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Boar proacrosin is a single-chain molecule which has the N-terminus of the acrosin A-chain (light chain)

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Boar proacrosin was isolated from spermatozoa by a novel procedure under conditions preventing proenzyme activation. The spermatozoal extract was fractionated by gel filtration and reversed-phase FPLC, all in acidic solutions. Isolated proacrosin had a molecular mass of 55/53 kDa (doublet) and was devoid of amidolytic activity. Its single N-terminal sequence corresponded to that of the 23-residue acrosin A-chain and continued with that of the acrosin B-chain. Autoactivation at pH 7.8 did not influence the molecular mass. However, activated material contained two parallel N-terminal sequences, those of the A- and B-chain. Thus, activation of proacrosin is analogous to that of other serine proteinase proenzymes.

Proacrosin; Acrosin; Enzyme activation; Fertilization; Serine proteinase; Amino acid sequence; (Boar sperm cell)

1. INTRODUCTION

Acrosin (EC 3.4.21.10), a serine proteinase from the mammalian sperm acrosome, is believed to play a key role in fertilization because of the special functional properties of the molecule. Thus, acrosin exerts proteolytic activity, which enables the spermatozoon to penetrate the zona pellucida of the ovum (cf. [1–6]), lectin-like activity, by which the sperm cell can bind to the zona pellucida [5–7], and hydrophobic activity, which anchors this membrane-associated enzyme on the sperm surface [2,3], so that acrosin may form a bridge between the male and female gamete.

Acrosin occurs in a large number of molecular forms. It is synthesized and occurs in the acrosome as an inactive precursor, proacrosin, with a molecular mass of 53–55 kDa (cf. [7–9]). Active, high- (50–55 kDa) and low-molecular-mass (35–38 kDa) forms have been characterized by N-terminal sequence and amino acid composition [1–6]. In this communication the corresponding data for proacrosin as well as a simple, novel procedure for proacrosin isolation are presented. The protein chemical nature of proacrosin activation is clarified.

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Abbreviations: FPLC, fast protein liquid chromatography; HPLC, high-performance liquid chromatography; BAPA, N^{α}-(benzoyl)-L-arginine-*p*-nitroanilide

2. EXPERIMENTAL

2.1. *Preparation of sperm extract*

Freshly collected boar spermatozoa were washed with 5 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 6.5, 0.264 M sucrose, 2 mM benzamidine and then extracted by stirring with 3% (v/v) acetic acid, 10% (v/v) glycerol, 5 mM benzamidine for 3 h at 4°C. After centrifugation (50000 × *g*, 10 min) the supernatant contained about 6 mg protein/ml (dye-binding assay [10]).

2.2. *Isolation of native proacrosin*

The sperm extract (1 ml) was fractionated by gel filtration chromatography on a 1 × 30 cm Superose 12 HR 10/30 column (Pharmacia), eluted with 7 M urea, 1 M formic acid at

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0.25 ml/min using an FPLC system (Pharmacia) and detection at 280 nm. The fractions (0.5 ml) were screened for proacrosin by activation to acrosin and application of a chromogenic substrate assay (below). Proacrosin-containing fractions were pooled and portions of 3 ml further fractionated by reversed-phase chromatography on a 1 × 10 cm ProRPC 15 μm HR 10/10 column (Pharmacia), eluted with a linear gradient of 0–60% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid during 65 min at 3 ml/min using the FPLC-system and detection at 280 nm. The fractions (1.5 ml) were screened for proacrosin by activation to acrosin and for homogeneity by SDS-PAGE (below).

2.3. *Isolation of modified proacrosin*

In an alternative approach the proacrosin-containing material from the Superose chromatography (above) was 'inactivated' by mercaptolysis and alkylation before the final isolation. A pool from several chromatographies (16 ml) was adjusted with solid Tris to pH 5, and incubated with 10 mg benzamidine and 0.8 ml mercaptoethanol for 2 h at 40°C. Then, 1.2 ml 4-vinylpyridine (Fluka) was added and allowed to react for 1.5 h at 25°C. The acidified, *S*-(pyridylethylated) material was fractionated in portions of 3 ml on the ProRPC column as above. The fractions were screened for homogeneity and proacrosin-like molecular mass by SDS-PAGE (below).

2.4. *Activation of proacrosin to acrosin*

Proacrosin was converted to acrosin by incubation at pH 7.8, and the resulting enzymatic activity measured as the hydrolysis of the chromogenic substrate N^{α}-benzoyl-L-arginine *p*-nitroanilide (BAPA). For detection of proacrosin in chromatographies 50 μl of each fraction was diluted with 2.5 ml of 0.2 M Tris chloride, pH 7.8, 0.05 M calcium chloride, 3.3% (v/v) dimethylformamide, containing 1 mg BAPA and 1 μg chondroitin sulfate (Sigma) as activator [11], the reaction being stopped after 10 min with 0.5 ml of 30% (v/v) acetic acid and the absorbance measured at 405 nm. For detection of preformed acrosin the same procedure was used, except for the exclusion of chondroitin sulfate. Progress of activation in pure proacrosin was monitored by incubation in 0.2 M Tris chloride, pH 7.8, 0.05 M calcium chloride at 25°C, mixing 2 ml aliquots with 1 ml of 0.1% BAPA in water after specified periods of time, and recording the absorbance increase at 405 nm and 25°C in a spectrophotometer (Beckman D-50). The amounts of active acrosin were calculated from the slopes, 10 absorbance units/min being defined as 1 U/ml [12].

2.5. *Gel electrophoresis*

Vertical slab gels (0.75 × 6.5 × 8 cm) containing gradients of 7.5–20% polyacrylamide and 0.1% SDS, but no mercaptoethanol, were used [6].

2.6. *Amino acid and sequence analysis*

Compositions were determined, after hydrolysis in 6 M hydrochloric acid at 110°C, using conditions where *S*-(pyridylethyl) cysteine and glucosamine are separated from other components [13]. For N-terminal sequence analysis the Edman degradation method was performed in a prototype spinning-cup sequenator [14]. Phenylthiohydantoin derivatives were identified in an isocratic HPLC system where the derivative of *S*-(pyridylethyl) cysteine also is separated [13].

3. RESULTS

Recently, a novel, highly simplified procedure for the isolation of boar acrosin from spermatozoal extracts was described [5,6]. The procedure has now been adapted to the isolation of the enzymatically inactive precursor, proacrosin. For this purpose washed boar spermatozoa were extracted under conditions where acrosin should be inactive and proacrosin activation negligible, i.e. at acidic pH and in the presence of a proteinase inhibitor, benzamidine. Proacrosin was purified from the extract by two chromatographic steps, gel filtration chromatography in acidic, 7 M urea solution (fig.1) and reversed-phase chromatography in a trifluoroacetic acid/water/acetonitrile system (fig.2), i.e. under circumstances where no activation would occur. The reversed-phase chromatography was carried out either with native, unmodified material (fig.2a) or with *S*-(pyridylethylated) material (fig.2b), the latter experimental variant with the dual purpose of annihilating any remaining enzymatic activity and converting half-cystine residues into an identifiable form for the sequence analysis.

The position of proacrosin in the chromatographies was established, when native material was processed (figs 1 and 2a), by activation to acrosin

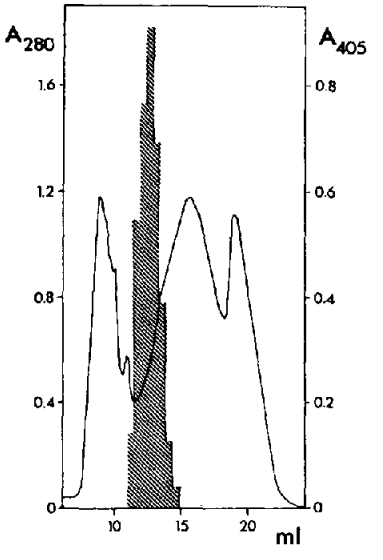

Fig.1. Chromatography of the boar sperm extract on a gel filtration (Superose) column in 7 M urea, 1 M formic acid; activatable proacrosin indicated by hatched area.

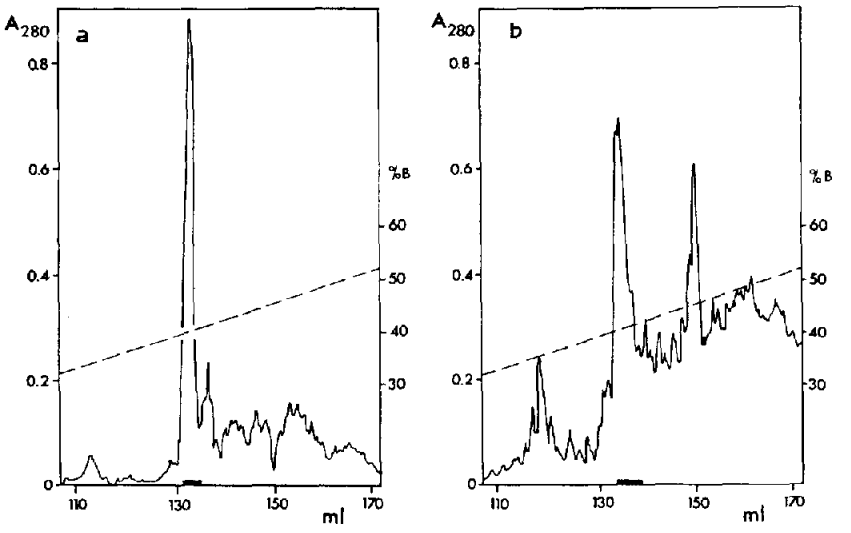

Fig.2. Chromatography of (a) native and (b) *S*-(pyridylethylated) proacrosin-containing material on a reversed-phase (ProRPC) column in 0.1% trifluoroacetic acid, 0–60% acetonitrile; pooled fractions indicated by horizontal bars.

and detection with the chromogenic substrate BAPA, and in the reversed-phase chromatographies (fig.2) by SDS-PAGE. No preformed acrosin activity could be detected. In the reversed-phase chromatographies proacrosin constituted the main component. It eluted at about 40% acetonitrile both in the native and the *S*-(pyridylethylated) form. SDS-PAGE of the proacrosin pools from both types of material showed a doublet of 55/53 kDa and the absence of contaminating proteins (fig.3).

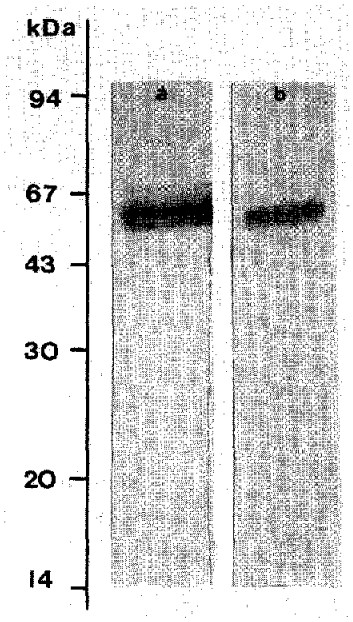

Fig.3. SDS-PAGE of proacrosin (a) before and (b) after activation to acrosin.

Isolated native and *S*-(pyridylethylated) proacrosin were analysed for N-terminal sequence. Only a single sequence, starting with Arg-Asp-Xaa-Ala-, was found in both types of material (fig.4). The sequence is identical in the first 23 positions with that of the 23-residue A-chain (light chain) of low-molecular-mass (38 kDa) and high-molecular-mass (53 kDa) acrosin [4–6]. It continues from position 24 with the N-terminal sequence of the B-chain (heavy chain) of low- and high-molecular-mass acrosin [2,5,6]. The absence of the B-chain N-terminal sequence, i.e. Val-Val-Gly-Gly-, in the isolated material indicates that it was uncontaminated by active acrosin. The amino acid composition of proacrosin is shown in table 1.

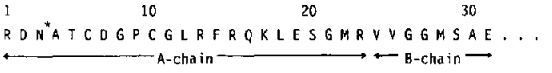

Fig.4. N-terminal amino acid sequence of boar proacrosin, identical with the total A-chain [4] and N-terminal B-chain [2] sequence as indicated. N^+ stands for N with carbohydrate side chain as established earlier [4].

Table 1

Amino acid composition of boar proacrosin

Amino acid	Mol residue/mol protein
D	32
T	27
S	27
E	42
P	67
G	41
A	31
C[a]	12
V	28
M	7
I	23
L	32
Y	13
F	14
K	26
H	9
R	30
W[b]	>5
Total	466

[a] Determined as *S*-(pyridylethyl) cysteine
[b] Estimated from sequence

The data are in good agreement with earlier analyses [3]. The hydrolysate contained glucosamine derived from the carbohydrate side chain in position 3 of the sequence [4].

The time-dependent autoactivation of the proacrosin preparation at pH 7.8 was evaluated in an amidolytic assay using BAPA as substrate (fig.5). The curve showed the characteristic sigmoidal shape. Maximal activity was observed after 2.5 h,

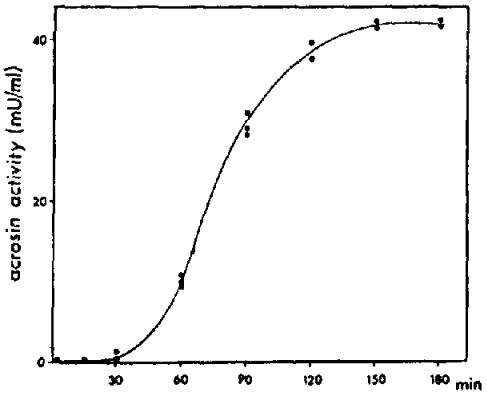

Fig.5. Autoactivation of proacrosin; amidolytic activity of acrosin on BAPA.

the final specific activity being 27 U/mg. The activated material had mainly retained the molecular mass of the unactivated form (fig.3). However, the N-terminal sequence had changed in the way that now the N-termini of both A-chain and B-chain were present, i.e. Arg-Asp-Xaa-Ala- and Val-Val-Gly-Gly- (cf. fig.4).

4. DISCUSSION

Acrosin, like several other serine proteinases, is only temporarily inactivated by exposure to low pH and reagents like urea, and this has been utilized to quench proteolytic degradation in recent purification procedures [5,6]. Proacrosin could be isolated under similar conditions as acrosin, but a low pH had to be used already during the initial sperm extraction. The resulting proacrosin preparation was pure as judged by the homogeneity in reversed-phase FPLC, SDS-PAGE (doublet) and N-terminal sequence and by the absence of enzymatic activity. The sequence differed from that of a previously described preparation, which may have been enzymatically active and therefore contained the acrosin sequence [15].

The proacrosin material of the present communication could be autoactivated to acrosin by means of a single proteolytic clip, the sequences around the cleavage site being homologous to those of other serine proteinases [2,4]. Proacrosin and the acrosin generated had similar molecular masses (53/55 kDa). However, a previously analysed 38 kDa form of acrosin and the present 53/55 kDa form had identical N-termini [2,4]. It can therefore be concluded that proacrosin undergoes proteolytic processing in the N-terminal region leading to active 53/55 kDa acrosin and additionally in the C-terminal region leading to active 38 kDa acrosin. The N-terminal cleavage has the obvious function of proenzyme activation, but also the C-terminal cleavage(s) may have a biological function during fertilization.

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