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# The polysulphate binding domain of human proacrosin/acrosin is involved in both the enzyme activation and spermatozoa–zona pellucida interaction

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## Summary

Mammalian acrosin is a protease present as a zymogen in the acrosome of a non-reacted mammalian sperm, and *in vitro* is able to carry out limited hydrolysis of homologous and heterologous zonae pellucidae. On the other hand, sulphated polymers and zona pellucida glycoproteins bind to acrosin on a domain different from the active site, named the polysulphate binding domain (PSBD). Thus it is believed that acrosome-reacted spermatozoa bind to glycan chains of the zona pellucida through PSBD participating as secondary binding receptor. The aim of the present work was to study the role of PSBD during both human gamete interaction and acrosin activation. In this work we present evidence that the anti-human acrosin monoclonal antibody C5F10 is directed to an epitope located on or near the PSBD on human proacrosin/acrosin. Moreover, we show that this antibody is able to inhibit both proacrosin activation induced by fucoidan and the sperm binding to the zona pellucida. Our results suggest that the same PSBD is involved in both sperm secondary binding, during zona pellucida penetration, and proacrosin activation.

Key words: Acrosin, Fucoidan, Proacrosin, Spermatozoon, Zona pellucida

## Introduction

The most studied enzyme in mammalian fertilisation has been the serine protease acrosin (EC 3.4.21.10) (for review see Barros *et al.*, 1996). The physiological significance of this enzyme in the mammalian fertilisation process has been inferred from studies showing that acrosin inhibitors inhibit both *in vitro* (De Ioannes *et al.*, 1990) and *in vivo* mammalian fertilisation (Dudkiewicz, 1983). In fact, purified acrosin is able to carried out specific digestion of some glycoproteins comprising the zona pellucida (Dumbar *et al.*, 1985; Urch *et al.*, 1985; Nakano *et al.*, 1989).

Mammalian acrosin is found as an inactive zymogen in the acrosomal matrix of non-reacted and non-capacitated spermatozoa (Polakoski & Parrish, 1979; Baba *et al.*, 1989*a*, *b*; Sillerico *et al.*, 1996). After activation and autocatalytic cleavage at amino acid 23, a two-chain active acrosin molecule is formed (α-acrosin) with a light and heavy chain held together by a disulphide bridge (Fock-Nuzel *et al.*, 1984; Baba *et al.*, 1989*a*; Topfer-Petersen *et al.*, 1990*a*). Further processing at the C-terminal generates β-acrosin, the mature acrosin (Polakoski & Parrish, 1977; Baba *et al.*, 1989*b*; Hardy *et al.*, 1991).

Earlier studies showed that the binding of a 53 kDa protein, isolated from boar spermatozoa, had a fucose-binding domain different from the active site (Töpfer-Petersen & Henschen, 1988). Thus, it was thought that the fucose-binding domain of that protein identified as proacrosin had a main role during sperm penetration. However, further studies indicated that sulphated polymers (fucoidan or polyvinylsulphate but not chondroitin sulphates A or C) also inhibit both sperm–zona pellucida and proacrosin–zona pellucida glycoproteins (ZPGs) binding

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*in vitro* (Huang & Yanagimachi, 1984; Jones *et al.*, 1988; Jones, 1991; Urch & Patel, 1991; Töpfer-Petersen *et al.*, 1990*b*). Non-sulphated polymers (desulphated fucoidan, polyvinylphosphate or dextran) do not inhibit this binding (Williams & Jones, 1990; Jones, 1991; Urch & Patel, 1991). In addition, Urch & Hedrick (1988) showed that binding of fucose or fructose to proacrosin may well be artifactual because of the presence of phenolic contaminants. Thus, whether the fucose-binding domain is different from the polysulphate binding domain (PSBD) remains to be determined.

There is evidence that one of the functions of proacrosin is to help to retain spermatozoa on the surface of the zona pellucida during or after the acrosome reaction (for review see Barros *et al.*, 1996). The mechanism of binding between proacrosin/acrosin and ZPGs is thought to involve a stereospecific recognition of polysulphate groups in a manner similar to that found for heparin–antithrombin III interactions (Jones, 1991; Urch & Patel, 1991). On the other hand, *in vitro* binding of proacrosin to high concentrations of fucoidan or ZPGs stimulates conversion of proacrosin to α- or β-acrosin, while simultaneously inhibiting amidase activity (Töpfer-Petersen & Cechova, 1990; Eberspaecher *et al.*, 1991; Lo Leggio *et al.*, 1994). However, there is no evidence that the PSBD involved as secondary binding molecule during zona pellucida penetration by sperm is the same one involved during proacrosin/acrosin activation induced by fucoidan.

On the other hand, acrosin has also been implicated in processes of membrane fusion such as (1) the induction of fusion between the outer acrosomal membrane and the overlying plasma membrane of the acrosomal cap (Meizel & Lui, 1976; Lui & Meizel, 1979) and (2) sperm–oocyte plasma membrane fusion (Wolf, 1977; Dravland & Meizel, 1982; Takano *et al.*, 1993).

Thus it appears that the proacrosin/acrosin system is involved in several steps during mammalian fertilisation, but there is no direct proof of its participation in any of them. Here we show that a monoclonal antibody against human acrosin is directed against the PSBD. This antibody is able to inhibit binding of human sperm to the zona pellucida, but not gamete plasma membrane fusion. Moreover, this monoclonal antibody is also able to block the activation of proacrosin induced by fucoidan.

## Materials and methods

### Reagents

Unless otherwise stated, all the reagents were purchased from Sigma (St Louis, MO) or Merck (Darmstadt, Germany).

### Sperm preparation

Human semen samples were obtained from an infertility clinic following the ethical normative of the Pontifical Catholic University of Chile. After liquefaction, the sample was washed twice in BWW containing 4 mg/ml bovine serum albumin (BWW-BSA) (Biggers *et al.*, 1971). The pellet was covered with 0.5 ml of fresh BWW culture medium containing 35 mg/ml of human serum albumin (BWW-HSA), and left in the incubator for 30 min to allow progressively moving spermatozoa to swim up into the culture medium (Barros *et al.*, 1979).

The highly motile swim-up spermatozoa were recovered and capacitated in drops of 100 µl of BWW-HSA containing 100 000 spermatozoa per drop, for 3 h at 37 °C in a 5% $CO_2$ atmosphere (Barros & Jedlicki, 1985).

### Effect of anti-acrosin monoclonal antibodies on human sperm binding to homologous zona pellucida

To determine the effect of anti-human acrosin monoclonal antibodies in sperm–zona binding, salt-stored human oocytes (2 M ammonium sulphate, 40 mM Hepes and 0.5% dextran, pH 7.0) were purchased from a local supplier. The oocytes were released into BWW culture medium containing 0.3% BSA, 0.1 mM soybean trypsin inhibitor and 10 µg/ml lima bean trypsin inhibitor, and remaining cumulus cells removed by passing through a narrow-bore pipette. As a result of treatment these oocyte were non-viable.

To capacitated human spermatozoa, mononoclonal antibodies C5F10 or E5E10 were added in different concentrations (100, 300 and 700 µg/ml), 15 min before the addition of human zonae pellucidae and the gamete mixture co-incubated for an additional 3 h at 37 °C under 5% $CO_2$. At the end of the incubation period the number of spermatozoa bound to each zona pellucida was recorded.

### Effect of anti-acrosin monoclonal antibodies on the fusion of human spermatozoa with zona-free hamster oocytes

Hamster oocytes without zona pellucida were obtained as described by Barros *et al.* (1984). Briefly, the oviducts of superovulated mature golden hamster females were isolated and placed in a tissue culture Petri dish with TALP-10K culture medium containing BSA at 4 mg/ml (Barros *et al.*, 1984). Oocytes in cumulus were recovered and treated with 0.1% hyaluronidase in BWW-BSA, to eliminate the

cumulus oophorus cells. They were then thoroughly washed by three changes of fresh BWW-BSA. Finally, to eliminate the zona pellucida, they were treated with 0.01% trypsin for 5–10 min and then extensively washed in BWW-BSA (Barros *et al.*, 1984).

To capacitated human spermatozoa the monoclonal antibodies C5F10 or E5E10, were added in different concentrations (100, 300 and 700 μg/ml), 15 min before the addition of zona-free hamster eggs to the drop. They were then co-incubated at 37 °C for 3 h, stained with 0.5% orcein in glacial acetic acid and examined for sperm penetration (Moreno & Barros, 1991).

**Extraction to obtain proacrosin**

Proacrosin as acid sperm extract was obtained according to the method described by Leyton *et al.* (1986). Briefly, semen samples were diluted 10-fold in 150 mM NaCl and incubated for 1 h at 37 °C. The sample was centrifuged at 200 *g* in a clinical centrifuge at room temperature, the pellet resuspended in 0.1% Triton X-100 and centrifuged again. Acrosin was obtained from this pellet in acid solution containing 1 mM HCl, 10% glycerol, 0.02% sodium azide and 50 mM benzamidine, pH 3.0. This suspension was centrifuged at 27 000 *g* for 15 min and the supernatant stored at −20 °C. Inmediately before continuing the experiment a 200 μl aliquot of frozen human acid sperm extract was dialysed overnight against 2 l of 1 mM HCl, pH 3.0.

**Gel electrophoresis and Western-blotting**

The molecular forms of proacrosin and acrosin were studied by one-dimensional polyacrylamide gel electrophoresis (SDS-PAGE) performed according to the method of Laemmli (1970). Samples for analysis were run in a 10% SDS-PAGE under reducing and denaturant conditions, and then transferred to nitrocellulose sheets at 100 V (200 mA) for 2 h, as described by Towbin *et al.* (1979). Nitrocellulose was blocked with 2% BSA in PBS, pH 7.4 (PBS-BSA) and then incubated overnight at 4 °C with a mixture of anti-human acrosin monoclonal antibodies C5F10, A8C10 and C2B10 (30 μg/ml of each) (Valdivia *et al.*, 1994; Sillerico *et al.*, 1996). After extensive washing with PBS plus 0.05% Tween 20 (PBS-Tween), they were incubated with rabbit anti-mouse IgG tagged with alkaline phosphatase at a dilution of 1:10 000 in PBS-BSA for 1 h at room temperature. After the incubation, the nitrocellulose membranes were washed with PBS-Tween and then the immunoreactive bands were visualised with nitro blue tetrazolium (NBT)-bromochloroindolyl (BCIP) substrates prepared in a buffer containing 100 mM Tris, 100 mM NaCl and 5 mM $MgCl_2$.

**Indirect immunofluorescence (IFI)**

Washed spermatozoa were plated on 8-well slides (50 000 spermatozoa per well) (Roboz Surgical Instruments). After drying they were fixed in PBS containing 4% paraformaldehyde for 10 min. They were then rinsed 3 times with PBS and permeabilised with 100% cold methanol for 10 min. These preparations were rapidly stored at 4 °C in a humid chamber.

Fourteen microlitres of anti-acrosin monoclonal antibody diluted in PBS-BSA were added to the wells and incubated for 1 h at room temperature in a humid chamber. After washing in PBS containing 150 mM NaCl, 14 μl of a solution of fluorescein isothiocyanate (FITC)-rabbit anti-mouse IgG diluted 1:200 in PBS-BSA was added and incubated for 1 h as described above. The samples were rinsed three times and mounted in PBS containing 70% glycerol and 2.5% 1,4-diazobicyclic(2,2,2)-octane pH 8.6. Finally, the samples were observed in a Nikon microscope equipped with epifluorescence (Japan).

In order to study the effect of fucoidan on C5F10 monoclonal antibody binding, permeabilised spermatozoa were preincubated with 0 (control), 1 and 10 mg/ml of fucoidan before and during incubation with the first antibody (C5F10, Valdivia *et al.*, 1994; or E5E10, Sepúlveda *et al.*, 1993) in PBS containing 1% BSA. After treating them with fluorescein-tagged second antibody, the number of labelled and non-labelled spermatozoa (between 100 and 150) was recorded and the percentage of fluorescent spermatozoa used as an indicator of antibody binding.

**Enzyme-linked inmunoassay (ELISA)**

Nunc-immuno plates (Nunc Co.) of 96 wells were incubated overnight at 4 °C with 50 μl of PBS, pH 7.0, containing $1 \times 10^6$ spermatozoa. The free reactive sites were blocked by incubating with PBS-BSA for 3 h at room temperature. Fifty microlitres of monoclonal antibody solution (C5F10 or E5E10) in PBS-BSA was added to the well and incubated for 2 h. Then the wells were washed with PBS-Tween and a rabbit α-globulin anti-mouse IgG conjugated to alkaline phosphatase in PBS-BSA was added to the wells and incubated for 1 h at room temperature. The immune complex was developed with the alkaline phosphatase reaction prepared with 1 mg/ml of *p*-nitrophenyl phosphate (PNPP) in 50 mM $NaHCO_3$, 50 mM $Na_2CO_3$, 1 mM $MgCl_2$. The reaction was stopped with 3 M NaOH and the absorbance measured at 405 nm (Harlow &Lane, 1988).

In order to study the effect of fucoidan, heparin and soybean trypsin inhibitor on anti-human acrosin monoclonal antibody binding, permeabilised human spermatozoa were incubated with different concentra-

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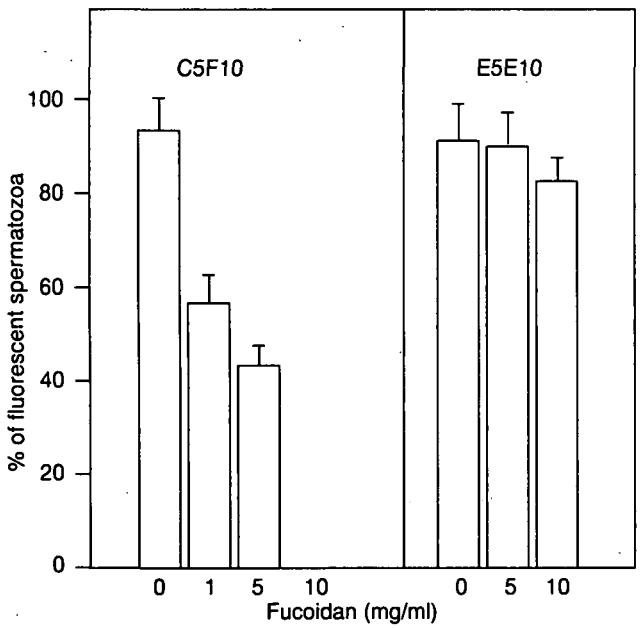


**Figure 1** Effect of fucoidan (1 or 10 mg/ml) on the percentage of fluorescent permeabilised human spermatozoa after treatment with anti human acrosin monoclonal antibody C5F10. Smears of permeabilised human spermatozoa were used to immunolocalise acrosin by indirect inmunofluorescence using the anti-human acrosin monoclonal antibody C5F10. The number of labelled and non-labelled spermatozoa was recorded, between 100 and 150 cells per experiment. The percentage of fluorescent spermatozoa in the control was taken as 100%. When another anti-human acrosin monoclonal antibody, E5E10, was used the fluorescence was not modified by fucoidan.

tions of fucoidan (0–50 mg/ml), heparin (0–50 mg/ml) or soybean trypsin inhibitor (SBTI; 0–50 μg/ml) and then treated with either of the two monoclonal antibodies C5F10 or E5E10.

## Results

### Effect of fucoidan on the binding of anti-human acrosin monoclonal antibodies to human spermatozoa

The fluorescence pattern of permeabilised human spermatozoa treated with anti-human acrosin monoclonal antibodies C5F10 and E5E10 was restricted to the acrosomal region (Plate 1*A*, *B* and *E*, *F* respectively; facing p. 80). When fucoidan was added to the spermatozoa prior to incubation with the antibody C5F10, the fluorescence label disappeared (Plate 1*C*, *D*), but not when antibody E5E10 was used (Plate 1*G*, *H*). Fluorescence inhibition of fucoidan was dose-dependent, reaching a total inhibition of the fluorescence at 10 mg/ml (Fig. 1).

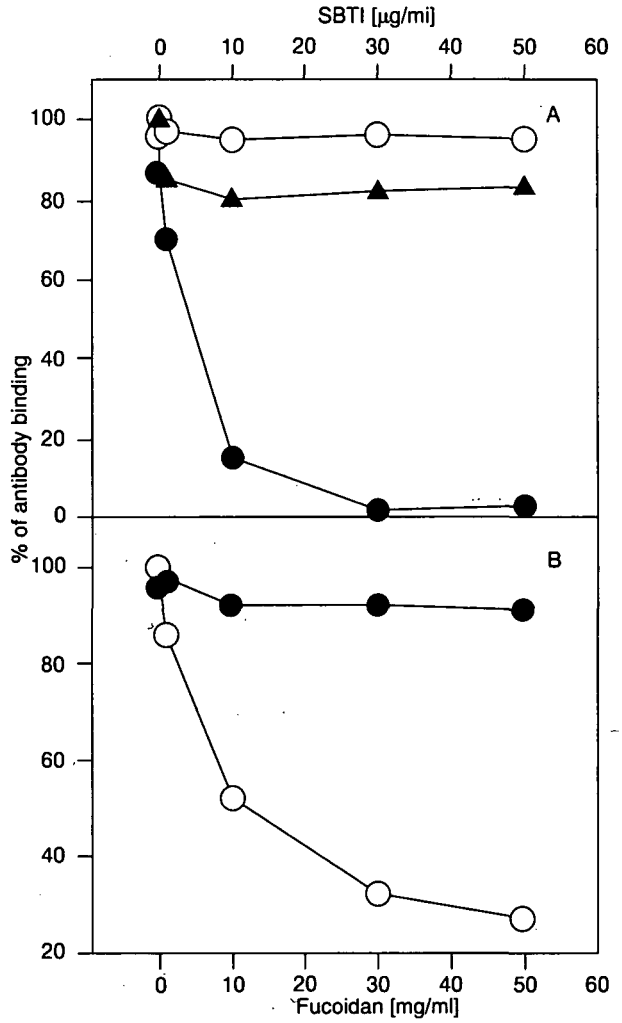


**Figure 2** ELISA of the effect of fucoidan, heparin and soybean trypsin inhibitor on anti-human acrosin monoclonal antibody binding. The binding of the monoclonal antibody C5F10 was inhibited in the presence of fucoidan (*A*, filled circles). On the other hand SBTI (*A*, open circles) or heparin (*A*, filled triangles) had no effect on binding of this antibody to permeabilised human spermatozoa. The binding of the anti-human acrosin E5E10 was inhibited by SBTI but not by fucoidan (*B*, open and filled circles, respectively).

In ELISA plates pre-incubated with fucoidan, the binding of the monoclonal antibody C5F10 was inhibited (Fig. 2*A*). When the spermatozoa were pre-incubated with heparin or the active site inhibitor of acrosin SBTI, the binding of the antibody was not affected (Fig. 2*A*). When human spermatozoa were pre-incubated with chondroitin sulphate A or C, the antibody was not inhibited (data not shown). As a control, the binding of another monoclonal antibody against acrosin (E5E10) was not affected in the presence of fucoidan but inhibited by SBTI (Fig. 2*B*).

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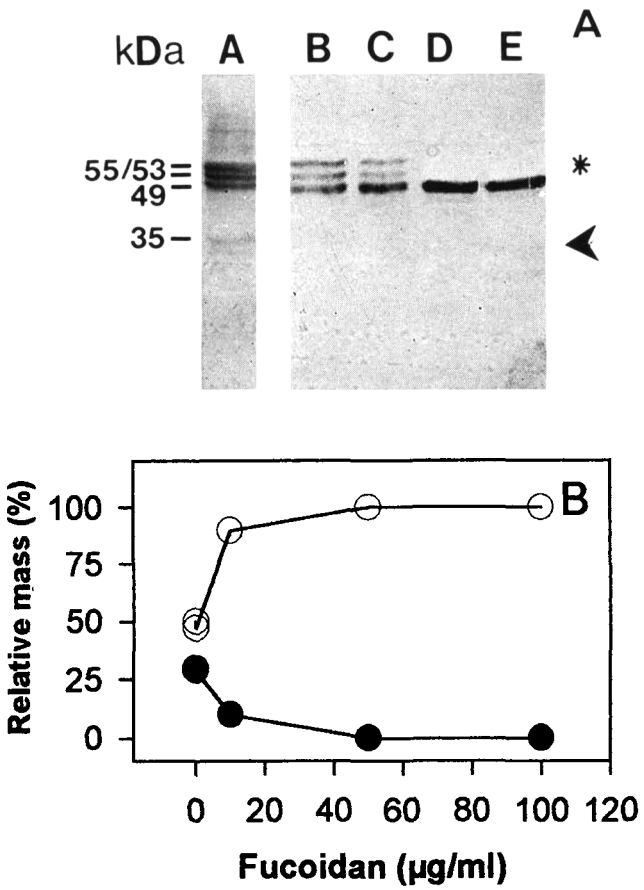


**Figure 3** Effect of fucoidan and anti-human acrosin antibody on proacrosin activation. Western blot of human (*A*) acid sperm extract incubated for 0 or 10 min at room temperature (lanes A, C) or in the presence of 25 and 50 µg/ml of fucoidan (lanes D, and, E., respectively). The molecular forms of acrosin were determined with an oligoclonal antibody against human acrosin. Quantification of band distribution showed that the bands at 55/53 kDa, corresponding to proacrosin, disappeared when the acid extract was incubated with 10 mg/ml or more of fucoidan (*A*, lanes D and, E., asterisk; *B*, filled circles). At the same time a band corresponding to α-acrosin appears (*B*, open circles). When acid extract was pre-incubated with the monoclonal antibody C5F10 (*A*, lane B), the bands at 55/53 kDa (proacrosin) remain as in the control. The arrowhead indicates the migration position of β-acrosin (35 kDa) and the asterisk indicates the position of proacrosin (55/53 kDa).

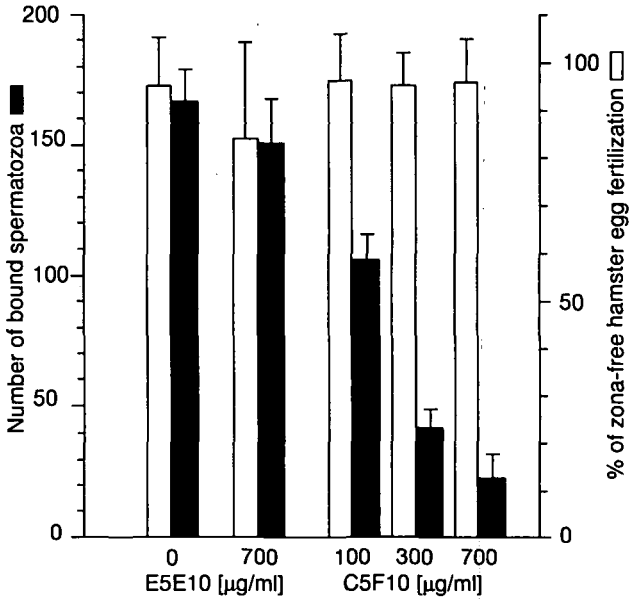


**Figure 4** Effect of anti-human acrosin monoclonal antibodies C5F10 and E5E10 on human sperm binding to homologous zona pellucida. Number of human sperm bound to human zona pellucida (black bars) or percentage of zona-free hamster egg penetration assay (empty bars) without antibody (0), or with monoclonal antibody E5E10 (700 µg/ml) or C5F10 (100, 300 or 700 µg/ml).

### Effect of fucoidan and C5F10 monoclonal antibody on the rate of proacrosin activation

Human sperm acid extract incubated with four different fucoidan concentrations showed progressive inhibition of amidase activity starting at 1–2 µg/ml; complete inhibition was obtained at 40 µg/ml (data not shown). At fucoidan concentrations that were able to inhibit acrosin activation, the Western blots showed that human proacrosin activation (55–53 kDa) into α-acrosin (49 kDa) was complete at 30 µg/ml (Fig. 3*A*, lane D, asterisk) or 50 µg/ml (Fig. 3*A*, lane E, asterisk). Human proacrosin activated for 10 min without fucoidan did not show formation of β-acrosin (35 kDa) as occurs in proacrosin activated for 30 min (Fig. 3*A*, lanes A and C, respectively). A densitometric analysis of the bands in each gel showed that at concentrations equal to or higher than 50 µg/ml, all the proacrosin had become converted into α-acrosin; meanwhile less than 30% of proacrosin is detected in the control where no fucoidan was added (Fig. 3*B*). It is important to mention that under these conditions we could not detect any formation of β-acrosin (Fig. 3*A*, lanes D and E, arrowhead).

On the other hand when the anti-human acrosin monoclonal antibody C5F10 was pre-incubated with human sperm acid extract containing mainly proacrosin and then incubated with fucoidan, 50 µg/ml for 10 min, proacrosin activation induced by fucoidan was completely inhibited (Fig. 4*A*, lane B).

### Effect of the C5F10 monoclonal antibody on sperm–zona pellucida interaction

Binding of capacitated human spermatozoa to homologous zona pellucida was inhibited in a concentration dependent manner when sperm were pre-treated with anti-human monoclonal antibody C5F10 (Fig. 4). The inhibition was not related to a non-specific

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detrimental effect of the antibody on the sperm function, since human spermatozoa treated similarly but with the anti-human monoclonal antibody E5E10 bound to human zona pellucida in numbers not different from the controls (Fig. 4). Moreover 96% of zona-free hamster eggs were fertilised when inseminated with similarly treated spermatozoa or with antibody solvent.

## Discussion

In the present work we have confirmed earlier reports that a monoclonal antibody (C5F10; Valdivia *et al.*, 1994) is directed against PSBD on human acrosin (Sepúlveda *et al.*, 1993). In fact, the binding of this antibody can be prevented by pre-incubation of spermatozoa with fucoidan but not with heparin or the active site inhibitor SBTI. Our results indicated that 50% inhibition ($IC_{50}$) was at 3 mg/ml of fucoidan. The binding of the antibody C5F10 was specific due to the fact that binding of another monoclonal antibody, E5E10, also directed against human acrosin, was not affected by fucoidan but was inhibited by SBTI. These results suggest that both the monoclonal antibody C5F10 and fucoidan are competing for the same binding site on human acrosin.

The role of PSBD of proacrosin/acrosin in mammalian gamete interaction has been inferred from an inhibitory effect in fertilisation exerted by fucose-sulphate-containing polysaccharides such as fucoidan, solubilised homologous zona pellucida glycoproteins or isolated glycan chains from zona pellucida glycoproteins (Huang & Yanagimachi, 1984; Berger *et al.*, 1989; Yurewicz *et al.*, 1991; Windt *et al.*, 1991; Yanagimachi, 1994). However, the localisation of the PSBD is unclear at present. On the other hand, biochemical and molecular biology studies on recombinant boar acrosin have shown that arginine, lysine and histidine residues located between Gly93 and Ala275, together with the participation of His47 and Arg50, are necessary for maximum polysulphate binding activity (Jansen *et al.*, 1995). These results suggest that in boar proacrosin the PSBD is formed by secondary and tertiary folding of the protein bringing together residues that are widely separated in the primary sequence.. On the other hand, site-directed mutagenesis of rabbit proacrosin pointed out that arginine residues Arg 47, Arg 50 and Arg 51 to alanine produced a recombinant without significant zona binding activity (Richardson & O'Rand, 1996). Our results suggest that PSBD on proacrosin/acrosin may be composed of more than one sub-binding site, that is to say, the binding site of fucoidan should be different from the binding site of heparin even though they both belong to the PSBD. Such a binding site has been proposed for other enzymes such as acetylcholinesterase, where an anionic peripheral site located on the surface of the molecule contains many different sub-sites each one with a different affinity for a specific ligand (Radic *et al.*, 1991; Kreinkamp *et al.*, 1991; Ordentlich *et al.*, 1995). Binding of different inhibitors or substrates to this anionic peripheral site modulates the enzyme activity of acetylcholinesterase (Berman *et al.*, 1981; Shafferman *et al.*, 1992). These results could explain why different sulphated polysaccharides such as heparin and fucoidan exert different activities on the inhibition α-acrosin (Lo Leggio *et al.*, 1994). Thus, different sub-sites in PSBD may have different roles in proacrosin activation, acrosin activity inhibition and sperm binding through zona pellucida penetration

The proacrosin/acrosin system has also been implicated in several membrane fusion events during mammalian fertilisation, such as the acrosome reaction (Meizel & Lui, 1976; Lui & Meizel, 1979) and sperm–oocyte fusion (Wolf, 1977; Dravland & Meizel, 1982; Takano *et al.*, 1993). In this work we have shown that monoclonal antibodies against acrosin inhibited only sperm penetration through zona pellucida and not gamete membrane fusion. Thus, we suggest that acrosin is not involved in sperm–oocyte fusion. Since other reports have used serine protease inhibitors and not specific acrosin inhibitor, it is possible that proteases other than acrosin could be involved in this process. Mammalian acrosome is a para-crystalline structure, and probably the molecules of acrosin are in very high concentrations. Therefore, during sperm penetration a high local concentration of acrosin may be acting at the acrosome–zona pellucida interaction (Olson & Winfrey, 1994). This may explain why such high antibody concentrations are needed to inhibit sperm binding.

In this work we have confirmed previous results (Töpfer-Petersen & Cechova, 1990; Eberspaecher *et al.*, 1991; Lo Leggio *et al.*, 1994) showing that fucoidan induces proacrosin activation, even at concentrations where the enzyme activity was completely inhibited. These observations could be explained as conformational changes in the protein induced by polysulphate binding, which expose the endoproteolytic cleavage sites while sterically compromising the active site for artificial substrates (Jones, 1991; Urch & Patel, 1991; Lo Leggio *et al.*, 1994). We have shown above that the epitope of the monoclonal antibody C5F10 is located on or close to the PSBD in proacrosin/acrosin, since C5F10 was able to block the activation rate of proacrosin induced by fucoidan in human proacrosin (Fig. 3). Therefore these results strongly suggest that the same binding domain located on the surface of proacrosin/acrosin is involved in proacrosin activation and binding to the zona pellucida.

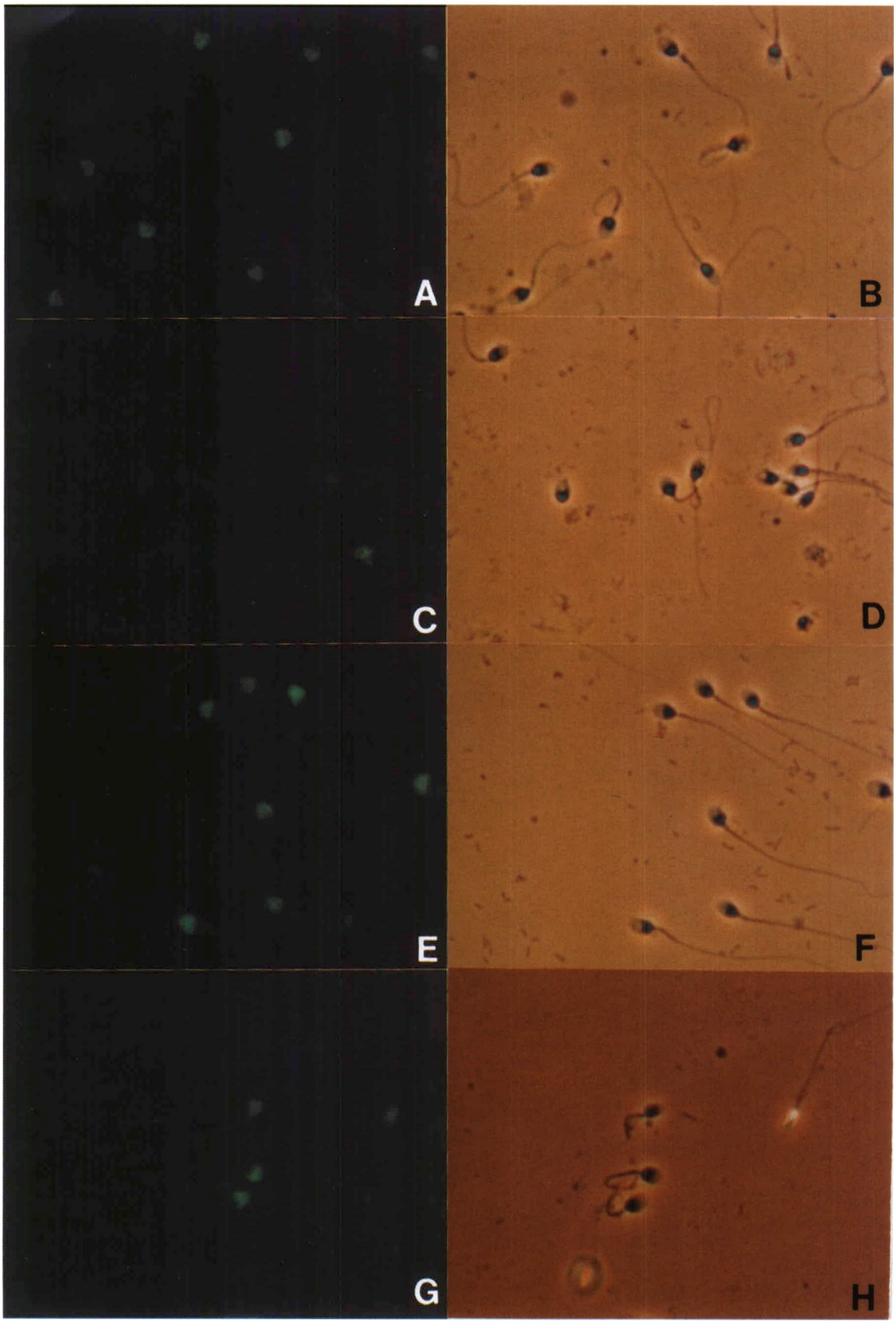


**Plate 1.** Immunofluorescence and phase-contrast micrographs of permeabilised human spermatozoa labelled with the anti-human acrosin monoclonal antibody C5F10 (*A*, *B*). No fluorescent label was observed on the acrosomal region of spermatozoa pre-incubated with fucoidan (10 mg/ml) (*C*, *D*). The use of the anti-human acrosin monoclonal antibody E5E10 (*E*, *F*) was not affected by fucoidan pretreatment (*G*, *H*).

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(*Facing p.* 80)

It is interesting that one domain of acrosin could be controlling both steps in the process of mammalian fertilisation. It could be possible that once the spermatozoon binds and reacts at the zona pellucida surface, the penetration rate could be controlled by the interaction between proacrosin and the sulphated polysaccharides of the zone. This process may stimulate the penetration rate, thus ensuring that the first bound reacted sperm crosses the zona pellucida to fuse with the egg. In addition, a glycosaminoglycan present in tubal fluid and others present in the matrix of cumulus–oophorus such as chondroitin sulphate, is also able to promote *in vitro* the activation of human proacrosin (Wincek *et al.*, 1979; Stambaugh & Mastroianni, 1980; Drahorad *et al.*, 1988, 1991). Thus there is a supporting role for the PSBD also during sperm–cumulus oophorus interaction.

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