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Low Trypsin and Chymotrypsin Inhibitor Mutants in Winged Bean (*Psophocarpus tetragonolobus* (L) DC)

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Abstract: Low trypsin inhibitor (LTI) and chymotrypsin inhibitor (LCTI) mutants have been identified and isolated from the M_5 mutant lines of winged bean. The LTI and LCTI lines demonstrated a true breeding feature in the subsequent generations. Three to five isoinhibitor bands of trypsin and chymotrypsin were absent in different mutants, indicating mutations in the respective gene loci. The LTI and LCTI mutants inhibited insect gut proteases as efficiently as their control counterparts. This could be attributed to a specific group of inhibitors present in all the mutant types.

Key words: trypsin inhibitor, chymotrypsin inhibitor, winged bean, mutants.

INTRODUCTION

The winged bean (*Psophocarpus tetragonolobus* (L) DC) is a potential crop offering scope to diversify the cultivation of high-protein legumes in tropical regions. All parts of the plant, including dry and green seeds, young pods, flowers, leaves, tubers, are edible and highly nutritious. The seed protein and oil content in winged beans (WB) have been reported to be 28–40% and 15–18%, respectively (Pospisil *et al* 1971; Masefield 1973).

Unlike soya beans, the WB contains a specific chymotrypsin inhibitor (CTI) along with trypsin inhibitors (TI) (Kortt 1980) and it becomes necessary to inactivate the TI and CTI before consumption. Autoclaving and boiling water treatments are known to destroy about 95% of the TI activity. The microwave heating for 10 min and the dry heat treatment (100°C for 60 min) on the other hand, could not produce any effect on WB protease inhibitors (Kadam *et al* 1987).

Inhibitors of trypsin and chymotrypsin have been implicated in reducing protein digestibility and in pancreatic hypertrophy (Liener 1976). The conventional cooking methods, effective against soya bean inhibitors, have been found to be ineffective as regards the WB inhibitors owing to their thermal stability (Chan and de Lumen 1982; Tan *et al* 1984) and cooking difficulties (King and Puwastein 1984; Kailasapathy *et al* 1985).

Thus, it is desirable to have WB genotypes with reduced levels of protease inhibitors. This communication reports mutants with reduced levels of TI and CTI with the hope that they would help improve the nutritional value of WB.

MATERIALS AND METHODS

Dry, mature seeds of WB, variety iiHp Sel-21 and its true breeding M_5 mutants obtained after treatments of ethyl methane sulphonate (EMS: 0·05 and 0·10%) and gamma-rays (5 and 10 kR), were ground to a fine powder, defatted with hexane and the meal was air dried. *N*-Benzoyl DL-arginine *p*-nitroanilide HCL (BAPNA), *p*-glutaryl-L-phenylalanine-4-nitroanilide (GLUPHEPA), polyvinylpolypyrrolidone (PVP), bovine trypsin treated with L-tosylamide-2-phenylethyl-chloromethyl ketone (TPCK) were obtained from Sigma Chemical Co (St Louis, MO, USA). The X-ray films were procured from Lazor (Selvasa, India).

Protein extraction

Defatted seed flour of WB was added to six volumes of distilled water and 2% PVP and allowed to stand for

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2 h at room temperature with intermittent shaking. The suspension was centrifuged at 6500 g for 30 min at 4°C to remove particulate matter and the clear supernatant was used for further studies. Protein concentration in the extracts was estimated by the Folin–phenol method (Lowry *et al* 1951) and occasionally by absorbance at 280 nm.

Enzyme inhibitor assay

For measuring trypsin inhibitory activity 20 μg of trypsin was mixed with appropriate quantity of inhibitor-containing extract (so as to inhibit 50–60% trypsin) and incubated at 25°C for 5 min. Residual trypsin activity was measured by using 1 ml of 1 mM BAPNA (Erlanger *et al* 1961). The reaction was terminated after 10 min by adding 200 μg of 30% acetic acid. To measure 100% trypsin activity, the inhibitor-containing extract was excluded from the above steps. The liberated *p*-nitroanilide was measured at 410 nm on a UV-160A Shimadzu spectrophotometer. The chymotrypsin inhibitory activity was measured by assaying residual enzyme activity by using GLUPHEPA as a chromogenic substrate (Mueller and Weder 1989). Both trypsin and chymotrypsin were assumed to be 100% active.

Electrophoresis

WB seed extracts were analysed by electrophoresis on 12% polyacrylamide gel slabs using Davis system (1964) in a Hoeffer SE 400 vertical slab gel apparatus. The protease inhibitor bands were visualised after electrophoresis by placing the gel in equilibration buffer (0·1 M Tris-HCl, pH 7·8) for 5–10 min. The gel was subsequently incubated in 0·1 mg ml^{-1} trypsin or chymotrypsin solution (in equilibration buffer) for 5 min, rinsed briefly in buffer and placed on an undeveloped X-ray film. The appearance of inhibitor bands on X-ray film was monitored visually after washing the hydrolysed gelatin with warm water (Pichare and Kachole 1994). For proteins the gels were stained with Coomassie brilliant blue R-250.

Inhibition of *Helicoverpa* protease

Helicoverpa larval guts stored at −20°C were extracted in 50 mM Tris-HCl buffer, pH 8, centrifuged and treated as protease solution. The inhibitor assay was carried out in glycine–NaOH buffer, pH 10 using BAPNA as the substrate (Godbole *et al* 1994).

RESULTS AND DISCUSSION

The WB mutants were analysed for the activity of trypsin and chymotrypsin inhibitors and against *Helicoverpa* gut proteases. The results are presented in Table 1. Significant changes were observed in different mutants for inhibitory activity against bovine trypsin and chymotrypsin. In case of the mutant showing flat pod and triangular leaves, the TI activity decreased to about 40% level. In linear leaf/flat pod mutant the CTI activity showed nearly four-fold reduction. The most important feature of all these LTI and LCTI mutants has been that, they all inhibited *Helicoverpa* protease to the same extent.

The electrophoretic profiles of the trypsin inhibitors (Fig. 1) revealed nine isoinhibitors in control (lane a). Except in linear leaf mutant (lane b), the isoinhibitor '1' was absent in all other mutants. The flat pod mutant (lane e) was lacking in isoinhibitor '7'. The triangular leaf mutant (lane f) exhibited least number of isoinhibitors (TI numbers 4, 5, 6 and 9 were present while the others were missing). The chymotrypsin isoinhibitors (Fig. 2) showed 8 isoinhibitors in control (lane d). The isoinhibitor '1' was absent in all the mutants, while inhibitors 6 and 8 were noticeable in all the mutants. The linear leaf mutant (lane b) showed a modified inhibitor with altered mobility (positioned between bands 3 and 4). The triangular leaf (lane f) and linear leaf/flat pod mutant (lane e) showed least number

TABLE 1
Protease inhibitor in winged bean mutants (mean ±SD)

Mutants	*Mutagen*	*Protein mg g^{-1} of seed*	*TIU per mg protein*	*CTIU per mg protein*	*HPIU[a] per mg protein*
Control	—	256·3 ± 0·26	64·2 ± 0·30	158·5 ± 0·55	97·8 ± 0·80
Flat pod	10 Kr gamma rays	230·4 ± 0·49	39·7 ± 0·63	144·2 ± 1·10	97·5 ± 0·95
Linear leaf	0·10% EMS	278·8 ± 1·07	63·3 ± 0·45	156·6 ± 0·30	97·9 ± 0·75
High yielding/long pod	0·05% EMS	234·4 ± 0·50	49·1 ± 0·60	102·3 ± 0·76	95·8 ± 0·40
Linear leaf/flat pod	5 Kr gamma rays	200·3 ± 1·27	42·7 ± 0·47	39·3 ± 0·58	82·9 ± 0·60
Triangular leaf	0·05% EMS	256·6 ± 0·85	38·3 ± 0·95	114·5 ± 0·70	93·4 ± 0·86

[a] HPIU, *Helicoverpa* protease inhibitor units.

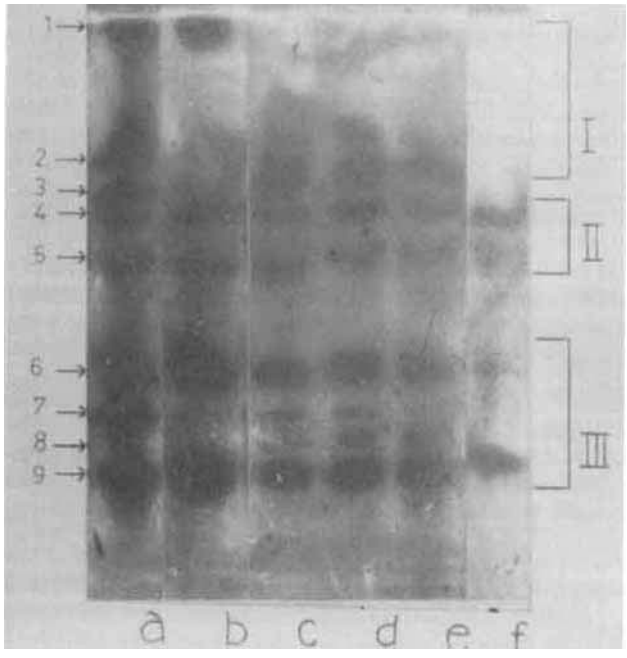

Fig 1. PAGE of TI in winged bean: (a) control, (b) linear leaf, (c) high yielding/long pod, (d) linear leaf/flat pod, (e) flat pod, and (f) triangular leaf. Protein (40 μg) was loaded in each lane. (X-ray contact print.)

of CTI (6, 7 and 8) while others were missing. Every missing inhibitor band indicated occurrence of mutations in the pertinent genes. The altered, relative proportions of isoinhibitors seemed quite evident from the changes in the sequence of their appearance on the X-ray film.

The lower activity shown by the mutants can be attributed to the lower protein content (Table 1). Although few mutants exhibited lower inhibitory activity, equal amount of protein in such mutants showed significant positive shift in inhibitor-protein proportion. Any change in extractibility of the inhibitor proteins could also result in altered inhibitor profile. According to Bacon *et al* (1995), the trypsin inhibitor genes respond greatly to the environmental conditions. If the mutants happen to respond differentially to the environment, it can be hoped that changes in TI activity could very well develop in them.

Very few reports are available with regard to mutations and TI–CTI genes in plants. Lazaro *et al* (1985) reported adverse action of the high lysine mutation on TI genes in barley. The same mutation, however, revealed increased chymotrypsin inhibitory activity by 20-fold (Williamson *et al* 1987). An 'opaque-2' mutation in maize has been found with enhanced trypsin inhibitor level (Halim *et al* 1973). Lowered levels of trypsin inhibitors have been reported in barley mutants induced by pesticidal treatments (Harsulkar 1994). It would not be possible to arrive at any conclusion regarding the nature of activities of TI and CTI genes after mutations until such studies in different systems would become available. Reports that are available at the moment provide conflicting evidence. In the present study no correlation could be established between lower/higher levels of TI, CTI and morphology of mutants. The mutability of trypsin and chymotrypsin inhibitor genes have been found to be different. The chymotrypsin locus has been found to be more prone to mutations. Some of the double headed trypsin–chymotrypsin inhibitors demonstrated loss of CTI activity and retention of TI activity (Figs 1 and 2). Such findings can be considered as an interesting feature of the present work. In WB the TI profile could be divided in to three groups, namely I, II and III (Fig 1) based on their mutability characteristics. The group I inhibitors (numbers 1–3) tend to get altered more frequently after the mutagenic treatments, followed by group III inhibitors (numbers 6–9). The group II inhibitors (numbers 4 and 5) on the other hand are extremely stable. Study of several mutants of winged bean has confirmed this fact. The constancy in inhibition of *Helicoverpa* proteases appears to be due to the group II inhibitors. It is felt that further studies of such inhibitors (group II) can prove extremely useful in understanding the defence proteins in plants, and can possibly be exploited to build resistance in them.

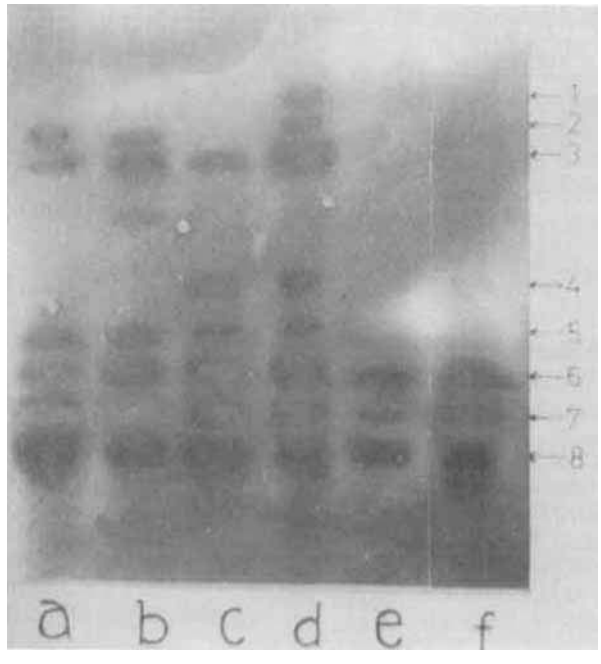

Fig 2. PAGE of CTI in winged bean mutants: (a) flat pod, (b) linear leaf, (c) high yielding/long pod, (d) control, (e) linear leaf/flat pod, and (f) triangular leaf. Protein (40 μg) was loaded in each lane. (X-ray contact print.)

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