Volume 244, number 1, 132–136 FEB 06797 February 1989

Activation of boar proacrosin is effected by processing at both N- and C-terminal portions of the zymogen molecule

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Received 26 October 1988; revised version received 20 December 1988

A mixture of 55 and 53 kDa boar proacrosins was autoactivated at pH 8.5 to produce a 43 kDa intermediate form and a 35 kDa mature acrosin, and each of four forms of (pro)acrosins was isolated. Analysis of the N-terminal sequences of the two proacrosins indicated the existence of a segment corresponding to the acrosin light chain at the N-terminal end of the zymogen. Two N-terminal sequences identical with those of the light and heavy chains were found in the intermediate form and mature acrosin. The proacrosins and the intermediate contained many more proline residues than the mature enzyme. These results indicate that the activation of boar acrosin zymogen is achieved by the removal of a C-terminal segment rich in proline residues and by the cleavage of the Arg^{23}–Val^{24} bond leading to the formation of the light and heavy chains.

Acrosin; Serine protease; Zymogen activation; (Boar spermatozoa)

1. INTRODUCTION

Acrosin, a serine protease occurring in sperm acrosomes, plays an important role in fertilization [1–3]. It is synthesized as an enzymatically inactive zymogen, proacrosin, which is activated to the mature protease by limited proteolysis [4,5]. Boar proacrosin has been reported to undergo the following sequential activation process [5,6]:

55 kDa proacrosin ⟶ 53 kDa proacrosin ⟶
49 kDa α-acrosin ⟶ 34 kDa β-acrosin ⟶
25 kDa γ-acrosin

where α-acrosin is an enzymatically active, labile intermediate and β-acrosin is the most stable mature form. Fridberger et al. [7] have shown that the N-terminal amino acid sequence of 55 kDa proacrosin is identical with that of a 38 kDa acrosin (probably identical with the β-acrosin and our 35 kDa acrosin described below) and suggested that the activation is achieved by the removal of the C-terminal portion of the zymogen. Fock-Nüzel et al. [8] have demonstrated that boar acrosin has a two-chain structure in which a 23-residue light chain is covalently linked by two disulfide bonds to a much larger heavy chain containing the catalytic site. More recently, it has been reported that 53 kDa proacrosin, which exhibits a lectin-like carbohydrate-binding activity [9–12], also possesses two N-termini due to the presence of the light and heavy chains connected by disulfide bonds [11,12]. These observations are consistent with the view that proacrosins and the mature enzyme are composed of two peptide chains and that the proacrosin activation is effected by the proteolytic removal of the C-terminal portion of the heavy chain. However, the activation mechanism is not yet fully understood. In particular, nothing is known of the origin of the light chain.

Here, we have purified 55 and 53 kDa proacrosins as well as a 43 kDa intermediate form and a 35 kDa mature acrosin from boar spermatozoa, and analyzed their N-terminal sequences and amino acid compositions. The results obtained provide evidence that both 55 and 53 kDa proacrosins are single-chain polypeptides and that the

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Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

activation of proacrosin is achieved not only by the removal of the C-terminal portion but also by the cleavage of the Arg^{23}–Val^{24} bond, an event leading to the generation of the light and heavy chains.

2. EXPERIMENTAL

2.1. *Purification of boar proacrosin*

Proacrosin was purified from freshly ejaculated boar spermatozoa according to [5] with some modifications. Acrosomal acid extract was filtered through a Sephadex G-200 column previously equilibrated with 2% acetic acid. Proacrosin in each fraction was assayed by determining the amount of acrosin activity which was produced from the zymogen after autoactivation, using *N*-benzoyl-L-arginine ethyl ester (Sigma) as a substrate [5]. Fractions containing proacrosin were combined and precipitated by addition of solid ammonium sulfate (0–50% saturation). The precipitate was collected by centrifugation, dissolved with 6 M guanidine hydrochloride (pH 2.5), and then applied to a Toyopearl HW-55 column (Tosoh, Japan) equilibrated with the same solution. The purified proacrosin was dialyzed thoroughly against 1 mM HCl and stored at 4°C until use.

2.2. *Isolation of each proacrosin, and intermediate/ mature acrosin*

Purified proacrosin (3–4 mg protein) was denatured on a boiling-water bath for 5 min in a solution (1 ml) of 1% SDS, 10 mM Tris-HCl (pH 6.8), 8% glycerol, 0.02% bromphenol blue and applied to a 10% polyacrylamide gel (0.2 × 20 × 40 cm). Electrophoresis was performed at a constant current of 15 mA. To visualize the location of proteins, either side of the gel (~1 cm) was cut off, stained for 5 min with Coomassie brilliant blue R-250, and destained with 7% acetic acid in 25% methanol. The unstained gels containing proteins were excised, cut into small pieces, and extracted at 4°C overnight in 0.1% SDS (5 ml) with constant shaking. The extract was passed through a Millex-GV filter (0.22 μm pore size, Millipore) and applied to an Ultra Pack C_4 column (6 × 100 mm, Yamazen) equilibrated with 0.1% trifluoroacetic acid using a Beckman 344 high-performance liquid chromatography (HPLC) gradient system. Proteins were eluted with a linear gradient of 0–60% acetonitrile in 0.1% trifluoroacetic acid at room temperature. The effluent was monitored at 214 nm, and the eluted proteins were collected manually and lyophilized. For isolation of the intermediate and mature forms of acrosin, purified proacrosin was activated at room temperature in 0.1 M Tris-HCl, pH 8.5, for 15 and 180 min, respectively. The mixtures were subjected to SDS-PAGE under nonreducing conditions followed by HPLC as described above.

2.3. *Analytical procedures*

Lyophilized proteins (400 pmol each) were dissolved with 0.1% SDS (usually 50 μl) and subjected to N-terminal sequence analysis using an Applied Biosystems pulse-liquid sequencer

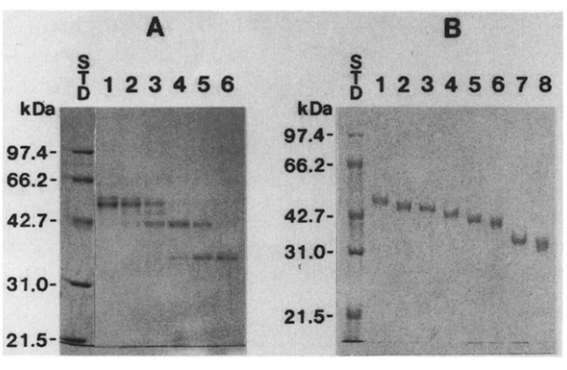

Fig.1. (A) Time course of proacrosin activation followed by SDS-PAGE. Purified proacrosin (0.1 mg/ml) was activated in 0.1 M Tris-HCl, pH 8.5, at room temperature. Aliquots (50 μl) sampled at 1, 3, 5, 10, 30 and 90 min (lanes 1–6, respectively) were treated at 100°C for 5 min with 50 μl of 20 mM Tris-HCl, pH 6.8, 2% SDS, 2% 2-mercaptoethanol, 16% glycerol, 0.04% bromophenol blue, and then subjected to SDS-PAGE. (B) SDS-PAGE of isolated 55 kDa proacrosin (lanes 1,2), 53 kDa proacrosin (lanes 3,4), 43 kDa intermediate (lanes 5,6), and 35 kDa mature acrosin (lanes 7,8). The non-reduced (lanes 2,4,6,8) and reduced (lanes 1,3,5,7) proteins (almost 2 μg each) were subjected to SDS-PAGE. The molecular mass standards (STD, Bio-Rad product) used were phosphorylase *b* (97.4 kDa), BSA (66.2 kDa), ovalbumin (42.7 kDa), carbonic anhydrase (31.0 kDa), and trypsin inhibitor (21.5 kDa). Protein bands were detected by Coomassie brilliant blue staining.

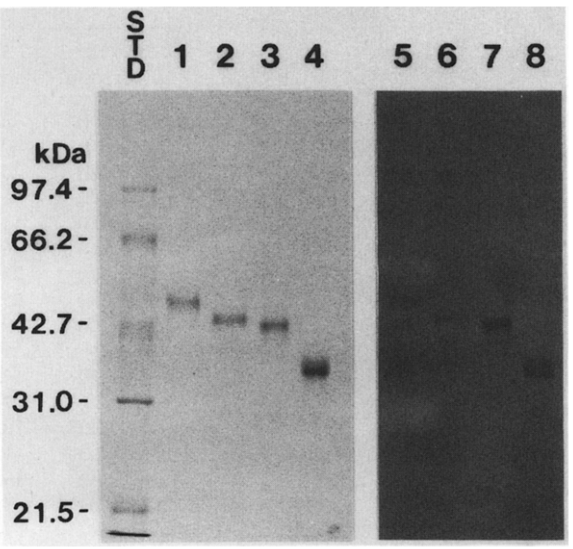

Fig.2. Western blot analysis of 55 kDa proacrosin (lanes 1,5), 53 kDa proacrosin (lanes 2,6), 43 kDa intermediate (lanes 3,7), and 35 kDa mature acrosin (lanes 4,8). Isolated proteins (almost 3 μg) were subjected to SDS-PAGE (10% polyacrylamide gel) under non-reducing conditions. The gel was either stained with Coomassie brilliant blue (lanes 1–4) or transferred to nitrocellulose membrane and reacted with anti-35 kDa boar acrosin (lanes 5–8).

Fig.3. N-terminal sequences of 55 and 53 kDa proacrosins (A) and 43 kDa intermediate and 35 kDa mature forms (B). Residues with asterisk (Asn^3, Cys^6, and Cys^{10}) were not identified in the present study but previously established in acrosin light chain [8]. Several residues, particularly Ser, Arg, and His, were identified qualitatively.

(model 477A/120A) with an on-line PTH analyzer. Proteins (15 μg each) were hydrolyzed in 6 M HCl for 24, 48, and 72 h at 110°C. The hydrolyzates were analyzed with a Hitachi L-8500 amino acid analyzer. Western blot analysis was performed using rabbit anti-35 kDa acrosin antibody and goat anti-rabbit IgG horseradish peroxidase conjugate (Jackson Immunoresearch Labs) as described [13]. Protein concentrations were determined by the method of Hartree [14].

3. RESULTS

Autoactivation of purified boar proacrosin (a mixture of 55 and 53 kDa forms) at pH 8.5 produced an intermediate and a mature acrosin, which migrated as 43 and 35 kDa proteins, respectively, on SDS-PAGE under reducing conditions (fig.1A). The two forms of proacrosin as well as 43 and 35 kDa acrosins were purified by SDS-PAGE in the absence of reducing agents followed by reversed-phase HPLC. The purified 55, 53, 43 and 35 kDa proteins migrated with apparent molecular masses of 46, 43, 41 and 34 kDa, respectively, on SDS-PAGE under non-reducing conditions (fig.1B). The differences in apparent molecular mass were most probably due to the unusual electrophoretic behaviour of non-reduced (pro)acrosins. Upon SDS-PAGE under reducing conditions, both the 43 kDa intermediate and

Table 1

Amino acid compositions of boar (pro)acrosins

Amino acid	Residues/molecule			
	55 kDa proacrosin	53 kDa proacrosin	43 kDa intermediate	35 kDa mature acrosin
Asx	23.7 (24)	22.5 (23)	22.7 (23)	20.7 (21)
Thr	23.0 (23)	20.6 (21)	19.7 (20)	18.3 (18)
Ser	24.4 (24)	20.9 (21)	21.1 (21)	16.6 (17)
Glx	43.2 (43)	35.9 (36)	33.6 (34)	28.2 (28)
Pro	63.7 (64)	64.7 (65)	64.4 (64)	34.8 (35)
Gly	40.0 (40)	36.5 (37)	34.8 (35)	32.5 (33)
Ala	28.3 (28)	25.8 (26)	24.4 (24)	19.7 (20)
Val[a]	25.7 (26)	25.9 (26)	25.8 (26)	20.5 (21)
Cys	11.5 (12)	10.4 (10)	11.5 (12)	10.5 (11)
Met	6.0 (6)	5.7 (6)	4.7 (5)	6.2 (6)
Ile[a]	18.6 (19)	18.8 (19)	17.7 (18)	17.1 (17)
Leu	28.4 (28)	27.6 (28)	24.9 (25)	21.1 (21)
Tyr	11.5 (12)	9.8 (10)	9.2 (9)	9.1 (9)
Phe	11.9 (12)	11.6 (12)	11.3 (11)	9.2 (9)
Lys	19.3 (19)	19.7 (20)	18.3 (18)	14.5 (15)
His	5.1 (5)	5.6 (6)	5.1 (5)	4.8 (5)
Arg	28.0 (28)	27.2 (27)	26.6 (27)	23.8 (24)
Trp	n.d.[b] (5)[c]	n.d. (5)[c]	n.d. (5)[c]	n.d. (5)[c]
Total	(418)	(398)	(382)	(315)

[a] Values of 72 h acid hydrolysis
[b] Not determined
[c] Data from [8]

The calculations of the amino acid compositions of 55, 53, 43, and 35 kDa (pro)acrosins are based on molecular masses of 46, 43, 41 and 34 kDa, respectively. The nearest integers are indicated in parentheses

35 kDa acrosin, but not the proacrosins, yielded a visible band in the low-molecular-mass region (<20 kDa); fig.1B, lanes 5,7). These bands were believed to represent the light chain. Another faintly stained band of apparent molecular mass 49 kDa was detected during the activation of proacrosin (fig.1A). However, this protein was not purified because of its low abundance. All the purified proteins reacted with anti-35 kDa boar acrosin antibodies on Western blot analysis (fig.2).

N-terminal sequence analysis indicated that both 55 and 53 kDa proacrosins had only a single N-terminus and that their sequences, at least up to residue 30, were entirely identical with each other (fig.3A). The sequence of the first 23 amino acids was the same with that of the light chain of boar acrosin sequenced previously [8]. Moreover, the sequence from residues 24 to 30, Val-Val-Gly-Gly-Met-Ser-Ala, coincided with the N-terminal 7-residue sequence of the acrosin heavy chain [8,15]. In contrast to the proacrosins, both 43 kDa intermediate and 35 kDa acrosin were found to have two N-terminal sequences identical with those of the light and heavy chains (fig.3B). These findings indicated that the N-terminal sequence of 55 kDa proacrosin was conserved during its activation, but the conversion of 53 kDa proacrosin to 43 kDa intermediate was accompanied by formation of a nick between Arg-23 and Val-24.

As shown in table 1, the 55, 53 and 43 kDa proteins were considerably rich in proline residues (64–65 residues per molecule). However, only 35 proline residues were found per molecule of 35 kDa acrosin, indicating that the conversion of 43 kDa intermediate to 35 kDa acrosin was effected by the removal of a proline-rich segment from the C-terminal portion. The amino acid composition of the 35 kDa acrosin was nearly identical to those of mature acrosins with molecular masses between 35 and 40 kDa reported by others [8,16,17].

4. DISCUSSION

In this study, activation of purified proacrosin produces a most predominant band for a 43 kDa intermediate and an extremely weak band for a 49 kDa intermediate on SDS-PAGE (fig.1A). It is not known at present whether the 43 kDa intermediate corresponds to the α-acrosin found by Polakoski and Parrish [5]. Assuming that our 49 kDa intermediate is identical with α-acrosin, the 43 kDa protein is considered to be a novel intermediate form of acrosin.

As shown in fig.3A, there is little doubt that both 55 and 53 kDa proacrosins are single-chain polypeptides, in contrast to recent reports that 53 kDa proacrosin has two N-termini [11,12]. The reason for this discrepancy is not clear. At any rate, based on our results, the process of boar proacrosin activation can be depicted as follows. First, 55 kDa proacrosin is converted to 53 kDa proacrosin by the removal of a small (~2 kDa) segment from the C-terminus. The next event, i.e. the conversion of 53 kDa proacrosin to a 43 kDa intermediate, is achieved not only by the removal of a C-terminal segment but also by the cleavage of the peptide bond between Arg-23 and Val-24. This latter cleavage generates a 23-residue light chain and a much larger heavy chain, which are covalently connected by two pre-existing disulfide bonds. The formation of 35 kDa mature acrosin from this intermediate is effected again by the removal of a C-terminal segment containing as many as 30 proline residues. This mechanism can easily account for the origin of the light chain found in the mature acrosin. The sequence corresponding to this chain is located at the N-terminal end of the 55 kDa polypeptide and separated from the rest of the polypeptide during the conversion of 53 kDa proacrosin to 43 kDa intermediate, as discussed above. In view of the fact that the zymogens belonging to the serine protease family are usually activated by removing a peptide segment(s) from the N-terminus, the activation of proacrosin is unique in that it is accomplished by the removal of C-terminal segments and by the nick formation near the N-terminus.

Finally, it should be noted that the N-terminal residue of 55 kDa proacrosin is not methionine, but arginine. This indicates that proacrosin is synthesized with an N-terminal extension segment of certain length. Our recent cloning and sequencing of boar acrosin cDNA have actually shown that the proacrosin is synthesized with an N-terminal 16-residue signal peptide (in preparation).

Acknowledgements: This work was supported in part by a research grant from the Scientific Research Funds of the Ministry of Education, Science, and Culture of Japan. The

authors wish to thank Dr Kazutomo Haraguchi for his technical help in N-terminal sequence analysis of proteins.

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