Vol. 138, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1986 Pages 139-145

BIOCHEMICAL STUDIES ON PROACROSIN AND ACROSIN FROM EPIDIDYMAL BOAR SPERMATOZOA: IN VITRO TRANSLATION OF BOAR TESTICULAR PROACROSIN mRNA

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Received May 23, 1986

SUMMARY An inactive form of acrosin was extracted from epididymal boar spermatozoa utilizing acid pH conditions. When subjected to activation in alkaline environment, this form turns into an enzymatically active species, which exhibits close-related electrophoretic characteristics. Both the precursor and the activated species, when incubated in the presence of thermolysin, give rise to two fastly moving acrosin molecular forms. In order to establish the nature of the true acrosin zymogen, we isolated poly(A^+)-RNA from boar testicles, performed its translation in vitro in the presence of [^{35}S] -methionine and reticulocyte lysate, immunoprecipitated the translation products with anti-boar acrosin antibody, and analyzed them by SDS-polyacrylamide gel electrophoresis and autoradiography. A single translation product of molecular weight 55,000 was detected. It is concluded that the polypeptide chain of the boar zymogen is of 55,000; increases in molecular weight are due to post-translational modifications, like glycosylation.

Acrosin (E.C.3.4.21.10), a spermatozoan trypsin-like enzyme involved in the mammalian fertilization, is stored in the acrosome in its precursor form, proacrosin (1). The activation of the zymogen appears to give rise to more acrosin active forms (2-3) and different molecular weight species both of proacrosin and of acrosin have been described often (2-6). Inhibition of the precursor activation has been proposed as a potential method of fertility control (7). However, the nature of the boar zymogen has not been yet well established (4,5,8).

In order to gain some insight into the molecular characterization of the components of the so-termed "proacrosin-acrosin system", we have sought to study this system using as starting material epididymal spermatozoa. Hopefully this will minimize, or completely avoid, the influence of factors (such as the presence of already "activated" or damaged sperm) that usually interfere with the interpretation of the results when ejaculated spermatozoa are employed.

Abbreviations: 1. N-benzoyl-DL-arginine-β-naphthylamide
2. sodium dodecyl sulfate-polyacrylamide gel electrophoresis
3. enzyme-linked immunosorbent assay

0006-291X/86 $1.50

Moreover, we hope to identify the polypeptide resulting from the synthesis directed by proacrosin mRNA. Towards this goal, we have performed the translation of boar testis poli(A^+)-RNA in a reticulocyte lysate system. The translation products have been immunoprecipitated with an anti-acrosin antibody, which recognizes different molecular species of acrosin (8-9), and the immunoprecipitated products have been analysed by gel electrophoresis and autoradiography.

Such studies have lead to the identification both of boar epididymal proacrosin and of the polypeptide synthesized by the proacrosin mRNA.

MATERIALS AND METHODS

Reagents: [^{35}S] -methionine (specific activity 1200 Ci/mmol) and rabbit reticulocyte lysate for performance of in vitro translation were purchased from Amersham International plc, Buckingampshire, England. Pansorbin (Staphylococcus aureus) was supplied by Calbiochem, La Jolla, CA U.S.A. Reagents for electrophoretic separation of proteins and RNAs were from BIO-RAD, Richmond, CA U.S.A. Oligo(dT)-cellulose, type 7, was purchased from Pharmacia Fine Chemicals,Uppsala, Sweden. Thermolysin (Protease type X), and BzArgNNab were from Sigma, St.Louis,MO. All other reagents were the best grade available.

Animal and collecion of epididymal sperm: A sexually mature boar was castrated at the Veterinary Department of the University of Milan. The excised testes were immediately dissected, the tunica albuginea removed with forceps, and parenchimal tissue fragments, if not used at once, frozen in liquid nitrogen and stored at -70°C. The epididymes were removed and an incision was made in the caudal region of each epididymis. This was then squeezed with a forcep and the sperm in the epididymal fluid appearing in the incision were removed with a Pasteur pipette. The sperm suspension was immediately transferred into a crio-tube and immersed in liquid nitrogen. The quick-frozen sperm were stored at - 70°C.

Extraction of sperm proacrosin/acrosin: Frozen epididymal sperm were thawed at room temperature and suspended in 0.3 M acetic acid, 50 mM NaCl, 50 mM benzamidine, pH 2.6. The pH of the suspension was re-adjusted to 2.6 and three sequential extraction procedures (15 min of stirring at 4°C/each) were carried out. The three supernatants (each from a centrifugation at 2,000g for 15 min at 4°C) were pooled and centrifuged at 25,000g for 30 min and 4°C. The resulting supernatant is referred to as the crude proacrosin/ acrosin preparation. Aliquots to be subjected to enzymatic assays (2), thermolysin-treatment (8), and activation (2) were dialyzed against 1 mM HCl, pH 3.0, to remove benzamidine.

Electrophoresis and Immunoblotting: Protein samples were subjected to analytical disc gel electrophoresis according to a modification of the cathodic gel system for basic proteins of Reisfeld et al. (10). Acrosin activity in the gel was determined with BzArgNNab as described by Muller-Esterl and Fritz (11). Proteins first subjected to the non-denaturating gel electrophoresis were also transferred to nitrocellulose paper according to Towbin et al. (12). The immunological detection of proteins was carried out essentially as previously reported (8).

Extraction of total RNA: Tissue fragments were homogenized in a guanidinium thiocyanate extraction medium (13) using the high-speed Polytron homogenizer (Brinkman Instruments, Westbury N.Y.). The homogenate was processed for the RNA separation by ultracentrifugation through a dense cushion of cesium chloride according to Glisin et al.(14). After removal of the cesium chloride cushion, the RNA pellets were dissolved in 10 mM Tris-HCl, pH 7.4, 5 mM EDTA, and 1% SDS and treated according to Maniatis(15).

Isolation of polyadenylated RNA: Poly(A^+)-containing RNA was separated by two cycles of binding to oligo(dT)-cellulose, essentially following the procedure of Aviv & Leder (16).The eluted poly(A^+)-RNA was recovered by centrifugation and redissolved in sterile water for storage at - 70°C.

Cell free translation of poly(A^+)-RNA: Translation of boar testis poly(A^+)-RNA was carried out with a reticulocyte lysate translation system. In a typical experiment, the reaction mixture (total 35 μl) consisted of 28 μl of lysate, 5 μl of exogenous RNA and 2 μl (10 μCi) of [^{35}S] -methionine. The translated products were analyzed by SDS-PAGE on 10% poly- acrylamide slab gels. These gels were prepared and run according to Laemmli (17) and then stained for protein with Coomassie-Blue. Autoradiography was performed with Kodak XR x-ray films.

Immunoprecipitation: Following translation, two reaction mixtures were diluted in a dilution medium (18). The gamma globulin fraction of a non immune rabbit serum was added to each reaction mixture. After 1 h incubation at 4°C, an excess of Pansorbin was added, and the incubation was continued for an additional 1h at 4°C. After removal of Pansorbin by centrifugation, one of the two resulting supernatants was incubated again with the rabbit non immune serum, whereas the other was incubated with the rabbit anti-boar acrosin antibody overnight at 4°C. The Pansorbin treatment was repeated and the two Pansorbin-bound antigen-antibody complexes were extensively washed in the dilution medium. Then, they were analyzed by SDS-PAGE on 7.5% polyacrylamide slab gel, which was stained and subjected to autoradiography as above reported

RESULTS AND DISCUSSION

The electrophoretic pattern of BzArgNNab hydrolyzing activity exhibited by the epididymal proacrosin/acrosin preparations, some of which were previously subjected to specific enzymatic treatments, is illustrated in Fig. 1,A. The

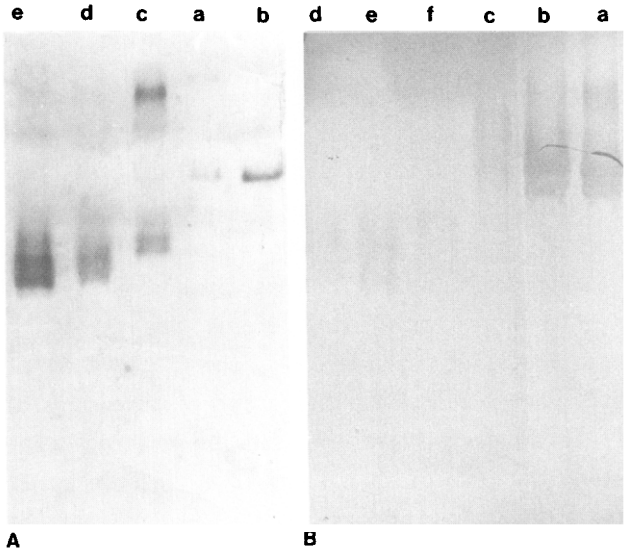


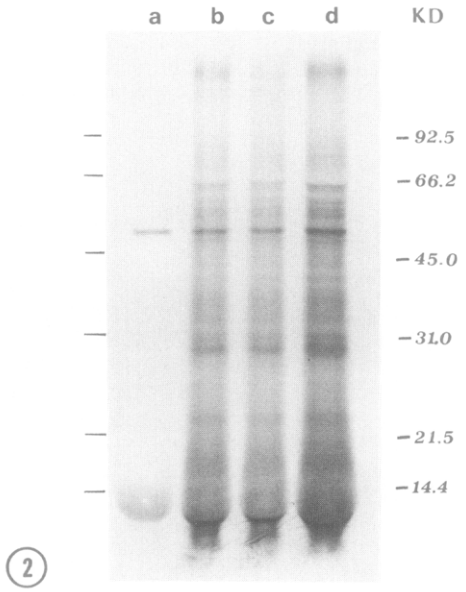
Figure 1. Electrophoretic patterns of epididymal proacrosin/acrosin preparations run under non-denaturating conditions. A: gel stained for BzArgNNab hydrolyzing activity. B: immunological detection of proteins transferred onto nitrocellulose paper. Lane: a, native preparation; b, acid-maintained (40 min at pH 3.0 and room temperature) native preparation; c, activated (40 min at pH 8.2 and 37°C) sample; d, thermolysin-treatment (10 min at pH 7.2 and 37°C) of the activated preparation; e, thermolysin-treatment of the native preparation; f, thermolysin-treatment of the acid-maintained sample

native preparation (lane a) showed no detectable activity; only after a 90 min incubation in the substrate medium, traces of activity seemed to become visible. The sample subjected to activation gave rise to a marked band (lane c), the electrophoretic mobility value of which corresponded to a RF=0.22. When the native and activated samples underwent the thermolysin-treatment, they showed similar activity patterns (lanes d and e). Fig. 1,B illustrates the correspective staining patterns obtained by the immunological detection performed on the same proacrosin/acrosin preparations. It appears that whereas the "treated" preparations gave rise to pictures that reflect the ones obtained by the enzymatic analysis, the native preparation showed a band, characterized by the lowest RF value found, i.e., 0.20, that was not enzymatically evidenced. Moreover, the native preparation, if kept at acidic pH (3.0) for 40 min and at room temperature, behaves in a peculiar manner as evidenced by both the two specific staining methods (Fig. 1, lanes b); this will be the subject for the next experimental analysis.

Previously, we have reported (8) the existence, in the acrosomal extract of freshly ejaculated boar spermatozoa, of an acrosin molecular species exhibiting in non-denaturating conditions an RF value of 0.21 and an apparent molecular weight of about 66 KD, calculated on SDS-PAGE data. This form, when first subjected to thermolysin treatment and then to analytical disc gel electrophoresis, appeared as a very thick band of activity, probably due to two close-migrating active acrosin forms, with an approximate RF=0.62 (8).

A similar behaviour, after analogous treatment, was shown both by the native epididymal sperm proacrosin/acrosin preparation (Fig. 1, lane e) and by the relative activated sample (Fig. 1, lane d). This suggests that the RF=0.20 reported here may be the true boar proacrosin since it appears to behave like a zymogen. In fact, it is evidenced immunologically, but not enzymatically; after prolonged activation (19) it turns into an enzymatically active molecular species of very similar electrophoretic mobility characteristics. In addition, both the putative zymogen and the "activated" precursor can originate two fastly moving molecular species of acrosin of similar RF values (=0.62 and 0.65). This occurs when the samples undergo a processament of proteolytic digestion that mimics that supposed by McRorie et al. (20) to take place "in vivo" by the action of acrolysin, the apparent thermolysin-like acrosomal protease. Such a result agrees with what we assumed to be the high molecular weight species of acrosin (RF=0.21) extracted from ejaculated spermatozoa (8). Indeed, earlier Mukerji and Meizel (21), in the rabbit, found a 68 KD proacrosin which after complete activation formed a 34 KD acrosin and these authors suggested therefore that proacrosin may contain two polypeptide chains of about the same molecular weight.

RNA prepared from intact testes gave ultraviolet spectra expected for nucleic acids and the RNA preparations were quantitated on the basis of A_{260}. Electrophoresis of RNA under denaturating conditions (15) showed the presence of high molecular weight species of RNA. Addition of total testicular RNA to the reticulocyte lysate system produced a substantial stimulation of polypeptide synthesis (Fig.2, lane d). Poly(A^+)-mRNA directed the synthesis of a heterogeneous population of polypeptides ranging in molecular weight from 10 to more of 100 KD (Fig.2, lanes b-c). Of the large number of the translated polypeptides only one polypeptide of molecular weight of 55 KD was detected in the specimen precipitated by the anti-acrosin antibody (Fig.3, lane a). This polypeptide was not observed when non immune rabbit serum was used for the immunoprecipitation (Fig.3, lane b). Thus, it would appear that the original proacrosin polypeptide chain is of about 55 KD. With the cell-free translation system here used the glycosilation of protein cannot occur; since boar acrosin is a glycoprotein and the carbohydrate portion represents approximately 10% of the total molecular weight (22), a molecular weight of 60 KD or more might be expected for the boar zymogen. To date, different molecular weights have been reported for boar proacrosin from ejaculated spermatozoa. Schleuning et al (5)



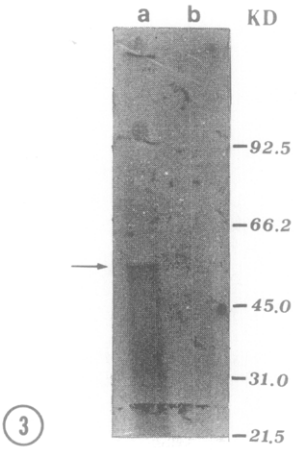


Figure 2. Autoradiography of the SDS-10% polyacrylamide gel electrophoresis of the products synthesized by the translation of: **endogenous mRNA** (lane a); **testicular poly(A^+)-RNA** (lane b: 2µg; lane c:1µg); **total testicular RNA** (lane d).

Figure 3. Autoradiography of the SDS-7.5% polyacrylamide gel electrophoresis of the translated products immuno- precipitated by: **anti-boar acrosin antibody** (lane a) and the gamma globulin fraction of a **non immune rabbit serum** (lane b). Of the large number of polypeptides synthesized by the translation of testicular poly(A^+)-RNA, only a 55 KD species (arrow) is specifically immunoprecipitated by the anti- acrosin antibody.

and Berruti (8) found precursor forms of more 60 KD; Polakoski and Parrish (4) reported the existence of two proacrosins of 55 and 53 KD (with RF values on non-denaturating gels of 0.36 and 0.38 respectively). These discrepancies as to the molecular weight of the precursor are probably due to the different extraction conditions employed and to the quality of the starting semen. As to our previous result (the calculated molecular weight was 66 KD), we cannot exclude a slight overestimation which might be due to the detection method used, i.e., a peroxidase type ELISA[3] test (23), which evidenced a rather diffuse band (8).

With the present investigation we supply two novel kinds of approach to the study of the "proacrosin/acrosin system" in the boar. The first, i.e., the use of epididymal spermatozoa has allowed us to identify an interesting molecular species, a plausible candidate for the true boar zymogen. The second, i.e., the research addressed to individualize the proacrosin mRNA has allowed us to verify that the translation product synthesized by such a mRNA is a single polypeptide of about 55 KD of molecular weight.

ACKNOWLEDGEMENTS

This work was supported by Bilateral Project n. 85.00412.04 from the Consiglio Nazionale delle Ricerche. The authors thank the equipe of the Veterinary Faculty of University of Milan for the surgical operation of the boar.

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