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Facile preparation of highly crystalline lamellae of (1 → 3)-β-D-glucan using an extract of *Euglena gracilis*

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ABSTRACT

In vitro synthesis of (1 → 3)-β-D-glucan was performed using laminaribiose phosphorylase obtained by an extraction of *Euglena gracilis* with sucrose phosphorylase. The synthetic product was a linear (1 → 3)-β-D-glucan with a narrow distribution of degree of polymerization (DP) centered on DP = 30. X-ray diffraction and electron microscopy revealed that the glucan molecules obtained were self-organized as highly crystalline hexagonal lamellae. This synthetic product has quite high structural homogeneity at every level from primary to higher-order structure, which is a great advantage for the detailed analyses of physiological functions of (1 → 3)-β-D-glucan.

1. Introduction

(1 → 3)-β-D-glucan is a polysaccharide occurring in many organisms such as fungi, bacteria, yeasts, and higher plants. It plays various physiological roles [1]. For several decades, this glucan has attracted much attention in the medical field because of its physiological effects such as immunomodulation, antiviral, and anticancer activities [2]. Such properties are considered to be sensitive to structural differences such as molecular conformation, degree of polymerization (DP), degree of branching (DB), and types of glycosidic linkage in the main chain as well as its aggregation structure [3–8], so that a highly homogeneous specimen should be used for functional analysis. Structural homogeneity is also a great advantage for better understanding of its detailed structure and physical properties such as molecular conformations in the solid and solution states and mechanical and thermodynamical properties.

Preparation of (1 → 3)-β-D-glucan with high structural homogeneity has been reported by a number of workers [9–12]. Naturally derived glucan, typically fungal glucan, is the most common material for structural and property analyses, but is generally obtained as a mixture with other substrates, proteins, and/or other saccharides, and has numerous structural diversity [3,11–15]. An organic synthesis is a powerful method to provide homogeneous saccharides, but the reaction pathways are generally complicated and the yields are too low to be useful [16]. Enzymatic synthesis is another available approach to artificial synthesis of saccharides *in vitro*. Proper selection of enzyme and reaction conditions provides saccharides with high purity, and potentially controls their structure as demonstrated with the other saccharides [17–22].

In vitro synthesis of (1 → 3)-β-D-glucan with glycoenzyme has been studied by several groups. Hrmova et al. [23] produced an unbranched (1 → 3)-β-D-glucan by using a so-called glycosynthase, mutated barley (1 → 3)-β-D-glucan endohydrolase with 3-thio-α-laminaribiosylfluoride as substrate, while Him et al. [24] and Pelosi et al. [25] demonstrated its *in vitro* synthesis by using glucan synthases extracted from cultured cells of higher plants and a fungus with uridine diphosphate glucose. Although these synthetic methods provide linear (1 → 3)-β-D-glucan with high efficiency, the preparations of enzymes and substrates are relatively difficult. On the other hand, Kitaoka et al. [26] reported the *in vitro* synthesis of oligosaccharides of (1 → 3)-β-D-glucan using a sugar phosphorylase, laminaribiose phosphorylase (LBP) obtained by extraction of *Euglena gracilis*. Because the cultivation of *E. gracilis* and the enzyme extraction are rather simple and straightforward, this method has

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great potential for both physiological and physical property analyses. In this study, therefore, we demonstrated the facile preparation method of water-insoluble (1 → 3)-β-D-glucans using LBP from an extract of *E. gracilis*. The combined use of sucrose phosphorylase (SP) with LBP allows a simple reaction system that requires only glucose and sucrose as the substrates with a catalytic amount of inorganic phosphoric acid [27]. This synthetic method provides linear (1 → 3)-β-D-glucan with a narrow DP distribution and a very homogeneous aggregation state.

2. Materials and methods

2.1. Samples

E. gracilis Z (NIES-48), obtained from National Institute for Environmental Studies (NIES, Tsukuba, Japan), was cultured in a medium containing 5 g of pepton, 2 g of yeast extract, 15 g of glucose, and 10 μg of cyanocobalamine in 1 L of water at 28 °C in the dark with strong aeration [28,29]. After cultivation for a week, the harvested cells were suspended in 10 mM Tris–HCl buffer (pH 7.2), buffer A, and sonicated to disrupt the cells. The suspension was centrifuged at 20,000 × *g* for 10 min at 4 °C, and the supernatant was used as the crude enzyme solution. Paramylon in the precipitate was purified as described elsewhere and stored in water until use [29]. Curdlan purchased from Wako Pure Chemical Industries Ltd. (Tokyo, Japan) was used without further purification.

2.2. Partial purification of LBP

All purification steps were done at 4 °C. The crude enzyme solution was saturated with 30% ammonia sulfate, and was centrifuged at 20,000 × *g* to discard the precipitate formed. The supernatant was then put on a hydrophobic column (2.5 cmφ × 10 cm) of butyl-Toyopearl 650 M (Tosoh, Tokyo, Japan) equilibrated with buffer A containing 30% saturated ammonium sulfate. The column was washed with 100 mL of buffer A containing 30% saturated ammonium sulfate and 150 mL of buffer A containing 20% saturated ammonium sulfate. Then the enzyme was eluted with a 300 mL linear gradient of ammonium sulfate (20–0% saturation) in buffer A [28]. The enzyme activity of each fraction was measured using the method of Saheki et al. [30]. Briefly, 20 μL of the fraction was added to 40 μL of a solution containing 125 mM Mops/KOH buffer (pH = 7.0), 25 mM glucose 1-phosphate (G1P), and 25 mM glucose, and incubated for 20 min at 37 °C. The reaction solution was diluted 10 times with water, then 100 mM zinc acetate and 0.25 mL of 10% ascorbic acid solution were added. The solution was incubated for 15 min at 37 °C, and the free inorganic phosphoric acid (Pi) in the solution was measured by using the absorbance at 850 nm detected by a UV spectrophotometer (UV-mini 1240, SHIMADZU Co. Ltd. Japan). Fractions eluted from the column at about 10% saturated ammonium sulfate had high activities, as observed in a previous paper [29], and this fraction was used as LBP. One unit (U) of activity was defined as the amount of the enzyme that produced 1 μmol of Pi per minute at 37 °C.

2.3. Synthesis of polysaccharide in vitro using LBP

LBP and SP are known to catalyze the following reactions (1) and (2), respectively [27,28,31]:

$$(\mathrm{G1c})_n + \mathrm{G1P} \leftrightharpoons (\mathrm{G1c})_{n+1} + \mathrm{Pi} \quad (1)$$

$$\mathrm{Suc} + \mathrm{Pi} \leftrightharpoons \mathrm{Fru} + \mathrm{G1P} \quad (2)$$

where $(\mathrm{Glc})_n$, G1P, Suc, Pi, and Fru denote a (1 → 3)-β-D-glucan molecule, glucose 1-phosphate, sucrose, inorganic phosphoric acid, and fructose, respectively. When LBP and SP coexist in the reaction system, therefore, the following net reaction is catalyzed:

$$(\mathrm{G1c})_n + \mathrm{Suc} \leftrightharpoons (\mathrm{G1c})_{n+1} + \mathrm{Fru} \quad (3)$$

This cooperative reaction of LBP and SP, therefore, proceeds with a constant concentration of Pi without the inhibition induced by the high concentrations of Pi, and enables a highly efficient glucan synthesis [27,32].

Partially purified LBP (0.3 U/mL) was incubated for 96 h at 37 °C in 50 mM Tris–HCl buffer (pH 7.2), buffer B, containing 200 mM sucrose, 0.1 mM glucose, 20 mM K_2HPO_4, and SP (0.1 U/mL, Oriental Yeast Co. Ltd., Japan). After reacting for four days, some white precipitate was observed at the bottom of the reaction tube. After centrifugal rinsing in water (1500 × *g*, 25 °C, 10 min), this precipitate was then treated in 2% sodium dodecyl sulfate (SDS) aqueous solution for 2 h at room temperature followed by further centrifugal rinsing (1500 × *g*, 25 °C, 10 min). The amount of synthetic product was roughly 50 mg from 50 mL reaction solution, but a yield measurement was not carried out in this study because of the difficulty of precise extraction of insoluble products from the protein and oligo- and monosaccharides in the solution. The purified product was stored in water until use.

2.4. ^{13}C NMR analysis

The freeze-dried synthesized product, paramylon and curdlan were dissolved in deuterated dimethylsulfoxide (DMSO-d_6), and ^{13}C NMR spectra of these solutions were recorded using a JEOL ALPHA-500 spectrometer (JEOL, Japan) at 500 MHz. 3-Trimethylsilyl-2,2,3,3-d_4-propionic acid sodium salt (Aldrich, USA) was used as an internal standard at 0 ppm.

2.5. Gel permeation chromatography (GPC)

The dried synthesized product was dissolved in 1% LiCl/*N*,*N*-dimethylacetamide (LiCl/DMAc) at 0.5% weight concentration by heating the mixture at 60 °C for several hours. The GPC was measured using a JASCO RI-1530 differential refractive index detector with a SHODEX LF-804 column (SHODEX, Japan) with a flow rate of 0.2 mL/min. The DP was then estimated from the chromatogram by calibration using the pullulan standard series with molecular weights of 5900–76,800 (Shodex Standard P-82, Shodex, Japan) and laminaribiose (Mw = 342), triose (Mw = 504), pentaose (Mw = 810), and heptaose (Mw = 1134) (Wako Pure Chemical Industries Ltd., Japan).

2.6. X-ray diffraction analysis

The never-dried synthetic product and paramylon, and damped curdlan were stored in an 85%-R.H. desiccator with saturated KCl aqueous solution at room temperature to remove free water on their surfaces [29]. Dried samples were prepared by freeze drying.

X-ray diffraction analyses were carried out using nickel-filtered Cu Kα radiation (λ = 0.15418 nm) from a rotating anode X-ray generator, RotaFlex RU-200BH (Rigaku, Japan), operated at 50 kV and 100 mA. All of the specimens were sealed in capillaries and mounted in a vacuum camera. The diffraction pattern was recorded on an imaging plate (BAS-IP SR127, Fuji Film, Japan) and was read and analyzed with the RAXIA-di software system (Rigaku, Japan).

2.7. Electron microscopy and electron diffraction analysis

Droplet of aqueous suspension of the purified synthetic product were deposited onto carbon-coated copper grids and allowed to dry in air. To estimate the thickness of the product, part of the sample