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Trypsin and chymotrypsin proteinase inhibitors of arid legumes

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ABSTRACT

Trypsin and chymotrypsin inhibitor activities were studied using cowpea, cluster bean, babul and algaroba seeds. Results of this study indicated that the mean value obtained for all the accessions examined was 52.84±3.05 TIU/mg in *Acacia senegal* which was significantly higher than that found for the other arid legumes studied. The majority of the cowpea accessions examined fell within the range of 15-45 TIU/mg and the significantly highest trypsin inhibitory activity (8.03±0.55 TIU/mg) was found in accession, IC-311403. In *Acacia senegal*, the trypsin inhibitors content ranged from 34.77±0.09 in IC-330522 to 85.45±7.02 TIU/mg in local cultivar. Algaroba and cluster bean have not shown any chymotrypsin inhibitory activity. Significant variability for chymotrypsin inhibitor content was observed among the various accessions of cowpea and *A. senegal* species. The accessions of arid legumes including tree legumes were found to be better and hence could be explored for further development and selection of good sources of PIs for insect resistance in transgenic crops.

Key words: *Acacia senegal*, Arid legumes, Chymotrypsin inhibitors, Cowpea, Transgenic crops, Trypsin inhibitors

Arid legumes are recognized for taming droughts, sustaining soil health and diversifying agriculture and for providing organic food, feed and variety of products of industrial use of export value (Kumar 2005). Pulses have nutritional and worldwide commercial importance because they are easy to store and are rich in protein and fiber. However, their nutritional value is limited by the presence of antinutritional factors such as proteinase inhibitors (PIs), saponins, lectins, phytate, tannins, L-DOPA, some of phenolic compounds and hydrogen cyanide act as barriers to the use of legumes as food or forage (Pugalenthi *et al*. 2005).

Proteinase inhibitors of plants are small molecular weight proteins that are natural, defense-related proteins often present in seeds and induced in certain tissues by herbivory or wounding. Thus, their main function is thought to be in plant defense, the regulation of endogenous proteinases, as storage proteins, as well as the prevention of unwanted proteolysis (Konarev *et al.* 2004). It comprises one of the most abundant classes of proteins in plants. Most storage organs such as seeds (*Leguminosae* and *Graminae*) and tubers (*Solanaceae*) contain 1 to 10% of their total protein as PIs, which inhibit different types of enzymes. There are a number of reports in the literature related to proteinase inhibitors in various legume species. The majority of reports discuss trypsin (TI) and chymotrypsin (CI) inhibitors. Only a limited number of articles have been reported about proteinase inhibitors in different germplasm accessions of arid legumes in India. So this study was undertaken to determine the presence of PIs in different accessions of arid legumes by using standard bovine trypsin and chymotrypsin enzymes for their further use in bioassay studies on important pest species.

MATERIALS AND METHODS

The present investigation was conducted during 2004–07 in the Division of Entomology, Indian Agricultural Research Institute, New Delhi. Seeds of various accessions were obtained from Regional Stations of National Bureau of Plant Genetic Resources (ICAR), New Delhi. Crude extract was obtained according to Hajela *et al.* (1999) with some modifications. The defatted flour was extracted with 0.01M Na-phosphate buffer (1:10 w/v) *p*H 7.0 containing 0.15M NaCl. It was homogenized for 10–15 min. by using homogenizer and then stirred for 2 hr at room temperature; the homogenate was centrifuged at 8 000 to 10 000 rpm for 30 min. The supernatant (crude extract) was diluted with distilled water and used as the initial source for proteinase

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inhibitors as well as for protein estimation in the screening studies. Different volumes of crude extract were added to 20 µg of trypsin in 200 µl of 0.01M Tris-HCl (*p*H 8.0) containing 0.02M $CaCl_2$ and incubated at 37°C in a water bath for 5–10 min. Residual trypsin activity was measured by adding 1 ml of 1mM BApNA (in 10% of DMSO) and incubated at 37°C for 10 min (Erlanger 1961). Reactions were stopped by adding 200 µl of 30% glacial acetic acid. After centrifugation, the liberated *p*-nitroaniline in the clear solutions was measured at 410 nm Inhibitor activity was calculated by the amount of crude extract required to inhibit 50% of trypsin activity, which is considered as one unit of trypsin inhibitor and expressed as trypsin inhibitor units per mg seed protein. Protein content in all extracts was determined according to the method of Lowry *et al.* (1951). The chymotrypsin inhibitor activity was also measured in a similar way except that the substrate used was BT*p*NA. One millimolar BT*p*NA was prepared in 0.01M Tris-HCl (pH 8.0) containing 40% ethanol. Data were subjected to both equal and unequal analysis of variance (ANOVA). The mean values of the chemical components were calculated from three replications. Data were statistically analyzed using statistical package. Correlation coefficients were calculated using statistical package of ICAR, Goa. Differences between any two means were determined using a least significant difference separation with a $P = 0.05$.

RESULTS AND DISCUSSION

Initial investigations were carried out to establish the presence of proteinase inhibitors (PIs) in the seeds of arid legumes including tree legumes. Emphasis was laid on two PIs, viz. trypsin inhibitors and chymotrypsin inhibitors as they are widely distributed in the seeds of legumes. Protein content and specific activity (TIU/mg protein and CIU/mg protein) were determined for each of the cultivar/accession of arid legumes. The inhibitory activities against trypsin and chymotrypsin in the phosphate buffer extracts are presented for all the arid legumes species and their accessions in Table 1. The activities are expressed as TIU/mg protein and CIU/mg protein. All the cultivars/accessions of different legume species contained trypsin inhibitory activity (TI). The mean value obtained for all the accessions examined was 52.84±3.05 TIU/mg in *Acacia senegal* which was significantly higher than that found for the other arid legumes studied and all the arid legumes species were compared using the same extraction procedure and analytical technique. The highest mean chymotrypsin inhibitory activity (CI) was studied in cowpea (13.16±0.69 CIU/mg) when compared to 1.83±0.09 CIU/mg in *Acacia senegal*. Whereas in the present study, algaroba and cluster bean have not shown any chymotrypsin inhibitory activity. The highest mean protein content was observed in accessions of *Acacia senegal* (228.21±3.81 protein/g seed) and the lowest in *Prosopis cineraria* (69.46±2.10 mg/g seed).

Table 1 Trypsin and chymotrypsin inhibitory activity of various accessions of arid legumes

Accession No.	Protein (mg)	TIU/mg	CIU/mg
	Cowpea (*Vigna unguiculata*)		
IC 249105A	76.62±2.10^{mno}	20.15±0.64^{fg}	10.53±0.62^{jklmno}
IC 4586	83.79±2.25^{jkl}	29.08± 0.75^{e}	10.50±0.25^{klmno}
IC 214753	82.27±0.46^{kl}	21.93± 0.07^{f}	16.00±1.37^{defg}
IC 249593	89.98±0.27^{h}	17.47± 0.15^{gh}	4.97±0.73^{q}
IC 44765	44.65±0.91^{q}	17.48±0.30^{gh}	8.54±2.13^{nop}
IC 249586	97.47±0.73^{g}	19.08±1.03^{fg}	9.77±0.39l^{mno}
IC 366776	117.33±0.54^{bc}	30.33±0.72^{e}	15.31±1.27^{defgh}
IC 202762	79.67±0.38^{lmn}	30.88±0.44^{e}	13.30±0.28^{fghijk}
IC 466882	77.13±1.10^{mn}	31.52±0.66^{e}	13.72±0.27^{efghij}
IC 331212	89.13±1.70^{hi}	29.95±0.88^{e}	11.82±0.07^{ijklmn}
IC 214751	107.41±2.48^{f}	22.68±0.38^{f}	12.76±1.25^{ghijkl}
IC 202787	115.15±0.76^{cd}	19.09±0.62^{fg}	16.26±0.22^{def}
IC 214835	87.56±1.16^{hij}	21.65±0.33^{fg}	5.34±0.10^{pq}
IC 326707A	86.66±0.30^{hijk}	52.79±1.91^{a}	26.21±1.68^{b}
IC 330996	87.12±0.22^{hij}	50.48±1.54^{ab}	18.57±0.10^{cd}
IC 331220	84.84±0.64^{ijk}	47.32±1.82^{bc}	8.35±0.08^{op}
IC 202790	89.14±1.21^{hi}	33.60±1.50^{e}	33.26±1.24^{a}
IC 249141	97.55±1.77^{g}	30.11±0.72^{e}	20.04±4.28^{c}
IC 202781	88.12±1.30^{hij}	22.78±1.07^{f}	9.40±0.14^{mno}
IC 249591	89.59±0.06^{h}	15.46±0.16^{gh}	4.34±0.12^{q}
IC 326293	106.1±1.71^{f}	46.12±4.33^{bc}	13.88±0.53^{efghij}
IC 202791	86.96±1.65^{hij}	49.39±2.58^{ab}	12.07±0.43^{hijklm}
EC 472262	90.25±1.38^{h}	22.71±0.22^{f}	10.75±0.70^{ijklmno}
EC 501045	112.28±1.76^{de}	18.06±0.43^{fg}	4.16±0.08^{q}
EC 367710	86.23±0.78^{hijk}	21.05±1.08^{fg}	16.68±1.12^{cde}
EC 20584	86.94±2.73^{hij}	21.11±.12^{fg}	8.02±0.70^{opq}
EC 50905	129.81±4.12^{a}	43.91±1.47^{cd}	17.89±1.95^{cd}
EC 249508	51.98±2.70^{p}	7.08±0.39^{i}	3.13±0.14^{q}
EC 240747	128.86±1.01^{a}	43.38+1.84^{cd}	20.00±0.60^{c}
EC 390287	121.11±2.16^{b}	33.57±0.60^{e}	18.51±1.72^{cd}
(Local)	132.10±0.25^{a}	44.01±5.51^{cd}	14.10±0.29^{efghi}
Mean	93.67±2.07	29.49±1.27	13.16±0.69
	Cluster bean (*Cyamopsis tetragonoloba*)		
IC 311403	90.26±6.06^{efgh}	8.03±0.55^{a}	
IC 311421	92.46±0.61^{efg}	6.81±0.10^{b}	
IC 311422	71.90±2.70^{ijk}	2.84±0.06^{fgh}	
IC 311428	120.70±2.27^{ab}	3.99±0.10^{cd}	
IC 311431	97.73±6.18^{def}	3.57±0.17^{de}	
IC 311432	61.23±0.16^{k}	2.63±0.01^{ghi}	
IC 311438	72.01±1.22^{ij}	3.55±0.07^{de}	
IC 311440	79.8+3.18^{hi}	2.84±0.15^{fgh}	
IC 311442	85.26±5.50^{gh}	1.91±0.15^{j}	
IC 311444	87.70±0.38^{fgh}	1.95±0.02^{ij}	
IC 311449	94.53±2.71^{defg}	3.49±0.05^{def}	
IC 329033	100.10±1.07^{de}	2.94±0.05^{efg}	
IC 329036	94.70±2.14^{defg}	2.52±0.04^{ghij}	
IC 329038	103.36±0.83^{cd}	2.02±0.13^{ij}	
IC 329681	113.76±2.02^{bc}	3.01±0.11^{efg}	
IC 370468	123.63±11.04^{ab}	2.02±0.19^{ij}	
IC 370478	87.43±1.77^{fgh}	4.57±0.31^{c}	

Continued

Table 1 *Concluded*

Accession No.	Protein (mg)	TIU/mg	CIU/mg
IC 370496	120.43±0.72^{ab}	2.42±0.01^{ghij}	
IC 311433	66.16±1.70^{jk}	2.15±0.06^{hij}	
IC 311441	125.20±2.80^{a}	2.35±0.05^{ghij}	
Mean	94.42±2.53	3.32±0.20	
	Babul (*Acacia senegal)*		
IC 330498	129.96±2.22^{m}	72.60±2.46*	2.90±0.03^{b}
IC 330505	232.43±2.73^{efgh}	60.31±8.07	1.50±0.00^{fgh}
IC 330508	269.36±7.28^{ab}	60.30±1.41	1.18±0.01^{jk}
IC 330510	232.16±5.86^{efgh}	51.44±1.20	3.66±0.13^{a}
IC 330500	217.80±2.73^{ijk}	69.93±0.81	1.19±0.09^{jk}
IC 330501	235.00±4.88^{efg}	65.94±37.55	2.11±0.08^{de}
IC 330502	233.50±5.60^{efg}	60.06±23.02	1.46±0.04^{fghi}
IC 330512	227.23±3.53^{fghi}	42.46±2.57	1.65±0.08^{f}
IC 330523	241.46±3.03^{de}	39.64±2.13	3.11±0.15^{b}
IC 330522	273.63±0.33^{a}	34.77±0.09	1.93±0.02^{e}
IC 330504	252.30±6.35^{cd}	52.08±23.88	2.32±0.20^{cd}
IC 330527	214.46±0.63^{jkl}	38.78±0.48	1.08±0.10^{k}
IC 330525	219.96±4.27^{ijk}	37.14±0.87	1.47±0.02^{fghi}
IC 330524	226.33±1.32^{fghi}	38.16±1.03	1.51±0.04^{fgh}
IC 330516	222.10±2.06^{l}	44.39±4.89	1.42±0.00^{ghi}
IC 330518	258.00±3.02^{hijk}	54.87±32.94	1.64±0.03^{fg}
IC 330519	224.56±4.36^{bc}	60.63±17.33	1.46±0.02^{fghi}
IC 330511	237.50±5.40^{ghij}	45.64±5.82	1.40±0.04^{hij}
IC 330521	212.10±2.60^{ef}	68.44±5.35	1.26±0.02^{ijk}
Local	203.10±1.80^{kl}	85.45±7.02	2.37±0.01^{c}
Mean	228.21±3.81	52.84±3.05	1.83±0.09
	Algaroba (*Prosopis cineraria)*		
IC 432188	47.56±0.87^{k}	10.34±0.28^{def}	
IC 432187	42.92±0.24^{l}	8.07±0.07^{fg}	
IC 432193	80.36±0.63^{d}	10.77±0.14^{def}	
IC 432196	63.50±0.46^{hi}	15.68±0.20^{bcd}	
IC 432195	62.50±0.64^{i}	15.87±0.30^{bcd}	
IC 432182	47.40±0.75^{k}	20.36±0.74^{ab}	
IC 432183	69.86±1.07^{f}	19.64±0.81^{abc}	
IC 432184	48.06±0.61^{k}	15.43±0.24^{bcd}	
IC 432185	66.33±1.55^{g}	3.57±0.03^{g}	
IC 432186	58.73±0.43^{j}	9.25±0.33^{efg}	
IC 432194	66.13±0.66^{gh}	17.65±0.24^{bc}	
IC 432192	69.16±0.41^{f}	7.81±0.13^{fg}	
IC 432198	76.36±2.31^{e}	19.93±3.40^{abc}	
IC 432200	103.83±0.60^{a}	14.14±0.61^{cde}	
IC 432197	71.86±0.34^{f}	20.17±6.64^{ab}	
IC 432201	94.43±1.73^{c}	16.65±0.18^{bc}	
IC 432189	97.83±0.50^{b}	15.10±0.75^{bcd}	
IC 432199	71.66±1.43^{f}	22.13±4.82^{a}	
IC 432190	75.32±0.29^{e}	21.91±0.90^{a}	
IC 432191	75.30±0.41^{e}	20.29±0.79^{ab}	
Mean	69.46±2.10	15.24±0.77	
LSD (5%)	7.36	4.60	1.72

In a column, means followed by a common letter(s) are not significantly different by LSD (P≤0.05), values represent mean of three replications ± SE * NS

Trypsin inhibitory activity

In all arid legume species, both large inter-specific as well as intra-specific variation in the trypsin inhibitory activity was found. The majority of the cowpea accessions examined fell within the range of 15-45 TIU/mg and the significant difference was observed between different accessions of cowpea. This high level of trypsin inhibitory activity is comparable to that of reported in velvet bean (Gurumoorthi *et al*. 2003 and Vadivel and Janardhanan 2005).

In the present study, the lowest TIU per mg was observed in accessions of cluster bean. The significantly highest trypsin inhibitory activity (8.03±0.55 TIU/mg) was found in accession, IC 311403 then followed by IC 311421 (6.81±0.10 TIU/mg) and IC 370478 (4.57±0.31 TIU/mg). All other accessions are fallen in the range of 1.91±0.15 to 3.99±0.10 TIU/mg. Though the mean protein content of cluster bean was comparable to other arid legumes studied but the inhibitory activity was low.

Tree legume of the genus *Acacia* and *Prosopis* are included in the study, as so far they were not subjected to such intensive study. In *Acacia senegal*, the trypsin inhibitors content ranged from 34.77±0.09 in IC 330522 to 85.45±7.02 TIU/mg in local cultivar. The variation observed among different accessions was non-significant. The specific activity of 138.99 TIU/mg was observed in *Acacia victoriae* Bentham (Ee *et al*. 2009). Trypsin inhibitory activity of *Prosopis cineraria* accessions ranged from 3.57±0.03 to 22.13±4.82 TIU/mg. The highest trypsin inhibitory activity was recorded in IC 432199 which was at par with IC 432190, IC 432190, IC 432191, IC 432182, IC 432183 and IC 432198 but significantly higher than noted in other accessions. Trypsin inhibitor activity was found to be significantly lower in IC 432185. The trypsin inhibitor activity from 13.48 to 65.43 TIU/mg protein was observed in different legume species (Vadivel and Janardhanan 2005).

All legumes, studied to date, have been found to contain trypsin inhibitors in varying amounts. Though the trypsin inhibitor activity has been studied in a number of pulses, the results obtained in the present investigation cannot be compared because the expression of trypsin inhibitor activity, nature, and concentration of the substrate etc., are different. However, based on the investigations carried out under similar experimental conditions for trypsin inhibitor activity in cultivated legumes like, *Cassia obtusifolia* (13.48 TIU/mg protein) and *Mucuna monosperma* (65.43 TIU/mg protein) (Vadivel and Janardhanan 2005); pigeonpea (21.74 and 35.43 TIU/mg) (Akande and Balogun 2009); chickpea (346.3 to 583.5 units/g in *desi* and 179.4 to 538.2 units/g in *kabuli*) (Oberoi *et al.* 2010). It may be inferred that the trypsin inhibitor activity obtained in the present investigation appears to be comparable as well as higher in case some accessions of *A. senegal* and lower in *P. cineraria* accessions.

Chymotrypsin inhibitory activity

Significant variability for chymotrypsin inhibitor content was observed among the various accessions of cowpea. The accession IC 202790, possessed the significantly highest CIU per mg protein (33.26±1.24), followed by IC 326707A (26.21±1.68), IC 249141 (20.04±4.28) and EC 240747 (20.00±0.60). The values for CIU content are more or less in agreement with those reported earlier by several workers in other legumes species (Gurumoorthi *et al*. 2003, Pugalenthi *et al*. 2005). The lowest CIU/mg protein was observed in accessions; EC-249508, EC-501045, IC-249591, IC-249593 and IC-214835 (3.13±0.14 to 5.34±0.10). Gurumoorthi *et al*. 2003 reported high level of trypsin inhibitor activity (45.2–49.6 TIU/mg protein) and chymotrypsin activity (26.2–30.1 CIU/mg protein) in velvet bean. Considerable trypsin inhibitory activity but showed relatively very low inhibition against α-chymotrypsin was found in the seeds of A. *victoriae* Bentham (Ee *et al*. 2009).

However, present investigation shows that the chymotrypsin inhibitory activity is very low and weak when compared to TI activity in all the accessions of *A. senegal*. Significant variation in chymotrypsin inhibitor content was observed in the accessions of *A. senegal*. The range of 1.08±0.10 to 3.66±0.13 CIU/mg was found in different accessions. The highest chymotrypsin inhibitory activity was found in IC-330510 while the lowest in IC-330527.

Protein content

The content of protein varied from 44.65±0.91 (IC 44765) to 129.81±4.12 (EC 50905) in cowpea. The accessions like, EC 240747, EC 390287, IC 366776, IC 202787¸ EC 501045, IC 214751 and IC-326293 also had the highest protein content when compared to other accessions. The mean protein content of 93.67±2.07 mg was observed in cowpea and at par with the mean protein content of cluster bean (94.42±2.53 mg). The accessions, IC 311441, IC 370468, IC 370496, IC 311428, IC 329681 and IC 329038 were recorded the highest protein content when compared to all other accessions of cluster bean. The lowest protein content of 61.23±0.16 mg was found in IC 311432 which was on par with IC 311433.

Of the two tree legumes evaluated, *Acacia* spp. has more protein content per gram of seed, which is comparable to that of the other legume species screened. *A. senegal* accession IC 330522 had the highest protein content of 273.63 mg/g seed, followed by IC 330508 (269.36 mg/g seed). In *P. cineraria* seed protein content varied from 42.92±0.24 mg (IC 432187) to 103.83±0.60 mg (IC 432200). The variation in protein content may result from both the genetic differences between species and within species and the effects of environment and method of analysis. The protein values are comparable to those reported for some cultivated legume seeds. The crude protein values ranged between 20.3% in *Cassia obtusifolia* and 35% in *Canavalia ensiformis* (Vadivel and Janardhanan 2005).

Table 2 Correlation coefficient between protein content, trypsin and chymotrypsin inhibitory activities of arid legumes

Crops	Parameters	TIU	CIU	Significant at 1% or 5%
Cowpea	Protein	r=0.628**	r=0.573**	1%
	TIU		r=0.679**	1%
Cluster bean	Protein	r=0.278*		5%
	TIU			
Acacia senegal	Protein	r=0.156	r=0.220	
	TIU		r=0.011	
Prosopis cineraria	Protein	r=0.604**		1%
	TIU			

Correlation between protein content and trypsin and chymotrypsin inhibitory activities of arid legumes

The results of correlation studies on protein content and the TI and CI activities of arid legume species are presented in Table 2. Cowpea, cluster bean and algaroba have significant positive correlation between protein content and TI activity. Whereas, Akande and Balogun (2009) observed that the Protein and trypsin inhibitor contents were significant and negatively correlated in pigeonpea. Protein content and CI activity are significantly and positively correlated (r=0.573**) in cowpea. Significant positive correlation between TI and CI activity was found in cowpea species. These results suggest the presence of a double-headed trypsin/chymotrypsin inhibitor analogous to the Bowman-Birk inhibitor of soybean. The significant correlations between protein and antinutritional may be helpful to identify good sources of resistant against insect pests.

The study inferred that the identification good sources of PIs are important tools to achieve a better understanding of fundamental principles of protein interaction and can be used to design new substances for the control of diseases and pathologic processes. Gupta *et al*. 2008 reported the effect of winged bean proteinase inhibitors on the growth and development of two lepidopteran insect-pests and this will help in developing transgenics to counter problematic lepidopterans.

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