PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 160, 449–452 (1979)

Characterization of Porcine Oocyte Zonae Pellucidae by Polyacrylamide Gel Electrophoresis[1] (40468)

ALFRED R. MENINO, JR. AND RAYMOND W. WRIGHT, JR.

Department of Animal Sciences, Washington State University, Pullman, Washington 99164

The zona pellucida of the mammalian egg has been determined to be glycoprotein in nature (1, 2). Zona pellucida solubilization in solutions of proteolytic enzymes (2, 3), acid pH (4, 5), denaturants (2), oxidizing agents (2), reducing agents (6, 7), and heated solutions (8) confirmed the presence of protein in the zona pellucida. Histochemical staining (1, 9, 10) and lectin-binding studies (11, 12) have demonstrated the presence of various saccharide residues as components of the zona pellucida. Lowenstein and Cohen (13) determined the dry mass of the murine zona pellucida to be 6.02 ng or 19% of the dry weight of the intact single cell embryo. These workers also calculated that only 30% or 1.8 ng of the murine zona pellucida is protein and the remainder is carbohydrate.

SDS (sodium dodecyl sulfate) polyacrylamide gel electrophoresis of rabbit zonae pellucidae showed four proteins to be present with no significant differences observed in mobility in the protein bands between embryos and unfertilized eggs (6). Inoue (14) localized several classes of polypeptides with molecular weights greater than 65,000 D (daltons) on SDS-polyacrylamide gel electrophoresis using zonae pellucidae from unfertilized mouse eggs. Repin and Akimova (15) conducted electrophoresis on rat and mouse zonae pellucidae isolated from both embryos and unfertilized eggs. Zonae pellucidae from unfertilized rat eggs showed an electrophoretic pattern consisting of five protein bands ranging in molecular weights from 20,000 to 182,000 D. Banding patterns of rat embryo zonae pellucidae showed six protein bands with molecular weights ranging from 10,000 to 35,000 D. Similarly, electrophoretic patterns of zonae pellucidae of unfertilized mouse eggs showed five protein bands ranging from 28,000 to 240,000 D, while mouse embryo electrophoretic patterns showed two protein bands of 100,000 and 140,000 D.

Although the zona pellucida of the laboratory animal has been studied for many years, little work has been performed on the zona pellucida from the farm species. Therefore, by using porcine ovarian oocytes the objectives of this study were to: (a) determine the protein organization in the zona pellucida by electrophoresis on SDS polyacrylamide gels with or without β-MSH (β-mercaptoethanol) and scanning densitometry and, (b) calculate the molecular weights of the bands visualized on SDS-β-MSH gels by plotting against protein standards of known molecular weights.

Materials and methods. Oocyte collection and recovery. Ovaries were collected from excised reproductive tracts of sows and gilts at a local abattoir, and were kept in ice during transport to the laboratory which took approximately 2 hr. Ovarian follicles were aspirated with a 23 gauge, ¾ in needle attached to a syringe. Aspirated follicular fluid was pooled and centrifuged. The supernatant was decanted and the pellet was vortexed for 30–60 sec and resuspended in PBS (Phosphate buffered saline). This procedure was repeated four times to free the oocyte and zona pellucida from surrounding follicle cells. Following the fourth washing, the cell suspension was diluted and dispersed with PBS into 15 × 60 mm culture plates, and the oocytes were recovered by suction into a finely drawn pipet. Recovered oocytes were washed three times in PBS and aranged in aliquots of 500 or 1000 oocytes. Only oocytes which were completely free of follicle cells were used in the study.

Solubilization of the zona pellucida. Denuded oocytes (free of follicle cells) were placed in solutions of 6 *M* urea. Dissolution of the zona pellucida was complete within 25 min. The oocyte-urea solution was either fil-

[1] Scientific Paper No. 5120. College of Agriculture Research Center, Washington State University. Project 0313.

0037-9727/79/040449-04$01.00/0

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tered through Whatman No. 1 filter paper or centrifuged for 5 min to separate oocytes from solubilized zonae pellucidae. Solutions of dissolved zonae pellucidae were examined under a dissecting microscope (70×) to insure the exclusion of oocyte or oocyte fragments by the separation technique.

Dialysis, lyophilization and storage of solubilized zonae pellucidae. Solutions of solubilized zonae pellucidae were dialyzed in #20 size dialysis tubing against a continuous flow of deionized water at 4° for 12–48 hr to insure complete urea removal. Dialyzed solutions were decanted into plastic conical tubes and frozen at −20° for storage before lyophilizing. Lyophilized zonae pellucidae were stored at −20° until electrophoresis.

Electrophoresis. Electrophoresis was conducted on 10% (5% Bis) horizontal polyacrylamide slab gels (16, 17) in a Beckman Microzone electrophoresis system. Gel dimensions were: 6.5 cm × 10.0 cm × 4.0 mm, and poured gels possessed eight sample wells for simultaneous analysis of protein standards and zona pellucida samples. Gels were prepared with a Tris–glycine buffer (pH 8.4) which also served as the electrode buffer. Electrophoresis was performed at 450 V and 55 mA for approximately 50 minutes. Following electrophoresis, gels were stained with Coomassie Brilliant Blue G (16) and destaining was performed electrolytically. Destained gels were either dried at 100° for 3 hr or stored at −4° in 2% acetic acid.

Analysis was conducted by solubilizing lyophilized porcine ovarian oocyte zonae pellucidae in one of three solutions. The treatments were (a) electrophoresis of three samples of 500 zonae pellucidae each conducted separately following solubilization in 6 *M* urea, (b) electrophoresis of three samples of 500 zonae pellucidae each conducted separately following solubilization in 2% SDS sample buffer (18) and (c) electrophoresis of seven samples of 500 or 1,000 zonae pellucidae each conducted separately following solubilization in 2% SDS-5% β-MSH sample buffer (18).

Molecular weight determination. Molecular weights of zona pellucida protein bands were determined by the method described by Weber and Osborn (19) using SDS-β-MSH polyacrylamide gels. Protein standards used for the Rm (relative mobility) vs. log mol wt plot were cytochrome C, chymotrypsinogen A, ovalbumin, bovine serum albumin, aldolase, catalase and ferritin (Boerhinger Mannheim).

Densitometry. Densitometric evaluation of the zona pellucida electrophoretic patterns was conducted on a Beckman CDS-F-100 computing densitometer at 550 nm. Relative percent of the area under each peak in the densitometric scan was computed.

Results. Electrophoresis of 500 zonae pellucidae dissolved in treatment 1 resulted in a single protein band. Densitometric evaluation of the separation pattern revealed that 93.3% of the dye bound to the polyacrylamide gel was due to the single protein band. Five hundred zonae pellucidae solubilized in treatment 2 and separated by electrophoresis produced a single protein band (Fig. 1). Evaluation by densitometry showed 96.6% of the dye bound to the polyacrylamide gel was due to the single protein band. Electrophoresis of 500 or 1,000 zonae pellucidae in treatment 3 resulted in four protein bands (Fig. 2). Densitometric evaluation of protein bands one, two, three and four showed that 6.3, 18.6, 23.7, and 50.3% respectively, of the dye bound to the polyacrylamide gel was due to the four bands.

Molecular weight determination. The plot of Rm vs. log mol wt for the protein standards used demonstrates the linearity of the relationship for molecular weight ranges from 1 × 10^4 to 10 × 10^4 D in 10% polyacrylamide gels (17, 19; Fig. 3). For each SDS-β-MSH gel, a plot similar to that presented was

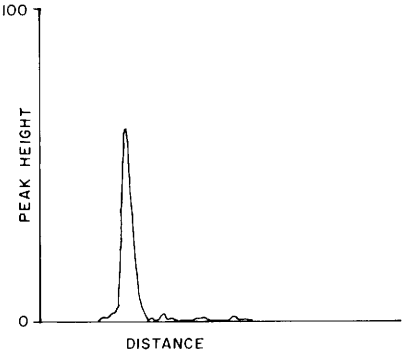

FIG. 1. Densitometric scan of electrophoretic pattern of 500 porcine oocyte zonae pellucidae dissolved in 2.0% SDS sample buffer and separated by electrophoresis in a 0.1% SDS-10.0% polyacrylamide gel.

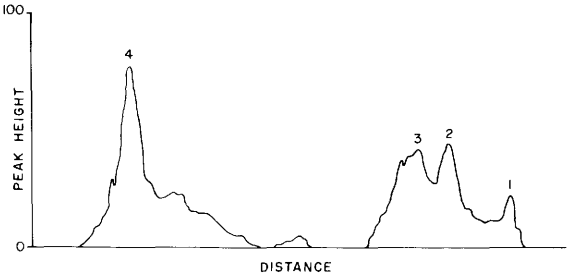

FIG. 2. Densitometric scan of electrophoretic pattern of 1000 porcine oocyte zonae pellucidae dissolved in 2.0% SDS-5.0% β-MSH sample buffer and separated by electrophoresis in a 0.1% SDS-10.0% polyacrylamide gel.

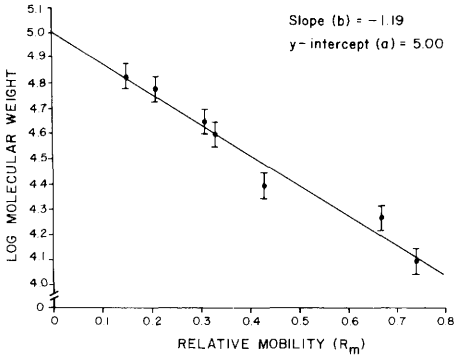

FIG. 3. Plot of mean relative mobility (Rm) vs. log molecular weight of protein standards dissolved in 2.0% SDS-5.0% β-MSH sample buffer and separated by electrophoresis in 0.1% SDS-10.0% polyacrylamide gel.

graphed and the calculated Rm values for the four zona pellucida bands were fitted into the equation of the line to obtain the log mol wt. The molecular weight of each band was then calculated and averaged with other respective molecular weights (Table I).

Discussion. This study suggests that the porcine oocyte zona pellucida is composed of a heterologous polypeptide substructure covalently bonded to form a single protein structure. When lyophilized porcine zonae pellucidae were dissolved in either urea or SDS and separated on polyacrylamide gels, only a single band was observed. These data suggest that the four protein bands visualized in SDS-β-MSH gels are interlinked by disulfide bonds to constitute a single complex. Urea and SDS are denaturants capable of destroying native quaternary, tertiary and secondary protein structure through the disruption of noncovalent bonds, however, both are incapable of cleaving covalent linkages. Treatment of the lyophilized protein with β-MSH, a disulfide reducing agent, would disrupt the covalent disulfide linkages maintaining the multimeric conformation of the zona pellucida protein. Electrophoresis following treatment with β-MSH would result in the separation of the reduced polypeptides as individual subunits, since the interchain disulfide bonding responsible for linking the four polypeptide species as a single multimeric unit have been degraded.

TABLE I. MEANS AND STANDARD DEVIATIONS OF RELATIVE MOBILITY (Rm), LOG MOLECULAR WEIGHT (LOG MOL WT) AND MOLECULAR WEIGHT (mol wt) FOR THE FOUR ZONA PELLUCIDA BANDS VISUALIZED ON SDS-β-MSH 10.0% POLYACRYLAMIDE GELS.

Zona pellucida band number[a]	Rm	log mol wt	mol wt (D)
1	0.06 ± 0.01	4.931 ± 0.030	85,000 ± 4,000
2	0.15 ± 0.01	4.817 ± 0.012	66,000 ± 2,000
3	0.20 ± 0.02	4.756 ± 0.026	57,000 ± 1,500
4	0.64 ± 0.02	4.235 ± 0.033	17,000 ± 1,000

[a] Band number assigned by distance from cathode to anode.

The observation of four polypeptides bonded covalently through disulfide linkages for the porcine oocyte zona ellucida is similar to the rabbit (6). In the study conducted by Gould *et al.* (6), electrophoresis of rabbit zona pellucida following solublization in β-MSH produced four polypeptide bands, suggesting disulfide bonding may be responsible for maintaining the zona pellucida structure. The suggestion of a single multimeric protein complex in the porcine zona pellucida is in contrast to that postulated by Repin and Akimova (15) for the mouse and rat zonae pellucidae. In their study, rat and mouse zonae pellucidae of unfertilized eggs were dissolved in SDS, then separated by electrophoresis on

SDS polyacrylamide gels. These investigators obtained five protein bands in the electrophoretic separation patterns for either rat or mouse unfertilized egg zonae pellucidae. The data presented by Repin and Akimova (15) suggested that the zona pellucida of the rat and mouse is a matrix of different proteins stabilized primarily by noncovalent interaction, such as hydrogen bonding. The results of this study also suggest a matrix of different proteins. However, it appears that these proteins are covalently linked to form a larger unit, which then interacts noncovalently with other such units to form the zona pellucida.

Summary. Polyacrylamide gel electrophoresis of zonae pellucidae isolated from porcine ovarian oocytes and dissolved in either 6 *M* urea or 2.0% SDS suggest that the zona pellucida is composed of a single protein in addition to a carbohydrate component. The structural organization of the protein in the porcine zona pellucida is postulated to consist of repeating units of this single protein maintained by noncovalent bonding. Electrophoresis in SDS-β-MSH polyacrylamide gels has shown the protein to be composed of four polypeptide or protein subunits with molecular weights of 17,000 D, 57,000 D, 66,000 D and 85,000 D. These proteins may be cross-linked by disulfide bridges comprising the single protein visualized in gels without β-MSH. The subunit proteins may be in a multimeric arrangement supported by covalent bonds which maintain the complex structure. Relative percentages of the subunit proteins determined by scanning densitometry were: 50.3% (17,000 D), 23.7% (57,000 D), 18.6% (66,000 D) and 6.% (85,000 D). The unequal percentages reported by densitometry may be an indicator of the number of molecular subunit species within a complex.

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Received September 5, 1978. P.S.E.B.M. 1979, Vol. 160.