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Kinetic Studies on the Hydrolysis of *N*-Acetylated and *N*-Deacetylated Derivatives of 4-Methylumbelliferyl Chitobioside by the Family 18 Chitinases ChiA and ChiB from *Serratia marcescens*

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Kinetic analyses of the hydrolysis reactions of *N*-acetylated and *N*-deacetylated derivatives of 4-methylumbelliferyl chitobioside [$(GlcNAc)_2$-UMB (1), GlcN-GlcNAc-UMB (2), GlcNAc-GlcN-UMB (3), and $(GlcN)_2$-UMB (4)] by ChiA and ChiB from *Serratia marcescens* were performed. Both enzymes released UMB from all compounds apart from 4. The S-v curves of the hydrolyses of 1 by ChiA and ChiB both exhibited atypical kinetic patterns, and the shapes of the two S-v curves were different from one another. However, both curve shapes were explained by assuming some of the enzyme present formed complexes with multiple molecules of the substrate. Conversely, the S-v curves generated in the cleavage of 2 and 3 by ChiA exhibited typical Michaelis-Menten profiles. Both enzymes hydrolysed 2 with an approximately 14-fold higher K_m value relative to 1, indicating that the *N*-acetyl group was recognised at the –2 subsite. The k_{cat} value obtained with ChiA was identical to the k_{cat} value observed for 1. However, the k_{cat} value for ChiB was one-fourth that of 1, suggesting that the removal of the *N*-acetyl group caused an increase in the formation of a non-productive ES-complex. ChiA and ChiB hydrolysed 3 with 5- and 20-fold greater K_m values relative to 1, respectively, and 60- and 30-fold smaller k_{cat} values relative to 1, respectively. The reaction mechanism of family 18 chitinases is discussed based upon the results obtained from the hydrolysis of these compounds.

Key words: chitinase, fluorogenic chitobioside, *Serratia marcescens*, steady state kinetics.

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

Chitinases [EC 3.2.1.14], the enzymes that hydrolyse chitin (a β-1,4–linked polysaccharide of GlcNAc), are classified into glycosidase families 18 and 19 based on their amino acid sequences (*1*). The major difference in the reactions of family 18 and 19 chitinases is found in the hydrolyses they perform, resulting in retention or inversion of the anomeric position, respectively (*2*, *3*). In general, the well known retaining glycosidases such as lysozyme, cellulase, xylanase and endo-1,3-1,4-β-D-glucan 4-glucanohydrolase have two acidic catalytic residues, one acting as a nucleophile and the other as a proton donor (*4*). However, in family 18 chitinases, the proton donor, glutamate, exists while the nucleophile is missing, as determined by mutational and X-ray diffraction analyses (*5*–*7*). Therefore, the reaction mechanism of family 18 chitinases must differ from that of the other retaining enzymes. A similar situation is also observed for a family 20 β-*N*-acetyl hexosaminidase (*8*). To explain the single glutamate-catalysed mechanism, a "substrate-assisted catalysis" model has been postulated and this represents the most plausible explanation put forward to date (*6*, *7*, *9*).

To facilitate the analysis of the hydrolytic reaction performed by chitinase, chitin, the natural substrate for the enzyme, is the most important substrate to study. However, it is difficult to evaluate the kinetic parameters obtained from this polysaccharide because chitin is an insoluble polymer and has a variable degree of polymerisation. Chitinases also hydrolyse the soluble, partially *N*-acetylated chitosan (the β-1,4 hetero-polysaccharide of GlcNAc and GlcN), which is a natural substrate for chitosanase [EC 3.2.1.132] (*10*–*12*). Thus, it is valuable to undertake kinetic analyses of chitinases by employing soluble low molecular weight substrates, such as oligosaccharides and chromophoric substrates.

The authors have previously reported the synthesis of 4-methylumbelliferyl-β-chitobioside (1) and its *N*-deacetylated derivatives; GlcN-GlcNAc-UMB (2), GlcNAc-GlcN-UMB (3), and $(GlcN)_2$-UMB (4) (*13*) (Fig. 1). These compounds retain the chair form in the pyranose rings, suggesting that they can be used as analogues of

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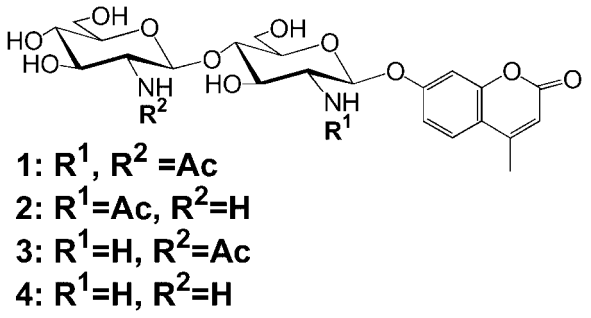

Fig. 1. **Structures of 4-methylumbelliferyl chitobioside and its partially *N*-deacetylated derivatives.**

the natural oligosaccharides (*13*). These fluorogenic substrates are considered to be versatile tools for the elucidation of the substrate specificity of family 18 chitinases. For example, kinetic studies on the hydrolysis of these compounds by chitinase A1 from *Bacillus circulans* WL-12, a family 18 chitinase, have demonstrated that the enzyme recognises *N*-acetyl groups at both the –1 and –2 subsites (*14*).

To elucidate the reaction mechanism of family 18 chitinases, it is desirable to study an enzyme for which the three-dimensional structure is available. Chitinase A and chitinase B (ChiA and ChiB) from *Serratia marcescens* are good candidates for such studies because high-resolution crystal structures of the enzymes, as well as the enzyme-$(GlcNAc)_n$ complexes, are available (*7*, *15*, *16*). In this report, a detailed kinetic analysis of the enzymatic reaction was performed employing compounds **1**–**4**; the anomeric form generated as well as the splitting pattern of the enzymatic products from $(GlcNAc)_6$, are described.

MATERIALS AND METHODS

Enzyme Production—*Serratia marcescens* 10428 strain was obtained from NCIMB Japan (Shimizu). The genes encoding ChiA and ChiB were amplified from the genomic DNA of *S. marcescens* by the polymerase chain reaction. The primers used for the reaction were as follows: ChiA forward primer 5′-**CATATG**CGCAAATTTAATAAACCGCTG-3′ (containing a *Nde*I site; bold characters), reverse primer 5′-**CTCGAG**TTATTGAACGCCGGCGCTGTT-3′ (containing a *Xho*I site and a stop codon; denoted by bold and underlined type, respectively), and ChiB forward primer 5′-**CATATG**TCCACACGCAAAGCCGTTATT-3′ (containing a *Nde*I site, bold characters), reverse primer 5′-**CTCGAG**TTACGCCAGGCGGCCCACCTT-3′ (containing a *Xho*I site and a stop codon, denoted by bold and underlined type, respectively). The amplified fragments were cloned into pCR®2.1-TOPO® (Invitrogen, USA) and the DNA sequence was confirmed. The plasmid was then digested with *Nde*I and *Xho*I, and the digested fragment was ligated into pET30b at the corresponding sites using a pET expression system (Novagen, USA). Next, the expression vectors containing the ChiA and ChiB genes were electroporated into *Escherichia coli* BL21GOLD(DE3), and the resulting transformants were incubated in Luria broth medium containing 0.05 mg/ml kanamycin at 37°C until the optical density at 600 nm reached a level of 0.6. Isopropyl-β-D-thiogalactopyranoside was then added to give a final concentration of 1 mM, and the cultures were incubated for 24 h at 25°C. The recombinant enzymes were purified by chitin affinity chromatography to completely purity on SDS–PAGE (*17*). Protein concentrations were determined from the absorbances at 280 nm based on the calculated molar absorption coefficients (ChiA; 105,500 M^{-1} cm^{-1}, ChiB; 112,040 M^{-1} cm^{-1}) determined from the amino acid compositions of ChiA and ChiB (*18*).

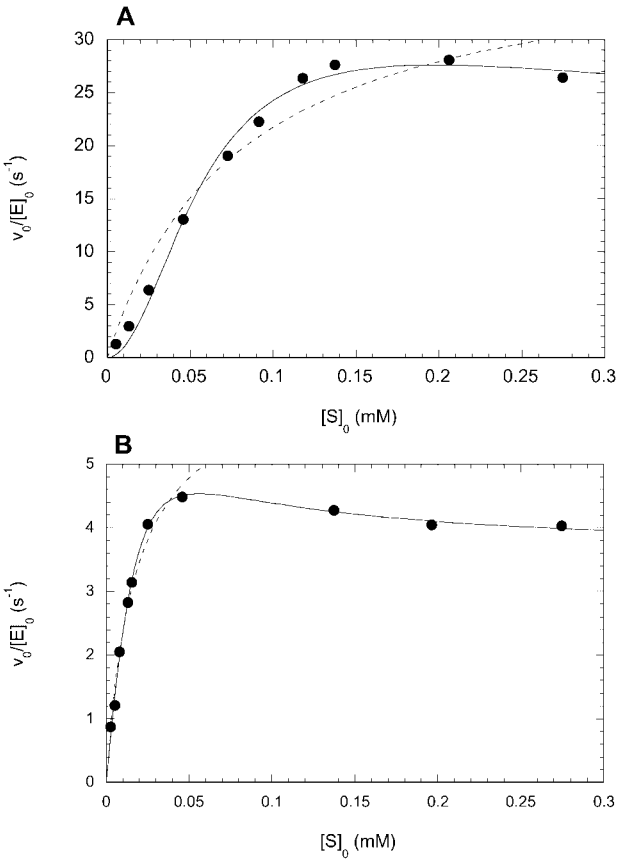

Fig. 2. ***S-v* plots of ChiA and ChiB obtained using $(GlcNAc)_2$-UMB as a substrate.** A, ChiA; B, ChiB. The dashed lines are the theoretical curves based on the Michaelis-Menten equation. The solid lines are calculated curves using eq. 1(ChiA) and eq. 2 (ChiB).

Kinetics for the Fluorogenic Substrates—Compounds **1**–**4** were synthesised as described previously (*13*). The hydrolyses of these compounds by ChiA and ChiB were carried out in 50 mM sodium phosphate buffer (pH 6.0) at 40°C. The reactions were monitored by measuring the increase in UMB concentration as described previously (*14*). The kinetic parameters were calculated by regressing the experimental data into each appropriate formula by the Kaleidagraph™ ver. 3.51 (Synergy Software) curve fitting method.

$(GlcNAc)_6$ Degradation—$(GlcNAc)_6$ was purchased from Seikagaku Kogyo Co. The hydrolysis of $(GlcNAc)_6$ was carried out at a concentration of 5.0 mM with an enzyme concentration of 0.1 μM at 40°C. The products were periodically analysed by HPLC as previously described (*19*).

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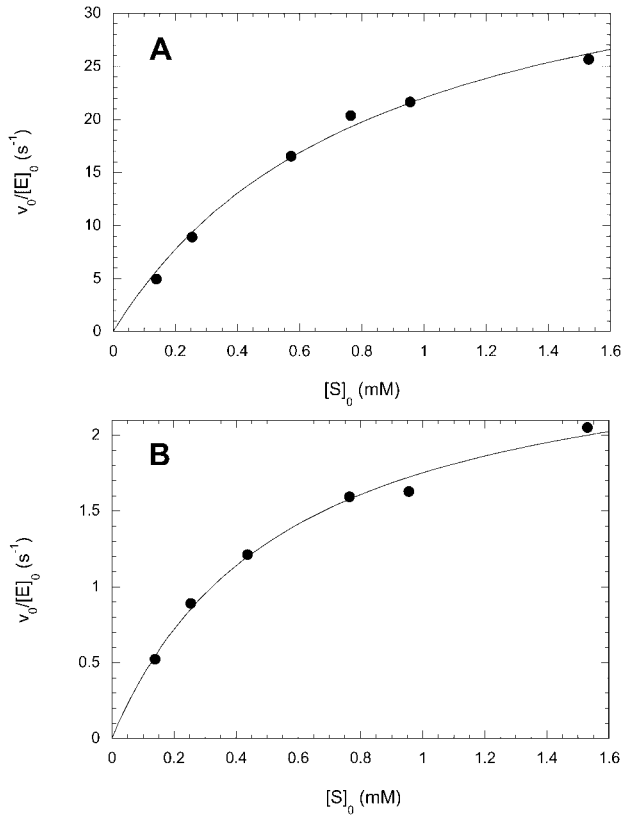

Fig. 3. ***S-v* plots of ChiA and ChiB obtained using GlcN-GlcNAc-UMB (2) as a substrate.** A, ChiA; B, ChiB. The solid lines are the theoretical curves based on the Michaelis-Menten equation. Other conditions are described in the text.

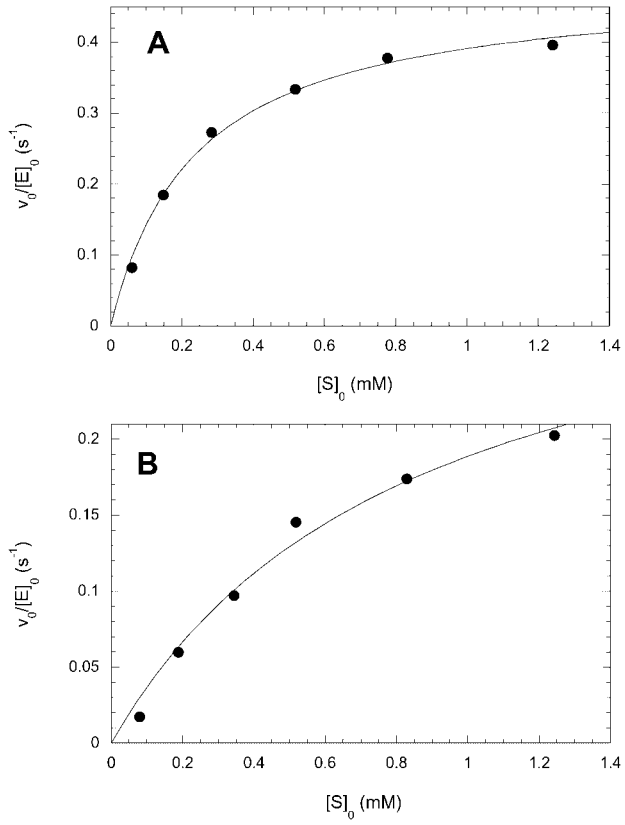

Fig. 4. ***S-v* plots of ChiA and ChiB obtained using GlcNAc-GlcN-UMB (3) as a substrate.** A, ChiA; B, ChiB. The solid lines are theoretical curves based on the Michaelis-Menten equation. Other conditions are described in the text.

RESULTS

Kinetics for $(GlcNAc)_2$-UMB (1) ChiA—Initially, the kinetic behaviour of the degradation reaction of **1** catalysed by ChiA was investigated to obtain standard properties toward the various $(GlcNAc)_2$-UMB derivatives. When the S-v curve of the degradation of **1** by ChiA was regressed with the Michaelis-Menten equation, the theoretical curve did not fit the experimental data well and this is shown in Fig. 2A. It appeared as though an allosteric effect was occurring at lower substrate concentrations and some substrate inhibition was occurring at higher concentrations. Taking these observations into account, the following Scheme 1 was assumed:

$$\mathrm{E} + 2\mathrm{S} \overset{K}{\rightleftharpoons} \mathrm{ES2} \overset{k_2}{\longrightarrow} \mathrm{E} + \mathrm{P} + \mathrm{S}$$
$$\mathrm{ES2} \rightleftharpoons \mathrm{ES3} \quad (K_i) \qquad \text{(Scheme 1)}$$

In this scheme, two molecules of **1** bind to one molecule of the enzyme to form an ES2 complex, followed by the hydrolysis of one of the substrates. The rate equation derived from scheme 1 is given by Eq. 1.

$$v_0 = \frac{k_2[E][S]^2}{K + [S]^2 + \frac{[S]^3}{K_i}} \qquad \text{(Eq. 1)}$$

In this equation, k_2 is a rate constant and K and K_i are dissociation constants, respectively. The theoretical curve generated with eq. 1 agreed well with the experimental data. The kinetic parameters in eq. 1 were calculated to be: $k_2 = 34.9\ s^{-1}$, $K = 0.0035\ mM^2$ and $K_i = 1.1$ mM.

ChiB—As shown in Fig. 2B, the S-v curve of ChiB also did not agree with the Michaelis-Menten type equation: The experimental data at lower substrate concentrations fitted the Michaelis-Menten type curve well but those at higher concentrations were lower than expected. If the data were regressed with an equation describing substrate inhibition, the theoretical curve shifted to a much lower position than the experimental data at higher concentrations. Taking these observations into account, the following Scheme 2 was assumed:

$$\mathrm{E} + \mathrm{S} \overset{Ks_1}{\rightleftharpoons} \mathrm{ES} \overset{k_2}{\longrightarrow} \mathrm{E} + \mathrm{P}$$
$$\mathrm{ES} \rightleftharpoons \mathrm{ES2} \quad (Ks_2) \qquad \text{(Scheme 2)}$$
$$\mathrm{ES2} \overset{k_2'}{\longrightarrow} \mathrm{E} + \mathrm{P} + \mathrm{S}$$

where Ks_1 and Ks_2 are the dissociation constants of the ES and ES2 complexes, respectively, and k_2 and k_2' are rate constants, respectively. In scheme 2, the formation of an ES2 complex followed by the hydrolysis of one of the substrates binding at a different rate was added to a typical Michaelis-Menten mechanism. The rate equation derived from Scheme 2 is given by Eq. 2.

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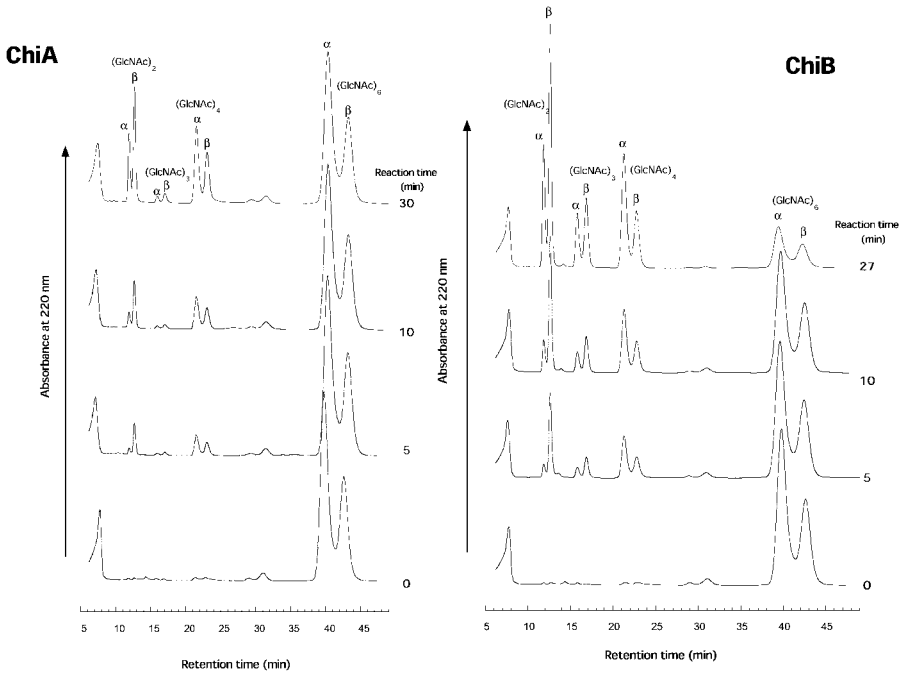

Fig. 5. **Anomer formation from $(GlcNAc)_6$ as catalysed by ChiA and ChiB.** Left-hand profiles indicate the ChiA catalysed reaction. Right-hand profiles indicate the ChiB catalysed reaction. Other conditions are described in the text.

$$v_0 = \frac{k_2[E]_0[S] + k_{2'}[E]_0\frac{[S]^2}{Ks_2}}{Ks_1 + [S] + \frac{[S]^2}{Ks_2}} \qquad \text{(Eq. 2)}$$

The kinetic parameters were determined as follows; k_2 = 11.4 s^{-1}, k_2' =3.6 s^{-1}, K_{s1} = 0.042 mM, and K_{s2}= 0.015 mM. The theoretical curve fits the experimental data well.

*Activity toward N-Deacetylated Chitobiosides (**2**–**4**)*—Both ChiA and ChiB released UMB from **2** and **3** but neither exhibited any activity toward the completely *N*-deacetylated chitobioside derivative (*4*), even at extended incubation times. From thin layer chromatographic analysis of the enzymatic products, cleavage occurs at the glycosidic bond linking the glyconic moiety with the UMB aglycon of **1**, **2,** and **3** (data not shown). Figures 3 and 4 show the S-v curves for **2** and **3** in the cleavage reactions performed by the enzymes. In contrast with the results obtained with **1**, typical Michaelis-Menten relationships were observed, and the kinetic parameters obtained are summarised in Table 1.

Hydrolysis of $(GlcNAc)_6$—The $(GlcNAc)_6$ degradation products of ChiA and ChiB were analysed by HPLC. Figure 5 shows the HPLC profiles of the hydrolytic products from $(GlcNAc)_6$ obtained with each of the two enzymes. A standard equilibrium ratio of α:β anomers for $(GlcNAc)_6$ was determined to be 1:0.6 from Fig. 5.

As shown in Fig. 5A, ChiA produced mainly $(GlcNAc)_2$ and $(GlcNAc)_4$ from $(GlcNAc)_6$. The α:β ratio of the ChiA reaction product, $(GlcNAc)_2$, was found to be 1:6.8 after a reaction time of 5 min. On the other hand, the ratio for the $(GlcNAc)_4$ reaction product was 1:0.6, a similar ratio to that of the substrate, $(GlcNAc)_6$. These results suggest that the reducing end of the newly created product has a β-configuration rather than α. These results indicate that ChiA mainly hydrolyses the second β-1,4 glycosidic bond from the non-reducing end of $(GlcNAc)_6$.

In contrast with ChiA, ChiB produced $(GlcNAc)_2$, $(GlcNAc)_3$, and $(GlcNAc)_4$ as shown in Fig. 5B. After the enzymatic reaction had proceeded for 5 min, the anomeric ratios (α:β) of the products, $(GlcNAc)_2$, $(GlcNAc)_3$, and $(GlcNAc)_4$ were 1:6.8, 1:2.4, and 1:0.6, respectively. These ratios suggest that the reducing ends of the enzymatic products are β in configuration. The anomeric configuration of the enzymatic products indicates that ChiB cleaves mainly the second and third glycosidic linkages

Table 1. **Kinetic parameters of the hydrolysis of 4-methylumbelliferyl chitobioside derivatives by ChiA and ChiB.**

Substrate	Enzyme	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})
$(GlcNAc)_2$-UMB	ChiA	34.9	0.059[a]	589.4
(1)	ChiB	11.4[b]	0.042[b]	270.3
GlcN-GlcNAc-UMB	ChiA	40.8	0.85	48.0
(2)	ChiB	2.7	0.56	4.9
GlcNAc-GlcN-UMB	ChiA	0.48	0.24	2.03
(3)	ChiB	0.35	0.85	0.41

[a]$K_m = K_{0.5}$. [b]$K_m = K_{S1}$, $k_{cat} = k_2$.

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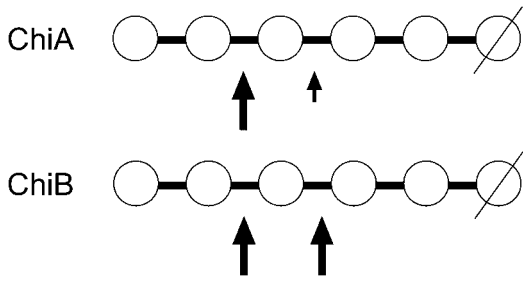

Fig. 6. **Schematic drawings of the cleavage patterns of $(GlcNAc)_6$ catalysed by ChiA and ChiB.** Arrows indicate cutting sites of the corresponding enzymes. The symbols on the right represent the reducing ends of $(GlcNAc)_6$.

from the non-reducing end of $(GlcNAc)_6$. The hydrolytic patterns of ChiA and ChiB are summarised in Fig. 6.

DISCUSSION

*Kinetic Properties toward **1***—It has been reported that the kinetic behaviour of the hydrolysis of **1** by family 18 chitinases does not obey the profile of a typical Michaelis-Menten mechanism (*14*, *19*). Thus, a careful evaluation is required in any such kinetic analyses of **1**. Previously, Brurberg et al. reported that ChiA exhibits sigmoidal kinetic properties in the hydrolysis of compound **1**, but they did not obtain sufficient kinetic parameters (*20*). As shown in Fig. 2A, the kinetic properties of ChiA toward **1** exhibited a sigmoidal shape with substrate inhibition apparent. This observation is explained by the formation of a 1:2 complex of ChiA with **1** as shown in scheme 1. It has been reported that ChiA has at least 8 subsites as evidenced by the 3-D structure of an inactive ChiA mutant (E315Q) complexed with $(GlcNAc)_8$ (*15*). To explain the substrate inhibition described by eq. 1 (the cubic term [S] in the denominator), a 1:3 complex of ChiA and **1** was putatively assumed in scheme 1. Thus, it is possible to assume that three molecules of **1** are capable of binding to ChiA because **1** is a mimic of $(GlcNAc)_3$. The existence of the cubic term may also be explained by assuming the presence of another 1:2 complex of ChiA and **1**, as reported in the analysis of the competitive substrate inhibition pattern of cellobiose phosphorylase (*21*). Although, it is not clear whether the 1:3 complex exists in the structural analysis, it is necessary to assume that ChiA forms a complex with multiple molecules of **1** in order to explain the observed phenomenon.

As shown in Fig. 2B, the hydrolysis of **1** by ChiB also exhibits an atypical kinetic pattern but different from that exhibited by ChiA. The 3-D structure of an inactive ChiB mutant (E144Q) complexed with $(GlcNAc)_5$ suggests that ChiB has more than 5 subsites (*7*). Therefore, it is reasonable to assume that ChiB forms a 1:2 complex with **1**. It should be noted that both the atypical kinetic patterns observed in the hydrolyses of **1** by ChiA and ChiB can be explained by assuming multiple-substrate-complex of the enzymes. The difference observed in the patterns of the hydrolyses of $(GlcNAc)_6$ by ChiA and ChiB suggests there is a difference in the subsite-affinity patterns of the enzymes, which may cause differences in the kinetic behaviour observed in the hydrolyses of **1**. We previously reported a strong substrate inhibition in the hydrolysis of **1** by *B. circulans* WL-12 chitinase A1, and that this inhibition was caused by the formation of an enzyme-multiple substrate complex (*14*). Such substrate inhibitions are frequently observed in the hydrolysis of small substrates by endo-type glycosidases that have multiple subsites in their binding sites (*22–24*). Taking into consideration all of the above results, it can be concluded that the formation of multiple-substrate complexes of the enzyme causes a variety of atypical kinetic patterns.

Kinetic Properties toward Partially N-Deacetylated Substrates—It is notable that the hydrolyse of **2** and **3** by both enzymes showed typical Michaelis-Menten kinetics. The atypical kinetics observed in the hydrolysis of **1** were basically explained by assuming enzyme–two substrate complexes. In that case, one substrate must be bound at a subsite apart from the active centre. Removal of an N-acetyl group may cause a deficiency of binding at that site, resulting in the Michaelis-Menten kinetics for **2** and **3**.

As shown in Table 1, the k_{cat} value obtained for ChiA toward **2** (40.8) was not significantly different from that obtained toward **1**, whereas the K_m value increased by a factor of 17 relative that for obtained with **1**. These results suggest that the –2 subsite of ChiA recognises the *N*-acetyl group of the substrate, resulting in the observed decrease in affinity. However, the absence of an *N*-acetyl group at the non-reducing end did not affect the reaction rate, implying that the *N*-acetyl group acts only as a determinant of the affinity of the substrate.

Conversely, the k_{cat} value for ChiB toward **2** decreased to a value 24% of that obtained with **1**, and the K_m value also increased to a level 13 times that obtained with **1**. The decrease in the k_{cat} value suggests that the lack of an *N*-acetyl group causes an increase in a non-productive complex and decreases the amount of productive complex formed. The differences observed between ChiA and ChiB can again be explained by differences in the subsite structures of the two enzymes.

ChiA cleaved **3** with a 5-fold higher K_m value and a 60-fold lower k_{cat} value compared with **1**, and, correspondingly, ChiB cleaved **3** with a 20-fold higher K_m value and a 30-fold lower k_{cat} value. That the *N*-acetyl group at the –1 subsite participates in substrate recognition is evidenced by the considerable increase in the K_m value. These results clearly indicate that the removal of the non-reducing end *N*-acetyl group causes a significant decrease in the observed catalytic activity. However, it is notable that the hydrolytic rates toward **3** (GlcNAc-GlcN-UMB) were not zero, indicating that both enzymes hydrolysed the β-glucosaminyl linkage between the glucosamine residue and the UMB moiety.

In the "substrate-assisted catalysis" mechanism, the *N*-acetyl group of the –1 subsite is essential for the hydrolytic reaction because the carbonyl oxygen is thought to act as the nucleophile (*7*). However, both enzymes released a UMB moiety from **3**, implying that the *N*-acetyl group is not essential for the reaction. Furthermore, chitinase A1 from *B. circulans* WL-12, another family 18 chitinase, also hydrolyses **3** (*14*). These results suggest that the reaction mechanism of family 18 chitinases cannot be fully explained by the substrate-assisted

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catalysis model in which there is anchemeric assistance of the *N*-acetyl group. Therefore, an alternative mechanism is necessary to understand the hydrolysis of **3**. Recently, Papanikolau *et al*. proposed a new reaction mechanism based on three-dimensional structures and mutational analysis (*15*). In this mechanism, Tyr390 of ChiA participates in the reaction by binding a water molecule with the aid of a nitrogen atom of the acetamide group of a GlcNAc residue at the –1 subsite. This mechanism may serve to explain the observed hydrolysis of **3**. However, the Y390F mutant still retains 0.42% of the corresponding activity compared with the wild type enzyme (*15*). Thus, this mechanism also does not fully represent the catalytic mechanism of family 18 chitinases. Therefore, in order to understand the reaction mechanism of family 18 chitinases, a better explanation for the hydrolysis of **3** is still required.

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