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A TRYPSIN AND CHYMOTRYPSIN INHIBITOR FROM SEEDS OF *BAUHENIA PURPUREA**

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Key Word Index—*Bauhenia purpurea*; Leguminosae; protease inhibitor; trypsin; chymotrypsin; purification; enzyme-inhibitor complexes.

Abstract—A trypsin and chymotrypsin inhibitor was partially purified from *Bauhenia purpurea* seeds and separated from a second inhibitor by Ecteola cellulose chromatography. The factor inhibited bovine trypsin and chymotrypsin as well as pronase trypsin and elastase. It formed a complex with trypsin and with chymotrypsin, but a ternary complex could not be detected. Differences were detected in the effect on trypsin and on chymotrypsin, although one enzyme interfered with the inhibition of the other. The results obtained point to two active centers on the inhibitor for the trypsin and chymotrypsin inhibition such that the one cannot complex with the inhibitor after this inhibitor had complexed with the other.

INTRODUCTION

THE PRESENCE of trypsin inhibitors in legume seeds has been reported for many types of legumes.[1] To the best of our knowledge, seeds from perennial plants were not investigated. It was thought useful to determine whether such inhibitors are present in the seeds of a perennial legume, *Bauhenia*. The work described below deals with the isolation and partial characterization of a trypsin and chymotrypsin inhibitor from *Bauhenia purpurea* seeds, and provides evidence of complex formation both with trypsin and chymotrypsin.

RESULTS

The inhibitor was extracted from the fat-free seed with water and purified by ammonium sulphate precipitation, followed by chromatography on CM-cellulose, Sephadex G25 and Ecteola cellulose (Table 1). Two components were found and the most active used for subsequent examination. The purified inhibitor (at 0·5 mg/ml) was soluble in water, 70% and 96% ethanol, acetone and 2·5% TCA. It was less soluble in higher concentrations of TCA and completely insoluble in typical fat solvents.

The inhibitor lost 72% of its activity after 1 hr in 0·1 N NaOH and 36% after 1 hr in 0·1 N HCl at room temperature. At the same temperature, it lost 50% of its activity after 4 hr at pH 1·5. At pHs 2–4, 30% of the activity was lost after 4 hr and 50% after 24 hr. At pHs 5 and 6, there was little loss of activity in 24 hr but it was reduced to 50% after 72 hr. At pHs 7–11, about 80% of the activity remained after 24 hr, decreasing to less than half after 48 hr with little left after 72 hr. At 0°, the inhibitor was much more stable, losing

* Based on the thesis of V.H.S. in partial fulfillment of the requirements for the M.Sc. degree.

[1] VOGEL, R., TRAUTSCHOLD, I. and WERLE, E. (1968) *Natural Proteinase Inhibitors*, p. 9, Academic Press, New York.

only 10–20% of its activity in 72 hr between pH 5 and 11. At 40 and 60°, the factor lost about 20% of its activity after 1 hr at pHs 2–11. At higher temperatures, the inhibitor lost its activity rapidly, retaining less than 10% at 100° at pHs 1·5–11.

TABLE 1. YIELD AND ENRICHMENT OF *Bauhenia* TRYPSIN INHIBITOR AT EACH STAGE OF PURIFICATION

Stage	Total no. of inhibitor units	Sp. act. (units per mg protein)	Yield %	Enrichment (fold)
Homogenate	820 250	50	100	1·0
Precipitate from 50% saturation with ammonium sulphate	787 200	155	96	3·1
Dialysate	672 200	162	82	3·2
After CMC chromatography	532 000	3800	65	76
After Sephadex G25	432 000	3950	53	79
Fraction B after ecteola cellulose chromatography	162 000	4500	20	90
Fraction B after Sephadex G25	154 000	4600	18	92

Inhibitor units were calculated as follows:

$$\frac{\text{Rise in Absorbance of enzyme product in absence of inhibitor} - \text{rise in Absorbance in presence of inhibitor per min}}{\text{Rise in Absorbance in absence of inhibitor per min}} \times 100.$$

The wave length used was according to the substrate as described in Experimental.
Sp. act.: inhibitor units/mg protein.

Urea (8M) caused a reduction in trypsin inhibitor activity at room temperature of 20% in 90 min and 35% in 120 min at pH 5·5. The corresponding figures at pH 7·6 were 20 and 25%. Chymotrypsin inhibition was virtually unaffected after 120 min at room temperature at pH 5·5. At 70°, the urea decreased chymotrypsin inhibition by 20% compared to a rise of 20% in inhibition in the absence of urea. Similar results were obtained at pH 7·6.

Table 2 summarizes the results obtained when the isolated inhibitor was incubated with various proteases and substrates as indicated. No effect was observed on pepsin, papain, pancreatic elastase or carboxypeptidase at their optimal pHs. A Lineweaver–Burk plot showed that the inhibitor was noncompetitive on trypsin with casein as substrate. K_i was 2×10^{-8}M. The inhibition of trypsin with BAPA was competitive with a K_i of 4×10^{-9}M. With BAEE, the inhibitor was noncompetitive with a K_i of 2×10^{-10}M. The action on chymotrypsin with casein or ATEE as substrate showed the inhibition to be mixed with a K_i of 2×10^{-5}M for the former and $3{\cdot}4 \times 10^{-10}$M for the latter.

Table 3 summarizes the results obtained when trypsin was added to chymotrypsin inhibitor complex or when chymotrypsin was added to trypsin inhibitor complex. The results of electrophoresis on cellulose acetate at pH 7·6 of trypsin, chymotrypsin and the inhibitor alone and in various combinations showed that the enzyme-inhibitor complexes had a different mobility from that of any separate component (Fig. 1). A ternary complex between trypsin, chymotrypsin and its inhibitor with a different mobility was not detected. Similar results were obtained on electrophoresis at pH 4·3.

Calcium ions (0·075 M $CaCl_2$) increased the inhibition of trypsin on BAPA by 33%. The increase was 15% when Ca^2 was at 0·05 M. At this latter concentration, the inhibition of chymotrypsin activity on ATEE was decreased by 17%. Magnesium ions, $MgCl_2$

TABLE 2. EFFECT OF *Bauhenia* TRYPSIN INHIBITOR ON VARIOUS PROTEASES

Enzyme	Substrate	pH	% Inhibition at the following inhibitor/enzyme ratios (w/w): 0·3	0·5	0·8	1·0
Trypsin	BAPA	7·6	32	60	80	100
	BAEE	7·6	28	57	70	85
	Casein	7·6	50	83	100	
	Haemoglobin	7·6	45	80	100	
Chymotrypsin	ATEE	7·6	50	63	88	96
	Casein	7·6	28	33	48	58
	Haemoglobin	7·6	33	55	65	78
Pronase trypsin	BAPA	7·6	35	63	82	100
	BAEE	7·6	35	60	75	90
	Casein	7·6	25	33	50	60
Pronase elastase	AAME	8·5	20	35	55	80

Inhibitor activity on trypsin, chymotrypsin and pronase trypsin was as described in Experimental. Pronase elastase was determined on AAME according to Gertler[2] with the reaction at pH 8·5 instead of 8·0.

BAPA, benzoyl DL-arginine *p*-nitroanilide; BAEE, benzoyl arginine ethyl ester; ATEE, *N*-acetyl *L*-tyrosine ethyl ester; AAME, tri-*L*-alanine methyl ester.

TABLE 3. THE INHIBITION OF TRYPSIN AND CHYMOTRYPSIN BY *Bauhenia* INHIBITOR, BY CHYMOTRYPSIN-INHIBITOR COMPLEX OR BY TRYPSIN-INHIBITOR COMPLEX RESPECTIVELY

Enzyme	Added	Substrate	Inhibitor units	Enzyme	Added	Substrate	Inhibitor units
T	I	BAEE	1200	C	T + I	BAPA	420
C	T + I	BAEE	1220	T	C + I	BAPA	280
T	C + I	BAEE	420	T + C	I	BAPA	400
C + T	I	BAEE	720	T	I	Casein	156
C	I	ATEE	1580	C	T + I	Casein	30
T	C + I	ATEE	1260	T	C + I	Casein	42
C	T + I	ATEE	440	T + C	I	Casein	44
T + C	I	ATEE	900	C	I	Casein	96
T	I	BAPA	460				

Trypsin (T) in each case (except BAEE) was 50 μl of 800 μg/ml; Chymotrypsin (C) was 50 μl of 250 μg/ml; and the inhibitor (I) was 50 μl of 140 μg/ml. When BAEE was the substrate, the trypsin concentration was 270 μg/ml. Substrate concentration was as described in experimental.

Inhibitor units calculated as described for Table 1. The final column shows the relative inhibitory activity of the same quantity of inhibitor under the various experimental conditions.

0·05 M, reduced the inhibition of trypsin on BAPA by 7% and of chymotrypsin on ATEE by 27%. The buffer used in these ion experiments was 0·1 M Tris(hydroxymethyl) aminomethane adjusted to pH 7·6 with HCl.

Papain at pH 8·0 or pepsin at pH 1·5 after 10 hr incubation had no effect at all on the inhibitor when tested on trypsin or on chymotrypsin. Trypsin, under the same conditions, greatly decreased inhibitor activity, the latter being virtually negligible after 10 hr. Similar

[2] GERTLER, A. and HOFMANN, T. (1970) *Can. J. Biochem.* **48**, 384.

results were obtained with chymotrypsin. Corroborative results were obtained by electrophoresis.

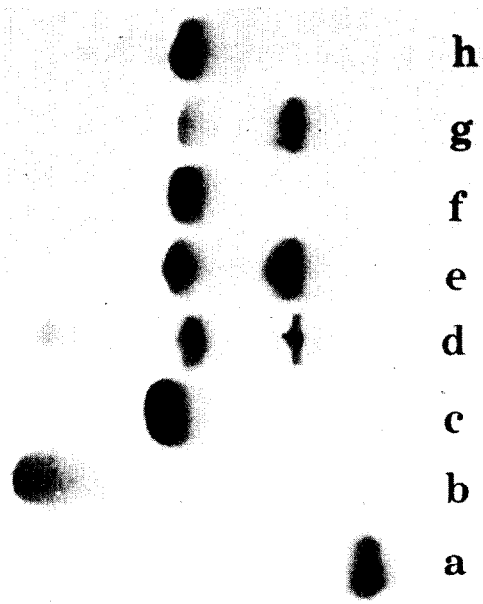

FIG. 1. ELECTROPHORETIC DIAGRAM OF BOVINE TRYPSIN, CHYMOTRYPSIN AND THE *Bauhenia* INHIBITOR IN VARIOUS COMBINATIONS.

The various combinations were incubated at 0° for 5 min and then subjected to electrophoresis at pH 7·6 as described in experimental. Staining was with nigrosin.
(a) inhibitor; (b) trypsin; (c) chymotrypsin; (d) trypsin and inhibitor; (e) chymotrypsin and inhibitor; (f) trypsin inhibitor to which chymotrypsin had been added; (g) chymotrypsin inhibitor to which trypsin had been added; (h) trypsin chymotrypsin mixture to which the inhibitor was added.

DISCUSSION

As mentioned above, Ecteola cellulose chromatography separated two protease inhibitors. The presence of a number of inhibitors in plants is a well known phenomenon. For example, four were detected in the soya bean[3] and four in the lima bean.[4]

The effect of urea indicates that the action of the inhibitor on trypsin differs from its action on chymotrypsin. Its stability to the action of pepsin at pH 1·5 for 10 hr indicates that the inhibitor may pass through the stomach without reduction of activity. It can therefore react in the duodenum with full activity.

Electrophoresis shows quite distinctly that a complex between inhibitor and trypsin or chymotrypsin is formed but a ternary complex could not be detected. Table 2 shows that with the exception of the action of trypsin on BAPA, chymotrypsin interfered with the inhibition of trypsin and trypsin with that of chymotrypsin. In addition, mixing the two enzymes before adding the inhibitor greatly reduced the inhibitory effect. The detection of differences between the inhibitor effect on trypsin and on chymotrypsin in response to

[3] RACKIS, J. J. and ANDERSON, R. L. (1964) *Biochem. Biophys. Res. Commun.* **15**, 230.
[4] JONES, G., MOORE, S. and STEIN, W. (1963) *Biochemistry* **2**, 66.

ions, urea and heat, points to the presence of different active centers for both enzymes. Two separate active centers have been reported for the AA inhibitor from the soya bean,[5] the lima bean inhibitor[6] and the black-eyed pea inhibitor.[7]

EXPERIMENTAL

Preparation. The *Bauhenia* seeds were ground to a powder and extracted with ether in a Soxhlet extraction apparatus. The fat-free powder was suspended in H_2O brought to pH 9·0 (pH of maximum extraction) with a small quantity of Na_2CO_3. After 30 min stirring, the suspension was centrifuged at 10 000 *g* at 0° for 15 min. The active inhibitor was in the supernatant. Inactive protein was removed by precipitation with $(NH_4)_2SO_4$ at 10% satn. The active factor precipitated when the $(NH_4)_2SO_4$ concentration was raised to 50% satn. The ppt. was dialysed against 0·005 M acetate buffer pH 5·5 and the insoluble residue was discarded. Further purification was accomplished by chromatography on CM-cellulose equilibrated with 0·005 M acetate buffer pH 5·5. On elution with rising acetate buffer concentrations pH 5·5 (0·05, 0·08 and 0·1 M), the active factor eluted with 0·1 M acetate with a 23-fold enrichment. Molecular filtration on Sephadex G25 slightly raised the specific activity. Subsequent Ecteola cellulose chromatography and elution with rising concentrations of phosphate buffer pH 6·5 (0·01 M, 0·03 M, 0·05 M) separated two factors eluting at 0·03 M and 0·05 M respectively. Fraction B eluting at 0·05 M was the most active. The yields and specific activities of each step are given in Table 1.

Protein determination. Protein concentration in the homogenate and in the $(NH_4)_2SO_4$ precipitates was by the method of Lowry *et al.*[8] Its concentration in the purified fractions was by determination of its absorption at 280 nm, an absorbance of 1·0 being equivalent to 1 mg/ml.

Enzyme activity determinations. Trypsin activity on BAPA was determined according to Erlanger *et al.*[9] except that the pH of the reaction was 7·6 instead of 8·15 (0·1 M phosphate buffer). The reaction mixture included 3 ml 0·016% BAPA and either 50 μl trypsin (800 μg/ml) or buffer. One activity unit was an increase in absorbance at 390 nm of 0·001 per min. Trypsin activity on BAEE was determined according to Schwert and Takenka.[10] The reaction mixture included 3 ml 0·02% BAEE in 0·1 M phosphate buffer pH 7·6 and either 50 μl trypsin (270 μg/ml) or buffer. One activity unit was an increase in absorbance at 253 nm of 0·001 per min. Chymotrypsin activity on ATEE was according to Schwert and Takenka.[10] The reaction mixture included 3 ml 0·02% ATEE in 0·1 M phosphate buffer pH 7·6 and either 50 μl chymotrypsin (250 μg/ml) or buffer. One activity unit was an increase in absorbance at 237 nm of 0·001 per min. Proteolytic activity on casein or hemoglobin was according to Kunitz[11] as described by Laskowski.[12] The reaction mixture included 3 ml of 1% casein or hemoglobin (dialysed against 0·1 M phosphate buffer pH 7·6) and 0·5 ml trypsin (40 μg/ml) or chymotrypsin (38 μg/ml). An increase in absorbance of 0·001 at 280 nm of the TCA soluble solution after incubation for 20 min at 37° was one activity unit.

Electrophoresis. The inhibitor, the various proteases and mixtures of the inhibitor with these proteases were subjected to electrophoresis on cellulose acetate in a Beckman microzone apparatus at 250V (5 mA) for 20–25 min. Staining was with amido black.

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