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^{1}H-NMR Spectroscopic Evidence on the Glycosidic Linkages of the Transglycosylated Products of Low-Molecular-Weight 1,4-β-D-Glucan Glucanohydrolase from *Trichoderma koningii*

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*Trichoderma koningii*에서 分離한 低分子 1, 4-β-D-Glucan Glucanohydrolase의 反應産物에 對한 核磁氣共鳴스펙트럼 分析

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ABSTRACT: The mode of transglycosylation reaction observed during the action of low-molecular-weight 1,4-β-D-glucan glucanohydrolase (EC 3.2.1.4) purified from *Trichoderma koningii* ATCC 26113 was investigated using ^{1}H-NMR spectroscopy. The H-1 proton resonances were analysed. After reaction of the enzyme with cellotriose, the reaction products were separated by high performance liquid chromatography. H-1 resonances of the products were consisted with those of cellobiose, cellotriose and cellotetraose, respectively. Therefore it was proved that all the reaction products formed by the action of the enzyme on cellooligosaccharides, including transglycosylation products, possess only β-1,4-glycosidic linkage(s).

KEY WORDS□*Trichoderma koningii*, 1,4-β-D-glucan glucanohydrolase, ^{1}H-NMR spectroscopy, transglycosylation

The mode of action of low-molecular-weight 1,4-β-D-glucan glucanohydrolase secreted from *Trichoderma koningii* was demonstrated with high performance liquid chromatography (Hong *et al.*, 1986): The formation of some transglycosylated products were suggested when the mixture of a cellooligosaccharide and a glycosyl acceptor such as p-nitrophenyl-β-D-glucoside or cellobiitol were incubated with the purified endoglucanase.

Transglycosylation action of the endoglucanases has been suggested also with various fungi, such as *Irpex lacteus* (Nisizawa and Hashimoto, 1959), *Trichoderma viride* (Okada and Nisi-

Note: H-1 means the proton attached to the C-1 carbon of each glucose monomer. Numbers of superscript (H-1^1, H-1^2, ···) mean the number of glucose monomer from reducing end, and α or β as subscripts (H-1_α, H-1_β, ···) means α-or β-anomer.

zawa, 1975; Maksimov, 1982), *Trichoderma koningii* (Maksimov, 1982), and *Aspergillus nidulans* (Maeng *et al.*, 1980). However, the nature of the glycosidic linkages formed by the transglycosylation reaction has not been clarified yet.

This report describes the identification of the glycosidic linkages of the reaction products formed by the action of the low-molecular-weight 1,4-β-D-glucan glucanohydrolase from *Trichoderma koningii* using ^{1}H-NMR spectroscopy.

MATERIALS AND METHODS

The low-molecular-weight 1,4-β-D-glucan glucanohydrolase was purified from the culture filtrate of *Trichoderma koningii* ATCC 26113 according to the method proposed by Hong *et al.* (1986).

Glucose, gentiobiose and D_2O (99.8% atom % of deuterium) were purchased from Sigma Chemical Co. MO. Sophorose was obtained from Koch-Light Laboratories, Colnbrook, Buchs and cellobiose and avicel from E. Merck, Darmstadt. Cellotriose, cellotetraose, and cellopentaose were prepared from HCl-hydrolysate of avicel according to the procedures proposed by Miller *et al.* (1960).

The reaction products were separated with an HPLC system operating with a Model 6000 A pump (Waters Associates, Milford, MA) and a Partisil PXS-10/25 PAC column (Whatman Inc., Clifton, NJ) according to the method described previously (Hong *et al.*, 1986). Each reaction product separated by HPLC was dried *in vacuo*.

All of the used sugars and the reaction products previously desiccated over P_2O_5 *in vacuo* for 2 days was dissolved in D_2O for ^{1}H-NMR spectroscopy. The dissociable protons of the sugars were exchanged twice for deuterium in D_2O. Finally, the sample solution were prepared in 0.5 *ml* of D_2O.

The ^{1}H-NMR spectra were taken with a Brucker FT-NMR Spectrometer operating at 200.1 MHz with a 5 mm insert (507-pp, Wilmad Glass, Buena, NJ.) at a probe temperature of 20°C. D_2O was provided as the field frequency lock and acetic acid as the internal standard. Sixteen scans with an aquisition time of 1.638 s were accumulated in most cases, while hundred scans were accumulated for some samples.

Table 1. *Chemical shifts of H-1 protons of glucose and its oligomers in D_2O*

Saccharides	Chemical shifts from internal D_2O (ppm)					
	H-1^1_α	H-1^1_β	H-1^2_β	H-1^3_β	H-1^4_β	H-1^5_β
α-glucose	5.19					
β-glucose		4.60				
α-sophorose β(1→2)	5.42		4.60			
β-sophorose β(1→2)		4.77*	4.70			
α-gentiobiose β(1→6)	5.20		4.47			
β-gentiobiose β(1→6)		4.63	4.49			
α-cellobiose β(1→4)	5.20		4.49			
β-cellobiose β(1→4)		4.64	4.49			
α-cellotriose**	5.19		4.51	4.48		
β-cellotriose**		4.63	4.51	4.48		
α-cellotetraose**	5.20		4.51	4.51	4.49	
β-cellotetraose**		4.64	4.51	4.51	4.49	
α-cellopentaose**	5.20		4.51	4.51	4.51	4.48
β-cellopentaose**		4.63	4.51	4.51	4.51	4.48

* This is an approximate value.
** The glycosidic linkages are (1→4).

RESULTS AND DISCUSSION

The resonance of H-1 proton, participated at β-1,4-glycosidic linkage of sophorose appeared at 4.60 ppm in α-anomer, and 4.70 ppm in β-anomer as shown in Table 1; and those of β-1,3-linkage of laminaribiose at 4.73 ppm in α-anomer, and 4.63 ppm in β-anomer (Bruyn *et al.*, 1975). The resonances of H-1 proton, participated at β-1,6-linkage of gentiobiose consisted of two doublets; one centered at 4.47 ppm (α-anomer), and the other at 4.49 ppm (β-anomer). Therefore, the resonances of H-1 protons of cellooligosaccharides with β-1,4-glycosidic linkages can be readily distinguished from those of glucose dimers and furthermore glucose oligomers which contain α-1,2-, β-1,2-, β-1,3-, and β-1,6-glycosidic linkage(s).

The reaction products produced from cellotriose by the endoglucanase were separated by HPLC. As shown in Figure 1, the chromatogram exhibited four separated peaks located at the positions of glucose, cellobiose, cellotriose and cellotetraose, respectively. The first peak can be readily assigned to glucose. However, it is not possible to clarify whether the second, third and fourth peaks just imply cellobiose, cellotriose and cellotetraose, respectively, because there exists some possibility that the reaction products involve some glucose oligomers containing the glycosidic linkage(s) other than β-1,4-glycosidic linkage. Thus the three peaks are designated G_2*, G_3* and G_4*, respectively.

The ^{1}H-NMR spectra of the reaction products ranging over 4.3-5.3 ppm are shown in Figure 2. The reaction products, G_2*, G_3* and G_4* can be clearly identified to be cellobiose, cellotriose and cellotetraose, respectively, with respect to the chemical shifts of H-1 protons (Table 1 and 2), and their coupling constants J(1,2) (Table 3 and 4). It leads to the conclusion that all the reaction products formed by the action of the purified endoglucanase on cellotriose, including transglycosylation

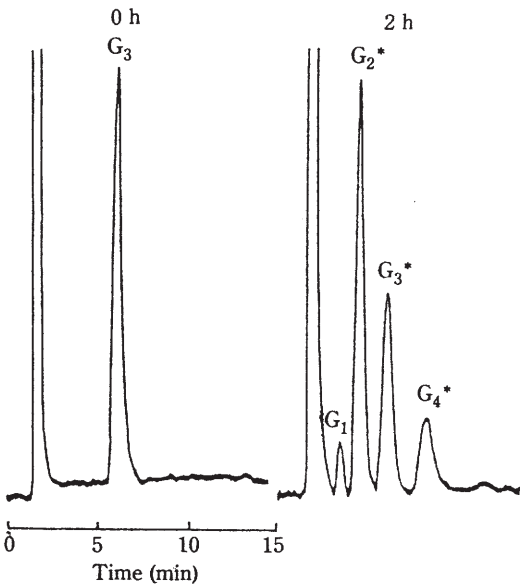

Fig. 1. *HPLC chromatogram of reaction products after 2 hour-reaction of 1,4-β-D-glucan glucanohydrolase to cellotriose (2 h), comparing to the substrate chromatogram (0 h). Reaction mixture consists of 1.000 μl of 19.8 mM cellotriose in 0.02 M acetate buffer, pH 5.0, and 140 μl of 2.3 units ml^{-1} enzyme solution. Injection volume, 35 μl. G_1, glucose; G_2*, glucose dimer; G_3*, glucose trimer; G_4*, glucose tetramer.*

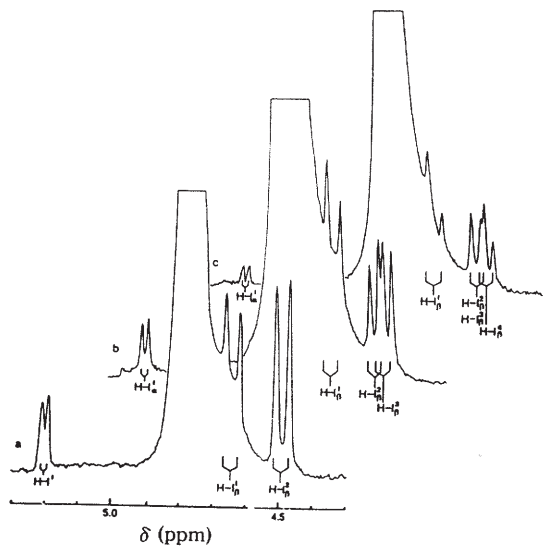

Fig. 2. *Partial 200 MHz ^{1}H-NMR spectra (4.3-5.3 ppm) or reaction products from cellotriose by 1,4-β-D-glucan glucanohydrolase. a. glucose dimer (G_2*); b, glucose trimer (G_3*); c, glucose tetramer (G_4*).*

Table 2. *Coupling constants, J(1,2), of glucose and its oligomers in D_2O*

Saccharides	Coupling constants (Hz)					
	$J(1^1_\alpha,2^1)$	$J(1^1_\beta,2^1)$	$J(1^2_\beta,2^2)$	$J(1^3_\beta,2^3)$	$J(1^4_\beta,2^4)$	$J(1^5_\beta,2^5)$
α-glucose	3.7					
β-glucose		7.9				
α-sophorose β(1→2)	3.6		7.8			
β-sophorose β(1→2)		7.9*	7.9			
α-gentiobiose β(1→6)	3.6		7.8			
β-gentiobiose β(1→6)		7.8	7.8			
α-cellobiose β(1→4)	3.7		7.8			
β-cellobiose β(1→4)		7.9	7.8			
α-cellotriose**	3.7		7.8	7.6		
β-cellotriose**		7.9	7.8	7.6		
α-cellotetraose**	3.6		7.8	7.8	7.6	
β-cellotetraose**		7.9	7.8	7.8	7.6	
α-cellopentaose**	3.2		7.7	7.7	7.7	7.5
β-cellopentaose**		7.9	7.7	7.7	7.7	7.5

* This is an approximate value.
** The glycosidic linkages are β(1→4).

Table 3. *Chemical shifts of H-1 protons of reaction products from cellotriose by 1,4-β-D-glucan glucanohydrolase in D_2O*

Saccharides	Chemical shifts from internal D_2O (ppm)				
	$H\text{-}1^1_\alpha$	$H\text{-}1^1_\beta$	$H\text{-}1^2_\beta$	$H\text{-}1^3_\beta$	$H\text{-}1^4_\beta$
G_2*	5.20	4.64	4.49		
G_3*	5.20	4.63	4.51	4.48	
G_4*	5.20	4.63	4.51	4.51	4.48

Table 4. *Coupling constants, J(1,2), of reaction products from cellotriose by 1,4-β-D-glucan glucanohydrolase in D_2O*

Saccharides	Coupling constants (Hz)				
	$J(1^1_\alpha,2^1)$	$J(1^1_\beta,2^1)$	$J(1^2_\beta,2^2)$	$J(1^3_\beta,2^3)$	$J(1^4_\beta,2^4)$
G_2*	3.7	7.9	7.8		
G_3*	3.6	7.9	7.9	7.7	
G_4*	3.6	7.9	7.8	7.8	7.6

products, possess only β-1,4-glycosidic linkage(s).

Transglycosylation reaction catalyzed by the β-glucosidase from *Aspergillus foetidus* was reported by Gusakov *et al.* (1984), who analyzed the products of enzymatic cellobiose hydrolysis using HPLC. The basic transglycosylation products were identified to be gentiobiose and isocellotriose, which enabled them to conclude that in the β-glucosidase-catalyzed transglycosylation, not a β-1,4-but a β-1,6-glycosidic linkage is formed after the transfer of a D-glucose residue to an acceptor molecule. In this respect, the possibility that glycosidic linkages other than β-1,4-linkage might be formed in the transglycosylation reaction catalyzed by the endoglucanase from *Trichoderma koningii* was carefully examined in this investigation. As described in the previous report (Hong *et al.*, 1986), the endoglucanase has different transglycosylation capacity from that of β-glucosidase in that it can transfer cellooligosaccharides such as cellobiose, cellotriose and cellotetraose instead of glucose. Thus various reaction products can be formed by the enzyme, which make some difficulties in isolation and identification of the products by HPLC.

적 요

Trichoderma koningii ATCC 26113에서 分離 精製한 低分子 1,4-β-D-glucan glucanohydrolase (EC 3.2.1.4)의 反應中에 나타나는 transglycosylation 樣相을 ^{1}H-NMR spectroscopy를 利用하여 分析하였다. 本 酵素를 cellotriose와 反應시킨 後, 反應生成物을 high performance liquid chromatography에 의해 分離하여 스펙트럼을 얻었다. 반응생성물의 H-1 resonances는 cellobiose, cellotriose, cellotetraose의 H-1 resonances와 一致하였기 때문에, 本 酵素가 cellooligosaccharides에 作用하였을 때 生成되는 產物들, 特히 transglycosylation에 의해 生成되는 產物들 모두가 β-1,4-linkage만을 가지고 있다는 사실을 증명 할 수 있었다.

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