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Note

Chitinase-catalyzed hydrolysis of 4-nitrophenyl penta-*N*-acetyl-β-chitopentaoside as determined by real-time ESIMS: The 4-nitrophenyl moiety of the substrate interacts with the enzyme binding site

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ABSTRACT

4-Nitrophenyl penta-*N*-acetyl-β-chitopentaoside [$(GlcNAc)_5$-pNP] was hydrolyzed by a family GH-19 class II barley chitinase, and the enzymatic reaction was monitored by real-time ESIMS. The wild-type enzyme hydrolyzed $(GlcNAc)_5$-pNP producing predominantly $(GlcNAc)_3$-pNP and a lesser amount of $(GlcNAc)_2$-pNP, indicating that the $(GlcNAc)_5$ portion of the substrate binds predominantly to subsites $-2 \sim +3$ and less frequently to $-3 \sim +2$. However, $(GlcNAc)_2$-pNP was mainly produced from $(GlcNAc)_5$-pNP by mutated enzymes, in which Trp72 and Trp82 located at +3/+4 were substituted with alanine (W72A and W72A/W82A), indicating that the $(GlcNAc)_5$ portion of the substrate binds predominantly to subsites $-3 \sim +2$ of the mutants. The mutations of the tryptophan residues resulted in a significant shift of the substrate-binding mode to the glycon side, supporting the idea that the indole side chain of Trp72 interacts with the 4-nitrophenyl moiety of the substrate at subsite +4.

For most glycoside hydrolases, oligosaccharides with chromophores, such as 4-nitrophenyl (pNP) and 4-methylumbelliferyl groups, have been used as substrates, and kinetic parameters have been determined based on rates of release of the chromophoric group.[1–3] These methods are both convenient and highly sensitive. Recently, however, several studies have reported problems resulting from the use of such chromophoric substrates.[4,5] The specificity data and kinetic parameters obtained from chromophoric substrates are not consistent with those obtained from the native oligosaccharide substrates, perhaps because the chromophores attached to the reducing end of the oligosaccharides undergo an additional interaction with the enzyme binding site. To correctly interpret the data obtained from chromophoric substrates, it is important to elucidate the interaction of the chromophoric moiety of the substrates with the enzyme-binding site.

We have been working on the structure and function of plant chitinases using the native chitin oligosaccharide substrates $[(GlcNAc)_n, n = 1–6]$. The mode of oligosaccharide hydrolysis was examined by HPLC,[6,7] and the data were compared between the wild-type and mutant chitinases to identify the amino acid residues responsible for substrate binding.[8,9] Recently, two tryptophan residues (Trp72 and Trp82) of a family 19 class II endochitinase from barley seeds (HvChiII) were mutated, and the mutated enzymes (W72A, W82A, and W72A/W82A) were characterized.[10] The enzyme is composed of two structural domains, which are rich in α-helices, and the substrate-binding cleft lies between these domains, as shown in Figure 1. The binding cleft of the chitinase consists of at least six subsites, −3, −2, −1, +1, +2, and +3. Trp72 and Trp82 are found in the flexible loop located at the end-most site (+3/+4 subsite) of the binding cleft. Since $(GlcNAc)_6$ was found to bind equally to subsites $-4 \sim +2$, $-3 \sim +3$, or $-2 \sim +4$ of the enzyme,[10] the pNP group of 4-nitrophenyl penta-*N*-acetyl-β-chitopentaoside [$(GlcNAc)_5$-pNP] might possibly interact with the tryptophan residues, when HvChiII acts on the artificial oligosaccharide. Thus, the wild-type and Trp-mutated forms of HvChiII can be regarded as the most appropriate enzymes for examining the interaction of the chromophoric moiety of artificial substrates with the enzyme-binding site. In this study, $(GlcNAc)_5$-pNP was hydrolyzed by the wild type and by Trp-mutated HvChiII (W72A, W82A, and W72A/W82A), and the enzymatic reaction was monitored by real-time ESIMS, which enables sensitive and speedy determination of the time-course of the reaction.[11,12]

Abbreviations: HvChiII, family GH-19 class II chitinase from barley seeds, *Horduem vulgare* L.; GlcNAc, 2-acetamido-2-deoxy-D-glucopyranose; $(GlcNAc)_n$, β-1,4-linked oligosaccharide of GlcNAc with a polymerization degree of *n*; $(GlcNAc)_5$-pNP, 4-nitrophenyl glycoside of $(GlcNAc)_n$; HPLC, high-performance liquid chromatography; ESIMS, electrospray ionization mass spectrometry.

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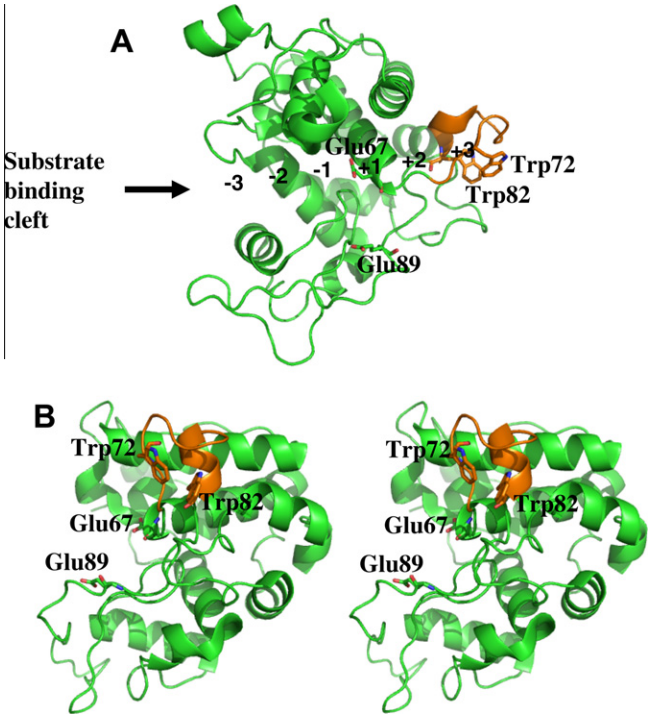

Figure 1. (A) Crystal structure of family GH-19 class II endochitinase from barley seeds (HvChiII, PDB code, 2BAA). Glu67 and Glu89 are the catalytic residues. Trp72 and Trp82 are the mutation targets in this study and are located at subsites +3/+4. (B) Stereoview from the tryptophan side.

The time-course of the enzymatic hydrolysis of $(GlcNAc)_5$-pNP was monitored by real-time ESIMS. The wild-type enzyme was found to hydrolyze $(GlcNAc)_5$-pNP, producing predominantly $(GlcNAc)_3$-pNP and to a lesser extent $(GlcNAc)_2$-pNP, as shown in Figure 2A. When the enzymatic reaction toward the same substrate was monitored by HPLC, $(GlcNAc)_3$-pNP and $(GlcNAc)_2$-pNP were found to be produced in similar proportions as shown in Figure 3. The results of HPLC were completely consistent with the data obtained by real-time ESIMS, suggesting the ESIMS data to be accurate. Based on these experimental data, the $(GlcNAc)_5$ portion of the substrate was found to bind predominantly to subsites $-2 \sim +3$ and less frequently to $-3 \sim +2$ of the chitinase-binding cleft, as shown in Figure 4B. On the other hand, we reported that the native substrate $(GlcNAc)_6$ binds to subsites $-4 \sim +2$, $-3 \sim +3$, or $-2 \sim +4$, with almost equal frequency, as shown in Figure 4A.[10] A comparison between Figure 4A and B suggests clearly that the chromophore linked to the reducing end of $(GlcNAc)_5$ affects the mode of binding to the chitinase. It appears that the 4-nitrophenyl group of $(GlcNAc)_5$-pNP interacts with the +3/+4 subsites, and the additional interaction brings about the shift of the binding mode to the aglycon side.

We mutated two tryptophan residues, Trp72 and Trp82, located at subsites +3/+4 of HvChiII and produced three mutant enzymes (W72A, W82A, and W72A/W82A). The side chain of Trp72 is exposed to the solvent, whereas Trp82 is buried inside (Fig. 1). As reported previously,[10] the rate of the enzymatic hydrolysis of native $(GlcNAc)_6$ significantly decreased in W82A, but was not affected by the W72A and W72A/W82A mutations. For the product distribution obtained from the native $(GlcNAc)_6$ substrate, a significant effect was observed only in W82A. When $(GlcNAc)_5$-pNP was

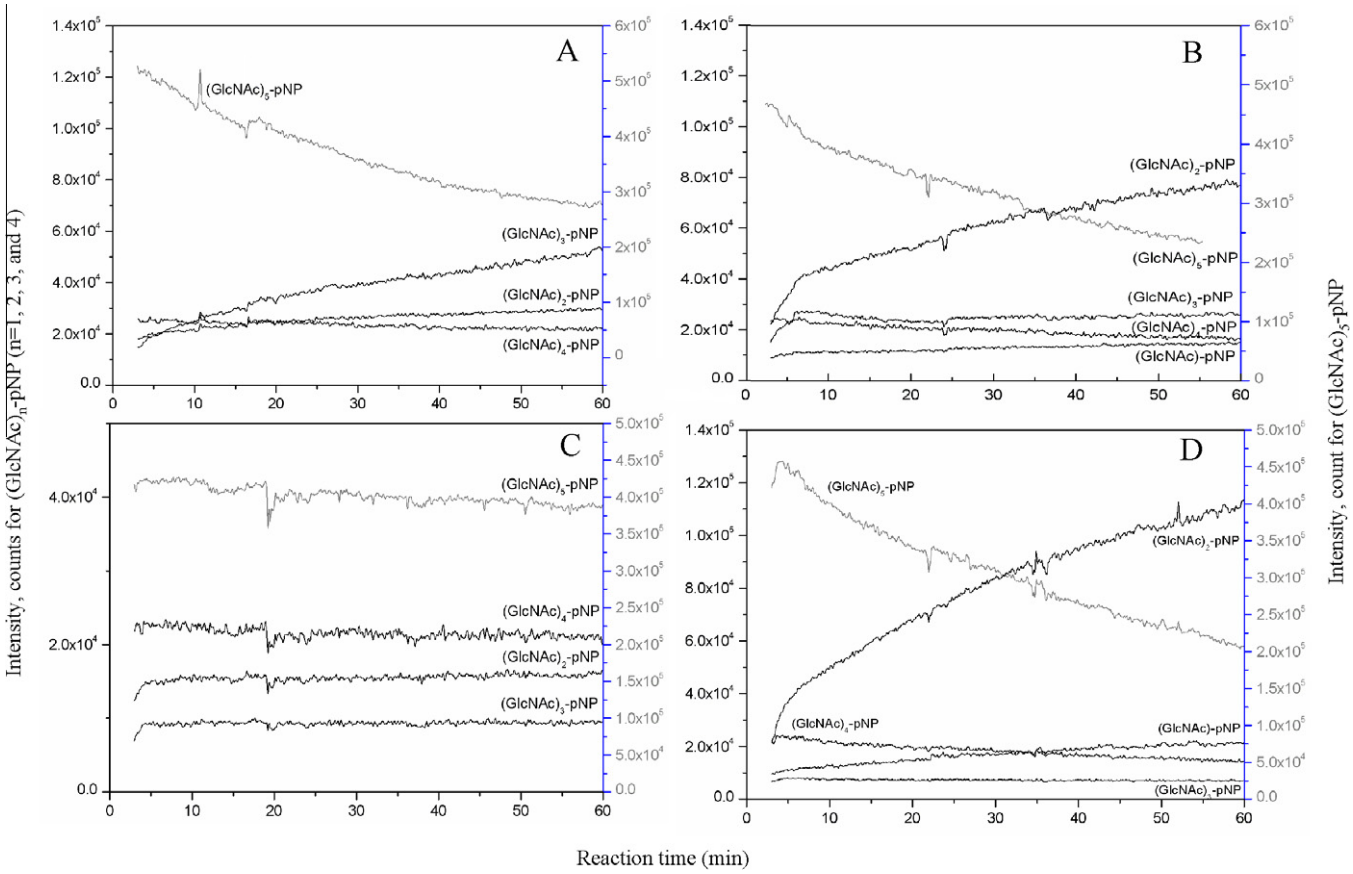

Figure 2. Time-courses of the enzymatic hydrolysis of $(GlcNAc)_5$-pNP as determined by real-time ESIMS. Left ordinates for individual figures represent the counts obtained for the products $(GlcNAc)_{1,2,3,4}$-pNP, while right ones represent those for the substrate $(GlcNAc)_5$-pNP. Reaction conditions are described in the text. (A) Wild type; (B) W72A; (C) W82A; and (D) W72A/W82A.