Fourier Transform Infrared Spectroscopic Analysis of the Intact Zona Pellucida of the Mammalian Egg: Changes in the Secondary Structure of Bovine Zona Pellucida Proteins During Fertilization

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The zona pellucida is the acellular transparent envelope surrounding the mammalian oocyte. An analysis of the changes in the structures of zona pellucida proteins is essential for understanding the molecular mechanisms underlying the important physiological roles of the zona during fertilization and preimplantation. The hardening of the zona caused by the structural changes during fertilization is generally accepted to be responsible for blocking polyspermy. In this study, we analyzed changes in the secondary structure of the zona during fertilization by Fourier transform infrared (FTIR) spectroscopy and transmission electron microscopy. The predominance of β-sheet structure in porcine ovarian egg zona proteins in water was ascertained using FTIR spectra. α-Helix structure was also present. The attenuated total reflection (ATR)-FTIR spectrum of intact, unsolubilized porcine zonae pellucidae from ovarian eggs indicated that the zona proteins in the native zona pellucida also have β-structure as the main constituent. Attenuated total reflection-FTIR spectroscopy of intact bovine zona pellucida obtained from ovarian and fertilized eggs at the blastocyst stage revealed that the β-structure content increased during fertilization. Furthermore, a reduction of the thickness of the zona during fertilization was observed using transmission electron microscopy. Therefore, the change in the zona architecture that causes hardening of the zona during fertilization is accompanied by changes in the secondary structure of the zona proteins. Exp Biol Med 231:166–171, 2006

Key words: zona pellucida; FTIR; ATR-FTIR; mammalian fertilization; zona hardening; secondary structure

Introduction

The effective functioning of a block to multiple sperm penetration into oocyte is decisive to mammalian development. The penetration of more than one sperm leads to abnormal embryogenesis and early death. The zona pellucida, a transparent envelope surrounding the mammalian oocyte, is one of the sites at which polyspermy is blocked after fertilization (1–4). At fertilization in most mammals, cortical granules in the oocyte rupture and the materials released into the perivitelline space act on the zona pellucida to harden it (5–8). The hardening of the zona pellucida is generally accepted to be responsible for blocking polyspermy, but the detailed zona architecture that produces "hardening" remains to be clarified.

The zona pellucida is composed of three glycoprotein components called ZPA, ZPB, and ZPC, listed in descending order of cDNA size (9). The properties and structures of the zona pellucida of fertilized and unfertilized eggs have been compared. Specific cleavage between Ala/Gly and Asp

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of ZPA is observed during fertilization in several mammals (10–13). Recently, specific cleavage of ZPA has also been found in frogs (14), and the consensus cleavage site of ZPA, including nonmammals, is between X (Ala, Gly, Asp, or Glu) and Asp–Asp/Glu. It has been suggested that the specific cleavage of ZPA induces supramolecular structural changes in the zona pellucida, thereby preventing sperm binding (15). Furthermore, cross-linkage formation between tyrosine residues of the zona proteins in the mouse (16) and disulfide bond formation between the zona protein components in the rat (17) and bovine (18) is suggested to be involved in the hardening of the zona pellucida. These results concerning changes in covalent bonds were obtained using solubilized zona proteins. To clarify the molecular mechanism involved in blocking polyspermy, it is indispensable to analyze the conformational changes of the zona pellucida in the intact state during fertilization. However, no spectroscopic analysis of the intact zona pellucida has been reported.

In this study, we first prepared porcine zona proteins by acid solubilization to prevent the exchange of disulfide cross-links and we ascertained that the acid-solubilized porcine zona protein mixture assumes predominantly β-structure in water, as measured using Fourier transform infrared (FTIR) spectroscopy. Attenuated total reflection (ATR)-FTIR spectroscopy is useful for analyzing protein structures in aqueous solution, because the ATR technique reduces the absorption by water. With a horizontal-type ATR plate, incident light is limited to the bottom of the sample solution (the depth of light is about 1 μm). Therefore, the ATR technique is promising for measuring insoluble material in a sample solution. In this study, we obtained ATR-FTIR spectra of bovine ovarian and fertilized-egg zonae pellucidae in the intact, unsolubilized state. The ATR-FTIR showed that the zona proteins in the intact porcine zonae pellucidae from ovarian eggs assume a secondary structure similar to that of the solubilized zona proteins. Attenuated total reflection-FTIR also revealed that the β-structure content of the zona proteins in the intact bovine zona pellucida increased during fertilization. Furthermore, using transmission electron microscopy, we showed that the thickness of the zona pellucida decreased during fertilization, forming a more compact supramolecular structure.

Materials and Methods

Preparation of Porcine and Bovine Zona Protein Mixture. Porcine and bovine zonae pellucidae were isolated from ovarian eggs as described previously (12, 19). Approximately 1×10^5 zonae were solubilized with 5 ml of 1.74 *M* acetic acid, and the pH of the solution was adjusted to 6.5 with aqueous ammonia. After centrifugation at 100 *g* for 3 mins, the supernatant was filtered through 0.45-μm Acro LC 13 (Gelman Scientific, Ann Arbor, MI). The filtrate was dialyzed successively against 25 m*M* ammonium acetate, pH 6.5, and water; the dialyzed materials were lyophilized.

Fertilized bovine eggs were prepared by the *in vitro* fertilization method, as described by Hamano and Kuwayama (20). The zona protein mixture from the fertilized eggs at blastocyst stage was prepared the same way as described above for the ovarian eggs.

Fourier Transform Infrared Spectroscopy. Fourier transform infrared measurements were carried out at 25°C on a PerkinElmer Spectrum-One FTIR spectrometer equipped with a TGS detector at a resolution of 2 cm^{-1} (PerkinElmer, Norwalk, CT). Interferograms from 200 scans were averaged to obtain one spectrum. Nitrogen gas was constantly pumped into the spectrometer to minimize water vapor, which absorbs in the spectral region of interest. About 12 μl of sample solution was placed between two CaF_2 plates separated by a 0.012-mm–thick Mylar spacer. The gap between the two CaF_2 plates was sealed with aluminum tape to suppress the evaporation of water. The FTIR spectrum of the solvent (water) was measured in the same way. The slight contributions from water vapor in each spectrum were completely removed by subtracting the water vapor spectrum. To eliminate the contributions of water from the spectrum of the sample solution, the spectrum of the solvent was subtracted from the spectrum of the sample solution after multiplying by an appropriate factor, so that the spectral line in the region of 2600 to 1800 cm^{-1} approached zero. Samples in solid film were prepared as follows: approximately 20 μl of sample solution was spread on a CaF_2 plate and spontaneously evaporated at 25°C. Second-derivative and difference calculations were performed using IGOR Pro 3.12 (WaveMetrics, Lake Oswego, OR) and the software supplied by PerkinElmer.

Attenuated total reflection-FTIR measurements were performed at 25°C on a PerkinElmer Spectrum-One FTIR spectrometer equipped with a universal ATR unit and a liquid nitrogen–cooled MCT-detector at 2 cm^{-1}. Interferograms from 2000 scans were averaged to obtain one spectrum. A suspension of about 10,000 unsolubilized zonae from porcine ovarian eggs (corresponding to about 300 μg of zona proteins) in 10 μl of water was placed on a Diamond/ZeSe crystal plate (PerkinElmer). In the cases of bovine, 3000 each of ovarian and fertilized zonae were used. The FTIR spectrum of water was measured in the same way. The procedures for vapor elimination, subtraction of the water spectrum, and the spectral calculation were as described above for the normal FTIR measurements.

Secondary Structure Composition of the Solubilized Zona Proteins. The circular dichroism (CD) spectra of the solubilized zona proteins were measured with a Jasco J-725 spectropolarimeter (Tokyo, Japan) at 25°C under constant nitrogen flush. The data were obtained in terms of mean residue ellipticity $[\theta]$ in deg cm^2 $dmol^{-1}$. The average molecular mass of the polypeptide backbones was estimated to be 46,100 Da for bovine proteins, using the values of molar ratios and molecular masses of three

Table 1. Molecular Masses, Residue Numbers, and Stoichiometry of the Protein Moieties of Bovine Zona Glycoproteins (ZPA, ZPB, and ZPC)

	ZPA	ZPB	ZPC	References
Molecular mass (daltons)[a]	67,578	48,617	35,450	9, 13
Residue number[b]	602	440	317	9, 13
Mean residue weight	112	110	112	
Weight ratio	1.21 : 1 : 1.45			13
Molar ratio	1 : 1.2 : 2.3			

[a] All cysteine residues are assumed to form disulfide linkages.
[b] C-termini of these components are assumed to be a furin cleavage site.

components shown in Table 1. Using these values, the mean residue weight of the bovine zona protein mixture was estimated to be 111. On the basis of CD data, the secondary structure composition was calculated with the CONTIN program (21).

Protein Concentration. The concentration of the bovine zona protein mixture was calculated from the molar extinction coefficients at 280 nm of bovine zona protein mixture (i.e., 4.2×10^4).

Transmission Electron Microscopy. The zonae isolated from ovarian and fertilized bovine eggs were prefixed with 3% paraformaldehyde, 2.5% glutaraldehyde, and 1% tannic acid in 0.1 *M* sodium cacodylate buffer, pH 7.4, for 1 hr; postfixed with 1% OsO_4 in 0.1 *M* sodium cacodylate buffer for 1 hr; dehydrated with graded ethanol; and embedded in epoxy resin. Ultrathin sections were stained with 2% uranyl acetate followed by 0.1% lead citrate and were observed with a JEOL 100CX electron microscope (Tokyo, Japan).

Results and Discussion

FTIR of a Mixture of Porcine Zona Pellucida Proteins. In these experiments, we dissolved the zona pellucida using a weak acid to prevent the exchange of disulfide linkages. Figure 1 shows the infrared absorption and second-derivative spectra for the porcine zona protein mixture, which was obtainable in large amounts. This absorption spectrum is quite complex, owing to absorption by the proteins and carbohydrates of the zona glycoproteins. The bands at 1640 and 1548 cm^{-1} are clearly from protein amide I and amide II, respectively (22). The bands at 1455 and 1401 cm^{-1} are from CH_2 bending and COO^- symmetric stretching modes of the protein side chains, respectively. The bands between 1200 and 1000 cm^{-1} result mainly from C-O or C-C stretching vibrations in the carbohydrates and proteins. In addition, the band at 1720 cm^{-1} may result from the C=O stretching mode of the protein COOH group, assuming that some COO^- groups are protonated at pH 6.5.

We focused on the amide-I patterns of the mixture to analyze the secondary structure of the porcine zona proteins. The second-derivative spectrum has two strong bands, at

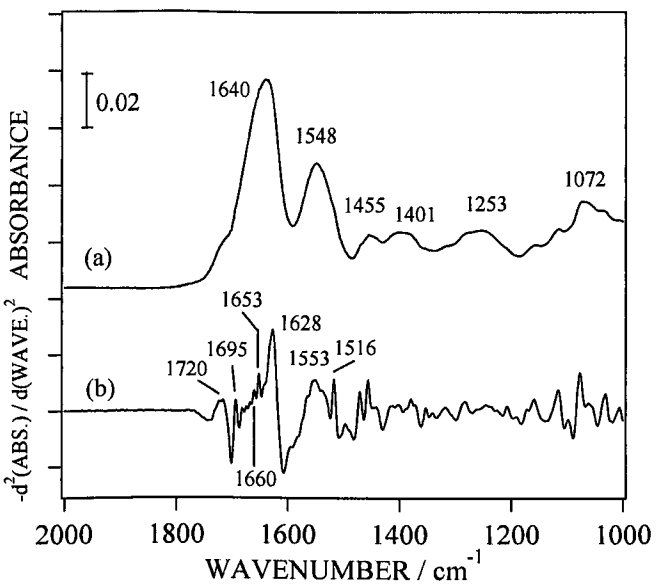

Figure 1. Infrared absorption (a) and second-derivative (b) spectra of solubilized porcine zona protein mixture in H_2O. The concentration of zona protein mixture was 1 mg/50 μl. A second-derivative is multiplied by −1.

1695 and 1628 cm^{-1} in the amide-I region (Fig. 1b), that can undoubtedly be assigned to β-sheet structure according to the empirical assignments for general proteins (22). We also observed the bands at 1660 and 1653 cm^{-1} as the minor components that are assigned to α-helix conformation and α-helix or unordered one, respectively (22). Aggregated proteins yield a CD spectrum similar to that of the β-structure (23). In a FTIR spectrum, as aggregated proteins have a strong band at 1620 to 1615 cm^{-1}, the aggregated proteins are distinguishable from the proteins with the β-structure (22). Therefore, the porcine zone proteins assume mainly intramolecular β-sheet structures in the solubilized state in water.

ATR-FTIR of the Intact, Unsolubilized Porcine Zona Pellucida. Using a FTIR spectrometer equipped with a universal ATR unit, infrared absorption spectrum of the zona pellucida in the intact, unsolubilized state was obtained. Figure 2a shows the ATR-FTIR spectrum of the intact porcine zona pellucida over the range 1900 to 1200 cm^{-1}, in which the absorption of water was subtracted completely. The bands at 1638, 1551, 1454, and 1401 cm^{-1} mainly reflect the zona proteins. The estimated volume occupied by the proteins and carbohydrates of the zona indicates that many water molecules hydrate the glycoproteins in the zona architecture (24). Note that the protein concentration was so low that the absorbance scale in Figure 2a is expanded relative to that observed in Figure 1a. Figure 2b shows the corresponding second-derivative spectrum. In the amide-I region, the bands at 1689 and 1632 cm^{-1} result from a β-sheet structure, and the band at 1653 cm^{-1} is probably from α-helix or unordered conformation. Therefore, the zona proteins mainly form β-sheet structure not only in the solubilized state but also in

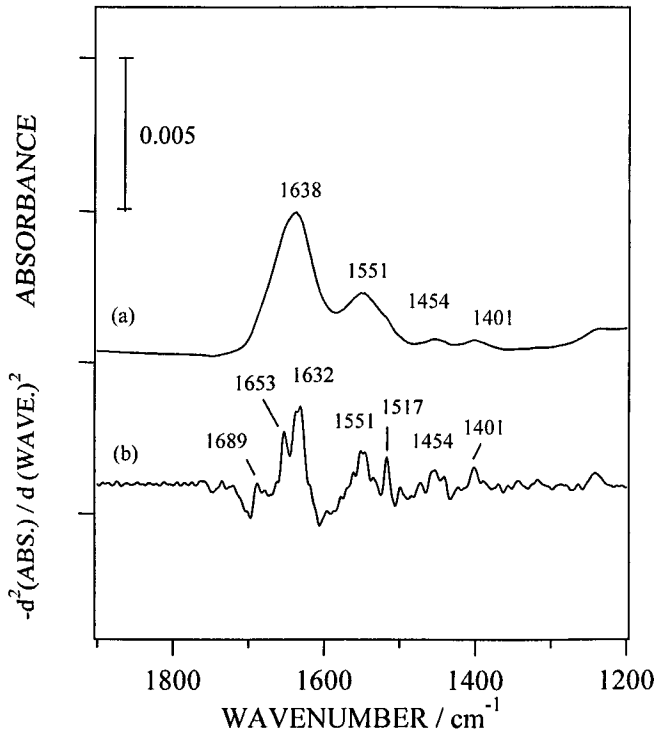

Figure 2. Attenuated total reflection-FTIR (a) and second-derivative (b) spectra of the intact, unsolubilized porcine zona pellucida. The suspension of about 10,000 zonae in 10 μl of water was measured. A second-derivative is multiplied by −1.

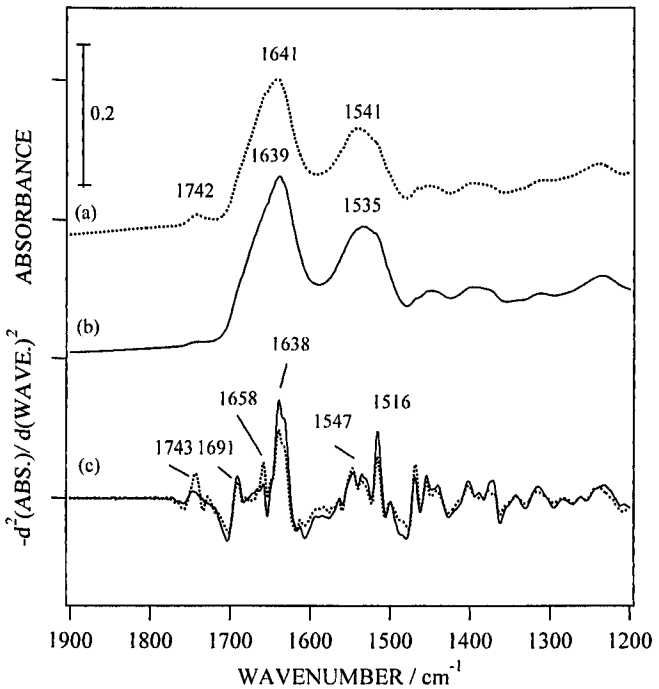

Figure 3. Infrared absorption (a) and (b) and second-derivative (c) spectra of bovine zona protein mixture from ovarian (dotted line) and fertilized (solid line) eggs in solid film. The concentration of zona pellucida was about 300 μg/50 μl. Second-derivatives are multiplied by −1. The reproducibility of the spectra was indicated by triplicate experiments.

the intact, unsolubilized state. The band at 1653 cm^{-1} is stronger in Figure 2b than in Figure 1b, indicating that the α-helix or unordered content was decreased slightly upon dissolution. It is difficult to determine whether α-helix or unordered conformation is responsible for the band at 1653 cm^{-1}, because this band can be assigned to either conformation, according to the empirical assignment (22) described above. In any case, the zona proteins assume secondary structures in the intact zona that are similar to those formed in water.

Changes in the Secondary Structure of Bovine Zona Proteins During Fertilization. As the large-scale preparation of fertilized porcine eggs is difficult, owing mainly to the frequent occurrence of polyspermy, we used bovine ovarian and fertilized eggs to investigate the changes in the secondary structures of the zona proteins during fertilization.

Figure 3 shows the infrared absorption and second-derivative spectra of the bovine zona protein mixtures from ovarian and fertilized eggs in solid film. The bands at 1641 cm^{-1} (Fig. 3a) and 1639 cm^{-1} (Fig. 3b) and the bands at 1541 cm^{-1} (Fig. 3a) and 1535 cm^{-1} (Fig. 3b) are from the amide-I and amide-II modes of zona proteins, respectively. It is noted that the weak band at about 1742 cm^{-1} is attributable to lipids, as mentioned later. We confirmed that the spectral pattern in the amide-I region, obtained using solid films (Fig. 3c), is the same as that obtained using aqueous solutions (data not shown). Differences are apparent in the amide-I region of the second-derivative spectra of zona protein mixtures from ovarian and fertilized eggs (Fig. 3c). The bands at 1691 and 1638 cm^{-1}, which are produced by β-sheet structure (22), were stronger in the spectrum of the fertilized zona proteins. Conversely, the band at 1658 cm^{-1}, which is the result of α-helix structure (22), was weaker in the spectrum of the fertilized zona proteins. Therefore, the β-sheet content increased at fertilization, while the α-helix content decreased.

Figure 4 shows the ATR and second-derivative spectra of intact, unsolubilized bovine zona pellucida from ovarian and fertilized eggs. The bands at 1642 and 1549 cm^{-1} of Figure 4a and the bands at 1639 and 1548 cm^{-1} of Figure 4b mainly reflect zona proteins. Unlike that observed in porcine zona pellucida (Fig. 2), bands were observed at 1737 cm^{-1} and 1743 cm^{-1} in Figures 4a and b, respectively. These bands are undoubtedly attributable to lipid ester C=O stretching and not to proteins (COOH, etc.), because these spectra also showed two strong bands owing to CH_2 stretching mode in the 3000 to 2800 cm^{-1} region (data not shown), as is usually observed for higher fatty acids or lipids.

The second-derivative spectrum of the zonae pellucidae from ovarian eggs showed three bands, at 1682, 1648, and 1632 cm^{-1} in the region of amide I, whereas that for fertilized eggs showed four bands, at 1689, 1658, 1640, and 1632 cm^{-1} in the same region. The bands at 1682, 1640, and 1632 cm^{-1} are assigned to the β-sheet according to the

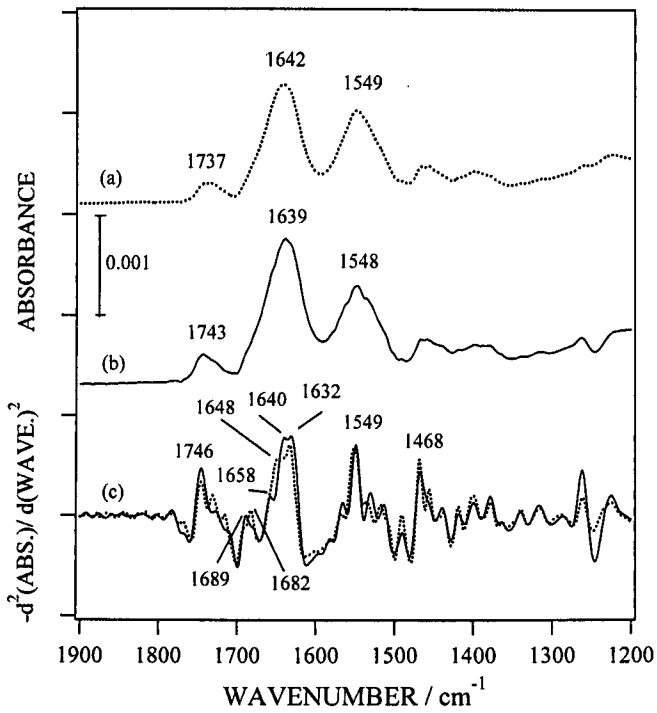

Figure 4. Attenuated total reflection-FTIR (a) and (b) and second-derivative (c) spectra of intact, unsolubilized bovine zona pellucida from ovarian (dotted line) and fertilized (solid line) eggs. The suspension of about 3000 zonae in 10 μl of water was measured. Second-derivatives are multiplied by −1. The reproducibility of the spectra was indicated by triplicate experiments.

empirical assignments for general proteins (22). Similarly, the bands at 1658 and 1648 cm^{-1} are assigned to α-helix and unordered structures, respectively. The bands at 1640 and 1632 cm^{-1} are stronger for the fertilized egg, whereas the band at 1648 cm^{-1} is stronger for the ovarian egg. This means that the β-sheet component increased and the unordered conformation decreased in the process of fertilization. Note that the spectral patterns observed in the 1750 to 1700 cm^{-1} region may reflect lipid components or the environments around esterified lipid compounds (25). The spectral differences observed in Figure 4 may be significant to future work discussing the lipid components of the bovine zona pellucida.

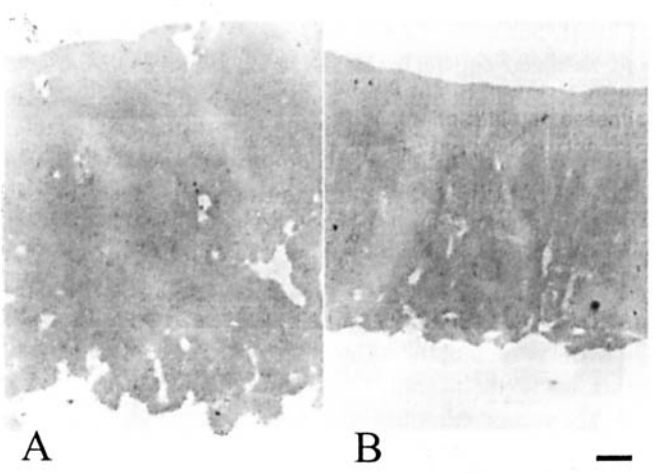

Figure 5. Transmission electron micrographs of bovine zona pellucida isolated from ovarian egg (A) and fertilized egg (B). A typical sectional view of five zonae observed was shown. The zonae are oriented with the oocyte toward the up side. Bar, 1 μm.

Table 2. Secondary Structure Compositions of Aqueous Solution of Bovine Ovarian and Fertilized Egg Zona Protein Mixtures Estimated on the Basis of CD Values with Program CONTIN (21)[a]

	α-Helix (%)	β-Sheet (%)	β-Turn (%)	Remainder (%)
From ovarian eggs	11	47	21	21
From fertilized eggs	7	54	24	15

[a] The reproducibility of the data was indicated by the values for duplicate experiments.

The CD spectra of the bovine zona protein mixture from ovarian and fertilized eggs in water were measured, and the mean residue ellipticity was calculated using the molecular masses and the molar ratio of the three components, as shown in Table 1. The composition of the secondary structures estimated from the ellipticities of the CD spectra using the CONTIN program indicates that 47% of the bovine zona protein mixture from ovarian eggs in water consisted of the β-sheet structure (Table 2). The CD spectra also indicated that the β-structure content increased, while the α-helix content decreased in the fertilized egg zona protein mixture. These results generally agreed with those obtained from FTIR spectroscopy in solid film (Fig. 3).

Change in the Thickness of Bovine Zona Pellucida Associated with Fertilization. An ultrastructural study revealed that the human zona is characterized by various filament networks (26). The filaments of the outer surface of the unfertilized egg zona are arranged in large, tight networks, whereas those of its inner surface are arranged in repetitive structures consisting of numerous short, straight filaments that cross each other. After fertilization, the filaments of the inner surface fuse (26). In our experiment, the thickness of the bovine zonae pellucidae isolated from ovarian and fertilized eggs was estimated using transmission electron microscopy. Five unfertilized and five fertilized egg zonae were observed, and typical sectional views are shown in Figure 5. A rigid surface oriented toward the oocyte was observed in the fertilized egg zona. The ovarian and fertilized egg zonae were about 11 ± 0.6 and 7 ± 0.2 μm thick, respectively, indicating that a more compact structure was formed associated with fertilization.

Conformational Changes of the Zona Pellucida Associated with Fertilization. When the bovine zonae are solubilized with 0.2% (w/v) pronase/phosphate-buffered saline, acidic solutions, or 5% (v/v) β-mercaptoethanol/7 *M*

urea, the time required for the solubilization of fertilized egg zonae is 10%–20% longer than for ovarian egg zonae (18). This zona "hardening" is thought to block polyspermy. In this study, we showed that the supramolecular structural change in the zona pellucida during fertilization is accompanied by changes in the secondary structure of the zona proteins. Differences in the FTIR spectra between ovarian and fertilized intact zonae were also observed over the ranges of 1570 to 1380 cm^{-1} and 1270 to 1220 cm^{-1} (Fig. 4). The assignment of these changes will contribute to understanding the molecular mechanisms underlying the functions of the zona pellucida.

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