Kinetic evidence related to substrate-assisted catalysis of family 18 chitinases

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Abstract The hydrolytic reaction of family 18 chitinase has been considered to occur via substrate assisted catalysis. To kinetically investigate the enzyme reaction mechanism, we synthesized compounds designed to reduce the polarization of the carbonyl in *N*-acetyl group, GlcNAc-GlcN(TFA)-UMB (2) and GlcNAc-GlcN(TAc)-UMB (3). Kinetic parameters in the hydrolysis of these compounds by chitinase A from *Serratia marcescens* (ChiA) were compared with those from the hydrolysis of $(GlcNAc)_2$-UMB (1). The k_{cat} of 2 was 3.4% of 1, but the K_m of 2 was 10-fold that of 1. In contrast, the k_{cat} of 3 was only 0.3% of that of 1, and the two reactions had an identical K_m. The drastic decreases in k_{cat} were probably due to the weak nucleophilic activity of the C2-*N*-trifluoroacetamide and *N*-thioacetamide groups at reducing ends of compounds 2 and 3, respectively. These results indicate that the anchimeric assistance of the C2 *N*-acetamide group at GlcNAc plays a key role in the hydrolytic reactions catalyzed by family 18 chitinases.

Keywords: Chitobiose phosphorylase; Chitinase; Glycoside hydrolase family 18; 4-Methylumbelliferyl chitobioside; Substrate-assisted catalysis; *Serratia marcescens*

1. Introduction

In general, the hydrolytic reactions of the retaining glycosidases, such as lysozyme, cellulase, xylanase and endo-1,3-1,4-β-D-glucan 4-glucanohydrolase, are thought to occur through the combined action of two acidic catalytic residues, one acting as a nucleophile and the other as a proton donor [1]. In family 18 chitinases, the proton donor residue has been shown to be a glutamate, whereas the residue that acts as the nucleophile has not been detected by either mutational or X-ray diffraction analyses [2–4], indicating that the reaction mechanism of family 18 chitinases differs from that of other retaining enzymes. Similar situations have also been observed with family 20 glycosidases (e.g., β-*N*-acetylhexosaminidase) and family 56 glycosidases (e.g., hyaluronidase) [5–8].

To explain the mechanism by which hydrolysis occurs via a single glutamate, a "substrate-assisted catalysis" model has been proposed [2,9,10]. According to this model, the catalytic residue (associated glutamate) donates a proton to the oxygen atom of the glycosidic linkage. The negatively polarized carbonyl oxygen of the C2-acetamide group of the substrate then attacks the C1 carbon in the same pyranose ring as the nucleophile from the α-direction, resulting in cleavage of the glycosidic linkage and the formation of "an oxazoline-like intermediate" on dissociation of the glutamate (Fig. 1). Finally, the hydroxyl ion formed through deprotonation of a water molecule by the dissociated glutamate attacks the C1 of the intermediate from the β-direction, resulting in retaining hydrolysis.

Using both kinetic and structural analyses, the enzymatic activity of family 20 glycosidases has been shown to occur by substrate-assisted catalysis. For example, a family 20 β-*N*-acetylhexosaminidase has been shown to hydrolyze GlcN(TAc)-UMB (**4**) at a rate 21-fold lower than its hydrolysis of GlcNAc-UMB (**5**) [11]. This is a strong support for the substrate assisted catalysis mechanism, because compound **4** was designed to reduce the polarization of the carbonyl (thionyl) group in order to weaken the nucleophilic effect of the oxygen (sulfur) atom. Furthermore, the 3D-structure of a family-20 β-*N*-acetylhexosaminidase from *Streptomyces plicatus* complexed with GlcNAc-thiazoline (**8**) suggested that the enzymatic hydrolysis proceeded via a cyclic intermediate [5,6].

In family 18 chitinases, this mechanism is supported well by the structural analyses of chitinase B (ChiB) of *Serratia marcescens* complexed with various ligands, which suggest that the carbonyl oxygen in *N*-acetyl group of the GlcNAc residue at subsite −1 acts as a nucleophile in the hydrolytic reaction [4,12,13]. Such structural evidence has also been obtained for several family 18 chitinases complexed with allosamidin [pseudo-$(GlcNAc)_3$] (**9**), which is an analog of the oxazolinium ion intermediate [2,4,12,14,15]. To date, however, there has been no kinetic evidence for the occurrence of this mechanism

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Abbreviations: ChBP, chitobiose phosphorylase; ESI-MS, electrospray ionization mass spectrometry; GlcNAc, 2-acetamido-2-deoxy-D-glucose; $(GlcNAc)_n$, β-1,4-linked oligosaccharide of GlcNAc with a polymerisation degree of *n*; GlcNAc-1-α-F, 2-acetamido-2-deoxy-α-D-glucopyranosyl fluoride; GlcNAc-GlcN-UMB, 4-methylumbelliferyl *O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1 → 4)-2-amino-2-deoxy-β-D-glucopyranoside; GlcNAc-1-P, 2-acetamide-2-deoxy-α-D-glucose 1-phosphate; GlcNAc-thiazoline, 2-methyl-(1,2-dideoxy-α-D-glucopyrano)-[2,1-*d*]-2-thiazoline; GlcNAc-UMB, 4-methylumbelliferyl 2-acetamide-2-deoxy-β-D-glucose; $(GlcNAc)_2$-UMB, 4-methylumbelliferyl *O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1 → 4)-2-acetamido-2-deoxy-β-D-glucopyranoside; GlcN(TAc)-UMB, 4-methylumbelliferyl-2-thioacetamido-2-deoxy-β-D-glucopyranoside; GlcNAc-GlcN(TFA)-UMB, 4-methylumbelliferyl*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1 → 4)-2-trifluoro-acetamido-2-deoxy-β-D-glucopyranoside; GlcNAc-GlcN(TAc)-UMB, 4-methylumbelliferyl *O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1 → 4)-2-thioacetamido-2-deoxy-β-D-glucopyranoside; UMB, 4-methylumbelliferone

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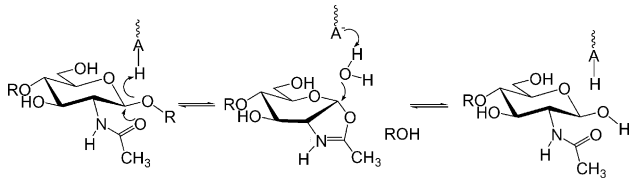

Fig. 1. Proposed substrate assisted catalysis mechanism for family 18 chitinases. The mechanism presented is based on the proposals in [4,14]. AH indicates a catalytic residue of family 18 chitinases (Glu315: ChiA).

in family 18 chitinases. An alternative reaction mechanism has recently been proposed for chitinase A (ChiA) from *S. marcescens*, a family 18 chitinase, based on three-dimensional structures and mutational analysis [16]. According to this mechanism, the Tyr390 of ChiA participates in the reaction by binding a water molecule with the aid of the nitrogen atom of the acetamide group of the GlcNAc residue at the −1 subsite, without a requirement for the anchimeric assistance of the carbonyl oxygen. Thus, to understand the reaction mechanism of family 18 chitinases, it is important to determine the mechanism of ChiA kinetically.

Although compound **4** is a powerful probe in the investigation of substrate-assisted catalysis, it is not a suitable probe for family 18 chitinases, because the enzymes do not effectively hydrolyze glycosides of monosaccharides. In contrast, $(GlcNAc)_2$-UMB (**1**), a glycoside of a disaccharide, is a suitable substrate for family 18 chitinases [17], suggesting that derivatives of compound **1** designed to reduce the electronegativity of the carbonyl oxygen of the *N*-acetyl group at the reducing end may be good probes. We therefore designed and synthesized GlcNAc-GlcN(TFA)-UMB (**2**) and GlcNAc-GlcN(TAc)-UMB (**3**), in each of which the *N*-acetyl group was selectively replaced by an *N*-thioacetamide or *N*-trifluoroacetamide group at the reducing end of the $(GlcNAc)_2$ moiety. In this report, we describe the synthesis of compounds **2** and **3** by chemo- and enzymatic procedures, and we show the effect of the replacement of the C2 acetamide group on ChiA activity.

2. Materials and methods

2.1. General methods

NMR spectra were determined using a Bruker DRX600 spectrometer operating at 298 K at a proton frequency of 600.13 MHz and a ^{13}C frequency of 150.15 Mz. Electrospray ionization mass spectrometry (ESI-MS) spectra were determined using a Bruker APEX II 70e Fourier transformed ion cyclotron resonance mass spectrometer.

2.2. Synthesis of GlcNAc-GlcN(TFA)-UMB (2)

Two drops of 28% (w/w) methanolic sodium methoxide were added to a solution of 4-methylumbelliferyl *O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-(1 → 4)-2-trifluoroacetamido-1,3,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside (20 mg, 0.024 mmol, [18]) in 8 ml of methanol. After reaction for 18 h at 20 °C, 2 drops of glacial acetic acid were added to neutralize the solution. Silica gel dry chromatography of the residue with 17:2:1 ethyl acetate/methanol/H_2O as the eluant yielded 14 mg (0.021 mmol, 89%) of compound **2**. ^1H NMR (methanol-d_4) δ 7.75 (d, $J = 8.8$ Hz, 1H, H-5) 7.02; (dd, $J = 2.5$ Hz, $J = 2.5$ Hz, 2H, H-6) 6.25 (d, $J = 1.3$ Hz, 1H, H-3) 5.28 (d, $J = 8.5$ Hz, 1H, H1) 4.56 (d, $J = 8.5$ Hz, 1H, H′1) 2.46 (d, $J = 1.2$ Hz, 3H, Ar-CH_3) 2.05 (s, 3H, NHCOCH_3); ^{13}C NMR (MeOH-d_4) δ 174.50 (NH*C*OCH$_3$) 23.13 (NHCO*C*H$_3$); ESI-MS m/z: 637.18, $[M+H]^+$ calcd. for $C_{26}H_{31}F_3N_2O_{13}$, 637.18.

2.3. Synthesis of GlcNAc-GlcN(TAc)-UMB (3)

Compound **4** was prepared as described elsewhere [11]. GlcNAc-1-α-F (**7**) was synthesized by reaction of tetra-*O*-acetyl GlcNAc with pyridinium poly(hydrogen fluoride), followed by *O*-deacetylation using sodium methoxide in methanol according to a standard procedure [19,20]. Pure chitobiose phosphorylase (ChBP) from *Vibrio proteolyticus* [21] (22 U, 7 mg) was added to a solution of compounds **4** (16 mg, 0.030 mmol) and **7** (31 mg, 0.14 mmol) in 30 ml of 50 mM Bis–Tris buffer (pH 7.5). After reaction for 2 days at 25 °C, the glycosides were isolated by Sep-pak C-18 (1 ml, Waters Co. USA), washed with H_2O and extracted with methanol. The reaction products were separated using preparative TLC (PLC plates 20 × 20 cm Silicagel 60 F_{254}, 1 mm, MERCK Germany). The final yield of compound **3** was 8 mg (0.013 mmol, 43%), ^1H NMR (methanol-d_4 and 1 drop of D_2O) δ 7.69 (d, $J = 8.8$ Hz, 1H, H-5) 7.00 (d, $J = 6.3$ Hz, 1H, H-8) 6.98 (d, $J = 2.4$ Hz, 1H, H-6) 6.21 (d, $J = 1.3$ Hz, 1H, H-3) 5.29 (d, $J = 8.2$ Hz, 1H, H1) 5.00 (dd, $J = 9.5$ Hz, $J = 9.0$ Hz, 1H, H2′) 4.55 (d, $J = 8.5$ Hz, 1H, H′1) 3.93-3.35 (m, 11 sugar protons) 2.50 (s, 3H, NHCSCH_3) 2.44 (d, $J = 1.2$ Hz, 3H, Ar-CH_3) 2.04 (s, 3H, NHCOCH_3); ^{13}C NMR (MeOH-d_4 and 1 drops of D_2O) δ 204.85 (*C*═S), 174.14 (*C*═O),163.58 (*C*═O in coumarin), 161.55 (C7″), 155.87 (C1″), 155.80 (C4″), 127.49 (C5″), 116.27 (C4″), 115.16 (C6″), 112.91 (C3″), 104.78 (C8″), 103.15 (C1), 99.94 (C1′), 80.63 (C5), 78.04 (C5′), 76.85 (C4), 75.63 (C3′), 74.07 (C3), 71.81 (C4′), 62.41 (C6′), 61.39 (C2 and C6), 57.21(C2′), 33.89 (NHCS*C*H$_3$), 23.12 (CH_3 in coumarin), 18.73 (NHCO*C*H$_3$); ESI-MS m/z: 599.19, $[M+H]^+$ calcd. for $C_{26}H_{34}N_2O_{12}S$, 598.18.

2.4. Kinetic analysis

ChiA from *S. marcescens* was prepared as previously described [17]. ChiA hydrolyses of fluorogenic substrates were performed in 50 mM sodium phosphate buffer (pH 6.0) and the reactions were monitored by measuring the increase in free UMB fluorescently [17].The kinetic parameters were calculated by regression of the experimental data into each appropriate formula using the curve fit method with Kaleidagraph™ ver. 3.51 (Synergy Software).

3. Results and discussion

3.1. Design of the probe substrates

Two derivatives of compound **1** were designed to reduce the polarity of the carbonyl at the reducing end *N*-acetyl group. The trifluoromethyl group in **2** withdraws an electron from the carbon atom of the carbonyl group, resulting in a decrease in polarization. In compound **3**, the thionyl group is less polarized than the carbonyl group because the electronegativity of the sulfur atom is less than that of the oxygen atom. Thus, the carbonyl (thionyl) of either **2** or **3** is less nucleophilic than that of compound **1** (see Fig. 2).

3.2. Synthesis of the probe substrates

The precursor of compound **2**, 4-methylumbelliferyl *O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-(1 → 4)-2-trifluoroacetamido-1,3,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside, is the precursor of GlcNAc-GlcN-UMB [18]. Changing the deacetylation method from ammonium gas/methanol to sodium methoxide/methanol resulted in an intact *N*-trifluoroacetyl residue, leading to the successful synthesis of **2**. To prepare compound **3**, we initially attempted direct thionization of the fully *O*-acetylated derivative of compound **1** using Lawesson's reagent [22]. This method, however, was not suitable, because there was no selectivity of thionization at each *N*-acetyl group (data not shown). We therefore attempted to synthesize **3** by the addition of a β (1 → 4)-linked GlcNAc to

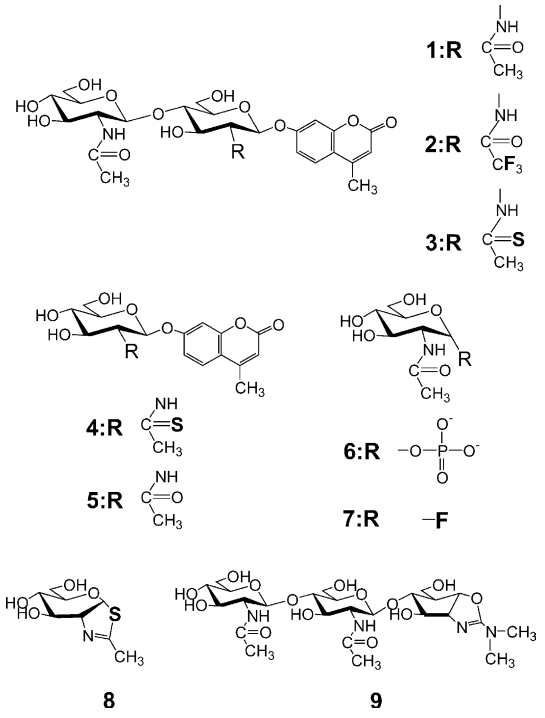

Fig. 2. Structures of the compounds used in this study. **1**, $(GlcNAc)_2$-UMB; **2**, GlcNAc-GlcN(TFA)-UMB; **3**, GlcNAc-GlcN(TAc)-UMB; **4**, GlcN(TAc)-UMB; **5**, GlcNAc-UMB; **6**, GlcNAc-1-P; **7**, GlcNAc-1-α-F; **8**, GlcNAc-thiazoline; and **9**, allosamidin.

compound **4**. Although chemical synthesis of **3** may be possible, it would require many steps for the selective protection and deprotection of functional groups. Thus, an enzymatic process was sought, since it would reduce the protection steps. We found that compound **1** could be synthesized from GlcNAc-1-P (**6**) and GlcNAc-UMB (**5**) using ChBP with perfect regio and stereo specificities at a good yield (47%) [21]. These results suggested that this enzyme was versatile and could add a β (1→4)-linked GlcNAc residue to compound **5** and its derivatives. We therefore attempted the enzymatic synthesis of compound **3** using compound **4** as the acceptor substrate. Due to the difficulty in obtaining compound **6**, we changed the donor substrate to compound **7**, which was much easier to synthesize. The enzymatic synthesis was successful, the yield of compound **3** being 43%. These results suggest that ChBP is a versatile tool in the synthesis of various derivatives of glycosides of $(GlcNAc)_2$.

3.3. Hydrolysis of the probe substrates by ChiA

To investigate the anchemeric effect of the *N*-acetyl group at the −1 subsite in the reaction, we performed kinetic analysis with compound **2**. The hydrolytic activity of enzyme toward 0.04 mM compound **2** was much lower than that toward compound **1**. The kinetic parameters were calculated by regressing the S-v data obtained at 0.02–0.2 mM concentrations with the Michaelis–Menten equation (Fig. 3A). As summarized in Table 1, the k_{cat} decreased to 3.4% of that of compound **1**, while the K_m increased 10-fold compared with compound **1**. The decrease in k_{cat} suggests that the less polarized carbonyl oxygen of the *N*-trifluoroacetyl group was less active in attacking the C1 carbon of the substrate to form the cyclic intermediate. In contrast, the increase in K_m suggests that the substitution disturbs the recognition by ChiA, making these results difficult to explain. It was infeasible to determine kinetic properties and parameters accurately because the K_m value was calculated to be much higher than the solubility of compound **2** (less than 0.2 mM).

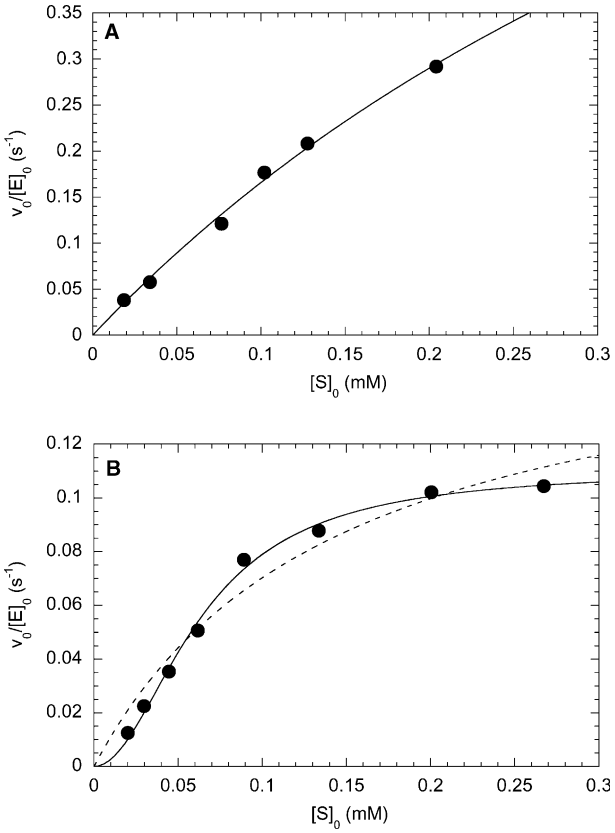

Fig. 3. S-v plots of ChiA obtained using GlcNAc-GlcN(TFA)-UMB (**2**) and GlcNAc-GlcN(TAc)-UMB (**3**) as a substrate. (A) Hydrolysis of **2**. The solid line is a theoretical curve based on the Michaelis–Menten equation. (B) Hydrolysis of **3**. The dashed line is a theoretical curve based on the Michaelis–Menten equation, whereas the solid line is the theoretical curve obtained by regressing the equation reported in [17]: $v_0 = k_2[E][S]^2/(K + [S]^2 + [S]^3/K_i)$.

To obtain further details of the catalytic reaction, we performed kinetic analysis with compound **3**. The time courses were linear at all the substrate concentrations examined within 10% hydrolyses, unlike those for the hydrolysis of **4** by the family 20 β-*N*-acetylhexosaminidase [11]. The S-v-curve of compound **3**

Table 1
Kinetic parameters of the hydrolysis of fluorogenic substrates by ChiA

Compound	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1}\,mM^{-1}$)
$(GlcNAc)_2$-UMB (**1**)	34.9 ± 3.7	0.059 ± 0.007[a]	589.4 ± 23.6
GlcNAc-GlcN(TFA)-UMB (**2**)	1.2 ± 0.3	0.6 ± 0.2	1.9 ± 0.1
GlcNAc-GlcN(TAc)-UMB (**3**)	0.11 ± 0.01	0.063 ± 0.006[a]	1.8 ± 0.1

[a] $K_m = K^{0.5}$.

hydrolysis by ChiA gave an atypical relationship of the Michaelis–Menten type (Fig. 3B). A similar sigmoidal kinetic tendency has been observed for the hydrolysis of **1** by ChiA [17,23]. This phenomenon can be explained well by an enzyme-multiple substrate complex [17]. The experimental data were regressed with the equation given in the legend of Fig. 4 and based on the competitive substrate inhibition model used for the hydrolysis of compound **1** [17]. The theoretical line was found to be in good agreement with the experimental data. Comparing the parameters, the k_{cat} of compound **3** was 0.3% of that of compound **1** with the identical K_m (Table 1). The drastic decrease in k_{cat} without a change in K_m for compound **3** compared with compound **1** strongly suggests that the decrease in polarity caused the drastic decrease in k_{cat}. These results indicate that the polarity of the carbonyl group is strongly related to the enzymatic activity and is strong kinetic evidence that the reaction mechanism of ChiA is substrate-assisted catalysis.

3.4. Reaction mechanism of family 18 chitinases

To date, several high resolution structures of family 18 chitinases complexed with ligands at their active site have been determined [2,4,12–15]. Allosamidin (**9**) in particular has been utilized to elucidate the catalytic mechanism of family 18 chitinases because it is considered to mimic a reaction intermediate.

In a recently proposed alternative mechanism for reactions of ChiA [16], Tyr390 was hypothesized as playing a key role in the reaction without considering the anchimeric assistance of the carbonyl oxygen. We believe that the substrate assistance model provides a better explanation of our kinetic results, because it is difficult to account for the substitution effect, that is the drastic decrease in k_{cat}, without presuming that the carbonyl oxygen assists in the formation of the oxazoline-like intermediate. The conserved tyrosine residue (Tyr390 in ChiA), however, must be involved in the catalytic reaction, as evidenced by the drastic decrease in the activity of Y→F mutants [14,16,24]. Most probably, this tyrosine residue assists in the formation and stabilization of the oxazoline-like intermediate, as described by Bokma et al. [14] and Bortone et al. [15].

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