Food Quality and Safety, 2023, **7**, 1–10
https://doi.org/10.1093/fqsafe/fyad019
Advance access publication 23 March 2023
Article

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Effects of HMW-GS Dx2 absence on the protein aggregation characteristics and thermal stability of wheat flour during maturation

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Abstract

Background: Wheat flour maturation affects the aggregation and structural stability of proteins. The number of high-molecular-weight glutenin subunits (HMW-GSs) differs in various wheat varieties. The effects of Dx2 absence on the protein aggregation characteristics and thermal stability of flour were investigated during 120 d of maturation using near-isogenic lines (NILs).

Results: The absence of Dx2 delayed and decreased the protein aggregation of flours during maturation, i.e. the maturation-induced increases were later and smaller for glutenin, glutenin macropolymer (GMP), glutenin/gliadin ratio, β-sheets, and β-sheet/α-helix ratio in HMW-D1a without Dx2 than in HMW-D1p with Dx2; these differences were ascribed to the weaker interactions between the sulfhydryl (-SH) groups, disulfide bonds (-S-S-), and hydrophobicity in the flours without Dx2. Flour maturation caused the dough microstructures to be more compact and denser, thereby increasing the flour thermal stability as observed by a higher denaturation peak temperature (T_p), enthalpy of thermal transition (ΔH), and degradation temperature (T_d), These changes led to better dough properties such as dough development time, dough stability time, and protein weakening, but the optimal stage in HMW-D1a without Dx2 was reached later.

Conclusion: These findings deepen the understanding of how HMW-GS Dx2 modifies protein structures during flour maturation.

Keywords: Dx2 absence; disulfide bond; glutenin macropolymer; secondary and micro structures; thermal stability; wheat flour maturation.

Introduction

Products prepared from freshly milled wheat flour usually have a sticky mouth feel and poor textural quality and require a maturation period to reach the optimal processing quality (Wang and Flores, 1999). Steamed bread prepared from flour stored for 40–50 d was shown to have the highest quality (Xu *et al.*, 2015). However, prolonged storage causes adverse effects on the processing quality and gluten quality of flour (Varzakas, 2016; Pooja *et al.*, 2018). Therefore, the maturation of wheat flour is important for improvement in the gluten network and rheological properties of dough, thereby improving product quality.

In the process of wheat flour maturation, a number of physical, chemical, and physiological changes occur until the final technological maturity is reached (Pyler, 1973), fostered by flour quality characteristics and storage conditions. Postmilling maturation influenced the contents of tocopherols, carotenoids (Hidalgo and Brandolini, 2008; Hidalgo *et al.*, 2009), and free amino groups of the flours (Hajnal *et al.*, 2014), thereby influencing their nutritional values. Examination during storage of high- and low-protein flour at 25 °C revealed that the free fatty acid content and pasting properties increased, and the content of sulfhydryl groups (-SH) was reduced, likely forming more extensive gluten (Fierens *et al.*, 2015). When wheat flour was stored at 28 °C and 68%–70% environmental humidity for 21 d, the water absorption and stability time of dough were increased to some extent (Temea *et al.*, 2016). In addition to the storage conditions, wheat variety is reportedly the strongest factor determining flour maturation. Compared with strong gluten wheat flour, the viscoelastic properties of weak gluten flour improved more markedly during short-term storage (Hadnađev *et al.*, 2015; Zhang *et al.*, 2021), and weaker flours required a longer time to reach the optimal quality characteristics (Pyler, 1973). However, a study by Wang *et al.* (2019) found complex changes using freshly milled wheat flour with high, medium, and low gluten during 90 d of maturation. The difference may be due to the diversity of wheat genotypes. Many wheat varieties have been used to study the effect of maturation on flour quality, but it is difficult to identify the role of gluten proteins during flour maturation due to the inconsistencies in genetic background between the varieties. Therefore, it is necessary to explore the effect of high-molecular-weight glutenin subunits (HMW-GSs) variations

Received 25 November 2022; Revised 13 March 2023; Editorial decision 16 March 2023

on protein polymerization during wheat flour maturation using near-isogenic lines (NILs).

Glutenin and gliadin are major components of wheat flour proteins, which govern its unique dough viscoelastic properties (Shewry *et al.*, 2003). Glutenin is subdivided into HMW-GSs and low-molecular-weight glutenin subunits (LMW-GSs) formed through inter- and intramolecular disulfide and hydrogen bonds (Lindsay and Skerritt, 1999). Although HMW-GS only accounts for 20% of whole glutenin, it makes major contributions to the gluten strength and elastic properties of dough (Shewry *et al.*, 2003). Several studies have reported that variations in the type and amount of HMW-GSs influence the polymerization of glutenin, thereby influencing dough properties (Liu *et al.*, 2016; Gao *et al.*, 2018). The absence of individual Ax1 or Dx2 subunits delayed and decreased the polymerization of glutenin during grain development and weakened the microstructure of gluten (Gao *et al.*, 2018). However, HMW-GS influencing protein aggregation during flour maturation is rarely reported, especially HMW-GS absence at the *Glu-D1* locus.

The glutenin macropolymer (GMP) content and size distribution are positively correlated with dough properties and bread loaf volumes (Weegels *et al.*, 1997; Lindsay and Skerritt, 1999). The changes in the quantity of GMP may be attributed to chemical breakdown and the rearrangement of gluten structures (Graveland *et al.*, 1985). The disulfide bonds (-S-S-) are responsible for the formation of the gluten network structure and are a key determinant of the rheological and baking properties of dough and flour (Delcour *et al.*, 2012). The oxidation of -SH to S–S bonds leads to protein aggregation (Bruneel *et al.*, 2011). A previous study found that the content of glutenin increased during flour maturation, accompanied by a decrease in gliadin and -SH (Rao *et al.*, 1978). Wang *et al.* (2014) and Zhang *et al.* (2021) found that the contents of glutenin macropolymers and disulfide bonds in freshly milled flour increased with progressive flour maturation in weak-, middle-, and strong-gluten wheat, thereby improving dough quality (Wang *et al.*, 2019). However, little information regarding the influence of the absence of a single Dx2 at the *Glu-D1* locus on the structural and thermal properties of flour during maturation is available.

The GMP, -SH and S–S bonds, surface hydrophobicity, and secondary structure of flour protein are frequently chosen as indicators reflecting the aggregation characteristics of protein and gluten quality (Wang et al., 2014, 2019). In this study, we determined the changes in GMP, free -SH and S–S bonds, surface hydrophobicity, protein secondary and micro structures, and thermal properties of flour during maturation, using two NILs with Dx2 absence/presence at the *Glu-D1* locus. The aims of this study were to investigate the effect of the absence of Dx2 on flour protein aggregation and thermal stability during flour maturation and to examine the underlying mechanism by which the absence of Dx2 changes the protein structure during maturation.

Materials and Methods

Materials and experimental design

Two wheat lines (HMW-D1a and HMW-D1p) were selected from HMW-GS NILs of Xinong 2208 with medium gluten strength in this study. A recent report confirmed that the two NILs only differ at the *Glu-D1* locus in terms of the absence/presence of HMW-GS Dx2 (Gao *et al.*, 2018). The HMW-D1a contains Ax1, Bx7, By9, and Dy12 subunits, while the HMW-D1p contains Ax1, Bx7, By9, Dx2, and Dy12 subunits. Our previous studies have suggested that HMW-D1p yields stronger dough and a better gluten network than HMW-D1a ascribed to the presence of Ax1 (Gao *et al.*, 2018; Song *et al.*, 2020).

HMW-D1a and HMW-D1p were planted individually in Yangling (108°40′E, 34°160′N), Shaanxi Province, China, during the 2019–2020 growing season. After harvest, the mature grains were sun-dried and tempered at 15% (mass fraction) for 20 h. Subsequently, the grains (moisture: 9.43% for HMW-D1a and 9.60% for HMW-D1p; Supplementary Table S1) were immediately ground using a Y400 multifunctional disintegrator (Laobenxing, Jinhua, China) and screened through an 80-mesh sieve. The fresh flour (6 kg) of HMW-D1a and HMW-D1p was evenly placed in three individual craft paper bags of uniform size to prevent internal heat dissipation. These bags were stored in a constant temperature and humidity incubator at 28 °C and 50% RH. The manually mixed samples were collected at 0, 30, 60, 90, and 120 d and then stored at 4 °C until further analysis concerning flour quality.

Extraction of flour protein fractions

Extraction of four compositions of flour was performed according to the method of Song *et al.* (2020). Samples of 0.05 g were suspended in 1 mL of 1.5% SDS solution and centrifuged at 18 100×*g* for 15 min at 20 °C to extract the GMP (Weegels *et al.*, 1997). The total protein content and composition were determined using the Kjeldahl method. All analyses were repeated in triplicate independently.

Determination of sulfhydryl group (-SH) and disulfide bond (-S-S-) contents

The -SH and -S-S- contents were determined according to the method described by Guo *et al.* (2018) with slight modifications. Flour (0.15 g) was dispersed in 8 mL Tris–Gly buffer (pH 8.0, consisting of 86 mmol/L Tris, 92 mmol/L Gly, 4 mmol/L EDTA, 8 mol/L urea, and 1% SDS), shaken and extracted for 1 h at room temperature. The suspension was centrifuged at 13 600×*g* for 10 min to remove the particulate and the supernatant was collected for further measurement of -SH and -S-S-. The following steps are consistent with Guo *et al.* (2018). Each sample was analysed in triplicate.

Determination of surface hydrophobicity

The surface hydrophobicity of flour was measured using an external fluorescence probe (8-anilinonaphthalene-1-sulfonic acid, ANS) according to the method of Wang *et al.* (2018) with some modifications. Flour samples (0.125 g) were dissolved in 30 mL of 50 mmol/L acetic acid solution and agitated at room temperature for 60 min before centrifugation at 10 000×*g* for 15 min. The supernatant was diluted to different concentrations (μg/mL). Next, 25 μL of ANS solution (pH 5.8, 8 mmol/L in 0.1 mol/L phosphate buffer) was added to 4 mL of extraction solution and incubated for 15 min in the dark. The fluorescence intensity was determined using a fluorescence spectrometer (LS 55, PerkinElmer, Waltham, MA, USA) at an excitation wavelength of 390 nm and an emission wavelength of 470 nm, with a slit width of 5 nm. Each sample was analysed in triplicate.

Secondary structure analysis using Fourier transform infrared (FTIR) spectroscopy

Infrared spectra of flour samples were recorded at 25 °C between 4000 cm^{-1} and 400 cm^{-1} using an FTIR spectrometer (Vertex 70, Bruker, Ettlingen, Germany) with a spectral resolution of 4 cm^{-1}, as described by Liu *et al.* (2016). The spectra were analysed using Omnic (version 8.0, Thermo Nicolet Inc., Waltham, MA, USA) and Peakfit (version 4.04, SPSS Inc., Chicago, IL, USA). The main structures and spectral band ranges were fitted as described previously (Georget and Belton, 2006; Zhu *et al.*, 2019), where they comprised intermolecular β-sheets at 1612–1620 cm^{-1}, β-sheets at 1625–1642 cm^{-1}, α-helices at 1650–1660 cm^{-1}, β-turns at 1660–1670 cm^{-1}, and antiparallel β-sheets at 1675–1695 cm^{-1}. Each sample was tested in triplicate.

Microstructural analysis of dough

Freshly prepared dough samples were used to quantify the microstructure of the dough by confocal laser scanning microscopy (CLSM) according to a previous method (Gao *et al.*, 2018). In brief, flour weighing 10 g was mixed with 4 mL deionized water and 1 mL of 0.1 g/L rhodamine B solution for 10 min. The dough samples were transferred to an object carrier and sealed with a cover glass before analyses using a CLSM system (Olympus, Tokyo, Japan). CLSM images were analysed using AngioTool64 (version 0.6a, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA) according to the method described by Gao *et al.* (2018).

Measurement of thermal properties

The thermal properties of flours were analysed with a thermal analysis instrument (STA7200RV, Hitachi, Tokyo, Japan) according to described methods (Zhu *et al.*, 2019). Briefly, each flour sample weighing 9 mg was placed in an aluminium pan and heated from 25 to 600 °C at a heating rate of 20 °C/min with nitrogen gas at a flow rate of 80 mL/min. The thermal curve was further analysed using TA7000 (version 10.41, Hitachi, Tokyo, Japan) provided with the instrument to quantify thermal properties comprising denaturation peak temperature (T_p), enthalpy of thermal transition (ΔH), degradation temperature (T_d), and weight loss. Each sample was tested in triplicate.

Dough mixing properties

The mixing properties of dough were analysed by using the Mixolab system (Chopin, Tripette and Renaud, France) according to the method described by Dhaka *et al.* (2012). The parameters recorded by the Mixolab system comprised (1) dough development time (min); (2) dough stability time (min); and (3) protein weakening based on mechanical mixing and temperature increase, C2 (Nm). Each sample was tested in triplicate.

Statistical analysis

Statistical analyses of the results were conducted using SPSS (version 22.0, SPSS Inc., Chicago, IL, USA). All of the data were subjected to single-factor analysis of variance and significant differences ($P<0.05$) among the parameters considered were tested using Duncan's multiple range test. Figures were generated using SigmaPlot 12.5 and Adobe Photoshop 7.0.

Results and Discussion

Dynamic changes in the protein fractions during flour maturation

As shown in Table 1, in the process of flour maturation, the total protein content exhibited no significant changes in HMW-D1a and HMW-D1p flour, while albumin and globulin contents showed a decreasing trend. There was a rapid increase in the contents of glutenin and GMP and the glutenin/gliadin ratio from 0 to 60 d for HMW-D1p and from 0 to 90 d for HMW-D1a, but the increase rates were higher in HMW-D1p than HMW-D1a. Changes in gliadin content in HMW-D1a and HMW-D1p were opposite to those in glutenin, ratio of glutenin to gliadin, and GMP.

Several studies found that protein aggregation in flours with different gluten strengths had various maturation patterns during flour maturation (Hadnađev *et al.*, 2015; Wang *et al.*, 2019). The absence of HMW-GS Dx2 decreased the intermolecular disulfide-bonded linkage formed by several

Table 1. The contents of protein fractions and GMP of HMW-D1a and HMW-D1p flour during maturation

Line	Maturation time (d)	Content of protein fraction (%)					Glutenin/gliadin ratio
		Total protein	Albumin and globulin	Gliadin	Glutenin	GMP	
HMW-D1a	0	13.130±0.508a	2.727±0.038a	6.987±0.107a	3.416±0.200 d	1.411±0.138 d	0.489±0.057d
	30	12.920±0.415a	2.658±0.035a	6.418±0.249b	3.844±0.209c	1.797±0.053 c	0.599±0.035c
	60	12.292±0.375a	2.497±0.029b	5.552±0.197c	4.242±0.181b	2.091±0.043a	0.764±0.029b
	90	12.406±0.602a	2.325±0.028c	5.529±0.194c	4.552±0.137a	2.197±0.122a	0.823±0.028a
	120	12.547±0.448a	2.356±0.062c	5.892±0.295c	4.299±0.109b	1.998±0.079b	0.730±0.062b
HMW-D1p	0	14.458±0.412a	2.692±0.133a	7.285±0.214a	4.481±0.178b	1.681±0.093c	0.615±0.033c
	30	14.833±0.695a	2.623±0.057a	6.928±0.038b	5.283±0.234a	2.197±0.114b	0.763±0.036b
	60	14.467±0.521a	2.398±0.040b	6.532±0.153c	5.537±0.406a	2.528±0.138a	0.848±0.024a
	90	14.469±0.665a	2.376±0.031b	6.792±0.108b	5.302±0.321a	2.489±0.085a	0.781±0.041b
	120	14.704±0.489a	2.370±0.075b	7.154±0.097a	5.180±0.133a	2.415±0.121a	0.724±0.048b

Values are expressed as mean±standard deviation ($n=3$). The results followed by a different letter in the same column are significantly different ($P<0.05$).

cysteine residues to reduce and delay the polymerization of glutenin (Shewry *et al.*, 2003), thereby weakening the structural and thermal characteristics of gluten (Gao *et al.*, 2018; Song *et al.*, 2020). In the present study, reaching the maximum glutenin content, glutenin/gliadin ratio, and GMP required a longer time in HMW-D1a than in HMW-D1p, indicating that the absence of Dx2 delayed the aggregation of protein during flour maturation, which was likely explained by the lower cysteine residue numbers of HMW-GS in HMW-D1a without Dx2 (Shewry *et al.*, 2003) and supported previous reports that weaker flour required a longer time to reach optimal quality characteristics (Pyler, 1973; Wang *et al.*, 2019). The change trend of gliadin was opposite to that of glutenin and the glutenin/gliadin ratio, indicating that the exchange of gliadin and glutenin occurs during maturation, which could be largely explained by the oxidation of free -SH to -S-S- between gliadins and glutenins, forming protein polymers with larger molecular weights (Huebner and Bietz, 1993).

Effects of Dx2 absence on the free -SH and -S-S- contents of flour proteins during the maturation

Figure 1 shows the free -SH (Figure 1A) and -S-S- (Figure 1B) concentrations in flours from the two NILs during flour maturation. The free -SH concentration decreased with progressive maturation, whereas the -S-S- concentrations first increased and then decreased. The minimum free -SH and maximum -S-S- concentrations occurred at the maturation stage of 60 d for HMW-D1p and at 90 d for HMW-D1a. Moreover, in the first 60 d, the change rates of free -SH and -S-S- concentrations were greater in HMW-D1p flour than in HMW-D1a flour.

HMW-GS is divided into two classes: x-type and y-type subunits. The x-type subunits generally have four conserved cysteine (Cys) residues, while the y-type subunits usually have seven conserved Cys residues (Shewry *et al.*, 2003). These Cys residues are involved in intermolecular disulfide bonding in the formation of GMPs and play an important role in the function of HMW-GSs (Shewry *et al.*, 2003; Delcour *et al.*, 2012). The present study showed that compared with HMW-D1p, HMW-D1a has fewer S–S bonds, which could be attributed to the absence of cysteine residues from Dx2 in HMW-D1a. Disulfide cross-links and sulfhydryl-disulfide interchange are considered major pathways of gluten protein aggregation (Wagner *et al.*, 2011). The decrease in the -SH group concentration and the increase in the -S-S- concentration in HMW-D1p and HMW-D1a flour indicated that the conversion occurred from -SH to -S-S- during maturation, thereby contributing to protein aggregation manifesting higher GMP contents (Table 1). Similar results have been reported in high-, medium-, and low-gluten wheat flour (Wang *et al.*, 2019; Zhang *et al.*, 2021). Therefore, it can be concluded that the absence of Dx2 decreased the formation of -S-S- during flour maturation, thereby decreasing the degree of protein aggregation.

Effects of Dx2 absence on the surface hydrophobicity of proteins during flour maturation

The changes in the surface hydrophobicity of flour proteins during maturation are shown in Figure 1C. The surface hydrophobicity reached a minimum at 60 d for HMW-D1p and at 90 d for HMW-D1a and subsequently did not significantly change. This change rate was greater in HMW-D1p than in HMW-D1a before reaching the minimum.

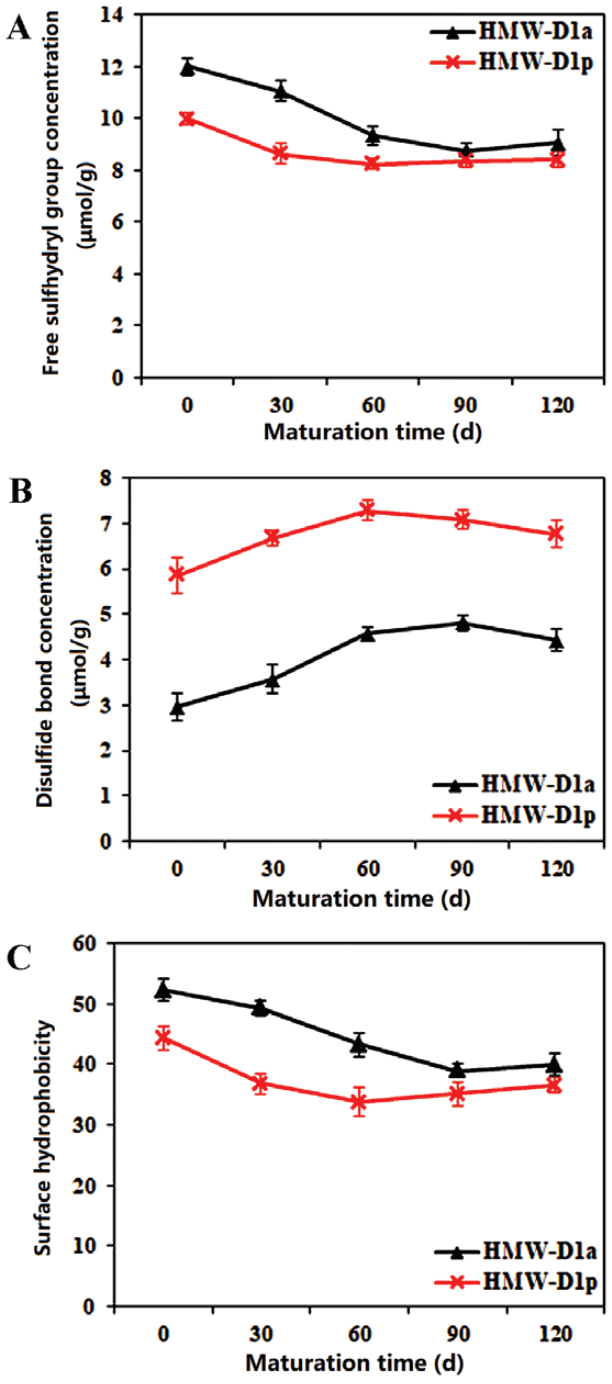

Figure 1. Changes in the sulfhydryl groups (A), disulfide bonds (B) concentrations, and surface hydrophobicity (C) of flour obtained from HMW-D1a and HMW-D1p during maturation.

Hydrophobic interactions are important for maintaining the tertiary conformations of proteins (Shewry *et al.*, 2003). Exposed hydrophobic moieties lead to less thermal stability and a disordered structure (Wang *et al.*, 2014). A decline in surface hydrophobicity indicates that the surface hydrophobic groups were buried (Wang *et al.*, 2018). Our results showed that the surface hydrophobicity of HMW-D1a was higher

than that of HMW-D1p during flour maturation, indicating that HMW-D1p proteins exhibit a more ordered structure and better thermal stability. The surface hydrophobicity decreased with the process of maturation, which suggested that maturation induced the redistribution of noncovalent forces and S–S bonds. Zhu *et al.* (2019) suggested that the compositions of HMW-GS affected the surface hydrophobicity of proteins. Thus, the difference in surface hydrophobicity between HMW-D1a and HMW-D1p was probably correlated with the absence and/or presence of Dx2, and the absence of Dx2 formed an unstable protein structure with more exposed hydrophobic moieties in HMW-D1a during postharvest maturation.

Effects of Dx2 absence on the secondary structure of proteins during flour maturation

The changes in the gluten secondary structure of the two NIL flours during maturation are shown in Table 2. The proportions of antiparallel β-sheets and β-turns hardly changed during flour maturation; however, the fractions of α-helices, β-sheets, intermolecular β-sheets, and the β-sheet/α-helix ratio significantly changed. There was an increase in the contents of β-sheets, intermolecular β-sheets, and the β-sheet/α-helix ratio from 0 to 60 d for HMW-D1p and to 90 d for HMW-D1a, but it exhibited a decline in the content of α-helices. However, with the extension of maturation, for HMW-D1a, the β-sheets and β-sheet/α-helix ratio exhibited a significant decline, but α-helices had a significant increase; for HMW-D1p, there was no significant difference.

The content of β-sheets in HMW-D1p reached a maximum prior to HMW-D1a and was higher than that in HMW-D1a during flour maturation. High β-sheet content indicates a stronger protein network and is positively correlated with the viscoelasticity of dough (Georget and Belton, 2006). Thus, gluten from HMW-D1p is expected to form a stronger network structure earlier than that from HMW-D1a during flour maturation. In contrast, an increase in α-helices induced by maturation enhanced the aggregation of gluten proteins, resulting in a denser and stronger network structure (Zhang *et al.*, 2021). This disagreement was probably attributed to differences among material genotype, sampling time, and processing conditions, which can be verified by further investigation. In addition, the increases in β-sheets and intermolecular β-sheets were at the expense of α-helices during flour maturation, which agreed with the results of a previous report (Wang *et al.*, 2019). An increase in β-sheets was attributed to the effect of covalent and noncovalent bonds between the glutenin molecules (Tuhumury *et al.*, 2014). Therefore, we concluded that flour maturation improved the secondary structure of gluten proteins by inducing changes in disulfide bonds and surface hydrophobicity. Moreover, with excessive prolongation of the maturation period, the proportion of β-sheets and β-sheet/α-helix ratio significantly decreased for HMW-D1a, whereas they exhibited no significant changes for HMW-D1p, suggesting that the absence of Dx2 was more likely to form a weaker network structure of gluten, thereby causing the lower stability of gluten protein in HMW-D1a. Significant correlations have been reported between non-extractable protein polymers with the β-sheet content and the β-sheet/α-helix ratio (Liu *et al.*, 2016). The changes in GMP and secondary structures of gluten proteins during flour maturation agreed with these correlations.

Effects of Dx2 absence on the gluten microstructure during flour maturation

As shown in Figure 2, the gluten network of HMW-D1p exhibited smaller apertures and more cross-linking than that of HMW-D1a at the maturation stage of 0 d. The network became denser with the progress of maturation, which was optimal at 60 d for HMW-D1p and at 90 d for HMW-D1a. In contrast, prolonged maturation resulted in more and larger apertures for HMW-D1a, while it caused a slight change for HMW-D1p. Quantitative analysis of the microstructure of gluten networks is shown in Table 3. The dough from fresh milled flour exhibited higher values in protein area, protein junctions, junction density, total protein length, lacunarity, and branching rate and had lower end-points and end-point rates for HMW-D1p than for HMW-D1a. When the maturation period was 60 d for HMW-D1p and 90 d for HMW-D1a, the protein area reached a maximum, as well as the protein junctions, junction density, total protein length, lacunarity, and branching rate, whereas the end-points and end-point rate were lowest.

Table 2. Secondary structure percentages of HMW-D1a and HMW-D1p flour proteins during maturation

Line	Maturation time (d)	Secondary structure composition (%)					β-sheet/α-helix ratio
		Intermolecular β-sheet	β-sheets	α-helice	β-turn	Antiparallel β-sheet	
HMW-D1a	0	7.33±0.12d	33.32±0.10d	27.82±0.13a	18.94±0.20a	12.60±0.21a	1.12±0.002e
	30	7.59±0.12c	34.05±0.13c	27.35±0.12b	18.87±0.14a	12.14±0.18ab	1.24±0.002d
	60	7.96±0.13b	34.22±0.18c	26.73±0.08c	19.06±0.20a	12.03±0.30b	1.28±0.003c
	90	8.31±0.09a	35.17±0.13a	25.04±0.13e	19.11±0.13a	12.38±0.13ab	1.41±0.002a
	120	8.23±0.14a	34.83±0.11b	25.69±0.10d	19.14±0.11a	12.11±0.24ab	1.36±0.001b
HMW-D1p	0	7.92±0.13d	34.12±0.09d	26.76±0.20a	18.79±0.11a	12.41±0.13a	1.28±0.007d
	30	8.47±0.14c	35.76±0.11c	24.39±0.18b	18.94±0.15a	12.44±0.24a	1.47±0.006c
	60	9.14±0.20a	36.45±0.13a	23.21±0.17d	18.92±0.11a	12.28±0.12a	1.57±0.006a
	90	8.70±0.13b	35.78±0.13bc	23.83±0.11c	19.09±0.13a	12.60±0.20a	1.50±0.002b
	120	8.89±0.13ab	36.01±0.14b	23.96±0.17c	18.88±0.12a	12.27±0.21a	1.50±0.004b

Values are expressed as mean±standard deviation (n=3). The results followed by a different letter in the same column are significantly different (P<0.05).

Relatively high protein area (indicating a low aperture area) and protein junction (related to the junction density and branching rate) values indicate a gluten network with greater connectivity (Bernklau *et al.*, 2016). The lacunarity represents the amounts of gaps and irregularities in a protein network and is positively correlated with dough characteristics, whereas the protein end-points and end-point rate are negatively correlated with those properties (Gao *et al.*, 2018). Therefore, flour maturation made the protein network of the dough more compact, which was consistent with the observation (Figure 2). The changes in the gluten network from HMW-D1p were quicker than those from HMW-D1a before reaching the densest networks, indicating that the absence of Dx2 delayed the formation of the gluten network during flour maturation, which further verified the finding that weak gluten flour required longer to reach optimal quality (Pyler, 1973). Moreover, we also found that the protein network of HMW-D1a dough exhibited more end-points and aperture

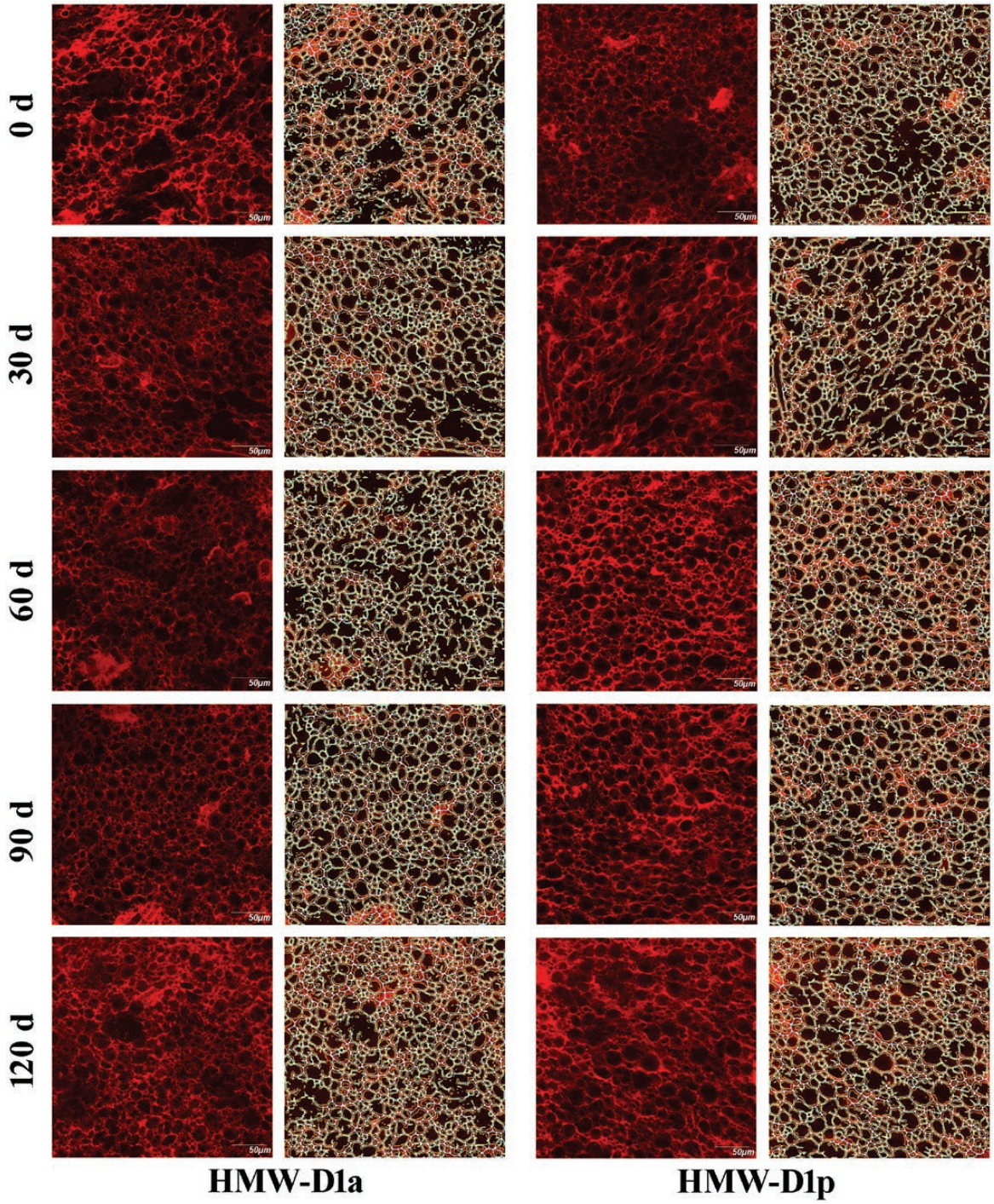

Figure 2. Protein network analysis of dough obtained from HMW-D1a and HMW-D1p flour during storage. The images in the first and third columns are the original CLSM images, and the scale bar represents 50 μm. The images in the second and fourth columns were processed with AngioTool. The junctions are shown in white, the protein skeleton in blue, and the protein outline/area in yellow.

area beyond the flour maturation period (90 d) than HMW-D1p (60 d), suggesting that the gluten structural stability of weaker dough obtained HMW-D1a was readily disturbed, which was consistent with free -SH, and -S-S- contents and hydrophobicity of flour proteins.

Effects of Dx2 absence on the thermal properties of flours during maturation

The thermal analyses of HMW-D1a and HMW-D1p flour during maturation are shown in Figure 3. In the process of maturation, HMW-D1p flour had higher values for

Table 3. Parameters determined by AngioTool for the protein network in dough obtained from HMW-D1a and HMW-D1d flour during maturation

Line	Maturation time (d)	Protein area ($\times10^4$ μm^2)	Protein junction ($\times10^2$)	Junction density ($\times10^{-3}$)	Total protein length ($\times10^3$ μm)	End-point ($\times10^2$)	Lacunarity ($\times10^{-2}$)	Branching rate ($\times10^{-3}$)	End-point rate ($\times10^{-3}$)
HMW-D1a	0	13.42±0.06d	11.60±0.16e	4.46±0.10e	22.65±0.14e	3.94±0.08a	3.66±0.10e	8.64±0.12d	2.93±0.07a
	30	13.88±0.13c	12.08±0.13d	4.83±0.06c	23.64±0.26d	3.74±0.04b	4.14±0.17c	8.67±0.05d	2.68±0.04b
	60	14.17±0.07b	13.47±0.15c	5.24±0.02b	24.17±0.15c	3.31±0.13c	4.49±0.01b	9.51±0.06c	2.34±0.08c
	90	14.62±0.08a	14.53±0.09a	5.59±0.11a	25.85±0.21a	2.93±0.09e	4.76±0.07a	9.93±0.05a	2.01±0.05d
	120	14.54±0.11a	14.12±0.05b	5.45±0.04ab	24.93±0.04b	3.12±0.11d	4.18±0.12c	9.71±0.03b	2.01±0.08d
HMW-D1p	0	13.53±0.12d	12.25±0.09d	4.53±0.10c	23.28±0.20e	2.54±0.02a	4.63±0.04d	9.05±0.03c	1.88±0.03a
	30	13.86±0.16c	13.37±0.11c	5.12±0.11b	24.06±0.11d	2.12±0.02b	4.993±0.13c	9.65±0.10b	1.53±0.01b
	60	14.67±0.03a	14.59±0.09a	5.52±0.16a	25.59±0.24a	1.99±0.02c	5.58±0.02a	9.95±0.08a	1.36±0.03d
	90	14.34±0.10b	14.19±0.11b	5.40±0.06a	25.11±0.17b	2.12±0.07b	5.31±0.03b	9.86±0.03a	1.47±0.04bc
	120	14.29±0.12b	14.03±0.09b	5.36±0.12a	24.72±0.21c	2.07±0.03b	5.28±0.08b	9.82±0.11a	1.45±0.03c

Values are expressed as mean±standard deviation (n=10). The results followed by a different letter in the same column are significantly different (P<0.05).

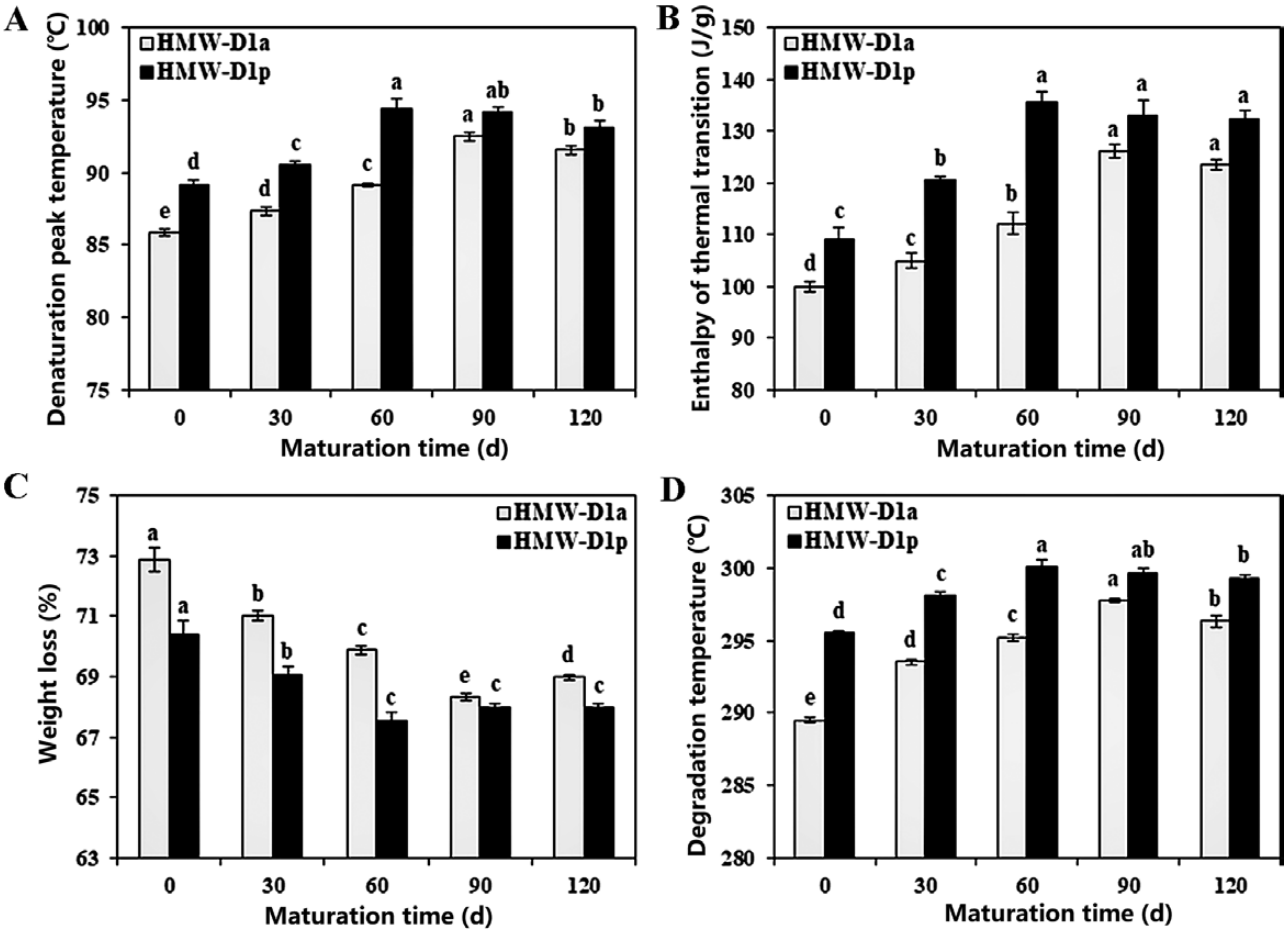

Figure 3. Changes in the thermal stability of the two NIL flours during maturation. (A) Denaturation peak temperature, (B) enthalpy of thermal transition, (C) weight loss, and (D) degradation temperature. Different letters for each indicate significant differences (P<0.05).

denaturation peak temperature (T_p), enthalpy of thermal transition (ΔH), and degradation temperature (T_d), but lower values for weight loss compared with HMW-D1a flour. The T_p, ΔH, and T_d reached a maximum at 60 d for HMW-D1p and 90 d for HMW-D1a, while the weight loss was lowest. The change rates were greater in HMW-D1p than in HMW-D1a. Subsequently, T_p, ΔH, T_d, and weight loss exhibited slight changes for HMW-D1p and obvious changes for HMW-D1a, except for ΔH.

Increases in T_p and ΔH are related to more regular and cross-linked gluten network structures (Wang *et al.*, 2014). Simultaneously, increased T_d and reduced weight loss also indicate the formation of a more compact and stronger gluten network in the model dough (Khatkar *et al.*, 2013). Maturation resulted in an increase in T_p, ΔH, and T_d and a reduction in the weight loss of HMW-D1a and HMW-D1p flours, indicating that the thermal stability of flour was improved during flour maturation. The thermal stability of gluten is closely correlated with the gluten network in dough (Khatkar *et al.*, 2013). HMW-GSs play an important role in the formation of the gluten network (Liu *et al.*, 2016; Gao *et al.*, 2018). Thus, the absence of Dx2 probably accounts for the difference in the thermal stability between HMW-D1a and HMW-D1p flours during maturation, which was explained by the results of secondary and microstructures of flour proteins in the present study.

Mixing properties of dough affected by flour maturation

Figure 4 shows the dough mixing properties reflecting gluten strength measured by Mixolab. The dough development time, dough stability time, and protein weakening significantly increased and reached a maximum at a maturation period of 60 d for HMW-D1p and 90 d for HMW-D1a. The increased rates were greater in HMW-D1p flour than HMW-D1a flour during flour maturation. However, the dough mixing properties exhibited a slower reduction in HMW-D1p than HMW-D1a.

The dough development time and dough stability time were used as indicators of gluten strength (Gao *et al.*, 2018), and protein weakening was positively correlated with the SDS sedimentation volume, dough extensibility, and loaf volume (Dhaka *et al.*, 2012). Our study showed that the maximum dough development time, dough stability time, and protein weakening in HMW-D1p occurred earlier than those in HMW-D1a during flour maturation, suggesting that the improvement in dough strength of HMW-D1a was delayed due to the absence of Dx2, which supported Pyler (1973), who suggested that weaker gluten flour needs a longer time to reach optimal quality. Moreover, there were higher increase rates in the above parameters in HMW-D1p than in HMW-D1a, which was consistent with the results of Wang *et al.* (2019) and inconsistent with those of Hadnađev *et al.* (2015). This divergence needs to be further verified using a number of wheat varieties in future studies. Prolonged maturation caused adverse effects on dough mixing properties, especially HMW-D1a without Dx2, and the absence of Dx2 accelerated the weakening of dough networks. Several studies have suggested that the GMP and -S-S- concentrations, surface hydrophobicity, secondary and microstructures, and thermal stability of gluten proteins are closely correlated with dough properties (Liu *et al.*, 2016; Gao *et al.*, 2018; Wang et al., 2018; 2019); therefore, we can conclude that the absence of Dx2 affected the flour protein structural characteristics, appearing as protein aggregation and thermal stability during flour maturation, thereby affecting dough quality.

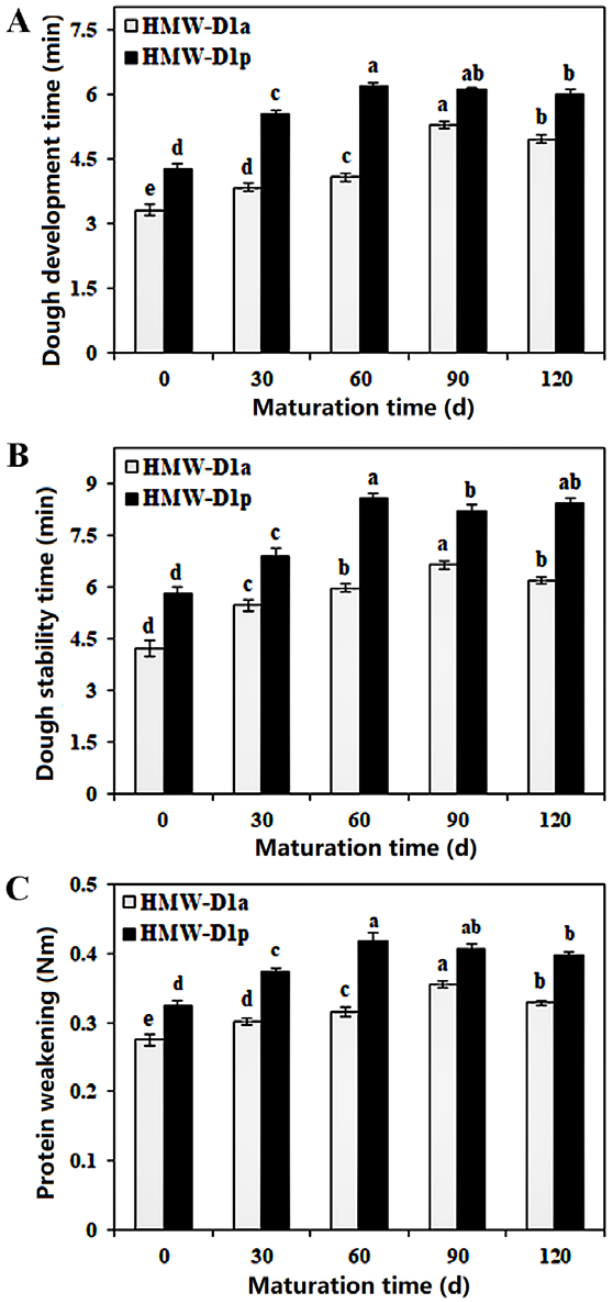

Figure 4. Changes in the dough mixing properties of the two NIL flours during maturation. (A) Dough development time, (B) dough stability time, and (C) protein weakening. Different letters above the bars indicate significant differences (P<0.05).

Conclusions

The absence of Dx2 delayed glutenin polymerization and the formation of protein secondary structures via the interaction between -SH groups, S–S bonds, and hydrophobicity, resulting in the looser microstructure of dough during

maturation, thereby contributing to the decline in flour thermal stability and dough mixing properties. These findings provide an understanding of how the absence of Dx2 changes protein structures during maturation.

Supplementary Material

Supplementary material is available at *Food Quality and Safety* online.

Funding

This study was supported by the Key Science and Technology Project of Shaanxi Agricultural Collaborative Innovation and Extension Alliance (No. LMZD202104) and the Introduction of Talents for Scientific Research of State Key Laboratory of North China Crop Improvement and Regulation (No. NCCIR2020RC-11).

Author Contributions

Liye Zhao and Lijun Song: Conceptualization, data curation, formal analysis, methodology, writing original draft, reviewing and editing. Liqun Li: Formal analysis, investigation, methodology, validation. Xuejun Li: Conceptualization, funding acquisition, resources, reviewing and editing. All the authors read and approved the manuscript.

Conflict of Interest

The authors declare no competing financial interest.

References

Bernklau, I., Lucas, L., Jekle, M., *et al*. (2016). Protein network analysis – a new approach for quantifying wheat dough microstructure. *Food Research International*, 89(1): 812–819.

Bruneel, C., Lagrain, B., Brijs, K., *et al*. (2011). Redox agents and N-ethylmaleimide affect the extractability of gluten proteins during fresh pasta processing. *Food Chemistry*, 127(3): 905–911.

Delcour, J. A., Joye, I. J., Pareyt, B., *et al*. (2012). Wheat gluten functionality as a quality determinant in cereal-based food products. *Annual Review of Food Science and Technology*, 3(1): 469–492.

Dhaka, V., Gulia, N., Khatkar, B. S. (2012). Application of Mixolab to assess the bread making quality of wheat varieties. *Scientific Reports*, 1(3): 183–190.

Fierens, E., Helsmoortel, L., Joye, I. J., *et al*. (2015). Changes in wheat (*Triticum aestivum* L.) flour pasting characteristics as a result of storage and their underlying mechanisms. *Journal of Cereal Science*, 65: 81–87.

Gao, X., Liu, T. H., Ding, M. Y., *et al*. (2018). Effects of HMW-GS Ax1 or Dx2 absence on the glutenin polymerization and gluten micro structure of wheat (*Triticum aestivum* L.). *Food Chemistry*, 240: 626–633.

Georget, D. M. R., Belton, P. S. (2006). Effects of temperature and water content on the secondary structure of wheat gluten studied by FTIR spectroscopy. *Biomacromolecules*, 7(2): 469–475.

Graveland, A., Bosveld, P., Lichtendonk, W. J., *et al*. (1985). A model for the molecular structure of the glutenins from wheat flour. *Journal of Cereal Science*, 3(1): 1–16.

Guo, X. F., Sun, X. H., Zhang, Y. Y., *et al*. (2018). Interactions between soy protein hydrolyzates and wheat proteins in noodle making dough. *Food Chemistry*, 245(15): 500–507.

Hadnađev, M., Hadnađev, D. T., Pojić, M., *et al.* (2015). Changes in the rheological properties of wheat dough during short-term storage of wheat. *Journal of the Science of Food and Agriculture*, 95 (3): 569–575.

Hajnal, E. J., Tomić, J., Torbica, A., *et al*. (2014). Content of free amino groups during postharvest wheat and flour maturation in relation to gluten quality. *Food Chemistry*, 164(3): 158–165.

Hidalgo, A., Brandolini, A. (2008). Kinetics of carotenoids degradation during the storage of einkorn *(Triticum monococcum* L. ssp. monococcum) and breadwheat (*Triticum aestivum* L. ssp. aestivum) flours. *Journal of Agricultural and Food Chemistry*, 56(23): 11300–11305.

Hidalgo, A., Brandolini, A., Pompei, C. (2009). Kinetics of tocols degradation during the storage of einkorn (*Triticum monococcum* L. ssp. monococcum) and breadwheat (*Triticum aestivum* L. ssp. aestivum) flours. *Food Chemistry*, 116(4): 821–827.

Huebner, F. R., Bietz, J. A. (1993). Improved chromatographic separation and characterization of ethanol-soluble wheat proteins. *Cereal Chemistry*, 70(5): 506–511.

Khatkar, B. S., Barak, S., Mudgil, D. (2013). Effects of gliadin addition on the rheological, microscopic and thermal characteristics of wheat gluten. *International Journal of Biological Macromolecules*, 53: 38–41.

Lindsay, M. P., Skerritt, J. H. (1999). The glutenin macropolymer of wheat flour doughs: structure-function perspectives. *Trends in Food Science & Technology*, 10(8): 247–253.

Liu, T. H., Gao, X., Li, L. Q., *et al*. (2016). Effects of HMW-GS at *Glu-B1* locus on the polymerization of glutenin during grain development and on the secondary and micro-structures of gluten in wheat (*Triticum aestivum* L.). *Journal of Cereal Science*, 72: 101–107.

Pooja, P., Shubhangi, S., Hari, N. M. (2018). Comparison of FT-NIR and NIR for evaluation of phyisco-chemical properties of stored wheat grains. *Food Quality and Safety*, 2(3): 165–172.

Pyler, E. J. (1973). Baking, Science and Technology. Seibel Publishing Company, Chicago, IL, USA, pp. 358–364.

Rao, V. S., Vakil, U. K., Sreenivasan, A. (1978). Comparative studies on physicochemical and baking properties of newly harvested and stored Indian varieties of wheat. *Journal of the Science of Food and Agriculture*, 29(2): 155–164.

Shewry, P. R., Halford, N. G., Lafiandra, D. (2003). Genetics of wheat gluten proteins. *Advances in Genetics*, 49: 111–184.

Song, L., Li, L. Q., Zhao, L., *et al*. (2020). Absence of Dx2 at Glu-D1 locus weakens gluten quality potentially regulated by expression of nitrogen metabolism enzymes and glutenin-related genes in wheat. *International Journal of Molecular Sciences*, 21: 1383.

Temea, A., Picoleta, N., Aimion, A., *et al*. (2016). Improvement of flour and dough rheological properties by maturation process. *Romanian Biotechnological Letters*, 21(2): 11381–11392.

Tuhumury, H. C. D., Small, D. M., Day, L. (2014). The effect of sodium chloride on gluten network formation and rheology. *Journal of Cereal Science*, 60(1): 229–237.

Varzakas, T. (2016). Quality and safety aspects of cereals (wheat) and their products. *Critical Reviews in Food Science and Nutrition*, 56(15): 2495–2510.

Wagner, M., Morel, M. H., Bonicel, J., *et al*. (2011). Mechanisms of heat-mediated aggregation of wheat gluten protein upon pasta processing. *Journal of Agricultural and Food Chemistry*, 59(7): 3146–3154.

Wang, L., Flores, R. A. (1999). The effects of storage on flour quality and baking performance. *Food Reviews International*, 15(2): 215–234.

Wang, P., Xu, L., Nikoo, M., *et al*. (2014). Effect of frozen storage on the conformational, thermal and microscopic properties of gluten: comparative studies on gluten-, glutenin- and gliadin-rich fractions. *Food Hydrocolloids*, 35: 238–246.

Wang, P., Zou, M., Tiao, M. Q., *et al*. (2018). The impact of heating on the unfolding and polymerization process of frozen-stored gluten. *Food Hydrocolloids*, 85: 195–203.

Wang, N., Ma, S., Li, L., *et al*. (2019). Aggregation characteristics of protein during wheat flour maturation. *Journal of the Science of Food and Agriculture*, 99(2): 719–725.

Weegels, P. L., Hamer, R. J., Schofield, J. D. (1997). Depolymerisation and repolymerisation of wheat glutenin during dough processing. II. Changes in composition. *Journal of Cereal Science*, 25(2): 155–163.

Xu, T., Liu, C., Zheng, X., *et al*. (2015). Influence of flour maturation time on the quality of Chinese steamed bread (in Chinese). *Modern Food Science and Technology*, 31(6): 264–271.

Zhang, X., Mu, M. Y., Tian, Y., *et al*. (2021). Aggregative and structural properties of wheat gluten during post-harvest maturation. *Journal of Stored Products Research*, 94: 101897.

Zhu, J., Li, L. Q., Zhao, L. Y., *et al*. (2019). Effects of freeze–thaw cycles on the structural and thermal properties of wheat gluten with variations in HMW-GS at the *Glu-B1* locus. *Journal of Cereal Science*, 87: 266–272.