Available online at www.sciencedirect.com
SCIENCE DIRECT®
Carbohydrate Research 340 (2005) 449–454
Carbohydrate RESEARCH

Enzymatic synthesis of a 2-*O*-α-D-glucopyranosyl cyclic tetrasaccharide by kojibiose phosphorylase

Hikaru Watanabe,* Takanobu Higashiyama, Hajime Aga, Tomoyuki Nishimoto, Michio Kubota, Shigeharu Fukuda, Masashi Kurimoto and Yoshio Tsujisaka

Amase Institute, Hayashibara Biochemical Laboratories, Inc., 7-7 Amase minami-machi, Okayama 700-0834, Japan

Received 6 October 2004; accepted 8 December 2004
Available online 13 January 2005

Abstract—The glucosyl transfer reaction of kojibiose phosphorylase (KPase) from *Thermoanaerobacter brockii* ATCC35047 was examined using *cyclo*-{→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→} (CTS) as an acceptor. KPase produced four transfer products, saccharides **1**–**4**. The structure of a major product, saccharide **4**, was 2-*O*-α-D-glucopyranosyl-CTS, *cyclo*-{→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-[α-D-Glc*p*-(1→2)]-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→}. The other transfer products, saccharides **1**–**3**, were 2-*O*-α-kojibiosyl-, 2-*O*-α-kojitriosyl-, and 2-*O*-α-kojitetraosyl-CTS, respectively. These results showed that KPase transferred a glucose residue to the C-2 position at the ring glucose residue of CTS. This enzyme also catalyzed the chain-extending reaction of the side chain of 2-*O*-α-D-glycopyranosyl-CTS.

Keywords: Cyclic tetrasaccharide; *Thermoanaerobacter brockii*; Kojibiose phosphorylase; Transglucosylation

1. Introduction

A cyclic tetrasaccharide, *cyclo*-{→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→} (abbreviated as CTS), has a unique structure consisting of four glucose residues joined by alternate α-(1→3)- and α-(1→6)-linkages. Côté and co-workers first reported that CTS was produced from a dextran-like polysaccharide, alternan, by its degradation enzyme.[1,2] Recently, we found a new enzymatic system to synthesize this saccharide from maltodextrins by a joint reaction of two glycosyltransferases, 6-α-D-glucosyltransferase and 3-α-isomaltosyltransferase.[3] We also succeeded in the mass production of CTS from starch in a high yield using both enzymes.[4,5] CTS has tolerance to the hydrolytic activity of glycosidases such as amylase or α-glucosidase; therefore, this saccharide is expected to be used as a low-calorie sweetener. Single-crystal X-ray structure analysis has shown that CTS has a shallow cavity in the center of its cyclic structure.[6] The cavity can bind with small inorganic ions.[7] CTS binds ethanol on both the concave and convex sides.[8] These properties of CTS open the potential for further applications, for example, as a carrier in drug delivery systems or a base that removes toxic metal cations.

Kojibiose phosphorylase (EC 2.4.1.230: KPase) catalyzes the reversible phosphorolysis of kojibiose (α-D-glucopyranosyl-(1→2)-D-glucopyranose) as follows: β-D-glucose-1-phosphate (β-G1P) + D-glucose ⇌ kojibiose + inorganic phosphate (Pi). We have reported on the purification and properties of KPase from *Thermoanaerobacter brockii* ATCC35047.[9] This enzyme also catalyzes transglucosylation using β-G1P as glucosyl donor to the appropriate acceptor. Therefore, KPase is expected to catalyze transglucosylation to CTS.

2. Results

2.1. Transglucosylation to CTS by KPase

A reaction mixture (1 mL) containing KPase (34.2 U/mmol for β-G1P), β-G1P (73 mM), CTS (154 mM)

* Corresponding author. Tel.: +81 86 231 6731; fax: +81 86 231 6738; e-mail: amaseken@hayashibara.co.jp

0008-6215/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carres.2004.12.012

in 50 mM sodium acetate buffer (pH 5.5) was incubated at 50 °C for 64 h. The reaction was stopped by heating in a boiling water bath for 10 min. A sample (50 μL) of the reaction mixture was removed for analysis by HPLC. As shown in Figure 1, KPase gave four transfer products, saccharide **1** (T_R 14.2 min: the HPLC retention time = T_R), **2** (T_R 15.6 min), **3** (T_R 17.4 min), and **4** (T_R 32.5 min). Figure 2 shows the time course of the reaction for the syntheses of saccharides **1**–**4**. Saccharide **4** seemed to be generated earlier than the other saccharides. The yield of saccharides **1**–**4** at a reaction time of 64 h reached 5.1%, 3.5%, 1.3%, and 22.5%, respectively.

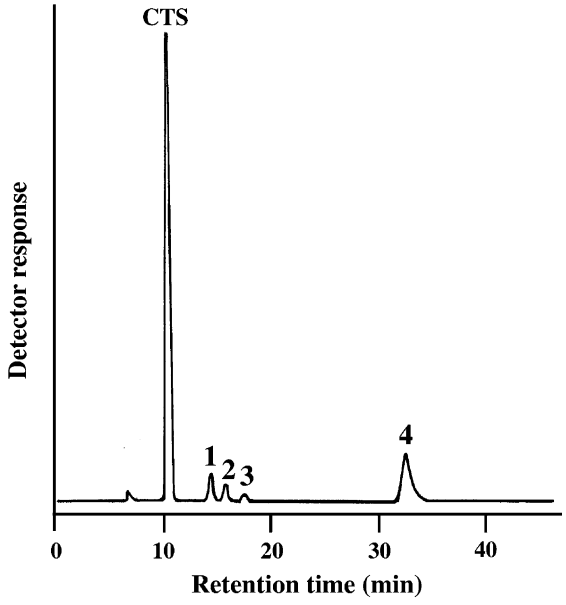

Figure 1. HPLC profile of reaction products on a mixture of β-G1P as the donor and CTS as the acceptor by KPase.

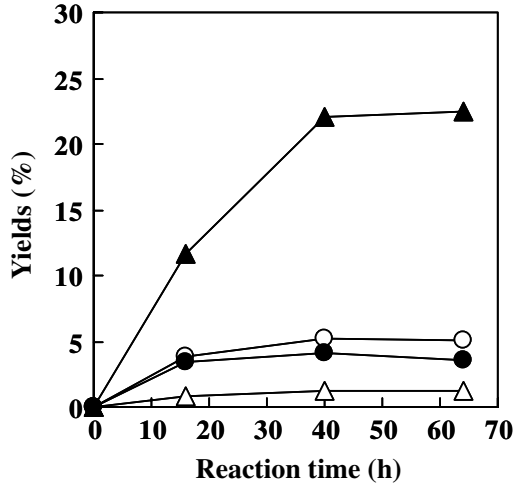

Figure 2. Time-course of formation of saccharides **1**–**4**. The yields of saccharides **1**–**4** are shown except β-G1P. ○, Saccharide **1**. ●, Saccharide **2**. △, Saccharide **3**. ▲, Saccharide **4**.

2.2. Preparation and isolation of saccharides 1–4

To prepare saccharides **1**–**4** on the gram scale, a reaction mixture containing 154 mM of CTS, 73 mM of β-G1P, and KPase (34.2 U/mmol for β-G1P) in 800 mL of 50 mM sodium acetate buffer (pH 5.5) was incubated at 50 °C for 64 h. The yields of saccharides **1**–**4** reached 5.1%, 3.5%, 1.3%, and 22.2%, respectively. After the enzyme reaction was stopped by heating, the mixture was centrifuged, and then the resultant supernatant was desalted by passing it through ion-exchange resins, 100 mL of Diaion SK1B (Mitsubishi Chem. Co., Tokyo, Japan), and 200 mL of Amberlite IRA411S (Japan Organo, Tokyo, Japan). The eluent was concentrated to 400 mL by evaporation at 40 °C. An 18-mL portion of the saccharide solution was put through a repeated preparative HPLC on an ODS-AQ R-355-15-AQ column (50 × 500 mm, YMC). Saccharides **1**–**4** were separated from CTS by elution with water as a solvent at a flow rate of 30 mL/min at 35 °C. The fractions containing saccharides **1**–**4** were separately collected and then evaporated at 40 °C. Saccharides **1**, **2**, and **4** were easily crystallized from their aqueous solutions. Saccharide **3** was obtained as a white powder by lyophilization. The amounts of purified saccharides **1**–**4** were 3.1 g (purity: 98.3%), 1.7 g (95.2%), 0.42 g (96.3%), and 18.6 g (98.3%), respectively.

2.3. Arsenolysis of saccharides 1–4 by KPase

KPase catalyzes the phosphorolysis of kojibiose. When arsenate is used in place of phosphate, kojibiose is converted into 2 mol of glucose.[10] As shown in Table 1, saccharide **4** was arsenolyzed to the equimolar of glucose and CTS. This result showed that saccharide **4** had a structure containing one glucose residue attached to CTS. When saccharides **1**–**3** were arsenolyzed, saccharide **4** was generated in addition to glucose and CTS. Therefore, saccharides **1**–**3** should have saccharide **4** in their structures.

2.4. Characterization of saccharide 4

The molecular mass of **4** was found to be 810 Da by measuring the $[M + Na]^+$ ion (*m*/*z* 833) by ESIMS. This value was identical to that of CTS linked to one glucose

Table 1. Arsenolysis of saccharides **1**, **2**, **3**, and **4**

Saccharide	Sugar composition (%, mol)[a]					
	Glucose	CTS	1	2	3	4
1	52.9	9.1	0.0	Nd	Nd	38.0
2	65.8	4.2	0.8	0.0	Nd	29.2
3	68.9	3.0	2.8	5.4	0.1	19.8
4	30.6	28.2	Nd	Nd	Nd	41.2

[a] Nd = not detected.