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Proteinaceous Inhibitors of Trypsin and of Amylases in Developing and Germinating Seeds of Pigeon Pea (*Cajanus cajan* (L) Millsp)

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Abstract: Inhibitors of trypsin and amylase in the extracts of developing seeds of 12 pigeon pea cultivars were analysed using a gel-X-ray film contact print technique and an enzyme-inhibitor assay, respectively. The inhibitors of amylase and trypsin in the extracts of germinating seeds of a pigeon pea cultivar (BDN2) were also studied. Nine trypsin inhibitor bands were detected in mature seeds of all the 12 cultivars. Inhibitory activities against amylase and trypsin were not detected in the extracts of seeds collected 11 and 27 days after flowering (DAF) by the enzyme-inhibitor assay. However, up to three trypsin inhibitor bands could be detected in the extracts of seeds collected 27 DAF by the gel-X-ray film contact technique. Two new slow-moving trypsin inhibitor bands were detected in the extracts of germinating seeds of BDN2 cultivar. These bands were prominent in extracts of seeds 10 days after germination (DAG). The amylase inhibitors and trypsin inhibitors in pigeon pea seeds are late synthesised proteins, their highest levels were observed in mature seeds and they were found to be slowly degraded during germination. Significant inhibitor activities were observed even 15 DAG. The amylases in developing seeds are insensitive to endogenous inhibitors.

Key words: amylase inhibitors, *Cajanus cajan*, developing seed, pigeon pea, seed germination, trypsin inhibitors.

INTRODUCTION

Pigeon pea (*Cajanus cajan* L) seeds contain inhibitor activity against trypsin, chymotrypsin and amylases (Singh and Eggum 1984; Singh *et al* 1984). At least two trypsin inhibitors and seven trypsin/chymotrypsin inhibitors (having inhibitory activities against both trypsin and chymotrypsin) were detected in pigeon pea seeds (Chavan and Hejgaard 1981; Pichare and Kachole 1994a,b). Recently, four amylase isoinhibitors were detected in the seed extracts of pigeon pea (Giri 1994). All these inhibitors are being extensively studied as antinutritional factors and as possible defence components against pests (Gatehouse *et al* 1986; Garcia-Olmedo *et al* 1987; Ryan 1990). Podborer (*Helicoverpa armigera*) and podfly (*Mellanagromyza obtusa* Malloch) are the most damaging pests of pigeon pea pods. These pests attack the host during seed development (Reed and Lateef 1990). We have initiated studies on the role of seed inhibitors in defence against insect pests. In this communication, we report the late appearance of proteinaceous inhibitors of proteases and of amylases in developing seeds of 12 pigeon pea cultivars and their degradation during seed germination in a cultivar (BDN2). Two of the cultivars chosen for the study are podborer tolerant (ICPL 332 and PPE45-2), others susceptible, and a few are resistant to fusarium wilt (Remanandan *et al* 1988).

EXPERIMENTAL

Mature and developing seeds of 12 pigeon pea cultivars (BDN2, BDN1, BDN7, BSMR175, BSMR736, BSMR380, C11, PPE45-2, Daithana Local, ICPL87119,

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ICPL87 and ICPL332) were obtained from the Agricultural Research Station (Marathwada Agricultural University, Badnapur, District Jalna, India). Two stages of developing seeds chosen for the study were based on weight distribution (stage I: 10–20 mg; stage II: 80–100 mg). BDN2 seeds gain this weight in about 11 and 28 DAF, respectively. Dry mature seeds constituted the third stage (stage III). BDN2 seeds were germinated in wet paper folds for up to 20 days.

Casein and trypsin were obtained from Sisco Research Laboratory (Bombay, India). Bovine trypsin type II, bovine chymotrypsin (TLCK-treated) type VII and polyvinylpolypyrrolidone (PVP) were from Sigma Chemical Company (St Louis, MO, USA). Human saliva was used as a source of amylase, except where mentioned otherwise. Starch was from Qualigen Fine Chemicals (India). X-ray films were obtained from Selvas Photographics Ltd (Silvassa, India). Other chemicals used were of the highest purity available.

Extraction of seed proteins

Mature seeds were washed with water, dried and ground to make a fine powder. The mature seed powder was defatted with a mixture of chloroform/methanol (2 : 1, v/v). Developing and germinating seeds were homogenised in, and washed with, acetone to remove coloured particles. Seed proteins from mature defatted powder and acetone powder were extracted in distilled water containing 1% PVP (w/v). Protein was estimated according to the procedure of Lowry *et al* (1951).

Inhibitor assay

Trypsin inhibitor assay was performed according to modified method of Kunitz as described earlier (Belew and Porath 1970; Pichare 1992). A suitable amount of trypsin was mixed with extract and the extract–enzyme mixture was preincubated at room temperature (25–28°C) for 30 min. It was then added to test tubes containing buffered casein (10 mg casein in 0·1 M Tris-Cl buffer pH 7·8) and incubated at 37°C. The reaction was terminated after 20 min by addition of 5% (w/v) trichloroacetic acid (TCA) to the assay mixture. Blanks were prepared by adding TCA to casein solution before the reaction mixture. The tubes were allowed to stand in room temperature (25–28°C) for 30 min and were centrifuged. Residual trypsin activity was estimated by measuring the absorbance of supernatant at 280 nm. One trypsin unit is defined as the activity resulting in an increase of one unit of absorbance at 280 nm of TCA-soluble casein hydrolysis products liberated by trypsin action at 37°C in 1 min under assay conditions. One trypsin inhibitor unit is the activity resulting in inhibition of one trypsin unit.

Amylase and amylase inhibitor activity assays were based on Bernfeld's method for amylase assay (1955). Seed extracts were mixed with amylase and incubated for 30 min at room temperature (25–28°C). The reaction was started by adding extract–enzyme mixture to test tubes containing buffered starch solution (2 mg starch in 20 mM phosphate buffer of pH 6·9 containing 6·7 mM NaCl) and incubated for 15 min. This reaction was terminated by adding 3,5-dinitrosalicylic acid (DNS) reagent to the assay mixture. The assay tubes were kept in a boiling water bath for 5 min, were cooled under tap water and the colour formed by maltose oxidation was measured at 530 nm. Activity of pigeon pea inhibitors towards endogenous amylases was also studied. The amylase activity was measured in the presence and absence of mature seed extract (amylase inhibitor) of some cultivars. One amylase activity unit is defined as the activity resulting in the liberation of 1 mg of maltose from starch at pH 6·9 at 37°C in 3 min. One amylase inhibitor unit is defined as activity resulting into decrease of one unit of absorbance (530 nm) of colour formed due to oxidation of maltose by DNS reagent at 37°C in 1 min under assay conditions.

Electrophoretic analysis

Extracts of developing and germinating pigeon pea seeds were analysed on native 10% polyacrylamide gel in vertical slab gel electrophoresis unit using the Davis buffer system (Davis 1964). Trypsin-inhibitor bands were visualised by gel-X-ray film contact print technique (Pichare and Kachole 1994a). After electrophoresis gels were placed in 0.1 M Tris-HCl, pH 7·8 for 5 min followed by incubation in 0·1 mg ml^{-1} trypsin in 0·1 M Tris-HCl buffer (pH 7·8) for 10 min. The gel was then briefly washed with buffer and placed on X-ray film for 5–10 min. Gelatin hydrolysis was monitored visually. The X-ray film was washed gently with tap water, dried and photographed.

RESULTS AND DISCUSSION

Marginal variations were observed in protein concentration, amylase activity and inhibitory activities against trypsin and amylase in the seed extract of 12 cultivars of pigeon pea at different stages of seed development (Table 1). Nine trypsin inhibitors and four amylase inhibitors (of which two had a very low activity) have been identified in the extracts of mature pigeon pea seeds (Giri 1994). The trypsin-inhibitor activity in the extracts is much higher than amylase-inhibitor activity. The units of trypsin-inhibitor activity and amylase-inhibitor activity are not comparable. Earlier reports from the authors' laboratory revealed that there are no significant differences in trypsin- and chymotrypsin-

TABLE 1
Inhibitors of trypsin and of amylases in seed extracts of different cultivars of pigeon pea (mean ± SD)[a]

Pigeon pea cultivars	*Protein mg g^{-1} of seed powder*			*TIU g^{-1} of seed powder*			*AIU g^{-1} of seed powder*			*AU g^{-1} of seed powder*		
	I	*II*	*III*	*I*	*II*	*III*	*I*	*II*	*III*	*I*	*II*	*III*
BDN2	4·43 ± 0·42	4·02 ± 0·24	10·43 ± 0·25	ND[b]	ND	40·02 ± 0·41	ND	ND	6·01 ± 0·16	52·12 ± 1·22	300·13 ± 1·08	ND
BDN1	3·61 ± 0·36	2·63 ± 0·25	8·04 ± 0·40	ND	ND	44·15 ± 0·02	ND	ND	5·19 ± 0·08	39·90 ± 0·49	296·40 ± 1·33	ND
BDN7	4·42 ± 0·32	3·62 ± 0·40	10·41 ± 0·73	ND	ND	36·33 ± 1·63	ND	ND	4·83 ± 0·24	28·42 ± 0·16	296·29 ± 1·41	ND
BSMR175	5·64 ± 0·28	3·21 ± 0·18	12·19 ± 0·86	ND	ND	48·41 ± 2·04	ND	ND	6·41 ± 0·16	47·94 ± 1·29	284·43 ± 2·25	ND
BSMR736	5·20 ± 0·56	4·09 ± 0·27	11·64 ± 0·73	ND	ND	36·44 ± 2·51	ND	ND	6·41 ± 0·33	44·42 ± 0·59	292·24 ± 1·16	ND
BSMR380	4·41 ± 0·65	2·41 ± 0·32	10·03 ± 0·29	ND	ND	44·02 ± 0·59	ND	ND	5·62 ± 0·41	72·23 ± 0·57	280·39 ± 2·20	ND
C11	5·18 ± 0·20	3·24 ± 0·37	10·84 ± 0·59	ND	ND	52·29 ± 0·20	ND	ND	4·82 ± 0·20	32·30 ± 0·33	188·43 ± 2·13	ND
PPE45-2[c]	6·84 ± 0·32	4·83 ± 0·30	11·21 ± 0·44	ND	ND	52·39 ± 0·02	ND	ND	6·01 ± 0·04	56·09 ± 1·73	219·87 ± 1·58	ND
Daithna (local)	3·62 ± 0·17	2·42 ± 0·16	10·02 ± 0·34	ND	ND	48·40 ± 0·33	ND	ND	7·21 ± 0·02	56·43 ± 1·17	284·34 ± 1·73	ND
ICPL87119	3·59 ± 0·33	2·1 ± 0·29	12·16 ± 0·51	ND	ND	44·23 ± 1·39	ND	ND	5·20 ± 0·08	40·22 ± 1·73	272·15 ± 0·10	ND
ICPL87	3·23 ± 0·12	1·61 ± 0·33	8·37 ± 0·19	ND	ND	40·28 ± 0·08	ND	ND	4·84 ± 0·42	52·38 ± 1·18	264·39 ± 1·22	ND
ICPL332[c]	5·41 ± 0·25	4·02 ± 0·32	10·04 ± 0·36	ND	ND	48·40 ± 1·22	ND	ND	6·37 ± 0·17	72·39 ± 1·73	236·24 ± 1·27	ND

[a] The above typical experiment was repeated at least three times.
[b] ND: not detectable.
[c] Pod borer tolerant cultivars.

inhibitor activities as well as in the inhibitor profiles of the different cultivars of mature seeds of pigeon pea (Pichare 1992; Pichare and Kachole 1994b). No detectable activities of these inhibitor proteins were found in the early developmental stages of the seeds in any of the cultivars analysed. The gel-X-ray contact print profiles of trypsin inhibitors did not show the presence of inhibitor bands in the seed extracts of early developing stage (stage 1, 11 DAF) in all the cultivars analysed (Fig. 1). One TI band was detected in the developing seed extract (stage II) of BDN1 (Fig. 1, lane h), two bands were detected in BDN2 (lane b) and three bands were detected in Daithna local (lane e). Profiles of the trypsin inhibitors present in the mature seeds are found to be identical (Fig. 1, lanes c, f, i and j). No significant differences were observed in trypsin-inhibitor profiles of these cultivars (results of all the cultivars are not shown). Only the intensities of trypsin-inhibitor bands varied in some of the cultivars on gel-X-ray contact print. This may be due to differences in extractibility or concentration of the individual inhibitor protein. However, total absence of any band was not detected in any of the pigeon pea cultivars studied. Thus, the results indicate that the trypsin-inhibitor profiles are identical in different pigeon pea cultivars and suggest that the inhibitors are late synthesising proteins. Substantial amylase activity insensitive to endogenous amylase inhibitors was also observed at these stages (Table 2). The authors have previously detected more than a dozen of amylase isozymes in developing seeds (Giri 1994). Developing seeds contain negligible amounts of proteases. Some samples were analysed for protease activity using caseinolytic method. Very low protease activity was detected in developing stage II (0·02, 0·04, 0·04 and 0·05 protease units per gram of seed powder of BDN2, BSMR175, BSMR380 and C11, respectively).

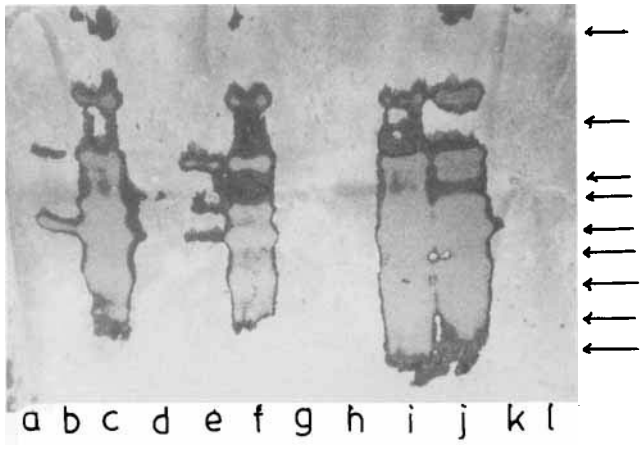

Fig 1. Photograph of gel-X-ray contact print of PAGE showing trypsin inhibitor bands in developing seed extracts of pigeon pea cultivars. Lane (a) BDN2 I, (b) BDN2 II, (c) BDN2 III, (d) Daithna Local I, (e) Daithna Local II, (f) Daithna Local III, (g) BDN1 I, (h) BDN1 II, (i) BDN1 III, (j) PPE45-2 III, (k) PPE45-2 II, and (l) PPE45-2 I. (About 100 μg protein was loaded in each lane.)

TABLE 2
Sensitivity of endogenous amylases to amylase inhibitors in mature pigeon pea seed in BDN2 cultivar

Extract	*SAIU*[a]	*AU*[b]
Mature seeds	6	ND[c]
Developing seeds (I)	ND[c]	52
Mature seed + developing seeds (I)	—[d]	53
Developing seeds (II)	ND[c]	300
Mature seeds + developing seeds (II)	—[d]	302

[a] SAIU: salivary amylase inhibitor unit.
[b] AU: amylase unit.
[c] ND: not detectable.
[d] —: not determined.

Two additional, slow-moving trypsin-inhibitor bands were detected in the extracts 10 DAG and were detectable up to 15 DAG in BDN2 (Fig. 2, lanes c and d). The appearance of new trypsin-inhibitor bands during germination may be due to partial degradation of pigeon pea trypsin inhibitors or these may be newly synthesised. Recently, it was reported that a protease is synthesised during seed germination in pigeon pea seeds (Godbole *et al* 1994b). The new trypsin inhibitors synthesised during germination may be involved in the regulation of protein hydrolysis. The trypsin inhibitor activity in germinating seeds is shown in Table 3.

Most insects attack pods at an early stage when pods contain developing seeds. The authors, therefore, feel that it is important to study the synthesis of these inhibitors, as well as other defence components while selecting a variety for pest/pathogen resistance. The

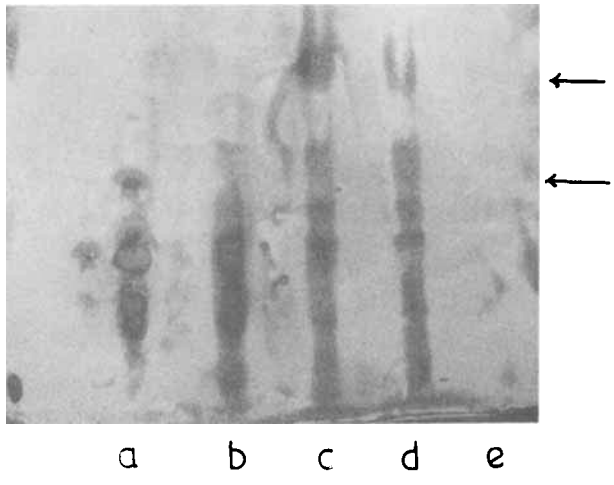

Fig 2. Photograph of gel-X-ray contact print of PAGE of trypsin inhibitor bands in extracts of germinating seeds of BDN2. Lane (a) 0 DAG, (b) 5 DAG, (c) 10 DAG, (d) 15 DAG, and (e) 20 DAG. (About 120 μg of protein in each lane.)

TABLE 3
Inhibitors of trypsin and amylases in germinating seeds of BDN2 cultivar of pigeon pea

Germination day	*TIU g^{-1} of powder*	*AIU g^{-1} of powder*
0	40	6·0
5	24	4·0
10	12	2·0
15	2·8	0·8
20	ND[a]	ND[a]

[a] ND: not detectable.

temporal and spatial synthesis of these proteins is very important because most of the insect attacks are on developing and mature seeds. It is obvious that a variety producing higher amounts of protease inhibitors and amylase inhibitors at early stages of seed development will be preferred in developing insect-pest resistant varieties provided these inhibitors are able to inhibit the pest midgut proteases and amylases.

Recently, Godbole *et al* (1994b) have reported detection of a protease inhibitor in a pigeon pea variety (TAT-10) after 7 days of flowering. However, in stage II (27 DAF), few inhibitor bands are seen but the activity is not detectable (Table 1). If the above-said early appearance of trypsin inhibitor is a unique feature of the variety TAT-10 of pigeon pea it may be exploited for defence against insect pests. TAT-10 is reported to have low resistance (51.50% pod damage) to *H armigera* (Anon 1990–1991). This method detects up to 10 ng of trypsin inhibitor. The method used by Godbole *et al* (1994a,b) was based on a solution assay using synthetic substrate. The possibility of interference of non-protein inhibitors of trypsin in the solution assay cannot be ruled out in this case, as homogenates of developing seeds were directly used for determination of inhibitor activity.

Most of the pigeon pea cultivars are susceptible to insect attacks and heavy damage occurs due to these pests. Interestingly all the cultivars contain inhibitors of proteases and amylases. The pests might have evolved in such a way that these inhibitors now fail to inhibit pest mid-gut proteases and amylases. Not only early developing seeds are vulnerable to the pests but the mature seeds are also heavily damaged by storage pests. Although the insect proteases and amylases are from the same group (serine proteases and alpha-amylases, respectively), they have different pH optima, temperature optima and other conditions (Gatehouse *et al* 1986; Purcell *et al* 1992). A purified inhibitor of pigeon pea has been reported to have very low affinity towards *H armigera* proteases as compared to soya bean and other inhibitors (Godbole *et al* 1994a). But these inhibitors strongly inhibit bovine trypsin. Similarly, Gatehouse *et al* (1986) have observed different specificities of wheat amylase inhibitor molecule towards amylases of different storage peats and found that they also have different effects *in vivo* and *in vitro*. It has also been studied that different classes of monomeric amylase inhibitors show high homology in their amino acid sequences despite differences in specificities against insect amylases (Gomez *et al* 1991).

Developing pigeon pea seeds contain abundant amylase activity (Table 1). Amylases involved in starch metabolism are synthesised in developing seeds and may be specific for the stage of development. Lower levels of amylases in early development indicate lower level of starch metabolism. Amylase activity was not detected in mature dry seeds. More than 10 amylase isozymes were identified in developing pigeon pea seeds (Giri 1994). If the seed amylases are active in insect guts, their contribution to the digestive capabilities of insect pests is likely to make up (at least in part) the losses of insect gut amylases due to seed inhibitors. This may foil the attempts of utilising amylase inhibitors as defence proteins in young developing seeds. Therefore, it would be interesting to see if the seed amylases are active in the presence of amylase inhibitors in insect guts. A similar contribution of low levels of endogenous seed proteases to protein digestion in insect gut may be significant as they are not inhibited by pigeon pea inhibitors (Godbole *et al* 1994b).

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