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*Protein Proteinase Inhibitors in Male Sex Glands and their Secretions**

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PROTEIN proteinase inhibitors occur in a variety of forms in plant and animal tissues and secretions,[1, 2] but only in a few cases have we some idea of their physiological function.[3–6] This is due, for example, to the relatively acid-stable trypsin inhibitors in male sex glands and their secretions.[6, 7] Trypsin inhibition capacities in tissues and seminal fluid of some species are shown in Table 1. Recently obtained results[8] showed us that spawn and testicle from herring also contain trypsin inhibitors (which inhibit also porcine acrosin, the sperm trypsin-like protease) in concentrations comparable to that of the seminal vesicles of mouse and sheep, respectively.

TABLE 1. TRYPSIN INHIBITION ACTIVITIES (mIU*) IN MALE SEX GLANDS AND THEIR SECRETIONS

Species	mIU per g tissue or ml plasma			
	Testes	Epididymis	Glandula vesicul.	Seminal plasma
Man	70–100	50–80	50–100	150–330
Cattle	40–70	50–80	900–1500	2400–3100
Pig	90–120	70–110	500–1000	800–1200
Sheep	–	–	250–500	–
Rat	100–200	90–130	1400–1600	–
Mouse	90–130	100–200	2200–2700	–
Guinea pig	100–220	300–400	3500–5000	–
Hamster	60–90	80–120	300–600	–

* One mIU inhibits the activity of about 1 μg trypsin Novo.

* Part of the work was done in collaboration with Dr. H. Tschesche, Technische Universität München and Prof. Dr. C. Schirren, Universitätskrankenhaus Eppendorf, Hamburg.

We isolated some of the seminal inhibitors by affinity chromatography.[7, 9] The mixtures of inhibitors obtained were separated into inhibitors with different characteristics – concerning the inhibition spectra, molecular weights, etc. – by gradient elution chromatography, equilibrium chromatography, and/or gel filtration. Examples were, the trypsin and trypsin-plasmin inhibitors from guinea pigs,[7] the trypsin and trypsin-chymotrypsin inhibitors from human sperm plasma,[10] and the different trypsin-plasmin inhibitors (Bdellins) from leeches.[11] These are presented in Table 2. All of these inhibitors also inhibit porcine acrosin, the acrosomal sperm protease.

TABLE 2. SEMINAL INHIBITORS ISOLATED BY AFFINITY CHROMATOGRAPHY

Source	Inhibition of				Mol. weight	Isolated	
	trypsin[a]	plasmin[b]	chymotrypsin[a]	acrosin		using	yield, %
Guinea pig seminal vesicles I	+	–	–	+[c]	6,600	EMA[e]–trypsin	80–95
Guinea pig seminal vesicles II	+	+	–	+[c]	6,700		
Boar sperm	+	+	–	+[b]	11,600	Biogel®–trypsin	63–85
Human sperm II	+	–	–	+[d]	5,400	CM–Cell.–trypsin*	83–100
Human sperm I	+	–	+	+[d]	12,700		
Leeches B	+	+	–	+[b]	4,800	EMA[e]–trypsin	69–76
Leeches A	+	+	–	+[b]	6,300		

[a] Bovine. [b] Porcine. [c] From rabbits. [d] Human. [e] Copolymer from ethylene and maleic acid.
* From E. Merck, Darmstadt.

The inhibitors with clearly defined inhibition spectra are not yet homogeneous; they are mixtures of isoinhibitors.[7, 11] For example, the inhibitor mixture isolated from guinea pig seminal vesicles by affinity chromatography was separable into two trypsin inhibitors (a modified form with the Arg-Ile bond in the reactive site broken and a native form with the intact bond) and five trypsin-plasmin inhibitors differing in their N-terminal amino acid sequences.[7]

In all cases investigated, the inhibitors form 1 : 1 equimolar complexes with the corresponding enzymes. This can be deduced from the titration curves.[7, 10, 11]

In Table 3 the amino acid compositions of some seminal inhibitors are given. Bdellin B-3, the trypsin-plasmin inhibitor from leeches, has the lowest molecular weight of all protein trypsin inhibitors found so far.

For the moment we are mainly interested in the inhibitors from human sperm plasma. The isolation procedure is summarized in Table 4. The inhibitor mixture obtained by affinity chromatography was separated into two fractions by gel filtration using Sephadex G-75.[10]

TABLE 3. AMINO ACID COMPOSITION (MOLE/MOLE) OF SEMINAL INHIBITORS

	Guinea pig sem. vesicles		Boar sperm plasma	Human sperm plasma		Bdellins from leeches	
	TI[a]	TPI[b]		I[c]	II[c]	B_3	A_4
Asp	6	6	11	12	7	5	8
Thr	1	4	6	5	3	4	3
Ser	2	5	7	8	5	2	3
Glu	10	4	8	10	6	6	5
Pro	5	2	4	9	4	–	3
Gly	6	5	8	11	7	4	4
Ala	1	–	4	4	2	4	4
Cys 1/2	6	6	8	12	6	6	10
Val	3	3	2	5	2	4	5
Met	–	1	2	2	1	–	1
Ile	4	1	4	3	4	–	1
Leu	5	3	4	6	4	2	1
Tyr	2	4	4	3	2	1	1
Phe	–	3	7	3	2	–	2
Lys	1	4	8	12	4	1	5
His	2	3	4	4	4	5	3
Arg	6	4	8	5	4	1	–
Trp	–	–	2			–	–
Glucos-amine	–	–	3			–	–
Galactos-amine	–	–	2			–	–
Total	60	58	104	114	67	45	59

[a] Trypsin inhibitor. [b] Trypsin-plasmin inhibitor. [c] Mixture of the isoinhibitors.

TABLE 4. ISOLATION OF PROTEASE INHIBITORS FROM HUMAN SPERM PLASMA

	Trypsin IU	Yield %	Spec. activ. IU/mg
Human sperm, 14.4 l	1260	(100)	0.00009
Sephadex CM-25	703[a] 380[b]	56[a] 30[b]	
Dialysis	620	49	
Desalting (2% HAc) Sephadex G-25, lyophil	583	46	0.053
Affinity chromatography:	778.5 IU were applied to the CM-Cellulose-trypsin* column in 4 successive runs.		
Affinity chromatography	752	97	
Dialysis (4 h, H_2O)	635	82	
Desalting Sephadex G-25 (2% HAc)	612	79	
Fractionation on Sephadex G-75 (2% HAc)	I 218 II 339	I[c] 28 II[c] 44	I 1.73 II 2.17

[a] Eluted with 5% NaCl after the adsorption step (batchwise, 886 IU adsorbed) and washing.

[b] Not adsorbed.

[c] Total : I + II + other fractions (54.6 IU = 7%) = 79%.

* From E. Merck, Darmstadt.

Fraction I (m.w. near 12,700) inhibits trypsin and chymotrypsin, fraction II (m.w. near 5400) only trypsin. Calculated from the specific activity of these fractions (cf. Table 4) a 20,000-fold purification was achieved. Due to our experience with other inhibitors we were not surprised to find that each of these inhibitors was a mixture of isoinhibitors. In Fig. 1 the separation of the isoinhibitors II by pH-gradient chromatography is shown.

Regarding the possible function of the seminal inhibitors, the results of the following experiments should be mentioned. Sperm acrosomal extracts contain a trypsin-like protease which was first characterized (from rabbit sperm) in more detail by Zaneveld *et al.*[6] This protease, named acrosin (for properties see ref. 6), seems to be necessary for penetration of the zona pellucida of the ovum by the spermatozoa.[6, 12]

We isolated the trypsin-like protease from boar spermatozoa. The sperm was centrifuged and adsorbed inhibitor was washed off by repeated suspension of the spermatozoa in salt-buffer

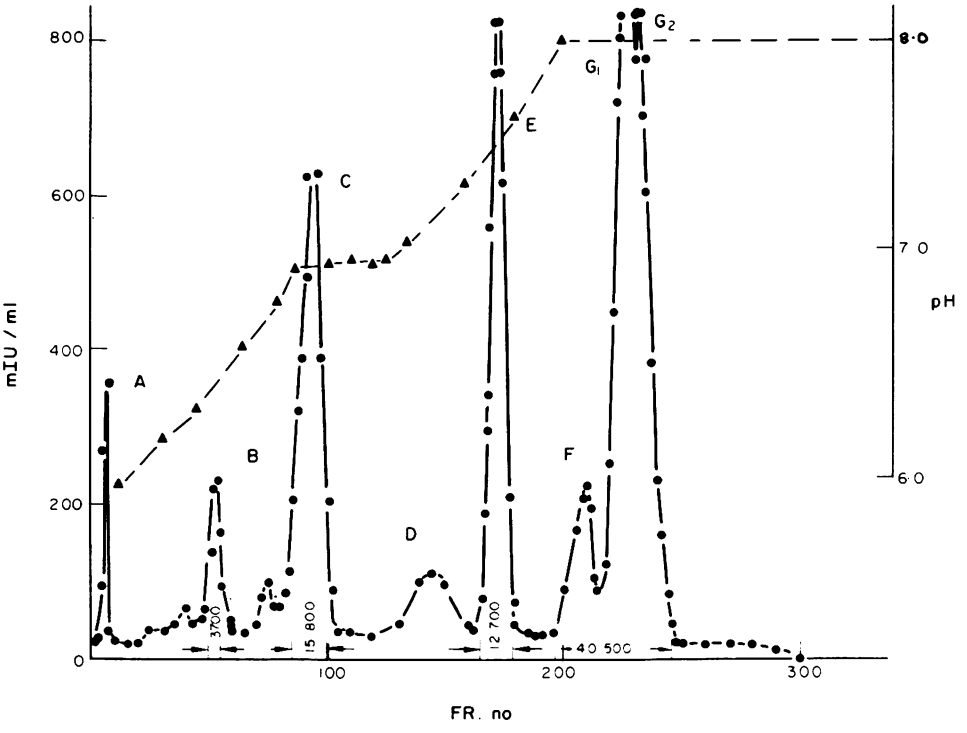

FIG. 1. Fractionation of the trypsin inhibitor II (m.w. near 5400) on CM-Sephadex C-25 by pH-gradient elution chromatography – separation of the isoinhibitors. 110 IU were applied to the column (1.0 × 25.5 cm) equilibrated with phosphate buffer, pH 6.1 and ionic strength $J = 0.2$. Left ordinate: Trypsin inhibitory activity in mIU per ml in the eluted fractions (abscissa); right ordinate: pH of the eluted fractions.

solutions. The sediment was extracted with dilute acetic acid, pH 2.6, and not with hyamine-containing solutions as reported by Zaneveld *et al.*[6, 13] Thus we obtained extracts with a very high benzoylarginine ethyl ester- and benzoylarginine *p*-nitroanilide-splitting activity. Our results[14] indicate that the acrosin-inhibitor complex is only partially soluble in neutral hyamine-containing solutions, whereas in slightly acidic solutions the acrosin is – dissociated from its complex with the inhibitor – very easily soluble. The acrosin isolated from boar spermatozoa by gel filtration and affinity chromatography using inhibitor resins has a very high specific activity: 5 U were found per mg acrosin at 50°C and pH 7.8 employing N-benzoyl arginine *p*-nitroanilide as substrate. Under the same conditions we found for purest trypsin preparations 1.2 U/mg.

The titration curves of boar acrosin with different trypsin inhibitors are shown in Fig. 2. From the titration curve with Bdellin B-3, the analytically pure trypsin-plasmin inhibitor from leeches,[11] we calculated the amount of acrosin present in the test samples to 10 pmole, i.e. the molarity of the acrosin solutions in the test system is near 3×10^{-9} M (for trypsin: 1×10^{-7} M). The dissociation constant K_i for the complex of acrosin with Bdellin B-3 is about 10^{-10} M and therefore extremely low compared with the other acrosin-inhibitor complexes (cf. Fig. 2).

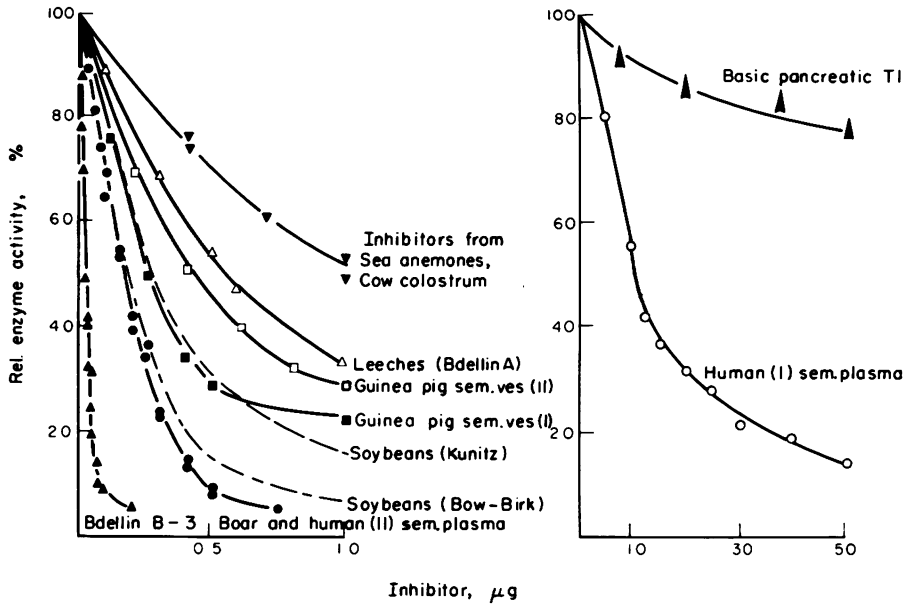

FIG. 2. Titration of boar sperm acrosin with trypsin inhibitors of different origin. About 10 pmole acrosin were incubated with increasing amounts of the inhibitors for a period necessary to reach equilibrium in a final volume of 2.0 ml in buffer solution, pH 7.8 at 25°C. The enzymatic reaction was started by addition of the substrate solution (1 mg N-benzoyl DL-arginine *p*-nitroanilide in 1.0 ml aqua dest.). Ordinate: Remaining acrosin activity in the test samples.

TABLE 5. EFFECT OF GUINEA PIG SEMINAL VESICLE TRYPSIN INHIBITORS ON THE FERTILIZING ABILITY OF CAPACITATED RABBIT SPERMATOZOA

According to Zaneveld *et al.* (6)

Additions per 10^5 sperm	Units of inhibitor activity (mIU)	No. of rabbits	No. of eggs	% fertilization
100 μg TPI*	170	4	14	14.3
0	0		10	100
250 μg TI*	750	5	15	73.5
0	0		10	90

* TPI, trypsin-plasmin inhibitor; TI, trypsin inhibitor.

Capacitated sperm were treated with inhibitor for 20 min at 37°C and 0.05 ml of the mixture (5×10^4 sperm) was inseminated into the oviducts of rabbits 12.5 hr after administration of HCG. The contralateral oviducts were inseminated with control sperm that were treated the same except that no inhibitor was added.

According to the concept developed by Zaneveld *et al.*[6, 15] the process of capacitation is accompanied by the removal of the inhibitor from the sperm acrosin, i.e. the dissociation of the acrosin-inhibitor complex during migration of the sperm in the female genital tract; consequently, the reactivation of acrosin. Capacitation is necessary for the spermatozoa to regain their fertilizing ability. Zaneveld *et al.*[6, 16] demonstrated that acrosin inhibitors can prevent fertilization, e.g. the trypsin and trypsin-plasmin inhibitors from guinea pig seminal vesicles isolated by Fink *et al.* (cf. Table 5). Therefore we expect that the physiological role of the acrosin inhibitors will be well known in the near future.

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