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ORIGINAL PAPER

Kunitz trypsin inhibitor in soybean: contribution to total trypsin inhibitor activity as a function of genotype and fate during processing

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Abstract
Detrimental effects of trypsin inhibitor activity (TIA) in soybean on human health have been attributed to Kunitz trypsin inhibitor (KTI) polypeptide. Literature is replete with reports pertaining to TIA in soybean seeds, however, the studies on the contribution of KTI polypeptide to total TIA have not been carried out. Further, very limited information is available on the quantitative changes occurring in this polypeptide due to different processing methods. In the present investigation, KTI polypeptide in soybean seeds was resolved by native polyacrylamide gel electrophoresis (PAGE) and substantiated by western blotting. Densitometry was employed to quantify KTI polypeptide in 102 soybean genotypes and assess the inactivation of this polypeptide due to boiling, autoclaving, microwave irradiation and sprouting. KTI concentration exhibited wide genetic variation, ranging from 0.07 to 15.9 mg/g soy flour, which corresponded to 1.0–79.8% of total TIA. Boiling and autoclaving for 15 min both resulted in complete inactivation of KTI. Microwave irradiation induced significantly higher reduction for KTI in soaked than dry seeds. Sprouting for 4 days caused 71.4% inactivation of KTI. The study showed that KTI contribution to total TIA was genotype-dependent and the inactivation of this polypeptide was a function of processing methods.

Keywords Kunitz trypsin inhibitor · Trypsin inhibitor activity · Genetic variability · Processing methods

Introduction

Soybean is increasingly being recognized as the 'functional food' of the century [1, 2]. The grains of this crop being packed with basic nutrients like protein, essential amino acids, fats, polyunsaturated fatty acids, minerals and vitamins have the potential to combat the malnutrition in developing countries. Several special bioactive ingredients, like tocopherols, isoflavones, saponins, present in soybean seeds can stave off the onset of chronic diseases like cancer, atherosclerosis, diabetes, Parkinson etc. However, soybean seed does have the limitation of presence of high concentration of anti-nutritional factor (trypsin inhibitor), which affects protein digestibility [3]. This protease inhibitor is considerably reduced in fermented soy products like *tempeh, miso* and *natto* [4] which are in the regular diet of the people in South-East Asia. In the recent past, soy food is gaining acceptance as 'health-food' beyond the countries of this region. In several countries like India, the masses are not accustomed to the consumption of soy-based fermented products, while unfermented soy products like *tofu* and soymilk, suit the local palate, and stand great potential for the wide acceptance. However, processing/manufacturing of these unfermented products must ensure the complete inactivation of trypsin inhibitor. In India, 9 parts of wheat and one part of soy grains are blended and ground in flour mill to prepare flour to be used as raw material in making soy-fortified *chapattis*-flat bread popular in Indian household. Prior to blending with the wheat grains, soybean grains are recommended to be boiled for minimum 20 min to ensure maximum inactivation of trypsin inhibitor. Trypsin inhibitor activity is attributed to the presence of 2 polypeptides, namely, Kunitz trypsin inhibitor (KTI) and Bowman-Birk inhibitor (BBI), the former is heat labile due to the presence of only 2 disulphide linkages, while the latter because of the presence of 7 disulfide linkages is considered relatively heat stable. Further, KTI is primarily responsible for trypsin inhibitor activity [5] and has detrimental effects on human health [6], while BBI has been reported to have nutraceutical properties [7].

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Several studies have been carried out for the estimation of total trypsin inhibitor activity (TIA) in soybean seeds and/or soy products using standard spectrophotometric methods [8, 9]. However, studies concerning the quantification of KTI polypeptide in soybean grains/soy products are limited. Pesic et al. [10] used densitometry for individual quantification of KTI in 12 soybean genotypes, while Zhou et al. [11] studied the variability of KTI in only 10 soybean genotypes using liquid chromatography-based mass spectroscopy. Kumar et al. [12] investigated 7 soybean genotypes for KTI using densitometry. In these studies, genotypes undertaken for the investigation were not sufficient to explore the genetic variability of KTI in soybean, and the contribution of KTI to total TIA was also not assessed, which were the major objectives of the present investigation. Further, numerous studies pertaining to the assessment of changes in the levels of total trypsin inhibitor activity (TIA) in soybean seeds as a result of boiling, autoclaving, microwave treatment and oven heating have been carried out [13, 14]. The investigations concerning the changes in the concentration of soybean KTI polypeptide on boiling and autoclaving are limited [15], and the studies investigating the effect of microwave irradiation on this polypeptide are not available. Changes in trypsin inhibitor activity in soybean and other crops at different intervals of sprouting have been reported in several investigations [16, 17]. However, the estimation of KTI polypeptide during the sprouting has been carried out in very limited studies [18, 19]. Kumar et al. [18] reported only qualitative changes in KTI polypeptide during soybean sprouting, but the authors did not quantify the changes in the concentration of KTI at different duration of sprouting. Dia et al. [19] reported no significant degradation in KTI polypeptide during sprouting, and suggested the need to determine optimum germination conditions, which would result in the maximum degradation of KTI. In the present investigation, it was thought worthwhile to assess the genotypic differences for contribution of KTI to TIA in a large number of soybean genotypes (102), and to investigate the changes in the levels of KTI due to boiling, autoclaving, microwave irradiation and sprouting for different duration.

Materials and methods

Material

Soybean genotypes (102) were raised in the field of ICAR-Indian Institute of Soybean Research, Indore (India) and freshly harvested seeds were used for exploring the genetic variability of KTI.

Chemical reagents and antibodies

Benzoyl DL-arginine-paranitroanilide-hydrochloride, Kunitz trypsin inhibitor, Nitrocellulose membrane (NCM), alkaline phosphatase conjugated anti-mouse antibody and readymade BCIP/NBT (5-bromo-4 chloro-3-indolyl phosphate/nitro blue tetrazolium) substrate were purchased from Sigma Aldrich, USA. KTI polyclonal detection antibodies were sourced from Abnova, Taiwan. All other chemicals were sourced from Sigma Aldrich, USA.

Soybean seeds (both control and treated) were freeze-dried in a freeze drier (Make Labconco) and finely ground for further analysis, and the data was expressed on dry weight basis.

Extraction and estimation of trypsin inhibitor activity

Trypsin inhibitor activity in finely ground soy flour was determined following standard procedure [9].

Extraction and quantification of KTI

Quantification of KTI was carried out using polyacrylamide gel electrophoresis (PAGE). For this purpose, finely ground soy flour of freshly harvested seeds of all the 102 varieties soy flour (50 mg) were homogenized in 30 volumes of double distilled water. The homogenized preparation was incubated for 3 h in shaker incubator at room temperature followed by centrifugation at 12,000 rpm for 10 min. The supernatants were collected and mixed with bromophenol blue dye. A fixed volume (10 µl) of this mixture containing approximately 25 µg of protein was loaded onto 10% polyacrylamide gel. Polypeptides, along with the standard KTI (2 µg) were resolved by applying 70 mA current for 45 min. The images were captured by Bio Rad imaging system GS-900. KTI on dry weight basis (mg/g flour) was calculated by comparing its density with KTI standard of known concentration using software *Image Lab 5.2.1* (Biorad, India). KTI activity (mg trypsin inhibited/g of soy flour) was calculated by multiplying KTI concentration by 2.51 as described elsewhere [12]. In the processing experiment, trypsin (7.0 µg) was added in the control and in treated samples to allow its binding with residual KTI, resulting in the formation of KTI-trypsin complex, which caused fading and disappearance of KTI band in the control and treated seed samples, respectively.

Western blotting

Protein bands resolved through native PAGE were transferred to pre-soaked NCM at 30 volts under semi dry conditions. Membrane was blocked with 5% bovine serum albumin (BSA) containing 1% Tween 20 in phosphate buffer saline and incubated overnight at 4 °C. KTI primary antibody (2000x) was added to blocking solution and incubated for 2 h with gentle agitation. This step was followed by the washing of the membrane for 10 min with PBS containing 0.1% Tween 20. The step was repeated 4–5 times. Alkaline phosphatase conjugated anti-mouse immunoglobulin (1:500 in 3% BSA) was added to the membrane and incubated for 2 h with gentle agitation. Washing of membrane was performed (4–5 times) as described above, followed by the addition of the soluble substrate system of conjugated enzyme alkaline phosphatase (BCIP/NBT). The membrane was incubated for 30 min in dark at room temperature. The completion of reaction resulted in the appearance of purple color. The reaction was stopped by adding the distilled water and the images were captured by Bio Rad imaging system GS-900.

Processing treatments

Seeds of soybean variety JS 97-52 were subjected to boiling, autoclaving, microwave irradiation and sprouting. For boiling treatment, dry seeds were boiled in distilled water at 100 °C for 5, 10 and 15 min. Autoclaving of soybean seeds was carried out at 121 °C and 15 psi for 15 min. For microwave treatment, both dry and pre-soaked seeds (immersed in distilled water for 30 min.) were irradiated in microwave oven (Samsung Model-MC28H5015VB, 0.6 KW power set at 2450 MHz frequency) for 1 and 2 min. of duration. For sprouting, soybean seeds were sprouted in germination paper at 28 °C and the seedlings were drawn for analysis after every 24 h till 4 days.

Statistical analysis

All steps and assays were performed in triplicate samples with satisfactory repetition of values. Data presented in Tables 1 and 2 are mean ± standard deviation of three independent replicates. All the statistical analyses were carried out through *SAS* 9.3 with significance at $P < 0.05$.

Results and discussion

Native PAGE of KTI positive genotype juxtaposed to null KTI genotype is presented in Fig. 1(Plate a), while plate b confirms the presence of KTI polypeptide in KTI positive genotype using anti-KTI polyclonal immunoglobulins. Densitometry analysis of concentration of KTI polypeptide resolved across 102 soybean genotypes showed that KTI concentration ranged from 0.07 (Punjab1) to 15.9 (PS1241) mg/g full fat soy flour (Table 1), with average value of 6.81 mg/g full fat flour. Zhou et al. [11] determined KTI concentration in 10 soybean genotypes and reported a range of 6.13–8.08 mg/g, with average concentration of 6.94 mg/g defatted flour, which is slightly lesser than the mean value of 7.96 mg KTI/g defatted flour (obtained after converting 6.81 mg/g full fat flour to its equivalent value in defatted flour, assuming 17% oil content in Indian soybean) noted across 102 soybean varieties in the present study. Pesic et al. [5] determined KTI concentration among 12 soybean genotypes and reported a range of 4.28–6.85 mg/g, with average value of 4.94 mg/g defatted flour, which is significantly lower than the average KTI concentration (7.96 mg/g defatted flour) observed in the present study.

As evident from Table 1, significant ($P < 0.05$) differences were observed for KTI polypeptide across 102 soybean genotypes. Genotypes PS 1241, NRC 7, PK 416, SL 96, Shivalik, PK 472, JS 20–34, NRC 12, MACS 450, MAUS 2 and VLS 2, VLS 63 exhibiting high level of KTI concentration (12 mg/g flour or more) were categorized as 'high KTI' genotypes, while the genotypes MACS 13, MAUS 81, Lee, SL 688, PS 1225, Pusa 24, MAUS 1 which showed relatively low KTI (6 mg/g flour or less) content, were categorized as 'low KTI' genotypes. Punjab 1 exhibited negligible level of KTI concentration.

Further, it was important to assess the contribution of KTI to the total TIA in soybean seeds to be used as raw material for the processing. For this purpose, KTI concentration of 102 soybean genotypes was multiplied by a factor of 2.51 to compute the KTI activity as described in our earlier study [12] which demonstrated that one mg KTI inhibited 2.51 mg of trypsin. Table 1 shows that KTI activity, across 102 soybean genotypes, ranged from 0.20 mg trypsin inhibited/g soy flour (Punjab1) to 40.0 (PS 1241), with average KTI activity of 23.6 mg trypsin inhibited/g soy flour; which corresponded to 1.0 (Punjab1) to 79.8% (Co-3), with mean value of 52.8% as contribution of KTI polypeptide to total TIA. Punjab 1, which showed very low level of KTI, is not a null KTI genotype unlike some of KTI free soybean genotypes which have been developed by crossing regular KTI positive soybean genotypes with donor parents (PI 542044, NRC 101) carrying null allele of KTI in countries like India [20] and Serbia [21]. The low activity of KTI in Punjab 1 may be because of the mutation in the *KTI3* gene or the differences in the regulatory *cis* sequences of this gene.

With regard to total trypsin inhibitor activity (TIA), it ranged from 18.6 (Punjab 1) and 74.8 (PK3 27) with average value of 45.9 mg trypsin inhibited/g soy flour. Srebric et al. [22] studied the variability of TIA among 7 soybean varieties and reported a range of 13.20 (Laura) – 33.93 mg

Table 1 Kunitz trypsin inhibitor (KTI) concentration and total trypsin inhibitor activity (TIA) of 102 soybean varieties

Variety	TIA	KTI conc. (mg/g)	KTI activity	Variety	TIA	KTI conc. (mg/g)	KTI activity
ADT-1	35.3 ± 1.7^{l}	9.5 ± 0.4^{f}	23.9 (67.7)	MAUS 32	49.2 ± 1.4^{h}	10.9 ± 0.5^{e}	27.4 (55.6)
Alankar	33.4 ± 1.3^{m}	8.0 ± 0.4^{h}	20.2 (60.3)	NRC2	42.2 ± 1.2^{j}	9.3 ± 0.3^{g}	23.4 (55.4)
Ankur	47.4 ± 1.42^{h}	9.2 ± 0.5^{g}	23.0 (48.1)	NRC 37	57.4 ± 1.1^{f}	7.3 ± 0.2^{i}	18.4 (32.0)
Birsa soybean	57.4 ± 1.14^{f}	8.5 ± 0.3^{h}	21.2 (36.9)	NRC7	68.4 ± 1.3^{b}	13.9 ± 0.6^{b}	35.0 (52.1)
Bragg	34.0 ± 1.36^{m}	9.2 ± 0.3^{g}	23.0 (67.9)	NRC12	46.3 ± 1.4^{i}	12.9 ± 0.6^{c}	32.4 (69.9)
Co-1	43.8 ± 1.31^{j}	10.0 ± 0.4^{f}	25.0 (57.5)	NRC86	46.5 ± 1.3^{i}	10.0 ± 0.5^{f}	25.3 (54.4)
Co Soya-2	40.1 ± 1.20^{k}	9.7 ± 0.5^{f}	24.4 (60.5)	PRS 1	35.0 ± 1.4^{m}	8.2 ± 0.4^{h}	20.7 (59.1)
Co -3	33.7 ± 1.34^{m}	$10.8 \pm 0.^{5e}$	27.0 (79.8)	Pusa16	62.5 ± 1.2^{d}	12.4 ± 0.5^{c}	18.6 (29.7)
DS 228	43.2 ± 1.30^{j}	10.6 ± 0.3^{e}	26.5 (61.1)	Pusa20	54.8 ± 1.6^{f}	11.9 ± 0.5^{d}	29.8 (54.3)
Pusa97-12	57.4 ± 1.14^{f}	11.5 ± 0.6^{d}	28.9 (50.1)	Pusa24	32.3 ± 1.2^{n}	6.3 ± 0.2^{j}	15.8 (48.9)
Davis	39.1 ± 1.56^{k}	11.4 ± 0.5^{d}	28.7 (73.1)	Pusa 37	41.4 ± 1.6^{j}	8.5 ± 0.2^{h}	21.4 (51.6)
Gujrat soybean1	32.6 ± 1.63^{m}	9.3 ± 0.4^{g}	23.4 (71.7)	Pusa 98-14	53.5 ± 1.5^{g}	12.9 ± 0.4^{c}	32.4 (60.6)
Gujrat soybean 2	39.3 ± 1.57^{k}	9.9 ± 0.2^{f}	24.9 (63.1)	PK 262	53.2 ± 1.0^{g}	9.3 ± 0.6^{g}	23.4 (43.9)
Harit soy	49.6 ± 1.48^{h}	11.6 ± 0.4^{d}	29.1 (58.4)	PK471	39.2 ± 1.5^{k}	10.4 ± 0.4^{f}	26.0 (66.3)
Hardee	38.1 ± 1.52^{i}	11.5 ± 0.5^{d}	28.8 (75.3)	Palam soya	42.8 ± 1.2^{j}	7.9 ± 0.3^{h}	19.8 (46.2)
Indira soya 9	49.4 ± 1.48^{h}	9.3 ± 0.4^{g}	23.4 (47.3)	PK 327	74.8 ± 1.4^{a}	6.6 ± 0.2^{j}	16.5 (22.1)
Improved Pelican	46.5 ± 1.39^{i}	7.1 ± 0.2^{i}	17.7 (37.8)	Pratap Soya 2	63.5 ± 1.2^{d}	11.3 ± 0.4^{e}	28.3 (44.5)
JS 2	49.1 ± 1.47^{h}	9.1 ± 0.3^{g}	22.6 (45.8)	Pusa22	76.4 ± 1.5^{a}	8.8 ± 0.2^{g}	22.1 (28.9)
JS20-34	64.1 ± 1.28^{d}	12.3 ± 0.6^{c}	30.9 (48.2)	Punjab1	$18.6 \pm 1.1^{\circ}$	0.07 ± 0.04^{m}	0.20 (1.0)
JS 71-05	43.1 ± 1.29^{j}	10.3 ± 0.4^{f}	25.9 (60.1)	Pusa40	48.4 ± 1.4^{h}	7.6 ± 0.0^{i}	19.1 (39.4)
Gaurav	44.3 ± 1.32^{i}	6.9 ± 0.2^{j}	17.2 (38.8)	PK416	51.4 ± 1.5^{g}	13.6 ± 0.6^{b}	34.2 (66.5)
Durga	31.4 ± 1.57^{i}	9.8 ± 0.5^{f}	24.6 (78.0)	PS1042	48.2 ± 1.4^{h}	8.5 ± 0.2^{h}	21.4 (44.3)
JS75-46	37.4 ± 1.49^{l}	11.8 ± 0.5^{d}	29.5 (78.8)	PS1024	39.5 ± 1.5^{k}	8.7 ± 0.1^{g}	21.9 (55.4)
JS76-205	57.0 ± 1.71^{f}	8.7 ± 0.3^{g}	21.9 (38.4)	PS 1225	34.6 ± 1.0^{n}	6.1 ± 0.1^{k}	15.4 (44.5)
JS79-81	38.8 ± 1.55^{k}	9.4 ± 0.3^{g}	23.5 (60.5)	PS1029	51.2 ± 1.5^{g}	9.2 ± 0.4^{g}	23.2 (45.3)
JS80-21	37.2 ± 1.48^{l}	9.8 ± 0.4^{f}	24.5 (65.5)	PK 308	68.0 ± 1.3^{c}	8.0 ± 0.4^{h}	20.2 (29.7)
JS90-41	36.9 ± 1.84^{l}	9.3 ± 0.3^{g}	23.4 (63.4)	PK472	40.4 ± 1.2^{k}	12.1 ± 0.3^{d}	30.4 (75.2)
JS93-05	36.6 ± 1.47^{l}	8.7 ± 0.2^{g}	21.9 (59.8)	PK564	51.6 ± 1.5^{g}	8.7 ± 0.3^{g}	21.8 (42.2)
JS95-60	37.6 ± 1.88^{l}	9.3 ± 0.3^{g}	23.2 (61.7)	PS1092	51.3 ± 1.4^{g}	9.2 ± 0.5^{g}	23.2 (45.2)
JS97-52	67.2 ± 1.34^{c}	11.2 ± 0.4^{e}	28.1 (41.8)	PS1241	56.6 ± 1.6^{f}	15.9 ± 0.6^{a}	40.0 (70.6)
JS 335	43.7 ± 1.74^{j}	11.4 ± 0.2^{d}	28.7 (65.6)	PS1347	33.2 ± 1.5^{m}	7.1 ± 0.2^{i}	17.9 (53.9)
JS2029	38.7 ± 1.54^{k}	9.1 ± 0.2^{g}	22.9 (59.1)	RVS2001-4	50.0 ± 1.2^{h}	7.0 ± 0.3^{j}	17.6 (35.2)
Kalitur	33.8 ± 135^{m}	9.0 ± 0.3^{g}	22.6 (66.8)	RKS24	40.8 ± 1.2^{k}	8.5 ± 0.4^{h}	21.4 (52.4)
KHSb2	48.8 ± 1.46^{h}	8.0 ± 0.1^{h}	20.1 (41.1)	RAUS5	50.1 ± 1.4^{h}	8.7 ± 0.5^{g}	21.9 (43.7)
KB79	45.8 ± 1.45^{i}	8.9 ± 0.3^{g}	22.3 (48.6)	SL4	41.9 ± 1.3^{j}	12.7 ± 0.6^{c}	32.0 (76.3)
Lee	42.9 ± 1.26^{j}	5.7 ± 0.1^{k}	14.2 (33.1)	Shilajeet	48.2 ± 1.4^{h}	7.3 ± 0.2^{i}	18.4 (38.1)
LSb1	51.2 ± 1.53^{g}	7.6 ± 0.1^{i}	19.0 (37.1)	SL96	35.2 ± 1.7^{m}	11.0 ± 0.4^{e}	27.5 (78.1)
MACS13	31.7 ± 1.26^{n}	4.3 ± 0.1^{l}	10.9 (34.3)	SL 688	48.2 ± 1.6^{h}	6.0 ± 0.2^{j}	15.1 (31.3)
MACS57	46.6 ± 1.86^{i}	8.4 ± 0.1^{h}	21.2 (45.4)	Shivalik	35.1 ± 1.4^{m}	11.0 ± 0.3^{e}	27.5 (78.3)
MACS58	45.4 ± 1.36^{i}	8.5 ± 0.2^{h}	21.4 (47.1)	SL295	36.7 ± 1.4^{l}	9.8 ± 0.3^{f}	24.6 (67.0)
MACS124	38.7 ± 1.54^{k}	7.9 ± 0.2^{h}	19.8 (51.1)	SL525	42.1 ± 1.2^{j}	7.5 ± 0.4^{i}	18.7 (44.4)
MACS450	50.0 ± 1.50^{h}	11.9 ± 0.5^{d}	29.0 (58.0)	Type 49	44.6 ± 1.3^{i}	9.2 ± 0.5^{g}	23.2 (52.0)
MAUS1	34.6 ± 1.38^{m}	6.7 ± 0.3^{j}	16.9 (48.8)	TAMS 38	41.3 ± 1.2^{j}	10.8 ± 0.5^{c}	27.1 (65.6)
MAUS2	47.5 ± 1.42^{h}	11.9 ± 0.6^{d}	29.8 (62.7)	TAMS 98-21	46.6 ± 1.2^{i}	8.6 ± 0.4^{h}	21.7(46.5)
MAUS61	42.3 ± 1.69^{j}	7.7 ± 0.2^{i}	19.3(45.6)	VLS1	41.8 ± 1.6^{j}	11.6 ± 0.6^{d}	29.2 (69.8)
MAUS61-2	36.4 ± 1.09^{l}	$10.6 \pm 0.4e$	26.6 (73.0)	VLS2	71.1 ± 1.2^{b}	13.2 ± 0.6^{b}	33.2 (46.6)
MAUS7	63.7 ± 1.27^{d}	8.2 ± 0.4^{h}	20.7 (32.4)	VLS21	60.7 ± 1.8^{e}	11.8 ± 0.6^{d}	29.5 (48.5)
Monett	65.9 ± 1.31^{c}	11.1 ± 0.7^{e}	27.9 (42.3)	VLS 47	40.9 ± 1.6^{k}	8.0 ± 0.4^{h}	20.0 (48.8)
MAUS158	36.7 ± 1.83^{l}	7.8 ± 0.4^{i}	19.5 (53.1)	VLS 63	$53.7 \pm 1.5g$	12.5 ± 0.6^{c}	32.0 (59.5)
MAUS47	46.7 ± 1.40^{i}	9.8 ± 0.4^{f}	24.7(52.8)	VLS 65	34.1 ± 1.3^{m}	9.8 ± 0.6^{f}	24.5(71.8)
MAUS 81	45.4 ± 1.36^{i}	5.6 ± 0.1^{k}	14.1 (31.0)	VLS59	41.0 ± 1.6^{k}	9.8 ± 0.4^{f}	24.7 (60.2)

Values given are mean of triplicate samples ± standard deviation. Total TIA = mg trypsin inhibited/g soy flour (dry weight basis). KTI activity [mg trypsin inhibited due to KTI per gram of soy flour] = KTI concentration × 2.51. Value given in parenthesis corresponds to percentage of total TI contributed by KTI. Values across the column with different superscripts are significantly different from each other at $P < 0.05$

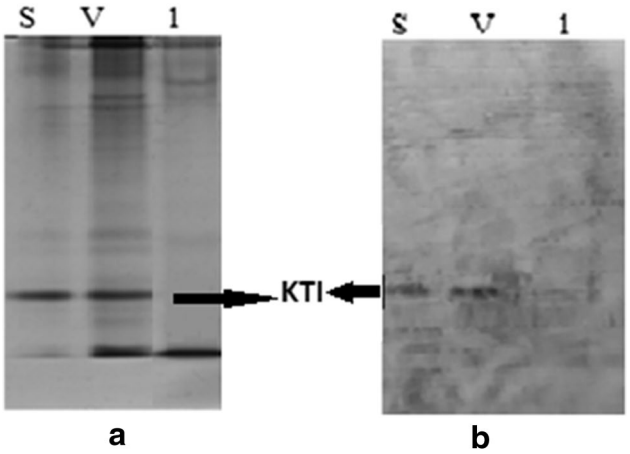

Fig. 1 Plate a: KTI polypeptide resolved through 10% Native PAGE. Plate b: confirmation of KTI polypeptide through western blot. Lane S, V, and 1 correspond to KTI standard, soy flour extract from KTI positive genotype 'JS 97-52' and soy flour extract of null KTI genotype (NRC 127), respectively

trypsin inhibited/g (ZP015). Laura (13.2 mg/g flour) and Lana (17.93 mg/g flour), reported to possess very low TIA by these authors in their study, were KTI-free soybean genotypes. Punjab 1, the genotype which exhibited very low level of TIA (18.6 mg trypsin inhibited/g soy flour) in the present investigation was not KTI-free genotype as faint band of KTI polypeptide, measurable by densitometry, was resolved through native PAGE and subsequently confirmed for this genotype. If Punjab 1 is not taken into consideration, 53.9% of total TIA is attributed to KTI across 101 soybean genotypes. This is the first report demonstrating the genotype-dependent contribution of KTI to total TIA in seeds of large number of soybean genotypes. Therefore, these results could not be compared from any other study in the literature. However, in an investigation pertaining to soy milk manufactured from a soybean variety, 74% of the total TIA was attributed to KTI [15].

Seeds of soybean variety JS 97-52 were selected and subjected to several processing methods to investigate their efficiency in inactivation of KTI. The results are presented and discussed below:

Inactivation of KTI polypeptide due to boiling

Table 2 depicts the changes in KTI due to boiling for 5, 10 and 15 min. The results showed that boiling of soybean seeds for 5 min caused 68.8% inactivation of KTI as evident from the reduction of this polypeptide from 11.2 to 3.5 mg/g soy flour. The boiling treatment extended up to 10 min caused 75.9% inactivation for this anti-nutritional inhibitor as KTI polypeptide plummeted to 2.7 mg/g from 11.2 mg/g soy flour. Figure 2 shows the changes in the density of KTI polypeptide in seeds due to boiling treatment. In comparison to the control (Lane 1), KTI polypeptide declined due to boiling for 5 min (Lane 3) and 10 min (Lane 5). The disappearing of residual KTI polypeptide (left after boiling for 5 min and 10 min) on the addition of trypsin in the extract (Lane 4 and Lane 6), substantiated the trypsin inhibitor activity of the residual KTI. Boiling for 15 min completely inactivated KTI polypeptide, as no residual KTI polypeptide could be detected by densitometer (Lane 7). We did not come across any earlier study pertaining to the effect of boiling of whole soybean seeds on the KTI content for comparing these results. For soy milk, [15] demonstrated that 30 min boiling is required to completely inactivate KTI. Our results also showed 63.0, 83.9 and 94% reduction in total TIA in soybean seeds due to boiling for 5, 10 and 15 min, respectively. The magnitude of reduction (83.9%) in TIA due to boiling of soybean seeds for 10 min in our study is lower than the reduction (97.5%) reported in *Phaseolus vulgaris* [13]. This difference may be because of the variation of the species undertaken for the boiling-induced inactivation of TIA in these two studies. Further, residual TIA (3.2 mg trypsin inhibited/g soy flour), which is equivalent to 5% of 67.2 mg/g in the control, observed after boiling soybean seeds for 15 min in our study may be attributed to the BBI only as the boiling for 15 min. duration completely inactivated KTI polypeptide.

Inactivation of KTI polypeptide due to autoclaving

Table 2 also depicts the changes in KTI and total TIA in soybean seeds on autoclaving (121 °C and 15 *psi*) for 15 min. The results showed that this treatment completely inactivated KTI, and TIA could not be detected through the standard spectrophotometric method [9]. Friedman et al. [23] reported 60.0 and 80.2% reduction of KTI in soy flour samples on autoclaving at 121 °C for 10 and 20 min, respectively.

Inactivation of KTI polypeptide through microwave irradiation

Table 2 presents the data with regard to quantitative changes in KTI due to the microwave irradiation of dry and soaked seeds for different times. Microwave irradiation of dry seeds for 1 min reduced KTI concentration from 11.2 to 5.4 mg/g soy flour, thereby causing 51.8% inactivation. In soaked seeds, microwave irradiation caused 86.6% inactivation (reducing from 11.2 to 1.5 mg/g full fat flour) for this polypeptide. Exposure of dry and soaked seeds for 2 min to microwave irradiation caused 94.6 and 99.1% reduction, respectively, which are significantly ($P < 0.05$) higher than the corresponding reduction observed due to irradiation for 1 min. Figure 3 shows the reduction of KTI in dry and

Table 2 Changes in concentration of KTI and total trypsin inhibitor activity of soybean seeds due to boiling, autoclaving, microwave irradiation, and sprouting

Treatment	KTI conc. mg /g flour	Total trypsin inhibitor activity TIA (mg trypsin inhibited/g flour)
Boiling		
Control	11.2 ± 0.3^{a} (28.1)	67.2 ± 1.3^{a}
Control + trypsin	2.2 ± 0.1^{f} (5.5)	–
5 min	3.5 ± 0.1^{e} (8.8)	20.2 ± 0.9^{g}
5 min + trypsin	ND	–
10 min	2.7 ± 0.1^{f} (6.8)	8.8 ± 0.5^{h}
10 min + trypsin	ND	–
15 min	ND	3.2 ± 0.3^{j}
15 min + trypsin	ND	–
Autoclaving		
15 min	ND	ND
Microwave-irradiation		
Dry seeds		
1 min	5.4 ± 0.21^{c} (13.6)	24.9 ± 1.2^{f}
1 min + trypsin	ND	–
2 min	0.6 ± 0.92^{h} (1.5)	5.7 ± 0.4^{i}
2 min + trypsin	ND	–
Soaked seeds		
1 min	1.5 ± 0.82^{g} (3.8)	18.2 ± 0.7^{g}
1 min + trypsin	ND	–
2 min	0.1 ± 0.74^{h} (0.25)	4.7 ± 0.4^{i}
2 min + trypsin	ND	–
Sprouting		
1 day	7.5 ± 0.3^{b} (18.8)	57.0 ± 0.9^{b}
2 days	5.7 ± 0.3^{c} (14.3)	37.6 ± 1.3^{c}
3″	4.2 ± 0.25^{d} (10.5)	30.2 ± 0.9^{e}
4″	3.8 ± 0.19^{d} (9.5)	33.7 ± 1.2^{d}

Values given are mean of triplicate samples ± standard deviation. Value given in parenthesis is Kunitz trypsin inhibitor activity i.e. mg trypsin inhibited due to KTI per g of full fat soy flour (dry weight basis). ND corresponds to 'not detected'

Values across the column with different superscripts are significantly different from each other at $P < 0.05$

soaked soybean seeds due to microwave irradiation as indicated by the decline in the density of KTI polypeptide. Compared to the control (Lane 1), Lane 2 and 4 depict changes due to microwave irradiation for dry seeds, while Lane 7 and 9 show changes in soaked seeds, for 1 and 2 min, respectively. On addition of trypsin, KTI polypeptide disappeared in dry seeds (Lanes 3 and 5) and soaked seeds (Lane 8 and Lane 10). It can be concluded from the results that microwave irradiation-induced inactivation of KTI was proportionate to exposure time, and the magnitude of inactivation was significantly ($P < 0.05$) high in soaked seeds than in dried seeds. Higher reduction observed in KTI concentration in soaked than dried seeds may be because of the higher electric dipole forms due to water molecules in the former case, which may result in intense heat energy transfer to proteins. Further, the reduction in KTI in dry and soaked seeds was in line with the decline in TIA due to microwave irradiation (Table 2). In literature, though the studies demonstrating the effect of microwave irradiation on TIA of soybean is available, however, the investigation concerning the effect of microwave irradiation on KTI polypeptide has not been carried out. Szmigielski et al. [21] reported 15.0 and 26.84% reduction in TIA of dry Polish bean flour on exposure to microwave irradiation for 1 and 2 min, respectively. This reduction noted for 1 and 2 min exposure of microwave in Polish bean was lower than observed in soybean seeds for the same exposure duration in the present study.

Changes in KTI polypeptide during germination of soybean seeds

Table 2 and Fig. 4 show the changes in KTI in soybean seeds and seedlings of different days. On comparison with control (non-germinated seeds), a continuous decline in the KTI

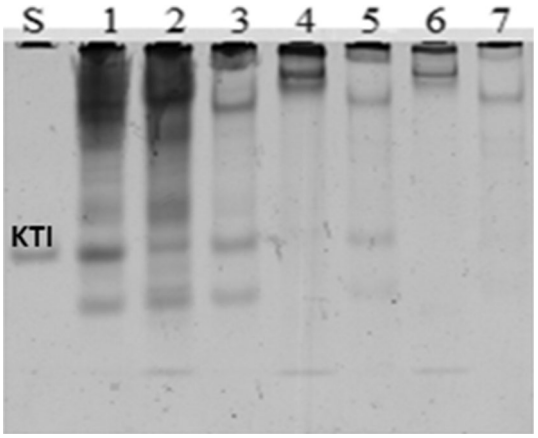

Fig. 2 Heat inactivation of KTI through boiling treatment. Lanes 1, 2, 3, 4, 5, 6, and 7 correspond to control (seed extract + with no boiling treatment), no boiling + trypsin, 5 min boiling, 5 min boiling + trypsin, 10 min boiling, 10 min boiling + trypsin, and 15 min. boiling, respectively. S denotes the KTI standard

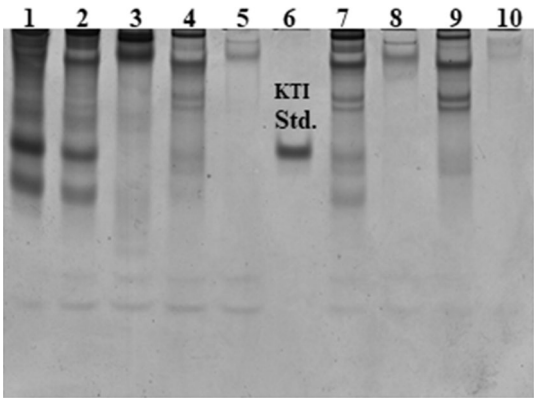

Fig. 3 Changes in the density of KTI polypeptide in dry and soaked soybean seeds of variety JS 97-52 due to microwave irradiation. Lane 1 and Lane 6 corresponds to control (no microwave treatment) and KTI standard, respectively. Lane 2 ,3 ,4, 5, corresponds to microwave irradiation of dry seeds for 1 min microwave irradiation, 1 min. microwave irradiation + trypsin, 2 min. microwave irradiation, 2 min. microwave irradiation + trypsin, respectively. Lane 7,8,9 and 10 corresponds to microwave irradiation of soaked seeds for 1 min microwave irradiation, 1 min. microwave irradiation + trypsin, 2 min. microwave irradiation, 2 min. microwave irradiation + trypsin, respectively

concentration was observed during sprouting. Dia et al. [19] reported non-significant changes in KTI on sprouting of soybean seeds for 72 h (3 days) at 25 °C. In our study, sprouting carried out at 28 °C for 4 days reduced KTI polypeptide concentration (66.1%) from 11.2 to 3.8 mg/g soy flour. Concomitant loss in total trypsin inhibitor activity, as evident in Table 2, during sprouting substantiates this reduction in KTI. The decline in KTI due to sprouting may be because of the *de novo* synthesis of proteases as suggested in an earlier

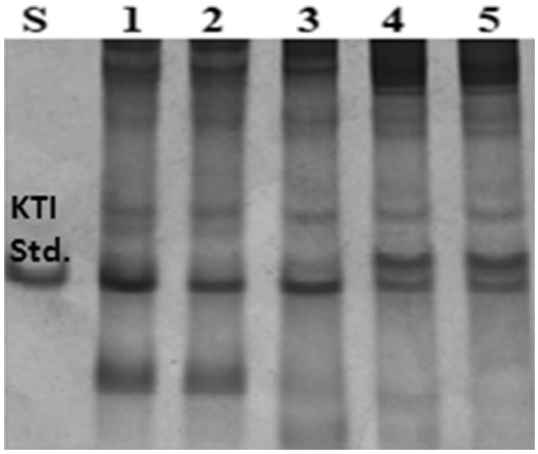

Fig. 4 Degradation of KTI polypeptide during sprouting in soybean seeds. Lanes S and 1 correspond to KTI std. and control. Lanes 2, 3, 4 and 5 correspond to 1 day, 2 day, 3 day and 4 day germinated seeds of soybean variety JS 97-52 respectively

study [24], which demonstrated the role of K1 protease in degradation of KTI during seed germination.

Among all the treatments, boiling for 15 min, autoclaving (at 15 psi and 121 °C) for 15 min, and microwave irradiation for 2 min led to the complete inactivation of KTI. Sprouting for 4 days induced 66.1% reduction in KTI.

Conclusions

In the backdrop of several reports demonstrating the detrimental effects of KTI in soybean on human health, it was important to assess the contribution of this antinutritional polypeptide to total trypsin inhibitor activity of soybean. Most of the studies conducted in the literature estimated total trypsin inhibitor activity in soybean, but none of them focused on the contribution of KTI to total TIA. Our results showed wide genotypic differences, ranging from 1.0 to 79.8%, for contribution of KTI to total TIA. Very low level of KTI was noted in some genotypes, which can serve as excellent raw material for processing soy products or can be exploited in plant breeding programme for development of low KTI soybean genotypes in combination with other desirable seed traits. The study showed that boiling, autoclaving and microwave irradiation significantly reduced KTI in soybean, and the efficiency of microwave radiation for inactivating KTI was enhanced on soaking soybean seeds. Sprouting, a processing method which involves no heat treatment also caused significant reduction of soybean KTI.

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Compliance with ethical standards

Conflict of interest The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

Ethical approval This article does not contain any studies involving human participants or animals performed by any of the authors.

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