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Trypsin-Plasmin Inhibitors from Leeches
Isolation, Amino Acid Composition, Inhibitory Characteristics

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Summary

Two groups of trypsin-plasmin inhibitors were isolated from commercially available "Hirudin" samples, extracts of the leech Hirudo medicinalis. The trypsin-plasmin inhibitors, named Bdellins*, were separated from the thrombin-specific inhibitor, named Hirudin, by chromatography (stepwise elution) on DEAE-cellulose. The Bdellins were further purified by affinity chromatography using trypsin resins and subsequently separated into two inhibitor fractions, named Bdellin A and B, by stepwise elution from DEAE-cellulose. Each fraction was separated into several components by equilibrium chromatography on DEAE-cellulose.

The amino acid compositions of the Bdellin-A inhibitors (Table 3) differ significantly from those of the Bdellin-B inhibitors (Table 4), whereas the amino acid values obtained for the different A (Fig. 1) and B (Fig. 2) components are partly very similar. The inhibitors form 1:1 complexes with bovine trypsin and porcine plasmin; the titration curves are shown in Figures 4 and 5. Some physiological and medical problems concerning the Bdellins are discussed.

Introduction

MARKWARDT et al. [1] and DE LA LLOSA et al. [2] isolated a thrombin-specific inhibitor from salivary glands of the leech Hirudo medicinalis. The polypeptide nature of this inhibitor, named Hirudin, has been well established by these authors; its molecular weight is near 10,000. Commercially available samples of Hirudin contain, in addition to the thrombin-specific inhibitor, appreciable amounts of trypsin-plasmin inhibitors [3]. The purification, the amino acid compositions, and some of the inhibitory characteristics of these inhibitors are presented in this paper.

* This name was suggested by R. MARX, Munich.

Methods and Results

Determination of Enzyme Activity and Enzyme Inhibition

The procedures described in the foregoing paper [4] were used for determination of the activity of bovine trypsin, bovine α-chymotrypsin, porcine plasmin, and subtilisin Novo as well as enzyme inhibition. The enzyme samples employed were also identical with the ones described [4]. The following substrates were

Abbreviations: Ac, acetate; DEAE, diethylaminoethyl; EMA, ethylene maleic acid copolymer; TRA, triethanolamine. IU (inhibition unit): 1 IU causes the reduction of substrate hydrolysis by 1μ mole per minute; cf. ref. [4].

used: N^{α}-Benzoyl-DL-arginine p-nitroanilide for trypsin and plasmin, N-3-(carboxypropionyl)-L-phenylalanine p-nitroanilide for chymotrypsin. Inhibition equilibrium was achieved at every degree of inhibition within the preincubation (enzyme plus inhibitor) period used (5 minutes, cf. Fig. 4 and 5).

Isolation Procedures and Properties

Separation of the Trypsin-Plasmin Inhibitors from Hirudin: DEAE-cellulose (acetate-form) columns, 4.2 × 44 cm, equilibrated and developed with 0.2M sodium acetate, pH 6.0, at 60 m*l* per hour were employed. 4.5 g "Hirudin" from Medimpex, Budapest, 270 ATE (anti thrombin units) and 0.9—1.0 IU (trypsin inhibition, cf. ref. [4]) per mg, dissolved in 6 m*l* of the elution buffer, were applied to each column. In three typical runs 94—95% of the trypsin-inhibiting activity applied were eluted in the inhibitor-containing fractions in a total buffer volume of 1.0—1.2 *l*.

Subsequently the thrombin inhibitor, which is completely retained on the cellulose under the conditions employed, was eluted using a linear gradient formed from 2.0 liters each of the starting buffer and 0.6M NaCl, 0.6M sodium acetate, pH 6.0. 74% of the inhibitory activity applied were recovered.

The trypsin-plasmin inhibitor fractions obtained from three column runs were combined and concentrated in vacuo until precipitation of salts started. The main portion of the salts was separated by dialysis for 4—5 hours against deionized water; afterwards concentration was continued, followed by dialysis as soon as salt crystals were formed. Thus a final concentration of 25 IU per m*l* was attained (total yield: 12,500 IU in 500 m*l*). Aliquots were diluted with 0.2M TRA-HCl, pH 7.8, to get the concentration desired for the trypsin-resin step.

Affinity Chromatography Using Water-Insoluble Trypsin Resins: The polyamphoteric trypsin EMA-resin applied was synthesized starting from 10 g trypsin according to the method given in ref. [5]. The isolation procedure described below was repeated four times using this same resin.

The trypsin resin was suspended in 70 m*l* of the trypsin-plasmin inhibitor solution containing 1000 IU (cf. the foregoing paragraph). The chilled (0—4° C) mixture was vigorously stirred for 60—120 minutes, followed by centrifugation. Calculated from the inhibitory activities in the supernatants, amounts of inhibitors corresponding to 910 up to 960 IU were bound to the trypsin resin. The insoluble complex was washed free from contaminating material by repeated suspension in chilled 0.2M TRA-HCl, pH 7.8 (cf. ref. [5]).

For dissociation the complex was suspended in 80—95 m*l* chilled 0.2M KCl-HCl, pH 2.0, for 60—90 minutes, followed by centrifugation. The supernatants contained 61—68% of the inhibitors bound to the resin. This procedure repeated yielded in each case another 8% of the bound inhibitor so that the overall recoveries amounted to 69—76%.

The combined acidic inhibitor solutions were adjusted to pH 6.0, concentrated by evaporation in vacuo, and desalted by gel filtration. Using Sephadex G-50 columns, equilibrated and developed with 0.02M ammonium acetate, pH 6.0, 82% of the inhibitory activity applied was found in the eluted inhibitor fractions; using Biogel P-2 columns, equilibrated and developed with 0.01M acetic acid, 83—88% of the inhibitory activities applied were recovered. The inhibitor-containing fractions were lyophilized. For the material thus obtained the following specific activities were found: 3.5—3.8 IU per mg using Biogel and 2.7—2.9 IU per mg using Sephadex. In the latter case the ammonium acetate was not sublimed off quantitatively during lyophilisation.

Recently, we repeated this isolation step applying 10 g of the trypsin-cellulose resin ("bovine trypsin polymer bound to CM-cellulose, 7—10 U/mg") from Merck AG, Darmstadt. The resin was suspended for 20 minutes in about 400 m*l* chilled 0.5M NaCl, 0.05M TRA-HCl, pH 7.8, containing 790—1070 IU of the trypsin-plasmin inhibitors. For separation of contaminations the

insoluble complex was suspended five times in the mentioned 0.55M salt-buffer solution and afterwards one time in 0.05M NaCl, 0.005M TRA-HCl, pH 7.8. Dissociation of the complex was achieved by suspending it three times in 300 m*l* chilled 1.0M KCl-HCl, pH 2.5, each time for 10 minutes. 82—71% of the inhibitor amount bound to the resin (878—526 IU) were thus recovered. The combined neutralized inhibitor solutions were desalted by repeated ultrafiltration using Amicon cells with UM-05 membranes or by gel filtration on Sephadex G-25. Specific activity of the inhibitor material obtained after lyophilisation: 4.2 IU per mg.
240 IU of the Bdellins were bound from 1 g of the trypsin resin (sample "2851 C") from Röhm & Haas, Darmstadt, under the same conditions; 66% of it were recovered after dissociation.

Separation of Bdellin Fraction A from Fraction B: DEAE-Cellulose (OH^--form) columns, 1.6 × 60 cm, equilibrated and developed with 0.1M sodium acetate, pH 6.0, at 65 m*l* per hour were employed. In 5 runs performed 350—539 mg of the Bdellin-A, B mixture (specific activity: 2.7—3.8 IU per mg), dissolved in about 3 m*l* of the equilibration buffer were applied. The inhibitor fractions eluted with the equilibration buffer in a total volume of 500—600 m*l* amounted in all runs to 55% of the inhibitory activity applied. The inhibitors contained in these fractions were named *Bdellin A*.

In each run 45% of the inhibitory activity applied were retained on the DEAE-cellulose column. These inhibitors, named *Bdellin B*, were subsequently eluted with 0.1M sodium acetate, 0.4M NaCl, pH 6.0, in a total volume of 170—240 m*l*. Cf. Table 2.
The inhibitor-containing fractions were concentrated by evaporation in vacuo or by ultrafiltration and desalted using Sephadex G-25 columns, equilibrated and developed with aqueous acetic acid (5%, V/V). The inhibitor fractions eluted (yields: 80—90% of the inhibitory activities applied) were lyophilized. The following specific activities were found: 3.3—3.5 IU/mg for Bdellin fraction A and 4.2—4.8 IU/mg for Bdellin fraction B.

Equilibrium Chromatography of Bdellin Fraction A: The DEAE-cellulose (OH^--form) columns (1.2 × 24 cm) employed were equilibrated and developed with 0.05M ammonium acetate, pH 6.3, at 6.5 m*l* per hour. 30 mg of Bdellin fraction A, dissolved in 1—2 m*l* of the elution buffer, were applied to each column. The distribution of the inhibitory activity among the fractions eluted is shown in Table 1, the elution diagram in Figure 1. Yields are given in Table 2. Equilibrium chromatography was repeated with the desalted (by repeated ultrafiltration) fractions 2, 3, and 4 shown in Figure 1 in separate runs using the same conditions. As expected from the form of the absorption line, fractions 2 and 3 turned out to be identical.

Table 1. Distribution of the Inhibitory Activity among the Bdellin Fractions obtained by Equilibrium Chromatography

Bdellin A				
Fraction (cf. Fig. 1)	1, (1a)	2, 3	4	5
% of inhibitory activity[a]	11—16	**33—40**	9—17	12—15
Bdellin B				
Fraction (cf. Fig. 2)	1	2	3	4, 5, 6
% of inhibitory activity[a]	5—7	14—20	**29—36**	9—15

[a] Related to the total inhibitory activity applied to the columns. Four columns running under identical conditions were evaluated.

Table 2. Isolation of the Trypsin-Plasmin Inhibitors Bdellins from Commercially Available "Hirudin" Samples

Purification step	Specific activity[a] IU/mg	Yield[b] %
Commercial samples	0.9—1.0	
Separation from Hirudin (thrombin inhibitor) on DEAE-cellulose (acetate-form): With 0.2M NaAc, pH 6.0, only the Bdellins were eluted.		94—95
Affinity chromatography using polyamphoteric trypsin EMA-resins	3.5—3.8[c]	69—76
Separation of Bdellin fractions A and B on DEAE-cellulose (OH^--form):		
Elution of Bdellin A with 0.1M NaAc, pH 6.0,	3.3—3.5	55 } 100
Bdellin B with 0.1M NaAc, 0.4M NaCl, pH 6.0	4.2—4.8	45 }
Fractionation of Bdellin A and B on DEAE-cellulose (OH^--form):		
Equilibrium chromatography of		
Bdellin A with 0.05M NH_4 Ac, pH 6.3,	3.8 (fractions 2, 3, 4)	75—85[d]
Bdellin B with 0.1M NaAc, pH 5.5	5.1 (fraction 3)	67—73[d]

[a] Trypsin inhibition; the values were determined of the desalted and lyophilized material.

[b] Related to the inhibitory activity applied to the column or resin; loss during desalting by gel filtration and ultrafiltration is not considered, see text.

[c] Material containing some ammonium acetate (see text): 2.7—2.9.

[d] All fractions together, cf. Table 1.

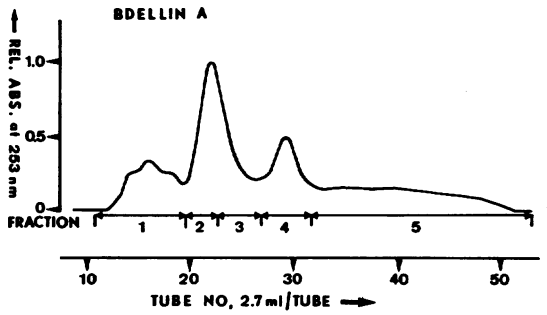

Fig. 1. Equilibrium Chromatography of Bdellin Fraction A.
For experimental details see Methods and Results. The inhibition curve paralleled that of the relative absorption, measured at 253 nm (ordinate).
Fraction 1a (cf. Table 3 and 1) corresponds to the last third of fraction 1, subsequent to fraction 2.

The inhibitor fractions thus obtained were desalted by repeated ultrafiltration employing Amicon cells with UM-05 membranes. About 5% of the inhibitor amount applied was lost during this step. The inhibitors obtained after lyophilisation with a specific activity of 3.8 IU per mg were used for all further investigations.

Equilibrium Chromatography of Bdellin Fraction B: 50 mg of Bdellin fraction B, dissolved in 1—2 ml of the equilibration buffer, were applied to each of the DEAE-cellulose (OH^--form) columns (1.2 × 55 cm). The columns were equilibrated and developed with 0.1M sodium acetate, pH 5.5, at 16.5 ml per hour. After elution of fraction B-3 (cf. Fig. 2) the residual inhibitor retained was eluted from the column with a buffer solution of higher molarity, 0.4M NaCl, 0.1M sodium acetate, pH 5.5. See Table 1 for distribution of the inhibitory activity among the fractions eluted and Table 2 for yields. After desalting equilibrium chromatography of Bdellin fraction B-3 was repeated under identical conditions.

The inhibitor fractions eluted were concentrated by ultrafiltration and desalted by gel filtration as described above. The inhibitor B-3 obtained

after lyophilisation with a specific activity of 5.1 IU per mg was used for further investigations.

In order to obtain enough material for sequential studies the isolation procedure was repeated on a larger scale. Fig. 3 shows the distribution of the inhibitory activity among the Bdellin fractions obtained employing inhibitors with 4.2 IU per mg purified with the trypsin-cellulose resin from Merck AG. The last fraction shown in Figure 2 can be separated into three fractions (B-4,-5,-6) using a linear gradient formed from 350 m*l* each

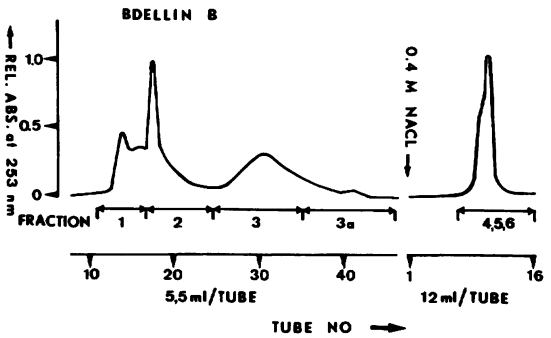

Fig. 2. Equilibrium Chromatography of Bdellin Fraction B.
For experimental details see Methods and Results. The absorption of the eluate was measured at 253 nm (ordinate).

into two fractions, A-2 and A-3, however both fractions turned out to be identical in their behaviour during chromatography and in the amino acid composition.

Only the nearest integer values are given for the other fractions not further purified by rechromatography, provided that the deviations

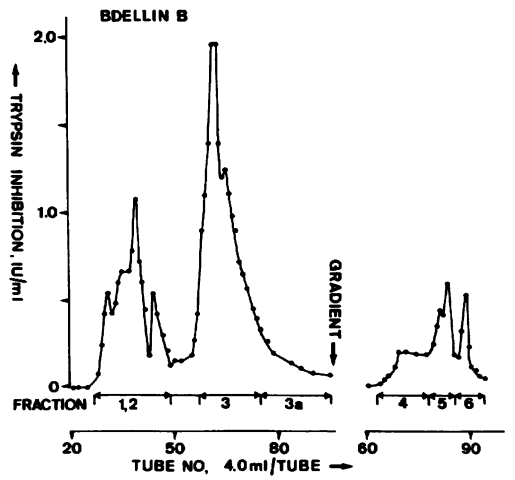

Fig. 3. Equilibrium and Gradient Elution Chromatography of Bdellin Fraction B.
Experimental details are given in Methods and Results. The gradient was started with tube number 1.

of the equilibration buffer (0.1M sodium acetate, pH 5.5) and 0.6M NaCl, 0.1M sodium acetate, pH 5.5 (cf. Fig. 3). By total yields of 82—84%, based on the inhibitory activity applied, 25—29% of the inhibitory activity were found in fraction B-1, 2, 35% in fraction B-3, 11—15% in fraction B-4, 5, 6 and 8—10% in the intermediate fractions. Inhibitor B-3 is used for the sequential studies under investigation.

Amino Acid Composition: The amino acid compositions of the different inhibitor components separated by equilibrium chromatography and of the starting mixtures, Bdellin fractions A and B, are presented in Tables 3 and 4. The inhibitors obtained after rechromatography were applied for analysis of components A-2, 3, A-4, and B-3. At first, inhibitor A-2, 3 was divided from the values of the corresponding main fraction were not larger than $\pm$ 0.1; otherwise the values found are shown.

The presence of carbohydrate residues and free sulfhydryl groups was not observed in the pure Bdellin fractions. Only about 91% of the cystine content of the Bdellins were recovered as cysteic acid after performic acid oxidation; some observations indicate that this is probably due to a small contamination of non-protein character which influences the oxidation reaction.

The inhibitor molecule of Bdellin B-3 contains no residue of the following amino acids: proline, methionine, isoleucine, phenylalanine, and tryptophan. For complete release of valine and lysine from the Bdellin-A fractions a longer hydrolysis time (70 hours) was necessary.

Table 3. Amino Acid Compositions of Bdellin-A Fractions

Bdellin-A: Mixture of inhibitors used as starting material for separation by equilibrium chromatography (cf. Fig. 1).
Bdellin A-1, A-1a (cf. legend of Fig. 1): Only the values deviating from the corresponding ones of inhibitor A-2, 3 are given.
Bdellin A-2, 3 and A-4: The inhibitors obtained after rechromatography were used for analysis

Bdellin fraction	A	A-1	A-1a	A-2, 3			A-4		
Hours hydrolyzed	20	20, 70	20, 70	20	70	Integer	21	70	Integer
Cysteic acid				9.10[a]		(10)	9.16[a]		(10)
Methionine sulfone				0.81[a]		(1)	0.95[a]		(1)
Aspartic acid	7.19	6	6	7.85	7.98	8	7.17	7.24	7
Threonine	3.01	2.6		3.01	2.88	3	2.91	2.82	3
Serine	2.97			3.06	2.64	3	2.90	2.77	3
Glutamic acid	6.45	6	6	5.18	5.27	5	5.04	5.06	5
Proline	2.80	2.3	2	2.95	2.73	3	2.76	2.74	3
Glycine	4.73	5	5	4.19	4.00	4	4.11	3.96	4
Alanine	3.96	3		3.98	3.96	4	3.83	3.88	4
Half-cystine	9.41			9.14	8.56	10	7.87	3.68	10
Valine	4.19[b]	3.4	4	4.61	4.95	5	4.35	4.89	5
Methionine	0.42			0.62	0.68	1	0.66	0.48	1
Isoleucine	1.01[b]			0.87	1.07	1	0.94	1.03	1
Leucine	1.58	2	2	1.19	1.12	1	1.14	1.12	1
Tyrosine	1.21			0.94	0.86	1	0.88	0.77	1
Phenylalanine	1.95		1	1.88	1.77	2	1.67	1.80	2
Lysine	4.82[b]	3	3	4.58	4.96	5	4.35	4.78	5
Histidine	2.80	2		2.95	2.94	3	2.93	2.83	3
Arginine	1.05	1.6	1	0.17	0.13	0	0.17	0.19	0
Tryptophan[c]	0.12			0.12		0			0
Total			56			59			58
Molecular weight[d]			5965			6339			6224

[a] After performic acid oxidation,
[b] Value from 70 hours hydrolysate,
[c] Spectrophotometric determination [7],
[d] Degree of amidation is not considered.

Molecular Weight: The molecular weights calculated from the amino acid compositions (cf. Tab. 3 and 4) are in good agreement with the values obtained by gel filtration experiments. Using Sephadex G-75 columns, equilibrated and developed with 0.15M NaCl, 0.05M citric acid, pH 2.5, at 17 m*l* per hour, the molecular weight found for Bdellin A (mixture) amounted to 7000, for Bdellin B (mixture) to 5600. From the specific activities similar values are calculated.

Inhibitory Characteristics: Both Bdellin groups A and B are strong inhibitors for bovine trypsin and porcine plasmin. The titration curves with Bdellin A-4 and Bdellin B-3 are shown in Figure 4 and Figure 5 respectively. In each case *one* enzyme molecule reacts with *one* inhibitor molecule to form the complex. No differences were observed applying the other Bdellin fractions in the titration experiments.

In both Bdellin groups lysine residues are located in the reactive site of the inhibitors: More than

Table 4. Amino Acid Compositions of Bdellin-B Fractions

Bdellin-B: Mixture of inhibitors used as starting material for separation by equilibrium chromatography (cf. Fig. 2). *Bdellin B-1, B-2, B-4, 5, 6:* Only the values deviating from the corresponding ones of inhibitor B-3 are given. *Bdellin B-3:* The inhibitor obtained after rechromatography was used for analysis.

Bdellin fraction	B	B-1	B-2	B-3			B-4, 5, 6
Hours hydrolyzed	20	20	20	20	70	Integer	20
Cysteic acid				5.60[a]		(6)	
Methionine sulfone				<0.05[a]		(0)	
Aspartic acid	5.21			5.13	5.14	5	7
Threonine	3.39	3	3.7	3.84	3.80	4	
Serine	1.93		2.7	1.97	1.96	2	4
Glutamic acid	5.81	5		5.93	6.02	6	8
Proline	0.60	1.4	1	<0.05	<0.05	0	1
Glycine	3.71			4.10	4.10	4	
Alanine	3.42	3.5		3.92	3.92	4	
Half-cystine	4.32			5.27	3.72	6	
Valine	4.08			4.09	4.17	4	
Methionine	0.19			<0.05	<0.05	0	
Isoleucine	0.19		0.3	0.16	0.09	0	0.3
Leucine	1.69	1.6		1.98	2.10	2	
Tyrosine	0.95			0.99	0.98	1	
Phenylalanine	0.35	0.4		0.09	0.10	0	0.5
Lysine	1.75	2	1.5	1.12	1.10	1	2
Histidine	6.15			4.94	5.02	5*	
Arginine	1.01	0.6		1.11	0.94	1	
Tryptophan[b]	0.12			0.25		0	
Total						45	
Molecular weight[c]						4830	

[a] After performic acid oxidation,

[b] Spectrophotometric determination [7],

[c] Degree of amidation is not considered.

* In Bdellin B-3, isolated recently (Fig. 3), 5.80 His were found. His may be destroyed to some extent during storage (cf. this volume, p. 228, Table 3, footnote c).

96% of the inhibitory activity against trypsin and plasmin are lost during maleylation [8]. The inhibitory activity is restored by deacylation in acidic solution [8]. The inhibitors are modified (reactive site bond split) to a smaller extent: The Bdellin fractions A, A-4, B, and B-3 lose 15—20% of their inhibitory activity during incubation with carboxypeptidase B under the following conditions: 0.3 mg inhibitor and 0.05 mg carboxypeptidase B in 0.43 m*l* TRIS-HCl, pH 8.0, 25°C, 29 hours (and 53 hours). The modification reaction occurs probably during contact with the trypsin resin [6]. In gel filtration experiments the formation of ternary complexes was not observed.

The following proteinases are not inhibited by the Bdellins: Porcine pancreatic kallikrein, bovine α-chymotrypsin, and subtilisin Novo. However, the commercial Hirudin samples contain about 25 mg (assuming a molecular weight near 6000) of an inhibitor for bovine α-chymotrypsin. Small amounts of this inhibitor are still determinable in both Bdellin mixtures, 2% by weight in Bdellin B and 5% in Bdellin A.

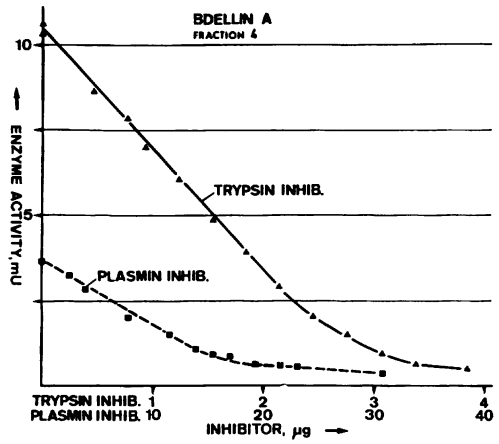

Fig. 4. Titration of Bovine Trypsin and Porcine Plasmin with Bdellin-A.

Bdellin fraction A-4 (cf. Fig. 1 and Table 3) with a specific activity of 3.8 IU (trypsin inhibition) per mg was used. Details of the procedure are presented in the foregoing paper [4] in Methods and in the legends of Figures 5 and 6.

1) *Titration of Trypsin*: Constant amounts of trypsin, 0.39 n mole titrated [4], were incubated with increasing amounts of the inhibitor in 2.0 m/ 0.2M TRA-HCl, pH 7.8, for 5 minutes at 25° C. The enzymatic reaction was started by addition of 1.0 m/ of the substrate solution. Measured at 94% inhibition, the degree of inhibition was constant using preincubation periods from 5 up to 15 minutes.

2) *Titration of Plasmin*: Constant amounts of plasmin, 2.5 n mole titrated [4], were incubated with increasing amounts of the inhibitor in 2.0 m/ 0.2M TRA-HCl, 0.05M L-lysine, pH 7.8, for 5 minutes at 25° C. The enzymatic reaction was started by addition of 1.0 m/ of the substrate solution.

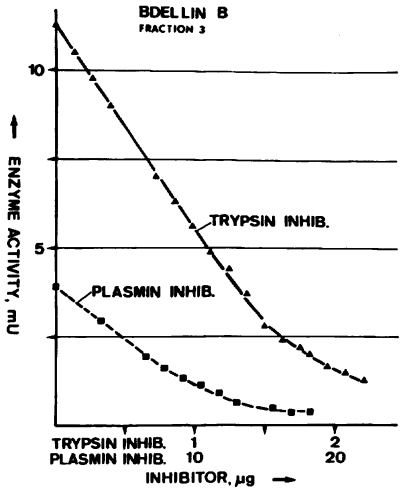

Fig. 5. Titration of Bovine Trypsin and Porcine Plasmin with Bdellin-B.

Bdellin fraction B-3 (cf. Fig. 2 and Table 4) with a specific activity of 5.1 IU (trypsin inhibition) per mg was used. Details of the procedure are presented in the foregoing paper [4] in Methods and in the legends of Fig. 5 and 6; cf. legende of Fig. 4.

1) *Titration of Trypsin*: Constant amounts of trypsin, 0.41 n mole titrated [4], were incubated with increasing amounts of the inhibitor in 2.0 m/ 0.2M TRA-HCl, pH 7.8, for 5 minutes at 25° C. The enzymatic reaction was started by addition of 1.0 m/ of the substrate solution.

2) *Titration of Plasmin*: Constant amounts of plasmin, 2.6 n mole titrated [4], were incubated with increasing amounts of the inhibitor in 2.0 m/ 0.2M TRA-HCl, 0.05M L-lysine, pH 7.8, for 5 minutes at 25° C. The enzymatic reaction was started by addition of 1.0 m/ of the substrate solution.

Discussion

Purification

A series of purification steps was necessary to obtain the trypsin-plasmin inhibitors Bdellins in highly purified form from commercially available "Hirudin" samples.

One gram of the starting material from Medimpex (270 ATE per mg) contains about 230 mg of the Bdellins and only 27 mg of the thrombin inhibitor Hirudin. This proportion to the disadvantage of Hirudin is probably the reason why the Bdellins were not easily separated during purification of Hirudin: We calculated from the results of our measurements (trypsin inhibition, electrophoresis) that one gram of the sterile "Hirudin" samples from Serva (3400 ATE per mg) contains, besides 300 mg Hirudin, still about 75 mg of the Bdellin-A, B mixture. The purest Hirudin samples isolated by MARKWARDT et al. [1] are free of Bdellins [3].

Separation of Bdellins and Hirudin is easily and completely achieved with satisfying yields by

stepwise elution of the inhibitors from the acetate form of DEAE-cellulose. The recovery of Hirudin is much lower if the OH^--form of DEAE-cellulose is employed. The Hirudin thus obtained was separated by gradient elution chromatography into four fractions, two main and two smaller fractions; the conditions used are described elsewhere.

The purification of the Bdellins is decisively simplified by the trypsin resin step. Many attemps to obtain pure Bdellins using only ion exchange chromatography were not successful. The recoveries obtained with the two different resins employed were similar, however, the specific activity of the Bdellins isolated with the trypsin-cellulose resin was higher (4.2 IU per mg instead of 3.5—3.8 IU per mg using a polyamphoteric EMA-resin). Probably this is due to reduction of unspecific adsorption by the cellulose carrier and to the use of 1.0M salt-buffer solutions for the binding and washing steps. If the complex was dissociated at pH 2 (EMA-resin), only two extractions were necessary, whereas at pH 2.5 (cellulose resin) some more extractions were made. In this case 63, 12, 4, and 1% of the bound inhibitors were recovered.

Loss of inhibitory activity was very low (only about 5%) during desalting of the inhibitor solutions by repeated (4—5 times) ultrafiltration in Amicon cells fitted with UM-05 membranes. The only disadvantage is that small soluble portions of the resin and of DEAE-cellulose are not separated during this procedure so that subsequent gel filtration may be necessary.

Multiple Inhibitor Forms

Both Bdellin groups A and B contain a relative high amount of disulfide bridges, a finding which is common to most of the proteinase inhibitors known till now [9]. The high content of histidine residues in the Bdellins is also remarkable. On the other hand, the amino acid compositions of the A-components (Table 3) are so different from those of the B-components that obviously no genetic relationship exists between the Bdellin components A and B. However, the differences within both inhibitor groups may be caused by enzymatic degradation during storage of perished animals and the first isolation steps; but also the synthesis of somewhat different components by mutated gens is possible [10]. It was expected that the number of bonds hydrolyzed during contact with the trypsin resin should be very small [11].

The isolation of at least four different components of the thrombin inhibitor may be explained in the same way.

The sequence of Bdellin B-3 is now under investigation. To our knowledge this is the first proteinase inhibitor which contains no proline residue [12]. It is noteworthy that lack of isoleucine, methionine, phenylalanine, and tryptophan is observed in this inhibitor. The only lysine residue present in Bdellin B-3 is located in the reactive site [8]. Therefore, the influence of chemical modification of this residue on trypsin and plasmin inhibition can be uniquely assigned.

Bdellin B-3 is the smallest naturally occuring inhibitor for trypsin and plasmin found until now. The inhomogeneity of Bdellin fraction B-3 shown in Figures 2 and 3 (shoulder in the right part of the peak!) is caused by modified inhibitor forms in which peptide bonds within the molecule are split without loss of the inhibitory activity.

Physiological and Medical Problems

The Bdellins occur in all zones of the body of the leech, but the highest concentration is found in the region of the outer sexual organs [13]. We assume therefore that the function of these inhibitors has to be seen in connection with the trypsin-plasmin inhibitors found in seminal vesicles and seminal plasma of many animals and of man [14, 15]. The Bdellins are strong inhibitors of the plasmin-like activity occurring in human sperm plasma [13]. Certainly, the Bdellins will also inhibit the trypsin-like proteinase isolated from rabbit sperms by ZANEVELD

et al. [15] which has an important function in fertilization.

The possible use of Bdellin-A in medical therapy (concerning plasmin inhibition) is under investigation [13]. An advantage may be the secretion of the Bdellins in the urine in contrast to the basic trypsin-kallikrein inhibitor which is stored in the kidneys shortly after intraveneous injection [16]. The reason why Hirudin is not widely used in medical therapy is perhaps caused by the fact that the purest samples available for medical use contain considerable amounts of the Bdellins so that both effects, thrombin inhibition and plasmin inhibition, supperimpose and thus no consistent results are obtained.

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