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Kinetic analysis of the reaction catalyzed by chitinase A1 from *Bacillus circulans* WL-12 toward the novel substrates, partially *N*-deacetylated 4-methylumbelliferyl chitobiosides

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Abstract The kinetic behavior of chitinase A1 from *Bacillus circulans* WL-12 was investigated using the novel fluorogenic substrates, *N*-deacetylated 4-methylumbelliferyl chitobiosides [GlcN-GlcNAc-UMB (2), GlcNAc-GlcN-UMB (3), and $(GlcN)_2$-UMB (4)], and the results were compared with those obtained using 4-methylumbelliferyl *N*,*N′*-diacetylchitobiose [$(GlcNAc)_2$-UMB (1)] as the substrate. The chitinase did not release the UMB moiety from compound 4, but successfully released UMB from the other substrates. k_{cat}/K_m values determined from the releasing rate of the UMB moiety were: 145.3 for 1, 8.3 for 2, and 0.1 s^{-1} M^{-1} for 3. The lack of an *N*-acetyl group at subsite (−1) reduced the activity to a level 0.1% of that obtained with compound 1, while the absence of the *N*-acetyl group at subsite (−2) reduced the relative activity to 5.7%. These observations strongly support the theory that chitinase A1 catalysis occurs via a 'substrate-assisted' mechanism. Using these novel fluorogenic substrates, we were able to quantitatively evaluate the recognition specificity of subsite (−2) toward the *N*-acetyl group of the substrate sugar residue. The (−2) subsite of chitinase A1 was found to specifically recognize an *N*-acetylated sugar residue, but this specificity was not as strict as that found in subsite (−1). **© 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Chitinase; Fluorogenic chitobioside; Steady-state kinetics; Chitin deacetylase

1. Introduction

Chitin is a β-1,4-linked polysaccharide of *N*-acetylglucosamine (GlcNAc), which is found abundantly in living organisms such as crustaceans, insects, and in the cell walls of fungi. *N*-Deacetylation of the chitin polysaccharide produces a glucosamine (GlcN) polysaccharide, chitosan, of which the physicochemical properties are quite different from those of chitin. The biological function of chitosan is also different from that of chitin, as evidenced by the strict specificity that occurs in most chitin- or chitosan-recognizing proteins such as chitinases and chitosanases. However, the molecular mechanistics of the specificity are somewhat complicated, for example, chitinases hydrolyze not only the β-1,4-glycosidic linkage of GlcNAc–GlcNAc, but also the GlcNAc–GlcN linkage [1–3]. A similar situation was found in the chitosanases, which can hydrolyze not only the GlcN–GlcN linkage but also GlcNAc–GlcN [4,5] or GlcN–GlcNAc [6] linkages. Such a complicated specificity has presented obstacles in the elucidation of the molecular mechanisms underlying GlcNAc and GlcN recognition. To facilitate this elucidation, it is highly desirable to establish a more quantitative method for determining the substrate specificities of chitinases and chitosanases.

To elucidate the molecular mechanism of the specificity, it is desirable to use an enzyme for which structural and functional data are available. Chitinase A1 from *Bacillus circulans* WL-12 has been studied from several aspects including its gene structure [7], catalytic mechanism [8,9], and three-dimensional structure [10]. This chitinase is a member of the family 18 chitinases according to the classification made by Henrissat and Bairoch [11]. Enzymes belonging to this family have been reported as catalyzing the hydrolysis of the glycosidic linkage through a 'substrate-assisted' mechanism [12–14]. In addition, chitobiase (family 20) has been postulated to hydrolyze the glycosidic bond via anchimeric assistance, as determined from inhibition analysis using selectively *N*-deacetylated chitooligosaccharides [15]. In the catalytic mechanism, the *N*-acetyl group of the substrate sugar residue at subsite (−1) was found to be important for facilitating the catalytic reaction. Chitinase A1 from *B. circulans* WL-12 has also been assumed to catalyze the hydrolysis through this mechanism [16]. However, as yet, a definitive confirmation of this has not been obtained. Thus, it is of interest to examine the substrate specificity of the *B. circulans* chitinase A1 not only from the viewpoint of substrate recognition but also from that of the catalytic mechanism.

Recently, we successfully synthesized $(GlcNAc)_2$-UMB (**1**), GlcN-GlcNAc-UMB (**2**), GlcNAc-GlcN-UMB (**3**) and $(GlcN)_2$-UMB (**4**) by chemical and enzymatic methods (Fig. 1) [17]. The substrates are well-characterized with respect to the location of the *N*-acetyl group, and as such afford an accurate source of information as to the *N*-acetyl group re-

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Abbreviations: GlcN, 2-amino-2-deoxy-D-glucopyranose; $(GlcN)_n$, β-1,4-linked oligosaccharide of GlcN with a polymerization degree of *n*; GlcNAc, 2-acetamido-2-deoxy-D-glucopyranose; $(GlcNAc)_n$, β-1,4-linked oligosaccharide of GlcNAc with a polymerization degree of *n*; UMB, 4-methylumbelliferone

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quirement at subsites (−1) and (−2) from the determination of the rate of 4-methylumbelliferone (UMB) release from the respective substrates. In the present investigation, we studied the kinetic behavior of chitinase A1 from *B. circulans* WL-12 toward the substrates, UMB glycosides of partially *N*-deacetylated chitobiosides, to elucidate the substrate recognition mechanism of the *B. circulans* chitinase.

2. Materials and methods

2.1. Materials

B. circulans chitinase A1 was obtained by the previously reported method [18]. Compound **1** was synthesized according to the method of Delmotte et al. [19]. Compound **2** was prepared by the enzymatic deacetylation of **1** with the chitin deacetylase from *Colletotrichum lindemuthianum*. Compound **3** was synthesized chemically from chitin via *O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-(1→4)-2-amino-1,3,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranose hydrochloride [20]. Compound **4** was obtained by the enzymatic deacetylation of compound **3** using the *C. lindemuthianum* chitin deacetylase. The structures of compounds **1**, **2**, **3** and **4** were confirmed by ^{1}H-nuclear magnetic resonance spectroscopy and matrix-assisted laser desorption ionization time of flight mass spectrometry analyses. Full synthetic details of the synthesis are described in our previous paper [17].

2.2. Enzymatic reaction

Each of the substrates was dissolved in 0.5 ml of 50 mM sodium acetate buffer (pH 5.5) to give 0.036 mM substrate solutions. A small amount of the enzyme solution was added to the substrate solution and the mixture was incubated at 40°C. As a control, the substrate solution was also incubated in the absence of chitinase. A portion (0.1 ml) of the reaction mixture was withdrawn after an appropriate reaction time and mixed with 0.5 ml of 0.4 M Na_2HPO_4–NaOH buffer, pH 11.9, to terminate the enzymatic reaction. The fluorescence intensity of the resultant solution was measured at 450 nm with excitation at 360 nm using a Shimadzu RF1500 spectrofluorometer. The concentration of UMB released from the fluorogenic substrates was calculated from a calibration curve obtained using authentic solutions of UMB after subtraction of the control value.

2.3. Steady-state kinetics

Solutions of compound **2** were prepared in concentrations ranging from 0.2 to 1.3 mM and solutions of compound **3** were prepared in concentrations from 0.2 to 2.8 mM in order to obtain steady-state kinetic constants. Each solution was mixed with the enzyme solution to give a final enzyme concentration of 3.1 nM. In the case of compound **1**, substrate solution concentrations ranging from 0.003 to 0.171 mM were prepared, and the final enzyme concentration was 0.9 nM. The initial velocity was determined from the increase in UMB concentration. The values of the kinetic constants were calculated from a plot of S/v_0 versus S, where v_0 and S are the initial rate of hydrolysis and the substrate concentration, respectively. In the case of **1**, the S versus v_0 plot exhibited a typical substrate inhibition profile. Thus, as described later, the kinetic constants were obtained with an appropriate reaction model for the substrate inhibition using a non-linear curve fitting method.

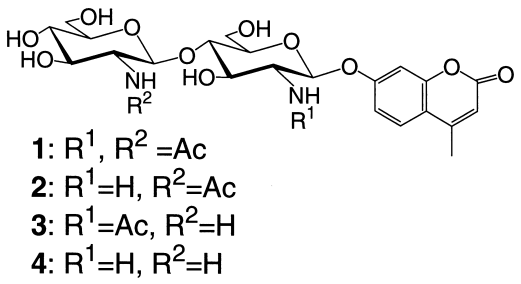

Fig. 1. Structure of $(GlcNAc)_2$-UMB and its partially *N*-deacetylated derivatives.

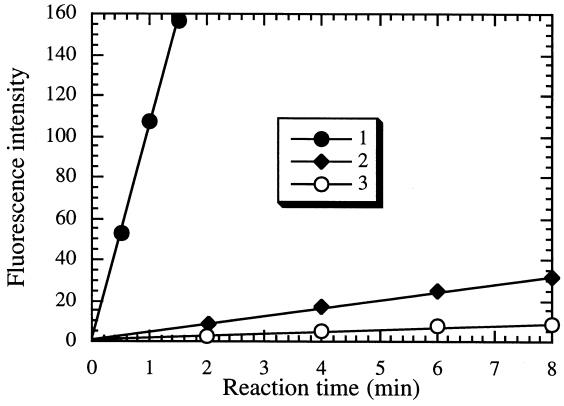

Fig. 2. Time courses showing the increase in fluorescence intensity from **1**, **2** and **3** in the presence of *B. circulans* WL-12 chitinase A1. Enzyme concentrations were 3.1 nM. Substrate concentrations were 0.036 mM. Other conditions are described in the text.

3. Results and discussion

3.1. Rates of UMB release from the substrates

When compound **4** was incubated with the chitinase, the fluorescence intensity did not increase. As expected, the chitinase did not exhibit any activity toward the completely *N*-deacetylated chitobioside (**4**). Fig. 2 shows time courses of the reaction of the chitinase with the fluorogenic substrates, producing increases in the fluorescence intensity. The rates of fluorescence increase obtained with compounds **2** and **3** were much slower than that with compound **1** (3.8% and 1.0% of that of compound **1**, respectively). From thin layer chromatographic analysis of the enzymatic products, the cleavage occurs at the glycosidic bond linking the glyconic moiety with the UMB aglycone of compounds **1**, **2** and **3** (data not shown). Therefore we were able to follow the total chitinase activity toward compounds **1**, **2** and **3** directly by measuring the increase in fluorescence intensity. As Fig. 2 demonstrates, the chitinase was even able to hydrolyze the glycosidic linkage between GlcNAc–GlcN and the UMB moiety of **3** at a very slow rate. According to a report of the chitinase cleavage specificity on 50% *N*-acetylated chitosan [3], the chitinase did not hydrolyze the glycosidic linkage of GlcN–GlcNAc in the chitosan. However, this report is not consistent with the data obtained with compound **3**. The apparently slow rate of the hydrolysis of the GlcN–GlcNAc linkage in 50% *N*-acetylated chitosan might not have afforded sufficient amounts of the enzymatic product to facilitate detection. Recently, a 'substrate-assisted' mechanism has been regarded as the most likely mechanism for the catalysis of the chitinase; that is, the oxocarbonium ion intermediate is stabilized by an anchimeric assistance of the *N*-acetyl group of the sugar residue at subsite (−1). From the viewpoint of the catalytic mechanism, the significant increase in fluorescence intensity from compound **3** (GlcNAc-GlcN-UMB) should be noted. To obtain further information about the hydrolytic reaction, kinetic constants for the chitinase were determined using the fluorogenic substrates.

3.2. Kinetics for the substrate, $(GlcNAc)_2$-UMB

Initially, we attempted to determine the kinetic constants for the chitinase toward substrate **1**. As shown in Fig. 3, a

plot of the substrate concentration versus the initial velocity indicated a strong substrate inhibition for substrate concentrations greater than 0.03 mM. The demonstrated kinetic behavior suggests that such a short oligomeric substrate might be able to bind to a vacant subsite, producing a 1:2 or 1:3 enzyme–substrate complex. Therefore, we tried to determine the kinetic parameters using several different models which accounted for this substrate inhibition phenomenon. Finally, the theoretical line obtained from the uncompetitive substrate inhibition model shown below [21] could be satisfactorily fitted to the experimental points, and the data fitting was used to obtain the kinetic constants.

$$\begin{array}{ccccc} [E] + [S] & \underset{}{\overset{K_m}{\rightleftarrows}} & [ES] & \overset{k_2}{\rightarrow} & [E] + [P] \\ & & \uparrow\downarrow K_i & & \\ & & [ES^2] & & \\ & & \uparrow\downarrow K_{ii} & & \\ & & [ES^3] & & \end{array} \qquad (1)$$

$$v_0 = \frac{V_{max}\,[S]}{K_m + [S] + ([S]^2/K_i) + ([S]^3/K_i K_{ii})}$$

$(V_{max} = k_2[E]_0;\ [E]_0 = 0.9\ \text{nM})$

where K_i and K_{ii} are dissociation constants of the 1:2 and 1:3 enzyme–substrate complexes, $[ES^2]$ and $[ES^3]$, respectively. From the theoretical line shown in Fig. 3, the kinetic constants were calculated to be $k_{cat} = 43.6\ s^{-1}$, $K_m = 0.3$ mM, $K_i = 0.2$ mM and $K_{ii} = 0.001$ mM. The substrate inhibition is frequently encountered in endo-type glycosidases which have a multi-subsite binding cleft when using low molecular weight oligomers as the substrates [21–23]. Chitinase 63 from *Streptomyces plicatus* also exhibits a substrate inhibition when using **1** as the substrate. Thus, such a substrate inhibition phenomenon would be predicted in family 18 chitinases because of the sequence homology of their active site clefts [24,25].

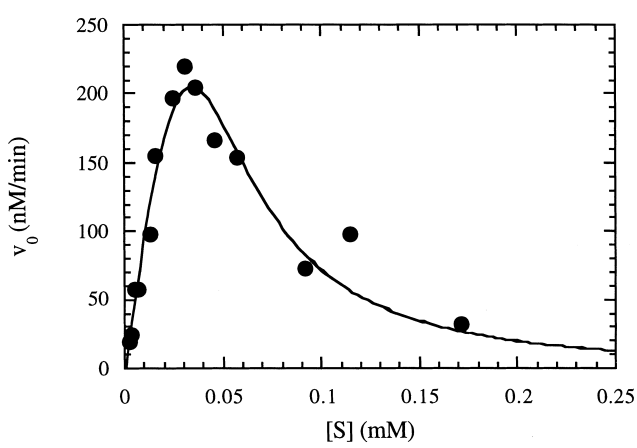

Fig. 3. The v_0 versus S plot for hydrolysis of **1** by *B. circulans* WL-12 chitinase A1. The enzyme concentration was 0.9 nM. The solid line is a calculated curve using Eq. 1. The kinetic parameters are described in the text.

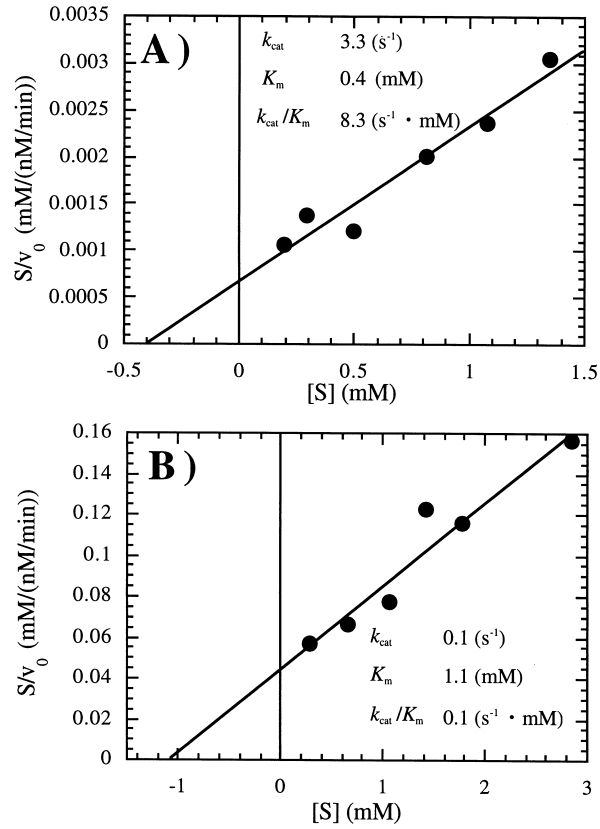

Fig. 4. S/v_0 versus S plots for hydrolysis of **2** and **3** by *B. circulans* WL-12 chitinase A1. A: GlcN-GlcNAc-UMB (**2**). B: GlcNAc-GlcN-UMB (**3**). The enzyme concentrations were 3.1 nM. Other conditions are described in the text.

3.3. Kinetics for GlcNAc-GlcN-UMB and GlcN-GlcNAc-UMB

In contrast to the findings for compound **1**, no substrate inhibition was observed when compounds **2** and **3** were used as the substrates, and kinetic constants were successfully obtained with a simple S/v_0 versus S plot (Fig. 4). It was expected that compounds **2** and **3** would not be able to bind to the vacant subsites because of their low affinities caused by the removal of the *N*-acetyl group. The kinetic constants obtained using compounds **2** and **3** as substrates were calculated from the fitted straight lines, and are summarized in Fig. 5. The removal of the *N*-acetyl group at subsite (−2) did not significantly affect the K_m value. It appears likely that the sugar binding ability is retained by hydrophobic or stacking interactions with the pyranose ring. The effect of the GlcN residue binding to subsite (−2) was, however, reflected in the catalytic constant, k_{cat}. The bound GlcN-GlcNAc-UMB at subsites (−2) and (−1) might be in an abnormal conformation, resulting in the lower k_{cat} value. The overall activity, k_{cat}/K_m, for compound **2** was 5.7% of that obtained with compound **1**. On the other hand, the removal of the *N*-acetyl group at subsite (−1) resulted in a considerable decrease in the k_{cat} value and a significant increase in the K_m value for compound **3**. The k_{cat}/K_m value decreased to 0.1% of that obtained with compound **1**. Therefore, the presence of the *N*-acetyl group at subsite (−1) was found to be very important in the catalytic reaction, supporting the idea that the catalysis of the chitinase takes place through a 'substrate-assisted' mechanism. In addition,

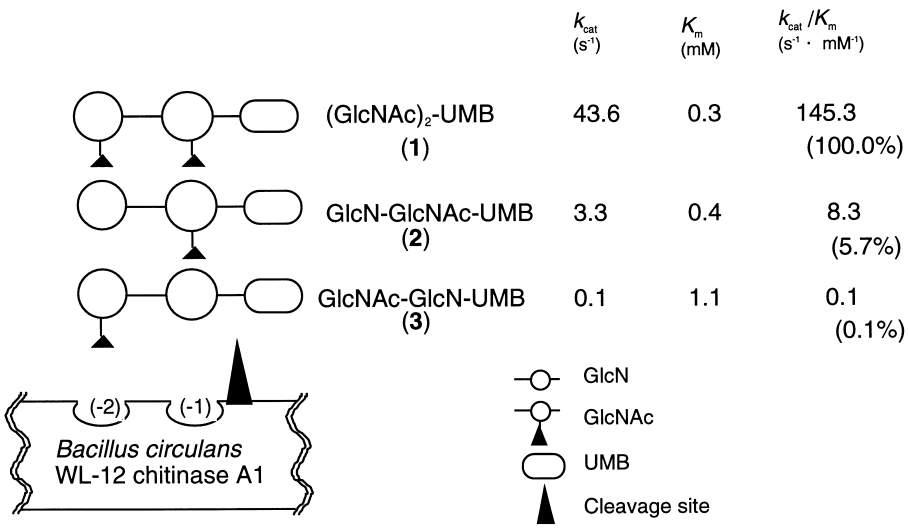

Fig. 5. Kinetic parameters for the hydrolysis of UMB glycosides of chitobiose (**1**, **2** and **3**) catalyzed by *B. circulans* WL-12 chitinase A1.

the *N*-acetyl group appears likely to participate not only in the catalysis but also in the binding of the sugar residue.

Conversely, the very low level of activity observed with compound **3** indicates that the hydrolytic reaction can still proceed without the assistance of the *N*-acetyl group. From kinetic analyses of mutated chitinases with various substrates, the Glu-204 residue in the chitinase has been considered to act as a proton donor in the catalytic reaction [8]. Further mutational analysis of other acidic amino residues, Asp-197, Asp-200 and Asp-202, has suggested that such residues might also participate in the hydrolytic reaction of the glucosaminide linkage. It appears likely that the *N*-acetylated residue of compound **3** binds to the subsite (−2) of the enzyme, resulting in an abnormal mode of the catalytic reaction [9].

In conclusion, the *N*-acetyl group of the substrate sugar residue was found to be very important in the catalysis of the chitinase from *B. circulans*, supporting the idea of a 'substrate-assisted' mechanism for the catalysis. The *N*-acetyl group is also a requirement for the recognition of the sugar residue at subsite (−2). Finally, this series of UMB chitobiosides has proved to be very useful substrates in the elucidation of recognition mechanisms of chitinolytic enzymes.

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