Quantification and varietal variation of low molecular weight glutenin subunits (LMW-GS) using size exclusion chromatography

Priya Dangi¹ · B. S. Khatkar¹

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Abstract Crude glutenin of four commercial wheat varieties viz. C 306, HI 977, HW 2004 and PBW 550 of diverse origin and breadmaking quality were fractionated by size-exclusion chromatography into three fractions of decreasing molecular weights. The relative quantity of peak II, containing LMW-GS specifically, varied considerably among the varieties as reflected from their discrete SEC profiles. The area % of peak II, containing protein of interest, was maximal for C 306 (22.08%) followed by PBW 550 (15.86%). The least proportion of LMW-GS were recovered from variety HW 2004 (9.68%). As the concentration of the sample extract injected to the column increased, the resolution of the peak declined in association with the slight shifting of retention time to the higher values. The best results were obtained for variety C 306 at 100 mg protein concentration with 3 M urea buffer. Consequently, the optimized conditions for purification of LMW-GS in appreciable amounts using SEC were established.

Keywords Size-exclusion chromatography · LMW-GS · Quantification · Concentration gradient

Introduction

Low molecular weight glutenin subunits (LMW-GS) are the most diverse and indiscriminate group of glutenins which accounts for 60% of wheat glutenins present in an endosperm. LMW-GS can be classified into different categories depending upon the structural and functional properties of subunits. Classically, LMW-GS can be divided into typical LMW-GS (also known as B-subunits) and gliadin-like LMW-GS (C- and D-subunits) which are structurally similar to gliadins, but functionally they are glutenins due to their ability to form intermolecular disulphide bonds (D'Ovidio and Masci 2004). The molecular weight estimations of LMW-GS designated for B-, C- and D-subunits were 40–50, 30–40 and 55–70 kDa, respectively (Khatkar and Schofield 1997). Quantitatively, B-subunits are recognized as the most important followed by C-subunits. The D-subunits expressed in some wheat cultivars represent a very minor fraction of LMW-GS.

The qualitative and quantitative aspects of the gluten proteins serves as an index for the prediction and evaluation of flour quality for end products (Chaudhary et al. 2016c). Wheat protein quality depends on the presence or absence of specific HMW-GS, LMW-GS, gliadins, ratio of monomeric to polymeric proteins and the molecular size distribution of glutenin (Khatkar et al. 1995; Khatkar and Schofield 1997; Khatkar 2006; Dhaka and Khatkar 2015). Contemporary, it is believed that the differences in wheat quality are most strongly determined by differences in glutenin quality. The wide disparity in the quality of glutenins may arise from fluctuation in its organization, size distribution and subunit composition. Therefore, an insight to the glutenin components is crucial for understanding the manner glutenin polymers collaborate with each other and with other flour constituents (Veraverbeke and Delcour

✉ B. S. Khatkar
bhupendarkhatkar@gmail.com

¹ Department of Food Technology, Guru Jambheshwar University of Science and Technology, Hisar, Haryana 125001, India

2002). However, the detailed perception of the molecular level phenomenon is yet largely unexploited. This comply the need to quantify each class of proteins: gliadins, LMW-GS and HMW-GS and thus, formulate models for predicting the utility of flour.

Despite indications that LMW-GS did not account for a major influence in breadmaking performance, their exact role in breadmaking remains highly under investigated. Lew et al. (1992) indicated B-type LMW-GS as ‘chain-extendors’, whilst C-type and D-type LMW-GS as ‘chain-terminators’ for the reason that the former possess at least two cysteine residues capable of forming inter-chain disulphide bonds, while the latter has only one cysteine residues. It can be concluded that a higher B-type or C-type LMW-GS proportion is correlated to larger glutenin size and superior glutenin quality. Larroque et al. (1997) further strengthened this fact by revealing SE-HPLC results which stated that a decrease in B-type/C-type LMW-GS indicated decrease in glutenin size.

The foremost experimental study in context to LMW-GS was laid down by Beckwith et al. (1966) who identified LMW-GS from the gel filtration extracts of wheat flour and termed them as high molecular weight gliadins linked by disulphide bonds. Larre et al. (1997) developed a method for the separation of HMW-GS and LMW-GS at preparative scale using Triton $\times$ 114 detergent on RP-HPLC. Nicolas et al. (1998) worked on the quantification of gliadins, HMW-GS and LMW-GS using RP-HPLC. Certain LMW-GS were partially purified using Ion-exchange chromatography and RP-HPLC followed by using preparative isoelectric focusing over a narrow pH range to obtain almost 90% purified B-LMW-GS (Sissons et al. 1998).

The advancement in the popularity of LMW-GS globally is particular due to their associations with the technological quality of the wheat flour. The isolation and purification of LMW-GS can be of great interest in predicting the structure and interactions of LMW-GS at molecular level and in turn the gluten functionality. The present investigation was intended to explore the differences in the proportions of LMW-GS in commercial wheat varieties with diverse baking quality. In addition, the effects of injecting varied concentrations of glutenin to the SEC column on the extraction of LMW-GS were studied. This study was further utilized to bring out the effects of purified LMW-GS on product development and in assessing the changes in the dough structure by the incorporation of these subunits.

Materials and methods

Selection of wheat varieties and flour extraction

Four commercial Indian wheat varieties, namely, C 306, HI 977, HW 2004 and PBW 550 were chosen for the study.

The varieties were procured from agricultural universities and regional centres. The varieties were cleaned, tempered to appropriate moisture level overnight and passed to Chopin laboratory mill (Model CD1, Villeneuve la Garonne, France) for milling. The wheat flours obtained were defatted using chloroform and stored at 5 °C in air-tight containers for further analysis (Dangi and Khatkar 2017b).

Extraction of wheat gluten and its fractionation

Standard ICC protocol was adopted to isolate gluten from defatted flour using Glutomatic instrument. Powdered freeze dried gluten samples (50 g) were dissolved in 200 ml of 70% ethanol and the mixture was stirred on a magnetic stirrer for 3 h at 25 °C followed by centrifugation for 30 min at $1000 \times g$, 4 °C. The whole process was repeated thrice. The resultant pellet, which is rich in glutenin was freeze dried. The powdered crude glutenin was stored at 5 °C (Chaudhary et al. 2017; Dangi and Khatkar 2017a).

Purification of LMW-GS

Crude glutenin of each variety in varying concentrations was dissolved in an optimized solvent system i.e. 3 M urea (pH 5.5). The mixture was passed through sonication in an ultrasonic chamber for 1 h and centrifuged for 30 min at $12,000 \times g$, – 15 °C. Sample extract (2 ml) was filtered through 0.22 μ m Millipore syringe filter (HV Millipore, DuraPore) and used for injection. SEC was performed in conjunction with Sephacryl S-200 column (HI PrepTM 16/60 Sephacryl S-200 HR, GE Healthcare) connected to a GE Healthcare and Lifesciences Fast Protein Liquid Chromatography system, comprising a model Äktaprime plus. The eluting buffer used was 3 M urea containing 0.15 M NaCl buffer (pH 5.5) at a flow rate of 0.5 ml/min. The elution buffer was filtered through 0.45 μ m filter (HV Millipore, DuraPore) and degassed under vacuum prior to use. The fractions were collected with an automated fraction collector, concentrated, dialyzed against 1% acetic acid and freeze-dried (Chaudhary et al. 2016b, c).

Data analyses

Mean and standard deviation were calculated using SPSS software version 16.0 (SPSS Inc.). The mean comparison was accomplished by Duncan’s multiple range test and the statistical significance was observed at $p < 0.05$. All the tests were carried out in triplicates.

Results and discussion

Quantification and varietal variation of LMW-GS

LMW-GS were quantified by size-exclusion chromatography protocol using a known quantity of glutenins as a quantitative standard. As far as diverse varieties are concerned, metaphorically congruent FPLC profiles were retrieved for the polymeric proteins for the sole discrepancy with reference to the amplitude of segregated peaks (as shown in Fig. 1). The proportion of each peak separated on SEC was quantified by integration of areas in the respective chromatographic profiles (Table 1) (Chaudhary et al. 2016a). The average quantity of proteins eluted in peak I, II, III and IV ranged from 65.19 to 70.76%, 9.68 to 22.08%, 9.6 to 17.36% and 1.07 to 2.8%, respectively. It was noteworthy that area % of peak I was the highest and

peak IV was the lowest for all the varieties with respect to a standard quantity of glutenin taken. The proportion of peak II that contained protein of interest (LMW-GS) was maximal for C 306 (22.08%), followed by PBW 550 (15.86%), HI 977 (14.44%) and HW 2004 (9.68%). Appreciable quantities of LMW-GS were recovered from varieties C 306 and PBW 550. Thereby, emphasizing the fact that this protocol can be of great importance in extracting a purified fraction of LMW-GS.

Effect of quantity injected

Table 2 presented the changes in area % of eluted peaks on injecting varied concentrations of glutenin to size-exclusion column. On elevating the concentration of glutenin subjected to SEC from 100 to 1000 mg, remarkable differences were observed in the area % of peaks.

Fig. 1 Fractionation of glutenins from four commercial wheat varieties by the optimized SEC process on FPLC **a** C 306, **b** HI 977, **c** HW 2004, **d** PBW 550

Table 1 Quantification of protein fractions of glutenin of wheat varieties eluted from SEC using 3 M urea buffer

Variety	Protein (%) recovery			
	Peak I	Peak II	Peak III	Peak IV
C 306	65.19 ± 3.57 ^a	22.08 ± 3.23 ^d	10.14 ± 2.13 ^b	1.07 ± 0.29 ^a
HI 977	66.09 ± 0.73 ^b	14.44 ± 1.13 ^b	17.36 ± 1.15 ^c	1.15 ± 0.29 ^a
HW 2004	68.18 ± 0.38 ^c	9.68 ± 2.34 ^a	17.03 ± 0.08 ^c	2.8 ± 0.57 ^c
PBW 550	70.76 ± 2.07 ^d	15.86 ± 1.87 ^c	9.6 ± 1.09 ^a	2.44 ± 0.78 ^b

Values followed by letters are significantly different at $p < 0.05$

Table 2 Distribution pattern of eluted peaks after loading varied quantities of glutenin on SEC

Sample size (mg)	Peak I	Peak II	Peak III	Peak IV
100	43.19 ^a	26.27 ^c	12.14 ^d	6.68 ^c
300	57.63 ^b	23.38 ^b	11.33 ^c	5.87 ^b
500	64.56 ^c	23.14 ^b	10.42 ^b	1.39 ^a
1000	69.74 ^d	19.24 ^a	8.85 ^a	1.85 ^a

Values followed by letters are significantly different at $p < 0.05$

The percentage of protein eluted in peak I increased considerably from 43.19% for 100 mg sample to 69.74% for 1000 mg sample. This is possibly due to the dissolution of more protein in the buffer. In addition, the resolution of the peaks declines implying the chances of cross-contamination. This presumably is responsible for the downturn in the proportions of peak II and III, with elevated levels of glutenin. As indicated, the proportion of protein eluted in peak II exhibited a downturn from 26.27% for 100 mg to 19.24% for loading of 1000 mg sample. Despite of changes in the proportion of eluted peaks, considerable advancement was perceived in terms of retention times as well. The retention time observed for respective peaks shifted slightly to the higher values as the concentration was changed from 100 to 500 mg. However, a drastic switch was observed on loading 1000 mg sample, with retention time being approximately doubled. It can be summarized that the quantitative distribution profile of glutenin may change with slight alterations in experimental conditions.

Conclusion

The increment in the quantity of sample size resulted in the extraction of exceeding levels of peak I proteins and decreased the levels of peak II. In addition, there was considerable loss of resolution of the chromatogram due to increased cross-contamination of peaks. With the increase in concentration of sample injected to the column, a shift in the retention time was observed. Appreciable amounts of LMW-GS of varieties C 306 and PBW 5550 were obtained following this protocol. Precisely, the results manifested

the efficiency of the optimized FPLC protocol in the purification of LMW-GS, which can be utilized further to explore their structural and techno-functional importance. Furthermore, SEC can be proved as a valuable tool in the purification and quantification of LMW-GS because of its speed, small sample size and reproducibility.

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