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Fabrication of doughnut-shaped particles from spheroidal paramylon granules of *Euglena gracilis* using acetylation reaction

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ABSTRACT

An anhydrous type of paramylon, the micro-sized granular storage carbohydrate (β-1,3-glucan) of *Euglena*, was transformed from a spheroidal to a doughnut-like shape by acetylation. Fourier transform infrared spectroscopic measurements suggested that the doughnut formation is due to removal of accessible regions of paramylon particles by acetylation of glucans. A time-course observation of the paramylon granules during acetylation by using field emission scanning electron microscopy revealed that the doughnut-making process begins with the removal of an outer membrane of the granule and that the central region of the granules is preferentially removed with the survival of a thick rim part to give the doughnut-like particles.

1. Introduction

Over the past few years, much attention has been paid to the production of useful materials from microalgae. This interest primarily hinges on the fact that microalgae can produce chemicals through carbon dioxide fixation and photosynthesis (Chisti, 2007; Kumar et al., 2010; Kurano & Miyachi, 2005; Raja, Hemaiswarya, Kumar, Sridhar, & Rengasamy, 2008; Schenk et al., 2008; Sydney et al., 2010; Ugwu, Aoyagi, & Uchiyama, 2008). While the primary object of current studies have been the production of biofuels, microalgae are a source of materials for other interesting applications. *Euglena*, microalgae that belong to both the plant and animal kingdoms, have been intensively studied as a possible source for various chemicals (Buetow, 1968; Chae, Hwang, & Shin, 2006; Hayashi, Toda, Ishiko, Komatsu, & Kitaoka, 1994; Kott & Wachs, 1964; Rodriguez-Zavala, Ortiz-Cruz, Mendoza-Hernandez, & Moreno-Sanchez, 2010; Yamane, Utsunomiya, Watanabe, & Sasaki, 2001). Our own interest in *Euglena* has focused on the use of paramylon, the storage carbohydrate (β-1,3-glucan) of the euglenoid algae (Barras & Stone, 1968; Brandes, Buetow, Bertini, & Malkoff, 1964; Clarke & Stone, 1960), for novel materials. Previous studies by other researchers have indicated that the paramylon of *Euglena gracilis*, the most well-researched euglenoid alga, has a micro-sized spheroidal shape and high crystallinity (more than 90%) (Buetow, 1968; Booy, Chanzy, & Boudet, 1981; Brandes et al., 1964; Clarke & Stone, 1960; Guttman, 1971; Holt & Stern, 1970; Kiss, Roberts, Brown, & Triemer, 1988; Kiss, Vasconcelos, & Triemer, 1987; Marchessault & Deslandes, 1979; Tamura, Wada, & Isogai, 2009). Despite these unique features, its applications have yet to be developed (Rodriguez-Zavala et al., 2010). We felt that while this granule is promising for novel useful materials, the creation of paramylon-based materials requires additional structural features and/or granule functions. With this consideration in mind, we initiated a program aimed at modifying the granule. In view of the spheroidal shape being the most significant feature, we originally thought that the best approach for bringing added values to this granule would be surface modification, which has previously been applied to other particles (Cigler, Lytton-Jean, Anderson, Finn, & Park, 2010; Huang et al., 2008; Ismaili, Lee, & Workentin, 2010; Kobayashi, Kimura, Togawa, Wada, & Kuga, 2010; Liu et al., 2008; Susumu et al., 2007).

In the course of examining the feasibility of surface chemical modifications, we found that *the granules were uniformly transformed from a spheroid into a doughnut-like shape under an acetylation condition*. We term the resulting granule a "paramylon doughnut." The main focus of this paper is a description of this finding. We also discuss the probable fabrication process for this unique doughnut.

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2. Experimental

2.1. Materials

All chemicals and reagents were commercially available and used without further purification.

2.2. Preparation of paramylon granules

E. gracilis NIES-48 (NIES Culture Collection) was cultured in an aqueous medium (27 L) containing 675 g of glucose, 135 g of corn steep liquor, 72.9 g of ammonium sulfate, 13.5 g of potassium dihydrogen phosphate, 13.5 g of magnesium sulfate heptahydrate, 5.4 g of calcium carbonate, 1.35 g of sodium ethylenediaminetetraacetate, 1.35 g of ammonium iron (II) sulfate hexahydrate, 486 mg of manganese (II) sulfate hydrate, 675 mg of zinc sulfate heptahydrate, 67.5 mg of vitamin B1, and 135 μg of vitamin B12 at 28 °C in the dark (pH 4.5). After cultivation for 72 h, the cells were collected by centrifugation (1500 × *g*, 10 min), redispersed in water (5.0 L), and sonicated for disrupting the cells. After centrifugation (1500 × g, 10 min), the pellet was dispersed in 20 L of water containing 1% (w/v) sodium lauryl sulfate, and heated in boiling water (50 L) for 30 min to remove proteins. The residual solid was washed with ion-exchange water twice, followed by air-drying at 80 °C to provide the desired product (165 g, yield 55%). The product was determined to be an anhydrous type by comparing its X-ray diffraction diagram with a previously reported one (Kobayashi et al., 2010). Hydrated granules were prepared by soaking the anhydrous ones in water for 10 days, and their successful preparation was confirmed by X-ray diffractometry (see Supplementary data).

2.3. Preparation of paramylon doughnuts

The paramylon doughnuts were prepared using a method similar to those for synthesizing cellulose acetate previously described (Meireles et al., 2010). Typically, 62.8 mg of the paramylon granules and 1.25 mL of acetic acid were placed into a round-bottomed flask. After the mixture was stirred for 1 h at ambient temperature under a nitrogen atmosphere, 1.5 mL of acetic acid, 1.5 mL of acetic anhydride, and 10 μL of sulfuric acid were added to this solution. After stirring for 4 h, the reaction mixture was treated in a centrifuge to afford a white precipitate. The solid residue was then thoroughly washed sequentially with acetic acid and methanol, followed by concentration under reduced pressure to give the desired product as a white powder (25.2 mg, yield 40%).

2.4. Scanning electron microscopy

Scanning electron microscopy was carried out under high vacuum at 5 kV accelerating voltage by using an S-4300 field emission scanning electron microscope (Hitachi). Granular samples required for observation were fixed on a microscope stage using a carbon conductive double-faced tape and sputter-coated with Pt–Pd using an E-1030 sputter coater (Hitachi).

2.5. Fourier transform infrared spectroscopy

Fourier transform infrared spectra of the granules were recorded using a JASCO FT/IR-480ST spectrophotometer equipped with an attenuated total reflectance accessory (ATR Pro 400-S, ZnSe prism, JASCO). All spectra were recorded between 4000 and 650 cm^{-1} with a resolution of 4 cm^{-1}.

2.6. Determination of degree of substitution

The degree of substitution (DS), which is the average number of acetyl groups attached to the glucose unit, was determined according to a method previously described (Rodrigues et al., 2008). In essence, ca. 50 mg of acetylated paramylon and 2.5 mL of ethanol were placed in a 50 mL conical flask. 2.5 mL of an NaOH aqueous solution (0.25 N) was added to the mixture and then it was mechanically stirred at ambient temperature overnight. After adding 5.0 mL of an HCl aqueous solution (0.25 N) and subsequent stirring for 30 min at ambient temperature, the mixture was titrated with the NaOH aqueous solution (0.25 N) using phenolphthalein as an indicator. This measurement was repeated in triplicate.

3. Results and discussion

3.1. Paramylon doughnuts formation

In our attempt to establish the paramylon surface modification method, we first examined acetylation as a model reaction to explore the reactivity of the granular surface. Our choice of acetylation was based on the expectation that this reaction is applicable to β-1,3-glucan because cellulose, which is structurally analogous to β-1,3-glucan, is easily acetylated (Cerqueira, Rodrigues, & Meireles, 2007; Meireles et al., 2010; Rodrigues et al., 2008; Sassi & Chanzy, 1995). The applicability of this reaction to paramylon was examined through the use of procedures similar to those previously described (Meireles et al., 2010). In brief, a reaction mixture made from paramylon (anhydrous type), acetic acid, acetic anhydride, and sulfuric acid was a heterogeneous solution in an early stage and then became transparent in approximately 10 h. Our original

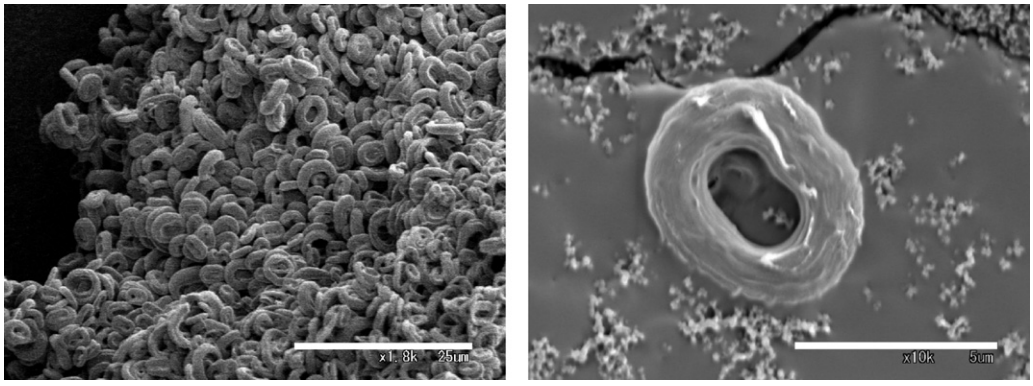

Fig. 1. Field emission scanning electron microscopic images of intermediate granules after treatment with acetylating reagents for 4 h: (a) low magnification image and (b) high magnification image. Bar represents 25 and 5 μm for (a) and (b), respectively.

expectation was that intermediate granules obtained by a centrifugal treatment of heterogeneous mixtures would have acetylated surfaces with maintenance of the spheroidal shape.

To examine this issue, we observed intermediate granules (4 h after starting the reaction) under a scanning electron microscope and measured its Fourier transform infrared spectrum. A typical microscopic image is shown in Fig. 1. It should be noted that the intermediate granules did not maintain the spheroidal shape inherent in the starting material but instead some granules developed a doughnut-like shape (i.e., a "paramylon doughnut"). The average major and minor axes of the doughnuts calculated based on the scanning electron micrographs were 2.92 ± 1.12 and 2.25 ± 1.07 μm, respectively, and those of the pore were 2.29 ± 0.73 and 1.13 ± 0.56 μm, respectively (average sample number was ca. 100). The infrared absorption spectrum of the doughnuts indicate a characteristic absorption band due to a carbonyl stretching vibration of acetyl groups at 1734 cm^{-1} (Fig. 2(a)). DS of the doughnuts, which was determined by titration, was 0.18 ± 0.09. These results imply that the doughnuts have some acetylated glucans on the granular surface. Unlike the anhydrous granules, hydrous ones did not provide the doughnuts but gels. The Fourier transform infrared spectrum of a gel obtained from the translucent solution (1 h after the start) exhibited a characteristic absorption band due to a carbonyl group at 1729 cm^{-1}, indicating that the gel consists of acetylated glucans (see Supplementary data). The high acetylation rate of the hydrated paramylon may be due to partial swelling of the paramylon crystallites with water molecules. Thus, this result indicates that the anhydrous form is essential for the doughnut fabrication.

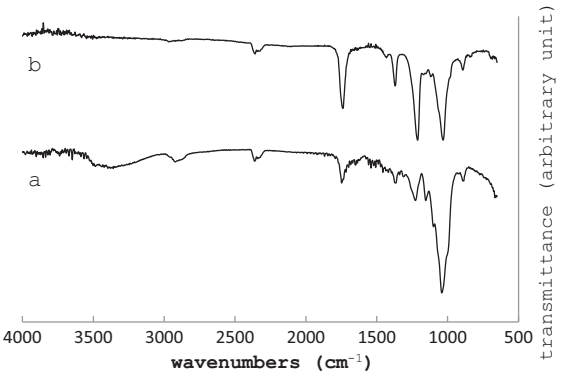

Fig. 2. Fourier transform infrared spectra of (a) intermediate granules (4 h reaction) and (b) a solid that was obtained by pouring a soluble part of the reaction mixture (4.5 h reaction) into methanol, followed by concentration under reduced pressure.

3.2. Glucan removal under acetylation condition

Our first basic question was *how was the doughnut shape formed?* Before attempting concrete discussions on this issue, we divided the question into two parts to clarify the nature of the problems involved in answering it. One was *how were the glucans removed?* The other was *how was the central portion of the granule preferentially removed?* The former question, we think, can be accounted for by removal of a part of accessible regions of the paramylon particle by acetylation. To confirm this idea, we measured Fourier

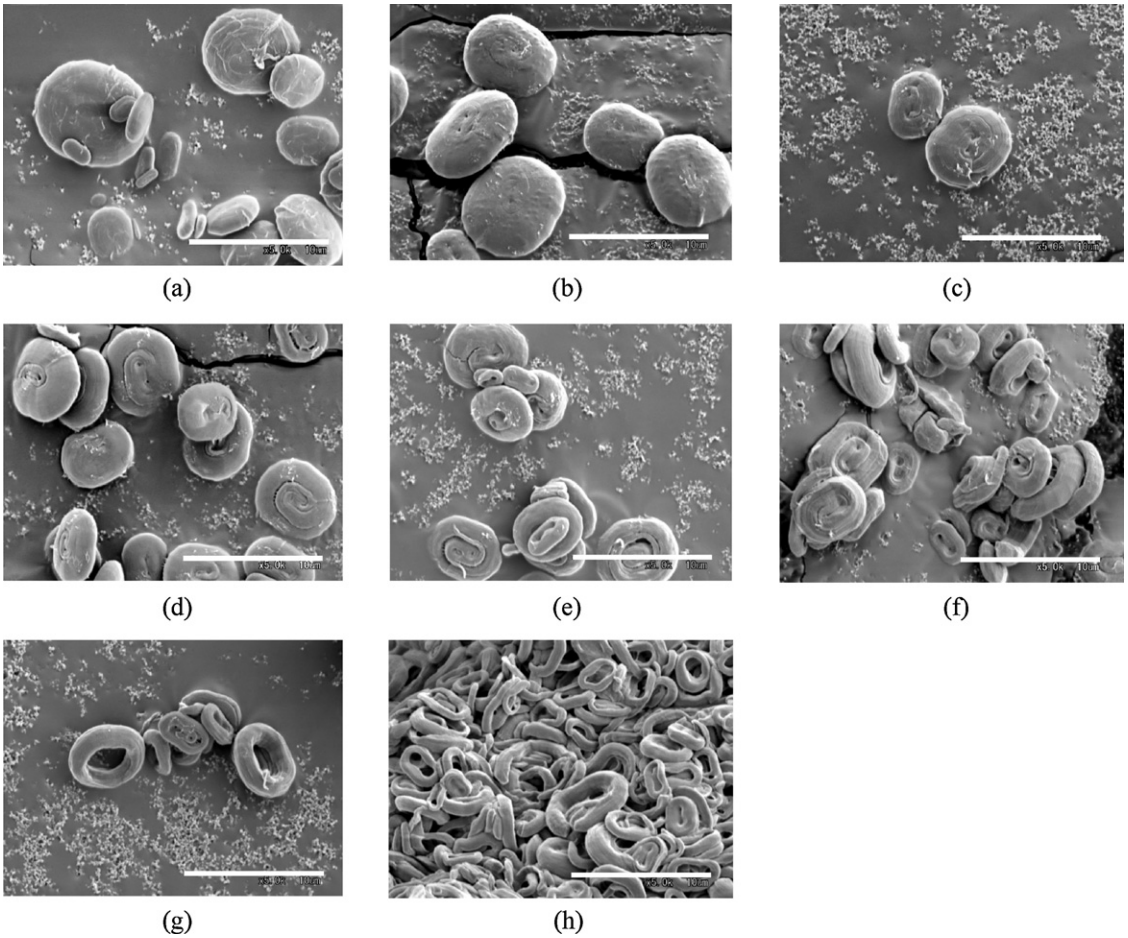

Fig. 3. Field emission scanning electron microscopic images of (a) the starting material and paramylon granules in the process of acetylation, (b) 10 min, (c) 30 min, (d) 1 h, (e) 2 h, (f) 3 h, (g) 4 h, and (h) 6 h after the start. Bar represents 5 μm.

transform infrared spectrum of solids obtained from a soluble part of the reaction mixture (4.5 h after the start). As shown in Fig. 2(b), the spectrum revealed characteristic absorption bands due to an acetyl group at 1735 cm^{-1} (carbonyl stretching vibration), 1365 cm^{-1} (methyl in-plane bending), and 1208 cm^{-1} (C–O stretching), indicating the glucans were acetylated to give soluble polysaccharides. Further, we determined the DS value of the acetylated glucans to be 2.62 ± 0.11. Thus, these results, together with the fact that the doughnuts have some acetylated glucans on the surface, imply that acetylation induced removal of glucans from the particle.

3.3. Preferential removal of granular central region under acetylation condition

To gain insight into preferential removal of the central area, we performed a time-course microscopic observation of the paramylon granules during acetylation in the expectation that this observation might enable us to obtain evidence for successive and preferential glucan removals. Experimental procedures were as follows. Aliquots (300 μL–1.5 mL) were withdrawn from a reaction mixture, which were prepared by dispersing 207 mg of paramylon in a mixture of acetic acid (7.0 mL), acetic anhydride (3.0 mL), and sulfuric acid (20 μL), as a function of time and treated in the centrifuge to separate insoluble solids. The residual solids were washed sequentially with acetic acid and methanol and then observed by field emission scanning electron microscopy after air-drying. Typical electron microscopic images of the solids obtained are shown in Fig. 3. Apparently, the granules changed their shape as the reaction progressed. Since there is a homogeneity in shape among the granules in each micrograph, we think that the breakdown process did not randomly occur but progressed in a well-regulated manner.

By comparing the image of the starting material (Fig. 3(a)), the second snapshot shows that after 10 min the granules more clearly exhibited geometric patterns (i.e., shallow dimples or a whorl-like structure) in the middle of the granular surface (Fig. 3(b)). As shown in the third and fourth snapshots, their clarity was enhanced by an additional stirring (Fig. 3(c and d)). A comparison among the first four snapshots suggests that the doughnut-making process began with the removal of an outer membrane of the paramylon granule, the presence of which has been pointed out by other researchers (Kiss et al., 1987). This comparison also suggests that the removal process was almost completed within 60 min.

The fifth and sixth snapshots show that within 3 h the granules obtained some deeply-etched patterns (i.e., deep dimples and a coiled structure) in the middle region (Fig. 3(e and f)). Prolonged incubation allowed some granules to obtain a doughnut shape, as shown in the seventh snapshot (Fig. 3(g)). A comparison of the granules in the sixth and seventh snapshots suggests that the rims could survive for a few hours probably because of its thickness, while the core moieties would be removed easier than the rim under the acetylation condition. As shown in the final snapshot (Fig. 3(h)), the surviving rim part became thinner with the progress of the reaction. Although the detailed mechanism for the preferential removal of the central portion remains to be resolved, possible explanations, which are based on a series of the snapshots, are as follows; (i) small holes in the core would allow the glucan removal reaction to progress outward from the center, (ii) the core part would be removed from the granule immediately when a joint moiety between the core and rim is dissolved.

4. Conclusions

The results presented in this paper highlight the fact that the anhydrous paramylon granules can be transformed into unique doughnut-like ones by treatment with acetylating reagents. Microscopic observations and infrared spectroscopic measurements clearly demonstrate that the transformation hinges on the preferential glucan removal of the central portion from the granule. According to the terminology in euglenoid paramylon morphology recently proposed, the paramylon doughnut is categorized as a small link (Monfils, Triemer, & Bellairs, 2011). Although it has been known that small links and structurally-related rings were the result of the dissolution and digestion of intact grains in the euglenoid cells (Gojdics, 1953; Heidt, 1937; Leedale, Meeuse, & Pringsheim, 1965), the doughnut preparation by the chemical reaction is significant in terms of mass and uniform production. In a broader context, the preparation of the paramylon doughnuts demonstrates the potential of *Euglena* as a resource for micro-sized materials. We are currently studying the preparation of materials made from the paramylon doughnuts. The results of these studies will be reported in due course.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carbpol.2011.08.012.

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