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Effect of freeze–thaw cycles on the molecular weight and size distribution of gluten

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ABSTRACT

Freezing processing is widely used in food industry, but some key scientific issue is still unclear. In this paper, the effects of freeze–thaw cycles on the molecular weight, radius of gyration, free amino group content, free sulphydryl group content and molecular conformation of gluten were investigated by size-exclusion chromatography in conjunction with multi-angle laser light scattering (SEC–MALLS), spectrophotometry and atomic force microscopy (AFM). The results showed that during the freeze–thaw cycles (frozen at −18 °C with cycling to 0 °C for 12 h and then back to −18 °C per 10 days) the molecular weight and radius of gyration of the gluten proteins decreased with the increase in freeze–thaw cycles, implying a depolymerisation in the high-molecular-weight fraction of the gluten. The free amino group content changed only slightly, and the free sulphydryl group content of the gluten increased from 9.8 μmol/g for the control to 13.87 μmol/g for the gluten stored for 120 days and submitted to 12 times freeze–thaw cycling, indicating that the depolymerisation of the gluten during freeze–thaw cycling was due to the breakage of disulphide bonds. AFM images showed that the gluten chain formed a fibril-like branched network which was weakened with increasing freeze–thaw cycles storage time. Some aggregation of the gluten chains was also observed in the AFM images.
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1. Introduction

The frozen food market has steadily grown in recent years due to consumer demand for convenient and high-quality products. With demand and market opportunities for frozen wheat-based products, frozen dough has become a valued alternative to its unfrozen counterpart. The dough freezing greatly affects the quality of final products such as bread, and should have a shelf-life of 16 weeks if the dough has not been temperature abused during transportation and storage (Phimolsiripol, Siripatrawan, Tulyathan, & Cleland, 2008). However, a certain amount of temperature fluctuation during frozen storage is unavoidable (Bhattacharya, Langstaff, & Berzonsky, 2003). It has been postulated that temperature fluctuations during storage and distribution accelerate the rates of protein quality deterioration, particularly due to water redistribution and ice recrystallisation (Phimolsiripol et al., 2008). Ice crystallisation in frozen dough greatly affects gluten proteins, reducing the viscoelastic properties of frozen dough (Simmons, Serventi, & Vodovotz, 2012; Zhao, Li, Liu, Liu, & Li, 2012b).

Dough is a complex mixture of water, gluten, starch, minerals and other components. Wheat gluten plays the most important role in the viscoelasticity of dough and in the formation of a network structure. Therefore, fully understanding wheat gluten and the factors that affect wheat gluten is quite crucial when the properties of dough and thus the quality of bakery products are being investigated (Kontogiorgos, 2011; Zhao et al., 2012a). It has often been reported that the molecular weight and molecular weight distribution (MWD) of gluten polymers are related to their functionality (Southan & MacRitchie, 1999).

During the freezing process and frozen product storage, the formation of ice is inevitable; accordingly, the investigation of the effects of the molecular weight of gluten on the quality of final products during frozen storage is of significance. Freezing and storage of dough at −18 °C generated loss in bread quality reflected by a lower loaf volume, longer fermentation time, an increment in the proportion of gas cells, and less elasticity in bread dough (Ribotta, León, & Anon, 2001). Therefore, it is of interest to discuss the change of molecular size of gluten in the frozen-storage process. Kennedy (2000) reported a considerable increase in the number of low-molecular-weight (LMW) oligomers, which presumably arose from the depolymerisation of glutenin, resulting in the weakening of the gluten protein structure and Ribotta et al. (2001) found that there was a decrease in the amount of glutenin subunits of high molecular weight between 88,700 Da and 129,100 Da during storage at −18 °C. However, the use of mercaptoethanol in this technique could break the disulphide

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bonds in gluten molecules and may not reflect the real molecular weight. In recent years, multiangle laser light scattering (MALLS) was proposed to characterise gluten proteins in conjunction with size-exclusion chromatography (SEC) (Monogioudi et al., 2009) and atomic force microscopy operated in noncontact mode (NCAFM) has been successfully applied in imaging isolated biological macromolecules because the technique offers the advantage of not damaging specimens on a substrate (Humphris et al., 2000).

There have been few studies reporting on the change of the wheat gluten proteins during frozen storage, especially in the case of temperature fluctuation. The temperature fluctuation greatly influences the gluten molecules and consequently the quality of final products. For example, the baking quality of wheat flour is closely related to the molecular weight and size distribution and content of glutenin (Mendichi, Fisichella, & Savarino, 2008), and the structure and interactions of the molecular chain are of interest in relation to understanding the processing functionality of gluten (Humphris et al., 2000). Therefore, the purpose of our work was to first investigate in detail the influence of freeze–thaw cycles on gluten molecular weight and size distribution in order to determine how molecules behave. Second, we concentrate more specifically on the impact of water redistribution and ice recrystallisation on gluten molecular chain structure.

2. Materials and methods

2.1. Materials

Untreated commercial wheat flour (protein 14.2%) from Canadian hard red winter wheat was obtained from Nanfang Co. Ltd. (Guangzhou, China), and stored in a 4 °C freezer (BCD-245, Bosch, Germany). NaCl, acetic acid, ethanol, di-sodium-tetraborate decahydrate, sodium dodecylsulphate, sodium phosphate, urea and tetrasodium ethylene diamine tetra-acetate were purchased from Sigma-Aldrich (St. Louis, MO, USA). All reagents were of analytical grade.

2.2. Wheat gluten extraction and freezing

Wet gluten was isolated according to the ICC standard No. 137/1 (ICC 1999), with some modifications. Six grammes of flour and 3 mL of distilled water were placed in the chamber of a gluten washing instrument (JJJM548, Jia Ding Cereals and Oil Instruments Co. Ltd., Shanghai, China) and mixed for 1 min. The dough was washed with 250 mL of a sodium chloride solution (20 g/L) for 5 min to remove the globulin and albumin and then with 250 mL of distilled water for 5 min to remove the residual starch and sodium chloride. To verify that the starch was completely removed from the washed gluten sample, an iodine solution was used. The washing procedure was continued until the blue colour disappeared. Two gluten samples were washed in parallel. The hydrated gluten was centrifuged at 6000 *g* for 10 min to obtain gluten with a hydration level of 60 ± 3% w/w. At low hydration levels, the sample was not homogeneous, whereas higher hydration levels resulted in extensive syneresis (Jiang, Kontogiorgos, Kasapis, & Douglas Goff, 2008).

After centrifuging, the hydrated gluten (approximately 4 g) was placed into cube pans (the side of 2 cm) and was frozen in a −80 °C freezer (ULT1386-5-V39, Revco., America) until the core temperature was measured to be −18 °C by a temperature probe (WS-106, Wason., China). Then, the hydrated gluten was stored in a −18 ±1 °C freezer (BCD-245, Bosch, Germany) for 120 days. The hydrated gluten was subjected to a freeze–thaw treatment. On the 5, 15, 25, 35, 45 … 115 days of frozen storage, the hydrated gluten was partially thawed at 0 °C freezer (BCD-245,Bosch., Germany) for 12 h, then back to the −18 °C freezer for frozen storage. This freezing, thawing and re-freezing treatment was repeated every 10 days. In other words, every 10 days frozen, the freeze–thaw treatment repeated 1 time. Three gluten samples were frozen in parallel. The control was a sample not subjected to freezing treatment.

At days 30, 60, 90 and 120, the frozen gluten (about 30 g) which undergoes 3, 6, 9 and 12 times freeze–thaw cycles respectively was sampled and lyophilised in a freeze-dryer (Wizard 2.0, VirTis Ltd., USA). The freeze-dried gluten was pulverised by a grinder into fine powders and screened using a 200 mesh sieve. Dried samples (protein 83.25%) were stored in sealed containers until use.

2.3. Gluten protein preparation

Gluten solution was prepared according to the procedure of Bean and Lookhart (2001), with minor modifications. During this process, the temperature of the gluten solution was controlled below 5 °C using an ice bath. Acetic acid was used as the solvent to dissolve the gluten proteins, since dilute acetic acid is a good solvent for cereal proteins, e.g., gluten and zein (Bean & Lookhart, 2001; Selling et al., 2007; Sessa, Eller, Palmquist, & Lawton, 2003; Sessa, Mohamed, Byars, Hamaker, & Selling, 2007), and normally is used as the solvent to characterise the molecular weight of the proteins by the SEC–MALLS technique, imparting little impact on the structure of protein molecular chain (Bean & Lookhart, 2001; Wu, Nakai, & Powrie, 1976). The concentration of the protein solution was determined by the micro-Kjeldahl method (ICC standard 105/2; N × 5.7% Dm). The concentration of the five protein samples (control, 30-day, 60-day, 90-day and 120-day) was approximately 8.0 mg/mL (acetic acid-to-gluten ratio, 50:1), which was then carefully adjusted to 3.0 mg/mL by adding acetic acid.

2.4. SEC–MALLS measurement

The supernatants of gluten solution (3.0 mg/mL) were separated using a Hewlett-Packard 1090 HPLC system consisting of a pump (1515, Waters corp., Milford, MA), a vacuum degasser, a thermostated autosampler (717, Waters corp., Milford, MA), a UV-detector (2478, Waters corp., Milford, MA) and a column compartment with a Biosep SEC-4000 column (Phenomenex, Torrance, CA). The SEC experimental conditions were: 0.5 M acetic acid mobile phase, 40 °C column temperature, 1.0 mL/min flow rate, 150 μL injection volume and 220 nm wavelength for UV detection.

MALLS data (molecular weight and radius of gyration) sampled at 1 s intervals were gathered with a multi-angle light scattering detector (DAWN HELEOSΓ, Wyatt Technology Corp., Santa Barbara, CA, USA) with 18 detection angles and a refractive index detector (DRI) (OptilabrEX, Wyatt Technology Corp., Santa Barbara, CA, USA) operating at a wavelength of 658 nm. The voltages of the photodiodes at each scattering angle were normalised by measuring the scattering intensity of bovine serum albumin (BSA). The light scattering detector was calibrated with toluene as recommended by the manufacturer. The delay volume between the light scattering detector and DRI was measured using BSA as a marker. The DRI detector voltage response was calibrated twice with five concentrations of sodium chloride at 40 °C. Four replicates were made for each determination and the parallel error was required to be less than 0.1%. The average of four measurements (molecular weight and radius of gyration) at the same sampling time is plotted in Fig. 2 and Fig. 5.

The value of d*n*/d*c* was measured online as described by Astafieva, Eberlein, and John Wang (1996). Five different concentrations of gluten dissolved in the 0.5 M acetic acid were analysed by a refractive index (RI) detector. Each sample was dissolved in duplicate, and nitrogen combustion was used to accurately determine the amount of protein dissolved. This procedure was repeated four times. The value of d*n*/d*c* was calculated using the software programme ASTRA 5.3.4.14 (Wyatt Technology Corp., CA). The value of d*n*/d*c* was 0.1767 ± 0.0028 mL/g.

2.5. Quantification of amino groups

Changes in free amino groups were determined spectrophotometrically using o-phthaldialdehyde (OPA) (Gujral & Rosell, 2004). An OPA solution was prepared by dissolving 40 mg OPA (dissolved in 1 mL ethanol first), 1.905 g of di-sodium-tetraborate decahydrate and 50 mg of sodium dodecylsulphate in 40 mL distilled water and then sequentially adding distilled water until the solution volume reached 50 mL. The OPA solution was stored in a dark bottle in a refrigerator.

Before measurement, the 2-mercaptoethanol solution and the OPA solution were mixed in a ratio of 1: 21.27 (v/v). Then, 0.4 mL of a gluten solution and 8 mL of the mixture were added to a microtitre plate and their absorbance was read at 340 nm. The results were calculated against a serine standard curve. Four replicates were made for each determination.

2.6. Free sulphydryl (SH) determination

Free SH groups were determined according to the procedure of Veraverbeke, Larroque, Békés, and Delcour (2000), with minor modifications. Free SH groups were determined spectrophotometrically after reaction with 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB). One millilitre of a gluten solution was shaken for 10 min in 1.0 mL of the sample buffer composed of 0.05 M sodium phosphate buffer, pH 6.5, containing 2.0%, v/v, SDS, 3.0 M urea and 1.0 mM tetrasodium ethylene diamine tetra-acetate. One hundred microlitres of DTNB reagent (0.1%, w/v, DTNB in the sample buffer) was mixed with the samples and the absorbance at 412 nm was read after 45 min. The absorbances were converted into amounts of free SH using a calibration curve with reduced glutathione (0–0.10 μmol). Four replicates were carried out for each determination. All reagents were of AR grade.

2.7. Atomic force microscopy (AFM)

A gluten protein solution with a concentration of 1 μg/mL was prepared using 0.5 M acetic acid. A 10-μL drop of solution was deposited on a freshly cleaved mica substrate. The substrate was placed in a controlled environment and the solvent was allowed to evaporate. In the controlled environment, the evaporation rate and thus the deposition rate of the protein onto the substrate were adjusted by changing the environmental conditions (atmospheric concentration of the solvent and the temperature).

AFM images were generated using a Nanoscope IIIa AFM (Digital Instruments, Veeco Metrology Group, Santa Barbara, CA) in noncontact mode. The microscope was operated in tapping mode in air using standard tapping-mode silicon cantilevers with a spring constant of 20–80 Nm^{-1}.

2.8. Statistical analysis

All data obtained in this study were analysed statistically. The results were expressed as the mean ± standard deviation. Differences among average values were estimated by analysis of variance with the application of Duncan's tests using SPSS 16.0. Average values were considered significantly different when $p \leq 0.05$.

3. Results and discussion

3.1. Molecular weight distribution determined by UV-detector

Once the d*n*/d*c* values were established for the solvent system, gluten proteins dissolved in the 0.5 M acetic acid presented quite different molecular weights; thus, an accurate and efficient fractionation of the fractions should be performed. Fig. 1 shows the changes in the SEC elution profile for gluten proteins with different storage times.

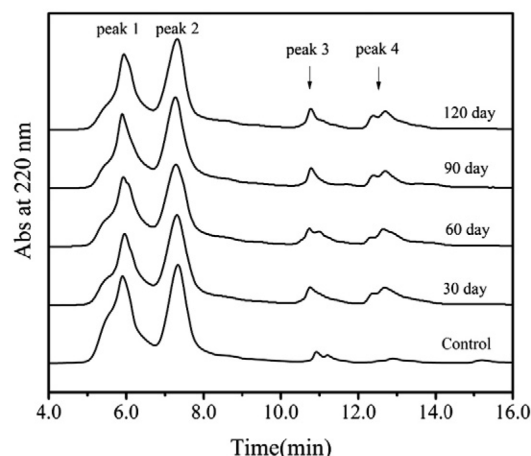

Fig. 1. SEC profiles showing changes in gluten protein after freeze–thaw storage for different times.

The profile indicates that the control gluten polymers are composed of three fractions, where the elution time ranges are 4.8–6.6 min (peak 1), 6.7–8.3 min (peak 2) and 10.4–11.4 min (peak 3). Area percentage refers to the percentage of the gluten polymer component in the UV chromatogram. The data in Fig. 1 clearly show that the peak area of the HMW fractions (peak 1) is the highest, reaching up to 49.57%.

As the storage time increased, the retention time of peak 1 and peak 2 of the frozen stored samples was negatively delayed compared with that of the control gluten, implying that the M_W represented by peak 1 and peak 2 decreased. Moreover, their areas decreased with storage time. After 120 days of frozen storage, the areas of peak 1 and peak 2 decreased to 37.83% and 39.93%, respectively. Evidently, the loss of protein was attributed to the depolymerisation of gluten polymeric proteins, possibly due to ice recrystallisation and water redistribution during frozen storage (Selomulyo & Zhou, 2007) or to enzymatic hydrolysis (Kawamura & Yonezawa, 1982; Redman, 1971; Wadhawan, 1988). However, there was little change in the retention time of peak 3 and its area increased with frozen storage time. For all the frozen stored samples, there was a new absorption peak whose elution time ranged from 12.0 min to 14.0 min (peak 4). The area of peak 4 increased with the frozen storage time. This indicates that there were gluten proteins with LMW in the elution solution, and their concentration increased with storage time. The increase in the number of gluten proteins with LMW is evidence of the depolymerisation of the gluten polymeric proteins during frozen storage. The damage to the gluten polymers increased with the storage time, i.e., the area of peak 4 for the sample stored for 120 days was higher than that of the others.

3.2. Effect of storage time on molecular weight

Fig. 2 shows the effect of the storage time on the M_W of the wheat gluten proteins. As shown in Fig. 2, the M_W of the gluten polymer is very broad (10^5–10^9 Da). It is noted that after 7.2 min of elution time the M_W of all of the samples increased sharply. This phenomenon contradicts the theory of SEC–MALLS, which holds that the M_W of proteins in elution decreases with increasing elution time. Bean and Lookhart (2001) reported that trace amounts of albumins and globulins with LMW were found in the gluten protein extract, which were eluted with LMW gluten proteins of similar M_W. The presence of albumins and globulins changed the measured value of d*n*/d*c*, leading to changes in the M_W and the abnormal upward

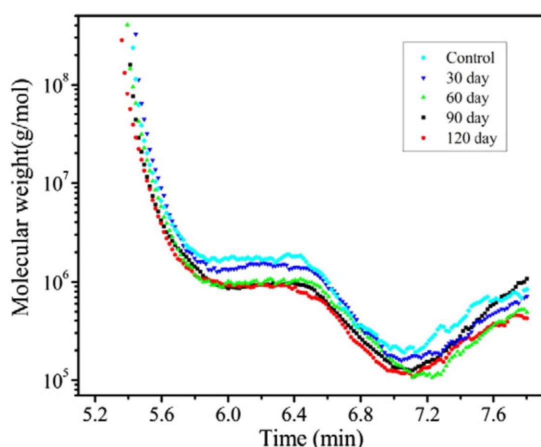

Fig. 2. Effect of freeze–thaw storage time on the molecular weight of wheat gluten protein.

swing in the molecular weight curves. However, the gluten protein extract undoubtedly contains the albumins and globulins; thus, an advanced purification technique is required to completely remove them.

The sizes of the gluten proteins were determined qualitatively by a UV detector as shown in Fig. 1, while their molecular weights were measured quantitatively by a refractive index detector as shown in Fig. 2. The M_W at an elution time of 5.3–7.2 min in Fig. 2 corresponds to peak 1 and 2 in Fig. 1. It is clear that peaks 1 and 2 fractions consisted of HMW polymers whose molecular weights ranged from approximately 10^5 Da to 10^9 Da. The sizes of peaks 3 and 4, which were composed of LMW polymers, cannot be determined accurately and are not illustrated in Fig. 2 due to the limitation of the MALLS detector. However, the baking quality of wheat flour is closely related to the molecular weight and size distribution and content of glutenin (Mendichi et al., 2008); therefore, knowing the HMW fraction (peaks 1 and 2) is quite adequate for understanding dough performance.

As shown in Fig. 2, with increasing storage time, the M_W of the gluten protein polymers clearly decreased. The M_W of the sample after 30 days of storage was slightly lower than that of the control, while the molecular weights after 60-, 90- and 120-day storage were significantly lower than the M_W of the control. Moreover, the M_W of the 60-day storage sample was similar to that of the 90-day storage sample, and the M_W of the sample stored for 120 days was the lowest; the M_W of this sample ranged from 3×10^5 Da to 4×10^8 Da. This implies that some depolymerisation of the gluten occurred during frozen storage. Kennedy (2000) analysed the protein structure of freeze-thawed dough and found a considerable increase in the number of LMW oligomers, which presumably arose from the depolymerisation of glutenin. The changes were particularly noticeable after several freeze–thaw cycles. Ribotta et al. (2001) measured the change in gluten proteins after storage at −18 °C for 120 days using the SDS gel electrophoresis technique and found that there was a decrease in the amount of glutenin subunits with HMWs between 88,700 Da and 129,100 Da, which were much lower than the M_W after 120 days of freeze–thaw storage, as described in our experiments. This was due to the difference in the method used to determine M_W. The SDS gel electrophoresis technique measures the size of glutenin subunits through the addition of mercaptoethanol. However, the conclusion of the researchers agrees with ours in that the HMW gluten polymer was depolymerised with the frozen storage. Additionally, Fig. 2 shows that between 5.9 and 6.3 min the M_W of the gluten polymers did not change with the elution time. This indicates that the molecular weight of these fractions was homogeneous.

3.3. Effect of storage time on free amino groups

Results from Fig. 1 and Fig. 2 indicate that depolymerisation occurs during freeze–thaw storage. The depolymerisation of gluten possibly originates from the breakage of bonds within gluten, or proteolysis. Although there have been a limited number of reports on the proteolytic enzymes associated with industrially produced vital wheat gluten (Bleukx, Roels, & Delcour, 1997), haemoglobinase and azocaseinase activities have been observed in vital wheat gluten (Wadhawan, 1988). Redman (1971) found that 90% of gluten hydrolysing activity was effective when extracted with 0.1 M NaCl. Kawamura and Yonezawa (1982) also suggested that gluten extracted with 0.2 M acetic acid retained 85% of haemoglobinase and 40% of N-carbobenzoxy-L-phenylalanyl-alanine hydrolase activity. These enzymes hydrolyse the gluten proteins during storage, resulting in an increase in the free amino group content. By monitoring the change in free amino group contents, the effect of the hydrolysis was determined.

In our experiment, to avoid the effect of the proteolytic enzymes, during the preparation of the gluten solution, the temperature was controlled to be less than 5 °C, and all samples were stored at −18 °C. Fig. 3 shows the effect of freeze–thaw storage time on the free amino group content of the wheat gluten. There was no significant difference ($p > 0.05$) in the amount of free amino groups among samples stored from 0 days to 120 days. The results of this study confirm that the observed depolymerisation is due to freeze-thawing rather than the hydrolysis caused by proteolytic enzymes.

3.4. Effect of storage time on SH

It is commonly known that there is a strong relationship between the structure of gluten and the disulphide bond content. The structure of glutenin is composed of disulphide-linked polymers of discrete polypeptides, while gliadin is a complex mixture composed largely of single-chain polypeptides bound by intramolecular disulphide bonds (Wrigley, 1996). Disulphide bonds play an important role in maintaining the structure of gluten.

Fig. 4 shows the effect of freeze–thaw storage time on the free SH group content of wheat gluten. The content of free SH groups increased with the frozen storage time; in other words, the number of disulphide bonds was reduced with storage time. After 30 days of freeze–thaw storage, the content of free SH groups increased slightly compared with that of the control gluten, from 9.86 μmol/g (for the control) to 10.22 μmol/g (for the samples stored for 30 days).

The results shown in Fig. 3 show that the slight increase in the molecular weight of gluten after 30 days of freeze–thaw storage is consistent with the slight change in the free SH content after 30 days

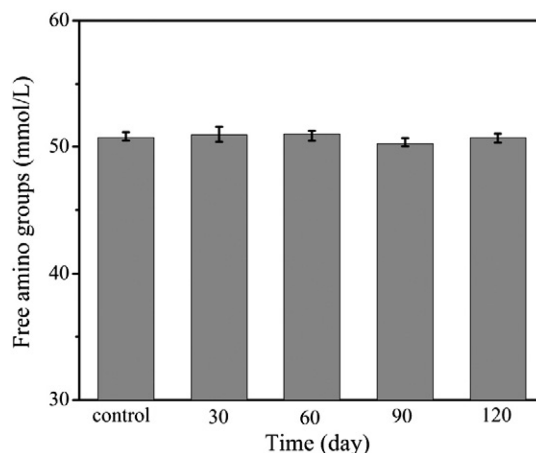

Fig. 3. Effect of freeze–thaw storage time on the free amino group content of wheat gluten.

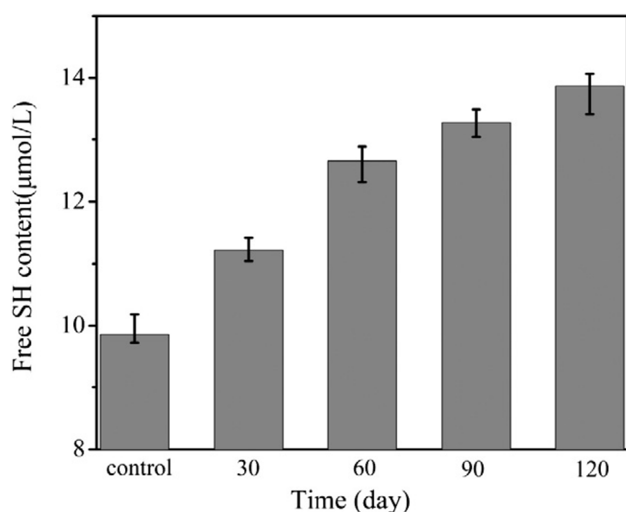

Fig. 4. Effect of freeze–thaw storage time on SH content of wheat gluten.

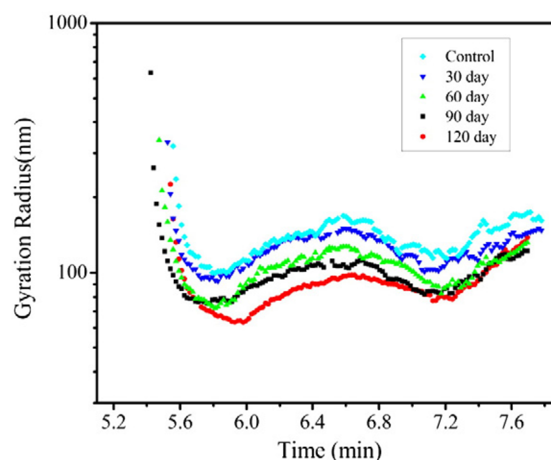

Fig. 5. Effect of freeze–thaw storage time on the radius of gyration of the wheat gluten solution.

of storage. This indicates that the short freeze–thaw storage time had small effects on the free SH content and molecular weight and thus on the quality of the dough. There was a significant difference in the free SH content between 30 days and 60 days of storage ($p < 0.05$); correspondingly, the molecular weights for the samples stored for 30 days and 60 days were significantly different (shown in Fig. 2). Similarly, the free SH content between 60 days and 90 days of storage is remarkably different ($p < 0.05$), however, the molecular weights of the samples stored for 60 days and 90 days were similar (shown in Fig. 2). This could be attributed to the increase in the breakage of intramolecular disulphide bonds, which would not lead to the depolymerisation of gluten; thus, the decrease in molecular weight was not obvious. During freeze–thaw cycles, the breakage of disulphide bonds is caused by the formation of ice crystals, which are represented by angular voids in the micrographs (Zounis, Quail, Wootton, & Dickson, 2002). The pressure induced by the formation of ice or water migration leads to the breaking of disulphide bonds and, furthermore, to the depolymerisation of gluten protein polymers. The breaking of disulphide bonds has also been observed during the mixing of dough, where the mechanical agitation disrupts the disulphide bonds in the glutenin macropolymers, which aggregate through the weak linkages between glutenin subunits, resulting in the liberation of oligomers and dimers of a defined composition. Some low-molecular-weight (especially type B) and *x*-HMW glutenins can be depolymerised if the doughs are extensively mixed (Lindsay & Skerritt, 1999; Weegels, Hamer, & Schofield, 1997; Weegels, VandePijpekamp, Graveland, Hamer, & Schofield, 1996).

3.5. Effect of storage time on radius of gyration (R_g)

Fig. 5 shows the change in R_g with freeze–thaw storage time. Only the data before an elution time of 7.8 min are illustrated. This is because the size of the fraction after an elution time of 7.8 min was not accurately obtained, limited by the lower limit of the MALLS detector (approximately 10 nm). Fig. 5 shows that the R_g of the freeze–thaw stored samples was lower than that of the control gluten. Additionally, the R_g of the gluten samples decreased with freeze–thaw storage time. The decrease in R_g is related to the breakage of disulphide bonds, which results in the disruption of part of the branched chains and a decrease in molecular weight.

3.6. Effect of storage time on molecular chain by NCAFM

AFM is a powerful tool that can provide topographic images of proteins in ambient atmosphere. The deposition of gluten on a mica substrate by the evaporation of a 0.01 μg/mL solution in 0.5 M acetic acid revealed a structure composed of an interlinked network (Fig. 6a) with inhomogeneous meshes measuring 60–100 nm in average diameter. The network is interlaced by fibril-like chains represented by the bright regions in Fig. 6a. McIntire and Brant (1997) and Humphris et al. (2000) reported the observation of apparent fibril networks formed by HMW glutenin subunits by AFM. The difference observed may be because the network composed of glutenin subunits was less complex than that composed of gluten molecules and because a higher magnification factor (1 μm × 1 μm scan) was employed in their experiments. As shown in Fig. 6a, some regions in the network were discontinuous, showing some branches. The cross-section of a network (look at the arrow in Fig. 6a) indicates that the protein chain had a characteristic minimum width of ~12 nm and minimum height of ~2.5 nm. The height was calculated by defining the lowest site in the cross-section as the base level because of the rough surface of the mica substrate. The calculated minimum height was a little higher than the diameter of an individual glutenin subunit (1.5–2.0 nm) (Miles et al., 1991). The gluten protein is mostly composed of disulphide-linked glutenin proteins rich in cysteine, which play an important role in disulphide bond formation. Interactions between intermolecular disulphide bonds and hydrogen bonding between the glutamine side chains and the amide groups in the peptide backbones of adjacent polypeptide chains occur, resulting in an interlinked branched network. The gluten proteins contain considerable amounts of glutamine and proline residues, which contribute to form intra- or intermolecular hydrogen bonds. At the same time, glutamine and proline residues favour interactions with water, especially through the hydrogen bonding with the glutamine side chains, which, in the absence of substrate interactions, will maximise H-bonding through intra- or intermolecular interactions (Humphris et al., 2000). Moreover, Fourier transform infrared studies of HMW subunits have shown that the content of β-sheet structures increases with increasing protein concentration, indicating that intermolecular hydrogen bonding can stabilise the fibrillar structures formed (Belton, Colquhoun, & Grant, 1995). A comparison with the fibrillar structure formed by the HMW subunit *1Dx5* (McIntire et al., 2005) shows that the branched network in Fig. 6a has inhomogeneous chain widths and more branches. This may be due to the presence of non-repetitive N- and C-terminal domains, amide groups from the gliadin connected to the glutamine residue from glutenin by hydrogen bonds.

Fig. 6b–e shows AFM images and cross-sections of gluten samples after freeze–thaw storage. The width and height of the gluten chains illustrated in cross-section in Fig. 6b and c are clearly lower than those in Fig. 6a, the minimum width being <10 nm and the minimum height being <2 nm (look at the arrows in Fig. 6b and c). Moreover,

the networks shown in Fig. 6b and c display less continuity than the network shown in Fig. 6a. This discontinuity was further exacerbated, as shown in Fig. 6d and e, but the width and height of gluten proteins after storage for 90 days and 120 days clearly increased. The minimum width of the gluten chains was measured to be approximately 10 nm. Intra- or intermolecular disulphide bonds or hydrogen bonds are the stabilising force for the interlinked network. However, due to the breakage of disulphide bonds during freeze–thaw storage, the gluten proteins were depolymerised and the molecular weight of gluten decreased. The branched network formed by gluten proteins after freeze–thaw storage was weakened compared with that formed by the control gluten proteins. After 90 and 120 days of storage, the weakening effect on the network was enhanced.

Bright spots can be clearly observed in Fig. 6b–e and are presumably the result of the aggregation of gluten chains. The size of aggregates increased with storage time. The diameter of the bright spot for gluten after 30 days of freeze–thaw storage was approximately 50–80 nm, while the diameters of the sample stored for 60 and 120 days were 120 nm and 160 nm, respectively. The brighter spots in the AFM images of Fig. 6d and e corresponding to the wider and higher gluten chains in the cross-section are likely due to the aggregation of several molecular chains or the folding of the molecule back onto itself. This observation

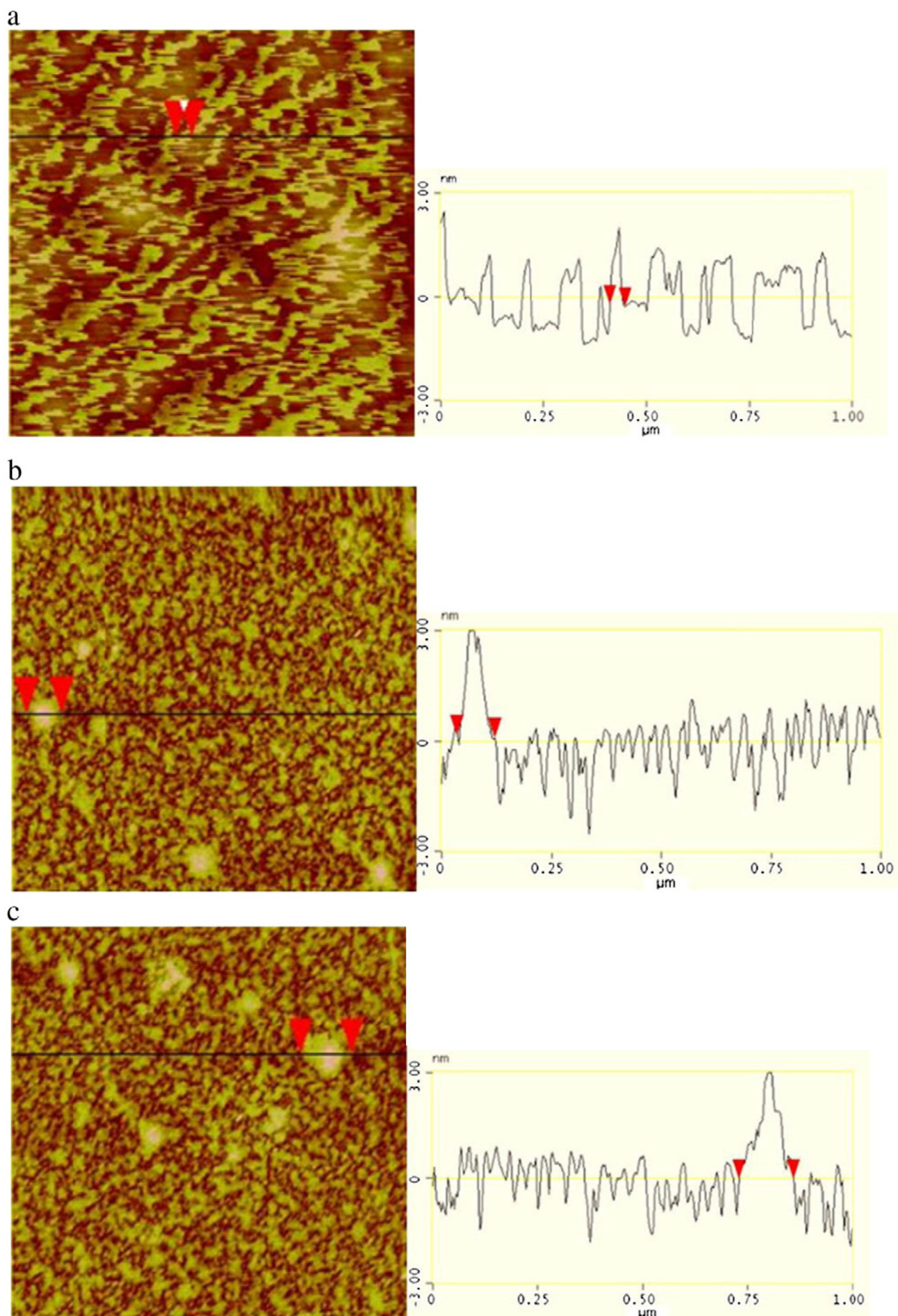

Fig. 6. Tapping-mode AFM images and cross-section of a network of gluten proteins. (a) Control gluten; (b) 30 day freeze–thaw sample; (c) 60 day freeze–thaw sample; (d) 90 day freeze–thaw sample; (e) 120 day freeze–thaw sample (scan size, 1 μm × 1 μm).

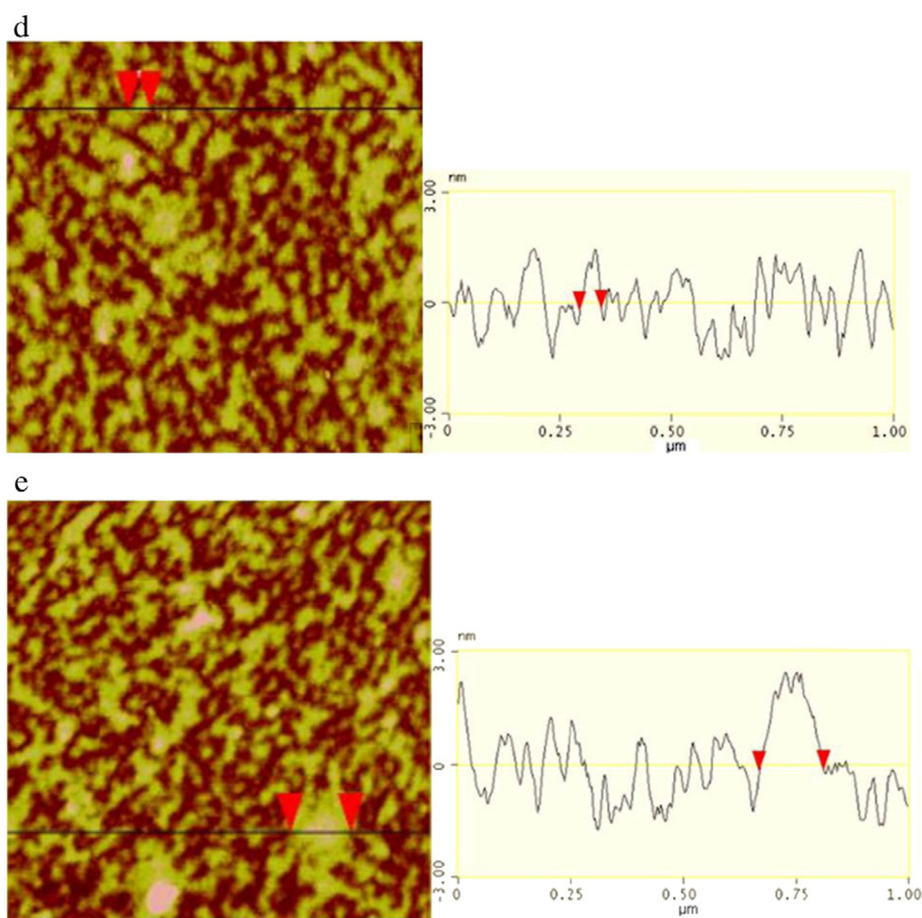

Fig. 6 (*continued*).

indirectly reflected the depolymerisation of gluten and the degree of depolymerisation during freeze–thaw storage. During preparation of the samples for AFM observation, gluten samples with higher degree of depolymerisation and having lower molecular weight and higher flexibility demonstrated higher tendency to aggregate in acetic acid, resulting in the bright areas in AFM photos. With increasing storage time, more disulphide bonds were disrupted and the M_W of gluten was reduced. After 90 and 120 days storage, the gluten molecules became smaller and were more flexible in acetic acid; this flexibility probably made the molecule fold more easily onto itself, and the smaller gluten molecules aggregated more easily.

4. Conclusions

The effects of freeze–thaw cycles on the molecular weight, radius of gyration, free amino group content, free sulphydryl group content and molecular conformation of gluten were investigated by SEC–MALLS, spectrophotometry and AFM. The SEC results show that the retention time of the gluten polymer peak during freeze–thaw storage is gradually delayed with increasing storage time. The molecular weight (ranged from 3×10^5 Da to 4×10^8 Da.) and radius of gyration of gluten decreased with increasing storage time under freeze–thaw conditions. Because of the water redistribution, ice recrystallization, and the mechanical agitation during water turning in to ice in freeze–thaw cycles, the M_W of the gluten decreased due to the break of the intermolecular disulphide bonds between the gluten polymers. The NCAFM results provide direct visual evidence of the gluten chains forming a fibril-like network and which is weakened during freeze–thaw storage. Description of the microstructure of molecular chain revealed that the change of the M_W affects the molecular chain structure. The lower molecular weight of the gluten has the higher flexibility which demonstrated higher tendency to aggregate in acetic acid. This research provides the general information on the molecule basis during the freeze–thaw cycles storage which may relate to their role in food frozen processing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.foodres.2013.04.013.

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