CHAPTER II

p-Aminobenzamidine-sensitive acrosomal protease(s) other than acrosin serve the sperm penetration of the egg zona pellucida in mouse

INTRODUCTION

Fertilization requires sperm to penetrate the zona pellucida (ZP), an extracellular glycoprotein matrix of the egg (1). In mouse, this matrix is composed of ZP1, ZP2, and ZP3 (125-127). Following the binding of acrosome-intact sperm to ZP3 (primary ZP binding), the acrosome reaction occurs, and the acrosome-reacted sperm penetrate the ZP (1). It has been also considered that the continued binding of the acrosome-reacting and acrosome-reacted sperm to ZP2 (secondary ZP binding) is necessary for the maintenance of the penetrating sperm (1, 84, 125-127).

Acrosin is a serine protease with a trypsin-like cleavage specificity, and is localized in the sperm acrosome as an enzymatically inactive zymogen, proacrosin. Around the time of the acrosome reaction, the zymogen is probably converted into the active form by autoactivation (43, 47, 53-62). Various trypsin inhibitors have been reported to cause a marked inhibition of sperm penetration through the ZP (96-101), thus suggesting that acrosin serves to cause limited hydrolysis of ZP to produce a penetration pathway for the motile sperm. However, previous studies using acrosin-deficient ($Acr^{-/-}$) mutant mice produced by homologous recombination provide the evidence that acrosin is not essential for the sperm penetration of ZP, since the mutant mice are all capable of producing healthy offspring (104). The observed phenotype of the $Acr^{-/-}$ mouse sperm was a delay in the sperm penetration of ZP at the early stages of *in vitro* fertilization (IVF) (104). Moreover, the release of several acrosomal proteins from the acrosome of the mutant mouse sperm was significantly delayed during calcium ionophore- and solubilized ZP-induced acrosome reaction, as described in CHAPTER I (128). Therefore, it is conceivable that the delayed sperm penetration of ZP in the $Acr^{-/-}$ mouse may derive from the altered rate of protein dispersal from the acrosome, and that the major role of acrosin is to accelerate the dispersal of acrosomal components during the acrosome reaction. If proteolytic enzyme(s) sensitive to trypsin inhibitors are essential for the sperm penetration of ZP, as described above, our findings (104, 128) lead to an additional question; does the sperm acrosome still contain trypsin-like protease(s), other than acrosin, that act on the limited proteolysis of ZP?

In this study, I have examined the effects of *p*-aminobenzamidine (pAB), a

competitive inhibitor of trypsin and acrosin (31, 95), on the function of $Acr^{+/+}$ and $Acr^{-/-}$ mouse sperm. An inhibitor-sensitive protease(s) other than acrosin is(are) present in the acrosome of sperm from wild-type and acrosin-null mice, and may contribute to the sperm penetration of the egg ZP.

MATERIALS AND METHODS

Materials

Monoclonal antibodies (mAb) against mouse sperm proteins, OBF13 (106) and MC101 (107), were the gifts from Drs. M. Okabe and K. Toshimori, respectively. mAb 7C5 against sp56 (11, 111) was purchased from QED Biologicals (La Jolla, CA), and was kindly provided by Dr. G. L. Gerton. Calcium ionophore A23187 was purchased from Dojindo Laboratories (Kumamoto, Japan). Protease inhibitors, pAB, benzamidine hydrochloride, diisopropyl fluorophosphate (DFP), N^{α}-tosyl-L-lysine chloromethyl ketone (TLCK), phenylmethanesulfonyl fluoride (PMSF), and *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), were purchased from Sigma (St. Louis, MO). $Acr^{+/+}$, $Acr^{+/-}$, and $Acr^{-/-}$ male mice were obtained by mating between $Acr^{+/-}$ males and females, as described previously (104).

Calcium ionophore-induced acrosome reaction

Fresh cauda epididymal sperm from male mice (2 to 3 months old) were capacitated in a 0.2-ml drop of modified Krebs-Ringer bicarbonate solution (TYH medium) containing glucose, sodium pyruvate, bovine albumin, and antibiotics (108) for 90 min at 37°C under 5% CO_2 in air. The capacitated sperm (4 x 10^6 sperm/ml) were induced to undergo acrosome reaction by addition of calcium ionophore A23187 at a final concentration of 5 μg/ml followed by incubation at 37°C under 5% CO_2 in air, as described in CHAPTER I (128). An aliquot (50 μl each) was taken at various time intervals after the addition of the ionophore, transferred into a 1.5-ml microcentrifuge tube, and centrifuged at 3,000 rpm for 10 min. The sperm pellets were resuspended in 50 μl of phosphate-buffered saline (PBS), and then subjected to immunocytochemical analysis.

Immunocytochemical analysis of sperm

Immunostaining was carried out by the avidin-biotin peroxidase complex (ABC) method (110) using a Vectastain Elite ABC kit (Vector laboratories), as described previously (128). Briefly, sperm suspension was placed onto glass slides that had been coated with Vectabond (Vector laboratories, Burlingame, CA), treated with PBS containing 4% paraformaldehyde, and washed with PBS. The fixed sperm samples on slides were treated with 0.3% hydrogen peroxide in methanol, washed with PBS containing 0.1% Tween 20, and blocked with 1.5% normal goat serum in PBS and with an avidin/biotin blocking kit (Vector laboratories). The slides were incubated with mAb, washed with the above blocking solution, and then treated with biotin-conjugated goat anti-mouse IgG + IgM (Jackson ImmunoResearch Laboratories, West Grove, PA) followed by an ABC solution containing horseradish peroxidase-conjugated avidin (Vector laboratories). After washing with PBS, the sperm samples were stained using 3, 3'-diaminobenzidine as a chromogen, mounted, and observed under an Olympus BX50 microscope.

In vitro fertilization (IVF)

Female ICR mice (2 months old, Japan Cler, Inc., Tokyo) were superovulated following intraperitoneal injections of PMSG and hCG at a 48-h interval, as described previously (128). Eggs associated with cumulus cells were recovered 15 to 16 h after hCG injection, and placed in TYH medium (0.2 ml) covered with warm mineral oil in a plastic petri dish. A drop (10 μl) of capacitated sperm suspension (3,000 sperm/μl) was added to the medium containing eggs. The eggs and sperm were incubated at 37°C under 5% CO_2 in air. After incubation for 6 h, the eggs were fixed with 0.25% glutaraldehyde and mounted on slides for whole-mount preparation. They were further fixed with 10% neutral formalin overnight, and then stained with 0.25% lacmoid in 45% acetic acid for the assessment of sperm penetration of ZP and fertilization.

Cytochemical detection of protease activity in sperm

Localization of protease activity in sperm was visualized by the silver proteinate method described by Yanagimachi and Teichman (1972) (129). A suspension of capacitated cauda epididymal sperm was spread over glass slides, air-dried, fixed in methanol (-30°C) for 30 min, and washed with water. The sperm on the slides were incubated at 37°C for 6 h in 80 mM Tris/HCl, pH 6.5, containing 0.2% mild silver proteinate (Ebisu Pharmaceutical Co., Tokyo) and 0.08% potassium bromide, thoroughly washed with water, and treated with a Fuji Film Pandol developer for 10 min. After rinsing with 2% sodium thiosulfate for 10 min, the sperm samples were treated with ethanol and with xylene, mounted, and viewed under an Olympus BX50 microscope.

Preparation of sperm extracts

Sperm extracts were prepared from cauda epididymal sperm by stirring gently in a pH 3.0 solution containing 10% glycerol, 50 mM benzamidine, and 0.02% sodium azide at 4°C for 6 h, as described previously (104). After centrifugation at 13,000 rpm for 10 min, the supernatant solution was dialyzed against 1 mM HCl to remove benzamidine, and used as acid sperm extracts.

Fresh cauda epididymal sperm in TYH medium free of bovine serum albumin were washed twice with the same medium by centrifugation at 3,000 rpm for 10 min. The acrosome reaction of the sperm cells was induced in the bovine serum albumin-free TYH medium by addition of calcium ionophore A23187, as described above. After incubation for 60 min at 37°C under 5% CO_2 in air, the sperm suspension was centrifuged at 3,000 rpm for 10 min. The supernatant solution was then dialyzed against 1 mM HCl, and used as a fraction of the released proteins from the sperm acrosome during the acrosome reaction. Protein concentration was determined using a Coomassie protein assay reagent kit (Pierce).

Detection of enzyme activity on SDS-PAGE

To detect proteins exhibiting gelatin-hydrolyzing activities, sperm extracts were analyzed by SDS-PAGE in the presence of 0.1% gelatin (130). After electrophoresis, the gels were washed twice with 2.5% Triton X-100 and with 0.1 M Tris/HCl, pH 8.0, and incubated in the same Tris buffer at 37°C overnight. The bands for the gelatin-hydrolyzing proteins were detected by staining the gels with Coomassie brilliant blue.

RESULTS

In CHAPTER I (128), I verified that pAB hinders acrosomal proteins from dispersing normally from sperm acrosome, and demonstrated the delay of the protein dispersal in *Acr*$^{-/-}$ mouse sperm during calcium ionophore A23187- and solubilized ZP-induced acrosome reaction. To examine whether the delayed protein dispersal is solely due to the absence of acrosin, or whether a pAB-sensitive protease(s) other than acrosin participates in the protein dispersal, immunostaining of *Acr*$^{-/-}$ mouse sperm following the ionophore treatment in the presence or absence of 1 mM pAB was carried out using mAb sp56, mAb MC101, and mAb OBF13 as probes. The mAb OBF13 was used as a control, since this mAb immunoreacted both with a protein located at the acrosome cap region of capacitated sperm, and with the same protein redistributed over the entire sperm head just after the acrosome reaction, as described before (106, 112, 128).

Immunostaining analysis using mAb OBF13 clearly showed that both *Acr*$^{+/+}$ and *Acr*$^{-/-}$ mouse sperm were acrosome-reacted by addition of calcium ionophore A23187, in spite that pAB was present in the incubation medium (Fig. 12). However, the disappearance of the signals immunostained by mAb sp56 and mAb MC101 in the *Acr*$^{+/+}$ sperm acrosome was obviously delayed in the presence of pAB, as described previously (53, 70-71, 128). In the *Acr*$^{-/-}$ mouse sperm, the number of the acrosome stained by these two antibodies further reduced in the absence of pAB. Most importantly, no significant difference in the patterns of the signal disappearance was found between the *Acr*$^{-/-}$ mouse sperm with the ionophore treatment in the presence and absence of pAB. These data imply that pAB-sensitive protease(s) other than acrosin may not contribute to accelerate the dispersal of acrosomal proteins from the acrosome during the ionophore-induced acrosome reaction.

Since *Acr*$^{-/-}$ mouse sperm are still capable of penetrating ZP, a protease(s) sensitive to trypsin inhibitors must be present in the acrosome if the limited proteolysis of ZP is essential for the sperm penetration (96-101). To ascertain this possibility, we carried out IVF assay in the presence of 1 mM pAB using cumulus- and ZP-intact eggs (Fig. 13). Most of the egg ZP (more than 80%) was penetrated in the absence of pAB

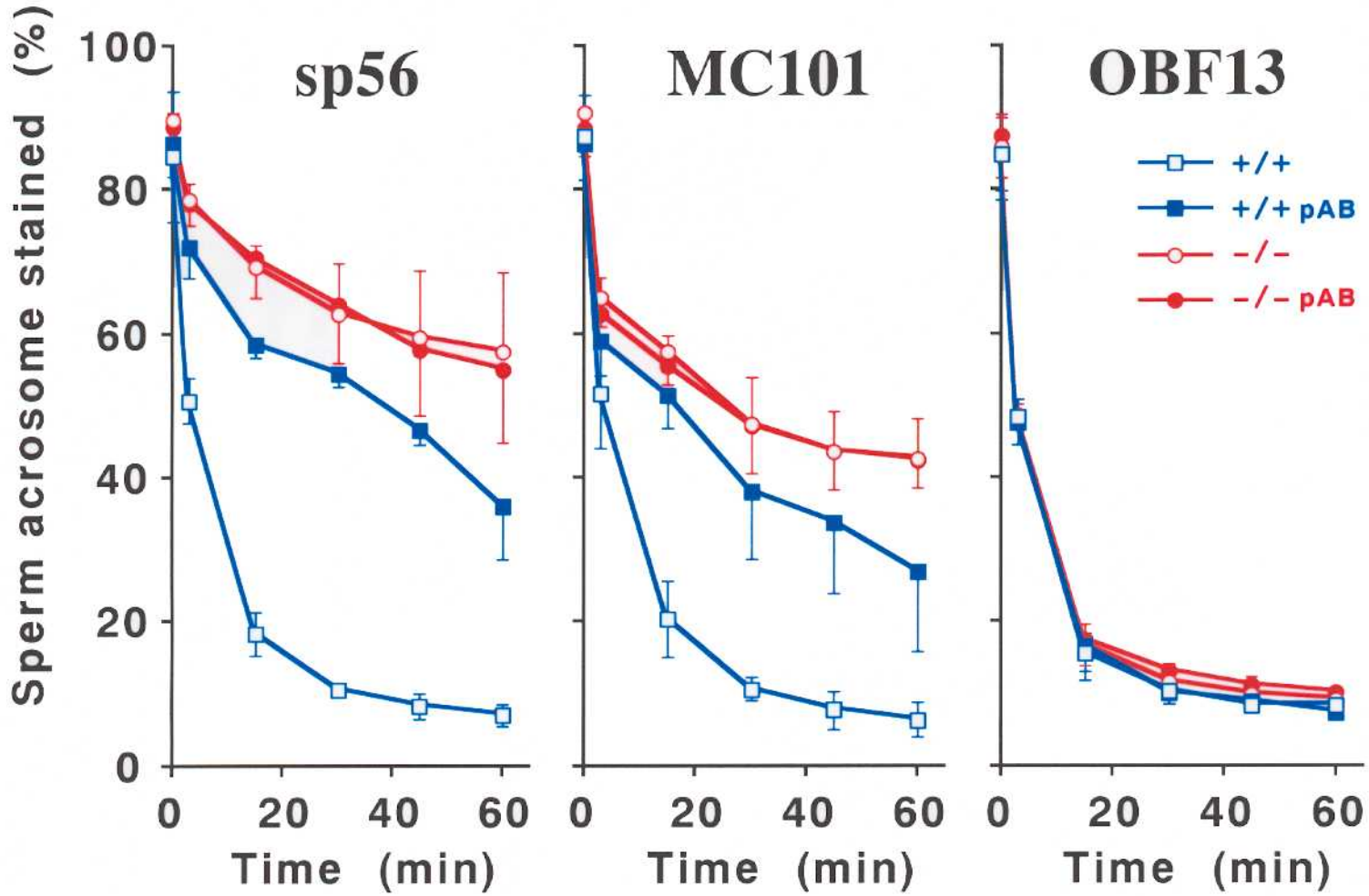

Fig. 12. Time course of protein dispersal from sperm acrosome following treatment of calcium ionophore A23187 in the presence of *p*-aminobenzamidine. Capacitated cauda epididymal sperm from wild-type (*Acr*$^{+/+}$, squares) and acrosin-deficient (*Acr*$^{-/-}$, circles) mice were treated with the ionophore in the presence (closed symbols) or absence (open symbols) of 1 mM *p*-aminobenzamidine, sampled at 3, 15, 30, 45, and 60 min after addition of the ionophore, and immunostained by the ABC method (110) using monoclonal antibody against sp56, MC101, or OBF13, as described in Materials and methods. The 300 sperm were selected at random and divided into two groups by the following criteria described previously (128): sperm which still contained the antigens recognized by the antibodies in the acrosome, in spite that they were acrosome-intact, initiated the acrosome reaction, or had already acrosome-reacted, and sperm which contained no signal of the antigens in the acrosome (the acrosome reaction had been already accomplished, and the antigens had been completely dispersed). The numbers of the sperm containing the immuno-positive signals in the acrosome were counted. Data are expressed as the means ± S.D., where n≥3.

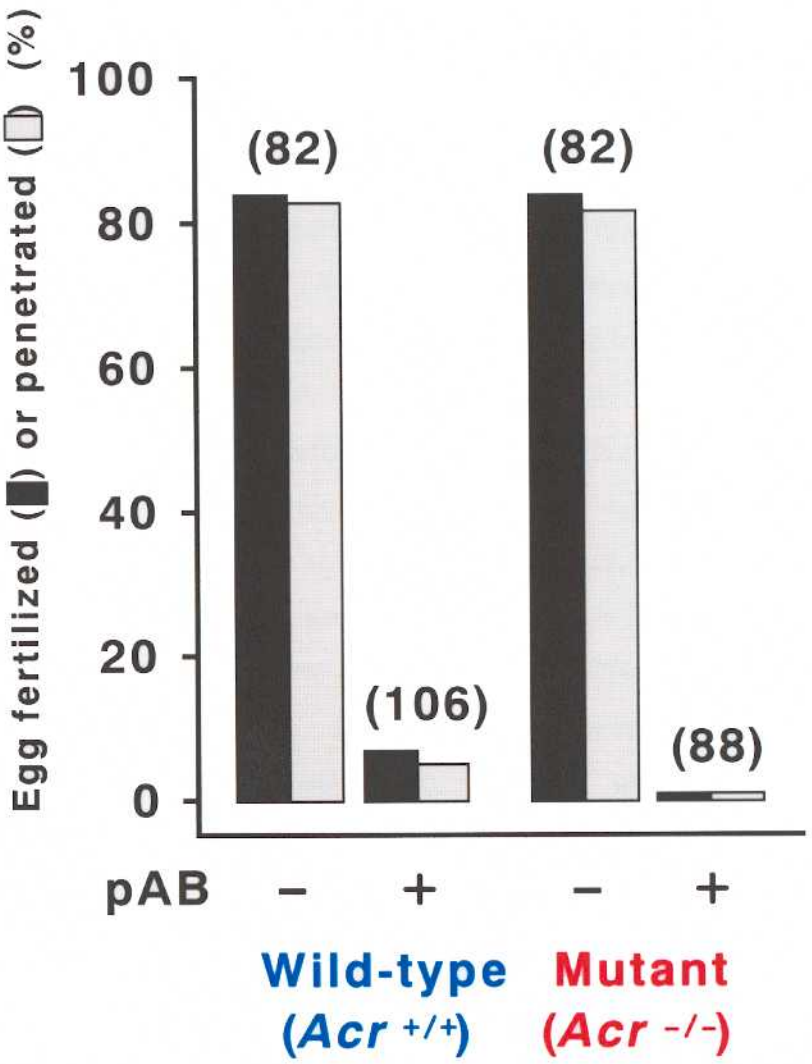

Fig. 13. ***In vitro*** **fertilization in the presence of *p*-aminobenzamidine.** Cauda epididymal sperm from wild-type ($Acr^{+/+}$) and acrosin-deficient ($Acr^{-/-}$) mice were capacitated in TYH medium and incubated with cumulus- and zona pellucida-intact eggs in the presence (+) or absence (-) of 1 mM *p*-aminobenzamidine (pAB). After the incubation for 6 h, the sperm penetration and fertilization were assessed. The eggs, which had sperm within their perivitelline space and/or on the vitellus, were defined as "egg (zona pellucida) penetrated" (black boxes). If the eggs penetrated had a female and male pronucleus(lei) with fertilizing sperm tail(s) in the vitellus, they were considered as "egg fertilized" (shadow boxes). Numbers in parentheses at the top of the boxes represent the egg numbers examined.

by capacitated epididymal sperm from $Acr^{+/+}$ and $Acr^{-/-}$ mice at 6 h after insemination under the experimental conditions employed. The rate of the sperm penetration in the presence of 1 mM pAB was negligibly low (1 to 7%) in either of the $Acr^{+/+}$ and $Acr^{-/-}$ mice. Thus, the sperm penetration of ZP most likely necessitates the protease activity, which is inhibited by pAB, even in the absence of the acrosin activity. It should be very important to note that the motility of the capacitated sperm is obviously normal in the presence of 1 mM pAB (data not shown), as described by Fraser (1982) (69).

To assess the presence of protease activity in cauda epididymal sperm, cytochemical analysis was performed according to the silver proteinate method (129) using $Acr^{+/+}$, $Acr^{+/-}$, and $Acr^{-/-}$ mice. It is known that many proteases, including trypsin, papain, ficin, and pronase, are able to hydrolyze the silver proteinate, a mixture of silver ions and milk casein hydrolyzate (129). As shown in Fig. 14, the brownish black signals representing the protease activity were detectable only in the acrosome of $Acr^{+/+}$, $Acr^{+/-}$, and $Acr^{-/-}$ mouse sperm. No significant signal was observed in other regions of the sperm, as described previously (129). The $Acr^{+/+}$ and $Acr^{-/-}$ mouse sperm exhibited the strongest and weakest signal intensities, respectively, as judged visually, possibly reflecting the amounts of the acrosin activity in the acrosome of $Acr^{+/+}$, $Acr^{+/-}$, and $Acr^{-/-}$ mouse sperm (104). These data demonstrate that mouse sperm likely possess a proteolytic enzyme(s) other than acrosin in the acrosome. However, it is not clear at the present time whether the protease(s) are located in the acrosomal matrix and/or on the plasma and acrosomal membranes of the acrosomal cap region. Since the positive signals of the protease activity entirely disappeared together with the complete dispersal of the acrosomal matrix due to the ionophore treatment of the mouse sperm, the proteolytic enzymes are probably released from the acrosomal matrix during the acrosome reaction.

Baba *et al.* (104) have previously demonstrated the presence of a 42-kDa protein exhibiting gelatin-hydrolyzing activity in acid sperm extracts of $Acr^{+/+}$, $Acr^{+/-}$, and $Acr^{-/-}$ mice, although $Acr^{-/-}$ mouse sperm lacked a 41-kDa gelatin-hydrolyzing protein which may correspond to (pro)acrosin. To characterize the 42- and 41-kDa proteins further, the time course of the gelatin-hydrolyzing activity in acid sperm extracts incubated at pH 8.5 was monitored by SDS-PAGE in the presence of gelatin (Fig. 15A). As

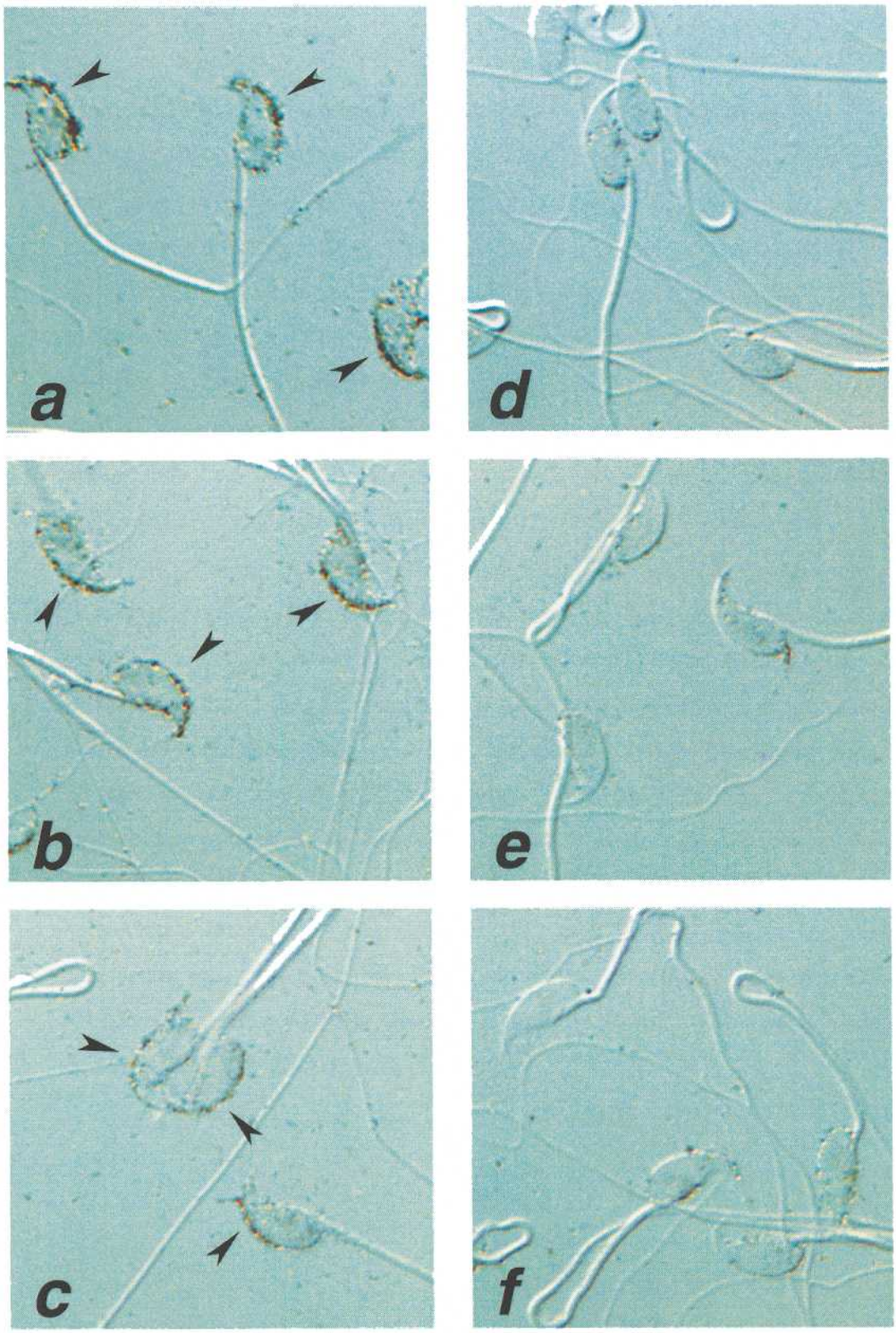

Fig. 14. Cytochemical detection of protease activity in mouse sperm. Capacitated cauda epididymal sperm from wild-type (*Acr*$^{+/+}$, a and d), heterozygous (*Acr*$^{+/-}$, b and e) and homozygous (*Acr*$^{-/-}$, c and f) mice for a disruptive mutation in the mouse acrosin gene were untreated (a, b, and c) or treated (d, e, and f) with calcium ionophore A23187 for 60 min, and subjected to cytochemical detection of protease activity in the sperm using the silver proteinate method (129). The enzyme activity in the sperm was visualized as brownish black signals (arrow heads).

expected from the data described previously (104), the levels of the activities of the 42- and 41-kDa proteins increased in $Acr^{+/+}$ mouse as the time elapsed. However, the activity level of the 42-kDa protein remained constant in $Acr^{-/-}$ mouse during the pH 8.5 incubation up to 3 h. These data indicate that formation of the active 42-kDa protein may be accelerated by (pro)acrosin produced during the incubation of the acid sperm extracts at pH 8.5. Furthermore, pre-treatment of the sperm extracts with DFP completely abolished the gelatin-hydrolyzing activities of the 42- and 41-kDa proteins (Fig. 15B). Both activities were also inhibited strongly by TLCK, partially by PMSF, and slightly by TPCK. Thus, the 42-kDa protein as well as 41-kDa protein probably belongs to a superfamily of trypsin-like serine proteases.

To examine whether gelatin-hydrolyzing protease(s) are present in protein fractions released from acrosome of $Acr^{+/+}$ and $Acr^{-/-}$ mouse sperm following calcium ionophore-induced acrosome reaction, SDS-PAGE in the presence of gelatin was carried out (Fig. 16). The patterns of the gelatin-hydrolyzing proteins released were consistent with those of sperm acid extracts, except that additional proteins with sizes of 35 and 32 kDa hydrolyzed the gelatin. Since both the 35- and 32-kDa gelatin-hydrolyzing proteins were missing in the $Acr^{-/-}$ mouse sperm, these two proteins may correspond to a processed and/or mature form of (pro)acrosin. The gelatin-hydrolyzing activities of the 42-, 41-, 35-, and 32-kDa proteins were all inhibited completely when the gels were incubated in the presence of 10 mM pAB after SDS-PAGE. Thus, the 42-kDa protein as well as the 41-, 35-, and 32-kDa forms of (pro)acrosin is sensitive to pAB.

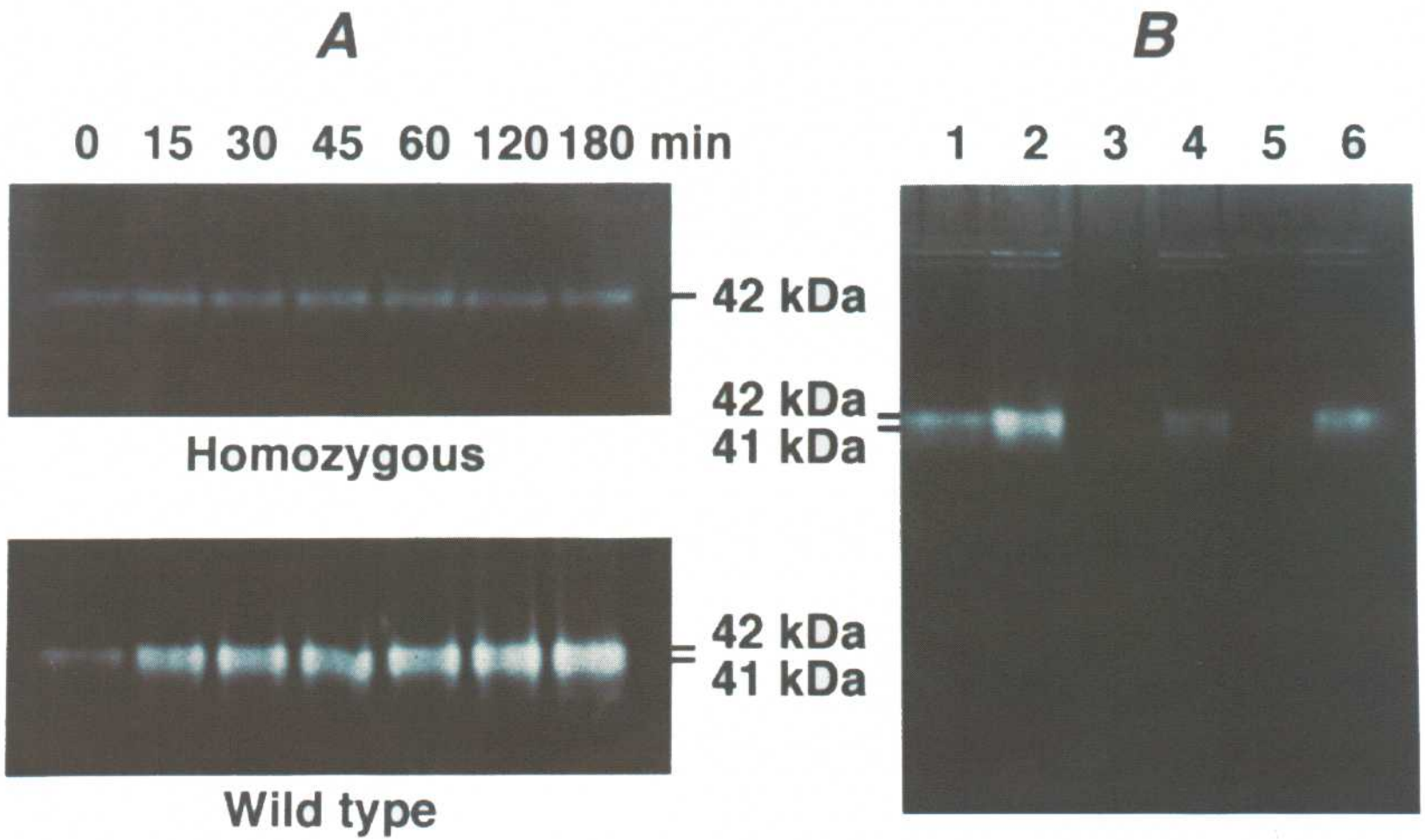

Fig. 15. Acceleration of a 42-kDa protease formation during pH 8.5 treatment of acid extracts from cauda epididymal sperm, and identification of the 42-kDa protein as a serine protease. A, Time course of formation of the active 42-kDa protease and acrosin. Acid extracts (10 μg of proteins) from wild-type (*Acr*$^{+/+}$) and acrosin-deficient (*Acr*$^{-/-}$) mouse sperm were incubated at 37°C in 0.1 M Tris/HCl, pH 8.5, and an aliquot was taken out at various time indicated and subjected to SDS-PAGE in the presence of 0.1% gelatin. Note that a 41-kDa protein probably corresponding to acrosin appears only in the *Acr*$^{+/+}$ mouse sperm after the 15-min incubation, and that in the *Acr*$^{-/-}$ mouse sperm, the level of the active 42-kDa protease remains constant during the pH 8.5 incubation up to 3 h. B, Treatment of sperm acid extracts with various protease inhibitors. The acid extracts (10 μg of proteins) from *Acr*$^{+/+}$ mouse sperm were untreated (lane 1) or treated in 0.1 M Tris/HCl, pH 7.5, containing no inhibitor (lane 2) or 10 mM each of DFP (lane 3), PMSF (lane 4), TLCK (lane 5), and TPCK (lane 6) for 60 min at room temperature, lyophilized, dissolved in 8 M urea, and then subjected to SDS-PAGE in the presence of 0.1% gelatin.

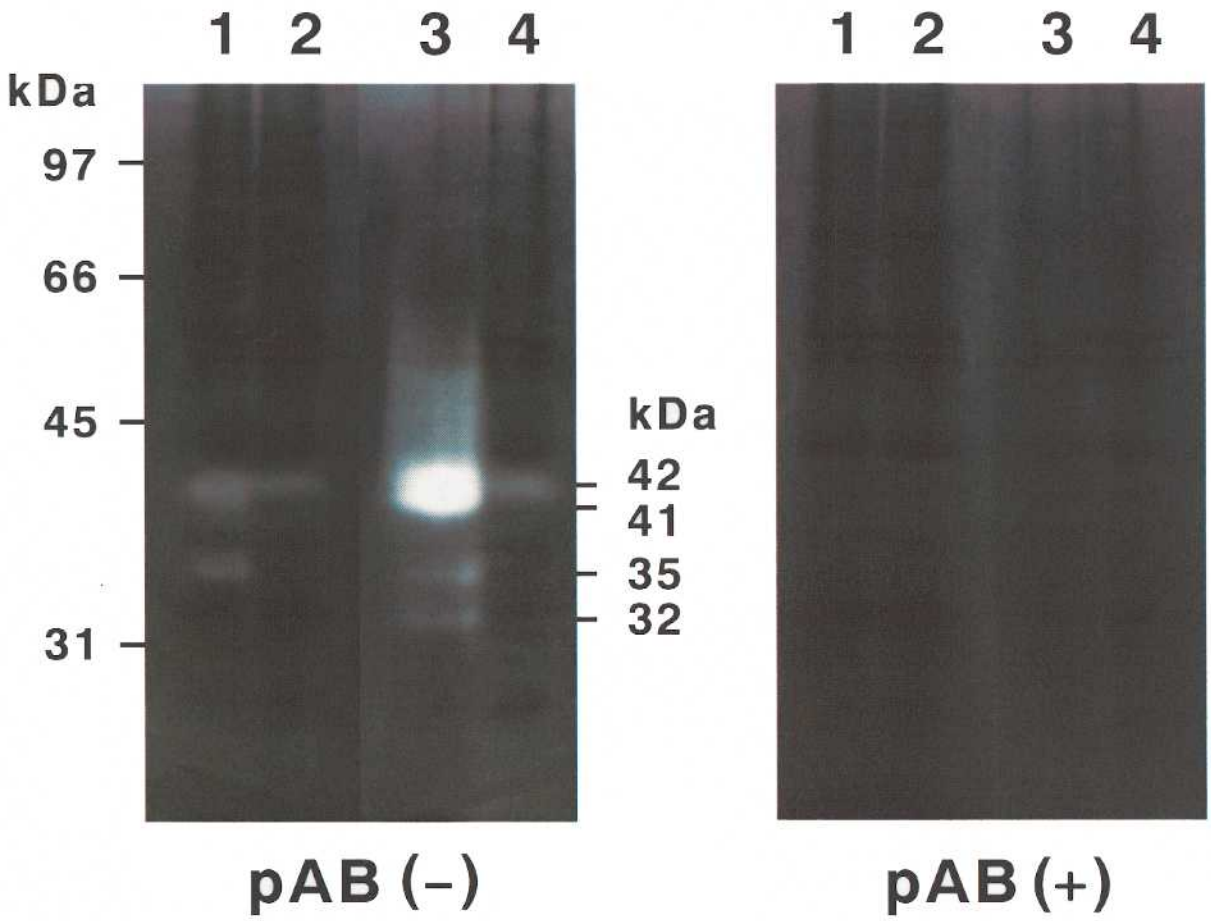

Fig. 16. Detection of gelatin-hydrolyzing activity in protein fractions released from sperm acrosome following calcium ionophore-induced acrosome reaction. Cauda epididymal sperm from wild-type (*Acr*$^{+/+}$, lanes 1 and 3) and acrosin-deficient (*Acr*$^{-/-}$, lanes 2 and 4) mice in TYH medium free of bovine serum albumin were induced to undergo the acrosome reaction with calcium ionophore A23187. The sperm suspension was centrifuged, and the supernatant was dialyzed against 1 mM HCl. The dialyzed solution (20 μg of proteins) was incubated in 0.1 M Tris/HCl, pH 8.0, at room temperature for 0 h (lanes 1 and 2) or 3 h (lanes 3 and 4), and then analyzed by SDS-PAGE in the presence of 0.1% gelatin. After electrophoresis, the gels were washed twice with 2.5% Triton X-100 and with 0.1 M Tris/HCl, pH 8.0, incubated in the same Tris buffer at 37°C overnight, and stained with Coomassie brilliant blue. The washing and incubation procedures were carried out in the presence (+) or absence (-) of 10 mM *p*-aminobenzamidine (pAB). Note that all proteins with the gelatin-hydrolyzing activity, including the 42-kDa protease, are completely inhibited by pAB.

DISCUSSION

This study demonstrates the existence of a pAB-sensitive protease(s) other than acrosin in the acrosome of mouse sperm. The proteolytic enzyme(s) may contribute to sperm penetration of the egg ZP without having a role in dispersing the acrosomal proteins during acrosome reaction. Thus, the data represented here appear to strengthen our previous proposal that the major role of acrosin in the sperm function is likely to accelerate the dispersal of the acrosomal proteins during the acrosome reaction (128). However, it is necessary to emphasize that the direct contribution of acrosin to the limited proteolysis of ZP can not be ruled out completely at the present time.

As far as I have determined, the rate of complete dispersal of acrosomal proteins from the acrosome was very low (19 to 25 and 7 to 9%) in $Acr^{+/+}$ and $Acr^{-/-}$ mouse sperm, respectively, using solubilized ZP as a trigger of the acrosome reaction (see Table II in CHAPTER I and ref. 128). On the basis of the data, I still used calcium ionophore to induce the acrosome reaction of sperm in this study. Although the same experiment using a physiological inducer of the acrosome reaction, egg ZP, is required, the protein dispersal from the acrosome of $Acr^{-/-}$ mouse sperm in the presence of pAB following the ionophore-induced acrosome reaction is consistent with that in the absence of the acrosin inhibitor (Fig. 12). This result supports that acrosin may be a sole protein responsible for accelerating the dispersal of acrosomal proteins during the acrosome reaction. However, there is a discrepancy that sp56 and MC101 in $Acr^{+/+}$ mouse sperm are slightly more readily released from the acrosome in the presence of pAB than found in $Acr^{-/-}$ mouse sperm in the presence and absence of the acrosin inhibitor (Fig. 12). This discrepancy may be explained by a possibility that acrosin has acted on the protein dispersal in a small degree just before pAB inhibits the enzyme in the acrosomal matrix of $Acr^{+/+}$ mouse sperm, since it is unlikely that pAB and benzamidine (95) are permeable to the sperm membranes.

Fraser (1982) (69) clearly demonstrated that pAB does not affect the motility of capacitated sperm and vesiculation between the plasma and outer acrosomal membranes in mouse sperm, and found that the removal of pAB from sperm suspension pre-treated with the inhibitor gives a fertilization rate highly similar to the untreated sperm. Thus,

it appears reasonable to consider that pAB is unlikely to be toxic to sperm cells up to at least 1 mM concentration, and has the following two effects on fertilizing sperm by inhibiting trypsin-like proteases, including acrosin: the delay in dispersing proteins from the acrosomal matrix, and the block of sperm penetration of ZP. In the present study, neither $Acr^{+/+}$ nor $Acr^{-/-}$ mouse sperm hardly penetrated ZP in the presence of 1 mM pAB until 6 h after insemination (Fig. 13). I can predict much higher rates of the sperm penetration, because such a long incubation time is presumably enough for the complete dispersal of the acrosomal matrix in some sperm following the ZP-mediated acrosome reaction, in spite of the delay due to the presence of pAB or the absence of acrosin. However, the penetration rates are negligibly low (1 to 7%) in the presence of pAB (Fig. 13). Therefore, these data imply that pAB effectively inhibits the protease activity responsible for creation of a penetration pathway for motile sperm, and provide direct evidence that a pAB-sensitive protease(s) other than acrosin may serve to enable sperm to penetrate the ZP. Although the attachment (adhesion) of $Acr^{+/+}$ and $Acr^{-/-}$ mouse sperm to ZP was totally normal on IVF in the presence of pAB (data not shown), we have not yet examined possible inhibitory effects of pAB on the primary and/or secondary binding of sperm to ZP. Thus, further experiments regarding the sperm binding to ZP are necessary to prove conclusively that the sperm penetration of ZP essentially requires the protease activity inhibited by pAB.

A 42-kDa protease exhibiting gelatin-hydrolyzing activity is present in the acrosome of $Acr^{+/+}$ and $Acr^{-/-}$ mouse sperm (Figs. 15, 16, and ref. 104). This protease most likely belongs to a superfamily of trypsin-like serine proteases, since the activity was inhibited by DFP, TLCK, and pAB (Figs. 15, 16). It is intriguing that this protein is enzymatically active without the treatment of the sperm acid extracts at pH 8.5, and is scarcely activated in the absence of acrosin (Fig. 15). Also, the molecular size of the 42-kDa protease is not changed even when it is activated by acrosin (Fig. 15). A possible explanation for these results is that the 42-kDa protease as well as acrosin may be present in the acrosome as an enzymatically inactive proprotein, a part of which is already activated by a processing enzyme(s) with a trypsin-like cleavage specificity. If so, the latent form (pro-form) of the 42-kDa protease can be converted into the active enzyme not by autoactivation but by other enzyme(s), including acrosin, during the acrosome reaction, and the active 42-kDa protease may then serve the limited

proteolysis of egg ZP. To prove this possibility, the structure/function relationship of the pro- and mature-forms of the 42-kDa protease remains to be examined. At any rate, the 42-kDa protease is a candidate enzyme involved in the sperm penetration of the ZP.

Accumulating data of acrosin indicate that the major role of the enzyme in the sperm function is not limited proteolysis of egg ZP but acceleration of protein dispersal from the acrosomal matrix. Moreover, we have recently isolated cDNA clones encoding each of four different serine proteases, TESP1, TESP2 (131), TESP3, and TESP4 (132), from a mouse testis cDNA library. These proteases appear not to be detected as gelatin-hydrolyzing proteins on SDS-PAGE in the presence of gelatin (Figs. 15, 16. and ref. 104). Thus, it may be also important to pay attention to other proteases that are present in very small quantities in the acrosomal matrix and/or on the acrosomal membranes.

SUMMARY

It has been reported that a significant delay of protein dispersal from the acrosomal matrix is observed in wild-type mouse sperm by adding *p*-aminobenzamidine, a trypsin/acrosin inhibitor, to the incubation medium. The pattern of this delayed release was similar to that of the acrosin-deficient mutant mouse sperm, as described in CHAPTER I (128). In the present paper, no further delay of the protein dispersal was found when the acrosin-deficient sperm were treated with *p*-aminobenzamidine, indicating that among the *p*-aminobenzamidine-sensitive protease(s), only acrosin may function to accelerate this process (Fig. 17). Although the acrosin-deficient sperm penetrated the zona pellucida (104), the addition of *p*-aminobenzamidine in the fertilization medium caused a significant inhibition of fertilization *in vitro*. The result indicates that there is a *p*-aminobenzamidine-sensitive protease(s) other than acrosin participating in the zona penetration step (Fig. 17). Indeed, we demonstrated that a non-acrosin protease with a size of 42 kDa was present in the supernatant of the acrosome-reacted sperm suspension (Fig. 17). The enzyme was inhibited by *p*-aminobenzamidine, diisopropyl fluorophosphate and *N*-tosyl-L-lysine chloromethyl ketone, and was apparently activated by acrosin (Fig. 17).

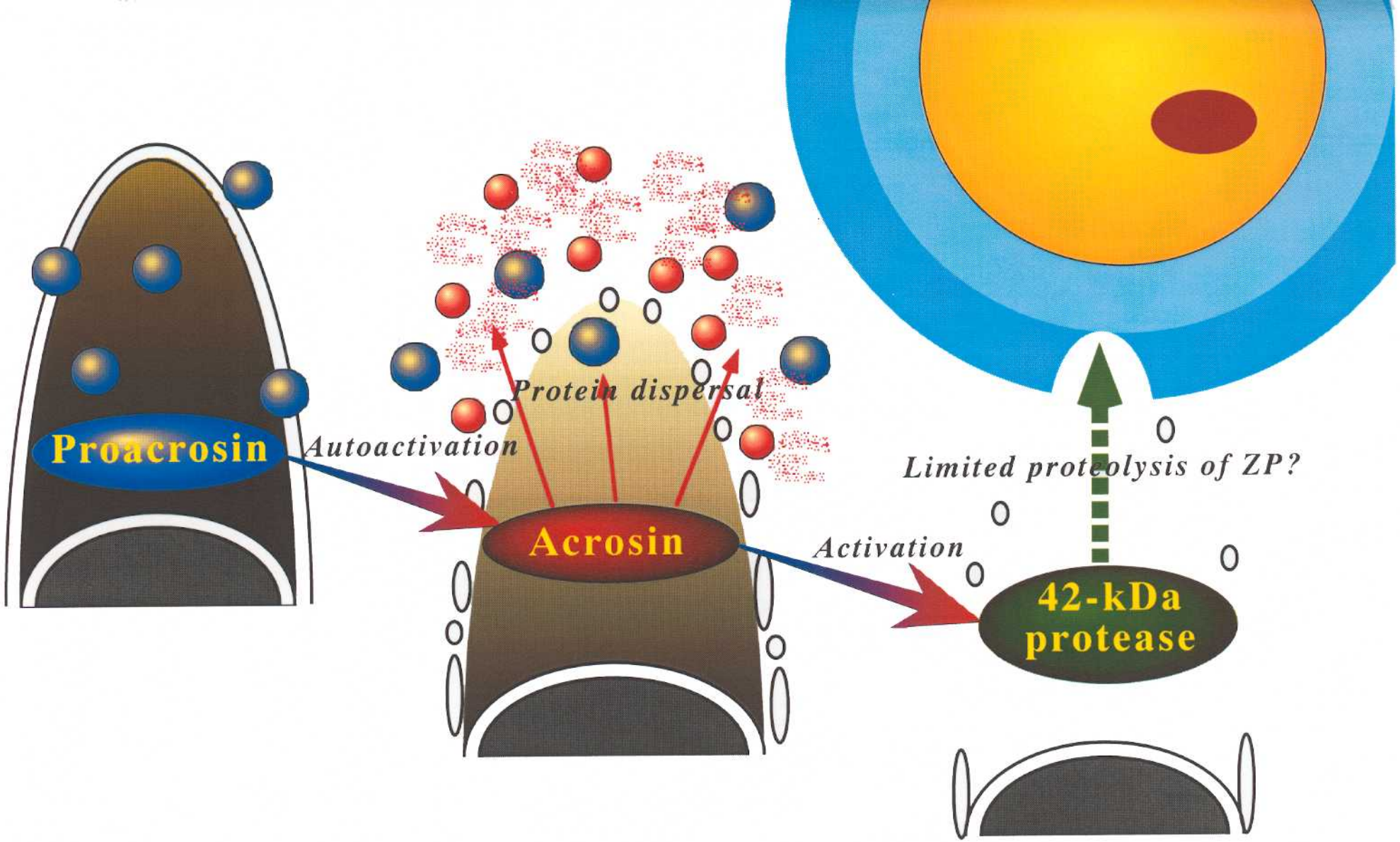

Fig. 17. Potential mechanism of limited hydrolysis of ZP. Among the *p*-aminobenzamidine-sensitive protease(s), only acrosin may function to accelerate the dispersal of acrosome proteins. There is a p-aminobenzamidine-sensitive protease(s) other than acrosin participating in the zona penetration step. Indeed, we demonstrated that a non-acrosin protease with a size of 42 kDa was present in the supernatant of the acrosome-reacted sperm suspension. The enzyme was inhibited by *p*-aminobenzamidine and was apparently activated by acrosin.