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Enzymic α-galactosylation of a cyclic glucotetrasaccharide derived from alternan☆

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Abstract

Alternanase catalyzes the hydrolysis of alternan, an α-(1→3)-α-(1→6)-D-glucan produced by *Leuconostoc mesenteroides*, resulting in the formation of a cyclic tetramer *cyclo*{→3)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→}$_2$ (*c*Glc$_4$). Two α-galactosidases, one from coffee bean and the other produced by a fungus, currently described as *Thermomyces lanuginosus*, were found to catalyze an efficient 6-*O*-α-D-galactopyranosylation of *c*Glc$_4$. The attachment of a nonreducing α-D-galactopyranosyl residue to the *c*Glc$_4$ molecule opens new possibilities for future applications of the cyclic tetramer, since the D-galactopyranosyl residue can be easily modified by D-galactose oxidase to introduce a reactive aldehyde group. The results also extend our knowledge about the synthetic potential of *T. lanuginosus* α-galactosidase. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: α-Galactosidase; Cyclic oligosaccharide; Alternan

1. Introduction

Alternan, an α-(1→3)-α-(1→6)-D-glucan produced by *Leuconostoc mesenteroides* is subject to enzymic hydrolysis by few enzymes, due to its alternating linkage structure.[1] There are only a few soil microorganisms that are known to degrade alternan in an endo fashion. These bacteria have been tentatively identified as a *Bacillus* species.[2] During growth of these species on alternan or on maltose, a unique α-D-glucanase called alternanase is secreted into the culture fluid. Alternanase catalyzes the hydrolysis of the polysaccharide, resulting in the formation of a cyclic tetramer *cyclo*{→3)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→}$_2$ (*c*Glc$_4$).[3,4] Since the discovery of this saccharide structure, no examples of its use or application have been presented. The recently established crystalline structure of *c*Glc$_4$[5] reveals a certain degree of asymmetry of the molecule and its ability to bind one molecule of water in its dish-shaped cavity.[5] The *c*Glc$_4$ molecule differs in both size and shape from much larger cyclodextrins and also from reducing or linear oligosaccharides. In order to explore possible applications of *c*Glc$_4$, e.g., in chiral or affinity chromatography, as an agent to regulate protein folding or bind small molecules, or as a structure mimicking some immunologically active saccharide groups, we

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have attached a D-galactopyranosyl residue enzymatically to cGlc$_4$. The presence of an α-D-galactopyranosyl residue may enable the oxidation of the primary hydroxyl group by galactose oxidase to afford a reactive cGlc$_4$ derivative having an aldehyde group. In this work we report on the α-D-galactosylation of cGlc$_4$ using two different α-galactosidases.

2. Experimental

Carbohydrates.—The cyclic tetrasaccharide *cyclo*{→3)-α-D-Glc*p*-(1→6)-α-D-Glc*p*-(1→}$_2$ (cGlc$_4$) was prepared by hydrolysis of alternan with alternanase as described previously.[4] However, the preparation was done on a larger scale, requiring changes in the isolation strategy as follows. The alternan hydrolyzate containing a mixture of reducing sugars and cyclic nonreducing products, including cGlc$_4$, was first passed through a Dowex 1 (OH^-) anion-exchange column. Reducing sugars bound to the anion-exchange resin while cyclic oligosaccharides passed through the column. The concentrated eluate was subsequently chromatographed on a column of Bio-Gel P-2 to separate cGlc$_4$ from its glucosylated forms that were also present in the mixture.[4] Lyophilized cGlc$_4$ was recrystallized three times from aq EtOH (~60% EtOH) to remove traces of its glucosylated forms, mainly 6-*O*-α-D-glucopyranosyl-cGlc$_4$.[4] This step was prerequisite to carrying out any enzymatic galactosylation of cGlc$_4$. Melibiose, used as a D-galactopyranosyl donor, was from Fisher Biotech (Pittsburgh, PA, USA). 4-Nitrophenyl α-D-galactopyranoside was from Sigma Chemical Co. (St. Louis, MO, USA).

Enzymes.—Green coffee bean α-galactosidase (suspension in 3.2 M $(NH_4)_2SO_4$) was from Sigma Chemical Co. Purified α-galactosidase from the fungus *Thermomyces lanuginosus* IMI 158 479 was prepared as described by Puchart et al.[6] It should be noted that a recent comparison of the strain with other *T. lanuginosus* strains[7] indicates that the strain does not show a complete identity with other *T. lanuginosus* strains, and, therefore, it may be reclassified [B.A. Prior, Z.B. Ögel, and P. Biely, unpublished results].

α-Galactosidase assay.—Assay mixtures (0.5 mL) containing enzyme and 2 mM 4-nitrophenyl α-D-galactopyranoside in 0.05 M NaOAc buffer (pH 5.5 for coffee bean enzyme and pH 4.5 for the fungal enzyme) were incubated at 40 °C. Reactions were stopped by addition of 1 mL of a saturated solution of $Na_2B_4O_7$ after which the absorbance of solutions was measured at 410 nm. One unit of α-galactosidase activity is defined as the amount of enzyme liberating 1 μmol of 4-nitrophenol in 1 min.

Galactosylation of c*Glc$_4$.*—After finding that melibiose can serve as a galactosyl donor superior to 4-nitrophenyl α-D-galactopyranoside in transgalactosylation reactions catalyzed by both coffee bean and fungal α-galactosidase, the galactosylation of cGlc$_4$ was done as follows: 342 mg of melibiose in 0.5 mL of 2 mM NaOAc buffer (pH 5.5 or 4.5 depending on the type of the enzyme used) was mixed with 0.5 mL of a solution of cGlc$_4$ (243 mg) in the same buffer and the reaction was started by addition of either 12 U of coffee bean α-galactosidase or 2.5 U of the fungal α-galactosidase. The reaction mixtures were incubated at 40 °C and the galactosylation of cGlc$_4$ was monitored by thin-layer chromatography (TLC, see below). Aliquots of the reaction mixtures were taken at intervals and diluted with 19 volumes of dimethyl sulfoxide (DMSO). Samples (1 μL) of diluted solutions were analyzed by TLC. The reaction catalyzed by coffee bean enzyme was terminated after 42 h, and that catalyzed by the fungal enzyme after 90 h, by passing the reaction mixture through a column of Dowex 1 (OH^-) to bind reducing sugars. As it will be shown below, this also served as the first step toward purification of galactosylated cGlc$_4$. Reaction mixtures containing melibiose without cGlc$_4$ were run in parallel to monitor the products formed by D-galactosyl transfer to melibiose, D-galactose and/or D-glucose.

Other methods.—Thin-layer chromatography was done on silica gel plates (E. Merck) in a solvent mixture consisting of 1:4:3:2 nitroethane–acetonitrile–ethanol–water. Carbohydrates were made visible with *N*-(1-naphthyl)ethylenediamine dihydrochloride reagent.[8] Total sugar concentration was

determined by the phenol–H_2SO_4 method.[9] ^1H and ^{13}C NMR spectroscopy measurements were done in ^2H_2O using a Bruker Avance 400 spectrometer equipped with a 5 mm $^1H/^{13}C$ dual probe, at 400.13 MHz (^1H) and 100.61 MHz (^{13}C). Methylation analysis was performed according to published procedures.[10,11] The resulting partially methylated alditol acetate derivatives were separated by gas–liquid chromatography on an instrument coupled to a Finnegan TSQ 700 mass spectrometer. Chemical ionization (CIMS) of molecules was done with NH_3 as the reagent gas. The molecular mass of each product was determined by electrospray mass spectrometry (ESIMS) done in a solution of 0.1% AcOH.

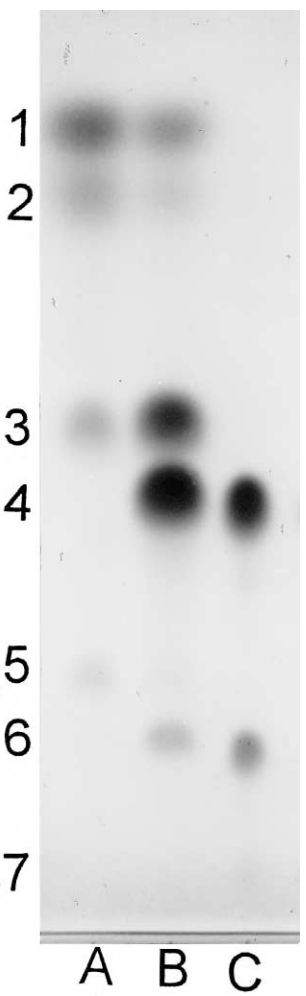

Fig. 1. TLC analysis of reaction mixtures: (A) melibiose + coffee bean α-galactosidase (42 h); (B) melibiose + *c*Glc_4 + coffee bean α-galactosidase (42 h); (C) reaction mixture B after removal of reducing sugars by anion-exchange. Compounds: 1, D-glucose; 2, D-galactose; 3, melibiose; 4, *c*Glc_4; 5, α-D-galactopyranosyl-melibiose; 6, 6-*O*-α-D-galactopyranosyl-*c*Glc_4; 7, di-*O*-α-D-galactopyranosyl-*c*Glc_4.

3. Results and discussion

Two different α-D-galactosidases were tested for their ability to galactosylate *c*Glc_4: commercially available coffee bean enzyme and recently purified α-galactosidase produced by *T. lanuginosus*.[6] The coffee bean enzyme was reported earlier to be efficient in α-D-galactosylation of cyclodextrins from melibiose,[12,13] while the *T. lanuginosus* enzyme has been shown to catalyze efficient transgalactosylation from 4-nitrophenyl α-D-galactopyranoside.[6] In preliminary experiments both enzymes catalyzed an efficient galactosylation of *c*Glc_4 using either 4-nitrophenyl α-D-galactopyranoside or melibiose. Limited solubility in water of the aryl glycoside (max. ~ 0.15 M) led us to use melibiose as the glycosyl donor (solubility ~ 2 M). This enabled us to use a higher concentration of D-galactopyranosyl donor, thus enhancing the overall yield of the desired product.

The preparative scale reactions were performed at 1 M melibiose concentration and 0.6 M concentration of *c*Glc_4. Monitoring of the reactions by TLC showed that a considerable portion of *c*Glc_4 was converted to slower-migrating compounds. The major galactosylated product showed chromatographic mobility lower than the product formed by galactosyl transfer to melibiose (Fig. 1). The galactosylated melibiose product was not studied, but is believed to represent a trisaccharide. The degree of conversion of *c*Glc_4 was estimated from the relative proportions of *c*Glc_4 and Gal-*c*Glc_4, as determined by phenol–sulfuric acid analysis after elution of the isolated compounds from dried TLC plates. In the mixtures containing coffee bean α-galactosidase, about 22% of *c*Glc_4 was converted to the major galactosylated product. A 15% conversion was recorded in the mixture with the fungal enzyme. Minor products were not isolated for rigorous structural analysis, but the mixtures also contained traces of two positional isomers of the major product. The minor isomers could be detected by TLC as zones migrating slightly ahead of the major product when larger samples were applied and as minor GLC peaks upon methylation analysis. GLC–MS analysis of the mixture indi-

Table 1
Methylation analysis of galactosylated cGlc$_4$ products [a]

Source of α-galactosidase	2,3,4,6-Tetra-*O*-methyl (Gal)	2,3,4-Tri-*O*-methyl (Glc)	2,4,6-Tri-*O*-methyl (Glc)	2,4-Di-*O*-methyl (Glc)
Coffee bean	1.0	2.0	1.1	1.3
T. lanuginosus	1.0	1.8	1.1	1.1

[a] Relative molar ratios of alditol acetate derivatives from per-O-methylated oligosaccharides.

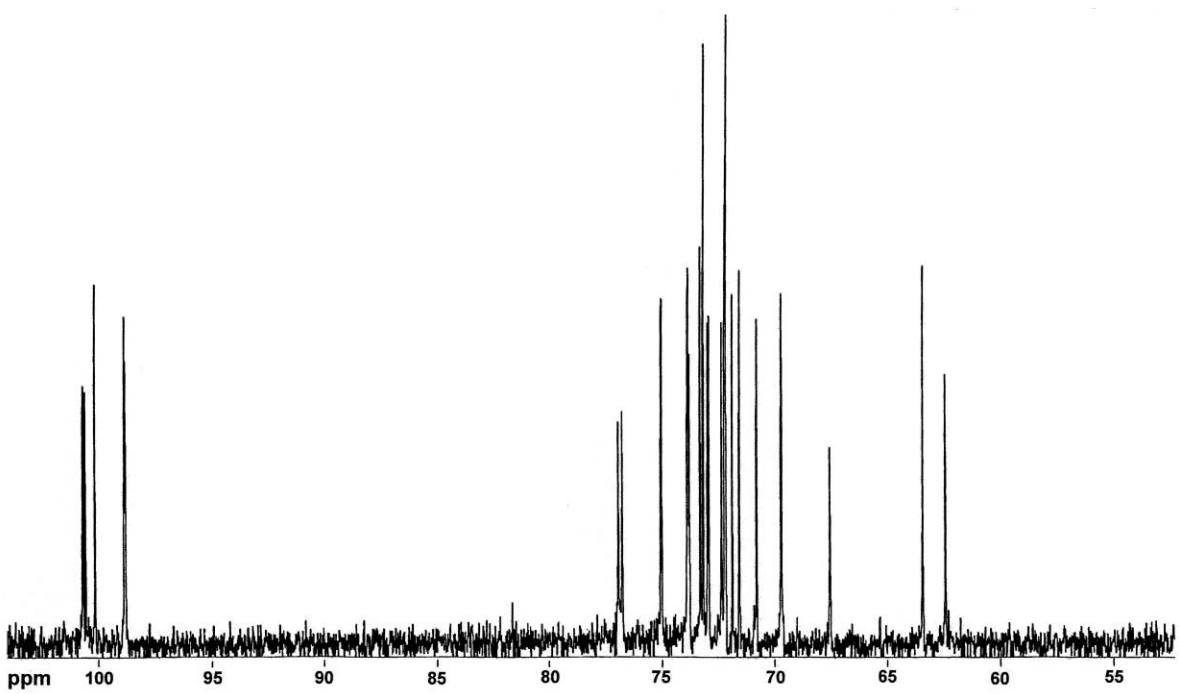

Fig. 2. Proton-decoupled ^{13}C NMR spectrum of 6-*O*-α-D-galactopyranosyl-cGlc$_4$.

cates the positional isomers to be 4-*O*-α-D-galactopyranosyl cGlc$_4$ and either 2- or 3-*O*-α-D-galactopyranosyl cGlc$_4$. A small amount of doubly galactosylated cGlc$_4$ was detected by mass spectrometry (see below).

The major product of cGlc$_4$ galactosylation by both enzymes was isolated from the reaction mixtures in two steps. The first step involved removal of reducing sugars on Dowex 1 (OH^-) (Fig. 1), the second gel filtration. The major products synthesized by both enzymes were identical. We present here the data for the product of cGlc$_4$ galactosylation by coffee bean enzyme. Although there were some differences in the pattern of minor products, since their content did not exceed 1–2% of the yield of the major product, they are not considered in this work. A 20 mM solution of the major product synthesized by coffee bean enzyme was hydrolyzed by both α-galactosidases to cGlc$_4$ and D-galactose. The fact that it contained only one D-galactopyranosyl residue was confirmed by electrospray mass spectrometry (ESIMS), which afforded a pseudomolecular ion, an $M + Na^+$ adduct of m/z 833.4, corresponding to a branched cyclic pentasaccharide. Traces of an ion of m/z 995.3 indicates the presence of doubly galactosylated cGlc4. The m/z value for the product glycosylated by the fungal enzyme was 833.5. Methylation analysis showed that the major product of cGlc$_4$ galactosylation contains one galactopyranosyl residue linked by a $(1 \rightarrow 6)$ linkage to one of the two α-$(1 \rightarrow 3)$-glucosylated residues of the cyclic tetramer (Table 1). The overall structure of the cyclic branched pentasaccharide and the α configuration of the galactopyranosyl linkage were confirmed by

Table 2
C-1 and C-6 resonances in the ^{13}C NMR spectrum of α-D-galactosylated cGlc$_4$

Sugar residue	C-1 (ppm)	C-6 (ppm)
6-*O*-α-D-Glucosylated α-D-glucopyranoside	99.0	69.7
3-*O*-α-D-Glucosylated α-D-glucopyranoside	100.8	62.5
3-*O*-α-D-Glucosylated-6-*O*-α-D-galactosylated α-D-glucopyranoside	100.8	67.6
α-D-Galactopyranoside	100.2	63.1

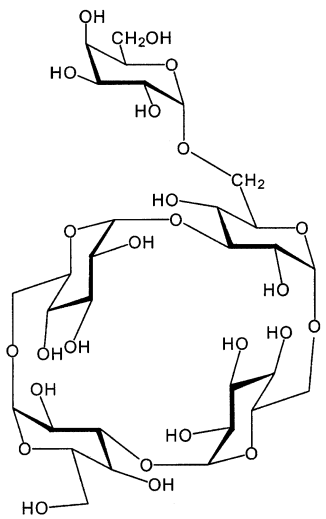

Fig. 3. Structure of the product formed on enzymic galactosylation of cyclic tetramer (cGlc$_4$) generated from alternan by alternanase.

^{13}C NMR spectroscopy (Fig. 2). The spectrum is very similar to that of 6-*O*-α-D-glucopyranosyl cGlc$_4$ previously described in the enzymic hydrolyzate of alternan.[4] The assignments of key resonances are shown in Table 2. The C-6 signals were confirmed by the distortionless enhancement by polarization transfer (DEPT) method.[14]

These results show that both α-galactosidases catalyze an efficient 6-*O*-α-D-galactopyranosylation of cGlc$_4$ to generate the structure depicted in Fig. 3. The results also extend our knowledge about the synthetic potential of *T. lanuginosus* α-galactosidase and suggest that the fungal enzyme could be used for galactosylation of cyclodextrins. Preliminary experiments confirm this suggestion. The attachment of a nonreducing α-D-galactopyranosyl residue to the cGlc$_4$ molecule opens new possibilities for future applications of the cyclic tetramer, since the D-galactopyranosyl residue can be easily modified by D-galactose oxidase to introduce a reactive aldehyde group.

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