



P-ISSN: 2349-8528
 E-ISSN: 2321-4902
 IJCS 2018; 6(4): 388-392
 © 2018 IJCS
 Received: 21-05-2018
 Accepted: 22-06-2018

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Identification of novel protein band (s) distinguishing hulled v.s hullless varieties in barley (*Hordeum vulgare* L.) and its characterization

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Abstract

Barley (*Hordeum vulgare*) is one of the cereal crops among top five major crops of the world which is widely used for food, feed stock, malting, brewing and distilling industry. It belongs to the Poaceae family. The barley is cultivated as a Rabi crop in India with sowing being under taken from October-December and harvesting from March-May. Our aim was to quantify the protein of the hulled and hullless barley varieties and identify the novel protein band in hulled barley varieties. The range of seed grain protein in both hulled and hullless barley varieties were found 49.63 mg/gm. to 105.63 mg/gm. Maximum protein was found in Dolma *i.e.*, 105.63 mg/gm, which minimum protein was found in NDB 1173 *i.e.*, 49.63 mg/gm. Variation in presence or absence and the relative mobility of some electrophoretic band was also observed among the hulled and hullless barley genotype. The highest variation in the electrophoretic profile of seed storage protein was observed around at 70 kDa and 20 to 40 kDa range of band. All the Twelve barley genotypes hulled and hullless also differed in intensity of bands as well as in number of protein bands. To raise antibody against identified protein, rabbit was selected for immunization. A subcutaneous method was used for making protein titer for production of antibody.

Keywords: SDS-PAGE, hulled barley, hull-less barley, protein profile, antibody raising

Introduction

Barley (*Hordeum vulgare* L.) is one of the most important in cereals and earliest domesticated crop plants of the world (Zohary and Hopf. 1993) [49]. It belongs to the Poaceae family and a member of the grass family. It is cultivated in almost all parts of the world except the tropical region. It is the major source of food for large number of people living in the cooler semi-arid areas of the world, where wheat and other cereals are less well adopted. The cultivated barley is a self-pollinating, diploid species ($2n=2x=14$) with a genome size of approximately 25.3 x 10⁹ bp equivalent to 5.5 pg DNA of a haploid nucleus (Bennett and Smith, 1976) [3]. The barley is used as animal fodder, as a source of fermentable material for beer and certain distilled beverages, and as a component of various health foods. Barley has beta-glucan and anti-cholesterol substance and acetylcholine which energies our nervous system that recover memory loss, barley flour have also a property of digestibility due to low gluten, soluble dietary fibers, high lysine, thymine and riboflavin. It also contains high levels of several antioxidants, which prevent many degenerative diseases such as cancer, heart diseases, stroke and premature ageing. Barley grain contains 12.5% moisture, 11.5% albuminoids, 74% carbohydrate, 1.3% fat, 1.5% curved fiber and ash (Anderson *et al.*, 1990). Barley grains occur in two forms, one is hulled and second one is hullless. Hullless or naked barley differs from hulled barley by the loose husk cover of caryopsis that is easily separable upon threshing in contrast to hulled barley (Bhatta, 1999) [5]. The hullless grain character is controlled by the single recessive gene 'nude' located on the long arm of chromosome 7 H (Kikuchi *et al.*, 2003). The identification of barley cultivars is of great commercial importance in the malting and brewing industry. The use of biochemical rather than morphological criteria for the identification of cultivars of different crop species has received considerable attention (Cooke and Cliff, 1983) [12]. In particular, the analysis of seed storage proteins by various electrophoresis techniques has been shown to be a valuable method of distinguishing cereal cultivars.

Polyacrylamide gel electrophoresis (PAGE) of hordein proteins is being used worldwide as a method for identifying barley cultivars (Shewry and Pratt, 1978) [43].

Traditional approach to varietal identification involves the study of morphological description, which is not only time consuming and resource requiring but is also affected by the environment. The gel electrophoresis is used and successful biochemical method applied to barley variety identification from the grain. In the particular, electrophoresis of the alcohol soluble seed storage protein fraction (Hordein) has been extensively researched (Chnapek *et al.*, 2007) to assess origin of cultivated plants, assign genome structure, realize genome analyses, identify varieties and detect lines and mutants (Gregova *et al.*, 1995). Barley is very well adapted and is produced commercially in both irrigated and dry land environments throughout the state. Worldwide, it can be grown successfully where other grain crops are poorly adapted, including high latitude and high elevation regions and even bordering desert. The aim of present study was to identify probable protein variant between hulled and hullless barley varieties for use in genetic modification.

Materials and Methods

Seed material

The different barley varieties six hull-less *i.e.*, (NDB- 943, HBL-276, BHS-352, Dolma, Gitanjali, Karan- 16) and six hulled *i.e.*, (Narendra Jau-1, Narendra Jau- 2, Narendra Jau-3, NDB- 1445, RD- 2552, NDB -1173) were collected from Department of Genetic and Plant Breeding, N. D University of Agriculture Technology Kumarganj, Faizabad.

Protein extraction

The total protein was extracted from individual seed samples. 0.5 g of each variety was taken and ground to fine powder using pestle and mortar and then, 1 ml Tris urea buffer (0.05 M Tris-HCl, 2% SDS, 5 M Urea, 1% β - mercaptoethanol with pH 8.0) was added and crude homogenate was centrifuged at Room temperature at 15,000 rpm for 10 min, and extracted protein sample was collected as supernatant and pellet were discarded and then stored at -20°C.

Protein quantification

The concentration of the extracted protein samples was determined using Lowry's method (1976) using different concentrations of the samples against the control. Relative concentrations of all samples were calculated using the formula from BSA standard chart.

Result and Discussion -

Estimation of protein by Folin Lowery's method

The levels of barley protein were measured using the method of Folin and Ciocalteu's reagent (Lowery *et al.*, 1951). Ciocalteu's reagent gave the light blue colour with protein. Protein optical density was observed in 660 nm against standard curve of BSA protein. The range of protein content in both hulled and hullless barley varieties grain from 49.63 mg/gm. to 105.63 mg/gm. Maximum protein was found in hulled barley variety NDB-1173 *i.e.*, 49.63 mg/gm and minimum protein in variety Dolma *i.e.* 105.63 mg/gm.

Identification of Barley Protein by SDS-PAGE

As shown in fig. 1,2 and 3 all the lane showing various electrophoretic on SDS-PAGE. Maximum numbers of bands were found in Narendra Jau- 1, Narendra Jau- 2, Narendra Jau- 3, NDB- 1173, NDB-1445 (hulled varieties) and HBL-

276, BHS- 352, NDB- 943, Gitanjali, Karan- 16 (hullless varieties). Narendra jau- 1 and Narendra Jau- 2 both has maximum number of band comparatively to other. (Fig.1 and 2).

HBL- 276, BHS- 352, NDB- 943, Gitanjali, Karan- 16, had seven bands which were common in both hulled and hullless but one extra band was present in most of the hulled barley varieties in upper portion of the SDS-PAGE gel, it was approximately 70 kDa, (fig. 1).

3.4 Identification of the novel protein band of hulled barley varieties

An extra novel protein band approximately 70 kDa in hulled barley varieties (NARENDRA JAU- 1, NARENDRA JAU- 2, NARENDRA JAU- 3, NDB- 1173, NDB- 1445 and RD 2552) was observed. As shown in fig. 1 and 2.

The highest variation in the electrophoretic profile of seed storage protein was observed around 70 kDa to 40-20 kDa range of bands. All the twelve barley genotypes (hulled and hullless) were differed in intensity of bands as well as in number of protein bands.

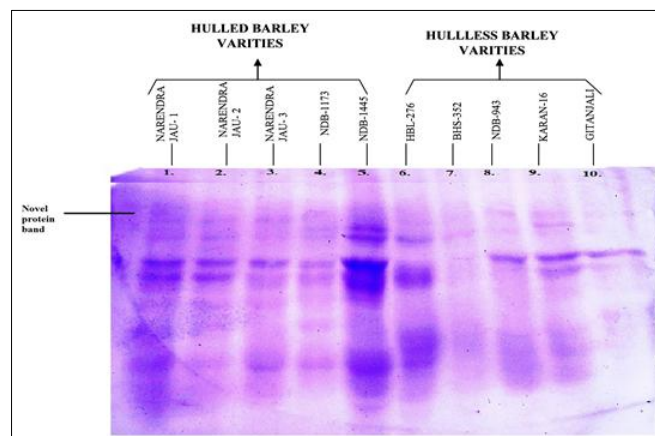


Fig 1: protein profiling of barley seed grain on 12%SDS-PAGE

4.1 Elution and injection protein of barley-

Eluted protein recovered in eppendrops tube roughly 100 μ g protein each gel was recovered. Each tube (100 μ g) was used for injection purpose. Protein was mixed with adjuvant was white in colour as shown in emulsion (fig. 4 A, B and C), until white emulsion was not formed and checked it in ice cold water.



Fig 4: A and B Freund's complete Adjuvant 150 μ l mixed with 150 μ l (150 μ g) of protein and emulsion was prepared



Fig 5: preparation and checking of emulsion if ice cold water for immunization

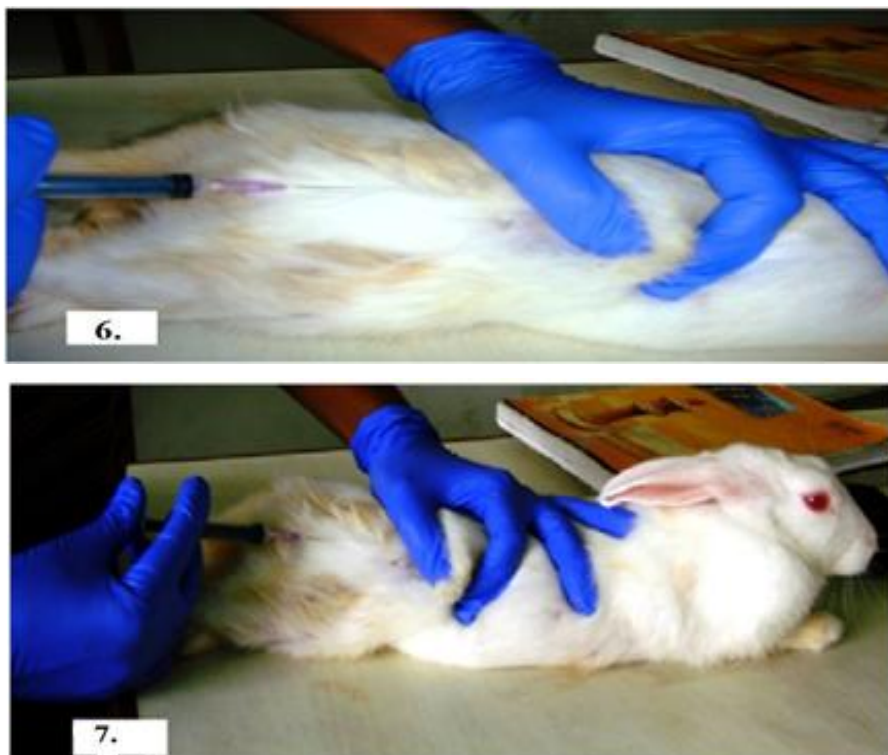


Fig 6 and 7: Injecting the identified novel protein with complete adjuvant in the body of rabbit by subcutaneous method

4.2 Harvesting the blood sample from rabbit

After the raising antibody blood collection was done. Before the bleeding rabbit was wrapped in towel and left for a while. Marginal vein of the ear was selected for collection of blood. For this tip of ear was gently holded, hair was shaved and rubbed with 70% ethanol. Blood collection was done with a sterile needle and syringe or butterfly catheter and syringe. The ear was held out horizontally and stabilized, the penetrated vein with needle bevel up. Only a small portion of the needle was inserted in parallel above the vessel with the tip directed into lumen along with longitudinal axis and inserted the needle into vein properly, and then withdrawn the amount of blood desired, but only small volume (less than 3 ml) could be obtained from the marginal ear vein. Later, the vein penetration site was covered with cotton ball and then applied gentle pressure to the site for up to 2 minutes. Later, rabbit was returned in the cage.

4.3 Storage the blood serum:-

After this process, blood serum was stored into eppendrops tube, and then put at room temperature for 1 hours and then overnight at 4°C. Next day, it centrifuged at 4000 rpm for 10 min, and then collected supernatant, was stored at -20°C.

5. N-terminal sequencing of identified novel protein band -

The purified desired protein band containing phosphate buffer saline (0.1M, pH- 7.0) was blotted on PVDF (Poly vinylidene di fluoride membrane) and transferred to PVDF membrane and used for the N- terminal sequencing from blotted protein, and this Purified protein is being sent for N- terminal sequencing from Rajeev Gandhi Centre for Biotechnology Trivandrum, Kerala.

Discussion-

According to the results obtained, it is concluded that our aim was to quantify the protein of the hulled and hullless barley varieties and identify the novel protein band in hulled barley

varieties. The barley protein is easily soluble in Tris-Urea buffer (Galani *et al.*, 2011) ^[17], and we used this method in our experiment for isolating the protein from barley seed grain, but some workers also have used different methods such Lontie and Voets (1959) ^[27] used 55% isopropanol with 1% (2-β-mercaptoethanol) for the extraction of barley seed protein at 60°C. Barley protein was also extracted from single dry seeds in buffered alcohol by Doll and Andersen. (1981). Since bands were satisfactory we stucked to this method. Some slight modification was also done by Landry *et al.*, (1972) ^[24] and Shewry *et al.* (1978) ^[43] for the extraction of barley protein. They used 55% propanol with 2% 2-β-mercaptoethanol at 30°C. For finding quantity of protein in both hulled and hullless barley Lowery method (Lowery *et al.*, 1951) was used for protein estimation. First protein optical density was observed at 660 nm against standard curve of BSA. The range of seed grain protein in both hulled and hullless barley varieties were found 49.63 gm/mg to 105.63 mg/gm and maximum protein was found in Dolma *i.e.*, 105.63 mg/gm and minimum protein was found in NDB 1173 *i.e.*, 49.63 mg/gm (Table 2). Thus, in hullless barley variety protein content was high. Results of this study were similar to the finding of Pandey *et al.*, (2013) ^[33]. Kumar and Matta (2011) ^[22] showed also similar result in barley *i.e.*, (12.1gm/100gm).

Variation in presence or absence and the relative mobility of some electrophoretic band was also observed among the hulled and hullless barley genotype. The highest variation in the electrophoretic profile of seed storage protein was observed around 70 kDa to 20 kDa range. All the Twelve barley genotypes hulled and hullless differed in intensity of bands as well as in number of protein bands. Kumar and Matta (2011) ^[22] showed also similar result in protein profiling in germinating barley seed grain.

In, Narendra Jau- 1, Narendra Jau- 2, and NDB-1173 minimum bands were observed. Whereas, NDB- 943 and Gitanjali 2-2 bands were seen respectively. Near about 50

kDa protein band with very high intensity was found in both hullless barley varieties. Approximately, a novel protein band of 70 kDa was observed in hulled barley seed grain and it was not observed in hullless barley seed grains. Results of this study were similar to those of (Shewry *et al.*, 1978) [43], who used an acidic PAGE system in barley seed grains, (Chmelik *et al.*, 2002) [9]. These findings are natural as the poly peptide band patterns and relative intensity of different bands were known to be influenced by a number of factors such as cultivar used, growing conditions extraction protocols etc. (Shewry *et al.*, 1978; Heisel *et al.*, 1986) [43, 19].

To raise antibody against identified protein, rabbit was selected for immunization. Rabbits are suited for the purpose of antibody production being suitable for easy handling, and were used for genetic manipulation of antibody. A subcutaneous method was used for making protein titer for production of antibody. Hulled variety was used for injection and making titer. Thus, the present study gave new insights into protein variation in hulled and hullless barley genotype. However, the authentic proteins difference in hulled vs. hullless can be further characterized by 2-D gel and N-terminal sequencing. The N-terminal sequencing followed by BLAST may throw some light in this direction.

Conclusion

Entitled on Identification of novel protein band (s) distinguishing hulled vs. hullless varieties in barley (*Hordeum vulgare* L.), and its characterization in which included total twelve barley varieties we found that the protein content in both hulled and hullless barley varieties grains ranged from 49.63 mg/gm to 105.63 mg/gm. Minimum protein was found in hulled barley variety NDB-1173 *i.e.*, 49.63 mg/gm and maximum protein in variety Dolma *i.e.* 105.63. Maximum numbers of bands were found in Narendra Jau- 1, Narendra Jau- 2, Narendra Jau- 3, NDB- 1173, NDB-1445 (hulled varieties) and HBL- 276, BHS- 352, NDB- 943, Gitanjali, Karan- 16 (hullless varieties).

Narendra jau- 1 and Narendra Jau- 2 both has maximum number of band comparatively to other. (Fig.1 and 2). HBL-276, BHS- 352, NDB- 943, Gitanjali, Karan- 16, had seven bands which were common in both hulled and hullless but one extra band was present in most of the hulled barley varieties in upper portion of the SDS-PAGE gel, it was approximately 70 kDa, (fig. 1). An extra novel protein band approximately 70 kDa in hulled barley varieties

NARENDRA JAU- 1, NARENDRA JAU- 2, NARENDRA JAU- 3, NDB- 1173, NDB- 1445 and RD 2552) was observed as shown in fig. 1 and 2. The highest variation in the electrophoretic profile of seed storage protein was observed around at 70 kDa and 20 to 40 kDa range of bands. All the twelve barley genotypes (hulled and hullless) also differed in intensity of bands as well as in number of protein bands. To raise antibody against identified protein, rabbit was selected for immunization. A subcutaneous method was used for making protein titer for production of antibody.

Acknowledgement

The author is grateful to Major Advisor and Head, Department of Plant Molecular Biology and Genetic Engineering for providing necessary facilities.

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