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Genetic variability and effect of heat treatment on trypsin inhibitor content in soybean [*Glycine max* (L.) Merrill.]

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ABSTRACT

Kunitz trypsin inhibitor (KTI), a major anti-nutritional factor present in soybean seed, is a major bottleneck in industrial use of soybean and public acceptance of soybean food products. Biochemical screening for trypsin inhibitor content was carried out in 145 genotypes of soybean comprising exotic collections and released varieties. The trypsin inhibitor content ranged from 14.15 to 186.27 mg g^{-1} of seed meal. Lowest level of trypsin inhibitor content was observed in genotype PI542044, (14.15 mg g-1 seed meal). Popular Indian soybean varieties viz., JS335, JS9305, NRC37, NRC7, PK1225, DS9712, JS9752, and DS9814 contained higher level of trypsin inhibitor. Heat treatment was able to reduce the TI content but to certain level leaving about 20% residual activity. The study indicated the need for other ways to create genotypes with reduced KTI for use in breeding program.

Key words: Biochemical screening, Kunitz trypsin inhibitor, Residual activity, Soybean, TI locus.

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INTRODUCTION

Soybean, containing about 20% oil and 40% protein, is one of the most important grain legumes in the world. Its multivariate uses as oil, feed, health supplement and other applications enabled it to occupy a coveted place among the oil seed crops across the countries. However, presence of some anti-nutritional factors in soybean seeds exerted a negative impact on nutritional quality of its protein (Liener 1994) and eventual acceptance of the soy food products. Protease inhibitor is one such anti-nutritional factor present in soybean seeds that cause pancreatic hypertrophy, hyperplasia, which ultimately results in inhibition of growth, as was observed in rats, chicks and mice fed with purified extracts from soybean rich in trypsin inhibitors (Liener and Kakade 1980). Therefore, elimination of trypsin inhibitor (TI) largely through heating or other approaches such as fermentation, precipitation, washing and filtration is practiced during manufacturing of soybean food products for general human consumption. The type and intensity of heating, however, affects the nutritional value of soy flour and its products (Bau *et al*., 2001). Moderate heat treatment causes partial denaturation of proteins (Anderson, 1992) while excessive heat-treatment makes loss of essential amino acids in soy proteins (Rios-Iriarte and Barnes, 1996). At high temperature, amino acids, such as lysine, bind with reducing sugar and are rendered unavailable, while cystine, a limiting amino acid in soybean seeds, is destroyed. Therefore, reducing trypsin inhibitor through genetic approach is considered to be the safe, cost effective and durable mechanism for enhancing acceptability of soy food products. A reduction in trypsin inhibitor activity by 79–87% is considered to be safe for human consumption (Leontowicz *et al.*, 1998).

Soybean trypsin inhibitor is composed primarily of Kunitz trypsin inhibitor (KTI) (about 80% of the total) and Bowman Birk trypsin inhibitor (about 20%). The KTI has 13 distinguishable electrophoretic forms, viz. Tia, Tib (Singh, *et al.* 1969), Tic (Hymowitz, 1973), Tid (Zhao and Wang 1992), Tie (Wang, *et al.* 1996, 2001), Ti-null type (Orf and Hymowitz 1979), Tif (Wang *et al.* 2004), Tibi5 (Wang and Li, 2005), Tiaa1, Tiaa2, Tiab1, Tig and Tik (Wang, *et al.* 2008, 2012). These electrophoretic forms are controlled by co-dominant multiple alleles at a single locus (Wang, *et al.* 2008, 2012). Therefore, genetic improvement of soybean genotype is possible through manipulation of KTI using appropriate breeding program. In India, limited efforts have been made to improve genetic potential of soybean genotypes for enhanced nutritional quality through reduction or elimination

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of KTI. Present investigation was therefore directed towards identifying genotypes containing little or no seed KTI for use in breeding program. Further, effectiveness of heat treatment on reduction of TI level was also investigated. Development of a genotype low in TI content shall be a useful benchmark in future soybean breeding programme.

MATERIALS AND METHODS

A set of 145 genotypes comprising varieties, breeding lines and exotic germplasm of soybean was used for biochemical screening of TI contents. Matured seeds of the genotypes were obtained from the Genetics Division, Indian Agricultural Research Institute, New Delhi-12, and were used for biochemical extraction of TI. The genotypes that contained higher amount of TI (>100mg/100 g seed wt.) were subjected to heat treatment to assess its impact on reduction of TI.

Trypsin inhibitor (TI) assay: Trypsin inhibitor activity was measured by using a synthetic substrate (for trypsin) N-u-benzoyl OL-arginine p-nitroanilide (BAPNA), according to the method described by Hamerstrand *et al* (1981). For its assay, trypsin working solution was prepared by dissolving 0.001g trypsin in 49ml of 0.001N HCl and volume was made up to 50ml. Tris- buffer was prepared from 0.30g Tris with 0.15g of $CaCl_2.2H_20$ and dissolved in 30ml of sterile double distilled water. BAPNA solution was prepared from 0.02g of BAPNA with 0.50ml of dimethyl sulphoxide (DMSO) and final volume was made to 50ml with Tris buffer.

Extraction of TI from seed: A soya sample of 0.125 g (2 seeds approx) was homogenised with 8ml of 0.01N NaOH (pH adjusted to 8.4-10.0 whenever required) for 3 hrs. Stirring was done sufficiently to keep the sample in suspension. It was then diluted to the extent that 2ml of the sample extract inhibited 40-60% of the trypsin used as standard in the analysis.

Statistical analysis: The data were subjected to analyses of variance (ANOVA). The actual content of trypsin inhibitor was calculated by the formulae given by the Hamerstrand *et al* (1981)

RESULTS AND DISCUSSION

Screening for TI content: Significant and wide variability was observed among the soybean genotypes for TI contents (Table 1) which ranged from 14.15 to 186.27 mg g^{-1} seed meal (Tables 2, 3 4). The ANOVA (Table 1) confirmed the significant genetic variability present among the genotypes. This variability would offer scope for the breeders to select genotypes for breeding program directed towards development of varieties with low TI content. PI542044, an exotic germplasm obtained from USA had the lowest TI content (14.15 mg g^{-1} seed meal), while highest TI content was detected in the genotype EC39779 (186.27 mg g^{-1} seed meal) followed by EC472164 (179.87 mg g^{-1} seed meal) and EC81655 (144 mg g^{-1} seed meal). PI542044 is a medium height, erect plant type with early maturity (90-95 days). Thus, it can be a potential donor of alleles for low TI content. The mean TI content in various genotypic groups *viz.*, exotic collection, breeding lines and varieties was 76.92 (range 14.15-186.27), 69.51(range 32-135.41) and 76.09 (range 40.93-123.96) mg g^{-1} seed meal, respectively. Although the mean among the groups are comparable, yet the range of TI contents widely varied. The exotic genotypes were more diverse in TI content than others. Out of the 61 exotic genotypes, 28 had TI content more than 70 mg g^{-1} seed meal. Two exotic collection germplasm *viz.*, EC457459 (34.36 mg g^{-1} seed meal) and EC471786 (37.73 mg g^{-1} seed meal) had moderate levels of TI content (Table 2). *Glycine Soja*, a progenitor of cultivated soybean found to contain higher amount of TI (134.74 mg g^{-1} seed meal). Among the 70 breeding lines analyzed, 29 found to have more than 70 mg $g^{-1}$of TI content, however, KDS256 (32.00 mg g^{-1} seed meal), MAUS2 (32.17 mg g^{-1} seed meal) and MRSD352 (35.03 mg g^{-1} seed meal) contained moderate level of TI (Table 3). Similarly, the 13 soybean varieties tested found to contain more than 40mg g^{-1} of TI. The most popular varieties *viz.*, JS335, JS9305, NRC37, NRC7, DS9712, JS9752, SL688 and DS9814 were found to have TI levels of 119.75, 58.78, 102.91, 63.66, 83.37, 62.82, 40.93 and 123.96mg g^{-1} of seed meal, respectively; thus no variety was identified with low or moderate level of TI content (Table 4).

TABLE 1: Analysis of variance (ANOVA) for the trypsin inhibitor content among the 145 soybean genotypes

Source	DF	SS	MSS	F cal
Replication	1	30.41	30.41	0.40
Genotypes	144	224440.23	1558.61**	20.65
Error	144	10867.84	75.47	-

While categorizing the genotypes based on TI content, it was found that out of 145 genotypes 68 contained TI in the range of 41 to 86 mg g^{-1} seed meal. Thus most of the genotypes contained TI in the medium range; genotypes with too low or high TI content were rare. No other genotype had TI content comparable to PI542044 making it to be the only potential donor for the trait of low TI contents.

Effect of heat treatment on TI content: A set of 24 genotypes with trypsin inhibitor content of more than 100mg $g^{-1}$of seed meal was selected for estimating the effect of heat treatment on TI content. The sample extract of these genotypes were boiled for 15 minutes at 100^0C before estimating the trypsin inhibitor content. The results indicated that the trypsin

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TABLE 2: Trypsin inhibitor (TI) content (mg g^{-1}seed meal) in exotic collections of soybean

Sl.No.	Germplasm	TI (mg/g)	Sl.No.	Germplasm	TI (mg/g)
1	EC 472342	68.04	32	EC 471938	101.05
2	EC471881	70.57	33	EC 471950	47.33
3	EC471849	92.13	34	EC 471955	45.81
4	EC457538	77.98	35	EC 472016	136.76
5	EC457514	59.62	36	EC 472065	113.18
6	EC39779	186.27	37	EC 472066	103.75
7	EC439619	85.89	38	EC 472123	134.74
8	EC439601	72.93	39	EC 472131	107.79
9	EC50082	50.19	40	EC 472164	179.87
10	EC439597	82.53	41	EC 472171	111.83
11	EC439617	75.45	42	EC 471972	63.83
12	EC458375	62.32	43	EC 471973	90.27
13	EC457074	66.02	44	EC 471979	55.75
14	EC457180	83.54	45	EC 472100	44.63
15	EC 472228	48.67	46	EC 472120	54.23
16	EC 472243	52.88	47	EC 472121	64.51
17	EC 456574	49.18	48	EC 472122	107.79
18	EC 472242	66.19	49	EC 472128	70.57
19	EC 471786	37.73	50	EC 472137	60.97
20	EC 456574	82.19	51	EC 472138	53.39
21	EC114526	61.31	52	EC 472141	45.81
22	EC 456639	40.42	53	EC 472163	64.00
23	EC 457222	86.40	54	EC 471998	142.15
24	EC 472250	67.87	55	EC 457184	74.78
25	EC 456597	45.31	56	EC 457322	92.13
26	EC 456618	42.78	57	EC389179	74.27
27	EC 456566	75.79	58	EC 457459	34.36
28	EC 447104	53.56	59	EC 457489	76.29
29	EC439599	58.44	60	EC471394	52.38
30	EC81655	144.00	61	PI542044	14.15
31	EC 457189	69.56	62	*G.soja*	134.74

*Mean= 76.92; Range=14.15-186.27; SE=±4.33; SD=34.07; CV=44.29

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inhibitor content got reduced to the tune of 66.50 to 88% depending on genotypes (Table 5). The minimum and maximum reduction was observed in the genotypes EC472123 (66.50%) and EC471938 (88.00%), respectively. The average inhibition of trypsin inhibitor was 79.33% whereas, the average residual activity was found to be 20.67%. Out of 24 genotypes analyzed, 12 were found to have more than 80% of TI reduction after the heat treatment. However, residues were found in many of the genotypes studied.

The soybean genotypes included in the study found to vary in the contents of TI. The wide range of genetic variability present among the genotypes would offer breeding opportunities for development of genotypes with lower TI content. The exotic germplasm PI542044 found to have lowest level of TI. Rani *et al.* (2011) also reported similar result. Currently, this genotype has been used in breeding programs aiming reduction of TI content. Molecular breeding efforts are underway in transferring the allele conferring lower level of TI in PI542044 to popular soybean varieties of India (Kumar *et al.* 2011). Molecular markers linked to the allele for lower TI content has also been reported (Kin *et al.*, 2006) which have made the molecular breeding effective.

Most of the Indian soybean varieties were found to contain higher level of trypsin inhibitor in its seeds. Two most popular varieties of India namely, JS335 and JS9305 contained 119.75 and 58.78 mg -1g seed meal, respectively rendering them unpopular in soy-food making industries. A number of other genotypes *viz.*, KDS252, MAUS2, EC457459, MRSB352, EC471786 and EC456639 were found to contain moderate level of TI. Kumar *et al.*, (2001) and Manjaya *et al.*, (2007) also reported moderate to higher level of TI content in a few soybean genotypes of India. Genotypes with moderate or lower level of TI would be fit to use in soyfood industries with little or no processing.

Total elimination of TI is often challenged due to its evolutionary significance. Presence of kunitz trypsin inhibitor reported to have a number of important biological functions including cytokine inducing, antitumor and HIV-1

TABLE 3: Trypsin inhibitor (TI) content (mg g^{-1}seed meal) in 70 soybean breeding line

Sl.No.	Breeding lines	TI (mg/g)	Sl.No.	Breeding lines	TI (mg/g)
1	AMS 353	40.42	36	M11913	101.39
2	DS9822	89.09	37	MRSD352	35.03
3	DS 2004	59.79	38	TS 14822	62.82
4	DS 2006	46.32	39	TGX185553D	77.14
5	DS 2007	68.21	40	SL432A	84.88
6	DS 2008	77.98	41	UPSL65A	69.05
7	DS 2009	82.19	42	UPSL769	76.13
8	DS 2010	67.54	43	UPSV19	58.27
9	JS9214	70.40	44	UPSL786	49.85
10	JS9029	61.98	45	UPSL94	55.58
11	JS9306	102.74	46	UPSL27	67.87
12	JS9466	42.78	47	UPSL163	103.58
13	JS9247	95.83	48	UPSL240	60.29
14	JS9307	81.85	49	UPSL149	44.80
15	JS9329	85.56	50	UPSL152	81.52
16	JS(SH)9526	80.67	51	UPSL340A	78.32
17	JS(SH) 9616	48.51	52	UPSV12	58.95
18	KDS 256	32.00	53	UPSL505	42.95
19	M135	100.55	54	UGM 20075	61.14
20	M253	94.48	55	VLS 57	53.39
21	M1094	87.58	56	VLS17	100.21
22	AVRDC5	44.46	57	V42	63.16
23	BR11	51.37	58	V19	67.54
24	MACS985	66.02	59	SL284	65.68
25	MACS52	117.56	60	SL444	60.97
26	MACS975	43.62	61	SL528	47.33
27	NRC66	63.16	62	SL459	61.64
28	PK 1387	63.83	63	SL 717	66.86
29	PK1141	90.95	64	SL 637	66.86
30	PK1080	78.32	65	MAUS158	71.58
31	PK1084	70.06	66	MAUS 164	78.82
32	PK 1343	106.78	67	MAUS81	45.47
33	PK7427B	59.28	68	MAUS 162	84.88
34	PK1225A	135.41	69	MAUS 222	80.17
35	PKS15	42.27	70	MAUS2	32.17

*Mean= 69.51; Range=32-135.41; SE=±2.52; SD=21.09; CV=30.33

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reverse transcriptase inhibitor activities (Evandro *et al.*, 2010). Bowman-Birk Inhibitor (BBI), the other form of TI present in soybean can suppress the growth of cancer cells (Armstrong *et al.*, 2000) and multiple sclerosis. Both *in vitro* and *ex vivo*, BBI inhibited myelin basic protein-specific proliferation of lymph node cells BBI reduced the activity of matrix metalloproteinase-2 and -9 in spleen cell supernatants and was detected in the central nervous system of treated rats. BBI suppresses experimental autoimmune encephalomyelitis in a safe and inexpensive way (Gran *et al.*, 2006). Moreover, trypsin inhibitor offers resistance to many of the lepidopteron and coleopteron insect (Michael *et al.*, 2007; Huma *et al.*, 2007). Therefore, development of genotypes with low TI content would be the most appropriate breeding approach so as to meet the need of the industries as well as survival of the genotype in the nature.

TABLE 4: Trypsin inhibitor (TI) content (mg g^{-1}seed meal) in soybean varieties

Varieties	TI (mg/g)
Bragg	48.00
CO 1	73.43
DS9712	83.37
DS9814	123.96
JS335	119.75
JS9305	58.78
JS9752	62.82
MACS450	43.45
NRC37	102.91
NRC7	63.66
PK1225	82.36
SL688	40.93
SL 525	85.73

*Mean= 76.09; Range=40.93-123.96; SE=±7.52; SD=27.13; CV=35.65

In an effort to understand the molecular basis of lower TI content in a mutant soybean line PI196168, Krishnan (2001) detected two deletions and one G to T transversion in the coding sequence of gene encoding TI. These mutations introduced four stop codons in the reading frame of the trypsin inhibitor locus resulting in a truncated protein which was non functional. Jofuku *et al.* (1989) confirmed the alteration of nucleotides resulting in frameshift within the KTi3- gene that causes premature termination during translation. Similar kind of observation was also made in Bowman–Birk protease inhibitor (BBI). Cloning and sequencing results of BBI gene from *Glycine microphylla* revealed a complete open-reading frame shift mutation in BBI gene region that resulted in lower accumulation of kunitz trypsin inhibitor (Krishnan and Kim 2003). Understanding the molecular basis of lower TI content may open up vistas for designing allele specific marker for use in marker-assisted selection (MAS). Such marker would pave the efficient transfer of allele for low TI to other important genotypes enhancing use of soybean in food industries.

Heat treatment of soy extract reduced the trypsin inhibitor level significantly (Kumar *et al*., 2006; Machado *et al*., 2008; Dipika *et al*., 2010). However, its effect was not uniform in all the genotypes had residual activities indicating the limitation of heat treatment in elimination of trypsin inhibitor contents to the safer level. The level of TI residues depended on the intensity and time of heating. Usually, it required 15 min thermal treatment to inactivate around 80 per cent of the protease inhibitors. This declines protein solubility, quality and the availability of other essential amino acids. Above all the heat treatment is cost-ineffective for soy processing units as it accounts for 25 per cent of total energy cost. It thus justifies the necessity of breeding efforts to reduce the TI content in order to popularize soybean and soy foods among the consumers across the world.

TABLE 5: The effect of heat treatment on trypsin inhibitor (TI) inhibition

Genotypes	TI mg g^{-1} (before heat treatment)	TI mg g^{-1} (after heat treatment)	Percent inhibition
EC 471938	101.05	12.13	88.00
EC 472016	136.76	22.23	83.74
EC 472065	113.18	14.82	86.91
EC81655	144.00	41.26	71.35
EC 472066	103.75	14.82	85.72
EC 472123	134.74	45.14	66.50
EC39779	186.27	35.87	80.74
EC 472131	107.79	23.58	78.12
EC 472164	179.87	49.85	72.29
EC 472171	111.83	18.86	83.14
EC 472122	107.79	21.56	80.00
EC 471998	142.15	29.64	79.15
PK 1343	106.78	33.68	68.46
JS9306	102.74	17.01	83.44
M135	100.55	28.29	71.86
M11913	101.39	16.34	83.88
MACS52	117.56	26.61	77.36
NRC37	102.91	21.05	79.54
VLS17	100.21	22.91	77.14
JS335	119.75	16.67	86.08
DS9814	123.96	28.97	76.63
PK1225A	135.41	27.62	79.60
UPSL163	103.58	18.19	82.44
G.soja	134.74	24.25	82.00

Mean: 79.33 (Range: 65.50 – 88.00); SD: 4.90; CV: 6.18; SE: ±1.00.

In the present study, no genotypes other than PI542044 could be identified with lower level of TI to use as donor in breeding programs. Since the basis of lower TI content is the mutation in the target gene, hence site directed mutagenic treatment may be applied to create more donor low TI content in soybean.

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