

Isolation of a Glycopeptide Fraction from the Surface of the Sea Urchin Egg that Inhibits Sperm-Egg Binding and Fertilization

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ABSTRACT The role of cell surface glycoproteins of the sea urchin egg in binding sperm has been examined by studying the biological activity of glycopeptides derived from these glycoproteins. Glycopeptides were produced from egg surface glycoproteins by Pronase digestion. After fractionation by gel filtration the glycopeptides were tested for their ability to inhibit the binding of sperm to eggs, presumably by competing with the egg surface glycoproteins for binding sites on the sperm. One glycopeptide fraction with an apparent molecular weight of ~6,000 was found to be a potent inhibitor of sperm-egg binding, as well as fertilization, even at nanomolar concentrations. This activity was heat stable and exerted its effect against the sperm and not the egg. Experiments with a radiolabeled form of the glycopeptide fraction directly demonstrated that at least one component of it bound to sperm. Specific binding of the radiolabeled glycopeptide occurred only to acrosome-reacted sperm. Because the isolated glycopeptide fraction has many of the characteristics that one would expect of a biologically active fragment of an egg surface receptor for sperm, these findings are consistent with the idea that one or more glycoconjugates on the surface of the egg are involved in sperm binding.

The sea urchin fertilization system has long been the subject of studies concerned with the specificity of cell-cell interactions (for a review, see Monroy [19]). It is well established that fertilization involves a specific adhesion of the acrosomal process of the sperm to the egg surface and that this interaction contributes to the species-specific fertilization observed in many but not all of these animals (13, 22, 23, 30). The high degree of specificity that characterizes this adhesive process, coupled with the observations that pretreatment of the egg with proteases or certain lectins results in loss of sperm binding capacity, has led to the hypothesis that sperm bind to eggs through the interaction between a receptor for sperm on the egg surface and a complementary binding molecule on the sperm acrosomal process (2, 6, 11, 15, 21, 29, 31). In its simplest form, this hypothesis would imply that both the adhesive strength and the specificity of the binding process reside in the interaction between these two molecules.

Evidence that would afford direct confirmation of this hypothesis has been very difficult to obtain. With respect to the adhesive molecule associated with sperm, Vacquier and co-

workers (3, 10, 28) have isolated a protein, bindin, that has many of the properties expected of the sperm component of such an adhesion system: (a) bindin is localized to the acrosomal process of the sperm; (b) it binds to eggs; and (c) it agglutinates eggs with some degree of species specificity. However, the fact that bindin forms large aggregates in aqueous solution has complicated efforts to demonstrate that it can compete with sperm for the receptor at the egg surface. In separate studies, Aketa and co-workers (1) have isolated, from sperm, large TCA-soluble polysaccharides that both agglutinate eggs and inhibit fertilization in a species-specific manner.¹

Several groups have attempted to isolate a component from the surface of the egg that binds to sperm or to bindin isolated from sperm. Previous work in this laboratory resulted in the solubilization of a receptorlike activity from egg membranes

¹ We have confirmed the existence of such polysaccharides but have been unable to demonstrate that they inhibit fertilization species specifically in *S. purpuratus* and *A. punctulata* (W. Kinsey, J. Rubin, and W. Lennarz, unpublished observations).

(21). Although this preparation could inhibit fertilization species specifically by binding to sperm, all attempts at purification failed because of the extreme lability of the inhibitory activity. Tsuzuki et al. (26) have also solubilized a receptorlike activity from sea urchin eggs by urea extraction. Although the crude preparation inhibits fertilization species specifically, this putative sperm receptor has not been obtained in a purified form that is biologically active. Glabe and Vacquier (11) obtained a high molecular weight glycoprotein fraction that was released from eggs upon fertilization and demonstrated that this material would bind to bindin. Some degree of species specificity was observed in this preparation; however, direct participation of this glycoprotein fraction in sperm-egg adhesion or bindin-mediated egg agglutination was not established. In addition to these studies on sea urchin gamete interaction, Bolwell et al. (5) have isolated a glycoprotein from *Fucus* (brown algae) eggs that inhibits fertilization species specifically. Similarly, Bleil and Wasserman (4) have isolated a glycoprotein from the mouse egg that inhibits fertilization, but specific binding of this molecule to the sperm has not yet been demonstrated.

One major problem with studies that use inhibition of fertilization to measure the activity of isolated preparations of the putative sperm receptor is that effects of such preparations on sperm-egg adhesion cannot be distinguished from effects on the acrosome reaction or on fusion of the two gametes. In fact, it is now apparent that agents that either induce or prevent the acrosome reaction can, under certain conditions, inhibit fertilization species specifically (14, 25, 27). In one approach to circumventing this difficulty, Glabe and Lennarz (9) have evaluated the ability of various cell surface components to inhibit bindin-mediated egg agglutination. This study demonstrated that glycopeptides released from the surface of *Strongylocentrotus purpuratus* eggs by Pronase treatment were effective inhibitors of egg agglutination, although the effect was not species specific. However, the fact that an end product of exhaustive proteolysis exhibited this biological activity was consistent with the possibility that sperm-egg adhesion occurs through the interaction of the carbohydrate side chain of the egg surface receptor for sperm with a lectinlike molecule on the sperm. Indeed, Vacquier and Moy (29) have presented evidence that bindin may have lectinlike properties.

In the present study, we have prepared and fractionated the peptides released from the surface of *Arbacia punctulata* eggs by digestion with Pronase. To obviate the problems with the fertilization assay discussed above, we assayed these fragments for receptor activity by measuring their ability to inhibit the binding to eggs of sperm that had been preinduced to undergo the acrosome reaction (13). Of the peptides examined, one glycopeptide fraction was found to inhibit the binding of sperm to eggs. This fraction has several of the properties that would be expected of a biologically active fragment of the sperm receptor.

MATERIALS AND METHODS

Preparation of Cell Surface Glycopeptides

A. punctulata were purchased and maintained as previously described (21). Eggs were collected by electric shock and suspended in artificial seawater (Instant Ocean, Aquarium Systems, Mentor, Ohio). To remove the jelly coat, a 10% (vol/vol) suspension of eggs was titrated to pH 5.5 with HCl, the eggs were allowed to settle, and the supernate was removed. This procedure was repeated once to remove as much jelly coat as possible. The de-jellied eggs were then washed twice with 10 vol of artificial seawater buffered to pH 8.0 with 10 mM Tris-HCl (TBSW) and finally adjusted to a 50% (vol/vol) suspension with TBSW.

Pronase (B grade, Calbiochem-Behring Corp., LaJolla, Calif.) was dissolved

in TBSW at a concentration of 1.0 mg/ml and preincubated for 30 min at 50°C to destroy any glycosidases. After cooling, this solution was added to 2 vol of a 50% (vol/vol) suspension of eggs and the mixture was incubated at 15°C with gentle stirring for 10–15 min. The treatment was terminated immediately if any sign of egg lysis (pigment release) became apparent. At the end of the treatment, the eggs were removed by centrifugation at 500 g and the supernate was then centrifuged in a Sorvall SS-34 rotor (DuPont Co., Wilmington, Del.) at 10,000 rpm for 20 min. The resulting clear supernate was transferred to a sealed container, a drop of toluene was added, and the mixture was digested at 50°C for 24 h. At the end of this period, additional predigested Pronase was added (to make the final concentration of Pronase 0.43 mg/ml) and the mixture was digested for an additional 24 h. At the end of this digestion, the mixture was boiled for 10 min to destroy the protease activity and then lyophilized.² The dried powder was dissolved in a minimum volume of distilled water, and insoluble material was removed by centrifugation in a Sorvall SS-34 rotor at 10,000 rpm for 30 min.

Gel Filtration

In cases where the biological activity of the fractions was to be measured directly, gel filtration was performed in columns (2.5 × 95 cm) of Bio-Gel P-4 or P-10 (200–400 mesh; Bio-Rad Laboratories, Richmond, Calif.) equilibrated and eluted with Millipore-filtered artificial seawater, pH 7.8–8.0. For preparative purposes, gel filtration columns were equilibrated and eluted with 0.1 M ammonium acetate, pH 7.5. The peptide content of the eluted fractions was estimated by measuring the absorbance at 280 nm or by the difference in absorbance at 215 and 225 nm. Neutral hexoses were determined by the phenol-sulfuric acid assay (18). Hexosamines were determined by the Svennerholm method (24). Sialic acids were determined by the periodate-resorcinol assay (12). Nucleic acids were estimated from the ratio of absorbance at 260 and 280 nm. Uronic acids were determined by the carbazole method (8). Protein was estimated by the method of Lowry et al. (16) or by the difference in absorbance at 215 and 225 nm using bovine serum albumin as a standard. The apparent molecular weight of the glycopeptide was estimated on a Bio-Gel P-10 using the following standards: α -lactalbumin, 10,000 daltons; cytochrome c, 10,000; cyanogen bromide fragment 1 of α -lactalbumin, 3,900; ovalbumin glycopeptide, 1,500.

Bioassays

To quantitate the binding of sperm to eggs, a modification of the assay by Vacquier and Payne (30) that has been described in detail in a previous publication (13) was used. In this procedure, sperm are first induced to undergo the acrosome reaction and then assayed for their ability to bind to eggs. Thus, possible inhibitory effects of a test substance on the acrosome reaction (a prerequisite to sperm binding) are avoided. Putative receptor fragments were assayed for their ability to inhibit sperm egg binding by adding the glycopeptide solution to the egg suspension before the addition of sperm. A final volume of 300 μ l was maintained. Sperm binding was then measured as previously described (13).

To test the effect of the putative receptor fragment on fertilization, the highly sensitive fertilization assay described by Schmel et al. (21) was used. As in the above assay, glycopeptide solutions were added to the egg suspension before the addition of sperm.

To study the effect of the glycopeptide on jelly-coat induction of the acrosome reaction, sperm (200 μ g of protein) were added to 100 μ l of TBSW containing 60 mM CaCl₂ with or without glycopeptide at a concentration of 3.3 μ g/ml (the highest concentration used in the sperm binding assays). After 30 s, 100 μ l of TBSW or 100 μ l of TBSW containing jelly coat (50 nmol fucose equivalents) was added. After 30 s, the sperm were fixed with glutaraldehyde and the percent of sperm that underwent the acrosome reaction was assessed as previously described (7).

The partially purified glycopeptide was radiolabeled by the reductive alkylation procedure of Means and Feeney (17). The glycopeptides were dissolved in 0.5 ml of 0.2 M sodium bicarbonate, pH 9.0, and 100 mCi of sodium borotritide (9.8 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) dissolved in 0.5 ml of the bicarbonate buffer was added. This was then followed by addition of four successive 50- μ l aliquots of 0.37% formaldehyde as described (17). After a 1-h incubation in ice, the mixture was dialyzed against distilled water and chromatographed on Bio-Gel P-10.

Binding of Radiolabeled Glycopeptides to Sperm

Binding of ³H-glycopeptides to sperm was determined by a filter assay. TBSW

² Omission of the heat treatment step had no effect on the elution properties of the inhibitory glycopeptide isolated by gel filtration.

containing 60 mM CaCl₂ was prepared by adding an aqueous solution of 0.5 M CaCl₂ to TBSW to reach a final concentration of 60 mM CaCl₂. An aliquot of sperm (180–200 μg of sperm protein) was added to 100 μl of TBSW containing 60 mM CaCl₂ and egg jelly (50 nmol fucose equivalents) to induce the acrosome reaction. After 20 s, an aliquot (10–100 μg of sperm protein) was quickly transferred to a tube containing the radiolabeled glycopeptide in 100 μl of TBSW and the final volume was adjusted to 200 μl. The cells were allowed to incubate with the glycopeptide for 30 s, and then the mixture was applied to a Millipore GFC filter presoaked in a solution of bovine serum albumin (1.0 mg/ml) in TBSW. The filter was immediately washed with two 10-ml aliquots of ice-cold artificial seawater. Nonspecific binding to the filter was determined by applying an equivalent mixture of TBSW containing 60 mM CaCl₂, egg jelly, and radiolabeled peptide to the filter. Binding to sperm that had not undergone the acrosome reaction was determined in an identical mixture in which jelly coat was omitted. Filters were counted in 20 ml of Liquiscint scintillation fluid (National Diagnostics, Inc. Somerville, N.J.) after the cells had been solubilized with 200 μl of 1 N NaOH and neutralized with 1 N HCl. The occurrence of the acrosome reaction was monitored by electron microscopy as previously described (7).

RESULTS

Isolation of Receptor Fragment

To determine whether the mixture of peptides released from the egg surface by Pronase treatment contained one or more components with receptorlike activity, the mixture was fractionated by gel filtration and the fractions were tested for inhibitory activity in the sperm-binding assay. A biologically active receptor fragment would be expected to compete with the egg surface receptor for binding sites on the sperm and thereby inhibit sperm-egg adhesion. Initial fractionation was carried out using Bio-Gel P-4 because this gel readily separates glycopeptides in the molecular weight range of 1,500–4,000 that are produced by Pronase digestion of well-studied glycoproteins such as ovalbumin, thyroglobulin, etc. Surprisingly, most of the egg glycopeptides were found to elute in the excluded volume of the column (Fig. 1). Repeated Pronase treatment did not result in an alteration of the elution profile, indicating that the glycopeptides were not susceptible to further proteolytic cleavage by the enzyme preparation. The excluded fraction (P-4 I) contained virtually all of the inhibitory activity for sperm-egg binding; essentially no activity was detected in the partially included material (P-4 II) (data not shown).

The results of subsequent gel filtration of fraction P-4 I on Bio-Gel P-10 is shown in Fig. 2. This column resolved a number of glycopeptides ranging in molecular weight from >10,000 to ~1,000 (Fig. 2, top). Assays of the fractions for their ability to inhibit the sperm-egg binding revealed that virtually all the activity was localized in the fractions eluting at 112–128

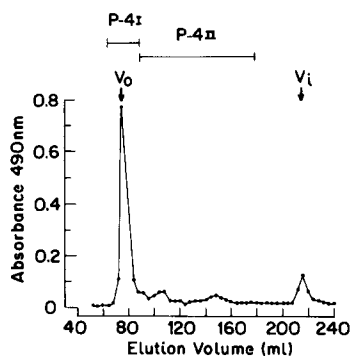


FIGURE 1 Elution profile of pronase glycopeptides released from intact *A. punctulata* eggs. Gel filtration was performed on a column of Bio-Gel P-4 equilibrated in 0.1 M ammonium acetate. Fractions P-4 I and P-4 II were pooled, lyophilized, and assayed for biological activity as described in Materials and Methods. V₀, void volume; V_i, included volume.

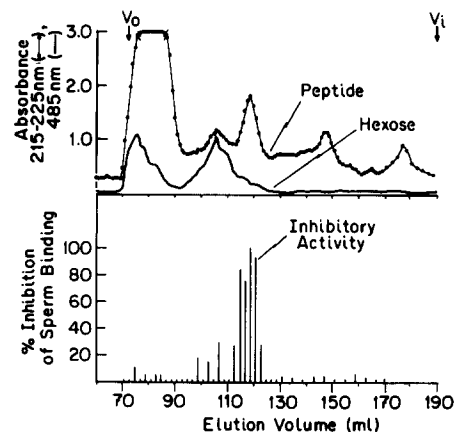


FIGURE 2 (top) Gel filtration profile of glycopeptide fraction P-4 I on a Bio-Gel P-10 column. Fractions were monitored for peptide and neutral hexose content as described in Materials and Methods. (bottom) Gel filtration profile of the biological activity of the egg surface peptides. Aliquots (25 μl) of the fractions were assayed for inhibitory activity in the sperm binding assay. Essentially identical profiles of activity were obtained in three separate experiments.

ml (Fig. 2 bottom). These fractions were pooled, lyophilized, and rechromatographed on Bio-Gel P-10. As shown in Fig. 3, a single peak containing peptide, neutral hexose, and hexosamine was recovered that contained the inhibitory activity. On the basis of a comparison with standards of known molecular weight, the elution position of this component indicated an apparent molecular weight of 6,000. Typically, 450 μg (peptide) of the partially purified glycopeptide could be obtained from 10 ml of packed, dejellied eggs (~1.1 g total egg protein).

It is clear that the glycopeptide is derived from the cell surface of the egg because exhaustive self-digestion of Pronase alone or of jelly coat did not result in production of a glycopeptide of the same molecular weight as the receptor fragment as determined by chromatography on Bio-Gel P-10.

Effect of Putative Sperm Receptor Fragment on Sperm-Egg Binding and on Fertilization

Because it was possible that the inhibitory effect of the glycopeptide was on induction of the acrosome reaction rather than on the subsequent binding event, sperm were preincubated with or without glycopeptide. These aliquots of the preincubated sperm were added to TBSW with or without jelly coat. The percentage of sperm that had undergone the acrosome reaction was then assessed as described in Materials and Methods. Less than 2% of the sperm that had been preincubated with or without glycopeptide underwent the acrosome reaction in the absence of jelly coat. However, when jelly coat was added, both preparations of preincubated sperm reacted identically. Both contained 54% reacted sperm (average of three experiments ± 5%). Thus it is clear that the glycopeptide does not inhibit the acrosome reaction.

To study further the biological properties of the partially purified glycopeptide fraction, we examined the relationship between the concentration of the glycopeptide and its ability to inhibit sperm-egg binding. The results shown in Fig. 4 demonstrate that the inhibitory effect of the glycopeptide is concentration dependent and that 50% inhibition of sperm binding occurs at a glycopeptide concentration of ~1 μg/ml. Because the molecular weight of the glycopeptide is estimated to be ~6,000 daltons, 50% inhibition of sperm binding occurs

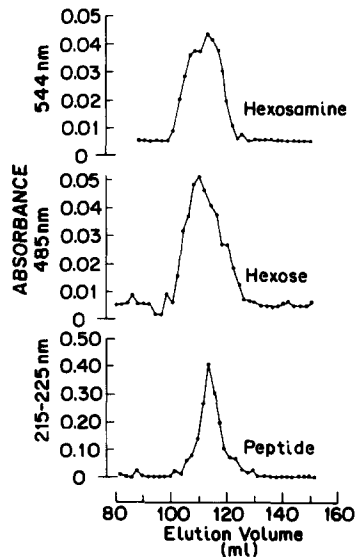


FIGURE 3 Elution profile obtained upon rechromatography of the biologically active glycopeptide fraction on Bio-Gel P-10. Hexose, hexosamine, and peptide content was monitored as described in Materials and Methods.

at ~200 nM glycopeptide. At higher concentrations, sperm binding was almost completely inhibited. The glycopeptide was stable to boiling and had no detectable effect on sperm motility as detected by phase-contrast microscopy. When the glycopeptide was tested against *S. purpuratus* gametes, it was found that sperm-egg binding was inhibited at glycopeptide concentrations similar to those used above against *A. punctulata*. Thus the effect of the glycopeptide fragment is not species specific, at least not between the two species tested so far.

Because sperm-egg binding is a component of the overall fertilization process, the effect of the glycopeptide on fertilization was studied. Using the fertilization assay described earlier (21), it was found that the glycopeptide fraction inhibits fertilization in a concentration-dependent manner, with 50% inhibition of fertilization being observed at a glycopeptide concentration of ~2.3 $\mu\text{g}/\text{ml}$ (Fig. 4). The observation that the fertilization assay is slightly less sensitive to inhibition than is the sperm binding assay may result because, in the fertilization assay, sperm do not undergo the acrosome reaction until they contact the egg.³

Binding of the Glycopeptide to Sperm

To determine whether the glycopeptide exerted its inhibitory effect on sperm-egg adhesion by binding to sperm, we tested the effect of separately pretreating eggs or sperm with the glycopeptide. Any excess, unbound glycopeptide was removed by washing the eggs or by diluting the sperm. The pretreated gametes were then tested for their ability to bind in a sperm-egg binding assay. The results presented in Table I show that pretreatment of eggs has no effect on their subsequent ability to bind sperm. This indicates that the glycopeptide does not act by binding tightly to, or otherwise modifying, the egg surface. However, when sperm previously induced to undergo the acrosome reaction were pretreated with the glycopeptide and subsequently tested in the sperm-binding assay, their

³ Studies with somewhat less purified preparations of the glycopeptide indicated that it also inhibits bindin-dependent egg agglutination (17), but it is not species specific (C. Glabe and W. J. Lennarz, unpublished studies).

capacity to bind to eggs was greatly reduced. These results suggest that, indeed, the glycopeptide exerts its biological effect through some interaction with the sperm.

To examine more quantitatively the interaction of the gly-

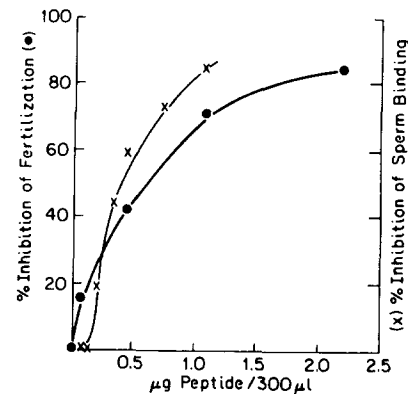


FIGURE 4 Inhibition of sperm binding and fertilization by the partially purified glycopeptide. Increasing concentrations of the glycopeptide were tested for inhibition of sperm binding against *A. punctulata* gametes using sperm preinduced to undergo the acrosome reaction as described in Materials and Methods. To assess for inhibition of fertilization, increasing concentrations of the glycopeptide were tested against *A. punctulata* gametes under conditions where sperm were limiting.

TABLE I
Binding of Inhibitory Glycopeptide to Sperm

Initial step	Subsequent step	Sperm-egg binding (% of control)*
Preaddition to egg‡		
None	3 washes	(100)
Glycopeptide (2.9 $\mu\text{g}/\text{ml}$)	No washes	2.5
Glycopeptide (2.9 $\mu\text{g}/\text{ml}$)	3 washes	100
Preaddition to sperm§		
None	Dilution of sperm	(100)
Glycopeptide (2.0 $\mu\text{g}/\text{ml}$)	Dilution of sperm	45
None	Dilution of sperm, addition of 0.16 $\mu\text{g}/\text{ml}$ of glycopeptide	114

* These values represent the average of three separate experiments.

‡ Both gametes were pretreated with the partially purified glycopeptide and then tested in the sperm binding assay. In the case of eggs, two 300- μl aliquots of a 1% (vol/vol) suspension of eggs were exposed to the glycopeptide for 30 s. One aliquot was not washed, whereas the other was washed with three 2-ml portions of TBSW. When the washing step was omitted, the ratio of glycopeptide to sperm was 174 μg of glycopeptide/mg of sperm protein.

§ In the case of sperm, 100 μg (10 μl) of sperm was added to 50 μl of TBSW containing 50 mM CaCl_2 and jelly coat (100 nmol fucose equivalents/ml), followed immediately by either 50 μl of TBSW or 50 μl of TBSW containing the partially purified glycopeptide (preaddition). After the cells were incubated for 25 s, 25 μl of the cell suspension was then used in the sperm binding assay. In the third assay tube, to control for carry-over of free glycopeptide with the sperm upon their dilution, the calculated amount of glycopeptide was added directly to the eggs. Then sperm was added and binding was assessed. To achieve sufficient dilution (low carry-over) of the glycopeptide in the sperm binding assay, we used a relatively low glycopeptide to sperm ratio (2.2 μg of glycopeptide/mg of sperm protein as compared with 174 μg of glycopeptide/mg of sperm protein in the egg pretreatment experiment) in the preaddition step. This was accomplished by increasing the sperm concentration. Consequently, under these experimental conditions, only 45% inhibition of binding was observed.

copeptide with sperm, the partially purified glycopeptide fraction was radiolabeled by reductive alkylation as described in Materials and Methods. The radiolabeled material was then rechromatographed on a column of Bio-Gel P-10, from which a single radioactive peak corresponding in elution volume to the untreated glycopeptide was recovered. After dialysis and concentration by lyophilization, the specific radioactivity of the labeled glycopeptide was found to be 332,000 cpm/ μg of glycopeptide. The biological activity (inhibition of sperm-egg binding) of the glycopeptide was found to be reduced by $\sim 50\%$ using the radiolabeling procedure.

Binding of the labeled glycopeptide to sperm was assessed using a filter assay. As shown in Fig. 5A, binding of the ^3H -labeled glycopeptide to sperm that have undergone the acrosome reaction increased linearly with glycopeptide concentrations up to 10–15 $\mu\text{g}/300\ \mu\text{l}$. Sperm that have not undergone the acrosome reaction bound only about half as much radioactivity. The acrosome reaction-dependent binding (cpm glycopeptide bound to reacted sperm minus cpm glycopeptide bound to unreacted sperm) is shown in Fig. 5B. The acrosome reaction-dependent binding is saturable at glycopeptide concentrations of 10 $\mu\text{g}/300\ \mu\text{l}$ or above, indicating that all of the acrosome reaction-dependent binding sites on the sperm are occupied at these concentrations.

To obtain a rough estimate of the proportion of the ^3H -labeled glycopeptide fraction that was capable of binding to sperm, the concentration of ^3H -labeled glycopeptide was held constant and the extent of binding was measured as a function of the concentration of prereacted sperm. As shown in Fig. 6, the amount of ^3H -labeled glycopeptide bound increased with increasing concentration of reacted sperm up to 1.25 mg of sperm protein/ml (250 μg per assay tube). At higher concentrations of sperm, no additional binding was observed, suggesting that most of the active ^3H -labeled glycopeptide was now bound to sperm. As expected from the previous results,

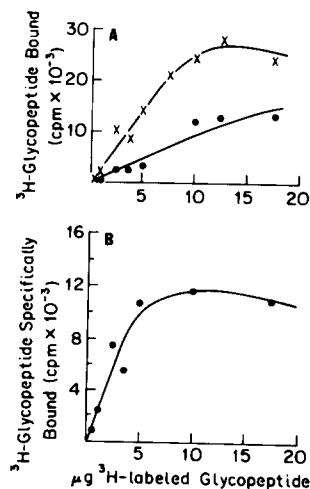


FIGURE 5 (A) Binding of the ^3H -labeled glycopeptide to unreacted and acrosome reacted sperm. 25 μg of control, untreated sperm (5% reacted) (●) or sperm preinduced to undergo the acrosome reaction (68% reacted) (X) were incubated with increasing concentrations of the radiolabeled glycopeptide as described in Materials and Methods. All values have been corrected for nonspecific binding to the filter. These background levels represented ~ 50 –60% of the counts bound to a filter with unreacted sperm. (B) Acrosome reaction-dependent binding. This curve was generated by subtracting the curve measuring binding of the ^3H -glycopeptide to unreacted sperm from that obtained measuring binding to reacted sperm.

the labeled glycopeptide also binds to unreacted sperm, but at only $\sim 50\%$ the level observed with reacted sperm. To determine whether or not the ^3H -labeled glycopeptide was binding to the same site as the unlabeled glycopeptide, we added excess unlabeled glycopeptide to the sperm along with the radiolabeled material. As seen in Fig. 7, the unlabeled glycopeptide can effectively compete with the ^3H -labeled glycopeptide for binding sites on the acrosome-reacted sperm. In several similar experiments, excess unlabeled glycopeptide consistently reduced the binding of the ^3H -labeled glycopeptide to acrosome-reacted sperm by $\sim 50\%$, that is, to the level found to bind to sperm that have not undergone the acrosome reaction.

To study this phenomenon in more detail we tested the ability of the unlabeled glycopeptide to compete with the ^3H -labeled glycopeptide for binding sites on sperm before and after induction of the acrosome reaction. As seen in Table II, binding of the ^3H -labeled glycopeptide to unreacted sperm was unaffected by addition of an excess of unlabeled glycopeptide; thus there is no specific binding under these conditions. In contrast, in the case of acrosome-reacted sperm, binding of the ^3H -labeled glycopeptide was decreased by 50% by simultaneous addition of excess unlabeled glycopeptide and is therefore specific binding. This result is consistent with the possibility

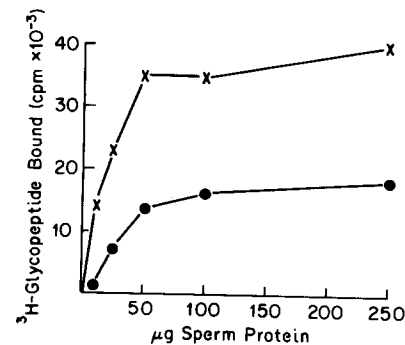


FIGURE 6 Effect of sperm concentration on binding of the radio-labeled glycopeptide. Increasing amounts of control, untreated (5% reacted), and prereacted sperm (68% reacted) were incubated with a constant level of radiolabeled glycopeptide (2.5×10^6 cpm; 7.5 μg). Unreacted sperm (●), acrosome-reacted sperm (X).

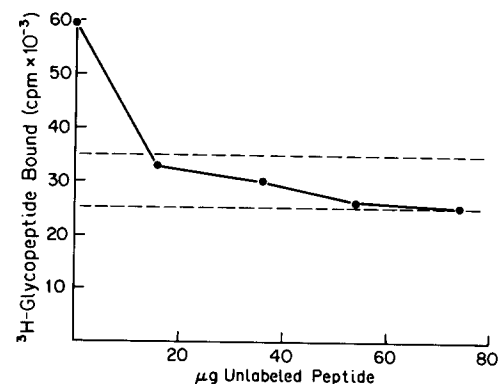


FIGURE 7 Effect of unlabeled glycopeptide on the binding of labeled glycopeptide to acrosome-reacted sperm. Sperm (50 μg of protein) were preinduced to undergo the acrosome reaction, then incubated with mixtures containing 10 μg of ^3H -labeled glycopeptide and increasing quantities of unlabeled glycopeptide. The region between the dashed lines represents the range of binding of the ^3H -labeled material to control, unreacted sperm observed in the absence of excess unlabeled glycopeptide.

TABLE II
Effect of Excess Unlabeled Glycopeptide on the Binding of
³H-Labeled Glycopeptide to Sperm before and after Induction
of the Acrosome Reaction

³ H-Labeled glycopeptide μg	Unlabeled glycopeptide μg	³ H-Labeled glycopeptide bound cpm	Specific ³ H-labeled glycopeptide binding cpm
Unreacted sperm			
0.8	0	204	
0.8	50	212	nil
1.6	0	377	
1.6	50	384	nil
Acrosome-reacted sperm			
0.8	0	412	
0.8	50	198	214
1.6	0	781	
1.6	50	308	473

In this experiment, 100 μg of control, untreated sperm (0% reacted) and acrosome-reacted (57% reacted) sperm were incubated with ³H-labeled glycopeptide (0.8 or 1.6 μg) to which had been added 0 or 50 μg of the unlabeled glycopeptide. The amount of ³H-labeled glycopeptide bound was then determined as described under Materials and Methods. Specific binding is defined as cpm of ³H-labeled glycopeptide bound in the absence of unlabeled glycopeptide minus cpm of ³H-labeled glycopeptide bound in the presence of 50 μg of unlabeled glycopeptide.

that nonspecific binding sites are present on both unreacted and acrosome-reacted sperm, and that a population of specific binding sites is available on the surface of the sperm only after induction of the acrosome reaction. An alternative possibility is that reductive alkylation of the glycopeptide fraction to label the putative receptor leads to chemical modification of about half of the biologically active glycopeptide, so that its binding is no longer specific for acrosome-reacted sperm. This would be consistent with the earlier noted observation that the labeled glycopeptide has only ~50% of the specific activity of unlabeled glycopeptide in inhibiting sperm-egg binding.

DISCUSSION

Earlier work from this laboratory established that the surface of *A. punctulata* eggs contains a protease-sensitive component that is involved in fertilization (21). Furthermore, experiments with cell surface membranes prepared from the eggs and a component solubilized from these membranes provided evidence that the protease-sensitive component was a glycoprotein and that it had the properties expected of a receptor for sperm, because it inhibited fertilization in a species-specific manner. However, as noted in the beginning of this paper, attempts to obtain a stable, soluble form of the receptor were unsuccessful.

Given the possibility that the receptor is a glycoconjugate and that its binding to the sperm could be mediated by lectin-type interactions (28), we have attempted to isolate a glycopeptide derived from the receptor. In taking this approach we set forth the following criteria that a glycopeptide fragment had to meet before it could be considered to be a functional component of the intact receptor: (a) the glycopeptide should be derived from a glycoconjugate located on the cell surface of the egg. (b) The glycopeptide should inhibit sperm-egg binding by competing with the egg surface receptor for specific binding sites on the sperm. (c) The glycopeptide should bind only to sperm that have undergone the acrosome reaction, thereby exposing the binding. Additionally, if the glycopeptide frag-

ment possesses all of the properties of the intact receptor, it should inhibit sperm-egg binding and, therefore, fertilization, in a species-specific manner.

The results presented here demonstrate that the mixture of proteolytic fragments released from the surface of intact *A. punctulata* eggs by Pronase treatment, and therefore derived from the vitelline layer or the plasma membrane, contains one fraction, separated on the basis of molecular size, that is a highly effective inhibitor of both sperm-egg adhesion and fertilization. This fraction neither induces nor inhibits the acrosome reaction. This inhibitory activity is heat stable and is present in a glycopeptide fraction that has an approximate molecular weight of 6,000. Although this glycopeptide fraction is inhibitory at nanomolar concentrations, its effects are not species specific when tested against *S. purpuratus* gametes. The biological activity of the glycopeptide fraction is manifested through an interaction with the sperm and not the egg. Studies of the glycopeptide fraction radiolabeled by reductive alkylation indicate that it contains at least one component that binds to sperm. Specific, competitive binding is observed only in sperm that have undergone the acrosome reaction. Thus the glycopeptide fragment meets three of the four criteria outlined above.

Additional studies with the labeled glycopeptide fraction indicate that, in the presence of excess sperm, binding is linearly dependent on the concentration of glycopeptide. Experiments in which the ratio of sperm to ³H-labeled glycopeptide was varied indicate that binding is dependent on the concentration of sperm until very high levels of sperm are present. Under the latter conditions, where no additional binding is observed, a maximum of ~0.75% of the radiolabeled material binds specifically to acrosome-reacted sperm. This strongly suggests that the active glycopeptide is grossly contaminated with inactive glycopeptide or other, unrelated peptides of similar molecular weight. Given this limitation, the fact remains that the properties of the partially purified, biologically active material are consistent with the hypothesis that an oligosaccharide side chain of the sperm receptor is involved in sperm-egg adhesion. That the activity is stable to boiling indicates that the tertiary structure of the peptide component of the glycopeptide is not essential for the binding properties. In this context, it is of interest that the glycopeptide fraction containing the biologically active material is unexpectedly large. Glycopeptides derived by Pronase treatment of typical avian and mammalian glycoproteins range in molecular weight from 1,500 to 4,000 and contain very few amino acids. The partially purified glycopeptide fraction described here is ~6,000 daltons and contains ~65% protein, 20% hexose, and 15% hexosamine. One possible explanation for the relatively high apparent molecular weight of the glycopeptide fraction is that it contains several oligosaccharide chains closely spaced along the polypeptide chain so as to protect it from more extensive proteolysis. In this context, it should be mentioned that, although amino acid analysis of the glycopeptide revealed that all 20 amino acids except cysteine were present, serine and threonine were found to represent 25 mol % of the total. The high content of these two residues is consistent with the possibility that oligosaccharide chains O-linked to the peptide via these two amino acids are present. However, because the biologically active component represents only a small proportion of the mass of the glycopeptide fraction, further chemical studies will be meaningful only after the biologically active component is isolated in pure form.

One of the most important observations of this study is that, although this novel, cell-surface-derived glycopeptide fraction contains a component that inhibits sperm egg binding and fertilization, and binds only to reacted sperm, it lacks the species specificity expected of an intact, functional receptor. One possible explanation is that the glycopeptide isolated is not a fragment of the sperm receptor but a peptide component that adventitiously binds to the acrosome-reacted sperm and sterically obstructs the specific binding sites. Although we cannot directly rule out this possibility at the present time, the fact that only one of the many egg surface-derived peptides has any biological effect and that this glycopeptide is effective at nanomolar concentrations argues strongly against it. A second possibility is that, in the intact sperm receptor, the functions of species recognition and generation of adhesive strength reside in different parts of the molecule. The glycopeptide fragment produced by proteolysis may no longer contain that portion of the receptor molecule that determines species specificity. Thus the only property retained intact and functional in the glycopeptide would be binding capacity. Although this hypothesis would explain our data, it does require a more complicated model for species-specific cell adhesion. A third possibility is that the glycopeptide retains all of the structural elements required for species-specific cell adhesion but that its conformation in solution lowers its binding ability to such an extent that differences in species specificity are not detectable. Examples exist in which molecules lose much of their activity upon solubilization. In some cases, the biological activity is restored upon incorporation into a lipid bilayer or attachment to a solid substrate (20). Finally, it should be noted that the binding and species-specifying properties need not reside in the same cell surface molecule. Indeed, in the approach taken in this study the assay used for detection of the receptor fragment is solely designed to measure binding. Therefore, if two components are involved, our method would be useful only to isolate the binding component.

As in the case of most other receptors, it is clear that a great deal is yet to be learned about the molecular properties of the receptor system that is responsible for species-specific binding of sperm to eggs. Because this particular system has been under study for >60 yr, it is useful to view the more recent findings in the context of efforts over the last 5 yr. Just as the fertilizin hypothesis (19) failed to recognize the complicated steps that we now know are involved in fertilization (the acrosome reaction, sperm-egg binding, and membrane fusion), the more recent lectin hypothesis discussed in the beginning of this paper may eventually have to be revised as more is learned about sperm-egg interactions. In any case, the results reported in this study provide additional evidence that the carbohydrate chains of one or more cell surface glycoproteins of the egg may play a role in sperm-egg binding. However, it is apparent that a detailed understanding of the factors that control species specificity in sperm-egg binding will require isolation of intact receptor in stable, biologically active form.

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