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Mechanism of maturation and nature of carbohydrate chains of boar sperm acrosin

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The acrosin zymogen proacrosin exists in two molecular forms which are believed to be single-chain polypeptides. During autoactivation in a cell-free system, the 55 and 53 kDa zymogens are sequentially converted into the 49, 36, 31 and 25 kDa forms. A similar mechanism of maturation was revealed, when the calcium ionophore A23187 was added to suspensions of boar spermatozoa. The 49 kDa form has been identified as the first active acrosin form in the maturation cascade. However, this form is indistinguishable from the 53 kDa zymogen in SDS-PAGE at nonreducing conditions. Two carbohydrate chains were evidenced on the acrosin molecule. The chain attached to the Asn^3 of the acrosin light chain was enzymatically cleaved without loss of acrosin activity. By contrast, the carbohydrate chain linked to the acrosin heavy chain could be cleaved only after acrosin denaturation. Based on the susceptibility of acrosin to endoglycosidases F and H, a biantennary structure of both carbohydrate chains is proposed.

Proacrosin activation; Acrosin conversion; Glycosylation; Carbohydrate structure; Boar spermatozoa

1. INTRODUCTION

Acrosin (EC 3.4.21.10) is a two-chain glycoprotein which plays an essential role in mammalian fertilization [1]. It is a trypsin-like serine proteinase which is initially present in the intact acrosome as an enzymatically inactive zymogen form, proacrosin [2]. Acrosin has been described to be responsible for the limited proteolysis of the oocyte zona pellucida glycoprotein matrix [3] and is suggested to be involved in sperm–egg interaction by its capacity to bind zona pellucida glycoproteins [4,5]. Boar proacrosin has been identified as a mixture of two forms with apparent molecular masses of 55 and 53 kDa and is sequentially autoconverted to enzymatically active forms of 49, 34 and 25 kDa [6,7]. The active enzyme consists of a light chain, 23 amino acids long, linked by two disulphide bridges to the heavy chain [8]. Baba et al. [9] postulated that proacrosin is a single-chain polypeptide containing a segment corresponding to the light chain at the N-terminus. The 55 kDa form is sequentially processed by liberation of a proenzyme segment from the C-terminus followed by cleavage of single peptide bond producing the heavy and light chains. In contrast to this hypothesis, Töpfer-Petersen et al. [10] suggested another mechanism of activation in which proacrosin forms an active, double-chain intermediate containing the full-length heavy chain. This intermediate then undergoes a maturation process leading to the most stable active form.

Fock-Nüzel et al. [11] reported that boar acrosin contains a single carbohydrate chain attached to Asn^3 in the light chain. On the other hand, Töpfer-Petersen et al. [10] found carbohydrate attached to the acrosin second glycosylation site situated on the acrosin heavy chain.

To overcome these inconsistencies we undertook the present study in which we revealed the number and nature of carbohydrate chains in acrosin molecules and highlighted the mechanism of activation.

Abbreviations: SDS-PAGE, sodium dodecylsulfate gel electrophoresis.

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2. EXPERIMENTAL

2.1. *Preparation of acrosin*

Crude acrosin fraction was prepared from freshly ejaculated boar spermatozoa as described previously [12]. The lyophilized acrosin was dissolved in 1 mm HCl, diluted in 100 mM Tris, 25 mM $CaCl_2$, pH 8 to a final concentration of 0.5 $mg \cdot ml^{-1}$ and allowed to react for 60 min at 37°C in the presence or absence of 20 mM benzamidine. The reaction was stopped by adding SDS to a final concentration of 20 $mg \cdot ml^{-1}$.

2.2. *Induction of the acrosome reaction*

The Tris-buffered medium, pH 7.7 [13], was used as a washing and incubation medium. Fresh boar ejaculates were washed twice and layered on 40–80% discontinuous Percoll gradient [13]. After centrifugation, the 80% layer was washed twice and spermatozoa were resuspended to a final concentration of 2.5×10^7 ml^{-1}. The acrosome reaction was induced by addition of the calcium ionophore A 23187 (Sigma) to a final concentration of 10 μM followed by 60 min incubation at 37°C. Sperm suspensions were then extracted [12] and the extract was subjected to SDS-PAGE.

2.3. *Deglycosylation of boar acrosin*

Acrosin was deglycosylated according to the method of Tarentino [14] with some modifications. Lyophilized acrosin was either dissolved in 1% SDS or denaturated in 100 mM mercaptoethanol, 1% SDS (boiled 3 min) to a concentration of 5 mg·ml^{-1}. Aliquots of 10 μl were taken from these solutions and added to 90 μl of 0.6% NP40, 20 mM EDTA, 20 mM benzamidine, 250 mM sodium acetate, pH 5. Deglycosylations were conducted in the presence of either endoglycosidase F (300 mU·ml^{-1}) or endoglycosidase H (60 mU·ml^{-1}) for 18 h at 37°C. Both enzymes were purchased from Boehringer (Mannheim, Germany).

2.4. *Electrophoresis and Western blotting*

SDS-PAGE was performed on 10% polyacrylamide slab gels according to Laemmli [15] and proteins were then transferred onto nitrocellulose sheets (Hybond C, Amersham) according to the method of Towbin [16]. Protein blots were stained with ACR.2 monoclonal antibody [17], recognizing an epitope located on the acrosin heavy chain. For identification of glycoproteins, the blots were probed with biotinylated concanavalin A (Sigma) and the bound lectin was identified by streptavidin-peroxidase (Radiochemical Centre Amersham, UK) using the protocol recommended by the manufacturer. Molecular mass of proteins were estimated by comparison with mobility of low-molecular weight markers (Pharmacia, Uppsala, Sweden).

2.5. *Gel enzymography*

Gelatinolytic activity in gels after SDS-PAGE was analyzed by the method of Heussen and Dowdle [18]. Nondenaturating acidic PAGE and detection of acrosin amidase activity in gels were carried out according to Garner [19], using *N*-α-benzoyl-arginine 2-naphtylamide (Sigma) and Fast Garnet salt for activity visualization. For comparison of amidase activity pattern with distribution of immunoreactive peptides, the acid PAGE gels were equilibrated in 1% (w/v) SDS, 0.3 M Tris, pH 8.8 for 60 min, followed by incubation in 0.1% SDS, 0.192 M glycine, 0.3 M Tris, pH 8.5, for 30 min, then electrotransferred onto nitrocellulose and stained with ACR.2 antibody as described above.

3. RESULTS AND DISCUSSION

It is generally known that after disulphide bond reduction and protein relaxation, electrophoretical mobility of single chain molecules decreases, whereas either component of double-chain molecules has a higher mobility than the nonreduced form. When boar acrosin was analysed by immunoblotting in the absence of any reducing agents, only two closely related bands in the 50 kDa region were observed (Figs. 1a, 2a). However, when the same sample was reduced, the 55, 53 and 49 kDa forms appeared (Fig. 1d, 2c). This finding suggests two possible explanations. The 55 kDa proacrosin is either converted into the 53 kDa single-chain form followed by activation into double-chain 49 kDa acrosin [9] (this molecular mass belongs to the heavy chain only and the compact double-chain molecule has the same electrophoretic mobility as the single-chain 53 kDa form) or it is transformed directly into the active, double-chain acrosin followed by further conversions [10]. If the later hypothesis is true, the full-length, double-chain acrosin having in nonreduced form the same electrophoretical mobility as the 55 kDa single-chain proacrosin should be expected. Considering the fact that the existence of active 49 kDa form has been well documented, the latter hypothesis also implicates the existence of two active high molecular forms of acrosin. This is not consistent with our findings. Only one zone of amidase activity has been found in the high-molecular weight region of acidic PAGE (Figs. 1e,f and 2f). This activity zone was still present even if all of the 55 and 53 kDa molecules were converted (compare Fig. 1b,e with Fig. 1f). A similar mechanism of acrosin conversions was achieved using the calcium ionophore A23187 to induce

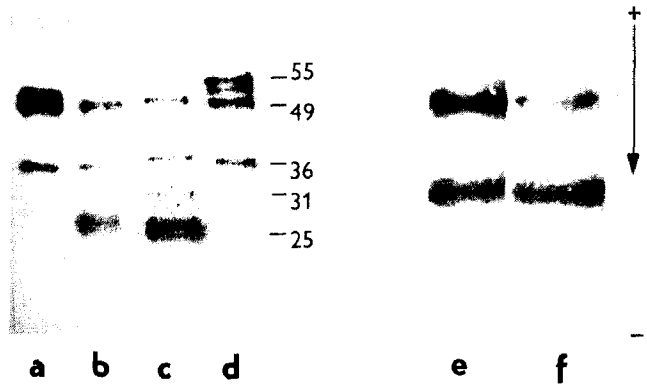

Fig. 1. Activation and conversions of boar acrosin in a cell-free system. Boar acrosin was incubated in the presence (a,d,e) or absence (b,c,f) of benzamidine and analysed either by SDS-PAGE followed by immunoblotting (a–d) or by acidic PAGE, followed by amidase activity staining (e–f). SDS-PAGE was run in parallel in the presence (c,d) or absence (a,b) of mercaptoethanol. Molecular masses of individual acrosin forms (reduced) are indicated (kDa).

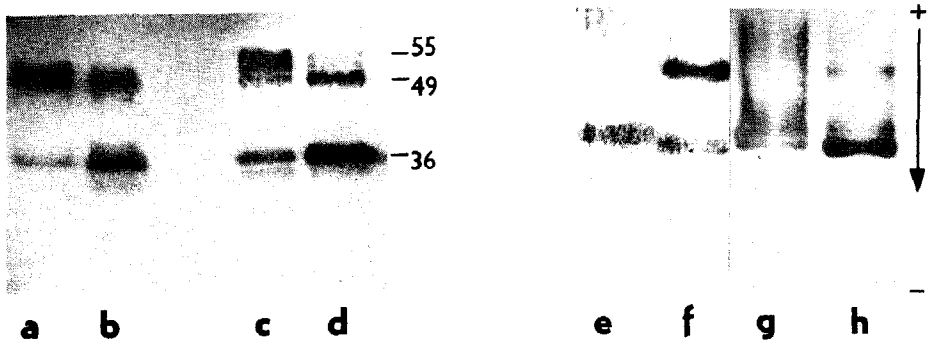

Fig. 2. Activation and conversions of boar acrosin in suspensions of living spermatozoa. Boar spermatozoa were incubated in the presence (b,d, and h) or absence (a,c,e and g) of calcium ionophore A23187 to induce the acrosome reaction, extracted and the extracts were analysed by immunoblotting (a–d) and by acidic PAGE, followed either by amidase activity staining (e–f) or by blotting and staining with ACR.2 antibody (g–h). Both the nonreduced (a,b) and reduced (c,d) samples were run in SDS-PAGE. The molecular masses of acrosin forms (reduced) are indicated

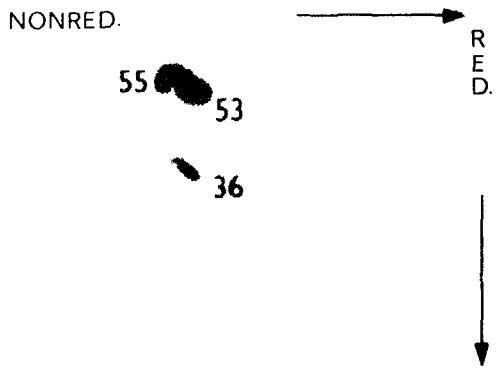

Fig. 3. Boar sperm acrosin was analysed by two-dimensional SDS-PAGE (the first dimension under nonreducing, the second under reducing conditions), followed by immunoblotting. The numbers represent molecular masses in kDa.

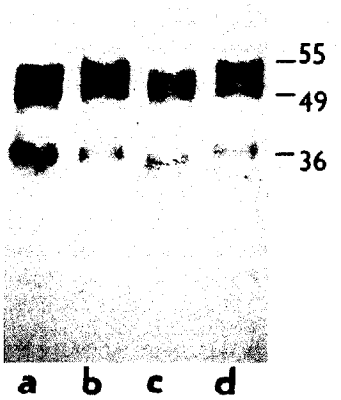

Fig. 5. Deglycosylation of reduced boar acrosin. Acrosin was boiled in the presence of SDS and mercaptoethanol, subjected to enzymatic deglycosylation and analysed by immunoblotting. (a) Control sample before any incubation. (b) Control sample incubated in the absence of glycosidases. (c) Deglycosylation of acrosin by endoglycosidase-F. Deglycosylation of acrosin by endoglycosidase-H.

the acrosome reactions in suspensions of living spermatozoa (Fig. 2). The only exception was the finding that no degradation of the 36 kDa acrosin to the 31 and 25 kDa forms was observed under these conditions. All these findings, and also those previously reported [6–8], strongly supported the mechanism of maturation suggested by Baba et al. [9]. Moreover, when a mixture of the 55 and 53 kDa forms was analyzed by two-dimensional SDS-PAGE (nonreduced versus reduced), the 55 kDa form was observed as a single spot, while the material migrated with the mobility of 53 kDa in the first dimension was partially converted into the 36 kDa form (probably via the less stable 49 kDa form) during the procedure. This also supports the suggestion that only the 53 kDa zymogen can be activated into the active acrosin.

Fock-Nüzel et al. [11] reported that boar acrosin contains only one carbohydrate chain, attached to Asn^3 of the acrosin light chain. On the other hand, Töpfer-Petersen et al. [10] have shown that peptide 10 derived from the heavy chain contains 3 mol glucosamine/mol peptide. Moreover, the heavy chain contains the typical sequon of *N*-glycosylation sites Asn-Ser-Thr [9,20]. When acrosin was subjected to enzymatic deglycosylation in a native state, the susceptibility to endoglycosidase-F but not to endoglycosidase-H was evidenced (Fig. 4e–h). This procedure did not destroy the acrosin enzymatic activity (Fig. 4i). However, when these deglycosylated and control samples were boiled in the presence of mercaptoethanol and the electrophoretical mobilities of heavy chains were then compared, no shift in mobility of heavy chains treated with EndoF was observed (compare Fig. 4a vs. Fig. 4c). Thus, only the carbohydrate attached to the acrosin light chain was cleaved under these conditions. On the other hand, when acrosin was reduced before enzymatic deglycosylation, the heavy chain relaxed and became susceptible to endoglycosidase-F (Fig. 5c). These findings, and the reactivity of acrosin heavy chain with concanavalin A (data not shown) suggest that *N*-linked carbohydrate chains are attached to both the light and the heavy acrosin chains. Based on the specificity of the endoglycosidases used [14], and on their reactivity with acrosin, we concluded that both acrosin carbohydrate chains are complex biantennary type. This suggestion is in agreement with the proposal of Töpfer-Petersen et

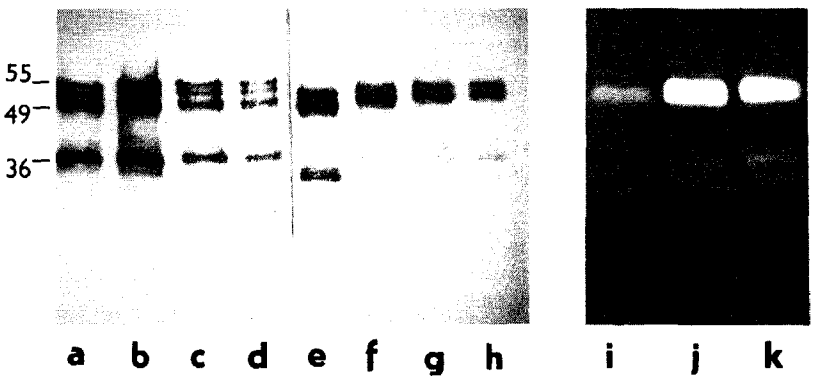

Fig. 4. Enzymatic deglycosylation of native boar acrosin. Acrosin was subjected to enzymatic deglycosylations followed by immunoblotting with ACR.2 antibody (a–h) or analysed for gelatinolytic activity in SDS gels (i–k). Samples run in lanes a–d were reduced before electrophoresis. (a,e,i) Deglycosylations were conducted in the presence of endoglycosidase-F (b,f,j) Deglycosylation was conducted in the presence of endoglycosidase-H. (c,g,k) Boar acrosin was incubated in the absence of any glycosidases. (d,h) Boar acrosin prior to any incubation.

al. [10]. However, the precise structure of acrosin carbohydrate chains remains to be clarified.

REFERENCES

[1] Yanagimachi, R. (1988) in: The Physiology of Reproduction (Knobil, E. and Neil, J. eds.) pp. 135–185, Raven Press, New York.

[2] Bhatacharyya, A.K. and Zaneveld, L.J.D. (1982) in: Biochemistry of Mammalian Reproduction (Zaneveld, L.J.D. and Chatterton, R.T. eds.) pp. 119–151. Wiley, New York.

[3] Dunbar, B.S., Dudkiewicz, A.B. and Bundman, D.S. (1985) Biol. Reprod. 32, 619–630.

[4] Töpfer-Petersen, E. and Henschen, A. (1987) FEBS Lett. 226, 38–42.

[5] Jones, R., Brown, C.R. and Lancaster, R.T. (1988) Development 102, 781–792.

[6] Polakoski, K.L. and Parrish, R.F. (1977) J. Biol. Chem. 252, 1888–1894.

[7] Parrish, R.F. and Polakoski, K.L. (1978) J. Biol. Chem. 253, 8428–8432.

[8] Cechova, D., Töpfer-Petersen, E. and Henschen, A. (1988) FEBS Lett. 241, 136–140.

[9] Baba, T., Kashiwibara, S., Watanabe, K., Itoh, H., Michikava, Y., Kimura, K., Takada, M., Fukamizu, A. and Arai, Y. (1989) J. Biol. Chem. 264, 11920–11927.

[10] Töpfer-Petersen, E., Calvete, J., Schäfer, W. and Henschen, A. (1990) FEBS Lett. 275, 139–142.

[11] Fock-Nüzel, R., Lottspeich, F., Henschen, A. and Müller-Esterl, W. (1984) Eur. J. Biochem. 14, 441 446.

[12] Moos, J., Peknicova, J., Surneva-Nakova, T.N. and Pavlik, M. (1990) FEBS Lett. 264, 243–245.

[13] Berger, T. and Horton, M.B. (1988) Gamete Res. 19, 101–119.

[14] Tarentino, A.M., Gomez, C.M. and Plummer, T.H. (1985) Biochemistry 24, 4665 4671.

[15] Laemmli, U.K. (1970) Nature 227, 680–685.

[16] Towbin, H., Stachelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.

[17] Peknicova, J. and Moos, J. (1990) Andrologia 22, 427–435.

[18] Heussen, C. and Dowdle, E.B. (1980) Anal. Biochem. 102, 196–202.

[19] Garner, D.A. (1975) Anal. Biochem. 67, 688–694.

[20] Adham, I.M., Klemm, U., Maier, W.M., Hoyer-Fender, S., Tsaousidou, S. and Engel, W. (1989) Eur. J. Biochem. 565–568.