Zona pellucida-binding of boar sperm acrosin is associated with the N-terminal peptide of the acrosin B-chain (heavy chain)

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Recently, it has been shown that boar acrosin exhibits a carbohydrate-binding activity with a specificity to fucose, by which it can bind to the oocyte zona pellucida. By limited autoproteolysis of a high-molecular mass acrosin (55/53 kDa), designated as α-acrosin, a 15 kDa fragment was generated which interacts strongly with the porcine zona pellucida. Zona-binding was demonstrated on protein blots and by the solid-phase zona-binding assay utilizing biotinylated zona proteins. The zona-binding peptide was isolated by reversed-phase HPLC and analyzed for amino acid sequence. Its single N-terminal sequence corresponded to that of the acrosin B-chain (heavy chain). These data indicate that the zona-binding properties of acrosin are associated with the N-terminal peptide of the acrosin heavy chain.

Sperm-egg interaction; Acrosin; Zona-binding; Amino acid sequence; Spermatozoa

1. INTRODUCTION

The sperm acrosome is a lysosome-like organelle which covers the anterior part of the sperm nucleus [1]. It contains a powerful lytic equipment which is released as a consequence of the acrosome reaction and which the spermatozoa can utilize to penetrate the egg's outer investment. One of the critical steps during mammalian fertilization is the penetration of the ovum's zona pellucida. The acrosomal serine proteinase, acrosin, may be the most likely candidate which contributes to sperm penetration by specific and limited proteolysis of the zona proteins thus facilitating the entry of the motile sperm into the zona pellucida [2–4]. Recent work has demonstrated that acrosin is identical to the major fucose-binding protein of boar spermatozoa [5,6] which also shows a strong affinity to the zona pellucida [6,7]. It has been suggested that acrosin is involved in the complex events of sperm-zona interactions by means of its fucose-binding sites [8,9].

Acrosin consists of a light chain (A-chain) and a heavy chain (B-chain) linked by two disulfide bridges. The heavy chain presenting the typical N-terminal sequence of an activated serine proteinase comprises the catalytic triad of the enzyme, and the light chain of 23 amino acids corresponds to the propart [10,11]. It has been shown that fucose- and zona-binding sites are located on the acrosin heavy chain and that the carbohydrate affinity is independent of the inherent proteolytic activity of the enzyme [12]. In this communication evidence is presented that the N-terminal peptide of the acrosin heavy chain (B-chain) generated by limited autoproteolysis of high-molecular mass acrosin accounts for the acrosin-zona binding.

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Abbreviations: FPLC, fast protein liquid chromatography; HPLC, high-performance liquid chromatography; SDS-PAGE, sodium dodecylsulfate polyacrylamide electrophoresis; BSA, bovine serum albumin

2. EXPERIMENTAL

2.1. *Isolation of boar acrosin*

Active high-molecular mass acrosin (55/53 kDa) was isolated from acidic boar sperm extracts by a two-step procedure including high-performance gel filtration and reversed-phase HPLC as recently described [12]. The protein was identified by SDS-PAGE and N-terminal amino acid analysis.

2.2. *Limited autoproteolysis of boar acrosin*

Purified high molecular mass acrosin (1.8 mg) was dissolved in 1 mM ammonium formiate, pH 6.0 (0.5 ml). For autoproteolysis the solution was adjusted to pH 8.0 with 0.1 M ammonium hydrogen carbonate, pH 8.0 (1 ml), and allowed to react for 30 min at 37°C. To terminate the reaction the mixture was acidified with formic acid to pH 2.0, and was immediately subjected to reversed-phase HPLC on a Nucleosil 300-10 μm C18 column (250 × 0.4 mm, Machery and Nagel). The column was eluted with a linear gradient of 0–60% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid during 120 min at 1 ml/min. The fractions were screened for zona-binding activity (see below), for molecular mass by SDS-PAGE, and N-terminal amino acid analysis.

2.3. *Solid-phase zona binding assay*

The isolated porcine zonae pellucidae [13] were biotinylated as described in [6] with one modification. The lyophilized zonae pellucidae were solubilized in 0.05 M Tris-HCl, pH 7.5, containing 0.3 M Li-3.5-J_2 salicylate (Sigma) for 15 min at 25°C [14]. After centrifugation at 43000 × *g* for 60 min at 4°C, the clear supernatant was extensively dialyzed against water and then biotinylated according to [6]. Zona-binding activity was tested by means of the solid-phase

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zona-binding assay as described [6,12]. Inhibition experiments were performed by reacting immobilized acrosin (0.05 μg/well) with biotinylated zona pellucida (0.19 μg/ml) in the presence of various dilutions of inhibitors. The binding step was carried out in the presence of 5 mM benzamidine hydrochloride (Sigma) to suppress proteolytic activity of the enzyme.

2.4. *Electrophoresis and blotting*

Analytical SDS-PAGE was performed on 7.5–20% polyacrylamide gradient slab gels (0.75 × 80 mm) in 0.19 M glycine, 25 mM Tris, 0.1% SDS, pH 8.3, at a constant voltage of 180 V for 1.5 h at 4°C using a Midget electrophoresis unit (LKB-Pharmacia). To visualize proteins the gels were stained with 0.25% Coomassie brilliant blue-250 (Serva) in 20% trichloroacetic acid and destained with 5% methanol/7% acetic acid. Alternatively, the separated proteins were electrophoretically blotted onto PVDF-membranes (Immobilon, Millipore) for 20 h at 6 mA using a Midget electrophoretic transfer unit (LKB-Pharmacia) according to [15]. Zona-binding activity was identified utilizing the biotinylated zona proteins as described in detail [12].

2.5. *Amino acid sequence analysis*

For N-terminal amino acid sequence analysis about 0.1–0.2 nmol of the proteins were subjected to solid phase Edman degradation by lysine coupling to DITC-glas [16]. The immobilized proteins were degraded with an initial yield of more than 95%.

3. RESULTS

For purification of an active high-molecular mass acrosin, designated as α-acrosin [17], a two-step procedure was used as recently described [12]. Acidic sperm extracts were subjected to high-performance gel filtration chromatography in acidic 7 M urea and then to reversed-phase chromatography in a trifluoroacetic acid/water/acetonitrile system, i.e. under conditions where proteolytic degradation of proteins was considerably diminished. Active high-molecular mass acrosin was identified by SDS-PAGE showing molecular masses of 55/53 kDa and by N-terminal sequence analysis demonstrating the typical two N-termini of the active enzyme due to the presence of the ligh chain (Arg-Asp-Xaa-Ala-) and heavy chain (Val-Val-Gly-Gly-) [6,11,12].

Limited autoproteolysis of the 55/53 kDa acrosin at pH 8.0 for 30 min generates a typical peptide pattern on SDS-PAGE under non-reducing conditions (Fig. 1). Proteolytic processing of α-acrosin results in the formation of β-acrosin, which migrates as a 38-kDa band. Additionally, a 24-kDa fragment and peptides with molecular masses of about 15, 11.5 kDa and a faint band at 13 kDa appear. During prolonged autoproteolysis the 15-kDa band disappeared whereas the 11.5-kDa band seems to be more stable against autoproteolysis (Fig. 1).

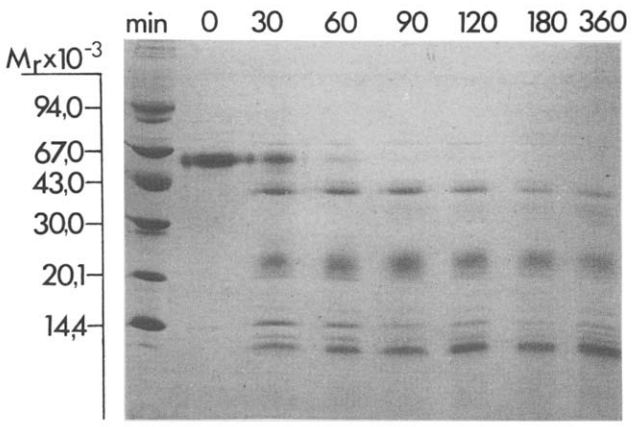

Fig. 1. Time course of the autoproteolysis of α-acrosin monitored by SDS-PAGE under non-reducing conditions.

Protein blots of the autolyzed acrosin probed with the biotinylated zona proteins show a strong zona-binding activity for the 55/53 kDa protein corresponding to the active α-acrosin, for the 38-kDa protein corresponding to the active β-form, and for the 15-kDa protein (Fig. 2b). The zona-binding was inhibited by the presence of unlabelled zona proteins (Fig. 2c) and fucoidan (Fig. 2d) indicating the specificity of the binding and its specificity to fucose. The 15-kDa fragment was isolated in low amounts by reversed-phase HPLC of the autolyzed acrosin. The presence of zona-binding activity was conveniently monitored by the solid-phase zona binding assay utilizing the biotinylated zona proteins [6,12]. As revealed by SDS-PAGE and N-terminal sequence analysis, the zona-binding peptide eluted at about 41–42% acetonitrile; however, separated from both forms of active acrosin, i.e. α-acrosin and β-acrosin, which eluted at rather higher acetonitrile concentration (43–44%; Fig. 3). The isolated fragment was analyzed for N-terminal sequence. Only a single sequence, starting with Val-Val-Gly-Gly was found (Fig. 4). The sequence is identical in the first 23 positions with the N-terminal sequence of the B-chain (heavy chain) [6,10,18] with the exception of the positions 1, 12 and 14. The absence of any identifiable products in position 1 is easily explained by the fact that the N-terminal amino acid is coupled to the DITC-glas. The positions 12 and 14 are occupied by tryptophan

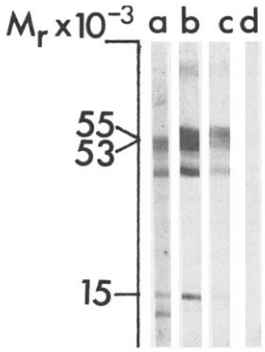

Fig. 2. Detection of zona-binding activity on protein blots of the autolyzed α-acrosin with (b) the biotinylated zona proteins (1.0–1.5 μg/ml), (c) in the presence of unlabelled zona proteins (15 μg/ml), and (d) in the presence of fucoidan (100 μg/ml). (a) Protein-labelling with 0.1% Coomassie blue in 40% methanol/10% acetic acid.

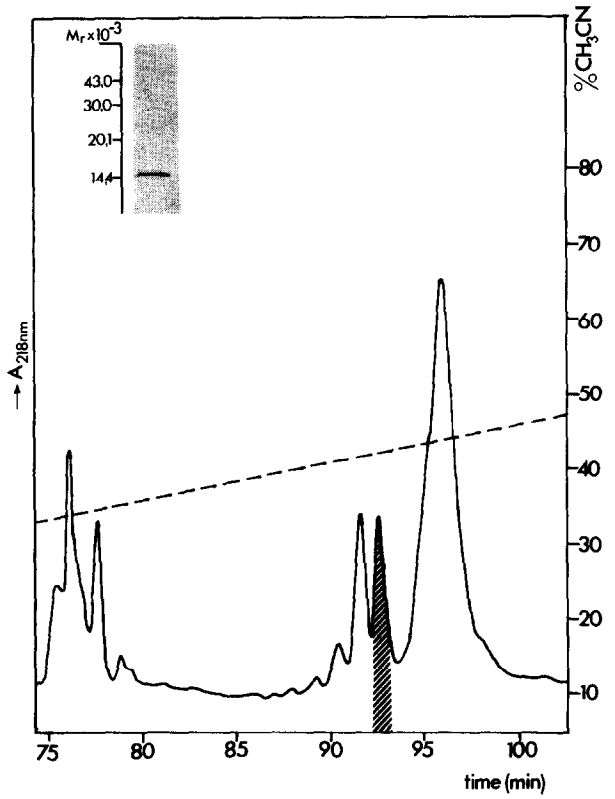

Fig. 3. Chromatography of the autolyzed α-acrosin on reversed-phase HPLC. Broken line, acetonitrile gradient in 0.1% (v/v) trifluoroacetic acid. Zona-binding activity was monitored by means of the solid-phase zona-binding assay; hatched area, pooled zona-binding fraction. (Inset) SDS-PAGE of the pooled zona-binding fraction.

[6,10] which were partly destroyed under Edman degradation conditions. The immobilized fragment was degraded with an initial yield of 0.12 nmol which is below the detection limit of tryptophan under the sequencing conditions used.

The zona-binding activity of acrosin (55/53 kDa) and the 15-kDa fragment was determined by their capacity to inhibit the acrosin zona-binding using the solid-phase zona-binding assay. Under standard conditions acrosin and the fragment inhibited 50% of the maximum binding at concentrations of 4.3 nmol and 6.6 nmol, respectively.

4. DISCUSSION

Acrosin shows distinct structural features as compared to other serine proteinases which may be related to its special role in fertilization [19]. It combines several functional properties within a single molecule – the catalytic triad of the proteinase, a special membrane-associating character [17,20] and the carbohydrate-binding sites by which acrosin can bind to the zona pellucida [6,12]. An interesting approach to study the structure-function relationships in acrosin is the isolation and characterization of the structural units which are responsible for the distinct functions of acrosin. Processing of α-acrosin to mature β-acrosin results in the loss of the proline-rich C-terminal peptide of the heavy chain [17,18,21] which could be isolated under certain conditions [17]. It is suggested that the affinity of acrosin for surfaces such as that of spermatozoa can be graded by progressive release of the enzyme from the C-terminal domain [17,20]. Further processing generates a N-terminal peptide of the acrosin heavy chain which shows zona-binding capacity. As determined by its inhibitory activity on the acrosin zona-binding this 15-kDa fragment has lost about one-third of its binding capacity compared to the whole molecule. This might be due to conformational change and/or partial destruction of binding sites during acrosin processing. The fragment represents the first 120–130 amino acids of the heavy chain as calculated from amino acid analysis and contains two of the amino acids forming the catalytic triad [18,21]. Thus, autoproteolysis creates different active acrosin forms or inactive fragments which combines the acrosin functions in different ways. The carbohydrate affinity can be separated from the proline-rich segment by C-terminal processing without concomitant loss of enzymatic activity or alternatively by N-terminal processing generating fragments which have lost their proteolytic activity. It is reasonable to suggest that specific processing of acrosin may be related to its function to assist a motile sperm to traverse the zona pellucida. O'Rand and coworkers [22] hypothesize that alternating cycles of binding to the zona, digestion of the zona and release from the zona together with the forward motility of the spermatozoon would be required to achieve penetration. Acrosin with its unique structural and functional properties may be instrumental in all 3 stages.

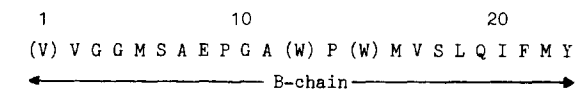

Fig. 4. Amino-terminal amino acid sequence of the acrosin zona-binding peptide, identical with the N-terminal sequence of the acrosin heavy chain (B-chain). Residues in parentheses were not identified in the present analysis but previously established in acrosin [6,12].

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