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Proteinase Inhibitors from Dog Submandibular Glands — Isolation, Amino Acid Composition, Inhibition Spectrum

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Summary

Proteinase inhibitors were isolated from aqueous and acidic extracts of dog submandibular glands employing gradient elution and equilibrium chromatography on CM-cellulose. Mainly four inhibitors were obtained which differ only slightly in amino acid composition (cf. Table 2 and 3). Molecular weights of 12750 up to 12878 were calculated for the different components.
The following enzymes are strongly inhibited: bovine trypsin and α-chymotrypsin, subtilisin Novo, porcine pancreatic elastase, Aspergillus Oryzae protease, plasmin (pig), and part of the proteolytic activity of pronase. No inhibition of collagenase was found. The dog submandibular inhibitor (DSI) is double-headed; the two different reactive sites do not overlap: The DSI-chymotrypsin and the DSI-subtilisin complex — both complexes contain equimolar amounts of enzyme and inhibitor — are able to bind an additional trypsin molecule so that ternary complexes are formed.
The potential physiological function as well as the significance of DSI for medical problems are discussed.

Introduction

The physiological role of pancreatic trypsin inhibitors, described in some foregoing papers [1—4], seems to be obvious: Inhibition of premature activation of trypsinogen with all its severe consequences in the gland. The high concentrations of proteinase inhibitors found in other organs of various animals [5] may protect them against the action of trypsin-like and chymotrypsin-like proteinases.
The highest concentration of a proteinase inhibitor found until now in animal tissues exists in submandibular glands of dogs [5]. Depending on the state of the gland 1 g of fresh tissue contains maximal 5 mg inhibitor. This inhibitor was discovered by TRAUTSCHOLD, WERLE, HAENDLE and SEBENING [6] and described in more detail by TRAUTSCHOLD [7] and HAENDLE [8]. Some of the results were presented in earlier lectures given by TRAUTSCHOLD [9, 10]. Similar to the pancreatic trypsin inhibitors [1—4] the dog submandibular proteinase inhibitor is also a secretory protein [8].

Abbreviations: BAPNA, N^{α}-benzoyl-DL-arginine p-nitroanilide; BPTI, basic pancreatic trypsin inhibitor (Kunitz-type) = trypsin-kallikrein inhibitor from bovine organs; CM, carboxymethyl; CPPN, N-3-(carboxypropionyl)-L-phenylalanine p-nitroanilide; M. W., molecular weight; TRA, triethanolamine; TRIS, trishydroxymethyl aminomethane.

Methods

Isolation Procedure

Extraction and Purification Steps: Dog submandibular glands from Peele Freeze, Biologicals (USA), containing 10—14 IU (trypsin inhibition) per g tissue, were thawed and homogenized in deionized ice water (2 *l* for 100 g glands). After centrifugation the supernatant was adjusted to pH 6.0—6.5 and stirred with 100 g CM-cellulose (H^+-form) for two hours in an ice bath. The CM-cellulose adsorbate was washed 3 times with 500 m*l* 0.01M sodium acetate, pH 5.0. In order to elute the inhibitor the adsorbate was suspended in 5% (w/w) NaCl, 0.01M TRA-HCl, pH 8.0, for 10 minutes. By repeated elution 90—95% of the inhibitory activity found in the homogenate was recovered in the supernatant eluates of the cellulose.

The main salt portions were separated by dialysation: 4 hours, deionized water, 0—4°C. Concentration (evaporation in vacuo) was followed by fractionation on Sephadex G-50 columns equilibrated and developed with aqueous (5%, V/V) acetic acid. Lyophilisation of the inhibitor containing eluates yielded a white powder with a specific activity of 1.4 up to 1.8 IU (trypsin inhibition) per mg. Loss of about 18% of the inhibitor was observed during these steps.

Another part of the inhibitor material was isolated from acidified extracts (perchloric acid, 3%, w/w) of the homogenates [6, 7, 8]. In this case the supernatant of the precipitated proteins was neutralized with 5M K_2CO_3 solution. Precipitated $KClO_4$ was separated by filtration, and the inhibitor solution was diluted with water 1:5 before adding CM-cellulose.

Chromatographic Separations: Depending on the foregoing isolation procedure somewhat different methods were employed.

1) The following chromatographic systems were used for further purification of the inhibitor material isolated from *perchloric acid extracts* of the homogenates:

1—1) Gradient elution chromatography on CM-cellulose (Fig. 1);

1—2) Gel filtration on Biogel P-2 (5.0 × 30 cm), equilibrated and developed with 0.01M acetic acid (followed by lyophilisation of the inhibitor-containing eluate);

1—3) Rechromatography on CM-cellulose of each of the two fractions I* and II* shown in Figure 1 under the same conditions as given in the legend of Figure 1, except that the slope of the NaCl-gradient was only 2/3 of the one described;

1—4) Gel Filtration on Biogel P-2 (for conditions see step 1—2) followed by lyophilisation of the eluted inhibitor containing fractions.

The amino acid composition of the two inhibitor fractions I* and II* thus obtained is given in Table 2.

2) For further purification of the inhibitor material isolated from *aqueous extracts* the following systems were employed:

2—1) Gradient elution chromatography on CM-cellulose as shown in Figure 1 yielding fractions 2-I and 2-II (a somewhat modified gradient was used: 0,5M NaCl to 0.05M NaCl, each in the elution buffer.);

2—2) Ultrafiltration of the inhibitor fractions in Amicon cells (membrane: UM-2) by repeated dilution with deionized water;

2—3) Equilibrium chromatography on CM-cellulose of fraction 2-I (Fig. 2) and fraction 2-II (Fig. 3) in separate runs;

2—4) Ultrafiltration of the inhibitor fraction A_2 shown in Figure 2 followed by rechromatography under identical conditions as given in Figure 2, except the length of the column used (twice as high, complete separation of fractions A_1 and A_2, cf. legend of Fig. 2, section 2-2).

2—5) Gel filtration of the rechromatographed inhibitor fraction A_2 and of the inhibitor fraction C (shown in Fig. 3) on Sephadex G-50 equilibrated and developed with aqueous acetic acid (5%, V/V) followed by lyophilisation of the inhibitor-containing fractions.

The amino acid composition of the two inhibitor fractions A_2 and C thus obtained is given in Table 3.

Determination of Enzyme Activity and Enzyme Inhibition

Trypsin: The activity of trypsin and trypsin inhibition was measured with N^{α}-benzoyl-DL-arginine p-nitroanilide (BAPNA) as substrate. Details are given in ref. [11, 12]. One mU corresponds to about 1 μg trypsin; bovine trypsin (Novo Industri A/S) was used throughout. One unit of inhibition activity (IU) causes the reduction of BAPNA hydrolysis by 1 μmole per minute, one mIU the 10^{-3} fold amount. The molarity of the trypsin solutions used in the titration experiments (Fig. 5) was determined according to CHASE and SHAW [13] and by inhibition tests with an inhibitor (BPTI) solution of known molarity [14].

Chymotrypsin: N-3-(carboxypropionyl)-L-phenylalanine p-nitroanilide (CPPN) was applied as substrate. Details are given in ref. [12, 15]. Definitions correspond to the ones mentioned above. Bovine α-chymotrypsin (Novo Industri A/S, 1100 NF per mg) was used throughout. One mU corresponds to about 20 μg α-chymotrypsin.

The molarity of the chymotrypsin solutions used in the titration experiments (Fig. 7) was determined by inhibition tests with an inhibitor (BPTI) solution of known molarity [14].

Plasmin: Plasmin activity and plasmin inhibition was measured with BAPNA as substrate. Assay conditions were the same as for trypsin [11, 12] except for the presence of 0.05M L-lysine in the buffer solution. Increase in extinction was observed for 10 minutes [12]. Plasmin from pig (batch 25-S-68, 2.68 Novo units per mg) was a gift from Novo Industri A/S. For the stock solution 10 mg plasmin were solved in 4.0 m*l* 0.0025N HCl. The molarity of the plasmin solution used in the titration experiments (Fig. 6) was determined by inhibition tests with an inhibitor (BPTI) solution of known molarity [14].

Subtilisin, Aspergillus Oryzae Protease, Pronase: Proteinase activity and enzyme inhibition was measured with azo-casein (Pentex-PP 6262, Fluka AG) as substrate. Constant amounts of the enzymes were incubated with increasing amounts of inhibitor in 1.0 m*l* 0.1M sodium potassium phosphate buffer, pH 7.6, for 5 minutes at 30°C. Afterwards 2.0 m*l* azo-casein solution (2%, w/w) in the same buffer was added and the mixture incubated for 10 minutes at 30°C. The enzymatic reaction was stopped by addition of 3.0 m*l* aqueous trichloracetic acid (5%, w/w). After 30 minutes at room temperature the extinction of the supernatant was read against a blank at 366 nm. The assay procedure is described in more detail in ref. [12], p. 1029.

Subtilisin (Crystalline Bacterial Proteinase, 22.0 Anson trypsin units per g, Batch 50-2) was a gift from Novo Industri A/S. Pronase E (lyophil., 70000 PUK/g from Streptomyces griseus) was purchased from Merck AG and alkaline Aspergillus Oryzae Protease (highly purified, 3500 PU (pH 8)/mg protein) from Röhm & Haas GmbH, Darmstadt.

Elastase: The activity of elastase from pig pancreas (cryst., suspension, 15 E/mg, from Merck AG) was measured according to the method published by SACHAR et al. [16] with elastin-orcein (Merck AG) as substrate. Elastase inhibition was determined in the following manner: A mixture of 0.15 m*l* of the elastase suspension (containing about 0.75 mg elastase in 0.2M TRIS-HCl, pH 8.8) and the inhibitor solution was filled up to 1.50 m*l* with 0.2M TRIS-HCl, pH 8.8. This incubation mixture was briefly (5 minutes) shaken and admixed with 20 mg elastin-orcein. The test sample was vigorously shaken for 30 minutes at room temperature. The enzymatic reaction was stopped by addition of 2.0 m*l* 0.5M phosphate buffer, pH 6.0. After centrifugation the extinction of the supernatant was read against a blank at 578 nm.

Results and Discussion

Isolation of Inhibitors

The developement of simpler isolation methods as described for dog submandibular inhibitor [6—10] was necessary in order to obtain enough material for sequential studies [18]. The method

presented includes only a few steps, repeated chromatography on CM-cellulose and gel filtration or ultrafiltration, each with high yield in inhibitory activity.

From *perchloric acid* extracts of the glands — in which all proteases are inactivated — two inhibitor fractions were obtained by gradient elution chromatography (Fig. 1) in about equal amounts (Tab. 1, 1[c]). Both fractions differ only slightly in amino acid composition: Fraction I* contains 1 more glutamic acid residue and 1 lysine residue less than fraction II* (Tab. 2).

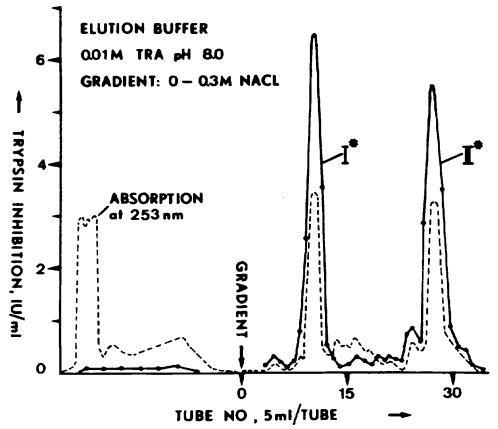

Fig. 1. Gradient Elution Chromatography on CM-Cellulose. Inhibitor material isolated from perchloric acid extracts (see Methods) was employed. 100—140 mg, dissolved in 1.0 ml of the starting buffer, were applied to the column (1.6 × 30 cm) which was equilibrated and developed with 0.01M TRA-HCl, pH 8.0, at 10.5 ml per hour. As soon as the protein content and the inhibitory activity in the eluate decreased, a linear gradient formed from 0.5 liters each of starting buffer and 0.01M TRA-HCl, 0.3M NaCl, pH 8.0, was used for elution. Inhibitor fraction I* appeared in the eluate at a NaCl concentration of about 0.04M, inhibitor fraction II* at 0.08M. Yields are given in Table 1.

Using the same chromatographic procedure from *aqueous* extracts of the glands an inhibitor fraction I (termed "2-I") was obtained in an amount that was about twice as high as that of fraction II (termed "2-II", cf. Tab. 1, 1[d]), however, both fractions were not homogeneous. Further purification by equilibrium chromatography was necessary (Fig. 2 and 3):

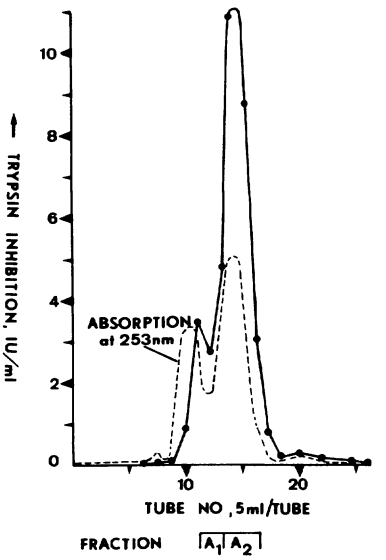

Fig. 2. Equilibrium Chromatography of Fraction 2-I, obtained by Gradient Elution Chromatography. Conditions were the same as given in Fig. 4; however columns with deviating dimensions were employed.

2-1) 228 IU (trypsin inhibition) were applied to the column (2.0 × 30 cm). The elution curve is shown in the Figure. Elution rate: 12 ml per hour.

2-2) Complete separation of fractions A_1 and A_2 is achieved by using a longer column (2.0 × 65 cm) and a smaller elution rate (6 ml per hour, cf. Table 1).

For distribution of inhibitory activity in the eluted fractions and yields see Table 1.

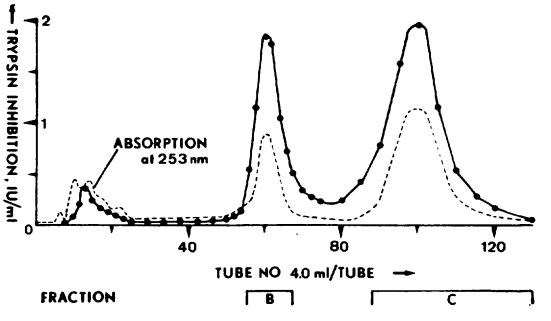

Fig. 3. Equilibrium Chromatography of Fraction 2-II, obtained by Gradient Elution Chromatography. The column and the conditions described in Fig. 4 were used. 255 IU (trypsin inhibition) were applied. For distribution of inhibitory activity in the eluted fractions see Table 1.

Table 1. Distribution of Inhibitory Activity among the Fractions obtained by Gradient Elution and Equilibrium Chromatography

Important intermediate fractions are put in parenthesis

Fractions from Fig.	Number of runs	Percent[a] of inhibitory activity (trypsin inhibition) found in the given fractions				Total yield[b] [%][a]
Gradient elution chromatography						
		I		II		
1[c]	3	32—38*	(10—12)	26—28*	(12—15)	97—100
1[d]	3	45—51	(9—25)	22—29	(4—8)	94—100
Equilibrium chromatography						
		A_1	A_2	B	C	
4	1	14	50	7	28	99
2[e]	3	17—27	53—71			87—97
2[f]	3	1—2	87—99*			95—100
3[g]	1		8	27	54*	95

[a] Related to the inhibitory activity applied to the column.

[b] Including all fractions containing inhibitory activity.

[c] Inhibitors isolated from perchloric acid extracts of the glands (see Methods).

[d] Inhibitors isolated from aqueous extracts of the glands (see Methods).

[e] Fraction I (from 1[d]) served as starting material; the short column described in Figure 2 was used.

[f] Fraction I (from 1[d]) and fraction A_2 (from 2[e]; rechromatography!) served as starting material; the long column described in Figure 2 was used.

[g] Fraction II (from 1[d]) served as starting material.

* These inhibitor fractions were used, after gel filtration (Sephadex G-50, 5% (w/w) acetic acid) and lyophilisation, for further investigations.

1) After complete separation of an inactive contamination A_1 from fraction I (see legend of Fig. 2 and Tab. 1, 2[e] and 2[f]) an inhibitor A_2 was obtained which has the same amino acid composition (Tab. 3) as inhibitor fraction I* which was isolated from acidic extracts.

2) Inhibitor C, obtained from fraction II (cf. Fig. 3 and Tab. 1, 3[g]) was found to lack a single residue each of glycine and proline, when compared to inhibitor fraction II[1]. Probably glycine and proline are split off by exopeptidases* in the aqueous extracts of the glands, whereas the glutamic acid residues is also absent in inhibitor fraction II* isolated from acidic extracts.

[1] Glycine was determined as the only N-terminal residue by H. TSCHESCHE and E. FINK.

Therefore the assumption is logical that submandibular glands of dogs contain *two* very similar inhibitors, one of them synthesized by a mutated gene, which differ only in one Glu and one Lys residue. This is astonishing in so far as the glands were collected from different breeds. The occurrence of isoinhibitors is also described by other authors [19—24].

In order to find out the proportions of the inhibitor fractions in the material obtained from aqueous extracts a sample was subjected directly to equilibrium chromatography (Fig. 4). The portion of the main fractions A_2 and C, which are identical with the corresponding inhibitor fractions shown in Figures 2 and 3, amounts to 92% of the total inhibitory activity found in this sample (cf. Tab. 1; the inhibitory activity in fraction A_1 belongs to A_2, see 2[e] and 2[f] in Tab. 1

Table 2. Amino Acid Composition (Residues per Molecule) of DSI-Fractions Isolated from Acidic Extracts Using Gradient Elution Chromatography

Fraction	I* (Fig. 1, Table 1)			II* (Fig. 1, Table 1)		
	20 hrs	70 hrs	Integer	20 hrs	70 hrs	Integer
Cysteic acid	12.06[a]		(12)	12.19[a]		(12)
Methionine sulfone	3.16[a]		(3)	2.84[a]		(3)
Aspartic acid	13.33	13.18	13	13.17	13.09	13
Threonine	6.71	6.59	7	6.70	6.45	7
Serine	7.81	6.99	8	7.64	6.79	8
Glutamic acid	8.88	9.33	**9**	8.02	8.16	**8**
Proline	5.87	6.03	6	5.84	6.27	6
Glycine	8.91	9.24	9	8.79	9.09	9
Alanine	6.07	6.19	6	5.86	6.14	6
Half-cystine	10.56	10.10	12[b]	11.60	9.87	12[b]
Valine	2.66	4.00	4	2.74	3.74[c]	4
Methionine	2.28	2.90	3[b]	2.22	2.47	3[b]
Isoleucine	4.33	5.27	5	4.45	5.24[d]	5
Leucine	5.96	6.23	6	5.82	6.18	6
Tyrosine	4.95	4.54	5	4.93	4.25	5
Phenylalanine	3.96	4.09	4	3.86	4.00	4
Lysine	9.90	9.87	**10**	10.91	10.89	**11**
Histidine	3.08	3.08	3	2.80	3.04	3
Arginine	4.84	5.17	5	4.69	4.84	5
Tryptophan[e]	0.3		0	0.4		0
Total			115			115
Mol. weight[f]			12750			12750

[a] After performic acid oxidation.
[b] Calculated from the values of the oxidized inhibitor (cf. a).
[c] 120 hrs: 3.89 residues.
[d] 120 hrs: 5.06 residues.
[e] Spectrophotometric determination [17].
[f] Degree of amidination is not considered.

and Fig. 2). Fraction B is not yet further investigated. These results show that enzymatic degradation in the aqueous extracts of the glands is limited and causes no serious disadvantages. On the other hand some of the inhibitor is adsorbed by the precipitated protein and must be eluted by repeated extractions if the extracts are acidified with perchloric acid.

Amino Acid Composition

The amino acid compositions of the isolated inhibitor fractions are shown in Tables 2 and 3. The small differences in the content of glutamic acid, lysine, glycine and proline are already discussed in the preceding paragraph. Furthermore, the following should be mentioned: When based on the molecular weight the number of disulfide bridges corresponds to that of many other inhibitors obtained from animal organs (e. g. pancreas glands, bovine organs, seminal vesicles, etc.). Values obtained by the one-column method were used to coordinate the numbers of basic and neutral or acidic amino acid residues. Release of arginine and isoleucine is finished only after a hydrolysis time of 70

Table 3. Amino Acid Composition (Residues per Molecule) of DSI-Fractions Isolated from Aqueous Extracts Using Gradient Elution and Equilibrium Chromatography

Fraction	A_2 (Fig. 2, Table 1) 20 hrs	70 hrs	Integer	C (Fig. 3, Table 1) 20 hrs	70 hrs	Integer
Cysteic acid	11.68[a]		(12)	11.75[a]		(12)
Methionine sulfone	2.90[a]		(3)	2.98[a]		(3)
Aspartic acid	13.13	13.17	13	13.22	13.18	13
Threonine	6.95	6.74	7	6.96	6.80	7
Serine	7.90	7.06	8	7.78	7.53	8
Glutamic acid	9.05	8.92	**9**	8.12	8.34	**8**
Proline	6.19	5.92	6	4.86	5.05	**5**
Glycine	8.99	8.97	9	8.25	8.43	**8**
Alanine	5.99	5.97	6	5.98	6.15	6
Half-cystine	11.41	9.89	12[b]	10.59	10.99	12[b]
Valine	2.75	3.82	4	2.90	3.84	4
Methionine			3[b]			3[b]
Isoleucine	4.64	5.12	5	4.90	5.08	5
Leucine	6.15	6.17	6	6.03	6.15	6
Tyrosine	4.82	4.46	5	4.68	4.53	5
Phenylalanine	3.85	3.93	4	3.75	4.02	4
Lysine	9.97	10.08	**10**	11.10	11.30	**11**
Histidine	3.06	3.09	3	2.98	3.09	3
Arginine	4.88	4.98	5	4.57	5.02	5
Tryptophan[c]	0.35		0			
Total			115			113
Mol. weight[d]			12750			12595

[a] After performic acid oxidation.
[b] Calculated from the values of the oxidized inhibitor.
[c] Spectrophotometric determination [17].
[d] Degree of amidination is not considered.

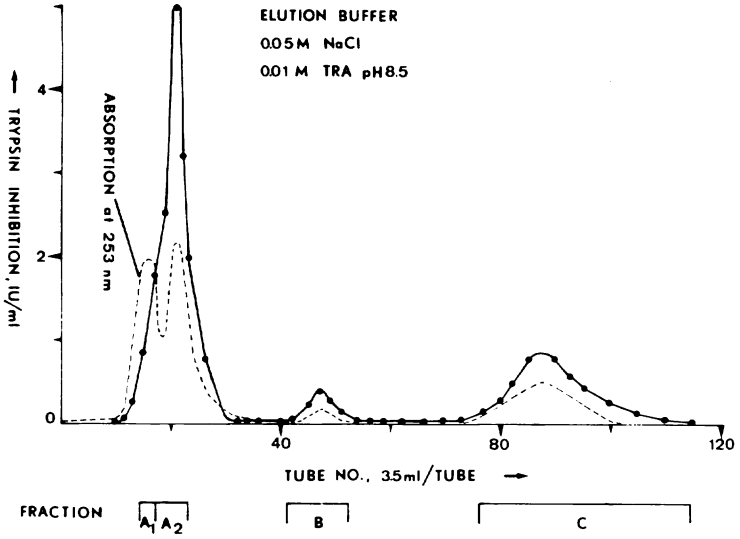

Fig. 4. Equilibrium Chromatography on CM-Cellulose. Inhibitor material isolated from aqueous extracts was employed. 100—150 mg (dissolved in 5.0 m*l* of the elution buffer) were applied to the column (2.0 × 30 cm), which was equilibrated and developed with 0.05M NaCl, 0.01M TRA-HCl, pH 8.5, at 12 m*l* per hour. Distribution of inhibitory activity in the eluted fractions and yields are presented in Table 1.

hours. Due to methodical difficulties the values of tryptophan and proline must be verified in the course of sequential studies. The presence of carbohydrate residues in the DSI-molecules was not observed.

Molecular Weight

For the molecular weight of DSI-fraction A_2 the following values were obtained by different methods:

1) Calculated from amino acid composition (Tab. 3) 12750
2) From gel filtration experiments (Tab. 4) 12000
3) From ultracentrifuge studies [25] 11900
4) Calculated from the specific activity (2.5 IU per mg, trypsin inhibition) 13200

The values are in good agreement with each other and with those reported in the literature [9, 10, 26, 27]. DSI forms a ternary complex with trypsin and chymotrypsin (Tab. 4). Dissociation of DSI into subunits during complex formation may therefore be excluded; it is also improbable regarding the amino acid composition.

Some observations indicate that less pure preparations of DSI may bind two trypsin molecules per molecule inhibitor. Perhaps in this state the reactive site for chymotrypsin can bind a trypsin molecule, too. But we have no evidence that the pure DSI-molecule binds two trypsin molecules as might be deduced from the specific activity of earlier obtained preparations [7—10].

Inhibition Spectrum

DSI inhibits strongly the following proteinases: Bovine *trypsin* (Fig. 5) and *α-chymotrypsin* (Fig. 7), *subtilisin* Novo (Fig. 8), porcine pancreatic *elastase* (Fig. 9) and alkaline *A. oryzae protease* (Fig. 10). In these cases *one* enzyme molecule reacts with *one* inhibitor molecule to form the complex under the conditions employed. This conclusion is based on the values given in Table 5 and — especially for elastase — on the linear shape of the titration curves. On the other hand from the titration curves it is possible to calculate the amount of active enzyme molecules present in the enzyme preparations, e. g. in subtilisin Novo, A. oryzae protease, and elastase (cf. Tab. 5). It is remarkable that the activity of subtilisin and elastase was not decreased by high amounts (0.3—0.5 mg) of BPTI under the same conditions.

Pronase also contains proteinases which are strongly inhibited by DSI: One third of the azocasein-splitting activity of pronase was inhibited by titration with DSI in a manner characteristic for 1:1 complex formation (Fig. 11). That is why it is possible to calculate that 1 mg of the pronase preparation employed contains 14 n mole of DSI-reactive proteinases.

Porcine *plasmin* is also inhibited by DSI, but much more weaker than the above mentioned enzymes (Fig. 6). The complex is already highly

Table 4. Estimation of M. W. by Gel Filtration of DSI and DSI-Enzyme Complexes

The chilled (10°C) Sephadex G-75 column used was equilibrated and developed with 0.05M TRA-HCl, 0.15M NaCl, pH 7.0. Of each protein or protein complex (containing equimolar amounts of enzyme(s) and inhibitor) about 3 mg were applied. Absorption at 253 nm as well as enzyme inhibition (after dissociation of the complexes with aqueous perchloric acid) were measured in the eluates

Substance	V/V_0	M. W.
Dextran blue	(V_0)	
Albumin (human)	1.27	68000
Chymotrypsinogen	1.57	25000
Cytochrom c	1.86	12000
BPTI	2.30	6500
DSI	1.86[a]	12000
DSI + trypsin	1.52	29000[c]
DSI + chymotrypsin	1.42	39000
DSI + trypsin and chymotrypsin	1.30[b]	60000

[a] Trypsin and chymotrypsin inhibition paralleled exactly in the eluted fractions.

[b] Degradation products of lower m. w. were also observed.

[c] Non additive molecular weights were also found with other examples [26].

Table 5. Enzyme/Inhibitor Ratio in the Complex Calculated from the Titration Curves

Enzyme	Fig.	Substrate used	Amount of enzyme titrated		Theoretical amount of inhibitor necessary for complete inhibition
			n mole	μg	n mole
Trypsin	5	BAPNA	0.28		0.26
Chymotrypsin	7	CPPN	0.78		0.78
Subtilisin	8	Azo-casein		25[a]	0.80
Elastase	9	Elastin-orcein		750[b]	0.80
A. oryzae protease	10	Azo-casein		25[c]	0.58
Pronase	11	Azo-casein		50[d]	0.70

[a] Assuming a m. w. of 27500 [43] the subtilisin preparation contains 22 μg of active enzyme.
[b] Assuming a m. w. of 28500 [43] the elastase used contains 23 μg of active enzyme.
[c] Assuming a m. w. of 19600 [43] the protease used contains 11 μg of active enzyme.
[d] Assuming a m. w. of 19000 [44] the pronase preparation contains 13 μg of an enzyme inhibited by DSI.

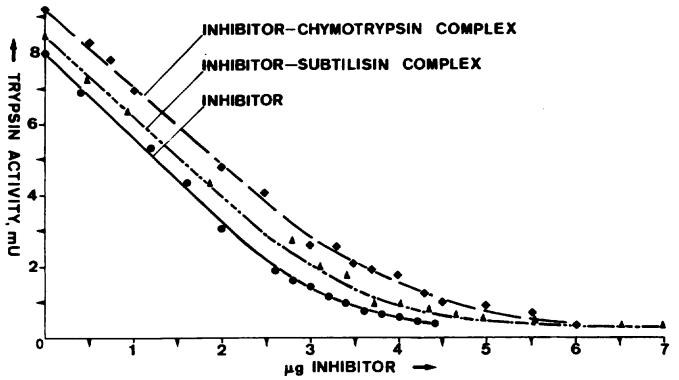

Fig. 5. Titration of Bovine Trypsin with Dog Submandibular Inhibitor (DSI), DSI-Chymotrypsin Complex, and DSI-Subtilisin Complex. DSI-fraction A_2 (cf. Fig. 2 and Table 1) was used throughout.

1) *Titration with DSI:* Constant amounts of trypsin, 0.28 n mole (i. e. about 10 μg by weight), titrated with BPTI, were incubated with increasing amounts of DSI in 2.0 m/ 0.2M TRA-HCl (without $CaCl_2$), pH 7.8, for 8 minutes at 25°C. The enzymatic reaction was started by addition of 1.0 m/ substrate solution (1 mg BAPNA in deionized water, cf. [11, 12]). Measured at 90% inhibition, no time dependence of the degree of inhibition was observed using preincubation periods from 1 up to 15 minutes.

2) *Titration with DSI-Chymotrypsin Complex:* Equimolar amounts of DSI (120 μg) and α-chymotrypsin (about 240 μg), calculated from the titration curve in Fig. 7 from measurements at 50% inhibition, were preincubated in 5.0 m/ 0.2M TRA-HCl, pH 7.8, for 15 minutes and longer at 0°C. Constant amounts (0.32 n mole) of trypsin were incubated with increasing amounts of the DSI-chymotrypsin complex in 2.0 m/ TRA-HCl for 8 minutes at 25°C; the enzymatic reaction was started as described above. No time dependence of the degree of inhibition of trypsin (by the DSI-chymotrypsin complex) was observed using preincubation periods from 1 up to 15 minutes. On the *abscissa* only the amount of DSI bound in the complex is given.

3) *Titration with DSI-Subtilisin Complex:* Equimolar amounts of DSI (103 μg) and subtilisin (about 250 μg), calculated from the titration curve in Fig. 8 from measurements at 50% inhibition, were preincubated in 2.24 m/ phosphate buffer, pH 7.6, for 10 minutes and longer at 0°C. Constant amounts (0.30 n mole) of trypsin were incubated with increasing amounts of DSI-subtilisin complex in 2.0 m/ 0.2M TRA-HCl, pH 7.8, for 8 minutes at 25°C. The enzymatic reaction was started by addition of the BAPNA-substrate solution (cf. Methods and trypsin titration). The amount of subtilisin employed caused no BAPNA-hydrolysis. On the *abscissa* only the amount of DSI bound in the complex is given.

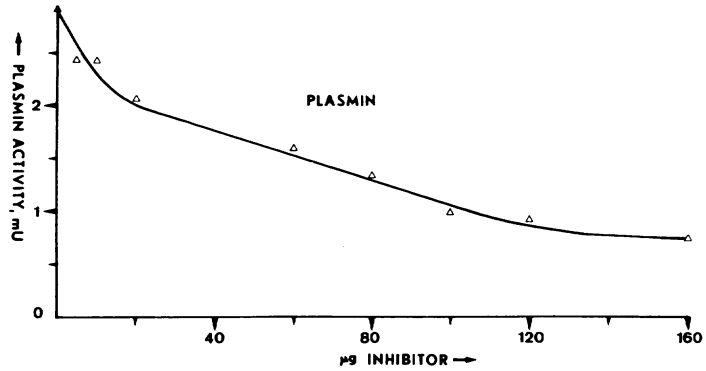

Fig. 6. Plasmin Inhibition. Constant amounts (250 µg, 1.8 n mole) of plasmin were incubated with increasing amounts of DSI (fraction A_2, cf. Fig. 5) in* 0.2M TRA-HCl, 0.05M L-lysine, pH 7.8, for 8 minutes at 25°C. The enzymatic reaction was started by addition of 1.0 ml BAPNA-substrate solution (cf. Methods and Fig. 5, trypsin titration). Measured at 71% inhibition, no time dependence of the degree of inhibition was observed using preincubation periods from 1 up to 15 minutes. * (2,0 ml).

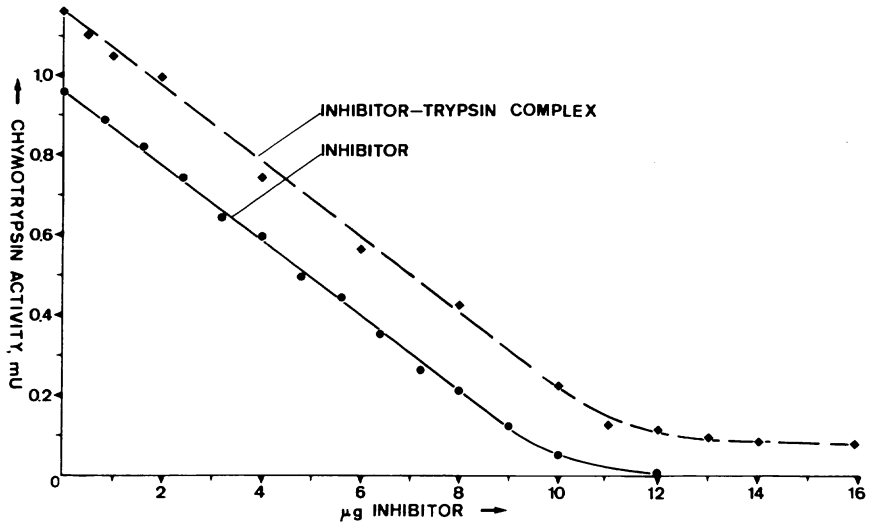

Fig. 7. Titration of Bovine α-Chymotrypsin with DSI and DSI-Trypsin Complex. DSI-fraction A_2 (cf. Fig. 2 and Table 1) was used throughout.

1) *Titration with DSI:* Constant amounts of α-chymotrypsin, 0.78 n mole (i. e. about 20 µg by weight), titrated with BPTI, were incubated with increasing amounts of DSI in 2.0 ml 0.2M TRA-HCl, 0.02M $CaCl_2$, pH 7.8, for 8 minutes at 25°C. The enzymatic reaction was started by addition of 1.0 ml substrate solution (5 mg CPPN in 1.0 ml 0.2M TRA-HCl, pH 7.8), cf. [12, 15]. Measured at 90% inhibition, the degree of inhibition is constant at preincubation periods from 1 up to 15 minutes.

2) *Titration with DSI-Trypsin Complex:* Equimolar amounts of DSI (80 µg) and trypsin (about 200 µg), calculated from the titration curve in Fig. 5 from measurements at 50% inhibition, were preincubated in 6.0 ml 0.2M TRA-HCl, 0.02M $CaCl_2$, pH 7.8, for 10 minutes and longer at 0°C. Constant amounts (0.96 n mole) of α-chymotrypsin were incubated with increasing amounts of the DSI-trypsin complex for 8 minutes at 25°C; the enzymatic reaction was started by addition of the substrate solution (see above). Measured at 90% inhibition, no time dependence of the degree of inhibition of α-chymotrypsin (by the DSI-trypsin complex) was observed using preincubation periods from 1 up to 15 minutes.

On the *abscissa* only the amount of DSI bound in the complex is given.

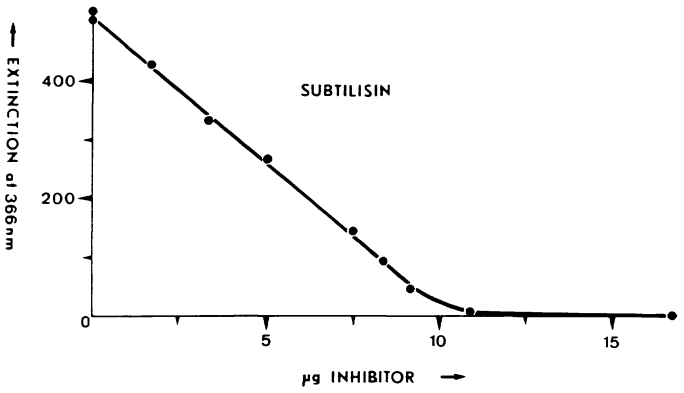

Fig. 8. Inhibition of Subtilisin Novo. To constant amounts (25 µg) of subtilisin increasing amounts of DSI (fraction A_2, cf. Fig. 5) were added. The procedure is given in the Methods. Employed substrate: Azo-casein.

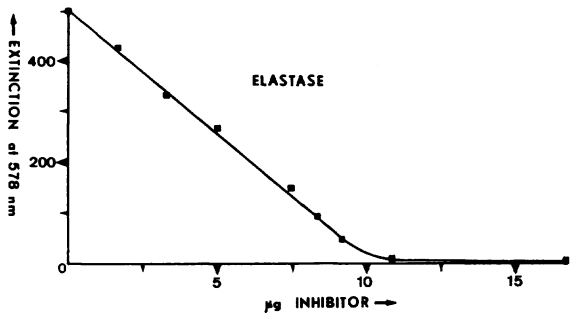

Fig. 9. Inhibition of Pancreatic Elastase. To constant amounts (0.75 mg) of elastase increasing amounts of DSI (fraction A_2, cf. Fig. 5) were added. The procedure is given in the Methods. Employed substrate: Elastin-orcein.

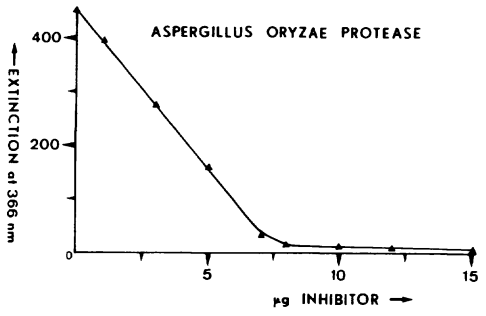

Fig. 10. Inhibition of Aspergillus Oryzae Protease. To constant amounts (25 µg) of the protease increasing amounts of DSI (fraction A_2, cf. Fig. 5) were added. The procedure is given in the Methods. Employed substrate: Azo-casein.

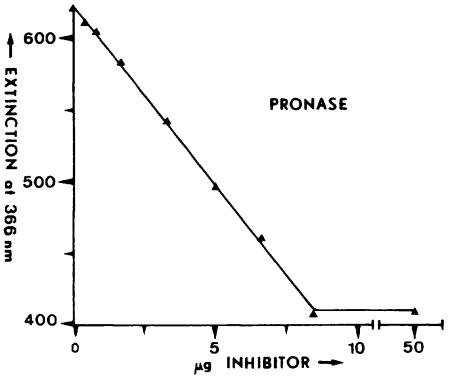

Fig. 11. Inhibiton of Proteolytic Activity of Pronase. To constant amounts (50 µg) of Pronase increasing amounts of DSI (fraction A_2, cf. Fig. 5) were added. The procedure is given in the Methods. Employed substrate: Azo-casein. Note, that only part of the proteolytic activity (ordinate) is inhibited

dissociated in the presence of BAPNA which is a substrate with only a small affinity to plasmin. The steeper slope of the titration curve at the beginning may be due to a small degree of contamination with trypsin which was used for the activation of plasminogen.

No inhibition of *collagenase* could be demonstrated. The activity of 0.1 mg of collagenase (from Worthington: 159 U/mg; substrate: p-phenylazobenzyloxycarbonyl-L-pro-L-leu-gly-L-pro-D-argOH from Fluka; method according to [28]) was neither diminished in the presence of 0.1 mg DSI nor by 0.5 mg BPTI. HAENDLE [8] reported that porcine *pancreatic kallikrein* is also not inhibited by DSI.

The results show clearly that DSI is a strong inhibitor of functional serine proteinases belonging to different families of this group [29]: In addition to proteinases of the trypsin-chymotrypsin family of mammals other serine proteinases from bacteria or mold fungus bearing no structural resemblance to the afore mentioned ones are also inhibited. It is most interesting that the inhibition spectra of ovoinhibitors [19] and of inhibitors from potatoes [30] are very similar to that of DSI. (See also the following paragraph.)

Reactive Sites

Different reactive sites on the DSI-molecule are responsible for the inhibition of trypsin and chymotrypsin. No decrease in activity against both enzymes is observed after exhaustive maleylation; however, if the maleylated DSI-derivative is reacted with the arginine-modifying butandion-2,3 reagent [31] it retains only its inhibitory activity for chymotrypsin. Therefore an arginine residue is located in the reactive site for trypsin inhibition [32].

The formation of ternary complexes is unambiguously demonstrated by the titration curves given in Figures 5 and 7 and the results of the gel filtration experiments (Tab. 4). The binding of trypsin therefore does not interfere with the binding of chymotrypsin or subtilisin, consequently the DSI-molecule is double-headed with not overlapping reactive sites [33]. Very similar properties were reported for the ovoinhibitors from egg white [19, 34], the inhibitor AA from soybeans [35], and the lima bean protease inhibitor LBI [21].

The subtilisin-DSI complex inhibits trypsin but not chymotrypsin. Therefore and from the specifity requirements of the proteinases [36] it may be deduced that the chymotrypsin-reactive site and the subtilisin-reactive site on the DSI-molecule are identical; this same site may also be responsible for the inhibition of elastase [36] and perhaps A. oryzae protease. Unfortunately DSI is not so easily modified in slightly acidic solutions as the Kunitz trypsin inhibitor [37], the Bowman-Birk inhibitor AA [35] from soybeans, and the inhibitor from lima beans [21]; investigations with altered conditions are in progress.

The results mentioned show that the inhibition of chymotrypsin by BPTI is a special case [38, 39]. The wide inhibition spectrum of DSI as well as the similar inhibition spectra of the ovoinhibitors are mainly caused by the chymotryptic reactive sites of these inhibitor molecules.

Physiological Function

The assumption that DSI protects mucosa cells in mouth and esophagus against the action of proteinases ingested with the food [5, 7—9] is supported by our findings: The inhibition of subtilisin, elastase and mold proteases by DSI. But also a special function of DSI in connection with proteinases found in submaxillary glands [40] etc. of some animals is possible.

The inhibition of elastase by DSI may be an important fact for the application of this inhibitor for medical therapy in future: The destruction of connective tissue cells caused by elastases during acute pancreatitis [41] or α_1-antitrypsin deficiency [42] is possibly prevented by DSI.

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