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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.