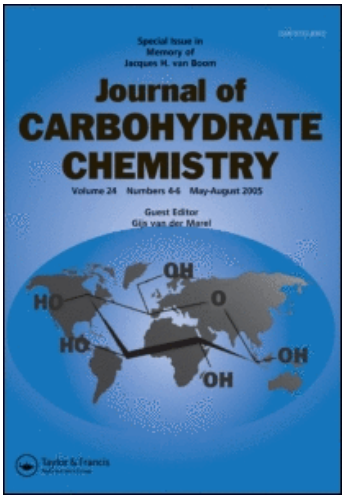

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A Bacterial Chitinase Acts as Catalyst for Synthesis of the *N*-Linked Oligosaccharide Core Trisaccharide by Employing a Sugar Oxazoline Substrate

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A chitinolytic enzyme, chitinase A1 from *Bacillus circulans* WL-12, was found to catalyze a glycosyl-transferring reaction to form the *N*-linked oligosaccharide core structure, Man(β1-4)-GlcNAc(β1-4)-GlcNAc, by employing Man(β1-4)-GlcNAc-oxazoline as glycosyl donor. When the reaction was carried out in the presence of 20 v/v% acetone, the trisaccharide was obtained in 32% yield. It has been shown for the first time that a chitinase behaves like an endo-β-*N*-acetylglucosaminidase in spite of low structural similarity between them.

Keywords Family 18 chitinase, Endo-β-*N*-acetylglucosaminidase, Sugar oxazoline, Transition state analog, *N*-linked oligosaccharide

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INTRODUCTION

Chitinases, which cleave the β-1,4-glycosidic bonds of chitin, have been the focus of recent attention due to their roles in chitin recycling in nature and in defense mechanisms of plants, and as prospects for biotechnological exploitation of natural chitinous materials.[1] Producing oligosaccharides with various biological activities (e.g., elicitor activity, antifungal activity) is one of the most important biotechnological applications for chitinase-catalyzed reactions, although glycosyl-transferring activity of chitinases are typically lower than that of other glycosidases.[2,3] In order to promote glycosyl-transferring reactions effectively, sugar oxazoline derivatives were employed as transition state analog glycosyl donors for glycosyl-transferring reaction catalyzed by glycosyl hydrolase family 18 chitinases.[4,5] For example, artificial chitin was produced by the chitinase-catalyzed glycosyl-transferring reaction using *N,N′*-diacetyl chitobiose oxazoline derivative.[6]

We have extended this concept of utilizing sugar oxazolines as glycosyl donors to synthesis of various functionalized oligosaccharides containing an *N,N′*-diacetyl chitobiose moiety. For instance, novel oligosaccharide macromonomers have been prepared by the chitinase-catalyzed addition reaction of *N*-acetyllactosamine oxazoline derivative (Gal(β1-4)-GlcNAc-oxazoline) 2 as a glycosyl donor and *N*-acetylglucosamine derivatives with a polymerizable group as glycosyl acceptor (Fig. 1).[7] These results show that the galactose unit of 2 can be accepted by the subsite -2 of chitinase, indicating that the

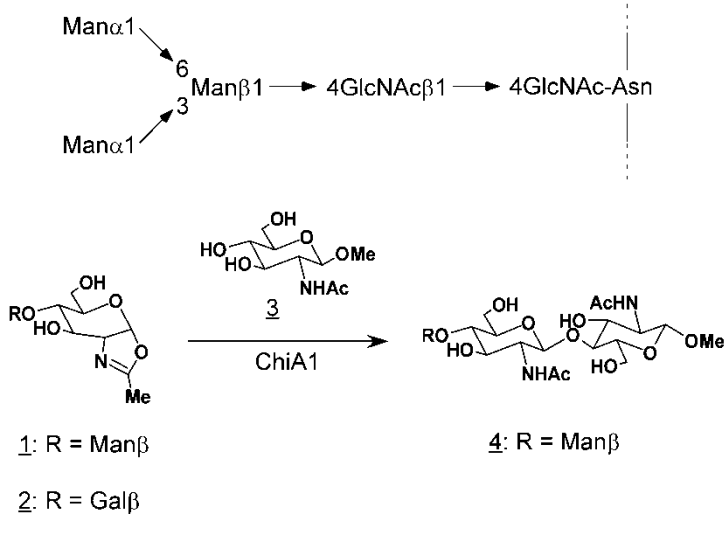

Figure 1: Structure of core pentasaccharide of *N*-linked glycoprotein and chitinase-catalyzed glycosyl-transferring reactions with sugar oxazolines.

recognition at the subsite -2 is low enough to accept hexoses other than *N*-acetylglucosamine (GlcNAc).

Based on this fact, we postulated that a disaccharide oxazoline derivative, Man(β1-4)-GlcNAc-oxazoline 1, would be recognized by the active site of the chitinase and then can produce the common trisaccharide core structure of *N*-linked oligosaccharides (Man(β1-4)-GlcNAc(β1-4)-GlcNAcβ) emerged in glycoprotein or glycopeptides, when reacted with an appropriate GlcNAc acceptor (Fig. 1). In this paper, we wish to report that chitinase A1 from *Bacillus circulans* WL-12 (ChiA1) catalyzes the glycosyl-transferring reaction of 1 to a glycosyl acceptor 3 (GlcNAcβ-*O*-Me) to produce the corresponding trisaccharide derivative 4 (Man(β1-4)-GlcNAc(β1-4)-GlcNAcβ-*O*-Me).

MATERIALS AND METHODS

Chemicals

We selected ChiA1 as catalyst because the catalytic behavior of this enzyme was well characterized.[8–10] ChiA1 was isolated according to the previous report.[7] The glycosyl donor substrate, Man(β1-4)-GlcNAc-oxazoline 1, was prepared by modifying the procedure described in the previous report.[11] All commercially available reagents were used without further purification.

Transglycosylation Reaction

The enzyme reactions with ChiA1 (30.4 μM) in 20 μL Tris-HCl buffer (50 mM, pH 8.0) containing 40 to 640 mM of 3 as a glycosyl acceptor and 10 to 200 mM of 1 as a glycosyl donor were performed at 37°C. The formation of the resulting trisaccharide 4 was monitored by absorption at 214 nm, and the product was separated by HPLC [LiChroCART (4.0ϕ × 250 mm) column with 100% H_2O as eluent at 40°C, 0.7 mL/min]. The structure of the product trisaccharide was analyzed with ^{1}H NMR (DPX-400, Bruker) and MALDI-TOF MS (Protein TOF, Bruker).

p-Nitrophenyl β-D-Chitobiosidase Assay

A reaction mixture of *p*-nitrophenyl β-D-chitobioside and the enzyme in 50 mM phosphate buffer (pH 6.0) was incubated at 37°C for 20 min in the presence or the absence of acetone. After the reaction was stopped by adding a 0.4 M solution of Na_2HPO_4 (pH 11.5), the amount of the liberated *p*-nitorophenol was determined by measuring the absorbance at 400 nm.

RESULTS AND DISCUSSION

Synthesis of *N*-Linked Oligosaccharide Core Trisaccharide Catalyzed by ChiA1

The typical reversed-phase HPLC chromatograms of the reaction mixture are shown in Fig. 2. After 1 h, the peak due to the glycosyl donor 1 has almost disappeared and a new peak corresponding to the trisaccharide 4 appeared. The molecular weight of the resulting trisaccharide was confirmed to be 600 for $C_{23}H_{40}N_2O_{16}$ by MALDI-TOF MS (Fig. 3). The newly formed glycosidic linkage was characterized to be β-1,4 type by the comparison with authentic sample. The peak of the product was almost disappeared after 8 h, indicating that the product 4 was hydrolyzed by the action of ChiA1, giving rise to the hydrolyzate (Man(β1-4)-GlcNAc-OH) and GlcNAc-*O*-Me or (Man(β1-4)-GlcNAc-GlcNAc-OH) and methanol (Fig. 4, route (a)).

Effect of Donor/Acceptor Ratio

In order to increase the yield of the product 4, the effect of the concentration ratio of donor and acceptor on the glycosyl-transferring efficiency was investigated (Fig. 5). When the donor concentration was fixed at 40 mM and the concentration of acceptor increased, the conversion to 4 increased and reached a maximum (22%) at 640 mM of acceptor (Fig. 5(a)). When the acceptor concentration was fixed at 640 mM and the donor concentration increased, the product yield increased to 24% at 20 mM and gradually decreased at higher concentration (>20 mM), indicating that higher donor concentration caused a side reaction (Fig. 5(b)). This phenomenon can be explained by the formation of a byproduct at higher donor concentration (>20 mM). The MALDI-TOF MS analysis showed that molecular weight of the byproduct was 965, which

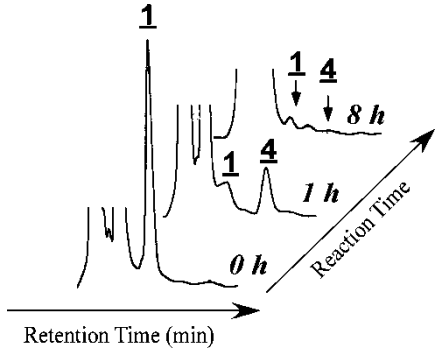

Figure 2: Reversed-phase HPLC chromatograms of the reaction mixture. Numbers in this figure correspond to compound numbers in the main text. Italicized numbers indicated the reaction time.

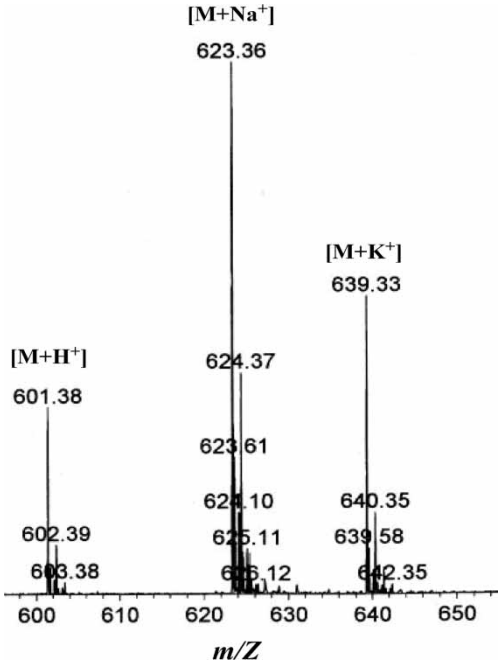

Figure 3: MALDI-TOF MS spectrum of the resulting trisaccharide (Man(β1-4)-GlcNAc(β1-4)-GlcNAc-β-*O*-Me).

corresponds to a pentasaccharide derivative ((Man-GlcNAc)$_2$-GlcNAc-*O*-Me). Although the precise structure and the formation mechanism of the byproduct have not been made clear, the byproduct was estimated to be a linear pentasaccharide, Man-GlcNAc-Man-GlcNAc-GlcNAc-*O*-Me (Fig. 4, route (b)). This compound can be produced by the enzymatic addition reaction of 1 to the mannose unit at the nonreducing end of the resulting trisaccharide derivative 4 located in the acceptor site. It may be assumed that the 4‴ hydroxy group of 4

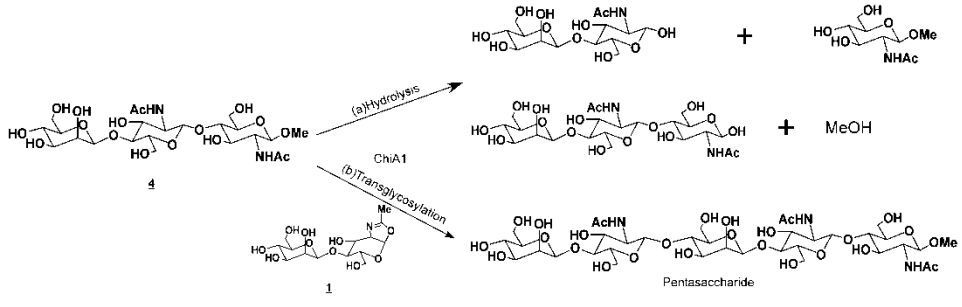

Figure 4: Plausible side reactions toward transglycosylated product 4. (a) Hydrolysis of 4 catalyzed by ChiA1. (b) ChiA1-catalyzed addition reaction of 4 to 1 at higher concentration of 1 (>20 mM).

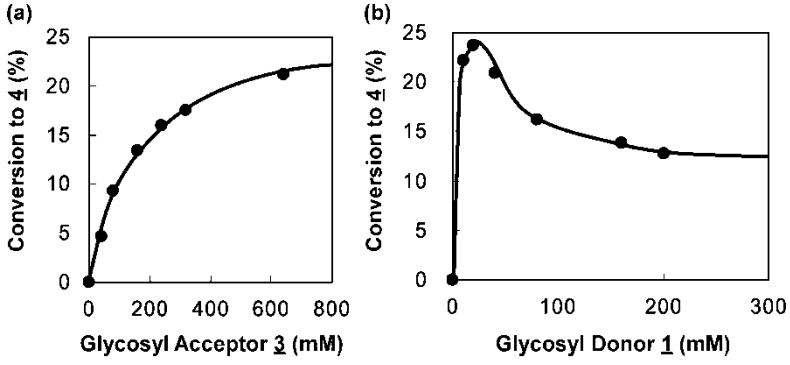

Figure 5: Effect of donor/acceptor ratio on the glycosyl-transferring efficiency. (a) The initial concentration of glycosyl donor is fixed at 40 mM. (b) The initial concentration of glycosyl acceptor is fixed at 640 mM. The conversion was defined as the amount of consumed glycosyl donor 1.

reacts preferentially because the mannose unit at the nonreducing end of 4 is most likely to be located at the +1 subsite, judging from the linear shape of 4 as well as that of the catalytic site of ChiA1. At the lower donor concentration (<20 mM), the byproduct pentassacharide could not be detected, probably because the acceptor site is predominantly occupied by GlcNAc-*O*-Me than Man-GlcNAc-GlcNAc-*O*-Me.

Effect of Organic Solvent

The effect of organic solvent on glycosyl-transferring efficiency was also investigated, since addition of organic solvent often increases the yield of hydrolase-catalyzed glycosyl-transferring reactions.[12] We tested the glycosyl-transferring reactions in the presence of seven water-miscible organic solvents, acetone, aectonitrile, dioxane, DMA, DMF, DMSO, and THF (Table 1). The concentration of the organic solvents were fixed at 50%

Table 1: Effect of organic solvent on the transglycosylation reaction.

Organic solvent	Relative activity (%)
Acetone	79
Acetonitrile	26
Dioxane	4
DMA	25
DMF	7
DMSO	0
THF	37

(v/v). The yield of trisaccharide 4 for 1 h in the presence of 40 mM donor and 40 mM acceptor in the absence of organic solvent was defined as a maximum level (100%) of enzyme activity. The reason why we employed this ratio of glycosyl donor and acceptor was to avoid a precipitation of saccharide substrate caused by the addition of organic solvent. A fairly high relative activity (79%) was observed for ChiA1-catalyzed glycosyl-transferring reaction in the presence of 50% acetone (v/v). On the contrary, less activity remained in the presence of DMF, dioxane, or DMSO. Thus, we employed acetone as cosolvent in further experiments.

Dose Effect of Acetone

We then evaluated the dose effect of acetone on the transglycosylation efficiency. The yield of trisaccharide 4 after 1 h incubation in the presence of 20 mM donor and 640 mM acceptor in the absence of organic solvent was defined as a standard level of enzyme activity (100%). When the reaction was carried out in the presence of 20% acetone, the activity of the ChiA1-catalyzed transglycosylation reaction showed 135% and the trisaccharide 4 was obtained in 32% yield (● in Fig. 6). On the contrary, hydrolytic activity of ChiA1 gradually decreased by increasing acetone concentration and almost diminished in the presence of 50% acetone (○ in Fig. 6). These results clearly indicate that the addition of acetone enhanced the glycosyl-transferring activity of ChiA1 toward the substrate 1.

Insight into Enzyme Evolution by Transition State Analog Substrate

It has been well known that endo-β-D-*N*-acetylglucosaminidases are efficient catalysts to cleave the β-1,4 glycosidic bond of the *N,N′*-diacetyl chitobiose moiety in *N*-linked oligosaccharides in glycoproteins or glycopeptides.[13,14]

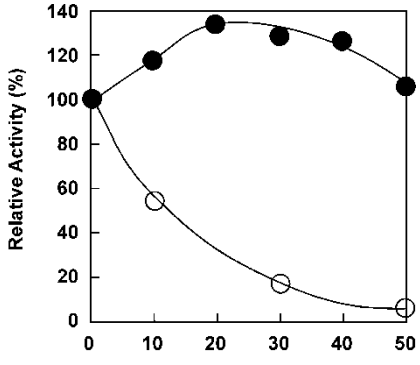

Figure 6: The dose effect of acetone on the hydrolyzing or the transglycosylating activity of ChiA1. ●, Transglycosylation. ○, Hydrolysis.

Recently, we have reported that endo-β-D-*N*-acetylglucosaminidases from *Arthrobactor protophormiae* (Endo-A) and *Mucor hiemalis* (Endo-M) were able to catalyze glycosyl-transferring reactions of the sugar oxazoline 1 to *p*-nitrophenyl (pNP) β-D-*N*-acetylglucosaminide, giving rise to core trisaccharide derivative, (Man(β1-4)-GlcNAc(β1-4)-GlcNAcβ-*O*-*p*NP) in 54% and 45% yield, respectively.[11] The resulting trisaccharide was not hydrolyzed by the enzyme catalyst, indicating that the resulting trisaccharide 4 has almost no affinity toward the catalytic site of Endo-A or Endo-M. In the present reaction, ChiA1 catalyzes the addition reaction of 3 to the oxazoline derivative 1, affording 4, which is hydrolyzed at longer reaction time, indicating that the trisaccharide 4 has stronger affinity toward the active site of chitinase.

Although it has already been reported that the amino acid sequence similarity between chitinase (glycosyl hydrolase family 18) and endo-β-D-*N*-acetylglucosaminidase (glycosyl hydrolase family 85) is low (e.g., sequence similarity between ChiA1 and Endo-A is 11.8%), their catalytic sites were highly conserved (Family 18, DGxxxDxE; and Family 85, DGxxxNxE). This conserved region was characteristic for the enzymes hydrolyzing β-1,4 *N*-acetyl-D-glucosaminide linkages in retaining mode. Nonetheless, substrates that can be recognized by both enzymes have not been identified so far by the study of substrate specificity using either *p*-nitrophenyl glycosides or polysaccharides as substrates. The fact that Man(β1-4)-GlcNAc-oxazoline was recognized by both enzymes suggests that family 18 chitinase and family 85 endo-β-*N*-acetylglucosaminidase have a common ancestor in the light of reaction-based inspection of molecular evolution.

In conclusion, we have demonstrated for the first time that ChiA1 can catalyze the glycosyl-transferring reaction using Man(β1-4)GlcNAc-oxazoline for constructing the *N*-linked core trisaccharide moiety. Further investigations for optimizing reaction conditions including site-directed mutagenesis or direct evolution are now in progress, aiming at the development of useful biocatalyst in glycotechnology.

ABBREVIATIONS

Man, D-mannopyranosyl; GlcNAc, *N*-acetyl D-glucosaminyl; Gal, D-galactopyranosyl; THF, tetrahydrofuran; DMA, *N,N*-dimethylacetamide; DMF, *N,N*-dimethylformamide; DMSO, dimethyl sulfoxide; dioxane, 1,4-dioxane.

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