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Influence of diurnal photosynthetic activity on the morphology, structure, and thermal properties of normal and *waxy* barley starch

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ABSTRACT

This study investigated the influence of diurnal photosynthetic activity on the morphology, molecular composition, crystallinity, and gelatinization properties of normal barley starch (NBS) and waxy barley starch (WBS) granules from plants cultivated in a greenhouse under normal diurnal (16 h light) or constant light photosynthetic conditions. Growth rings were observed in all starch samples regardless of lighting conditions. The size distribution of whole and debranched WBS analyzed by gel-permeation chromatography did not appear to be influenced by the different lighting regimes, however, a greater relative crystallinity measured by wide-angle X-ray scattering and greater crystalline quality as judged by differential scanning calorimetry was observed under the diurnal lighting regime. NBS cultivated under the diurnal photosynthetic lighting regime displayed lower amylose content (18.7%), and shorter amylose chains than its counterpart grown under constant light. Although the relative crystallinity of NBS was not influenced by lighting conditions, lower onset, peak, and completion gelatinization temperatures were observed in diurnally grown NBS compared to constant light conditions. It is concluded that normal barley starch is less influenced by the diurnal photosynthetic lighting regime than amylose-free barley starch suggesting a role of amylose to prevent structural disorder and increase starch granule robustness against environmental cues.

1. Introduction

Starch is a water-insoluble polyglucan, packaged as discreet granules, and produced in green algae and higher plants as a major carbon reserve. In plants carbon is temporarily produced in leaves during the day and degraded at night to support nocturnal growth and metabolism, whereas in the seeds of many agriculturally important crop plants starch is stored on a long-term basis to support germination and seedling growth for the next generation. Such storage starches form the basis for much of the world's food supply, providing calories for humans and livestock alike, and in addition are utilized in many non-food industries. Although common structural elements exist in all starches [1], structural differences exist between species and genotypes within species which are exploited in the many uses of starch products [2].

A common structural feature present in starch granules is the presence of alternating semi-crystalline and amorphous structures. The alternating structures, commonly referred to as 'growth rings', can be viewed in native starch, but are more readily observed following treatment with dilute acid or amylolytic enzymes using a variety of microscopic techniques such as light, scanning electron, and transmission electron microscopy [3,4]. The growth rings represent alternating layers of increasing and decreasing levels of crystallinity, refractive index, density, and resistance to enzymatic attack [3]. Although the presence of growth rings appears to be a universal feature of starch granules, the underlying mechanisms responsible for their appearance are still uncertain. Prior investigations on the nature of growth rings suggested their occurrence may be the result of plants following either a diurnal or circadian rhythm; Meyer [5] hypothesized that in starch granules

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one growth ring per day is formed due to the diurnal rhythm. In 1925, Bakhuyzen [6] reported that wheat starch grown under constant light conditions did not display growth rings. A similar result was obtained by Buttrose [7] wherein growth rings were not observed in barley starch when grown under constant light conditions. The underlying theory for the disappearance of growth rings according to Buttrose [7] is that during sunlight, when the generation of precursor for starch synthesis is high, crystalline growth rings are synthesized. During the night, when essential precursors for starch synthesis cannot be produced via photosynthesis, the plant synthesizes the amorphous components. However, when potato (tuber) starch was developed in constant light conditions, growth rings were observed [3]. The growth rings found in starch from potato grown under constant light differed from those grown under diurnal conditions, as prominent 'major' rings, in which the enzymatically digested amorphous zone was large, alternated with 'minor' rings with a narrower digested zone [3]. It should be noted that similar patterns of alternating 'major' and 'minor' rings were also observed in starch granules in other studies on potato starch grown under normal conditions [8].

To the authors' knowledge, the effect of diurnal photosynthetic activity on the physicochemical and molecular characteristics of starch has not been previously reported. This research therefore investigated the effect of diurnal and constant light growing conditions on selected morphological, chemical and physical properties of normal and *waxy* barley starch through light and confocal microscopy, gel-permeation chromatography, X-ray diffraction, and differential scanning calorimetry analysis. Barley was selected as a model cereal as this plant has previously been investigated in other comparative studies on diurnal activity *versus* constant light conditions [7]. We examined the influence of amylose in determining starch structure under the different light regimes by comparing a normal barley line with a *waxy* cultivar lacking amylose. Results from this research will allow for a greater understanding of the influence of diurnal activity on the physical and molecular characteristics of starch.

2. Materials and methods

2.1. Barley genotypes and growth conditions

Two varieties of barley, Cinnamon (*waxy* barley starch; WBS) and Golden Promise (normal barley starch; NBS), were cultivated under normal diurnal (16 h light) or constant light growing conditions in a greenhouse at the University of Copenhagen (Copenhagen, Denmark). The barley samples grown in constant light were shielded from natural sunlight and grown for three months from planting until maturation under constant 180 μE artificial light using mercury lamps. Diurnal samples were grown under ambient conditions, with supporting 90 μE artificial mercury light from 4 a.m.–8 p.m. The temperature was 18–20 °C.

2.2. Enzymes

β-Amylase (10,000 U/mL) from barley [(1,4)-α-D-glucan maltohydrolase: EC 3.2.1.2], pullulanase (700 U/mL) from *Klebsiella planticola* (amylopectin 6-glucoanhydrolase; EC 3.2.1.41), isoamylase (1000 U/mL) from *Pseudomonas sp.* (glycogen 6-glucoanhydrolase; EC 3.2.1.68), and lichenase (1000 U/mL) from *Bacillus subtilis* (*endo*-1,3-β-D-glucanase: EC 3.2.1.73) were sourced from Megazyme International Ireland (Bray, Wicklow, Ireland).

2.3. Starch extraction

Starch was extracted from barley flour based on the method by Carciofi et al. [9], with modifications as described by Goldstein et al. [10]. Briefly, 5 g of milled barley was mixed with 25 mL of 5 mM dithiothreitol containing 1% (w/v) sodium dodecyl sulphate for 30 min at room temperature, and subsequently centrifuged at 3300 × *g* for 15 min. The pellet was washed twice with water and filtered through a 70 μm mesh cloth. The filtrate was centrifuged (3300 × *g* for 15 min) and 50 mL of 20 mM Na phosphate buffer (pH 6.5) was added. The mixture was incubated in a 50 °C water bath for 5 min before the addition of 100 μL lichenase enzyme, after which the sample was incubated for 1 h, with stirring every 15 min. After centrifugation (3300 × *g* for 15 min) the pellet was washed twice with distilled water, once with ethanol, followed by air drying overnight.

2.4. Isolation and analysis of starch granule-bound proteins

For analysis of starch-granule bound proteins, barley starch was isolated from barley according to the method of Ahmed et al. [11]. Isolation of starch granule-bound proteins (*i.e.* proteins trapped inside the granule matrix as opposed to proteins more loosely attached to the granule surface) was performed as follows. Starch granules (approximately 50 g) from barley flour were resuspended in 150 mL cold aqueous washing buffer (50 mM tris (hydroxymethyl) aminomethane (Tris)-acetate, pH 7.5, 1 mM Na_2-EDTA, and 1 mM DTT) and centrifuged at 3000 × *g* for 1 min at 4 °C. This washing step was repeated 5 times. The pellet was then washed 3 times with acetone followed by 3 washes with 2% (w/v) SDS. Starch granule-bound proteins were extracted by boiling the washed starch in SDS loading buffer (62.5 mM Tris-HCl, pH 6.8, 2% [w/v] SDS, 10% [w/v] glycerol, 5% [v/v] β-mercaptoethanol, 0.001% [w/v] bromophenol blue) (approximately 50 mg starch in 1 mL buffer). Boiled samples were centrifuged at 13,000 × *g* for 5 min and the supernatant was used for SDS-PAGE analysis of granule-bound proteins.

2.5. SDS-PAGE

Protein samples were separated on 1D-gels using precast 4–12% Bis-Tris gradient gels (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) with 3-(N-morpholino)-propanesulfonic acid running buffer and following the manufacturer's instructions for sample preparation and electrophoresis. Gels were stained with colloidal Coomassie Brilliant Blue G 250 (50% (v/v) methanol, 10% (v/v) acetic acid, 0.25% (w/v) Coomassie Blue) as described by Shevchenko et al. [12].

2.6. Starch granule morphology

Morphology of barley starch granules was observed by light-, polarized light-, and confocal microscopy. For light microscopy analysis, starch granules were lightly treated with dilute HCl (10 mg starch in 400 μL 2.2 M HCl, 12 h duration) to gently hydrolyse amorphous material and viewed under an Olympus BX40 light microscope (Melville, NY, USA) connected to a digital camera (Olympus DP11-N) and a monitor (Sony PVM-14N5U; Tokyo, Japan) to obtain digital images. Polarized light images were acquired with the same imaging system using a polarized light filter.

Confocal laser scanning microscopy (CLSM) was conducted on starch granules without prior treatment in dilute HCl according to methods described by Glaring et al. [13], using a TCS SP2 confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany). Granules were stained prior to scanning with 20 mM 8-amino-1,3,6-pyrenetrisulfonic acid (APTS) as described by Glaring et al. [13]. Transmission light recordings were made simultaneously with the fluorescence readings.

2.7. Wide-angle x-ray diffraction

X-ray measurements of hydrated samples were acquired using a GANESHA-SAXS/WAXS instrument from SAXSLAB (Denmark) equipped with a 100XL+ micro-focus sealed X-ray tube (Rigaku-Denki, Co., Tokyo, Japan) producing a photon beam with a wavelength of 1.54 Å. The scattering patterns were recorded with a 2D 300 K Pilatus detector from Dectris (Baden, Switzerland). The samples were measured in the wide-angle X-ray scattering (WAXS) setting covering a q-range from about 0.06 to 2.8 $Å^{-1}$ where the scattering vector q is defined by $q = 4\pi/\lambda \sin(\theta)$, λ is the X-ray wavelength and (θ) is half of the scattering angle. The exposure time was 600 s. The latter corresponds to an upper 2θ value of 40.15° or 2.24 Å. The water content of samples was adjusted by water phase sorption for 10 days in desiccators at a relative humidity of 90% using a saturated salt solution of barium chloride. Hydrated samples were then sealed between thin mica films to prevent any significant change in water content during the measurement, which was performed in vacuum. Relative crystallinity was calculated according to the methods described by Brückner [14] and Frost et al. [15]. Amorphous background scattering was estimated using an iterative smoothing algorithm in MATLAB (Natick, Massachusetts, USA). The relative crystallinity was then estimated from the peak and total areas as:

$$Relative\ Crystallinity = \frac{Area\ of\ Peaks}{Total\ Area}$$

where the areas are numerically integrated using built in MATLAB functions. The relative amounts of different crystal polymorph types were determined following subtracting the estimated amorphous background and fitting with a series of Gaussian peak profiles. From these fits, the amounts of V-type polymorph were estimated as the ratio of the area under the characteristic V-type peaks at roughly $2\theta = 13°$ and 20° compared to the total peak area of all fitted Gaussian peaks, whereas the A-type polymorph was estimated utilizing the fitted characteristic main peaks at $2\theta = 15°$ and 23° and the unresolved doublet at 17° and 18° 2θ.

2.8. Differential scanning calorimetry

The thermal properties of native starch samples were analyzed by differential scanning calorimetry (DSC) using a Discovery DSC from TA instruments (New Castle, DE, USA). Scans were performed from 25 °C to 85 °C at a rate of 5 °C/min. All starch samples were analyzed in slurries of 2 mg starch and 10 μL 10 mM NaCl in duplicates. Onset temperature (T_o), peak temperature (T_p), completion temperature (T_c) and enthalpy change (ΔH) were derived from the thermal profiles.

2.9. Starch molecular structure

To determine the size distribution of barley starch with gel-permeation chromatography (GPC), duplicate starch samples (8 mg) were dissolved in 90% (v/v) DMSO (200 μL) and gently stirred overnight at room temperature. Following dilution with warm water (800 μL, 80 °C), the starch sample (400 μL) was applied to a Sepharose CL 2 B (GE Healthcare, Uppsala, Sweden) column (1.6 × 32 cm) and eluted with 0.01 M NaOH at 0.5 mL/min. Even numbered fractions (1 mL) were collected and analysed for carbohydrate content with the phenol-sulfuric acid method [16]. The maximum wavelength of absorption (λ_{max}) of the iodine-glucan complex was determined in the collected odd-numbered fractions. 1 mL of 0.01 M HCl was added to the fractions and following neutralization, 0.1 mL of 0.01 M I_2/0.1 M KI solution was added. Wavelength spectra were recorded from 300 to 800 nm with a WPA Spectrawave S800 diode array spectrophotometer (Harvard Bioscience, Holliston, MA, USA).

β-Limit dextrins of barley starches were prepared by dissolving starch in 90% (v/v) DMSO and stirring overnight as described above. The following day, 100 μL of 0.01 M Na acetate buffer (pH 6.0) along with 2 μL of β-amylase were added and the mixture was stirred overnight. The sample was diluted to 1 mL with water, heated in order to deactivate the enzyme, and 400 μL was applied to the Sepharose CL 2B column. Fractions were analysed for carbohydrate content and λ_{max} as described above.

Chain length analysis of barley starch with GPC was completed according to methods described by Kalinga et al. [17]. Briefly, duplicate samples of barley starch were debranched with isoamylase and pullulanase whereafter the debranched sample was applied on a column (1 × 90 cm) of Sepharose CL 6 B (GE Healthcare, Uppsala, Sweden) and eluted with 0.5 M NaOH at 1 mL/min. Fractions (1 mL) were analysed for carbohydrate content as above. The relative content of amylose and amylopectin were determined from the chromatograms according to Sargeant [18]. In order to further characterize the long chain and short chain components of amylose, the amylose fraction was divided into the fraction eluted at the void of the gel, and the fraction which eluted between the void volume and amylopectin chain fraction, respectively.

2.10. Statistical analysis

All analyses were conducted in duplicate and analyzed using SPSS (IBM Corporation, Armonk, NY, USA). Significant differences were determined by comparing means by Tukey's test at a significance level of $p < 0.05$.

3. Results

3.1. Morphology of starch granules

Following the treatment of isolated starch granules with dilute HCl, growth rings were observed in starch granules from WBS and NBS grown under both diurnal and constant light conditions (Fig. 1). This observation was unexpected, and was not consistent with earlier findings reported by Buttrose [7], and Tester et al. [19] wherein growth rings were not observed in barley starch when grown under constant illumination conditions. The presence of the "Maltese cross" in all starch samples regardless of growing conditions observed under polarized light indicated that the ordered, radial arrangement of starch molecules [20] was preserved under both growing conditions (Fig. 2). The appearance of growth rings in WBS and NBS barley grown under constant light conditions was also confirmed by CLSM analysis without prior treatment of the granules in dilute HCl (Fig. 3). In this analysis, the reducing ends of starch components were labelled with the fluorescent probe APTS, with smaller molecular components exhibiting greater fluorescence intensity due to their higher molar ratio of reducing ends per anhydrous glucose residues, and consequent greater amount of fluorescent labelling by weight [21]. NBS granules exhibited strong fluorescence in the hilum regardless of lighting regime, whereas this was not observed in WBS granules. Transmission light recordings of the granules revealed the presence of tangentially and radially arranged lighter spots especially in NBS granules and when grown in constant light (Fig. 3), whereas similar spots were absent or only weakly indicated in WBS granules (not shown).

3.2. Architecture of barley starch granules grown in diurnal and constant light

The crystalline polymorphism of barley starch granules cultivated under diurnal or constant light photosynthetic conditions

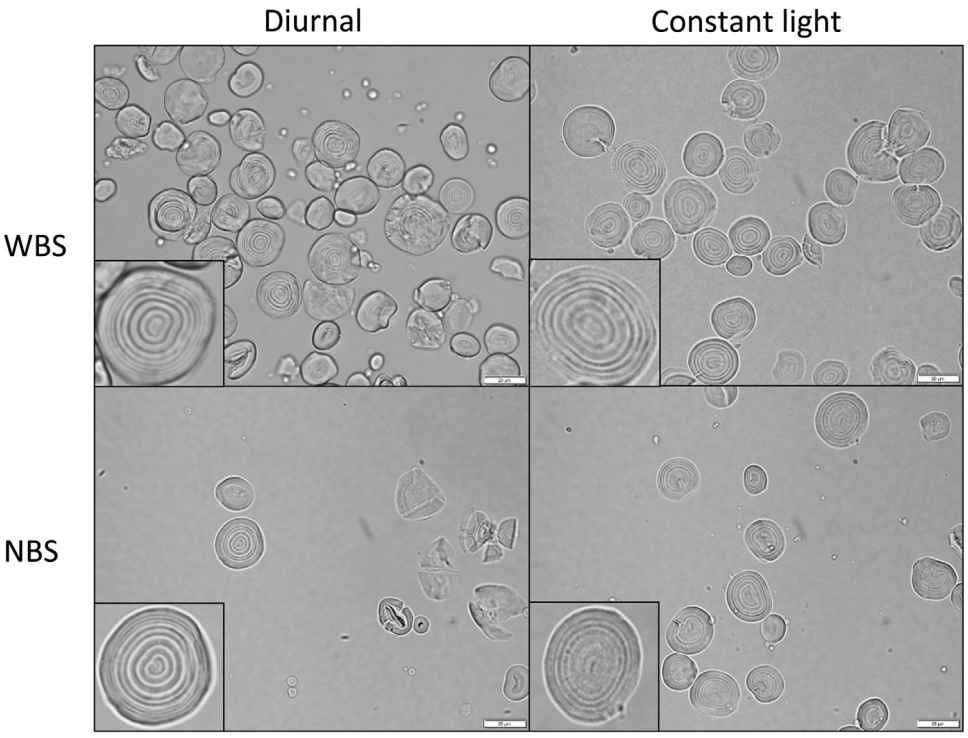

Fig. 1. Acid treated NBS and WBS granules grown in diurnal or constant light displaying presence of growth rings viewed by light microscopy. Scale bar represents 20 μm. Inset displays granules at higher magnification.

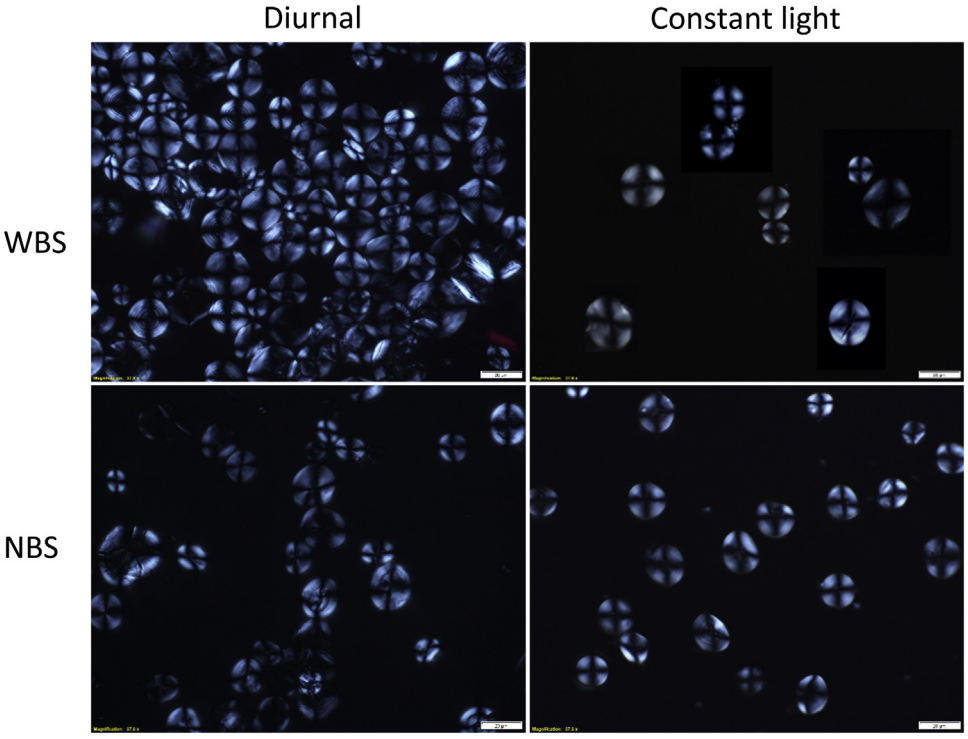

Fig. 2. Diurnal and constant light grown NBS and WBS granules viewed by polarized light microscopy. Scale bar represents 20 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Relative crystallinity (RC) of WBS and NBS granules grown in diurnal and constant light.

	RC (%)	A-type (% of total)	V-type (% of total)	V-type (% of crystals)
Diurnal NBS	19.5	16.9	2.5	12.9
Constant Light NBS.	19.2	16.7	2.4	12.6
Diurnal WBS	20.4	16.7	3.6	18.1
Constant Light WBS	15.7	13.9	1.7	11.1

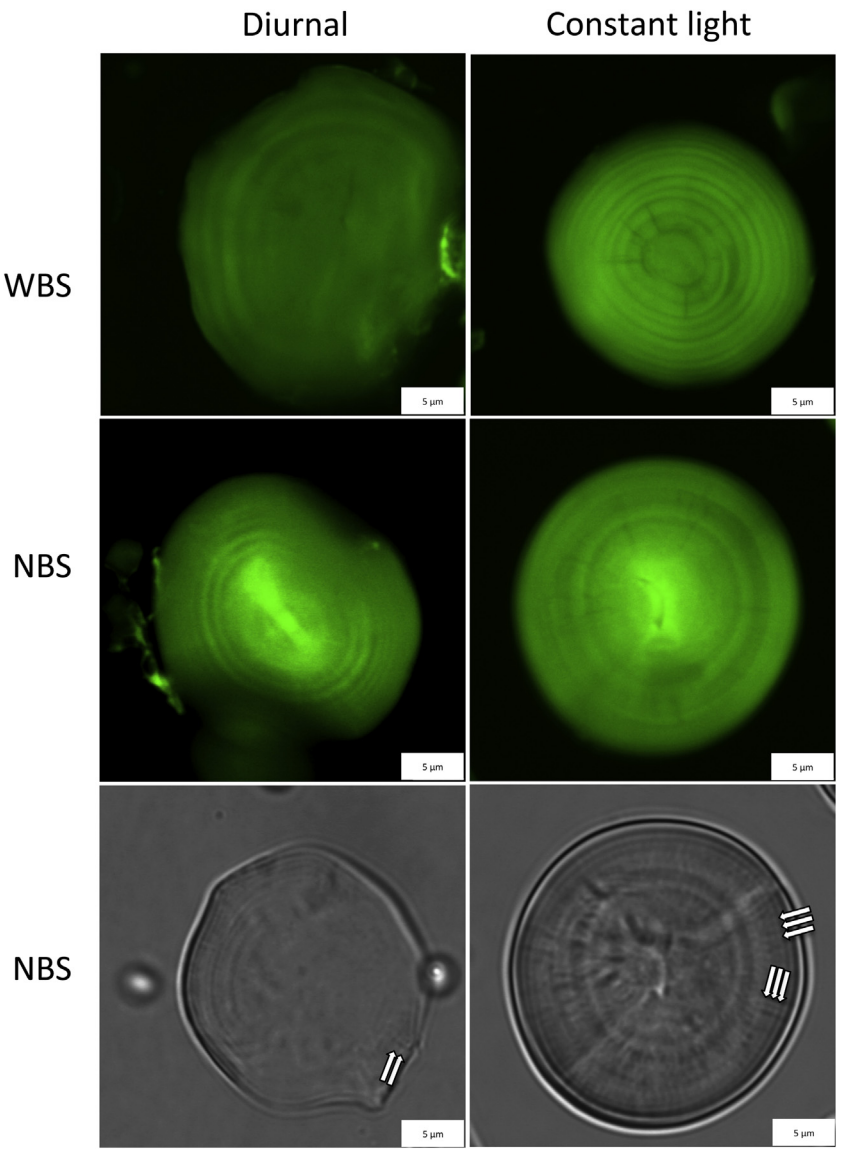

Fig. 3. Diurnal and constant light grown NBS and WBS granules viewed by CLSM. In the two lower figures the same granules were viewed in transmission light mode. Arrows highlight light spots, possibly blocklets. Scale bar represents 5 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

was determined by wide angle X-ray crystallography (WAXS). The X-ray diffraction patterns (Fig. 4) demonstrate that all barley samples exhibited primarily A-type and V-type crystallinity [4]. Estimation of the relative crystallinity was based on the WAXS data as illustrated in Fig. 5. The relative crystallinity of NBS was not influenced by diurnal or constant light growing conditions (Table 1), whereas the relative crystallinity of WBS was reduced when grown under the constant lighting regime (15.7%) compared to diurnal conditions (20.4%). Diurnally grown WBS also exhibited a greater contribution of V-type crystals to the relative crystallinity (18.1%) compared to WBS grown under constant light conditions (11.1%).

The thermal properties of gelatinization of WBS and NBS cultivated under diurnal or constant light conditions were determined by DSC analysis, and the gelatinization transition temperatures (T_o, T_p, T_c) and enthalpy of gelatinization (ΔH) were recorded. It is well accepted that T_p is an indicator of crystalline perfection, whereas ΔH represents the energy required to rupture hydrogen bonds within double helices [22]. Illumination conditions appeared to influence the gelatinization profiles of both WBS and NBS (Fig. 6). NBS grown under diurnal conditions exhibited lower T_o, T_p, T_c and ΔH values compared to its counterpart cultivated under continuous illumination. Interestingly, the opposite trend was observed in WBS, as diurnal WBS exhibited higher T_o and T_p values indicating higher crystalline perfection in diurnally grown WBS, whereas WBS grown under constant light conditions displayed a broader, more heterogeneous distribution of crystalline quality, as shown by the broader temperature range ($T_c - T_o$).

3.3. Molecular structure of starch

Effects of light conditions and amylose deposition in the starch granules on its molecular structure were investigated by analysis of the size distribution of native and β-amylase treated WBS and NBS using GPC on Sepharose CL 2B (Figs. 7 and 8, respectively). The non-treated native starch reveals size distributions of amylose and amylopectin components while the β-amylase-treated samples give information on the internal chain structure of these molecules. WBS grown under both diurnal and constant light conditions displayed a single peak eluting at the void volume, with the diurnal sample displaying a slightly broader size distribution. The maxi-

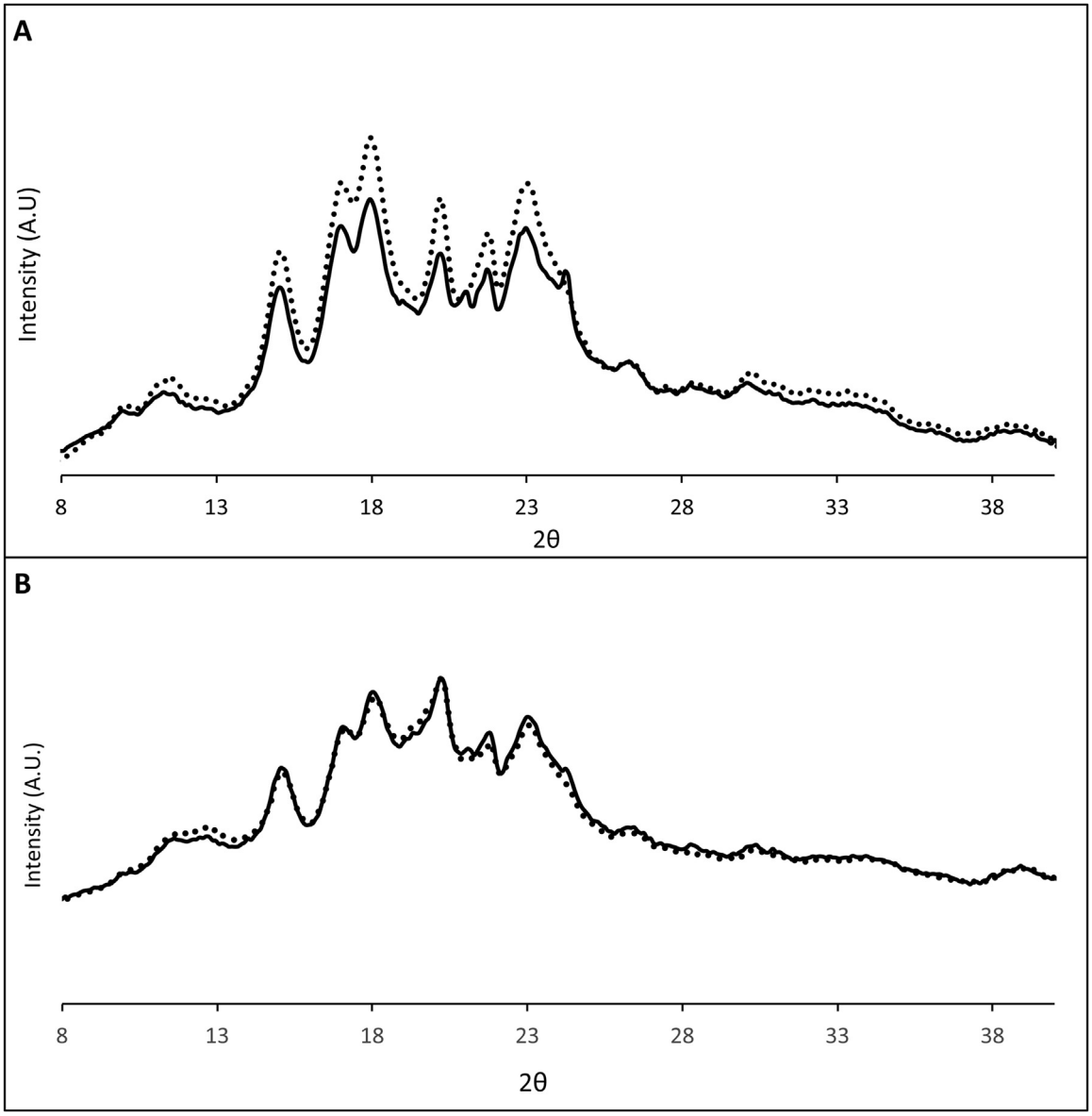

Fig. 4. Wide angle X-ray scattering pattern of WBS (A) and NBS (B) grown under diurnal (·····) and constant light conditions (––).

mum absorbance of the iodide-glucan complex (λ_{max}) suggests the presence of pure amylopectin and this value was similar under diurnal and constant light growing conditions (approximately 540 nm). When WBS was treated with β-amylase, which cleaves successive maltose units from the non-reducing ends of glucan polymers until a branch point is reached, the size of the residual β-limit dextrin (β-LD), as well as the quantity of maltose produced, can be determined. The β-LD profile of diurnal and constant light WBS was comparable as the size of the β-LD remained very large and was eluted at the void volume. In addition, similar quantities of maltose were produced (approximately 63 wt%), which was seen as a peak at the total volume of the gel. The λ_{max} of the β-LD of WBS was similar to that of the original WBS (i.e. 540 nm regardless light conditions). NBS displayed a bimodal (amylopectin and amylose) molecular size distribution profile, which also was similar regardless the light conditions (Fig. 8). Amylopectin components of NBS, which were eluted at or near the void volume, exhibited similar λ_{max} (approximately 560 nm) regardless of lighting regime. The larger amylose components of NBS, which possibly co-eluted with smaller amylopectin molecules following the major amylopectin peak, displayed similar λ_{max} values independent of lighting conditions during growth. However, constant light NBS maintained greater λ_{max} values than diurnal NBS for the smaller amylose components eluting between fractions 47–59. Similar quantities of maltose were produced following the addition of β-amylase to NBS (approximately 41 wt%) regardless the lighting regime. As for WBS, the λ_{max} values of the β-LD of the NBS amylopectin were similar under the two lighting regimes. However, the λ_{max} of the β-LD of branched amylose components, which eluted after fraction 35, were slightly higher in constant light NBS than in diurnal NBS.

Following the addition of isoamylase and pullulanase, which hydrolyze α-(1,6)-glucosidic linkages present in starch, the size distribution of the debranched components were determined by GPC on a column of Sepharose CL 6B (Fig. 9). In this analysis, the long chains of amylose eluted in the early fractions, followed by the elution of the shorter amylopectin chains in later fractions. The chain length distribution of WBS appeared similar when grown under the two light regimes, and the same was true for the amylopectin component in NBS. However, the apparent amylose content of NBS markedly decreased from 23.1% when grown under constant light

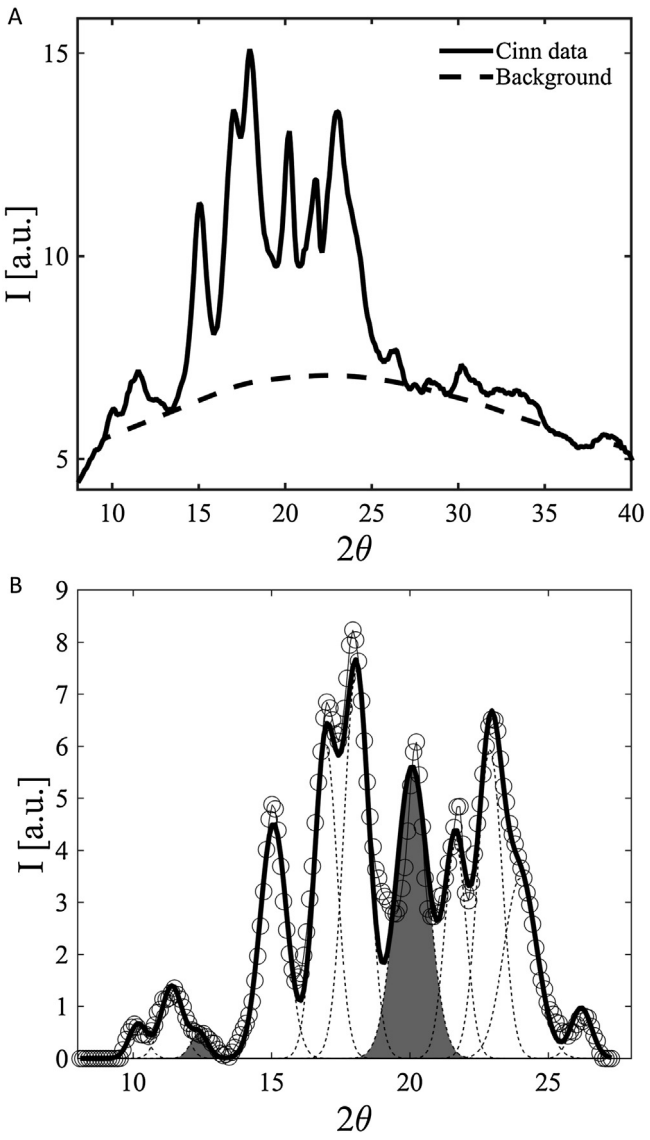

Fig. 5. Estimation of crystallinity measures using the WBS/diurnal sample as example. (A) Estimation of the amorphous background using the smoothing procedure from [15]. (B) Fitting a series of Gaussians to the peak profile obtained by subtracting the amorphous contribution. The shaded peaks are those originating from the V-type polymorph and used for the calculation of the percentage of this crystal type.

Table 2
Amylose content and composition of WBS and NBS cultivated under diurnal or constant light conditions.

	Apparent Amylose (%)	Long chain amylose: short chain amylose ratio
Diurnal WBS	0	–
Constant Light WBS	0	–
Diurnal NBS	18.7[a]	2.7[a]
Constant Light NBS	23.1[b]	4.2[b]

Values with same letters are not significantly ($p < 0.05$) different.

conditions to 18.7% when grown under diurnal conditions (Table 2). Moreover, the molecular structure of the amylose component was altered by the lighting conditions as the ratio of long:short amylose chains decreased from 4.2 in NBS cultivated under constant light to 2.7 when grown diurnally, indicating that the diurnal cycle influences the structure and composition of amylose in NBS granules.

3.4. Analysis of starch granule-bound proteins

In order to investigate whether differences in granule bound starch proteins influenced differences in starch molecular composition, granule bound starch proteins were isolated, stained and visualized following electrophoresis (Fig. 10). No difference in protein complement was observed in NBS and WBS regardless of light treatment during cultivation. As expected, the most abundant protein band (approx. 56–60 kDa) in NBS was granule bound starch synthase I (GBSS I), which is responsible for the synthesis of amylose [23]. This band was also present in WBS, suggesting that this particular *waxy* line expresses a non-active GBSSI protein as demonstrated for other *waxy* lines [24]. Other starch granule-associated proteins known to be present in barley include starch synthase (SS) I (75 kDa), SSII (87 kDa), starch branching enzyme (SBE) IIa (83 kDa), and SBEIIb (83 kDa) [11]. Interestingly, a starch granule-associated protein of >150 kDa was detected in NBS grown in 24 h light (Fig. 10). MS analysis of the polypeptide failed to identify this protein.

4. Discussion

The occurrence of growth rings irrespective of diurnal or constant light growing conditions (Figs. 1 and 3) suggests that the growth ring formation is not controlled by diurnal photosynthetic activity. It is not clear why growth rings were observed in barley starch cultivated under constant light in this study, but not observed in starch granules from barley cultivated under constant light conditions by Tester et al. [19] and Buttrose [7]. Previous studies on this issue utilized different light sources i.e. Bakhuyzen [6], and Buttrose [7] used mercury lamps while Tester et al. [19] used either mercury or Na lamps and Pilling and Smith [3] did not specify the light source. In our investigation mercury lamps were used and it remains thus unclear how the light source, or the intensity of illumination, influences the appearance of growth rings. Furthermore, there were variations in the appearance and visibility of growth rings. We investigated approximately 100 starch granules from each source by microscopy and all these had varying intensely growth rings, however, some of which were only vaguely visible, others very clear. Hence, sampling can be important and biased sampling can result in analysis of starch granules with less clear growth ring structures impeding the interpretation of the data.

Alternative explanations for the occurrence of growth rings in all samples regardless of lighting conditions include circadian rhythms and physical mechanisms [3]. Circadian rhythms may influence the appearance of growth rings through the periodic regulation of starch synthesizing enzymes such as GBSS and SSIII, which have been shown to directly influence the presence of growth rings [3]. However, the regulation of growth ring formation by circadian rhythms is unlikely in the case of the plants grown in continuous light for the majority of their life cycle. Growth ring formation may also be influenced by physical restraints as it is hypothesized that during the packing of double helices from adjacent external amylopectin chains, it may become energetically unfavorable to continue synthesizing crystalline components, resulting in a generation of the amorphous components [3]. The generation of amorphous component may then relieve any stresses induced on the matrix and allowing for the granule to synthesize new semi-crystalline areas [3].

Structural analysis of NBS indicated that under constant light growing conditions the concentration of amylose increased (Table 2 and Fig. 9). It is documented [25,26] that increasing amylose content results in an increased size of the crystalline lamellae and a corresponding decreased size of the amorphous lamellae, as amylose acts to disrupt the packing of amylopectin double helices. A

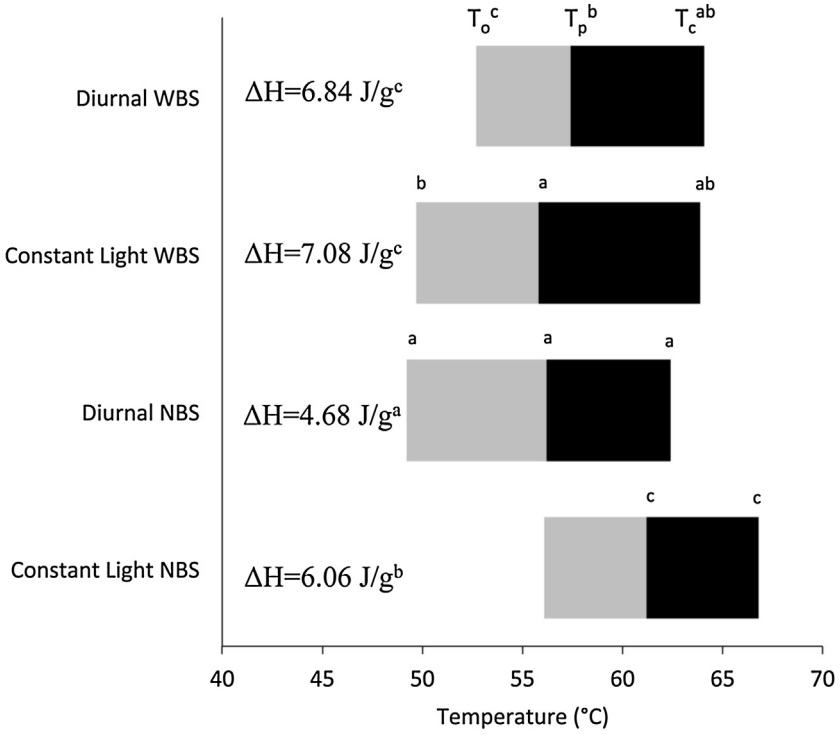

Fig. 6. Gelatinization parameters of NBS and WBS cultivated under diurnal or constant light conditions. T_o = Onset temperature; T_p = Peak melting temperature; T_c = Conclusion temperature; ΔH = Enthalpy of gelatinization. Values with same letters are not significantly ($p < 0.05$) different.

small angle x-ray study of a large number of different starch types [27] has revealed that for A-type crystalline polymorphic starches, increasing amylose content affects the crystalline packing of the double helical mesogens and increases the thickness of the crystalline lamella. Therefore, as amylose apparently influences the organization of the amorphous and crystalline lamellae, and one semi-crystalline growth ring may contain a zone of ~10 lamellar repeats [3] followed by an amorphous growth ring, it is a possibility that the altered organization of lamellae due to increased amylose may interfere with the contrast of semi-crystalline and amorphous rings, making them difficult to decipher without treatment with enzymes or dilute acid. In this study, growth rings present in NBS were not readily visible in their native state, and were only revealed following treatment with dilute (2.2 M) HCl. Similarly, Pilling and Smith [3] treated cracked starch granules with α-amylase from porcine pancreas to reveal the presence of growth rings in potato starch cultivated under constant light. It is essential to note that certain previous studies on the effect of growth rings under continuous illumination conditions, which reported their absence [19,28], did not treat the granules with acids or enzymes prior to visualization by light microscopy.

APTS has been demonstrated to almost fully penetrate the starch granule matrix [13,21], hence the non-even distribution of fluorescence after washing out the excess dye is most likely due to differential distribution of reducing ends in the starch granule. The structural properties of amylose were postulated to influence the appearance of growth rings in NBS when viewed by CLSM, as amylose is a smaller molecule in relation to amylopectin and therefore contains a greater molar ratio of reducing ends per anhydrous glucose residue, resulting in a greater amount of fluorescent labelling by weight compared to amylopectin [21]. The presence of alternating bright and darker growth rings observed in NBS by CLSM analysis (Fig. 3) can therefore be rationalized as the greater quantitative binding of the fluorescent probe by the amylose component compared to amylopectin, and the supposedly predominant deposition of amylose in the amorphous growth rings [29] compared to the semi-crystalline growth rings composed mainly of amylopectin. However, there are conflicting views on the location of amylose within the starch granule. Our previous study [21] found a high concentration of amylose in the center of the granule, which was supported in this analysis as normal barley starch displayed strong APTS fluorescence in the hilum (Fig. 3), whereas Jane and Shen [30] postulated that amylose components were more concentrated at the periphery of the granule than in the core following chemical gelatinization of normal potato starch with calcium chloride. As WBS is devoid of amylose (Table 2), the appearance of growth rings in WBS cannot be explained by the greater fluorescent label binding by weight of the amylose component, but rather must be explained by differences in fluorescent label binding by the amylopectin component.

An interesting notion is that NBS granules possessed a regular pattern of light spots arranged radially and tangentially when viewed by transmission light mode in the confocal microscope, especially in granules formed in constant light conditions (Fig. 3). The size of the spots was roughly 200–400 nm with somewhat smaller sizes towards the periphery of the granules. Badenhuizen and Katz [31,32] described similar patterns of structural entities, which they named blocklets, in wheat and potato starch granules. Since then, blocklets ranging in size from approximately 20 to 500 nm have been observed by several authors using atomic force microscopy in a range of different starch types including both *waxy* and non-*waxy* granules [33–36]. Recently, Herrera et al. [37] isolated starch nanoparticles (SNPs) from maize starch granules by acid treatment and they suggested the SNPs were individual blocklets. The yield of SNPs increased with the amylose content of the granules, however, *waxy* maize granules did not yield any SNPs. Interestingly, in this work the light spots, which possibly were visualized blocklets inside the intact granules, were readily observed in NBS, but not in WBS. In addition, the spots were more apparent in the NBS granules grown under constant light, which possessed

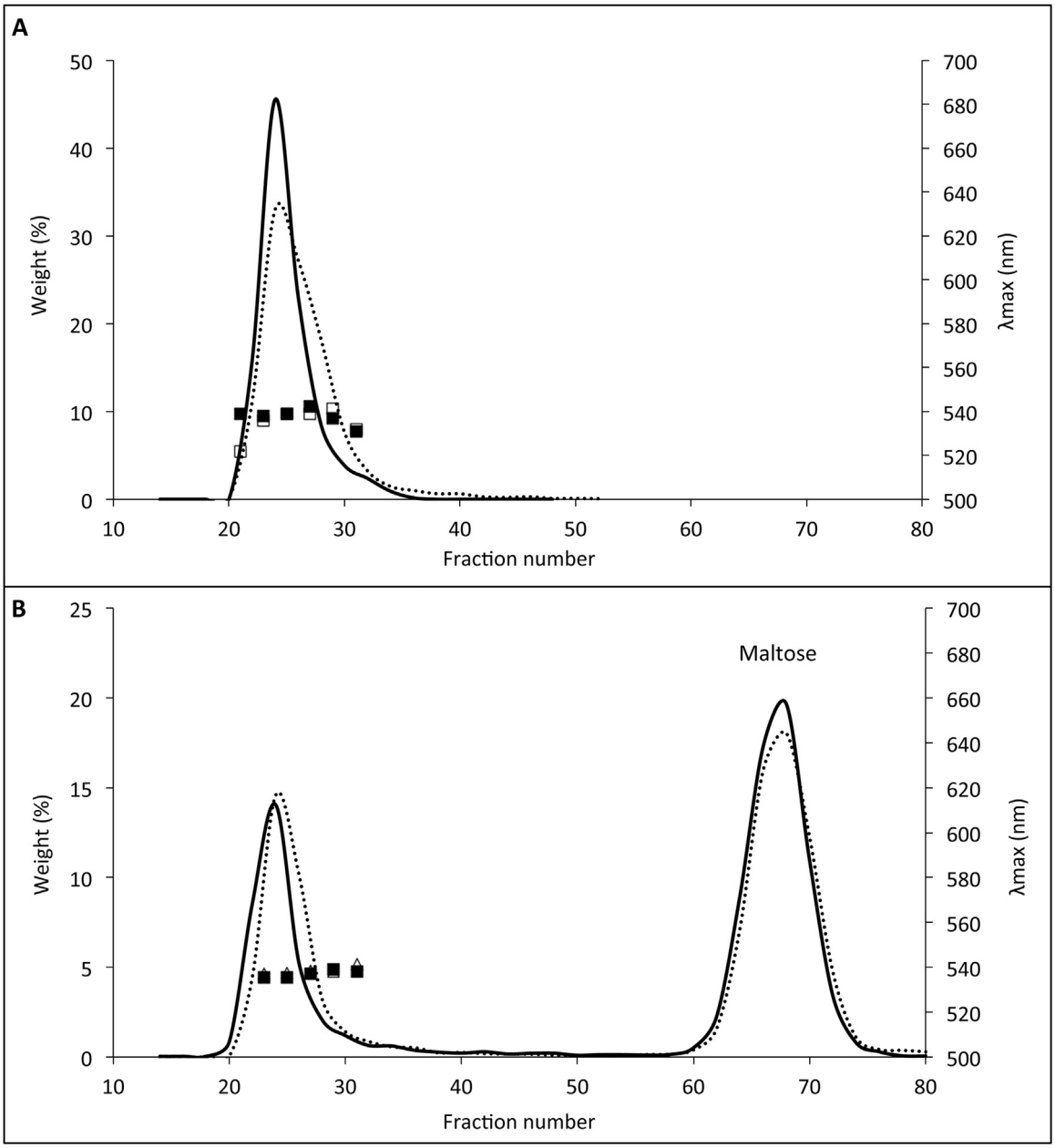

Fig. 7. Molecular size distribution of native (A) and β-amylase treated WBS (B) grown under diurnal (·····) and constant light conditions (––) determined by GPC on Sepharose CL 2B, with corresponding λ_{max} for diurnal (□) and constant light (■) conditions.

higher amylose content than their counterparts grown in diurnal conditions (Table 2). The actual nature of such blocklet structures remains unclear, however.

The physical and thermal characteristics of NBS and WBS were determined by X-ray diffraction (WAXS, Fig. 4) and DSC analysis (Fig. 6), respectively. For NBS, diurnal or constant light regimes did not influence the diffraction patterns (Fig. 4). The elevated amylose content (+4.4%), and consequently lower relative amylopectin content in NBS exposed to constant light did not appear to have an impact on the relative crystallinity. This observation supported findings reported elsewhere wherein similar differences in apparent amylose content of normal barley starch did not have a noticeable influence on the relative crystallinity [38]. While the relative crystallinity determined by WAXS provides an indication of the percentage of crystallinity with respect to the total material [39], the DSC endotherm obtained during the gelatinization of starch provides information on the quality and organization of the crystalline lamella [25,40]. The lower onset and peak melting temperatures of diurnally grown NBS can be interpreted that the starch was of lower crystalline quality than NBS cultivated under constant light conditions, as it has been suggested that the peak melting temperature represents a measure of starch crystalline perfection [25,41]. The lower enthalpy values of the diurnally grown NBS indicated that a lower quantity of double helices was synthesized. For the WBS sample, a substantial drop in relative crystallinity of the constant light samples compared to the diurnally grown sample was observed. The presence of the V-type crystal polymorph in WBS, which is typically associated with amylose-lipid complexes, was surprising, although its presence has been reported in other *waxy* barley starches, with the complex postulated to be formed between the outer branches of amylopectin and native lipids [38]. Numerous reports have documented such relationships between low relative crystallinity and low onset gelatinization temperatures [42,43]. This relationship was confirmed for the WBS sample in our

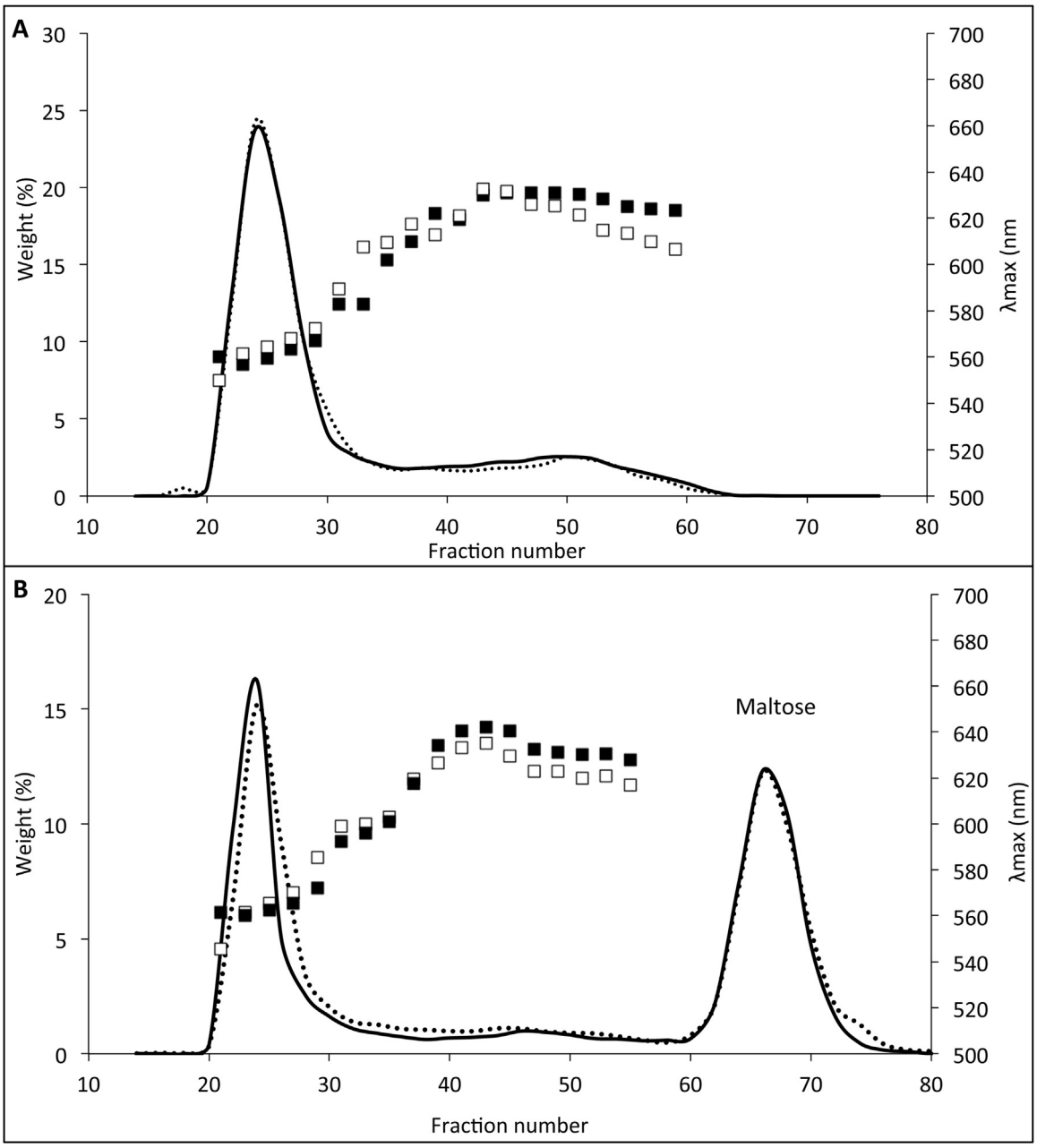

Fig. 8. Molecular size distribution of native (A) and β-amylase treated NBS (B) grown under diurnal (·····) and constant light conditions (––) determined by GPC on Sepharose CL 2B, with corresponding λ_{max} for diurnal (□) and constant light (■) conditions.

study. It has been suggested by Tester et al. [44] that the crystalline lamellae restricts the hydration of amorphous regions. Therefore, a higher relative crystallinity might imply that the amorphous region hydrates less efficiently resulting in a delayed initiation of swelling and gelatinization [43]. The fact that the enthalpy remained similar for the diurnal and the constant light exposed WBS, despite predicted differences in granule crystallinity (as discussed above), implied that a similar quantity of double helices were synthesized, although in constant light growing conditions a population of those helices may have been disoriented (unpacked) or the ordering at the ends of existing double helices were unpacked in the crystallites [45].

The molecular size distribution of WBS (Fig. 7) did not appear to be largely impacted when grown under constant light compared to diurnal conditions. In addition, when the starches were treated with β-amylase to produce β-LDs, a similar quantity of remaining internal components was observed. The similar quantities of maltose produced independent of light regime are indicative of a similar proportion of external chains independent of growing conditions. Moreover, similar λ_{max} values were observed for WBS and its β-LDs regardless of the light conditions. λ_{max} of the iodide-glucan complex positively correlates with the length of the glucan chain [46,47] and amylopectin can interact with iodide to form inclusion complexes with both the external amylopectin chains and internal amylopectin chains involved in the connection of clusters [17,48,49]. The observed WBS λ_{max} values implied that the nature of the complex-forming segments in the external and/or internal parts of the macromolecule were similar and independent of the light conditions indicating that the core structural features of amylopectin in the starch granule are not influenced by changes in photoperiod.

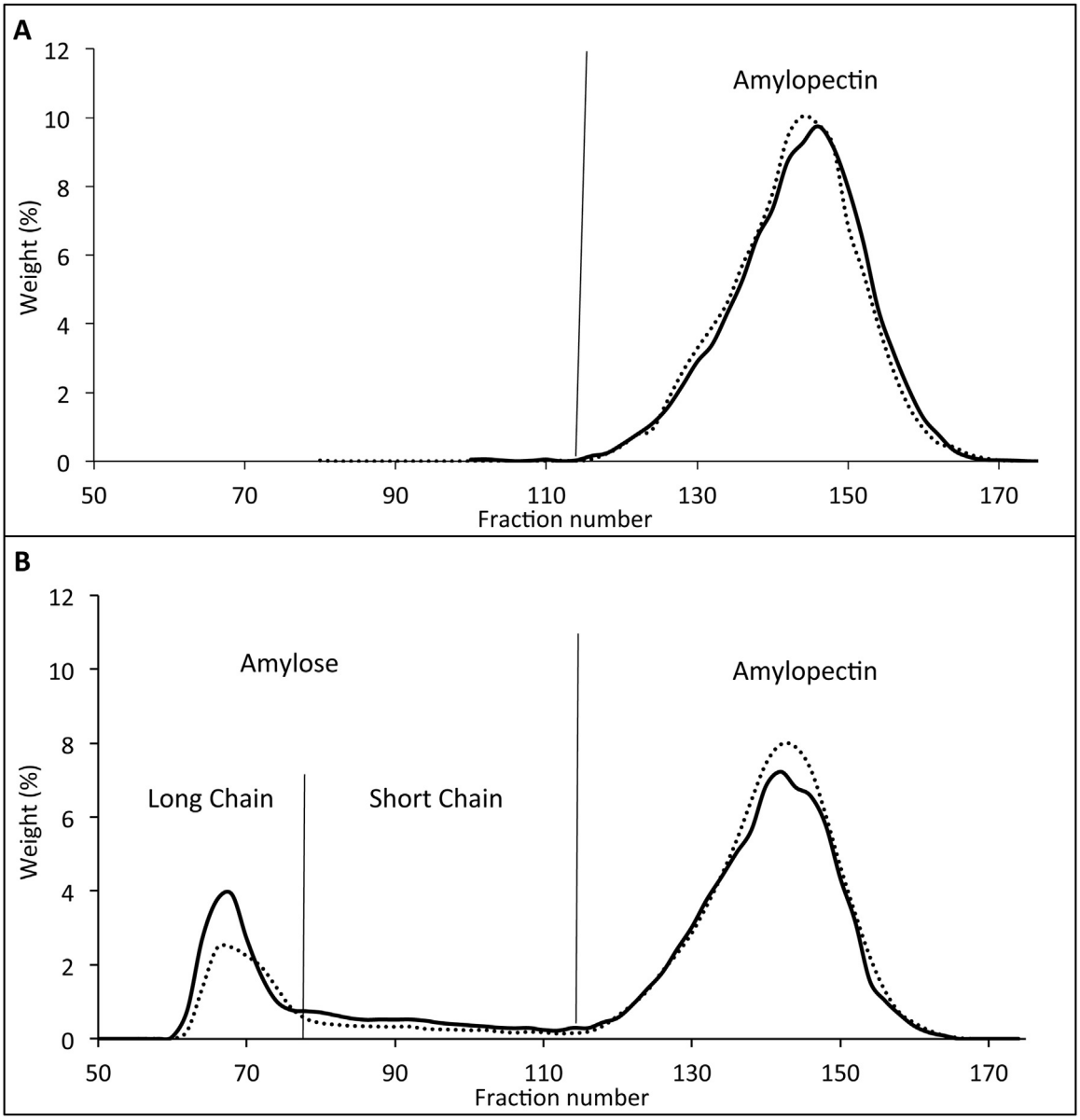

Fig. 9. Debranched profiles of diurnal (·····) and constant light conditions (––) cultivated WBS (A) and NBS (B) determined by GPC on Sepharose CL 6B.

The β-limit value of WBS (~61 wt%) was greater than that of NBS (42 wt%), implying that the amount and structure of branched amylose components, known to be present in barley starch [50], influenced the quantity of maltose produced by β-amylase. Like WBS, NBS displayed a similar molecular size distribution profile and λ_{max} values independent of light regimes (Fig. 8), although under constant light the λ_{max} values for smaller amylose molecules, (fractions 49–59) were higher. These differences in λ_{max} values suggest that the amylose component, but not the amylopectin, was affected by the lighting conditions. The differences in λ_{max} values of the amylose component complement and support the different size distribution profiles of debranched NBS when studied by GPC on Sepharose CL 6B (Fig. 9). NBS cultivated under diurnal light conditions exhibited a decreased amylose content, as well as a decreased ratio of long:short amylose chains (Table 2) compared to constant light conditions, inferring that diurnal photosynthetic activity influences the structure of the amylose component.

Following visualization of granule-bound starch proteins (Fig. 10) it was apparent that no differences in protein compliment of the major starch granule bound proteins were observed in NBS and WBS following cultivation in diurnal or constant light growing conditions. Therefore, differences in the molecular structure of NBS cannot be explained by differences in the quantity of granule-bound starch proteins. Amylose content is strongly influenced by available cellular ADP-glucose pools [51] and it is possible that these pools are greater (or are present throughout the growth period for longer time periods) in plants grown under continuous illumination, since ADP-glucose pyrophosphorylase (AGPase), responsible for ADP-glucose synthesis [52] is activated in the light through redox modulation [53]. Albeit the activation state and ADP-glucose content in the plants used in this study were not determined, it can be postulated that NBS grown under constant light conditions may contain a greater concentration of ADP-glucose in the plastid compared to the diurnal sample due to continually ongoing photosynthesis. Clarke et al. [51] reported that the rate of starch synthesis and the amylose:amylopectin ratio is correlated with the concentration of ADP-glucose in developing pea embryos. It is broadly agreed that the affinity of GBSSI, the key enzyme involved

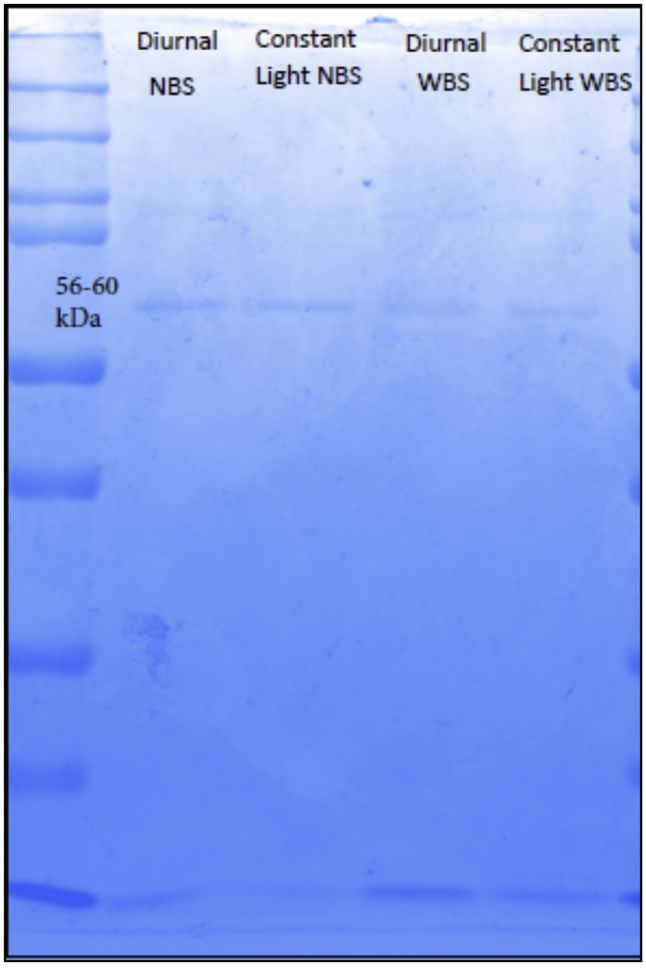

Fig. 10. Analysis of starch granule-bound proteins in NBS and WBS cultivated under diurnal or constant light conditions by SDS-PAGE followed by Coomassie Blue staining. 56–60 kDa corresponds to band representing GBSS proteins. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in the synthesis of amylose, for ADP-glucose is lower than that of the key amylopectin synthesizing enzyme soluble starch synthase [48]. Therefore, an increase in ADP-glucose will potentially increase the amylose:amylopectin ratio regardless the presence of the GBSS I enzyme.

5. Conclusions

The observation of growth rings in all barley samples does not support earlier reports of the absence of growth rings in barley starch cultivated under constant light, and argues against the notion that growth rings in barley starch are due to diurnal photosynthetic rhythms. Decreased amylose content, decreased long amylose chain components, and altered gelatinization profiles obtained from NBS cultivated under diurnal conditions compared to constant light conditions provides direct evidence that diurnal photosynthetic activity influences the structure and organization of NBS. For WBS, crystallinity and melting temperatures were higher when grown under diurnal conditions than under constant light implying that the diurnal photosynthetic oscillation facilitates crystalline registration and order. For NBS, mainly the crystalline order was increased with diurnal light. Hence, amylose can prevent structural flexibility to the granule and impart starch granule robustness against environmental cues.

References

[1] P.J. Jenkins, R.E. Cameron, A.M. Donald, A universal feature in the starch granules from different botanical sources, Starch/Stärke 45 (1993) 417–420.
[2] I.J. Tetlow, Starch synthesis in developing seeds, Seed Sci. Res. 21 (2011) 5–32.
[3] E. Pilling, A.M. Smith, Growth ring formation in the starch granules of potato tubers, Plant Physiol. 132 (2003) 365–371.
[4] S. Pérez, E. Bertoft, The molecular structures of starch components and their contribution to the architecture of starch granules: a comprehensive review, Starch/Stärke 62 (2010) 389–420.
[5] A. Meyer, Untersuchungen über die Stärkekörner, Fischer, Jena, Germany, 1895.
[6] H.V.D.S. Bakhuyzen, Starch grains of wheat considered as partially dehydrated amylose, Exp. Biol. Med. 23 (1925) 195–197.
[7] M.S. Buttrose, The influence of environment on the shell structure of starch granules, J. Cell Biol. 14 (1962) 159–167.
[8] V.A. Frey-Wyssling, M.S. Buttrose, Makromolekulare feinstlamellen in den körnern der kartoffelstärke, Makromol. Chem. 44 (1961) 173–178.
[9] M. Carciofi, S.S. Shaik, S.L. Jensen, A. Blennow, J.T. Svensson, É. Vincze, K.H. Hebelstrup, Hyperphosphorylation of cereal starch, J. Cereal Sci. 54 (2011) 339–346.
[10] A. Goldstein, G. Annor, J.L. Putaux, K.H. Hebelstrup, A. Blennow, E. Bertoft, Impact of full range of amylose contents on the architecture of starch granules, Int. J. Biol. Macromol. 89 (2016) 305–318.
[11] Z. Ahmed, I.J. Tetlow, R. Ahmed, M.K. Morell, M.J. Emes, Protein–protein interactions among enzymes of starch biosynthesis in high-amylose barley genotypes reveal differential roles of heteromeric enzyme complexes in the synthesis of A and B granules, Plant Sci. 233 (2015) 95–106.
[12] A. Shevchenko, M. Wilm, O. Vorm, M. Mann, Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels, Anal. Chem. 68 (1996) 850–858.
[13] M.A. Glaring, C.B. Koch, A. Blennow, Genotype-specific spatial distribution of starch molecules in the starch granule: a combined CLSM and SEM approach, Biomacromolecules 7 (2006) 2310–2320.
[14] S. Brückner, Estimation of the background in powder diffraction patterns through a robust smoothing procedure, J. Appl. Crystallogr. 33 (2000) 977–979.
[15] K. Frost, D. Kaminski, G. Kirwan, E. Lascaris, R. Shanks, Crystallinity and structure of starch using wide angle X-ray scattering, Carbohydr. Polym. 78 (2009) 543–548.
[16] M. Dubois, K.A. Gilles, J.K. Hamilton, P. Rebers, F. Smith, Colorimetric method for determination of sugars and related substances, Anal. Chem. 28 (1956) 350–356.
[17] D.N. Kalinga, R. Waduge, Q. Liu, R.Y. Yada, E. Bertoft, K. Seetharaman, On the differences in the granular architecture and starch structure between pericarp and endosperm wheat starches, Starch/Stärke 65 (2013) 791–800.
[18] J. Sargeant, Determination of amylose: amylopectin ratios of starches, Starch/Stärke 34 (1982) 89–92.
[19] R.F. Tester, J.B. South, W.R. Morrison, R.P. Ellis, The effects of ambient temperature during the grain-filling period on the composition and properties of starch from four barley genotypes, J. Cereal Sci. 13 (1991) 113–127.
[20] J.L. Jane, Z. Ao, S.A. Duvick, M. Wiklund, S.H. Yoo, K.S. Wong, C. Gardner, Structures of amylopectin and starch granules: how are they synthesized? J. Appl. Glycosci. 50 (2003) 167–172.
[21] A. Blennow, M. Hansen, A. Schulz, K. Jørgensen, A.M. Donald, J. Sanderson, The molecular deposition of transgenically modified starch in the starch granule as imaged by functional microscopy, J. Struct. Biol. 143 (2003) 229–241.
[22] D. Cooke, M.J. Gidley, Loss of crystalline and molecular order during starch gelatinisation: origin of the enthalpic transition, Carbohydr. Res. 227 (1992) 103–112.
[23] J. Luo, R. Ahmed, B. Kosar-Hashemi, O. Larroque, V.M. Butardo Jr., G.J. Tanner, M.L. Colgrave, N.M. Upadhaya, I.J. Tetlow, M.J. Emes, A. Millar, S.A. Jobling, M.K. Morell, The different effects of starch synthase IIa mutations or variation on endosperm amylose content of barley, wheat and rice are determined by the distribution of starch synthase I and starch branching enzyme IIb between the starch granule and amyloplast stroma, Theor. Appl. Gen. 128 (2015) 1–13.
[24] E.K. Asare, M. Baga, B.G. Rossnagel, R.N. Chibbar, Polymorphism in the barley granule bound starch synthase 1 (GBSS1) gene associated with grain starch variant amylose concentration, J. Agric. Food Chem. 60 (2012) 10082–10092.
[25] S.S. Kozlov, A. Blennow, A.V. Krivandin, V.P. Yuryev, Structural and thermodynamic properties of starches extracted from GBSS and GWD suppressed potato lines, Int. J. Biol. Macromol. 40 (2007) 449–460.
[26] P. Jenkins, A. Donald, The influence of amylose on starch granule structure, Int. J. Biol. Macromol. 17 (1995) 315–321.
[27] J.S. Sanderson, R.D. Daniels, A.M. Donald, A. Blennow, S.B. Engelsen, Exploratory SAXS and HPAEC-PAD studies of starches from diverse plant genotypes, Carbohydr. Polym. 64 (2006) 433–443.
[28] H.V.D.S. Bakhuyzen, The structure of starch grains from wheat grown under constant conditions, Exp. Biol. Med. 23 (1926) 302–305.
[29] E. Montgomery, F. Senti, Separation of amylose from amylopectin of starch by an extraction-sedimentation procedure, J. Polym. Sci. 28 (1958) 1–9.
[30] J.L. Jane, J.J. Shen, Internal structure of the potato starch granule revealed by chemical gelatinization, Carbohydr. Res. 247 (1993) 279–290.
[31] E.A. Hanson, J.R. Katz, Abhandlungen zur physikalischen Chemie der Stärke und der Brotbereitung. XVII. Über Versuche die gewachsene Struktur der

Stärkekorns mikroskopish sichtbar zu machen besonders an lintnerisierter Stärke, Z. Phys. Chem. (A) 168 (1934) 339–352.
[32] E.A. Hanson, J.R. Katz, Abhandlungen zur physikalischen Chemie der Stärke und der Brotbereitung. XVIII. Weitere Versuche die gewachsene Struktur der Stärkekorns mikroskopisch sichtbar zu machen, Z. Phys. Chem. (A) 16 (1934) 135–142.
[33] D.J. Gallant, B. Buochet, P.M. Baldwin, Microscopy of starch: evidence of a new lever of granular organization, Carbohydr. Polym. 32 (1997) 177–191.
[34] A.A. Baker, M.J. Miles, W. Helbert, Internal structure of the starch granule revealed by AFM, Carbohydr. Res. 330 (2001) 249–256.
[35] J.M.C. Dang, L. Copeland, Imaging rice grains using atomic force microscopy, J. Cereal Sci. 37 (2003) 165–170.
[36] H. Park, S. Xu, K. Seetharaman, A novel in situ atomic force microscopy imaging technique to probe surface morphological features of starch granules, Carbohydr. Res. 346 (2011) 847–853.
[37] M.P. Herrera, T. Vasanthan, R. Hoover, Characterization of maize starch nanoparticles prepared by acid hydrolysis, Cereal Chem. 93 (2016) 323–330.
[38] R. Waduge, R. Hoover, T. Vasanthan, J. Gao, J. Li, Effect of annealing on the structure and physicochemical properties of barley starches of varying amylose content, Food Res. Int. 39 (2006) 59–77.
[39] A. Lopez-Rubio, B.M. Flanagan, E.P. Gilbert, M.J. Gidley, A novel approach for calculating starch crystallinity and its correlation with double helix content: a combined XRD and NMR study, Biopolymers 89 (2008) 761–768.
[40] V. Vamadevan, E. Bertoft, K. Seetharaman, On the importance of organization of glucan chains on thermal properties of starch, Carbohydr. Polym. 92 (2013) 1653–1659.
[41] R.F. Tester, W.R. Morrison, Swelling and gelatinization of cereal starches. I. Effects of amylopectin amylose, and lipids, Cereal Chem. 67 (1990) 551–557.
[42] S. Gomand, L. Lamberts, L. Derde, H. Goesaert, G. Vandeputte, B. Goderis, J. Delcour, Structural properties and gelatinisation characteristics of potato and cassava starches and mutants thereof, Food Hydrocol. 24 (2010) 307–317.
[43] G. Vandeputte, R. Vermeylen, J. Geeroms, J. Delcour, Rice starches. I. Structural aspects provide insight into crystallinity characteristics and gelatinisation behaviour of granular starch, J. Cereal Sci. 38 (2003) 43–52.
[44] R.F. Tester, S.J.J. Debon, X. Qi, M.D. Sommerville, R. Yousuf, R.M. Yusuph, Amylopectin crystallisation in starch, Spec. Publ.–R. Soc. Chem. 271 (2001) 97–102.
[45] S. Kozlov, T. Noda, E. Bertoft, V. Yuryev, Structure of starches extracted from near isogenic wheat lines, J. Therm. Anal. Cal. 86 (2006) 291–301.
[46] J.M. Bailey, W.J. Whelan, Relationship between iodine stain and chain length, J. Biol. Chem. 236 (1960) 969–973.
[47] W. Banks, C.T. Greenwood, K.M. Khan, The interaction of linear: amylose oligomers with iodine, Carbohydr. Res. 17 (1971) 25–33.
[48] F. Chauhan, K. Seetharaman, On the organization of chains in amylopectin, Starch/Stärke 65 (2013) 191–199.
[49] X. Shen, E. Bertoft, G. Zhang, B.R. Hamaker, Iodine binding to explore the conformational state of internal chains of amylopectin, Carbohydr. Polym. 98 (2013) 778–783.
[50] Y. Takeda, C. Takeda, H. Mizukami, I. Hanashiro, Structures of large: medium and small starch granules of barley grain, Carbohydr. Polym. 38 (1999) 109–114.
[51] B.R. Clarke, K. Denyer, C.F. Jenner, A.M. Smith, The relationship between the rate of starch synthesis: the adenosine 5′-diphosphoglucose concentration and the amylose content of starch in developing pea embryos, Planta 209 (1999) 324–329.
[52] H.P. Ghosh, J. Preiss, Adenosine diphosphate glucose pyrophosphorylase: a regulatory enzyme in the biosynthesis of starch in spinach leaf chloroplasts, J. Biol. Chem. 241 (1966) 4491–4504.
[53] A. Tiessen, J.H.M. Hendriks, M. Stitt, A. Branscheid, Y. Gibon, E.M. Farré, P. Geigenberger, Starch synthesis in potato tuber is regulated by post-translational redox modification of ADP-glucose pyrophosphorylase, Plant Cell 14 (2002) 2191–2213.