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Original Article

Analysis of polysulfate-binding domains in porcine proacrosin, a putative zona adhesion protein from mammalian spermatozoa

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ABSTRACT Proacrosin is one of the major proteins found within the acrosomal vesicle of mammalian spermatozoa. Previous work has shown that it binds non-enzymically and with high affinity to polysulfate groups on zona pellucida glycoproteins (ZPGPs) thereby leading to the hypothesis that at fertilization it functions as a secondary ligand molecule to retain acrosome-reacted spermatozoa on the surface of the egg. In the present work we have investigated the nature and extent of the polysulfate binding domain on boar sperm proacrosin using a combination of group-specific modifying reagents, fragmentation analysis, peptide synthesis and expression of deletion recombinants in *E. coli* bacteria. Taken overall, our results show that arginine, lysine and histidine residues located between Gly 93 and Ala 275, together with the participation of His 47 and Arg 50, are necessary for maximum polysulfate binding activity. The secondary and tertiary structure of this central peptide domain is also important to ensure correct alignment of basic residues with complementary sulfate groups on ZPGPs. Proacrosin, therefore, has many properties in common with other polysulfate binding proteins, such as antithrombin III and sea urchin sperm bindin, in having a conformation-dependent domain containing basic amino acids that mediates specific protein-protein interactions. These observations strengthen the hypothesis that proacrosin is a multifunctional protein with a major role as a ligand molecule at fertilization.

KEY WORDS: *fertilization, zona pellucida, spermatozoa, adhesion molecules*

Introduction

Information on the disposition and structure of the ligand-receptor complexes that mediate gamete recognition and binding is fundamental to an understanding of the molecular mechanisms of fertilization. Much is now known about these molecules in unicellular organisms and in several invertebrate species in which chemotactic peptides, mating factors and adhesion proteins have been characterised and sequenced (reviewed by Snell, 1990). However, the situation in mammals is less well defined and more difficult to interpret. The most definitive evidence to date on the nature of the sperm receptors present on the zona pellucida (ZP) is based on extensive studies in the mouse by Wassarman and co-workers who have shown that different zona glycoproteins (ZPGPs) have specific functions (reviewed by Wassarman, 1990). Glycoprotein ZP3, for example, contains primary receptors and mediates attachment of acrosome-intact spermatozoa whereas ZP2 carries secondary receptors that retain acrosome-reacted spermatozoa on the zona surface. Information on ZPGPs in other species is more fragmentary but it seems likely that a similar situation will emerge.

Identification of the complementary ligand molecule(s) on spermatozoa on the other hand, has proved more problematic. The difficulty is not that potential ligands have not been described; rather, the plethora of disparate candidates has made it impossible to discern common mechanisms or underlying themes. In the mouse, for example, putative ligands include galactosyltransferase (Shur, 1986), a trypsin inhibitor-sensitive site (Benau and Storey, 1987), autophosphorylating protein p95 (Leyton and Saling, 1989), protein sp56 (Bleil and Wassarman, 1990) and a 15 kDa protease inhibitor-binding protein (Poirier *et al.*, 1986). Even allowing for the possibility that different antigens

Abbreviations used in this paper: ZP, zona pellucida; ZPGPs, zona pellucida glycoproteins; PCR, polymerase chain reaction; IPTG, isopropyl ß-D-thiogalactopyranoside; L-BAPNA, L-benzoyl-arginine p-nitroanilide; CNBr, cyanogen bromide; FPLC, fast protein liquid chromatography; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride.

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-16  MLPTAVLLVL AVSVAA
  1  RDNATCDGPC GLRFRQKLES GMRVVGGMSA EPGAWPWMVS LQIFMYHNNR
 51  RYHTCGGILL NSHWVLTAAH CFKNKKKVTD WRLIFGANEV VWGSNKPVKP
101  PLQERFVEEI IIHEKYVSGL EINDIALIKI TPPVPCGPFI GPGCLPQFKA
151  GPPRAPQTCW VTGWGYLKEK GPRTSPTLQE ARVALIDLEL CNSTRWYNGR
201  IRSTNVCAGY PRGKIDTCQG DSGGPLMCRD RAENTFVVVG ITSWGVGCAR
251  AKRPGVYTST WPYLNWIASK IGSNALQMVQ LGTPPRPSTP APPVRPPSVQ
301  TPVRPPWYFQ RPPGPSQQPG SRPRPPAPPP PPPPPPPPPP PPPPPPPPPP
351  QQVSAKPPQA LSFAKRLQQL IEALKGTAFS SGRSYYETET TDLQELPAS
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Fig. 1. Amino acid sequence of porcine proacrosin (from Adham *et al.*, 1989 and Baba *et al.*, 1989). *Arrows indicate internal cleavage sites during autoactivation to ß-acrosin and regions underlined represent synthesised peptides. Six disulphide bridges are present between Cys 6-Cys 136, Cys 10-Cys 144, Cys 53-Cys 71, Cys 159-Cys 228, Cys 191-207 and Cys 218-248. Potential glycosylation sites are found at Asn 3 and Asn 192.*

are required for different facets of the fertilization process, it is difficult to relate this information with knowledge of the sperm receptors on the zona referred to above. A possible exception to this rule is proacrosin. Proacrosin is the zymogen form of the serine protease acrosin (EC 3.4.21.10) present within the acrosomal vesicle of all mammalian spermatozoa and exposed only during the acrosome reaction. It binds selectively and non-enzymically to stereochemically-aligned polysulfate groups on ZPGPs with a Kd of 10^{-8} M (Jones and Brown, 1987; Jones and Williams, 1990; Jones, 1991; Urch and Patel, 1991). On the basis of this and other data (reviewed by Jones, 1990), proacrosin has been assigned a putative function as a secondary ligand molecule to retain acrosome-reacted spermatozoa on the zona surface. Such a hypothesis correlates well with the role of mouse ZP2 as the secondary sperm receptor (Bleil *et al.*, 1988; Mortillo and Wassarman, 1991) since ZP2 is more heavily sulfated than ZP1 or ZP3 (Shimizu *et al.*, 1983) and hence, would have appropriate binding sites for proacrosin.

Sulfate binding is a well-known mechanism for protein-protein and protein-hapten recognition and is found in such diverse systems as binding of leech hirudin to thrombin (Huttner *et al.*, 1991), sea urchin sperm bindin to egg fucans (De Angelis and Glabe, 1988) and heparin to antithrombin III (Evans *et al.*, 1990). A common feature of these proteins is that the sulfate binding site contains a concentration of basic amino acids brought together by tertiary folding of the protein thereby creating a localized region of positive charge on the surface of the molecule. Such a site is usually distinct from other active centers. In antithrombin III for example, the heparin- and thrombin-binding sites are located on different sides of the molecule (Schreuder *et al.*, 1994) and in sea urchin bindin the regions responsible for sulfate recognition, membrane association and species specificity of fertilization are separate from one another (De Angelis and Glabe, 1990; Lopez *et al.*, 1993; Minor *et al.*, 1993; Miraglia and Glabe, 1993). Thus, antithrombin III and bindin are examples of multifunctional proteins with different sites on the molecule having different activities.

In this communication we have investigated the nature and extent of the polysulfate-binding site on boar sperm proacrosin using a combination of group-specific modifying reagents, fragmentation analysis, peptide synthesis and expression of deletion recombinants in *E. coli* bacteria. Results show that a restricted number of basic residues within the central domain of the molecule are crucial for optimal binding activity and further suggest that folding of the protein is necessary to ensure their correct alignment with polysulfate groups on ZPGPs.

Results

Validation of probes

Throughout this work we have used a mixed ZPGP probe rather than purified individual glycoproteins. This is justified on the grounds that (a) all components of the pig ZP bind to proacrosin (Brown and Jones, 1987; Jones 1991). This is not unexpected as all of the ZPGPs are sulfated (Nakano *et al.*, 1990); (b) pig ZPGPs M_r $90x10^{-3}$ and M_r $55x10^{-3}$ (the equivalent of mouse ZP2 and ZP3 respectively) both have a significant inhibitory effect on sperm-

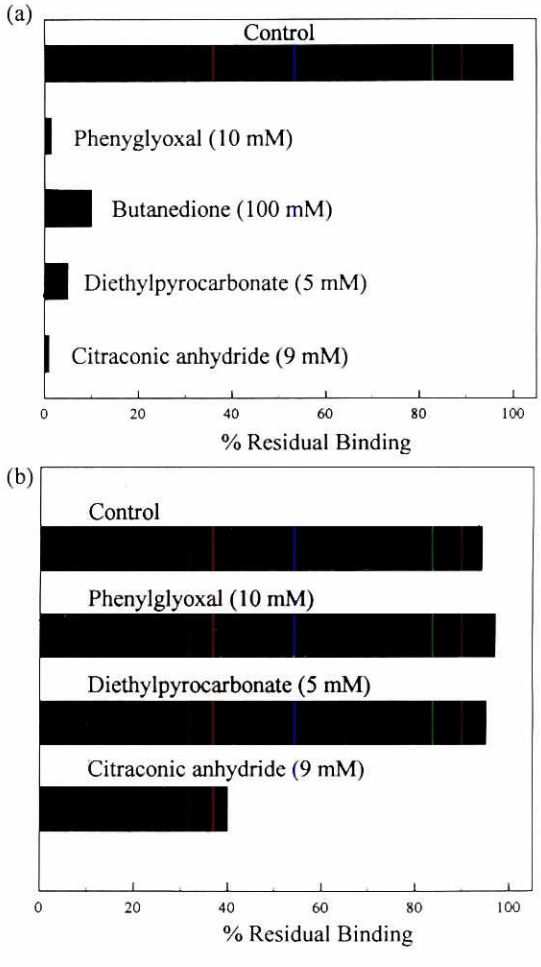

Fig. 2. Effects of group-specific modifying reagents on binding of ^{125}I-ZPGPs to proacrosin. (a) *Pretreatment of proacrosin with 10 mM phenylgloxal, 100 mM butanedione, 5 mM dethylpyrocarbonate and 9 mM citraconic anhydride on binding of ^{125}I-ZPGPs.* **(b)** *Protection of basic amino acids in proacrosin with pre-bound ^{125}I-ZPGPs against group-specific modifying reagents as shown.*

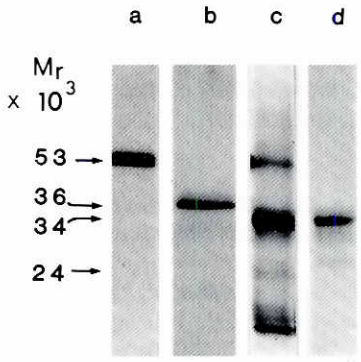

Fig. 3. Fragmentation of proacrosin. *SDS-PAGE of* **(a)** *purified sperm proacrosin;* **(b)** *purified ß-acrosin;* **(c)** *CN-Br cleavage products of proacrosin;* **(d)** *purified M_r 34x10³ CN-Br fragment of proacrosin.*

zona binding *in vitro* (Berger *et al.*, 1989). This suggests that receptor activity does not reside exclusively in one particular ZPGP; (c) a mixture of ZPGPs is closer to the physiological situation on the surface of the intact zona as there may be co-operativity between different ZPGPs for sulfate binding sites on proacrosin.

Binding of ^{125}I-fucoidan was used as an additional check that any observed differences between proacrosin and its expressed fragments were not caused by heterogeneity of the ZPGP probe but by the ability of the target proteins or peptides to recognize polysulfate groups. Fucoidan is a polysulfated polymer that has been shown to bind avidly to proacrosin and to inhibit sperm-zona binding *in vitro* at µM levels comparable to those found for inhibition of ZPGP probes on dot-blots (Jones *et al.*, 1988).

Effects of group-specific modifying reagents on polysulfate-binding activity of proacrosin

To assess whether basic amino acids were important for the polysulfate binding activity of sperm proacrosin, preliminary experiments were carried out with group-specific modifying reagents; phenylglyoxal and 2,3-butanedione (for arginines), diethylpyrocarbonate (for histidines) and citraconic anhydride (for amino lysines). These reagents are not site-specific so it is to be expected that all the 27 arginines, 17 lysines and 5 histidines in proacrosin (Fig. 1) will be derivatized accordingly. As shown in Fig. 2, binding of ^{125}I-ZPGPs to proacrosin was reduced to less than 10% of control values by pretreatment with 10 mM phenylglyoxal, 100 mM 2,3-butanadione, 5 mM diethylpyrocarbonate or 9 mM citraconic anhydride. Preincubation of proacrosin with ^{125}I-ZPGPs on the other hand, protected arginine and histidine residues against inactivation by 10 mM phenylglyoxal and 5 mM diethylpyrocarbonate to the extent that less than 10% of the probe was displaced. Protection of amino lysines against 9 mM citraconic anhydride was less efficient with the result that only ~40% of ^{125}I-ZPGPs remained bound after 1 h incubation. Proacrosin, therefore, is similar to many other polysulfate-binding proteins in having basic residues close to or within the binding site.

Identification of polysulfate-binding domains on sperm proacrosin by fragmentation analysis

Activation of proacrosin to its enzymically stable form, ß-acrosin, takes place by endoproteolytic processing at both the N- and C-terminal ends (Baba *et al.*, 1989). Sequential internal cleavages at the C-terminus removes 3 fragments (a total of 77 amino acids) that are rich in prolines and which are then lost from the molecule. At the N-terminus a single cleavage between residues Arg 23 and Val 24 produces a peptide (the so-called "light chain") that remains cross-linked to the remainder of the molecule (the "heavy chain") by 2 disulfide bridges (Fig. 1). The net result is a reduction in size of the protein from M_r ~55x10³ to ~36x10³ (Fig. 3). This "natural" fragmentation process affords a ready means of assessing the relative importance of the proline-rich C-terminus for polysulfate binding. Purified ß-acrosin, which had a specific activity of 162±7.4 mU/µg protein (1 Unit enzyme will convert 1 µmole substrate/min at 30°C) and gave a single band on SDS gels at M_r ~36x10³, had similar relative binding capacities for ^{125}I-ZPGP and ^{125}I-fucoidan probes as its zymogen form, suggesting that the 77 residue proline-rich C-terminus is not crucial for polysulfate-binding activity either directly or indirectly (Table 1). Furthermore, reduced and alkylated ß-acrosin retained >90% of its ^{125}I-ZPGP-binding activity implying that the N-terminal light chain, which would be removed under these circumstances, is of minor importance for sulfate binding.

Further fragmentation of proacrosin was then achieved by CNBr cleavage. Proacrosin contains a total of 6 methionine residues, 4 of which occur within the first 45 amino acid residues from the N-terminus (Fig. 1). The 2 largest peptides produced as a result of CNBr treatment had M_r of 34x10³ and 24x10³ after SDS-PAGE (Fig. 3). N-terminal sequencing of the M_r 34x10³ peptide produced 2 sequences, RDNAT.... and YHNNR...., representing residues 1-5 and 46-50 respectively. This indicates that cleavage had taken place at Met 24 and Met 45 with loss of the intervening peptide. It was not possible to establish precisely the length of the M_r 34x10³ peptide by C-terminal sequencing but the fact that the M_r 24x10³ peptide had the sequence VGLGT.... suggests that cleavage had also taken place at Met 278. The exact size of the peptide comprising residues 1-22 plus 46-278 calculates as 34.075x10³ which is close to the estimated value from SDS-

TABLE 1

RELATIVE BINDING OF ^{125}I-ZPGP AND ^{125}I-FUCOIDAN PROBES TO SPERM PROACROSIN, ß-ACROSIN, M_r 34X10³ FRAGMENT, POLYARGININE AND SYNTHETIC PEPTIDES

	^{125}I-ZPGPs	^{125}I-fucoidan
Proacrosin	100.0	100.0
ß-acrosin	97.7	105.6
34x10³ CN-Br fragment	84.0	73.0
Proacrosin+SDS (1%)	17.3	21.9
Polyarginine (Sigma)	9.6	-
Arginine x5	0.1	-
Arginine x20	0.3	-
Arginine x25	1.3	-
Arginine x30	5.8	-
Peptide P2 (F44-C55)	0.2	0.4
Peptide P3 (R1-R23)	0.4	1.7
Peptide P4 (W64-L83)	0.7	2.4
Peptide P6 (V24-M45)	0.2	0.6
Peptide P11 (G247-G272)	0.4	1.8
Peptide P12 (S222-V246)	0.8	2.4
Peptide P13 (Y197-G220)	0.3	0.9
Peptide P14 (G171-W196)	0.4	1.1

Proacrosin is taken as 100

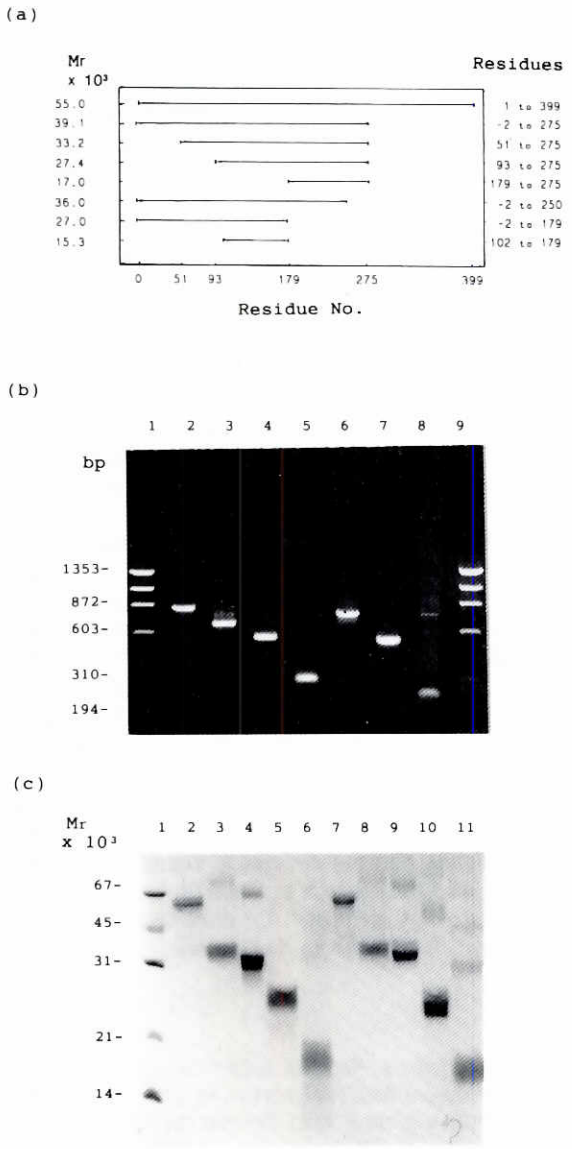

Fig. 4. Bacterial expression of recombinant forms of proacrosin. (a) *Diagram indicating relative lengths of fragments expressed in bacteria. The top bar at M_r 55x10³ represents native sperm proacrosin.* **(b)** *Agarose gel of proacrosin DNA inserts amplified by PCR. (1) and (9) ø 174. Hae III size markers. Remaining inserts represent fragments as follows: (2) residues -2 to 275; (3) residues 51 to 275; (4) residues 93 to 275; (5) residues 179 - 275; (6) residues -2 to 250; (7) residues -2 to 179; (8) residues 102 to 179.* **(c)** *SDS-PAGE of purified expressed proteins. (1) M_r marker proteins; (2) and (7) native sperm proacrosin; (3) and (8) M_r 39.1x10³ fragment; (4) M_r 33.2x10³ fragment; (5) M_r 27.4x10³ fragment; (6) M_r 17x10³ fragment; (9) M_r 36x10³ fragment; (10) M_r 27x10³ fragment; (11) M_r 15.3x10³ fragment.*

PAGE. The M_r 34x10³ fragment retained 73%-84% of the binding capacity of whole proacrosin towards ^{125}I-ZPGPs and ^{125}I-fucoidan probes (Table 1) suggesting that while regions lying between residues Met 278 and Arg 322 may have some polysulfate-binding activity, the principal sites are between Met 45 and Met 278 with its attached light chain. To assess the contribution of the light chain directly, a peptide representing residues 1 to 23 (peptide P3; calculated pI 8.6) was synthesised and tested in the dot-blot assay against both probes. Less than 1% of the binding capacity of native sperm proacrosin was observed (Table 1).

Analysis of polysulfate binding domains in sperm proacrosin by expression of deletion-recombinant proteins in E. coli bacteria

The previous results have shown that although basic amino acids in proacrosin are involved in polysulfate binding, not all of the arginines, lysines and histidines are necessary. From the fragmentation analysis it would seem that 4 arginines and 1 lysine in the N-terminal "light" chain and 3 arginines and 3 lysines in the 77 residue C-terminus, make little or no contribution. To delineate more precisely the regions of the protein that are responsible for polysulfate binding, selected fragments of proacrosin cDNA were amplified by PCR, ligated into a pRSET T7 vector system and expressed in *E. coli* JM109 as a fusion protein. A total of 7 cDNA fragments of varying lengths (Fig. 4a and b) were expressed, the proteins of predicted size identified on SDS-PAGE and purified by electroelution (Fig. 4c). After removal of SDS and renaturation through guanidine HCl, proteins were tested on dot blots for binding of ^{125}I-ZPGP and ^{125}I-fucoidan probes. An internal control consisting of purified sperm proacrosin, was subjected to the same SDS-PAGE, elution and renaturation conditions as the expressed fragments. This control was necessary in view of the earlier observation that traces of SDS (an anionic detergent) strongly inhibit uptake of ^{125}I-ZPGP and ^{125}I-fucoidan probes (Table 1).

The longest fragment expressed contained residues -2 to 275 (Fig. 4a) with an M_r 39.1x10³ taking into account the pRSET T7 leader sequence. This fragment bound ^{125}I-ZPGP and ^{125}I-fucoidan probes to the same, or slightly higher, extent as did native sperm proacrosin (Fig. 5). To verify that this binding was

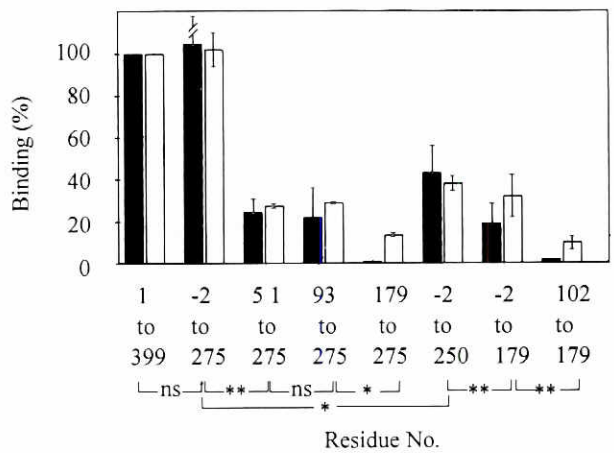

Fig. 5. Relative binding (%) of ^{125}I-ZPGPs and ^{125}I-fucoidan probes to native sperm proacrosin (residues 1 to 399) and bacterial recombinant fragments (residues as indicated). ■, *^{125}I-ZPGP probe.* ❐, *^{125}I-fucoidan probe. ns, not significant. *P<0.05; **P<0.01. Values are means± SEM.*

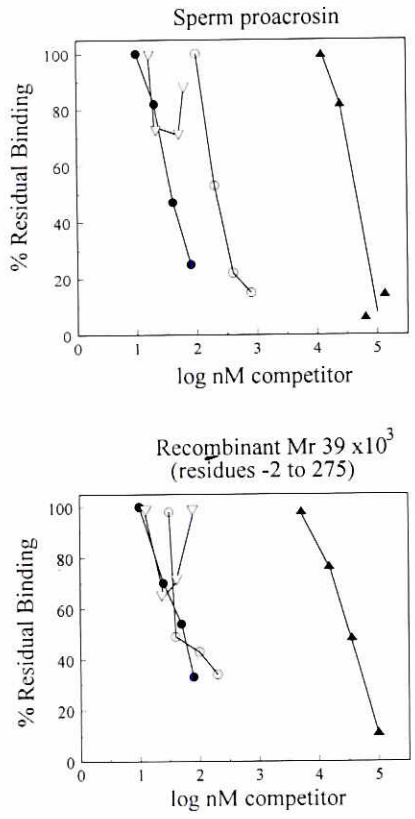

Fig. 6. Mechanism of binding of ^{125}I-ZPGPs to sperm proacrosin and bacterial fragment M_r 39.1x10³. *Inhibition of binding of ^{125}I-ZPGPs to native proacrosin and bacterial M_r 39.1x10³ fragment by fucoidan (●-●); dextran sulfate (M_r 500x10³) (○-○); polyvinylsulfate (▲-▲); dextran (M_r 500x10³) (∇-∇).*

not caused by the polyhistidine metal binding site in the vector, (a continuous sequence of 6 histidines) the same fragment was expressed in pET 23a vector which lacks the polyhistidine sequence. The affinity of this recombinant protein for ^{125}I-ZPGPs and ^{125}I-fucoidan probes was indistinguishable from that produced by the pRSET T7 vector (results not shown) indicating that short stretches of basic residues on their own do not have significant sulfate-binding activity.

To confirm that the mechanism of binding of ^{125}I-ZPGPs and ^{125}I-fucoidan probes to the M_r 39.1x10³ protein involved polysulfate groups and was similar to that described for native sperm proacrosin, inhibition assays were carried out with fucoidan, dextran sulfate, polyvinylsulfate and dextran. As shown in Fig. 6, the results for proacrosin and the M_r 39.1x10³ expressed protein were quantitatively similar with an IC_{50} for fucoidan of 0.36 μM versus 0.13 μM, for dextran sulfate (M_r 500, x10³) 0.04 μM versus 0.05 μM, for polyvinylsulfate 47.0 μM versus 54.0 μM and for dextran >400 mM versus >400 mM (in each case the values refer to proacrosin versus M_r 39.1x10³ protein, respectively). Scatchard plot analysis of the affinity of ^{125}I-fucoidan probe for proacrosin gave a Kd= 1.2x10^{-7} M and for the M_r 39.1x10³ protein Kd= 1.1x10^{-7} M.

Therefore, the C-terminal 124 amino acids in proacrosin do not participate directly in polysulfate binding despite containing 8 arginines and 3 lysines. However, removal of a further 25 residues from the C-terminus (fragment -2 to 250) caused a sharp decrease in binding of ^{125}I-ZPGP and ^{125}I-fucoidan probes to 43% and 38% respectively of controls. Truncating this fragment again by an additional 71 amino acids (residues - 2 to 179) reduced binding further to levels as low as 20% to 30% of controls. The small fragment spanning residues 179 to 275 contained little or no activity on its own (Fig. 5).

Shortening the protein from the N-terminus by 51 residues (fragment 51 to 275) severely reduced its binding activity for ^{125}I-ZPGP and ^{125}I-fucoidan probes to 24% and 27% respectively of control levels (Fig. 5). Since loss of the first 23 N-terminal amino acids (the "light" chain) appears to have only a marginal effect (Table 1, see earlier), the intervening peptide (residues 24 to 49) containing 1 histidine at position 47 and 1 arginine at position 50 would seem to be important. Extending the N-terminus to residue 93 (fragment 93 to 275) did not cause any further reduction in binding of either probe but beyond residue 93 (fragment 179 to 275) binding activity fell to <10% of controls. Again the short intervening fragment spanning residues 102 to 179 had very low activity on its own.

Polysulfate binding activity of synthetic peptides derived from proacrosin

Recently, it has been shown that polymers of basic amino acids (polylysine, polyhistidine and polyarginine) have weak binding activity for ^{125}I-ZPGPs and ^{125}I-fucoidan probes whereas other polyamino acids (e.g. polyproline) have little or no ability to bind (Table 1; see also Williams and Jones, 1993). We speculated that regions of the primary sequence of proacrosin containing clusters of lysines, arginines or histidines are potential polysulfate binding sites. Since commercial preparations of polyamino acids are very heterogeneous with respect to chain length (for "Sigma" polyarginine the range is 28 to 84 residues), we assessed the minimum polymer size required for significant binding of ^{125}I-ZPGP and ^{125}I-fucoidan probes. Linear polymers of arginine containing 5, 20, 25 and 30 residues were synthesized and tested in the dot-blot assay. As shown in Table 1, significant binding could only be detected with chain lengths of >25 arginines and none of these were comparable to that of sperm proacrosin. On this basis therefore, it seems unlikely that short linear stretches of basic amino acids would, on their own, constitute a sulfate binding site. However, the conformation of these peptides is not known and probably bears little relationship to that of the native protein. Therefore, synthetic peptides 20 to 25 residues long representing various regions of the proacrosin sequence (Fig. 1) were tested for their relative binding activity for ^{125}I-ZPGPs. All peptides, P2, P3,

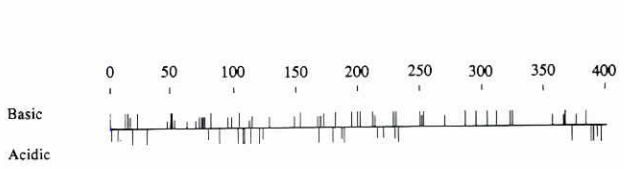

Fig. 7. Distribution of basic and acidic residues in porcine proacrosin. *Height of vertical bar indicates residue. Basics; (|) arginine; (|) lysine; (|) histidine. Acidics; (|) glutamic acid; (|) aspartic acid.*

P4, P6, P11, P12, P13 and P14, showed <10% binding activity irrespective of the region from which they were derived (Table 1).

Discussion

The major objectives of this work were first, to identify the types of amino acids in boar sperm proacrosin that interact with polysulfate groups and constitute the ZPGP-binding site, and second, to define the position and limitations of this site within the primary sequence. For this purpose we have used a combination of group-specific modifying reagents, fragmentation analysis, peptide synthesis and expression of deletion recombinants in *E. coli* bacteria. Results show that arginine, lysine and histidine residues are responsible for polysulfate binding and although these amino acids are distributed evenly throughout the primary sequence, the binding site is restricted to a central domain lying between Gly 93 and Ala 275 with important contributions from His 47 and Arg 50. This suggests that protein folding is important for the organization of such a site in order to achieve the correct spatial configuration of positively charged residues.

The involvement of polysulfate groups in the mechanism of binding of ZPGPs to sperm proacrosin has been demonstrated in the ram (Williams and Jones, 1990), guinea pig (Jones and Williams, 1990) and boar (Jones, 1991; Urch and Patel, 1991) using competition assays with sulfated and non-sulfated polymers. Our current working hypothesis predicts that the polysulfate "receptors" are located on the ZPGPs and the complementary binding site(s) on proacrosin. Evidence to support this interpretation comes from direct analysis of sulfate in purified pig ZPGPs (Nakano *et al.*, 1990) and the incorporation of $^{35}SO_4$ in the ZP of growing mouse oocytes cultured *in vitro* (Shimizu *et al.*, 1983). The sulfate groups are associated primarily with the carbohydrate moiety (Nakano *et al.*, 1990) which would provide the repeating polymeric framework necessary for their correct spatial alignment. This alignment determines to a large extent the specificity of the protein-sulfate interaction as ZPGPs show considerable selectivity for proacrosin, even when challenged with very basic proteins such as histone H1 or cytochrome c (Williams and Jones, 1993). By contrast, proacrosin is less discriminating and will bind a variety of sulfated polymers (e.g. fucoidan, dextran sulfate, galactan sulfate, polyvinyl sulfate), albeit with different affinities. At present, the spatial orientation of sulfate groups on the ZPGP sugar chains is not known so it is not possible to make predictions regarding the dimensions of the binding site on proacrosin. However, earlier studies have shown that more than one sulfate group is necessary implying that the binding site on proacrosin contains several "contact" points (Jones, 1991; Urch and Patel, 1991).

Chemical modifying reagents have been widely used to demonstrate the importance of positively charged amino acids in proteins that bind polyanionic substrates and ligands (Borders and Riordan, 1975; Riordan *et al.*, 1977). Arginine for example, is present within the catalytic site of many glycolytic enzymes and it has been suggested that this has evolved as a mechanism for distinguishing between phosphorylated and non-phosphorylated substrates (Riordan *et al.*, 1977). The modification conditions used in the present investigation are those shown previously to optimise the group specificity of each reagent (Lunblad and Noyes, 1984; De Angelis and Glabe, 1988) although the results should be interpreted with caution as we have not investigated the kinetics of the reactions or characterised the derivatized products. Nonetheless, results clearly show that 10 mM phenylglyoxal, 100 mM 2,3-butanedione, 5 mM diethylpyrocarbonate and 9 mM citraconic anhydride quantitatively inactivate ^{125}I-ZPGP binding to proacrosin, suggesting that the polysulfate binding site contains at least 1 arginine, 1 lysine and 1 histidine. Protection experiments using ZPGPs pre-bound to proacrosin confirmed this conclusion although we were not able to identify unmodified (i.e. protected) residues because of the difficulty of dissociating the ZPGP probe from its binding site. Therefore, in the remaining experiments we sought to define the extent of this site using synthetic peptides, fragmentation of purified sperm proacrosin and expression of deletion recombinants in *E. coli* bacteria.

The results obtained with reduced ß-acrosin indicate that the first 23 amino acids at the N-terminus and the last 77 amino acids at the C-terminus are unlikely to be involved in polysulfate binding as their removal during proacrosin activation has no significant effect on uptake of either ^{125}I-ZPGPs or ^{125}I-fucoidan. Similarly, the absence of residues 22 to 44 in the M_r $34x10^3$ protein generated by CNBr cleavage of proacrosin has only a small effect (~15%). These results were confirmed and extended with the use of recombinant proteins which allowed a more controlled definition of the dimensions of each fragment. Proacrosin contains a total of 27 arginines, 17 lysines and 5 histidines out of 399 residues. These amino acids are distributed uniformly throughout the primary sequence with only occasional clusters (Fig. 7). Deletion of a 124 residue peptide containing 8 arginines and 3 lysines from the C-terminus (recombinant fragment -2 to 275) to yield a M_r $39.1x10^3$ protein has no significant effect on binding of either I-ZPGP or ^{125}I-fucoidan probes. However, truncating the protein any further from position 275 caused a sharp drop in binding activity. At the N-terminus, deletion of a 50 residue peptide lowered binding to approximately 25% of controls. Since removal of the first 23 residues containing 4 arginines and 1 lysine has little effect, it may be deduced that the intervening peptide between positions 24 and 50 containing 1 histidine (His 47) and 1 arginine (Arg 50) is important for maximum binding activity. Furthermore, the recombinant fragments representing residues 51 to 275 and residues 93 to 275 have similar binding activities which suggests that the 2 arginines, 4 lysines and 4 histidines lying between positions 51 and 93 are not crucial for the polysulfate binding site. Beyond residue 93 however, any further deletion caused a precipitous drop in ZPGP-binding activity. On this basis it can be deduced that not more than 12 arginines, 9 lysines and 1 histidine constitute the polysulfate binding site in boar proacrosin and that they lie between positions Gly 93 and Ala 275 with the participation of His 47 and Arg 50.

In an attempt to delineate this site further, synthetic peptides, 20 to 25 residues long, were selected from different regions of the sequence and measured for relative ZPGP-binding activity. The low binding activity of peptides P2, P3, P4 and P6 was not unexpected since they are derived from the extreme N-terminus but peptides P11, P12, P13 and P14 are all from within the putative polysulfate binding domain, yet their activity was <5% of intact proacrosin. This situation contrasts with that found in sea urchin sperm bindin in which an internal nonapeptide containing only 2 arginines and 2 histidines had a similar level of fucan binding as the whole protein (De Angelis and Glabe, 1990).

Treatment of this peptide with phenylglyoxal or diethylpyrocarbonate completely inactivated fucan binding confirming the validity of using these modifying reagents. Significantly, replacing one of the histidines with a serine caused a 6- to 16-fold decrease in fucan binding depending on the position of the substitution. Minor *et al.* (1993) found that a 20-residue peptide containing the bindin nonapeptide sequence was a potent inhibitor of fertilization in 2 species of sea urchins reinforcing the view that specific regions of the molecule are involved in sperm-egg contact. Preliminary experiments to investigate the biological effects of the proacrosin peptides on fertilization *in vitro* using live pig eggs have shown that peptide P12 significantly reduces sperm binding to the zona whereas peptide P4 inhibits zona penetration (M. Yoshida and R. Jones, unpublished observations). P12 contains only 2 arginines but, by analogy to the bindin nonapeptide, it may have sufficient affinity for appropriately aligned sulfate groups on the ZPGPs to cause an effect at a cellular level that is not readily detectable biochemically. This may be a reflection of the stereochemistry of the interaction. Unfortunately, the bacterially expressed fragments are not soluble above pH 6.0, precluding an assessment of their effects on fertilization.

Overall, we interpret our results to mean that in proacrosin the polysulfate binding site is created by secondary and tertiary folding of the protein bringing together residues that are widely separated in the primary sequence. This would help to explain why synthetic peptides and short recombinant fragments show poor binding activity; they are of insufficient length to fold into the correct configuration. A precedent for this is found in human antithrombin III in which the primary heparin-binding site is formed by close alignment of two α-helices that allow Arg 47, Lys 114, Lys 125, Arg 129, Arg 132 and Lys 133 to come into close proximity and form a localised region of positive charge (antithrombin III has an overall pI of 5.75). A recent X-ray crystallographic analysis of antithrombin III shows that the dimensions of this region are approximately equal to the size of the heparin pentasaccharide (Schreuder *et al.*, 1994). Computer modelling studies of sperm proacrosin have revealed several clusters of basic residues along one face of the protein that are a potential polysulfate binding site but without accurate information on its crystal structure such predictions must remain speculative (S. Jansen, R, Jones and J. Coadwell, unpublished observations). This information, however, would be valuable for the design of future contraceptive agents that, by utilizing the stereochemistry of the polysulfate binding site to achieve specificity of recognition, could block the action of proacrosin and hence prevent fertilization. Such a drug is currently under investigation in our laboratories.

An interesting observation made in the course of these experiments was that the bacterial M_r 39.1×10^3 recombinant fragment had consistently higher (~10%) binding activity than the native sperm protein. One obvious explanation for this difference is that the bacterial protein is not glycosylated. Sperm proacrosin has 2 potential N-glycosylation sites at Asn 3 and Asn 192, the latter lying well within the polysulfate binding domain. The presence of a carbohydrate chain in this region could conceivably have steric effects, especially if the terminal sugar was negatively charged. Precisely this situation has been found in mutant forms of human antithrombin III in which loss of carbohydrate at Asn 135 increases heparin affinity (Brennan *et al.*, 1987) whereas gaining an oligosaccharide chain at Ile 7 (by conversion to Asn) decreases affinity (Evans *et al.*, 1990). Both of these residues flank the heparin binding site in the native folded protein.

Materials and Methods

Chemicals

All routine chemicals were of the highest purity available commercially and were purchased from Sigma Chemicals, British Drug Houses or Pierce. Restriction enzymes and molecular biology grade chemicals were obtained from Boehringer Corporation, Pharmacia-LKB, Promega and Stratagene. Nitrocellulose membranes were supplied by Schleicher and Schuell and ^{125}I-Na by Amersham International.

Extraction and purification of proacrosin from boar spermatozoa

Proacrosin was extracted from washed ejaculated boar spermatozoa into 10% glycerol/1 mM HCl pH 3.0 for 16 h at 4°C as described previously (Polakoski and Parrish, 1977), and purified to homogeneity by chromatography on an FPLC Superose 12 column in 6 M guanidine HCl/0.5 M sodium acetate pH 3.0 (Lo Leggio *et al.*, 1994). Purified ß-acrosin was obtained by allowing proacrosin to autoactivate in 0.1 M Tris/HCl pH 8.5 at 4°C for 24 h followed by FPLC on Superose 12 column as described above.

Preparation of ^{125}I-fucoidan and ^{125}I-zona pellucida glycoprotein probes

Whole zona pellucidae were recovered from minced pig ovaries onto 75 μm nylon mesh screens (Hedrick and Wardrip, 1987) and further purified on Percoll step gradients (10%/20%/30% Percoll in 130 mM NaCl, 10 mM Na_2HPO_4, 2 mM EDTA, 10 mM Na citrate, 0.2% polyvinylalcohol) by centrifugation at 2000g for 15 min. Zonae were solubilized in 5 mM NH_4HCO_3 pH 9.0 at 65°C for 30 min, centrifuged at 10000g for 10 min and supernatant proteins (~10 μg) radiolabeled with 50 μCi ^{125}I in 300 μl sterile PBS and 50 μg 1,3,4,6-tetrachloro-3α, 6α-diphenylglycouril ("Iodogen"; Markwell and Fox, 1978; Jones 1991) for 20 min at room temperature. ^{125}I-ZPGPs were stored frozen in Eppendorf tubes that had previously been blocked with 0.1% BSA to prevent non-specific loss of probe. The specific activity of ^{125}I-ZPGP probes was 20,000-30,000 cpm/μg protein.

Fucoidan was purified from commercial material (Sigma) by ß-elimination and pronase digestion (De Angelis and Glabe, 1987; Williams and Jones, 1990), conjugated with fluoresceinamine and iodinated with "Iodogen" as described above. A composition analysis showed that this material contained 75% fucose, 8% xylose, 5% galactose, 1% mannose, 1% arabinose, 2% glucose, 8% uronic acid and 2% unidentified material (Williams and Jones, 1990). Fucoidan concentrations were estimated colorimetrically with orcinol-H_2SO_4 reagent (Vasseur, 1948).

Expression of proacrosin cDNA and derived fragments in E. coli bacteria and purification of recombinant proteins

The proacrosin cDNA clones, λBA_2, λBA_3 and λBA_5, (Adham *et al.*, 1989) were a generous gift from Prof. W. Engel, University of Göttingen. Amplification of proacrosin inserts was achieved by PCR. The PCR-derived products were spliced by overlap extension polymerase chain reaction (Horton *et al.*, 1989) to give the full-length proacrosin sequence. Fragments of a defined length were generated by PCR to amplify the desired regions using the full length proacrosin sequence as a template. Where necessary, mutagenic primers containing a *Bam* HI or in one case, an Asp 718 restriction site in the forward primer and a *Hind* III restriction site in the reverse primer, were used to obtain in-frame cloning into the multicloning site of a pRSET T7 expression vector. The reverse primer also contained a double TAA stop codon adjacent to the 5' end of the restriction site to control the ending of the translation. The choice of the most appropriate vector from the three reading frames available depended on the initial selection of the mutagenic primers that fitted best with the proacrosin sequence.

E. coli JM 109 cultures were transformed with pRSET T7 plasmids containing the corresponding proacrosin DNA fragment. Cultures were grown to saturation and plasmid DNA was recovered following the miniprep protocol of Sanders and Burke (1990). Small amounts of this plasmid solution were used for restriction enzyme analysis to confirm the presence and correct length of the insert. The remainder was treated with the restriction enzyme *Nco* I and subsequently used to transform *E. coli* BL 21 (DE3). Although there is no restriction site within the proacrosin DNA insert for *Nco* I, it cleaved the multicloning site that is replaced by the insert after ligation. This served to linearize those plasmids without the appropriate insert in order to eliminate growth of unwanted plasmids after transformation of BL 21 (DE3) bacteria. This strain also contains the pLysS plasmid that produces small amounts of T7 lysozyme. The pLysS plasmid conveys resistance to chloramphenicol. T7 lysozyme is the natural inhibitor of T7 polymerase and is supposed to reduce unspecific background transcription before induction of bacteria (Studier *et al.*, 1990). This serves to prevent loss of plasmids during the growth phase when expressing genes that are toxic to *E. coli*. Cultures of 100 ml were grown to an OD_{600} of 0.7 and induced with 0.3 mM IPTG. Bacteria were harvested by centrifugation for 10 min at 2500g, resuspended in 6 ml H_2O, frozen at -20°C overnight and slowly thawed to room temperature the next day. Ethanol (6 ml) was added to the bacterial suspension, vortexed for 20 sec and sonicated (W-225 Sonicator at medium output for 1 min). Inclusion bodies were pelleted by centrifugation for 10 min at 8000g and washed once in a 0.1% Triton X-100 solution. After two subsequent washes in H_2O to remove traces of Triton X-100 pelleted inclusion bodies were extracted with 2% SDS in 62.5 mM Tris/HCl pH 7.2. Solubilized proteins were separated on preparative non-reducing SDS polyacrylamide gels, side tracks stained with Coomassie Blue R-250 and recombinant proteins of interest excised from the unstained portion of the gel for electroelution (14 h in an ISCO model 1750 concentrator). Eluted proteins were precipitated with 4 vols acetone on dry ice to remove SDS, pellets solubilized in 6 M guanidine HCl pH 5.8 and protein renatured by rapid dilution into 1 mM HCl pH 3 as described by Hager and Burgess (1980). Control material consisted of previously purified sperm proacrosin treated in an identical fashion. Preliminary experiments showed there was no difference in binding activity between the original purified proacrosin and denatured-renatured proacrosin indicating that the protein had re-folded successfully.

Dot-blot assay for binding of ^{125}I-ZPGPs and ^{125}I-fucoidan to proacrosin and recombinant proteins

Binding of iodinated probes to purified proacrosin and recombinant proteins was measured quantitatively by dot-blot assays (Jones, 1991). Non-specific binding is defined as the amount of probe remaining on dot-blots in the absence of target protein and specific binding as the amount of probe bound to target protein minus non-specific background. All assays were carried out in duplicate and experiments repeated at least twice. The dot-blot assay has been validated by reference to parallel experiments using Western blots and has given similar results to those of Urch and Patel (1991) who used proacrosin conjugated to agarose beads. Thus, the protein retains its native conformation when immobilised on different solid supports. The dot-blot assay was preferred as proacrosin does not transfer quantitatively from SDS-gels during Western blotting.

For assessing specificity of binding by recombinant proteins, dot-blots were blocked with a variety of sulfated and non-sulfated polymers before application of the labeled probe (Jones, 1991).

Group specific modification of basic residues in proacrosin

Chemical modification of basic amino acids in proacrosin was performed using the reagents phenylglyoxal or 2,3-butanedione for arginines (Cheung and Fonda, 1979; Riordan, 1973), diethylpyrocarbonate for histidines (Church *et al.*, 1985) and citraconic anhydride for amino lysines (Atassi and Habeeb, 1972) essentially as described by De Angelis and Glabe, (1988). Purified sperm proacrosin (0.3 nmols) was dot-blotted onto nitrocellulose membrane and incubated with modifying reagent or appropriate buffer control at room temperature for 1 h. Stock solutions of phenylglyoxal (0.5 M) and diethylpyrocarbonate (1 M) were prepared in ethanol and diluted to the appropriate concentration in 0.1 M $NaHCO_3$/0.15M-NaCl, pH 7.8 (for phenylglyoxal) or PBS (for diethylpyrocarbonate). The final concentration of ethanol was 2%. Butanedione was used as supplied and diluted into 0.1 M sodium borate/0.15 M-NaCl, pH 8.3. Citraconic anhydride was added directly to dot-blots in 0.2 M sodium borate and the pH titrated to 8.0 with 1 M-NaOH. After incubation with modifying reagents blots were washed 3 times in 10 ml PBS, blocked with 5% BSA for 1 h and probed with ^{125}I-ZPGPs as before. In protection experiments, dot-blots containing proacrosin were probed first with ^{125}I-ZPGPs and then incubated with modifying reagents for 1 h at room temperature. Blots were counted before and after addition of reagents and percentage displacement calculated. Residual binding is defined as specific binding in the presence of modifying reagents relative to specific binding in absence of reagents.

Peptide synthesis and protein sequencing

Peptides of known sequence were synthesised on a Millipore 9500 peptide synthesizer using standard t-Butoxycarbonyl derivatives according to the manufacturer's recommendations. The peptide was cleaved from the solid support and the side chain protecting groups removed with anhydrous hydrogen fluoride/anisole at 0°C for 2 h. Purification and desalting of the crude peptide was accomplished on a Sephadex P2 polyacrylamide gel filtration column in 65% acetic acid followed by lyophilization and storage under vacuum.

N-terminal amino acid sequence analysis was carried out on an Applied Biosystems gas phase sequencer (Model 470A) equipped with on-line phenylthiohydantoin amino acid derivative analyser (Model 120A). The sequencing program was run as recommended by the manufacturer.

Electrophoresis

Proteins were separated by reducing or non-reducing SDS-PAGE (Laemmli, 1970) and either transferred to PVDF membranes ("Immobilon"; Millipore) by electroblotting or stained with 1% Coomassie Brilliant Blue R-250 in methanol acetic acid/water (40%:7%:53%, respectively). Relative molecular masses were calculated by reference to the mobility of known protein standards (Pharmacia low molecular weight kit).

Other procedures

Cleavage of proacrosin at methionine residues was carried out with CNBr. Approximately 4 nmol dry protein in an Eppendorf tube was solubilized in 200 µl 70% formic acid containing ~2 mg CNBr, flushed with N_2 and incubated 16 h at 23°C. The CNBr was removed under vacuum and the protein lyophilised twice more from distilled water. Peptides generated were purified by preparative SDS-PAGE or blotted onto "Immobilin" for N-terminal amino acid sequencing. Total protein was quantified using the dye binding reagent of Bradford (1976) with BSA or purified proacrosin as standard. Amidase activity of ß-acrosin was measured spectrophotometrically at 405 nm and 30°C with L-BAPNA as substrate (Schleuning and Fritz, 1976).

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