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Discovery of cellobionic acid phosphorylase in cellulolytic bacteria and fungi

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ABSTRACT

A novel phosphorylase was characterized as new member of glycoside hydrolase family 94 from the cellulolytic bacterium *Xanthomonas campestris* and the fungus *Neurospora crassa*. The enzyme catalyzed reversible phosphorolysis of cellobionic acid. We propose 4-*O*-β-D-glucopyranosyl-D-gluconic acid: phosphate α-D-glucosyltransferase as the systematic name and cellobionic acid phosphorylase as the short names for the novel enzyme. Several cellulolytic fungi of the phylum Ascomycota also possess homologous proteins. We, therefore, suggest that the enzyme plays a crucial role in cellulose degradation where cellobionic acid as oxidized cellulolytic product is converted into α-D-glucose 1-phosphate and D-gluconic acid to enter glycolysis and the pentose phosphate pathway, respectively.
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1. Introduction

Cellulose degradation is one of the most crucial bioprocesses in the natural carbon cycle, because cellulose is an abundant organic compound on Earth. The hydrolytic enzymes, generally called cellulase including *endo*-glucanase (EC 3.2.1.4), cellobiohydrolase (EC 3.2.1.91 or EC 3.2.1.176), and β-glucosidase (EC 3.2.1.4), have major roles in the process [1,2]. The contribution of oxidative enzymes such as cellobiose dehydrogenase (EC 1.1.99.18) [3,4] and lytic polysaccharide monooxygenase [5–7] has lately attracted considerable attention in enzymatic saccharification of cellulosic biomass [8–10]. Although the oxidized cellulolytic products often occur during cellulose degradation, their metabolic pathway has not been elucidated yet because the process is not simply connected to glycolysis as they are a mixture of neutral and oxidized sugars.

Cellobionic acid (4-*O*-β-D-glucopyranosyl-D-gluconic acid) is an intermediate in mycotic cellulose degradation [1,2]. It is obtained by spontaneous hydrolysis of cellobiono-1,5-lactone, which is extracellularly converted from cellobiose by cellobiose dehydrogenase [3,4], as well as from cellulose by the combined action of lytic polysaccharide monooxygenase and cellobiohydrolase [5–7]. The resultant cellobionic acid has been believed to be hydrolyzed by intracellular or extracellular β-glucosidase to form D-glucose and D-gluconic acid at the beginning of the discovery of cellobiose dehydrogenase [3], whereas cellobionic acid is a worse substrate than cellobiose as typical substrate for β-glucosidase [11]. This is because the hydrolysate D-gluconic acid is a strong non-competitive inhibitor of β-glucosidase [12], clearly suggesting another metabolic scheme should be applied for the cellobionate metabolism.

Phosphorylases are members of enzymes involved in the intracellular catabolism of particular glycosides [13–15]. These enzymes reversibly phosphorolyze glycosides, to produce sugar 1-phosphates with strict substrate specificities. The catabolic pathway, including the phosphorolysis that enables direct production of phosphorylated sugars without consuming ATP, is energetically efficient. However, there is little variation among phosphorylases with only 21 known activities [15]. It is, thus, desired to identify phosphorylases with previously unreported substrate specificities. Phosphorylases have been classified as members of glycoside hydrolase families (GH) 13, 65, 94, 112, and 130, or glycosyltransferase families 4 or 35 in the Carbohydrate-Active Enzymes database (http://www.cazy.org/) based on amino acid sequence similarity [16]. Among these families, GH94 is primarily comprised of phosphorylases that catalyze reversible phosphorolysis of β-D-glucosides to form α-D-glucose 1-phosphate (αGlc1*P*) with

Abbreviations: GH, glycoside hydrolase family; αGlc1*P*, α-D-glucose 1-phosphate; PAGE, polyacrylamide-gel electrophoresis

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inversion of the anomeric configuration. At present, cellobiose phosphorylase (EC 2.4.1.20), cellodextrin phosphorylase (EC 2.4.1.49), laminaribiose phosphorylase (EC 2.4.1.31), *N*,*N*′-diacetylchitobiose phosphorylase (EC 2.4.1.280), and cyclic β-1,2-glucan synthase (EC 2.4.1.–) are categorized into GH94.

In this study, we discovered cellobionic acid phosphorylases (XCC4077 and NCU09425) from the plant pathogenic bacterium *Xanthomonas campestris* and red bread mold of the phylum Ascomycota *Neurospora crassa*, respectively, as new members of GH94. We propose a new metabolic pathway of cellobionic acid that it is converted by energetically-efficient phosphorolysis in the cytoplasm of cellulolytic organisms into αGlc1*P* and D-gluconic acid to enter the glycolysis and pentose phosphate pathway, respectively.

2. Materials and methods

2.1. Construction of expression plasmids

Two genes encoding XCC4077 and NCU09425 (GenBank ID: AAM43298.1 and EAA28929.1, respectively) were amplified by PCR from genomic DNA of *Xanthomonas campestris* pv. *campestris* ATCC33913 and *Neurospora crassa* OR74A, respectively, using KOD-plus DNA polymerase (Toyobo, Osaka, Japan) with oligonucleotide primers (Table S1) constructed based on the genome sequences (GenBank ID: AE008922 and AABX020000076, respectively). Each amplified gene was inserted into pET24a (+) (Novagen, Madison, WI, USA) with the restriction endonuclease sites (Table S1) using Ligation high Ver.2 (Toyobo). Each expression plasmid was propagated in *Escherichia coli* DH5α (Toyobo), purified by a FastGene Plasmid Mini Kit (Nippon Genetics Co., Tokyo, Japan), and verified by sequencing (Operon Biotechnologies, Tokyo, Japan).

2.2. Recombinant enzyme preparation

E. coli BL21 (DE3) (Novagen) transformant harboring the expression plasmid was grown at 37 °C in 200 ml of Luria–Bertani medium containing 50 μg/ml kanamycin, until the absorbance reached 0.6 at 600 nm. The expression was induced by 0.1 mM isopropyl β-D-thiogalactopyranoside and continued at 18 °C for 24 h. Cells were harvested by centrifugation at 20000×*g* for 20 min and suspended in 50 mM HEPES–NaOH (pH 7.0) containing 500 mM NaCl (buffer A). The suspended cells were disrupted by sonication (Branson sonifier 250A, Branson Ultrasonics, Emerson Japan, Kanagawa, Japan). The supernatant collected by centrifugation at 20000×*g* for 20 min was applied to a HisTrap FF column (GE Healthcare, Buckinghamshire, UK), equilibrated with buffer A containing 10 mM imidazole, by using an ÄKTA prime (GE Healthcare). After washing with buffer A containing 22 mM imidazole and subsequent elution by using a 22–400 mM imidazole linear gradient in buffer A, fractions containing the target proteins were pooled, dialyzed against 10 mM HEPES–NaOH buffer (pH 7.0), and concentrated (AMICON Ultra; Millipore, Billerica, MA, USA). The protein concentration was determined spectrophotometrically at 280 nm using theoretical extinction coefficients of $\varepsilon = 168,110$ and $161,120\ cm^{-1}\ M^{-1}$, based on the amino acid sequences of XCC4077 and NCU09425, respectively [17]. The molecular masses of purified proteins were estimated by SDS–polyacrylamide-gel electrophoresis (SDS–PAGE, Mini-PROTEAN Tetra electrophoresis system; Bio-Rad Laboratories, Hercules, CA, USA) and by gel filtration (HiLoad 26/600 Superdex 200 pg; GE Healthcare) equilibrated with 10 mM HEPES–NaOH buffer (pH 7.0) containing 150 mM NaCl at a flow rate of 0.5 ml/min, using Marker Proteins for molecular Weight Determination on High Pressure Liquid Chromatography (Oriental Yeast Co., Tokyo, Japan) as standards.

2.3. Measurement of enzymatic activity

The phosphorolytic activity was routinely determined by quantifying αGlc1*P* released during the reaction in 40 mM HEPES–NaOH (pH 7.0) or 40 mM MOPS–NaOH (pH 7.0) for XCC4077 or NCU09425, respectively, containing 10 mM substrate and 10 mM inorganic phosphate (P_i) at 30 °C by using the phosphoglucomutase-glucose 6-phosphate dehydrogenase method [18] as described previously [19].

The synthetic activity was routinely determined by measuring the increase in P_i using a reaction mixture containing 10 mM αGlc1*P* (α-D-glucose 1-phosphate disodium salt hydrate; Sigma–Aldrich, St. Louis, MO, USA) and 10 mM D-gluconic acid (gluconic acid sodium salt; Nacalai Tesque, Kyoto, Japan) in 40 mM MES–NaOH (pH 6.0) or 40 mM MOPS–NaOH (pH 6.5) for XCC4077 or NCU09425, respectively, at 30 °C by following the method of Lowry and Lopez [20] as described previously [21].

2.4. Temperature and pH profiles

The effects of pH on the phosphorolytic and synthetic activities using 9.9 nM XCC4077 or 7.9 nM NCU09425 were measured under the standard conditions described above, using the following 40 mM buffers: sodium citrate (pH 3.0–5.5), Bis–Tris–HCl (pH 5.5–7.0), HEPES–NaOH (pH 7.0–8.5), and glycine–NaOH (pH 8.5–10.5). The thermal and pH stabilities were evaluated by measuring the residual synthetic activity under the standard conditions after incubation of 3.9 μM XCC4077 or 6.6 μM NCU09425 at a temperature range of 30–90 °C for 15 min and at various pH values at 4 °C for 24 h, respectively.

2.5. Substrate specificity analysis

The phosphorolytic activities of XCC4077 (78 μM) on β-linked glucobioses (cellobiose, sophorose, laminaribiose, gentiobiose) and *N*,*N*′-diacetylchitobiose were examined under the standard conditions described above.

To investigate the acceptor specificities of XCC4077 (78 nM), synthetic reactions were performed under the standard conditions described above, by substituting D-gluconic acid with carbohydrate acceptor candidates (Table S2).

2.6. Structural determination of reaction product

Reaction products for structural determination were generated in 1 ml of reaction mixture (pH 6.0) containing 500 mM αGlc1*P* and 500 mM D-gluconic acid or D-glucuronic acid with 20 μM XCC4077. The reaction mixtures were incubated at 30 °C for 24 h, followed by treatment with α-D-glucose 1-phosphatase from *E. coli* at 30 °C for 24 h, as described previously [19]. The reaction products were separated on a Toyopearl HW-40S column (26 mm internal diameter × 700 mm; Tosoh, Tokyo, Japan), equilibrated with distilled water, at a flow rate of 1.0 ml/min. Fractions containing the reaction products were collected and lyophilized. The amount of product obtained was 58 and 39 mg from D-gluconic acid and from D-glucuronic acid, respectively. The structures of the products were identified by comparing their ^{1}H and ^{13}C NMR spectra acquired in D_2O with 2-methyl-2-propanol as an internal standard using a Bruker Avance 800 spectrometer (Bruker Biospin, Rheinstetten, Germany) with those of authentic data.

2.7. Kinetic analysis

The initial velocities of the phosphorolytic reactions were determined under the standard conditions with 54 nM XCC4077 or 2.0 nM NCU09425 and a combination of initial concentrations of

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