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Purification and Characterization of 1,3-β-D-Glucan Phosphorylase from *Ochromonas danica*

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1,3-β-D-Glucan phosphorylase (BGP) is an enzyme that catalyzes the reversible phosphorolysis of 1,3-β-glucosidic linkages to form α-D-glucose 1-phosphate (G1P). Here we report on the purification and characterization of BGP from *Ochromonas danica* (OdBGP). The purified enzyme preparation showed three bands (113, 118, and 124 kDa) on SDS-polyacrylamide gel electrophoresis. The optimum pH and temperature were 5.5 and 25 °C–30 °C. OdBGP phosphorolysed laminaritriose, larger laminarioligosaccharides, and laminarin, but not laminaribiose. In the synthesis reaction, laminarin and laminarioligosaccharides served as good acceptors, but OdBGP did not act on glucose. Kinetic analysis indicated that the phosphorolysis reaction of OdBGP follows a sequential Bi Bi mechanism. The equilibrium of the enzymatic reaction indicated that OdBGP favors the reaction in the synthetic direction. Overnight incubation of OdBGP with laminaribiose and G1P resulted in the formation of precipitates, which were probably 1,3-β-glucans.

Key words: 1,3-β-D-glucan phosphorylase; *Ochromonas danica*; laminarin; laminarioligosaccharides; 1,3-β-D-glucan

Carbohydrate phosphorylases catalyze the phosphorolysis of glycosidic linkages at the nonreducing ends of oligosaccharides and polysaccharides to form sugar phosphates.[1] Twenty phosphorylases have been identified, each of which cleaves a specific glycosidic linkage.[2] Because phosphorylase reactions are reversible, the synthesis reactions of phosphorylases using sugar phosphates as donor substrates are useful in preparing oligosaccharides, polysaccharides, and glycosides.[1]

Three phosphorylases related to laminarioligosaccharides or 1,3-β-glucans are known: laminaribiose phosphorylase (LBP; EC 2.4.1.31), 1,3-β-oligoglucan phosphorylase (BOP; EC 2.4.1.30), and 1,3-β-D-glucan phosphorylase (BGP; EC 2.4.1.97). LBP and BOP were found in *Euglena gracilis* in the 1960s.[3,4] Both of these enzymes catalyze the following reversible reaction: $(1,3\text{-}\beta\text{-D-glucosyl})_n + Pi \leftrightarrow (1,3\text{-}\beta\text{-D-glucosyl})_{n-1} +$ α-D-glucose 1-phosphate (G1P). However, they have different substrate preferences. LBP prefers laminaribiose to larger laminarioligosaccharides, whereas BOP degrades laminaritriose and larger laminarioligosaccharides faster than laminaribiose.[5] Previous studies have investigated the properties of these enzymes in detail.[3–7] Furthermore, Kitaoka *et al.* have developed a method of synthesizing a series of laminarioligosaccharides using these two enzymes.[8] Recently, LBPs were found in the bacteria *Paenibacillus* sp. and *Acholeplasma laidlawii*, and their biochemical properties and amino acid sequences were reported.[9,10]

Similarly to LBP and BOP, BGP is an enzyme catalyzing the reversible phosphorolysis of 1,3-β-glucosidic linkages to form G1P. BGP was found in 1969 by Kauss and Kriebitzsch[11] in *Poterioochromonas malhamensis* (previously known as *Ochromonas malhamensis*), which belongs to the class Synurophyceae. It was partially purified from a cell-free extract, and its acceptor specificity was studied in the synthesis reaction. BGP from *P. malhamensis* acts on side-chain-branched (1,3;1,6)-β-glucans (laminarin and chrysolaminarin), but not on glucose; these properties are different from those of LBP and BOP, derived from *E. gracilis*.[4,6] Albrecht and Kauss examined other basic properties of BGP from *P. malhamensis*, including optimum pH, optimum temperature, and kinetic parameters,[12] but there have been no further detailed characterizations or suggested applications for this enzyme in the last four decades. Therefore, important characteristics such as reaction mechanism, molecular mass, primary structure, and reaction products remain unknown.

Ochromonas danica belongs to the class Chrysophyceae (golden algae). It is a unicellular freshwater flagellate that can show both photoautotrophic and heterotrophic growth. It synthesizes chrysolaminarin as an intracellular storage polysaccharide.[13,14] Although its genome sequence has not been determined, *O. danica* is the only species of Chrysophyceae whose expressed sequence tags have been reported.[15] Here we report the purification and characterization of BGP from *O. danica* (OdBGP). In addition, we found that OdBGP appears to synthesize 1,3-β-glucan from G1P and laminaribiose.

Materials and Methods

Chemicals. Laminaribiose, laminaritriose, laminaritetraose, laminaripentaose, laminarihexaose, laminariheptaose, cellobiose, cellotriose, cellotetraose, and cellopentaose were purchased from Seikagaku Biobusiness (Tokyo). Laminarin, sophorose, methyl β-D-glucopyranoside, paramylon, and α-D-glucose 1,6-bisphosphate were from Sigma-Aldrich (St. Louis, MO). Isomaltose, *p*-nitrophenyl α-D-glucopyrano-

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Abbreviations: BGP, 1,3-β-D-glucan phosphorylase; BOP, 1,3-β-oligoglucan phosphorylase; DP, degree of polymerization; G1P, α-D-glucose 1-phosphate; LBP, laminaribiose phosphorylase; OdBGP, BGP from *Ochromonas danica*; PAGE, polyacrylamide gel electrophoresis; TLC, thin-layer chromatography

side, *p*-nitrophenyl β-D-glucopyranoside, and CM-cellulose were from Nacalai Tesque (Kyoto, Japan). All other chemicals used were from Wako Pure Chemical Industries (Osaka, Japan), unless otherwise indicated.

Algal strain and growth conditions. O. danica NIES-2142 was obtained from the Microbial Culture Collection at the National Institute for Environmental Studies (Tsukuba, Japan). *O. danica* was precultured at 22 °C for 7 d in 250 mL of O medium containing 1 g/L of glucose, 1 g/L of polypeptone, 1 g/L of yeast extract, and 0.5 g/L of beef extract under fluorescent illumination (25 μmol photons/m^2/s) under a 12-h light/dark cycle. This culture was inoculated into 5 L of E medium containing 15 g/L of glucose, 5 g/L of peptone, and 2 g/L of yeast extract. After cultivation at 30 °C for 3 d with shaking at 160 rpm without illumination, the cells were harvested by centrifugation at 6,000 × *g* for 10 min and rinsed with distilled water. The cell pellet was stored at −30 °C until used.

Purification of OdBGP. The cells were suspended in 250 mL of buffer A (20 mM Tris–HCl, pH 8.0, 1 mM EDTA, and 1 mM dithiothreitol) plus 1 mM phenylmethylsulfonyl fluoride, and were disrupted by ultrasonication. After centrifugation at 40,000 × *g* at 4 °C for 30 min, the supernatant was fractionated with $(NH_4)_2SO_4$ from 30% to 70% saturation. The precipitate was dissolved in 50 mL of buffer A. The solution was dialyzed against buffer A and applied to a column (16 × 200 mm) of Q Sepharose FF (GE Healthcare, Buckinghamshire, UK) equilibrated with the same buffer. The column was washed with buffer A, and the proteins were eluted with a 600-mL gradient of 0–1 M NaCl in buffer A. The active fractions were pooled and brought to 30% saturation with $(NH_4)_2SO_4$. The solution was applied to a column of HiTrap Phenyl HP (5 mL, GE Healthcare) equilibrated with buffer A plus 30% $(NH_4)_2SO_4$. The column was washed with the same buffer, and the proteins were eluted with a 250-mL gradient of 30–0% $(NH_4)_2SO_4$ in buffer A and, finally, with 50 mL of buffer A. The active fractions were pooled and dialyzed against buffer B (20 mM Tris–HCl, pH 7.5, 1 mM EDTA, and 1 mM dithiothreitol). The solution was applied to a column of RESOURCE Q (1 mL, GE Healthcare) equilibrated with buffer B. The column was washed with buffer B, and the proteins were eluted with a 10-mL gradient of 0–190 mM NaCl and a 200-mL gradient of 190–230 mM NaCl in buffer B. The active fractions were pooled and stored at 4 °C until used.

Protein analysis. Protein concentrations were determined by Bradford's method[16] using bovine serum albumin as standard. SDS-polyacrylamide gel electrophoresis (PAGE) was carried out by the method of Laemmli[17] with a 7.5% polyacrylamide gel, and the gel was stained with Bio-Safe CBB G-250 stain (Bio-Rad Laboratories, Hercules, CA). The native molecular mass of OdBGP was determined by size-exclusion chromatography by HiPrep 16/60 Sephacryl S-300 HR column (GE Healthcare). The column was calibrated with a Gel Filtration Calibration Kit HMW (GE Healthcare). Native-PAGE was performed by the method of Davis[18] with a 6% polyacrylamide gel, and the gel was stained with Bio-Safe CBB G-250 stain. Activity staining was carried out by the method described by Miyatake and Kitaoka,[19] with minor modifications, as follows: After protein separation by Native-PAGE using a 6% polyacrylamide gel containing 1 mM laminaribiose, the gel was incubated in 100 mM sodium citrate buffer (pH 4.0) containing 10 mM G1P and 10 mM $CaCl_2$ at 30 °C for 1 h, and then washed with distilled water. The gel was soaked in 70 mM Tris–HCl buffer (pH 7.2) containing 3 mM $Pb(NO_3)_2$ for 1 h, washed with distilled water, and stained with 5% $(NH_4)_2S$.

Enzyme assays. Synthetic activity was measured by quantifying the amount of Pi by the method described by Saheki *et al.*,[20] as follows: The reaction mixture (100 μL), containing 100 mM sodium citrate buffer (pH 5.5), 10 mM G1P, 1 mM laminaripentaose, and the enzyme, was incubated at 30 °C for 10 min, unless otherwise indicated. The reaction was terminated by adding 1 mL of molybdate reagent (15 mM ammonium molybdate and 100 mM zinc acetate, pH 5.0) and 250 μL of 10% ascorbic acid reagent (pH 5.0). The mixture was incubated at 30 °C for 15 min, and then the concentration of Pi was determined by measuring A_{850}. One unit (U) of enzyme activity was defined as the amount of enzyme that produces 1 μmol of Pi per min.

Phosphorolytic activity was measured by quantifying the amount of G1P by the method described by Michal,[21] as follows: The reaction mixture (50 μL), containing 100 mM sodium acetate buffer (pH 5.5), 50 mM KH_2PO_4, 1 mM laminaripentaose, and the enzyme, was incubated at 30 °C for 10 min, unless otherwise indicated. After boiling for 5 min, an aliquot (20 μL) was mixed with 20 μL of 1 M Tris–HCl buffer (pH 7.5) containing 10 mM $MgCl_2$, 10 μL of 10 mM $NADP^+$, 4 μL of 5 μM α-D-glucose 1,6-bisphosphate, 4 μL of 5 U/mL glucose-6-phosphate dehydrogenase (Oriental Yeast, Tokyo), and 2 μL of 25 U/mL phosphoglucomutase (Oriental Yeast). The mixture was incubated at 30 °C for 10 min, and then the concentration of G1P was determined by measuring A_{340}.

Kinetic analysis. To identify the reaction mechanism and to determine the kinetic parameters for the phosphorolysis reaction of OdBGP, initial velocities were measured at various concentrations of laminaritriose (1, 2, 4, 8, and 10 mM) and KH_2PO_4 (2, 5, 10, 25, and 40 mM). Kinetic parameters were determined by fitting the data to the following equation for a sequential Bi Bi mechanism with GraFit 7 software (Erithacus Software, Surrey, UK):

$$v = k_{cat}[E_0][A][B]/(K_{iA}K_{mB} + K_{mB}[A] + K_{mA}[B] + [A][B])$$

where A is laminaritriose and B is Pi.

Laminarioligosaccharide determination. The concentrations of laminarioligosaccharides were measured by high-performance liquid chromatography, as described previously.[22]

Product analysis. A reaction mixture containing 50 mM sodium acetate buffer (pH 5.5), 100 mM G1P, 2.5 mM laminaribiose, 5 mM dithiothreitol, and 50 mU/mL OdBGP was incubated at 30 °C. Samples were collected at intervals and boiled for 5 min. They were analyzed by thin-layer chromatography (TLC) and 1,3-β-glucan assay, as described below.

For TLC analysis, the sample (50 μL) was mixed with 50 mg of Amberlite MB-20 (Sigma-Aldrich, $H^+/CH3COO^-$ form) for 5 min and centrifuged at 14,000 × *g* for 5 min to remove G1P and salts. An aliquot (10 μL) of the supernatant was concentrated to 1 μL by vacuum centrifugation and spotted onto a silica gel 60 TLC plate (Merck, Darmstadt, Germany). The plate was developed twice in AcOEt/AcOH/H_2O (2:2:1, v/v/v), dried, and soaked for 10 s in a solution containing 5% (v/v) H_2SO_4 in MeOH. After removal of MeOH by evaporation, the plate was heated at 100 °C for 10 min to visualize spots.

1,3-β-Glucan assay was performed by the method described by Shedletzky *et al.*,[23] with minor modifications. The sample (50 μL) was mixed with 10 μL of 6 M NaOH and kept at 80 °C for 30 min. After the addition of 210 μL of the aniline blue mix,[23] the mixture was incubated at 50 °C for 30 min and then at room temperature for 30 min. The fluorescence intensity (excitation, 360 nm; emission, 530 nm) of the mixture was measured with a fluorescence multi-well plate reader, CytoFluor II (PerSeptive Biosystems, Framingham, MA).

Results and Discussion

Purification of OdBGP

OdBGP was purified from a cell-free extract of *O. danica* NIES-2142 by ammonium sulfate fractionation and 3 steps of column chromatography (Table 1). The overall purification was 93.3-fold with a yield of 16.7%, and the specific activity (measured in the synthetic direction) of the purified OdBGP was 26.5 U/mg. Approximately 2 mg of purified OdBGP was obtained from 5 L of culture.

The purified enzyme preparation showed three bands on SDS–PAGE, with molecular masses of 113, 118, and 124 kDa (Fig. 1A). These polypeptides could not be separated by further chromatography. Extraction and purification in the presence of various protease inhibitors also gave the same band pattern (data not shown),

Table 1. Summary of the Purification of OdBGP

Procedure	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification (-fold)
Crude extract	349	1,230	0.284	100	1
$(NH_4)_2SO_4$ (30–70%)	241	348	0.693	69.1	2.44
Q Sepharose FF	159	45.0	3.53	45.6	12.4
HiTrap Phenyl HP	78.8	8.00	9.85	22.6	34.7
RESOURCE Q	58.4	2.20	26.5	16.7	93.3

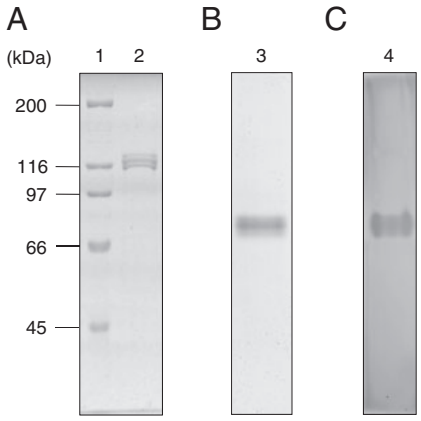

Fig. 1. Gel Electrophoresis of Purified OdBGP.
The purified enzyme preparation was analyzed by SDS–PAGE (A), native-PAGE (B), and activity staining (C). Lane 1, molecular mass markers; lanes 2–4, purified OdBGP.

suggesting that the multiple bands were probably not due to a proteolytic artifact. The native molecular mass of the purified enzyme was estimated to be approximately 250 kDa by size-exclusion chromatography, indicating that OdBGP is a homodimer and/or a heterodimer. Because a broad band, which may be a set of closely overlapping bands, was observed on native-PAGE (Fig. 1B), the dimeric protein species were thought to be structurally similar. Activity staining after native-PAGE indicated that the band(s) had OdBGP activity in the synthetic direction (Fig. 1C). The molecular masses of the polypeptides in the preparation of purified OdBGP were similar to those of LBPs from *E. gracilis* (120 kDa)[7] and *Paenibacillus* sp. (100 kDa).[9] Moreover, both LBPs were found to be dimeric. Structural analysis of OdBGP is currently in progress.

Effects of pH and temperature

The effects of pH and temperature on OdBGP activity and stability were examined by assaying synthetic activity. The optimum pH for OdBGP was approximately 5.5 (Fig. 2A), similar to that of BGP from *P. malhamensis* (pH 5.5)[12] but lower than those previously reported for LBPs (pH 6.0–7.2)[7,9,10,24] and BOP (pH 7.0–7.5).[4] The activity of OdBGP was low at neutral pH, and almost no activity was observed at pH 8.5. The enzyme was stable between pH 4.7 and 9.1 at 4 °C for 15 h (Fig. 2B). The optimum temperature for OdBGP was 25 °C–30 °C (Fig. 3A), higher than that of BGP from *P. malhamensis* (22.5 °C).[12] OdBGP was stable at up to 40 °C at pH 7.5 for 30 min (Fig. 3B). The effects of pH and temperature on phosphorolytic activity

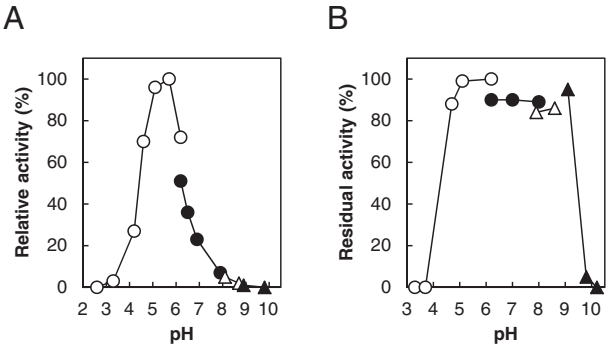

Fig. 2. Effects of pH on OdBGP Activity.
(A) Optimum pH. The synthetic activity of OdBGP was measured at 30 °C for 10 min in 100 mM buffer at various pH values (hollow circle, sodium citrate; solid circle, 3-morpholinopropanesulfonic acid-NaOH; hollow triangle, Tris–HCl; solid triangle, glycine-NaOH). (B) pH Stability. The enzyme was incubated at 4 °C for 15 h in 100 mM buffer at various pH values (hollow circle, sodium citrate; solid circle, 3-morpholinopropanesulfonic acid-NaOH; hollow triangle, Tris–HCl; solid triangle, glycine-NaOH), and then residual synthetic activity was measured.

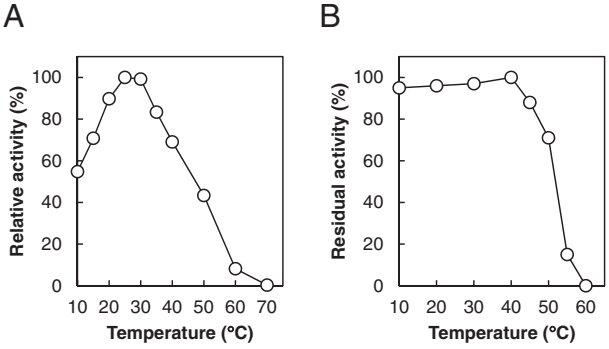

Fig. 3. Effects of Temperature on OdBGP Activity.
(A) Optimum temperature. The synthetic activity of OdBGP was measured at various temperatures for 10 min in 100 mM sodium citrate buffer (pH 5.5). (B) Thermal stability. The enzyme was incubated at various temperatures for 30 min in 20 mM Tris–HCl buffer (pH 7.5), and then residual synthetic activity was measured.

were almost the same as those on synthetic activity (data not shown).

Substrate specificity

The phosphorolytic activity of OdBGP toward various substrates was examined (Table 2). Kauss and Kriebitzsch have reported that BGP from *P. malhamensis* catalyzes the phosphorolysis of laminarin, but they did not examine the phosphorolytic activity of this enzyme toward other substrates.[11] In addition to laminarin, laminaritriose and the larger laminarioligosaccharides were degraded by OdBGP. The activity toward laminaripentaose and laminarihexaose was particularly high. On the other hand, we found that OdBGP showed no phosphorolytic activity toward laminaribiose, which is the best substrate for LBPs[5,7,9,10] and a good substrate for BOP.[5] Furthermore, OdBGP did not catalyze the phosphorolysis of the other glucobioses, glucosides, or cellooligosaccharides listed in Table 2.

The acceptor specificity of OdBGP in the synthesis reaction was also investigated (Table 2). Previous studies have indicated that laminarin is a good acceptor for BGP from *P. malhamensis*,[11] whereas it did not

Table 2. Relative Activity of OdBGP toward Various Substrates

Substrate (1 mM[a])	Relative activity (%)[b]	
	Phosphorolysis	Synthesis
Glucose	0	0
Laminaribiose	0	69
Laminaritriose	70	81
Laminaritetraose	73	99
Laminaripentaose	100	100
Laminarihexaose	94	95
Laminarin	89	359
Cellobiose	0	3
Cellotriose	0	6
Cellotetraose	0	9
Cellopentaose	0	13
Sophorose	0	2
Gentiobiose	0	0
Trehalose	0	0
Kojibiose	0	0
Nigerose	0	0
Maltose	0	0
Isomaltose	0	0
Methyl α-D-glucopyranoside	0	0
Methyl β-D-glucopyranoside	0	1
p-Nitrophenyl α-D-glucopyranoside	0	0
p-Nitrophenyl β-D-glucopyranoside	0	56

[a]But 10 mg/mL for laminarin.
[b]Activity toward laminaripentaose was taken to be 100%.

serve as an acceptor for LBP[6] or BOP[4] from *E. gracilis*. Similarly to *P. malhamensis* BGP, OdBGP showed high activity with laminarin as acceptor in the synthesis reaction. Furthermore, we found that various laminarioligosaccharides also functioned as acceptor substrates, though this was not examined in a previous study of BGP isolated from *P. malhamensis*.[11] OdBGP showed high activity toward laminaritetraose, laminaripentaose, and laminarihexaose. Similarly to BGP from *P. malhamensis*,[11] OdBGP did not act on glucose, which is a good acceptor for LBPs and BOP.[5,7,9,10] Thus, the substrate specificity of OdBGP toward laminarioligosaccharides and glucose in the synthesis reaction was compatible with that in the phosphorolysis reaction. Slight activity was observed toward cellobiose and sophorose, but gentiobiose did not serve as an acceptor. Moreover, OdBGP showed low activity toward cellotriose and the larger cellooligosaccharides. The α-linked glucobioses and glucosides listed in Table 2 did not function as acceptor substrates. In contrast to some LBPs[6,7,9,24] and BOP,[4] OdBGP was virtually inactive toward methyl β-D-glucopyranoside, but OdBGP showed high activity toward *p*-nitrophenyl β-D-glucopyranoside. Thus, we confirmed that the substrate specificity of OdBGP is distinctly different from those of LBPs and BOP.

Kinetic analysis

Double reciprocal plots of the initial velocity of OdBGP against the laminaritriose concentration at several concentrations of Pi showed a series of straight lines intersecting at a point (Fig. 4), which indicates that the phosphorolysis reaction of OdBGP follows a sequential Bi Bi mechanism, consistently with the reactions of other phosphorylases that act on β-linked glucooligosaccharides, including LBPs[7,9,10] and cellobiose phosphorylases (EC 2.4.1.20).[25–29] The kinetic

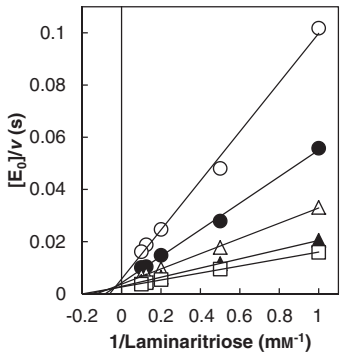

Fig. 4. Double-Reciprocal Plot of the Phosphorolysis of Laminaritriose.

The concentrations of Pi were as follows: hollow circle, 2 mM; solid circle, 5 mM; hollow triangle, 10 mM; solid triangle, 25 mM; hollow square, 40 mM.

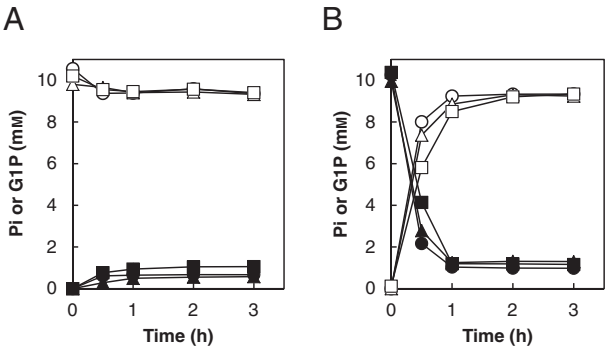

Fig. 5. Changes in the Concentrations of Pi and G1P during the Enzymatic Reaction.

OdBGP (50 mU/mL) was incubated at 30 °C in 100 mM sodium acetate buffer (pH 5.5) with substrates. Samples were collected at intervals and boiled for 5 min. The concentrations of Pi (hollow symbols) and G1P (solid symbols) in the samples were then determined. The substrates in the reaction mixture at the start of the reaction were as follows: (A) circles, 10 mM laminaritriose and 10 mM Pi; triangles, 10 mM laminaripentaose and 10 mM Pi; squares, 20 mg/mL laminarin and 10 mM Pi; (B) circles, 10 mM laminaritriose and 10 mM G1P; triangles, 10 mM laminaripentaose and 10 mM G1P; squares, 20 mg/mL laminarin and 10 mM G1P.

parameters obtained were as follows: $k_{cat} = 400\,s^{-1}$, $K_{mA} = 4.1$ mM, $K_{mB} = 4.4$ mM, $K_{iA} = 16$ mM (A, laminaritriose; B, Pi).

Equilibrium of the enzymatic reaction

OdBGP can catalyze reactions in both synthetic and phosphorolytic directions, as described above. To study the equilibrium of the OdBGP reaction, changes in the concentrations of Pi and G1P during incubation of OdBGP (50 mU/mL) at pH 5.5 and 30 °C were measured. When 10 mM Pi and 10 mM laminaritriose were used as substrates, only 0.7 mM G1P was formed after 1 h of reaction (Fig. 5A). A longer incubation period did not increase the amount of G1P, indicating that the reaction reached equilibrium. On the other hand, when 10 mM G1P and 10 mM laminaritriose were used as substrates, the concentration of G1P fell to 2.2 mM after 30 min of reaction, and then it decreased to a steady value of 1.0 mM after 1 h (Fig. 5B). In both experiments, the sum of G1P and Pi was constant at 10 mM throughout the reaction. Furthermore, similar changes in Pi and G1P concentrations were observed when 10 mM laminaripen-

taose or 20 mg/mL of laminarin was used as substrate instead of 10 mM laminaritriose (Fig. 5). Thus, under these experimental conditions, the amount of G1P was much smaller than that of Pi at the equilibrium state. Equilibrium constants were calculated for the following reactions:

$$\text{Laminaritriose} + \text{Pi} \leftrightarrow \text{Laminaribiose} + \text{G1P} \quad (1)$$

$$\text{Laminaritetraose} + \text{Pi} \leftrightarrow \text{Laminaritriose} + \text{G1P} \quad (2)$$

$$\text{Laminaripentaose} + \text{Pi} \leftrightarrow \text{Laminaritetraose} + \text{G1P} \quad (3)$$

$$\text{Laminarihexaose} + \text{Pi} \leftrightarrow \text{Laminaripentaose} + \text{G1P} \quad (4)$$

The constants (K_1, K_2, K_3, and K_4) were 0.13, 0.11, 0.11, and 0.17, respectively. These results indicate that OdBGP favors the reaction in the synthetic direction. This is similar to the properties of other phosphorylases related to β-linked glucooligosaccharides, including LBPs,[6,24] BOP,[4] cellobiose phosphorylases,[25,26,30] and cellodextrin phosphorylase (EC 2.4.1.49).[31]

Previous reports have stated that LBP and BOP from *E. gracilis*[5,6] and BGP from *P. malhamensis*[11] might be involved in the degradation of intracellular 1,3-β-glucan (paramylon or chrysolaminarin), although no evidence was presented, but it is difficult to determine the physiological role of phosphorylases. For example, plant starch phosphorylase (EC 2.4.1.1) catalyzes the reversible phosphorolysis of 1,4-α-glucan, and it has been suggested that it functions mainly in the mobilization of starch in view of its low affinity to G1P and the relatively high Pi/G1P ratio in plant cells,[32] but recent studies indicate that starch phosphorylase from rice endosperm strongly favors synthesis over the degradation of 1,4-α-glucan even under conditions of excess Pi, and that it plays a crucial role in starch biosynthesis *in vivo*.[33,34] Further studies are necessary to assess the physiological function of OdBGP.

Product analysis

As mentioned above, OdBGP catalyzes the reversible phosphorolysis of 1,3-β-glucosidic linkages and favors the reaction in the synthetic direction. In addition, it uses laminarioligosaccharides of various sizes and laminarin as acceptors in the synthesis reaction. On the basis of these findings, we expected that OdBGP would produce large laminarioligosaccharides or 1,3-β-glucans by oligomerization or polymerization of glucose when we used a large amount of G1P and small amounts of low molecular mass laminarioligosaccharides as starting materials. To confirm this, OdBGP was incubated with 2.5 mM laminaribiose and 100 mM G1P for various periods (the longest being overnight), after which the reaction products were analyzed.

After removing G1P and other salts by means of ion-exchange resins, the products were analyzed by TLC (Fig. 6). During the first 1 h of the reaction, several spots thought to be a series of small laminarioligosaccharides were observed. A spot at the origin of the TLC plate appeared after 2 h, indicating that high molecular mass products had formed. Spots corresponding to laminaribiose (the initial substrate) and small laminarioligosaccharides (*e.g.*, laminaritriose and laminaritetraose) almost disappeared after 4 h. After more than 8 h, only a single spot was observed at the origin. Thus, incubation of OdBGP with laminaribiose and G1P under these

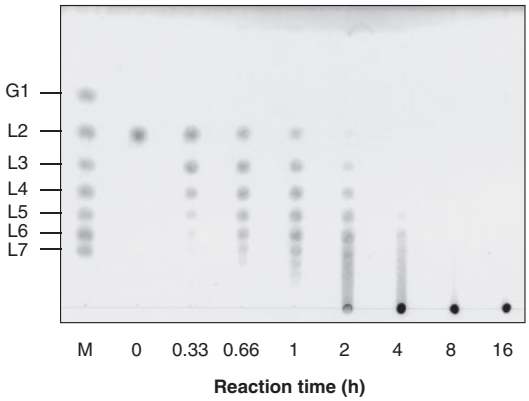

Fig. 6. TLC Analysis of the Products Synthesized by OdBGP.
OdBGP (50 mU/mL) was incubated at 30 °C in 50 mM sodium acetate buffer (pH 5.5) with 100 mM G1P and 2.5 mM laminaribiose. Samples were collected at intervals and boiled for 5 min. Then they were desalted, concentrated, and analyzed by TLC. M, marker; G1, glucose; L2, laminaribiose; L3, laminaritriose; L4, laminaritetraose; L5, laminaripentaose; L6, laminarihexaose; L7, laminariheptaose.

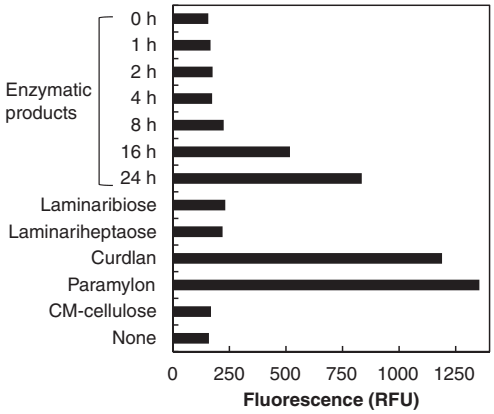

Fig. 7. 1,3-β-Glucan Assay of the Products Synthesized by OdBGP.
OdBGP (50 mU/mL) was incubated at 30 °C in 50 mM sodium acetate buffer (pH 5.5) with 100 mM G1P and 2.5 mM laminaribiose. Samples were collected at intervals and boiled for 5 min. Enzymatic products (or 10 mg/mL of commercial carbohydrates for comparison) were stained with aniline blue, and the fluorescence intensity was measured.

experimental conditions resulted in the formation of various laminarioligosaccharides and larger products. The short-term products contained only small laminarioligosaccharides and not high molecular mass products, which suggests that OdBGP elongates laminarioligosaccharides or 1,3-β-glucans distributively (non-processively) rather than processively.

The products were also analyzed by 1,3-β-glucan assay with aniline blue dye. It is well known that 1,3-β-glucan forms a fluorescent complex upon interaction with a fluorochrome present in this dye.[35] The fluorescence intensity of the samples during the first 8 h of reaction was low, comparable to the background (Fig. 7). In contrast, the intensity increased several fold after an overnight reaction, indicating that 1,3-β-glucan was synthesized.

Overnight incubation of OdBGP with these substrates resulted in the formation of precipitates (data not shown). Ogawa *et al.* prepared laminarioligosaccharides and 1,3-β-glucans with a degree of polymerization (DP) of 10–170 by formic acid hydrolysis of curdlan, a gel-

forming linear 1,3-β-glucan of DP > 200.[36] They reported that 1,3-β-glucans with DP 30–170 were insoluble in water, whereas laminarioligosaccharides with DP < 20 were soluble. Therefore, it appears that the products formed after overnight incubation were 1,3-β-glucans of DP > 30. The characteristics of the enzymatic products, including structure, molecular mass, and morphology, will be reported elsewhere in detail.

References

1) Kitaoka M and Hayashi K, *Trends Glycosci. Glycotechnol.*, **14**, 35–50 (2002).
2) Nakai H, Kitaoka M, Svensson B, and Ohtsubo K, *Curr. Opin. Chem. Biol.*, **17**, 301–309 (2013).
3) Maréchal LR and Goldemberg SH, *Biochem. Biophys. Res. Commun.*, **13**, 106–109 (1963).
4) Marechal LR, *Biochim. Biophys. Acta*, **146**, 417–430 (1967).
5) Marechal LR, *Biochim. Biophys. Acta*, **146**, 431–442 (1967).
6) Goldemberg SH, Marechal LR, and De Souza BC, *J. Biol. Chem.*, **241**, 45–50 (1966).
7) Kitaoka M, Sasaki T, and Taniguchi H, *Arch. Biochem. Biophys.*, **304**, 508–514 (1993).
8) Kitaoka M, Sasaki T, and Taniguchi H, *Agric. Biol. Chem.*, **55**, 1431–1432 (1991).
9) Kitaoka M, Matsuoka Y, Mori K, Nishimoto M, and Hayashi K, *Biosci. Biotechnol. Biochem.*, **76**, 343–348 (2012).
10) Nihira T, Saito Y, Kitaoka M, Nishimoto M, Otsubo K, and Nakai H, *Carbohydr. Res.*, **361**, 49–54 (2012).
11) Kauss H and Kriebitzsch C, *Biochem. Biophys. Res. Commun.*, **35**, 926–930 (1969).
12) Albrecht G and Kauss H, *Phytochemistry*, **10**, 1293–1298 (1971).
13) Stone BA, "Chemistry, Biochemistry, and Biology of (1→3)-β-Glucans and Related Polysaccharides," eds. Bacic A, Fincher GB, and Stone BA, Academic Press, San Diego, pp. 5–46 (2009).
14) Gibbs SP, *J. Cell Biol.*, **15**, 343–361 (1962).
15) Terauchi M, Kato A, Nagasato C, and Motomura T, *Phycological Res.*, **58**, 217–221 (2010).
16) Bradford MM, *Anal. Biochem.*, **72**, 248–254 (1976).
17) Laemmli UK, *Nature*, **227**, 680–685 (1970).
18) Davis BJ, *Ann. NY Acad. Sci.*, **121**, 404–427 (1964).
19) Miyatake K and Kitaoka S, *Bull. Univ. Osaka Pref., Ser. B*, **32**, 49–54 (1980).
20) Saheki S, Takeda A, and Shimazu T, *Anal. Biochem.*, **148**, 277–281 (1985).
21) Michal G, "Methods of Enzymatic Analysis," ed. Bergmeyer HU, Wiley-Blackwell, Hoboken, pp. 185–191 (1984).
22) Isono N, Kimoto T, and Hisamatsu M, *Bull. Grad. Sch. Bioresour. Mie Univ.*, **39**, 55–60 (2013).
23) Shedletzky E, Unger C, and Delmer DP, *Anal. Biochem.*, **249**, 88–93 (1997).
24) Manners DJ and Taylor DC, *Arch. Biochem. Biophys.*, **121**, 443–451 (1967).
25) Kitaoka M, Sasaki T, and Taniguchi H, *Biosci. Biotechnol. Biochem.*, **56**, 652–655 (1992).
26) Nidetzky B, Eis C, and Albert M, *Biochem. J.*, **351**, 649–659 (2000).
27) Kim Y-K, Kitaoka M, Krishnareddy M, Mori Y, and Hayashi K, *J. Biochem.*, **132**, 197–203 (2002).
28) Rajashekhara E, Kitaoka M, Kim Y-K, and Hayashi K, *Biosci. Biotechnol. Biochem.*, **66**, 2578–2586 (2002).
29) Hamura K, Saburi W, Abe S, Morimoto N, Taguchi H, Mori H, and Matsui H, *Biosci. Biotechnol. Biochem.*, **76**, 812–818 (2012).
30) Alexander JK, *J. Bacteriol.*, **81**, 903–910 (1961).
31) Sheth K and Alexander JK, *J. Biol. Chem.*, **244**, 457–464 (1969).
32) Preiss J and Sivak M, "Photoassimilate Distribution in Plants and Crops: Source-Sink Relationships," eds. Zamski E and Schaffer AA, Marcel Dekker, New York, pp. 139–168 (1996).
33) Satoh H, Shibahara K, Tokunaga T, Nishi A, Tasaki M, Hwang S-K, Okita TW, Kaneko N, Fujita N, Yoshida M, Hosaka Y, Sato A, Utsumi Y, Ohdan T, and Nakamura Y, *Plant Cell*, **20**, 1833–1849 (2008).
34) Hwang S-K, Nishi A, Satoh H, and Okita TW, *Arch. Biochem. Biophys.*, **495**, 82–92 (2010).
35) Wood PJ and Fulcher RG, *Carbohydr. Polym.*, **4**, 49–72 (1984).
36) Ogawa K, Tsurugi J, and Watanabe T, *Carbohydr. Res.*, **29**, 397–403 (1973).