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Characterization of the highly branched glycogen from the thermoacidophilic red microalga *Galdieria sulphuraria* and comparison with other glycogens

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ABSTRACT

The thermoacidophilic red microalga *Galdieria sulphuraria* synthesizes glycogen when growing under heterotrophic conditions. Structural characterization revealed that *G. sulphuraria* glycogen is the most highly branched glycogen described to date, with 18% of α-(1 → 6) linkages. Moreover, it differs from other glycogens because it is composed of short chains only and has a substantially smaller molecular weight and particle size. The physiological role of this highly branched glycogen in *G. sulphuraria* is discussed.

1. Introduction

Energy storage molecules are important in the physiology of cells during periods of transient nutrient starvation often encountered in nature. Starch (present in plants and various species of green and red algae) and glycogen (present in some bacteria, yeast and animal cells) are two of the most common energy reservoirs molecules. Both are glucose polymers with a linear backbone of α-(1 → 4) linked units and branches attached at certain points through α-(1 → 6) linkages. However, they differ in the percentage and distribution of branches, which at the end influences the structure and properties of the molecule itself [1]. Starch is composed of two types of polymers: amylose, with virtually no branching points, and amylopectin, with 5–6% of α-(1 → 6) linkages distributed in a non-random manner [2]. The branched regions are arranged in clusters separated by linear regions, leading to the appearance of crystalline sections and the formation of insoluble starch granules [3]. Glycogen contains shorter linear chains compared to amylopectin and a higher number of random branching points arranged in tiers [4]. The consequence is that glycogen adopts a globular form which is highly soluble in water because it prevents chain interaction and granule formation. The branching degree of glycogen depends on its source and ranges from 7 to 13% [5–9].

Red algae (Rhodophyta) are a group of photosynthetic eukaryotes that include unicellular and multicellular species. The energy storage compound of red algae is generally called floridean starch, named after one of the taxonomic groups in which Rhodophyta is classified [10]. Earlier research was focused on describing the differences in the precursor molecule and the intracellular location of the floridean starch pathway with respect to the plant and green algae starch [11]. However, more recent studies have shown that the composition of floridean starch, regarding the presence or absence of amylose [12], and the structure, regarding the level of branching, varies among species [13–15]. *Galdieria sulphuraria* is an unicellular red alga that is considered to be one of the most primitive eukaryotes on earth [16]. It is able to thrive in acidic environments with pH values from 0 to 4 at temperatures up to 56 °C, conditions found in hot sulfur springs and volcanic areas around the world. The harsh conditions in which this alga lives have pushed *G. sulphuraria* towards adaptation through the development of metabolic flexibility [17], being able to grow autotrophically, performing photosynthesis when light is available, and heterotrophically, using

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several organic compounds as carbon source in the absence of light [18]. The glucan accumulated by *G. sulphuraria* as energy reservoir has been analyzed in a previous work whose aim was to point out the differences in the structure of this molecule between several red algae species belonging to the same order [13]. This study showed that a strain of *G. sulphuraria* isolated from Japan and grown autotrophically in a concentrated mineral medium accumulated a glycogen-type of molecule, differing from the other members of its order, in which a plant-like starch or semi-amylopectin type of molecule was synthesized.

In this study, the structure of glycogen extracted from the red microalga *G. sulphuraria* strain SAG 108.79 native to Yellowstone National Park growing under heterotrophic conditions is characterized in detail and compared to prokaryotic and eukaryotic glycogens using the same experimental procedures.

2. Materials and methods

2.1. Materials

Glycogen from Oyster type XI was purchased from Sigma-Aldrich. Glycogens from *Pseudomonas* V-19, *Sphaerotilus natans* and *Arthrobacter viscous* were a kind gift from dr. M. Breedveld (Groningen), who obtained the samples from late dr. L. Zevenhuizen (Wageningen University).

2.2. Strains, media and growth conditions

G. sulphuraria strain SAG 108.79 was obtained from the culture collection of the University of Göttingen (Sammlung von Algenkulturen, Germany). Cells were maintained growing on 1.5% agar plates of Allen's mineral medium [19] at pH 4 and 40 °C with constant illumination of 100 $\mu E/m^2 s^{-1}$. Colonies were transferred to a fresh plate once a month. For glycogen extraction, *G. sulphuraria* cells were grown in 1 L flasks containing 500 mL of Allen medium at pH 2 supplemented with 1% (w/v) glycerol at 40 °C in darkness on a rotary shaker at 150 rpm.

Escherichia coli strain DSM 10235 and *Arthrobacter globiformis* strain DSM 20124 were purchased from the culture collection of the Leibniz Institute (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany). For glycogen extraction, *E. coli* and *A. globiformis* cells were grown in 1 L flasks containing 200 mL of M9 mineral medium at pH7 supplemented with 0.5%(w/v) tryptone (BD Bacto) and 1% D-glucose at 37 °C (for *E. coli*) or 30 °C (for *A. globiformis*) on a rotary shaker at 180 rpm.

2.3. Glycogen extraction from G. sulphuraria

G. sulphuraria cells were grown until late exponential phase and harvested by centrifugation at 10,000 × *g* for 5 min at room temperature. The cell pellet was washed twice with ultra-pure water and freeze-dried. Dry cells were resuspended in 20 mL of ultra-pure water, mixed with 10 mL of glass beads (425–600 μm, Sigma-Aldrich) and disrupted by shaking for 10 min in a Mixer Mill (MM400, Retsch) at a frequency of 30 Hz. The cell lysate was transferred to a new tube and centrifuged at 20,000*g* for 10 min. The supernatant, free of cell-debris and unbroken cells, was incubated in a waterbath at 100 °C for 15 min to denature and precipitate proteins and then centrifuged at 20,000*g* for 20 min. The supernatant was transferred to a new tube and 0.1 volumes of 50% trichloroacetic acid (TCA) were added to further precipitate residual proteins. The sample was incubated on ice for 10 min and then centrifuged at 20,000*g* for 20 min. The clear supernatant was mixed with 1 volume of absolute ethanol to precipitate the glycogen at 4 °C. Precipitated glycogen was recovered by centrifugation at 10,000*g* for 10 min and freeze-dried. The dry glycogen was resuspended again in ultra-pure water, precipitated with 1 volume of absolute ethanol and freeze-dried.

To exclude a possible influence of intracellular amylase activity during extraction on the final structure of the glycogen, two additional versions of the extraction protocol were also tested. In one of the versions, cell disruption was performed at low temperature and the sample was always kept on ice in between steps. The dry cells were resuspended in cold (4 °C) ultra-pure water and disrupted by bead beating in pre-cooled (−20 °C) jars containing glass beads. The cell lysate was centrifuged at 20,000*g* for 10 min at 4 °C to remove cell debris and the supernatant was then boiled at 100 °C for 15 min to denature and precipitate proteins. From this point on, the sample was further treated as described above. In the other version, intracellular enzymes were inactivated prior to cell disruption by boiling the cell suspension at 100 °C for 15 min. Cell disruption and glycogen extraction was continued as described above.

2.4. Glycogen extraction from E. coli and A. globiformis

E. coli and *A. globiformis* were grown on M9 media containing tryptone and glucose until late exponential phase. Cells were harvested, washed with sterile water, resuspended in the same volume of M9 media containing glucose but not tryptone and incubated under the same conditions for the same amount of time as before. Then, cells were harvested by centrifugation at 20,000*g* for 10 min at 4 °C, the cell pellet washed twice with ultra-pure water and freeze-dried. The dry pellet was resuspended in ultra-pure water and cells were disrupted by passing them 5 times through an EmulsiFlex B-15 disruptor (Avestin) at a homogenizing pressure of 36,000 psi, cooling down on ice between disruptions. Cell debris and unbroken cells were removed by centrifugation at 20,000*g* for 10 min at 4 °C and the cell lysate was further treated in the same way as described in Section 2.2 to precipitate the glycogen, with the exception that no boiling step was performed.

2.5. ^1H NMR analysis

5 mg of glycogen was dissolved in 600 μL of deuterium oxide (D_2O, 99.9%$_{atom}$, Sigma–Aldrich), freeze-dried and exchanged in the same solvent two more times. Samples were measured in a NMR 500 Hz spectrometer (Varian) with the probe temperature set at 80 °C to avoid overlapping of the peaks of interest with the signal originating from residual water in the sample, thus allowing a more precise integration. For an accurate quantification of the branched and linear linkages in the molecule based on the integration of the signals, special attention was payed to the delay time between pulses in order to allow all protons to fully relax before a new pulse was applied. An experiment to measure the T1 (spin-lattice) relaxation times of the protons of interest was carried out following the inversion-recovery method with 10 tau values. The longest T1 (that of the H1 in the α-(1 → 6) linkage) was 1.49 s and, in consequence, the relaxation delay during spectra acquisition was set to 25 s, thus more than 5 times the longest T1value, as recommended in literature [20]. The spectra were acquired by running 16 scans with a 90° pulse. Acetone (δ^1H 2.225 ppm in D_2O) was used as internal reference for chemical shift assignment. Data were analyzed using MestReNova 9.1.0 (Mestrelab Research S.L.). The percentage of each type of linkage was calculated setting the total integral value of both peaks to 100.

2.6. Chain length distribution

Glycogen samples were dissolved in ultra-pure water to a final concentration of 20 mg/mL and then 500 μL of this solution were mixed with 3 volumes of sodium acetate buffer 200 mM pH 4.5 con-