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The complete primary structure of three isoforms of a boar sperm-associated acrosin inhibitor

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Acrosin inhibitors of seminal vesicle origin, after binding to their acceptor molecules on the anterior part of ejaculated sperm, are thought to be important capacitation factors, protecting zona binding sites during sperm uterine passage, and then dissociating to allow sperm binding to the zona pellucida of the oocyte. Each species so far tested possess an heterogeneous population of isoinhibitors which may display overlapping but not identical biological functions. Here we report the complete primary structure of three isoforms of a boar sperm-associated acrosin inhibitor, whose sequences are 90% identical to the seminal plasma counterpart. Despite this high analogy, the differences between the sperm-associated and the seminal plasma inhibitors may confer to them different physico-chemical properties which are postulated to be of functional importance.

Boar acrosin isoinhibitor; Amino acid sequence; Mass spectrometry

1. INTRODUCTION

Acrosin inhibitors are seminal vesicle secretory polypeptides structurally related to the serine proteinase inhibitors of the Kazal-type [1], a large family of homologous proteins ubiquitous in all vertebrate species so far investigated [2]. The biological role of acrosin inhibitors have not been rigidly defined, however. At ejaculation, sperm is mixed with, and binds to a number of seminal components from the secretions of the accessory sexual glands, including acrosin inhibitors. They may protect the male and/or female genital tract epithelia against the proteolytic degradation by acrosin liberated from occasional damaged spermatozoa [3,4]. Additionally, by binding to acceptor molecules on the sperm surface, they may play a role as capacitation factors stabilizing receptors necessary for the sperm–oocyte interaction, i.e. zona pellucida-binding sites, which may become exposed after release of the inhibitor during sperm residence in the female tract [5,6]. Seminal plasma acrosin inhibitors of the Kazal-type have been isolated and sequenced from boar [1], bull [7–9] and human [10]. In the boar, as in other species, isoinhibitors, thought to be natural mutants and gene products of different genes, have been identified [1]. In this report we have isolated and sequenced three isoforms of a boar sperm-associated acrosin inhibitor. The sequences of these isoforms differ only at their N- and C-terminal parts, and are 90% identical to the known sequence of the boar seminal plasma acrosin inhibitor [1]. The differences between the seminal plasma and the sperm-associated isoinhibitor forms show that they may be different gene products. Moreover, these structural differences may confer to both molecules different physico-chemical properties which, most probably, are of functional importance. A group of low molecular weight boar sperm proteins mediating the binding of the isoinhibitors to the sperm surface was also identified.

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2. ANALYTICAL METHODS

Sperm-associated acrosin inhibitors were isolated and identified as in [11]. Briefly, fresh ejaculated boar semen was separated from seminal plasma by centrifugation at 600 × g for 20 min. Spermatozoa were resuspended and washed twice by centrifugation with 50 mM *N*-morpholinoethane sulfonic acid, 0.264 M sucrose, pH 6.5. The final pellet was extracted overnight at 4°C with one volume of 2.5% (v/v) acetic acid containing 10% (v/v) glycerol and 50 mM benzamidine. After centrifugation at 14,000 × *g* for 10 min at 4°C the clear supernatant was fractionated on a Sephadex G-50 column (3.9 × 78 cm) in 1 mM HCl. The fractions were neutralized with 1 M Tris, diluted 1:1 (v/v) with 50 mM Na_2CO_3/$NaHCO_3$ pH 9.6, immobilized onto ELISA plates, and tested with an anti-boar seminal inhibitor polyclonal antibody [12]. In parallel, each fraction was tested for acrosin inhibitory activity as in [13]. The positive fractions of both assays were pooled, lyophilyzed, dissolved in 5 mM ammonium acetate, pH 6.0, and applied to a DE-Cellulose 52 column (2.5 × 14 cm) equilibrated with the same buffer. After washing, the retained material was eluted with a 5–200 mM ammonium acetate gradient at pH 6.0. The flow-through fractions, which contained acrosin inhibitor(s), were concentrated and desalted by ultrafiltration using an Amicon cell with a UM5

membrane. Further purification of acrosin inhibitors was performed by reverse-phase chromatography using a Vydac C18 RPC analytical column, equilibrated with a mixture of 0.1% (v/v) trifluoroacetic acid in water (A) and 0.1% trifluoroacetic acid in acetonitrile (B) and eluted first isocratically (20% B) for 5 min followed by a linear gradient up to 35% B in 30 min, at a flow rate of 1 ml/min. The fractions containing acrosin inhibitor(s), which eluted at 26% B, were lyophilized.

SDS-gel electrophoresis was done according to Laemmli [14].

Reduction of cystine residues was accomplished with 2-mercaptoethanol (5% (v/v), final concentration), and derivatization of the newly exposed cysteines was done with a 2-fold molar excess of 4-vinylpyridine over reducing agent.

Cleavage of the acrosin inhibitor (10 mg/ml in 70% (v/v) formic acid) at methionine residues was done with cyanogen bromide (100 mg/ml final concentration) for 4 h at room temperature in the dark. The reaction mixture was then diluted with water and lyophilyzed.

Native or cyanogen bromide-treated acrosin inhibitor (5 mg/ml in 50 mM ammonium bicarbonate pH 8.0) was further degraded with chymotrypsin (Sigma) at an enzyme/substrate ratio of 1:100 (w/w) overnight at 37°C.

Isolation of peptides was done by reverse-phase HPLC on a Lichrospher RP-100 (Merck, Darmstadt) C18 column (25 × 0.4 cm, 5 μm particle size) eluting at 1 ml/min with a linear stepwise gradient of 0.1% TFA in (A) water and (B) acetonitrile.

N-terminal sequence analyses were done with an Applied Biosystems gas-phase sequencer model 473A, following the manufacturer's instructions.

Fast bombardment mass spectra were recorded with a mass spectrometer MAT 900 (Finnigan MAT, Bremen) equipped with a liquid secondary ion ionization system. Time-of-flight plasma desorption mass spectrometry was done using a Bioion (Uppsala, Sweden) spectrometer with a ^{252}Cf ionization source.

3. RESULTS AND DISCUSSION

Acrosin inhibitors tightly associated to washed boar spermatozoa were isolated following the procedure developed by Jonáková et al. [11], as described above. The final HPLC-isolated product showed a single broad electrophoretic band of about 8 kDa. Molecular weight determination by time-of-flight mass spectrometry clearly showed the existence of two major components with a molecular weight average of 7691±8 and 7924±8 Da (Fig. 1). N-terminal sequence analysis confirmed the presence of two polypeptides whose sequences were:

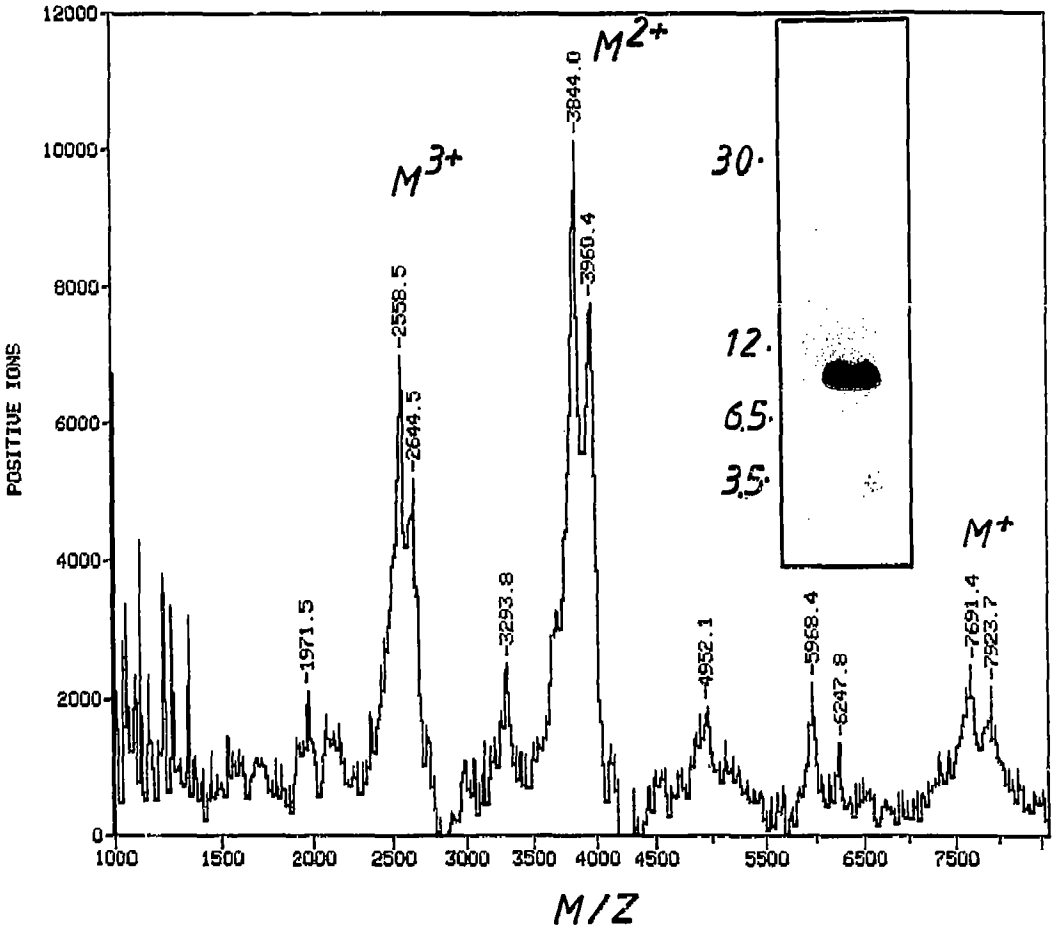

Fig. 1. Determination of the molecular weight of the acrosin isoinhibitors. The molecular weight of the components present in the sperm-associated acrosin isoinhibitor sample was measured by time-of-flight plasma desorption mass spectrometry using ^{252}Cf-induced ionization. Species corresponding to the mono-, di- and tri-protonated (M^+, M^{2+} and M^{3+}, respectively) quasimolecular ions were observed. The spectrum was recorded at an acceleration voltage of 17 kV and corresponded to the average of 20 × 10^6 primary ions. Inset: analysis of the HPLC-purified isoinhibitor fraction by SDS-polyacrylamide (15%) gel electrophoresis; at the left, molecular weight markers in kDa.

ARSKKTRKEPDCDVYRSHLFFCTREMDICGTNG..., and SKKTRKEPDCDVYRSHLFFCTREMDICGTNGKS...

This result indicated that the inhibitor fraction actually contained a mixture of two structurally related peptides which may differ solely in their N-terminal ends, since the mass difference between ARS and S (227 Da) corresponds to the mass difference betweent the two major molecular ions identified by mass spectrometry (Fig. 1).

The complete amino acid sequence of the acrosin isoinhibitors was obtained by N-terminal sequencing of the whole fraction and of a set of HPLC-purified cyanogen bromide cleavage products and chymotryptic peptides derived from reduced and ethylpyridylated samples (Fig. 2). Sequence assignment was confirmed by fast atom bombardment mass spectrometric analysis of chymotryptic peptides obtained from underivatized samples. In addition, three intramolecular disulphide bridges were identified (Fig. 2). The disulphide bridge pattern (C^1–C^5, C^2–C^4, C^3–C^6) is typical for serine proteinase inhibitors of the Kazal-type [1].

Among the cyanogen bromide peptides characterized, a minor component was found whose amino acid sequence was: GHCREYTSAARS. Thus, beside the two major isoinhibitors a third minor component with an extended C-terminal end was present (Fig. 2).

When the amino acid sequences of the sperm-associated acrosin isoinhibitors were compared with all the protein sequences deposited in the MIPS data bank, only significant analogy with other Kazal-type serine proteinase inhibitors was found. The strongest analogy (90% amino acid sequence identity) was with the boar seminal plasma acrosin inhibitor [1]. Thus, their amino acid sequences only differ (Fig. 2) in that (a) the sperm-associated counterpart contains N-terminal extensions of 3 or 5 amino acids; (b) five positions showing glutamine or asparagine in the seminal plasma isoinhibitor contain the corresponding carboxylic acid in the sperm-associated isoform; (c) two adjacent residues have their relative positions changed; and (d) the minor component of the sperm-associated acrosin isoinhibitor possesses an extra alanine residue at its C-terminal end. These differences (shown in boldface in Fig. 2) indicated that the sperm-associated and the seminal plasma acrosin isoinhibitors may be products of different genes. On the other hand, the three sperm-associated isoinhibitors may arise by alternative proteolytically processing of the same gene product.

Despite the apparently small sequence differences, a closer analysis indicated that the seminal plasma and the sperm-associated isoinhibitors may possess different physico-chemical properties. Thus, whereas the seminal plasma inhibitor is a glycoprotein [1], the isoinhibitors characterized here are not glycosylated. Furthermore, the sperm-associated forms possess isoelectric points of about one pH unit lower than that of the seminal plasma counterpart (8.2–8.4 vs. 9.5). Interestingly, all amide/carboxylic acid substitution, except the one adjacent to the active arginine residue (shown in shadow

Fig. 2. Alignment of the amino acid sequence of the seminal plasma and the sperm-associated acrosin isoinhibitors. The seminal plasma acrosin inhibitor (upper sequence) is from [1]. The complete primary structure of the sperm-associated isoinhibitors (this work) was obtained by protein-chemical and mass spectrometric analyses. The sequence is numbered taking the longest form as reference. CB, cyanogen bromide fragment; C, chymotryptic derived peptide. The numbers below a given fragment correspond to its molecular weight ($M+H^+$) determined by fast atom bombardment mass spectrometry. The differences between the seminal plasma [1] and the sperm-associated acrosin isoinhibitors are indicated in boldface. The active argine residue is shown in shadow. The C-terminal extension found in a minor component is in italics between brackets. S-S, disulphide bridge.

in Fig. 2), lie on the same face of the molecule, when the structure of the sperm-associated acrosin inhibitor was modelled with the known three-dimensional coordinates of its homologous elastase inhibitor (courtesy of Dr. W. Bode, Martinsried). These molecular features may be of functional significance regarding its binding to sperm acceptor molecules. In this respect it may be noticed that we have not found the seminal plasma inhibitor [1] in extracts of washed sperm.

Experiments in our laboratory show that the acrosin isoinhibitors characterized here specifically recognize low molecular weight (14–18 kDa) boar sperm-associated component(s), some of which have been implicated in the binding of the spermatozoon to its homologous egg's zona pellucida [15,16] (data not shown). This interaction does not inhibit the subsequent binding of the sperm protein(s) to isolated zona pellucida.

Mammalian sperm is mixed with, and binds to a number of seminal components at ejaculation. The subsequent removal of at least some of these components is a necessary feature of capacitation. In the mouse, a low molecular weight (6.4 kDa) acrosin inhibitor of seminal plasma origin binds at ejaculation to the sperm plasma membrane covering the apical portion of the acrosome [17]. The inhibitor interacts with a 15 kDa acceptor molecule [18], and inhibits the binding of sperm to zona pellucida [19–21]. The authors postulate that binding of the inhibitor to its acceptor molecule may stabilize specific sites necessary for sperm–egg interaction, and that during sperm residence in the female genital tract, the inhibitor is released from the sperm surface and the zona-binding sites are exposed. Our data agree with this hypothesis. Further studies are, however, needed to precisely identify the inhibitor-binding boar sperm component(s), and its relationship to the mouse 15 kDa acceptor molecule. Since sperm–zona pellucida binding is mediated through a (sperm) protein–(zona) carbohydrate recognition mechanism, it would be interesting to delineate the relationship between the inhibitor binding site and the zona binding site within the sperm protein(s). Another exciting question which remains to be solved is whether or not the inhibitor utilizes the protease-binding site for interaction with its sperm acceptor.

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