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AMYLASE INHIBITORS OF PIGEONPEA (*CAJANUS CAJAN*) SEEDS

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Key Word Index—*Cajanus cajan*; Leguminosae; Pigeonpea; *Helicoverpa armigera*; seed development; germination; amylase inhibitors.

Abstract—Pigeonpea (*Cajanus cajan* L) seeds were analysed quantitatively for amylase inhibitor (AI) activity and qualitatively, by an in-gel-detection method on polyacrylamide gels. At least four AI isoforms were identified in pigeonpea seeds. The AIs inhibit human salivary and bovine pancreatic amylase but fail to inhibit bacterial, fungal and endogenous amylase. Pigeonpea AIs were found to be active over a pH range of 4.5 to 9.5 and were heat labile. The isoelectric point of a major inhibitor is 6.2. AIs were tolerant to proteolysis by trypsin, chymotrypsin, bromelain and endogenous pigeonpea proteases. Pigeonpea AIs were synthesized during late seed development and also degraded during late germination. Addition of AIs or protease inhibitors (PIs) alone to a diet of *Helicoverpa armigera* larvae did not increase mortality. However, the larvae reared on a diet containing AIs and PIs in combination, showed increased mortality and adverse effects on larval growth and development. *In vitro* inhibition of *Helicoverpa* gut amylase revealed that only 22% activity is sensitive to inhibitors. Further investigations on interactions of pigeonpea AIs and PIs with *Helicoverpa* gut enzymes is necessary to develop strategies to strengthen defense mechanisms in pigeonpea against *H. armigera*

INTRODUCTION

Pigeonpea is a multipurpose grain legume crop grown by resource-poor farmers in many developing countries of semi-arid tropics and subtropics. It is important in human nutrition as a rich source of dietary protein [1, 2]. Preharvest damage due to insect pests on developing seed and postharvest losses due to storage pests are severe in pigeonpea [3]. Therefore, we are biochemically studying in-built defense mechanism of pigeonpea which could help to develop strategies to strengthen natural mechanism to protect huge losses of this pulse grain.

The amylase inhibitors (AIs) and protease inhibitors (PIs) have gained attention as possible tools of natural and engineered resistance against pests and pathogens [4, 5]. Two approaches are fast emerging in order to develop resistance against insect infestation, employing inhibitors of amylases and proteases. The first approach is to induce hyper synthesis of inhibitors at the most vulnerable stage of insect attack in the target tissue, while the second involves development of transgenic plants expressing heterologous inhibitors. The most studied AIs of common bean (*Phaseolus vulgaris*) among the legumes were shown to have toxic effects on several insect pests [6–8]. Various strategies were built around them to reinforce defense system in plants. Transgenic tobacco, pea and azuki bean showed total resistance against important pests when AI gene of common bean was over expressed in them [9–12].

Only a few reports are available on pigeonpea PIs [13–18] and AIs. Singh *et al.* [2] studied AI activity in the vegetative parts and grains of pigeonpea, and showed that it accumulates only in mature seeds. Our earlier reports deal mainly with pigeonpea PIs [16–18]. We have developed a simple and sensitive in-gel-detection method for AIs using starch–polyacrylamide gels [19] and have extensively used this method to characterize pigeonpea AIs. The present communication consists of the study of the nature, number, specificities and distribution of AIs. Inhibition potential of AIs on *Helicoverpa armigera* gut amylases was assayed *in vitro* while their *in vivo* effects were evaluated by feeding larvae on a diet containing pigeonpea AIs.

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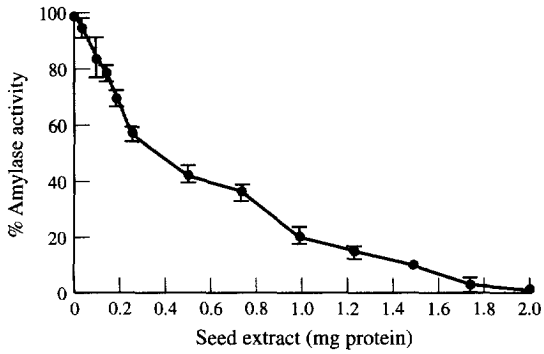

Fig. 1. Inhibition of human salivary amylase by pigeonpea seed proteins (AIs)

RESULTS AND DISCUSSION

AIs of pigeonpea seeds

The inhibition of human salivary amylase by pigeonpea buffered extract is shown in Fig. 1. Amylase activity in the absence of extract was taken as 100%. Preincubation of amylase with the extract for at least 20 min is necessary for optimal inhibition. The pigeonpea extract did not exhibit any differences in the inhibition of human salivary amylase and bovine pancreatic amylase but failed to inhibit bacterial, fungal and endogenous (from developing and germinating seeds of pigeonpea) amylases. Extracts of 18 individual seeds did not show a significant difference in the degree of inhibition (content of AI activity, 20.7 ± 2.4 units g^{-1} dry wt). PVP is known to precipitate polyphenols. The extracts in presence and absence of PVP had similar AI activity. This indicates that the AI activity in the extract of pigeonpea seeds is not related to free poly phenols [20].

$(NH_4)_2SO_4$ fractionation of seed extracts yielded fractions (0–40%, fraction I; 40–60%, fraction II; 60–80%, fraction III and 80–100%, fraction IV) which were analysed quantitatively for AI activity by assay and qualitatively on electrophoretic gel. The fraction I had 71% AI activity and fraction IV had the remaining 29% activity. The fractions II and III did not have detectable AI activity. However, fraction II (40–60%) had major PI activity. The fractions possessing AI activity produced four AI bands on starch–polyacrylamide gel (Fig. 2). Densitometric scanning of activity-stained gel revealed that a fast moving band in fraction I contributes only 10% activity, the rest of the activity was shared by slow moving AI band of fraction I. However, two broad zones of AI activity were observed in the fraction IV (Fig. 2, lane 2) These results indicate that the slow moving AI in the 0–40% fraction pigeonpea amylase inhibitor-I (PAI-I) is the major amylase inhibitor of pigeonpea seed (Fig. 2, lane 1).

Biochemical properties of pigeonpea AIs

Pigeonpea AIs were found to be stable at 37°C for several h. A consistent increase in inhibitor activity

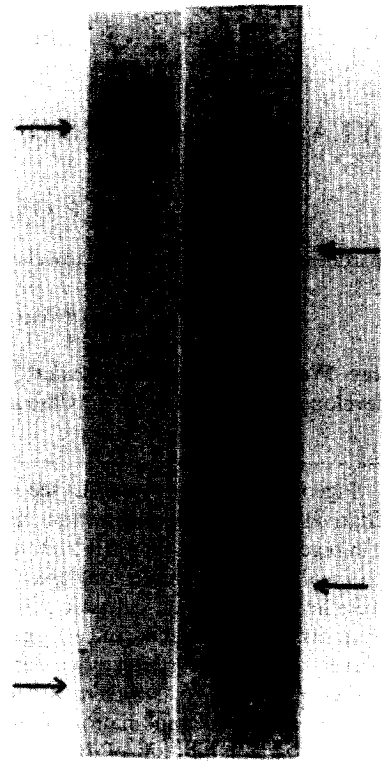

Fig. 2. Scanned print of starch–polyacrylamide gel of amylase inhibitor bands of pigeonpea. Starch–PAGE were performed on 7% native PA gel containing 0.5% soluble starch. After electrophoresis gel was equilibrated in the assay buffer for 10 min followed by incubation in salivary amylase solution for 30 min. The gel was washed in the water and stained with iodine solution as positive staining of unhydrolysed starch in the gel for AI activity bands [19] (a) 0–40% $(NH_4)_2SO_4$ fraction of amylase inhibitor (200 μg protein); (b) 80–100% $(NH_4)_2SO_4$ fraction of amylase inhibitors (200 μg protein). Proteins are migrating downwards.

was observed after subjecting AIs at 60° for 10 min (Table 1). However, heating at 80° for 10 min tends to destroy inhibitor activity. Heating the extract in boiling water for 8 min totally destroyed inhibitor activity (Fig. 3).

The pigeonpea AI activity in seed extract was found to be stable and active over a wide range of pH (4.5 to 9.5). Maximum inhibition of salivary amylase was observed at pH 7.0 (results not shown). The isoelectric

Table 1. Effect of heat on amylase inhibitor activity of pigeonpea seed extract

	Amylase inhibitor activity units X 10^{-2}		
	Heat time (min)		
Temp.	0	5	10
40°	27 ± 0.8*	30 ± 1.1	30 ± 1.5
60°	27 ± 0.8	34 ± 0.7	39 ± 1.6
80°	27 ± 0.8	30 ± 0.7	23 ± 1.2

* Each value is mean of five replicates $\pm$ S.D.

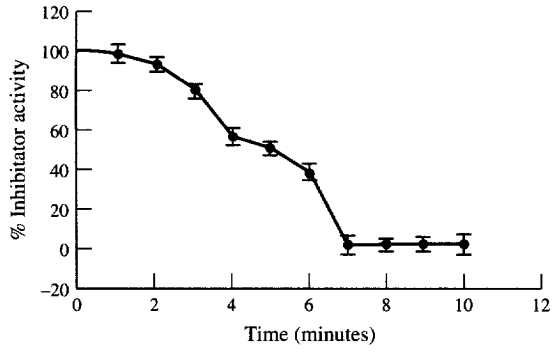

Fig. 3. Heat inactivation of AI activity in the seed extract of pigeonpea as function of incubation time at 80°.

point of the major AI determined by activity staining after IEF gel was 6.2. Incubation of AIs with 2-mercaptoethanol destroys inhibitor activity (results not shown). During the electrophoretic analysis of crude extracts on starch–polyacrylamide gel, the inhibitor activity was observed to streak conspicuously on the gel (Fig. 4, lane 1). Different protease treatments of the extracts resulted in the formation of new active AI fragments (Fig. 4, lanes 2, 3 and 4). Longer incubation of AIs with a higher concentration of proteases failed to further hydrolyse these active fragments indicating that these fragments are resistant to proteases. Similar protease resistant AIs of pearl millet (*Pennisetum typhoideum*) [21] and of white bean (*Phaseolus lunatus*) [22] have been reported.

Fig. 4. Starch–PAGE of amylase inhibitor of the pigeonpea seed extract. AI bands were visualized as described in the legend of Fig. 2. and in the text. (a) crude extract (b) bromelain treated seed extract (c) trypsin treated seed extract (d) chymotrypsin treated seed extract (All 210 μg protein). For details of proteases treatment to the extract see text. Proteins are migrating downwards.

Physiological changes in AI activity

AI activity was not detectable in the seed extracts at 20 days after flowering (DAF). However, at 40 DAF inhibitors were detectable which increase further at 50 DAF and in mature seeds sequentially (Table 2). In the extracts of developing seeds at 40 and 50 DAF, significant amylase activity was observed. The amylase activity of developing seeds was insensitive to endogenous inhibitors. The activity reached its maximum at 40 and 50 DAF and which was drastically reduced in mature (dry) seed (Table 2). The electrophoretic analysis of these extracts on starch–polyacrylamide gels reveals at least 10 amylase isoenzymes which hydrolysed starch during the electrophoretic run. Although the pigeonpea AIs appeared to be ineffective against endogenous amylases, the specificity of the inhibitors against individual amylase was not determined. Proteins released during soaking of seed, extracts of seed coat, leaves and flowers did not show detectable inhibitor activity. However, AI activity was observed in the extracts of cotyledons and in embryo. The inhibitor activity decreases with time during germination of seeds. Inhibitor profiles on starch–polyacrylamide gel at 5, 10, 15 and 20 days after germination (DAG) are shown in Fig. 5. The area and intensity of the activity bands reduced progressively during germination. This may be due to proteolysis of AIs. The action of endogenous proteases synthesized during germination on inhibitor was very similar to the action of bromelain, trypsin or chymotrypsin as all of them produced similar population of active fragments (Figs 4 and 5).

Role of AIs in insect–pest resistance

In order to understand the contribution of AIs in pigeonpea defense mechanism, their effects on growth and development of *Helicoverpa* larvae were assessed by feeding them on a diet formulated with $(NH_4)_2SO_4$ fractionated extract, fraction I (0–40%) containing AIs and fraction II (40–60%) containing PIs. The critical observations are presented in Table 3. The larval growth and development were affected in the group fed on the diet containing both fractions (fraction I and II, AIs and PIs) in combination. Higher mortality was also evident in this group. However, AIs or PIs alone did not have any adverse effect on *Helicoverpa* larvae (Table 3).

In vitro inhibition of *Helicoverpa* gut amylase (HGA) by pigeonpea AIs reveals that only 22% activity was sensitive to the inhibitors. HGA analysis of starch–polyacrylamide gel showed at least four amylase isoenzymes (unpublished). The low inhibition of HGA by pigeonpea seed extract may be due to some inhibitor-insensitive amylase in gut extract or due to degradation of AIs by *Helicoverpa* gut proteases. The higher mortality of larvae reared on a diet containing both AIs and PIs in combination, than either AI of PI alone, may be due to protection of AIs

Table 2. Endogenous amylase and salivary amylase inhibitor activity in developing seed extracts of pigeonpea seeds (BDN2)

Days after flowering	Protein (mg)*	Endogenous amylase activity units*	Salivary AI activity units*
20	55.4±4.2	0.6±0.1	ND†
40	68.4±5.2	90.8±7.2	14.8±1.8
50	79.0±4.5	87.5±5.4	25.6±2.0
Dry seed	90.0±6.6	2.6±0.3	32.5±2.2

* Each value is mean of three replicates ± S.D., per gram of seed powder.
† ND = No activity detectable.

Fig. 5. Starch–PAGE of amylase inhibitors in germinating seed extracts. Visualization of AI bands were described in legend of Fig. 2 and in the text. (a) dry seed extract (b) 5 DAG (c) 10 DAG (d) 15 DAG (e) 20 DAG (all 200 μg protein). Proteins are migrating downwards.

from gut proteases and/or their synergetic effect on *Helicoverpa* larvae.

The presence of AIs in beans was noted as early as 1945 [23] but only recently have they received attention in other legumes [19, 24, 25]. Common bean AIs are well documented and have shown their tremendous potential to increase plant defense against insect pests using various approaches [6–12]. Two studies on the presence of AI activities in pigeonpea and chickpea were reported earlier [2, 24]. However, detailed investigations on AIs have not been carried out. This is mainly because of the difficulty of detecting AI activity in presence of endogenous amylases and the non-availability of a in-gel-detection method to study various isoforms. The only method available for detection of AI isoforms was immunostaining after electrophoresis. This requires anti-bodies which could be obtained only after purification of the protein. Moreover, in some cases the immunodetection gives false signals due to cross reactivity with other closely related proteins. This report is the first attempt to visualize and characterize pigeonpea AIs using a novel in-gel-detection method [19].

At least four AIs were identified in pigeonpea seeds. The isoelectric point of the major AI (PAI-1) is 6.2. The inhibitors were found to be heat labile. However, a slight increase in the inhibitor activity was observed at 60° for 10 min, this may be due to irreversible change(s) in conformation leading to an increase in the number of available active sites rather than native inhibitor(s). Release of conformation constraints followed by activation after proteolytic cleavage in bean

Table 3. Effect of feeding pigeonpea seeds AIs, PIs and both (AIs and PIs) in combination on *Helicoverpa* larva

Days	Control	AIs	PIs	AI + PI in combination
No. of larvae	33	33	33	33
6th day				
Dead larvae	2 (6%)	2 (6%)	4 (12%)	5 (15%)
Surviving larvae	31	31	29	28
12th day				
Dead larvae	9 (27%)	10 (30%)	14 (42%)	20 (60%)
Surviving larvae	24	23	19	13

The experiments were repeated thrice. The parentheses figures are mortality of larvae in percent.

AIs has been reported [26]. Changes in pigeonpea AIs associated with heating are not known. AIs with activity against endogenous amylases involved in metabolic regulation of starch are known in many cereal plants [27]. However, present results indicate that endogenous amylases are insensitive to the inhibitors. The possibility of having activity against some amylases is not ruled out as endogenous amylase preparation contains several amylase isoenzymes.

AIs showed toxicity to a variety of insects when fed on AI-rich diet, however, their toxic effects vary among the species of insects and also with AI isoforms [4, 7, 28, 29]. This reveals divergence in the properties of AIs prevail although they are closely related [30]. In the present experiment, a diet containing AIs does not show any adverse effect on larval growth. The pigeonpea AIs could inhibit only 22% of HGA activity. Four amylase isoenzymes were detected in HGA (data not shown). It seems from these results that some amylases may not be inhibited by pigeonpea AIs. However, possibility of having inhibitor-insensitive amylases in HGA needs further investigation. Adaptation of *Zabrotes subfasciatus* to AIs of common bean by producing inhibitor insensitive amylases is known [7]. Ishimoto and Chrispeels [8] recently reported that *Z. subfasciatus* protect themselves from common bean AIs by secreting protease that are able to degrade inhibitors. Similarly, *Helicoverpa* larvae adapt to chickpea PIs by producing proteases that are not only insensitive to protease inhibitors but have ability to digest them (Giri *et al.*, unpublished).

EXPERIMENTAL

Plant material. Pigeonpea seeds of cultivar BDN-2 were obtained from Agricultural Research Station (ARS) of Marathwada Agricultural University, Parbhani at Badnapur, District Jalna, India. Seeds were germinated in paper folds and cotyledon and other tissues were harvested after 5, 10, 15 and 20 days. Opened flowers were tagged on 0 day and developing pods of 20, 40, 50 and 60 DAF were harvested. Fresh full grown leaves and flowers of *ca* 100-day-old plant were collected. Plant tissues were stored at −20° until further use.

Protein extraction. Dry pigeonpea seeds were ground to obtain a fine flour. The flour was defatted with Me_2CO and hexane. Defatted flour with added 1% PVP was suspended in H_2O or in 0.02 M Na-Pi buffer, pH 6.9 (six vol.) and was stirred for about 2 hr. The suspension was centrifuged at 12000 *g* for 30 min and dialysed against extracting soln with 3 changes. The ppt. obtained was removed by centrifugation and the supernatent was used for analysis. Extract was processed for fractionation by adding solid $(NH_4)_2SO_4$ for 40% satn and left at 4° overnight. The ppt. pellet was collected by centrifugation. Supernatant was used for further fractionation at 60, 80 and 100% $(NH_4)_2SO_4$ satn. Protein pellets obtained at each step were dissolved in minimum vol., dialysed and analysed for AIs and PIs.

BDN-2 seeds were surface sterilized with EtOH and allowed to soak in (1:5) H_2O with added 1% PVP at 55° for 2 hr. Seeds were removed and the H_2O used for soaking was centrifuged and clear supernatant was analysed for released AI activity. Germinating and developing seeds, seed coat, cotyledons and embryos of mature seeds, leaves and flowers were homogenized with chilled Me_2CO in Polytron Homogenizer, filtered, washed with Me_2CO and hexane and allowed to dry at room temp. (27°). The dry powder was extracted in H_2O or in the buffer as described above. The protein concn was measured according to ref. [31].

Extraction of Helicoverpa *gut amylase.* Actively feeding *Helicoverpa armigera* larvae of 4th or 5th instar were dissected and midguts were collected. The midguts were extracted (1:3) in 0.02 M Na-Pi buffer, pH 6.9 containing 10 mM NaCl for 2 hr and centrifuged. The supernatant was dialysed against the buffer and was used as source of larval gut amylase.

Inhibitor assays. The amylase and amylase inhibitory activities were estimated using a modification of the methods in ref. [32] as described in ref. [19]. One amylase activity unit is defined as the activity resulting into liberation of 1 mg of maltose from starch at pH 6.9 at 37° in 3 min. One amylase inhibitor unit is one amylase-unit inhibited under the given assay conditions. Protease inhibitor activity of the extract and $(NH_4)_2SO_4$ ppt. protein was estimated using caseinolytic assay as described earlier [17, 18].

Inhibitor stability. The extracts of pigeonpea seeds were incubated at 100° up to 10 min, and at 40, 60 and 80° for 5 and 10 min. The extracts were centrifuged and supernatants were analysed for inhibitor activity. pH stability of amylase inhibitors was studied by subjecting the extracts to different pH (4.5 to 9.5) for 24 hr (0.1 M NaOAc buffer, pH 4.5 and 5.0; 0.1 M Na-Pi buffer, pH 6.0 and 7.; 0.1 M Tris–HCl buffer, pH 8.0 and 9.0 and 0.1 M Tris–glycine buffer, pH 9.5). Similarly salivary amylase was incubated in the buffer at respective pH and assayed. Extracts containing AIs were incubated with different protease soln (bromelain 1.6 mg ml^{-1} in 0.1 M NaOAc buffer, pH 4.5; trypsin and chymotrypsin 0.8 mg ml^{-1} in 0.1 M tris-HCl buffer pH 7.8) for 30 min at 37° and analysed electrophoretically for AI activity fragments.

Electrophoretic analysis and visualization of AI bands. Extracts and $(NH_4)_2SO_4$ fractionated proteins were analysed by electrophoresis in non denaturing, basic, 7% polyacrylamide gels containing 0.5% soluble starch using Davis buffer system [33]. After electrophoresis the gels were equilibrated in amylase assay buffer for 5 min, incubated in amylase soln (20 units ml^{-1}) for 30 min at 37°, washed with H_2O and stained in I_2 soln (10 mM I_2 in 14 mM KI) for 5 min [19]. Excess I_2 was washed off with H_2O. The gels were then photographed, or stored in polyethylene sheets. The stained gel was wrapped in cellophane and scanned

by Laser densitometer for quantitative determination of AI activity (The Helium–Neon Laser source of Ultrascan XL, LKB, Sweden produces a beam of monochromatic light at 633 nm).

Diet preparation. Composition of diet for larvae was as follows: for 300 ml diet, seed flour (non-defatted), 31.5 g; yeast extract, 1.25 g; ascorbic acid, 1.08 g; sorbic acid, 0.60 g; methylparahydroxybenzoate, 0.60 g; tetracycline, 0.67 g; B-complex, 2 tablets; formalin (40%), 0.2 ml were dissolved in 250 ml H_2O or aq. extracts containing AI, PI or both in combination. Agar (4.9 g) was melted in 50 ml of H_2O and mixed with components of diet. About 10 ml diet was poured into disinfected plastic containers and allowed to solidify. One larva was placed in each container having perforated (pin holes) plastic cap [34]. In a typical experiment, 30–40 larvae were reared on each diet composition. The experiments were repeated at least $\times 2$. A meticulous record of observations was maintained.

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