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Effects of cold temperature on starch molecular structure and gelatinization of late-maturity alpha-amylase affected wheat

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ABSTRACT

The synthesis of late-maturity alpha-amylase (LMA) occurs when wheat experiences a cold-temperature ‘shock’ during the post-anthesis, grain-filling period. This markedly increases the high isoelectric point (pI) alpha-amylase in wheat grains in susceptible varieties. The affected grain has low Falling Numbers (FN) and is rejected at receival point or downgraded to feed grade, as low FN is associated with inferior end-product quality. However, several studies have reported the lack of correlation between low FN-LMA and end-product quality. Here, we characterize, for the first time, starch molecular structure and gelatinization properties of cold-treated wheat grains; starch structure has significant influence on flour functionality and end-product quality. Results show that the cold-treatment during post-anthesis has minimal effect on starch structure. While there was a small decrease in the gelatinization temperature for every wholemeal flour samples from cold-treated wheat grains, this is unlikely to cause any undesirable effect on end-product. The present findings suggest that the supposition that LMA is a major contributor to inferior end-product quality should be reconsidered.

1. Introduction

Late maturity alpha amylase (LMA) is a field condition which is a consequence of weather variability during the growing season, whereby a high pI isoform of α -amylase is produced during the mid-late stage of grain development (Mares and Mrva, 2008). Generally, the level of LMA expression is higher when wheat plants experience a greater temperature ‘shock’. However, studies have shown LMA activity can vary significantly among grains from the same plant and some wheat genotypes may also exhibit a constitutive patterns of LMA expression under a wide range of environmental conditions (Mares and Mrva, 2014): some genotypes may or may not always express LMA, and even when they do express LMA, the level of expression can be very different. LMA is primarily triggered by a sudden drop in temperature during plant growth, between the post-anthesis and ripening stages (Mares and Mrva, 2008). There are also other factors influencing LMA, such as the wheat genotype and the magnitude of the temperature change (Mrva et al., 2008). In most cases, LMA does not occur in wheat grain when grown under

normal conditions. High-pI α -amylase is also synthesized during the initiation of seed germination, where its primary function is to break down starch in the endosperm into simple sugars to fuel the early stage of seedling growth (Ritchie et al., 2000). The enzymatic activity peaks around 4–5 days after the start of germination and then begins to decline progressively. High-pI α -amylase activity remains low throughout the rest of plant development and only remnant amounts of the enzyme are left in mature grains (Mares and Mrva, 2008). When wheat plants experience a sudden 7–15 °C drop in temperature between 20 and 30 days post-anthesis, high-pI α -amylase synthesis is initiated and the enzyme can be retained in matured grains through harvest (Barrero et al., 2013). To identify these high-pI α -amylase occurrences by when they are manifest, they are also known as LMA or pre-maturity α -amylase (PMAA) in the UK.

LMA grains contain excessive amounts of high pI α -amylase and hence, its flour has a lower falling number (FN) than flour milled from normal grains. The FN test is a standardized method (AACCI Method 56–81.03) used widely in the wheat industry, primarily to evaluate grain

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quality. The test was designed to simulate starch gelatinization during baking, and measures the extent of α -amylase hydrolysis on starch. However, it does not directly measure α -amylase activity. It involves heating the whole wheat flour slurry to initiate starch gelatinization, followed by measuring its viscosity as a means of relating to α -amylase activity (Perten, 1964). The FN is the time taken (in seconds) for a standard plunger to fall through the gelatinized slurry. Many countries have different FN standards and is largely dependent on their intended end-product usage. The requirement to pass the FN test in most countries ranges between 250 and 450 s, with 300 s being the reference value (He et al., 2019). Due to the elevated amount of α -amylase in LMA wheat flour, the starch is hydrolyzed more rapidly. This liquefaction leads to a decrease in the viscosity of the wheat flour slurry. Hence, the plunger can fall through the slurry in a shorter amount of time and hence LMA wheat grains generally have lower FNs. Mares and Mrva (2008) state that LMA flour can have FN below 100 s, but typically it ranges from 150 s to close to the FN threshold value. The wheat industry views low FN flour as an indication of unsound grains which carry a risk of adversely impacting on processing and end-product qualities. Therefore, LMA affected grain is either sold at a discounted price or downgraded to feed grade when FN falls below the receival limit (Kingwell and Carter, 2014).

Many studies have reported a correlation between low FN flour and poor end-product quality. It has been shown that end-products made from wheat flour with $\text{FN} \leq 200$ have sticky crumb, poor crumb texture, over-darkening of bread crust and lower loaf volume, among other characteristics (Edwards et al., 1989; Every and Ross, 1996; Olaerts et al., 2018). However, most of these studies assess the quality of end-product on pre-harvest sprouting (PHS) grains instead of LMA grains. PHS is also another cause of low FN in flour. However, PHS grains contain high levels of other hydrolytic enzymes such as β -amylase and endo-xylanases in addition to α -amylase, due to germination of physiologically mature grains occurring in the ears as a consequence of rain prior to harvest (Corder and Henry, 1989). Furthermore, starch is broken down by the hydrolytic enzymes during PHS, whilst LMA amylolysis only occurs during starch gelatinization.

FN test is widely used to discriminate PHS grains from sound grains and many studies have demonstrated low FN flours caused by PHS produce poor quality end-products. However, there is no evidence for low FN flour due to LMA having the same adverse outcome. Furthermore, the rapid gelatinization process in the FN test is very different from the conditions of large-industrial scale processing and its test result may not an absolute indicator of wheat flour quality (Mares and Mrva, 2008). Besides, viscosity is not only determined by α -amylase liquefaction of starch (the largest component of flour), but is also dependent on factors such as the starch granule morphology, their molecular composition and effects of other macromolecules in wheat (He et al., 2019).

At this time, no study has reported on any detrimental effects of LMA on end-product quality. The first comprehensive evaluation of LMA on bread quality (Newberry et al., 2018) reported a lack of correlation between bread baking traits, FN and LMA affected grain. These authors demonstrated that an elevated level of α -amylase in grains reduced the FN values of flour; however, no significant correlation was found between low LMA-FN and poor end-product quality, in terms of oven spring, loaf weight, loaf volume or any other quality parameters of breads processed using low and high FN wheat flours (ranging from 157 to 234 s, and from 250 to 300 s respectively). These findings suggest that the FN test may not be relevant to grain quality evaluation for all situations and perhaps not the most ideal method to evaluate the soundness of grains in the case of LMA.

To date, very few studies have elucidated the actual impact of LMA on end-product quality. Furthermore, the effects of cold-temperature 'shock' on starch molecular structure seems to have been overlooked. Starch is the main component of wheat flour and its structural features, which are greatly influenced by environmental conditions, contribute significantly to its functionality. Functional properties in this regard

include starch gelatinization, pasting properties and retrogradation (Jane et al., 1999).

Starch is a branched glucose polymer with two main components: amylose (with moderate molecular weight and largely linear with a few long-chain branches), and amylopectin (with much higher molecular weight and a very large number of short-chain branches). Here, starch molecular structure is measured using size-exclusion chromatography (SEC) and fluorophore-assisted carbohydrate electrophoresis (FACE) for acquiring the whole starch molecules weight distribution and debranched starch molecules chain-length distribution (CLD). The crystallinity was determined by wide-angle X-ray diffraction (XRD) analysis and starch gelatinization was evaluated using differential scanning calorimetry (DSC). These analyses can provide information on the relationship between structure and functional properties of starch specific to LMA, which is needed for reconsidering the assumption that LMA has detrimental effects on end-product quality because of the associated low FN.

The present study has three aims: (1) to characterize the starch molecular structure of LMA affected wheat grains, (2) to determine the starch gelatinization properties of LMA-induced wheat flour to assess the impact of cold temperature and LMA on the thermodynamic performance of LMA grains, and (3) to relate (1) and (2) to each other.

2. Materials and methods

2.1. Materials

Sandwich enzyme-linked immunosorbent assay (ELISA) was employed for LMA determination. This uses polyclonal and monoclonal antibodies that bind specifically to LMA; the high pI isoform of wheat α -amylase. Both antibodies are from South Australia Research and Development Institute (Hartley Grove, SA, Australia), colour developer (3,3',5,5'-Tetramethylbenzidine substrate) was purchased from ELISA Systems (Windsor, QLD, Australia). Protease from *Streptomyces griseus* (type XIV) and isoamylase from *Pseudomonas* sp. were purchased from Sigma-Aldrich Pty. Ltd. (Castle Hill, NSW, Australia) and Megazyme International, Ltd. (Wicklow, Ireland) respectively. Pullulan standards with known molecular weight ranging between 342 and 2.35×10^6 Da were from Polymer Standards Services (PSS) GmbH (Mainz, Germany). All other chemicals were reagent grade and used as received.

2.2. Plant growth and LMA induction

Two Australia wheat (*Triticum aestivum* L.) cultivars, Hartog (LMA-tolerant) and Kennedy (LMA-susceptible) were used for this study, based on their LMA characteristics previously reported by Mrva et al. (2006). The plant growth method was adapted from Mrva and Mares (2001) with minor modifications. Wheat was grown in a climate-controlled glasshouse under natural light supplemented with high intensity LED lights at the University of Queensland, Brisbane, Australia, between July and November 2018. The temperature in the glasshouse was programmed on a diurnal temperature cycle of 25 °C and 15 °C during the day and night, respectively, 12/12 h day/night cycle. Ten sound grains of either Hartog or Kennedy were sown in a 125 mm ANOVA pot and hand-watered daily as required. On day 28 post-anthesis (DPA, days past anthesis), any secondary tillers were removed, and the pots for LMA induction were relocated to a 13 °C cold room chamber (again with 12 h/12 h day/night cycle) to initiate 'cold-temperature shock' for 10 days before transferring back to the glasshouse until maturity and harvest. Control pots remained in the glasshouse throughout the experiment. Upon ripeness, spikes were threshed using a Wintersteiger LD 180 laboratory thresher (Wintersteiger Co., Ried, Austria) and whole grains were milled on a Perten Lab Mill 3310 (PerkinElmer Co., Hägersten, Sweden). Grain from each plant (i.e. a single primary tiller) was harvested and threshed separately, providing biological (between plants in a pot) and statistical replications (between pots).

2.3. Wheat flour LMA determination

LMA content was measured in 50 mg of wheat flour, as described in [Barrero et al. \(2013\)](#). All spectrophotometric measurements were taken using a FLUORstar OPTIMA (BMG LABTECH Co. Ortenberg, Germany) microplate reader. LMA content in flour samples was expressed as its absorbance reading (OD) value.

2.4. Starch extraction for structural analysis

Starch was extracted from flour samples following the method described by [Nguyen et al. \(2019\)](#) with minor modifications, as follows. Approximately 7–9 mg of flour were mixed with 0.5 mL of tricine buffer (pH 7.5, 250 mM) containing protease at 37 °C for 30 min to remove proteins. Subsequently, the samples were centrifuged and the supernatant was discarded. Thereafter, 0.45% (w/v) sodium bisulfite solution was mixed with the precipitates at 37 °C for another 30 min followed by a 4000 g centrifugation for 10 min. After centrifugation, the starch residue in the precipitate was dissolved in dimethyl sulfoxide containing 0.5% (w/w) LiBr (DMSO/LiBr) overnight at 80 °C using a thermomixer. Ethanol was then added to the DMSO/LiBr solution and the precipitated starch were collected after centrifugation. The precipitated starch was re-dissolved in DMSO/LiBr and transferred to a chromatography vial for whole branched starch molecule SEC analysis. For debranched starch analysis, the precipitated starch was dissolved in warm 0.9 mL deionized water followed by mixing with 0.1 mL acetate buffer (0.1 M, pH 3.5), 5 µL sodium azide solution (0.04 g/mL) and 2.5 µL isoamylase (1000 U/mL). The mixture was incubated at 37 °C for 3 h and the pH was neutralized with 0.1 M NaOH solution. The pH-neutralized mixture was incubated again at 80 °C for 1 h before freeze-drying overnight to obtain dried starch. The dried starch was then re-dissolved in DMSO/LiBr solution for debranched starch molecular structure SEC analysis.

2.5. Fluorophore-assisted carbohydrate electrophoresis (FACE)

FACE analysis was performed on a PA-800 PLUS FACE System, equipped with a solid-state laser-induced fluorescence (LIF) detector and an argon-ion laser as the excitation source (Beckman-Coulter, Brea, CA, USA). The starch samples for FACE analysis were prepared in the same way as the debranched starch for SEC analysis. Before analysis, samples were labelled using 8-aminopyrene-1,3,6-trisulfonic acid (APTS). A carbohydrate separation medium was used as supplied by equipment manufacturer. Analytes were injected into a 40 cm long and 50 µm diameter N-CHO-coated capillary at 25 °C using a voltage of 30 kV. The CLD of the debranched starch, being the number distribution of chains containing X monomer units, is denoted $N_{de}(X)$. Note that FACE is only accurate for $X \lesssim 180$, i.e. amylopectin chains and very short amylose chains.

2.6. Size-exclusion chromatography (SEC)

SEC separates polymers by molecular size (not by molecular weight), the separation parameter being the hydrodynamic radius R_h . With a differential refractive index detector as used here, the result is the SEC weight distribution w ($\log R_h$). There is no relation between R_h and molecular weight for a complex branched polymer such as whole starch. For a linear polymer such as debranched starch, there is such a relation, and using the Mark-Houwink equation, one can obtain the SEC debranched distribution w ($\log X$) ([Vilaplana and Gilbert, 2010](#)).

SEC analyses were carried out using the Agilent 1100 series SEC system (Waters, Wyatt) equipped with a differential refractive index detector (RID-10A, Shimadzu Corp, Kyoto, Japan) and GRAM 100 pre-column (PSS). GRAM 1000 and 3000 (PSS) were also equipped for debranched and branched starch samples respectively. DMSO/LiBr solution was used as the mobile phase with a flow rate of 0.3 and 0.6 mL/min for branched and debranched SEC analysis respectively. A series of

pullulan standards with peak molecular weights ranging from 342 to 2.35×10^6 were from PSS. The amylose content of the debranched starch samples were calculated as the percentage of the area under curve (AUC) of the amylose region from degree of polymerization DP 100 to that of the entire amylose and amylopectin region ([Vilaplana et al., 2012](#)).

2.7. Wide-angle X-ray diffraction (XRD)

Starch crystallinity analyses were performed using a Bruker D8 Advance MKII X-ray diffractometer (Bruker, Inc., Madison, WI, USA) with Cu K α radiation generated at 40 kV and 30 mA. Isolated starch samples were scanned at 1.2°/min with step interval of 0.005° in the region of diffraction angle 2θ between 5° and 30°. Relative crystallinity was calculated as percentage of total peak area to the total diffraction area using TOPAS 4.1 software.

2.8. Differential scanning calorimetry (DSC)

Gelatinization properties of the wholemeal flour were determined using a Differential Scanning Calorimetry Model Q2000 (TA Instruments, New Castle, DE, 19720, USA). Samples between 3 and 4 mg were weighed into aluminum pans before suspending in a 1:3 wt ratio of sample to deionized water. Pans were sealed hermetically and equilibrated at room temperature for 1 h before analysis. Samples were heated from 30 to 120 °C at 10 °C/min. An empty pan was used as a reference and the onset temperature (T_o), peak temperature (T_p), conclusion temperature (T_c) and enthalpy ΔH were calculated using TA Universal Analysis 2000TM software.

2.9. Statistical analysis

Experimental values are expressed as mean $\pm$ standard deviation. Data were analyzed by one-way ANOVA and Tukey test used to detect significance difference of mean values (p value < 0.05) using SPSS version 16 (SPSS Inc., Chicago, USA). All experiments were performed in duplicate unless stated otherwise.

3. Results

3.1. LMA content in cold-treated wheat grains

LMA content was determined to ensure that LMA had been successfully expressed in the glasshouse before conducting subsequent analyses. Two different LMA characteristic cultivars were included in this study to examine for any difference in their starch molecular structure and thermodynamic characteristic in response to cold-temperature treatment during the post-anthesis stage. As shown in [Table 1](#), there was almost no difference in LMA content between the control and cold-treated samples of Hartog. The trend we observed between wholemeal flour and isolated starch was the same. This indicates that LMA was not expressed during cold-temperature treatment and presumably, the low level of enzymatic activity detected is the typical residual amount in matured grains ([Mares and Mrva, 2014](#)). In contrast, for Kennedy the cold-treated samples had significantly higher enzymatic activity than the control sample. There was also no visible sign of PHS; this is an indication that high pI isoform of alpha-amylase detected by ELISA was attributed by the presence of LMA induced in wheat grains after the 10-day cold-temperature treatment. The result from this study is also in agreement with the findings reported by [Mrva et al. \(2008\)](#) for these two cultivars.

3.2. Molecular starch CLD structural analysis

While both the amylopectin and amylose CLDs can be quantified non-empirically in terms of the parameters from the underlying

Table 1

LMA content (OD value), DSC parameters for gelatinization properties and XRD parameters for starch molecular arrangement and crystallinity.

	Wheat cultivars			
	Hartog (LMA tolerant)		Kennedy (LMA susceptible)	
	Control	Cold-treated	Control	Cold-treated
LMA content				
OD value	0.16 ± 0.01 ^A	0.17 ± 0.01 ^A	0.17 ± 0.01 ^A	0.59 ± 0.01 ^B
DSC parameters				
Onset temperature (°C)	56.8 ± 0.11 ^A	55.0 ± 0.02 ^B	58.7 ± 0.18 ^A	56.4 ± 0.03 ^B
Peak temperature (°C)	61.9 ± 0.06 ^A	60.4 ± 0.05 ^B	63.6 ± 0.10 ^A	61.7 ± 0.05 ^B
Conclusion temperature (°C)	68.6 ± 0.02 ^A	66.9 ± 0.08 ^B	70.2 ± 0.19 ^A	68.4 ± 0.11 ^B
ΔH (J g ⁻¹)	6.38 ± 0.11 ^A	5.90 ± 0.01 ^B	6.60 ± 0.01 ^A	6.10 ± 0.01 ^B
XRD parameters				
XRD pattern	A	A	A	A
Crystallinity (%)	29.4 ± 0.04 ^A	30.1 ± 0.09 ^B	28.8 ± 0.01 ^A	30.0 ± 0.05 ^B

Values represent mean ± SD of duplicate test. Different letters marked within the row of the same cultivar has significant difference at $P < 0.05$.

biosynthesis (Tao et al., 2019), for the purposes of the present study, it is sufficient to use just a few parameters such as relative numbers of longer and shorter chains.

Amylopectin chains $DP < 37$ are here categorized as short and $DP \geq 37$ as long. The amylopectin CLD distribution of controls and cold-treated samples determined by FACE in the low DP region up to 100, normalized to their global maximum (Fig. 1). All samples showed a typical polymodal distribution, the interpretation of which is well known. The first peak, which was also the global maximum region, ranged between DP 6 to 19; a small shoulder occurred at $\sim DP 14$ attributed to the first enzyme set (starch synthases, starch branching enzymes and debranching enzymes) producing the amylopectin chains spanning a single lamella. The second peak covered the DP region between DP 20 and 36, where the amylopectin chains spanned two crystalline lamellae. The third peak covered the remaining region from DP 37 onwards and is for chains spanning three or more lamellae. All samples had similar amylopectin CLD profile. A small increase in short amylopectin chains ($DP < 37$) was observed in cold-treated samples for both cultivars but no significant difference was found (Table 2).

SEC weight distributions of the debranched starch for all wheat

samples are presented in Fig. 2. It is noted that SEC suffers from unavoidable band-broadening (Cave et al., 2009), so the $w(\log X)$ for the amylopectin chains will not be discussed, as the FACE data do not suffer from this problem. For Hartog, the maximum in the amylose region showed small differences in peak height and DP between the control and

Table 2

Starch molecular structure parameters of amylopectin and amylose from FACE and SEC CLDs.

	Wheat cultivars			
	Hartog (LMA tolerant)		Kennedy (LMA susceptible)	
	Control	'Cold-temperature shock'	Control	'Cold-temperature shock'
FACE analysis				
Amylopectin peak maximum DP	12 ± 0.0 ^A	12 ± 0.0 ^A	12 ± 0.0 ^A	12 ± 0.0 ^A
Average CLD DP	30 ± 0.0 ^A	30 ± 0.0 ^A	30 ± 0.0 ^A	30 ± 0.0 ^A
% of short chains (DP < 36)	88.8 ± 0.1 ^A	89.2 ± 0.0 ^A	87.7 ± 0.2 ^A	88.6 ± 0.3 ^A
SEC – whole molecule analysis				
Amylose peak R_h /nm	30.3 ± 0.5 ^A	29.70 ± 0.3 ^A	30.7 ± 0.1 ^A	30.4 ± 0.1 ^A
Amylose peak height (log R_h)	0.67 ± 0.0 ^A	0.69 ± 0.0 ^A	0.75 ± 0.0 ^A	0.77 ± 0.0 ^A
Amylopectin peak R_h /nm	319 ± 0.5 ^A	317 ± 0.7 ^A	319 ± 9.3 ^A	310 ± 7.6 ^A
Average branched starch molecule R_h /nm	43.4 ± 4.8 ^A	40.7 ± 2.0 ^A	53.6 ± 1.5 ^A	51.4 ± 0.7 ^A
Average amylopectin R_h /nm	255 ± 2.5 ^A	254 ± 1.0 ^A	249 ± 0.6 ^A	244 ± 2.1 ^A
Average amylose R_h /nm	17.8 ± 0.8 ^A	16.6 ± 1.7 ^A	18.8 ± 0.9 ^A	17.1 ± 1.5 ^A
SEC – debranched analysis				
Amylose peak maximum DP	1590 ± 37 ^A	1622 ± 83 ^A	1299 ± 34 ^A	1421 ± 103 ^A
Amylose peak maximum height (log X)	0.36 ± 0.0 ^A	0.39 ± 0.0 ^A	0.32 ± 0.0 ^A	0.32 ± 0.0 ^A
Amylose content (%)	28.9 ± 0.0 ^A	29.6 ± 0.0 ^A	24.5 ± 0.0 ^A	24.6 ± 0.0 ^A

Values represent mean ± SD of duplicate test. Different letters marked within the row of the same cultivar group indicates significant difference at $p < 0.05$. Amylopectin chains $< DP 36$ are classified as short chains for this study.

Fig. 1. FACE CLDs, $N_{de}(X)$, of debranched starch as a function of DP (X). All CLD distributions were normalized to the same global maximum. The actual data comprise individual points for each DP, but for visual ease, these are replaced by connected lines.

Fig. 2. SEC weight CLD of debranched starch, $w_{de}(\log X)$, as a function of DP (X). All weight CLD were normalized to the same global maximum.

cold-treated samples. No significant difference was found in their amylose content. For Kennedy, the control and cold-treated samples had almost identical peak height, CLD and amylose content. However, their amylose peak DP's difference was noticeably greater for Kennedy than

Hartog. Similarly, no significant differences were found in any of the SEC debranched starch parameters between the two sample groups for both cultivars.

SEC weight distributions, normalized to their global peak maxima, of

Fig. 3. SEC weight distribution of branched starch, $w_{br}(\log R_h)$ as a function of R_h . (Upper panel) whole DP region, normalized to the same global maximum and (lower panel) an enlargement of the amylose region, normalized to the amylose maximum.

the whole branched starch molecules for all wheat samples are presented in Fig. 3. As is commonly seen, there are distinct regions corresponding to the small whole molecules of amylose and the larger whole molecules of amylopectin. Note that amylose content was only determined using debranched starch data by measuring the AUC, because this cannot be quantified accurately from the whole branched starch size distribution due to band-broadening. No significant differences were found in any of the SEC whole branched parameters but it was observed that for both cultivars cold-temperature treated samples experienced a decrease in their molecular sizes for both polymers (Table 2). The minor difference can be seen more clearly between controls and cold-treated samples in the amylose region shown in Fig. 3, which is an enlargement of the SEC chromatogram in which the distributions were normalized to their amylose maximum. Hartog cold-treated samples have less of the larger-sized amylose and vice versa for the smaller-sized amylose for their overall distribution as compared to the control samples. In contrast, all Kennedy samples had almost identical weight size distribution regardless of their growing temperature.

3.3. Starch crystallinity determination

Fig. 4 shows the diffractogram of the scattering patterns of the whole branched starch for all the wheat starch samples measured by the XRD. All samples of both cultivars exhibited a typical A-type crystallinity diffraction pattern characterized by four distinctive peaks, with main diffraction doublet at 17° and 18° and two other peaks at 15° and 23° 2θ . A smaller peak at 20° 2θ revealed the presence of a V-type polymorph starch formed by single helices of amylose and their inclusion complexes with lipids within the semi-crystalline lamellae. For both cultivars the cold-treated samples were found to have higher crystallinity compared to their respective control samples (Table 1).

3.4. Starch gelatinization

The endothermic changes during starch gelatinization were examined using DSC to assess the thermal characteristic of the samples. The DSC parameters T_o , T_p , T_c and ΔH are given in Table 1. These parameters are associated with the order and stability of the crystallites within the starch granules, which is a major determinant of their functional properties such as water uptake and granule swelling capacity (Copeland et al., 2009). All wholemeal flour samples exhibited a typical range of gelatinization temperature. Although significantly different between treatments, both cultivars cold-treated samples had slight decreases in T_o , T_p , T_c and ΔH . Evidently, the higher LMA content in the Kennedy cold-treated samples did not have any marked impact on starch gelatinization, as Hartog cold-treated samples also had a similar decrease in their gelatinization temperature and enthalpy in comparison to its control samples. Interestingly, both cultivars cold-treated samples with higher crystallinity, an indication of a greater amount of double helices, have lower gelatinization enthalpy than their respective controls.

4. Discussion

4.1. Influence of cold temperature on starch molecular structure and crystallinity

Amylopectin and amylose composition in starch granules are instrumental in the formation of crystalline and amorphous structures, and this molecular architecture contributes significantly to thermodynamic and mechanical properties of starch. In this study, the weight distributions of whole starch molecules and CLDs have been examined to study the effects a cold temperature shock during post-anthesis on starch structure of two different wheat cultivars (one expressing LMA in response to the cold shock, and the other which does not), and to examine how the difference in structure (if any) impacted on their functionality as measured by the gelatinization properties. Both Hartog and Kennedy cold-treated samples showed a slight decrease in all molecular parameters but these differences were not significant (Table 2). These findings suggest that the starch molecular structure is not affected by the 'cold-temperature shock' during the late post-anthesis stage regardless of the LMA characteristics of the cultivar. Many studies have reported more severe effects of growing temperature on starch biosynthesis activities, and consequently affecting different levels of starch structure such as the amylose to amylopectin ratio, starch structure crystallinity and CLD distribution (Liu et al., 2011; Lu et al., 1996). Despite the lack of agreement between the present study and previous findings of similar research, it is reasonable to postulate the generalization on the effects of growing temperature on starch biosynthesis activities may not be of absolute relevance to the case of LMA-starch structure incident. The previous research focus mainly on elucidating the effects of different climatological conditions on cereal grain development during the grain filling period. However, LMA phenomenon is specific to the occurrence of cold temperature stress during the late post-anthesis stage. In view of the wheat biological development timeline, starch biosynthesis may have already passed its maximal during this late stage of grain filling (Thitisaksakul et al., 2012). A study by Zhang et al. (2010) has shown that the starch synthase activity in wheat decreased sharply after 24 DPA, suggesting that temperature fluctuations during late stage of post-anthesis may have minimal impact on starch biosynthesis activity and the starch molecular structure.

The polymorph type and crystallinity are influenced by the starch molecular structure; amylopectin is generally considered to be the major determinant for crystallinity. Although there was a decrease in the overall amylopectin content in cold-treated samples, the small increase in crystallinity is attributed to the increase in short amylopectin chains (Table 2). Starch crystallinity is largely dependent on the ratio of long ($DP \geq \sim 36$) and short ($DP \leq \sim 36$) amylopectin chains, which are responsible for the formation of double helices that are confined specifically to the crystalline clusters (Copeland et al., 2009). These XRD results further demonstrate that the cold temperature during late stage of post-anthesis has no effect on starch molecular structure and

Fig. 4. XRD spectra diagram of branched starches illustrating A-type polymorph diffraction.

crystalline polymorphism; hence the crystallinity between cold-treated samples and control was very similar. The observed small increase in crystallinity in both cultivars for cold-treated samples is unlikely to have any significant effects on wheat flour functionality to influence end-product quality.

4.2. Impact of LMA and cold temperature on starch gelatinization

Wholemeal flour was used in this study for evaluating starch gelatinization in preference to isolated starch, as flour is more representative of the real-world scenario, being used for a wide array of food products rather than isolated starch. Although the LMA content was approximately 3.5-fold higher in the Kennedy cold-treated samples, both Hartog and Kennedy cold-treated samples had approximately the same decrease for their phase transition temperature (1–2 °C) and gelatinization enthalpy ($\sim 0.5 \text{ J g}^{-1}$) (Table 1). This result suggests that the presence of LMA has hardly any impact during starch gelatinization, in agreement with the findings of Ral et al. (2016). It is likely that the LMA hydrolysis efficiency is limited by the initial substrate availability and enzyme accessibility was constrained at the start of gelatinization (Baks et al., 2007). In addition, the presence of starch-lipid complexes, the V-type polymorph shown in the XRD results, may have also reduced the susceptibility of starch to enzymatic hydrolysis (Copeland et al., 2009). This present study showed that in both cultivars the cold-treated samples had slightly lower phase transition temperatures and gelatinization enthalpies than their respective control samples. However, it is unlikely this small difference in molecular structure between the control and cold-treated samples had any substantial relevance to the decrease of starch thermal parameters observed in the DSC analysis. The higher gelatinization temperature and enthalpy for the control samples of both cultivars is potentially the outcome of greater thermal effects on granule structural annealing at a longer length scale compared to the cold-treated samples. Annealing facilitates the molecular reorganization of starch granules below gelatinization temperature, causing the crystalline and amorphous domains to rearrange into a more 'ideal' (lower free energy) configuration. In this experiment, it appeared that the both cultivars control samples annealed into a more thermodynamically stable structure as compared to their cold-treated samples, which explains the increased gelatinization enthalpy and peak transition temperature. However, it is striking that this goes in the opposite direction to crystallinity. Thus, this indicates that the annealing effect was greater in the amorphous domain of the control starch granules, in which the increased amylose mobility during annealing may have facilitated the optimization of crystalline order in the semi-crystalline region between the crystalline and amorphous domains (Ratnayake and Jackson, 2008).

5. Conclusions

At present, LMA affected grain is portrayed to be of poorer quality, as indicated by a low FN. As such, various stakeholders of the wheat industry are suffering from economic losses due to market rejection of these LMA affected grains. However, this study has shown that (1) cold temperature during late stage of post-anthesis did not have any significant effects on the molecular starch structure and (2) LMA does not have any detrimental effects on gelatinization properties. Hence, it is unconvincing that LMA-affected wheat grains or flour are of inferior quality solely based on its low FN. These results also provide further evidence supporting earlier findings that have raised questions on the alleged impact of LMA on end-product quality.

Declaration of competing interest

The authors declare no conflict of interest.

CRedit authorship contribution statement

GalexK.S. Neoh: Data curation, Formal analysis, Investigation, Validation, Visualization, Writing - original draft. **Xiaoyan Tan:** Investigation. **MarkJ. Dieters:** Visualization, Formal analysis, Funding acquisition, Supervision. **GlenP. Fox:** Formal analysis, Investigation, Validation, Visualization, Funding acquisition, Supervision. **RobertG. Gilbert:** Formal analysis, Investigation, Validation, Visualization, Supervision.

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