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Research Article

Assessment of processing and nutritional parameters in soybean genotypes with contrasting level of protein content

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Abstract
In the present investigation, two soybean genotypes with 35.43% and 44.40% protein content were evaluated for glycinin (7S), β-conglycinin (11S), and also assessed for anti-nutritional factors like kunitz trypsin inhibitor (KTI), off-flavour-generating lipoxygenase, *in vitro* protein digestibility (IVPD), and amino acid composition. Concentration of α', α and β subunit of β-conglycinin (7S) and the acidic subunit of glycinin (11S) was significantly (P<0.05) less, while the basic subunit was higher in low-protein genotype compared to the high-protein genotype. However, no significant difference was noted for the ratio of 11S to 7S fraction. KTI was significantly (P<0.05) less in low-protein genotype (LPN7N101), though no significant difference was noted between low- and high-protein soybean genotype (EC 468447) for lipoxygenase. IVPD of low-protein genotype was significantly (P<0.05) higher than the high protein genotype. Concentration of arginine (4.27%), phenylalanine (2.69%), valine (2.02%), glutamate (8.98%), aspartate (4.83%), and glycine (2.11%) in high-protein genotype was significantly (P<0.05) higher than the corresponding values (2.81, 1.81, 1.48, 4.99, 3.13 and 1.47%, respectively) of these amino acids in low-protein genotype.

Keywords
Soybean, Protein, Glycinin and β-conglycinin, Kunitz trypsin inhibitor, Amino acid composition, *In vitro* protein digestibility

Introduction

Besides being one of the most economical sources of quality protein, soybean is also enriched with basic nutrients, namely, essential amino acids, oil, essential fatty acids, vitamins, minerals, which can combat mal- and under-nutrition in developing countries. Globally, people are becoming increasingly aware of the special nutraceutical components like isoflavones, tocopherols, lecithin, Bowman-Birk factor, saponins *etc.* associated with soybean, which reduces the risk of onset of breast cancer in women, Alzheimer's disease, cardiovascular diseases, osteoporosis, diabetes, *etc* (Messina *et al.*, 2010). In India, in the wake of meteoric rise in cardiovascular diseases and diabetes in recent years, inclusion of soybean in daily diet is being advocated by the nutritionists and medical practitioners. High-protein soybean genotypes are the preferred raw material for processing good quality soy-food products like soy milk and *tofu* (Stanojevic *et al.*, 2011). Regular soybean varieties, in general, contain 38-40% protein content, but genotypic variation ranging from 31 to 46% for this trait has been reported by several workers (Garcia *et al.*, 1997; Lee *et al.*, 2013; Arefrad *et al.*, 2014). In India, commercial cultivation of soybean was initiated primarily to combat the rampant malnutrition among masses. Paradoxically, to meet the edible oil requirement of the burgeoning middle-class population, soybean is the major oilseed crop, and at present accounts for 11.5 million tons of the total oil seed production (37.76 million tons) (DES, 2016; USDA, 2017). Further, in the backdrop of 70% of the edible oil requirement of the country being met through import, development of high-oil soybean genotypes is being accentuated to increase the oil yield per hectare. However, continued focus on increasing the yield and the emphasis on development of high-oil varieties in soybean breeding programme, may affect the levels of the protein in newly released varieties as reported in an earlier study (Arnason, 2017) because of the purported negative correlation of protein with oil and yield (Ifrim *et al.*, 2012, Stobaugh *et al.*, 2017;). The decline in protein content in soybean may cause changes in storage protein fractions, amino acid composition, kunitz trypsin inhibitor -an antinutritional factor that affects protein digestibility- and off-flavour generating lipoxygenase.

In soybean, β-conglycinin (7S) and glycinin (11S) are two major storage proteins which account for approximately 40% and 25% of the total seed proteins, respectively (Nielsen *et al.*, 1989). Of the total storage protein, glycinin content is about 60% of storage proteins while the remaining 40% is β-conglycinin (Taski-Ajdukovic *et al.*, 2010). β-Conglycinin is a trimeric glycoprotein, consisting of α', α, and β subunit; while glycinin is composed of acidic and basic polypeptides. These storage

protein fractions vary significantly in functional properties like gel-making and emulsification capacity, important for soya food manufacturing (Wagner *et al*., 1999). Glycinin content and 11S/7S protein fraction ratio in soybean seeds have been reported to correlate positively with *tofu* gel firmness (Mujoo *et al*., 2003). Poysa *et al.* (2006) reported that genetic elimination of 11S subunit caused non-coagulation of protein required for manufacturing *tofu*. Level of protein in soybean seeds may affect the level of different fractions of β-conglycinin (7S) and glycinin (11S), thereby influencing the ratio of 11S and 7S fraction, which can influence the gelling property of soy proteins; and hence, the quality of processed soy-products. Further, the concentration of protease inhibitors like kunitz trypsin inhibitor, which inhibits digestion of protein (Horton *et al*., 2006), and lipoxygenase, which is responsible for the off-flavour generated in soy-products, may also vary in soybean genotypes with extreme levels of protein content. A variation in the concentration of kunitz trypsin inhibitor in soybean seeds used as raw material can affect the digestibility of soy-products processed therefrom, especially when the heat treatment during the processing is less than 15 min. More importantly, significant variation in protein content in seeds can also impact the levels of amino acids in the final product. The studies focusing on the relationship of protein content in soybean seeds with storage protein subunits and amino acid composition are limited (Krishnan *et al*., 2007), while the literature pertaining to assessment of soybean genotypes with extreme levels of protein in relation to *in vitro* protein digestibility is not available. Recently, Mourya *et al.* (2016) screened a large number of soybean genotypes from different countries for protein content and identified low- and high-protein genotypes. In the present investigation, 2 genotypes with extreme level of protein were assessed for storage protein fractions *viz.* glycinin and β-conglycinin, amino acid composition, kunitz trypsin inhibitor, lipoxygenase and *in vitro* protein digestibility.

Material and Methods

In our laboratory, a large number of soybean genotypes (1210) from 18 countries, namely India (851), USA (94), Taiwan (48), Philippines (20), Sri Lanka (11), China (10), Brazil (9), Hungary (8), Nigeria (6), Argentina (5),Germany(5), Australia (4), Thailand (4), Myanmar (3), Japan(2), Nepal (2), Canada (2), Russia (1) and 125 genotypes of unknown origin were screened recently for protein content using Near-Infrared Reflectance Spectroscopy (Mourya *et al*., 2016). From the low- and high-protein group identified in this study, an advanced breeding line, namely LPN7N101 derived from the cross NRC 7×NRC 101 with low protein content (35.43%); and genotype EC 468447, with high protein content (44.40%) were selected for the present investigation. NRC 7 is a soybean variety released for cultivation in India; while NRC 101 is a kunitz trypsin inhibitor free genotype developed at ICAR-Indian Institute of Soybean Research, India. Both low- and high-protein genotypes were raised in randomized block design with three replicates in the experimental fields of ICAR-Indian Institute of Soybean Research, Indore, Madhya Pradesh, India in cropping season 2016. Each plot consisted of 3 rows for each of the 2 genotypes. The rows were 3 m long and 45 cm apart, while plant-to-plant distance was maintained at 5 cm. The freshly harvested seeds were subjected to resolution and quantification of storage protein fractions and estimation of oil, protein, kunitz trypsin inhibitor, lipoxygenase and amino acid composition and *in vitro* protein digestibility (IVPD).

Oil from finely ground 500 mg flour (30 mesh) was extracted with 180 ml hexane in an automated Soxhlet unit (Pelican Equipments, Chennai, India) for 3 h. Percentage oil content was determined by weight differences (Soxhlet, 1879). For estimation of protein content, dried soy-flour was subjected to the estimation of nitrogen content through Kjeldahl method (Kjeldahl, 1883). *In vitro* protein digestibility of all the genotypes was determined by pepsin digestion method (Kayembe *et al.*, 2013). Moisture-free flour (350 mg) was taken and 5 ml of 0.075 N HCl and 0.5 ml of pepsin solution (2 mg dissolved in 1 ml of 0.075 N HCl) was added to it. The tubes were incubated at 37°C for 24 h. Subsequently, enzyme action was stopped by adding 5 ml of 10% (w/v) trichloroacetic acid (TCA). The digest was passed through Whatman No. 2 filter paper and the residue was washed with warm water. Nitrogen content in the residue was estimated by the Kjeldahl method. *In vitro* protein digestibility was obtained by calculating the difference between the content of nitrogen in the sample before and after *in vitro* digestion with pepsin. Nitrogen content was multiplied by the conversion factor (6.25) to obtain crude protein.

Protein from soybean seeds were resolved using Tricine SDS-PAGE as given by Schagger (2006) with slight modification. A fixed amount of soluble protein was loaded onto the SDS-PAGE (Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis) consisting of 3 layers, namely, separating/running (16%), spacer (10%) and stacking (4%) gel. The voltage employed during the movement of the protein in stacking (5%) and spacer (10%) was 30 V while separating gel (16%) was run under 200 V current. Cathode buffer (10X,

pH 8.2) in upper buffer chamber was prepared using 1 M Tricine, 1 M Tris and 1% SDS; while anode buffer (10X, pH 8.9) in the lower chamber was composed of 1 M Tris and 0.2 M HCl. After completing the run, gel was stained with 0.25% coomassie brilliant blue followed by destaining using methanol: water: acetic acid in the ratio of 45:45:10, respectively. The storage protein profile pattern, concentration of kunitz trypsin inhibitor and lipoxygenase was scanned and the fractions of storage protein *viz.* glycinin, β-conglycinin and polypeptides of kunitz trypsin inhibitor and lipoxygenase were quantified through densitometer Bio-Rad G900 using the software, *Image Lab 5.2.1*. The data was expressed as percentage of total extractable proteins.

Dried soybean flour (1 g) was hydrolysed in 6 N HCl at 105 °C for 24 h and filtered. The supernatant so obtained was subjected to amino acid analysis through HPLC based amino acid analyser procured from Waters India Private Limited following the method given by Seo (2005). ACCQ-Fluor reagent kit (WAT052880) of Waters India Private Limited was used for derivatization of the amino acids in the hydrolysed samples. First of all, ACCQ-Fluor reagent was reconstituted. For this purpose, the vial (2A) containing ACCQ- Fluor reagent powder was tapped so that the compound is settled completely on the bottom, followed by addition of 1ml of ACCQ- Fluor reagent diluent (vial 2B) and the mixture was incubated on heating block till the reagent powder completely dissolved. Borate buffer (70 µl) was added to 10 µl of the diluted hydrolysed sample and vortex. Subsequently, 20 µl of reconstituted ACCQ-Fluor reagent was added and heated for 10 min at 55 °C. A 5 µl of pre-derivatized amino acid mixture sample, standard and blank was loaded into HPLC, which was equipped with Waters 510 binary pump and Waters 2475 fluorescent detector. Separation of amino acids was carried out on ACCQ Tag column through mobile phase (ACCQ Tag Eluent as solvent A: 60% Acetonitrile as solvent B) at a flow rate of 1.0 ml/min in a multi-step gradient. Concentration of each amino acid in sample was computed by comparing the peak area of a particular amino acid in the sample chromatogram with the corresponding amino acid peak in the standard chromatogram. Table 2 depicts the concentration of all the essential and non-essential amino acids except tryptophan and cysteine, which are prone to acid-hydrolysis (Ohta *et al.*, 1979)

All the statistical analyses were carried out in triplicate samples using software GraphPad Prism 7.03 (GraphPad Software, Inc. USA).

Results and Discussion

Over the years, selection of soybean genotypes based upon yield and yield-related components has diverted the attention of plant breeders from maintaining protein content, an important criterion to assess the economic and processing value of the crop. Further, to maximise the recovery of oil from soybean grains in countries facing edible oil crunch, soybean breeding programme for the development of high-oil genotypes has resulted in genotypes with low protein content (Arnason, 2017). Significant variation in protein content may trigger subtle changes in the levels of storage protein (11S and 7S) subunits, essential amino acids, undesirable components like trypsin inhibitor and off-flavour generating lipoxygenase and protein digestibility.

In the current study, seeds of low-protein (LPN7N101) and high-protein (EC 468447) soybean genotype were tested for protein content and the results are presented in Table 1. Low-protein genotype exhibited 35.43% protein; while high-protein genotype showed 44.40% protein. Oil content of low- and high-protein genotype was 23.48% and 17.58%, respectively (data not presented in Table 1), conforming to the negative correlation between oil and protein content (Ifrim *et al*., 2012; Stobaugh *et al*., 2017). Further, *in vitro* protein digestibility of low-protein genotype (57.57%) was significantly (P<0.05) higher than high-protein genotype (51.21%).

Estimation of concentration of storage protein subunits in low- and high-protein genotypes was done using SDS-PAGE (Fig.1). Protein bands were resolved into two major storage protein fractions, namely, 11S (Glycinin) and 7S (β-conglycinin). Glycinin fraction (11S) was further resolved into acidic (40-43 kDa) and basic (22-23 kDa) subunit; while β-conglycinin fraction separated into 3 bands, namely, α' (78 kDa), α (75 kDa) and β subunit (47 kDa). Besides these major storage protein subunits, other polypeptides corresponding to kunitz trypsin inhibitor and lipoxygenase activity were also distinctly separated as shown in Fig. 1. Results obtained through densitometry analysis of protein bands of both low- and high-protein genotype are presented in Table 1. The value for each protein band in a lane was expressed as the percentage of total extractable protein. With regard to subunits of β-conglycinin (7S) fraction, concentration of α' (7.50%) and α (11.90%) subunit of low-protein genotype was significantly (P<0.05) lower than the corresponding values (9.60% and 12.80%, respectively) in high-protein genotype. Similarly, concentration of β subunit

(9.10%) in low-protein genotype was also significantly (P<0.05) lower than high-protein genotype (10.30%). Concentration of β-conglycinin (7S) fraction computed from the summation of α', α, and β subunits in low-protein genotype (28.50%) was significantly (P<0.05) lower than high-protein genotype (32.70%). With regard to subunits of glycinin (11S) fraction, concentration of acidic subunit (13.80%) in low-protein genotype was significantly (P<0.05) lower than high-protein genotype (18.60%). On the contrary, concentration of basic subunit (19.10%) of low-protein genotype was significantly (P<0.05) higher than high-protein genotype (17.10%). Therefore, as noted for 7S fraction, 11S fraction computed by summation of acidic and basic subunit in high-protein genotype (35.70%) was also slightly higher than low-protein genotype (32.90%). As a result, no significant difference in the ratio of 11S to 7S fraction was noted in low-protein (1.15) and high-protein soybean genotype (1.09).

Table 1 also depicts the concentration of kunitz trypsin inhibitor and lipoxygenase quantified using densitometry. In India, soybean is recommended to be ground with wheat (1:9) to make soy fortified flour to make *chapatti*, flat Indian bread. For this purpose, soybean grains must be boiled for 15-20 min to inactivate kunitz trypsin inhibitor (KTI) followed by sun-drying and, subsequently, blended with wheat grain prior to milling. However, these processing steps being time-consuming and cumbersome, in general, followed by the consumers at household level. Kunitz trypsin inhibitor is a 20 kD polypeptide, which is primarily responsible for trypsin inhibitor activity in soybean, in its active form affects the protein digestibility. The polypeptide was significantly (P<0.05) high (1.30%) in high-protein genotype compared to the low-protein genotype (0.20%). However, concentration of off-flavour generating lipoxygenase protein in low-protein genotype was not significantly different from high-protein genotype.

Table 2 presents the levels of essential and non-essential amino acids of low- and high-protein soybean genotype. Among all the amino acids analysed, the difference between low- and high-protein genotype was the largest for glutamic acid followed by aspartic acid, but these are non-essential amino acids. Among essential amino acids, concentration of arginine (2.81%), phenylalanine (1.81%), valine (1.48%), glycine (1.47%) was significantly (P<0.05) less in low-protein genotype compared to their corresponding values (4.27, 2.69, 2.02 and 2.11%, respectively) in high-protein genotype. Among non-essential amino acids, concentration of aspartate (3.13%) and glutamate (4.99%) were significantly (P<0.05) low in low-protein genotype compared to their corresponding values (4.83% and 8.98%, respectively) in high-protein genotype. No significant differences were noted for isoleucine, leucine, threonine, serine, alanine, proline, tyrosine, histidine and methionine between low- and high-protein genotype.

Further, our results showed that low-protein genotype, exhibited lesser accumulation of all 3 subunits of 7S fraction (α', α, and β) and acidic subunit of 11S fraction compared to the high-protein genotype. Krishnan *et al.* (2007) analysed high-protein lines (>50%), namely, PI427138, LG00-13260, BARC6 and a regular soybean genotype William 82. Higher levels of α', α and β subunits of 7S fraction and acidic subunit of 11S fraction in high-protein genotypes than regular genotype reported by these authors supports our observation. However, the higher value of basic subunit of 11S fraction in high-protein genotypes in their study is in contrast to the present study which showed lower value of basic subunit in high-protein soybean genotype. This contrasting observation for the basic subunit may be because of the fact that in the study of Krishnan *et al.* (2007), high-protein genotypes investigated possessed >50% protein content, while EC 468447 used as high-protein genotype possessed 44.40% protein. The results were also compared with the study of Taski-Ajdukovic *et al.* (2010) who analysed storage protein subunits in high-protein soybean genotypes from different maturity group *vis-à-vis* genotype with regular level of protein (38-40%). The authors demonstrated that the level of concentration of different subunits of 7S and 11S in high-protein soybean genotypes was a function of the maturity group they belonged. Further, the ratio of 11S (glycinin) to 7S (β-conglycinin) is important with regard to the functionality of soy proteins in manufacturing soy-food products. This ratio was not significantly different in low- and high-protein genotype in the present study. In an earlier study (Arefrad *et al*., 2014), soybean genotype DPX with 34.90% protein showed slightly higher value of 11S to 7S ratio than in soybean genotype Sahar with 40.94% protein. Further, correlation analyses showed significant (P<0.05) positive correlation of protein content with 7S fraction (P<0.05, r = 0.729*) and its β-subunit (P<0.05, r = 0.747*), but significant (P<0.05) negative correlation (P<0.05, r = -0.842*) with basic subunit of 11S fraction. Moreover, significantly (P<0.05) low arginine content noted in low-protein genotype than high-protein soybean genotype is in consonance with the earlier study (Krishnan *et al.*, 2007) which demonstrated increased level of arginine in high-protein genotypes. Further, significant (P<0.05) negative correlation (P<0.05, r = -0.803*) was

noted between protein content and *in vitro* protein digestibility. We did not come across any study investigating the relationship of protein content with *in vitro* protein digestibility in soybean, though a positive relationship between seed protein content and *in vitro* protein digestibility has been reported in raw seeds of *Pisum sativum* (Park *et al*., 2010).

Results from the current study showed that soybean genotypes with extreme levels of protein may have different concentration of subunits of storage protein fractions, namely, glycinin and β-conglycinin but not necessarily possess different ratio of glycinin to β-conglycinin, higher level of which is important for processing good quality and yield of soy-processed products. The current study also showed that compared to high protein soybean genotype, low-protein soybean genotype may also be low in protein quality due to the low levels of some of the essential amino acids, but may have better IVPD, due to the reduced level of protease inhibitors like kunitz trypsin inhibitor.

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Table 1. Protein, *in vitro* protein digestibility (IVPD), lipoxygenase, kunitz trypsin inhibitor and storage protein fraction in low- and high-protein soybean genotype

Genotype	Protein (%)	IVPD (%)	Lipoxygenase (%)	KTI (%)	Storage protein fractions (%)							
					β-Conglycinin (7S) subunits			Total extractable β-Conglycinin	Glycinin (11S) subunits		Total extractable glycinin	Ratio of 11S to 7S
					α´	α	β		Acidic	Basic		
Low-protein (LPN7N101)	35.43±0.36[b]	57.57±0.24[a]	0.92±0.02[a]	0.20±0.02[b]	7.50±0.15[b]	11.90±0.15[b]	9.10±0.08[b]	28.50±0.02[b]	13.80±0.05[b]	19.10±0.10[a]	32.90±0.02[b]	1.15±0.07[a]
High-Protein (EC 468447)	44.40±0.41[a]	51.21±0.26[b]	1.02±0.13[a]	1.30±0.05[a]	9.60±0.10[a]	12.80±0.25[a]	10.30±0.04[a]	32.70±0.07[a]	18.60±0.02[a]	17.10±0.10[b]	35.70±0.10[a]	1.09±0.09[a]

The data are expressed as mean ± S.D. of triplicate analyses. Mean followed by the same superscript within the same column are not significantly different at $P \leq 0.05$ probability.

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Table 2. Amino acid composition of low- and high-protein soybean

	Amino acids composition (%)															
Genotype	**Phe**	**Ile**	**Leu**	**Lys**	**Met**	**His**	**Thr**	**Val**	**Ser**	**Ala**	**Arg**	**Asp**	**Glu**	**Gly**	**Pro**	**Tyr**
Low-protein (LPN7N101)	1.81±0.09 b	1.93±0.08 a	2.31±0.09 a	1.81±0.07 a	0.38±0.01 a	0.87±0.04 a	1.06±0.05 a	1.48±0.02 b	1.28±0.05 a	2.42±0.12 a	2.81±0.14 b	3.13±0.15 b	4.99±0.24 b	1.47±0.07 b	1.86±0.09 a	1.55±0.07 a
High-Protein (EC468447)	2.69±0.13 a	1.74±0.10 a	2.52±0.15 a	1.96±0.11 a	0.36±0.01 a	1.27±0.07 a	1.29±0.07 a	2.02±0.10 a	1.74±0.08 a	2.80±0.16 a	4.27±0.25 a	4.83±0.28 a	8.98±0.53 a	2.11±0.08 a	1.82±0.10 a	1.42±0.07 a

The data are expressed as mean ± S.D. of triplicate analyses. Mean followed by the same superscript within the same column are not significantly different at $P \leq 0.05$ probability.

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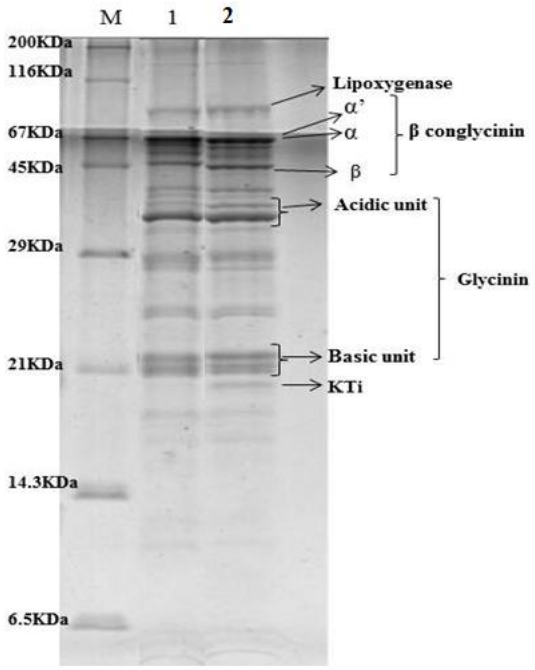

Fig. 1. SDS-PAGE analysis of low- and high-protein soybean genotypes. Lane M-standard protein marker. Lane 1-LPN7N101, lane 2- EC 468447