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Specific binding of sulphated polymers to ram sperm proacrosin

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Sulphated polysaccharides and zona pellucida glycoproteins have been shown to bind non-enzymatically to proacrosin, the protein found within the acrosomal vesicle of mammalian spermatozoa. The mechanism of this interaction has been investigated using ^{125}I-fucoidan to probe purified ram sperm proacrosin. Results show that (a) binding of ^{125}I-fucoidan to proacrosin is inhibited only by sulphated polymers and (b) recognition is mediated by poly(sulphate) groups and is largely independent of the composition of the polymer chain. It is suggested that a similar mechanism is responsible for the interaction between proacrosin and zona pellucida glycoproteins during the early stages of fertilization in mammals and this process mediates firm binding of spermatozoa to the egg.

Proacrosin; Sulphate group; Adhesion molecule; Zona pellucida; Fertilization

1. INTRODUCTION

Proacrosin, the zymogen form of the serine proteinase acrosin (EC 3.4.21.10), is a glycoprotein found within the acrosomal vesicle of mammalian spermatozoa (reviewed in [1]). Recently, it has been shown that in addition to its nascent proteolytic properties, proacrosin can also bind non-enzymatically to receptors on the zona pellucida, the extracellular matrix that surrounds all mammalian eggs [2–4]. There is evidence that this non-enzymatic binding displays selectivity in the sense that certain sulphated polymers (e.g. dextran sulphate, fucoidan) can inhibit the process whereas others (e.g. chondroitin sulphates A and C) have little or no capacity to do so [5]. Sulphated polymers can also inhibit amidase activity of active acrosin [6] implying that they bind close to the active site. In several respects therefore, proacrosin has properties similar to cell adhesion proteins such as fibronectin, laminin and 'bindin' [7,8]. Bindin is the protein isolated from the acrosomal granule of *Strongylocentrotus purpuratus* spermatozoa that binds with high affinity ($K_d = 10^{-8}$ M) to fucoidan and glycoprotein receptors on the vitelline layer of Echinoderm eggs [9]. It has been suggested that bindin and proacrosin are functionally analogous [1,4,5].

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Abbreviations: GLC, gas liquid chromatography; BSA, bovine serum albumin; SDA-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; PBS, phosphate buffered saline pH 7.2; CNBr, cyanogen bromide

In previous work we have shown that ^{125}I-fucoidan and ^{125}I-zona pellucida glycoproteins can bind selectively to proacrosin on Western blots [5,10]. To understand more about the mechanism of this interaction, we have used ^{125}I-fucoidan as a probe to assess the properties of polysaccharides and synthetic polymers that mediate their specific binding to ram sperm proacrosin. Results show that poly(sulphate) groups are the critical factors involved and that recognition is largely independent of the composition of the polymer chain.

2. MATERIALS AND METHODS

2.1. *Chemicals*

Na[^{125}I] and *N*-succinimidyl 3-(4-hydroxy, 5-[^{125}I]iodophenyl)propionate (Bolton and Hunter reagent) were supplied by Amersham. 1,3,4,6, Tetrachloro-3α-6α-diphenylglycouril (IODO-GEN) was obtained from Pierce, pronase from Calbiochem and poly(vinyl phosphate), poly(acrylic acid, ammonium salt) and poly(styrene sulphonic acid) from Polysciences. All other chemicals used were of the highest purity commercially available and were purchased from Sigma or BDH.

2.2. *Preparation of ^{125}I-labelled polysaccharides and ^{125}I-zona pellucida glycoproteins*

Crude fucoidan (from *Fucus vesiculosis*; Sigma) was purified by β-elimination and pronase digestion as described by DeAngelis and Glabe [11]. The final digest was passed through a Sephadex G-75 column in 0.1 M ammonium formate, pH 7.4 and fractions assayed for carbohydrate content using the orcinol reaction [12]. Appropriate fractions were pooled, dialysed extensively against distilled water and freeze-dried. A portion of the purified material was subjected to acid hydrolysis and released sugars detected by gas liquid chromatography (GLC). We are grateful to Dr. R.R. Selvendran, AFRC Institute of Food Research, Norwich, for carrying out the compositional analysis. Galactan (from larch; Kodak Laboratories) and mannan (from yeast; Sigma) were used without further purification. To facilitate iodina-

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tion, polysaccharides were activated with cyanogen bromide (CNBr) and then conjugated with fluoresceinamine [13]. Approximately 50 μg conjugated polysaccharide was iodinated with 100 μCi IODO-GEN in 300 μl phosphate-buffered saline (PBS) pH 7.2 [14] and stored frozen in aliquots at −20°C. The specific activity of the ^{125}I-fucoidan probe was 2.7×10^5 cpm · μg^{-1}. ^{125}I-Galactan and ^{125}I-mannan had much lower specific activities and were used only in a qualitative sense.

Eggs were released from sheep ovaries by breaking open swollen follicles into PBS. Cumulus cells were removed using a fine-bore pipette and eggs (88–100) radio-labelled with 50 μCi ^{125}I-Bolton and Hunter reagent [15]. Eggs were washed 5 times in PBS to remove unbound label and ^{125}I-zonae pellucidae dissolved by the careful addition of 0.1 N HCl to pH 3.0. Iodinated, solubilized zona pellucida glycoproteins were collected by centrifugation (10 000 × g for 5 min) and stored frozen at −20°C. The amount of protein in these preparations was too low for accurate measurement, hence this probe was used only in qualitative experiments.

2.3. *Purification of proacrosin from ram spermatozoa*

Ejaculated semen was collected from several rams, diluted with 3 vols PBS and spermatozoa washed by centrifugation (1500 × g for 15 min) through 0.264 M sucrose/10 mM Hepes/2 mM pAB, pH 7.4. Sperm pellets were resuspended in 4 vols of 0.264 M sucrose/2 mM pAB, adjusted to pH 3 with 0.1 N HCl and stirred overnight at 4°C. Cells were pelleted by centrifugation at 30 000 × g for 30 min and the supernatant ('pH 3 extract') sorted at −20°C. Proteins in pH 3 extracts were separated by preparative sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) [16] under non-reducing conditions, the band containing proacrosin excised and protein electroeluted (ISCO model 1750). Protein concentrations were determined by the method of Bradford [16] using bovine serum albumin (BSA) as standard. Molecular masses were calculated by reference to mobility of known standards ('Rainbow' markers, Amersham).

2.4. *Western blotting*

Proteins in pH 3 extracts were separated by SDS-PAGE and either stained directly with Coomassie brilliant blue R-250 or electroblotted onto cellulose nitrate membrane (0.45 μm pore size). Blots were blocked with 5% BSA (Fraction V, Sigma) in PBS for 2 h, rinsed and probed with ^{125}I-fucoidan or ^{125}I-galactan or ^{125}I-mannan (~200 000 cpm · ml^{-1} in PBS) or ^{125}I-zona glycoproteins ($1–2 \times 10^4$ cpm · ml^{-1} in PBS) for 1 h. Bound probe was detected by autoradiography using pre-flashed Fuji X-ray film. Alternatively, blots were probed with a rabbit polyclonal antibody raised to a synthetic dodecapeptide of boar proacrosin and visualized with a peroxidase-linked goat anti-rabbit IgG using 4-chloronaphthol as substrate. Preliminary experiments showed that this antibody cross-reacted with ram proacrosin (Jones, R., unpublished work). The dodecapeptide had the following sequence: -FMYHNNRRYHTC-. This represents residues 21–32 inclusive of proacrosin heavy chain [18].

2.5. *Solid phase binding assay*

Purified proacrosin (2 μg) was dot-blotted onto 1 cm^2 squares of cellulose nitrate membrane, dried, blocked with 5% BSA/PBS for 2 h and then probed with 100 μl ^{125}I-fucoidan (200 000 cpm · ml^{-1}) in PBS, pH 7.2, for 60 min. Preliminary experiments showed that $t_{1/2}$ for maximal binding was 10 min. Inhibition studies were carried out by exposing the blot to a variety of saccharides and polymers (see Results) at different concentrations for 1 h before addition of the probe. Radioactivity remaining on the membrane was measured in an LKB multigamma counter. The effect of ionic strength on binding ^{125}I-fucoidan to proacrosin was investigated by adding 0–0.8 M NaCl to the probe suspended in 20 mM Na HPO_4 buffer, pH 7.2. Specific binding is defined as the amount of probe bound to target protein minus non-specific background. Non-specific background is defined as the amount of probe remaining on the nitrocellulose in the absence of target protein.

3. RESULTS

3.1. *Composition of probes*

A compositional analysis of the purified fucoidan preparation yielded 72.8% fucose, 4.9% galactose, 7.4% xylose, 1.4% mannose, 1.4% arabinose, 2.1% glucose, 8.2% uronic acid and 1.9% unidentified material. These values are close to those reported by Medcalf and Larsen [19] for the major fucoidan fraction (fraction 2) purified from *Fucus vesiculosus*. Fucoidan also contains a small degree of branching at C-3 of fucose residues and up to 25% sulphate [19,20]. Galactan contained 72.0% galactose, 13.6% arabinose, 8.7% glucose, 2.3% uronic acid, 1.3% rhamnose, 0.9% xylose and 0.4% mannose; the polysaccharide is also sulphated [20]. The purity of the mannan probe was not investigated.

SDS-PAGE of the ^{125}I-zona probe under non-reducing conditions revealed two major labelled proteins at 122 kDa and 100 kDa and two minor components at 86 kDa and 66 kDa (Fig. 1j). Some very high mass material (~225 kDa) always remained near the top of the separating gel. This profile of a few high mass components is typical of zona pellucida glycoproteins from mammalian eggs in general, e.g. ZP1 (200 kDa), ZP2 (120 kDa) and ZP3 (83 kDa) in the mouse [21] and one 90 kDa and two 55 kDa glycoproteins in the pig [22].

3.2. *Identification of zona- and polysaccharide-binding proteins in extracts of ram spermatozoa*

pH 3 Extracts of ram spermatozoa contained a wide range of proteins when separated by non-reducing SDS-

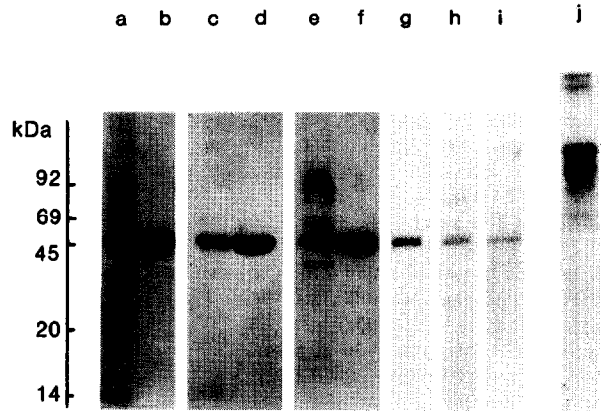

Fig. 1. SDS-PAGE/Western blots/autoradiographs of proteins in pH 3 extracts (a,c,e,g,h and i), purified ram proacrosin (b,d,f) and ^{125}I-zona pellucida glycoproteins (j). Lanes (a) and (b) show Coomassie blue-stained gels. Lanes (c) and (d) show a Western blot probed with anti-proacrosin antibody. Lanes (e) to (i) are autoradiographs taken from Western blots probed with ^{125}I-fucoidan (e and f), ^{125}I-mannan (g), ^{125}I-galactan (h) and ^{125}I-zona pellucida glycoproteins (i). Lane (j) is an autoradiograph after SDS-PAGE of the ^{125}I-zona probe. Mobility of known protein standards is as shown.

PAGE and stained with Coomassie blue R-250 (Fig. 1a). The major protein present had a mass of 46 kDa and was identified as proacrosin on the basis of its cross-reactivity with anti-boar proacrosin antibody (Fig. 1c). Purified proacrosin was > 90% homogeneous (Fig. 1b) and gave a strong reaction with the antibody (Fig. 1d). When Western blots from parallel gels were probed with ^{125}I-fucoidan, the probe bound strongly to a protein in pH 3 extracts with an average mass of 46 kDa (Fig. 1e) and weakly to 4 other proteins at ~90 kDa, 60 kDa, 38 kDa and 14 kDa. ^{125}I-Fucoidan also recognized the purified proacrosin preparation (Fig. 1f). ^{125}I-Mannan, ^{125}I-galactan and ^{125}I-zona pellucida glycoproteins all bound strongly and selectively to the 46 kDa protein in pH 3 extracts (Fig. 1g–i).

Therefore, these experiments have shown that ram sperm proacrosin is a major binding protein for sulphated polysaccharides and homologous zona pellucida glycoproteins. In the experiments described below the mechanism of this interaction has been investigated using ^{125}I-fucoidan as a probe.

3.3. *Kinetics of ^{125}I-fucoidan binding to ram proacrosin*

The binding of ^{125}I-fucoidan to proacrosin approached saturation under the conditions described for the solid phase assay (results not shown). One μg of proacrosin bound a maximum of 16 ng fucoidan. Scatchard plot analysis of these data gave a $K_d = 3.5 \times 10^{-8}$ M.

Table I

Concentration of competitor to achieve 50% inhibition (IC_{50}) of ^{125}I-fucoidan binding to proacrosin

Competitor	IC_{50}
'Cold' fucoidan (~100 kDa)	19.1 nM
Dextran sulphate (500 kDa)	31.6 nM
Dextran sulphate (5 kDa)	251.0 nM
Galactan (~100 kDa)	22.4 μM
Xylan (~10 kDa)	299.0 μM
Mannan (~34.9 kDa)	1.0 mM
Poly(vinyl sulphate) (~100 kDa)	326.0 nM
Poly(styrene sulphonic acid) (~70 kDa)	1.8 μM
Poly(vinyl phosphate) (~100 kDa)	>100.0 μM*
Poly(acrylic acid) (~250 kDa)	> 40.0 μM*
Chondroitin sulphate A (~30–50 kDa)	>400.0 μM*
Chondroitin sulphate C (~30–50 kDa)	>400.0 μM*
Dextran (500 kDa)	>160.0 μM*
D(+)-fucose	>800.0 mM*
D(+)-galactose	>800.0 mM*
D(+)-mannose	>800.0 mM*
Lactose	>400.0 mM*
Glucosamine	>500.0 mM*
Galactosamine	>500.0 mM*
Glucose-6-sulphate	>200.0 mM*
Sodium sulphate	>200.0 mM*

*Where no inhibition was observed the highest concentration tested is shown

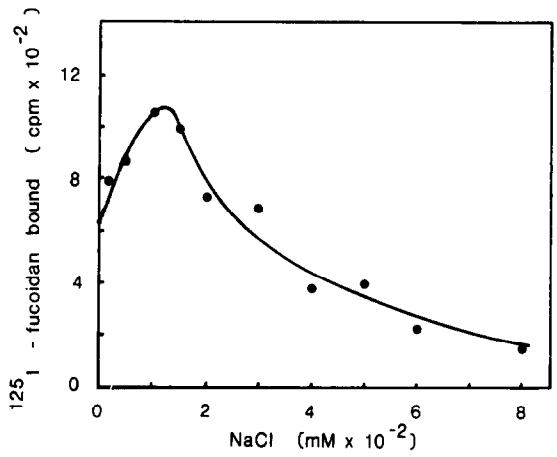

Fig. 2. Effects of ionic strength on specific binding of ^{125}I-fucoidan. Immobilized proacrosin was probed with ^{125}I-fucoidan in 100 μl of 20 mM sodium phosphate buffer, pH 7.2, to which was added 0–800 mM NaCl. Specific binding was measured and plotted against salt concentration.

3.4. *Inhibition of binding of ^{125}I-fucoidan to proacrosin by saccharides, polysaccharides and synthetic polymers*

To assess the properties of fucoidan (size, composition, charge density, etc.) that are critical for binding to proacrosin, inhibition experiments were carried out with a variety of monosaccharides, polysaccharides, glycosaminoglycans and synthetic polymers. The concentration for 50% inhibition (IC_{50}) was calculated from semi-log plots of inhibitor concentration against maximum specific binding activity. Results are summarized in Table I. Binding of ^{125}I-fucoidan to immobilized proacrosin was inhibited strongly by 'cold' fucoidan, dextran sulphate (500 kDa and 5 kDa), poly(vinyl sulphate), poly(styrene sulphonic acid), galactan, xylan and mannan but not by dextran (500 kDa), chondroitin sulphates A or C, poly(vinyl phosphate), poly(acrylic acid) or any monosaccharide, disaccharide or amino sugar tested.

Since many of the above polymers have a high charge density [10], the importance of electrostatic forces in the binding mechanism was investigated by carrying out the assay in the presence of varying concentrations of NaCl. As shown in Fig. 2, uptake of ^{125}I-fucoidan increased rapidly in the presence of low levels of NaCl to reach maximum binding between 100 mM and 150 mM. Thereafter, it declined progressively with 50% inhibition at 325 mM NaCl.

4. DISCUSSION

Fertilization in mammals involves specific recognition and fusion between complementary gametes. A critical early stage in this process is the binding of spermatozoa to the zona pellucida that surrounds the egg. Operationally, two levels of recognition can be

distinguished; an initial loose attachment phase that in some species is Ca^{2+}-dependent and probably involves ligand molecules on the plasma membrane overlying the sperm head (e.g. galactosyltransferase [23]), followed by a secondary binding phase that is characterized by its tenacity and specificity. It has been suggested that secondary binding is mediated by components of the acrosomal vesicle since at this stage vesiculation of the acrosome and exposure of the acrosomal matrix has taken place [24]. If secondary ligand molecules did not exist then the sperm would be able to detach itself from the zona surface and swim away.

In sea urchin spermatozoa 'bindin' fulfils the role of a secondary ligand [9] while in mammalian spermatozoa proacrosin is the putative zona adhesion molecule. Bindin has the capacity to agglutinate rabbit red cells and initially was considered to be a lectin with preference for fucose residues [25]. However, it is now apparent from the studies of DeAngelis and Glabe [10,26,27] that this definition may be an oversimplification. The composition of the polysaccharide is not as important as the degree of sulphation since synthetic compounds that lack any saccharide structure, e.g. poly(vinyl sulphate), are equally efficient in competition assays. The results presented here on ram proacrosin are in good agreement with these concepts. The importance of sulphate groups is demonstrated clearly by dextran (500 kDa) versus dextran sulphate (500 kDa) and by poly(vinyl phosphate) versus poly(vinyl sulphate). However, the stereochemistry of attached sulphate groups also appears to be important as glucose-6-sulphate and Na_2SO_4 do not compete with fucoidan in the solid phase assay. As far as is known, fucoidan contains $\alpha(1->2)$ linked L-fucose residues that are sulphated mainly at C-4; $<10\%$ of sulphate is on C-2 or C-3 [25]. Chondroitin sulphate A is also sulphated at C-4 of *N*-acetylgalactosamines and chondroitin sulphate C is sulphated at C-6 yet both poor inhibitors of fucoidan binding. Thus, at present it is difficult to discern any consistent pattern for the position of sulphate groups that would enable predictions to be made regarding binding affinity. More information is required on the 3-dimensional structure of these polymers as it is likely that the distance or span between 2 or more sulphates is the important factor. An illustration of the subtlety of such a recognition system is shown by the interaction between heparin and antithrombin III [27]. The antithrombin III binding region in heparin resides in an internal pentasaccharide that contains 4-*O*-sulphate and 2-*N*-sulphate groups. At least 3 of these sulphate groups are required for recognition, especially the *O*-sulphate on C-3 of the central glucosamine; absence of a sulphate group at this position leads to low affinity binding heparin. If a similar situation applied to the interaction of fucoidan and proacrosin, then it would explain why polymers with similar charge densities have different binding affinities; it depends on the stereochemistry of their projecting sulphate groups.

The remarkable selectivity of proacrosin for sulphate or sulphonic ester groups as opposed to phosphates suggests that the binding site on the protein is equally important. Oxygen atoms on sulphate are slightly less ionized than those on phosphate but otherwise their tetrahedral geometry and dimensions are similar. X-ray diffraction studies on the sulphate binding protein from *Salmonella typhimurium* indicate that ionization of sulphate oxygens is suppressed and that instead of salt bridges, co-ordinated hydrogen bonds are formed between sulphates and specific residues (probably basic amino acids) on the surface of the protein [29]. This hypothesis is supported by chemical mutagenesis experiments on bindin which have shown that arginines are particularly important [11]. It has been further suggested that guanido moieties on arginine form resonating cyclic hydrogen-bonding systems with sulphate oxygens [26]. If so, then it would explain why the interaction of fucoidan with bindin is resistant to ionic strength (the IC_{50} = 1.2 M NaCl). Since the binding of fucoidan to ram proacrosin is more sensitive to ionic strength of the medium (IC_{50} = 0.325 M NaCl), electrostatic forces may be involved. In this context the biology of the system is relevant; fertilization in sea urchins is external in sea water ($\sim$0.55 M NaCl) whereas in mammals it takes place internally in oviduct fluid ($\sim$0.11 M NaCl).

In conclusion, our results indicate that ram sperm proacrosin has affinity for poly(sulphate) containing polymers and that the specificity and avidity of its interaction with these compounds is dependent on the degree of complementarity between aligned sulphate groups on the polymer and binding sites on the surface of the protein. It is suggested that a similar mechanism is responsible for the binding of proacrosin to zona pellucida glycoproteins since the latter proteins are sulphated [30] and interaction can be blocked by fucoidan [5,10].

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