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Aqueous size-exclusion chromatographic method for the quantification of cyanobacterial native glycogen

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ABSTRACT

Cyanobacterial glycogen has gained interest as a valuable biomass feedstock for biofuel production. However, an ideal method for native glycogen quantification has not been developed. Here, we have proposed a simple methodology that enables the quantitative determination of cyanobacterial glycogen concentration with high repeatability using aqueous size-exclusion chromatography with a differential refractive index detector (SEC/RID). Our SEC/RID system also allows size distributions for native glycogen based on hydrodynamic volumes (V_h), which is proportional to the product of the molecular mass (M) and intrinsic viscosity [η], obtained by universal calibration using linear homopolymers of known M with Mark–Houwink–Sakurada parameters. The universal calibration curve achieved a broad linear range (V_h parameter $[\eta]M = 2 \times 10^2$–8×10^8 mL g^{-1}) with a high correlation coefficient ($R^2 = 0.9942$), because the developed system is equipped with an OHpak SB-806M HQ aqueous column containing four types of polyhydroxy methacrylate-based particles with different particle and pore sizes. Based on the SEC/RID system, response of molecular size distribution of glycogen in microalgae to the cultivation condition was first observed. Our established SEC/RID method has several advantages over conventional techniques, including the simultaneous quantitative and size distribution analyses of glycogen, and represents a potentially useful tool to elucidate the relationship between structural properties and the roles of glycogen in metabolism.

1. Introduction

Recently, prokaryotic cyanobacteria and eukaryotic microalgae, including green and red algae, and diatoms, have attracted attention as feedstock for biofuel production [1,2], as they are able to convert captured solar energy into biomass more efficiently than higher plants [3]. Cyanobacteria, which are oxygenic phototrophic prokaryotes, produce the carbohydrate glycogen, which can serve as a substrate for ethanol production through microbial fermentation [4]. Accordingly, grain biomass, including starch and sugar cane juice, which is currently used in biofuel production, could potentially be replaced by cyanobacterial glycogen.

Many researchers have screened for carbohydrate-rich microalgae and developed cultivation systems for improving glycogen production [4–9]. Among cyanobacteria, the filamentous species *Arthrospira* (*Spirulina*) *platensis* is a promising carbohydrate source for industrial bioethanol production. *A. platensis* thrives under alkaline (pH 9.5–11) and high-salinity (1.2 M sodium carbonate) conditions [10,11], which would reduce the risk of microbial contamination in open pond reactors to promote the high productivity of glycogen [9,12]. Moreover, filamentous microalgae are particularly suited for industrial applications, as single-celled cyanobacteria are difficult to harvest due to their small size, which limits scaled approaches [3].

Glycogen, which is composed of monomeric glucose units with highly branched (1 → 4)(1 → 6)-linked α-D-glucan, is an important biological macromolecule with a molecular weight (MW) of 10^5–10^9 [13,14]. To date, the quantification of microalgal native

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glycogen has been performed by the measurement of glucose chemically or enzymatically released from glucan using strong acids or glucoamylase. However, the use of hydrolysis prevented information to be obtained for glycogen molecules. According to a previous report [15], the molecular size and conformation of native glycogen produced by the cyanobacterium *Synechocystis* sp. PCC6803 undergoes drastic changes in response to gene mutation of glycogen-branching enzyme. Accordingly, the development of a novel quantitative glycogen analytical system that enables the structural information of glycogen with high repeatability is needed for the selection of microalgae capable of high-level glycogen production, as well as for biological studies on glycogen metabolism.

Size-exclusion chromatography (SEC) is the most developed and widely used technique to determine the characterization of glycogen [16]. It is essential to recall that SEC methods of branched macromolecules including glycogen and starch in solution separate on size or hydrodynamic volume (V_h), not on MW or molecular mass (M) [17]. Therefore, various types of detection, such as differential refractive index, multiple-angle laser light scattering, in line viscometry, and fluorescence, are required for the detailed structural characterization of branched polysaccharides at a macromolecular level, since different detectors provide information about different types of distributions of molecules [18]. Among them, differential refractive index detector (RID) is a concentration-sensitive detector widely employed for the concentration of polymers in solution. In addition, SEC combined with a RID (SEC/RID) system makes it possible to see molecular size (i.e., V_h) by universal calibration principle using Mark–Houwink–Sakurada (MHS) parameters [19]. Thus, SEC is a promising tool for the analysis of microalgal carbohydrate. However, only a few reports have described the application of these SEC techniques to microalgal glycogen [15], which cannot acquire detailed information about V_h and quantitative values of the glycogen because of the fact that a suitable methodology for both glycogen quantification and molecular size distribution (MSD) has not been developed in conjunction with SEC.

The objective of the present research was to establish a simple and practical methodology for the quantitative analysis of microalgal native glycogen based on SEC combined with a most widely used detector (RID). To develop such a system, we selected a unique SEC column, the Shodex OHpak SB-806M HQ, which contains four types of polyhydroxy methacrylate-based particles with different sizes and pore sizes. After the separation performance of this column was determined using pullulan standards, microalgal glycogen was successfully quantitated with the SEC/RID analytical system. Using the SEC/RID system developed here, the effects of culture temperature on glycogen accumulation and its molecular size alteration in *A. platensis* were successfully evaluated.

2. Materials and methods

2.1. Chemicals and reagents

Information for the pullulans, maltotriose, maltose, and glucose standards is listed in Table 1. All pullulans were purchased from Shoko Co., Ltd. (Tokyo, Japan). Maltotoriose, maltose, glucose, purified oyster glycogen, ammonium carbonate, sodium nitrate, sodium acetate, and hydrochloric acid were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Potassium hydroxide, acetic acid, and ethanol were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). LC/MS-grade distilled water (Wako) and Milli-Q water (Millipore Co., Billerica, MA, USA) were used for sample preparation and the mobile phase of HPLC, respectively. All constituent materials of the mineral salts medium of Ogawa and Terui (SOT) [20] were purchased from Nacalai Tesque. *Pseudomonas* sp. isoamylase with a specific activity of 280 U mg^{-1} (40 °C, pH 3.5, oyster glycogen) and *Rhizopus* sp. glucoamylase having a specific activity of 30 U mg^{-1} (60 °C, pH 4.5–5.0, soluble starch) were obtained from Megazyme International Ireland (Wicklow, Ireland) and Toyobo Co., Ltd. (Osaka, Japan), respectively.

2.2. Strain and growth conditions

A. platensis NIES-39 was obtained from Global Environmental Forum (Tsukuba, Japan). *A. platensis* NIES-39 was grown in slightly modified SOT liquid medium [20], which contained 16.8 g $NaHCO_3$, 0.5 g K_2HPO_4, 2.5 g $NaNO_3$, 1.0 g K_2SO_4, 1.0 g NaCl, 0.2 g $MgSO_4{\cdot}7H_2O$, 0.04 g $CaCl_2{\cdot}2H_2O$, 0.01 g $FeSO_4{\cdot}7H_2O$, 0.08 g Na_2 EDTA, and 1.0 mL A5 solution (2.86 g H_3BO_3, 2.5 g $MnSO_4{\cdot}7H_2O$, 0.222 g $ZnSO_4{\cdot}7H_2O$, 0.079 g $CuSO_4{\cdot}5H_2O$, and 0.021 g $Na_2MoO_4{\cdot}2H_2O$ per liter) per liter.

All cultivations were carried out in 500 mL flasks containing 250 mL SOT medium in an NC350-HC plant chamber (Nippon Medical and Chemical Instruments Co., Ltd., Osaka, Japan) under continuous illumination of 50 μmol photons m^{-2} s^{-1} using white fluorescent bulbs (Life Look HGX and NHG; NEC Corp., Tokyo, Japan) with 100 rpm agitation. To study the effects of cultivation temperatures on the qualitative (molecular size) and quantitative variations of native glycogen produced by *A. platensis*, cells were first pre-cultured at 30 °C for 5–6 days and then inoculated into fresh medium at a biomass concentration of 0.03 g dry weight (DW) L^{-1}. The cells were further incubated at 27, 30, 36, or 39 °C and harvested at late log-phase (10-day-old cultures). Temporary nitrogen starvation was performed using SOT medium lacking sodium nitrate. For the experiment, *A. platensis* cells were pre-cultured in SOT medium at 30 °C for 7 days and then inoculated into normal or nitrate-depleted (N-depleted) SOT medium at a biomass concentration of 0.45 g DW L^{-1}. Each culture was maintained at 30 °C for 3 days.

2.3. Sample preparation

2.3.1. Glycogen extraction

A. platensis cells were harvested with a polytetrafluoroethylene membrane filter (Omnipore, 10 μm pore size, 47 mm diameter; Millipore). Immediately after filtration, the cells were washed with 260 mM pre-chilled ammonium hydrogen carbonate in 32.5% (v/v) methanol at −25 °C at a ratio of 1:2 (v/v; washing solution to cell suspension). After washing, cells on the membrane filter were immediately frozen in liquid nitrogen, freeze-dried using a freeze dryer (Labconco Co., Kansas, MO, USA), and stored at −80 °C until sample extraction was performed.

Glycogen extraction was performed as described previously [5] with minor modifications. Briefly, glycogen was extracted from 10 mg DW cells with 200 μL KOH (30%, w/v) by incubation in a 95 °C heat block for 90 min, followed by placement on ice. To precipitate glycogen, 600 μL ethanol pre-chilled at 4 °C was added to the cooled extracts, which incubated on ice for 1 h. The extracts were then centrifuged at 3000 × *g* for 5 min at 4 °C. The resulting pellets were washed twice with cold ethanol and then dried for 10 min at 60 °C in a heat block. The dried sample was reconstituted in 100 μL water. Prior to analysis or enzymatic treatment, the reconstituted sample was centrifuged at 10,000 × *g* for 5 min at 4 °C, and the resulting supernatant was recovered.

2.3.2. Enzymatic hydrolysis of glycogen

The isoamylase treatment described in [21] was used with minor modifications, as described below. A 250 μL reaction mixture containing 10 mg mL^{-1} purified oyster glycogen or glycogen extracts (50 μL) obtained from *A. platensis* cells (10 mg DW) and isoamylase