SUMMARY

Membrane fractions have been isolated from eggs of *Fucus serratus* which inhibit fertilization in a species-specific manner. This activity is destroyed by α-fucosidase and α-mannosidase. Some 6% of the protein of this membrane fraction binds to Con A-agarose, following SDS solubilization, and when eluted with α-methyl mannoside inhibits fertilization when preincubated with sperm, but not eggs. This inhibitory activity is species-specific and destroyed by α-fucosidase but not by trypsin. SDS-gel electrophoresis reveals 1 band staining strongly with Coomassie Brilliant Blue G and weakly with the PAS reagent. This major band represents a glycoprotein with an approximate molecular weight of 30000 Daltons.

Membrane fractions from sperm of *Fucus serratus* solubilized in KCl yielded a protein-containing fraction, after affinity chromatography on desulphated fucoidan-Sepharose. This fraction is 100-fold more effective in inhibiting fertilization after preincubation with eggs than either Con A or fucose-binding protein. It is species-specific and inhibition is reversed when pretreated eggs are washed with fucoidan. Activity is destroyed by heat and trypsin and only one diffuse band is apparent on SDS gels. This stains positively with Coomassie Brilliant Blue G but not with PAS and has a molecular weight of approximately 60000 Daltons.

Tentative calculation of the numbers of putative receptor molecules gives a figure of 2.5 × 10⁶ receptors per egg and 1.8 × 10⁶ receptors per sperm.

INTRODUCTION

Although fertilization involves a complex series of events it is now evident from a number of studies that the initial specific recognition between gametes is mediated by surface-localized complementary macromolecules. Studies in this laboratory utilizing lectins, polysaccharides and enzymes have produced indirect evidence that gamete recognition in the brown alga *Fucus serratus* is based upon the association between fucosyl- and mannosyl-containing ligands on the egg surface and complementary carbohydrate-binding proteins on the sperm surface (Bolwell, Callow, Callow & Evans, 1979). More direct proof requires the isolation and characterization of the appropriate receptors.

Studies on the receptors involved in oogamous recognition in the analogous sea-urchin system are well advanced. Recently, large protease-sensitive, surface-localized glycoproteins with affinity for the complementary sperm receptor have been isolated.

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from eggs of several species (Glabe & Vaquier, 1978; Schmell, Earles, Breaux & Lennarz, 1977; Tsuzuki, Yoshida, Onitake & Aketa, 1977). In contrast, a pure protein receptor has been isolated from sperm of Strongylocentrotus purpuratus (Vaquier & Moy, 1977) and a predominantly carbohydrate-containing receptor from sperm of Hemicentrotus pulcherrimus (Aketa, Miyazaki, Yoshida & Tsuzuki, 1978). The similarities to the putative receptors involved in recognition in Fucus serratus revealed by indirect studies are therefore apparent. Furthermore, the oogamous form of fertilization in the dioecious species, Fucus serratus, may have more in common with the marine echinoids than those systems which have been studied in lower plants so far. These studies have focussed mainly on isogamous species of green algae, particularly Chlamydomonas (reviewed by Wiese & Wiese, 1978) and the yeast Hansenula wingei (Crandall, 1978). However, in both these cases the receptors involved in recognition were similar, in that they have been shown to be glycoproteins of high molecular weight.

Receptors have hitherto been isolated from gametes on the basis of either their ability to cause species-specific agglutination or to inhibit fertilization. This report describes the isolation of receptors from eggs and sperm of F. serratus demonstrated on the basis of species-specific inhibition of fertilization. These receptors have the characteristics predicted by the previous indirect studies (Bolwell et al. 1979).

MATERIALS AND METHODS

Fertile plants of Fucus serratus, Fucus vesiculosus and Ascophyllum nodosum were collected from several locations on the N.E. coast of Yorkshire and stored moist at 4 °C for up to 10 days. Gametes were released as required and, in the case of eggs, washed to remove any adhering mucilage (Callow, Coughlan & Evans, 1978).

Concanavalin A (Con A) insolubilized on beaded agarose, epoxy-activated Sepharose 6B, yeast mannan, α-mannosidase, α-L-Fucosidase, and trypsin were purchased from Sigma; Amberlite XAD-2 from B.D.H.; fucoidan from K & K Laboratories Inc., New York. Fucoidan was desulphated by the method of Kantor & Schubert (1957). Coupling of saccharides to epoxy-activated Sepharose 6B was accomplished by the method of Sundberg & Porath (1974).

Preparation of receptor from eggs

Sperm receptor from eggs was isolated by affinity chromatography of solubilized plasma-membrane enriched fractions prepared by a modification of the method of Thom, Powell, Lloyd & Rees (1977). One packed volume of eggs was slowly added with stirring to 40 vol. of 0.02 M borate, 0.2 mM EDTA pH 10.2. After 15 min 4 vol. of 0.5 M borate, pH 10.2 were added and stirring continued for a further 5 min giving a final pH of 9.5. These conditions gave optimum lysis of eggs and plasma membrane could be observed by phase-contrast microscopy budding off the cell surface into large vesicles. The suspension was then centrifuged at 430 g for 10 min followed by centrifugation of the resulting supernatant at 30000 g for 30 min. The final pellet was gently suspended in 10 cm³ phosphate-buffered saline, pH 7.2, layered on to a 10-cm³ cushion of 1 M sucrose in the same buffer and centrifuged at 24000 g for 1 h. The plasma membrane-enriched fraction from the interface (Callow, Coughlan & Evans 1978) was harvested with a Pasteur pipette, reuspended in phosphate-buffered saline and pelletted at 100000 g x 15 min.

Membranes derived from 1 cm³ packed vol. of eggs were reuspended in either 0.5 cm³ 0.1 M Tris-HCl pH 7.2, 1 mM CaCl₂, 1 mM MnCl₂, 1% sodium dodecyl sulphate (SDS) and 1 mM mercaptoethanol for preparative work or 10 mM phosphate buffer pH 7.2, 1% SDS and 1% mercaptoethanol for polyacrylamide gel-electrophoresis (PAGE) and incubated at 37 °C for 2 h. SDS was removed prior to affinity chromatography by adding 50 mm 3 M KCl to 0.5 cm³ solubilized membrane preparation. Following removal of the SDS precipitate by centrifugation,
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aliquots (100 mm³) of the solubilized membrane were added to a 2-cm³ column of Amberlite XAD-2 which had been extensively washed with distilled water before equilibration with 0.1 M Tris-HCl, pH 7.2, 1 mM MnCl₂ and 1 mM CaCl₂. Elution of the sample was carried out in this buffer. This removed any remaining SDS (Fox, Stevens, Taylor & Poulson, 1978) which would otherwise have interfered with the fertilization bioassay, either directly or indirectly, by causing release of inhibitory Con A from the subsequent affinity column step (Lotan, Beattie, Hubbell & Nicolson, 1977).

Affinity chromatography of the solubilized membrane was carried out on a Con A-agarose column (1–3 cm³) which had previously been extensively washed successively with distilled water, 0.5 M NaCl, 0.1 M acetate buffer, pH 4.5, and 0.05 M borate buffer, pH 9.0, before use. The column was then equilibrated with 0.1 M Tris-HCl, pH 7.2, containing 1 mM CaCl₂ and 1 mM MnCl₂. Samples of solubilized membrane were added and allowed to equilibrate for at least 40 min before washing with Tris-buffer. Specific fractions bound to Con A were eluted with 20 mM α-methylmannoside in Tris buffer.

Preparation of receptor from sperm

Egg receptor from sperm was prepared by affinity chromatography of soluble fractions washed from the sperm surface by hypertonic salt solution. Sperm were harvested after release by centrifugation at 1000 g for 20 min. The sperm pellet was gently resuspended in 2 vol. of seawater, and 3 M KCl gradually added with stirring to give a final concentration of 2.5 M KCl. The suspension was allowed to stand with occasional stirring for 1 h at 4 °C (Stage 1, Table 3, p. 218) before centrifugation at 10000 g for 1 h at 4 °C. The supernatant was then filtered through a 0.45-μm Millipore filter to remove any floating whole sperm debris (Stage 2, Table 3). This fraction soluble in hypertonic salt was then dialysed against distilled water at 4 °C until a white precipitate formed which was collected by centrifugation at 10000 g for 15 min. The precipitate was resuspended in distilled water and solid KCl added to the stirred suspension to a concentration of 1 M. The solution was equilibrated for 30 min at 4 °C and then centrifuged at 10000 g for 15 min. The resultant supernatant (Stage 3, Table 3) contained all the receptor activity. For affinity chromatography 1 cm³ of the solubilized receptor was allowed to equilibrate for 1 h at room temperature on a 3-cm³ column of desulphated fucoidan bound to Sepharose 6B previously equilibrated with 1 M KCl. The sample bound to the column was then extensively washed with 1 M KCl and active fractions eluted with 0.1 M Tris-HCl and 1 M KCl buffer, pH 8.8 (Stage 4, Table 3).

Fertilization assay

Routine assessment of the ability of putative receptor fractions to inhibit fertilization was quantitatively estimated by the standard fertilization assay (Callow, Evans, Bolwell & Callow, 1978). Sperm fractions were preincubated with eggs for 10 min before adding whole sperm while egg fractions were added to eggs at the same time as sperm, as described for inhibition studies by Bolwell et al. (1979). Since gametes of Fucus serratus are extremely sensitive to a wide range of chemicals all reagents used were tested for non-specific inhibition of fertilization in the bioassay. Some controls contained identical amounts of column eluent to assess the effects of any possible salinity changes following the addition of extracts. Not more than 250 mm³ of each fraction to be tested was added to eggs at the same time as sperm in the standard 2-cm³ assay (5000 eggs plus enough sperm to give 50–60% fertilization in untreated controls after 5 min at 22 °C) or, in the case of preincubations, either 500 eggs or 4 x 10⁵ sperm were exposed to the fraction for a specified length of time at 22 °C in a total volume of 2 cm³. Pretreated eggs were allowed to settle and washed twice with Millipore-filtered seawater before adding untreated sperm. Pretreated sperm were collected by centrifugation at 1000 g for 5 min. The supernatant was removed before resuspending the sperm in seawater and adding to untreated eggs in the standard assay. Controls were subjected to the same procedures. Species specificity of inhibitory activity was tested on gametes of other dioecious fucoids, either Fucus vesiculosus or Ascophyllum nodosum, depending on availability.
Other procedures

Heat lability was examined by heating the receptor fraction at 100 °C for 10 min before cooling and testing. Trypsin sensitivity of extracts from sperm was tested by incubating 250-mm³ receptor fraction with 25 μg trypsin for 30 min before preincubating with eggs for 5 min. Trypsin at this concentration has no effect on the capacity of eggs for fertilization (Bolwell et al. 1979). Eggs were then washed with seawater before the addition of untreated sperm. The effect of carbohydrases and protease enzymes on extracts from eggs was examined by first incubating the fraction with 0.005 I.U. of α-mannosidase, α-fucosidase or 100 μg cm⁻³ trypsin in a total of 250 mm³ of buffer at the appropriate pH at 22 °C for 30 min. The composition and pH of each treatment is given in Table 2, p. 214. In the case of unsolubilized egg membrane, pellets following centrifugation of the incubation mixture were resuspended in 250 mm³ seawater before assaying for inhibition of fertilization as described above at the same time as the gametes were mixed. Soluble fractions of receptor from eggs were added directly to the standard assay following enzyme treatment, giving an eightfold dilution. Since the degradative enzymes used also inhibit fertilization (Bolwell et al. 1979) a control incubation containing only enzyme was included. At these dilutions not enough extraneous enzyme was available to cause more than 10% inhibition over the 5-min incubation when compared with untreated controls.

SDS-Polyacrylamide gel electrophoresis (PAGE) was carried out by the method of Weber & Osborne (1966). Fractions specifically eluted by affinity chromatography were pooled, dialysed overnight against distilled water and then freeze-dried. Receptor fractions or membranes were solubilized in 10 mM Na₂HPO₄-NaOH buffer pH 7 containing 1% SDS and 1% mercaptoethanol and incubated for 2 h at 37 °C. Samples were then subjected to electrophoresis on 10% acrylamide gels at 2 mA/tube until the tracking dye (Bromophenol blue) had reached the lower end of the tube. Gels were stained for protein with Coomassie Brilliant Blue G (Fairbanks, Steck & Wallach, 1971) and for carbohydrase using the PAS procedure (Zaccharias, Zell, Morrison & Woodlock, 1969), and were scanned in a Joyce-Loeb Chromoscan 200 at 530 nm and 550 nm respectively. Molecular weight markers (BDH) were used to calibrate the gels.

Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951) after first precipitating in 10% TCA, with bovine serum albumin as standard. Protein from column eluates and nucleic acids was estimated spectrophotometrically from the data of Warburg & Christian (1941). Chlorophyll was determined in 80% (v/v) acetone extracts by the method of Bruinsma (1961); carbohydrate by the phenol-sulphuric method (Dubois et al. 1956) using glucose as a standard.

RESULTS

Isolation of receptor from eggs

Table 1 summarizes the stages in the purification of the receptor from eggs of Fucus serratus. Crude extracts of eggs were extremely effective in inhibiting fertilization. This inhibitory activity was present both in a particulate form (30000 g pellet) and in a soluble form in the supernatant. The amount of activity recovered in either fraction varied over the period of storage of the parent plants. The total sedimentable activity decreased by 80% over a period of 14 days while there was a concomitant 10-fold increase in the inhibitory activity recovered in the supernatant. However, the bulk of the soluble activity was not species-specific in its action, fertilization in Fucus vesiculosus being inhibited to the same extent as in Fucus serratus. Furthermore in attempts to isolate the inhibitory principle from the soluble extract it was found to bind to both Sephadex and Biogel, turning brown, and 70% of the activity was precipitated by insoluble PVP. Although the component responsible for the inhibition has not been identified these results suggest that polyphenols, which are prominent
Table 1. Stages in the purification of sperm receptor from eggs of Fucus serratus

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total protein, mg</th>
<th>Total carbohydrate, mg</th>
<th>Chlorophyll, mg</th>
<th>Sp. act., I.U. protein⁻¹</th>
<th>Species-specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Crude homogenate</td>
<td>30.25</td>
<td>195</td>
<td>0.585</td>
<td>240</td>
<td>—</td>
</tr>
<tr>
<td>(2) 30000-g supernatant</td>
<td>26.5</td>
<td>184</td>
<td>0.320</td>
<td>150</td>
<td>—</td>
</tr>
<tr>
<td>(3) 30000-g pellet</td>
<td>3.75</td>
<td>9.0</td>
<td>0.270</td>
<td>58</td>
<td>17</td>
</tr>
<tr>
<td>(4) Plasma membrane-enriched</td>
<td>0.75</td>
<td>2.2</td>
<td>0.002</td>
<td>20</td>
<td>28</td>
</tr>
<tr>
<td>(5) Eluate from affinity chromatography (Fraction 10, Fig. 2)</td>
<td>0.08</td>
<td>—</td>
<td>0</td>
<td>25</td>
<td>310</td>
</tr>
</tbody>
</table>

A typical fractionation as described in Methods of 1 cm³ packed volume of eggs (1.5 x 10⁸ cells; 1.2 g fresh weight) released from parent plants stored for 4 days is shown. 1 I.U. is the amount of extract required to give 50% inhibition of fertilization under standard assay conditions. Fractions were prepared and assayed as described in Methods.

constituents of fucoid eggs, were responsible. In contrast the sedimentable activity was species-specific in its action when tested against Fucus vesiculosus. The apparent loss in activity during storage may be significant in the light of the finding that species specificity is less stringent when gametes are released from stored plants (Bolwell, Callow, Callow & Evans, 1977).

Fractionation of the 30000 g pellet on a sucrose cushion enabled the recovery of a membrane fraction banding at 1 M sucrose and containing 2.5% of the total cell protein, 1% of the total carbohydrate, 0.003% of the total chlorophyll and 35% of the sedimented inhibitory activity (Table 1). Membranes banding at the 1 M sucrose interface from eggs and zygotes of Fucus serratus are plasmalemma enriched (Callow, Coughlan & Evans, 1978; Schroeter, personal communication) and are morphologically similar to those seen to bud off from the egg surface during lysis. Membranes prepared in this way were effective in inhibiting fertilization in Fucus serratus but had no effect on fertilization in Fucus vesiculosus or Ascophyllum nodosum (Fig. 1). Sperm of Fucus serratus were observed to bind to membrane fragments when incubated together in seawater. A similar binding of sperm to echinoid cell surface complexes has been noted by Decker & Lennarz (1979). The inhibitory activity of the Fucus membranes was destroyed by preincubation with α-L-fucosidase or α-mannosidase but not by trypsin (Table 2). These properties taken together are consistent with the hypothesis that receptor present on the egg membrane surface is responsible for the inhibition.

Since Con A can inhibit fertilization by competing with sperm for binding sites on the egg surface (Bolwell et al. 1979) the receptor was isolated from membranes dispersed in SDS by affinity chromatography on Con A-agarose. However, before affinity chromatography it was first necessary to reduce the SDS concentration to a level at which gametes were unaffected, as they are extremely sensitive to detergent, and which minimized leakage of Con A from the column (Lotan et al. 1977). This was achieved by salt-precipitation of SDS followed by elution of the supernatant through a
Fig. 1. Species-specific inhibition of fertilization by membranes derived from eggs of *Fucus serratus*. Membranes were added to eggs at the same time as sperm as described in Methods. ○, inhibition of gametes of *Fucus serratus*; ●, inhibition of gametes of *F. vesiculosus* or *A. nodosum*.

Table 2. The effect of various treatments on the inhibitory activity of plasma membrane-enriched preparations and soluble sperm receptor from eggs of *Fucus serratus*

<table>
<thead>
<tr>
<th>Species</th>
<th>Treatment</th>
<th>Treatment medium</th>
<th>Inhibition of fertilization</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fraction added: (1) 40 µg membrane protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>F. serratus</em></td>
<td>None</td>
<td></td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>0.005 U α-L-fucosidase</td>
<td>Seawater pH 6.5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>0.005 U α-mannosidase</td>
<td>Seawater pH 5.2</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>100 µg cm⁻² trypsin</td>
<td>Seawater</td>
<td>63</td>
</tr>
<tr>
<td><em>F. vesiculosus</em></td>
<td>None</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td><em>A. nodosum</em></td>
<td>None</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Fraction added: (2) 3.2 µg soluble protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>F. serratus</em></td>
<td>None</td>
<td></td>
<td>56</td>
</tr>
<tr>
<td></td>
<td>Pre-incubation with eggs</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Pre-incubation with sperm</td>
<td></td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>Enzyme control*</td>
<td></td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>0.005 U α-L-fucosidase*</td>
<td>Tris buffer pH 7.2</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>100 µg cm⁻² trypsin*</td>
<td>Tris buffer pH 7.5</td>
<td>59</td>
</tr>
<tr>
<td><em>F. vesiculosus</em></td>
<td>None</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td><em>A. nodosum</em></td>
<td>None</td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>

Plasma membrane-enriched fractions were prepared, treated and assayed as described in Materials and methods. See also Methods for a detailed description of enzyme treatments of soluble preparations (marked*).

column of Amberlite XAD-2 (Allen & Crumpton, 1976; Fox et al. 1978). This procedure did not entail a significant loss of protein, carbohydrate or inhibitory activity (Table 1). Buffer containing 1% SDS subjected to the same procedure did not inhibit fertilization, showing that this procedure was effective in removing the SDS. Follow-
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ing removal of SDS, the solubilized membrane fraction was then subjected to affinity chromatography on Con A-agarose.

Since Con A itself is very effective in inhibiting fertilization even at low concentration (Bolwell et al. 1979), considerable precautions were taken to prevent the leakage of Con A from the column, which can be significant even in the absence of detergent (Cohen, Ponce de Lion, Hessle & Eisenburg, 1976). The column was washed extensively as described in the Methods and SDS was removed from extracts as outlined above. The possibility that further leakage was responsible for any inhibition of fertilization was countered by performing fertilization assays in the presence of 10 mM α-methylmannoside which prevents Con A inhibition of fertilization but has no effect itself (Bolwell et al. 1979). Furthermore, the possible presence of Con A in column eluates subjected to PAGE was examined by running additional control gels containing native Con A in the presence or absence of the specific receptor fraction.

The majority (85%) of the protein in the SDS-treated membrane fraction eluted from the Con A-agarose column without binding (Fig. 2). This fraction had no effect on fertilization. In contrast the fraction released from the column with 20 mM α-methylmannoside in Tris-buffer was highly effective in inhibiting fertilization (Table 1). As little as 2 μg protein cm⁻³ was sufficient to give 50% inhibition of fertilization (Fig. 3) compared with the 120 μg cm⁻³ of fucoidan required to give the same level of inhibition (Bolwell et al. 1979). The inhibitory activity was destroyed by the α-L-fucosidase treatments but not by trypsin and was effective when preincubated with sperm but not eggs (Table 2). It was not effective against gametes of Ascophyllum nodosum or Fucus vesiculosus. The characteristics of Con A-binding, sensitivity to α-L-fucosidase and species-specificity are in accordance with the presence of a sperm receptor.

SDS-gel electrophoresis of components of the dialysed, freeze-dried, α-methylmannoside-eluted fraction from Con A-agarose gave a number of bands all faintly staining with Coomassie Brilliant Blue G and one strongly staining major band of 30000 Daltons molecular weight which also stained weakly with the PAS procedure (Fig. 4). Repeat experiments showed some variation in the minor components but only the 30 Kd glycoprotein consistently appeared in all active samples. Some of the minor bands may have represented aggregates of 30 Kd glycoprotein due to inadequate digestion. Shorter time of digestion did show that this protein forms dimers and higher aggregates. Con A itself had a mobility on gels similar to that of the membrane glycoprotein (Fig. 4) but the 2 were always distinguishable even by co-electrophoresis. It is highly unlikely, therefore, that the results reported here represent spurious effects of Con A leached from the affinity column.

Isolation of receptor from sperm

The initial treatment of whole fresh sperm with 2.5 M KCl left the majority of the individual gametes intact when viewed microscopically, although flagella were dissolved (Stage 1, Table 3). Considerable inhibitory activity was released and the fraction was also very effective in agglutinating eggs. If the extraction was carried out on
Fig. 2. Fractionation of SDS-treated membranes on ConA-agarose. Membrane components were dissolved in 1% SDS and the SDS removed by precipitation and treatment with Amberlite XAD-2 before applying to a 3-cm³ column of ConA-agarose. ●, elution profile (O.D. 280 nm) with 0.1 M Tris pH 7.2, 1 mM Ca²⁺, 1 mM Mn²⁺ only; ○, elution profile when 20 mM α-methyl mannose in Tris buffer was added at fraction 8. There was no elution after a subsequent 1 M NaCl wash.

Fig. 3. Inhibition of fertilization by soluble receptor from eggs of Fucus serratus. Soluble receptor was prepared by affinity chromatography of solubilized membranes derived from eggs and inhibition was determined by adding receptor at the same time as the gametes were mixed as described in Methods.

sperm that had been stored as a frozen pellet, however, there was approximately a 50% reduction in the inhibitory activity released, flagella remained intact and no agglutination activity was exhibited.

From the sperm preparation 96% of the inhibitory activity was recovered in the 100000 g supernatant (Stage 2, Table 3); most of the protein, carbohydrate and the
component responsible for the agglutination were sedimented. This is pertinent since some workers (Vaquier & Moy, 1977; Wiese & Wiese, 1978) have used agglutination as a marker for solubilized receptor. However the agglutination activity from *Fucus serratus* sperm was not species-specific; whole sperm extracts agglutinated eggs of *Fucus serratus*, *Fucus vesiculosus* and *Ascophyllum nodosum* to the same extent. The nature of the agglutination agent is not known, but it was not trypsin sensitive.

On storage of the 100,000 g supernatant at 4 °C for 6–7 days the inhibitory activity became irreversibly insoluble. However, this was prevented by first dialysing the supernatant prepared from sperm released not more than 4 days after collecting the plants, against distilled water overnight at 4 °C. This precipitated the inhibitory activity which was completely recovered in soluble form by salting into 1 M KCl after centrifugation. The inhibitor remained fully active for up to a month in this form. Ninety per cent of the inhibitory activity in the 2.5 M supernatant was recovered in this heat-labile and trypsin-sensitive form. Furthermore the activity was species-
Table 3. Purification of the egg receptor from sperm of Fucus serratus

<table>
<thead>
<tr>
<th>Stage</th>
<th>Total protein, mg</th>
<th>Total carbohydrate, mg</th>
<th>Nucleic acid, %</th>
<th>Agglutination, %</th>
<th>Inhibition, total I.U.</th>
<th>Sp. act., i.u./mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) 2.5 M KCl extract</td>
<td>288</td>
<td>45</td>
<td>10</td>
<td>100</td>
<td>1872</td>
<td>6.5</td>
</tr>
<tr>
<td>(2) 100,000-g supernatant</td>
<td>36</td>
<td>6.48</td>
<td>0</td>
<td>0</td>
<td>1300</td>
<td>50</td>
</tr>
<tr>
<td>(3) 1 M KCl-soluble</td>
<td>2.7</td>
<td>4.32</td>
<td>4</td>
<td>0</td>
<td>1200</td>
<td>600</td>
</tr>
<tr>
<td>(4) Eluate from affinity chromatography (fraction 8, Fig. 6)</td>
<td>0.29</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1008</td>
<td>3475</td>
</tr>
</tbody>
</table>

Details of the purification procedure, estimation of components and inhibition of fertilization are given in the Methods section. 1 i.u. is the amount of extract required to give 50% inhibition of fertilization after preincubation with eggs for 5 min, under standard assay conditions. The details of a typical preparation starting with 2 g of sperm (3 x 10⁹ cells) are shown.

The inhibitory activity in the 1 M KCl-soluble fraction (Stage 3, Table 3) was removed by affinity chromatography on fucoidan-Sepharose 6B. This is consistent with the previous demonstration (Bolwell et al. 1979) that fucoidan, a fucose-containing heteropolysaccharide, inhibited fertilization by binding to sperm. Fucose-Sepharose 6B did not bind the inhibitory activity, suggesting that binding is not a function of a simple terminal sugar residue. Furthermore, elution of the bound protein from fucoidan-Sepharose 6B was only possible using 10 mM NaOH in 1 M KCl. Gel electrophoresis showed this eluted fraction to be heterogeneous. However, since fucoidan is highly sulphated (Percival & MacDowell, 1967) it is probable that ion-exchange effects were taking place in addition to the affinity binding. Fucoidan was therefore desulphated (Kantor & Schubert, 1957) before coupling to Sepharose 6B. The desulphated form was found to be as effective as native fucoidan in binding to sperm and inhibiting fertilization (Bolwell et al. 1979), indicating that the affinity of the receptor for fucoidan was not due to ionic binding to the sulphate.

When 1 M KCl fractions containing inhibitory activity (Stage 3, Table 3) were allowed to equilibrate with the desulphated fucoidan-Sepharose 6B at room temperature for 1 h before washing with 1 M KCl, the fractions eluted contained 90% of the protein applied and all the carbohydrate and nucleic acid. The bound protein was eluted by 0.1 M Tris buffer in 1 M KCl pH 8.75 (Fig. 6) but not with 20 mM α-L-fucose in 1 M KCl, further indicating an oligosaccharide requirement for the binding. The activity also bound to yeast-mannan-Sepharose 6B. The eluted fractions (Stage 4, Table 3) contained all the inhibitory activity (Fig. 7) and were 100-fold more specific when tested against gametes of Fucus vesiculosus and Ascophyllum nodosum (Fig. 5).
Fig. 5. Inhibition of fertilization by 1 M KCl soluble extract derived from sperm of *Fucus serratus* (Stage 3, Table 3). Inhibition of fertilization assayed by preincubation of extract with eggs as described in Methods. •, with eggs of *Fucus serratus*; ○, with eggs of *F. vesiculosus* or *A. nodosum*, followed by fertilization of either with homologous sperm. Δ, with eggs of *F. serratus*, following heat or trypsin treatment of the receptor as described in Methods.

Fig. 6. Elution profile of the affinity chromatography of the 1 M KCl-soluble fraction (45 i.u. cm$^{-2}$) on desulphated fucoidan coupled to Sepharose 6B (Stage 4, Table 3). Fractions 1–6 were washed with 1 M KCl then active fractions were eluted with 0·1 M Tris-HCl in 1 M KCl, pH 8·75. 1-cm$^3$ fractions were collected. •, $E_{280}$ nm; ○, inhibitory activity tested with *F. serratus* gametes (1 i.u. the amount of extract required to give 50% inhibition of fertilization under standard assay conditions as described in Methods).

effective in inhibiting fertilization after preincubation with eggs for 5 min than Con A or fucose-binding protein (Miles) (Bolwell et al. 1979). Extended incubations of eggs with this fraction for up to 30 min gave no increases in inhibition (Table 4). The activity was heat-labile, trypsin-sensitive and species-specific when tested against
Fig. 7. Inhibition of fertilization by column eluate (28 I.U. cm⁻³) from affinity chromatography of soluble sperm extract from Fucus serratus (Fraction 8, Fig. 6). ●, preincubation with eggs of Fucus serratus. Preincubation with eggs of either F. vesiculosus (○) or A. nodosum (△) followed by the homologous sperm.

Table 4. The effect of various treatments on inhibition of fertilization by egg receptor from sperm

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Preincubation with eggs, min</th>
<th>Wash</th>
<th>Inhibition of fertilization, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>5</td>
<td>Seawater</td>
<td>50</td>
</tr>
<tr>
<td>None</td>
<td>30</td>
<td>Seawater</td>
<td>54</td>
</tr>
<tr>
<td>Heat 100 °C, 10 min</td>
<td>5</td>
<td>Seawater</td>
<td>0</td>
</tr>
<tr>
<td>Trypsin, 100 μg cm⁻³, 30 min</td>
<td>5</td>
<td>5 min 100 μg cm⁻³ desulphated fucoidan, then seawater</td>
<td>0</td>
</tr>
</tbody>
</table>

Details of the treatments on sperm receptor and fertilization assays are given in Methods. ‘Wash’ refers to the post preincubation wash which preceded addition of sperm under standard assay conditions.

gametes of Ascophyllum nodosum or Fucus vesiculosus. Furthermore, inhibition was reversed when eggs pretreated for 5 min with receptor were washed with 100 μg cm⁻³ of the desulphated fucoidan in seawater (Table 4).

Polyacrylamide gel electrophoresis showed that while at least 9 proteins were added to the desulphated fucoidan-Sepharose 6B column, only 1 bound. This was specifically eluted as a disperse band which may possibly represent 2 species with similar molecular weights of about 60000 Daltons (Fig. 8). Since only a single band can be detected in this position on gels of the 1 M KCl fraction before affinity chromatography, it is possible that protease activity during storage before affinity chromatography was...
DISCUSSION

Studies in this laboratory on the processes of egg-sperm recognition in fertilization in brown fucoid algae have focussed on the isolation and characterization of the interacting surface molecules. This paper provides evidence for the successful isolation from egg membranes of *Fucus serratus* of a glycoprotein with the characteristics of a sperm receptor, and a lectin-like protein which may serve as the egg receptor counterpart from the sperm.

The assumption that the glycoprotein from eggs represents the sperm receptor is based on several lines of evidence. Firstly, it inhibits fertilization in a species-specific manner, and although binding experiments *per se* have yet to be carried out, the evidence from preincubation experiments would suggest that the glycoprotein reacts with the sperm surface. This specificity is to be expected, since *Fucus serratus* does not normally crossfertilize with other fucoids (Bolwell et al. 1977). The putative receptor appears to compete with eggs for binding sites on the sperm surface in the same way as some polysaccharides (Bolwell et al. 1979). Sperm receptors from sea-urchin eggs have responsible for splitting the receptor into two bands. This has been observed in the isolation of other proteins (Ellis, 1973; Kerby, 1979). The fraction binding to the affinity column stained positively with Coomassie Blue but not with PAS. The receptor constituted 0.15% of the total cell protein.
been identified by the same criterion (Schmell et al. 1977; Tsuzuki et al. 1977). Secondly, the Fucus egg glycoprotein is derived from membrane fractions enriched in plasma membranes (Callow et al. 1978). Thirdly, this glycoprotein has the characteristics of the receptor indicated in a previous study (Bolwell et al. 1979). Thus, the receptor bound to Con A and was sensitive to both $\alpha$-fucosidase and $\alpha$-mannosidase and insensitive to trypsin. Fourthly, although the final product showed some heterogeneity on PAGE, only the 30 Kd glycoprotein consistently appeared in all active fractions examined.

The egg receptor from sperm was also isolated on the basis of species-specific inhibition of fertilization. This criterion has been used by other workers (Aketa et al. 1978) while others have used species-specific egg agglutination (Glabe & Vaquier, 1977; Vaquier & Moy, 1977). Sperm of Fucus serratus washed with hypertonic 3 M KCl released both types of activity but only the inhibitory activity was species-specific when assessed carefully according to the strict kinetic considerations described by Bolwell et al. (1979). Subsequent fractionation procedures permitted the isolation of a pure protein of molecular weight of 60000 Daltons. This protein has the required properties of fucan- and mannan-binding and trypsin sensitivity indicated by previous indirect experiments on whole gametes (Bolwell et al. 1979). Gel electrophoresis showed the product of affinity chromatography to be 2 bands of almost identical molecular weight. The reasons for regarding the receptor as being homogeneous, however, have been described above. Although the evidence presented for this protein being the receptor is somewhat circumstantial, other interpretations can be eliminated. Many of the results could be explained if the protein was a fucosidase, since $\alpha$-fucosidase pretreatments of eggs inhibit fertilization in Fucus serratus (Bolwell et al. 1979). However, the putative receptor does not inhibit fertilization in Ascophyllum while pretreatments of eggs of Ascophyllum with 0.01 I.U. of bovine fucosidase reduces their subsequent fertilization by 60% when compared with untreated controls (unpublished results). Furthermore, extended preincubations do not cause any increase in inhibition and the activity is fully reversible by washing with desulphated fucoidan, which would not be expected if the inhibition were due to degradative activity. Finally, the receptor binds to mannan insolubilized on Sepharose-6B demonstrating additional specificity. Effects due to endocytosis can also be eliminated since the reversibility of the inhibition by washing with fucans is consistent with surface-localized events.

Assuming that molecules isolated do indeed represent surface-localized gamete receptors, it is possible to calculate tentatively the numbers of receptor molecules on both gamete surfaces. From Table 1, taking the number of specific inhibitory units in the egg membrane 30000 g pellet as representing 100%, then the fraction eluting from the affinity column represents $186 \mu g$ from $1.5 \times 10^9$ eggs. This gives $2.5 \times 10^6$ receptors per egg. In contrast, from Table 3, $539 \mu g$ of egg receptor from $3 \times 10^9$ sperm gives $1.8 \times 10^4$ receptor molecules per sperm.

The above calculations are clearly tentative. Studies currently in progress on the association of the putative receptors, with each other, and with gamete surfaces, will establish the precise biological significance and location of these molecules, and will permit quantitative comparisons with the above figures.
REFERENCES


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