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STARCH GRAIN SURFACE AND STARCH DEGRADATION IN TURIONS OF THE DUCKWEED *SPIRODELA POLYRHIZA (LEMNACEAE)*

Summary

Turions are survival organs of aquatic plants such as the great duckweed (*Spirodela polyrhiza*). They consist of approximately 50% storage starch (per dry weight) used to support the growth of newly formed sprouts following germination. They could be employed as a good model system for investigations of the storage starch degradation in plants. To induce starch degradation in the plant cells turions must be irradiated for a few days with continuous light absorbed by the plant photoreceptor phytochrome. During such treatment changes in the profile of proteins associated with the starch grain surface have been observed. It was shown by *in vitro* binding studies that several proteins (α-amylase, starch dikinase R1, β-amylase) are desorbed from the surface or lose the ability to bind to it. This effect was especially obvious when starch grains from turions irradiated for 4 days (irradiated samples) were compared to those from turions kept in darkness (dark control). A hypothesis was presented that unknown changes in the surface properties of starch grains might be very important in the mechanism of starch degradation, by altering the binding of proteins.

The aim of the study was to investigate these properties immediately before and after the start of the starch degradation. Precise structural analysis of the starch grain surface was performed using a non-contact atomic force microscopy (nc-AFM). The grain surface revealed increasing roughness and a reduced density of the structural elements in the samples after irradiation. Two different kinds of randomly organized surface elements were detected by nc-AFM: the one type of a globular structure and the other one more oblong. They could be considered as the carbohydrate lamellas situated in the different way at the starch granule surface. Both were observed to become larger after irradiation. This might be a result of binding of water molecules to the carbohydrate lamellas or bending the surface carbohydrate helices into superhelices by new inter-carbohydrate hydrogen bonds. Such a modification of the starch granule surface could be a consequence of events started by the photoreceptor phytochrome involving starch phosphorylation / dephosphorylation, perhaps mediated by the newly discovered starch dikinase.

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Intoduction

Turions are resting fronds of aquatic vascular plants. In *Spirodela polyrhiza* (duckweed), turions have an important function in the survival strategy of the plants as vegetative fronds cannot tolerate low temperatures, and, therefore, usually die during late autumn. These resting fronds overcome unfavourable seasons by sinking to the bottom of the ponds or lakes [6]. Turions contain two meristematic pockets from which new vegetative sprouts can develop following germination [1]. In spring, after rise of the temperature, turions germinate. The main storage compound in turions is starch [4]. It has been shown previously that starch does not seem to contribute to early events in germination. Instead, starch fulfils two distinct functions [7]. Firstly, starch secures the survival of turions during periods of unfavourable germination conditions by very slow degradation lasting for months or even years. Secondly, it supports accelerated growth of the newly formed sprouts following germination, in a faster degradation response lasting for a few days. This second response is regulated by light and this light effect is mediated by the plant photoreceptor phytochrome. Turions could be considered as a model system for investigation of the mobilisation of storage starch in plants. Starch degradation in turions could be induced by repetitive red light pulses (Rp) as reported by Dölger et al. [4]. Whereas one Rp per day, applied for a period of 6 days, shows already a measurable effect, the full response has only been observed after hourly applied Rps [3]. These results were explained in terms of a developing source-sink system and by the existence of two separate steps in the process of starch degradation in turions: formation of a sprout (= sink) during the Rp-induced germination, and starch degradation in the storage tissue (= source) induced by the second light treatment.

Following various light pre-treatments on *Spirodela polyrhiza* turions, native starch granules were isolated and two fractions of starch-related proteins were distinguished: proteins enclosed within the starch particles (starch-internalised proteins) and those attached to the surface (starch-associated proteins). Two starch associated proteins were identified immunochemically as α-amylase (EC 3.2.1.1) and the R1 protein [8, 9]. Continuous illumination with red light induces a rapid degradation of starch. Within the first 24 h of illumination the level of starch-associated α-amylase transiently increased and subsequently decreased rapidly. Similarly, the amount of the starch-associated R1 also decreased during illumination. The dissociation of both α-amylase and R1 from the starch granules preceded the decrease in starch content [9]. However, binding of the two proteins to starch granules remained unchanged when the turions did not perform net starch degradation as observed during continuous darkness. Thus, during net starch degradation so far unidentified changes are postulated to occur at the surface of the starch particles that are relevant for protein binding. This conclusion was supported by *in vitro* studies. The enzyme did bind to starch granules pre-

pared from dark-stored turions (in which starch degradation had not been initiated), but not to those isolated from illuminated (starch degrading) turions [9].

The aim of the study was to investigate the starch granule surface properties immediately before and after the start of the starch degradation and to detect possible changes in the presence of proteins at the starch grain surface.

Material and methods

Material

Formation of the turions

All experiments were performed using etiolated turions of the duckweed *Spirodela polyrhiza* (L.) Schleiden, strain SJ cultivated in the way described elsewhere by Appenroth *et al.* [2]. To obtain non-dormant turions (that are capable of phytochrome-induced germination), cold treatment (5 ± 1°C) was carried out for additional 28 days in continuous darkness. Except the cold period, turions were kept at 25.0 ± 0.1°C.

Isolation of starch granules

Following various light treatments, turions and newly formed sprouts were frozen in liquid nitrogen and stored at – 80°C until use. The samples were prepared according to the method described by Ritte *et al.*(2000). The obtained starch fraction was washed twice with buffer 0.5 M HEPES-KOH, pH 7.0 (5 ml and 1 ml, respectively), and dried to dryness under vacuum for approximately 8 h. The samples were stored at – 80°C until use. Starch was quantified according to Ley *et al.* [7].

Methods

Light sources and irradiation

For the irradiation experiments the following light sources and filters were used: red light pulses (λ_{max} = 683 nm, half-bandwidth 63 nm, 490 μmol m^{-2} s^{-1}, 5 min) were applied using a slide projectors (Diafant 250, Liesegang, Düsseldorf, Germany; 24 V / 250 W) equipped with a glass filter RG645, 3 mm thick (Schott, Mainz, Germany) and a dichroic filter IR7, 3 mm thick (OptoChem, Stromberg, Germany); for illumination with continuous red light (λ_{max} = 658 nm, half-bandwidth 25 nm, 12 μmol m^{-2} s^{-1}) red fluorescence tubes (36 W/ 60; Osram, München, Germany) plus a red Plexiglas (GS501, 3 mm thick; Röhm, Darmstadt, Germany) were used. All manipulations of the turions were carried out in dim green light (λ_{max} = 553 nm, half-band width 8 nm, < 0.2 μmol m^{-2} s^{-1}) as described before by Appenroth *et al.* [2].

Non-contact atomic force microscopy

High-resolution non-contact Atomic Force Microscopy (nc-AFM) was performed using a Park Scientific Instrument Autoprobe CP model (California, USA) of the Regional Laboratory for Physicochemical Analyses and Structural Research at the Jagiellonian University, described elsewhere [5, 12]. Starch granules were spread onto an adhesive tape fixed onto an AFM sample holder, and observed at ambient conditions. The granules were partially embedded in the "sticky tape" to overcome problems with the large height variation in granule topography. For each starch sample images of several starch granules were collected.

Results and discussion

The surface of control starch grains isolated from dark kept turions of *Spirodela polyrhiza* and analyzed by nc-AFM is shown in Fig. 1. The starch grain surface revealed increasing roughness and a reduced density from ca.160/μm^2 to 50/μm^2 of its structural elements after irradiation with continuous red light for 4 days. Two kinds of randomly organized surface species were detected at the starch granule surface by nc-AFM: the one type of a globular structure and the other more oblong (Fig. 1 and Fig. 2). They could be considered as the carbohydrate lamellas situated in different ways at the starch granule surface. Those, densely packed were visible from the top-side (so detected as globular elements), while the other-loosely packed, laying at the surface were side-viewed by the microscope (and detected as oblong species). This observation indicates that the structure elements were not uniformly distributed at the grain surface. After the irradiation the surface species became larger. It was estimated that the granular elements of the dark control samples (no starch degradation) have a diameter of approximately 60 nm whereas the same elements from samples irradiated for 4 days (starch degradation) have a diameter of 100 nm (Fig. 3 and Fig. 4). The oblong elements were approximately 50 nm thick and 120 nm long before irradiation. Following a red light irradiation, the size of these elements increased to approximately 70 nm and 170 nm, respectively (Fig. 5 and Fig. 6). The observed modification of the grain surface is most probably not the result of a physical interaction of starch and light but, more indirectly, a consequence of events started by the plant photoreceptor phytochrome involving starch phosphorylation / dephosphorylation. The function of the so-called R1 protein as starch dikinase, postulated already several years ago [8], was recently revaeled by Ritte *et al.* [11]. The phosphorylation level may enhance the binding of more water molecules to the carbohydrate helices or may induce the formation of new hydrogen bonds between lamellar helices present at the granule surface, which bend together into superhelices. Such a process, involving of some surface OH-groups, supported by the decreasing of the surface element density, might result in lowering of

the granule surface capacity towards proteins. The decreased protein binding under starch degrading conditions was shown in several systems [9, 10]. A number of problems still remains to be investigated to understand the role of the starch grain surface properties in the starch degradation process.

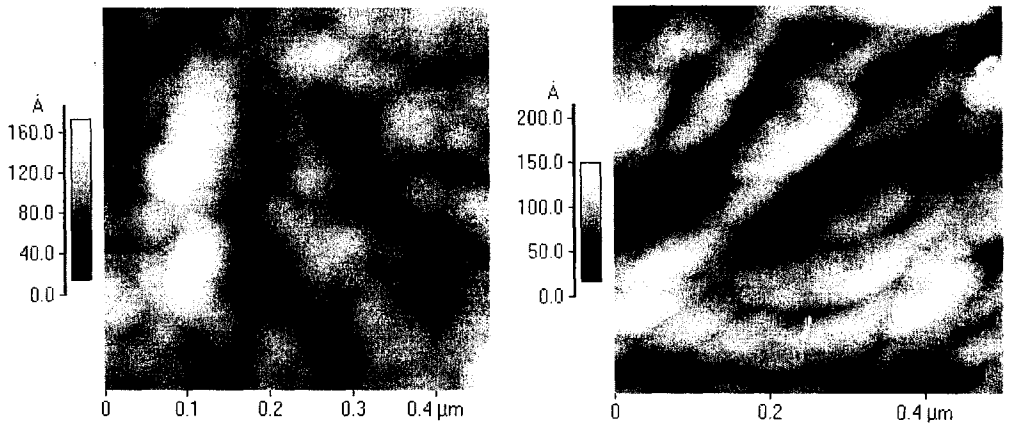


Fig. 1. Nc-AFM images of the surface elements of the dark control (native) starch samples.

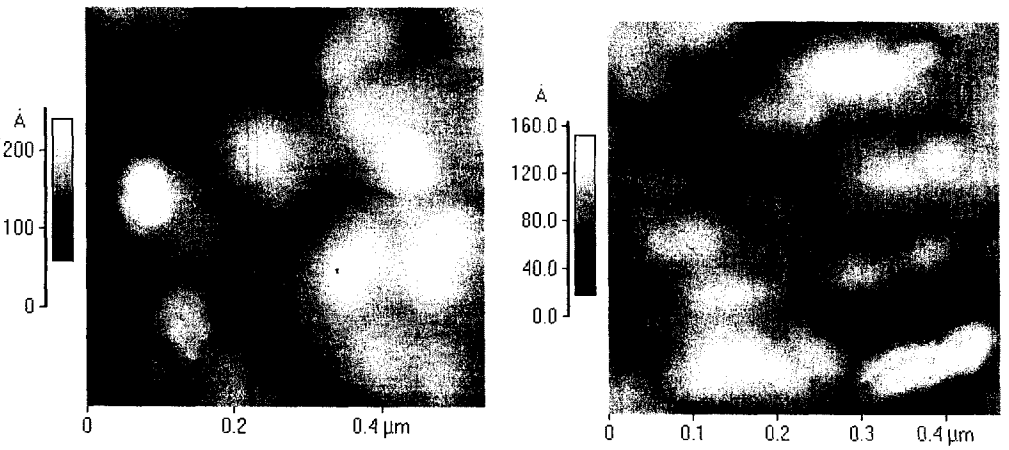
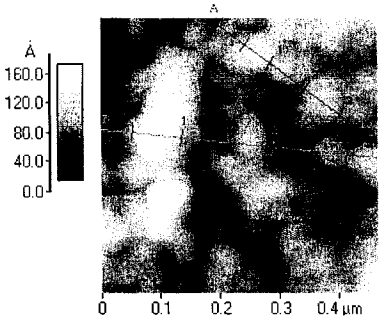
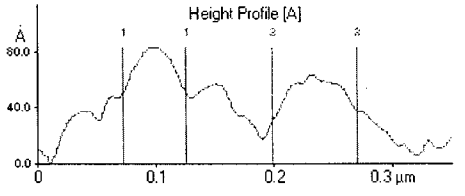


Fig. 2. Nc-AFM images of the surface elements of the irradiated starch samples.



Line	Distance [nm]
[A]	1: 52,2 2: 73,0
[B]	1: 82,7 2: 75,4 3: 60,7



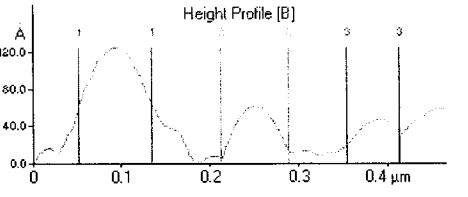
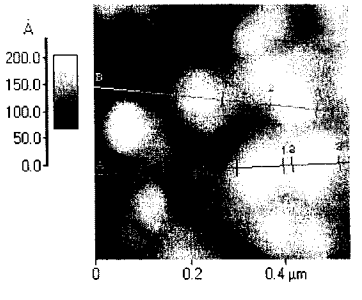
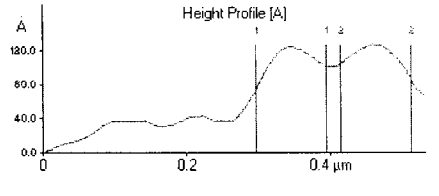


Fig. 3. Dimensions estimated for the granular species observed at the surface of the dark control starch grains.



Line	Distance [nm]
[A]	1: 98,9 2: 98,9
[B]	1: 97,1 2: 99,4



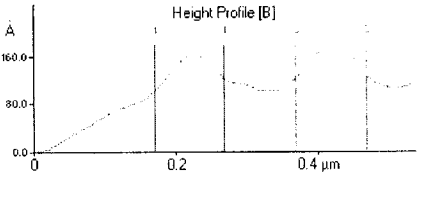
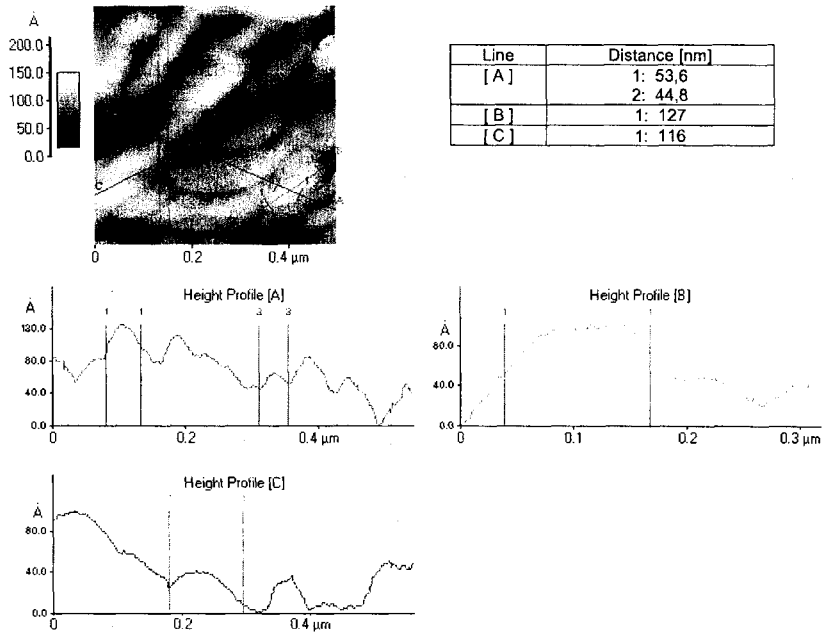
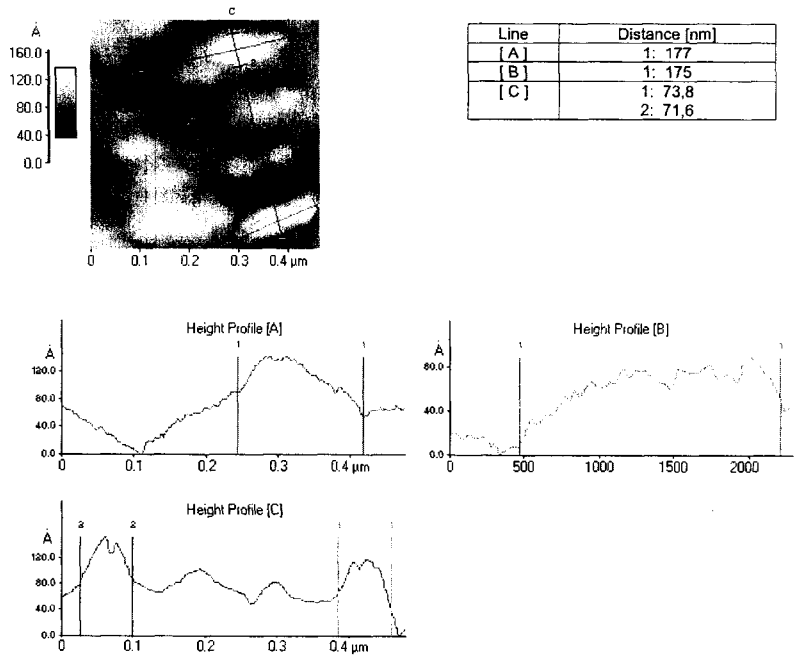


Fig. 4. Dimensions estimated for the granular species revealed at the surface of the irradiated starch grains.



Line	Distance [nm]
[A]	1: 53,6 2: 44,8
[B]	1: 127
[C]	1: 116

Fig. 5. Dimensions estimated for the oblong species revealed at the surface of the dark control starch grains.



Line	Distance [nm]
[A]	1: 177
[B]	1: 175
[C]	1: 73,8 2: 71,6

Fig. 6. Dimensions estimated for the oblong species observed at the surface of the irradiated starch grains.

Conclusions

1. Red light irradiation caused an increasing of the granule surface roughness and reduced density of the structural elements. These structural elements became larger after irradiation probably due to binding of water molecules to the carbohydrate lamellas or bending the surface carbohydrate helices into superhelices by new inter-carbohydrate hydrogen bonds.
2. Observed modification of the starch granule surface could be a consequence of events started by the photoreceptor phytochrome involving phosphorylation / dephosphorylation of starch. This could result in lowering of the granule surface capacity towards binding proteins.
3. A number of problems still remains to be investigated to understand the role of the starch grain surface properties in the starch degradation process.

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ZMIANY POWIERZCHNI GRANULI SKROBIOWEJ A DEGRADACJA SKROBI W TURIONACH ROŚLINY WODNEJ *SPIRODELA POLYRHIZA (LEMNACEAE)*

Streszczenie

Turiony są organami przetrwalnikowymi roślin wodnych, jak *Spirodela polyrhiza* Zawierają ok. 50% skrobi, która jest zużywana na wspomaganie rozwoju nowo powstających kiełków. Turiony mogą służyć jako modelowy system do obserwacji procesu degradacji skrobi w roślinach. Rozpoczęcie tej degradacji, po kilkudniowym naświetleniu turionów światłem ciągłym, absorbowanym przez fotoreceptor roślinny – fitochrom. Początek degradacji skrobi jest związany z desorpcją protein (alfa-amylaza, beta- amylaza czy skrobiowa dikinaza R1) z powierzchni granul skrobiowych. Efekt ten jest szczególnie wyraźny po porównaniu skrobi pochodzącej z turionów roślin naświetlanych przez 4 dni ze skrobią turionów roślin trzymanych w ciemności. Założono więc, że naświetlanie powoduje nieznane dotychczas zmiany na powierzchni granuli skrobiowej, które wpływając na wiązanie protein decydują o mechanizmie degradacji skrobi. Celem badań było obserwowanie powierzchni gałeczek skrobiowych przed i po naświetlaniu, czyli tuż przed i po starcie degradacji skrobi. Precyzyjna analiza powierzchni gałeczek skrobi była wykonana metodą bezkontaktowej mikroskopii sił atomowych (nc-AFM). W próbkach skrobi naświetlanej stwierdzono większą chropowatość powierzchni gałeczki skrobiowej i luźniejsze upakowanie jej elementów. Na badanych powierzchniach zaobserwowano przypadkowo rozmieszczone elementy o dwu rodzajach kształtów: bardziej okrągłe lub podłużne. Można uważać je za węglowodanowe łańcuchy w różny sposób usytuowane na powierzchni granuli. Stwierdzono, że po naświetlaniu powierzchniowe „cegiełki" zwiększają swoje rozmiary. Może to być spowodowane przyłączaniem cząsteczek wody do łańcuchów glukozowych albo też efekt łączenia się tych łańcuchów w większe poprzez międzycząsteczkowe wiązania wodorowe. Obserwowana modyfikacja powierzchni jest prawdopodobnie skutkiem zdarzeń zapoczątkowanych przez fotoreceptor fitochromowy, a obejmujących fosforylację/defosforylację skrobi przy współudziale nowoodkrytej dikinazy skrobiowej.