Disulfide formation in bovine zona pellucida glycoproteins during fertilization: evidence for the involvement of cystine cross-linkages in hardening of the zona pellucida

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The time for solubilization of the bovine zona pellucida in a hypotonic buffer containing 5% (v/v) β -mercaptoethanol and 7 mol urea l⁻¹ increased by 10% after fertilization. Coupling with a specific fluorescent thiol probe, monobromobimane (mBBBr), was markedly greater in the zona pellucida of ovarian eggs compared with fertilized eggs, indicating that the cysteine residues in the zona pellucida of unfertilized eggs are oxidized to cystines during fertilization. After endo- β -galactosidase digestion to remove *N*-acetylglucosamine repeats of the carbohydrate chains, three zona pellucida glycoproteins (ZPA, ZPB and ZPC) coupled with the fluorescent bimane groups were fractionated efficiently by reverse-phase HPLC. Estimation of bimane groups in the three components and SDS-PAGE revealed that intramolecular disulfide bonds in ZPA and intra- and intermolecular disulfide bonds in ZPB were formed during fertilization, but oxidation of cysteine residues in ZPC was low. Specific proteolysis of ZPA during fertilization was also observed. These results indicate that the formation of disulfide linkages together with specific proteolysis result in the construction of a rigid zona pellucida structure, which is responsible for hardening of the zona pellucida.

Introduction

The zona pellucida is one of the two sites at which polyspermy is blocked (Wolf, 1981; Stewart-Savage and Bavister, 1988; Yanagimachi, 1994). After fertilization in most mammals, cortical granules in the oocyte are broken and the materials released into the perivitelline space act on the zona pellucida, resulting in hardening (Braden *et al.*, 1954; Austin and Braden, 1956; Barros and Yanagimachi, 1971; Drobnis *et al.*, 1988). The hardening of the zona pellucida together with cortical granule-independent changes in the plasma membrane are generally accepted to be responsible for blocking polyspermy. After fertilization in mice, a protein component of the zona pellucida, ZP2, is cleaved specifically by a protease released from the cortical granules (Bleil *et al.*, 1981; Moller and Wassarman, 1989). The specific cleavage of a component during fertilization is also observed in the pig and bovine zona pellucida (Hedrick *et al.*, 1987; Hatanaka *et al.*, 1992; Noguchi *et al.*, 1994). However, correlation of the specific cleavage with hardening of the zona pellucida remains to be determined. Schmell and Gulyas (1980), using an indirect method in a study of mice, proposed that the

cross-linkages between tyrosine residues of the zona pellucida proteins formed by ovoperoxidase caused the hardening. In contrast to sea urchins (Foerder and Shapiro, 1977), dityrosyl residues have not been found in the zonae pellucidae of fertilized eggs in mice. However, cross-linkages of disulfide bonds in zona pellucida proteins in rats are thought to be involved in hardening of the zona pellucida (Zhang *et al.*, 1991).

It is necessary to compare the properties and structure of the zona pellucida of a fertilized egg with that of an unfertilized egg to understand the function of the zona pellucida. However, the limited availability of fertilized mammalian eggs has prevented molecular analysis. In the present study, a large number of fertilized bovine eggs were produced by *in vitro* fertilization (IVF) and the properties of the zona pellucida were compared with those of ovarian eggs.

The nomenclature for zona pellucida proteins from different species is confusing (Hedrick, 1996). Bleil and Wassarman (1980) designated three glycoproteins from the mouse zona pellucida as ZP1, ZP2 and ZP3 from the highest molecular mass to the lowest, respectively. However, the size of the ZP1 gene, which forms a dimeric structure after biosynthesis, is smaller than that of ZP2. Harris *et al.* (1994) proposed that the protein genes should be termed ZPA, ZPB

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and ZPC according to the size of the cDNA: ZPA for the largest and ZPC for the smallest. Three native glycoproteins of the bovine zona pellucida have similar molecular masses due to heterogeneous carbohydrate chains, although the sizes of their protein skeletons are different (Noguchi *et al.*, 1994). The terminology used in the present study is as follows: ZPA, ZPB and ZPC proteins that are gene products of ZPA, ZPB and ZPC genes, respectively. Incorporation of the bimane group into these three zona pellucida proteins was also investigated in this study.

Materials and Methods

Preparation of ovarian, matured and fertilized bovine eggs

Ovarian, matured and fertilized bovine eggs and their zonae pellucidae were prepared as described by Hamano and Kuwayama (1993) and Noguchi *et al.* (1994). Ovaries were obtained from an abattoir. Within 1 h after animals were killed, cumulus-egg complexes were isolated from the ovaries and were matured to metaphase II in TCM 199 containing 5% (w/v) fetal bovine serum at 38.5°C for 21 h under 2% CO₂ in air. Matured eggs in BO medium (Brackett and Oliphant, 1975) containing 1% (w/v) BSA were fertilized with spermatozoa capacitated in BO medium, and were incubated at 37°C. Differentiation of the embryos was terminated by freezing at the two-cell, four-cell, eight-cell, 16-cell and morula stages. The embryos were stored at -80°C until use. In the chromatographic and electrophoretic analyses, a mixture of embryos from the two- to the 16-cell stages was used as the fertilized eggs, whereas ovarian eggs before fertilization were used as the unfertilized group.

Zona pellucida solubilization assay

Ten ovarian eggs and ten fertilized eggs at each stage were placed separately in various media (Table 1) at 25°C and the time required for complete lysis of the zona pellucida was recorded.

Modification of isolated zonae pellucidae with mBBr

Zonae pellucidae were isolated by removing the ooplasm from the eggs by pipetting with a narrow bore pipette. The isolated zonae pellucidae were modified with monobromobimane (mBBr) (Calbiochem, La Jolla, CA) using a stock solution of 50 mmol l⁻¹ mBBr in acetonitrile. mBBr was added to the isolated zonae pellucidae in 50 ml PBS containing 0.3% (w/v) EDTA to a final concentration of 0.5 mmol mBBr l⁻¹ and the suspension was incubated at 25°C for 1 h in the dark. The modified zonae pellucidae were washed three times with fresh PBS by centrifugation at 1000 g for 5 min.

Examination by fluorescence microscopy of the isolated zonae pellucidae modified with mBBr

The features of the modified zonae pellucidae were observed and photographed with an Olympus BH2-QRFL fluorescence microscope using Kodak Tri-X film.

Incorporation of bimane group into the zona pellucida glycoproteins of ovarian and fertilized eggs

The zonae pellucidae isolated from approximately 3000 ovarian and fertilized eggs were modified with 0.5 mmol mBBr l⁻¹ in PBS containing 0.3% (w/v) EDTA for 1 h. After removal of the excess reagents by several transfers to fresh PBS, the zonae pellucidae were solubilized by heating at 70°C in H₂O. The heat-solubilized zonae pellucidae were digested with endo-β-galactosidase (1 mU) at 37°C for 48 h to remove N-acetyllactosamine regions in the carbohydrate chains (Noguchi *et al.*, 1994). Desalting was performed by Nucleosil 300-7C18 HPLC using 0.1% (w/v) trifluoroacetic acid in 90% (w/v) acetonitrile as an eluent. Glutathione (reduced form) modified with mBBr was used as a control and the excess reagents were removed by elution with 20 mmol ammonium acetate l⁻¹ on a Bio-Gel P2 column. The amount of bimane group incorporated into the zona pellucida proteins was estimated by measuring the fluorescence intensity at 483 nm with excitation at 405 nm of the mBBr-modified (mB-) proteins against the modified glutathione (mB-glutathione).

Table 1. Solubilization time of bovine zonae pellucidae of ovarian and fertilized eggs

Medium	pH	Solubilization time (s) ^a	
		Ovarian eggs	Fertilized eggs
0.2% (w/v) pronase + PBS	7.4	98 ± 9	106 ± 14
Lactic acid + PBS	3.0	Insoluble ^b	Insoluble
Acidic Tyrode's solution	2.5	258 ± 24	300 ± 24
Acidic Tyrode's solution	2.0	88 ± 7	107 ± 11
5% (v/v) β-mercaptoethanol + buffer A ^c	7.4	Insoluble	Insoluble
7 mol urea l ⁻¹ + buffer A	7.4	Insoluble	Insoluble
5% (v/v) β-mercaptoethanol + 7 mol urea l ⁻¹ + buffer A	7.4	678 ± 21	743 ± 22

^aTwenty eggs were placed in the medium at 25°C and the time (mean ± SD) required for complete lysis of the zonae pellucidae was recorded.

^bInsoluble: no change in the zonae pellucidae was observed after 24 h.

^cBuffer A: 10 mmol Tris-HCl l⁻¹ + 0.4% (w/v) polyvinylpyrrolidone.

Fractionation of endo- β -galactosidase digests of mBr-zona pellucida proteins into three components

After mBr-zona pellucida proteins from approximately 3000 ovarian and fertilized eggs were digested with endo- β -galactosidase as described above, the digests were subjected to chromatography on a reverse-phase HPLC column (Nucleosil 300-7C18 column, 4 mm $\times$ 150 mm) by an increasing concentration of acetonitrile in 0.1% (w/v) trifluoroacetic acid at a flow rate of 1.0 ml min⁻¹. An identical amount of protein estimated by amino acid analysis was applied in each chromatography. Absorbance at 210 nm and fluorescence intensity at 483 nm with excitation at 405 nm were monitored. The fractions including the zona pellucida proteins were subjected to SDS-PAGE under non-reducing and reducing conditions, and three zona pellucida glycoproteins (ZPA, ZPB and ZPC) were identified by the molecular masses of the bands.

Gel filtration HPLC of the last peak on the reverse-phase HPLC

The last peak on reverse-phase HPLC (fraction 5) was lyophilized and applied to a TSK-gel G3000SW column equilibrated with 3.5 mmol SDS l⁻¹, 100 mmol sodium sulfate l⁻¹ and 50 mmol sodium phosphate l⁻¹ (pH 7.0). The elution was performed with the equilibration buffer at a flow rate of 0.4 ml min⁻¹.

Estimation of incorporation of the bimane group into the three components

The amount of bimane group incorporated into the three components was estimated using mB-glutathione as the standard as described above.

Amino acid and N-terminal sequence analyses

The amounts of proteins and glutathione were determined by amino acid analysis (Nakano *et al.*, 1987). N-terminal amino acid sequences were determined by automated Edman degradation using an on-line model 120A phenylthiohydantoin amino acid analyser.

Results

Changes in solubility of the zona pellucida during fertilization

The time for lysis of the zona pellucida of fertilized eggs in 0.2% (w/v) pronase-PBS was slightly greater than that for ovarian eggs (Table 1). Without pronase, the zonae pellucidae were not solubilized at pH > 3.0. At pH 2.5 and 2.0, the duration of lysis of the zona pellucida of fertilized eggs was slightly greater than that of ovarian eggs. Thus, in PBS containing pronase and acidic solutions, the increase in the

Fig. 1. Relative times required for complete lysis of the bovine zona pellucida at various stages of development. Ovarian eggs (Ov), matured eggs (Ma), and embryos at the two-cell (2), four-cell (4), eight-cell (8), 16-cell (16) and morula (Mo) stages were suspended separately in medium containing 5% (v/v) β -mercaptoethanol, 7 mol urea l⁻¹, 0.4% (w/v) polyvinylpyrrolidone and 10 mmol Tris-HCl l⁻¹ (pH 7.4), and the times required for lysis relative to lysis of the zonae pellucidae of ovarian eggs were calculated. Duplicate experiments using a total of 20 eggs for each stage were performed. Standard deviations of the solubilization time were within 3%.

time of solubilization of the zona pellucida after fertilization was small. The zonae pellucidae were not solubilized in 5% (v/v) β -mercaptoethanol or 7 mol urea l⁻¹, but were solubilized in a mixture of both reagents, in which the time of solubilization of zonae pellucidae from fertilized eggs was greater than those from ovarian eggs (743 versus 678 s). The times for lysis of the zonae pellucidae of matured eggs and embryos at various stages of development in the β -mercaptoethanol-urea solution relative to that of ovarian eggs are shown (Fig. 1). From the two-cell to the morula stage, about a 10% increase in the time for lysis was observed. The standard deviation of the solubilization time of the zonae pellucidae at each stage was within 3%. It is unclear why there was a small increase in solubilization time for zonae pellucidae from matured eggs compared with ovarian eggs.

Modification of the isolated zonae pellucidae of unfertilized and fertilized eggs with mBBr

The zonae pellucidae isolated from ovarian eggs were strongly coupled with mBBr, while those from the fertilized eggs at the two-cell stage were very weakly coupled (Fig. 2).

Incorporation of bimane group into the zona pellucida protein mixture from ovarian and fertilized eggs

The bovine zona pellucida is composed of three glycoproteins termed ZPA, ZPB and ZPC from the highest

Fig. 2. Paired phase-contrast (a,c) and fluorescence (b,d) photomicrographs of representative bovine zona pellucidae of a total of 30 zona pellucidae modified with monobromobimane (mBBBr) after isolation. The isolated zona pellucidae from ovarian eggs (a,b) and embryos at the two-cell stage (c,d) were fixed with 3% (v/v) glutaraldehyde. Duplicate experiments were performed at each stage. Scale bars represent 15 μm .

molecular mass of protein skeleton to the lowest, respectively (Harris *et al.*, 1994). The molecular masses of these protein skeletons are 63, 41 and 34 kDa, respectively (Noguchi *et al.*, 1994). The molar ratio of the protein moiety of three bovine zona components (ZPA:ZPB:ZPC) calculated from their molecular masses and amino acid composition was about 1:1:2. The amount of the bimane group incorporated into the protein mixture was estimated on the basis of the fluorescence intensities of bimane groups coupling to the protein mixture relative to that of mB-glutathione (Table 2). The incorporation into the zona pellucidae of fertilized eggs was low.

Fractionation of the zona pellucida proteins coupled with the bimane group

Separation of the three components of native bovine zona pellucida protein is difficult due to a prominent heterogeneity of carbohydrate chains (Noguchi *et al.*, 1994). When the digests of the heat-solubilized zona pellucidae with endo- β -galactosidase were applied to a reverse-phase HPLC column, a good and reproducible separation of the mBr-zona pellucida protein mixture into three components was achieved (Fig. 3). SDS-PAGE of every peak (Fig. 4) revealed that the three components coupled with the bimane group were eluted in the following order: endo- β -galactosidase digested-ZPC, -ZPB and -ZPA and the last peak (fraction 5) which contained ZPA and high molecular mass multimers (HMM) of the zona pellucida protein. Fluorescence of ZPA and ZPB was almost undetectable after fertilization, although there was not a marked change in the amount of protein, indicating that intramolecular disulfide

Table 2. Number of bimane groups incorporated into the zona pellucida proteins of ovarian and fertilized bovine eggs

	Bimane groups introduced (mol mol ⁻¹)
Zona pellucida protein mixture of ovarian egg	0.37
ZPA	0.25
ZPB	0.09
ZPC	0.07
Zona pellucida protein mixture of fertilized egg	0.05
ZPA	0.01
ZPB	0.00
ZPC	0.06

Values are the average of duplicate analyses.

bonds in ZPA and ZPB are formed during fertilization. However, a similar amount of the bimane group was incorporated into ZPC of the zona pellucida of fertilized and ovarian eggs (Fig. 3 and Table 2), indicating that during fertilization oxidation of cysteine residues in ZPC is low.

N-terminal peptide (21 kDa) and C-terminal peptide (63 kDa) are cross-linked through disulfide bonds in a portion of ZPA (76 kDa) of the zona pellucida of ovarian eggs (Noguchi *et al.*, 1994). In addition to a band of 76 kDa, the bands of both peptides were present in SDS-PAGE under reducing conditions (Fig. 4c, lane 4). No band of 76 kDa was detected in the zona pellucida of fertilized eggs (Fig. 4d, lane 4), indicating that ZPA underwent complete cleavage to 21 and 63 kDa peptides during fertilization. N-terminal sequences of the 21 and 63 kDa peptides were determined to

Fig. 3. Fractionation of monobromobimane (mBBBr)-modified bovine zona pellucida proteins after digestion with endo- β -galactosidase. The digests of mBBBr-zona pellucida proteins from ovarian eggs (a) and fertilized eggs (b) ($n = 3000$ in each group) were subjected to chromatography on a Nucleosil 300-7C18 column (4 mm $\times$ 150 mm) by an increasing concentration of acetonitrile (---) in 0.1% (w/v) trifluoroacetic acid at a flow rate of 1.0 ml min⁻¹. An identical amount of protein estimated by amino acid analysis was applied in each chromatography. Absorbance at 210 nm (—) and fluorescence intensity at 483 nm (---) with excitation at 405 nm were monitored. The numbers indicate fractions 1–5.

Fig. 4. SDS-PAGE of bovine zona pellucida protein fractions from reverse-phase HPLC. Zona pellucida proteins were isolated from ovarian eggs (a,c) and fertilized eggs (b,d). Fractions 1–5 from the HPLC (Fig. 3) were subjected to electrophoresis in lanes 1–5, respectively, on an 11% acrylamide gel under non-reducing (a,b) and reducing conditions (c,d). HMM: high molecular mass multimers; M: molecular mass markers. Proteins were silver stained.

be IleAspGlyValAsnGlnLeu and AspAspThrAlaGlyProLys, respectively. The sequence of the 76 kDa protein (ZPA) was the same as the 21 kDa peptide.

The last peaks in the reverse-phase HPLC (Fig. 3) were separated into three fractions by gel filtration HPLC (Fig. 5a,b). The incorporation of the bimane group into the multimers also decreased after fertilization (Fig. 5b, fractions 4 and 5). SDS-PAGE of each fraction under non-reducing conditions showed that the ZPA monomer was eluted in fractions 3 and 6 (Fig. 5c, lanes 3 and 6). Under reducing conditions, only one main band was released from the multimers of the zona pellucida proteins in fertilized eggs (Fig. 5d, lanes 4' and 5'). The molecular mass of ZPB digested with endo- β -galactosidase is 68 kDa (Noguchi *et al.*, 1994) which is similar to the 63 kDa peptide from ZPA (Fig. 5d). The position of the dense band in the separation of the high molecular mass multimers (Fig. 5d, lanes 4' and 5') indicates that the multimers formed during fertilization are constructed mainly from ZPB.

Estimation of the amount of bimane group incorporated into the three components

The amount of the bimane group incorporated into the three components was estimated as described earlier. Incorporation of the bimane group into ZPA and ZPB was almost undetectable after fertilization, whereas incorporation into ZPC was similar in the zona pellucidae of fertilized and ovarian eggs (Table 2).

Discussion

The bovine zona pellucida was insoluble in isotonic medium at pH 3.0, although the zona pellucida of pig ovarian eggs is solubilized within about 20 s in lactic acid-PBS at pH 3.6 (Hatanaka *et al.*, 1992). In acidic Tyrode's solution, pH 2.5, the time for lysis of the hamster zona pellucida is 30–60 s and for mouse and rat zona pellucida is 5–10 s (Nicolson *et al.*,

Fig. 5. Separation of fraction 5 from reverse-phase HPLC of bovine zona pellucida proteins (Fig. 3) into high molecular mass multimers and ZPA by gel filtration HPLC. Fraction 5 (Fig. 3) was subjected to chromatography on a TSK-gel G3000SW column (7.5 mm $\times$ 60 cm) by elution with 3.5 mmol SDS l^{-1} , 100 mmol sodium sulfate l^{-1} and 50 mmol sodium phosphate l^{-1} (pH 7.0) at a flow rate of 0.4 ml min^{-1} . Fraction 5 from the zona pellucida proteins of ovarian eggs (a) (Fig. 3a), and fertilized eggs (b) (Fig. 3b). Absorbance at 210 nm (—) and fluorescence intensity at 483 nm (---) with excitation at 405 nm were monitored. SDS-PAGE of the fractions of gel filtration HPLC on a 11% acrylamide gel under non-reducing conditions (c) and on a 12.5% acrylamide gel under reducing conditions (d). Lanes 1–6 in (c) and lanes 4'–6' in (d) correspond to fractions 1–6 and 4–6, respectively. Arrowheads a, b and c indicate the position of bands of ZPA, ZPB and the 63 kDa fragment of ZPA, respectively. HMM: high molecular mass multimers; M: molecular mass markers. Proteins were silver stained.

1975). These observations indicate that the bovine zona pellucida has a more rigid structure than that of rodents and pigs. The bovine zona pellucida was also resistant to β -mercaptoethanol and urea; the time for solubilization in a hypotonic buffer containing 5% (v/v) β -mercaptoethanol and 7 mol urea l^{-1} was > 11 min. For solubilization of the zona pellucida, cleavage of hydrogen bonds as well as reduction of disulfide linkages must be necessary. The time for lysis of the zona pellucida of fertilized eggs was about 10% greater than that of ovarian eggs. This increase in solubilization time did not change markedly from the two-cell stage to the morula stage.

The presence of free thiols in the unfertilized and fertilized eggs was investigated using the fluorescent probe, mBBR, which reacts specifically with thiol groups under physiological conditions (Kosower *et al.*, 1979; Kosower and Kosower, 1987). mBBR has been used mainly for the analysis of thiol and disulfide status in mammalian spermatozoa (Seligman *et al.*, 1991; Seligman *et al.*, 1994; Yossefi *et al.*, 1994). This probe is suitable for modification of the zona pellucida, since there is a possibility that the architecture of the zona pellucida is changed in hypotonic and hypertonic solutions (Hatanaka *et al.*, 1988). The zonae pellucidae isolated from ovarian eggs were strongly coupled with bimeane group in PBS, while those from fertilized eggs were weakly coupled.

In neutral and alkaline media at 37°C, disulfide interchange in peptides occurs and this reaction is triggered by the thiol group (Jensen, 1959). An intramolecular thiol-disulfide interchange is followed by intermolecular exchange, leading to polymerization which is accelerated at higher temperatures. A similar reaction might be induced during solubilization of the zonae pellucidae of ovarian eggs. During solubilization, free thiols in the zonae pellucidae of ovarian eggs probably trigger the formation of high molecular mass multimers cross-linked through intermolecular disulfide linkages. The multimers detected as an immobile band in the SDS-PAGE were eluted in the last peak in the reverse-phase HPLC together with a portion of ZPA.

The free thiols in the isolated zonae pellucidae are modified with mBBR and the modified residues no longer trigger the formation of multimers during solubilization. Treatment with mBBR for 1 h may be insufficient for complete modification of the zona pellucida, and the residual unmodified thiols trigger the formation of multimers during solubilization. Treatment of zonae pellucidae from ovarian eggs with mBBR for 6 h significantly reduced the amount of multimers and their fluorescence intensity (data not shown).

Measurement of the relative fluorescence intensity compared with mB-glutathione revealed that the thiol content of zonae pellucidae of fertilized eggs was about 14% of that of ovarian eggs. The multimers were also produced in the zonae pellucidae of fertilized eggs, although the amount was smaller than in the ovarian eggs.

The results of this study indicate that the disulfide bonds in ZPA formed during fertilization are predominantly intramolecular linkages. The oxidation may be induced after specific cleavage by a protease released from the oocyte. The findings of the present study indicate that the specific cleavage site of ZPA is the amino group side of the Asp

residue. During fertilization in pigs, ZPA (90 kDa) protein is specifically cleaved at the peptide bond between Ala and Asp residues into 25 and 65 kDa peptides (Hatanaka *et al.*, 1992; Hasegawa *et al.*, 1994). A protease with similar specificity may be released at fertilization in both species. In ZPB, intra- and intermolecular disulfide bonds are formed. In contrast to ZPA and ZPB, oxidation of cysteine residues in ZPC during fertilization is low.

The positions of cysteine residues in the zona pellucida proteins are highly conserved (Harris *et al.*, 1994) and most of these residues form disulfide linkages, contributing mainly to the maintenance of the zona pellucida structure (Dunbar, 1983; Dunbar *et al.*, 1994). Although the amino acid sequence of bovine ZPA has not been determined, this component, which is homologous to pig ZPA, is thought to have 20 cysteine residues. The results of the present study indicate that a small number of the cysteine residues are present in the reduced state in the unfertilized egg. It is postulated that the formation of intra- and intermolecular disulfide linkages during fertilization induces the construction of a more rigid structure, so that more time is required for solubilization of the zona pellucida in β -mercaptoethanol-urea solution.

Bovine ZPA is cleaved specifically by proteases during fertilization (Noguchi *et al.*, 1994). The mechanism by which the proteolysis induces the conversion of thiols to disulfides in the native zona pellucida structure remains unclear. It is likely that the conversion causes the hardening of the zona pellucida which is responsible for blocking polyspermy.

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