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Synthesis of β-D-glucopyranosyl-(1 → 4)-D-arabinose, β-D-glucopyranosyl-(1 → 4)-L-fucose and β-D-glucopyranosyl-(1 → 4)-D-altrose catalysed by cellobiose phosphorylase from *Cellvibrio gilvus*

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Abstract

β-D-Glc*p*-(1 → 4)-D-Ara*p*, β-D-Glc*p*-(1 → 4)-L-Fuc*p*, and β-D-Glc*p*-(1 → 4)-D-Alt *p* were synthesised using a partially purified cellobiose phophorylase from *Cellvibrio gilvus*. The disaccharides were purified, and analysed by ^{1}H and ^{13}C NMR. © 1998 Elsevier Science Ltd.

Keywords: β-D-Glucopyranosyl-(1 → 4)-D-arabinose; β-D-glucopyranosyl-(1 → 4)-L-fucose; β-D-glucopyranosyl-(1 → 4)-D-altrose; Cellobiose phosphorylase; *Cellvibrio gilvus*; Disaccharide synthesis

1. Introduction

Cellobiose phosphorylase (EC 2.4.1.20) catalyses the reversible phosphorolysis of cellobiose to form α-D-glucose-1-phosphate (G-1-P) and D-glucopyranose; thereby playing an important role in cellulose breakdown. Our laboratory has previously purified the enzyme from *Cellvibrio gilvus* to an electrophoretically homogeneous state and characterised it extensively [1–3]. We are currently evaluating different acceptors in the synthetic reaction of cellobiose phosphorylase as a means of gaining information regarding the enzyme's active site. In addition, we hope that the synthesis of novel oligosaccharides may prove to have applications in the food and health areas as well as for their use as substrates in future enzyme characterisation. Synthesis using the reversible reaction of phosphorylases is advantageous over other enzymatic methods, such as the transglycosylation reaction of glycosidases, due to the regioselectivity of the enzyme resulting in only one product and because of the ready availability of donor substrate. The broad specificity of cellobiose phosphorylase from *C. gilvus* makes it a particularly useful enzyme in oligosaccharide synthesis.

We have reported the synthesis and characterisation of β-D-glucopyranosyl-(1 → 4)-D-xylose [4], β-D-glucopyranosyl-(1 → 4)-D-glucosamine, β-D-glucopyranosyl-(1 → 4)-2-deoxy-D-glucose [5], β-D-glucopyranosyl-(1 → 4)-6-deoxy-D-glucose, β-D-glucopyranosyl-(1 → 4)-D-mannose and β-D-glucopyranosyl-(1 → 4)-D-mannosamine [6]. Acceptor specificity has been shown to require that the configuration of β-D-glucopyranose is retained at the C-1, C-3, C-4,

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and C-5 positions [3]. However, cellobiose phosphorylase from *Clostridium thermocellum* can also catalyse the reaction of G-1-P with D-arabinose [7,8], L-fucose [7,9] and D-altrose [9]. Unlike D-glucose when these sugars are in the $^4C_1$ chair form they possess axial hydroxyl groups at C-2 and C-3. The structure of the resulting β-D-Glc*p*-(1 → 4)-D-Ara*p* was confirmed by periodate oxidation and methylation analyses and hydrolysis using β-glucosidase [8]. The structures of the L-fucose and D-altrose products have not been determined but based on the other disaccharides obtained from the enzymatic synthesis reaction were presumed to be β1,4 linked. β-D-Glc*p*-(1 → 4)-D-Alt*p*, has been given the trivial name celtrobiose, and has been synthesised chemically [10]. However, to our knowledge, the above cases are the sole instances of synthesis of β-D-Glc*p*-(1 → 4)-D-Ara*p* and β-D-Glc*p*-(1 → 4)-L-Fuc*p*.

In this study, we investigated the ability of *C. gilvus* cellobiose phosphorylase to utilise acceptors with configurations different to D-glucose at the C-3 position, and confirmed the structures of several disaccharides produced using NMR spectroscopy.

2. Experimental

Chemicals.—L-Fucose, D-allose, 3-deoxy-D-glucose, 3-*O*-methyl-D-glucose, 3-fluoro-3-deoxy-D-glucose, G-1-P (dipotassium salt), BSA, and MOPS were obtained from Sigma Chemical (St. Louis, USA). D-Arabinose, NaOH, $NaH_2PO_4 \cdot 2H_2O$, $Na_2HPO_4 \cdot 12H_2O$, $(NH_4)_2SO_4$, and ethanol (HPLC grade) were obtained from Nacalai Tesque (Kyoto, Japan). Acetonitrile (HPLC grade) was purchased from Wako Pure Chemical Industries (Osaka, Japan). D-Altrose was obtained from Aldrich (Wisconsin, USA).

General methods.—HPLC was performed with Shimadzu LC9A pump system (Kyoto, Japan) and GL Sciences RI model 504 detector (Tokyo, Japan), using either a TSK–gelG–oligo–PW column (7.8 × 300 mm; Tosoh Tokyo, Japan) or a TSK–gel–Amide-80 column (4.6 × 250 mm; Tosoh). HPAEC was carried out using a Dionex DX300 model (Dionex, CA, USA) equipped with Dionex CarboPac PA1 column (4 × 250 mm). Samples (10 μL) were injected using a Shimdazu SIL-10A*i*. The eluate (1 mL min^{-1}) was monitored using a Pulsed Amperometric Detector (Dionex). The column was eluted as follows; the first 7.5 min isocratic 100 mM NaOH–50 mM NaOAc; followed with a linear gradient of NaOAc to 100 mM NaOH–500 mM NaOH at 10 min; isocratic conditions to 14 min, then at 14.5 min the column was equilibrated at 100 mM NaOH–50 mM NaOAc (isocratic). Column chromatography was performed using a hydrophobic interaction column (TSK–gel Butyl-650 M Tosoh; 2.6 × 235 mm), a size exclusion column of Biogel P2 (Bio-Rad, CA, US; 16 × 500 mm), an activated charcoal column (Wako; 16 × 500 mm) and ion exchange columns with SP Sepharose Fast Flow (Pharmacia, Uppsala, Sweden; 7 × 15 mm) and Q Sepharose Fast Flow (Pharmacia; 7 × 15 mm).

^1H, ^{13}C, DQF–COSY, TOCSY and ^{13}C–^1H COSY NMR spectra were recorded using a Bruker DRX600 spectrometer (^1H, 600 MHz; ^{13}C, 150 MHz). D_2O was used as the solvent and the spectra was recorded at 303 K. Chemical shifts were expressed in ppm (δ) downfield from tetramethylsilane using acetone or 2-methyl-2-propanol as internal referencing.

Preparation of cellobiose phosphorylase from C. gilvus.—*C. gilvus* cells were cultivated and cellobiose phosphorylase partially purified according to the method of Kitaoka et al. [2] with the following modifications. To the supernatant obtained from $(NH_4)_2SO_4$ at 40% saturation, additional $(NH_4)_2SO_4$ was added until the solution reached 70% saturation. The precipitate formed was dissolved in 0.5 M phosphate buffer (pH 7.0) and $(NH_4)_2SO_4$ was added to 20% saturation. This step was repeated twice. The combined supernatants were applied to a hydrophobic interaction column (TSK–gel Butyl-650 M; flow rate 10 mL min^{-1}) previously equilibrated with 0.5 M phosphate buffer (pH 7.0) containing $(NH_4)_2SO_4$ (20% saturation). Proteins were eluted with this buffer for 400 mL, followed by a linear gradient with the concentration of $(NH_4)_2SO_4$ decreasing from 20% saturation to 7.6% saturation over 2100 mL. Active fractions were pooled and dialysed against 1 mM MOPS buffer (pH 7.0). No further purification was performed.

Disaccharide synthesis.—Enzymatic synthesis was performed with 50 mM sodium phosphate buffer, 0.02% BSA, 10 mM G-1-P, 0.04 U mL^{-1} cellobiose phosphorylase and 100 mM monosaccharide at 37° for 24 h. Exceptions to this were the synthesis of fucose disaccharide with 50 mM MOPS buffer, and the synthesis of altrose disaccharide with 300 mM D-altrose. One unit of enzyme activity was defined as the amount of enzyme which produces 1 μmol of D-glucose per min from 10 mM cellobiose and 10 mM Pi at 37°.

Purification of β-D-glucopyranosyl-(1 → 4)-L-fucose.—The crude reaction mixture (50 mL) was applied to an activated charcoal column. The column was washed with 20 volumes of water followed by a linear gradient of 0 to 15% ethanol over 30 column volumes. Fractions were analysed by Dionex HPLC. Product-containing fractions were concentrated and applied to an HPLC size exclusion column (TSK–gelG–oligo–PW) for further purification.

Purification of β-D-glucopyranosyl-(1 → 4)-D-arabinose.—The crude synthesis reaction mixture (100 mL) was lyophilised, and a reduced volume was applied in two lots to a Biogel P2 column and eluted with MilliQ water. Fractions were analysed by Dionex HPLC. The product-containing fractions were concentrated by rotary evaporation and applied once more to Biogel P2. The fractions containing the disaccharide were combined, concentrated and applied to an HPLC column, TSK–gelG–oligo–PW. Analysis by ^{1}H NMR showed the disaccharide was not completely pure. The sample was then applied to a TSK–gel–Amide-80 column and eluted with 82% acetonitrile/water at 30°. Product-containing fractions were concentrated by rotary evaporation and passed through a washed Millipore filter to remove silica obtained during the HPLC process.

Purification of β-D-glucopyranosyl-(1 → 4)-D-altrose.—The reaction mixture (3.9 mL) was lyophilised and the reduced volume applied to a Biogel P2 column and eluted with MilliQ water. The fractions were analysed by Dionex HPLC and the product-containing fractions were combined and concentrated by rotary evaporation and re-applied to the Biogel P2 column. The sample was applied to a column of SP Sepharose Fast Flow (OH^-), eluted with MilliQ water and adjusted to neutral pH with dilute NaOH. The remaining G-1-P was removed by eluting the sample through a column of SP-Sepharose Fast Flow (OH^-) followed by a column of Q Sepharose Fast Flow (H^+).

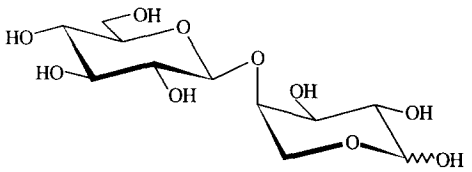
Table 1
Disaccharide synthesis by cellobiose phosphorylase

Glucosyl acceptor	Disaccharide concentration[a]
L-Fuc	0.11 mg mL^{-1}
D-Ara	0.10 mg mL^{-1}
D-Alt	1.1 mg mL^{-1}
D-All	0.02 mg mL^{-1}
3-Deoxy-D-Glc	0.02 mg mL^{-1}
3-*O*-methyl-D-Glc	0 mg mL^{-1}
3-Fluoro-3-deoxy-D-Glc	1.2 mg mL^{-1}

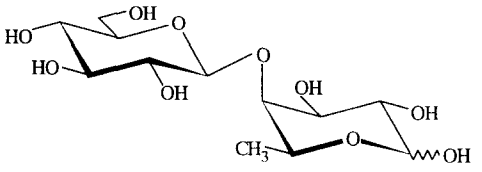
[a]Measured by size exclusion HPLC.

3. Results and discussion

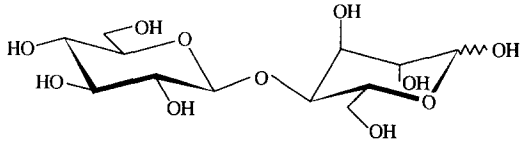
Previous work on *C. gilvus* cellobiose phosphorylase has shown that monosaccharides with axial hydroxyl groups on the C-3 are not effective substrates [3]. In this study, the glucosyl acceptor concentration was raised to 10 or 30 times that of G-1-P and the enzyme was partially purified to avoid interference from other enzymes. The monosaccharides tested were incubated with cellobiose phosphorylase and G-1-P for 24 h at 37°. The amount of disaccharide produced determined by size exclusion HPLC and the results are presented in Table 1. The moderate acceptor ability of 3-fluoro-3-deoxy-D-glucose (1.2 mg dis-



β-D-Glc*p*-(1→4)-D-Ara



β-D-Glc*p*-(1→4)-L-Fuc



β-D-Glc*p*-(1→4)-D-Alt

Fig. 1. Structures of disaccharides formed in the study.

Table 2
^{1}H NMR shift data and coupling constants from the disaccharides β-D-Glc*p*-(1 → 4)-D-Ara*p*, β-D-Glc*p*-(1 → 4)-L-Fuc*p*, β-D-Glc*p*-(1 → 4)-D-Alt*p*

Compound	Residue	H-1 ($J_{1,2}$)	H-2 ($J_{2,3}$)	H-3 ($J_{3,4}$)	H-4 ($J_{4,5a}$) ($J_{4,5b}$)	H-5a ($J_{5a,5b}$)	H-5b	H-6a	H-6b
β-D-Glc*p*-(1 → 4)-D-Ara*p*	α-Ara*p*	4.57 (7.7)	3.52 (9.9)	3.72 (3.6)	4.14 (0.5) (2.1)	4.06 (13.4)	3.65	–	–
	β-Ara*p*	5.27 (3.5)	3.83 (9.9)	3.95 (3.5)	4.14 (1.1) (2.8)	4.01 (13.2)	3.82	–	–
	Glc*p*	4.53 (7.9)	3.36 (9.5)	3.51 (8)	3.42	3.46	–	3.73	3.91
β-D-Glc*p*-(1 → 4)-L-Fuc*p*	α-Fuc*p*	5.22 (3.9)	3.75 (10.5)	3.84 (3.2)	4.02 (~ 0)	4.27	–	1.29	–
	β-Fuc*p*	4.59 (7.7)	3.42 (10.1)	3.62 (3.2)	3.96 (~ 0)	3.86	–	1.32	–
	Glc*p*	4.47 (7.8)	3.39 (9)	3.50 (8)	3.42 (10)	3.45	–	3.93	3.73
β-D-Glc*p*-(1 → 4)-D-Alt*p*	α-Alt*p*	4.98 (7.9)	3.82 (9.4)	4.11 (3.4)	4.04 (7.4)	4.17	–	3.82	n.d.[a]
	β-Alt*p*	5.23 (1.3)	3.81 (4.2)	4.29 (2.8)	3.94 (9.9)	3.90–3.94	–	3.77	3.89–3.94
	Glc*p*	4.53, 4.52[b] (7.9, 7.9)	3.32, 3.29[b] (9.4, 9.4)	3.49, 3.48[b] (8.8, 8.9)	3.40 (9.8)	3.42–3.47	–	3.73	3.89

[a] n.d. = not determined.
[b] Different values were observed for the α and β anomers. The values reported are for the α and β anomers, respectively.

Table 3
^{13}C NMR data from the disaccharides β-D-Glc*p*-(1 → 4)-D-Ara*p*, β-D-Glc*p*-(1 → 4)-L-Fuc*p*, β-D-Glc*p*-(1 → 4)-D-Alt*p*

Compound	Residue	C-1	C-2	C-3	C-4	C-5	C-6
β-D-Glc*p*-(1 → 4)-D-Ara*p*	α Ara*p*	99.2	74.7	74.2	78.4	66.4	–
	β Ara*p*	95.2	71.5	70.5	78.9	62.7	–
	Glc*p*	103.3, 103.5[a]	75.5	78.1, 78.2[a]	72.2	78.5	63.3
β-D-Glc*p*-(1 → 4)-L-Fuc*p*	α Fuc*p*	94.0	70.5	70.3	82.7	68.3	17.1
	β Fuc*p*	97.9	73.9	73.8	81.7	72.5	17.2
	Glc*p*	104.9	75.2, 75.1[a]	77.1, 77.2[a]	71.1, 71.2[a]	77.6	62.3
β-D-Glc*p*-(1 → 4)-D-Alt*p*	α Alt*p*	95.3	72.1	71.5	76.1	71.5	61.9
	β Alt*p*	93.4	72.3	71.9	75.8	74.6	62.8
	Glc*p*	104.5, 105.3[a]	74.7, 74.8[a]	77.2, 77.3[a]	71.2, 71.1[a]	77.5, 77.4[a]	62.3, 62.2[a]

[a]Different values were observed for the α and β anomers. The values reported are for the α and β anomers respectively.

accharide mL^{-1}) and the negligible acceptor ability of 3-deoxy-D-glucose (0.02 mg mL^{-1}) and 3-*O*-methyl-D-glucose (0 mg mL^{-1}) indicates that the C-3 hydroxyl group is involved in hydrogen bonding to the enzyme. Cellobiose phosphorylase was able to utilise D-Alt as an acceptor (1.1 mg mL^{-1}) while D-All was a poor acceptor. This suggests the enzyme may accommodate an axial C-3 hydroxyl group providing the C-2 hydroxyl group is also axial. This is reflected in the acceptor ability of L-FuC (0.11 mg mL^{-1}) and D-ara (0.10 mg mL^{-1}). The absence of a hydroxymethyl group at the C-6 of the two sugars would contribute to their lower acceptor ability compared to D-Alt [3]. In addition, L-FuC and D-All are predominantly in the $^1C_4$ chair form in solution and this would also account to the low yield relative to D-Alt.

After the reactivity of the substrates was established, the disaccharides formed using D-Ara L-Fuc and D-Alt as acceptors were synthesised in larger quantities for characterisation. Following purification using a range of chromatography procedures, the yields for β-D-Glc*p*-(1 → 4)-D-Ara*p*, β-D-Glc*p*-(1 → 4)-L-Fuc*p*, and β-D-Glc*p*-(1 → 4)-D-Alt*p* were 1.2 mg (0.6% yield based on added G-1-P), 1 mg (0.7% yield) and 1.2 mg (19.1% yield) respectively.

The disaccharides were characterised by NMR. DQF–COSY spectra were used to determine coupling constants; and TOCSY spectra were used to identify individual spin systems. Using these methods, almost all ^1H signals were assigned (Table 2). Coupling constants were determined in all but a few cases in which there was extreme signal overlap (Table 2). Based on the ^1H NMR assignments, all ^{13}C signals were assigned by ^{13}C–^1H COSY experiments (Table 3). The ^1H NMR spectra of the disaccharide synthesised using D-Ara was compared to that of D-Ara. The downfield shift of signals at H-4 signal of the arabinose residue indicated the disaccharide was 1 → 4 linked. The coupling constant $J_{1,2}$ of the glucose residue was 7.9 Hz, reflecting a β linkage. The coupling constants of the arabinose residue showed it to be in the $^1C_4$ chair conformation (Fig. 1). The structures of β-D-Glc*p*-(1 → 4)-L-Fuc*p*, and β-D-Glc*p*-(1 → 4)-D-Alt*p* were determined in the same manner and are also shown in Fig. 1. The L-Fuc and D-Alt residues were shown to be in the $^1C_4$ and $^4C_1$ chair forms respectively. It is presumed that L-Fuc and D-Ara bind to the enzyme and react in the $^4C_1$ chair forms, and later adopt the more stable $^1C_4$ chair forms in solution.

We conclude that *C. gilvus* cellobiose phophorylase can use acceptors with C-3 hydroxyl group in axial position providing the C-2 hydroxyl is also axial. In all cases a β-(1 → 4)-linkage was formed.

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