Food Analytical Methods
https://doi.org/10.1007/s12161-018-1227-9

Comparative Assessment of Trypsin Inhibitor vis-à-vis Kunitz Trypsin Inhibitor and Bowman-Birk Inhibitor Activities in Soybean

Vineet Kumar[1] **· Anita Rani**[1] **· Mohd Shuaib**[1] **· Priyanka Mittal**[1]

Received: 13 November 2017 / Accepted: 6 March 2018
© Springer Science+Business Media, LLC, part of Springer Nature 2018

Abstract

Trypsin inhibitor activity (TIA) in soybean is attributed to two polypeptides, namely, Kunitz trypsin inhibitor (KTI) and Bowman-Birk inhibitor (BBI). Standard spectrophotometric protocol widely followed for estimation of TIA is cumbersome and does not distinguish KTI from BBI. In the present investigation, extraction conditions for KTI were optimized and different forms of this polypeptide were resolved in 180 soybean genotypes of Indian and exotic origin through native PAGE. This led to the identification of three KTI alleles, namely, Ti^a, Ti^b, and Ti^c, with Ti^a occurring in most of the Indian genotypes. Trypsin-KTI complex assay exhibited binding of Ti^a polypeptide with 2.51 fold concentration of trypsin. Subsequently, seeds of selected genotypes were subjected to estimation of KTI and BBI activity through densitometry and enzyme-linked immunosorbent assay (ELISA), respectively; and total TIA through standard spectrophotometric protocol. Summation of KTI and BBI was significantly ($P < 0.05$) lower than that of TIA determined through the spectrophotometric method.

Keywords Trypsin inhibitor · Kunitz trypsin inhibitor · Bowman-Birk inhibitor · Densitometry · ELISA

Introduction

Soybean being rich in basic nutrients, namely, protein, essential amino acids, essential fatty acids, vitamins, and minerals can combat mal- and under-nutrition in developing countries. Besides, consumption of soy-based products has been associated with the reduced risk of onset of several life-style diseases like obesity, diabetes, and oxidative-stress complications like breast cancer, Parkinson, and Alzheimer in the developed world. This has been attributed to the presence of molecules of nutraceutical significance such as isoflavones, tocopherols, saponins, and lecithin in soybean seeds. However, trypsin inhibitor present in soybean seeds affects the digestibility of protein (Onesti et al. 1991; Brune et al. 2010). Trypsin inhibitor activity is attributed to two polypeptides, namely, Kunitz trypsin inhibitor (KTI) and Bowman-Birk inhibitor (BBI). The latter polypeptide (8 kDa) containing seven disulfide linkages is a heat-stable molecule and has been reported to have anti-oral cancer property (Galati and Brien, 2004). It is the former polypeptide (KTI) which is primarily responsible for total trypsin inhibitor activity (Peric et al. 2014) and is considered detrimental to human health (Liener, 1994). KTI is heat labile due to presence of only two disulfide linkages, but requires minimum 15–20-min boiling for its complete inactivation in soybean seeds (Chen et al. 2014). Several methods for inactivation of trypsin inhibitor in soybean and legumes have been reviewed (Vagadia et al. 2017; Aviles-Gaxiola et al. 2018). However, due to faulty processing and insufficient temperature and duration of the heating, KTI remains active in the final food and feed products (Brandon et al. 1991).

Trypsin inhibitor is estimated spectrophotometrically through the method given by Hammerstrand et al. (1981), which is the modification of Kakade et al. (1969). This method is not only cumbersome but does not differentiate Kunitz trypsin inhibitor (KTI) from Bowman-Birk inhibitor. With the advent of densitometry, it is now possible to quantify the specific individual polypeptide resolved through polyacrylamide gel electrophoresis by running the known quantity of standard for this polypeptide. Enzyme-linked immunosorbent assay (ELISA)-based methods have also been used to quantify protease inhibitors. Brandon et al. (2004) used polyclonal antibody to quantify KTI, and monoclonal antibody for estimation of BBI in soybean seeds and products. In the present

✉ Vineet Kumar
vineetksahni@yahoo.com

[1] ICAR-Indian Institute of Soybean Research, Khandwa Road, Indore, Madhya Pradesh 452001, India

Published online: 15 March 2018

investigation, extraction conditions for KTI were optimized and large number of genotypes comprising Indian soybean cultivars and exotic genotypes were screened through native PAGE to identify the allelic differences for KTI. Subsequently, binding capacity of the major (the most frequently found) KTI allele with trypsin was assessed to quantify KTI through densitometry in selected genotypes. The same genotypes were also analyzed for BBI concentration through immunoassay so that sum of activities of both these protease inhibitors can be compared with that of total trypsin inhibitor activity (TIA) determined through a conventional spectrophotometric method given by Hammerstrand et al. (1981).

Material and Methods

Of the 180 soybean genotypes undertaken for the study, 102 are Indian soybean varieties (SL 96, PK 564, MAUS 158, RAUS 5, VLS 21, PK 472, VLS 63, NRC 86, MAUS 81, Monetta, MACS 124, RVS 2001-14, JS 20-34, JS 335, NRC 7, NRC 2, JS 20-29, JS 95-60, JS 75-46, JS72-44, JS 71-05, JS 72-280, Pusa 40, JS 93-05, Shivalik, JS 97-52, Pusa 20, Pusa 24, KHSb 2, JS 79-81, PK 327, VLS 1, SL 688, PRS 1, MACS 58, VLS 59, JS 90-41, Pusa 22, PS 10-92, PS 10-24, SL 295, PK 262, MAUS 47, T 49, MAUS 61, PS 1241, Alankar, Gujrati Soya 1, Gujrati Soya 2, VLS 47, Lee, Co 1, Co 2, Co 3, JS 80-21, MAUS 2, Pusa 16, VLS 2, Pb 1, MACS 13, MAUS 1, Bragg, MACS 450, Kalitur, Pratap Soya 2, PS 1347, Birsa Soya 1, ADT 1, JS 2, SL 4, Palam Soya, I-pelican, Shilajeet, PS 12-25, PS 10-29, PS 10-42, NRC 37, TAMS 98-21, VLS 65, Pusa 37, Pusa 98-14, Ankur, SL 525, Hardee, Indira Soya, PK 471, Harasoya, NRC 12, DS 228, Pusa 97-12, PK 416, MACS 57, MAUS 71, MAUS 32, MAUS 61-2, LSb 1, RKS 24, KB 79, JS 76-205, PK 308, TAMS 38, Davis), 24 are germplasm accessions (NRC 121**,** AGS 610, AGS 458, Karune, Akiyoshi, AGS 406, IC 210, Sawarnvasundhara, Hougyoku, Dadacha 2001, AGS 439, GC 8501, NRC 107, AGS Whydox, AGS 330, New Terpian, GC 693508, AGS 380, Boiling type, Kegone, AGS 447, Dada cha mame, Karune, AGS 406), and 54 are advanced breeding lines developed for food-grade traits like lipoxygenase-2 free, lipoxygenase-3 free, lipoxygenase-1 free, high oleic acid content. Most of the 102 soybean varieties have one parent derived from maturity group VII, while most of the 24 germplasm accessions are vegetable soybean accessions procured from Asian Vegetable Research and Development Centre, Taiwan, and many of the 54 advanced breeding lines for food-grade traits have been developed with Indian soybean variety as the recipient parent. All the genotypes were raised in the field in the cropping season 2016 and freshly harvested seeds were subjected to analysis.

Chemical Reagents and Antibodies

Benzoyl DL-arginine-paranitroanilide-hydrochloride, Kunitz trypsin inhibitor, Bowman-Birk inhibitor (trypsin/chymotrypsin inhibitor) lyophilized powder, alkaline phosphatase conjugated anti-mouse and anti-rabbit antibody, and alkaline phosphatase yellow were purchased from Sigma Aldrich, USA. BBI monoclonal detection antibodies were sourced from Agdia, Inc., Elkhart, USA. Maxisorb Nunc immunoplates and plate seal were purchased from Thermofisher India Pvt. Limited, India.

Extraction of Kunitz Trypsin Inhibitor and Its Estimation Using Densitometry

Soy flour (50 mg) was dissolved in 30 volumes of solvents, namely, double distilled water/0.01 N sodium hydro-oxide/1.5 M Tris buffer (pH 8.0) and incubated for 3 h at room temperature in a shaker incubator followed by centrifugation at 12,000 rpm for 10 min. Pellet was re-extracted with solvent. The supernatant obtained from both the events was pooled, which was mixed with bromophenol blue dye (3:1) and loaded on to the 10% native PAGE gel. In one of the lanes, 2 μg of standard KTI was loaded. Polypeptides were resolved by applying a 70-mA current for 45 min (Fig. 1). The images were captured by Bio Rad imaging system GS-900. The density of KTI polypeptide relative to the concentration of standard was quantified using software *Image Lab 5.2.1* (Biorad, India).

Validation and Limit of Detection of Densitometry Method for Estimation of KTI

Validation of densitometry method for the estimation of KTI was carried out using increasing concentration of internal standard, i.e., purified Kunitz trypsin inhibitor (0.125 to 2 μg) in the extract of a soybean variety and assessing the changes in the density through a densitometer. Standard curve so obtained showed satisfactory linearity and very strong positive correlation between internal standard concentration and relative density over the studied standard (KTI) range with the determination co-efficient of $R^2 = 0.977$ ($y = 0.3161x + 0.0479$). To determine the detection limit of KTI through densitometry, varying concentrations (10, 20, 100, and 500 ng) of KTI were subjected to native PAGE, and resolution obtained showed that minimum 20 ng of this polypeptide is required to be detected through a densitometer.

Trypsin-Kunitz Trypsin Inhibitor Complex Assay

Proteins extracted from the seeds of an Indian soybean variety (VLS 1) and 2 μg Kunitz trypsin inhibitor standard along with bromophenol dye were loaded onto adjacent lanes of 10% native PAGE gel (Fig. 3, lanes V and S, respectively). In the subsequent lanes (Fig. 3, from lanes 1–6), seed extract of the same variety (pre-incubated with

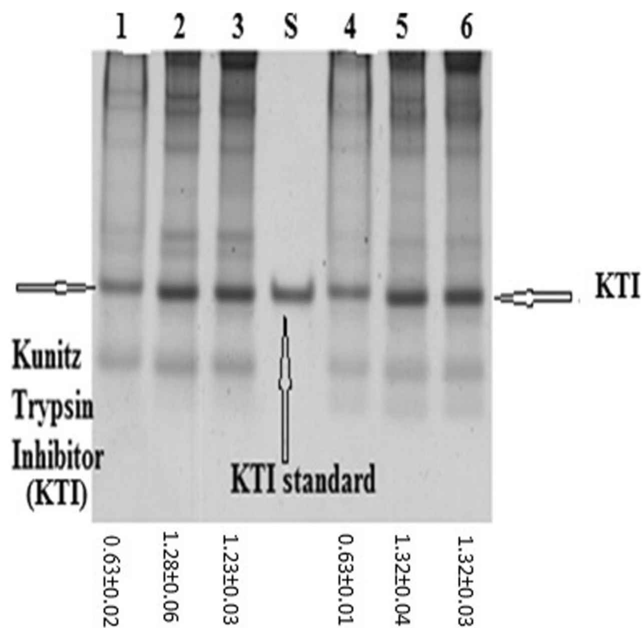

Fig. 1 Kunitz trypsin inhibitor (KTI) polypeptide extraction using different solvents. Lanes 1, 2, 3 and 4, 5, 6 correspond to KTI polypeptide in the seeds of JS97-52 and JS95-60 extracted from 1.5 M Tris buffer (pH 8.0), double distilled water (ddH_2O), 0.01 N NaOH, respectively, and resolved on 10% native polyacrylamide gel electrophoresis. Lane S corresponds to KTI standard (2 μg). Density of KTI polypeptide in lanes 1, 2, 3, 4, 5, and 6 was computed from the density of KTI polypeptide in the standard. The values given on the bottom of the lane is the density of KTI polypeptide ± SD relative to the KTI standard in lane S

anti-BBI immunoglobulins) mixed with trypsin in increasing concentrations (4.25, 4.45, 5.56, 6.2, 7.5, and 7.82 μg) was loaded. Gel was run at 75 mA for 45 min. Density of KTI polypeptide in each lane compared to that of the standard (lane S) was determined using Bio Rad imaging system GS-900 through *Image Lab 5.2.1* (Biorad, India).

Extraction and Estimation of Bowman-Birk Inhibitor Through Indirect ELISA

Extraction

BBI was extracted following the method given by Paucar-Menacho et al. (2010) and quantified through indirect ELISA method (Cucu et al. 2012) with slight modifications. For this purpose, 50 mg of defatted soy flour was suspended in 1 ml of 50 mM Tris buffer (pH 8.2) and homogenized using a polytron homogenizer (PT2100 by Kinematica, Switzerland) followed by the sonication for 70 min. During this period, samples were vortexed after every 10 min to ensure adequate suspension. The samples were then centrifuged at 20,000*g* for 35 min at 4 °C. The resultant supernatant was diluted 25,000 times to reach a final dilution of 5×10^5 times using phosphate-buffered saline. Standard curve ($y = 0.03x + 0.3146$, $R^2 = 0.996$) was drawn using purified BBI. Working range for standard was from 1 to 100 ng.

Immunoassay for Quantification

Diluted samples were coated on 96-well Maxisorb Nunc immunoplates in triplicate (100 μl/well) by incubation at 4 °C for overnight (15–18 h) followed by repeated washing (4 times) with buffer (PBS + 0.05% Tween 20) using a microplate washer (Immunowash 1575, Biorad Laboratories India Private Ltd). To block uncoated "sticky" sites on wells, 5% bovine serum albumin + 1% Tween 20 in phosphate-buffered saline was added to wells and incubated for 2 h (with interval shaking) at 37 °C followed by washing as carried out in the previous step. A 50-μl volume of diluted (1:1000 in 3% BSA buffer solution) Bowman-Birk primary antibody was added to each well and incubated for 2 h with shaking at an interval. After washing, 50-μl volume of diluted (1:1000 in 3% BSA buffer) alkaline phosphatase-conjugated anti-mouse antibody was added to each well and incubated for 2 h at room temperature. Unbound conjugated anti-mouse antibodies were removed by repeated washing followed by addition of 100 μl of *p*-nitrophenyl phosphate (pNPP). The plate was immediately sealed with black polyvinyl seal and incubated at 37 °C. Reaction was stopped at 30 min using 3 N NaOH (100 μl/well). Absorbance was recorded using an ELISA plate reader (Multiscan Go from Thermo Scientific Pvt. Ltd., India) at 405 nm.

Extraction and Estimation of Trypsin Inhibitor Activity

Trypsin inhibitor activity was determined following the standard spectrophotometric procedure (Hammerstrand et al. 1981). The sieved soy flour samples were extracted with 50 ml NaOH (0.01 N) for 4 h with constant stirring at 125 rpm in an orbital shaker so as to keep the samples in suspension. The suspension so obtained was appropriately diluted so that 2 ml of the extract inhibited 40–60% of the trypsin. For each sample, 2-ml aliquots of the diluted extract, 2 ml trypsin solution (prepared by dissolving 0.004 g of the trypsin in 0.001 N HCl), was added and the tubes were maintained in a constant temperature in water bath at 37 °C for 10 min. Five milliliters of benzoyl DL-arginine-paranitroanilide-hydrochloride [prepared by dissolving 0.08 g benzoyl DL-arginine-paranitroanilide-hydrochloride in 2 ml of dimethylsulfoxide, diluting to 200 ml with 50 mM Tris buffer (pH 8.2) containing 20 mM $CaCl_2$ and pre-warmed to 37 °C] was rapidly added to each tube. The contents were stirred immediately on a vortex mixer and the tubes were placed in a water bath at 30 °C. The reaction was terminated after 10 min by the rapid addition of 30% acetic acid. The fourth tube containing a sample extract (sample blank) was prepared by the same procedure except that the trypsin solution in this tube was added after the reaction was terminated by the addition of glacial acetic acid. The absorbance of each solution was determined at 410 nm against the sample blank. Values obtained

from each of the two sample extracts were subtracted from the trypsin standard. These values were averaged and the trypsin content inhibited was determined as follows:

$$\text{TI of defatted sample (mg/g)} = (\text{Differential absorbance} \times \text{dilution factor}) / \text{`}0.019 \times 1000$$
$$\text{Inhibition\%} = (100 \times \text{differential absorbance}) / \text{absorbance of the standard}$$

Statistical Analysis

All the statistical analysis was carried out using SAS *9.3*.

Results and Discussion

Extraction Under Different Solvents

In our study, three different solvents, viz. 1.5 M Tris buffer (pH 8.0), 0.01 N NaOH, and double distilled water, were used for optimizing the extraction of Kunitz trypsin inhibitor polypeptide from soybean flour. Figure 1 depicts the density of the KTI polypeptide of two varieties, namely, 'JS 97-52' and 'JS 95-60'extracted with three different solvents. In both the varieties 'JS 97-52' and 'JS 95-60', the density of KTI polypeptide (compared to KTI standard in lane S) extracted through 1.5 M Tris buffer (pH 8.0) (lanes 1 and 4) was significantly ($P < 0.05$) lesser than that of double distilled water (lanes 2 and 5) and 0.01 N NaOH water (lanes 3 and 6); however, no significant ($P < 0.05$) differences were noted when extraction of KTI was carried out using double distilled water and 0.01 N NaOH. Liu and Markakis (1989) used four extraction solvents, viz. 0.01 N NaOH (pH 10.0), 0.001 N HCl (pH 2.5), Tris buffer (pH 8.2), and distilled water (pH 6.5) for the extraction of trypsin inhibitors. These authors reported distilled water as the most efficient solvent for extracting trypsin inhibitor from raw soybean; however, in our results, both 0.01 N NaOH and distilled water were equally efficient for extraction of KTI. Zhou et al. (2017) reported that 20 mM Bis-Tris HCl (pH 6.4) was the most appropriate buffer for extraction of KTI with solvent to soybean flour ratio as 30:1, with a 3 h extraction time. In our study, 0.01 N NaOH and double distilled water both gave maximum extraction of KTI, with the same ratio of solvent to soy flour (30:1) and extraction time (3 h).

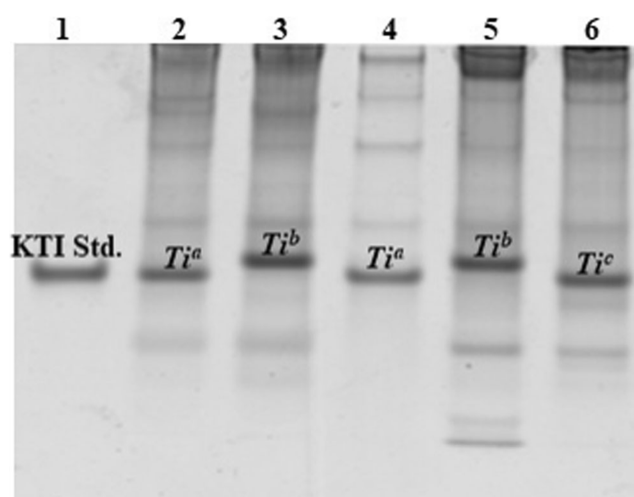

Fig. 2 Different isoforms of Kunitz trypsin inhibitor (KTI) through polyacrylamide gel electrophoresis. Lane 1 represents the band of standard KTI (20 kDa). Lanes 2, 3, 4, 5, and 6 represent KTI polypeptide (with varying R_f values) in JS 20-34, lipoxygenase-1 free advanced breeding line (JS 335 × PI 133226), boiling-type, NRC 121, and Karune, respectively. Lanes 2 and 4 represent the Ti^a isoform of KTI in two soybean genotypes, namely, JS 20-34 and boiling-type, with R_f value of 0.75. Lanes 3 and 5 represent the Ti^b isoform of KTI in lipoxygenase-1 free breeding line developed from JS 335 × PI 133226 and NRC 121 at R_f value of 0.70. Lane 6 represents Ti^c isoform of KTI in genotype Karune which shows R_f value 0.76

Allelic Forms of KTI in Indian and Exotic Soybean Genotypes

In the present investigation, 180 genotypes comprising Indian, advanced breeding lines, and exotic genotypes resolved for three KTI alleles, viz. Ti^a, Ti^b, and Ti^c were observed, with R_f values of 0.75, 0.70, and 0.76, respectively. Of these 180

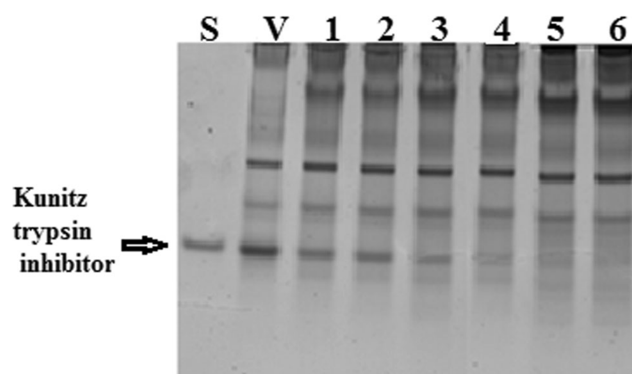

Fig. 3 Trypsin-Kunitz trypsin inhibitor (KTI) complex assay, i.e., trypsin binding capacity of KTI: Lanes V and S correspond to KTI polypeptide density in the extract (7.5 μl) of soybean varieties VLS 1 and KTI (2 μg), respectively. Lanes 1, 2, 3, 4, 5, and 6 correspond to KTI polypeptide when 4.25, 4.45, 5.56, 6.20, 7.5, and 7.82 μg trypsin added in 7.5 μl of supernatant obtained after extraction of 50-mg flour of VLS 1 in 1 ml of ddH_20. KTI in variety VLS 1 (1.56 × 2 = 3.12 μg) inhibited 7.82 μg of trypsin completely, i.e. 1 g of KTI binds with 2.51 g of trypsin

Table 1 Trypsin-Kunitz trypsin inhibitor (KTI) complex assay with increasing concentration of trypsin in soy flour extract

Lane	Trypsin (μg) added in 50-μl seed extract	KTI density relative to standard KTI (2 μg in lane S)
V	0	1.56 ± 0.05 (3.12 μg)
1	4.25	0.65 ± 0.03 (1.30 μg)
2	4.45	0.58 ± 0.04 (1.16 μg)
3	5.56	0.40 ± 0.02 (0.80 μg)
4	6.20	0.25 ± 0.01 (0.5 μg)
5	7.5	0.03 ± 0.01 (0.06 μg)
6	7.82	0.00

Values given in the parenthesis correspond to KTI concentration (μg)

accessions, 171 genotypes exhibited *Ti*^{a}, 6 genotypes showed *Ti*^{b}, and 3 genotypes showed presence of *Ti*^{c}. Figure 2 depicts the resolution of *Ti*^{a} (JS 20-34), *Ti*^{b} (lipoxygense-1 free advanced breeding line derived from JS 335 × PI 133226 and NRC 121), and *Ti*^{c} (Karune) allele. Very high frequency of *Ti*^{a} compared to that of the other two alleles in the present investigation is similar to the frequency of the three alleles reported in an earlier study (Hymowitz and Hadly 1972) which showed that of 3038 soybean accessions investigated, 88.8, 10.9, and 0.3% carried *Ti*^{1}, *Ti*^{2}, and *Ti*^{3} allele, respectively. KTI alleles demonstrated as *Ti*^{1}, *Ti*^{2}, and *Ti*^{3} by PAGE were subsequently designated as *Ti*^{a}, *Ti*^{b}, and *Ti*^{c} (Orf and Hymowitz 1977). These three variants (*Ti*^{a}, *Ti*^{b}, and *Ti*^{c}) have been reported to contain same number of amino acids (181) with varying sequences (Kim et al. 1985), having conserved amino acids (Arg-63 and Ile-64) at their reactive site. *Ti*^{a} and *Ti*^{c} have identical amino acid sequence except at the position-55, with Gly and Glu in *Ti*^{a} and *Ti*^{c}, respectively. In *Ti*^{a} and *Ti*^{b}, amino acids at positions 12, 62, 71, 74, 114, 120, 137, and 176 are different (Kim et al. 1985). In wild soybean (*Glycine soja*) germplasm, Wang et al. (2008) reported four new allelic variants of KTI, viz. *Tia*^{a1}, *Tia*^{a2}, *Tia*^{b1}, and *Ti*^{g}; *Tia*^{a1}, *Tia*^{a2}, and *Tia*^{b1} have same R_f values as *Ti*^{a} in *Glycine max* but have different nucleotide base pair sequences.

Trypsin-Kunitz Trypsin Inhibitor Complex Assay

Of the 180 soybean genotypes analyzed, 95% of the genotypes exhibited *Ti*^{a} allele. Therefore, it was important to find the binding capacity of this polypeptide with trypsin polypeptide. Figure 3 and Table 1 depict the decline in KTI with increasing conc of trypsin. At zero level of trypsin, density of KTI for variety VLS 1 was 1.56 (compared to the KTI standard) which corresponded to 3.12 μg compared to the density of KTI standard (2 μg). Increasing conc of trypsin in the seed extract caused higher decline in the density of KTI polypeptide. A 4.25, 4.45, 5.56, 6.20, 7.5, and 7.82 μg of

Table 2 Kunitz trypsin inhibitor (KTI) and Bowman-Birk inhibitor (BBI) concentration in selected soybean genotypes and the comparison of their summation with TIA

Genotypes	KTI concentration (mg/g defatted flour) A	KTI activity (mg trypsin inhibited/g defatted four) B (B = A × 2.51)	BBI Concentration (mg/g defatted flour) C	BBI activity (mg trypsin inhibited/g defatted four) 1 g of BBI binds with 0.5 g of trypsin by weight D = ½ C	TIA (KTI activity by densitometry + BBI activity by ELISA E = B + D	TIA (mg trypsin inhibited/g defatted flour) F
PS 1241	18.53 ± 0.4	46.7	22.0 ± 1.8	11.0	57.7	67.0 ± 2.3
NRC 12	14.97 ± 0.2	37.7	13.5 ± 0.1	6.7	44.4	55.6 ± 3.3
NRC 37	8.70 ± 0.3	21.9	19.7 ± 1.3	9.9	31.8	67.34 ± 2.9
JS 20-34	14.41 ± 0.2	36.3	12.5 ± 0.7	6.3	42.6	75.00 ± 2.6
New Terapian	9.03 ± 0.2	22.7	23.4 ± 1.6	11.7	34.4	59.30 ± 3.9
JS 97-52	14.00 ± 0.3	35.3	7.4 ± 0.3	3.7	39.0	72.78 ± 4.1
SL 525	10.03 ± 0.4	25.3	10.2 ± 0.7	5.1	30.4	50.00 ± 1.1

TIA was determined following Hammerstrand et al. (1981)

trypsin in the seed extract (7.5 μl), reduced the density of KTI polypeptide to 0.65, 0.58, 0.40, 0.25, and 0.03 respectively, compared to the standard. Finally, an addition of 7.82 μg of trypsin in the seed extract caused disappearance of KTI band, indicating thereby complete inactivation of KTI (3.12 μg). This showed that KTI polypeptide binds 2.51 times of concentration of trypsin. Orf and Hymowitz (1977) also performed trypsin-Kunitz trypsin inhibitor binding assay to confirm the presence of trypsin inhibitor polypeptide in soybean. However, these authors did not quantify trypsin binding with this polypeptide.

KTI and BBI in Selected Genotypes

Table 2 presents the KTI, BBI, and total trypsin inhibitor activity in selected genotypes through densitometry, ELISA, and standard spectrophotometric method given by Hammerstrand et al. (1981), respectively. KTI concentration ranged from 8.70 mg/g for NRC 37 to 18.53 mg/g of defatted flour for PS 1241, with average KTI content of 12.8 mg/g defatted soy flour. Pesic et al. (2007) investigated 12 soybean genotypes for KTI content through densitometry but expressed the KTI value as percentage of total extractable protein, which ranged from 4.28 to 6.85%, with most of the genotypes having KTI value of 4.5% of total extractable proteins. In the present study, assuming 39% average protein content and 80% as the extractable protein in the genotypes undertaken for analysis, average KTI content is 12.8 mg/g defatted soy flour equivalent to 4.1% of total extractable protein, which is comparable to the average KTI concentration (4.5%) reported by Pesic et al. (2007). Recently, Zhou et al. (2017) determined KTI concentration in the seeds of ten soybean genotypes through two-dimensional chromatography and reported a range of 6.13–8.08 mg/g of defatted flour, with average value of 6.94 mg/g of defatted flour, which was significantly lower than the average value of KTI (12.8 mg/g defatted flour) observed in seven soybean genotypes in the present study as determined by densitometry. This variation in KTI level between our results and that of Zhou et al. (2017) may be because of the genotypic differences and different methods of extraction and estimation.

Considering the results of trypsin-KTI complex assay of Ti^a allele that KTI can inhibit 2.51 concentration of trypsin, trypsin inhibitor activity of the genotypes in the present study ranged from 21.9 (NRC 37) to 46.7 (PS 1241) mg/g of defatted soy flour.

Bowman-Birk inhibitor, as determined through ELISA using monoclonal antibodies, ranged from 7.40 for JS 97-52 to 23.4 mg/g defatted soy flour for New Terapian, with average value of 15.54 mg/g of defatted soy flour. Pesic et al. (2007) analyzed BBI content in 12 soybean genotypes using PAGE based densitometry. The authors reported 1.79% of total soy protein as average BBI content. The author did not express the data in milligram per gram. Assuming 39% average protein content in the genotypes selected for the study and 80% as the extractable protein as mentioned above, average BBI in our study was 4.96% of total extractable protein, which is significantly higher than that reported by Pesic et al. (2007). Pauchar-Menacho et al. (2010) determined BBI in the germinated soybean flour through ELISA method. The authors reported a range of 2.1–3.5% of total soy protein extracted from the soy flour (manufactured from germinated seedlings for different period), which is lower than the average value for BBI (4.96%) observed in our study. The lesser value of BBI in the study of Pauchar-Menacho et al. (2010) may be because the soy flour was manufactured from germinated seedlings, which have higher levels of protease activity which may degrade the BBI.

Table 2 also shows that TIA obtained by the summation of KTI by densitometry and BBI by ELISA ranged from 30.4 (SL 525) to 57.7 (PS 1241) mg/g of defatted soy flour, with average value of 40.0 mg/g of defatted soy flour. Assuming 39% average protein content and 80% as the extractable protein, average TIA content of all the genotypes investigated in the present study was 15.1% of total extractable protein. Pesic et al. (2007) investigated 12 soybean genotypes and reported 2.32 and 13.17% of total extractable protein as the lowest and the highest total trypsin inhibitor activities, respectively, with average value of 6.32% of total extractable protein, which was significantly lower than average (15.1%) observed in our study. However, TIA of all the genotypes determined spectrophotometrically by Hammerstrand et al. (1981) method was significantly ($P > 0.05$) higher than the values obtained by the summation of KTI and BBI.

Conclusion

Trypsin inhibitor activity in soybean seeds is one of the major challenges for promoting soybeans as "health food." It is important to estimate TIA in soybean grains meant for raw material for the processing and the residual level in the final processed products. Heat-labile KTI polypeptide is primarily responsible for trypsin inhibitor activity. The remaining TIA is attributed to heat-stable BBI, which has lately been acclaimed as a nutraceutical molecule. The most widely used spectrophotometric method for the estimation of TIA does not distinguish KTI from BBI. The results showed that spectrophotometric method overestimates the TIA value as evident from the lower summation value of KTI and BBI determined through densitometry and ELISA, respectively, than the TIA obtained through spectrophotometric method. The results indicated that the soybean grains to be used as raw material and the final processed soy food products may be assessed for KTI activity than total TIA value.

Funding This work was supported by Food Safety and Standards Authority of India (FSSAI), Ministry of Health and Family Welfare, Government of India, under Sanction No. F.No.65/R&D/Soybean/RARD/2016/FSSAI.

Compliance with Ethical Standards

Conflict of Interest Vineet Kumar declares that he has no conflict of interest. Anita Rani declares that she has no conflict of interest. Mohd Shuaib declares that he has no conflict of interest. Priyanka Mittal declares that she has no conflict of interest.

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

Informed Consent Not applicable as this study does not include any human participants.

References

Aviles-Gaxiola S, Chuck-Hernandez C, Serna Saldivar SO (2018) Inactivation methods of trypsin inhibitor in legumes: a review. J Food Sci 83(1):17–29. https://doi.org/10.1111/1750-3841.13985

Brandon DL, Bates AH, Friedman M (1991) ELISA analysis of soybean trypsin inhibitors in processed foods. Adv Exp Med Biol 289:321–337. https://doi.org/10.1007/978-1-4899-2626-5

Brandon DL, Bates AH, Friedman M (2004) Immunoassays for Bowman–Birk and Kunitz soybean trypsin inhibitors in infant formula. J Food Sci 69(1):vi–67. https://doi.org/10.1111/j.1365-2621.2004.tb17849.x

Brune MFSS, Pinto MO, Peluzio MCG, Moreira MA, Barros EG (2010) Biochemical and nutritional evaluation of a free soybean line Kunitz trypsin inhibitor and lectins. Food Sci Technol 30(3):657–663. https://doi.org/10.1590/s0101-20612010000300014

Chen Y, Xu Z, Zhang C, Kong X, Hua Y (2014) Heat-induced inactivation mechanisms of Kunitz trypsin inhibitor and Bowman-Birk inhibitor in soymilk processing. Food Chem 154:108–116

Cucu T, Deverese B, Kerkaert B, Rogge M, Vercruysse L, De Meulenae B (2012) ELISA based detection of soybean proteins: a comparative study using antibodies against modified and native proteins. Food Anal Methods 5:1121–1130. https://doi.org/10.1007/s1261-011-9341-y

Galati G, Brien PJO (2004) Potential toxicity of flavonoids and other dietary phenolics: significance for their chemo preventive and anticancer properties. Free Radic Biol Med 37(3):287–303. https://doi.org/10.1016/j.freeradbiomed.2004.04.034

Hammerstrand GE, Black LT, Glover JD (1981) Trypsin inhibitors in soy products. Modification of the standard analytical procedure. Cereal Chem 58(1):42–45

Hymowitz T, Hadly HH (1972) Inheritance of trypsin inhibitor variant in seed protein of soybean. Crop Sci 12(2):197–198. https://doi.org/10.1086/336690

Kakade ML, Simons N, Liener IE (1969) An evaluation of natural VS synthetic substrates for measuring antitryptic activity of soybean samples. Soybeans: natural vs synthetic substrates 46:518–526

Kim SH, Hara S, Hase S, Ikenaka T, Toda H, Kitampura K, Kaizuma N (1985) Comparative study on amino acid sequences of Kunitz-type soybean trypsin inhibitors, Ti^a, Ti^b, and Ti^c. J Biochem 98(2):435–448

Liener IE (1994) Implications of anti-nutritional components in soybean foods. J Critical Rev in Food Sci Nutr 34:31–67. https://doi.org/10.1086/336690

Liu K, Markakis P (1989) An improved colorimetric method for determining antitryptic activity in soybean products. Cereal Chem 66(5):415–422

Pesic MB, Vacelic-Radovic BV, Barac MB, Stanojevic SP, Nedovie VA (2007) Influence of different genotype on trypsin inhibitor levels and activity in soybeans. Sensors 7:67–74

Onesti S, Brick P, Blow DM (1991) Crystal structure of a Kunitz-type trypsin inhibitor from Erythrina caffra seeds. J Mol Bio 217(1):153–176. https://doi.org/10.1016/00222836(91)90618-G153-176

Orf JH, Hymowitz T (1977) Inheritance of a second trypsin inhibitor variant in seed protein of soybeans. Crop Sci 17(5):811–813

Peric V, Srebric M, Dragicevic V, Nikolic A, Mikic A, Drinic SM (2014) Development of soybean varieties with specific nutritional composition of grain. J Hyg Eng Design 8:174–177

Pauchar-Menacho LM, Berhow MA, Mandarino JMG, Gonzalez DE, Mejia E, Chang YK (2010) Optimization of germination time and temperature on the concentration of bioactive compounds in Brazilian soybean cultivar BRS 133 using response surface methodology. Food Chem 119:636–642. https://doi.org/10.1016/j.foodchem.2009.07.011

Vagadia BH, Vanga SK, Raghavan VSS (2017) Inactivation methods of soybean trypsin inhibitor—a review. Trends in Food Sci Tech 64:115–125. https://doi.org/10.2016/j.tifs.2017.02.003

Wang KJ, Takahata Y, Kono Y, Kaizuma N (2008) Allelic differentiation of Kunitz trypsin inhibitor in wild soybean (*Glycine soja*). Theor Appl Genet 117:565–573. https://doi.org/10.1007/s00122-008-0800-9

Zhou T, Han S, Li Z, He P, (2017) Purification and quantification of Kunitz trypsin inhibitor in soybean using two-dimensional liquid chromatography. Food Anal Methods doi:https://doi.org/10.1007/s1216-017-0902-6