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PROTEASE INHIBITORS FROM BROAD BEAN ISOLATION AND PURIFICATION

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Abstract—Four protease inhibitors were demonstrated and two, BBPI-1 and BBPI-2, were purified from broad bean seeds using a combination of $(NH_4)_2SO_4$ fractionation, ion-exchange chromatography on CM 52-cellulose and CM 50 Sephadex. BBPI-1 and 2 had broad specificity and inhibited trypsin, chymotrypsin, thrombin, pronase and papain. Both inhibitors were heat stable, had a wide pH tolerance, a MW of approximately 11000 and contained 14·5% N_2. BBPI-1 and 2 had a pI of 8·5 and 7·5 respectively.

INTRODUCTION

PROTEIN inhibitors of trypsin and other proteases are widespread in both the plant and animal kingdom. The seeds of the *Leguminosae* are especially rich sources of these inhibitors [1–3] and a number have been purified and characterized as far as the primary structure.[4,5] These inhibitors have been well studied, chiefly because of their importance in the digestibility of protein by animals and humans.[6] The physiological role of these inhibitors in the plant remains obscure but involvement in the control of protein hydrolysis in germinating seeds[7] and as protective agents against insects and fungal pests[3] have been suggested.

In this country seeds of *Vicia faba*, including field and broad bean, have traditionally been consumed by both man and animal, but in recent years the field bean has increased in importance as the source of protein for production of textured protein products for human consumption.[8] Information on the deleterious effects of protease inhibitors (PI) in the seeds of *Vicia faba* are scarce, indeed Borchers *et al.*[2] failed to detect any inhibitors in the seeds. The presence of PI in double bean was demonstrated by Sohonie and Ambe[9] in 1955, who described a method for its purification. Using a modified procedure a crystalline inhibitor was prepared which was shown to be soluble and stable in 2·5% TCA, heat stable and tolerant to extreme pH.[10] The inhibitor which had a broad specificity was active

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against trypsin, chymotrypsin, acetyl trypsin and papain, contained 13% N_2 and had an isoelectric point of 8·6.[10] A similar inhibitor was found in field bean by Nitsan,[11] which also proved to be stable to autoclaving, while the purified inhibitor isolated by Wilson *et al.*[12] was found to be heat labile and had a low percentage nitrogen (8·1%).

In view of the paucity and conflicting information on the PI from *Vicia faba*, an investigation was initiated to isolate, purify and characterize the inhibitors from broad bean. The present communication is concerned with the isolation, purification and partial characterization of the inhibitors from this bean.

RESULTS

Isolation of pure protease inhibitors

2·5% TCA and 0·05 M H_2SO_4 proved to be selective extractants of PI (antitrypsin activity) and similar proteins while the other reagents tested had the disadvantage of being effective protein solvents thus giving PI extracts of low specific activity (Table 1). In the extraction procedure finally adopted, TCA was used in preference to H_2SO_4 because it gave extracts of slightly higher specific activity. A 4-fold increase in the PI specific activity of the inhibitors in TCA extract could be obtained either by dialysis against distilled H_2O for 48 hr or by adjusting the pH to 4·5. The latter procedure was the one generally adopted in the purification process.

TABLE 1. SURVEY OF EXTRACTANTS FOR THE ISOLATION OF PROTEASE INHIBITORS FROM BROAD BEAN*

Extractant	pH	Total protein† (g)	Total [TU]^{eas} inhibited‡	Specific activity§
0·1 M KCl–HCl	2·6	95	475	5·0
0·1 M acetate	4·0	50	550	11·0
0·1 M acetate	5·6	110	645	5·9
0·1 M phosphate	7·0	150	450	3·0
0·1 M phosphate	8·0	150	450	3·0
0·1 M Tris–HCl	8·0	220	550	2·5
0·05 M H_2SO_4	1·5	30	600	19·2
0·05 M NaOH	11·5	250	465	1·8
0·1 M NaCl	6·5	105	550	6·0
2·5% TCA	1·9	29	615	21·2
Dist. H_2O	5·5	213	550	2·6

* 1 kg Meal.
† Protein determined by Biuret.[17]
‡ Inhibitor units expressed as Kunitz Units.
§ 10^{-3}[Tu]^{eas} inhibited mg inhibitor.

$(NH_4)_2SO_4$ fractionation of the pH 4·5 extract after clarification produced three PI rich fractions namely between 30–40%, 40–50% and 50–60% saturation, which accounted for almost 90% of the total PI units of the original TCA extract (Table 2). Each fraction possessed almost equal amounts of Trypsin Inhibitor Units (TIU) but the 50–60% fraction had the highest specific activity. Acrylamide gel electrophoresis of each fraction revealed a similar qualitative pattern of the inhibitors but different quantitative protein distribution. The mobility of the inhibitors BBPI 1–4 on the gels relative to the marker dye was 0·15, 0·40, 0·05 and 0·30 respectively.

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TABLE 2. AMMONIUM SULPHATE FRACTIONATION OF TCA EXTRACT FROM BROAD BEANS

Fraction	Total protein* (mg)	Total [TU]^{cas} inhibited†	Specific activity‡
0–30	380	46	116·6
30–40	1800	182	101·0
40–50	2200	185	84·1
50–60	1040	174	167·3
60–100	2000	15	7·5

* Protein determined by Folin–Lowry method[23] For other details see Table 1.

TABLE 3. YIELD AND POTENCY OF FRACTIONS OBTAINED DURING THE PURIFICATION OF PROTEASE INHIBITORS FROM BROAD BEAN

Fraction	Protein (mg)	[TU]^{cas} inhibited	Specific activity	Yield (%)
TCA extract	29000	615	21	100
Dialysis or pH 4·5 fraction	7500	615	80	100
$(NH_4)_2SO_4$ fractionation				
(a) 30–40%	1800	182	101	30
(b) 40–50%	2200	185	84	30
(c) 50–60%	1040	174	167	28
CM-Cellulose				
(a) Peak **1** + **2**	847	46	54	18·6
Peak **4**	95	68	712	
(b) Peak **1**	211	38	365	
Peak **2**	51	20	399	27
Peak **4**	109	109	1000	
(c) Peak **1**	51	8	158	
Peak **2**	40	9	202	
Peak **3**	33	11	328	20
Peak **4**	129	103	799	
CM-Sephadex				
Peak **3**				
(c) **BBPI-2**	9	7	753	1·1
Peak **4**				
(a) **BBPI-1**	58	61	1047	10·0
(b) **BBPI-1**	92	96	1047	15·6
(c) **BBPI-1**	87	91	1047	14·8

Chromatography on CM-cellulose resolved each $(NH_4)_2SO_4$ fraction into four components possessing PI activity. These were numbered **1–4** in order of elution. Components **1** and **2** were eluted at 0·235 M and 0·25 M NaCl respectively, while **3** and **4** emerged between 240–400 ml and 400–880 ml 0·25 M NaCl respectively. Each $(NH_4)_2SO_4$ fraction contained different proportions of each component e.g. in the 50–60% fraction, component **4** accounted for about 75% of the PI activity eluted from the column while the remainder contained equal amounts of **1**, **2** and **3** (Table 3). Acrylamide gel electrophoresis revealed that **1**, **2**, **3** and **4** each contained several proteins but only **3** and **4** were purified further on CM-Sephadex. Component **4** was found to contain a single inhibitor which was eluted between 160–400 ml 0·25 M NaCl and was named **BBPI-1**. Component **3** contained equal proportions of **BBPI-1** and a second inhibitor which was eluted at 0·25 M NaCl and which was designated **BBPI-2**. **BBPI-1** and **2** were shown to be single proteins on acrylamide gels.

The specific activity of BBPI-1 and 2 determined as antitrypsin activity using casein as substrate was found to be 1047 and 753 mg protein thus giving a purification of 50- and 35-fold respectively.

Properties of the broad bean protease inhibitors

BBPI-1 and 2 were found to inhibit a wide range of proteases from widely different sources including trypsin, chymotrypsin, pronase, thrombin and papain albeit only slightly (Table 4). Both inhibitors must possess at least two inhibitor sites since chymotrypsin was inhibited by the *BBPI*-trypsin complex and vice versa. Pepsin, carboxypeptidase A and B, amino peptidase and subtilisin were not affected by either inhibitor. Pepsin was found not to hydrolyse either inhibitor over a period of 24 hr at pH 2.

In acid media (pH 2·5) heating at 100° for 1 hr had no effect on the antitrypsin activity and at 120° for the same periods only 10% of the activity was lost. At pH 12 however, the inhibitors were rapidly inactivated at 70° and above.

Preliminary estimates of 11000 for the MW of *BBPI*-1 and 2 were obtained by means of electrophoresis on SDS-acrylamide gel. The isoelectric pH of BBPI–1 and 2 by isoelectric focussing were found to be 8·5 and 7·5 respectively. Generally the nitrogen content of both inhibitors was not lower than 14·5%.

TABLE 4. PERCENTAGE INHIBITION OF PROTEASES BY BBPI

Inhibitor (I)	Ratio E/I mol	Inhibition (%) Trypsin (Bovine)	Trypsin (Hog)	Chymo trypsin	Thrombin	Ratio E/Iwt	Pronase	Acetyl trypsin
BBPI-1	1·0	60	40	32	25	1·0	100	85
	0·5	75	54	55	50	0·5	100	86
BBPI-2	1·0	40	39	17	—	1·0	94	59
	0·5	60	53	30	—	0·5	94	69

DISCUSSION

Four PI have been shown to occur in the broad bean seeds and two of these have been isolated in a highly purified state. The properties of both inhibitors are very similar with regard to specificity, stability, MW and nitrogen content, but both possess different activity towards trypsin, pI and other physical characteristics, indicating differences in composition. Because both inhibitors have similar properties they may be regarded as iso-inhibitors. Multiple inhibitors have been isolated from a number of legume seeds including soybean,[13] Lima bean,[14] garden bean[15] and mung bean.[16]

BBPI-1 and 2, like all the inhibitors previously described, appear to have a characteristic low MW. Both inhibitors are stable at low pH but are extremely labile at neutral and alkaline pH. This observation is contrary to those of Sohonie *et al.*,[10] who showed the crystalline inhibitor from double bean was stable to autoclaving for 1 hr at pH 7·6 and Wilson *et al.*[12] who found a purified inhibitor from field bean dissolved $2·5 \times 10^{-3}$ M HCl was inactivated by autoclaving for 30 min.

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The inhibitors purified here also conform to the general pattern of legume inhibitors in possessing a broad specificity to both plant and animal proteases, and in being capable of the simultaneous and independent inhibition of both trypsin and chymotrypsin. Further they are not destroyed or broken down by pepsin. As yet it is now known whether the BBPI are effective against the proteases of *Vicia faba*. With all this work, the ultimate aim is to determine the physiological role of the inhibitors both in the developing and germinating seeds. Only by characterising the pure inhibitors fully will these functions be completely understood.

EXPERIMENTAL

Materials. Seeds of *Vicia faba* cv. Long Pod were dehulled by hand and ground in a bench hammer mill to pass through a 0·2 mm sieve. The meal was defatted by several extractions with petrol b.p. 40–60° (1:4 w/v) for over 24 hr. After removal of the petrol, the defatted meal was stored at 4°.

Survey of extractants. Crude extracts of the antitrypsin factors were prepared by extracting 1 g with 5 vol. of the following solns for 2 hr at 20°; H_2O, 0·05 M NaOH, 0·05 M H_2SO_4, 0·1 M KCl–HCl buffer pH 2·4; 0·1 M Na acetate buffer pH 4 and 5·6, 0·1 M phosphate buffer pH 7 and 8, 0·1 M Tris–HCl buffer pH 10, 0·1 M NaCl and 2·5% TCA. The supernatant obtained after centrifuging the extract at 15000 *g* for 30 min was used as the crude extract which was assayed for protein by the biuret method[17] and for antitrypsin activity by the Kunitz method.[18] TCA was the most effective agent for extracting the antitrypsin factors of high sp. act. (Table 1).

Purification procedure. Bean meal (100 g) was extracted 2× with 5 vol. of 2·5% TCA for 2 hr at 20°. After centrifugation at 15000 *g* for 30 min the combined supernatants (pH 1·9) were adjusted to pH 4·5 with 2 M NaOH. After standing for 1 hr at 20°, the suspension was clarified by centrifugation at 15000 *g* for 30 min. The supernatant was fractionated at 30, 40, 50, 60 and 100% $(NH_4)_2SO_4$ saturation and the protein ppts in each case were collected by centrifugation at 15000 *g* for 30 min. The pellets were dissolved in the minimum vol. of H_2O and dialysed for 60 hr at 4° against several changes of H_2O. The dialysed proteins were lyophilized and stored at −14° until required.

Chromatography on CM-52 cellulose. CM-cellulose, after equilibration with 0·05 M acetate buffer pH 4, was packed in a column 2·5 × 90 cm by pumping 5 mM acetate buffer pH 4 through the column at 80 ml hr for 24 hr. Approximately 300–400 mg of the $(NH_4)_2SO_4$ isolate was dissolved in 20 ml starting buffer (5 mM acetate buffer pH 4). Elution of the antitrypsin factors was effected by the following buffers at 80 ml/hr: (a) 1 litre starting buffer; (b) linear gradient elution between 0–0·25 M NaCl in starting buffer using a total vol. of 4 l.; (c) 1 l. 0·25 M NaCl in starting buffer. Fractions (4 ml) were collected and every 5th tube was checked for A at 280 nm and assayed for antitrypsin activity. Numbering from the start of the gradient, the fractions eluted between 960–990; 1000–1055; 1060–1100 and 1101–1220 were pooled to yield fractions **1, 2, 3** and **4** respectively. These fractions were dialysed against several changes of H_2O at 4° and then lyophilized. Each fraction was examined by polyacrylamide gel electrophoresis.

Chromatography on CM Sephadex C-50. A 2·5 × 90 cm column of pre-swollen CM-Sephadex was packed by pumping 50 mM phosphate buffer pH 6 through it for 24 hr at a flow rate of 20 ml/hr. Fraction **3** and **4** from CM-cellulose chromatography was dissolved in 10 ml (*ca* 100 mg) of 50 mM phosphate buffer pH 6 and applied separately to the column which was eluted as follows:
(a) 200 ml 50 mM phosphate buffer pH 6; (b) linear gradient from 0–0·25 M NaCl in starting buffer using a total vol. of 1 litre; (c) 500 ml of 0·25 M NaCl in starting buffer. Fractions (4 ml) were collected and every 5th tube was checked for A at 280 nm and assayed for antitrypsin activity. Following the chromatography of fraction **4** the fractions eluted between 340 and 400 were pooled to yield antitrypsin Factor 1 (**BBPI-1**) (vol. 240 ml). Similarly fraction **3** gave antitrypsin Factor **2** (**BBPI-2**) between 280 and 330 (vol. 200 ml) and **BBPI-1** as above. The pooled fractions were dialysed against H_2O for 78 hr and lyophilized. The purity of both inhibitors was checked by acrylamide gel electrophoresis at pH 4 and 9·4.

Activity of protease inhibitors. The inhibitory activity against trypsin, chymotrypsin, pronase, acetyl-trypsin and papain was determined by the caseinolytic procedure described by Kunitz.[18] Under the conditions of the assay, Kunitz[18] defined 1 unit of antitrypsin activity (TIU) as the amount of inhibitor which depressed the trypsin or chrymotrypsin activity by one trypsin or chymotrypsin unit. Sp. act. of the inhibitor was defined as the number of trypsin-inhibitor units/mg inhibitor protein. (The trypsin unit (TU)^{cas} is defined as the amount of enzyme which results in an increase of 1 unit of A at 280 nm per min at 37° and pH 7·6). Antithrombin activity was assayed by the method of Hummel [19] using TAME as substrate.

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Polyacrylamide gel disc electrophoresis was performed according to the method of Ornstein and Davies[20] and vertical flat gel electrophoresis according to the method described by Roberts.[21]

Isoelectric focussing was effected[22] with ampholine giving a pH gradient between 3 and 10, stabilized against convection by means of a polyacrylamide gel matrix. The process was carried out in disc electrophoresis tubes using a conventional apparatus.

Protein determination. Protein was determined either by the Biuret procedure[17] or by Lowry's method.[23] Total N was determined by micro-Kjeldahl method as modified by Lillevick.[24]

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