Ring-Opening Glycosylation of a Chitobiose Oxazoline Catalyzed by a Non-Chitinolytic Mutant of Chitinase

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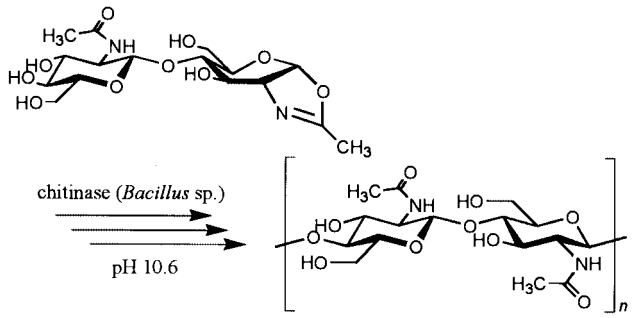
Ring-opening glycosylation of a chitobiose oxazoline was exclusively achieved by catalysis of a mutated chitinase that is only active for the glycosylation and not for chitinolysis.

Sugar oxazoline derivatives have been found as a novel artificial substrate for some glycosyl hydrolases and their enzymatic glycosylation was demonstrated.[1,2] This glycosylation involves a ring-opening process of the oxazoline to generate acetamide group as well as a nucleophilic addition at the anomeric position.[3] When a chitobiose oxazoline (Chi_2NAc-oxa) was subjected to chitinase catalysis (*Bacillus* sp.), the enzymatic ring-opening polyaddition was induced in a perfect regio- and stereoselective manner, affording artificial chitin having β(1→4) glycosidic linkages. This is the first successful in vitro synthesis of chitin via a nonbiosynthetic pathway (Scheme 1).[4]

Scheme 1.



Recent X-ray crystallographic studies shed a light on the active-center structures of some isolated chitinases. Chitinase A1 from *Bacillus circulans* WL-12 is one of the chitinases, whose amino acid sequence,[5a,b] catalytic residues[5c] and three-dimensional structure[5d] were reported. In this study, first we have investigated interaction of Chi_2NAc-oxa with the well-defined active center of Chitinase A1. Secondly, on the basis of mechanistic consideration, a mutant Chitinase A1 has been designed as a novel catalyst for the ring-opening glycosylation of Chi_2NAc-oxa.

Purified wild-type Chitinase A1 (ChiA1-Wt, 80 μg, 1.1×10^{-9} mol) was added to a solution of Chi_2NAc-oxa (8.5 mg, 2.1×10^{-5} mol) in a phosphate buffer (pH 9.0, 40 mM, 125 μL) and incubated at 30 °C. HPLC analysis of the solution revealed that Chi_2NAc-oxa was consumed to produce chitooligosaccharides as the water-soluble product.[6] Not only chitotetraose (Chi_4NAc) and chitohexaose (Chi_6NAc) but also chitotriose (Chi_3NAc) and chitopentaose (Chi_5NAc) were produced (Figure 1a). They were all degraded into chitobiose (Chi_2NAc) and *N*-acetyl glucosamine (GlcNAc) eventually. On the other hand, when Chi_2NAc was used instead of Chi_2NAc-oxa, no product was formed. These results demonstrate that ChiA1-Wt catalyzed both the ring-opening glycosylation of Chi_2NAc-oxa and the hydrolysis (chitinolysis) of the chitooligosaccharide products.

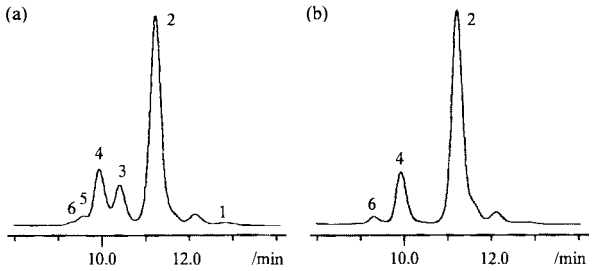
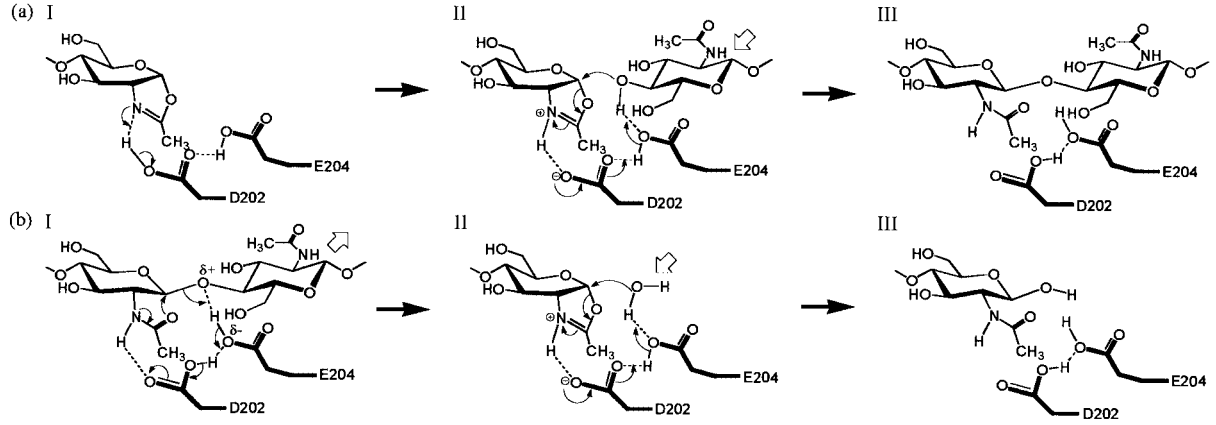


Figure 1. HPLC traces of chitooligosaccharide products from Chi_2NAc-oxa (a) with ChiA1-Wt after 5 h and (b) with ChiA1-E204Q after 5 days; GlcNAc(1), Chi_2NAc(2), Chi_3NAc(3), Chi_4NAc(4), Chi_5NAc(5), Chi_6NAc(6). Sugar KS-802 column (Shodex, 8.0 × 300 mm) used for HPLC analysis (water eluent, 0.7 mL/min, 80 °C) with RI detector. A peak at 12.1 min is due to an impurity .

Catalytic functions of two specific carboxylic residues of ChiA1-Wt, D202 and E204, are now focused to explain the two different reactions at the same active center involving oxazolinium species.[1c–g] The ring-opening glycosylation of Chi_2NAc-oxa (Scheme 2a) proceeds via activation to the corresponding oxazolinium ion. D202 would be responsible for the *N*-protonation to the oxazoline ring. Then, 4-OH group of a glycosyl acceptor nucleophilically attacks the anomeric position of the activated glycosyl donor. This brings about ring-opening of the oxazoline to generate acetamide group and glycosylation to form a β(1→4)-glycoside with inversion of the anomeric configuration from α to β. E204 would mediate proton-transfer from the glycosyl acceptor to D202. Repetition of the ring-opening glycosylation results in oligomerization of Chi_2NAc-oxa. Hydrolysis of the chitooligosaccharide products should occur owing to the chitinolytic activity of wild chitinase. The chitinolytic mechanism by ChiA1-Wt (Scheme 2b) can be described as follows: a target *O*-glycosidic bond is protonated by E204, which causes the bond cleavage and formation of an oxazolinium-ion intermediate. D202 stabilizes the intermediate until a water molecule nucleophilically attacks the anomeric position from the β-side.[7]

The former mechanism for the ring-opening glycosylation of Chi_2NAc-oxa employs D202 as a proton donor to initiate the reaction, which is a clear difference from the latter mechanism for chitinolysis using E204 as a proton donor. It is speculated that acidic character of E204 should be less important for the ring-opening glycosylation of Chi_2NAc-oxa, while the general-acid catalysis by E204 is essential for chitinolysis. Thus, for

Scheme 2.



suppressing the chitinolysis selectively, Chitinase A1 was modified to diminish the acidic character of E204, where the COOH group was replaced with $CONH_2$ group by site-specific mutation from Glu to Gln (E204Q). Then, it was anticipated that the mutant Chitinase A1 (ChiA1-E204Q) could catalyze the ring-opening glycosylation of Chi_2NAc-oxa exclusively.

Purified ChiA1-E204Q was prepared according to the previous procedure.[5c] ChiA1-E204Q (80 μg, 1.1×10^{-9} mol) was added to a solution of Chi_2NAc-oxa (8.5 mg, 2.1×10^{-5} mol) in a phosphate buffer (pH 9.0, 40 mM, 125 μL) and incubated at 30 °C. The HPLC analysis indicated the formation of Chi_4NAc and Chi_6NAc as the water-soluble product (Figure 1b). Formation of GlcNAc, Chi_3NAc and Chi_5NAc was not detected. The concentrations of Chi_4NAc and Chi_6NAc increased monotonously along with the reaction time and did not decrease even after Chi_2NAc-oxa disappeared. By addition of ChiA1-Wt to the solution, however, the products were all hydrolyzed into Chi_2NAc and GlcNAc eventually. Similar results were obtained by using six buffer solutions of Chi_2NAc-oxa at different pH values between 7.1–10.2. These results demonstrate that ChiA1-E204Q still holds the catalytic activity for the ring-opening glycosylation of Chi_2NAc-oxa, while it is no more active for chitinolysis.

In conclusion, it has been demonstrated that the ring-opening glycosylation of Chi_2NAc-oxa is catalyzed by Chitinase A1 in the same active center with chitinolysis. The mechanistic consideration on the catalytic function of E204 allowed to prepare a nonchitinolytic mutant of Chitinase A1 for the exclusive glycosylation of Chi_2NAc-oxa. These results provide useful information for in-depth understanding of the enzymatic catalysis as well as clues for developing a new "glycosynthase"[8] and for the design of artificial substrates. Further studies are under progress in our laboratories.

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References and Notes

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6 Little amount of water-insoluble products was produced by using ChiA1-Wt. This result indicates that the polymerization activity of ChiA1-Wt is much lower than that of the previously utilized chitinase (*Bacillus* sp.), which afforded polysaccharide chitin very efficiently as the major product.

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