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Mode of action and specificity of a chitinase from unicellular microalgae, *Euglena gracilis*

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Abstract. The unicellular alga, *Euglena gracilis*, produces a chitinase consists of two GH18 catalytic domains (Cat1 and Cat2) and two CBM18 chitin-binding domains (CBD1 and CBD2). Here, we produced a recombinant protein of Cat2 domain, and the mode of action as well as specificity were determined using *N*-acetylglucosamine (A) oligomers (A_n, n = 4, 5, and 6) and partially *N*-acetylated chitosans, which are hetero-polymers composed of A-unit and glucosamine (D) unit, as the substrates. Cat2 hydrolyzed chitin oligosaccharides and partially *N*-acetylated chitosans with a non-processive/endo-splitting mode of action. NMR analysis of the product mixture revealed that the reducing end residues were found to be exclusively A-unit, while both A-unit and D-unit were found at the non-reducing end. This indicated that subsite -1 exclusively binds A-unit, while +1 binds A-unit as well as D-unit. To further analyze the specificities of other subsites, the products were separated by size-exclusion chromatography, and the sugar sequences of the individual products were identified by NMR spectroscopy. Based on the sugar sequences of the products, we found that subsite -2 prefers to bind A-unit but not exclusively while subsite +2 have no preference to A-unit or D-unit. The specificities of Cat2 binding subsites are similar to those of GH18 human chitotriosidase, but significantly differ from those of plant and bacterial GH18 chitinases investigated to date.

Keywords: *Euglena gracilis*, chitinase, specificity, partially *N*-acetylated chitosan

Introduction

Euglena gracilis is a unicellular microalga that is extensively used as a model organism for studying cell biology and biochemistry in laboratories. Biotechnological applications of *E. gracilis* have also been conducted for production of numerous important compounds such as α-tocopherol (vitamin E), paramylon, wax esters, polyunsaturated fatty acids, biotin, and amino acids (Krajčovič et al., 2015; Inui et al, 2017). Therefore, an enormous amount of biochemical data has been accumulated for this microalga. In higher plants and mammals, it is well known that the chitin-mediated defense system plays an important role in their immunity responses (Schlumbaum et al., 1986; Broglie et al., 1991; Brunner et al., 1998; Bueter et al., 2013; Kasprzewska, 2003; Arakane et al., 2012). However, the defense system in *E. gracilis*, which exhibits both plant and animal characteristics, has not yet been described. Since chitinases are major enzymes involved in the chitin-mediated defense system, it is now highly desirable to investigate the structure and function of chitinases from *E. gracilis*. Taira et al. (2018) have recently reported gene cloning, expression, purification, and characterization of a family GH18 chitinase from *E. gracilis* (EgChiA). The enzyme consists of two GH18 catalytic domains (Cat1 and Cat2) and two CBM18 chitin-binding domains (ChBD1 and ChBD2), which are arranged in the order of Cat1, ChBD1, Cat2, ChBD2 from the N-terminus, as shown in Fig. 1. The most interesting aspect of EgChiA is that a transmembrane domain is attached to the C-terminus of the enzyme. EgChiA may play some specific role in chitin-mediated defense system in this microalga.

Chitosans are water-soluble binary polysaccharides composed of *N*-acetyglucosamine (A-unit) and glucosamine (D-unit). Previous investigations reported that the subsite specificities of chitinolytic enzymes could be defined using chitosans as the substrates. (Vårum et al., 1996; Sasaki et al.. 2006; Heggset et al., 2009, 2010, and 2012). The sugar residues on the reducing end, non-reducing end and nearest neighbors

of them were identified by analyzing the NMR spectra of the enzymatic products from the chitosans based on the assignments of the NMR chemical shifts. Subsite specificities toward A-unit or D-unit for individual chitinolytic enzymes were deduced from the chemical compositions and the sugar sequences of the enzymatic products. Among these works, Sasaki et al. (2006) reported the specificities of GH18 and GH19 chitinases from rice, and three contiguous subsites, -2, -1, and +1, in GH19 chitinases were found to be specific to A-unit, while in GH18 enzymes only subsite -1 was specific to A-unit. This suggested that the enzyme targets for GH19 enzymes under physiological conditions are different from those of the GH18 enzymes. A study of the enzyme specificity may provide the needed insights into the physiological functions of the plant chitinases, which is unclear.

Here we focused on one of the catalytic domains from EgChiA, Cat2. The recombinant protein was characterized with respect to its mode of action and the specificity by degrading chitin oligomers and chitosans with varying degree of acetylation (F_A). The generated products were characterized using chromatographic and NMR methods. Here we found that Cat2 hydrolyzes water-soluble partially *N*-acetylated chitosans with a non-processive manner. More Interestingly, Cat2 was found to recognize two A-units at subsites -2 and -1, whereas the other GH18 chitinases investigated to date recognize A-unit only at subsite -1.

Results

Mode of action defined from the chitin oligosaccharide hydrolysis.

The recombinant Cat2 protein was expressed in *Escherichia coli* Rosetta-gami(DE3) cells using the pGEM plasmid containing the Cat2 gene by the method of Taira et al. (2018). The protein was subsequently purified by three-step chromatography using an anion-

exchange, hydrophobic, and gel-filtration columns. The yield of the purified protein was 20.4 mg/L culture. Chitin oligosaccharides, A_4, A_5, and A_6, were hydrolyzed by Cat2, and the time-courses of the enzymatic reaction were followed by HPLC. As shown in Fig. 2, A_2 was predominantly produced from A_4. Small amounts of A_3 and A were also produced from A_4 substrate, and the product A_3 was further hydrolyzed into A+A_2 (Fig. 2A). Equal amounts of A_2 and A_3 were produced from A_5, and A_3 was hydrolyzed into A+A_2 (Fig. 2B). A_6 appeared to be hydrolyzed into A_3+A_3 and A_2+A_4, and the products, A_3 and A_4, were subsequently hydrolyzed into A and A_2 (Fig. 2C). These time-course experiments indicated that Cat2 hydrolyzes the chitin oligomers mostly in an endo-splitting manner, while there was no indication of a processive mode of action for Cat2.

Mode of action defined from the degradation of partially-*N*-acetylated chitosans.

Chitosans with different F_As (0.18, 0.32, 0.48 and 0.66) were thoroughly hydrolyzed, i.e. to a maximal degree of scission (α), by repeatedly adding the Cat2 solution into the reaction mixture until no further increase in α value was observed. The size distribution of degraded chitooligosaccharides (CHOS) mixture was determined by size-exclusion chromatography (SEC), and the extent of degradation (α value) was determined by NMR and calculated according to the equation (1) described in Methods section. The size distribution showed a continuum of the oligomer sizes for all chitosan substrates with various F_As, as shown in Fig. 3. The greater the F_A value of the chitosan substrate, the larger the extent of degradation (α value). This indicated that Cat2 prefers to act toward A-units in the chitosan polysaccharide chains. The size distribution of the enzymatic products was also examined by following the time-course for enzymatic degradation of chitosan with $F_A = 0.66$. As it can be seen from Fig. 4, the size distribution again displays a continuum of the size of the oligomers at all α values. The oligomers with no preference for even- or odd- numbered DP were produced, while the void peak (eluted at ca. 6.9 h) decreases rapidly to be hardly detected at an early stage of degradation,

i.e. α as low as 0.13, indicating that Cat2 hydrolyzes the chitosan with a non-processive/endo-splitting mode of action (Horn et al., 2006). Cat2 was regarded as suitable for defining specificity of the individual subsites from sugar residue identities of the reducing end, non-reducing end, and their nearest neighbors of the enzymatic products.

Sugar residue identity of the enzymatic products deduced from NMR analysis

The ^{1}H-NMR spectra of the CHOS mixtures obtained from incubation of partially *N*-acetylated chitosan (F_A = 0.66) with Cat2 to α values of 0.03, 0.05, 0.13, and 0.34 are shown in Fig. 5. The results obtained for the products with an α = 0.13, the α-anomer signal of the reducing end GlcNAc was found at 5.19 ppm, whereas no signal for the reducing end GlcN (α-form) could be detected (5.43 ppm). The β-anomer signal of the reducing end GlcNAc was found to split into two doublets depending upon the neighboring unit to the reducing end; –A**A** (4.71 ppm) and –D**A** (4.75 ppm), as previously described (Sørbotten, et al., 2005). The ratio between signal intensity for –A**A** compare to –D**A** was higher the product (0.81) than the value of F_A for the chitosan substrate (0.66). This indicated that A-unit is more likely to be found as the nearest neighbor of the reducing end residue than D-unit. Further analysis was performed using ^{13}C-NMR spectra of the enzymatic products as the chemical shift for C-3 and C-5 are sensitive the A and D sequence (Vårum et al., 1996). The spectra in the range between 75.5 and 79.5 ppm of the products obtained from partial degradation (α = 0.1 and 0.2) of chitosan with F_A=0.66 are shown in Fig. 6. The C5 signals of A- and D-units at non-reducing ends were detected at 78.4 and 79.0 ppm, respectively. The intensity of A-unit was higher than that of D-unit, and the intensities of both signals increased with progress of the degradation of chitosan without changing the ratio of the intensities. The intensity ratio was very close to F_A (0.66) of chitosan as the substrate, indicating that A- and D-units are located at the non-reducing end of the CHOS with equal probabilities. The non-reducing end C3 signal of the acetylated unit was found to split into two signals (76.1 and 76.0 ppm) depending

upon the neighboring residue; **A**A– or **A**D–. The ratio of the intensities appeared to reflect F_A (0.66) of chitosan as the substrate, indicating that both A- and D-units are found at the nearest neighbor to the non-reducing end A-unit.

Separation and characterization of individual enzymatic products of chitosan

To confirm the results obtained from the NMR analysis of the enzymatic product mixture, we tried to separate the product mixture obtained after degradation of F_A = 0.66 chitosan to α = 0.13 and α = 0.34 by SEC and characterize the individual fractions by ^{1}H-NMR spectroscopy. A low molecular weight region (DP ≤ 10) of the chromatogram for α = 0.13 is shown in Fig. 7. Individual peak fractions were assigned as indicated in the figure based on the retention times of the authentic standards from A_2 to A_6. Individual fractions shaded by grey were collected and their degree of polymerizations (*DP*) and acetylated fractions (F_A) were calculated based on the anomeric region of ^{1}H-NMR spectra. The calculations were also conducted for the individual degraded fractions of F_A = 0.66 chitosan with α = 0.34 (Fig. 4), and the results are listed in Table 1. At α=0.13, the *DP* values increased in proportion to the fraction number, while the F_A values were almost constant in any fractions tested. In contrast, at α=0.34, the F_A values gradually decreased with increase in the *DP* values. The products at α=0.13 may be regarded as the oligosaccharides derived from the first preferential cleavages, indicating that the data at α=0.13 may provide information on the specificities for more preferable cleavages, while less specific cleavages may be included in the data at α=0.34.

Sugar sequence of individual enzymatic products defined by NMR spectroscopy

Sugar sequences of the products at α = 0.13 were initially analyzed by NMR spectroscopy. ^{1}H-NMR spectrum of the fraction 2 in Fig. 7 exhibited an identical profile to what was reported for mono-*N*-acetylchitobiose, in which the reducing end residue is *N*-acetylated, DA (Fukamizo et al., 1986, 1991). Anomeric proton regions of the ^{1}H-NMR spectra of

the fractions 3 and 4 are shown in Figs. 8A and 8B, respectively. *DP* and F_A of fraction 3 was 3.10 and 0.68, respectively (Table 1), indicating that most trimers in this fraction are composed of one D-unit and two A-units. As shown in Fig. 8A, the relative intensities of two doublets at 4.70 ppm –A**A** (major) and 4.75 ppm –D**A** (minor) suggested that the dominating trimer was identified as DAA with a minor amount of ADA in trimer fraction. Based on the chromatographic peak intensities of fractions 3 and AAA (Fig. 7), the relative amount of AAA to the total amount of trimers was calculated to be 48 %. The remaining 52 % was divided into DAA and ADA based on the relative intensities of the two doublets at 4.70 and 4.75 ppm (Fig. 8A), and the relative amounts of AAA, DAA, and ADA were finally calculated to be 48, 44, and 8 %, respectively (Table 2).

DP and F_A of fraction 4 was 4.11 and 0.71, respectively (Table 1), indicating that most tetramers in this fraction are composed of one D-unit and three A-units; that is, DAAA, ADAA, or AADA. AAAD can be ruled out, because of absolute necessity of *N*-acetyl group at subsite -1 of GH18 enzymes. The F_A value of 0.71 indicated that di-*N*-acetylated tetramers are also included in this fraction but that the amounts of mono-*N*-acetylated tetramers are negligible. From the chromatogram (Fig. 7), relative amounts of fractions 4 and AAAA to the total amount of tetramer products were calculated to be 75 % and 25 %, respectively. The fraction 4 (75 %) was subdivided into tri-*N*-acetylated and di-*N*-acetylated tetramers, based on the F_A value (0.71) as follows,

$$\text{(Relative amount of di-}N\text{-acetylated tetramers)} = 75 \times \frac{(0.75 - 0.71)}{(0.75 - 0.50)}\ (\%)$$

where the 0.75 and 0.50 represent F_As of tri- and di-*N*-acetylated tetramers, respectively. Thus, the relative amounts of tri- and di-*N*-acetylated tetramers were calculated to be 63 % and 12 %, respectively. β-anomer signal of A-unit at the reducing end split into a major doublet, –A**A** (4.70 ppm) and a minor doublet–D**A** (4.75 ppm) as shown in Fig. 8B.

Based on these signal intensities, tri-*N*-acetylated tetramers (63 %) were further subdivided into DAAA+ADAA and AADA, of which the relative amounts were calculated to be 52 % and 11 %, respectively. As summarized in Table 2, the relative amounts of AAAA, DAAA+ADAA, AADA, and DDAA were finally calculated to be 25, 52, 11, and 12 %, respectively, from the F_A values, chromatographic peak intensities (Fig. 7), and the NMR-signal intensities (Fig. 8B). As shown in this NMR spectrum, four doublets were observed at the anomeric proton region of D-units (4.85-4.92 ppm), corresponding to the number of de-*N*-acetylated components in fraction 4.

The products at $\alpha = 0.34$ were analyzed for their sugar sequences in a similar manner to that conducted in the fractions at $\alpha = 0.13$, and the results are presented in Table 2. The distribution for the dimer fractions (AA and DA) at $\alpha = 0.34$ was similar to that at $\alpha = 0.13$. For the trimer fractions, AAA was completely hydrolyzed, and DDA (89 %) was predominant over ADA (11 %). AAAA and DAAA were completely hydrolyzed and missing in tetramer fractions, in which DDAA, ADAA, and AADA were calculated to be 64, 29, and 7 %, respectively. The products, AAA, AAAA, and DAAA, were missing in the products at $\alpha = 0.34$, indicating that the enzymatic hydrolysis was almost completed at this stage.

Overall, only A-unit was found at the reducing end residues of the enzymatic products, while the second residues from the reducing ends were preferentially A-unit but not exclusively. No preferences were observed for the non-reducing end residues and their nearest neighbors.

Discussion

Size distribution of CHOS after extended hydrolysis of various chitosans with Cat2 (Fig. 3) represents the relationship between the F_A of chitosans and the extents of

degradation after complete hydrolysis. The greater F_A value for chitosan substrate, the higher the extent of degradation. Cat2 clearly prefers to hydrolyze the *N*-acetylglucosaminide linkages. It is convincing because chitinases of the GH18 family hydrolyze *N*-acetylglucosaminide linkages through the substrate-assisted mechanism, in which the acetamido group of the A-unit bound to subsite -1 forms an oxazolinium ion intermediate to stabilize the transition state of the catalytic reaction (Tews et al., 1997; van Aalten et al., 2001). As such, *N*-acetyl group of the sugar residue at subsite -1 is essential for the hydrolytic reaction catalyzed by Cat2 belonging to the GH18 family.

Sugar recognition specificity for individual subsites of chitinases can be deduced from the sugar residue identity of the reducing and non-reducing end residues of the enzymatic products. However, this analysis is only suitable for chitinases acting in a non-processive/endo-splitting mode, because most products from the other mode of actions (processive/exo-splitting) complicate the interpretation of the NMR spectra with respect to identification of reducing and non-reducing end residues. The reason is that the composition of degradation products are convolution of the preference and the topological relationship between enzyme and substrate as Sørbotten (Sørbotten et al., 2005) proposed that "catalytically important N-acetyl groups are positioned correctly in every second sugar only" in the case of processive chitinase. Here, we initially examined the mode of action of Cat2 (endo or exo, processive or non-processive) using chitin oligomer A_n (n = 4, 5, and 6) and partially *N*-acetylated chitosan (F_A=0.66) as the substrates. The product distributions obtained from these substrates (Figs. 2 and 4) clearly suggested that Cat2 hydrolyzes chitosan with a non-processive/endo-splitting mode of action. Therefore, Cat2 is suitable for specificity analysis of the individual subsites based on the sugar residue identity of the enzymatic products.

NMR analysis of the enzymatic product mixture (Figs. 5 and 6) indicated that the reducing end residues of the products are exclusively A-unit, while non-reducing end residues are both A- or D-unit. Thus, subsite -1 exclusively prefers A-unit while subsite

+1 display equal preference for both A-unit and D-unit. These findings are consistent with the fact that *N*-acetyl group of the sugar residue is essential for the catalytic action at subsite -1. Further information on the recognition specificity was obtained from the same NMR spectra. The nearest neighbor of the reducing end residues was predominantly A-unit, but a small amount of D-unit was also found at this position (Fig. 5); indicating that subsite -2 prefers to bind A-unit but not absolutely. Both A-unit and D-unit were found at the nearest neighbor of the non-reducing end residues (Fig. 6), indicating that no preference was found at subsite +2. To confirm these findings, we separated the reaction products (Fig. 7), and the sugar sequences were determined by NMR spectroscopy (Fig. 8). The sugar sequence data obtained for the reaction products are summarized in Table 2. The structures of the reaction products are almost consistent with the findings from NMR spectroscopy of the product mixture (Figs. 5 and 6). Thus, we concluded that Cat2 specifically recognizes A-unit at subsites -2 and -1, but subsite -2 is less specific than -1.

As described in Introduction, three contiguous subsites, -2, -1, and +1 of GH19 rice chitinases were found to be specific to A-unit, while in GH18 rice enzymes only subsite -1 was specific to A-unit (Sasaki et al., 2006). Similar studies were also conducted for bacterial GH18 chitinase, SmChiB (Sørbotten et al., 2005) and bacterial GH19 chitinase, ChiG from *Streptomyces coelicolor* A3(2) (Heggset et al., 2009), and the specificities are basically identical to those reported for rice chitinases. On the other hand, the specificities of human chitotriosidase (HCHT) for A-unit were assessed by sugar sequence analysis of enzymatic products, showing a strong, absolute, and a relative weak preference for A-unit at subsites −2, −1, and +1, respectively (Eide et al., 2012). The specificities of Cat2 from *Euglena* chitinase reported in this study are mostly similar to those of human enzyme HCHT. Although Cat2 belongs to the GH18 family, the specificity at subsite -2 of Cat2 is different from those of GH18 rice chitinases and SmChiB, but similar to HCHT belonging to GH18. This may be derived from the *Euglena*'s intermediate characteristics between plants and animals.

A chitinase having two catalytic domains was first found in the hyperthermophilic archaeon *Pyrococcus kodakaraensis* KOD1 (Tanaka et al., 1999). The N-terminal catalytic domain of *Pyrococcus* enzyme is similar to *Bacillus circulans* WL-12 GH18 chitinase A1 (36% identity, 403 amino acids), and the C-terminal catalytic domain has similarity to *Streptomyces erythraeus* GH18 chitinase (30% identity, 283 amino acids). These two catalytic domains have different cleavage specificities, suggesting a synergy between the two domains in chitin degradation (Tanaka et al., 2001). On the other hand, EgChiA has two catalytic modules, Cat1 and Cat2, amino acid sequences of which are very similar to each other; only three substitutions were found outside the consensus regions for GH18 chitinases (Taira et al., 2018). Thus, Cat1 may recognize the GlcNAc residues of the enzyme targets in a similar manner to that of Cat2. However, some differences in enzymatic properties between Cat1 and Cat2 may result from anchoring by a transmembrane domain attached to the C-terminus of Cat2; accordingly, some cooperative effects may exist in two catalytic domains in EgChiA. Further experiments are now under progress to produce and characterize a full-length protein of EgChiA.

Materials and methods

Materials

Chitin oligosaccharides $(GlcNAc)_n$ (n=1-6) were obtained by acid hydrolysis of chitin (Rupley, 1964), and purified by gel filtration on Cellufine GcL-25m (JNC Co., Tokyo). Chitosans (F_A= 0.18, 0.32, 0.48 and 0.66) were prepared by homogeneous de-*N*-acetylation of chitin (Sannan T., 1976). The intrinsic viscosity were determined to be 800, 820, 790 and 843 mL/g, respectively. *E. coli* Rosetta-gami(DE3) cells and pGEM were purchased from Novagen (Madison, WI). Toyopearl Butyl-650 M was from Tosoh (Tokyo,

Japan), while HiTrap Q FF and 16/60 Sephacryl S-100 HR were from GE Healthcare (Tokyo, Japan). All other reagents were of analytic grade and commercially available.

Expression and purification of Cat2 from EgChiA

E. coli Rosetta-gami (DE3) was transformed with the pGEM expression plasmid in which the Cat2 gene was inserted as described in the previous paper (Taira et al. 2018). Transformed *E. coli* cells were inoculated into LB medium containing 50 μg/mL of ampicillin and incubated at 37 ºC in a shaking incubator at 200 rpm overnight. The culture medium was transferred to 1 L medium and incubated at 37 ºC in a shaking incubator at 200 rpm until OD_{600nm} of the cell suspension reached 0.7. Isopropyl-β-D-thiogalactopyranoside (1 mM) was added to induce the expression of the enzyme, and the culture medium was then incubated at 18 ˚C in a shaking incubator at 200 rpm for 2 days. Cell culture was centrifuged at 6,500 rpm for 15 min to harvest the cells, which were resuspended in 20 mM Tris-HCl, pH 7.5 and disrupted with a sonicator. After centrifugation at 12,000 rpm for 15 min, the supernatant was dialyzed against 10 mM sodium acetate, pH 4.0 for overnight, and the precipitate was removed by centrifugation at 12,000 rpm for 15 min. The supernatant containing Cat2 was again dialyzed against 20 mM sodium phosphate, pH 6.0, and applied onto a HiTrap Q FF column (5 mL) equilibrated with the dialysis buffer. After washing the column twice with the same buffer, the proteins adsorbed were eluted with a linear gradient system from 0 to 0.5 M NaCl in the same buffer. Then, ammonium sulfate was added to the protein solution to give 1.0 M, and the resultant solution was applied onto a Toyopearl Butyl-650M column (ca 20 mL) equilibrated with 20 mM sodium phosphate, pH 6.0 containing 1.0 M of ammonium sulfate. After washing with the same buffer containing 0.8 M ammonium sulfate, the protein was eluted with the same buffer without ammonium sulfate. The Cat2 fractions were pooled, and applied onto a HiPrep 16/60 Sephacryl S-100 HR column equilibrated with 20 mM sodium phosphate, pH 6.0 containing 0.1 M NaCl. The column was

isocratically eluted with the same buffer at a flow rate of 0.7 mL/min. Fractions containing Cat2 was collected and the purity was verified by SDS-PAGE (Laemmli, 1970). Protein concentration was determined based on the molar extinction coefficient calculated by the equation proposed by Pace et al. (1995).

HPLC-based reaction time-courses for chitin oligosaccharide degradeation

The reaction products from the chitinase-catalyzed hydrolysis of A_n (n=4, 5, or 6) were quantitatively determined by gel-filtration HPLC (Sasaki et al., 2002 and 2003). The enzymatic reaction was performed in 20 mM sodium acetate buffer, pH 5.0, at 40˚C. Enzyme and substrate concentrations were 0.25 μM and 4.8 mM, respectively. To completely terminate the enzymatic reaction at a given incubation time, a portion of the reaction mixture was mixed with an equal volume of 0.1 M NaOH solution, and immediately frozen in liquid nitrogen. The resultant solution was applied to a gel-filtration column of TSK-GEL G2000PW (Tosoh, Tokyo), and eluted with distilled water at a flow rate of 0.3 mL/min. Oligosaccharides were detected by ultraviolet absorption at 220 nm. Peak areas obtained for individual oligosaccharides were converted to molar concentrations, which were then plotted against reaction time to obtain the reaction time-course.

Chitosan degradation experiments

Chitosan solution (final chitosan concentration, 1 %) dissolved in 50 mM sodium acetate, pH 4.0 containing 0.1 M sodium chloride was pre-incubated in a shaking water bath at 37 ºC for 10 min, and the Cat2 solution was added to give 0.14 μM of the final enzyme concentration. After a given incubation period, hydrochloric acid was added to adjust pH to 2.0 and the reaction mixture was immediately transferred to boiling water for 5 min to completely terminate the reaction followed by adjusting pH of the solution up to pH 4.5.

For a complete digestion, the enzyme solution was repeatedly added until no further increase in the reducing sugars in the reaction mixture was observed. Reducing sugars were determined by the method of Imoto and Yagishita (1971).

Size-exclusion column chromatography (SEC)

Superdex 30 resin (Prep Gradem Amersham Pharmacia Biotech) packed in sequentially connected columns with overall dimensions of 2.60 x 180 cm was used to analyze the product distribution of the enzymatic reaction mixture with continuously eluting 0.15 M ammonium acetate buffer, pH 4.5 with a flow rate of 0.8 mL/min, while refractive index of eluent was continuously recorded to obtain chromatogram. Fractionation was conducted (3.2 mL/tube), and the individual fractions exhibiting satisfactory separation and corresponding to oligomers with *DP* of 2-10 were collected, lyophilized, and characterized by NMR spectroscopy.

NMR spectroscopy

For ^{1}H- and ^{13}C-NMR measurements, individual oligomer fractions were dissolved in D_2O and the pD was adjusted to 4.2 with concentrated DCl or NaOD. NMR spectra were recorded at 355 K for ^{1}H and 300 K for ^{13}C on Bruker AVIII HD 400 MHz equipped with 5mm SmartProbe. For ^{1}H-NMR, the α-anomer resonance of reducing end H-1 of A-unit was set at 5.19 ppm for calibration, and the other resonances were assigned referring to the previous assignments (Vårum et al., 1991, Ishiguro et al., 1992). For ^{13}C-NMR spectra, assignments of enzymatically degraded chitosan (Vårum et al. 1996) were used.

Sugar sequences of enzymatic products from chitosans were determined based on sequence dependent shift of ^{1}H-NMR spectra as is described previously (Sørbotten et al. 2005). To determine the non-reducing end unit which was accommodated in +1 subsite upon hydrolysis, C-5 resonance of non-reducing end sugar unit in ^{13}C-NMR spectrum was used.

Determination of F_A of chitosan

Degree of *N*-acetylation, F_A, was determined based on the ratio of ^{1}H-NMR resonance intensities of *N*-acetylated to de-*N*-acetylated unit as is described elsewhere (Vårum *et al*. 1991).

Determination of extent of degradation, α

Degree of degradation, α, was calculated based on the following equation,

$$\alpha = \frac{A_{\text{H-1}}}{A_{\text{REH-1}}} = \frac{1}{DP_{\text{n}}} \qquad (1)$$

where $A_{\text{H-1}}$ and $A_{\text{REH-1}}$ represent the total area of H-1 resonances and area of reducing end H-1 resonances, respectively, and were determined based on ^{1}H-NMR spectra of chitosans or the degradation product mixture as described before (Sørbotten et al. 2005).

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References

Arakane, Y., Taira, T., Ohnuma, T., Fukamizo, T. (2012) Chitin-related enzymes in agro-biosciences. *Curr. Drug Targets*, 13, 442-470

Broglie, K., Chet, I., Holliday, M., Cressman, R., Biddle, P., Knowlton, S., Mauvais, C.J., and Broglie, R. (1991) Transgenic plants with enhanced resistance to the fungal pathogen *Rizoctonia solani*. *Science*, 254,1194-1197.

Brunner, F., Stintzi, A., Fritig, B., and Legrand M. (1998) Substrate specificities of tobacco chitinases. *Plant J.*, 14, 225-234.

Bueter, C.L., Specht, C.A., Levitz, S.M., (2013) Innate sensing of chitin and chitosan, *PLoS Pathog*., 9, e1003080.

Eide KB, Norberg AL, Heggset EB, Lindbom AR, Vårum KM, Eijsink VG, Sørlie M. (2012) Human chitotriosidase-catalyzed hydrolysis of chitosan. *Biochemistry*. 51, 487-495.

Fukamizo T, Minematsu T, Yanase Y, Hayashi K, Goto S. (1986) Substrate size dependence of lysozyme-catalyzed reaction. *Arch Biochem Biophys.* 250, 312-321.

Fukamizo T, Ohtakara A, Mitsutomi M, Goto S. (1991) NMR spectra of partially deacetylated chitotrisaccharides. *Agric Biol Chem.* 55, 2653-2655.

Heggset EB, Hoell IA, Kristoffersen M, Eijsink VG, Vårum KM. (2009) Degradation of chitosans with chitinase G from Streptomyces coelicolor A3(2): production of chito-oligosaccharides and insight into subsite specificities. *Biomacromolecules*. 10, 892-899.

Heggset EB, Dybvik AI, Hoell IA, Norberg AL, Sørlie M, Eijsink VG, Vårum KM. (2010) Degradation of chitosans with a family 46 chitosanase from Streptomyces coelicolor A3(2). *Biomacromolecules.* 11, 2487-2497.

Heggset EB, Tuveng TR, Hoell IA, Liu Z, Eijsink VG, Vårum KM. (2012) Mode of action of a family 75 chitosanase from Streptomyces avermitilis. *Biomacromolecules.* 13, 1733-1741.

Horn, S.J., Sørbotten, A., Synstad, B., Sikorski, P., Sorlie, M., Vårum, K.M., and Eijsink, V.G. (2006) Endo/exo mechanism and processivity of family 18 chitinases produced by *Serratia marcescens*. *FEBS J.*, 273, 491-503.

Imoto T, Yagishita K (1971) A Simple Activity Measurement of Lysozyme. Agric. Biol. Chem., 35,1154~1156.

Inui H, Ishikawa T, Tamoi M. (2017) Wax ester fermentation and its application for biofuel production. in *Euglena: Biochemistry, Cell and Molecular Biology* (Ed. by Schwartzbach S, Shigeoka S.) Springer International Publishing, pp. 269-283.

Ishiguro K, Yoshie N, Sakurai M, Inoue Y. (1992) A 1H NMR study of a fragment of partially n-acetylated chitin produced by lysozyme degradation. *Carbohydr Res.* 237, 333-338.

Kasprzewska, A. (2003) Plant chitinases—Regulation and function. *Cell. Mol. Biol. Lett.*, 8, 809-824.

Krajčovič J, Matej Vesteg, Schwartzbach SD. (2015) Euglenoid flagellates: a multifaceted biotechnology platform. *J Biotechnol.* 202, 135-145.

Laemmli, UK. (1970) Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature* 227, 680–685.

Pace, C.N., Vajdos, F., Fee, L., Grimsley, G., and Gray, T. (1995) How to measure and predict the molar absorption coefficient of a protein. *Protein Sci.*, 4, 2411-2423.

Rupley JA, (1964) The hydrolysis of chitin by concentrated hydrochrolic acid, and the preparation of low-molecular-weight substrates for lysozyme. *Biochim Biophys Acta* 83, 245-255.

Sannan, T., Kurita, K., and Iwakura, Y. (1976) Study on chitin 2, Effect of deacetylation on solubility. *Makromol.Chem.*, 177, 3589-3600.

Sasaki, C., Yokoyama, A., Itoh, Y., Hashimoto, M., Watanabe, T., and Fukamizo, T. (2002) Comparative study of the reaction mechanism of family 18 chitinases from plants and microbes. *J. Biochem.*, 131, 557-564.

1 Sasaki, C., Itoh, Y., Takehara, H., Kuhara, S., and Fukamizo T. (2003) Family 19 chitinase
2 from rice (*Oryza sativa* L.): substrate-binding subsites demonstrated by kinetic and
3 molecular modeling studies. *Plant Mol. Biol.*, 52, 43-52.
4 Sasaki C, Vårum KM, Itoh Y, Tamoi M, Fukamizo T. (2006) Rice chitinases: sugar
5 recognition specificities of the individual subsites. *Glycobiology.* 16, 1242-1250.
6 Schlumbaum A, Mauch, F, Vögeli, U, Boller T. (1986) Plant chitinases are potent
7 inhibitors of fungal growth. *Nature* 324, 365–367.
8 Sørbotten, A., Horn, S.J., Eijsink, V.G.H., and Vårum, K.M. (2005) Degradation of
9 chitosans with chitinase B from *Serratia marcescens*. Production of chito-
10 oligosaccharides and insight into enzyme processivity. *FEBS J.*, 272, 538-549.
11 Taira T, Gushiken C, Sugata K, Ohnuma T, Fukamizo T. (2018) Unique GH18 chitinase
12 from *Euglena gracilis*: Full-length cDNA cloning and characterization of its catalytic
13 domain. Biosi. Biotechnol. Biochem. in press
14 Tanaka T, Fujiwara S, Nishikori S, Fukui T, Takagi M, Imanaka T. (1999) A
15 unique chitinase with dual active sites and triple substrate binding sites from the
16 hyperthermophilic archaeon Pyrococcus kodakaraensis KOD1. *Appl Environ*
17 *Microbiol.* 65, 5338-5344.
18 Tanaka T, Fukui T, Imanaka T. (2001) Different cleavage specificities of the dual catalytic
19 domains in chitinase from the hyperthermophilic archaeon Thermococcus
20 kodakaraensis KOD1. *J Biol Chem.* 276, 35629-35635.
21 Tews, I., van Scheltinga A.C.T., Perrakis, A., Wilson, K.S., and Dijkstra, B.W. (1997)
22 Substrate-assisted catalysis unifies two families of chitinolytic enzymes. *J. Am. Chem.*
23 *Soc.* 119, 7954-7959.
24 van Aalten DM, Komander D, Synstad B, Gåseidnes S, Peter MG, Eijsink VG. (2001)
25 Structural insights into the catalytic mechanism of a family 18 exo-chitinase. *Proc*
26 *Natl Acad Sci U S A.* 98, 8979-8984.

Vårum K.M., Anthonsen, M., Grasdalen, H., and Smidsrød, O. (1991) Determination of the degree of *N*-acetylation and the distribution of *N*-acetyl group in partially *N*-acetylated chitins (chitosans) by high-field n.m.r. spectroscopy. *Carbohydr. Res.*, 211, 17-23.

Vårum, K.M., Holme, H.K., Izume, M., Stokke, B.T., and Smidsrod, O. (1996) Determination of enzymatic hydrolysis specificity of partially *N*-acetylated chitosans. *Biochim Biophys Acta.*, 1291, 5-15.

Figure legends

Fig. 1. Schematic representation of the domain organization of EgChiA. EgChiA is composed of two GH18 catalytic domains (Cat1 and Cat2) and two CBM18 chitin-binding domains (CBD1 and CBD2), which are alternatingly linked with each other. A transmembrane domain (TM) is connected to the C-terminus of EgChiA.

Fig. 2. HPLC-based reaction time-courses for degradation of $(GlcNAc)_4$ (**A**), $(GlcNAc)_5$ (**B**), and $(GlcNAc)_6$ (**C**).. The enzymatic reaction was performed in 20 mM sodium acetate buffer, pH 5.0, at 40˚C. Enzyme and substrate concentrations were 0.25 μM and 4.8 mM, respectively. A portion of the reaction mixture was applied to a gel-filtration column of TSK-GEL G2000PW (Tosoh, Tokyo), and eluted with distilled water at a flow rate of 0.3 ml/min. Oligosaccharides were detected by ultraviolet absorption at 220 nm.

Fig. 3. Size-exclusion chromatograms (SEC) of the enzymatic products from complete digestion of chitosans with various degrees of acetylation (F_A). The effluent was monitored by a refractive index detector. Corresponding F_A-values and the fraction of scission (α-value) are indicated at the upper left of each chromatogram. Peaks are labeled with *DP*–values or corresponding sequences.

Fig. 4. Size-exclusion chromatograms (SEC) of enzymatic products from chitosan (F_A = 0.66) at different degrees of Cat2 degradation (α). The α values determined based on the equation (1) from ^{1}H-NMR spectra were calculated to be 0.03 to 0.34.

Fig. 5. Anomeric proton region of the ^{1}H-NMR spectra of the enzymatic products from chitosan (F_A = 0.66) at α = 0.03-0.34. The resonance of α-anomer of reducing end A-

units was found at 5.19 ppm and those of the β-anomer were at 4.70 - 4.76 ppm. Two-doublet resonances of the latter are derived from the difference in the sugar identity (A or D) at the nearest neighbor of the reducing end residue. The resonances of anomeric protons of A- and D-units except the reducing ends resonate were observed at 4.58-4.66 and 4.86-4.93 ppm, respectively.

Fig. 6. C-5 and C-3 regions of the ^{13}C-NMR spectra of enzymatic products from chitosan (F_A = 0.66) at α = 0.1 and 0.2. C-5 resonances of A- and D-units at the non-reducing end were found at 78.4 and 79.0 ppm, respectively, and internal C-5 resonances were at 76.9 - 77.7 ppm. The resonances at 76.0-76.2 were derived from C-3 of the non-reducing end A-unit.

Fig. 7. Low-molecular weight region of SEC of the enzymatic products from the chitosan (F_A = 0.66) at α=0.13. Fractions collected are shaded by grey. Numbers (2-10) labeled for individual fractions represent the estimated DP_n. A, A_2, A_3, and A_4 correspond to fully *N*-acetylated oligomers.

Fig. 8. Anomeric proton region of the ^{1}H-NMR spectra of fraction 3 (**A**) and fraction 4 (**B**) shown in Fig. 7. The assignments are the same as in Fig. 5.

Table 1. DP_n and F_A of chitooligomers separated from degraded chitosan (F_A = 0.66) at different extents of degradation.

Fractions		3	4	5	6	7	8	9	10
α=0.13	DP_n	3.10	4.11	5.20	5.94	6.94	8.07	9.05	9.93
	F_A	0.68	0.71	0.74	0.73	0.70	0.69	0.67	0.64
α=0.34	DP_n	3.35	4.45	5.45	6.61	7.75	8.36	9.43	N/D
	F_A	0.66	0.59	0.51	0.47	0.44	0.38	0.40	N/D

Table 2. Sugar sequences of enzymatic products from partially *N*-acetylated chitosan ($F_A = 0.66$).

Extent of degradation	Dimer fractions ($2 + A_2$)	Trimer fractions ($3 + A_3$)	Tetramer fractions ($4 + A_4$)
$\alpha = 0.13$	73 % AA	48 % AAA	52 % ADAA
			DAAA
	27 % DA	44 % DAA	25 % AAAA
		8 % ADA	12 % DDAA
			11 % AADA
$\alpha = 0.34$	72 % AA	89 % DAA	64 % DDAA
	28 % DA	11 % ADA	29 % ADAA
			7 % AADA