Molecular directionality in crystalline β -chitin: hydrolysis by chitinases A and B from *Serratia marcescens* 2170

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β -Chitin microfibrils were treated with ChiA and ChiB (chitinases A and B respectively) from *Serratia marcescens* 2170. The β -chitin microfibrils were shortened, and the tips appeared narrowed and sharpened at both ends, after either consecutive or simultaneous degradation by ChiA and ChiB. Increased production of reducing sugars by simultaneous degradation (by ChiA and ChiB) of β -chitin, but not of glycol chitin, suggests synergistic interactions between the two enzymes. A combined analysis using the tilt microdiffraction method to determine the crystallographic axes, together with the biotin–streptavidin–gold–labelling method

specific to the reducing ends, was used to investigate the polarity of the degraded β -chitin microcrystals. The digestion of the β -chitin fibrils by ChiA occurred from the reducing end to the non-reducing end, whereas digestion by ChiB occurred from the non-reducing end to the reducing end. The results are in agreement with the previously determined three-dimensional structures of these enzymes.

Key words: β -chitin, chitinase a, chitinase B, reducing-end labelling, *Serratia marcescens*.

INTRODUCTION

Chitinases are a widespread group of enzymes which degrade chitin, a linear polysaccharide consisting of β (1–4)-linked *N*-acetylglucosamine residues. Chitinases belong to families 18 and 19 in the sequence-based classification system of glycoside hydrolases [1–3]. ChiA and ChiB (chitinases A and B respectively) from *Serratia marcescens* 2170 belong to family 18. The carbohydrate-binding and catalytic modules of both enzymes seem to generate a continuous substrate-binding site formed by a surface of aromatic residues, which guide the substrate into the enzyme's active site. The topologies of these unusual extended binding sites apparently lead to opposite chain-end specificities for the two enzymes [4–7]. This prompted us to investigate ChiA and ChiB chain-end preferences for β -chitin crystals.

Recently, a labelling technique that introduces biotin–streptavidin–gold tags to the reducing ends of polysaccharides, in combination with TEM (transmission electron microscopy), verified experimentally that degradation by ChiA1 (*Bacillus circulans* chitinase A1) proceeds from the reducing ends [8]. The result was in agreement with the enzyme structure [4,9], which provides a binding platform promoting hydrolysis from the reducing end. Since ChiA and ChiA1 have similar overall structures [4,9], such a mode of action is likely to be observed in ChiA.

In the present study, we have investigated the degradation pattern of β -chitin microcrystals by ChiA and ChiB in order to determine the molecular directionality of their digestion. In order to show whether the digestion of ChiA occurs from the reducing end, and whether the digestion of ChiB occurs from the non-reducing end, we have used the biotin–streptavidin–gold–labelling technique in combination with TEM and a series of tilting diffraction diagrams.

MATERIALS AND METHODS

Substrate

Tubes of vestimentiferan (*Lamellibrachia satsuma*) were collected in Kagoshima Bay, Kyushu, Japan. The tubes were soaked

in 5% (w/v) KOH at room temperature overnight, and then placed in 0.3% (w/v) NaClO₂ solution buffered at pH 4.9 in an acetate buffer at 70 °C for 4 h. This procedure was repeated twice. The purified tubes were hydrolysed at 100 °C in 2.5 M HCl for 4 h. The resultant microcrystalline suspension was neutralized by several centrifugal washes (18 800 g for 15 min) in distilled water, and finally dialysed overnight against distilled water. Glycol chitin was prepared by the method of Yamada and Imoto [10].

Production and purification of ChiA and ChiB

Both recombinant ChiA and ChiB from *S. marcescens* 2170 were produced in *Escherichia coli* cells harbouring plasmids pNCA112 and pMCB7, carrying the cloned *chiA* and *chiB* genes respectively. The recombinant enzymes were purified from the culture supernatant as described previously [9,11].

Enzymatic treatment of *L. satsuma* β -chitin for electron microscopy

Hydrolysis was performed using 0.5 mg/ml β -chitin and 1 mg/ml of ChiA or ChiB in 20 mM phosphate buffer, pH 6.0, at 37 °C for 45 h or 65 h respectively. These conditions were used both for enzyme treatment with ChiA or ChiB individually, and for treatment with the enzymes consecutively, where treatment with ChiA was followed by that with ChiB. For simultaneous treatment, β -chitin was treated with a mixture of ChiA (0.17 mg/ml) and ChiB (0.26 mg/ml) in 20 mM phosphate buffer, pH 6.0, at 37 °C for 16 h; the lower enzyme concentrations used and the reduced period of incubation were due to faster digestion in comparison with the other treatments.

At the end of each incubation period, the suspension was centrifuged (18 800 g for 15 min), and the digestion was terminated by washing with 0.2% (v/v) NaOH in order to denature the enzyme. The samples were then washed thoroughly by repeated centrifugation with distilled water, and subjected to reducing-end labelling where applicable (see below).

Abbreviations used: BXH-SG, biotinamidocaproyl hydrazide–streptavidin–gold; ChiA/B, chitinases A and B respectively; ChiA1, *Bacillus circulans* chitinase A1; TEM, transmission electron microscopy; 3D, three-dimensional.

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Figure 1 Crystallographic 2D structures of ChiA 1EHN (top) and ChiB 1E6N (bottom), showing the catalytic domain co-crystallized with an oligosaccharide and the position of the aromatic residues

Solubilization of β -chitin by ChiA and ChiB

The extent of degradation of *L. satsuma* β -chitin was deduced from the amount of soluble reducing sugars in the supernatant after centrifugation (20 000 *g* for 10 min) of the digestion mixture, following a modification of Schales' procedure [12]. For synergistic degradation, assays were performed by mixing the two chitinases on the basis of their activities. Each chitinase showed a linear relationship between the amount of enzyme used and the reducing sugars released, up to 0.2 μ mol of reducing sugars: therefore enzyme mixtures with various ChiA:ChiB ratios were adjusted to produce 0.2 μ mol of reducing sugars, unless synergistic effects were observed.

Assays were performed with 20 mM phosphate buffer, pH 6.0, at 37 °C for 30 min. The ChiA/ChiB mixture was added to 1 mg of crystalline *L. satsuma* β -chitin and soluble glycol chitin [13] respectively.

Labelling method

BXH-SG (biotinamidocaproyl hydrazide–streptavidin–gold) labelling specific to the reducing ends of polysaccharide chains was carried out as described by Imai et al. [8].

Electron microscopy

The samples were deposited on to carbon-coated grids, and allowed to dry. All the micrographs were taken with a Jeol 2000EXII electron microscope operated at 100 kV and recorded on Mitsubishi electron-microscope film. The images were taken at magnifications of $\times 2500$.

The diffraction diagrams were obtained in microdiffraction mode. A small condenser aperture of 20 μ m was inserted, and the

first condenser lens was fully overfocused to achieve an electron probe of approx. 100 nm. The samples were observed at 2500 $\times$ under extremely low dose conditions using an image intensifier (Fiber Optics Coupled TV; Gatan, Pleasanton, CA, U.S.A.). When a microcrystal was found, it was rotated to align with respect to the tilt axis by using a rotation-tilt holder (SRH holder; Jeol, Akishima, Japan). Diffraction patterns were recorded from the microcrystals with two different tilting procedures, which are explained in the Results and Discussion section below.

RESULTS AND DISCUSSION

Comparison of the 3D (three-dimensional) structures of ChiA and ChiB

The 3D structures of ChiA and ChiB with their active sites are shown in Figure 1. *S. marcescens* ChiA comprises three domains: an N-terminal domain, a catalytic $(\beta/\alpha)_8$ barrel domain and a small $(\alpha + \beta)$ domain, which is inserted into the $(\beta/\alpha)_8$ barrel domain. *S. marcescens* ChiB comprises a catalytic $(\beta/\alpha)_8$ barrel domain and a C-terminal putative chitin-binding domain. The positioning of the carbohydrate-binding modules relative to the enzyme active site is different in ChiA and ChiB (Figure 1). The support loop and the chitin-binding domain extend the substrate-binding cleft of ChiB on the reducing side of the active site, whereas the chitin-binding domain of ChiA extends the substrate-binding cleft at the non-reducing side, suggesting opposite chain-end specificities for the β -chitin chain [5]. This is illustrated in Figure 1, where the co-crystallized chito-oligosaccharide is inserted into the catalytic cleft either from the reducing end when digested by ChiA, or from the non-reducing end when digested by ChiB.

Figure 2 Bright-field diffraction contrast micrographs of *L. satsuma* β -chitin crystals

Micrographs are shown (a) after treatment with ChiA and (b) after treatment with ChiB. Black arrows show the tapered ends, and the white arrows show the non-degraded end.

Asymmetrical pattern of degradation on β -chitin microcrystals

The β -chitin microfibrils isolated from the protective tubes of *L. satsuma* are highly crystalline, as demonstrated by electron microdiffraction analysis [14,15]. The microfibrils were treated with ChiA (Figure 2a) or ChiB (Figure 2b) individually, or with both enzymes either consecutively (Figure 3a) or simultaneously (Figure 3b), and the morphological changes were monitored by TEM. The initial β -chitin microfibrils exhibited a well-defined and uniform shape; they were more than several micrometers in length, and 30–45 nm in width [15]. The length of the microcrystals apparently became shorter after both enzyme treatments.

The morphology of the microfibrils treated with ChiA showed a tapered end, which has been demonstrated on degradation by *B. circulans* WL-12 ChiA1 [9], and is shown in Figure 2(a). After treatment with ChiB, again the tips of the microfibrils were narrowed and sharpened at one end (Figure 2b). The hydrolysis of β -chitin microfibrils by both ChiA and ChiB occurs in a unidirectional manner, which substantiates further the view that the chains are parallel in β -chitin [8,14–16].

As predicted from the protein crystallography data [4–6], ChiA and ChiB degrade the β -chitin microcrystals from opposite ends, i.e. from the reducing and non-reducing ends respectively. This is illustrated in Figure 3, where the microfibrils were shortened and the tips appeared narrowed and sharpened on both ends after treating β -chitin either with ChiA followed by ChiB (Figure 3a) or with both enzymes simultaneously (Figure 3b).

The synergy between ChiA and ChiB is illustrated in Figure 4, which corresponds to an assay where a constant activity of protein per 1 mg of substrate was used, i.e. 0.2 μ mol of reducing sugar production per mg of chitin substrate. By comparing the two chitin substrates tested, the synergistic effect was only found with crystalline β -chitin degradation, and not at all with soluble glycol chitin degradation. The optimum synergistic condition was found at a ratio of ChiA to ChiB of 40 % to 60 %, which corresponded to a ChiA:ChiB ratio of 1:2 in terms of molar protein concentration.

Figure 3 β -Chitin microcrystals degraded by ChiA and ChiB (a) consecutively and (b) synergistically

The arrows show typical morphological features of the double enzymatic attack.

Figure 4 Synergistic degradation of crystalline β -chitin and soluble glycol chitin by various ratios of ChiA and ChiB at a constant total activity load to 1 mg of substrate

The enzyme mixture was not on the basis of the amount of enzyme in the assay, but on the enzyme activity. The enzymes were mixed in such a way that the sum of the activity of each enzyme corresponds to the production of 0.2 μ mol of reducing sugars.

The opposite directionality of these enzymes in relation to crystalline β -chitin digestion is clearly demonstrated from the microscopic study. However, we could not visualize the actual co-ordination of these enzymes to explain the synergistic effect, such as the pattern degrading perpendicularly to the length of the microcrystals, as observed in cellulose degradation by cellobiohydrolase I (CBH I; Cel7A) [17]. A rational explanation for the synergy between ChiA and ChiB could be the endo-activity of one of these enzymes, which has been suggested to exist for ChiA only [6]. The microscopic experiments here have so far failed to visualize the generation of new ends in the middle of the chitin crystals by either enzyme, and technical improvements may be required to achieve the demonstration of such (presumably rare) events.

Molecular directionality of ChiA and ChiB degradation

Figures 5(a) and 5(b) present strategies to determine the crystallographic c -axis of a given β -chitin microfibril by taking microdiffraction patterns with a precise tilt. We have tested two independent ways that have been reported previously: a rotational tilt about the long axis (c -axis) of the β -chitin microcrystals [15], as shown in Figure 5(a), and a tilt perpendicular to the c -axis [14], as shown in Figure 5(b).

The former method was applied to observe the directionality of the tapering brought about by ChiA degradation. A microcrystal showing clearly a tapering effect was first selected, and rotated in the microscope to adjust the microcrystal axis parallel to the tilt axis. When the $[1\bar{1}0]$ pattern (the pattern taken by the incident electron beam parallel to the $[1\bar{1}0]$ zone axis) is observed and the c -axis is upward as in Figure 5(a), the $[100]$ pattern can be taken after a clockwise rotation, whereas the $[010]$ pattern appears after an anticlockwise rotation, thus allowing us to determine the directionality of the c -axis.

The latter method was applied to observe the directionality of the tapering by ChiB degradation. Again, the microcrystal showing clear tapering was selected, but rotated in the microscope to adjust the microcrystal axis perpendicular to the tilt axis. When the $[1\bar{2}0]$ pattern is observed and the c -axis is upward, as in Figure 5(b), the (013) spots on the third layer line appear on the first and third quadrant after backward tilt (left in Figure 5b), while the corresponding spots ($0\bar{1}3$) appear on the second and fourth quadrant after forward tilt (right in Figure 5b), and therefore we are able to determine the directionality of the c -axis.

Given this background, a series of tilting diffraction experiments was carried out. The results obtained for ChiA are shown in Figure 6(a). A microcrystal showing equatorial (110) spots (the pattern in the centre panel) was tilted around its long axis, and the diagrams after 55° anticlockwise (bottom) and 27° clockwise (top) rotations were recorded respectively. This situation is just the opposite as that depicted in Figure 5, and accordingly the directionality of the c -axis was determined: the c -axis points downwards, and therefore the tapered end in this case is assigned as the reducing end. The result is identical with that which we reported previously for ChiA1 from *B. circulans* [14].

A typical example of the tilt-diffraction diagrams of ChiB-treated microfibrils is shown in Figure 6(b). A microcrystal showing equatorial (120) spots was tilted perpendicularly to its long axis, and the diagram after 19° in the plus (backward) direction was recorded. On the basis of theoretical considerations in Figure 5(b), it is possible to determine the directionality of the c -axis in such a way that the (013) spots must appear on the first and third quadrant if the c -axis points downwards, which was true

Figure 5 Schematic representation of two tilt microdiffraction experiments used in the present study

(a) A rotation about the c -axis [15] is shown. At a given tilt angle, one can obtain a characteristic diffraction pattern from a single β -chitin crystal. Three of these typical patterns are shown. From the left, the patterns taken by the incident electron beam parallel to the zone axis $[010]$, $[1\bar{1}0]$ and $[100]$ are shown, which in between rotational angle differences are 27° ($[010]$ to $[1\bar{1}0]$) and 55° ($[1\bar{1}0]$ to $[100]$) respectively. By monitoring the change of patterns and the rotatory direction, the directionality of the c -axis can be determined. (b) A tilt perpendicular to the c -axis [14] is shown. By tilting a crystal giving equatorial (120) diffraction, one can bring (013) planes to be diffracted on the third layer line. Since the position of the (013) spot is clearly off from the meridional (vertical) line, one can determine if it appears in the first and third quadrants or the second and fourth quadrants. Depending on the position of the (013) diffraction together with the tilting direction, the directionality of the c -axis can be determined.

in this case. Therefore the tapered end in this case was assigned as the non-reducing end.

In order to confirm the results obtained by the diffraction technique, the chain-end preferences of ChiA and ChiB were investigated by TEM and a method which labels the reducing ends of the β -chitin microcrystal. By introducing a hydrazide derivative of biotin at the reducing end of a chitin chain, followed by specific interaction between biotin and streptavidin coupled with a colloidal gold particle (i.e. the BXH-SG technique [8]), the chain-end specificities of ChiA and ChiB may be visualized using TEM [9]. When applying the BXH-SG technique, gold particles appeared at the tapered end in the case where β -chitin was degraded by ChiA, and at the intact end of the β -chitin microcrystals when degraded by ChiB, as can be seen in Figure 7.

Figure 6 Typical tilt-microdiffraction experiment on the β -chitin microcrystal hydrolysed by ChiA (a) or ChiB (b)

(a) A typical tilt microdiffraction experiment performed on the β -chitin microcrystal hydrolysed by ChiA is shown. The diagram was recorded at various tilt angles ($+38^\circ$, $+10^\circ$ or -45° , as shown in the Figure insets). The fibre direction is vertical, and the crystals were rotated about the fibre axis. The plus tilting corresponds to the clockwise rotation. As shown in Figure 5(a), the c -axis is determined to point downwards. (b) A typical tilt microdiffraction experiment on the β -chitin microcrystal hydrolysed by ChiB is shown. The diagram was recorded without tilt (inset a) and after tilting (inset b). The plus tilting allows the tapered tip to move downwards from the observer, while the minus tilt brings it up to the observer. In both diagrams, the fibre direction is vertical. The (120) spot is the equatorial diffraction in this case, meaning that the chitin microfibril is tilted around the $a^* + 2b^*$ vector. By tilting the crystal by approx. $+19^\circ$, the diffraction spots (013) appear on the first and third quadrant; thus the c -axis is determined to point upwards.

Although the labelling frequency was not excellent, it was consistent, and the data obtained thus provide the first experimental demonstration that ChiA from *S. marcescens* degrades β -chitin from the reducing end to the non-reducing end (Figure 7a), whereas ChiB degrades β -chitin from the non-reducing end to the reducing end (Figure 7b). These results are in agreement with the 3D structures of the different chitinases [4–7].

Figure 7 β -Chitin microcrystals degraded by ChiA (a) and ChiB (b), and subsequently labelled by the BXH-SG method

Gold particles are observed at the tapered end in the case where β -chitin was degraded by ChiA (a; inset), and at the intact end in the case where β -chitin was degraded by ChiB (b; inset). [The encircled areas of (a) and (b) are shown magnified in the insets for the respective Figure panels.] The black arrows show the reducing ends, whereas the white arrows show the non-reducing ends.

Conclusion

By a combined analysis using a tilt microdiffraction method to determine the crystallographic axes and the BXH-SG labelling method specific to the reducing ends of the enzymatically degraded β -chitin microcrystals, it was possible to experimentally demonstrate the chain end specificities for ChiA and ChiB from *S. marcescens*. ChiA degrades β -chitin microfibrils from the reducing end to the non-reducing end, whereas Chi B degrades β -chitin microfibrils from the non-reducing end to the reducing end.

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