Serine protease activity in boar seminal vesicles and its immunological similarity to sperm acrosin

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A mouse monoclonal antibody against boar acrosin and antiserum prepared to highly purified acrosin in female rabbits were used to detect the antigen in various fluids and tissues of boars using an indirect immunofluorescence technique. A strong reaction was found in fluid and epithelial tissue of the seminal vesicles as well as in the germinal cells in the testis. No immunoreactivity was detected in tissues of the epididymides and other organs of the boar. The antigens present in seminal vesicle fluid of boars were partially purified by column chromatography. It was demonstrated that two antigens differing in molecular mass were present and both possessed protease and amidase activity. The higher molecular mass antigen eluted from a gel filtration column in a volume identical to that of proacrosin. The same result was obtained in polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE). The low molecular mass antigen was eluted from Sephadex G-75 column together with natural protease inhibitors corresponding in molecular mass to less than 20 kDa. The mobility of the antigen in SDS-PAGE was greater than that of chymotrypsin. It is assumed that the protease from seminal vesicle epithelia resembled acrosin in structure and function. Acrosin may therefore not be specific for spermatozoa.

Introduction

Acrosin is one of the hydrolases released from the sperm acrosome as a result of the acrosomal reaction and is considered to play an essential role in fertilization, in recognition, binding and penetration of the zona pellucida of the ovum (for review, see Klemm *et al.*, 1991), and in cleavage of fibronectins and other proteins of basal membranes (Planchenaut *et al.*, 1991). Acrosin is initially present in the acrosome as a zymogen, proacrosin, (for review see Parrish and Polakoski, 1979), the activation of which takes place after acrosome disruption (Brown and Harrison, 1978). The localization of the site of acrosin synthesis has been the subject of intensive research. Proacrosin–acrosin is found exclusively within the acrosome of mammalian spermatozoa synthesized post-meiotically in round spermatids (Flörke *et al.*, 1983). The proacrosin gene is expressed only in the testes, and all other tissues assayed were found to possess neither immunoreactivity against antibodies to acrosin nor features of proacrosin expression (Nayernia *et al.*, 1992). The seminal vesicle fluid and tissues have not been tested for the presence of acrosin.

In the present report, we provide evidence for the presence of proacrosin in both fluid and epithelial cells of the seminal vesicles of boars.

Materials and Methods

Preparation of tissues and fluids

Blood serum and secretions from boar seminal vesicles, testes, prostate and cauda epididymides, and pig follicular fluid were obtained from slaughtered animals. The testis connected with the epididymis was separated from the connective tissue and washed with saline. The epididymis was cut from the testis, and testis secretion collected by puncturing the rete testis duct with a glass capillary. The epididymis was filled with air by means of an injection syringe and a needle inserted into the vas deferens. By inserting another needle into the cauda epididymis duct a mixture of spermatozoa and fluid was obtained. The prostate and seminal vesicles separated from the connective tissue were cut away from the urethra, and the secretions were collected by applying pressure. After centrifuging the secretions for 15 min at 3500 g, the supernatants were frozen and stored at -70°C . The tissues from reproductive and other organs were obtained from slaughtered animals, frozen in liquid nitrogen and stored at -70°C . The isolation of boar α - and β -acrosin forms was as described by Železná and Čechová (1982) and Čechová *et al.* (1988).

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Materials

Peroxidase-conjugated rabbit anti-mouse IgG was obtained from Sigma (St Louis, MO); goat anti-rabbit IgG coupled to peroxidase and pig either anti-mouse or anti-rabbit globulin coupled with fluorescein isothiocyanate (FITC) were from Sevac (Prague). Sephadex G-75 and reversed-phase column were from Pharmacia (Uppsala). Micro-enzyme-linked immunosorbent assay (ELISA) plates were from Dynatech (Denkendorf), ultrafiltration membrane YM 10 from Amicon (Lexington, MA). All the other reagents were purchased from standard commercial sources. Rabbit antiserum to acrosin was prepared by immunizing female rabbits, each with an intracutaneous injection of 1 mg α -acrosin (Železná and Čechová, 1982) dissolved in 0.1 ml saline mixed with complete Freund's adjuvant. Two months later, rabbits were injected with 200 μ g of the antigen in complete Freund's adjuvant. Two weeks after the last injection, blood samples were collected from the ear vein. Antiserum was heat inactivated for 30 min at 56°C. The titre of antibodies checked with 10 μ g of antigen by ELISA was 1:50 000. Preparation and characterization of the monoclonal antibody (MO.AKR1) were as described by Pěkníková *et al.* (1986) and Tesařík *et al.* (1988). The mouse monoclonal antibody and rabbit serum against acrosin did not react with pig trypsin, chymotrypsin, plasmin or kallikrein.

Enzyme-linked immunosorbent assay

The acrosin concentration in blood serum, seminal plasma and reproductive tract fluids was assayed by priming wells in microELISA plates with antigens (1 μ g per well) in phosphate-buffered saline (PBS) at 4°C. After extensive washing (PBS, 1% BSA, 0.05% Tween 20), anti-acrosin rabbit serum (serial dilutions in PBS–Tween) was added to each well and the plates were incubated at 22°C for 1 h. After thorough washing, the anti-acrosin antibodies were reacted at 37°C for 1 h with 100 μ l of 1:1000 (v:v) goat anti-rabbit globulin coupled to peroxidase, washed again and the bound peroxidase activity was detected using *o*-phenylene diamine and hydrogen peroxide as substrate. The absorbance was determined at 495 nm using an RM ELISA reader (Dynatech AG, Branch Germany, Denkendorf). For detection of the antigen in column effluent the wells in microELISA plates were primed overnight at 4°C with either 50 μ l column effluent (after Sephadex G-75) and 50 μ l coating buffer (0.05 mol sodium carbonate l^{-1} plus 0.5 mol sodium hydrogen carbonate l^{-1} , pH 9.6), or 25 μ l column effluent (reversed-phase chromatography), 25 μ l 1 mol Tris l^{-1} , and 50 μ l coating buffer. After extensive washing (PBS, 0.5% BSA, 0.05% Tween 20), 100 μ l of antibody preparation diluted either 1:1000 (monoclonal) or 1:10 000 (polyclonal) was added to each well and the plates were incubated at 37°C for 2 h, as described above.

Fluorescence labelling assays

An indirect immunofluorescence technique was applied to detect the acrosin in slices (3 μ m thick) of frozen pig tissues of vesicular gland, caput, corpus and cauda of epididymides,

testes, prostate, urethra, bulbourethral gland, liver, kidney, heart, spleen, pancreas, intestines, lungs, lymphatic gland and muscle. The tissue sections were obtained from seven animals. At least four sections of any tissue sample per animal were evaluated. Frozen tissue sections were incubated with a drop of antiserum (either mouse monoclonal or rabbit polyclonal) diluted 1:30 (v:v) or 1:60 (v:v) respectively with PBS for 1 h at 22°C. After washing (0.2% BSA in PBS), pig, either anti-mouse or anti-rabbit, globulin coupled with fluorescein isothiocyanate (FITC) diluted 1:15 (v:v) and preabsorbed with tissue homogenate was used as the second incubation medium. The culture supernatant of parental Sp 2/0 Ag14 myeloma cells and normal mouse serum served as controls for the monoclonal antibody. Both normal rabbit serum and antiserum to α -acrosin preabsorbed with the same compound (4 mg ml^{-1}) were used as controls for rabbit anti-acrosin serum. The slides were observed with an Orthoplan-Leitz microscope (Westlar) equipped with a halogen-quartz lamp using an FITC interference filter.

Purification of protease from seminal vesicle fluid

Seminal vesicle fluid (135 mg of proteins in 7.5 ml) was fractionated at 4°C on a Sephadex G-75 column (2.6 cm $\times$ 68 cm) equilibrated with 1 mmol hydrochloric acid l^{-1} (flow rate 0.18 ml min^{-1}). Fractions, 3.6 ml, were screened for absorbance at 280 nm, presence of amidase activity and acrosin inhibitors and for immunoreactivity (see below). Both high and low molecular mass antigen-containing fractions were separately pooled, concentrated with an ultrafiltration membrane to 30% of the volume, and used for electrophoretic studies, or further purified by reversed-phase chromatography. The material (2 ml) was applied to a 1 cm $\times$ 10 cm ProRPC 15 μ m HR 10/10 column and eluted with a linear gradient of 0–60% (v/v) acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 3 ml min^{-1} for 65 min, using the fast-protein liquid chromatography (FPLC) system. Proteins were detected by absorbance at 280 nm. The fractions (1.5 ml each) were screened for activable zymogen, for immunoreactivity by ELISA.

Zymogen activation, enzyme and acrosin inhibitor assays

Zymogens were converted to active enzymes by incubation at 25°C in pH 7.8, and the resulting enzymatic activities were measured at 25°C in terms of hydrolysis of the chromogenic substrate *N*- α -benzoyl-DL-arginine nitroanilide. After gel filtration, 0.5 ml of each fraction was diluted with 2.0 ml of 0.2 mol Tris-chloride l^{-1} , pH 7.8, with 0.025 mol calcium chloride l^{-1} , and 500 ng trypsin was added as activator. After incubation for 10 min, 1 mg *N*- α -benzoyl-DL-arginine nitroanilide in 0.1 ml dimethylformamide was added: the reaction was stopped after 15 min with 0.5 ml of 30% (v/v) acetic acid, and absorbance was measured at 405 nm against a blank (with buffer instead of fraction solution). For detection of trypsin-like activity after reversed-phase chromatography, the assay was performed in the absence of calcium chloride and trypsin; 0.2 ml of each fraction was tested. The same procedure was

Fig. 1. Immuno-electrophoretic pattern of the reaction of antisera to α -acrosin with acrosin and seminal vesicle fluid of boars. (a) α -acrosin (A – 0.5%; B – 0.25%); (b) seminal vesicle fluid (A – 0.5%; B – 1.0%).

used to estimate preformed enzymes, except that the substrate was added before the zymogen. Determination of acrosin inhibitors was based on the assay of the residual amidase activity of acrosin after incubation for 10 min with inhibitor (Železná and Čechová, 1982). The protein concentration was estimated spectrophotometrically at 280 nm, assuming that 1 mg protein ml^{-1} exhibits an absorbance of 1.0 at 280 nm in a 1 cm light path.

Gel electrophoresis and protein and enzyme detection

For protease zymographic detection, 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (slab gels 1 mm) containing 0.08% casein was used (Čechová *et al.*, 1990). The samples were prepared in Laemmli sample buffer (Laemmli, 1970) without reduction. After SDS-PAGE, the gels were washed for 1 h at room temperature in 2.5% (v/v) Triton X-100 in water and then three times with distilled water. After incubation in 100 mmol glycine l^{-1} , adjusted with sodium hydroxide to pH 8.3, at 37°C for 2 h, the gels were stained for 1 h in 0.1% Amido Black 10B. Proteolytic digestion was detected by observing clear zones of casein hydrolysis. Proacrosin, β -acrosin, plasmin and chymotrypsin were used as enzyme standards.

Other methods

Immuno-electrophoresis was performed according to Williams (1971). Zona-binding activity was tested by means of the solid phase zona-binding assay as described by Töpfer-Petersen and Henschen (1987). Sperm immobilization test was performed according to Isojima *et al.* (1987).

Results

Preparation and characterization of antisera

A rabbit antiserum against α -acrosin was obtained by immunizing rabbits with a highly purified protein sample. A single precipitation line was seen when the antibody was tested

Fig. 2. Reactivity of anti-acrosin monoclonal antibody to antigen in frozen tissue sections detected by an indirect immunofluorescence assay. (a) Seminal vesicle tissue ($\times 240$). Strong immunoreactivity was localized in secretory epithelium (se). (b) Testicular tissue ($\times 240$). The positive reaction of antibody was evident in all seminiferous tubules of the testis at the level of spermatids (sp) in tubular lumen. No fluorescence was seen in interstitial tissue (it). (Controls exposed to the action of normal mouse serum and anti-mouse globulin labelled with fluorescein isothiocyanate were negative.)

against the antigen, i.e. acrosin (Fig. 1a). The same precipitation line was observed when the antibody was tested against seminal vesicle fluid (Fig. 1b), but when a high concentration of seminal vesicle fluid was used a second faint band was also found. The antigen did not react with any of the other fluids tested.

In ELISA, the antiserum titre in the reaction with 10 μg of the α -acrosin was 1:50 000, and with proacrosin sample was 1:200 000. The sperm immobilization test gave negative results.

Localization of antigen

In ELISA, both monoclonal and polyclonal antibodies against acrosin reacted with seminal vesicle fluid. The titre of polyclonal antibodies was 1:25 000. The reactions with blood serum and the other fluids tested (epididymal, testicular, prostatic and follicular) were negative.

After application of either mouse monoclonal or rabbit polyclonal antibodies against acrosin to tissue sections, strong fluorescence of the secretory epithelium in boar seminal vesicle

Fig. 3. Patterns of zymograph SDS polyacrylamide gel electrophoresis of protease-containing material. Samples were subjected to electrophoresis in SDS polyacrylamide gels containing casein and then analysed for protease digestion. Caseinolytic activity appeared as a clear band on the dark background. Electrophoretic mobility of (a) plasmin, (b) proacrosin, (c) β -acrosin and (d) chymotrypsin are indicated. A: Lane (1) fluid of boar seminal vesicle (100 μ g); lane (2) high molecular mass antigen fraction after gel filtration (50 μ g); lane (3) low molecular mass antigen fraction after gel filtration (200 μ g). B: Pooled fractions contained proacrosin after reversed-phase column chromatography (2 μ g).

tissue was detected (Fig. 2a). The control with a normal serum was negative. The testicular tissue (Fig. 2b) was negative, but a strong fluorescence of germ cells in seminiferous tubules was found. No fluorescence was observed when the antiserum was applied to pig erythrocytes, lymphocytes or any other tissue tested (prostate, epididymides, bulbourethral gland, liver, pancreas, heart, spleen, lungs, intestine, kidney and muscle).

Purification of antigen from seminal vesicle fluid

Ultrasensitive protease detection zymography was used to follow the presence of proteases in seminal vesicle fluid. Two digestion bands were observed (Fig. 3), one with relative molecular mass similar to that of plasmin, and the other with relative molecular mass similar to that of proacrosin. Gel filtration on a Sephadex G-75 column was used as the first step of purification. Both polyclonal and monoclonal antibodies to boar acrosin were tested for immunoreactivity in column effluent. On the elution profile (Fig. 4), two peaks of immunological activity to anti-acrosin serum were visible. No difference was found when either monoclonal or polyclonal antibodies were used in microELISA tests. The higher molecular mass antigen emerged from the column in the same effluent volume as did proacrosin. The enzyme eluted as zymogen which had to be activated with a small amount of trypsin. The ELISA maximum corresponded very well to the level of

Fig. 4. Gel filtration of boar seminal vesicle fluid on Sephadex G-75. (·····) absorbance at 280 nm; (---) amidase activity after activation with trypsin; (○—○) enzyme-linked immunosorbent assay (ELISA) reaction with monoclonal antibody; (●—●) ELISA reaction with rabbit polyclonal antibodies expressed as absorbance at 492 nm; (▨) fractions containing trypsin inhibitor activity; (▨) pooled fractions A and B.

amidase activity obtained after activation. The lower molecular mass antigen was eluted after gel filtration together with proteinaceous acrosin inhibitors present in seminal vesicle fluid. The amidase activity in the low molecular mass substance could not be detected even in the presence of trypsin. The fractions corresponding to both antigens were pooled (Fig. 4). Pooled fractions A and B contained proteolytic activity, as evidenced by a positive reaction using ultrasensitive protease detection zymography (Fig. 3). Caseinolytic activity (clear area) of the major high molecular mass fraction A occurred in the proacrosin region. The proteolytic activity of the minor low molecular mass fraction B was apparent in a region with relative molecular mass lower than 25 kDa (chymotrypsin). The elution profile obtained from reversed-phase chromatography of the high molecular mass fraction A is shown (Fig. 5a). The latent protease activity (zymogen) was eluted with 40% acetonitrile. The zymogen was autoactivated at pH 8.0 with sigmoidal kinetics (Čechová *et al.*, 1988). No preformed amidase activity could be detected before autoactivation. An acrosin inhibitor eluted at 37% acetonitrile (Fig. 5a). The maximum of microELISA and zona-binding assays corresponded well to the maximum of amidase activity obtained after activation. The elution profile obtained from reversed-phase chromatography of pooled low molecular mass antigen-containing fraction B from Sephadex G-75 is shown (Fig. 5b). Amidase activity eluted at 40% acetonitrile and could be detected without autoactivation. The maximum of amidase activity and microELISA tests was in excellent agreement. Zona-binding test and zymographic detection of this sample were done and negative results were obtained. A large amount of acrosin inhibitor was eluted with 24% acetonitrile.

Fig. 5. Elution patterns of chromatography of the crude proacrosin/acrosin fractions eluted from Sephadex G-75 column on a reversed-phase column in 0.1% trifluoroacetic acid and 0–60% acetonitrile. (a) Chromatography of the A fraction. (b) Chromatography of the B fraction. (·····) Absorbance at 280 nm; (---) percentage of acetonitrile; (○—○) amidase activity (in (a) after autoactivation, in (b) without autoactivation), and (●—●) acrosin inhibitor activity indicated as percentage of maximum activity; (△—△) microELISA activity and (▲—▲) zona-binding activity expressed as absorbance at 492 nm.

Discussion

The trypsin-like serine protease, acrosin, appears to have a specific function at the molecular level of fertilization, presumably by facilitating the penetration of spermatozoa through the zona pellucida of the ovum (for review see Hedrick *et al.*, 1988) or in the acrosome reaction (Tesařík *et al.*, 1990). Acrosin is considered to be highly specific for mammalian spermatozoa. There is no evidence indicating that proacrosin or acrosin is present in any other body cell or fluid, but the fluid of seminal vesicles had not been tested. Using sensitive zymographic detection after SDS-PAGE of boar seminal vesicle fluid, we observed a faint zone of proteolysis that appeared at a position corresponding to that of proacrosin. To characterize that protease, we partially purified the enzyme using the method recently described for boar proacrosin or sperminogen isolation (Čechová *et al.*, 1988, 1990). The elution pattern of a Sephadex G-75 column demonstrated that in seminal vesicle fluid of the boar two compounds crossreacted with antibodies to acrosin and differed in their molecular masses. The faint amidase activity after activation with trypsin could be determined only

in the high molecular mass material. Both materials, however, showed proteolytic activity to casein on zymograph electrophoresis. After the reversed-phase chromatography of the high molecular mass material, a zymogen of trypsin-like protease emerged from the column in 40% acetonitrile, as did proacrosin (Čechová *et al.*, 1988). The zymogen was separated from acrosin inhibitor. The presence of that inhibitor in the acrosin-like fraction eluted from Sephadex G-75 could account for the fact that the zymogen did not autoactivate. Inhibitors of acrosin prevent the activation of proacrosin (for review see Parrish and Polakoski, 1979). The results indicated that the fluid of boar seminal vesicles expressed a zymogen that was electrophoretically and antigenically indistinguishable from proacrosin. We do not have amino acid sequence evidence that the zymogen is proacrosin, but the strong similarities in molecular mass, immunological and chemical properties, zymogen activation, and enzymatic activity, with proacrosin and acrosin support the contention that the zymogen detected in seminal vesicle fluid and tissue is proacrosin. The information about the presence of proacrosin in boar seminal vesicle fluid is new and it is not consistent with a view of high

specificity of the acrosin for sperm cells only. It has not been detected in any other cell or fluid in the mammalian body. The results presented here, using immunofluorescence, indicate that proacrosin-like antigen is probably synthesized by seminal vesicle epithelia. It is difficult to determine whether the same structural gene is expressed both in spermatids and in epithelial cells of seminal vesicles or whether two different structural genes are expressed in different tissues. A protease separated from seminal acrosin inhibitors was obtained after reversed-phase chromatography of the low molecular mass immunoreactive fraction. The existence of the low molecular mass acrosin-like antigen in boar seminal fluid is unexpected. The protease was eluted from the column in 40% acetonitrile as was the acrosin, but the explanation that the low molecular mass acrosin is an artefact of proacrosin autodigestion does not seem satisfactory. The autodigestion of the proacrosin proceeds in a stepwise fashion (Parrish and Polakoski, 1979) and passes through a very stable 35 kDa product (Železná and Čechová, 1982). We could not detect the 35 kDa acrosin in seminal vesicle fluids of any of the six boars tested. Furthermore, the autodigestive 24 kDa product (Parrish and Polakoski, 1979) has never been observed under physiological conditions (Moos *et al.*, 1991). We conclude that the low molecular mass acrosin-like protease in seminal vesicle fluid has a special function. The low molecular mass antigen was detected in the seminal fluids of all six boars tested (unpublished observations).

Proteases serve as biological regulators of a variety of physiological processes. Seminal plasma contains a variety of proteases, together with a number of protease inhibitors and activators (for review see Morton, 1977). At present, the physiological roles of most of these proteases are not known. It has been suggested that limited proteolysis may play a role in the apparent loss or modifications of sperm surface molecules that occur during epididymal maturation and capacitation. The action of seminal plasma proteases on bulk proteins in seminal plasma results in the formation of vasoactive small polypeptides, and in maturation of a peptide hormone. Saling *et al.* (1981) proposed that an enzyme of tryptic specificity is involved in the binding of mouse spermatozoa to zona pellucida and Meizel and Lui (1976) and Meizel (1985) proposed that it is involved in the acrosome reaction. It is not known which gland produces seminal plasma proteases. In prostatic fluids of some species, proteases with kallikrein-like specificities were detected (Lazure *et al.*, 1984; Lilja and Laurel, 1985) and their physiological function was determined (Lilja and Laurel, 1985). Data about proteases in secretions of seminal vesicles are even more scarce. In the human seminal vesicle, a zymogen of a serine protease has been demonstrated, the activation of which was inhibited by an inhibitor of both urokinase and protein C (Laurell *et al.*, 1992). It is possible that the zymogen might be an extracellular proacrosin, similar to that found in seminal vesicles of boars. The role of acrosin bound to the intra-acrosomal membrane in the secondary phase of sperm-egg binding has been suggested by Töpfer-Petersen and Henschen (1987) and Jones and Brown (1987), but the proacrosin-acrosin system present on the surface of the plasma membrane of the sperm head (Tesařík *et al.*, 1988, 1990) might also be involved in the primary phase of sperm-egg binding. In the light of the new data we consider

that the proacrosin-like antigen detected on the surface of sperm plasma membrane (Tesařík *et al.*, 1988) originated from seminal vesicle fluid rather than from the sperm acrosome, where it had to traverse the barrier of two membranes. The role of acrosin-like protease in seminal vesicle fluid may also be connected with a limited cleavage or solubilization of fibronectins (Planchenault *et al.*, 1991).

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