

# INVOLVEMENT OF AN ACROSINLIKE PROTEINASE IN THE SULFHYDRYL-INDUCED DEGRADATION OF RABBIT SPERM NUCLEAR PROTAMINE

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## ABSTRACT

Previous studies demonstrated that proteolytic activity is associated with isolated rabbit sperm nuclei and is responsible for the degradation of nuclear protamine that occurs during thiol-induced in vitro decondensation of the nuclei (Zirkin and Chang, 1977; Chang and Zirkin, 1978). In this study, we present the results of experiments designed to characterize this proteolytic activity. Basic protein isolated from rabbit sperm nuclei incubated with 5 mM dithiothreitol (DTT) and 1% Triton X-100 for increasing periods of time exhibited progressively faster migrating bands on acid-urea polyacrylamide gels, reflecting the progressive degradation of protamine. Ultimately, a specific and characteristic peptide banding pattern resulted. When sperm nuclei were treated with the esterase inhibitor nitrophenyl-*p*-guanidino benzoate (NPGb) to inhibit the nuclear-associated proteolytic activity and then incubated with one of several exogenous proteinases in addition to DTT and Triton X-100, characteristic peptide banding patterns were seen for each exogenous proteinase employed. For trypsin, chymotrypsin, pronase, and papain, the peptide banding patterns differed from one another and from the pattern characteristic of protamine degradation by the nuclear-associated proteinase. By contrast, when rabbit acrosin served as the exogenous proteinase, the peptide banding pattern seen was identical to the pattern characteristic of the nuclear-associated proteinase. These results demonstrate directly that the proteinase associated with rabbit sperm nuclei and involved in sperm nuclear decondensation in vitro is acrosinlike.

Striking changes occur in spermatid nuclear morphology and protein composition during spermiogenesis in eutherian mammals. The nuclei of differentiating spermatids condense during progressive stages of spermiogenesis, ultimately transforming into the highly condensed nuclei of mature spermatozoa. In addition, the somatic histones in nuclei of early spermatid stages are replaced entirely by protamine, a basic protein unusually

rich in both arginine and cysteine (1, 3, 13, 21, 23). Upon release from the testis, mammalian spermatozoa migrate through the epididymis, during which time sulfhydryl groups of the cysteine-rich protamine become oxidized (4, 18). This results in unusually large numbers of disulfide cross-links in the nuclei of mature spermatozoa (4, 18). During fertilization, the sperm protamine is replaced by histones (9, 14) and the highly condensed sperm

nucleus decondenses, eventually forming the male pronucleus (2, 6, 25, 26).

Previous studies *in vitro* have shown that disulfide bond reduction is required to decondense nuclei of mature spermatozoa (4, 7, 10, 15, 16, 22, 30). Recently, however, the possibility that sulfhydryl-induced proteolytic activity also is involved in sperm nuclear decondensation *in vitro* has been suggested (7, 19, 20, 30). For example, recent studies have shown that protamine of the sperm nuclei is degraded when spermatozoa or isolated sperm nuclei are incubated *in vitro* with sulfhydryl compounds (7, 19, 20), and that substantial protamine degradation must occur before the sperm nuclei decondense (7, 30). From these *in vitro* studies, it is tempting to speculate that proteolytic degradation of protamine also might be involved in protamine replacement and/or sperm nuclear decondensation during fertilization. However, there is no evidence as yet to indicate whether proteolytic activity, either from the sperm or from the egg, plays a role in these processes.

Indeed, the possible involvement of sperm nuclear proteolytic activity in the *in vitro* decondensation of sperm nuclei has been considered as yet in only few studies (7, 19, 20, 27, 30). Moreover, the chemical nature and the localization of the proteinase involved in protamine degradation have received even less attention (19, 20). In this study, we have employed an experimental approach based upon the hydrolysis of sperm nuclear protamine by exogenous proteinases to characterize the proteolytic activity associated with rabbit sperm nuclei *in vitro* and responsible for nuclear protamine degradation and decondensation. We present evidence demonstrating directly that this proteinase is acrosinlike.

## MATERIALS AND METHODS

### *Isolation of Rabbit Sperm Nuclei*

Ejaculates were obtained from New Zealand white rabbits by means of an artificial vagina and washed twice with 4 vol of ice-cold Tris buffer (50 mM, pH 7.4). To separate sperm heads from tails, the washed sperm suspensions were sonicated  $3-4 \times$  for 1-min intervals at  $0^{\circ}-4^{\circ}\text{C}$  using an Ultrasonics sonicator (Heat Systems-Ultrasonics, Inc., Plainview, N. Y.) at 60 W power output. The sonicated preparations were then made 1.0 M in sucrose, layered into tubes containing 2.0 M sucrose, and centrifuged at 27,000 g for 20 min. The resulting pellets, containing essentially pure sperm nuclei, were treated twice (10 min at  $0^{\circ}-4^{\circ}\text{C}$ ) with 1% Triton X-100 (Triton), then washed twice with 30 ml of Tris buffer. Isolated nuclei were used on the day of preparation; storage of frozen nuclei or of nuclei maintained at  $0^{\circ}-4^{\circ}\text{C}$  for even 1 d resulted in significant loss of nuclear-associated proteolytic activity.

### *Sulfhydryl Incubation of Isolated Sperm Nuclei*

Nuclei ( $2-4 \times 10^6$ ) were incubated for 0-90 min at  $37^{\circ}\text{C}$  in 5 ml of Tris buffer (50 mM, pH 7.4) or Tris buffer containing 5 mM dithiothreitol (DTT) and Triton. At intervals, the reactions were terminated by dilution with large volumes of ice-cold Tris buffer. For examination of the hydrolysis of sperm nuclear protamine by nuclear-associated proteinase, basic protein was extracted from the sulfhydryl-incubated nuclei and electrophoresed on acid-urea polyacrylamide gels (see below).

In some experiments, rabbit sperm nuclei were incubated at  $37^{\circ}\text{C}$  for 30 min in 5 ml of Tris buffer (50 mM, pH 7.4) containing  $1 \times 10^{-4}$  M nitrophenyl-*p*-guanidino benzoate (NPGb) and 0.6% dimethyl sulfoxide, or  $4.5 \times 10^{-5}$  M soybean trypsin inhibitor (SBTI), to inhibit nuclear-associated proteinase, after which excess inhibitor was removed by centrifuging  $3 \times$  in 30 ml of Tris buffer. Sperm nuclei were then resuspended in Tris buffer and incubated in DTT and Triton. Basic protein extracted from these nuclei was then electrophoresed.

### *Incubation of Sperm Nuclei with Exogenous Proteinases*

To examine the pattern of hydrolysis of sperm nuclear protamine by exogenous proteinases of differing specificities (17), we prepared nuclei in which the nuclear-associated proteolytic activity was inhibited with NPGb as above. These nuclei were incubated at  $37^{\circ}\text{C}$  for increasing times (0-180 min) in 5 ml of (a) Tris buffer (50 mM, pH 7.4), (b) Tris buffer containing DTT and Triton, or (c) Tris buffer containing DTT and Triton with either trypsin (2.1 U/ml), chymotrypsin (0.04 U/ml), papain (0.3 U/ml), pronase (2.2 U/ml), or rabbit acrosin containing 10-15 U/ml of esterase activity (see below for acrosin preparation method). 1 mU of acrosin activity is here defined as the amount of enzyme which hydrolyzes 1 nmol/min of  $\alpha$ -*N*-benzoyl-L-arginine ethyl ester (BAEE) at  $25^{\circ}\text{C}$ . In all experiments, the reactions were stopped by dilution of the incubation solutions with large volumes of ice-cold Tris buffer. The nuclei were then pelleted by centrifugation, and nuclear basic protein was extracted and electrophoresed (see below).

### *Preparation of Rabbit Acrosin*

Acrosin extracts were prepared according to procedures modified slightly from those of Huang-Yang and Meizel (12). Testes from 10 rabbits were placed in saline at room temperature and the tunicae were removed by dissection. Acrosin was extracted by homogenizing the testes in 40 ml of ice-cold 0.25 N  $\text{H}_2\text{SO}_4$  with a VirTis Omnimixer (VirTis Co., Gardiner, N.Y.). The resulting homogenates were kept at  $0^{\circ}-4^{\circ}\text{C}$  for 1 h, then centrifuged at 8,000 g for 20 min, and the sediment was discarded. The supernates, containing acrosin, were dialyzed for 72 h against three changes of 4 liter of 1 mM HCl. The dialyzed samples were lyophilized and the resulting powders were dissolved in 5 ml of 1 mM HCl. To remove acrosin inhibitors present in these preparations (29), the acrosin preparations were eluted in 1.5-ml aliquots through a Sephadex G-100 column ( $60 \times 1.5$  cm) equilibrated with 1 mM HCl. Fractions in the molecular weight range  $>40,000$  (using ovalbumin as standard) were combined and lyophilized to dryness. The powder was dissolved in 1 mM HCl and assayed for esterase activity using *p*-tosyl-L-arginine methyl ester (TAME) or BAEE substrates as described by Zanvelid et al. (28). The ratio of the rate of hydrolysis of BAEE

and TAME in our acrosin preparations was ~2.6:1 (units per milliliter). Huang-Yang and Meizel (12) and Zaneveld et al. (28) reported that purified rabbit acrosin hydrolyzed BAEE and TAME in a ratio of 2.5:1 and 2.6:1, respectively.

### Extraction of Sperm Nuclear Basic Protein

Protamine was extracted from sperm nuclei according to the procedures of Marushige and Marushige (19). Nuclei were pelleted by centrifugation and incubated in 1.1 M NaCl, 6 M urea, and 0.1 M 2-mercaptoethanol for 90 min at 37°C to dissociate protamine from DNA. The free sulfhydryl groups of protamine were then blocked by the addition of 0.25 M iodoacetamide in 50 mM Tris buffer at pH 8.0. Incubations were continued in the dark for an additional hour. DNA was precipitated from these solutions by addition of ice-cold dilute HCl to a final concentration of 0.16 M and the solutions were placed on ice. After 30 min, the samples were centrifuged and supernates treated with 20% trichloroacetic acid to precipitate proteins. The precipitates were recovered by centrifugation, dissolved in 0.9 N acetic acid, and dialyzed against 0.9 N acetic acid overnight. To concentrate the samples for electrophoresis, the dialyzed proteins were lyophilized to dryness and the resulting powders were redissolved in 100–150  $\mu$ l of 0.9 N acetic acid containing 1 M urea.

### Electrophoresis

Basic protein samples (15–20  $\mu$ g) dissolved in 0.9 N acetic acid and 1 M urea were electrophoresed in a Hoefer SE 500 slab electrophoresis unit (Hoefer Scientific Instruments, San Francisco, Calif.) using acid-urea polyacrylamide gels modified from those described by Panyim and Chalkey (24). An 11.5-cm separating gel containing 15% acrylamide and 6.25 M urea at pH 3.2 was initially electrophoresed at 320 V constant voltage for 3 h, then overlaid with a 0.5-cm stacking gel, pH 4.0, containing 2.5% acrylamide and 0.6% bisacrylamide cross-linker. After polymerization, the stacking gel was electrophoresed for 5 min at 200 V. The samples were then applied and electrophoresis was continued at 13 mA constant current for 3.5 h. The gels were stained with 0.1% Amido Schwartz (Sigma Chemical Co., St. Louis, Mo.) for 1–1.5 h, then destained overnight in a solution of 45% ethanol and 7% acetic acid. The stacking gel permitted electrophoresis of large samples with significantly greater resolution of peptide bands than that obtained with standard acid-urea gels.

### Reagents

DTT, BAEE HCl, TAME HCl, 3  $\times$  crystallized type II  $\alpha$ -chymotrypsin, and 2  $\times$  crystallized type III papain were obtained from Sigma Chemical Co. Soybean trypsin inhibitor and trypsin were obtained from Worthington Biochemical Corp., Freehold, N.J. Pronase was purchased from Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.

### RESULTS

Basic protein isolated from rabbit sperm nuclei incubated with DTT and Triton at 37°C for increasing periods of time exhibited progressively faster migrating bands on acid-urea polyacrylamide gels (Fig. 1). Protamine isolated from control (untreated) or zero-time nuclei exhibited one major peptide band (Fig. 1a). After incubation of the nuclei in DTT and Triton for 5–10 min, six

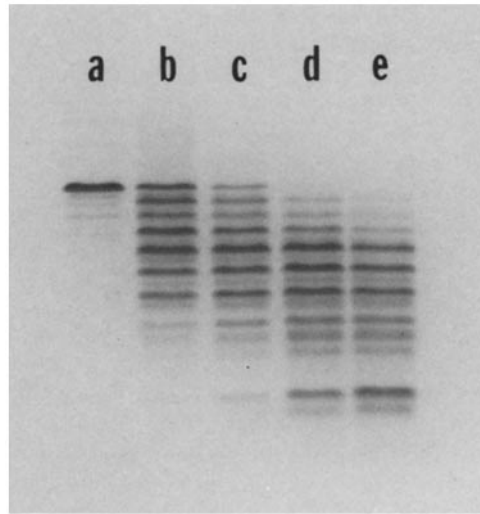
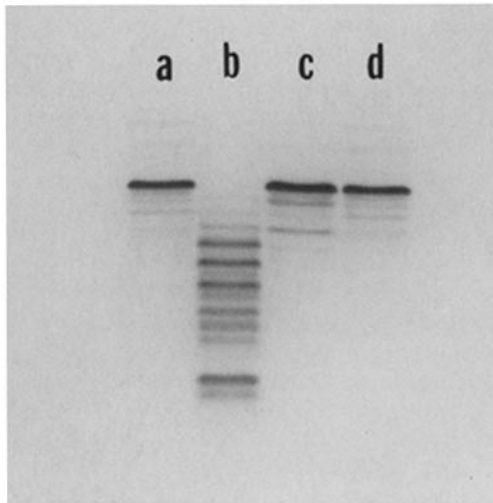


FIGURE 1 Protamine degradation resulting from incubation of isolated rabbit sperm nuclei in 5 mM DTT and 1% Triton at 37°C. Basic protein isolated from nuclei that were incubated in DTT and Triton for 0 (a), 5 (b), 15 (c), 30 (d), and 90 (e) min. The peptide banding pattern at 30 min was identical to that following incubation for 90 min or longer. Migration is from top to bottom on all figures.

major faster-migrating bands in addition to the main protamine band were resolved (Fig. 1b). Degradation of protamine continued through 15 min, with the native protamine band substantially diminished at this time (Fig. 1c). By 30 min, degradation reached its maximum extent (~15 new bands) and the native protamine band no longer was apparent (Fig. 1d). The peptide banding pattern observed at 30 min was identical to that observed following incubation for 90 min or longer (Fig. 1e), although the proportions of individual bands varied somewhat.

Basic protein isolated from sperm nuclei treated first with the esterase inhibitors NPGB (Fig. 2c) or SBTI (Fig. 2d) and, after extensive washing, incubated with DTT and Triton for 90 min, exhibited one major band identical in mobility to that of protamine isolated from control nuclei incubated in the absence of DTT (Fig. 2a). Thus, treatment of sperm nuclei with these esterase inhibitors prevented the extensive degradation of protamine that occurred when uninhibited nuclei were treated with DTT and Triton (Fig. 2b). This indicated that the degradation of sperm nuclear protein seen in uninhibited nuclei resulted from enzymatic proteolysis.

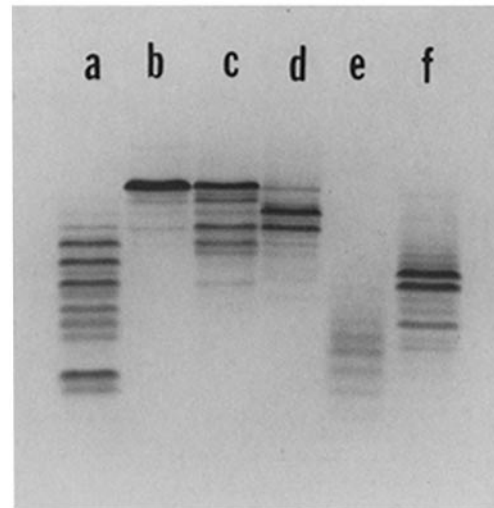


**FIGURE 2** Inhibition of protamine degradation by proteinase inhibitors. (a) Basic protein isolated from sperm nuclei that were incubated for 90 min in Tris buffer. (b) Basic protein from sperm nuclei incubated in DTT and Triton for 90 min. (c) Basic protein from nuclei that were treated first with  $1 \times 10^{-4}$  M NPGB and, after extensive washing, with DTT and Triton for 90 min. (d) Basic protein from nuclei treated first with soybean trypsin inhibitor and, after extensive washing, with DTT and Triton. Treatment of sperm nuclei with esterase inhibitors prevented the extensive degradation of protamine that occurred when uninhibited nuclei were incubated with DTT and Triton.

Previous reports have shown that nuclear protein of somatic cells is degraded differently by proteinases of varying substrate specificities (17). In our experiments, the formation of a specific peptide banding pattern after sperm nuclei were incubated in DTT and Triton for 30 min or more suggested that the sperm protamine was degraded in a manner characteristic of the proteinase associated with the isolated nuclei. This suggestion was supported by the results of experiments in which sperm nuclei were incubated with exogenous proteinases of differing substrate specificities. Isolated rabbit sperm nuclei were incubated with the esterase inhibitor NPGB to inhibit the nuclear-associated proteinase, washed to remove any unbound inhibitor, and then incubated with DTT and Triton in the absence or presence of one of several exogenous proteinases. Basic protein isolated from nuclei incubated first with NPGB and subsequently with DTT and Triton for 90 min exhibited only the native protamine band (Fig. 3*b*); the banding pattern characteristic of sperm nuclear

protamine degradation by the nuclear-associated proteinase (Fig. 3*a*) was not seen. By contrast, when NPGB-inhibited nuclei were incubated with trypsin (Fig. 3*c*), chymotrypsin (Fig. 3*d*), papain (Fig. 3*e*), or pronase (Fig. 3*f*), in addition to DTT and Triton, protamine degradation occurred. A time-course of protamine degradation was determined for each of these proteinases. In each case, a specific peptide banding pattern characteristic of protamine degradation by that proteinase was obtained by 30 min. These characteristic banding patterns differed substantially from one another and, in each case, differed from the peptide banding pattern characteristic of the nuclear-associated proteinase (Fig. 3*a*).

In contrast to these results, when NPGB-inhibited sperm nuclei (Fig. 4*a*) were incubated in DTT and Triton with rabbit acrosin as the exogenous proteinase, a peptide banding pattern was obtained (Fig. 4*b*) that was strikingly similar to the banding pattern characteristic of protamine



**FIGURE 3** Protamine degradation resulting from incubation of sperm nuclei with exogenous proteinases. (a) Protamine degradation characteristic of nuclear-associated proteinase. (b) Basic protein from nuclei that were treated first with NPGB and subsequently incubated with DTT and Triton. NPGB-inhibited nuclei incubated in DTT and Triton with either trypsin (c), chymotrypsin (d), papain (e), or pronase (f) for 90 min. With each exogenous proteinase, a specific banding pattern characteristic of protamine degradation by that proteinase was obtained. These characteristic banding patterns differed from one another and from the peptide banding pattern characteristic of the nuclear-associated proteinase.

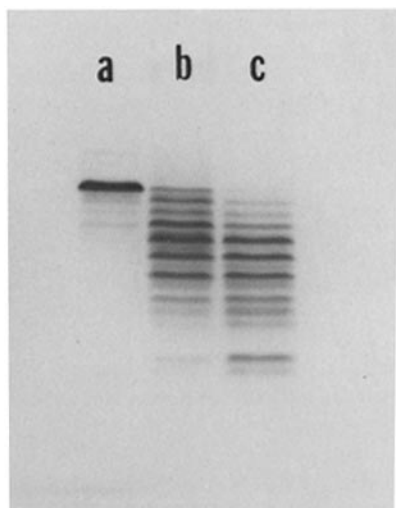


FIGURE 4 Protamine degradation resulting from incubation of sperm nuclei with rabbit acrosin. (a) Basic protein from NPGB-inhibited nuclei that were incubated with DTT and Triton for 90 min. (b) Basic protein from NPGB-inhibited nuclei incubated with DTT, Triton, and rabbit acrosin for 90 min. (c) Basic protein from uninhibited nuclei treated with DTT and Triton for 90 min. The peptide banding pattern with acrosin as the exogenous proteinase was strikingly similar to the banding pattern characteristic of protamine degradation by the nuclear-associated proteinase.

degradation by the nuclear-associated proteinase (Fig. 4c); band-for-band similarity was seen, although the proportions of the individual bands varied somewhat.

## DISCUSSION

Young (27) recently reported that rabbit spermatozoa centrifuged through sucrose or incubated with Triton did not decondense when subsequently incubated in DTT and Triton, and concluded that centrifugation through sucrose or treatment with Triton removed proteolytic activity from the spermatozoa. In marked contrast, our present and previous (7, 30) results demonstrate clearly that sperm nuclei isolated through sucrose and then incubated with Triton retain the proteolytic activity involved in proteolytic degradation of protamine and in sperm nuclear decondensation.

The results of the studies presented herein demonstrate that incubation of rabbit sperm nuclei for increasing times in DTT and Triton resulted in degradation of nuclear basic protein in a specific manner, observed as characteristic banding on

acid-urea polyacrylamide gels. We interpret the formation of this characteristic banding pattern as reflecting, in part, the substrate specificity of sperm-associated proteinase. This interpretation is supported by the results of studies in which exogenous proteinases of differing substrate specificities were employed. Thus, when sperm nuclei were treated with the esterase inhibitor NPGB to inhibit the nuclear-associated proteinase, washed extensively, and then incubated either with trypsin, chymotrypsin, pronase, or papain in addition to DTT and Triton, specific and characteristic banding patterns were seen for each of the proteinases employed. In each case, these banding patterns differed from one another and from the pattern characteristic of protamine degradation by the nuclear-associated proteinase.

We used this specificity of protamine degradation by nuclear-associated and exogenous proteinases to determine whether the nuclear-associated proteinase might be similar or identical to rabbit acrosin. Acrosin was isolated from rabbit testes and used as an exogenous proteinase as in the experiments above. In contrast to the results obtained with trypsin, chymotrypsin, papain, and pronase, however, when NPGB-inhibited sperm nuclei were incubated with a combination of DTT, Triton, and rabbit acrosin, a characteristic banding pattern was obtained that was essentially identical to that obtained by incubating control (uninhibited) nuclei with DTT and Triton. These results indicate that the nuclear-associated proteinase is acrosinlike.

The results of these experiments permit at least two quite different interpretations: (a) an acrosinlike proteinase is associated with sperm chromatin *in situ*; or (b) the proteolytic activity associated with sperm chromatin *in vitro* is of acrosomal origin and becomes bound to the chromatin during the *in vitro* treatments of the spermatozoa. Proteolytic activity has been reported to be associated with chromatin isolated from mouse and bull testes (8) and from somatic cell nuclei (5, 11). Thus, it is conceivable that the rabbit sperm nucleus itself is the source of the activity. That the proteolytic activity associated with both rabbit and bull (19, 20) sperm nuclei has properties in common with acrosin also suggests the possibility that the activity associated with isolated nuclei might be derived from the acrosome during the isolation procedure. Experiments under way in our laboratory are designed to determine which of these possibilities is correct.

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