JPMB | Journal of Plant Molecular Breeding
Published by Genetics and Agricultural Biotechnology Institute of Tabarestan
2024 | Volume 12 | Issue 2

KTI and BBI protease inhibitors in seed storage protein of Iranian soybean cultivars

Mehdi Arefrad*[1], Nadali Babaian Jelodar[2], Ghorbanali Nematzadeh[2], Ali Dehestani[1] and Elahe Gorji[2]

[1] Genetics and Agricultural Biotechnology Institute of Tabarestan (GABIT), Sari Agricultural Sciences and Natural Resources University (SANRU), Sari, Iran
[2] Department of Biotechnology, Sari Agricultural Sciences and Natural Resources University (SANRU), Sari, Iran

Original paper

OPEN ACCESS

Edited by
Prof. Ahmad Arzani,
Isfahan University of Technology, Iran

Date
Received: 26 December 2024
Accepted: 15 January 2025
Published: 26 February 2025

Correspondence
Dr. Mehdi Arefrad
Mehdiarefrad@yahoo.com.

Citation
Arefrad, M., Babaian Jelodar, N. A., Nematzadeh, Gh.A., Dehestani, A. and Gorji, E. (2024). KTI and BBI protease inhibitor activity in seed storage protein of Iranian soybean cultivars. *J Plant Mol Breed.* 12 (2): 95-107.
doi: 10.22058/jpmb.2025.2048750.1324.

Abstract: Kunitz (KTI) and Bowman-Birk (BBI) protease inhibitors are considered the most significant factors that decrease the quality of soybean proteins. In this study, the levels of these proteins were investigated using the 2D-PAGE, and their activity was evaluated through trypsin and chymotrypsin proteases in ten Iranian soybean cultivars. These results indicated that the Katol cultivar has the lowest concentration of both KTI and BBI proteins and the lowest trypsin and chymotrypsin inhibitory levels. Therefore, this cultivar is an ideal choice for soybean protein-based diets and could also serve as a valuable parent in breeding programs aimed at improving protein quality in soybean . Moreover, no significant correlation was found between KTI with BBI proteins, nor between these proteins with protease activity. Therefore, it seems that genetic control targeting either KTI or BBI proteins alone may not be an effective approach to improving the quality of soybean proteins. Additionally, no correlation was found between KTI and BBI proteins with agronomic traits. This also suggests that reducing protease inhibitors in soybean proteins does not adversely affect overall soybean performance.

Keywords: Soybean, kunitz protein, bowman-birk protein, trypsin inhibitor, chymotrypsin inhibitor, agronomic traits.

Introduction

Among oilseeds, soybean with 40% protein and 20% oil, is considered a strategic plant for oil and protein production. Soybean is the top oil producer globally and its seed protein competes with other sources of animal protein. Currently, Brazil leads in production with 169 million tons, followed by the United States with 118 million tons, and Argentina with 49 million tons. Iran ranks 34th in the world with an estimated 25,000 hectares, of soybean cultivation area and an annual production of 53,000 tons (USDA, 2024). The nutritional values of soybeans play a prominent role in human and livestock nutrition. Soy protein is increasingly consumed by humans and it also makes a relatively inexpensive protein source for livestock (Medic et al., 2014). Nevertheless, raw soybeans contain several antinutritional factors such as protease inhibitors, lipoxygenase, tannins, and phytates that could potentially impact the body's metabolism of consumers (Yasothai, 2016; Luthria et al., 2018). Among them, protease inhibitors such as trypsin and chymotrypsin inhibitors are typically found in soybean meal at very high levels (Natarajan et al., 2016; Park et al., 2023; Varga et al., 2023; Vorster et al., 2023; Kim et al., 2024). These antinutritional factors regulate the consumer's endogenous proteases and can interfere with protein digestion resulting in a negative impact on the utilization of soybean-based protein products (Varga et al., 2023). The inhibitors have been shown to induce pancreatic enzymes, hypersecretion, and a rapid stimulation of pancreas growth, which is histologically described as pancreatic hypertrophy and hyperplasia (Palacios et al., 2004). Several protease inhibitors have been identified in soybean storage proteins. Much of their activity is thought to be attributed to Kunitz (KTI) and Bowman-Birk (BBI) proteins, which represent the majority of the bioactive proteins that strongly inhibit trypsin and trypsin-chymotrypsin respectively (Gillman et al., 2015; Mittal et al., 2021; Kim et al., 2024).

The KTI is a monomeric and non-glycosylated protein encoded by a single gene in cotyledon. In contrast, the BBI is controlled by three genes and has two independent sites of inhibition, one at Lys 16-Ser 17 against trypsin and the other at Leu 43-Ser 44 against chymotrypsin (Livingstone et al., 2007; Mittal et al., 2021). It has been suggested that the levels of KTI and BBI vary among different genotypes (Pesic et al., 2007; Gu et al., 2010; Natarajan et al., 2016; Mittal et al., 2021).

A more comprehensive understanding of the variability of protease inhibitors among different soybean varieties could enhance current efforts to improve seed protein quality. However, there is limited information available regarding the relationship between genotype-specific seed levels of KTI and BBI proteins, particularly BBI, and their impact on trypsin and chymotrypsin inhibitory. Pesic et al. (2007) evaluated 12 commercial soybean cultivars and found no relationship between KTI and BBI proteins and trypsin activity. However, Zilic et al. (2011) observed a negative correlation between KTI and trypsin activity. Further evaluation of KTI and BBI protease inhibitors and activities, as well as exploring possible correlations among them could be useful for enhancing the health impacts of soybean proteins.

Unfortunately, given the importance of protease inhibitor activity in soy protein quality, complete information about the protein quality of different Iranian soybean cultivars is unavailable. Therefore, this study aimed to investigate the relationship between the level of KTI and BBI protease inhibitors in seed protein with trypsin and chymotrypsin inhibitory, as well as the most important agronomic characteristics in 10 soybean cultivars currently cultivated in Iran. Awareness of the relationship between protease inhibitors and these characteristics could enhance our knowledge of how to improve new soybean cultivars with superior quantitative and qualitative traits.

Materials and Methods

Plant material

The 10 adopted Iranian soybean cultivars: Hill, Sahar, Nekador, Caspian, Katol, Sari, Telar, Tapor, Arian, and Parto were provided by the Mazandaran Agriculture Research Center and planted in the experimental field of the Genetic and Agricultural Biotechnology Institute of Tabarestan (GABIT). The names of current Iranian soybean cultivars, breeding pedigree, year of introduction, and their grope maturity are presented in Table 1. Field experiments were conducted using a randomized

complete block design with three replications. Each plot consisted of five rows, with a distance of 50 cm between rows and 10 cm between plants. After the plants reached full maturity and any marginal effects were eliminated, ten plants were randomly chosen from each plot and assessed for key agronomic characteristics including plant height, number of branches, 100-grain weight, and seed yield.

2D-PAGE analyses

The seeds from each cultivar were peeled and finely powdered using a mortar and pestle in the presence of liquid nitrogen. 10 mg of each sample was washed with TCA/acetone 10% at -20 °C for 1 hour. The precipitated pellet was dried under vacuum and suspended in Isoelectric Focusing (IEF) buffer (7M urea, 2M Thiourea, 4% CHAPS, 1% DTT, and 0.2% ampholyte pH 3.0-10.0 as a carrier) in a ratio of 1:15 (v/w) (Natarajan, 2014; Natarajan et al., 2016). Samples were mixed in a vortex mixer for 30 minutes at room temperature. The insoluble tissue was removed by centrifugation at 14000 g for 15 minutes. Protein concentration was determined according to the Bradford method, with bovine serum albumin (BSA) as the standard (Bradford, 1976). 2D-PAGE was performed according to the instructions in the Bio-Rad IPG Ready Strips manual. A 1000 µg protein sample was loaded onto 24 cm IPG strips pH: 4.0-7.0 and rehydrated for 1 hour. The strips were then covered with mineral oil and allowed to rehydrate overnight.

The first dimension (isoelectric focusing) was performed with the following program: 50 V for 20 min, 200 V for 1 h, 500 V for 1 h, 1000 V for 1 h, 4000 V for 2 h, 8000 V for 2 h and 9500 V for 2.5 h, using the protean IEF electrophoretic chamber (Bio-Rad). For the second dimension, the IPG strips were initially incubated for 2×15 minutes with 2% DTT to reduce sulfhydryl groups, followed by alkylation with 2.5% Idoacetamide in 50 mM Tris-HCl pH: 8.8, 6M urea, 30% glycerol and 2% SDS. Equilibrated strips were then placed onto 12% polyacrylamide gels and covered with molten 0.5% Agarose in 0.375M Tris pH: 8.8, 0.192M glycine, 2% SDS and 0.01% bromophenol blue as sample buffer. They were then electrophoresed using Tris/Glycine/SDS running buffer (Bio-Rad PROTEAN Plus Dodeca). The electrophoresed gels were stained in a solution of 0.1% (w/v) Coomassie Brilliant Blue R250, 40% methanol, and 10% acetic acid for 1 hour. Subsequently, they were destined several times using a solution of 40% methanol and 10% acetic acid.

The gels were scanned separately at a resolution of 600 dpi and 16-bit TIFF grayscale pixel depth using the Bio-Rad Calibrated Densitometer GS-800. Image analysis was then conducted using SameSpot Progenesis software (TotalLab, Newcastle, England) which enables spot detection, quantification, and spot matching across multiple gels. The KTI and BBI proteins were identified using the Soybean Proteome Database, SoyProDB (Tavakolan et al., 2013; Natarajan, 2014), as well as TagIdent and ExPASy software tools (SIB Bioinformatics Resource Portal) (Table 2).

Table 1. Characteristics of the current Iranian soybean cultivars.

No.	Line	Cultivar	Breeding method	Growth type	Maturity group	Year of release
1	.....	Hill	Imported	Determinate	4	1961
2	Pershing	Sahar	Imported	Semi-determinate	5	1993
3	BP	Telar	Selection from Pershing	Determinate	5	2001
4	JK	Sari	Imported	Determinate	5	2001
5	032	Nekador	BP×Hood (Gorgan-3)	Determinate	5	2010
6	DPX	Katol	Imported	Semi-determinate	5	2010
7	033	Caspian	BP×Hill	Determinate	5	2011
8	.....	Arian	Imported	Determinate	5	2015
9	032-240-P1	Parto	Mutation from Nekador	Determinate	5	2016
10	2002	Tapoor	Sahar×JK	Determinate	5	2016

Reference: Seed and Plant Certification and Registration Institute (SPCRI)

Table 2. Characterization of protease inhibitor proteins based on soybean proteome data.

No.	Protein name	Accession number	Theoretical pI	Molecular weight (Da)
1	Kunitz trypsin inhibitor (KTI1)	P25272	4.97	22545.94
2	Kunitz trypsin inhibitor (KTI1)	NP_001237705.1	4.85	22446.81
3	Kunitz trypsin inhibitor (KTI1)	NP_001236275.2	5.39	22686.88
4	Kunitz trypsin inhibitor (KTI2)	NP_001237786.1	5.24	22676.01
5	Kunitz trypsin inhibitor (KTI3)	NP_001238611.2	4.95	24076.37
6	Kunitz trypsin inhibitor (KTI3)	NP_001237751.1	5.18	23655.24
7	Bowman-Birk protease inhibitor (BBI)	NP_001238367	5.15	12062.88
8	Bowman-Birk protease inhibitor (BBI)	KAH1233928	6.85	24020.93
9	Bowman-Birk protease inhibitor (BBI)	CAA48657	4.92	11496.28

Trypsin and chymotrypsin inhibition assay

Total protein soluble was extracted using an extraction buffer (100 mM Tris-HCl pH: 8.0 containing 20 mM $CaCl_2$) at a ratio of 1:10 (w/v) of seed powder. The suspension was shaken for 30 minutes and then centrifuged at 15000 rpm for 30 minutes at 4 °C. The clear supernatant was collected and used for the protease inhibitor assay.

The level of protease inhibition was determined by measuring the activity of trypsin (T1426) and chymotrypsin (C3142) protease using specific substrates, BAPNA (N-α-Benzoyl-DL-Arginine p-Nitroanilide) and GPNA (N-Glutaryl-L-Phenylalanine p-Nitroanilide) respectively, from Sigma Aldrich (Livingstone et al., 2007).

Preparations of trypsin and chymotrypsin were made at concentrations of 0.002% and 0.006% respectively, in 1 mM HCl at 4 °C. To prepare the substrate, dissolve 10 mg of BAPNA and 20 mg of GPNA in 200 µL of DMSO then dilute to 100 mL with 100 mM Tris-HCl pH: 8.0 containing 20 mM $CaCl_2$. To measure protease activity, 20 µl of extracted protein was incubated with 300 µl of each protease for 5 minutes. Protease activity was then measured by adding 500 µl of specific substrate and incubating for 20 minutes at 37 °C. The reaction was stopped by adding 100 µl of 30% acetic acid. For the control sample, protease activity was tested with a specific substrate in the presence of a protein extraction buffer. The absorbance of the entire reaction solution was measured at 405 nm against a reagent blank (where the enzyme and extract were replaced with the extraction buffer) using a spectrometer. The inhibition of trypsin and chymotrypsin was calculated using the following equations: Percent inhibitors = 1 – (OD_{Sample} / OD_{Enzyme}) × 100

Statistical analysis

All experimental samples were evaluated with three replications. The field experiment was designed using a randomized complete block design, while the laboratory samples were assessed using a completely randomized design. Quantitative analysis of protein subunits was conducted using the SameSpot Progenesis software program. Data analysis was carried out using the SAS statistical software and the EXCL program. After conducting an analysis of variance test (ANOVA) for independent samples, all means were compared using Duncan's multiple range test at a 5% probability level. Correlation coefficient calculations for traits were determined using Pearson's simple correlation analysis to assess the strength and significance of trait associations.

Results

Agronomic characteristics

The results of the comparison of mean agronomic traits showed that the Katol cultivar had the highest plant height, distinguishing it from the other cultivars (Table 3). Meanwhile, Parto and Sari cultivars had the lowest plant height. The Katol, Hill, and Arian cultivars had the highest number of branches. The highest number of pods was observed in Parto followed by Arian. The highest pod weight was observed in Katol followed by Telar and Arian. The highest 100-grain weight was also obtained in Arian, with the lowest in the Parto cultivar. Finally, the Katol cultivar with the highest plant height, number of branches, pod weight, and

grain weight, and the Telar cultivar with the highest number of seeds and pod weight, had the highest grain yield (Table 3).

Quantification of KTI and BBI proteins

The seed storage protein pattern and protease inhibition subunits are displayed in Figure 1. The results of the quantification of protease inhibitor proteins indicated that the studied cultivars were divided into two groups based on KTI protein content (Figure 2). Cultivars such as Hill, Sahar, Nekador, Caspian, Sari, Telar, Arian, and Parto had the highest content of KTI protein and were placed in the first group, while Katol and Tapor cultivars with the lowest content were placed in the second group. For BBI protein, the studied cultivars were divided into four groups. Sahar ranked first with the highest amount, while Caspian and Katol were ranked last with the lowest amount (Figure 2).

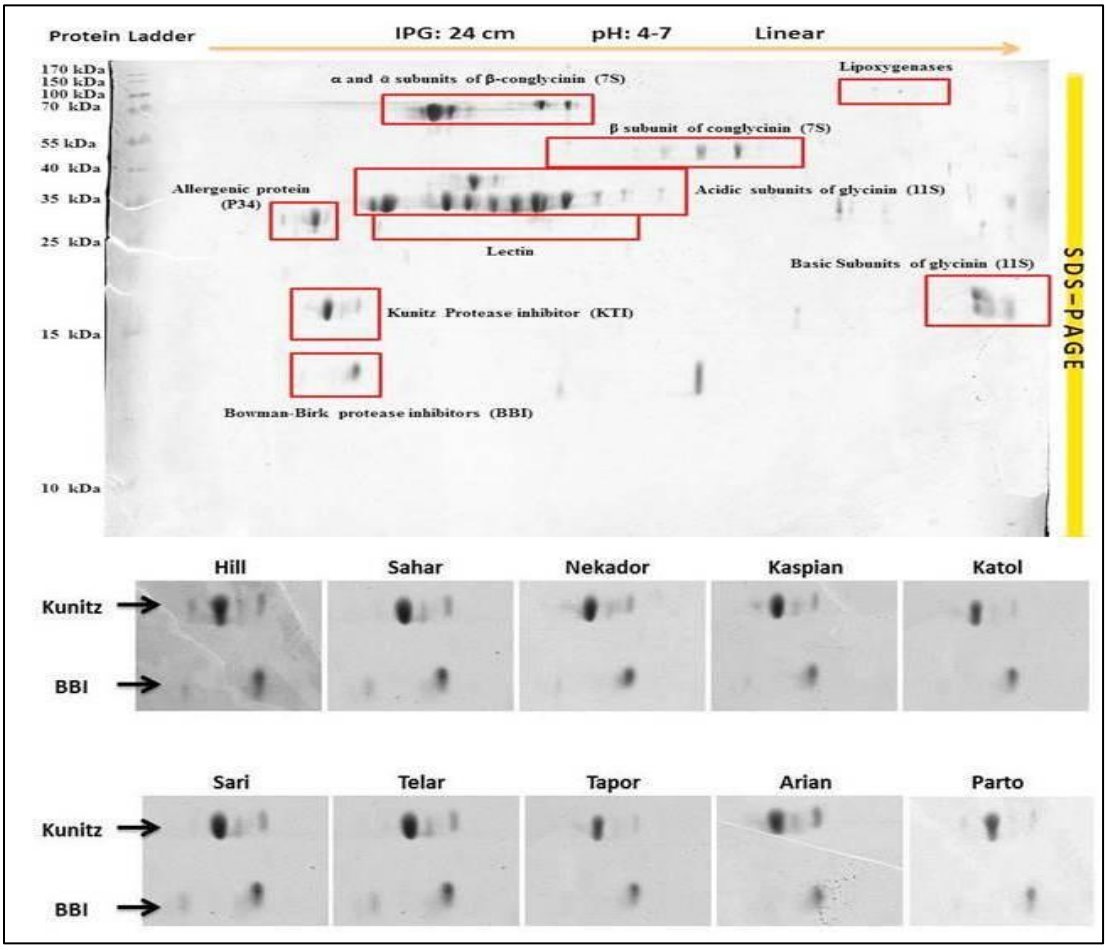

Figure 1. Seed storage proteome pattern of soybean and KTI and BBI proteins position in different Iranian soybean cultivars based on soybean proteome data.

Finally, Hill, Nekader, Sari, Tellar, and Arian cultivars which had the highest levels of KTI protein, and Sahar cultivar which had the highest levels of KTI and BBI proteins, also had the highest levels of protease inhibitors (Figure 2). Conversely, Katol with the lowest levels of KTI and BBI proteins followed by Tapor which has the lowest levels of KTI protein showed the lowest content of protease inhibitors (Figure 2).

Table 3. Mean comparison of agronomic traits of soybean cultivars.

Plant traits	Soybean cultivars									
	Hill	Sahar	Telar	Sari	Nekador	Katol	Caspian	Arian	Parto	Tapour
Plant height (cm)	121.4 cd	130.0 bc	120.6 cd	98.5 e	136.5 b	183.8 a	125.5 cd	123.3 cd	95.0 e	118.5 d
No. of branches	3.7 a	1.7 d	2.8 bc	2.8b c	1.1 d	3.7 a	1.1 d	3.5 ab	2.7 c	1.7 d
No. of pod.	67.2 cd	82.6 a-d	90.3 ab	77.2 b-d	64.3 d	80.7 b-d	62.3 d	88.3 a-c	103.3 a	77.0 b-d
Pods weight (gr)	31.0 c	35.0 bc	42.2 ab	36.3 bc	35.6 bc	46.7 a	32.2 c	38.7 a-c	32.1 c	30.8 c
100 seed weight (gr)	16.0 de	16.2 cd	15.2 de	19.0 b	17.5 c	22.0 a	16.4 cd	19.9 b	12.5 f	14.7 e
Seed yield (gr)	20 cd	22.44 b-d	27.17 ab	26 abc	22.94 b-d	32.22 a	21.22 b-d	24.83 b-d	19.28 d	21.06 b-d

Means in each row with similar letter(s) are not significantly different at 5% probability level, using Duncan's multiple range test

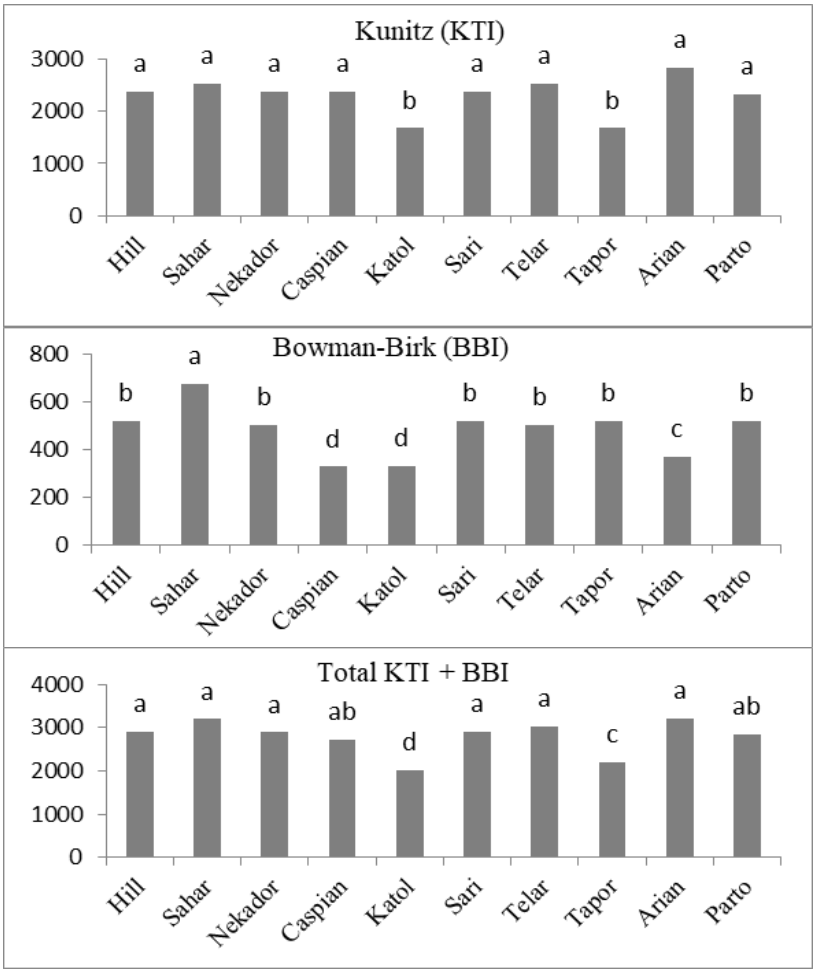

Figure 2. Quantified of Kunitz (KTI) and Bowman-Birk (BBI) protease inhibitors of 10 soybean cultivars by densitometry analyses.

Trypsin and chymotrypsin inhibition assay

The results of the protease inhibitors showed that the studied cultivars were divided into five groups based on trypsin inhibition (Figure 3). Sahar, Caspian, and Tapor cultivars had the highest levels of trypsin inhibition and were placed in the first group, while Katol, Telar, and Arian cultivars with the lowest levels were in the last group. For chymotrypsin inhibition, the Sahar, Caspian, and Tapor cultivars showed the highest inhibition rate, while the Katol was followed by Nekador, Telar, and Arian cultivars with the lowest inhibition rates. In conclusion, the Sahar, Caspian and Tapor cultivars exhibited the highest levels of inhibition for both trypsin and chymotrypsin proteases, while Katol was followed by Nekador, Telar and Arian with the lowest levels of inhibition for both trypsin and chymotrypsin proteases. Finally, Sahar, Caspian, and Tapor cultivars which have high levels of trypsin and chymotrypsin inhibition showed the highest protease inhibition. In contrast, Katol followed by Nekador, Telar, and Arian which have low levels of trypsin and chymotrypsin inhibition showed the lowest protease inhibition (Figure 3).

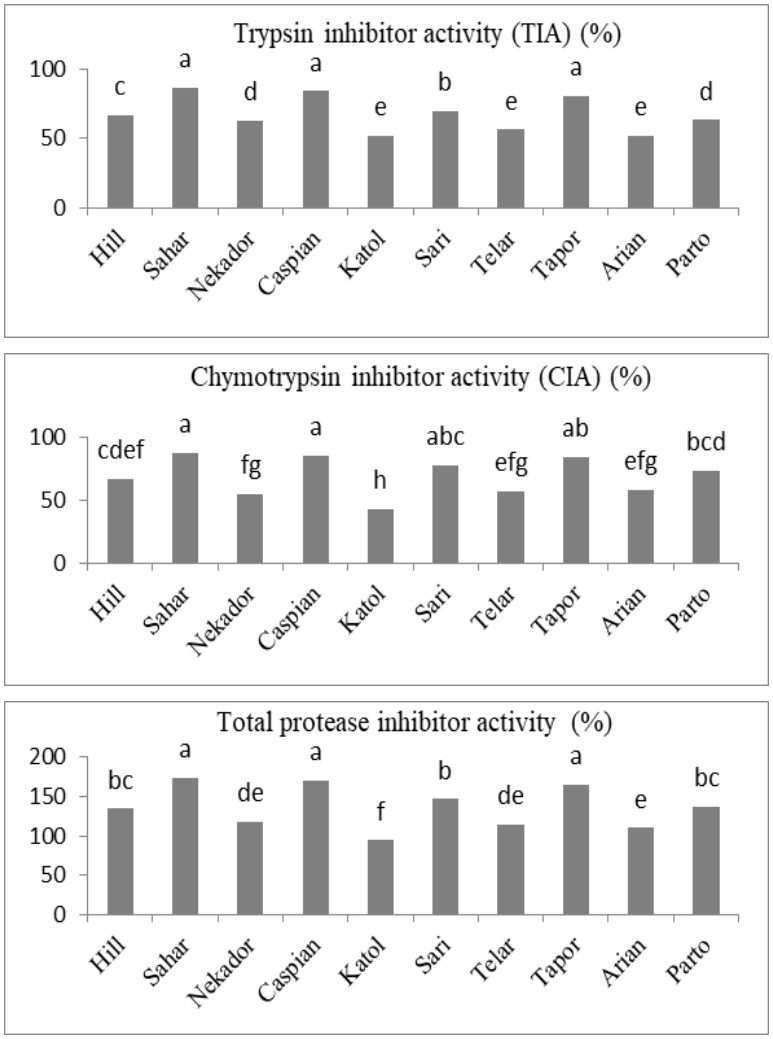

Figure 3. Protease inhibitor activity in the presence of the seed storage protein extracts of 10 soybean cultivars.

Correlation analysis

The relationships between protease inhibitory with protease activity as well as agronomic traits, were studied using correlation coefficient analyses (Table 4). These results demonstrate that the impact of the KTI protein on the level of protease inhibitor is more significant than that of the BBI protein (0.96).

Although no significant association was found between KTI with BBI protease inhibition, a very positive and significant association was observed between trypsin with chymotrypsin activity (0.92). Moreover, KTI and BBI protease inhibitory did not show a significant correlation with vegetative and reproductive traits. However, protease activity had a significant negative correlation with the number of branches, number of pods and grain yield.

Table 4. Correlation coefficients between protease inhibitors and agronomic traits.

Protease inhibitors /agronomic traits		Protease inhibitors			Protease activity		
		KTI	BBI	KTI + BBI	TIA	CIA	TIA + CIA
Protease inhibitors	KTI	1					
	BBI	0.15	1				
	KTI + BBI	0.96**	0.41	1			
Protease activity	TIA	-0.10	0.42	0.02	1		
	CIA	0.05	0.43	0.17	0.92**	1	
	TIA + CIA	0-.01	0.44	0.10	0.97**	0.98**	1
Agronomic traits	Plant height	-0.45	-0.43	-0.53	-0.29	-0.57	-0.45
	Branch	0.01	-0.24	-0.05	-0.70*	-0.57	-0.64
	Number of Pods	0.12	0.18	0.16	-0.38	-0.11	-0.24
	Pod weight	-0.09	-0.38	-0.19	-0.68*	-0.75*	-0.73*
	Seed weight	-0.08	-0.52	-0.22	-0.44	-0.54	-0.50
	Yield	-0.23	-0.40	-0.32	-0.57	-0.65*	-0.63*

* and **: Significant at 1% and 5% probability levels, respectively. KTI; Kunitz protein; BBI; Bowman-Birk protein; TIA; Trypsin inhibitor activity; CTI; Chymotrypsin inhibitor activity

Discussion

In recent years, soybean cultivars have been bred based on maximizing seed yield and oil content. Unfortunately, this strategy has led to limited genetic diversity, which may hinder advancements in enhancing seed yield, protein content, and oil content (Kisha et al., 1998). Soybean meal is increasingly being consumed by humans, and serves as a relatively inexpensive protein source for animal husbandry. Today, improving the quality of storage proteins has become one of the most important goals in soybean breeding programs (Medic et al., 2014). The quality of soybean proteins is limited by the high content of protease inhibitors, which have a negative nutritional impact in both food and feed applications (Natarajan et al., 2016; Vorster et al., 2023; Kim et al., 2024).

Several protease inhibitors have been identified in soybean storage proteins, with most of their activity attributed to Kunitz (KTI) and Bowman-Birk (BBI) proteins (Livingstone et al., 2007; Mittal et al., 2021). These proteins are known to strongly inhibit trypsin and trypsin-chymotrypsin activity respectively (Gillman et al., 2015; Mittal et al., 2021; Kim et al., 2024). These inhibitors can also cause an increase in the secretion of pancreatic enzymes and stimulate the rapid growth of the pancreas, resulting in histologically described pancreatic hypertrophy and hyperplasia (Palacios et al., 2004). Soybean KTI proteins are encoded by three genes: KTI-1, KTI-2, and KTI-3. The KTI-1 and KTI-2 genes are mainly expressed in leaves, roots, and stems, while the KTI-3 gene is specifically expressed in seeds (Krishnan, 2001). Unlike the KTI protein, which is encoded by

a single gene (21 kDa), the BBI proteins are encoded by a family of related genes and are generally expressed in seeds (8-15 kDa) (de Almeida Barros et al., 2012). Protease inhibitors play a defensive role in plants. The KTI-1 and KTI-2 proteins in vegetative tissues provide resistance to pests, while KTI-3 in seeds plays an anti-nutritional role for consumers (Gillman et al., 2015; Sultana et al., 2023; Vorster et al., 2023). The transfer of these defense genes and their expression in the leaves through genetic engineering strategies has enabled many plants to become resistant to a variety of pests (Sultana et al., 2023).

It is possible to inactivate the protease inhibitors through heat treatment or eliminate them by fractionation during food processing. Nonetheless, the BBI protein is heat stable due to the presence of seven disulfide bridges, unlike KTI which only has two disulfide bonds (Friedman and Brandon, 2001; Perez-Maldonado et al., 2003). Moreover, heat treatment reduces the solubility of proteins and availability of amino acid. Unfortunately, this approach is not only costly, but also not completely effective (Luthria et al., 2018). Many studies have reported variations in the content of KTI and BBI proteins among different soybean cultivars (Pesic et al., 2007; Mittal et al., 2020). Cultivars lacking these proteins have also been identified and introduced (Mittal et al., 2021; Dhaliwal et al., 2022). Therefore, identifying and improving new cultivars with low concentration of KTI and BBI proteins will help enhance the nutritional quality of soybean proteins, making it more suitable for consumers (Dhaliwal et al., 2022).

The evaluation of protease inhibitory proteins revealed significant differences in KTI and BBI protein concentrations among the current Iranian cultivars. The results of this study indicated that among the Iranian soybean cultivars the Sahar with the high concentration of both KTI and BBI proteins, exhibits a high level of trypsin and chymotrypsin inhibitory. Additionally, Katol with the low concentration of both KTI and BBI proteins, shows the lowest levels of trypsin and chymotrypsin inhibitory. Therefore, Katol cultivar can be an ideal cultivar for soybean protein-based diets and could also be utilized as a valuable parent in a breeding program to improve the quality of soybean proteins. It has been suggested that the KTI and BBI proteins, especially BBI, possess anticarcinogenic and cancer chemopreventive properties (Kennedy and Szuhaj, 1994; Isanga and Zhang, 2008; Krishnan et al., 2012). Therefore, the Sahar cultivar, which had the highest concentration of BBI and a high percentage of trypsin-chymotrypsin inhibition, may be a suitable source for this purpose. Although Sahar with the high level of KTI and BBI proteins and Katol with the low level of KTI and BBI proteins, characterized by the high and low activity of trypsin and chymotrypsin inhibitory respectively, nevertheless, no significant relationship was found between KTI and BBI proteins with trypsin and chymotrypsin inhibitory. For example, the Tapor cultivar, which had a low content of KTI, exhibited high trypsin inhibitory. In contrast, the Arian cultivar, which had a high content of KTI, showed low trypsin inhibitory. There have been several reports on the relationship between KTI protein with protease activity. For example, Zilic et al. (2011) observed a positive correlation between KTI and trypsin inhibitory. Moreover, Dhaliwal et al. (2022), studied soybean isolines with a null kti allele and reported that these isolines showed significant differences in protease inhibitor activity. However, Pesic et al. (2007) reported no association between KTI with trypsin inhibitory. A study also demonstrated that mutants of KTI-1, KTI-2 and KTI-3 were unable to produce adequate levels of protease activity in soybean protein (Kim et al., 2024). Therefore, there seems to be an interaction between KTI and BBI proteins for protease inhibitors in soybean storage protein. For example, the evaluation of a soybean genotype with frameshift mutations in both the KTI-1 and KTI-3 proteins showed a significant increase in BBI protein levels (Gillman et al., 2015). Whereas, an evaluation of genetic diversity in a wide range of genotypes that exhibited significant differences in trypsin inhibitors, revealed that there was no significant association between KTI and BBI proteins (Mittal et al., 2021). On the other hand, it has been reported that soybean lipoxygenase protein, which is considered a defense protein due to its ability to oxidize unsaturated fatty acids, is also capable of inhibiting the proteases trypsin and chymotrypsin (de Carvalho et al., 1999; Barros et al., 2008). In addition, unlike the Kunitz protein, the BBI protein plays an important role in inhibiting both

trypsin and chymotrypsin proteases (Livingstone et al., 2007; Mittal et al., 2021). Therefore, based on the results of this study and other reports, it appears that genetic control alone of a protease inhibitor protein cannot be an effective approach to increase the quality of soybean proteins.

Based on our results, no correlation was found between KTI and BBI proteins with agronomic traits. However, protease activity showed a negative correlation with pod weight and seed yield. Limited information is available on the relationship between protease inhibitors with agronomic characteristics in soybean. Nevertheless, Dhaliwal et al. (2022), studied soybean isolines with a null kti allele and reported that there were no significant differences in agronomic traits such as days to maturity, plant height, seed weight, protein content and oil content. Livingstone et al. (2007) also demonstrated that reducing the protease inhibitors in soybean seed protein can be achieved without making substantial modifications to seed composition or nutritional quality. Therefore, the lack of correlation between KTI and BBI proteins with agronomic traits, as well as the negative correlation between protease activity with grain yield, indicate that reducing protease inhibitors in soybean proteins has no negative impact on soybean breeding programs. On the other hand, some studies have reported that new modern cultivars of soybean have fewer protease inhibitor proteins compared to the wild and older varieties (Natarajan et al., 2007). This feature contributes to the greater resistance of wild soybean varieties to pests and diseases. For instance, studies have identified several distinct protease inhibitors in wild soybean seeds that are not as prevalent in cultivated varieties (Natarajan et al., 2007). Therefore, in order to improve new soybean cultivars, it is essential to increase the levels of KTI-1 and KTI-2 proteins in vegetative tissues while reducing the presence of KTI-3 and BBI proteins in seed storage proteins.

Conclusion

The results of this study demonstrated that, among the current Iranian soybean cultivars, Sahar with the highest concentration of both KTI and BBI proteins, exhibits a high level of trypsin and chymotrypsin inhibitory. Additionally, Katol which has the lowest concentration of both KTI and BBI proteins, exhibits the lowest levels of trypsin and chymotrypsin inhibitory. Therefore, Katol cultivar can be an ideal cultivar for soybean protein-based diets and could also be utilized as a valuable parent in a breeding program to improve the quality of soybean proteins. Based on correlation analysis there was no significant relationship between KTI and BBI proteins, as well as between these proteins with protease activity, it appears that genetic control of either KTI or BBI proteins alone may not be an effective approach to improving the quality of soybean proteins. Furthermore, the lack of correlation between KTI and BBI proteins with agronomic traits suggests that reducing protease inhibitors in soybean proteins does not have a negative impact on soybean breeding programs.

Supplementary Materials

No supplementary material is available for this article.

Author Contributions

Conceptualization, M. A., N. B. J., G. N. and A. D.; methodology, M. A. and E. G.; software, M. A. and E. G.; validation, M. A. and E. G.; formal analysis, M. A. and E. G.; investigation, M. A. and E. G.; resources, M. A., N. B. J., G. N. and A. D.; data curation, M. A.; writing—original draft preparation, M. A.; writing—review and editing, M. A.; visualization, N. B. J., G. N. and A. D.; supervision, M. A., N. B. J., G. N. and A. D. All authors have read and agreed to the published version of the manuscript.

Funding

This research was conducted with the cooperation and financial support by Genetics and Agricultural Biotechnology Institute of Tabarestan (GABIT).

Acknowledgments

The authors of this article would like to express their gratitude to the Genetics and Agricultural Biotechnology Institute of Tabarestan (GABIT) for their cooperation and funding.

Conflict of Interest Statement

The authors declare no conflict of interest.

References

Barros, J.G.d.A., Moraes, R.M.A.d., Piovesan, N.D., Barros, E.G.d., and Moreira, M.A. (2008). Effect of protease inhibitor on the levels of lipoxygenase in soybean seeds. *Cienc. Agrotec.* 32: 1126-1132.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72(1-2): 248-254.

de Almeida Barros, B., Da Silva, W.G., Moreira, M.A., and de Barros, E.G. (2012). In silico characterization and expression analysis of the multigene family encoding the Bowman–Birk protease inhibitor in soybean. *Mol. Biol. Rep.* 39: 327-334.

de Carvalho, W.L., de Almeida Oliveira, M.G., de Barros, E.G., and Moreira, M.A. (1999). Lipoxygenases affect protease inhibitor levels in soybean seeds. *Plant Physiol. Biochem.* 37(6): 497-501.

Dhaliwal, S.K., Dhillon, S.K., Gill, B., Kaur, G., Sirari, A., and Sharma, S. (2022). Effect of Genetic Elimination of Kunitz Trypsin Inhibitor on Agronomic and Quality Traits in Soybean [*Glycine max* (L.) Merril]. *Legume Res-An Int. J.* 45(1): 32-38.

Friedman, M., and Brandon, D.L. (2001). Nutritional and health benefits of soy proteins. *J. Agric. Food Chem.* 49(3): 1069-1086.

Gillman, J.D., Kim, W.-S., and Krishnan, H.B. (2015). Identification of a new soybean kunitz trypsin inhibitor mutation and its effect on bowman− birk protease inhibitor content in soybean seed. *J. Agric. Food Chem.* 63(5): 1352-1359.

Gu, C., Pan, H., Sun, Z., and Qin, G. (2010). Effect of soybean variety on anti-nutritional factors content, and growth performance and nutrients metabolism in rat. *Int. J. Mol. Sci.* 11(3): 1048-1056.

Isanga, J., and Zhang, G.-N. (2008). Soybean bioactive components and their implications to health—a review. *Food Rev. Int.* 24(2): 252-276.

Kennedy, A., and Szuhaj, B. (1994). Bowman-Birk inhibitor product for use as an anticarcinogenesis agent. *Patent NumberUS Patent 5, 338, 547*.

Kim, W.-S., Gillman, J.D., Kim, S., Liu, J., Janga, M.R., Stupar, R.M., and Krishnan, H.B. (2024). Bowman–Birk Inhibitor Mutants of Soybean Generated by CRISPR-Cas9 Reveal Drastic Reductions in Trypsin and Chymotrypsin Inhibitor Activities. *Int. J. Mol. Sci.* 25(11): 5578.

Kisha, T., Diers, B., Hoyt, J., and Sneller, C. (1998). Genetic diversity among soybean plant introductions and North American germplasm. *Crop Sci.* 38(6): 1669-1680.

Krishnan, H.B. (2001). Characterization of a soybean [*Glycine max* (L.) Merr.] mutant with reduced levels of Kunitz trypsin inhibitor. *Plant Sci.* 160(5): 979-986.

Krishnan, H.B., Jang, S., Baxter, I., and Wiebold, W.J. (2012). Growing Location has a Pronounced Effect on the Accumulation of Cancer Chemopreventive Agent Bowman‐Birk Inhibitor in Soybean Seeds. *Crop Sci.* 52(4): 1786-1794.

Livingstone, D., Beilinson, V., Kalyaeva, M., Schmidt, M.A., Herman, E.M., and Nielsen, N.C. (2007). Reduction of protease inhibitor activity by expression of a mutant Bowman-Birk gene in soybean seed. *Plant Mol. Biol.* 64: 397-408.

Luthria, D.L., Maria John, K.M., Marupaka, R., and Natarajan, S. (2018). Recent update on methodologies for extraction and analysis of soybean seed proteins. *J. Sci. Food Agric.* 98(15): 5572-5580.

Medic, J., Atkinson, C., and Hurburgh, C.R. (2014). Current knowledge in soybean composition. *J. Am. Oil Chem.' Soc.* 91: 363-384.

Mittal, P., Kumar, V., Anita, R., and Gokhale, S. (2021). Bowman-Birk inhibitor in soybean: genetic variability in relation to total trypsin inhibitor activity and elimination of Kunitz trypsin inhibitor. *Not Sci Biol* 13(1): 10836-10836.

Mittal, P., Kumar, V., and Rani, A. (2020). Effect of genotype, seed development stages, and processing treatments on Bowman-Birk inhibitor in soybean and its level in commercial soy products. *Acta Aliment.* 49(3): 348-355.

Natarajan, S., Khan, F., Song, Q., Lakshman, S., Cregan, P., Scott, R., Shipe, E., and Garrett, W. (2016). Characterization of soybean storage and allergen proteins affected by environmental and genetic factors. *J. Agric. Food Chem.* 64(6): 1433-1445.

Natarajan, S., Xu, C., Bae, H., and Bailey, B.A. (2007). Proteomic and genomic characterization of Kunitz trypsin inhibitors in wild and cultivated soybean genotypes. *J. Plant Physiol.* 164(6): 756-763.

Natarajan, S.S. (2014). Analysis of soybean seed proteins using proteomics. *J. Data Min. Genom. Proteom.* 5(1).

Palacios, M., Easter, R., Soltwedel, K., Parsons, C., Douglas, M., Hymowitz, T., and Pettigrew, J. (2004). Effect of soybean variety and processing on growth performance of young chicks and pigs. *J. Anim. Sci.* 82(4): 1108-1114.

Park, A., Kang, S.-H., Kang, B.-H., Chowdhury, S., Shin, S.-Y., Lee, W.-H., Lee, J.-D., Lee, S., Choi, Y.-M., and Ha, B.-K. (2023). Identification of a novel KTi-1 allele associated with reduced trypsin inhibitor activity in soybean accessions. *Agriculture* 13(11): 2070.

Perez-Maldonado, R., Mannion, P., and Farrell, D. (2003). Effects of heat treatment on the nutritional value of raw soybean selected for low trypsin inhibitor activity. *Br. Poult. Sci.* 44(2): 299-308.

Pesic, M.B., Vucelic-Radovic, B.V., Barac, M.B., Stanojevic, S.P., and Nedovic, V.A. (2007). Influence of different genotypes on trypsin inhibitor levels and activity in soybeans. *Sensors* 7(1): 67-74.

Sultana, M.S., Mazarei, M., Jurat-Fuentes, J.L., Hewezi, T., Millwood, R.J., and Stewart Jr, C.N. (2023). Overexpression of soybean trypsin inhibitor genes decreases defoliation by corn earworm (*Helicoverpa zea*) in soybean (*Glycine max*) and Arabidopsis thaliana. *Front. Plant Sci.* 14: 1129454.

Tavakolan, M., Alkharouf, N.W., Khan, F.H., and Natarajan, S. (2013). SoyProDB: A database for the identification of soybean seed proteins. *Bioinformation* 9(3): 165.

USDA, S.e. (2024). https://ipad.fas.usda.gov/cropexplorer/cropview/commodityView.aspx?cropid=2222000.

Varga, A., Camelia, U., Galben, R.D., Varban, D., Muntean, S., Moldovan, C., Balaș, S., and Marcel, D. (2023). Soybean Trypsin Inhibitor Activity-a review. *Hop Med. Plants* 31(1-2): 151-160.

Vorster, J., Westhuizen, W.v.d., Plessis, G.d., Marais, D., Sparvoli, F., Cominelli, E., Camilli, E., Ferrari, M., Le Donne, C., and Marconi, S. (2023). In order to lower the antinutritional activity of serine protease inhibitors, we need to understand their role in seed development. *Front. Plant Sci.* 14: 1252223.

Yasothai, R. (2016). Antinutritional factors in soybean meal and its deactivation. *Int J Sci Environ Technol.* 5(6): 3793-3797.

Zilic, S.M., Barac, M.B., Pesic, M.B., Mladenovic Drinic, S.D., Ignjatovic‐Micic, D.D., and Srebric, M.B. (2011). Characterization of proteins from kernel of different soybean varieties. *J. Sci. Food Agric.* 91(1): 60-67.

JPMB
Journal of Plant Molecular Breeding
Published by Genetics and Agricultural Biotechnology Institute of Tabarestan
2024 | Volume 12 | Issue 2

مقاله پژوهشی

بازدارنده‌های پروتئازی KTI و BBI در پروتئین ذخیره دانه ارقام سویا ایرانی

مهدی عارف‌راد[1]، نادعلی بابئیان جلودار[2]، قربانعلی نعمت زاده[2]، علی دهستانی[1] و الهه گرجی[2]

(1) پژوهشکده ژنتیک و زیست فناوری کشاورزی تبرستان، دانشگاه علوم کشاورزی و منابع طبیعی ساری، ساری، مازندران، ایران

(2) دانشکده بیوتکنولوژی، دانشگاه علوم کشاورزی و منابع طبیعی ساری، ساری، مازندران، ایران

چکیده: بازدارنده‌های پروتئازی Kunitz (KTI) و Bowman-Birk (BBI) مهمترین فاکتورهای کاهش دهنده کیفیت پروتئین‌های دانه سویا محسوب می‌شوند. در این مطالعه، سطوح مختلف این پروتئین‌ها با استفاده از تکنیک 2D-PAGE بررسی و فعالیت آن‌ها نیز از طریق پروتئازهای تریپسین و کیموتریپسین در ۱۰ رقم سویای ایرانی ارزیابی شد. این نتایج نشان داد که رقم کتول با کمترین غلظت هر دو پروتئین KTI و BBI و کمترین سطوح بازدارندگی تریپسین و کیموتریپسین از دیگر ارقام متمایز شده بود. بنابراین این رقم می‌تواند یک منبع ایده‌آل برای جیره‌های مبتنی بر پروتئین سویا باشد و همچنین می‌تواند به عنوان یک والد ارزشمند در برنامه‌های اصلاحی برای بهبود کیفیت پروتئین‌های دانه سویا مورد استفاده قرار گیرد. علاوه بر این، هیچ ارتباط معنی‌داری بین پروتئین‌های KTI با BBI و همچنین بین این دو پروتئین با فعالیت پروتئازها یافت نشد. بنابراین، به نظر می‌رسد که کنترل ژنتیکی هر یک از پروتئین‌های KTI یا BBI به تنهایی نمی‌تواند رویکرد مؤثری برای بهبود کیفیت پروتئین‌های دانه سویا باشد. علاوه بر این، هیچ ارتباطی بین پروتئین‌های KTI و BBI با صفات زراعی یافت نشد. این همچنین نشان می‌دهد که کاهش بازدارنده‌های پروتئازی در پروتئین‌های دانه سویا تأثیر منفی در برنامه‌های اصلاحی سویا ندارد.

کلمات کلیدی: سویا، پروتئین KTI، پروتئین Bowman-Birk، بازدارنده تریپسین، بازدارنده کیموتریپسین، صفات زراعی.

ویراستار علمی
دکتر احمد ارزانی،
دانشگاه صنعتی اصفهان، ایران،

تاریخ
دریافت: ۶ دی ۱۴۰۳
پذیرش: ۲۶ دی ۱۴۰۳
چاپ: ۸ اسفند ۱۴۰۳

نویسنده مسئول
دکتر مهدی عارف راد
Mehdiarefrad@yahoo.com

ارجاع به این مقاله
Arefrad, M., Babaian Jelodar, N. A., Nematzadeh, Gh.A., Dehestani, A. and Gorji, E. (2024). KTI and BBI protease inhibitor activity in seed storage protein of Iranian soybean cultivars. *J Plant Mol Breed*. 12 (2): 95-107.
doi: 10.22058/jpmb.2025.2048750.1324.