Trypsin and Chymotrypsin Proteinase Inhibitors from Cereal Seed Extracts

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Proteinase inhibitory activity was assayed from the seeds of 57 accessions of five species of cereal germplasms. Barley and maize had both trypsin inhibitory (TI) and chymotrypsin inhibitory (CI) activity. Chymotrypsin inhibitory activity is not found in the accessions of rice, wheat and oats studied. TI and CI activities in barley and maize are higher than those of the other cereals tested. Oat accessions contained the lowest trypsin inhibitory activity. TI protein was highest in barley accession IC82680 followed by maize CIMMYT line 51705 and lowest in rice cultivar, Pusa Sugandha. Protein and CIU content were positively correlated in barley ($r=0.877^{**}$). TI and CI activity are positively correlated in barley seed extracts.

Key Words: Cereals, Trypsin, Chymotrypsin inhibitory activity, Correlation

Introduction

Proteinase inhibitors (PIs) are present in multiple forms in numerous tissues of animals and plants as well as microorganisms. The defensive capacities of plant PIs rely on inhibition of proteases present in insect guts or secreted by microorganisms, causing a reduction in the availability of amino acids necessary for their growth and development (De Leo *et al*., 2002). Proteinase inhibitors comprise one of the most abundant classes of proteins in plants. Most storage organs such as seeds from families Leguminosae and Graminae and tubers from family Solanaceae contain only 1 to 10% of their total proteins as proteinase inhibitors, which inhibit different classes of enzymes (Ryan, 1990). These inhibitors are also widely distributed among different botanical families.

Soybean trypsin inhibitor was the first PI isolated and characterized. The presence of high levels of protease inhibitors on a continual basis can lead to chronic hypersecretion by the pancreas, loss of proteolytic activity in the gut, loss of appetite, starvation and eventual death. The decline of PI content during germination correlated with increased activation of these proteases in the mobilization of storage proteins (Richardson, 1977).

Members of the serine and cysteine proteinase inhibitor families have been more relevant to the area of plant defense than metallo and aspartyl proteinase inhibitors (Laskowski *et al*., 1988). Serine Proteinase Inhibitors are universal throughout the plant kingdom and have been described in many plant species. The limited studies made so far indicate a wide variability of proteinase inhibitors in cereals in India. Keeping this in view, the present study was undertaken to find out the presence of proteinase inhibitors (Trypsin and chymotrypsin) in various germplasm collections of cereals.

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Materials and Methods

The present investigation was conducted in the Division of Entomology, Indian Agricultural Research Institute, New Delhi during 2004-2007.

Seed Materials and Chemicals

Seeds of different cereal germplasms were procured from Regional Stations of National Bureau of Plant Genetic Resources, Division of Genetics (IARI), Directorate of Maize Research and Directorate of Rice Research (DRR). Bovine trypsin (EC 3.4.21.4) and chymotrypsin (EC 3.4.21.1) were purchased from SRL (India). Standard substrates, *viz*., Benzoyl-DL-Arginine para-Nitroanilide (BA*p*NA) and Benzoyl-DL-Tyrosine-para-Nitroanilide (BT*p*NA) were procured from Sigma, Chemical CO. (St. Louis, Mo, U.S.A.). All other chemicals and reagents used were of analytical grade.

Extraction of Proteinase Inhibitors from Legumes and Cereals

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) pH 7.0 containing 0.15M NaCl. It was homogenized for 10-15 minutes by using homogenizer and then stirred for 2 h at room temperature; the homogenate was centrifugate at 8,000 to 10,000 rpm for 30 minutes. The supernatant (crude extract) was diluted with distilled water. The supernatant (crude extract) was precipitated with ammonium sulfate at concentrations of 0-30, 30-60 and 60-90%. The fraction obtained at 30-60% ammonium sulfate saturation showed high inhibitory activity against bovine trypsin. This fraction was used for the estimation of protein content as well as for the presence of PI activity.

Measurement of Inhibitor Activity

Different volumes of ammonium sulfate precipitated at 30-60% fractions (25 to 100 µl) were added to 20 µg of

trypsin in 200 µl of 0.01M Tris-HCl (pH 8.0) containing 0.02M $CaCl_2$ and incubated at 37^{0}C in a water bath for 5-10 minutes. Residual trypsin activity was measured by adding 1 ml of 1mM BApNA (in 10% of DMSO) and incubated at 37^{0}C for 10 minutes (Erlanger *et al*., 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 using UV/VIS-double beam Perkin Elmer λ3B spectrophotometer. Only 20 µg of trypsin in 200 µl of buffer without ammonium precipitated fractions was considered as control. Inhibitor activity was calculated by the amount of ammonium precipitated fractions 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 (Ignacimuthu, 1999). Protein content in all extracts was determined according to the method of Lowry *et al*. (1951) using bovine serum albumin, fraction V (M.wt. 66kDa, Sigma) as the standard.

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 (Hajela *et al.,* 1999).

Statistical Analysis

Data were subjected to analysis of variance (ANOVA) and compared by using LSD. Tests were carried out at the 1% and 5% level of significance.

Results and Discussion

The inhibitory activities in cereals against trypsin and chymotrypsin are presented in Table 1 (a, b) and Fig. 1. Most of the investigated accessions/cultivars of cereals have trypsin inhibitory activity (TI) while only barley and maize contained chymotrypsin inhibitory activity (Table 1b). In this experiment, results revealed that the TI and CI activities in barley and maize are generally higher than in wheat, rice and oats. Protein content in all the accessions/cultivars was below the expected level because in the present study, the fractions obtained from ammonium precipitated at 30-60% were used for the assay of protein and PI activity in all the cereals tested. The other fractions precipitated at 0-30 and 60-90% was not included in the assay. Only part of the protein was used for the testing the PI activity.

Rice, wheat and oat accessions recorded the lowest TI values (7.4-170.50 TIU/g seed). These accessions also failed to show chymotrypsin inhibitory activity. In the present study, the concentration of the ammonium sulfate precipitated fractions from rice, wheat and oat was increased to a maximum of 200 µl for testing the presence of chymotrypsin activity but assay failed to show the presence of CI activity. Barley and maize have both TI and CI activity (Hejgaard and Boisen, 1980). Similar results are given in the review paper of Boisen (1983). He mentioned that both TI and CI activity in barley, TI and weak CI activity in wheat, TI and no CI activity in rice and low TI and CI activity in oat. Lekes *et al*. (1989) also reported the presence of chymotrypsin inhibitory activity in different barley accessions.

Of the twenty-three rice accessions evaluated, the highest trypsin inhibitory activity was found in Vikas which was significantly different from all other accessions of rice. Of the wild species of *Oryza* tested, *officinalis* contained the highest TIU of 57.1/g seed. Among the wheat accessions evaluated, significantly higher activity was recorded in the cultivar, Pusa T-1336 and *T. abysinicum*. Oat accessions had the lowest TIU/g seed. Accession IC82680 of barley had the highest activity of 785.0 TIU/g seed. Maize CIMMYT line 51705 had the highest TIU value of 483.40/g seed, which was significantly different from all the other CIMMYT lines and cultivars.

The specific activity of the trypsin inhibitory protein was the highest in barley accession IC82680 whereas in rice cultivar, Pusa Sugandh it was very low. All the five accessions of barley had higher TIU per mg protein when compared to all other cultivars/accessions of cereals tested. Accessions of oat recorded low TIU per mg protein. Barley accession IC82680 had CI activity of 301.0 units/g seed, which was higher than that of all the other 4 accessions. Among CIMMYT lines of maize, 51705 had the highest CIU/ g seed compared to all other CIMMYT lines and cultivars.

Very few literature on the studies on the assay of proteinase inhibitors in cereals are available.

Table 2 presents the results of correlation studies in cereals. Rice, wheat, oats and maize had significant positive correlation between protein and TI activity. No significant correlation was observed in barley. Protein and CI activity was positively correlated in barley and maize though the correlation is statistically not significant. Correlation between TI and CI activity was positively significant at 1% in barley (r= 0.877**). Wheat also had positive correlation between TI and CI activities but not significant (Table 2).

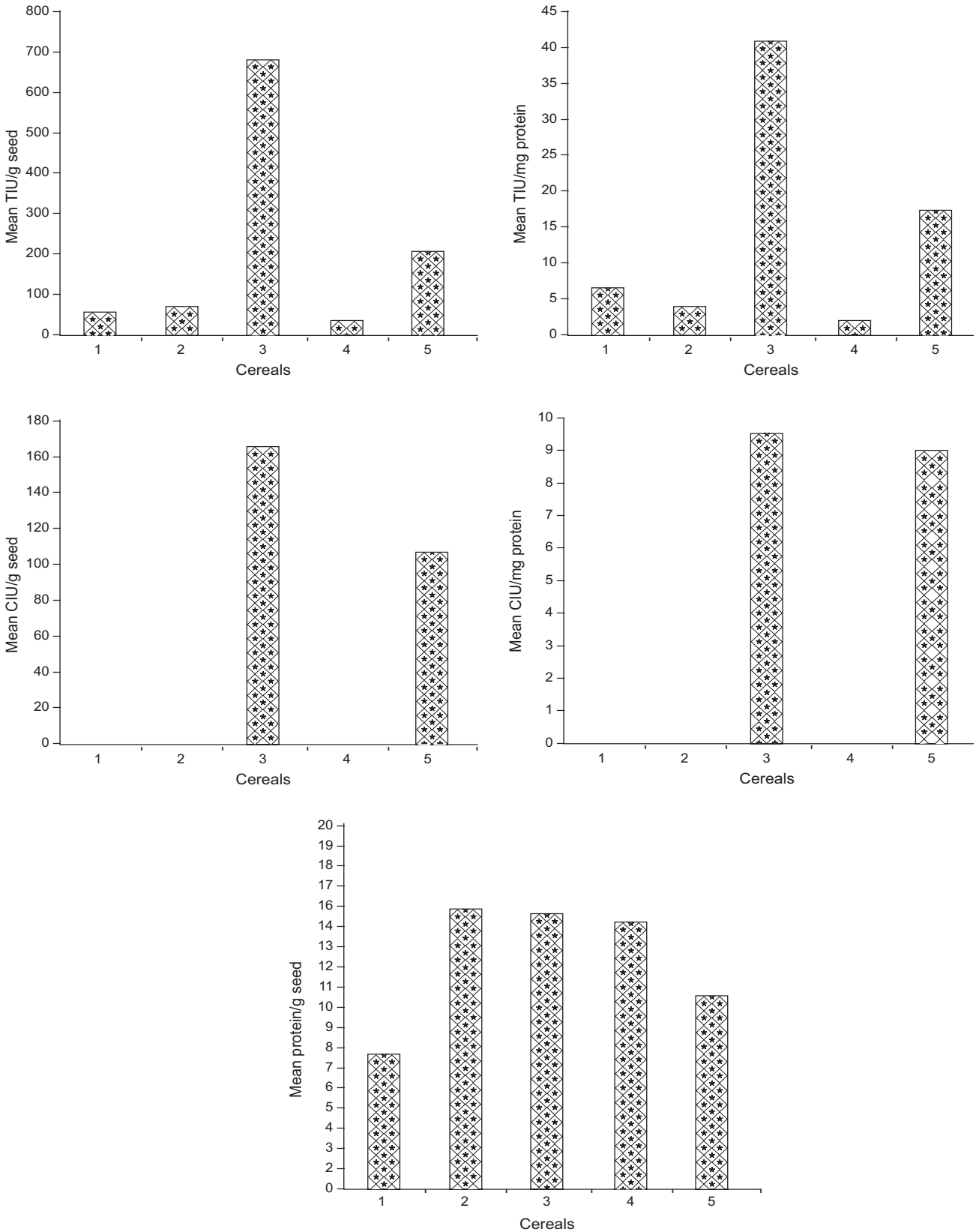

Fig. 1: Species mean values of TIU/g seed, TIU/mg protein, CIU/g seed, CIU/mg protein and protein content of various cereals
1. *Oryza sativa,* 2. *Triticum aestivum*, 3. *Hordeum vulgare*, 4. *Avena sativa*, 5. *Zea mays*

Table 1a. Trypsin inhibitory activity and protein content of germplasm accessions of rice, wheat and oats

S.No.	Accession no/cultivars	Protein mg/g seed	TIU/g seed	TIU/mg protein
Rice (*Oryza sativa*)				
1	Pusa Sugandh	8.7	7.4	0.8
2	IR-64	8.0	53.3	6.8
3	P-44	9.3	62.1	6.6
4	Jaya	8.8	46.4	5.2
5	P-1121	8.8	52.6	5.9
6	Phalguna	8.5	57.3	6.7
7	TKM-6	10.6	65.1	6.1
8	Suraksha	7.1	51.5	7.2
9	Kavya	9.1	20.8	2.2
10	9313 (Javanica)	11.0	112.5	10.2
11	Taroari Basmati	7.4	51.9	6.9
12	Mugad sugandha	8.6	91.0	10.5
13	MTU-1001	8.5	59.3	6.9
14	Abhaya	8.8	54.8	6.1
15	Taipei-309	7.7	75.1	9.7
16	IET-14590	9.8	48.7	4.9
17	Vikas	11.6	67.0	5.7
18	W-1263	6.9	70.6	10.2
19	FR-13A	5.6	44.5	7.9
20	TN-1	8.0	73.0	9.1
21	Ajaya	8.2	73.9	8.9
22	BSI-115	9.3	36.6	3.9
23	Chaitanya	8.0	70.7	8.8
	Wild Species			
24	*O. nivara*	4.7	26.3	5.5
25	*O. officinalis*	8.7	57.1	6.5
26	*O. rufipogon*	4.4	20.9	4.7
	Mean	8.31	55.78	6.68
Wheat (*Triticum aestivum* L.)				
1	IC138900	11.80	15.20	1.28
2	IC138895	14.50	40.51	2.79
3	IC138896	13.23	38.90	2.93
4	IC118796	11.10	21.18	1.90
5	IC118774	16.5	51.10	3.11
6	Pusa T-1336	20.16	107.87	5.34
7	WR-544	16.20	96.46	5.97
	Wild Species			
8	*T. amplissifolium*	19.36	89.20	4.60
9	*T. compactum*	15.86	52.06	3.28
10	*T. sphaerococum*	21.06	138.60	6.59
11	HD	19.33	61.03	3.16
12	*T. durum*	12.83	41.76	3.25
13	*T. dicocoides*	23.03	64.96	2.82
14	*T. dicocum*	23.30	55.53	2.39
15	*T. abysinicum*	16.0	170.50	10.66
16	Synthetic wheat	22.56	99.56	4.41
	Mean	17.30	71.52	4.03
Oat (*Avena sativa*)				
1	EC159067	13.0	29.3	2.2
2	EC96553	20.0	35.1	1.7
	Mean	16.5	32.2	1.95
	L.s.d. (P<0.05)	0.76	19.16	1.77

Table 1 b. Trypsin inhibitory activity and chymotrypsin inhibitory activity and protein content of germplasm accessions of barley and maize

S.No.	Accession no /cultivars	Protein mg/g seed	TIU/g seed	TIU/mg protein	CIU/g seed	CIU/ mg protein
Barley (*Hordeum vulgare* L.)						
1	IC82680	17.8	785.0	43.9	301.0	16.91
2	IC82512	15.0	644.7	43.0	137.9	9.19
3	IC82506	17.2	659.5	38.2	91.3	5.30
4	IC79590	17.7	704.6	39.8	156.7	8.85
5	IC60712	17.5	622.6	35.4	126.1	7.20
	Mean	17.04	683.28	40.06	162.60	9.49
5. Maize (*Zea mays*)						
1	51701	10.87	146.13	13.44	126.70	11.65
2	51702	9.20	112.26	12.54	131.10	14.50
3	51703	11.53	147.90	12.82	115.30	10.00
3	51704	14.20	147.2	10.49	109.70	7.74
4	51705	15.66	483.40	30.88	134.50	8.59
5	Surya	12.33	148.73	12.06	68.30	5.53
6	Pro-311	11.63	237.36	20.40	71.56	6.15
7	PMZ-150	10.03	225.66	22.50	80.80	8.07
	Mean	11.93	206.08	16.89	104.71	9.02
	L.s.d. (P<0.05)	0.76	19.16	1.77	12.27	1.37

Note: Specific information on IC or EC of wild species of rice, wheat and maize (lines) was not given at the time of collection from DRR, Division of Genetics (IARI) and DMR.

Table 2. Correlation coefficient between protein content, trypsin and chymotrypsin inhibitory activities of different cereals

Crops	Parameters	TIU	CIU	Significant at 1% or 5%
Rice	Protein	R=0.371**	–	1 %
	TIU	–	–	–
Wheat	Protein	R=0. 472**	–	1 %
	TIU	–	–	–
Barley	Protein	r=0. 445	r=0.206	–
	TIU	–	r=0.877**	1%
Oat	Protein.05	r=0.837*	–	5 %
	TIU	–	–	–
Maize	Protein	R=0.607**	r=0.157	1 %
	TIU	–	r=0.193	

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References

Boisen S (1983) Protease inhibitors in cereals. *Acta Agriculture Scandinavica*, **33**: 369-381.

De Leo F, F Volpicella, S Licciulli, R Liuni, L Gallerani and LR Ceci (2002) PLANT-PIs: a database for plant protease inibitors and their genes. *Nucl. Acids Res*. **30**: 347-348.

Erlanger BF, N Kokowsky and W Cohen (1961) The preparation and properties of two new chromogenic substrates of trypsin. *Arch. Biochem. Biophys*. **95**: 271-278.

Hajela N, AH Pande, S Sharma, DN Rao and K Hajela (1999) Studies on double-headed protease inhibitors from *Phaseolus mungo*. *Plant Biochem. Biotech*. **8**: 57-60.

Hejgaard M and S Boisen (1980) High-lysine proteins in Hiproly barley breeding: Identification, nutritional significance and new screening methods. *Hereditas*, **93**: 311-320.

Ignacimuthu S (1999) Protein antimetabolites in legume seed defense. *Indian J. Exp. Biol*., **37**: 215-222.

Laskowski M Jr, I Kato, WJ Kohr, SJ Park, M Tashiro and HE Whatley (1988) Positive Darwinian selection in evolution of protein inhibitors of serine proteinases. *Cold Spring Harbor Symp. Quant. Biol*., **52**: 545-553.

Lekes J, K Vaculova and K Hubik (1989) Measurement of the activities of beta -amylases and chymotrypsin inhibitors in the grain of fodder barley genotypes. *Sbornik UVTIZ Genetika a Slechteni*. **25**(2): 87-93.

Lowry OH, HJ Rosebrought, AL Farr and RJ Randall (1951) Protein measurement by folin phenol reagent. *J. Biol. Chem*. **193:** 265-275.

Richardson M (1977) The protease inhibitors of plants and micro-organisms. *Phytochem.* **16**: 159-169.

Ryan CA (1990) Protease inhibitors in plants: Gene for improving defences against insect and pathogens. *Annu. Rev. Phytopathol*., **28**: 425-49.