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GLYCOPROTEINS OF MOUSE ZONA PELLUCIDA: ANALYSIS OF THEIR REACTIVITY TO LECTINS

Satoru Shimizu, Mie Ito and Jurrien Dean*

Laboratory of Ultrastructure Research
Aichi Cancer Center Research Institute
Chikusa-ku, Nagoya 464, Japan
and
*Laboratory of Chemical Biology, National Institute of Arthritis,
Diabetes, and Digestive and Kidney Diseases
National Institutes of Health, Bethesda, Maryland 20205, U.S.A.

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Summary: Zonae pellucidae were isolated as an intact structure from super-ovulated mouse eggs using a new simplified method, and the proteins were fractionated by SDS-polyacrylamide gel electrophoresis. Location of proteins by silver stain showed three bands previously designated as ZP-1, ZP-2 and ZP-3. The reactivities of these proteins to specific lectins were examined after transferring them to nitrocellulose paper by the "Western" blotting technique. Ricinus communis agglutinin-I, Triticum vulgaris agglutinin, Dolichos biflorus agglutinin and Glycine max agglutinin reacted to all three zona proteins. However Concanavalin A reacted only to ZP-2 and Arachis hypogaea agglutinin only to ZP-1 and ZP-3. These results indicate that the carbohydrate of each zona protein is unique.

The zona pellucida surrounds the mammalian egg and mediates the species-specific binding of sperm to egg (1). Mouse zona is composed of three glycoproteins designated as ZP-1, ZP-2 and ZP-3 with average molecular weights of 200,000, 120,000 and 83,000, respectively (2). Following successful fertilization, the zona remains morphologically unchanged but is modified by a hardening reaction to prevent further sperm binding (1). Analysis of the zona proteins from two-cell embryos has suggested that ZP-2 is proteolytically cleaved such that its average molecular weight is reduced from 120,000 to 90,000 daltons (3). The exact role that this modification has on the block to polyspermy is as yet unclear.

Early estimates suggest that 20-30% of the intact zona pellucida is carbohydrate (4,5) and more recent studies using radioactive fucose have demonstrated that all three zona proteins contain sugar side chains (6). Analysis of the carbohydrate composition of the zona pellucida is greatly

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complicated by the small amount of available biological material. Each murine zona contains only 5 ng of protein (2) and yet there is no tissue culture source of this material. In this communication we report the detection of the three individual zona proteins using a sensitive silver stain and make use of lectins as specific probes of the carbohydrate composition of each.

Materials and Methods

Reagents: Concanavalin A and peroxidase-labeled lectins were purchased from EY Laboratory (San Mateo, Calif.). Biotinyl lectins, avidin D and biotinyl peroxidase were the products of Vector Laboratory (Burlingame, Calif.) Horseradish peroxidase (type I, Sigma Chemical Co., St. Louis, Mo.) was further purified by Sephadex G-200 (Pharmacia, Uppsula). Crystalline bovine serum albumin (BSA) was purchased from Povite Producten, B.V. (Amsterdam) and 3,3'-diaminobenzidine tetrahydrochloride from Wako Pure Chemical Industry Ltd (Osaka, Japan).

Preparation of mouse zona pellucida: Female ICR mice (5-8 week old) were intraperitoneally injected with 10 units of pregnant mare serum gonadotropin (Teikokuzoki Pharmaceutical Co., Tokyo) followed 48 hours later by 10 units of human chorionic gonadotropin (Mochida Pharmaceutical Co., Tokyo). After 14 to 16 hours, the animals were sacrificed and superovulated eggs were isolated from the oviducts. The cumulus-mass surrounding the eggs was dispersed by incubation with hyaluronidase (300 unit/ml, Sigma type VI) for 15 min in Hanks medium containing 0.02% BSA (w/v). The eggs were extensively washed in the same media without hyaluronidase and suspended in Hanks medium containing 0.02% BSA, 0.2% (v/v) Nonidet P-40 (NP-40, Shell Chemical Co., West Orange, N.J.), 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride and 10 μg/ml of lima bean trypsin inhibitor (P-L Biochemicals, Inc., Milwaukee, Wisc.). After three cycles of freezing in an ethanol-dry ice bath and thawing at 20°C, the suspension was examined microscopically and morphologically pure zonae were collected by micropipet and washed ten times with Hanks medium.

Gel electrophoresis and silver staining: Zona proteins were solubilized and separated by SDS-polyacrylamide gel electrophoresis using a 6 x 9 x 0.1 cm running gel (7). The gels were fixed and the proteins visualized by silver stain (8) modified by the deletion of glutaraldehyde fixing and the subsequent washing with distilled water.

Blotting to nitrocellulose paper and lectin reactions: Proteins were transferred by the "Western" blotting technique (9) to nitrocellulose paper (Schleicher and Schuell, Keene, N.H.) in 0.025 M Tris, 0.192 M glycine and 20% methanol using a constant voltage of 6 volt/cm for 20 hours. The nitrocellulose paper was then treated with 5 ml of 0.15 M NaCl, 0.01 M Tris-HCl containing 1% BSA for 1 hour at room temperature. Excess buffer was removed and the paper was incubated with 3 ml of Con A, peroxidase-labeled lectin or biotinyl lectin as follows (see Table I): For Con A, the solution was made 0.5 mM $CaCl_2$, and the nitrocellulose paper was incubated first with Con A (100 μg/ml) and then, after three washes, with 3 ml of peroxidase (100 μg/ml) (11). For RCA-I-peroxidase, PNA-peroxidase and SBA-peroxidase, the lectin conjugate was 50 μg/ml (12). For DBA-biotin and WGA-biotin, the nitrocellulose strip was incubated first with biotinyl lectin (10 μg/ml), washed as above, and then reacted with 3 ml of avidin-biotinyl peroxidase complex for 30 min at 20°C (13). All of the lectin-reacted nitrocellulose strips were then washed three times with 15 ml of 0.01 M Tris-HCl, pH 7.4, 0.15 M NaCl containing 0.1% BSA and once with the same buffer without BSA. The presence of the lectin conjugates was visualized by incubation for 1-5 min at 20°C with 10 ml of 0.05%

diaminobenzidine and 0.01% H_2O_2 in 0.1 M Tris-HCl, pH 7.4 (11-13). For control experiments the lectin binding was carried out in the presence of 0.1-0.3 M of the competitive sugar and all of the above washes and reactions performed.

Results and Discussion

Zonae pellucidae were readily isolated as an intact structure from super-ovulated eggs after freezing and thawing in the presence of NP-40. They appeared pure both morphologically and by the absence of contaminating proteins in SDS gels after silver staining which has a sensitivity well under 1 ng/mm^2 of gel (8). This isolation method is simple, reproducible and can be applied to oocytes and eggs of any size. As few as 40 zonae were sufficient to give distinct bands after SDS-polyacrylamide gel electrophoresis representing the three zona proteins, ZP-1, ZP-2 and ZP-3 (see Figure 1). Using a "Western" blotting technique (9) the three glycoproteins were transferred to nitrocellulose paper and their individual carbohydrate composition probed by specific lectins. The transfer efficiency of the zona proteins was very high. After blotting, no protein was found remaining in the gel as determined by staining with silver (see Figure 1).

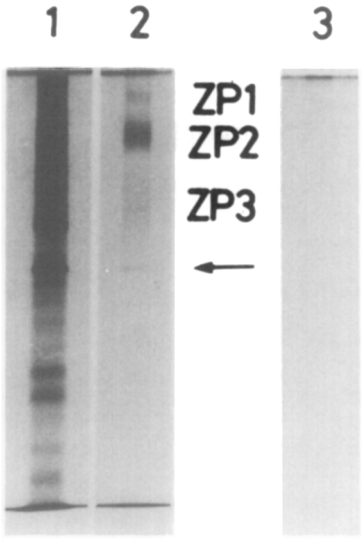

Figure 1. SDS-polyacrylamide slab gel electrophoresis of mouse eggs and zonae pellucidae. Samples were dissolved in 0.125 M Tris-HCl, pH 6.8 containing 2% SDS and 10% glycerol, and incubated at 37°C for 30 min. After electrophoresis at 14 mA for 2 h, proteins were visualized by silver stain. (1) 50 eggs, (2) 40 zonae pellucidae, (3) same as lane 2 except that the gel was stained after the proteins were transferred to nitrocellulose paper. Arrow indicates BSA from washing media.

Table I

Lectin	Abbreviation	Sugar Specificity (10)
Concanavalin A	Con A	α-D-Man > α-D-Glc > α-D-GlcNAc
Arachis hypogaea agglutinin	PNA	β-D-Gal-(1→3)-D-GalNAc > D-GalN = α-D-Gal
Ricinus communis agglutinin	RCA-I	β-D-Gal > α-D-Gal
Glycine max agglutinin	SBA	α-D-GalNAc > β-D-GalNAc >> α-D-Gal
Triticum vulgaris agglutinin	WGA	β-D-GlcNAc-(1→4)-β-D-GlcNAc-(1→4)-D-GlcNAc β-D-GlcNAc-(1→4)-D-GlcNAc
Dolichos biflorus agglutinin	DBA	α-D-GalNAc >> α-D-Gal

The three proteins appear as wide bands with the majority of the stain concentrated in ZP-2. All three bands are labeled after incubation with radioactive fucose (6) and the broadness of the bands is most likely due to carbohydrate side chain heterogeneity. The role of the carbohydrate side chains is not clear either in the species-specific binding of sperm to the zona pellucida or in the modification that the zona undergoes to prevent polyspermy. Earlier studies have shown that the intact unfractionated zona stains histochemically for carbohydrate (14) and that several lectins (Con A, RCA, WGA) bind to the zona pellucida of multiple species; mouse, rat, hamster and pig (5,15-17). However, other lectins discriminate among species, e.g. DBA binds to mouse and hamster but not to pig and PNA binds to mouse but neither to pig nor hamster. Intact mouse zona pellucida reacts with Con A, RCA, WGA, DBA and PNA (16,17). Thus, lectins appear to be effective probes of species-specific differences of the carbohydrate moieties.

We have taken advantage of our ability to separate the three murine zona glycoproteins, transfer them to nitrocellulose strips by "Western" blotting and have probed them with specific peroxidase conjugated lectins. Parallel studies conducted in the presence of individual sugars which inhibit these reactions, further enhance the interpretation of the specificity of lectin binding. RCA-I, SBA, DBA and WGA (see Table I for sugar specificity) bound to all three zona proteins although the relative intensities of the reaction

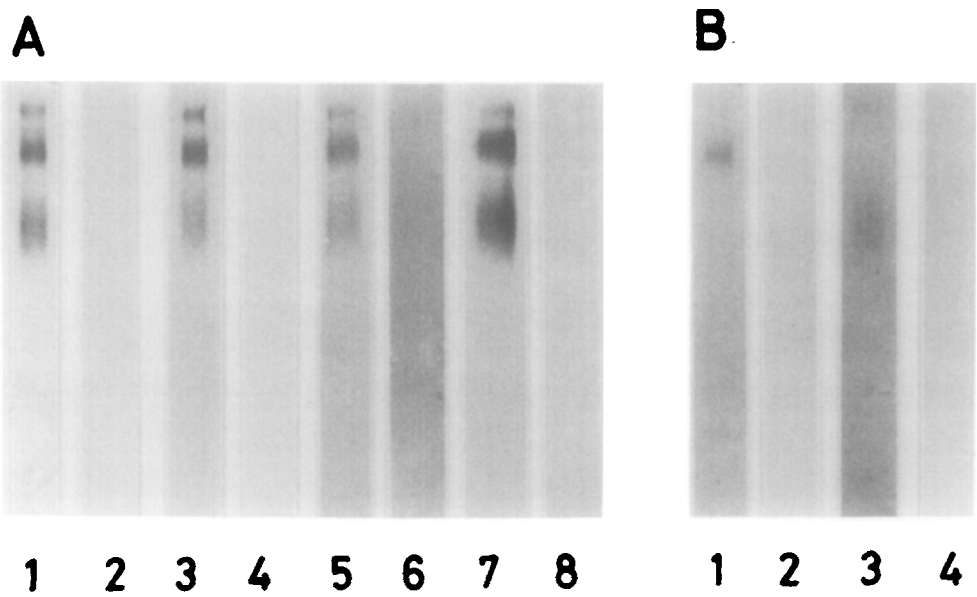

Figure 2. Glycoprotein patterns of zona pellucida as detected by lectins. A (1) RCA-peroxidase, (2) RCA-peroxidase with 0.3 M D-galactose, (3) SBA-peroxidase, (4) SBA-peroxidase with 0.2 M N-acetyl-D-galactosamine, (5) DBA-biotin, (6) DBA-biotin with 0.2 M N-acetyl-D-galactosamine, (7) WGA-biotin, (8) WGA-biotin with 0.3 M N-acetyl-D-glucosamine. All smaples contained 50 zonae pellucidae.

B (1) Con A-peroxidase, (2) Con A-peroxidase with 0.1 M α-methyl-D-mannoside, (3) PNA-peroxidase, (4) PNA-peroxidase with 0.2 M D-galactose. All samples contained 100 zonae pellucidae.

among the three glycoproteins were different from one lectin to another (see Figure 2A). This in itself suggests that the sugar side chains differ from one another. Furthermore, Con A which binds preferentially to D-mannose and D-glucose (10) reacted only with ZP-2 (see Figure 2B) even when both the concentration of zonae pellucidae and of lectin were doubled. In a complementary fashion PNA with a relative preference for terminal β-galactose bound only to ZP-1 and ZP-3 and not to ZP-2 (see Figure 2B). Thus, it appears that all three zona proteins contain N-acetylglucosamine, N-acetylgalactosamine and internal galactose residues but only ZP-1 and ZP-3 have terminal galactose residues. Furthermore, ZP-2 is unique in containing α-D-mannose or glucose which is not present in either ZP-1 or ZP-3.

In these present studies small quantities of zona material have been separated into their component glycoproteins, detected by silver stain and analyzed by sensitive and specific lectins. By identifying the difference in

the carbohydrate side chains of the three zona proteins we have demonstrated that each glycoprotein has a unique carbohydrate content. These results will be useful in determining the role that the sugar moiety plays both in mediating species-specific sperm binding to the zona pellucida and the post-fertilization block to polyspermy.

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