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# Isolation of a High Molecular Weight Glycoconjugate Derived From the Surface of S purpuratus Eggs That Is Implicated in Sperm Adhesion

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Sea urchin sperm–egg adhesion is mediated by bindin, a sperm surface protein that has lectin-like activity. Bindin agglutinates eggs, and this interaction has been shown to be inhibited by glycopeptides released from the egg surface by protease treatment. In this study, we report the purification and properties of such an egg surface glycoconjugate that may be involved in sperm adhesion. The glycoconjugate was partially purified by gel filtration and affinity chromatography on bindin particles. Upon gel filtration on Sepharose CL 4-B, the glycoconjugate elutes near the void volume, suggesting that it has a molecular weight in excess of one million. In addition, we have found that the egg surface glycoconjugate agglutinates bindin particles, indicating that it is multivalent. Carbohydrate analysis indicates that the glycoconjugate is composed primarily of fucose, xylose, galactose, and glucose. This purified egg surface component is the most potent inhibitor of bindin-mediated egg agglutination yet described.

**Key words: egg receptor for sperm, cell surface glycoconjugate, sea urchin fertilization, sperm–egg binding, bindin–receptor interaction**

Carbohydrate containing components or glycoconjugates of cell surfaces are believed to play an important role in a wide variety of cellular recognition and adhesive phenomena [1]. In sea urchin fertilization, the first step in gamete interaction is induction of the acrosome reaction in sperm by a sulfated fucose polymer component of the egg jelly coat [2]. This interaction has been shown to be species-specific in several species of sea urchins. It has also been proposed that the specific adhesion of sperm to the egg vitelline layer is also mediated by egg surface glycoconjugates [3, 4]. The

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evidence that supports this hypothesis is that periodate oxidation of formaldehyde fixed eggs inhibits the agglutination of eggs by the sperm adhesive protein, bindin [3]. Furthermore, it has been reported that egg surface glycopeptides block the agglutination of eggs by bindin [3, 4], that bindin agglutinates erythrocytes, and that this agglutination can be specifically inhibited by galactose [5]. These observations are consistent with the hypothesis that sperm–egg interaction is mediated by a carbohydrate binding protein, bindin, interacting with egg surface glycoconjugates. Although we have reported that S purpuratus and A punctulata egg surface glycoconjugates, prepared by exhaustive pronase digestion, inhibit bindin-mediated egg agglutination, we also observed that this inhibition does not display the same degree of species specificity observed for the agglutination of eggs by bindin [4]. A similar lack of species specificity has been obtained using a glycopeptide from the surface of A punctulata eggs that inhibits sperm–egg binding and fertilization [6]. In this report, we describe the affinity purification and some properties of the glycoconjugate derived from the surface of S purpuratus eggs by pronase treatment that may play a role in sperm adhesion. In the accompanying paper efforts to identify the intact receptor are reported [7].

## MATERIALS AND METHODS

Arbacia punctulata was maintained at 18°C and Strongylocentrotus purpuratus at 7°C in aquaria containing artificial seawater (Instant Ocean, Aquarium Systems, Eastlake, Ohio). Gametes were obtained by injection of 0.5 M KCl or by electric shock. Eggs from S purpuratus were dejellied by titrating a 10% suspension of eggs to pH 5 with 0.1 M HCl for 2 min and readjusting the pH to 7.9 with 1 M Tris-HCl. Egg surface glycoconjugates were prepared from dejellied S purpuratus eggs by Pronase digestion as previously described [4]. Inhibition of bindin-mediated egg agglutination by these glycoconjugates was determined by addition of these compounds to bindin particles and incubation for 5 min before addition of eggs. The extent of agglutination was assessed as previously described [4]. A unit of inhibitory activity is defined as the amount required to inhibit by 50% the extent of agglutination of eggs produced by 1 μg of bindin in the standard assay.

Agglutination of bindin particles by egg surface glycoconjugates was examined by measuring the change in turbidity as reflected in the light scattering properties of the suspension of bindin particles. Changes in light scattering were quantitated at 660 nm at an angle of 90° in an Aminco fluorimeter. Two hundred μg of sonicated bindin particles was added to 1 ml of seawater containing 22 nmole hexose equivalents of egg surface glycoconjugate, and the change in turbidity was recorded.

Gel filtration was carried out on 2 × 60 cm columns of Biogel P-6 (Biorad, Richmond, California) or Sepharose CL 4-B (Pharmacia, Piscataway, New Jersey), in 0.1 M $NH_4COOH$, pH 7.4. Carbohydrate was determined by the phenol-sulfuric acid method [8]. Primary amino groups were quantitated by reaction with fluorescamine (Pierce, Rockford, Illinois). Saccharides were quantitatively analyzed by gas chromatography of the alditol acetate derivatives on 1% OV 2340 with a Hewlett Packard model 5702A gas chromatograph. After hydrolysis of the sample in 2 M trifluoroacetic acid at 121°C for 1 hr, the sample was lyophilized and the monosaccharides were reduced with $NaBH_4$ as previously described [2]. The resulting alditols were acetylated with acetic anhydride: pyridine, 1:1 (vol/vol), and heated at 90° for 20 min. Inositol

was used as an internal standard. A sample containing only bindin and Pronase was analyzed in parallel to serve as a control for any material contributed by these components. Sugars were found to be absent from this control sample.

## RESULTS

Pronase digestion of 100 ml of packed, dejellied eggs as previously described [4] yielded a glycopeptide fraction containing 11 μmole of hexose equivalents as determined by the phenol sulfuric acid method. After concentration by lyophilization, the sample was dissolved in 2 ml of 0.1 M $NH_4COOH$ and fractionated on a column of Biogel P-6. As shown in Figure 1, this procedure separates the egg surface glycoconjugates into two major peaks of carbohydrate containing material. The first peak (peak I) elutes at the void volume of the column and represents large oligosaccharides or glycopeptides. The second peak (peak II), contained in the fractionation volume of the column, represents smaller oligosaccharides or glycopeptides. The two carbohydrate-rich peaks contain relatively little protein or aromatic amino acids as judged by the low absorbance at 280 nm. Most of the material absorbing at 280 nm elutes in the included volume as expected for small peptides and amino acids.

The carbohydrate-containing peaks were pooled, lyophilized, and dissolved in seawater, and their activity in inhibiting bindin-mediated egg agglutination was determined. As shown in Figure 2, the material in the high molecular weight fraction (peak I) inhibits egg agglutination by 90% at high concentrations, and inhibits by 50% at approximately 20 nmole of hexose equivalents. The material in the lower molecular weight fraction (peak II) shows no inhibition at up to 300 nmole of hexose equivalents. The apparent large size of peak I is not due to incomplete Pronase digestion, since additional Pronase digestion of this pooled material does not result in any decrease in the size as judged by gel filtration.

To obtain a better estimate of the size of the inhibitory egg surface glycoconjugates, we further fractionated the pooled peak I on Sepharose CL 4-B, which has a published exclusion limit of 5 million daltons for dextrans. As shown in Figure 3, chromatography on Sepharose CL 4-B further fractionates the peak I material into several carbohydrate-containing peaks. The peaks were pooled, and the inhibitory activity was determined as indicated in the upper panel. Only the high molecular weight material in or near the void volume of the column contains inhibitory activity. Since the large size of the inhibitory material is comparable to that expected for some glycosaminoglycans, we examined the effect of hyaluronic acid, chondroitin sulfate, and heparin on the agglutination of eggs by bindin. None of these compounds was found to have any effect on bindin-mediated egg agglutination at concentrations exceeding 2.5 mg/ml. Furthermore, treatment of the peak I egg surface material with the enzymes chondroitin ABCase, hyaluronidase, or endo-B-galactosidase had no effect on the inhibitory activity of this material.

In addition to inhibiting bindin-mediated egg agglutination, we observed that the egg surface glycoconjugates in peak I also agglutinated bindin particles. This agglutination could be detected as a change in the light scattering of a suspension of bindin particles. The kinetics of the interaction of peak I glycoconjugates and bindin particles is shown in Figure 4. In the absence of added glycoconjugates, there is relatively little change in the light scattering of the suspension. This observation, suggesting that the

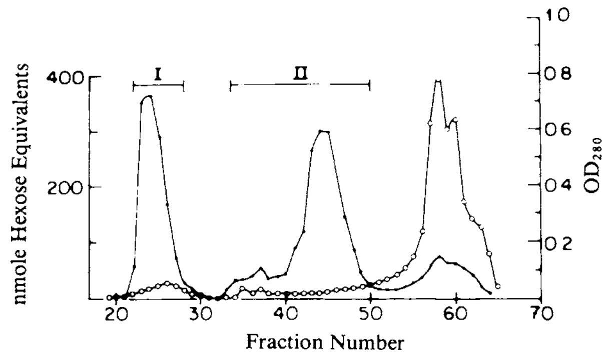


Fig. 1. Fractionation of egg surface glycopeptides by gel filtration Biogel P-6. Glycopeptides were prepared from 100-ml dejellied eggs by exhaustive Pronase digestion as previously described [4]. The digest was concentrated to a volume of 2 ml and applied to a column of Biogel P-6 as described in methods. Five-ml fractions were collected, and aliquots were analyzed for carbohydrate (●——●) and for peptide content at 280 nm (○——○). Carbohydrate-rich fractions I and II were pooled as indicated.

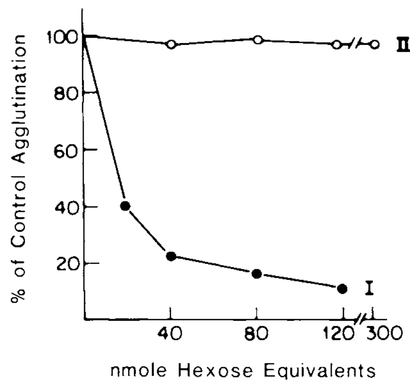


Fig. 2. Inhibition of bindin-mediated egg agglutination by egg surface glycocojugates. Varying amounts of fractions I and II were mixed with 1 μg of bindin particles in a total volume of 200 μl and incubated at 22°C for 5 min. After the incubation period, 200 μl of a 1% vol/vol dejellied egg suspension was added, and the extent of agglutination was determined as previously described [4]. The inhibition is expressed relative to the extent of agglutination observed with 1 μg of bindin in the absence of additions.

peak I glucoconjugates are multivalent, formed the basis for an affinity purification of the inhibitory glycoconjugates. The agglutinated bindin particles were found to be flocculent after addition of glycoconjugates, and could be removed by centrifugation at 2,000g for 5 min.

More bindin particles were added to the absorbed supernatant, and the agglutination cycle was repeated twice, after which the supernatant no longer agglutinated bindin particles. This finding suggests that the bindin-inhibition activity is stiochiometric, and can be absorbed by excess bindin.

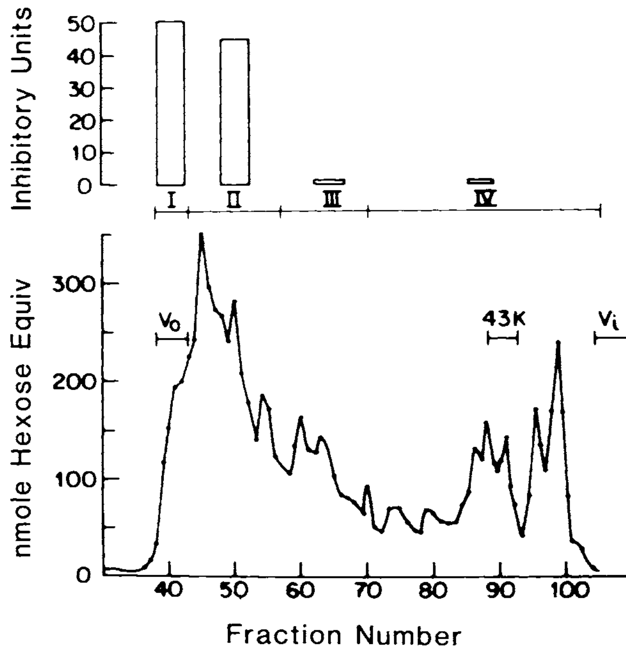


Fig. 3. Fractionation of peak I egg surface glucoconjugates on Sepharose CL 4-B. Peak I material was concentrated to a volume 2 ml and applied to a Sepharose CL 4-B column as described in Methods. In the lower panel, 3 ml fractions were collected and the carbohydrate content of an aliquot was determined (●——●). In the upper panel, fractions were pooled as indicated, and the total number of inhibitory units was determined. A unit of inhibitory activity is defined as the amount required to inhibit by 50% the extent of agglutination induced by 1 μg of bindin.

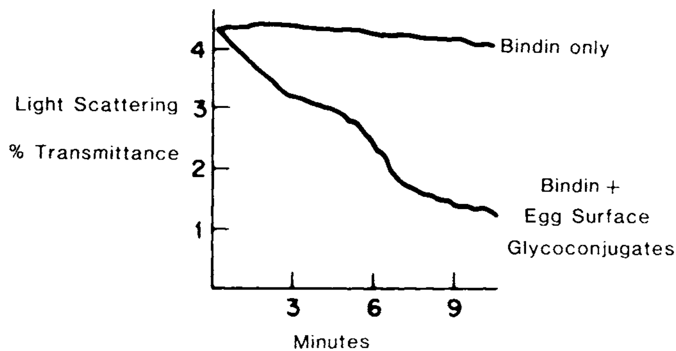


Fig. 4. Kinetics of agglutination of bindin particles by egg surface glycoconjugates. The agglutination of bindin particles, as reflected by changes in turbidity, was determined by measuring the decrease in light scattering at 90° as described in Methods.

The results of affinity purification of the inhibitory activity by agglutination of bindin particles is shown in Table I. The inhibitory activity in the bindin pellet could be recovered by Pronase digestion followed by dialysis of the digest against distilled water, boiling to inactivate any remaining protease activity, lyophilization, and resuspension in seawater. Approximately 50% of the inhibitory units were recovered in the bindin pellet, which contains 1.4% of the starting amount of hexose equivalents. A small amount of the starting amino groups is also detected in this fraction, but this value is approaching the error in the estimate. The crude glycopeptide fraction had a specific activity value in egg agglutination inhibition of 16.7 units/μmole hexose equivalents, whereas the value for the purified material was 460 units/μmole hexose equivalents. The affinity purified glycoconjugate was found also to inhibit the agglutination of Arbacia eggs by Arbacia bindin at similar concentrations to those required to inhibit agglutination of S purpuratus eggs by homologous bindin (data not shown).

We have quantitatively analyzed the carbohydrate composition of the affinity-purified egg surface glycoconjugates to gain some insight into their structure. In the starting peak I fraction obtained from the egg surface Pronase digest, nearly all saccharides common to glycoconjugates are detected (ie, fucose, xylose, mannose, galactose, galactosamine, and glucosamine). In contrast, the affinity-purified material contains only fucose, xylose, galactose, glucose, and an unidentified sugar (Table II). The complete absence of amino sugars suggests that this material is not related to the gly-

**TABLE I. Purification of Inhibition Activity by Affinity Chromatography on Bindin Particles**

| | Units of inhibitory activity[a] | μmoles Hexose equivalents | nmoles Amino group equivalents |
|---|---|---|---|
| Crude egg surface pronase digest | 333 | 20 | ND[b] |
| Affinity purified by bindin absorbtion | 166 | 0.37 ± 0.005 | 5.4 ± 3.7 |
| Remaining in supernatant | 77 | 14 | 815 |
| Percent adsorbed by bindin | 50% | 1.4% | 0.66% |
| Total recovered | 72% | 74% | ND |

[a]Activity in inhibiting bindin-dependent egg agglutination was assessed as previously described [4].
[b]ND, not determined.

**TABLE II. Carbohydrate Composition of Affinity-Purified Egg Surface Glycoconjugate**

| Saccharide | Mole percent |
|---|---|
| Fucose | 32 |
| Xylose | 12 |
| Galactose | 11 |
| Glucose | 13 |
| Glucosamine | nd[a] |
| Galactosamine | nd |
| Unidentified (not arabinose or ribose) | 32 |

[a]nd, Not detected.

cosaminoglycan class of polymers or oligosaccharides that are N-glycosidically linked to asparagine residues. The ratio of total monosaccharides to amino groups in the affinity-purified glycoconjugates is approximately 200:1 (Table I). Assuming one amino group per polysaccharide chain, a molecular weight of 360,000 can be estimated for the polysaccharide chain.

## DISCUSSION

We have isolated and partially purified by gel filtration and affinity chromatography, a proteolytically derived component of the egg surface that was previously shown to inhibit bindin-mediated egg agglutination [3, 4]. This egg surface glycoconjugate is the most potent inhibitor of bindin-mediated egg agglutination yet reported. We have also observed that this egg surface component agglutinates bindin particles, suggesting that it interacts directly with bindin and that it is multivalent. The only other material that has been demonstrated to interact with bindin is a very high molecular weight egg surface glycoprotein [9]. The interaction of this material with bindin was measured by the binding of $^{125}$I-labeled egg surface material to bindin particles, and this interaction was found to be inhibitable only by similar unlabeled egg surface material. As reported in the accompanying paper [7], we have found that the high molecular weight egg surface glycoconjugates prepared by Pronase digestion also inhibits the binding of labeled egg surface material to bindin particles, suggesting that these surface components are structurally related. Our results suggest that the egg surface glycoconjugate that inhibits bindin-mediated egg agglutination may be a relatively minor component of the total egg surface glycoconjugates. After affinity purification, 50% of the total inhibitory activity is recovered in a fraction that contains only 1.4% of the total starting hexose equivalents.

Although the proteolytically derived egg surface glycoconjugate that inhibits bindin-mediated egg agglutination has been purified by affinity chromatography on bindin particles, this glycoconjugate does not display the species specificity that might be expected from the properties of the interaction of bindin with the intact cell surface component [4]. Perhaps species specificity is a property of another egg surface component that is distinct from the molecules involved in producing the adhesive forces. Species specificity could also result from the precise three-dimensional orientation of the intact egg surface glycoconjugate in vivo, as has been previously considered [10]. The participation of this egg surface glycoconjugate in sperm adhesion has not been directly demonstrated, and must await further experimental evidence.

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