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A Modified Sequential Extraction Procedure for Glutenin (HMW & LMW), Gliadin and Other Proteins from Wheat Seed

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ABSTRACT

The detection of Celiac disease responsible gluten protein fraction and development of antiglidin and antiglutenin antibody, require suitable and reliable extraction protocol. In the present study, the protocol is based on the sequential combination of extraction of albumin (water), globulin (0.5M NaCl), gliadin (50% 1-propanol) and glutenin (50% 1-propanol; 0.08 M Tris-HCl) from single seed. The composition of resulting protein fractions were analyzed by SDS-PAGE. The strength of our extraction protocol is that it starts with the pre-extraction to remove albumin and globulin. However, reducing agents had not used to be lethal for the T-cell cultures. Sequential extracted gluten proteins had less contamination of other fraction. It will be useful to investigate the toxicity of gluten (gliadin and glutenin) in improved wheat cultivars that affect the health of celiac patients. Consequently, it will be also fruitful for identification of wheat varieties on the basis of grain quality.

Key word *Wheat seed, Gluten, SDS-PAGE, Quality, Celiac disease and antibody.*

Gluten is the main structural protein of wheat with equivalent toxic proteins found in other cereals viz., rye and barley (Shewry and Halford 2002). Gluten proteins play active role in celiac disease, as ingestion of glutens lead to damage of villi of small intestine (See *et al.* 2015). The toxic protein fractions of gluten are gliadin (monomeric) and glutenin (aggregated) proteins (Shewry and Tathum 2016). Toxic amino acid fragments of gluten proteins have varied in distribution between different groups of gluten proteins. However, the quality of bread also depends on gluten content and is the main factor to screen wheat varieties (Payne *et al.* 1982 and Gupta *et al.* 2016).

Celiac disease is a malabsorptive disorder which affected more than 1% world population, and at present available cure is only a gluten-free diet (GFD). Generally, celiac patient spends more money on cure instead of their diet. Untreated celiac disease is a bowl of disease, causes excess mortality (See *et al.* 2015). However, there are many barriers, include accessibility of GFD, cost and cross-contamination of gluten. Major challenge in development of gluten-free foods or low gluten containing wheat variety is large investment of fund, time and suitable protocol of gluten extraction to screen the varieties. In India, the number of patients with celiac disease increases gradually (WGO Practice Guideline). In future prospect, Food Safety and Standards Authority of India (FASSI) and Indian Council of Medical Research (ICMR) are being search of specific antibodies. Specific monoclonal antibodies are required to test for screening of toxic gluten proteins in food products marketed as safe GFD. Pure extracted gliadin and glutenin fragment will be used as target antigens for the development of specific antibodies (Ellis1998).

First time gluten was extracted by Osborne (1907) on the basis of cereal protein solubility. After that, gluten extraction protocol from durum and hexaploid wheat, were optimized by Jones *et al.* 1959; Woychik *et al.* 1964; Beckwith *et al.* 1966; Kasarda *et al.* 1976, Payne and Corfield1979; Bietz and Wall 1980; Payne *et al.* 1982; Burnouf and Bietz (1984); Bietz *et al.* 1984; Bietz and Burnouf 1985; Marchylo *et al.* 1989; Graybosch and Morris 1990; Gupta *et al.* 1990; Singh *et al.*1991; Fu and Sapirstein 1996; Tatham *et al.* 2000; Dupont *et al.* 2005, 2008 and Van den Broech *et al.* 2009, used different reagents according to their objective. Marchylo *et al.* (1989) extracted gluten by removing starch and albumins/globulins from wheat flour. The extractions of toxic gluten fraction require suitable and reliable protocol (Van den Broech *et al.* 2009) without use of reducing agents. Reducing agents containing gluten extracts are harmful for the development of antibodies, which are used to identify the toxic fractions.

MATERIAL AND METHODS

Wheat seeds (viz. C306) were collected from net house of NRC on Plant Biotechnology that grown in aseptic condition under various regimens of fertilizer and optimum temperature (Altenbach *et al.* 2003).

In the present study, the sequential extraction of different fractions from seed sample was standardized after comparative study of three methods: (I)- Tatham *et al.* 2000, (II)- 50% 1-Propanol with 1% DTT extract and (III)- Modified sequential extraction protocol. Total gluten was extracted by earlier used method of Payne *et al.* (1980).

(I)- Tatham *et al.* (2000):

According to Tatham *et al.* (2000), wheat flour (100 mg) was continuously mixed with 1 ml of butanol-1 for 1 hour at 20^{0}C. Mixture of flour-butanol was centrifuged and supernatant was discarded. First residue was mixed with 1 ml of 0.5 M NaCl for 1 hour at 20^{0}C and supernatant was discarded. Remaining residue was washed with water. Second residue was mixed with1 ml of 70% Ethanol to extract gliadin. For the extraction of glutenin from third residue, added 1 ml of 50% propane, 2% 2-ME and 1% acetic acid, incubated at 20^{0}C for 1hour. After incubation, mixture of residue was centrifuged and supernatant containing glutenin was collected in fresh tube.

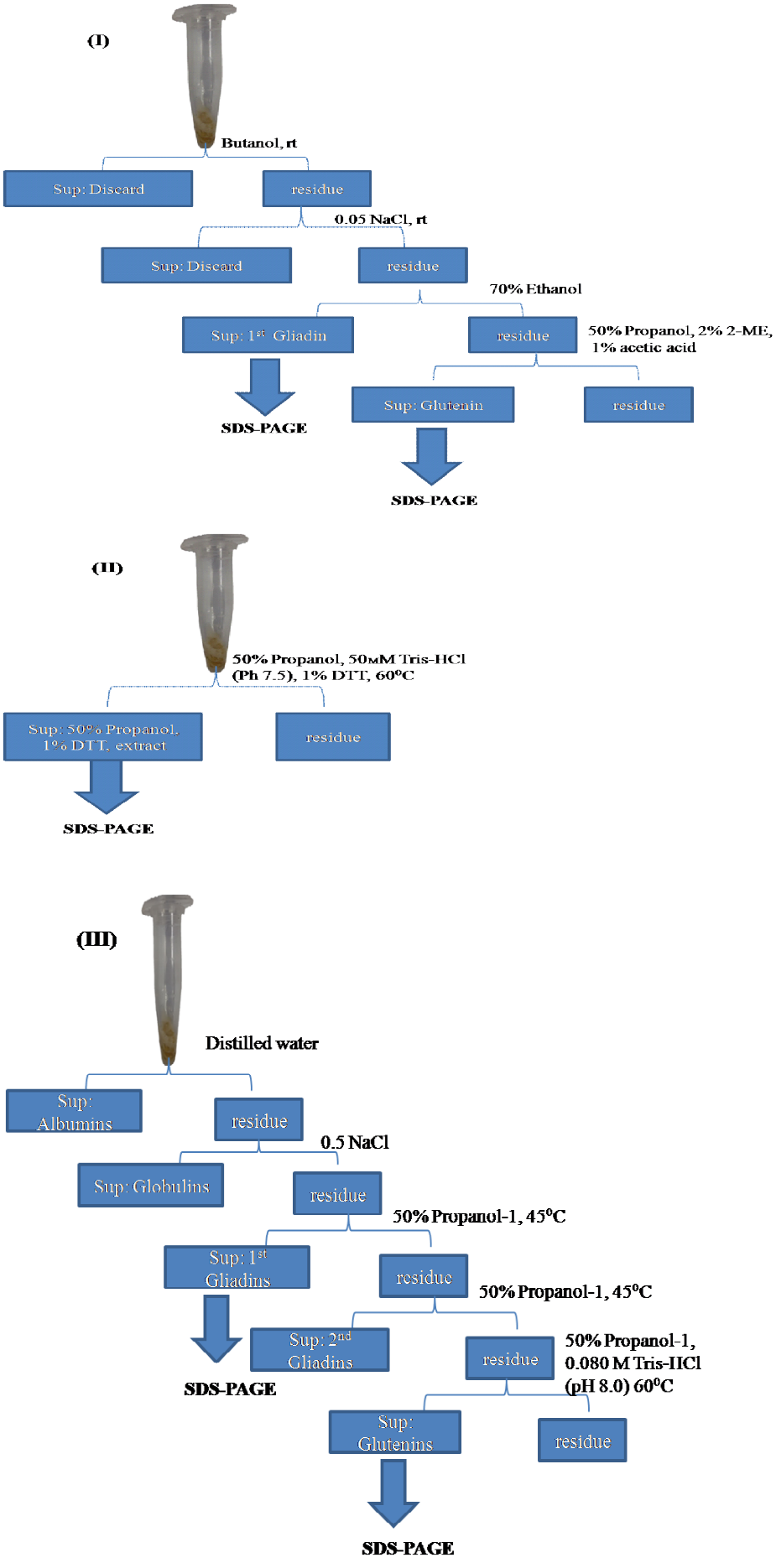

Fig. 1. Graphical representation of protocol used for extraction of gliadins and glutenins from wheat seed by using (I)- Tatham *et al.* 2000, (II)- 60% Ethanol extract and (III)- Modified sequential extraction protocol

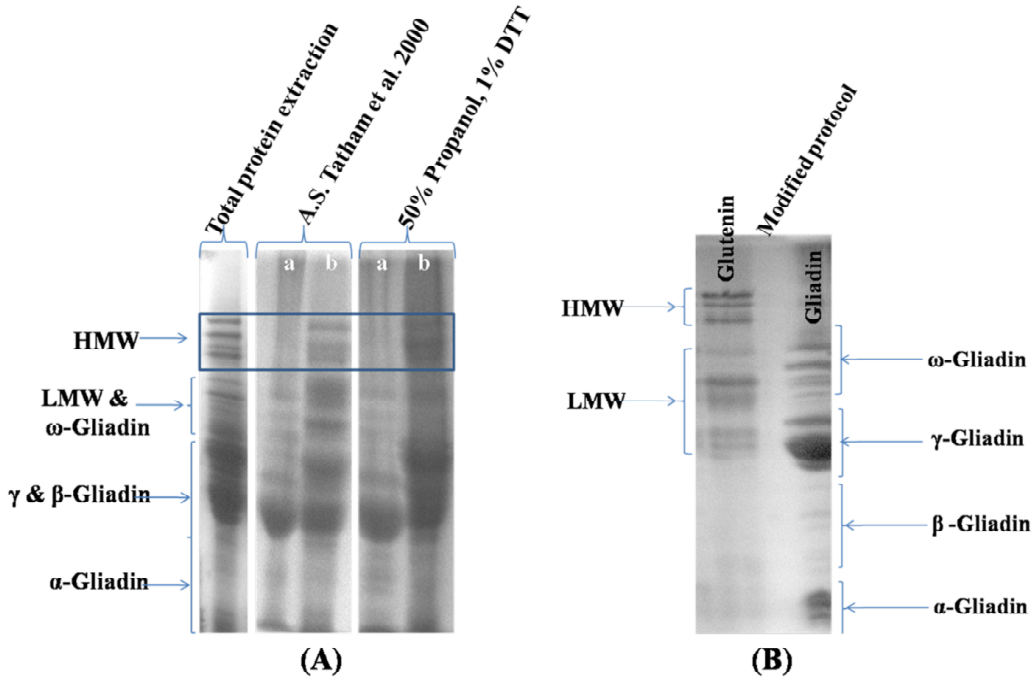

Fig. 2. SDS pattern of sequential extracted proteins by three different protocols. **(A)-**Total Gluten protein, (**a**) Gliadin and (**b**) Glutenin proteins extracted by Tatham *et al.* 2000 and 50% 1-Propanol with 1% DTT respectively; **(B)-** Glutenin and Gliadin proteins by modified protocols.

(II)- 50% 1-Propanol with 1% DTT:

In this method, 50% 1-Propanol, 50mM Tris-HCl (pH 7.5) and 1% DTT was used to extract alcohol soluble proteins at 60^{0}C. After centrifuged, supernatant containing glutenin was collected in fresh tube.

(III)- (III)- Modified sequential extraction protocol

Modification of sequential extraction is based on the extraction method of Marchylo *et al.*, 1989 and NK Singh *et al.* 1991. According to present protocol, seven stock solutions are required: (1)- Distilled (RO) Water, (2)- 0.5M NaCl, (3)- 50% 1-Propanol, (4)- Gluten extraction buffer (50% 1-Propanol, 80 mM Tris- HCl, pH 8.0), (5)- Loading sample buffer contain 2% SDS, 40% glycerol, 20 mg bromophenol blue, 0.08M Tri-HCl, pH 8.0 and (6)- 4-Vinylpyridine, (7)- Staining solution (6 % (w/v) trichloroacetic acid, 18 % methanol, 6 % glacial acetic acid and 0.025 % coomassie brilliant blue R250). The endosperm of seed was crushed into fine powder.

Procedure of modified sequential extraction protocol:

Extraction of Albumin

In this step, a crushed single seed containing endosperm kept in eppendorf tube and added 200 µl, RO water. Sample was mixed and incubated for 60 minutes at room temperature. After incubation, centrifuged at 12000 rpm for 10 minutes and collected 100 µl, supernatant (**Albumin**) in fresh tube. Step was repeated twice.

Extraction of Globulin

After transfer of supernatant, Added 100 µl, 0.5M NaCl to first residue. The sample was properly mixed by vortex and kept at 37^{0}C for 30 minutes. After incubation, centrifuged for 10 min at 12000 rpm and transferred 100µl, supernatant (**Globulin**) to fresh tube. Step was repeated twice.

Extraction of Gliadin

After globulin extraction, added RO water to second residue and centrifuged for 2 minutes at 12000 rpm. Supernatant was discarded and added 50% 1- Propanol to remaining residue. The sample was mixed and again incubated at 45^{0}C for 30 min. After incubation, the sample was centrifuged (residue) at 12000 rpm for 10 min and collected supernatant (**Gliadin**) in fresh tube. Step was repeated twice.

Extraction of LMW and HMW

After extraction of gliadin, added 100 µl Gluten extraction buffer (50% propanol-1 and 0.08M Tris-HCl, pH 8.0) in remaining third residue. Mixed the sample (residue) by vortex and incubated at 60^{0}C for 30 minutes, then centrifuge at 12,000 rpm for 5 minutes at room temperature. Without transferred the supernatant, added 1.4%, 4-vinylpyridine and 100 µl, Gluten extraction buffer in sample. Sample was mixed properly by vortex and again incubated at 60^{0}C for 30 minutes. After incubation, centrifuged the sample at 12000 rpm for 10 minutes at room temperature and Collected supernatant (**LMW & HMW**) in fresh tube. Step was repeated twice. Sample loading buffer (100 µl) was added in 100 µl of supernatants. Mixed the sample and kept in water bath at 85^{0}C for 20 minutes and centrifuged at 12000 rpm for 10 min before loading.

Wheat Gluten protein and different polypeptides fractions like Glutenin (HMW and LMW) and Gliadin extracted from single seed of wheat were loaded on SDS Polyacrylamide gel containing 6% stacking gel and 12% resolving gel and as allowed to run on 40mA for longer

period of 4 hours. After running the gel, was stained with coomassie brilliant blue solution (6 % (w/v) TCA, 18 % methanol, 6 % glacial acetic acid and 0.025 % CBB R250) as described by Sreeramalu and Singh (1995) for overnight and next day destained in water for overnight.

RESULT AND DISCUSSION

European and North American have mainly affected by Celiac disease (Green *et al.* 2003; West *et al.* 2014; Altobelli *et al.* 2014 and Catassi *et al.* 2014). However, prevalence data of Ramakrishna *et al.* (2016) has showed that 1% population of northern part of India is affected by Celiac disease. For the detection of Celiac disease in community, monoclonal antibodies of antiglidin and antiglutenin are required which is developed by pure gluten protein fraction. So, developing countries require a suitable and reliable gluten extraction protocol.

The sequential extraction protocol is based on the solubility of wheat storage proteins (Osborne *et al.* 1907). The current method used to extract HMW, LMW and gliadin subunits from single seed. All these protein fractions were analyzed by SDS-PAGE and nonspecific background bands were not observed (figure 2b). Subsequently, Glutenin lane contains four and eight protein fragments protein fragments encoded by HMW, LMW subunit genes respectively. Gliadin genes encoded about 20 protein fragments and which will be differ according to wheat varieties. This procedure is easy to use, highly reproducible and gives clear banding patterns of glutenin and gliadin subunit genes. Earlier similar banding pattern of HMW (3-8 fractions), LMW and Gliadin (10-24 fractions) had also reported by Payne *et al.* 1982, Gupta *et al.* 1990, Singh *et al.* 1991, Tatham *et al.* 2000 and Dupont *et al.* 2008. Although earlier extraction method had used reducing agent to be lethal for the T-cell cultures (Van den Broech *et al.* 2009).

We standardized four-step extraction protocol (Figure 1-III) without using any regents. However, pure extracted glutenin and gliadin subunits may be used for development of monoclonal antibody to detect gluten protein dependent diseases and gluten free food products. Cross contamination of endosperm protein represents the main problem in preparation of pure protein fractions for analysis and study. The strength of our extraction protocol is that it starts with the pre-extraction to remove albumins and globulins. Van den Broech *et al.* (2009) also observed that pre-extraction of gliadin prevents cross-contamination with LMW. LMW glutenin and gliadin subunits are controlled by tightly linked genes (Singh *et al.* (1991). There are many albumins and globulins are in the same molecular weight as gliadins and also observed similar banding pattern (Vensel *et al.* 2005; Hurkman *et al.* 2007 and Singh *et al.* 2001 and Dupond *et al.* 2005). Allelic variation of glutenin and gliadin Subunits affect the breadmaking quality in wheat (Payne *et al.* 1987). HMW subunits are used for screening of bread making cultivars in wheat breeding programs (Payne *et al.* 1987). However, LMW subunits have quite difficult for screening of cultivars and gliadin bands can only be used as an indicator for the presence of specific LMW subunit alleles (Singh *et al.* 1991). Total albumin/globulin, gliadin, and glutenin estimation fluctuate with extraction procedure, wheat varieties and may also vary with field situation (Wieser *et al.* 1998 and Triboi *et al.* 2003).

Present modified protocol will be used to screen the wheat genotypes/germplasm containing low content of gluten for the development of new wheat variety by conventional breeding. Celiac safe wheat can be developed by molecular breeding approaches to mutate or down regulate toxic proteins. It will be broadly useful for the routine screening of glutenin and gliadin subunit composition of breeding lines produced by wheat breeders.

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