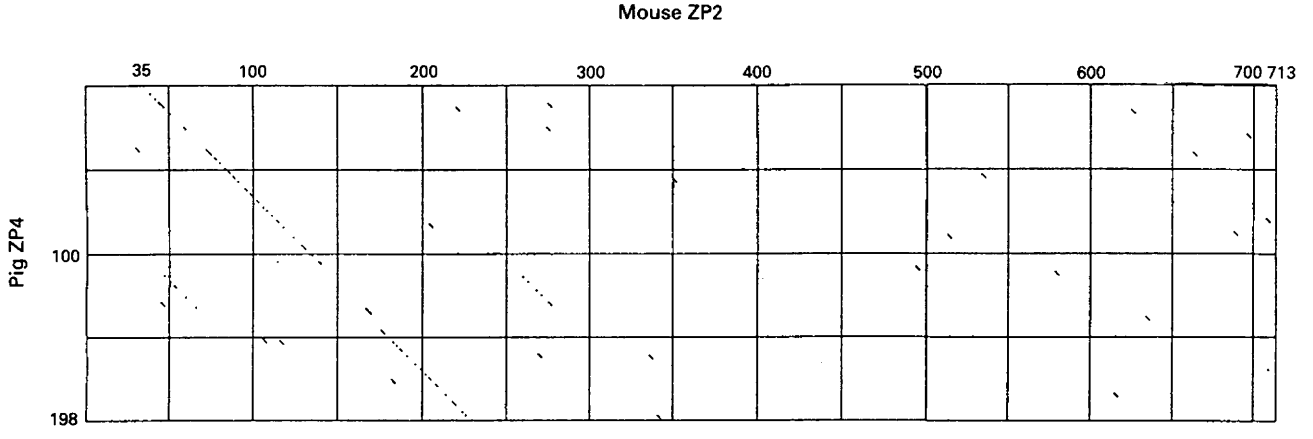


**Fig. 7.** Amino acid sequence homology between the pig 198-amino acid sequence of zona pellucida polypeptide ZP4, deduced from cloned DNA and mouse ZP2 peptide (713 amino acids). In mouse ZP2, the amino acid sequence (1–34) is the signal peptide sequence. This dot matrix indicated the homology between porcine ZP4 plus amino-terminal region of porcine ZP2 and the amino-terminal region of mouse ZP2.

were increasingly released into the reaction mixture. It was, therefore, concluded that two different molecules with 128 amino acid residues and 133 amino acid residues were included in the preparation of pZP4. The fact that the yield of Phe was 3–4 times greater than that of the other amino acids is consistent with the presence of four Phe residues in the carboxy-terminal region (124–133) of pZP4 (Fig. 5).

### *Computer analyses*

The hydropathicity plot and the predicted secondary structure of the 198 amino acid sequence deduced from the cloned cDNA are shown (Fig. 6). When the deduced amino acid sequence was compared with the published sequence of mouse ZP2 (Liang *et al.*, 1990) and ZP3 (Ringuette *et al.*, 1988), it was found that pZP4 resembled the amino-terminal portion of mouse ZP2. The amino acid sequence similarity between the 198 amino acid sequence determined in this study and mouse ZP2 by dot matrix analysis are shown (Fig. 7). A comparison of amino acid sequences between pZP4 (133 amino acids) and mouse ZP2 in their amino-terminal 132 residues was made (Fig. 8). All five Cys residues were located in identical positions.

## Discussion

In the study reported here, we determined the entire amino acid sequence of pZP4 core protein and also part of the amino-terminal sequence of pZP2 by sequence analysis with Edman degradation and cDNA cloning with polymerase chain reaction. The results showed that two potential *N*-linked glycosylation sites Asn49, 58 were present in the pZP4 polypeptide. The first, Asn49, was determined from the cDNA sequencing but not from the peptide analysis, probably owing to *N*-linked glycosylation, while the second, Asn58, was determined by both methods. This finding suggests that the first, Asn49, is glycosylated but that the second, Asn58, is not.

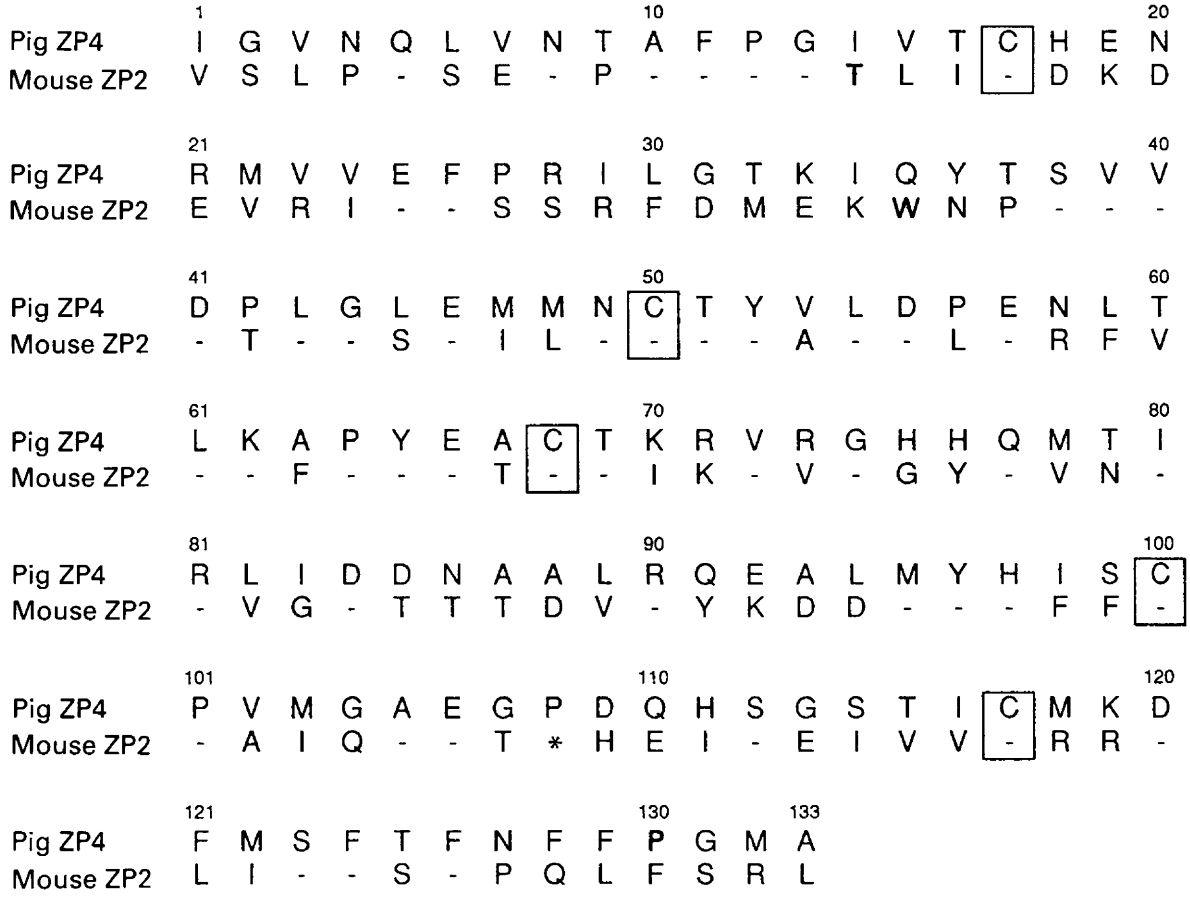


**Fig. 8.** Comparison of amino acid sequences between zona pellucida polypeptides porcine ZP4 and mouse ZP2. The single-letter amino acid code is used. Mouse and porcine polypeptides are aligned so as to provide maximum similarity between the polypeptides. For mouse ZP2, only those positions where the amino acid sequence differs from porcine ZP4 (133 amino acids) are shown. An amino acid deletion in mouse ZP2 is indicated by *. The location of cysteine residues are boxed. All five cysteine residues are located in identical positions (17, 50, 68, 100, 117).

The heterogeneity of $M_r$ and pI in pZP4 is considered to be due to the various carbohydrate compositions; however, the number of *N*-linked glycosylation sites contributing to the heterogeneity is unknown. The presence of *O*-linked oligosaccharide chains contributing to the heterogeneity must also be considered, although Hedrick and Wardrip (1986) showed that pZP4 glycoprotein does not contain *N*-acetylgalactosamine to link to the OH group of Ser/Thr.

In the amino-terminal amino acid analysis of pZP4, two different sequences were found, one starting with Ile1 and the other starting with the next Gly2. It should be possible to determine whether this is due to alteration by post-translational modification, or due to diversity at the gene level by determining the entire DNA sequence of pZP1. At this moment, we consider the amino-terminal amino acid of pZP4 Ile. Sequencing of the two peptide fragments (CNBr-P2, AspN-P2) derived from pZP4 showed that the carboxy-terminal amino acid of pZP4 was Phe128, as no extending amino acid was detected. However, the results from the carboxypeptidase Y method and the cDNA cloning indicated that there is another pZP4 peptide that contains five more amino acids at the carboxy-terminal region. Since the Edman degradation method is not as efficient for determining the carboxy-terminal amino acid sequence as the amino-terminal sequence, it is considered that pZP4 contains two kinds of polypeptide comprising 128 and 133 residues. The relative molecular masses corresponding to these polypeptides (14 579; 15 081) agreed satisfactorily with the $M_r$ values as determined by SDS–PAGE of deglycosylated pZP4 (Hedrick and Wardrip, 1987; Henderson *et al.*, 1987; Koyama *et al.*, 1991). The functional significance of the existence of two types of pZP4 (128 amino acids and 133 amino acids) is unknown. However, the fact that only one amino acid sequence starting with Asp was detected in the pZP2 sequence indicates that cleavage occurs between positions 133 and 134 but not between positions 128 and 129. After this cleavage, the additional five amino acids located in the carboxy-terminal region are likely to be lost in the half of pZP4 composed of 133 amino acids. This process might involve the removal of a five amino acid peptide with a specific proteolytic enzyme, or the successive removal of single amino acids with an enzyme like a carboxypeptidase. Another possibility is that some pig populations lack the DNA region encoding this five amino acid sequence. In fact, it has been shown that there is variety between Caucasians and Japanese in the DNA sequence encoding human ZP3 (van Duin *et al.*, 1992).

We previously reported that solubilized porcine zona pellucida was separated into two components ($M_r$: 92 000 and 55 000) under non-reducing conditions, while the same material was separated into four components ($M_r$: 92 000; 69 000; 55 000; 23 000) under reducing conditions (Koyama *et al.*, 1991). In diagonal SDS–PAGE, the non-reduced, 92 000 component (pZP1) was partially divided into two components with $M_r$ values of 69 000 and 23 000 (pZP2 and pZP4) under reducing conditions. These results suggest that pZP1 is partially cleaved into pZP2 and pZP4, and the latter two components are connected by S–S bonds. Immunological analysis has shown that a monoclonal antibody (3A4) which reacted with pZP1 under non-reducing conditions reacted with pZP1 and pZP4 under reducing conditions (Koyama *et al.*, 1991). Similar observations were reported by other researchers (Hedrick and Wardrip, 1986; Henderson *et al.*, 1987; Timmons *et al.*, 1987). In addition, the fact that the cDNA cloned in this study is longer at the 3′ end than the length predicted from the 133 residues of pZP4 polypeptide shows that pZP2 and pZP4 originate from a larger common polypeptide product (pZP1); the presence of a cDNA sequence coding for pZP4 and some pZP2 peptides indicates that pZP1 is not a heterodimer of pZP2 and pZP4 but a parent polypeptide of pZP2 and pZP4.

There are several possible mechanisms for the formation of pZP2 and pZP4 from pZP1. First, the proteolytic cleavage of pZP1 might occur in the cells during transport and secretion as a post-translational modification. Second, the secreted pZP1 protein might be modified after deposition around the oocytes. In this situation, extracellular enzymes released from cortical granules of oocytes or follicular granulosa cells might contribute to the modification. The contents of the cortical granules are thought to be released not only after fertilization but also during maturation of the oocytes (Ducibella *et al.*, 1990). Several reports have shown that pZP1 is sensitive to the cortical granule enzymes released during fertilization (Hedrick *et al.*, 1987; Hatanaka *et al.*, 1992). In addition, it has been reported that mouse ZP2 ($M_r$: 120 000) was converted to ZP2f ($M_r$: 90 000) and a smaller glycoprotein ($M_r$: 23 000) by the enzymes released following activation of eggs (Bleil *et al.*, 1981). Thus, it is likely that pZP1 converts to pZP2 and pZP4 in the porcine ovary, although the possibility that cleavage of pZP1 occurred during the process of preparing the zona pellucida materials cannot be ruled out.

Comparison of the amino acid sequence of pZP4 with published amino acid sequences revealed 39.1% similarity to the amino-terminal portion of mouse ZP2 (Liang *et al.*, 1990). In particular, the position of five Cys residues were identical between these two species. Since Cys residues are involved in S–S bonds, the principal structure of pZP4 and mouse ZP2 might be conserved. One of the two potential *N*-linked glycosylation sites of Asn49 is also the same in the two species. The amino acid sequence of rabbit 55K protein shows similarity to mouse ZP2 (Schwoebel *et al.*, 1991). However, no significant homology of the sequences between pZP4 plus a part of pZP2 (198-amino acid sequence) and rabbit 55K was detected. pZP4 thus corresponds to the amino-terminal region of mouse ZP2 and rabbit 55K protein corresponds to the carboxy-terminal region of mouse ZP2.

The pZP3 glycoprotein family, which exhibits apparent electrophoretic homogeneity, is reported to contain two distinct glycoproteins (pZP3α, pZP3β) as shown by chemical deglycosylation (Sacco *et al.*, 1986; Henderson *et al.*, 1987; Hedrick and Wardrip, 1987). In this study, the glycoprotein possessing the larger core protein is termed pZP3α, and the other component possessing a smaller core protein is termed pZP3β. Hedrick and Wardrip (1987) named the acidic component pZP3α and the basic component pZP3β, so our terms of ZP3α and ZP3β mean opposite components in their terminology. It would be interesting to determine which component in pigs corresponds to which component in mice. *O*-linked oligosaccharide has been shown to function as a primary sperm receptor in mice and to be present only in ZP3 in mice (Wassarman, 1990). However, several *O*-linked oligosaccharide chains were included in pZP3α and pZP3β in pigs (Yurewicz *et al.*, 1992). In addition, the ligand–receptor assay showed that only pZP3α inhibited boar sperm binding to porcine zona pellucida (Yurewicz *et al.*, 1983; Sacco *et al.*, 1989), whereas partial amino acid sequence analysis showed that pZP3β included a similar amino acid sequence to mouse ZP3 (Yurewicz *et al.*, 1992). Moreover, in sperm-binding inhibition experiments with antibodies, polyclonal and monoclonal antibodies, against pZP3α and pZP3β, inhibited primary sperm binding to the zona pellucida (Henderson *et al.*, 1988; Berger *et al.*, 1989; Bagavant *et al.*, 1993). Collectively, it is difficult to determine which glycoprotein, pZP3α or pZP3β, is the counterpart of mouse ZP3 that possesses the primary sperm receptor function. Spermatozoa–zona pellucida interactions in pigs may be more complicated events than those in mice.

Active immunization with pZP4 has been shown to induce temporary infertility in hamsters (Hasegawa *et al.*, 1992), and a monoclonal antibody (5H4) that recognized a sequential peptide epitope of pZP4 was found to strongly inhibit human sperm binding to zona pellucida *in vitro* (Koyama *et al.*, 1992). These reports suggest that pZP4 contains an antigen epitope useful for developing a contraceptive vaccine. In addition, other researchers reported that antisera raised to deglycosylated porcine ZP3 (of pZP3α and pZP3β) interfered with fertility (Dunbar *et al.*, 1989; Keenan *et al.*, 1991; Paterson *et al.*, 1992). Moreover, Millar *et al.* (1989) reported that a synthetic peptide consisting of eight amino acids, deduced from mouse ZP3 gene, could induce infertility in female mice by active immunization. Recently, Schwoebel *et al.* (1992) analysed the antigenicity and immunogenicity of recombinant zona pellucida antigens in rabbits and demonstrated that recombinant zona pellucida proteins could elicit a humoral immune response that recognizes native zona pellucida proteins. The results of these reports encourage us to apply recombinant DNA technology to produce zona pellucida antigens that may be used in the development of contraceptive vaccines. We are preparing a recombinant pZP4 polypeptide to examine its antifertility effect by active immunization in animals.

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