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## Determination of degradability of germinated and heated soybean seeds and its proteins fractions

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### ABSTRACT

A study on the sensitivity of watermelon variety Bojura to mutagenic agents was carried out in 2013-2014. The goal was to establish effective doses for mutagenic treatment of dry seeds with <sup>60</sup>Co gamma rays (80, 100, 200, 250, 350 and 450 Gy) and swollen seeds with water for 24 hours were treated with ethyl methanesulfonate (EMS) at a concentration of 2%. Dominant mutations were not observed in the M<sub>1</sub> generation. Morphological changes in 14 of 1395 M<sub>2</sub> plants were observed. Phenotypic variations changes were the colour of the seed coat, chlorophyll disorders of cotyledons, leaves, petals, and alterations of the location of the fruit set in the central stem. Visible changes of the morphological characteristics of the fruit were not observed. The doses induced certain morphological changes, however, higher doses or combined gamma rays <sup>60</sup>Co and EMS treatments would induce mutations more efficiently. Subsequent experiments are required to obtain mutants with changes that affect flowers and fruits. The results are important for increasing mutation efficiency in watermelon breeding.

**Keywords:** Soybean Seed, Germination, heat treatment, Degradability, SDS-Page

## Introduction

Full-fat soybean, which contains 40% CP and 17% of fat, is interesting as a source of protein and energy in rations for cows in the first part of lactation (Grummer *et al.*, 1994), but the protein is highly degradable by rumen microbes (Krishnamoorthy *et al.*, 1982). Various chemical and physical processing has been suggested to decrease ruminal protein degradability. Attempts to decrease the rumen degradability of proteins have involved treatment with heat (Mir *et al.*, 1984; Nakamura *et al.*, 1994), formaldehyde (Nishimuta *et al.*, 1974; Thomas *et al.*, 1979; Cooker *et al.*, 1983; Mir *et al.*, 1984), acetic acid (Robinson *et al.*, 1994), tannic acid (Driedger and Hatfield, 1972), lignosulfonate (Windschitl and Stern, 1988; McAllister *et al.*, 1993) and xylose treatments (Harstad and Prestlokken, 2000; Sacakli, 2001; Nobar *et al.*, 2009). Heat processing is the most commonly used physical method (Plegge *et al.*, 1985; Pena *et al.*, 1986). More recently, treatment of SBM by germination was successful in reducing degradation of soybean protein by rumen microorganisms.

Germination processes have been developed in some countries to overcome some of the disadvantages associated with ungerminated soybeans, such as undesirable flavour and odour and the presence of trypsin inhibitors (McKinney *et al.*, 1958; Suberbie *et al.*, 1981; Vanderstoep, 1981). Germination may also result in an increase in nutritive value relative to

ungerminated seeds (Fordham *et al.*, 1975). Germinated seeds were rated high in protein, appearance, flavor and texture and could be consumed uncooked in salad, boiled in water with suitable seasoning or fried in fat (Smith and Circle, 1978).

Also germination can increase amount of vitamin, mineral availability and decreased tannic acid and phytic acid in legumes (Ghavidel and Prakash, 2007; Hussein and Ghanem, 1999; Kaushik *et al.*, 2010).

As mentioned earlier, various chemical and physical processing has been suggested to decrease ruminal protein degradability. Heat processing is the most commonly used physical method (Plegge *et al.*, 1985; Pena *et al.*, 1986). This improved production has been attributed primarily to an increase in escape of protein from ruminal degradation, thereby providing more amino acids (AA) for intestinal digestion and absorption (Nasri *et al.*, 2008).

Use of SDS-PAGE allows direct visualization of protein degradation, isolation and quantification of feed proteins. Thus it can be used to predict ruminal degradation directly and to assess the type of proteins that will escape the rumen (Messman and Weiss, 1994). Moreover, this technique could detect differences in ruminal degradability of true proteins between untreated and treated protein supplements (Sadeghi *et al.*, 2006).

The objectives of this study were to evaluate the effect of germination and heating treatments in the *in situ* disappearance of the dry matter (DM) and crude protein (CP)

fractions of soybean meal and their sub-unit fractions by a SDS-PAGE discontinuous system.

## Material and Method

### Sample preparation and treatment

In this study one of Iranian cultivars of soybeans (L17) was used. This is the most common cultivar used in ruminant diets in Iran.

For Germination soybean seeds were soaked in distilled water (1:5 w/v) for 6 h at room temperature. The water was drained off, and the seeds transferred to a moisture adherent flax cloth (tarts) to germinate for 4 days in the dark at 22 °C. Every 24 h, the seeds were moistened with distilled water and carefully shaken (Yasmin *et al.*, 2008). For heat treatment, after 4 days of germination, a part of germinated grains placed in an autoclave at 121 °C with steam pressure (100 kPa). At the end, seeds were ground and dried in an air oven at 60 °C for analysis.

### Chemical analyses

Feed samples were analyzed for dry matter, crude protein, ether extract, crude fiber and crude ash content of soybean grains after treatment and rumen incubation by using the procedures of AOAC (2005).

### In situ ruminal procedure

Three wethers fitted with rumen cannula were used to measure rumen degradability of feeds. The wethers were fed a diet composed of (on DM basis), 385g/day alfalfa hay, 280g/day barley grain, 35 g/day wheat bran and 1.5 g/day lime stone at maintenance (NRC, 1985). The wethers were kept in individual tie-stalls with individual feed bins in an animal house and had continuous access to water. Diets were given as total mixed ration with fresh feed offered twice each day (08:30 and 15:30 h). The nylon bag technique (Orskov and McDonald, 1979) was used to measure the DM degradation of feeds in the rumen. Nylon bags (4 cm×8 cm polyester bag; pore size 45-50 μm) containing 3 g of seeds ground through a 2 mm screen were incubated in the rumen for 2, 4, 8, 16, 24 and 48 h for seeds, immediately after the morning feeding. As a whole, there were six replicates for each feed sample and for each incubation time (3wethers × 2 bags). Immediately after removal from the rumen, the bags were washed in cold water and frozen at -18 °C. At the end of the collections, they were unfrozen and washed together with the zero time bags (not incubated in the rumen) for 20 min and then dried at 80°C for 24 h. The residues were weighed and submitted for analysis.

### Determination of protein sub-units

Protein sub-units were fractionated by a SDS-PAGE discontinuous system (Laemmli, 1970). All ruminal undegradable fractions from each incubation period were dried, ground (0.25 mm particle size) and replicate

samples pooled. Twenty micrograms of untreated or treated SBM were placed into 750 μl SDS-PAGE sample buffer. After 30 min of mixing (i.e., vortex and inverse), samples were immersed at 90°C for 3 min, and then centrifuged at 10000 × g for 1 min. A 25 μl aliquot of each sample was loaded into the sample well. Electrophoresis of proteins was on 1 2.5% resolving gel (1.0 × 190 × 150 mm) with 3.75% acrylamide stacking gel. The gels were kept at a constant current of 30 mA until the bromophenol blue marker dye reached the bottom of the gel. Protein fixation and staining were completed simultaneously using a solution of Coomassie brilliant blue. Gel destaining was accomplished by using a 150 ml/l methanol and 100 ml/l acetic acid solution. One standard protein mixture including β-galactosidase (116 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), lactate dehydrogenase (35.0 kDa), REREase Bsp98I (25 kDa), β-Lactoglobulin (18.4 kDa) and Lysozyme (14.4 kDa) were used.

### Calculations and statistical analysis

In situ Dry Matter (DM) rapidly degradable fraction (a), potentially degradable fraction (b), and rate of degradation of fraction b (c), were calculated according to model of Orskov and McDonald (1979) as  $y=a+b(1-e^{-ct})$  that y is the actual degradation of DM after t, a is the intercept of the degradation curve at time zero, b is the potential degradability of the component of the insoluble but degradable DM, which will in time be degraded, c represents the constant of degradation rate of b at time, t is incubation time.

Data were subjected to one-way analysis of variance using the analysis of variation model (ANOVA) of SAS (1999). Multiple comparison tests used Duncan's multiple-range test (Snedecor and Cochran, 1989).

## Results and Discussion

Soybean is used worldwide for animal nutrition and it is important sources of protein, carbohydrates and energy due to their high contents of oil. Germination and heating as separated processes have proved partially beneficial for the nutritional quality such as chemical composition and rate rumen degradability of soybean seeds. The study of the effect of combined germination and heating may provide useful information for optimization of use of soybean seeds as feed in ruminant rations.

### Chemical composition

Table 1 shows the results of proximate analyses, as well as dry matter, crude protein, ether extract, crude fiber and crude ash. Germination increased DM and CP content of seeds ( $p<0.05$ ) and had no effect on ether extract and crude ash. The heat treatment did not have a considerable effect on the DM, CP, EE and ash of the germinated soybean seeds.

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The small variations observed were considered to be within the range of the method error.

Germination during 4 days modify the proximate composition of the soybean seeds on a dry matter basis such as DM, CP and ether extract, except for a small decrease crude fiber and ash. Increasing seed crude protein and change amino acid composition due to germination reported (Ghavidel and Prakash, 2007; Mostafa *et al.*, 1987). A significant change in the oil content, due to the germination process, and slight decreases in the oil content of the beans amounting to 5.37% and 7.69%, respectively previously reported by Mostafa *et al.* (1987). This decrease could probably be ascribed to consumption of oil as energy and/or synthesis of certain structural constituents in the young seedling (Singh *et al.*, 1968).

#### Rumen degradability

Dry matter losses from the nylon bags incubated in the rumen and in situ DM degradability characteristics are presented in Table 2. There were differences ( $p < 0.05$ ) among treatment of soybean seeds in dry matter degradability after several incubation times. Total dry matter washing losses (zero time bags) represented 17.25 to 21.79% of DM in heated seeds and untreated seeds, respectively. Dry matter disappearance from nylon bags incubated in the rumen increased with increasing incubation time. The 48 h incubation time was sufficient for test seeds to be degraded. After 48 h incubation, untreated soybean seeds had highest

DM rumen degradability. Results show that DM rumen degradability was reduced by heating treatment.

Crude protein rumen degradability and in situ CP degradability characteristics are presented in Table 3. There were differences ( $p < 0.05$ ) among treatment of soybean seeds in dry matter degradability after several incubation times. Crude protein washing losses (zero time bags) represented 9.87 to 20.55% of CP in heated seeds and untreated seeds, respectively. Dry matter disappearance from nylon bags incubated in the rumen increased with increasing incubation time. The 48 h incubation time was sufficient for test seeds to be degraded. After 48 h incubation, untreated and germinated soybean seeds had highest DM rumen degradability 86.77% and 83.14% respectively).

A large range of dry matter degradation characteristics was obtained: the 'a', 'b' and 'c' values ranged from 8.58 to 11.48% (for HGSS and GSS), 78.14 to 87.17% (for HGSS and NSS) and 0.046 to 0.052 %h<sup>-1</sup> (for HGSS and GSS), respectively. Results show that heat treatment reduced CP effective degradability in rumen.

The relationship between the degradability parameters a, b and c and the chemical composition of 60 test feeds was reported by Woods *et al.* (2003). They reported that the slowly fermented structural carbohydrates are thought to play a dominant role in the degradation characteristics in the rumen. It seems that germination and heating can be changed amount and component of carbohydrate content of seeds.

**Table 1.** Chemical composition of soybean seeds with and without germination and heat treatment (% DM) †

Item	Soybean seeds		
	NSS	GSS	HGSS
Dry Matter	92.54 <sup>b</sup>	93.06 <sup>a</sup>	93.05 <sup>a</sup>
Crude Protein	35.32 <sup>b</sup>	38.66 <sup>a</sup>	39.32 <sup>a</sup>
Ether Extract	17 <sup>b</sup>	16.3 <sup>a</sup>	16.2 <sup>a</sup>
Crude Fiber	6.00	5.80	5.68
Ash	6.02	6.01	6.02

† Three samples analyzed for each feed

NSS: untreated soybean seeds, GSS: Germinated soybean seeds and HGSS: Heated and Germinated soybean seeds

<sup>a, b</sup> Means within a column with different subscripts differ ( $P < 0.05$ ).

**Table 2:** The in situ disappearance of dry matter (%) and DM degradation characteristics of test feeds in the rumen

Feeds	DM degradability (%DM)							Degradation characteristics			
	0 h	2 h	4 h	8 h	16 h	24 h	48 h	a (%)	b (%)	c (%.h <sup>-1</sup> )	ED
NSS	21.7 <sup>a</sup>	22.7 <sup>b</sup>	27.1 <sup>b</sup>	34.8 <sup>a</sup>	53.0 <sup>b</sup>	79.5 <sup>a</sup>	83.9 <sup>a</sup>	16.77 <sup>b</sup>	80.99 <sup>a</sup>	0.042 <sup>b</sup>	72.9 <sup>a</sup>
GSS	21.0 <sup>a</sup>	26.8 <sup>a</sup>	31.6 <sup>a</sup>	37.3 <sup>a</sup>	58.6 <sup>a</sup>	69.8 <sup>b</sup>	80.4 <sup>ab</sup>	19.71 <sup>a</sup>	68.46 <sup>b</sup>	0.049 <sup>a</sup>	68.3 <sup>b</sup>
HGSS	17.2 <sup>b</sup>	22.5 <sup>b</sup>	25.1 <sup>b</sup>	30.6 <sup>b</sup>	45.1 <sup>c</sup>	70.3 <sup>b</sup>	79.4 <sup>b</sup>	15.37 <sup>b</sup>	82.19 <sup>a</sup>	0.034 <sup>c</sup>	67.8 <sup>b</sup>
SEM (n=6)	0.581	0.696	0.811	0.878	1.091	1.139	1.018	0.608	0.648	0.0006	1.22

NSS: untreated soybean seeds, GSS: Germinated soybean seeds and HGSS: Heated and Germinated soybean seeds.

a, b, c Means within a column with different subscripts differ ( $P < 0.05$ ).

ED = Effective degradability ( $k=0.02$ )

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**Table 3:** *The in situ disappearance of crude protein (%) and CP degradation characteristics of test feeds in the rumen*

Feeds	DM degradability (%DM)							Degradation characteristics			ED
	0 h	2 h	4 h	8 h	16 h	24 h	48 h	a (%)	b (%)	c (%.h <sup>-1</sup> )	
NSS	12.4 <sup>a</sup>	17.4 <sup>b</sup>	21.8 <sup>b</sup>	35.7 <sup>b</sup>	57.5 <sup>b</sup>	75.0 <sup>a</sup>	86.7 <sup>a</sup>	9.17 <sup>b</sup>	87.17 <sup>a</sup>	0.050 <sup>b</sup>	71.5 <sup>a</sup>
GSS	12.0 <sup>a</sup>	20.5 <sup>a</sup>	25.4 <sup>a</sup>	38.4 <sup>a</sup>	52.5 <sup>b</sup>	74.6 <sup>a</sup>	83.1 <sup>a</sup>	11.48 <sup>a</sup>	79.71 <sup>b</sup>	0.052 <sup>a</sup>	43.3 <sup>a</sup>
HGSS	9.8 <sup>b</sup>	17.2 <sup>b</sup>	20.2 <sup>c</sup>	31.5 <sup>c</sup>	40.4 <sup>c</sup>	72.8 <sup>a</sup>	75.2 <sup>b</sup>	8.58 <sup>b</sup>	78.14 <sup>b</sup>	0.046 <sup>c</sup>	37.1 <sup>b</sup>
SEM (n=6)	0.22	0.36	0.44	0.69	0.92	1.51	1.59	0.190	1.626	0.00004	1.34

NSS: untreated soybean seeds, GSS: Germinated soybean seeds and HGSS: Heated and Germinated soybean seeds.

a, b, c Means within a column with different subscripts differ ( $P < 0.05$ ).

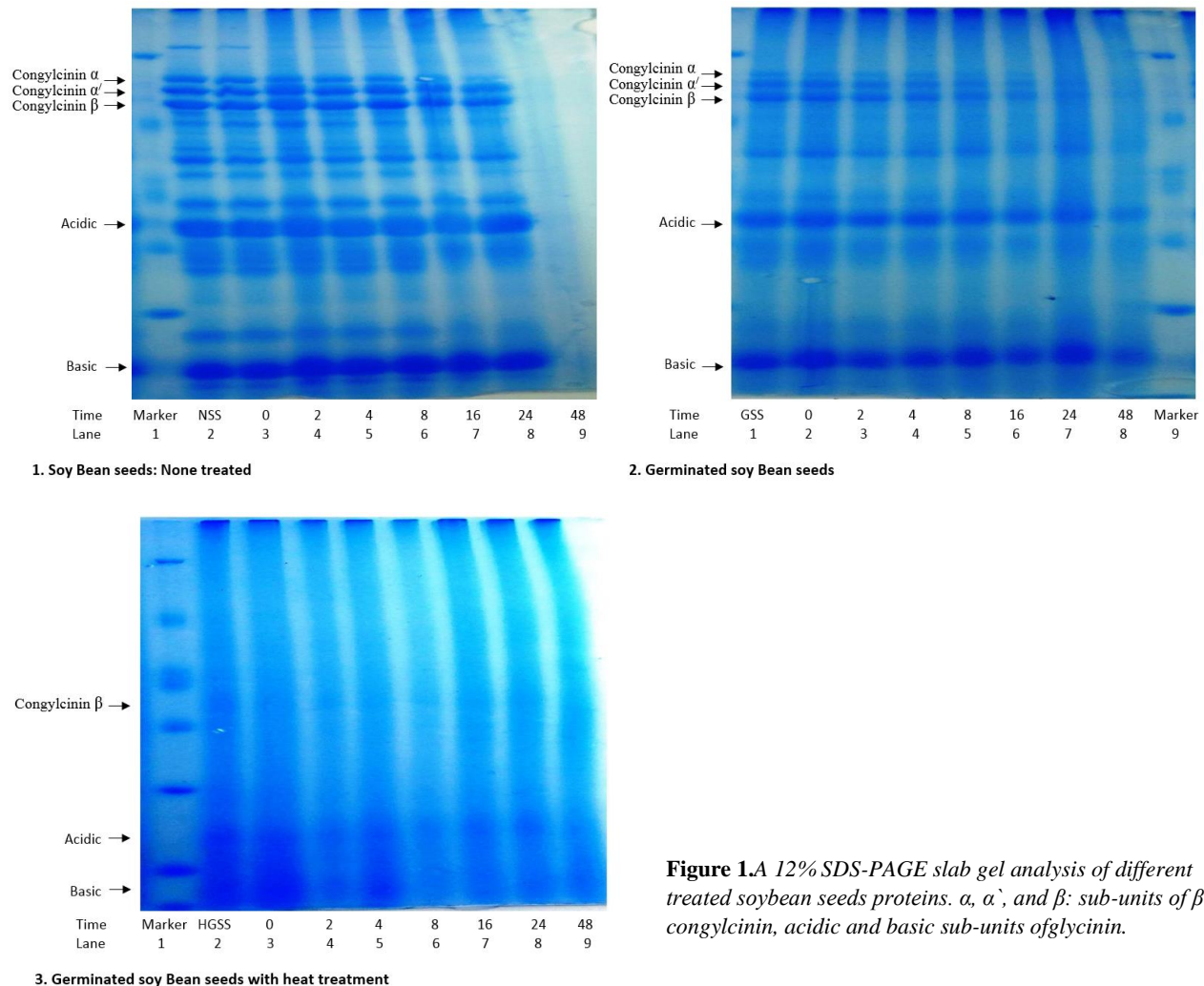
ED = Effective degradability ( $k=0.02$ )

The significant reduction in the rate of degradation(c) and hence ED of N by heat processing showed it appeared to increase by-pass protein from soybean and thereby increase the amount of available AA in the small intestine, provided that the digestibility of by-pass protein does not decrease with heat treatment. Previous studies also reported that heat processing reduced the rate of disappearance of DM and N of whole soybean (Nasri *et al.*, 2008; Faldet and Satter, 1991; Hsu and Satter, 1995; Aldrich *et al.*, 1997).

Heat facilitates the Maillard or nonenzymatic

brow reaction between sugar aldehyde groups and free amino acid groups of protein to yield an amino-sugar complex (Canbolat *et al.*, 2005; Lin and Kung, 1999).

This complex is more resistant than normal peptides to enzymatic hydrolysis and reversibility of this reaction is dependent on temperature and duration of heat exposure (Lin and Kung, 1999). However, some precautions must be taken when heat treatments are employed because the Maillard reactions might render the protein and the carbohydrate unavailable in the small intestine if excess heat was employed



**Figure 1.A** 12% SDS-PAGE slab gel analysis of different treated soybean seeds proteins.  $\alpha$ ,  $\alpha'$ , and  $\beta$ : sub-units of  $\beta$ -conglycinin, acidic and basic sub-units of glycinin.

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(Lin and Kung, 1999). In the Maillard reaction excess heat causes losses of sugar and amino acids, especially lysine, which can be 5 to 15 times greater than for the other amino acids (Andrian, 1974). Therefore, heat treatment should be kept to a minimum due to its cost and the possibility of destroying essential amino acids such as lysine and methionine and reducing the availability of other nutrients (Kratzer *et al.*, 1990; Van der Poel *et al.*, 1995; Qin *et al.*, 1998). The effect of heat treatment on the rumen degradability of protein sources was not consistent.

**SDS-Page**

The SDS-PAGE analysis of different treated soybean meal protein is presented in Figure 1. Two major components were observed:  $\beta$ -conglycinin and glycinin. Three components of  $\beta$ -conglycinin  $\alpha$ ,  $\alpha'$ , and  $\beta$  were separated with estimated molecular weights of 97.86, 88.86 and 78.74 kDa, respectively. Results show that all protein sub units such as  $\alpha'$ -conglycinin in untreated seed, degraded in 48 h after incubation. Also, in germinated seed, glycinin sub units resistance to degradation in all incubation time.

Two main polypeptide bands were identified as acidic and basic sub-units of glycinin with estimated molecular weights of 26.58 and 14.40 kDa, respectively. The estimated molecular weights for acidic and basic glycinin sub-units are in approximately agreement with those previously reported (Nobar *et al.*, 2005; Kella *et al.*, 1989; Van der Aar *et al.*, 1983).  $\beta$ -conglycinin is more susceptible to rumen degradation than the glycinin sub-units were (Figure 1) and this in agreement by Nobar *et al.* (2005) reporting. The resistance to ruminal degradation of glycinin compared with  $\beta$ -conglycinin is probably associated with its chemical and physical structure. Its acidic and basic subunits are associated through intermolecular disulfide bridges and most of the S-S links are buried in the interior part of the glycinin molecules (Langan, 1972). In addition, electrostatic and hydrophobic associations are involved in maintaining the tertiary structure of glycinin (Nobar *et al.*, 2005).

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