Studies on Mouse Sperm Binding Molecule (Ligand) to Egg Zona Pellucida. II. Purification of Ligand Molecule from Cauda Epididymal Sperm

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One of the ligand molecules was purified from mouse cauda epididymal sperm for an indication of its inhibitory activity in sperm-egg binding. It was isolated in an electrophoretically homogeneous form by ionophore A23187 treatment of sperm, sedimentation by ultracentrifugation, solubilization by 2% Nonidet P-40 (NP-40), repeated ion exchange chromatography on DEAE-Sephacryl CL-6B and gel filtration on Sephacryl S-200. The purified ligand exhibited a molecular mass of 60000 dalton by gel filtration and 67000 dalton by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). This molecule was radioiodinated by the lactoperoxidase method and assayed the binding ability to egg zona pellucida. The labeled ligand specifically bound to both cumulus-free egg zona pellucida and isolated-zona pellucida of unfertilized egg in a similar extent, but didn't bind to 2-cell egg zona pellucida. The maximal binding site of ligand per egg zona pellucida was estimated about 660000 molecules by Scatchard plot analysis.

Keywords — sperm-egg binding; ligand purification; zona pellucida; acrosome reaction

Introduction

The initial interactions between sperm and egg may be viewed as a form of cell-cell recognition and adhesion specific to fertilization. In fertilization of mammalian eggs *in vitro*, this process is involved in sperm capacitation and the acrosome reaction. Although an understanding of capacitation from a molecular viewpoint remains to be achieved in the mouse, it is well established that spermatozoa located within the perivitelline space have undergone the acrosome reaction.¹⁻³⁾ In addition to, only acrosome-intact sperm could bind to the zona pellucida. And the acrosome reaction occurred after the binding to egg.⁴⁾ Accordingly, a sperm surface recognition molecule (ligand) should exist on the surface of the acrosome. Recently, Shur and coworkers showed that sperm surface galactosyltransferases were involved in the recognition of the zona pellucida.⁵⁻⁸⁾

In this study, we purified one of the ligand molecules from mouse cauda epididymal sperm. The purified ligand molecules were radioiodinated. And then, by using the labeled molecules, the number of binding site of the purified ligand per egg was estimated by Scatchard plot analysis.

Materials and Methods

Reagents — Phenylmethylsulfonylfluoride (PMSF) and ionophore A23187 were purchased from Calbiochem (La Jolla, CA). Crystallized bovine serum albumin (BSA), ovalbumin (crystallized and lyophilized, grade V) and α -chymotrypsinogen-A were obtained from Sigma Chemical Co. (St. Louis, MO). Nonidet P-40 (NP-40), 4-methylumbelliferyl-*N*-acetyl- β -D-glucosaminide (4-MU-GlcNAc) and 4-methylumbelliferyl-*N*-acetyl- β -D-galactosaminide (4-MU-GalNAc) were from NaCalai TESQUE, Inc. (Kyoto, Japan). Other reagents were purchased from Wako Pure Chemical Industry Ltd. (Osaka, Japan).

Collection of Gametes and Sperm-Egg Binding Assay — Sperm and eggs were collected from ICR mice (Japan SLC, Inc., Japan) as previously described.⁹⁾ Eggs were freed from their cumulus cells by hyaluronidase treatment and washed in modified Krebs-Ringer bicarbonate medium containing 0.4% BSA (m-KRB) at room temperature. Sperm (10^6 cells/ml) were capacitated by incubation in m-KRB for 60 min. For competitive binding assay, cumulus-free eggs were preincubated with 25 μ l of each sample or HNC buffer (20 mM *N*-2-hydroxyethyl-

piperazine-*N'*-2-ethane-sulphonic acid (HEPES), 126 mM NaCl and 1.7 mM CaCl₂, pH 7.4) in 45 μ l drops of m-KRB for 30 min, followed by 5 μ l of capacitated sperm (10⁶ cells/ml) suspension and incubated in 5% CO₂/95% air for 30 min. At the end of the culture periods, the gametes were centrifuged through a discontinuous dextran gradient at room temperature. Eggs and bound sperm were fixed in the lowest layer by glutaraldehyde (stop-fix technique).¹⁰ The number of bound sperm per egg was determined with phase contrast microscopy (Type IMT, Olympus). All procedure were done at 37 °C unless otherwise indicated.

Preparation and Solubilization of Granule

— Sperm (10⁶ cells/ml) were preincubated in 3 ml of calcium-free m-KRB for 60 min at 37 °C to avoid a spontaneous acrosome reaction and washed with m-KRB by centrifugation (600 *g*, for 10 min). Washed sperm were incubated in 3 ml of m-KRB containing 5 μ M A23187 and 1 mM PMSF for 60 min at 37 °C. After incubation, the sperm suspension was centrifuged at 1500 *g* for 15 min. The supernatant was carefully removed and ultracentrifuged at 105000 *g* for 60 min. The resultant pellet fraction (granules) was collected and resuspended in 1 ml of 2% NP-40 in HNC buffer, followed by sonication as above and solubilized for overnight. After removal of non-solubilizable materials by ultracentrifugation, the solubilized granule was passed through a column of Bio-Beads SM-2 (Bio-Rad Laboratories, Richmond, California) to remove most of the detergent,¹¹ and then dialyzed against 500 volumes of HC buffer (consist of 20 mM HEPES and 1.7 mM CaCl₂, pH 7.4). All operations were performed at 4 °C unless otherwise noted.

Purification of Ligand — The solubilized granule was applied on DEAE-Sepharose CL-6B column (1 $\times$ 10 cm) equilibrated with HC buffer. The column was washed with equilibration buffer and eluted with a linear gradient of 20 ml of equilibration buffer as the starting buffer and 20 ml of 500 mM NaCl in the same buffer as seen in Fig. 1. Each fraction was dialyzed against 1000 volumes of HNC buffer and assayed both for an inhibitory activity of sperm-egg binding (competitive binding assay) and *N*-

acetylhexosaminidase activity.¹² The sperm-egg binding inhibitory fractions (fraction 48—52) were pooled and dialyzed against HC buffer prior to rechromatography. After rechromatography on a smaller column, the active fractions were pooled and applied on a Sephacryl S-200 column (1.5 $\times$ 45 cm) previously equilibrated by HNC buffer (Fig. 2). Each fraction was assayed for sperm-egg binding inhibitory activity. A molecular weight calibration of the active peak was carried out by BSA (67000), ovalbumin (43000) and α -chymotrypsinogen (25000). The purity of the purified ligand was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Enzyme Assay of *N*-Acetylhexosaminidase Activity — An aliquot (100 μ l) of enzyme solution was used for determining *N*-acetylhexosaminidase activity. *N*-Acetylglucosaminidase and *N*-acetyl-galactosaminidase activity was assayed using 4-MU-GlcNAc and 4-MU-GalNAc as substrate, respectively. The released 4-methyl-umbelliferone was determined by a fluorescence spectrophotometer (Hitachi F3000) at 365 nm for excitation and 450 nm for emission. One unit of enzyme activity is defined as that amount which will hydrolyze 1 μ mol of substrate per min.

Electrophoretic Analysis of Purified Ligand — SDS-PAGE was performed in 7.5% gel as described by Laemmli.¹³ The gels were stained in 0.2% Coomassie Brilliant Blue R-250. The molecular weight was calibrated by BSA, catalase (60000) and ovalbumin. All the reagents were obtained from Bio-Rad Laboratories.

Preparation of Labeled Ligand — [¹²⁵I]Iodine was obtained from DuPont-New England Nuclear. ¹²⁵I-Labeled preparation of ligand was prepared by a modification of the lactoperoxidase method.¹⁴ Briefly, 20 ng of purified ligand protein, 1.25 μ g of lactoperoxidase (Sigma) and 1 mCi Na [¹²⁵I] (17.4 Ci/mg) were mixed in 90 μ l of phosphate buffered saline (PBS, pH 7.4). The reaction was started by the addition of 10 μ l of 0.88 mM H₂O₂ and incubated for 20 min at room temperature. The reaction was terminated by the addition of 0.5 ml of 5 mM of cysteine solution in PBS. Unbound-

TABLE I. Purification of Ligand from Mouse Sperm

Purification step	Protein (mg)	ID ₅₀ ^{a)} (μg/50 μl)	Total ^{b)} activity	Purification fold
Solubilization	8.7	25	348	1.0
DEAE-Sepharose (Peak II)	0.9	7	129	3.6
Sephacryl S-200	0.2	2	100	12.5

a) 50% inhibition dose of sperm-egg binding.

b) Total activity was defined as (total protein)/(ID₅₀).

[¹²⁵I] was separated by pass through a Sephadex G-50 column (0.7 × 18 cm) equilibrated by PBS containing 0.1% gelatin.

Binding of Labeled-Ligand to Egg — The binding activity of radiolabeled-ligand to egg zona pellucida was measured as follows. Various concentrations of labeled ligand were incubated with cumulus-free, zona-intact eggs or mechanically isolated-zonae pellucidae and 2-cell eggs for 3 h at 4 °C in 50 μl of 20 mM HEPES-buffered-m-KRB (H-KRB) in the presence (20 mM) or absence of MnCl₂. After washing with chilled HNC buffer containing 0.4% BSA (HNC-BSA), the radioactivities associated with eggs or isolated-zonae were estimated by auto well γ-counter (Aloka, model ARC-300, Tokyo, Japan). Nonspecific binding was determined in samples containing a 400 fold excess of the un-

labeled ligand. The data from binding studies were analyzed by the method of Scatchard.

Results

Purification of the inhibitory activities of sperm-egg binding (*i.e.*, ligand) from mouse cauda epididymal sperm is summarized in Table I. The ligand was purified 12.5-fold over crude extracts. The yield of purified ligand was 0.2 mg protein from 100 mice. The 50% inhibition dose (ID₅₀) of sperm-egg binding was estimated 2 μg per 50 μl.

In ion-exchange chromatography, sperm-egg binding inhibitory activities appeared in three peaks (Fig. 1). The first peak (fraction 42—44) contained *N*-acetyl-hexosaminidase (both *N*-acetyl-glucosaminidase and *N*-acetyl-galac-

Fig. 1. DEAE-Sepharose CL-6B Chromatogram of Solubilized Granule

Bound proteins were eluted with a 0 to 0.5 M gradient of NaCl in HC buffer. Each fraction (1.5 ml) was monitored by conductivity (◇) and an absorbance at 280 nm (□), and dialyzed against HNC buffer followed by assay of an inhibitory activity (●) of sperm egg binding and *N*-acetylhexosaminidase activity (■).

Fig. 2. Gel Filtration of DEAE Peak-II Fraction on Sephacryl S-200

Pooled DEAE Peak-II fractions were placed on a column (1.5×45 cm) of Sephacryl S-200, and eluted with HNC at a flow rate of 2.5 ml/h. Each fraction was monitored at 280 nm (□) and the inhibitory activity (○) of sperm-egg binding assayed as given in the Materials and Methods.

tosaminidase) activity. The former activity was shown about 4-times compared with the latter activity, so we showed only *N*-acetylglucosaminidase activity. The second peak (fraction 48—52) showed the greatest inhibition of sperm-egg binding. These fractions were pooled and applied to a smaller column of DEAE-Sephacryl (data not shown). The active fractions were concentrated to 1 ml and placed on a Sephacryl S-200 column (Fig. 2). A large protein peak emerged with inhibitory activity of sperm-egg binding and with a molecular weight of 60000. The molecular weight of the purified ligand was analyzed by SDS-PAGE. As shown in Fig. 3, the molecular weight was estimated at about 67000. The band accounts for greater than 95% of the Coomassie blue staining on the gel by densitometric analysis (data not shown).

The purified ligand (fraction 44) was radioiodinated by the lactoperoxidase method. The specific radioactivity obtained was 2×10^7 cpm per 20 ng protein. The binding of radiolabeled-ligand to eggs and to isolated-zonae pellucidae were examined. The maximal binding to unfertilized egg was reached at 30 min and maintained up to 6 h (data not shown). When 50000 cpm

of labeled-ligand and eggs were incubated for 3 h at 4°C, about 2000 cpm of labeled-ligand bound to 50 unfertilized eggs, specifically, and it bound to 2-cell eggs to such a background level. Nonspecific binding level was 5—10 cpm per egg (Table II). Furthermore, we examined the

Fig. 3. SDS-PAGE of Purified Ligand

Purified ligand (5 μ g) was electrophoresed in 7.5% polyacrylamide gel. The protein bands were stained by 0.2% Coomassie Brilliant Blue R-250.

TABLE II. Binding of [¹²⁵I]Ligand to Unfertilized and 2-Cell Eggs

Egg		Total cpm	No. of eggs	cpm/egg	Mean ± S.E. (cpm/egg)
Unfertilized egg	Total	3850	57	68	
	binding	1248	46	27	47 ± 12
		2785	60	46	
	Nonspecific	550	49	11	
	binding	563	48	12	11 ± 1
		479	56	9	
Fertilized (2-cell) egg	Total	1111	58	19	
	binding	576	51	11	16 ± 3
		1139	60	19	
	Nonspecific	554	57	10	
	binding	170	53	3	5 ± 3
		135	55	2	

Unfertilized eggs and 2-cell embryos were incubated with [¹²⁵I]ligand (50000 cpm) in 50 μl of H-KRB buffer for 3 h at 4 °C and washed with HNC-BSA. The radioactivities of ligand bound to eggs were counted by γ-counter as described in Materials and Methods. Nonspecific binding was done in the presence of 400 fold excess of non-labeled ligand.

TABLE III. Binding of [¹²⁵I]Ligand to Egg Zona Pellucida

		Total cpm	No. of z.p. ^{a)}	cpm/z.p. ^{a)}	Mean ± S.E. (cpm/egg or cpm/z.p.)
Egg (oocyte + zona)	Total binding	4099	50	82	
		9474	75	126	100 ± 13
		6529	72	91	
	Nonspecific	311	30	10	
	binding	574	35	16	21 ± 9
		1459	40	38	
Isolated-zona pellucida	Total binding	1224	32	38	
		7935	69	115	93 ± 27
		10386	83	125	
	Nonspecific	268	30	9	
	binding	342	33	10	14 ± 4
		840	38	22	

Zona intact eggs and isolated zonae were incubated with [¹²⁵I]ligand (100000 cpm) for 3 h at 4 °C in H-KRB and washed with HNC-BSA. The radioactivity associated with eggs or zonae pellucidae were estimated as Table II. ^{a)} Zona pellucida.

binding of labeled-ligand to isolated zonae pellucidae. Both the specifically binding levels (total binding level minus nonspecific binding level) to isolated zonae and to eggs were identical (79 cpm per egg and per zona pellucida) (Table III).

Figure 4 shows a representative Scatchard plot of purified ligand binding level to unfertilized egg. [¹²⁵I]Ligand bound to egg at a concentration dependent manner in the presence or absence of MnCl₂ (20 mM) with a $K_a = 3.8 \times 10^{15}$

M⁻¹ and a binding capacity of 660000 molecules per egg.

Discussion

A variety of hydrolytic enzymes (*e.g.*, acrosin and *N*-acetylhexosaminidase) which have been identified in the acrosome are released during the acrosome reaction. Recognizing the possibility that the ligand molecule(s) might be

Fig. 4. Scatchard Plot of [¹²⁵I]Ligand Binding to Zona Intact Egg

Purified ligands were incubated with cumulus-free eggs for 3 h at 4 °C in the presence (●) or absence (○) of 20 mM of Mn²⁺ as described in Materials and Methods. After the incubation, the radioactivities associated with eggs were counted by γ -counter.

hydrolyzed by acrosin during the purification procedure, we added PMSF to the acrosome reaction-inducing medium. Shur *et al.*⁷⁾ indicated that by pretreatment of egg with *N*-acetylglucosaminidase, the sperm binding to egg zona pellucida was decreased. Accordingly, we assayed the *N*-acetylglucosaminidase activity in all fractions (Fig. 1). In DEAE-Sepharose CL-6B chromatography, sperm-egg binding inhibitory activities appeared in three peaks. The first peak contains *N*-acetylhexosaminidase activity. The second peak showed the most effective inhibition of sperm-egg binding but showed no *N*-acetylhexosaminidase activity. In this study, the second peak was carried out to a further purification. A highly active ligand was purified following repeated ion-exchange chromatography and gel filtration. Estimation of the molecular weight of the ligand molecule yielded values of 60000 and 67000 based upon gel filtration and SDS-PAGE, respectively (Figs. 2, 3). Furthermore, by high performance liquid chromatography on G3000SW column (Toyo Soda, Japan), the molecular weight was estimated at about 67000 (data not shown).

To determine a binding ability of purified ligand to egg zona pellucida, the ligand was radiolabeled by the lactoperoxidase method ($2 \times$

10^7 cpm per 20 ng of ligand). We found that [¹²⁵I]ligand bound to zona-intact eggs and to isolated-zonae pellucidae to a similar extent, but it bound to fertilized egg (2-cell) to some extent (Tables II, III). The extent of binding of ¹²⁵I-labeled ligand was concentration dependent (Fig. 4) and unlabeled ligand competed with radiolabeled ligand (Tables II, III). These results suggested that zona pellucida contain a finite number of binding sites for ligand. By assuming the molecular weight of ligand molecules as 67000, in Scatchard plot analysis, a maximal number of binding sites per egg zona pellucida was estimated *ca.* 660000 molecules (Fig. 4).

Bleil and Wassarman reported that ZP3, one of three zona pellucida glycoproteins, showed both a sperm receptor activity¹⁵⁾ and acrosome reaction-inducing activity.¹⁶⁾ Furthermore, they reported that radioiodolabeled-ZP3 bind to acrosome-intact sperm but not to acrosome-reacted sperm.¹⁷⁾ In other words, sperm surface receptor (ligand) is present on the surface of acrosome-intact sperm but not acrosome-reacted sperm.

On the other hand, Lopez *et al.* reported that sperm surface galactosyltransferase mediates sperm-egg binding.⁸⁾ Lopez and Shur showed that galactosyltransferase was retained during the

acrosome reaction on sperm surface, no galactosyltransferase activity was detected on plasma membrane vesicles released during the acrosome reaction.¹⁸⁾ These results suggested that ZP3 was not recognized by galactosyltransferase, but recognized by another molecule.

In this study, we purified one of the ligand molecules from mouse sperm. A binding level of labeled-ligand was not affected by the presence of 20 mM of Mn^{2+} (Fig. 4). However, Lopez *et al.* reported⁸⁾ that sperm-egg binding was inhibited by galactosyltransferase only in the presence of Mn^{2+} . These results suggested that we purified one of the ligand molecules which differs from galactosyltransferase.

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