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ORIGINAL ARTICLE

Microstructural, thermal and IR spectroscopy characterisation of wheat gluten and its sub fractions

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Abstract The gluten and its sub-fractions of good and poor bread quality wheat varieties were studied using scanning electron microscopy, differential scanning calorimetry (DSC) and IR spectroscopy techniques. The gluten of good bread quality wheat variety showed organized foam like matrix, whereas that of poor demonstrated an open gluten matrix. The glutenin of good bread quality wheat (HI 977) exhibited a more striated, organised texture in contrast to a dense, unorganised structure visible in C306. Gliadins of poor bread quality wheat were self-assembled to form a sheet like structure, whereas the gliadin proteins of good bread quality wheat variety showed more open microstructure. DSC thermal profiles of gluten and glutenin proteins of poor bread quality wheat showed exothermic peaks at around 200 °C. A distinct endothermic peak was detected in the glutenin fraction of good bread quality wheat, suggesting greater thermostability. Amide I peak at $\sim$1668 cm^{-1} for gluten of good bread quality wheat variety showed higher relative intensities of β-turn as compared to observed for gluten of poor bread quality.

Keywords Gluten · Glutenin · Gliadin · Microstructural analysis · Thermal analysis · IR Spectroscopy

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Introduction

Wheat gluten is a complex protein composed primarily of a three-dimensional network of linearly cross-linked glutenin subunits and gliadin proteins through hydrogen, hydrophobic, and disulfide bonds (Margarida Falcao-Rodrigues et al. 2005; Korableva and Kasymova 2010; Dua et al. 2009). Gluten proteins play a vital role in bread quality (Dhaka and Khatkar 2015a, 2015b; Sivam et al. 2010; Dong et al. 2009). They affect the stability of the dough and bread volume and form the skeleton of wheat dough. It is widely accepted that gliadins confer viscous properties to gluten (Khatkar et al. 2002) and glutenins impart strength and elasticity which is essential to hold gases produced during the fermentation and early stages of baking (Dhaka and Khatkar 2015a, b; Singh et al. 2011; Peighambardoust et al. 2011). Thus, variation in microstructural, thermal and spectral characteristics of gluten and its sub-fractions is expected to cause variation in bread quality baked for different varieties. Scanning Electron Microscopy (SEM) has been used in the recent years to study the microscopic structures of wheat flour dough and gluten (Kontogiorgos 2011). It provides stereoscopic images with high magnification. Differential Scanning Calorimetry (DSC) is considered an eloquent and powerful analytical tool for studying the thermal behaviour of gluten proteins (Bengoechea et al. 2007). Fourier Transform Infrared (FTIR) spectroscopy is recognized as a valuable tool for the examination of protein conformation in denatured form and dried states resulting in its greatly expanded use in studies of protein secondary structure and protein dynamics (Kong and Yu 2007). Therefore, these techniques were used in the present study to understand the microstructure and thermal characteristics of gluten and its sub-fractions of

good and poor bread quality wheat varieties. Efforts were also made to correlate the results obtained with the secondary structure of these fractions and to explain the wide variation in bread making potential of the two selected varieties.

Materials and methods

Materials

Grains of two different wheat varieties of diverse bread making potential were procured from Chaudhary Charan Singh Haryana Agricultural University, Hisar. The varieties HI 977 and C 306 had *Glu-1* score of 10 and 4, respectively. The high molecular weight glutenin subunit composition corresponding to variety HI 977 was 2*, 17 + 18, 5 + 10 and Null, 20, 2 + 12 for wheat variety C 306. The grains of the varieties were milled on a Chopin laboratory mill (Model CD1, Villeneuve la Garenne, France) into straight grade flours. The wheat flour samples were further analysed for protein (N × 5.7) content (AACC 2000). SDS sedimentation volume was estimated by the method of Axford et al. (1979).

Protein fractionation

Modified Osborne method was used to separate gluten into gliadins and glutenin. Gluten was separated from dough manually using distilled water. The gluten obtained was then freeze-dried and ground uniformly to a free flowing powder. Freeze dried gluten powder (50 g) was suspended in 1 L of 70 % (v/v) ethanol and stirred on a magnetic stirrer for 3 h at room temperature (~22 °C) followed by centrifugation at 1000×*g* for 30 min in cooling centrifuge at 4 °C. The extraction was repeated. The precipitant was collected as glutenin and the supernatant was subjected to rotary evaporator at 30 °C to remove ethanol to get the gliadins. Gliadin and glutenin fractions were freeze dried and powdered.

Differential scanning calorimetry

Calorimetric behaviour of gluten and its sub fractions were carried out using TA instruments, Q_{10} DSC model, USA, using aluminium pans. Powdered samples (3.0 ± 0.2 mg) of freeze dried gluten, glutenin and gliadin were weighed in aluminium pans, and covers were hermetically sealed into place. An empty, hermetically sealed aluminium pan was used as reference. The change in heat flow of samples was analysed over a temperature range of 40–250 °C at a rate of 5 °C/min.

Scanning electron microscopy

Scanning electron micrographs of the gluten and its fractions were acquired using Scanning Electron Microscopy (SEMTRAC Mini, Nikkiso, Germany). The samples were mixed with an optimum amount of water and the dough was formed and freeze dried. The structure obtained after freeze drying was fractured with a sharp knife to expose the inner structure and placed on to the aluminium stubs using double sided sticky tape. The exposed surface was coated with gold using a sputter coater to make sample conductive and then the inner surface was scanned at an accelerating potential of 5 kV and 100×*g* magnifications.

IR spectroscopy

Gluten, glutenin and gliadin samples of two wheat varieties were studied by Fourier transform infrared spectroscopy (FTIR). IR-spectral studies were performed on Shimadzu IR affinity-I 8000 FT-IR spectrometer under dry air at room temperature using KBr pellets. Sample (1 mg) was mixed with 300 mg of KBr supplied with FTIR unit. The samples were pressed directly on to attenuated reflectance KBr crystal into the sampling unit. Spectra were scanned between 4000 and 400 cm^{-1}, acquired at 4 cm^{-1} and signal averaged over 32 scans. The amide I band was examined critically for gluten and its fractions of both varieties, in terms of band intensities in relation to 1650 cm^{-1} band.

Results and discussion

Flour characteristics analysis

The good (HI 977) and poor (C 306) bread making wheat varieties had protein contents of 12.4 and 9.2 %, respectively. The flours made from hard wheat varieties were considered suitable for bread making are those and generally have high protein contents in the range of 10–14 % (Ktenioudaki et al. 2010). The importance of protein content lies in the ability of gluten to produce dough with the desired rheological properties. Moreover, higher amount of good quality protein is required for gas retention and dough rise during fermentation or early stages of baking. SDS sedimentation volume of 62 ml was recorded for HI 977, whereas C 306 had a substantially lower value of 37.5 ml. There has been evidence to suggest that the SDS sedimentation test can be independently used to predict the bread baking potential and strength of wheat varieties (Moonen et al. 1982; Greenaway et al. 1966). On the basis of SDS sedimentation test, HI 977 could be classified as a good bread quality variety, while C 306 as being poor bread wheat quality.

Table 1 Chemical analysis of wheat flour

Chemical analysis of flour	Wheat variety	
	HI 977	C 306
Protein (%)	12.4	9.2
Sedimentation volume (ml)	62	37.5
Dry gluten (%)	9.6	8.4
Gluten index (%)	99.3	65.1
Glutenin (%)	57.2	45.9
Gliadin (%)	43.5	53.1
Specific loaf volume (cm^3/g)	4.4	3.2

The gluten proteins impart unique bread making properties to wheat. Wheat varieties varied significantly for their dry gluten contents as shown in Table 1. C 306 demonstrated lower dry gluten content of 8.4 % and HI 977 had comparatively higher (9.6 %). The gluten of HI 977 was stronger, more elastic and was difficult to pass through glutomatic sieve exhibiting a higher gluten index of 99.3 %. On the other hand, gluten from C 306 was sticky, crumbly and easily passed through the sieve, depicting a lower gluten index of 65.1 %. The protein content, SDS volume and gluten index indicated HI 977 contained more 'good quality' protein than C 306. Glutenin and gliadin contents significantly varied among the two varieties. HI 977 had a higher proportion of glutenin (57.2 %), whereas C 306 showed 53.1 %. Specific loaf volumes (SLV) of HI 977 and C 306 were 4.4 and 3.2 cm^3/g, respectively. On the basis of chemical analysis, gluten quality parameters and baking test, HI 977 and C 306 were considered suitable for this study, HI 977 representing a good and C 306 a poor bread quality wheat variety.

Microstructural analysis of gluten and its fractions

Bread quality of a wheat variety is primarily attributed to its gluten proteins. Gluten is a macromolecular complex of alcohol soluble gliadins and alcohol insoluble glutenins. Interactions among these proteins fractions may be held responsible for the properties of gluten network and its baking potential. Gluten properties of good and poor bread quality HI 977 and C 306, respectively were compared to understand the critical difference between their gluten at microstructural level which might be responsible for the wide variation in their bread quality. Gluten microstructure of good bread quality HI 977 revealed that its proteins had foam like matrix (Fig. 1 aI), whereas gluten of poor bread quality wheat C 306 (Fig. 1aII) demonstrated extensively open gluten matrix. Open, non uniform structure of gluten proteins of poor bread quality wheat (C 306) may be held responsible for poor gas retention capacity and hence lesser bread loaf volume.

Glutenin fraction consists of high and low molecular weight glutenin subunits (HMW-GS and LMW-GS) cross-linked by disulfide bonds. HMW subunits in particular have been associated with bread making performance of flours (Wieser 2007) and viscoelastic properties of gluten matrix (Khatkar 2006). Therefore, understanding the microstructure of this fraction is of paramount importance in relation to its functionality. Glutenin matrix of HI 977 exhibited a number of aligned and unidirectional fibres (Fig. 1bI). It was interpreted from the image that HMW–GS subunits form the backbone of glutenin network and LMW-GS were seen as clustered structures in the glutenin matrix (Kontogiorgos 2011; Peighambardoust et al. 2005). On the contrary, micrograph of glutenin fraction of C 306 revealed a denser and unorganised structure with large gaps (Fig. 1bII).

Gliadins are monomeric proteins that interact by non-covalent interactions forming transient binding partners and their role in gluten network is to act as plasticizers and reduce the elasticity of the network. Distribution of gliadins varies depending on wheat variety as well as growing conditions. Gliadin fraction, after being visualized using scanning electron microscopy revealed that the gliadin of C 306 was self-assembled to form a sheet like structure (Fig. 1cII). In contrast, the gliadin proteins of good bread quality wheat HI 977 showed more open and ajared microstructure (Fig. 1cI).

Thermal characterisation of gluten and its fractions

Another area of interest during this research was the assessment of the thermal behaviour of two wheat varieties having different bread making qualities. The gluten proteins, like all polymers, are characterized by a temperature dependent equilibrium between two phases- a semi liquid phase prevalent at high temperature, and a glassy solid phase prevalent at low temperature. This type of physical change is termed 'glass transition' (Castellia et al. 2000). The glass transition temperature (Tg) is an indicator of thermostabilty. It is also correlated with the content of ordered secondary structure of a protein. Proteins with higher Tg are less susceptible to unfolding and denaturation at lower temperatures.

DSC thermograms of gluten of good (HI 977) and poor (C 306) bread quality wheat varieties are shown in Fig. 2a. Distinct endothermic peaks of gluten of HI 977 and C 306 were observed at 74.2 and 69.8 °C, respectively along with significant differences in their enthalpies (Table 2; Fig. 2a). It may be interpreted from the thermograms that more energy was needed for gluten HI 977 than that of C 306; specifying that gluten of former had more ordered secondary structure, furthur strengthening the results obtained by scanning electron microscopy. Another interesting point noted from the thermal profile of gluten of C

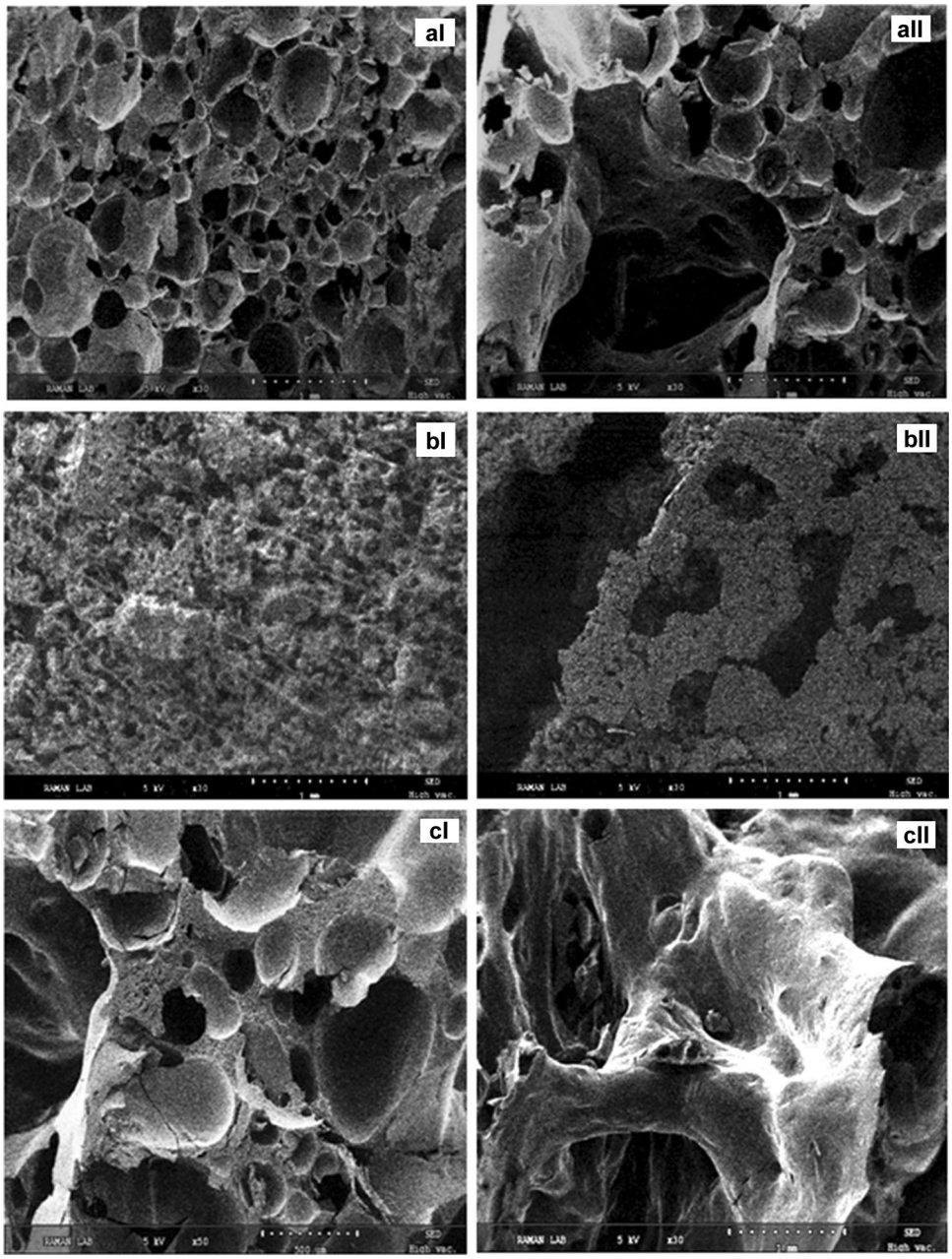

Fig. 1 Scanning electron micrographs of gluten (**a**), glutenin (**b**) and gliadin (**c**) of good (I-HI 977) and poor (II-C 306) bread quality wheat varieties

306 showed exothermic peak at high temperature around 200 °C, suggesting decomposition of proteins. This type of behavior may occur due to the rupture of bonds between the gluten molecules. On the other hand, thermogram of gluten of HI 977 did not show an exothermic peak at higher temperature, demonstrating greater thermostability at baking temperature in comparison to the gluten of poor bread quality wheat variety C 306. In a study by Levine and Slade (1990), it was indicated that the ability of wheat gluten to thermoset when heated above the glass transition temperature was critical to the final specific loaf volume for white pan bread. In addition, higher proportion of gliadin (Table 1) in C 306 might have played an important role in rendering less thermal stability, presumably by diluting the glutenin polymer and thus preventing inter and intra molecular bonding; and consequently inhibiting the formation of a strong gluten network.

Glutenins of HI 977 and C 306 showed distinct thermal behaviour in terms of glass transition temperature and enthalpies. Higher Tg i.e. 88.4 °C of glutenin HI 977 was observed, indicating more ordered and compact structure (Fig. 2b) as compared to that of C 306 having an endothermic peak at 63.6 °C (Fig. 2b). Glutenin fraction of HI 977 showed high enthalpy (89.6 J/g) than that of same fraction C 306 (71.1 J/g) signifying that the glutenin fraction of good bread quality wheat variety was more

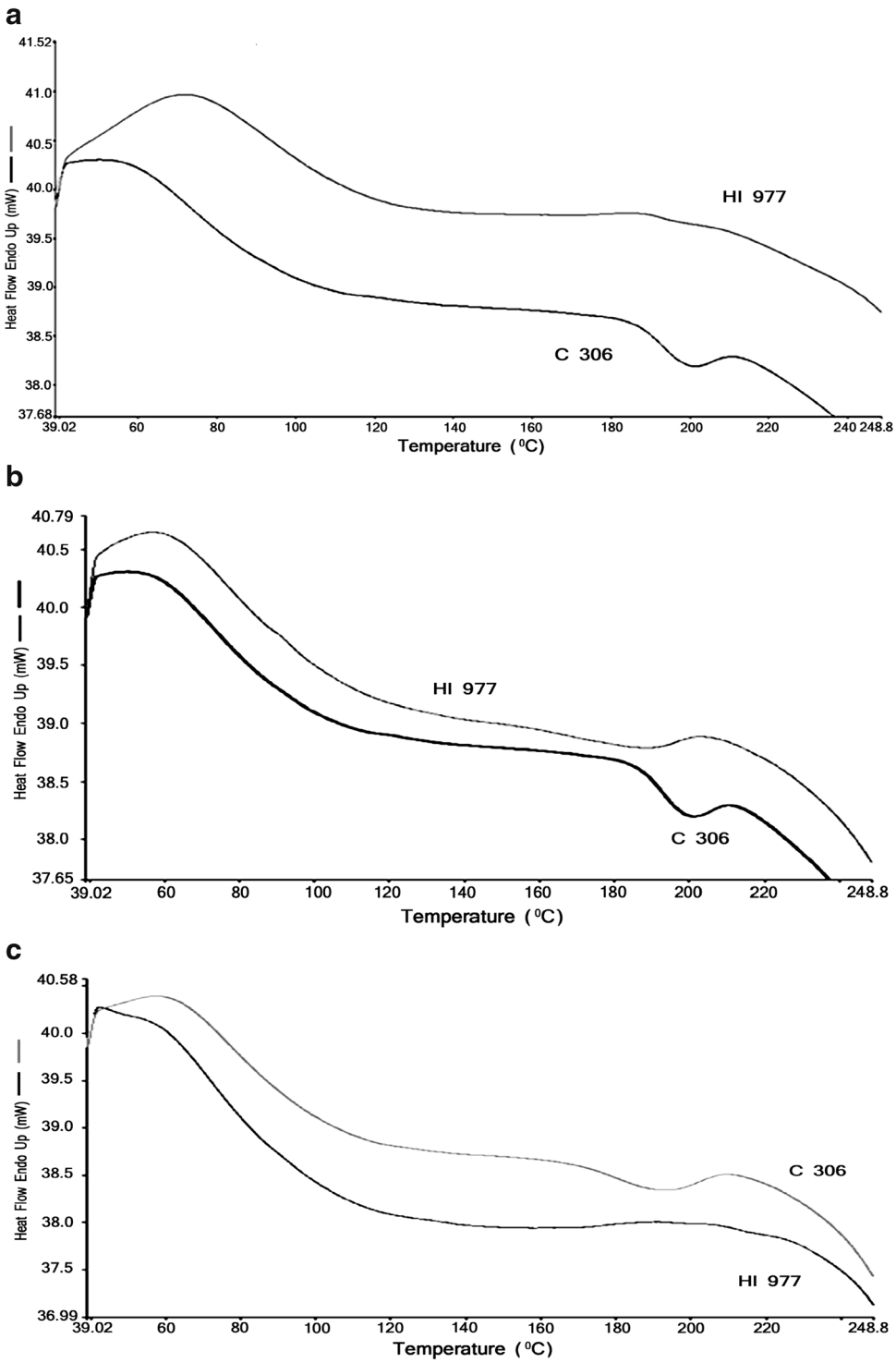

Fig. 2 **a** DSC thermogram of gluten of good (HI 977) and poor (C 306) bread quality wheat varieties. **b** DSC thermogram of glutenin of good (HI 977) and poor (C 306) bread quality wheat varieties. **c** DSC thermogram of gliadin of good (HI 977) and poor (C 306) bread quality wheat varieties

thermostable. A distinguished endothermic peak was detected at ~210 °C in glutenin HI 977 indicative of disulphide/sulphydryl exchange reaction, which was accountable for the stabilisation of the glutenin polymer, and hence was responsible for the formation of glutenin aggregates and good loaf volume. In contrast, an exothermic peak was observed for glutenin fraction of C 306 at temperature 210 °C indicative of low thermostability of its glutenin polymer. Schofield et al. (1983) have reported that the denaturation of gluten proteins at higher temperature lead to conformational changes particularly in the glutenin proteins, which were prompted due to the formation of new intermolecular disulphide bonds, resulting in larger disulphide-linked gluten protein aggregates.

Table 2 Thermal behaviour of gluten and its sub fractions of good (HI 977) and poor (C 306) bread quality wheat varieties

Samples	Glass transition temperature (°C)	Enthalpy (J/g)
Gluten		
HI 977	74.2	83.3
C 306	69.8	77.3
Glutenin		
HI 977	88.4	89.6
C 306	63.6	71.1
Gliadin		
HI 977	45.4	46.3
C 306	48.1	52.6

Gliadins are monomeric proteins linked by inter-chain disulphide bonds. Upon hydration, the gliadins behave as a viscous liquid (Singh and Khatkar 2005; Song and Zheng 2008) which imparts extensibility to dough. Thermal profile of gliadins (Fig. 2c) illustrated that the peak obtained for C 306 was symmetric; in contrast asymmetric curve with less enthalpy was obtained for HI 977. As evident from the thermogram, the glass transition temperature and enthalpy of gliadin fraction for poor bread quality wheat (C 306) was 48.1 °C and 52.6 J/g, respectively (Table 2). On the contrary, lower values of glass transition temperature (45.4 °C) and enthalpy (46.3 J/g) were observed for gliadin fraction of good bread quality wheat (HI 977).

IR spectroscopy characterisation of gluten and its fractions

Proteins exhibit three characteristic absorption bands in the mid infra-red spectrum designated as the amide I (1580–1720 cm^{-1}), amide II (1480–1580 cm^{-1}) and amide III (1430–1480 cm^{-1}) bands and the positions of these bands are sensitive to protein secondary structure (Kong and Yu 2007). The most sensitive spectral region of protein was the amide I band, which mainly arose due to amide carbonyl stretching of peptide linkages. High sensitivity to small variations in molecular geometry and hydrogen bonding patterns makes the amide I band uniquely useful for the analysis of protein secondary structural composition and conformational changes. The components of amide I band such as β-sheet, α-helix, and β-turns can be enlightened by studying their frequency in which they exist and contribute to protein secondary structure. A peak centred between 1658 and 1650 cm^{-1} is associated with α-helical and random structure, the shoulder at $\sim$1668 cm^{-1} is associated with β-turns and may also be related to glutamine side chains. Bands at 1612 and 1633 cm^{-1} are assigned to intermolecular and intramolecular β-sheet structure, respectively (Wellner et al. 2005). From the assessment of spectra presented in Fig. 3a, it was observed that gluten of good bread variety HI 977 had higher relative intensities of β-sheet at 1633 and 1612 cm^{-1} as compared to gluten of C 306. This may be ascribed to more ordered structural conformation of good bread quality variety HI 977 (Fig. 3a). It was also noticed that β-turn intensity at $\sim$1668 cm^{-1} for gluten was also greater in good bread quality wheat (HI 977). Hence, it can be concluded that the gluten of good bread quality wheat variety HI 977 was more elastic and stable as β-turn and β-sheet structure of gluten is considered to be related to elasticity and stability in gluten, which is an essential property of gluten for good bread quality.

The conformations of glutenin fractions of good (HI 977) and poor bread quality wheat (C 306) varieties are shown in Fig. 3b. It was noticed from the results that good variety HI 977 had more pronounced β-turn intensity at 1665 cm^{-1} indicating that glutenin fraction was very rich in β-turns (Parchment et al. 2001). β-turn structure of HMW-GS organize to give a regular β-spiral structure. Wellner et al. (2005) have postulated that the β-spiral structure of the central repetitive region of the HMW-GS was related to the elasticity of gluten. Such behaviour is important to baking technology as the ability of glutenin to form viscoelastic gluten and a gluten film network is essential for gas retention (Khatkar 2006). Furthermore, glutenin was rich in highly polar amino acids, in particular glutamine, which, acts as an H-bond donor and acceptor. Therefore, it participate in intra-molecular H-bond formation with other donor and acceptor amino acids (Khatkar and Schofield 1997). It has been observed that a large amount of glutamine facilitates formation of β-structures in synthetic polypeptides of glutamine (Krull et al. 1965). In this study, it was observed that the glutenin fraction of C 306 had weaker intensity for β-turns (band shift from 1665 to 1660 cm^{-1}) suggesting shorter β-turn region, leading to less elastic region of glutenins, resulting in poor bread making potential. The FTIR spectra for gliadin of C 306 possessed more β sheet structure (peaks at 1612 and 1633 cm^{-1}) than that of good bread quality wheat variety HI 977 (Fig. 3c). These conformational and network structure differences in gluten, glutenin and gliadin of good (HI 977) and poor (C 306) bread quality wheat varieties may be held responsible for the variations in the baking performance between the flours of these two varieties.

Conclusion

Microstructural, thermal and conformational study of gluten and its sub fractions of good (HI 977) and poor (C 306) bread quality wheat varieties divulged

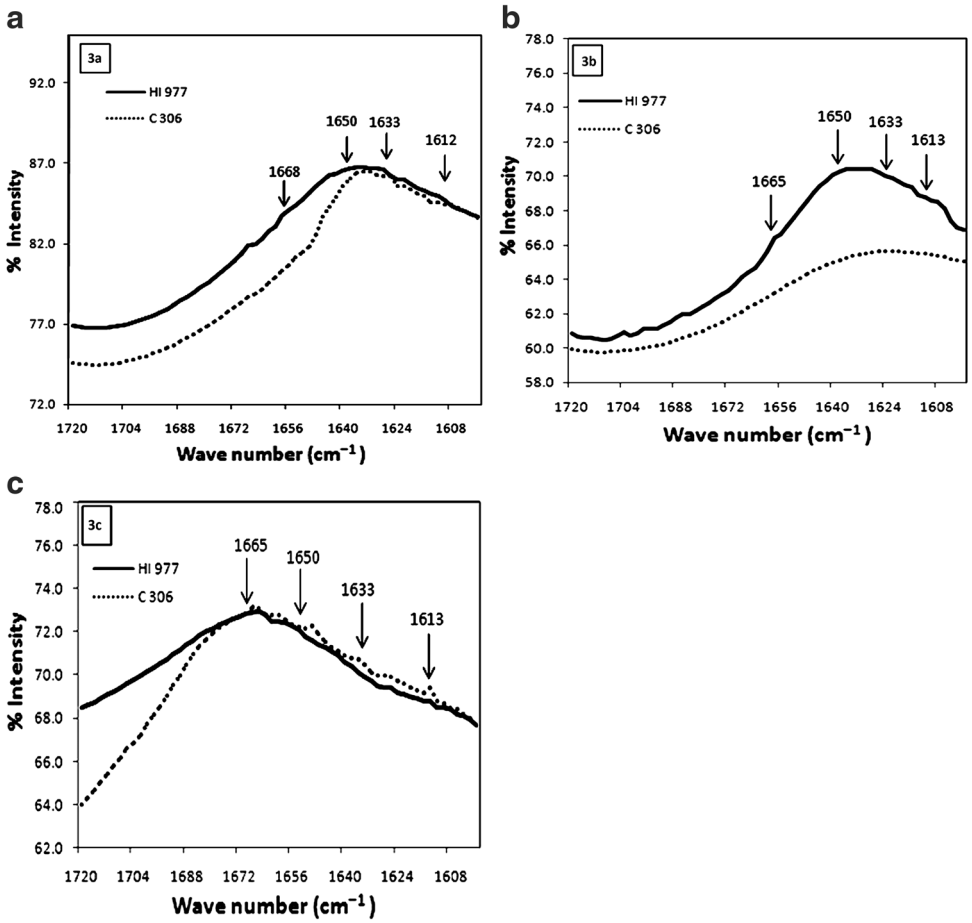

Fig. 3 **a** Amide I region for FTIR spectra of gluten of good (HI 977) and poor (C 306) bread quality wheat varieties. **b** Amide I region for FTIR spectra of glutenin of good (HI 977) and poor (C 306) bread quality wheat varieties. **c** Amide I region for FTIR spectra of gliadin of good (HI 977) and poor (C 306) bread quality wheat varieties

considerable differences. SEM micrograph of HI 977 revealed foam like structure which could be responsible for its good gas retention capacity. Glutenin of this variety depicted a striated and well ordered network suggesting an intense inter and intra molecular bonding leading to the formation of a strong gluten network. DSC studies validated greater thermal stability at baking temperature of gluten and glutenin of HI 977. A distinguished endothermic peak was detected at ~210 °C in glutenin HI 977 indicative of disulphide/sulphydryl exchange reaction, which was accountable for the stabilisation of the glutenin polymer, and hence was responsible for the formation of glutenin aggregates and good loaf volume. IR spectroscopy disclosed that the gluten and glutenin fraction of HI 977 had more β-turns and β-sheet structure hence were more elastic and stable than that of poor bread quality wheat variety further confirming the results exhibited from microstructures and DSC. Comparatively, glutenin fraction of C 306 had weaker intensity for β-turns indicating shorter β-turn region, leading to less elastic region of glutenins, resulting in poor bread making potential.

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