ISOLATION AND PARTIAL CHARACTERIZATION OF BOAR PROACROSIN

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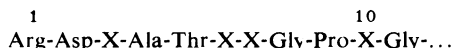
Boar proacrosin was purified to apparent homogeneity by a three-step procedure: gel filtration on Sephadex G-50 medium, ion-exchange chromatography on CM-Cellulose 32, and reversed-phase high-performance liquid chromatography on a C_4 column. The relative molecular mass (M_r) of the proacrosin estimated by gel filtration was about 70 000, whereas the results of an electrophoretic experiment on SDS-polyacrylamide gel with copolymerized casein under non-reducing conditions indicated an M_r of 55 000—60 000. The proacrosin reproducibly migrated on the gel as a double band. When purified, it remained stable at pH 8·0 for 30 min. The amino-acid composition of the homogeneous proacrosin was determined, the N-terminal amino-acid sequence being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Arg-Asp-X-Ala-Thr-X-X-Gly-Pro-X-Gly-...

Acrosin (EC 3.4.21.10) is a key proteinase localized in the acrosome of the spermatozoon. It is a three-domain glycoenzyme possessing a serine proteinase active site, a hydrophobic binding domain, and a carbohydrate binding domain (for review see ref.[1]). Acrosin is involved in the binding of the spermatozoon to the zona pellucida of the ovum[2–4] and penetration of the zona pellucida by the male gamete[5,6]. In the intact spermatozoon, acrosin is present in the form of an inactive precursor, proacrosin[7–10], and is activated to acrosin after disruption of the acrosome[11]. The activation of proacrosin gives rise to several active forms[8,9]. Some active forms from boar spermatozoa have been described[12,13]. Recently a very unstable α-form of boar proacrosin was isolated in our laboratory[14]. For the study of the molecular basis of the proacrosin activation process, purified intact proacrosin is essential. Here we describe a procedure of proacrosin isolation from boar spermatozoa and characterize the purified zymogen.

EXPERIMENTAL

Materials

Sephadex G-50 medium and Sephadex G-100 superfine were from Pharmacia Fine Chemicals (Uppsala, Sweden). CM-Cellulose 32 was a product of Whatman Biochemicals Ltd. (Maidstone, England). N_α-benzoyl-DL-arginine *p*-nitroanilide.HCl, sodium dodecyl sulfate, acrylamide, N,N,N',N'-tetramethylenediamine, human serum albumin, egg albumin, bovine chymotrypsinogen, bovine chymotrypsin, human plasmin, chondroitin sulfate. Na salt, carbonic anhydrase, were from Serva (Heidelberg, F.R.G.). N,N'-methylene-bis(acrylamide) was from Koch–Light (Colnbrook, England). Benzamidine.HCl and casein were purchased from Fluka (Buchs, Switzerland), β-galactosidase from Sigma (St. Louis, U.S.A.). Amidoblack 10 B was from Merck (Darmstadt, F.R.G.). Vydac 214 TP 24 column (4·6 × 250 mm), 5 μm particle size, was from Chromatopack (Middleburg, Tne Netherlands). Elongation factor T_u from *Escherichia coli* was a gift from J. Jonák.

Methods

Preparation of acrosomal extract. Fresh boar semen, 170 ml (15·3 . 10^9 spermatozoa), was centrifuged at 600 × g for 20 min at 15°C to remove seminal plasma. The spermatozoa were washed twice in sucrose medium pH 6·5 (0·264M sucrose, 0·005M N-morpholinoethane sulfonic acid) and centrifuged at 1 000 × g for 20 min at 15°C. The pellet of spermatozoa (20 ml) was extracted overnight with the same volume of 2% acetic acid, 10% glycerol and 50 mM benzamidine at 4°C. The acrosomal extract was centrifuged at 13 000 × g for 20 min at 4°C. The clear supernatant (24 ml), the source of crude proacrosin, was purified by a three-step procedure.

STEP 1. Gel filtration

Crude proacrosin was applied on a column of Sephadex G-50 medium (2·5 × 68 cm) equilibrated with 0·01M-HCOONa (pH 3·0). Fractions with acrosin activity eluted with same buffer were pooled (65 ml, 3·6 U/ml).

STEP 2. Ion-exchange chromatography

The fraction with acrosin activity (65 ml, 234 U) obtained from STEP 1 was applied to a column of CM-Cellulose 32 (2·8 × 6·5 cm) equilibrated with 0·01M-HCOONa (pH 3·0). The column was washed with 282 ml of the equilibration buffer and then a salt gradient (0—0·5M-NaCl) in equilibration buffer was applied. Pooled fractions with acrosin activity (48 ml, 4 U/ml) were divided in small portions (1 ml) and stored at −70°C.

STEP 3. High-performance liguid chromatography

The fraction with acrosin activity from the ion-exchange chromatography column was further purified by HPLC on a prepacked Vydac 214 TP 54 column (4·6 × 250 mm). A 0·250 ml sample was applied in each run and the column was eluted at a flow rate of 1·0 ml/min with a linear gradient of 0—32% acetonitrile in 0·1% trifluoroacetic acid (TFA) (v/v) for 10 min and a linear gradient of 32—42% acetonitrile in 0·1% TFA for 35 min. The fractions with acrosin activity were pooled, concentrated, evaporated 2 times with 0·1% TFA, and lyophilized in a Savant Speed Vac Concentrator.

Protein determination. Protein concentration was determined either by the method of Lowry et al.[15] or by the method of Bradford[16].

Amino acid analysis. The amino acid composition of the proacrosin was determined on a Durrum D-500 amino acid analyser (Palo Alto, CA., U.S.A.) (ref.[17]). Methionine and cysteine were determined after oxidation with performic acid.

Molecular mass determination. The apparent molecular mass of proacrosin was determined on Sephadex G-100 superfine column (1·88 × 48·5 cm) in 0·001M-HCl containing 0·2M-NaCl, pH 3·0. The chromatography was performed at a flow rate of 6 ml/h. Fractions of 1·5 ml/15 min were collected.

Casein-SDS-PAGE *zymography*. An ultrasensitive proteinase detection zymography was used to detect proacrosin and determine its degree of purification and molecular mass. The slab-gel electrophoresis was performed in 10% acrylamide gel copolymerized with 0·08% casein in the presence of sodium dodecyl sulfate (SDS) under nonreducing conditions[18–20]. Following the electrophoresis, SDS was removed from the gel by soaking the gel for 60 min with Triton X-100 (2·5% v/v). The Triton X-100 was removed with several washes of distilled water and the gel was incubated for 4 to 6 hours at 37°C in 0·1M glycine–NaOH (pH 8·3) to renature the proteinase. After incubation, the buffer was removed by washing with distilled water (3 times). Gel was stained with Amidoblack 10B (0·1% v/v) and destained with methanol–acetic acid–distilled water (3 : 1 : 6). Proteinase activity was detected as a clear area of gel on a darkly stained background.

Measurement of enzymatic activity. Activity of the purified proacrosin was determined as acrosin activity after zymogen activation. Conversion of proacrosin to acrosin was carried out by autoactivation: a 0·1 ml fraction from the column was incubated at 25°C in 2·3 ml buffer containing 0·2M-Tris.HCl pH 8·0, 0·025M-$CaCl_2$ either for 30 min (autoactivation) or for 5 min and in the presence of 1% (w/v) proacrosin activator chondroitin sulfate[21] (activation). Acrosin activity was measured spectrophotometrically after hydrolysis of a chromogenic substrate, N_α-benzoyl-DL-arginine *p*-nitroanilide (BzArgNan), at 405 nm as described in detail elsewhere[14]. Acrosin and proacrosin activities were expressed in international units (U). One acrosin unit corresponds to the hydrolysis of 1 μmol BzArgNan/min. Specific activity is expressed as U/mg of protein.

The N-*terminal sequence of boar proacrosin*. The N-terminus of native proacrosin (2 nmol) was sequenced automatically by Edman degradation on an Applied Biosystems 470 A Protein Sequencer. Program 03R PTH was used and phenylthiohydantoin derivatives of amino acid residues were analysed by HPLC (ref.[22]).

RESULTS AND DISCUSSION

The greater part of seminal proteinase inhibitors was removed from fresh boar semen by spinning off the seminal plasma and repeatedly washing the sperm. Proacrosin was extracted from sperm with 2% acetic acid, being protected against activation by a low-molecular-mass inhibitor, 50 mM bezamidine. Proacrosin was purified to apparent homogeneity by the three-step procedure outlined in the previous section:

STEP 1, gel filtration on Sephadex G-50 medium separated natural low-molecular--mass acrosin inhibitors from the proacrosin extract (unpublished results). Approximately 234 U total acrosin activity was obtained, with specific activity 1·2 U/mg.

STEP 2, ion-exchange chromatography on CM-Cellulose 32 (Fig. 1), 194 U acrosin activity (4·3 U/mg) was recovered.

STEP 3, approximately 2 mg of purified proacrosin was obtained from 20 reversed-phase high-performance-liquid-chromatography runs on a C_4 column. Pure proacrosin was eluted with 38–39% acetonitrile in 0·1% TFA (Fig. 2).

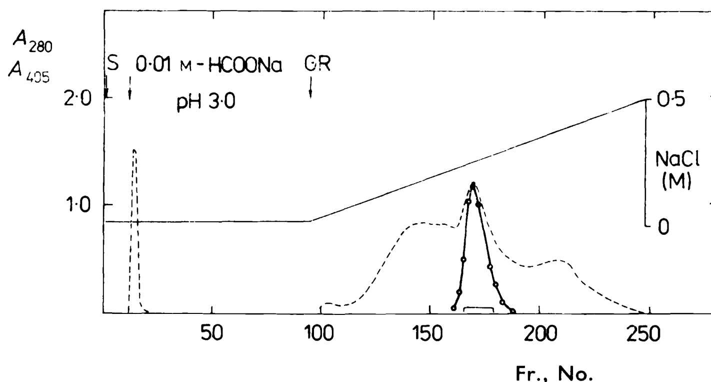


FIG. 1

Ion-exchange chromatography on CM-Cellulose 32. The chromatography was performed as described in Experimental, at a flow rate of 36 ml/h. Fractions of 3 ml/5 min were collected. (⌐‒‒¬) Pooled fractions with acrosin activity

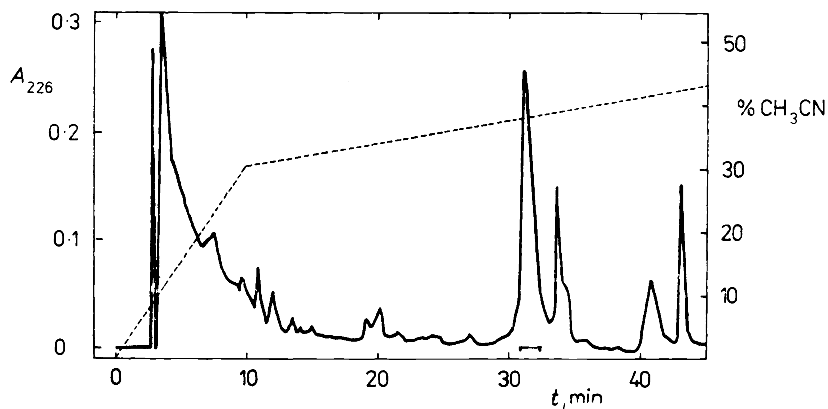


FIG. 2

Reversed-phase high-performance liquid chromatography. The HPLC was performed as described in Experimental, A_{226} was measured. (⌐‒‒¬) Pooled fractions with acrosin activity

The relative molecular mass of proacrosin estimated by gel filtration on Sephadex G-100 superfine in the presence of 0·2M-NaCl was about 70 000 (Fig. 3). We explain this high M_r by the presence of a carbohydrate moiety bound in the molecule of

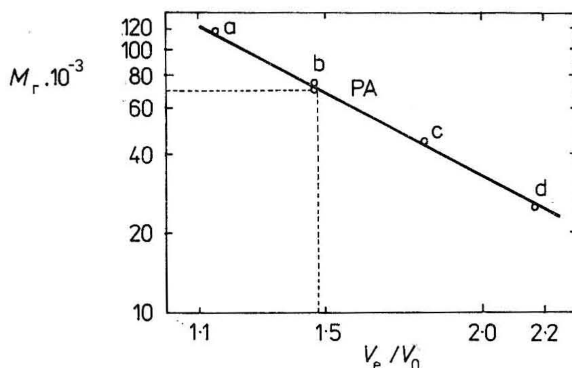


FIG. 3

Determination of molecular mass of boar proacrosin by gel filtration on Sephadex G-100 superfine. The gel filtration was performed as described in Experimental. Standards: a β-galactosidase (M_r = 116 000); b human serum albumin (M_r = 69 000); c egg albumin (M_r = 45 000); d bovine chymotrypsinogen (M_r = 25 000); PA proacrosin

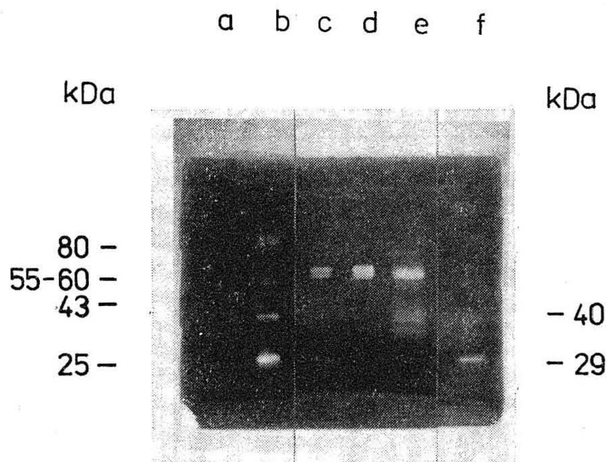


FIG. 4

SDS-PAGE 10% gel with copolymerized casein-zymographic detection of proacrosin. The following samples were applied from left to right: a human serum albumin (10 μg), elongation factor T_u (7·3 μg); b human plasmin (0·5 μg), bovine chymotrypsin (3 ng); c, d purified proacrosin (STEP 2) (10 μg, 20 μg); e crude proacrosin (STEP 1) (20 μg); f egg albumin (20 μg) carbonic anhydrase (15 μg), bovine chymotrypsin (1·5 ng)

boar proacrosin. Comparable values obtained by gel filtration have been reported by other laboratories as well[8,14]. Determinations of proteinase activity and M_r of proacrosin in electrophoretic experiments on SDS-polyacrylamide gel with copolymerized casein under nonreducing conditions gave an $M_r \sim 55\,000-60\,000$ (Fig. 4). This method associates detection of proteinase activity with molecular-mass determination. Proacrosin migrated on the gel reproducibly as a double band. Human serum albumin, having a high content of intramolecular S—S bonds, migrates electrophoretically at $M_r \sim 69\,000$ under reducing conditions and at $M_r \sim 55\,000$ to 60 000 under nonreducing conditions[23]. Similar migration differences are displayed by relatively large intramolecular S—S bonds possessing proteins that have been prepared under reducing or nonreducing conditions (bovine serum albumin, egg albumin). Proacrosin belongs among proteins with rather numerous S—S bonds

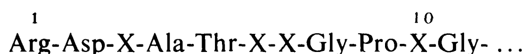
TABLE I

Comparison of amino acid composition of boar proacrosin, some other proacrosins, and α-acrosin. The values were obtained with 20-h hydrolysates and were not corrected

Amino acid	Boar proacrosin		α-Acrosin[14]	Proacrosin[27]	Proacrosin[28]
	residue mol	nearest integer			
Asp	34·2	34	26	32	32
Thr	27·4	27	22	26	27
Ser	34·5	35	24	29	27
Glu	49·5	50	33	40	42
Pro	67·6	68	67	65	67
Gly	52·0	52	39	37	41
Ala	32·0	32	25	27	31
Cys[a]	11·9	12	12	12	12
Val	28·1	28	27	26	28
Met[b]	6·3	6	6	6	7
Ile	21·4	21	18	20	23
Leu	35·9	36	26	33	32
Tyr	13·4	13	12	13	13
Phe	16·0	16	13	16	14
Lys	22·8	23	19	28	26
His	7·1	7	6	10	9
Arg	33·8	34	27	30	30
Trp	[c]		11	3	>5
Total		494	413	453	466

[a] Determined as cysteic acid; [b] determined as methionine sulfone; [c] not determined.

and also shows a mobility difference between its reduced and nonreduced forms. Under nonreducing conditions it migrates with equal mobility as nonreduced human serum albumin (Fig. 4). As a standard, we used elongation factor T_u ($M_r \sim$ $\sim$ 43 000), a protein whose mobility does not change under reducing or nonreducing conditions since it does not have intramolecular S—S bonds. The M_r of the protein moiety of the boar proacrosin molecule (55 000 – 60 000) coincides with the M_r of the acrosin zymogen obtained by translation of proacrosin mRNA isolated from boar testicles[24]. Acrosin splits BzArgNan as substrate at pH8·0 very rapidly[25]. Our preparation, on the other hand, was enzymatically inactive and only preincubation at pH 8·0 for at least 30 min (autoactivation) or the addition of chondroitin sulfate as activator[21] activated it. The time course of boar proacrosin activation followed a classical sigmoidal curve as described in detail elsewhere[26]. These findings served as evidence that the purified (STEP 3) preparation was the zymogen proacrosin and not acrosin. The amino acid composition of homogenous proacrosin (STEP 3), based on $M_r \sim$ 55 000, is shown in Table I. It does not differ much from α-acrosin[14] or the boar proacrosins isolated in other laboratories[27,28], which have been included in the table for comparison. Proacrosin has a high content of proline, shown to be accumulated in the C-terminal hydrophobic part of the chain in α-acrosin[29]. This region is apparently responsible for proacrosin binding to membrane. As compared with other laboratories, we found a higher content of glycine and glutamic acid in our proacrosin isolate. The following N-terminal amino acid sequence of native proacrosin was determined:



Sequence analysis of the N-terminal part showed that boar proacrosin has the N-terminal sequence identical with that of the boar acrosin light chain[30]. In position 3 from the N-terminus the amino acid could not be identified; it is asparagine with a sugar moiety bound onto it. In positions 6 and 10 are cystein residues, which in the native proacrosin molecule are incorporated into disulfide bridges and are unidentifiable in sequencing. This finding suggests that the light chain represents the N-terminus of proacrosin, and activation of proacrosin into acrosin may thus involve only splitting of a specific peptide bond inside the proacrosin molecule. This is in agreement with some independently obtained and recently published results[28].

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