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Protein Proteinase Inhibitors in Male Sex Glands

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Summary

Trypsin-specific and trypsin-plasmin inhibitors were isolated from seminal vesicles of guinea pigs. Two different procedures were used: 1. Inhibitor material obtained from perchloric acid extracts was purified by affinity chromatography (using water insoluble trypsin resin) and gradient elution chromatography on Sulfoethyl Sephadex. Mainly two very similar trypsin-specific inhibitors and five somewhat different trypsin-plasmin inhibitors were obtained. (The amino acid compositions are given in Table 3). 2. Also by avoiding the trypsin resin step several inhibitor fractions were obtained which were differing considerably in their amino acid compositions.

Inhibitors containing a lysine residue in the reactive site are reversibly inactivated by acylation with maleic anhydride; arginine inhibitors are inactivated by reaction with a butandion-2,3 reagent.

In the reactive site of the trypsin-specific inhibitor the sequence Arg-Ile is present. The modified inhibitor (Arg-Ile bond is broken) is inactivated by incubation with carboxypeptidase B or reaction with excessive maleic anhydride. The native inhibitor (Arg-Ile bond intact) is converted into the modified form both during contact with the trypsin resin and by incubation with 2.3 mole percent trypsin.

From acidic extracts of boar seminal plasma a trypsin-plasmin inhibitor was isolated by affinity chromatography. The preliminary amino acid and amino sugar composition is given in Table 8. The calculated molecular weight (11,607) is in good agreement with the value found by gel filtration experiments (12,000). In the reactive site of the inhibitor an arginine residue is present.

Introduction

Inhibitors for trypsin in male sex glands and in seminal plasma were first discovered in 1965 by Haendle [1], when he was looking for the origin of inhibitory activity in urine [2]. In Table 1 trypsin-inhibiting activities found in sexual glands and seminal plasma of man and of some animals are shown [3]. From these values it is obvious why we have first chosen guinea pig for our studies: Seminal vesicles of this animal contain by far the highest inhibitory activity.

This article contains parts of E. Fink, Dissertation, Naturwissenschaftliche Fakultät der Universität München, 1970.

One inhibition unit, IU, is the amount of inhibition that causes the reduction of enzymatic BAPNA hydrolysis by 1 μmole per minute.

The abbreviations used are: BAEE, N^{α}-benzoyl-L-arginine ethyl ester; BAPNA, N^{α}-benzoyl-D,L-arginine p-nitroanilide hydrochloride; TRA, triethanolamine hydrochloride.

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	—

The values were adopted from H. Haendle [3]. For definition of mIU see Fritz et al. [19]. One mIU inhibits the activity of about 1 g trypsin Novo.

Inhibitors from Guinea Pig Seminal Vesicles

Inhibitors Isolated from Acidic Extracts Using Trypsin Resin. The seminal vesicles were freshly collected and immediately frozen. Greater portions were thawed and extracted with ice water. Proteins of higher molecular weights in these extracts were precipitated by addition of perchloric acid to a final concentration of 3% (w/w). After neutralisation of the acidic supernatants with potassium carbonate, the inhibitors were adsorbed from the extracts onto water insoluble trypsin resin [4]. After dissociation of the insoluble complex, the degree of purity of the resulting inhibitor preparations was about 60%, calculated from the specific activity of desalted and lyophilized fractions [4, 5].

The inhibitor material was subsequently chromatographed on Sulfoethyl Sephadex, using an ammoniumacetate concentration gradient with constant pH 5.4 (Fig. 1). In the molarity range 0.05 to 0.15, there are mainly two peaks due to a trypsin-specific inhibitor, and from 0.15 to 0.4 there are about eight peaks due to a trypsin-plasmin inhibitor. We [5, 6] have studied the last three fractions (d, e and f) thoroughly. Fraction d is the component with the lowest molecular weight while fractions e and f both contain two components. The components e_1, e_2, f_1 and f_2 are derived from component d by addition of 1 to 5 amino acids at the N-terminal residue valine of component d (see Tab. 2). Component d might be produced from the other fractions during the isolation step with the trypsin resin.

Both components, a and b, of the trypsin-specific inhibitor have the same amino acid composition (Tab. 3); but as shown later in fraction a the Arg-X-bond in the reactive site is split.

Comparison with Inhibitors Isolated from Aqueous Extracts Avoiding the Trypsin Resin Step. In order to avoid loss of inhibitory activity by acidification of the aqueous extracts with perchloric acid as well as modification reactions during

Table 2. N-Terminal Amino Acid Sequences of Five Components of the Trypsin-Plasmin Inhibitor Isolated by Use of Trypsin Resin

N-Terminal sequence	Inhibitor fraction[a]
Val	d
Lys-Val	f_1
Ser-Lys-Val	f_2
Ala-Pro-Ser-Lys-Val	e_1
Phe-Ala-Pro-Ser-Lys-Val	e_2

[a] See Fig. 1.

The sequences indicated were obtained by investigation of the tryptic peptides of the inhibitor components. Experimental details are given in ref. [6].

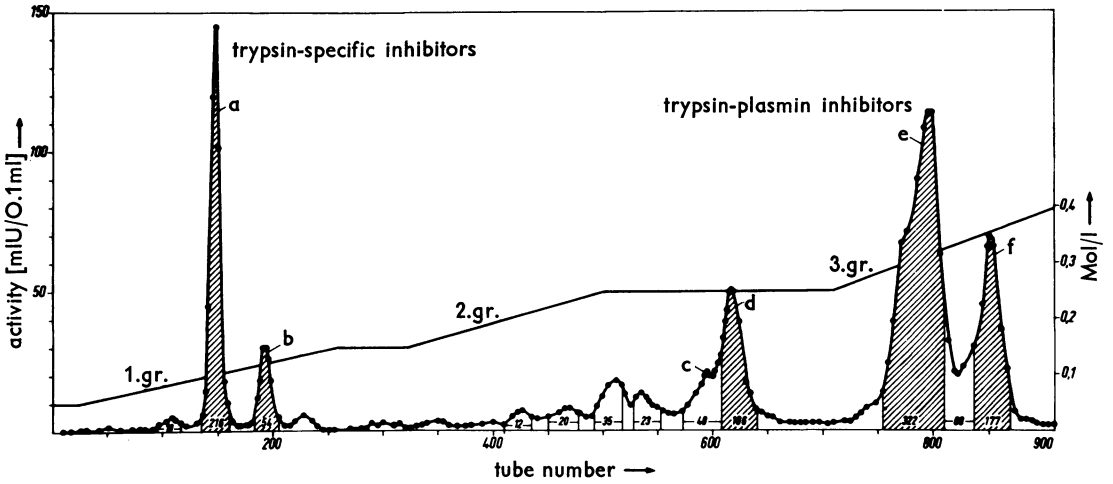
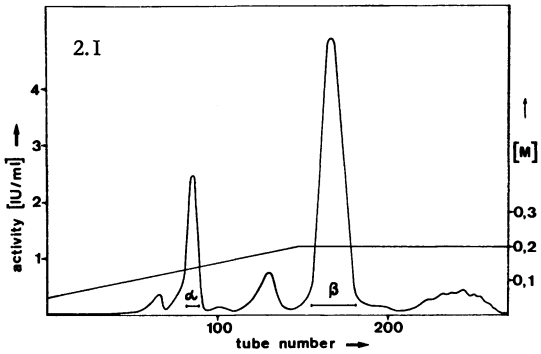


Fig. 1. Fractionation on Sulfoethyl Sephadex C-25 of an Inhibitor Preparation Isolated by Affinity Chromatography (Use of Trypsin Resin [4, 5]).

Left ordinate: Trypsin inhibition in mIU; right ordinate: Molarity of the elution buffer. The numbers in the peak areas indicate the total inhibitory activities found in these fractions.

10.9 IU (trypsin inhibition) of the inhibitor preparation were applied to the SE-Sephadex C-25 column (1.8 × 120 cm) equilibrated with 0.05M ammonium acetate buffer, pH 5.4. Elution was done with buffers and/or linear concentration gradients of ammonium acetate, pH 5.4, as shown in the figure. Elution rate: 10 cm/h; volume per fraction: 10 ml.

the trypsin resin step, part of the seminal vesicles were processed under more moderate conditions: The aqueous extracts obtained after centrifugation of the homogenates were concentrated in vacuo and fractionated by gel filtration on Sephadex G-50 equilibrated and developed with collidine acetate buffer, pH 7.8. The inhibitor-containing fractions were lyophilized. The material thus obtained was separated into



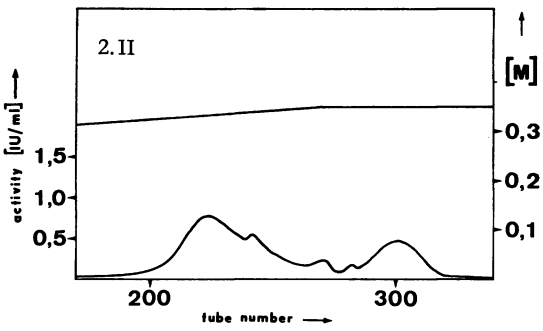


Fig. 2.I and 2.II. Fractionation on Sulfoethyl Sephadex C-25 of an Inhibitor Preparation Isolated by Conventional Chromatographic Methods [6].

For description of the figure and performance of the experiment see Fig. 1.

Fig. 2. I. 1880 IU (trypsin inhibition) of the trypsin-specific inhibitor preparation (see text) were applied to the column. In fraction α were eluted 297 IU, in fraction β 919 IU, and in other fractions 450 IU.

Fig. 2. II. 500 IU of the trypsin-plasmin inhibitor preparation were applied to the column. 482 IU were eluted in all fractions together.

Table 3. Amino Acid Composition of Trypsin-Specific Inhibitors from Guinea Pig Seminal Vesicles
Samples were hydrolyzed in 6N HCl for different times at 110°C and analyzed in the "Beckman Unichrom" analyzer in the two-column system

Isolation	With trypsin resin						Without trypsin resin					
Fraction	a (Fig. 1)			b (Fig. 1)			α (Fig. 2. I)			β (Fig. 2. I)		
Amino acid	Residues per molecule											
	20 hrs	120 hrs	Integer	20 hrs	120 hrs	Integer	20 hrs	120 hrs	Integer	20 hrs	120 hrs	Integer
Cysteic acid[a]	5.90		(6)	5.75		(6)	3.44			3.96		
Methionine sulfone[a]	0.00		0	0.00		0	0.35			0.33		
Aspartic acid	5.96	6.01	6	5.98	5.95	6	6.03	6.11	6	5.01	5.01	5
Threonine	1.04	1.01	1	1.17	1.11	1	1.82	1.70	2	1.06	1.01	1
Serine	2.15	1.91	2	2.26	2.01	2	6.63	5.17	7	2.89	2.41	3
Glutamic acid	10.26	10.26	10	10.00	10.05	10	16.93	16.81	17	9.32	9.22	9
Proline	5.16	5.14	5	5.29	5.19	5	5.58	5.19	5—6	4.16	4.12	4
Glycine	6.10	6.26	6	5.97	6.01	6	8.01	7.98	8	5.24	5.20	5
Alanine	1.03	1.11	1	1.14	1.12	1	1.92	1.93	2	1.10	1.18	1
1/2 Cystine	5.16	4.22	6	5.27	3.38	6	3.13	1.46	6[b]	3.64	3.32	6[b]
Valine	1.82	2.96	3	1.76	2.56	3	5.47	6.97	7	2.31	3.18	3
Methionine	—	—	0	—	—	0	—	—	0	—	—	0
Isoleucine	2.57	3.87	4	2.31	4.02	4	2.36	3.57	4	1.95	3.01	3
Leucine	5.11	5.14	5	4.87	5.08	5	4.67	4.58	5	4.01	3.90	4
Tyrosine	1.68	1.32	2	1.80	1.47	2	1.09	0.85	1	1.22	1.13	1
Phenylalanine	0.00	0.00	0	0.00	0.00	0	0.96	0.92	1	0.26	0.28	0
Lysine	0.91	1.03	1	1.14	1.17	1	4.88	5.06	5	2.11	2.20	2
Histidine	1.61	1.25	(1)2[c]	1.75	1.70	2[c]	5.10	5.12	5	2.57	2.58	2—3
Arginine	5.96	5.94	6	6.00	6.00	6	9.19	9.01	9	5.88	6.16	6
Tryptophan[d]	0.2		0	0.2		0	0.2		0	0.2		0
Total			(59)60			60			86—91			55—56
Molecular weights[e]			6772			6772			9931—10254			6275—6412

[a] After performic acid oxidation.
[b] Related to the number of aspartic acid residues in this fraction and in fractions a and b only a value of 6 half-cystine residues is possible. The low yield by performic acid oxidation cannot be easily explained.
[c] By analyzing this fraction shortly after isolation a value near 2 was found, by analyzing the same sample 1 year after storage (4°C) only about 1.25 histidine residues per molecule could be detected. Part of the histidine therefore may be destroyed during storage.
[d] Spectrophotometric determination [20].
[e] Amide content has not been considered.

two fractions — the trypsin-specific inhibitors and the trypsin-plasmin inhibitors — by chromatography on Sulfoethyl Sephadex C-25 under the conditions described in the legend of Figure 1, but with more specific gradients. Afterwards both fractions were desalted and rechromatographed individually under the conditions given in Figures 2. I and 2. II. Surprisingly Figure 2. I now shows the presence of at least four components of the trypsin-specific

Table 4. Amino Acid Compositions of Trypsin-Plasmin Inhibitors from Guinea Pig Seminal Vesicles
The inhibitors were isolated by the trypsin resin procedure and then fractionated as shown in Fig. 1. Samples were hydrolyzed in 6N HCl for different periods at 110° C and analyzed in the "Beckman Unichrom" analyzer in the two-column system

Fraction (Fig. 1)	d			e*			f*		
Amino acid	Residues per molecule								
	20 hrs	120 hrs	Integer	20 hrs	120 hrs	Integer	20 hrs	120 hrs	Integer
Cysteic acid[a]	5.92		(6)	5.79		(6)	5.92		(6)
Methionine sulfone[a]	0.93		(1)	0.92		(1)	0.96		(1)
Aspartic acid	6.00	6.05	6	6.01	6.00	6	6.00	5.99	6
Threonine	3.91	3.74	4	3.75	3.67	4	3.84	3.58	4
Serine	5.00	4.26	5	5.73	4.79	6	5.14	3.91	5
Glutamic acid	4.11	4.28	4	4.11	4.14	4	4.07	4.19	4
Proline	2.11	2.10	2	3.01	3.14	3	2.27	2.36	2
Glycine	5.14	5.27	5	4.96	5.01	5	4.89	4.92	5
Alanine	0.16	0.16	0	1.41	1.45	1	0.01	0.02	0
1/2 Cystine	5.17	4.96	6	5.20	5.29	6	4.85	3.03	6
Valine	2.63	2.99	3	2.23	2.98	3	2.45	2.90	3
Methionine			1			1			1
Isoleucine	0.96	1.36	1	0.98	1.09	1	0.85	1.03	1
Leucine	3.13	3.15	3	3.07	3.03	3	3.03	2.96	3
Tyrosine	3.66	3.42	4	4.05	3.67	4	3.49	3.11	4
Phenylalanine	2.67	2.92	3	3.78	3.59	4	2.59	2.90	3
Lysine	4.13	4.04	4	4.65	5.32	5	5.38	4.99	5
Histidine	2.89	2.94	3	2.78	3.08	3	2.96	2.90	3
Arginine	3.93	3.98	4	3.83	4.19	4	4.06	3.98	4
Tryptophan[b]	0.20		0	0.20		0	0.20		0
Total			58			63			59
Molecular weights[c]			6687			7217			6815

* Mixture of 2 inhibitors (see Tab. 2).
[a] After performic acid oxidation.
[b] Spectrophotometric determination [20].
[c] Amide content has not been considered.

inhibitor. The amino acid compositions of the two main components α and β are very different (Tab. 3); yet the compositions of component β and the trypsin inhibitors obtained using the resin method (Tab. 3, components a and b) are very similar. Either component α is very sensitive to acidification and therefore was lost during the isolation procedure previously mentioned, or peptides were split off during the resin step to yield fraction a or b. From these results it can be deduced that there are at least two different trypsin-specific inhibitors in seminal vesicles of guinea pigs.

Figure 2. II shows the elution diagram of the trypsin-plasmin inhibitors gained in this procedure. The amount of inhibitors obtained is much lower than in the former procedure. Further studies of these fractions are in progress.

By comparing the two described isolation procedures striking differences are noticed: The activity ratio of trypsin-specific inhibitors to trypsin-plasmin inhibitors changes drastically

from 10:25 (following perchloric acid precipitation and the trypsin-resin step) to 10:3 (when avoiding these steps). Loss of the big amount of trypsin-specific inhibitors during the first procedure is, as we know now, mainly due to their low solubility in acidic solutions. The last mentioned ratio therefore reflects the amounts of inhibitors existing in the tissue of the vesicles. We cannot exclude that the several components of the trypsin-specific inhibitor (as well as some components of the trypsin-plasmin inhibitors shown in Figure 1 and Table 2) shown in Figure 2.I are produced from one component by proteolysis during the first isolation steps. But it is more likely that some of them are synthesized by the action of mutated gens. Several similar examples are mentioned in the course of this conference [7—10].

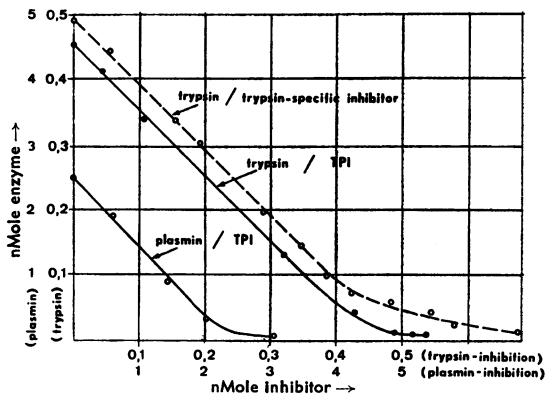


Fig. 3. Inhibition of Trypsin and Plasmin by Inhibitors from Guinea Pig Seminal Vesicles: To constant amounts of the enzymes increasing amounts of inhibitors (abscissa) were added and the remaining activities (ordinate) tested. As substrates served N^{α}-benzoyl-DL-arginine p-nitroanilide hydrochloride for trypsin and plasmin and also p-nitrophenyl p'-guanidinobenzoate for trypsin. For experimental details see ref. [5]. The following inhibitor preparations were used (see Fig. 1): Trypsin-specific inhibitor, fraction a; trypsin-plasmin inhibitor (TPI), fraction d. With the other inhibitor fractions identical curves were obtained.

Figure 3 illustrates the relationship between inhibitor concentration and inhibition of trypsin and plasmin. One molecule of each inhibitor reacts with one molecule of the enzyme to form the complex. The inhibition curves of the different components of each inhibitor are identical.

Reactive Sites of Proteinase Inhibitors

Influence of Lysine- and Arginine-modifying Reagents. By acylation of an inhibitor protein with maleic anhydride it can be elucidated whether a lysine or an arginine residue is located in the reactive center [11]. Lysine-inhibitors were inactivated by this reaction (Tab. 5), but not the arginine-inhibitors (Tab. 6). If the polymaleyl derivative of an inhibitor was still active, we incubated this derivative with the butandion reagent of GROSSBERG and PRESSMAN [12]; this reagent reacts rapidly with the guanidino group of arginine (Fig. 4).

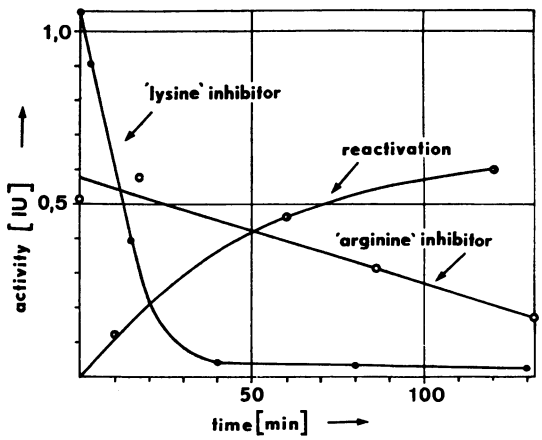


Fig. 4. Inactivation Rate of Lysine- and Arginine-Inhibitors by a Butandion-(2,3) Reagent and Reactivation Rate of a Maleylated Lysine-Inhibitor: The inhibitory activities of the samples (ordinate) were tested after different incubation periods (abscissa). A trypsin inhibitor with lysine (from Phaseolus vulgaris) and another with arginine (from sheep pancreas) in the reactive site were incubated with the butandion-(2,3) reagent used by GROSSBERG and PRESSMAN [12]. — The maleylated basic pancreatic trypsin inhibitor with a lysine residue in the reactive site was incubated in perchloric acid (3%, w/w; temperature: 60° C). For experimental details see ref. [11].

In all cases investigated [11] inhibitors which were fully active after maleylation lost their activity when they were treated with the butandion reagent. With inhibitors inactivating besides

Table 5. Lysine-Inhibitors[a]

Trypsin inhibitor from	Reversible inactivation by maleylation of the inhibitory activity for			
Pancreas: pig, dog, cat	trypsin			
Seminal vesicles of guinea pigs:				
Modified[b] trypsin-specific inhibitor TI′	trypsin			
Trypsin-plasmin inhibitors	trypsin	plasmin		
Leeches[c] A	trypsin	plasmin		
B	trypsin	plasmin		
Lung[d]: cattle, sheep	trypsin	plasmin	chymotrypsin	kallikrein

[a] Inhibitors bearing a lysine residue in the reactive site; see also reference [10].
[b] See Table 7.
[c] See reference [9].
[d] Identical with the basic pancreatic Kunitz inhibitor.

Table 6. Arginine-Inhibitors[a]

Trypsin inhibitor from	Maleylated inhibitor + butandion-(2,3) reagent: Loss of activity against			
Pancreas: sheep, cattle	trypsin			
Seminal vesicles of guinea pigs:				
native[b] trypsin-specific inhibitor TI	trypsin			
Boar seminal plasma	trypsin	plasmin		
Submandibular glands of dogs	trypsin		(not chymotrypsin)	
Human serum: inter-α-trypsin inhibitor	trypsin			
Sea anemone (actiniaria)	trypsin	plasmin	chymotrypsin	kallikrein

[a] Inhibitors bearing an arginine residue in the reactive site; see also reference [10].
[b] See Table 7.

trypsin other proteinases as well, the following results were found: The loss of activity against trypsin after one of these modification reactions is accompanied also by the loss of activity against the other proteinases (Tab. 5 and 6). Only the inhibitor from submandibular glands of dog did not lose its activity against chymotrypsin when the arginine residues of the polymaleyl derivative were modified. This result is discussed more extensively by FRITZ et al. [10].

The trypsin-plasmin inhibitors of seminal vesicles of guinea pigs are inactivated by reaction with maleic anhydride whether they are isolated by use of trypsin resin or not; i. e. they bear a lysine residue in their reactive center.

Reactive Site of the Trypsin-Specific Inhibitor from Seminal Vesicles.

Of the trypsin-specific inhibitors from seminal vesicles isolated with the trypsin resin method the main fraction (component a in Fig. 1) is inactivated by maleylation; noticeably, for complete inactivation a large excess of reagent is necessary (Tab. 7). When the same fraction is incubated with carboxypeptidase B the loss of inhibitory activity is paralleled by the liberation of arginine (0.9 mole per mole inhibitor); i. e. in component a the Arg-X-bond of the reactive center is split. Inactivation of this inhibitor fraction by maleylation is therefore due to acylation of the α-amino group of the X-

Table 7. Reactive Site of the Trypsin-Specific Inhibitor
MA = maleic anhydride
TI = native inhibitor (Arg-X-bond intact)[a]
TI′ = modified inhibitor (Arg-X-bond broken)[b]

Inhibitor	Treatment with	Remaining activity %
TI	MA	104
TI′	MA (big excess)	7
TI	Cpdase B	96
TI′	Cpdase B	6
TI	trypsin-resin + Cpdase B	10

[a] Fraction β from Figure 2. I.
[b] Fraction a from Figure 1.

residue in the reactive site. These findings are in full agreement with the results of LASKOWSKI, Jr. [13] obtained in similar experiments with the modified form of the soybean trypsin inhibitor. In the light of these results our former conclusions drawn from similar experiments with plant proteinase inhibitors [11] may not be correct. We failed to determine the exact amount of modified forms in these inhibitors.

In the same experiments, but using the trypsin-specific inhibitors isolated by avoiding the resin step (Fig. 2. I), different results were obtained: Inactivation did not occur by reaction with excess maleic anhydride or carboxypeptidase B. The same was true for component b in Figure 1. Samples of none-modified inhibitor (fraction β of Fig. 2. I) were applied to a column filled with a trypsin-polyacrylamide resin [14]. After dissociation of the complex in acidic solution 90% of the eluted inhibitor were modified for they lost their activity during maleylation or incubation with carboxypeptidase B. TSCHESCHE by means of mass spectrometry found that the newly formed α-amino group in the reactive site of this inhibitior belongs to isoleucine [16]. The reactive site bond of the none-modified inhibitor is also split — with different velocities depending on the acidity of the incubation mixture — in the presence of only 2.4 mole % trypsin [5, 6].

The reaction mechanism of the modification reactions will not be discussed; it is most elegantly elucidated in the lecture of LASKOWSKI, Jr. et al. [13] preceding this paper. The fact that the trypsin-specific inhibitors are obtained in almost completely modified form using the resin method and the trypsin-plasmin inhibitors are unmodified may be explained by different velocity constants of the individual modification steps (see ref. [13]). The reasons for this may be found in differences of the tertiary and primary structure of the reactive sites of these inhibitors.

Inhibitor from Seminal Plasma of Boar

Isolation Procedure. From acidic extracts of boar seminal plasma we isolated a trypsin-plasmin inhibitor using a trypsin-polyacrylamide resin. These studies are still in progress and we wish to point out that we can present preliminary results only.

Seminal fluid, collected at weekly periods, was pooled in aqueous perchloric acid (3%, w/w). After centrifugation the supernatants were neutralized, concentrated and fractionated on Sephadex G-25 columns, equilibrated and developed with aqueous acetic acid (5%, w/w). The eluted clear inhibitor fraction contained 90% of the original activity. The next steps were: Adsorption of the inhibitor to Sulfoethyl Sephadex and desorption with sodium chloride solutions (5%, w/w), pH 7, in 85—90% yield, dialyzation against deionized water (6 hrs), concentration and fractionation on Sephadex G-50 columns, equilibrated and developed with aqueous acetic acid. Lyophilisation of the eluted inhibitor fraction yielded a white powder with a specific activity which varied with different preparations from 50 to 150 mIU/mg.

This material was further purified by the trypsin-resin method [17]: The inhibitor (300 IU in 10 ml) was adsorbed to a trypsin-polyacrylamide resin column [15] from 0.4M NaCl, 0.1M TRA-buffer solution, pH 7.8. After washing the resin with the same salt-buffer solution the

inhibitor-trypsin complex was then dissociated and the inhibitor eluted with 0.4M KCl/HCl buffer, pH 2, in 85% yield. The resulting inhibitor solution was neutralized, concentrated by ultrafiltration and fractionated on Sephadex G-50, equilibrated and developed with aqueous acetic acid. After lyophilization of the eluted inhibitor fraction a white powder with a specific activity of 2.0 IU/mg (trypsin inhibition) was obtained. This material was used for the following investigations. (Some attempts to further purification by ion exchange chromatography were unsuccessful.)

Inhibition Properties and Composition. It can be seen from Figure 5 that the inhibitor reacts stoichiometrically with both trypsin and plasmin. Dependent on the degree of inhibition 5—8 minutes are necessary to reach inhibition equilibrium. The inhibitor has no effect on the activity of chymotrypsin (substrate: N-3-[carboxy-propionyl]-L-phenylalanine p-nitroanilide), thrombin (substrate: BAEE) and kallikrein (substrate: BAEE) from pig pancreas.

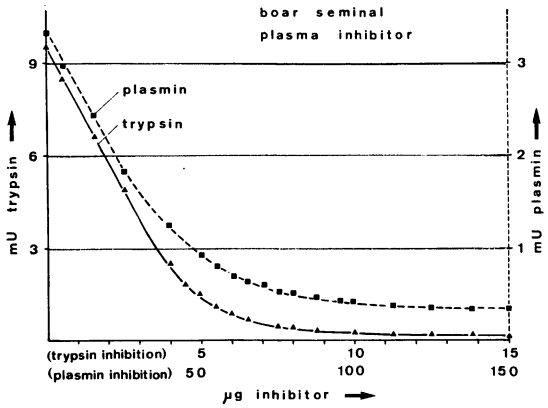


Fig. 5. Inhibition of Trypsin and Plasmin by a Seminal Plasma Inhibitor from Boar. To constant amounts of the enzymes increasing amounts of inhibitor (abscissa) were added and the remaining activities (ordinate) tested. As substrate served N^{α}-benzoyl-DL-arginine p-nitroanilide hydrochloride. Enzyme and inhibitor were incubated (for trypsin inhibition: 8 min, for plasmin inhibition: 10 min) in 2.0 ml 0.2M buffer solution, pH 7.8, afterwards 1.0 ml substrate solution was added, see ref. [5].

From the specific activity a molecular weight of about 13,000 is calculated. In gel filtration experiments in slightly acidic (pH 2.2) buffer solution the molecular weight was found to be approximately 13,500, in neutral solution near 12,000. The molecular weight calculated from

Table 8. Amino Acid and Amino Sugar Composition[a] of the Trypsin-Plasmin Inhibitor from Boar Seminal Plasma

Samples were hydrolyzed in 6N HCl at 110°C and analyzed in the "Beckman Unichrom" analyzer in the two-column system

Amino acid, amino sugar	Residues per molecule			
	20 hrs	70 hrs	122 hrs	Integer
Cysteic acid	7.2[b]			
Methionine sulfone	1.4[b]			
Aspartic acid	10.75	10.75	10.84	11
Threonine	5.95	5.71	5.60	6
Serine	6.82	6.00	5.34	7
Glutamic acid	8.26	8.11	8.09	8
Proline	4.56	4.08	4.28	4
Glycine	7.82	7.92	7.89	8
Alanine	4.32	4.32	4.32	4
1/2 Cystine	5.85	8.16	2.04	8
Valine	1.54	1.87	1.88	2
Methionine		1.10		2[c]
Isoleucine	2.83	3.97		4
Leucine	5.47	4.18	3.87	4
Tyrosine	4.12	4.37	3.05	4
Phenylalanine	7.25	7.34	7.33	7
Lysine	8.00	8.04	7.99	8
Histidine	3.98	3.97	3.90	4
Arginine	8.25	8.13	8.10	8
Tryptophan	(1.57)[d]			2
Glucosamine	2.3	1.1	0.6	3
Galactosamine	1.5	0.7	0.3	2
Total[a]				104
Mol. weight[a]				11607

[a] Investigations for other residues (e. g. neutral sugars, sialic acid etc.) were not performed.

[b] After performic acid oxidation.

[c] Calculated from the oxidized form of the inhibitor.

[d] Determined by a spectrophotometric method [20].

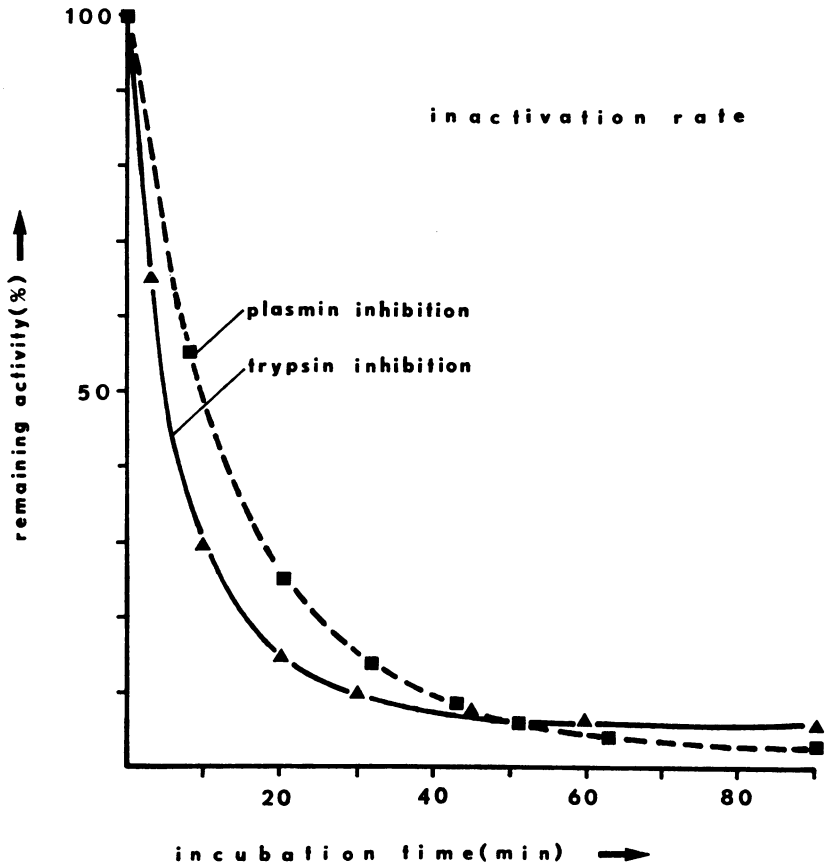


Fig. 6. Inactivation of Maleylated Inhibitor by a Butandion-(2,3) Reagent. The inhibitor from boar seminal plasma was maleylated as described in ref. [11]. To the maleylated inhibitor the butandion-(2,3) reagent was added; the decrease in inhibitory activity (ordinate) against trypsin and plasmin was tested after different incubation periods (abscissa). For methods see Fig. 4 and ref. [11].

the amino acid and amino sugar composition given in Table 8 is in good agreement with these values. Nevertheless, the calculation of other compositions may also be possible. We have not yet investigated if the inhibitor contains also some other residues (e. g. neutral sugar, amino sugar and sialic acid residues) not mentioned in Table 8.

The inhibitor is not inactivated by maleylation. After reaction with the butandion reagent, the polymaleyl inhibitor lost its activity against both trypsin and plasmin in a manner characteristic of arginine-inhibitors (Fig. 6). The inhibitor must be in the unmodified form (Arg-X-bond intact in the reactive site), because maleylation or incubation with carboxypeptidase B has no effect on its inhibition ability. No temporary inhibition could be demonstrated.

From the physiological point of view it is interesting that we only found the trypsin-plasmin inhibitor in seminal plasma of boar. The inhibitor with similar properties from seminal vesicles of guinea pigs seems to be more active in inhibition of fertilization than the trypsin-specific inhibitor as shown by Dr. ZANEVELD [18]. Further investigations are necessary to clear up whether only the trypsin-plasmin inhibitor exists in seminal fluid and the trypsin-

specific inhibitor is a non-excretable constituent of the vesicle cells. But it is also possible that our group and Dr. ZANEVELD [18] precipitated the trypsin-specific inhibitor by acidification or that this inhibitor is not existing in the species under investigation.

The inhibitor isolated by Dr. ZANEVELD et al. [18] from boar seminal plasma differs significantly in amino acid composition and molecular weight from the one presented in this paper. We assume that both groups isolated different inhibitors. But as we have not yet examined further criteria for homogeneity of the inhibitor isolated in our laboratory, we cannot exclude that further chromatographic procedures yield an inhibitor with somewhat different analytical data.

Experiments which show the significance of proteinase inhibitors from seminal plasma in fertilization are elegantly demonstrated and discussed in detail by Dr. ZANEVELD et al. [18].

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