Purification and Characterization of an Extracellular Fragment of the Sea Urchin Egg Receptor for Sperm

Kathleen R. Foltz and William J. Lennarz

State University of New York at Stony Brook, Department of Biochemistry and Cell Biology, Stony Brook, New York 11794-5215

Abstract. Fertilization in the sea urchin involves species-specific interaction between the ligand bindin on the surface of acrosome-reacted sperm and a receptor of high molecular weight on the surface of the egg. Efforts to understand this interaction and the resultant signal transduction events leading to egg activation have been limited because of the large size and extreme insolubility of the intact receptor on the egg surface. Earlier work suggested that an alternative strategy would be to isolate proteolytic fragments of the extracellular domain of this receptor. Consequently, we have treated *S. purpuratus* eggs with a specific protease, lysylendoproteinase C. This enzyme treatment abolished the ability of eggs to bind sperm and resulted in the release of proteolytic fragments that bound to sperm and showed inhibitory activity in a fertilization bioassay. One of these fragments, presumed to be a fragment of the extracellular domain of the receptor, was purified to homogeneity by gel filtration and anion exchange chromatography and shown to be a 70-kD glycosylated protein. Several lines of evidence support the contention that this fragment is derived from the receptor. First, the fragment inhibited fertilization species specifically. Second, species specific binding of the 70-kD glycoprotein to acrosome-reacted sperm was directly demonstrated by using ^{125}I-labeled receptor fragment. Third, the fragment exhibited the same species specificity in binding to isolated bindin particles. Species specificity was abolished by Pronase digestion of the fragment. This observation supports the hypothesis that although binding is mediated by the carbohydrate moieties, species specificity is dependent on the polypeptide backbone. The availability of a structurally defined fragment of the receptor will facilitate further studies of the molecular basis of gamete interaction.

ACCUMULATING evidence supports the idea that fertilization is initiated by a series of specific interactions between ligands on the surface of gametes (Wasserman, 1987; Yanagimachi, 1988; Ruiz-Bravo and Lennarz, 1989). In the sea urchin, fertilization is a species-specific event involving a ligand on the surface of the sperm (bindin) and a receptor on the surface of the egg (Schmell et al., 1977; Moy and Vacquier, 1979; Rossignol et al., 1984; Ruiz-Bravo and Lennarz, 1989). It has been hypothesized that upon sperm binding, the receptor transduces a signal, probably through a G protein-mediated pathway, that leads to a rise in internal calcium resulting in cortical granule exocytosis and other early events of egg activation (Whitaker and Irvine, 1984; Turner et al., 1986; 1987; Kline, 1988; Kline et al., 1988; Shilling et al., 1990). Clearly, an understanding of this species-specific receptor-ligand interaction and the resulting signal transduction events requires a detailed analysis of the structure-function relationship between the ligand and the receptor.

With respect to bindin, the ligand on the surface of the sperm, considerable information is available. Bindin is a 30.5-kD protein stored in the acrosomal vesicle that becomes localized to the surface of the acrosomal process upon activation of the sperm by egg jelly (Vacquier and Moy, 1977; Moy and Vacquier, 1979). Purified bindin binds to and agglutinates eggs species specifically (Glabe and Lennarz, 1979; Glabe et al., 1981). Bindin has been cloned, sequenced (Gao et al., 1986; Minor et al., 1989), and shown to have a strong affinity for sulfated, fucose-containing polysaccharides (Glabe et al., 1982; DeAngelis and Glabe, 1987). Studies have shown that binding facilitates the fusion of liposomes (Glabe, 1985; Kennedy et al., 1989), leading to the hypothesis that it may be involved in the actual fusion of the sperm and egg membranes. However, purified bindin does not activate unfertilized sea urchin eggs (Glabe et al., 1981), suggesting that the egg activation process requires more than a simple ligand-receptor interaction.

In contrast to bindin, relatively little is known about the egg surface receptor for sperm. Studies from this laboratory using eggs of *Strongylocentrotus purpuratus* and *Arbacia punctulata* provided the first evidence that a membrane-bound glycoconjugate on the egg surface functioned as a receptor (Schmell et al., 1977). Subsequently, the intact receptor of *S. purpuratus* was isolated and partially characterized (Rossignol et al., 1981; 1984). Using an in vitro bindin binding assay, it was determined that the receptor for bindin was a glycoconjugate of extraordinarily high molecular weight (>10^7) that exhibited some proteoglycan-like proper-

ties. Isolated receptor bound to sperm and inhibited fertilization species specifically. Pronase digestion resulted in a carbohydrate-rich fragment that bound sperm, but had lost species specificity. This finding led to the hypothesis that the carbohydrate chains are the adhesive element of the receptor and that the intact glycoconjugate is required for species-specific binding (Rossignol et al., 1984; Ruiz-Bravo and Lennarz, 1986; Ruiz-Bravo et al., 1986*b*).

Conclusions about the structure of the receptor were limited by the large size and extreme insolubility of the preparation. Even after refined purification (Ruiz-Bravo et al., 1986*a*), the receptor was not truly soluble, and NH_2-terminal sequence information could not be obtained. Polyclonal and monoclonal antibodies raised against the intact isolated receptor were directed toward the carbohydrate moieties (Ruiz-Bravo et al., 1986*b*, 1989; Ruiz-Bravo and Lennarz, 1989). The antibodies were used to localize the receptor to the surface of the egg plasma membrane and to determine that the receptor was synthesized during oogenesis. However, because they were directed against the carbohydrate they could not be used to screen expression libraries or to further define the structure of the receptor and its interaction with bindin.

An alternative approach to study the receptor was to isolate a biologically active fragment from the products of protease treatment of egg surfaces. Analyses of *S. purpuratus* egg surface trypsin digests revealed that soluble receptor fragments that bound sperm and prevented fertilization were produced (Ruiz-Bravo and Lennarz, 1986). The polypeptide content of these glycoconjugate fragments was a critical parameter in species specific inhibition of fertilization. Fragments retaining at least 30% polypeptide by weight retained species specificity in the bioassay; further reduction in the polypeptide content yielded material that was no longer species specific (Ruiz-Bravo and Lennarz, 1986). This observation lent further support to the hypothesis that some portion of the polypeptide backbone of the receptor was responsible for the species specificity of bioactivity, whereas the carbohydrate chains served as the adhesive element. Unfortunately, the tryptic digest was very heterogeneous and attempts to isolate a pure receptor fragment(s) met with limited success (Ruiz-Bravo and Lennarz, 1986, 1989).

We report here the purification of a biologically active fragment of the extracellular domain of the *S. purpuratus* egg receptor for sperm. After treatment of the egg surface with the specific protease, lysylendoproteinase C (LysC),[1] a 70-kD proteolytic fragment derived from the sperm receptor was purified to homogeneity. We report the biochemical and biological properties of this fragment. Its inhibitory properties and its ability to species specifically bind to acrosome-reacted sperm and to purified binding indicate that it is a fragment of the extracellular domain of the sperm receptor.

Materials and Methods

Isolation of Gametes and Preparation of LysC Digests

Adult *Strongylocentrotus purpuratus* and *Lytechinus pictus* were obtained from Marinus, Inc. (Long Beach, CA). Adult *Strongylocentrotus drobachiensis* were collected by Don Bryant (Bath, ME). Gametes were isolated by intracoelomic injection of 0.5 M KCl. Eggs were collected in millipore-filtered (22 μm) artificial sea water (FASW; Instant Ocean, Aquarium Systems, Mentor, OH), washed thrice at 14°C, and dejellied by passage through nitex (120 μm for *S. purpuratus*, 210 μm for *L. pictus*, and 250 μm for *S. drobachiensis*; Tekton, Inc., Elmsford, NY). Egg jelly was stored on ice before use in the bioassays. Dejellied eggs were washed three times in FASW and maintained as a 1% (vol/vol) solution in FASW at 14°C. Sperm were collected dry and stored on ice for up to 24 h.

For digestion of egg surfaces with lysylendoproteinase C (LysC; Boehringer Mannheim, Indianapolis, IN), dejellied, washed eggs were suspended as a 20% solution in FASW at room temperature. Based on preliminary experiments, we determined that the optimal condition for release of receptor fragments was 6 U of enzyme per 50 mL of packed eggs (200 ml total vol) for 15 min at 21°C. The digestion was stopped by the addition of 20 U of aprotonin (all chemicals and reagents were from Sigma Chemical Co., St. Louis, MO unless otherwise indicated) and the eggs were allowed to settle on ice. The supernatant was collected by aspiration, centrifuged at 10,000 rpm to remove debris, and concentrated 10-fold in an Amicon Corp. (Danvers, MA) ultraconcentrator using a P10 filter at 4°C. This concentrated, crude digest was the starting material for subsequent experiments described below.

Fertilization Bioassays

Fertilization bioassays were conducted essentially as described (Kinsey and Lennarz, 1981; Rossignol et al., 1984; Ruiz-Bravo and Lennarz, 1990). In all cases, parallel assays were conducted to control for buffer conditions. Briefly, sperm were diluted so that they were limiting and no more than 85–90% of the eggs would be fertilized in a control situation. To induce the acrosome reaction, an aliquot of diluted sperm was transferred to a tube containing egg jelly for 10–15 s. Then an aliquot of this sperm suspension was added to a 1% suspension of eggs containing buffer with or without the sample. In some instances, the sample was contained in the egg jelly solution such that the sperm were acrosome reacted in its presence (see Results). After 7 min at 14°C, the gametes were fixed by the addition of an equal volume of 2% glutaraldehyde. Fertilization was assessed by determining the percentage of eggs that exhibited elevated fertilization envelopes. Sperm binding to eggs was assessed by light microscopy (Kinsey and Lennarz, 1981). Duplicate samples were analyzed and 250–350 eggs were examined per duplicate. Values were normalized to control values obtained in FASW or buffer alone. The normalized values are expressed as the percentage of eggs fertilized or as the percentage of eggs failing to elevate fertilization envelopes (percent inhibition of fertilization).

Eggs treated with LysC as described above were washed three times and then assayed for their ability to be fertilized. Before this treatment, an aliquot of these eggs was removed and subjected to mock treatment without LysC. The percentage of eggs fertilized was normalized to this mock treatment value.

Purification of the 70-kD LysC Fragment

All steps of the purification were performed at 4°C unless noted otherwise. Samples were assayed for protein content by absorbance at 280 nm, carbohydrate content (see below), and for activity in the fertilization bioassay. As a first step, the concentrated LysC digest was chromatographed in a 1.5 × 40 cm column of Sepharose CL-2B-300 in 0.5 M NaCl, 10 mM Tris, pH 8 (nondissociating buffer). Usually, 5 mL of a 1.5-mg/mL digest was loaded on the column. The bioactive fractions (1.0 mL each) were pooled and dialyzed against 10 mM NaCl, 10 mM Tris, pH 8.

Gel filtration on Sepharose CL-2B-300 also was performed under dissociative conditions. The crude digest was dialyzed against 4 M guanidine-HCl, 0.5 M sodium acetate, pH 6.8, and then eluted with the same buffer. Fractions were dialyzed extensively against 10 mM NaCl, 10 mM Tris, pH 8, and then passed over a Bio-gel P-6 column (BIORAD, Rockville Center, NY) to remove any remaining guanidine-HCl. These fractions were assayed for bioactivity using a mock buffer sample that had been carried through the same procedures as the control.

After dialysis, the bioactive preparation was applied to DEAE-Sephacel; the column (1.75 × 12.5 cm) was washed with 10 vol of the same buffer. Initial, step-wise washing of the column revealed that a bound, bioactive component was eluted with 0.5 M NaCl, 10 mM Tris, pH 8. To purify this component, the proteins bound to the column were eluted with a gradient of 10 mM NaCl to 1 M NaCl in 10 mM Tris, pH 8 (200 ml of each). A 2 M NaCl final wash removed jelly coat contaminants. As the final step, the DEAE-purified bioactive sample was applied to a Sepharose CL-2B-300 column in either 0.5 M or 10 mM NaCl (no difference was observed)

in 10 mM Tris, pH 8, and the bioactive fractions were combined and lyophilized. The recovered material is referred to as the final purification product.

Apparent M_r determination of the DEAE-purified and final purification product was carried out using Sepharose CL-6B-200 in 0.5 M NaCl, 10 mM Tris, pH 8. After determining the positions of the LysC product and then the standards (carbonic anhydrase, albumin, alcohol dehydrogenase, β-amylase), the V_i was determined using ^{3}H-leucine and the V_o using Dextran blue 2000.

Sucrose gradient centrifugation was performed to confirm the coincidence of bioactivity with polypeptide and carbohydrate components. Briefly, 2 ml of crude LysC digest or the bioactive peak (V_o) from the nondissociating Sepharose CL-2B-300 column was layered on 25 ml of a 5–20% linear sucrose gradient in 0.5 M NaCl, 10 mM Tris, pH 8, and centrifuged for 18 h in a rotor (model SW28; Beckman Instruments, Inc., Palo Alto, CA) at 25,000 rpm. Fractions were collected and assayed for carbohydrate content and bioactivity as described above. Standards were catalase (11.3 S) and sea urchin sperm flagellar dynein (21 S; Gibbons and Fronk, 1979).

Protein Determination, Carbohydrate Analyses, and Sulfate Analysis

Protein concentration was determined by the method of Lowry et al. (1951) using BSA as the standard. 6-deoxy-hexose content was determined by the method of Dische (1947) using fucose and galactose as standards. Microanalysis of the sulfate and carbohydrate content of the purified 70-kD protein was performed by Dr. George Bousfield (University of Texas Cancer Center, M. D. Anderson Hospital). A CarboPac #76 column on a Dionex System was used for carbohydrate determination. For sulfate analysis, the sample was hydrolyzed and passed over a Dowex column to remove amino acids. The effluent was dried, dissolved in water, and chromatographed.

Purified 70-kD LysC fragment was subjected to treatment of Endoglycosidase H, Endoglycosidase F, and PNGase F essentially as suggested by the supplier (Boehringer Mannheim). Trifluoromethylsulfonic acid (TFMS; Aldrich Chemical Co., Milwaukee, WI) was used as described (Edge et al., 1981; Karp et al., 1982) in an attempt to remove O-linked carbohydrate moieties.

SDS-PAGE and Gel Purification of Proteins

SDS-PAGE was performed by the method of Laemmli (1970) using 8.75% acrylamide. After fixation in 50% methanol/0.1% acetic acid for at least 4 h, gels were rinsed in water for 15 min and incubated in DTT (10 μg per L) for 30 min. Gels were then stained by incubation in 0.1% silver nitrate for 30 min, briefly rinsed in water, and developed in sodium carbonate (30 g/L) and formaldehyde (500 μl of 37%/L). Development was stopped by immersing the gel in 2 M citrate.

For gel purification, reference lanes were stained in order to assess the positions of various polypeptides. Gels were incubated at room temperature in 0.5 M NaCl, 10 mM Tris, pH 8 for 5–7 h. Bands were excised and the gel was processed for silver staining for comparison to reference lanes. Excised bands were minced with a razor blade, frozen on dry ice, thawed, and 500 μl of buffer added. The sample was freeze-thawed a second time and spun through glass wool to collect the eluted protein. For controls, BSA and other proteins were excised along with blank portions of the gel.

Pronase Treatment

Preparations were treated with Pronase essentially as described (Ruiz-Bravo and Lennarz, 1986). Control digestions were Pronase alone and BSA treated with Pronase.

Iodination of the 70-kD Fragment and Sperm Binding Assays

Purified 70-kD LysC fragment was labeled with ^{125}I (Na salt; 100 mCi/mL; ICN, Costa Mesa, CA) using Iodogen (Pierce Chemical Co., Rockford, IL) as per the supplier's suggestions. Unincorporated ^{125}I was removed by gel filtration on Sephadex G-25 (medium) in 10 mM NaCl, 10 mM Tris, pH 8, and dialysis against the same buffer. The specific activity was 5×10^8 cpm/mg 70-kD protein. To confirm the labeling and to ensure the purity of the sample, a portion was electrophoresed and the gel subjected to autoradiography.

Sperm were diluted in FASW and an aliquot was then diluted into a 100-μl solution of buffer or of egg jelly containing increasing amounts of the labeled 70-kD fragment. After 2 min, the sperm were pelleted by centrifugation at 10,000 rpm in a microfuge. The supernatant was saved. Sperm were washed three times and the final pellet and supernatants were counted in a gamma counter (LKB Instruments, Inc., Gaithersburg, MD). Controls were buffer only, sperm only, and ^{125}I-labeled fragment alone. The results are reported as μg of ^{125}I-labeled 70-kD bound (i.e., pelleted) with the background subtracted.

Bindin was purified by established methods (Vacquier and Moy, 1977) and was tested for the ability to agglutinate eggs species specifically (Glabe and Lennarz, 1979, 1981). To assay directly for 70-kD protein binding to bindin, increasing amounts of the ^{125}I-labeled protein were incubated with bindin for 20 min at room temperature with gentle shaking (80 rpm). Triton X-100 was added to a final concentration of 1.5%, a 50-μl aliquot was layered in duplicate on a 30% sucrose cushion, and the samples were spun in a microfuge at 10,000 rpm for 10 min (Rossignol et al., 1984). The pellet of bindin was washed three times and counted, along with the supernatants. The controls were bindin alone and ^{125}I-labeled 70-kD protein alone.

Results

LysC Treatment Renders Eggs Unfertilizable

To obtain a fragment of the receptor, *S. purpuratus* eggs were treated with the specific protease LysC. As shown in Fig. 1, after 15 min this treatment resulted in the complete loss of the ability of the eggs to be fertilized by sperm. Three observations suggested that this loss of fertilizability was due to the inability of the eggs to bind sperm and not to an effect on gamete viability. First, 97% of the LysC–treated eggs were able to elevate fertilization envelopes upon treatment with the calcium ionophore A23187, indicating that the eggs were capable of being activated. Second, no sperm were observed by light microscopy to be associated with the egg surface (data not shown). Third, nearly 100% of the untreated eggs placed in the medium resulting from the final wash of treated eggs could be fertilized. From this last observation we concluded that there was no remaining protease or products of the treatment that would interfere with fertilization.

LysC Treatment Releases Fragments That Inhibit Fertilization

The mixture of LysC-generated egg surface proteolytic fragments was concentrated and tested for bioactivity. When incubated with either acrosome-reacted sperm, or with eggs upon the addition of acrosome-reacted sperm, this mixture inhibited fertilization in a concentration-dependent manner. As shown in Fig. 2, this inhibitory activity was species specific; the crude mixture did not inhibit fertilization of either *Lytechinus pictus* or *Strongylocentrotus drobachiensis* eggs.

Several observations eliminated the possibility that the inhibition of fertilization was due to toxic effects. First, eggs incubated with the crude fragments released by LysC treatment and then washed three times could be fertilized normally. Second, eggs could be activated by the addition of the calcium ionophore A23187 in the presence of the fragments. Third, sperm that were not acrosome reacted that were incubated with the preparation and then washed were able to fertilize eggs upon induction of the acrosome reaction by egg jelly or calcium ionophore. Dialysis against FASW or distilled water did not abolish the bioactivity of the mixture. The inhibitory activity was stable; it did not decrease over a period of 1 mo on ice.

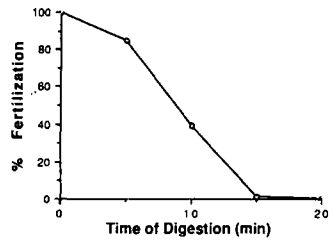

Figure 1. LysC treatment of eggs prevents fertilization. Washed, dejellied *S. purpuratus* eggs were treated with LysC and at various times an aliquot was removed, the inhibitor aprotonin was added, and the eggs were washed three times. Acrosome-reacted sperm were then added and after 5 min the gametes were fixed and the eggs assayed for fertilization by the presence of elevated fertilization envelopes.

Purification of Receptor Fragments

Based on the above results, we undertook to enrich for and purify the proteolytic fragment(s) that were presumed to bind to sperm in a species specific manner. First, the crude mixture was fractionated on Sepharose CL-2B-300 in a nondissociating buffer. Approximately 70–80% of the fertilization-inhibiting activity eluted from the column in the excluded volume. A minor peak of bioactivity was detected in the partially included fractions. Sucrose gradient centrifugation of the crude LysC digest and of the V_o peak from the Sepharose CL-2B-300 column in nondissociating buffer also resulted in the cosedimentation of bioactivity with a carbohydrate- and protein-containing peak. The calculated S-value of the bioactive fractions was large (>21 S), correlating with the observed behavior of the bioactivity on the gel filtration column in the nondissociating buffer (data not shown).

In contrast, gel filtration on Sepharose CL-2B-300 under dissociative conditions (4 M guanidine-HCl, 0.5 M Na Acetate, pH 6.8), revealed that the majority of the bioactivity was not in V_o (Fig. 3 *A*), but was included and eluted in fractions 24–28 (Fig. 3 *B*). This observation suggested that before treatment with the dissociative buffer the bioactivity was a component of a high M_r aggregate; this supposition was later confirmed (see below). The fractions (24–28) from the gel filtration column containing inhibitory activity were pooled, dialyzed, applied to DEAE Sephacel, and eluted with a salt gradient. All of the detectable bioactivity eluted in a single peak coincident with a peak of protein and carbohydrate at ~200 mM NaCl (Fig. 4).

We found that dialysis and subsequent DEAE chromatography of the bioactive excluded fraction obtained under nondissociative conditions also resulted in a single peak of bioactivity at 200 mM NaCl but the yield was considerably reduced. When this material was rechromatographed on a Sepharose CL-2B-300 column it eluted in the included volume (i.e., was no longer eluted in the V_o as an aggregate; data not shown).

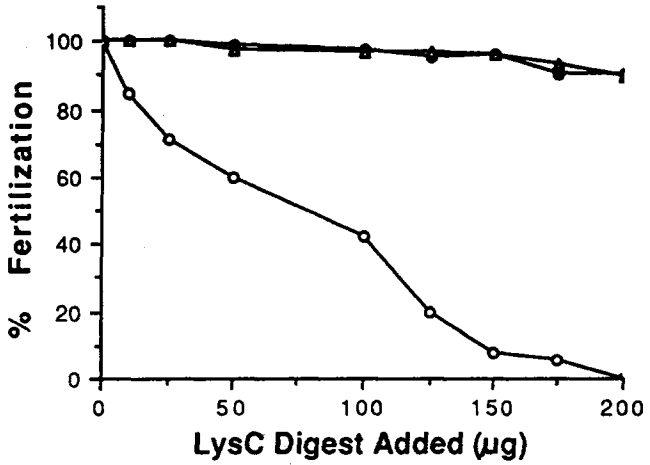

Figure 2. The LysC digest species specifically inhibits fertilization. Acrosome-reacted sperm were added to dejellied eggs of *S. purpuratus* (○–○), *L. pictus* (△–△), and *S. drobachiensis* (●–●) in the presence of increasing amounts of the crude LysC digest. Eggs were scored for fertilization by the presence of elevated fertilization envelopes.

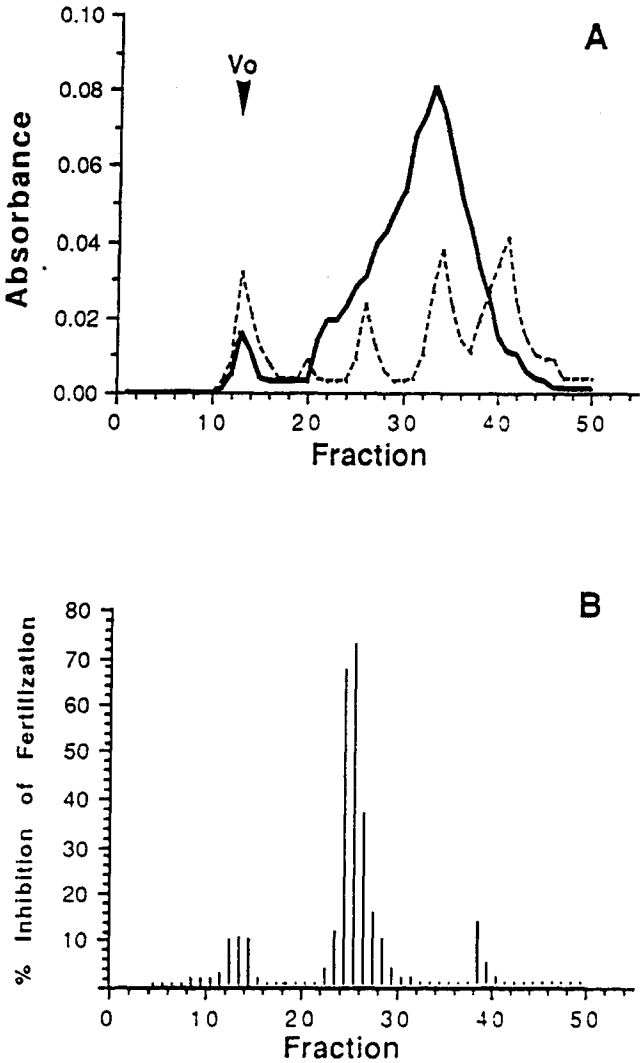

Figure 3. Fractionation of the LysC digest by gel filtration chromatography under dissociative conditions. Concentrated crude LysC digest was applied to a Sepharose CL-2B-300 column in dissociative buffer. Fractions were collected and assayed for protein content by absorbance at 280 nm (—) and 6-deoxy-hexose content by absorbance at 396 and 427 nm (---) expressed as the difference between the two readings (*A*). The void volume (V_o) is indicated. Fractions were also assayed for the ability to inhibit fertilization (*B*).

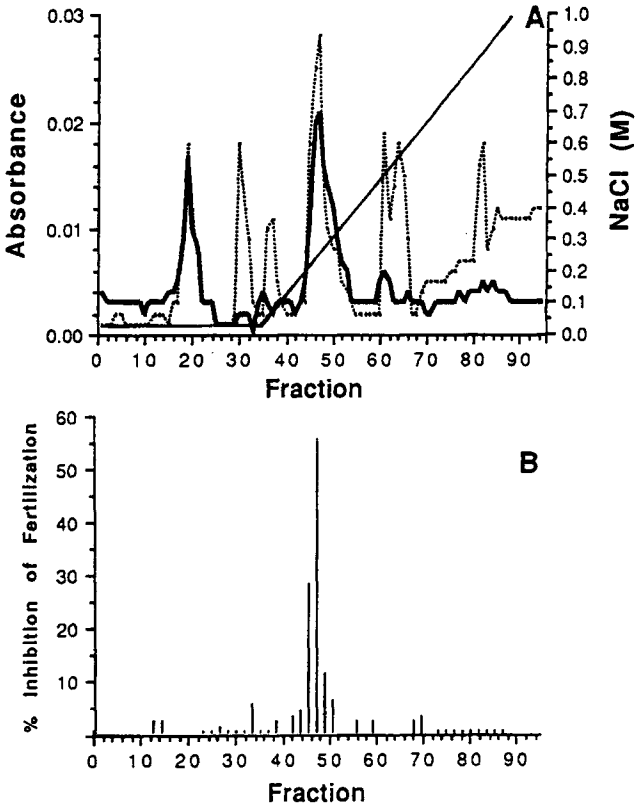

Figure 4. Purification of the 70-kD protein by DEAE chromatography. Bioactive fractions from the Sepharose CL-2B-300 column (Fig. 3 *B*, fractions 24–28) were pooled, dialyzed, and applied to a DEAE-Sephacel column. Fractions were eluted with a salt gradient (—) and assayed for protein (——) and 6-deoxy-hexose (···) content (*A*), as well as for the ability to inhibit fertilization in the bioassay (*B*).

A summary of the purification and recovery of protein and bioactivity is presented in Table I. Purification was 95-fold on average. When the various preparations were assayed by SDS-PAGE, it was observed that the amount of a 70-kD protein increased in proportion to the increase in specific activity. Shown in Fig. 5 is a silver-stained SDS polyacrylamide gel of the various preparations. After fractionation on DEAE Sephacel, the inhibitory fraction contained a single detectable protein (Fig. 5, lane *10*). When the purified fragment was subjected to gel filtration on a calibrated column of Sepharose CL-6B-200, it eluted in a single peak; the estimated size was judged to be ~100 rather than 70 kD. This

Table I. Purification of the 70-kD Receptor Fragment

Step	Inhibitory activity*	Fold purification‡	Protein	Recovery of activity
			mg	%
Crude	97.0	–	50.1	–
Sepharose CL-2B-300	7.8	12.4	3.4	85
DEAE	0.84	115	0.36	83

Data are given for a representative experiment using dissociative conditions for gel filtration.
* Inhibitory activity is defined as the amount of protein (μg) required to achieve 50% inhibition of fertilization in the bioassay.
‡ The fold purification of activity ranged from 50 to 134 for 18 experiments.

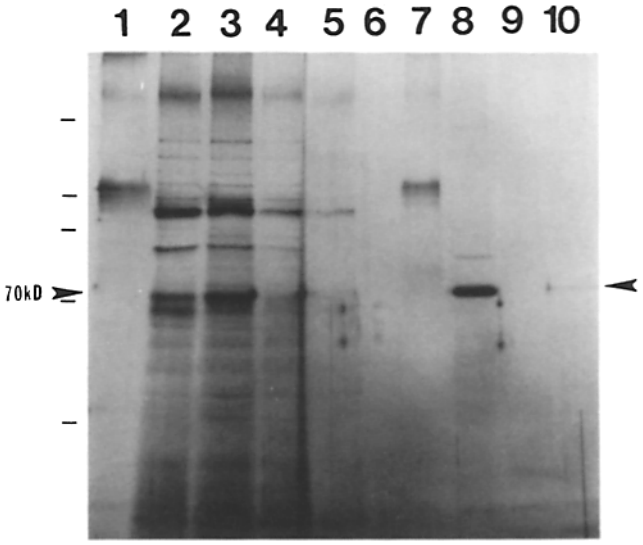

Figure 5. SDS-PAGE of LysC preparations. Samples were electrophoresed and the gel was stained with silver. Molecular mass standards (205, 116, 97.4, 66.2, and 45 kD) are indicated by bars. The 70-kD protein is denoted. Lanes *1–5* correspond to pooled fractions from the Sepharose CL-2B-300 column (see Fig. 3 *A*) and are: (lane *1*) void volume; (lane *2*) fractions 20–23; (lane *3*) bioactive fractions 24–28; (lane *4*) fractions 29–33; and (lane *5*) fractions 33–37. Lane *6* is blank. Lanes *7–10* correspond to the anion exchange steps (see Fig. 4 *A*), and are (lane *7*) the unbound fractions; (lane *8*) bound material eluted by a 0.5 M NaCl step wash; (lane *9*) blank; and (lane *10*) the purified bioactive peak obtained by elution with the salt gradient (see Materials and Methods).

peak contained a single polypeptide that was of M_r 70 kD by SDS-PAGE. This discrepancy most likely is due to the carbohydrate content of the fragment (see below and Discussion). Analysis of the 70-kD protein by reverse phase HPLC resulted in a single protein peak, indicating the purity of the preparation. Amino acid sequence analysis (Dr. Tom Fischer, Center for Analysis and Synthesis of Macromolecules, SUNY at Stony Brook) revealed a blocked NH_2-terminus. Cyanogen bromide digestion of the 70-kD protein resulted in three distinct cleavage products (data not shown) in agreement with amino acid composition analysis (Dr. George Bousfield, University of Texas Cancer Center, M. D. Anderson Hospital), indicating the presence of two to three methionine residues. In addition, a polyclonal antibody prepared against the purified 70-kD LysC fragment was found to specifically immunoprecipitate the 70-kD protein from crude LysC digests (data not shown).

The 70-kD Proteolytic Fragment Is a Sulfated Glycoprotein

The purified 70-kD proteolytic fragment was analyzed for carbohydrate and fucose content by Dr. George Bousfield. Fucose, mannose, galactose, and galactosamine were found to be the major sugars. The fragment also contained sulfate, but no sialic acid. The negative charge conferred by the sulfate may explain the ability of the fragment to bind the anion exchange resin, while the existence of carbohydrate side chains may explain the discrepancy between the estimated M_r by SDS-PAGE (70 kD) and gel filtration (100 kD). It should be noted that the glycoconjugate was resistant to

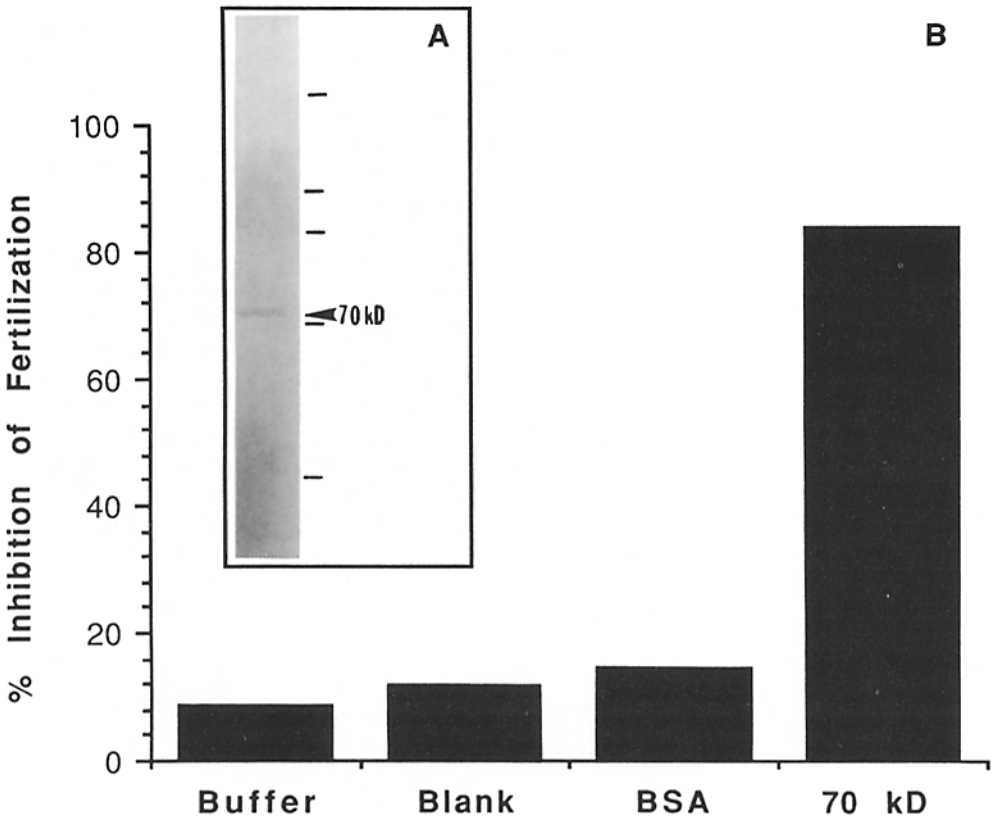

Figure 6. Inhibition of fertilization by the gel-purified 70-kD glycoprotein. Proteins were electrophoresed on an SDS polyacrylamide gel and the bands excised and tested for their ability to inhibit fertilization. *A* shows a duplicate sample of purified 70-kD fragment that was excised from an adjacent lane. Molecular mass standards are denoted and are the same as in Fig. 5. *B* shows the inhibitory activity of various samples compared to the buffer control. Approximately 50 μg of BSA and 15 μg of the LysC 70-kD protein were recovered from the gel and used in the fertilization bioassay. The blank is a region of the polyacrylamide gel that contained no protein.

digestion by Endo H, Endo F, and PNGase F; this resistance may be due to either the presence of sulfate on N-linked oligosaccharide chains or because the chains are not N-linked. TFMS treatment of the fragment resulted in a defined shift in M_r from 70 to 41 kD on SDS-PAGE, suggesting the presence of O-linked oligosaccharide chains. This reduction in M_r was also observed upon treatment with 70% formic acid and 1% trifluoroacetic acid as well. Since acid treatment did not result in the production of a new amino terminus (see above), we concluded that the M_r difference was due to removal of oligosaccharides or other acid-labile moieties and not to acidic cleavage of a peptide bond(s).

The 70-kD Glycoprotein Fragment Species Specifically Inhibits Fertilization

We established that the 70-kD glycoprotein was the inhibitory component by recovering it from SDS polyacrylamide gels and demonstrating that it inhibited fertilization, whereas blank regions of the gel and other gel-purified proteins did not (Fig. 6). A second proteolytic fragment of $\sim M_r$ 180 kD, contained in the crude digest and the initial gel filtration step, also inhibited fertilization. This is not surprising since it might be expected that more than one fragment would be generated by treatment of the surface receptor with LysC.

In the purification scheme, fractions were assayed for inhibitory activity at every step and the active fractions tested for species specific activity. In all cases, the bioactive fraction were species specific; i.e., they inhibited fertilization of *S. purpuratus* but not *S. drobachiensis* or *L. pictus* eggs. As shown in Fig. 7 *A*, the purified 70-kD glycoprotein fragment exhibited species specific inhibitory activity. To test the hypothesis that the polypeptide component of the receptor fragment confers species specificity (Ruiz-Brazo and Lennarz, 1986, 1989*b*), the 70-kD protein was digested extensively with Pronase. The resulting digest lost species specificity (Fig. 7 *B*).

The purified 70-kD protein was tested for gamete toxicity in the bioassays by the criteria outlined above for the initial LysC digest and found to be nontoxic. Several observations excluded the possibility that the 70-kD was a component of the jelly coat. First, DEAE chromatography removed contaminating jelly coat (see Materials and Methods). Second, none of the LysC preparations were found to induce the acrosome reaction, as would be expected of jelly coat. Third, sperm that had not been acrosome reacted did not bind the 70-kD protein (see Fig. 8 *B* and below).

The 70-kD Glycoprotein Binds Acrosome-reacted Sperm and Bindin

The above results implied that the 70-kD glycoprotein fragment inhibited fertilization by binding competitively to sperm. To directly test this idea, ^{125}I-labeled 70-kD fragment was prepared. The radiolabeled, purified 70-kD glycoprotein bound only to acrosome-reacted sperm (Fig. 8 *A*); binding to nonreacted sperm was negligible. A sizable percentage (70%) of the fragment bound and this binding was species specific, i.e., the *S. purpuratus* 70-kD receptor fragment did not bind to *L. pictus* acrosome-reacted sperm (Fig. 8 *B*).

We also tested the ability of the labeled 70-kD glycoprotein to bind purified bindin particles from *S. purpuratus*, *S. drobachiensis*, and *L. pictus*. The fragment bound to and

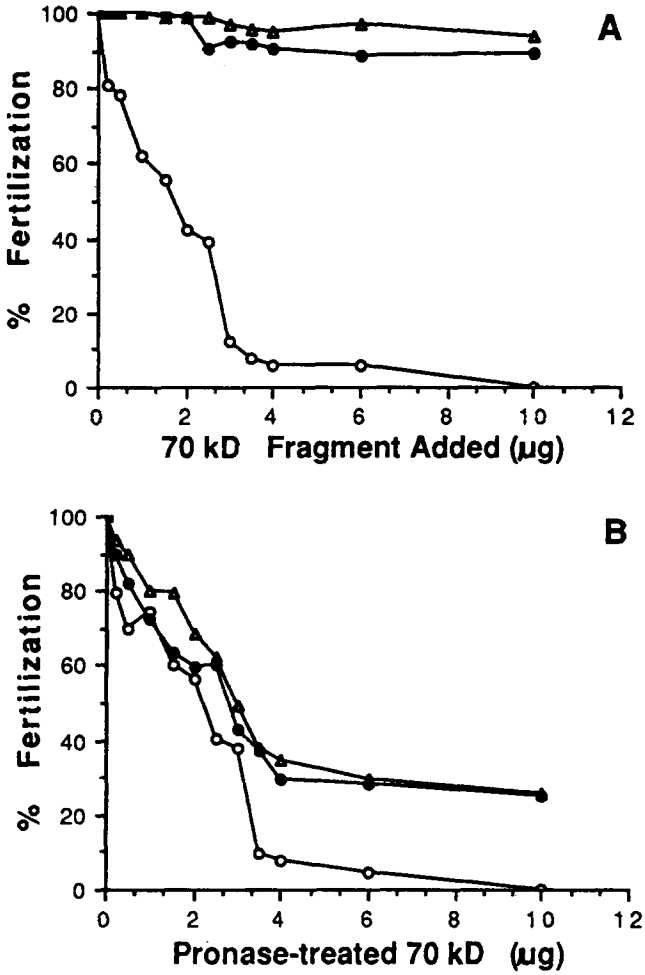

Figure 7. Species specificity of the purified 70-kD glycoprotein. Sperm were added to dejellied eggs of *S. purpuratus* (○–○), *L. pictus* (△–△), and and *S. drobachiensis* (●–●) in the presence of increasing amounts of the purified 70-kD fragment isolated from *S. purpuratus* LysC digests (*A*) or an equivalent amount of Pronase-digested fragment (*B*).

sedimented preferentially with *S. purpuratus* bindin, although significant binding to the bindin from the other two species could be detected; this binding was concentration dependent (Fig. 9) and was inhibited by addition of an excess of unlabeled fragment (data not shown).

Discussion

Previous work has established that the sperm receptor is a cell surface glycoconjugate of high molecular weight associated with the vitelline layer-plasma membrane complex (Schmell et al., 1977; Rossignol et al., 1984; Ruiz-Bravo et al., 1986*a*). The large size and extreme insolubility of the receptor precluded obtaining the structural information necessary to elucidate the molecular basis of species-specific binding and resultant signal transduction events. Trypsin treatment of egg surfaces resulted in the release of soluble, bioactive receptor fragments (Ruiz-Bravo and Lennarz, 1986) that retained species specificity, but these were heterogeneous and could be recovered only in low yield (Ruiz-Bravo and Lennarz, 1986, 1989).

We have overcome these problems by using a more specific protease, LysC, and gel filtration under dissociating conditions, followed by ion exchange chromatography, to obtain a homogeneous glycoprotein in high yield. The purity of this preparation was demonstrated by the following. (*a*) The active fraction purified by gel filtration and ion exchange chromatography contained a single protein of $\sim M_r$ 70 kD as judged by silver stain SDS polyacrylamide gel analyses. (*b*) Reverse phase HPLC revealed a single peak of protein containing the 70-kD protein. (*c*) Gel-purified 70-kD protein was active in the fertilization bioassay. (*d*) CNBr treatment of the 70-kD protein yielded only three peptides. Several lines of evidence indicate that this 70-kD glycoprotein is a proteolytic fragment of the extracellular domain of the sperm receptor of *S. purpuratus* eggs. Treatment of eggs with LysC abolished their ability to bind sperm and to be fertilized. One of the more predominant fragments released by this treatment was the 70-kD protein. This fragment was shown to inhibit fertilization species specifically. The glyco-

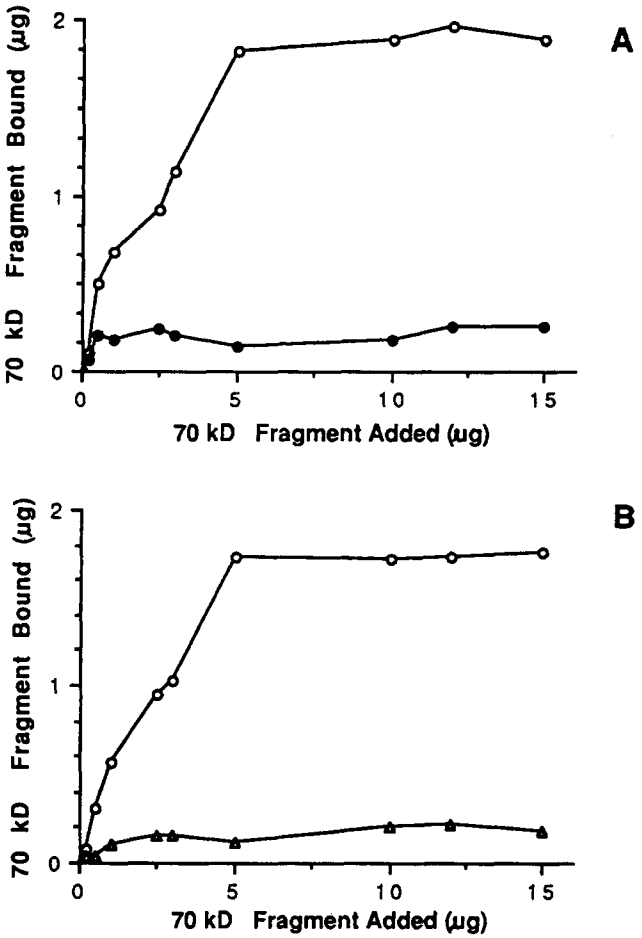

Figure 8. The 70-kD glycoprotein binds species specifically to acrosome-reacted sperm. Increasing amounts of purified, radiolabeled 70-kD fragment were incubated with nonacrosome-reacted (●–●) or acrosome-reacted (○–○) *S. purpuratus* sperm (*A*). Sperm were washed three times and counted. Alternatively, acrosome-reacted *S. purpuratus* (○–○) or *L. pictus* sperm (△–△) were incubated with increasing amounts of labeled fragment (*B*) and the level of labeled protein bound was determined.

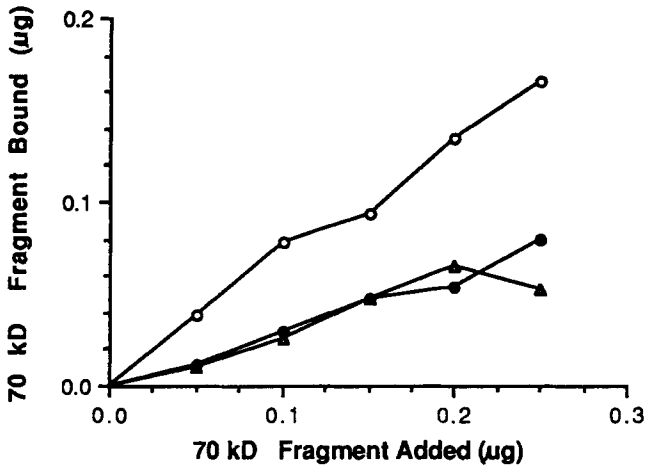

Figure 9. The purified 70-kD glycoprotein binds species specifically to purified bindin. Increasing amounts of purified, radiolabeled 70-kD glycoprotein were incubated with 50 μg of purified bindin particles from *S. purpuratus* (○–○), *L. pictus* (△–△), or *S. drobachiensis* (●–●) sperm. The amount of labeled protein bound was determined.

protein contained fucose, mannose, galactose, galactosamine, and sulfate. Use of labeled 70-kD protein provided direct proof that this fragment inhibited fertilization by binding competitively to sperm. ^{125}I-labeled glycoprotein bound only to acrosome-reacted sperm and to isolated bindin from *S. purpuratus*; it did not bind to sperm from other sea urchin species. Glabe and Lennarz (1979) demonstrated that purified bindin showed preferential binding to homotypic eggs although the level of specificity was much lower than that exhibited in the binding of acrosome-reacted sperm to eggs. Our results with purified bindin are in agreement with these earlier findings. Thus, the *S. purpuratus* 70-kD protein showed a preference for purified bindin from *S. purpuratus*, but also bound to bindin from *L. pictus* and *S. drobachiensis*. In comparison, binding of the purified 70-kD receptor fragment to acrosome-reacted sperm was highly species specific even at high concentrations.

In agreement with the earlier finding that glycoconjugates of high molecular weight were generated by trypsin treatment (Ruiz-Bravo and Lennarz, 1986), gel filtration of the LysC digest yielded biologically active fragments of molecular weight $>2 \times 10^6$. However, when this digest was treated with a chaotropic, dissociating buffer and subjected to purification, one component of the biologically active material was the 70-kD glycoprotein that could be separated from the high molecular mass aggregates. In view of these results, it is likely that the tryptic fragments generated in these earlier studies were components of high molecular weight aggregates (Ruiz-Bravo and Lennarz, 1986).

As noted above, carbohydrate analyses of the 70-kD bioactive protein revealed the presence of mannose, galactose, fucose, galactosamine, and sulfate. The intact receptor contained a higher percentage of hexosamines and iduronic acid along with sulfate (Rossignol et al., 1984). It is reasonable to assume that since the 70-kD glycoprotein represents only a small portion of the intact receptor ($M_r > 10^7$), it would not necessarily exhibit the same carbohydrate composition. Indeed, the 70-kD fragment may represent only one of potentially several domains for sperm binding on the receptor and not all of the carbohydrate content of the 70-kD fragment may be involved in binding. Glabe and Vacquier (1978) reported a receptor-like fragment of similar carbohydrate composition from egg surfaces. Although we do not yet know the structure of the polysaccharide chains on the 70-kD protein, the presence of fucose and sulfate in the receptor is particularly interesting because bindin, the ligand on the sperm surface, has been shown to have a high affinity for sulfated fucose moieties (Glabe et al., 1982; DeAngelis and Glabe, 1987).

Evidence has been presented that the carbohydrate component of the receptor is responsible for the sperm adhesiveness to the egg surface and that the polypeptide core confers the species specificity of the adhesion process (Ruiz-Bravo and Lennarz, 1986). In support of this hypothesis, we found that although the 70-kD glycoprotein exhibited species specificity in inhibiting fertilization and in binding to sperm and bindin, this specificity was lost upon Pronase treatment. A similar situation has been described for the mouse egg sperm receptor, ZP3. The carbohydrate chains of ZP3 are responsible for sperm binding, but the polypeptide core plays the role of species specifically inducing the acrosome reaction (Bleil and Wasserman, 1983). Thus, the two-part binding function of egg sperm receptors may be a common feature of gamete interactions.

Preliminary investigations indicate that it should be possible to use this approach to isolate sperm receptor fragments from other species. Treatment with LysC abolished the ability of *S. drobachiensis* and *L. pictus* eggs to bind sperm and resulted in a mixture of proteolytic fragments that inhibited fertilization. As in the case of *S. purpuratus*, this inhibitory activity was species specific and this specificity was destroyed by Pronase treatment.

The isolation of a homogeneous glycoprotein fragment of the *S. purpuratus* egg receptor for sperm should enable us to clone and sequence this domain of the receptor. This in turn will open the door to studies on the nature of species-specific gamete interaction, as well as the structure and function of the intact receptor and its possible role in the signal transduction process that culminates in fertilization.

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