Directional degradation of β-chitin by chitinase A1 revealed by a novel reducing end labelling technique

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Abstract A novel procedure for labelling the molecular ends of β-chitin crystals has been established. By introducing a hydrazide derivative of biotin at the reducing end of a chitin chain, followed by a specific interaction between biotin and streptavidin coupled with a colloidal gold particle, the chain directionality of β-chitin microcrystals could be directly visualized by transmission electron microscopy. This method allowed to certify the parallelism of the chitin chains in the β-chitin microcrystals, and also to label the reducing tips of β-chitin microcrystals degraded by *Bacillus circulans* chitinase A1. With these substrates, the labelling occurred only at their tapered tip, which indicates that the digestion of these crystals proceeded from their reducing end. The generalization of this new labelling method to other polysaccharide crystals is discussed. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: β-Chitin; Biotin–streptavidin interaction; Chitinase A1; *Bacillus circulans*; *Lamellibrachia satsuma*

1. Introduction

Chitinase A1 – a retaining chitinase of family 18 – is a major chitinase produced by *Bacillus circulans* WL, which is responsible for liberating primarily chitobiose residues upon degradation of crystalline chitin [1]. This enzyme is recognized as 'processive' in the sense that it catalyzes the cleavage of several consecutive bonds without dissociating from the substrate. A biochemical analysis using soluble chitodextrins labelled at their reducing ends by *p*-nitro-phenol residues has indicated that the enzyme was attacking at the non-reducing ends of these soluble substrates. This degradation scheme seemed substantiated by the peculiar morphology of chitinase A1-treated β-chitin microcrystals, where an unidirectional tapering of the substrates was clearly visualized by electron microscopy [2].

A recent three-dimensional (3D) structure of chitinase, together with that of several mutated enzymes co-crystallized with substrate analogues has shown unambiguously that this enzyme had directional preference for the reducing ends of the substrates and not for the non-reducing ends proposed earlier [3]. This discrepancy prompted us to check further the degradation pattern of β-chitin crystals by chitinase A1 in order to ascertain the molecular directionality of their digestion.

A transmission electron microscopy (TEM) method for staining with silver the reducing ends of polysaccharide crystals has been developed and applied to cellulose microcrystals [4,5]. This method has not only confirmed the parallel packing of the cellulose chains in the crystal lattice of cellulose I, but also revealed the directional degradation of cellobiohydrolases Cel7A and Cel6A [6,7]. With the silver labelling, we could prove that Cel7A attacked cellulose crystals from their reducing ends whereas Cel6A started the digestion of the crystals from their non-reducing ends. Unfortunately, the silver labelling technique that works well with cellulose is not operative on chitin crystals. It seems that this lack of reactivity is due to the presence of the *N*-acetyl moiety at the C2 of the glycosyl residues of chitin. Thus, for chitin, there is a need for a new method that would stain the chain ends and therefore reveal the directionality of the action of chitinases. In this work, we address this problem by describing a new reducing end labelling technique designed to analyze by TEM the tapering of β-chitin microcrystals after chitinase A1 treatment and to decide whether the digestion occurred at the reducing or non-reducing ends of chitin chains in their crystalline environment.

2. Materials and methods

2.1. Microcrystalline β-chitin sample

Tubes of vestimentiferan, *Lamellibrachia satsuma*, were collected in Kagoshima Bay, Kyushu, Japan [8]. Formaldehyde-fixed tubes were purified by two cycles of alkali and bleaching treatments. The samples were soaked in 5% (w/v) KOH at room temperature overnight, and then exchanged at 70°C for 4 h in 0.3% (w/v) $NaClO_2$ buffered at pH 4.9 in acetate buffer. Purified tubes were hydrolyzed by boiling in 2.5 M HCl under continuous strong stirring for 4 h. The resultant microcrystalline suspension was neutralized by several centrifugal washes with distilled water and finally dialyzed overnight against distilled water.

2.2. Degradation of β-chitin microcrystals by chitinase A1

Chitinase A1 is the major enzyme in the *B. circulans* chitinase system. The enzyme was purified from the periplasmic proteins produced by *Escherichia coli* harboring recombinant plasmids [9].

A part of the suspensions of microcrystalline β-chitin was degraded by this enzyme. The hydrolysis was achieved with 1 mg/ml of β-chitin and 1 mg/ml of enzyme in 20 mM phosphate buffer (pH 6.0) at 37°C for 3 h. At the end of this incubation period, the suspension was centrifuged and the digestion was terminated by washing with 0.2% NaOH in order to denature the enzyme. The sample was then washed thoroughly by repeated centrifugation with distilled water and subjected to the labelling procedures.

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2.3. Periodic acid treatment of β-chitin microcrystals

A part of either the initial or the digested β-chitin microcrystals was first reduced by 2% $NaBH_4$ and then subjected to periodic oxidation. With cellulose, this method is known to introduce aldehyde groups all along the C2 and C3 of the cellulose chains in addition to the non-reducing C4 [10]. In contrast to cellulose, one may expect that the periodic oxidation will bring aldehyde moieties only at the C3 and C4 of the non-reducing together with the C5 of the reducing ends, whereas the adjacent C6 is liberated as formic aldehyde (Fig. 1a). It is the aldehyde groups attached to the chain ends of chitin that are the targets for the labelling procedure.

To apply the periodate oxidation, the β-chitin microcrystals in suspension were first reduced by 2% $NaBH_4$ at 40°C for 72 h. After several centrifugal washes, the sample was treated by 2% $NaIO_4$ at 30°C for 6 h. These oxidized samples were then washed by repeated centrifugation in distilled water and subjected to the labelling procedures.

2.4. Biotinamidocaproyl hydrazide streptavidin–gold (BXH–SG) labelling procedure method

The non-covalent interaction between biotin and streptavidin is known to be very selective and efficient. Biotin is commercially available in a number of derivatives dedicated to attachment to a variety of functional groups. We selected a hydrazide compound of biotin, the biotinamidocaproyl hydrazide (Sigma Co., USA), hereafter abbreviated as BXH, assuming that this compound would react selectively with the aldehyde groups located at the tips of the β-chitin crystals. This fixed BXH should then be the target of the streptavidin–gold conjugate (Sigma Co., USA), hereafter abbreviated as SG, for visualization by TEM.

The labelling of the crystals included their suspension in solution of 1 ml absolute methanol and 50 μl acetic acid, with 3 mg BXH and 0.3 mg $NaBH_3CN$. This suspension was heated at 50°C for 14 h in the presence of molecular sieves 3A. After thorough centrifugal washing by methanol and distilled water, blocking of the non-specific sites was achieved for 30 min in 10 mM phosphate-buffered saline (PBS–0.15 M NaCl, 0.02% Tween 20 and 0.5% serum albumin, buffered at pH 7.0). The microcrystals were then incubated for 1 h at 37°C with 1 ml of SG solution, which was prepared by diluting 100-fold the commercial preparation with PBS. The resultant microcrystals were washed twice with 50 mM phosphate buffer (pH 7.0) including 0.02% Tween 20, and then twice with distilled water. All the reactions were performed in glass vials pre-washed by nitric acid.

2.5. TEM observation

All the micrographs were taken with a TEM Jeol-2000EXII operated at 100 kV and recorded on Mitsubishi MEM film. Diffraction contrast imaging in bright field mode was used to visualize the sample without further contrast enhancement. The images were taken at 1000–6000× under low dose exposure with the use of a Minimum Dose System (MDS, Jeol).

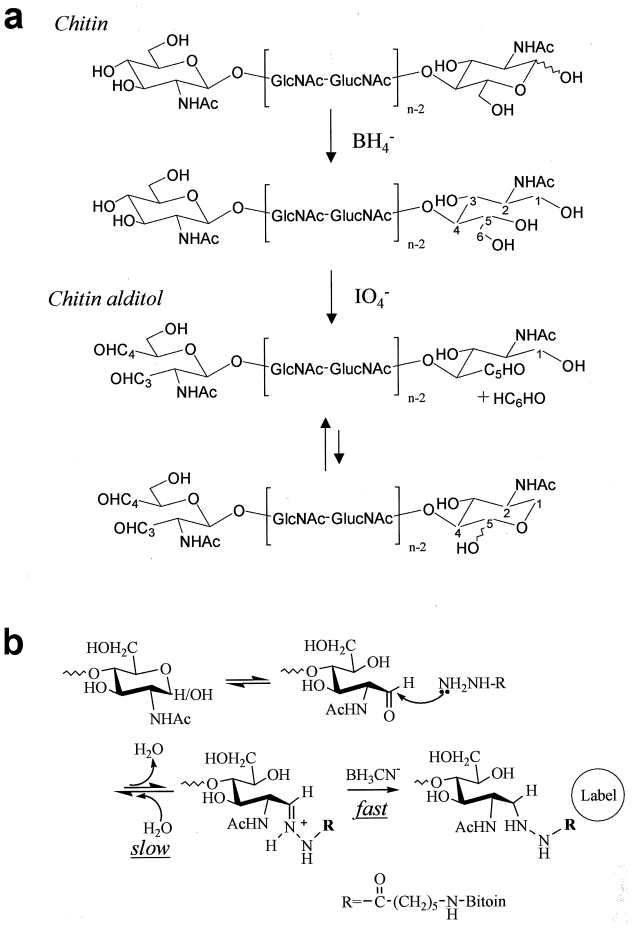

Fig. 1. a: Scheme describing the introduction of aldehyde groups both at reducing and non-reducing ends of chitin by periodic acid oxidation. b: Action of BXH on the aldehyde located at the reducing end of chitin. Nucleophilic addition of a hydrazide compound to the α-carbon of aldehyde occurs by attacking the lone electron pair in the primary amine with dehydration to provide an iminium moiety, which is subsequently reduced in the presence of BCH_3CN^- at a rapid rate (reductive amination). In this mechanism, biotin was introduced specifically to the reducing ends of chitin. Finally, the biotin introduced at the reducing end of chitin is visualized by SG.

3. Results and discussion

3.1. BXH–SG labelling

The reaction scheme leading to the labelling is shown in Fig. 1b. The first step consists of the biotinylation of the aldehyde groups located at the chitin crystal tips: the reducing end tips in the case of the initial or digested crystals and both tips in the case of the oxidized crystals. The biotinylation was achieved by reductive amination with $NaBH_3CN$. In this reaction, the first step is the rate determining formation of the iminium ion. This reaction is identical to a 'pyridyl amination' of the reducing ends of sugars [11]. In this study, the recipe for biotinylation was established by modifying the standard procedure of pyridyl amination. As it was found that the biotinylation step was critical to achieve a good probability of labelling, the parameters of biotinylation were systematically varied to obtain the optimum conditions described in Section 2.

One of the critical factors in the reductive amination is the adjustment of the pH [12,13]. For this, the concentration of acetic acid was carefully controlled for determining the optimum condition. It was determined that the reaction needed to be achieved in 1 ml of absolute methanol and 50 μl of acetic acid with 3A molecular sieves. The pH of this solution was around 4–5 (measured by pH test paper at room temperature).

One needs also to remember that the reductive amination is complete after a relatively long time, e.g. 18–36 h, as described by Borch et al. [12]. In our case, biotinylation for longer time (>24 h) did not improve the probability of the final labelling. A temperature lower than 75°C provided better results, while at much lower temperature (e.g. room temperature) the reaction did not work at all. By trial and error, we were able to set the time and the temperature of biotinylation as 14 h and 50°C, respectively.

Other biotinylation reagents that have different lengths of linker groups between hydrazide and biotin have also been tried, but BXH gave the best results.

Typical electron micrographs of initial β-chitin treated as

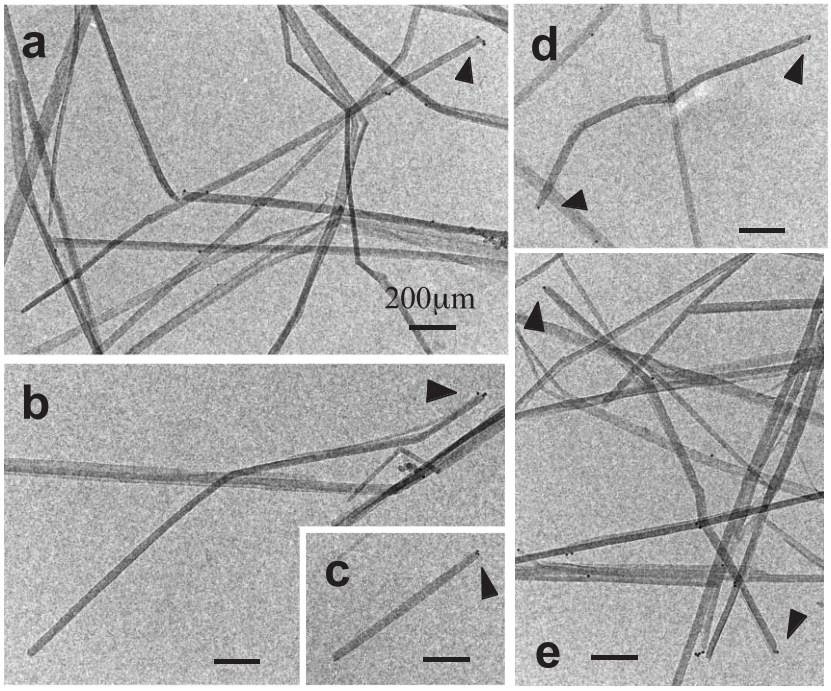

Fig. 2. β-Chitin microcrystals labelled by the BXH–SG method (arrowheads: the labelled ends). a–c: Microcrystals without any pre-treatment prior to the labelling procedure. Always only one tip is labelled, indicating the parallel structure in β-chitin microcrystal. Some labels on the side are also observed. d and e: Microcrystals oxidized by periodic acid prior to the labelling. In some crystals, both tips are labelled, a phenomenon which has never been observed in the initial microcrystals.

described are shown in Fig. 2a–c. Among the numerous pictures that were recorded, there was no case where both crystal tips were labelled by colloidal gold particles. Thus, the parallel structure in β-chitin was verified. The particles on the side of microcrystals were also frequently seen. However, when microcrystals whose aldehydes are at the reducing end were deactivated by BH_4^- reduction, gold particles were seldom seen (data not shown). These observations imply that the labels are almost specific to the reducing ends and therefore it is likely that some reducing ends are also exposed at the surface of a given initial microcrystal.

The specificity of BXH–SG technique was further verified by staining periodic acid-treated microcrystals where dialdehyde groups were generated at both ends of chitin molecules (Fig. 1a). As expected, gold particles could be detected at both crystal tips (Fig. 2d,e). Such dual labelling was never observed in the crystals that had not been subjected to periodate oxidation. Thus, we believe that our reaction is indeed specific of the aldehyde groups and that in the initial crystals, we are only targeting the reducing end tips. It is also noteworthy that our methodology can introduce functional moieties to both ends of a fibrillar colloidal β-chitin microcrystal as well as that of chitin molecules.

It can be noted that this BXH–SG technique could also be applied to other polysaccharides. For instance, applying it to cellulose I microcrystals (data not shown) provided consistent results with previous reports where the silver staining was applied [4,7]. Thus, it is suggested that the present reducing end labelling may be general for all polysaccharides.

3.2. BXH–SG labelling of the microcrystals degraded by chitinase A1

β-Chitin microcrystals degraded by chitinase A1 are tapered at only one of their tips [2]. When applying the BXH–SG technique, gold particles appeared specifically at the tapered tips (Fig. 3a,b). On the other hand the microcrystal pretreated by periodic acid became labelled at both ends (Fig. 3c). These experiments clearly indicate that the tapering caused by chitinase A1 must be directed from the reducing toward the non-reducing tips of the crystals. This conclusion is, however, contradictory to the previous results obtained from hydrolysis rate measurement using soluble phenyl β-chito-oligosaccharides as substrate analogues [2].

3.3. Substrate specificity of chitinase A1

The 3D structure of the catalytic domain of chitinase A1 co-crystallized with chitoheptaose is reproduced in Fig. 4 [3,14]. It indicates that chitin molecules are introduced from their reducing ends and are cut at the glycosidic linkage located at the second position from the reducing end. Such directional preference toward the reducing ends of crystalline substrate agrees well with the present study. In the structure of the mutant E204Q enzyme co-crystallized with (GlcNAc)7, the aromatic amino acids, Trp122 and Trp134 on the surface of this enzyme, are located along the cleft, quite closely next to the non-reducing ends of a heptamer. It seems therefore that these residues are responsible for directing the chitin molecules into the catalytic cleft. When these residues were replaced by partial mutation, the activity of the mutated enzyme toward *Lamellibrachia* β-chitin was dramatically reduced. This observation is therefore consistent with the suggestion that the chitin molecules are directionally introduced to the catalytic site from their reducing end [14].

From the work dealing with the action of chitinase A1 on chitodextrins substituted by *p*-nitro-phenol residues [2], it was shown that the second glycosidic bond from the non-reducing

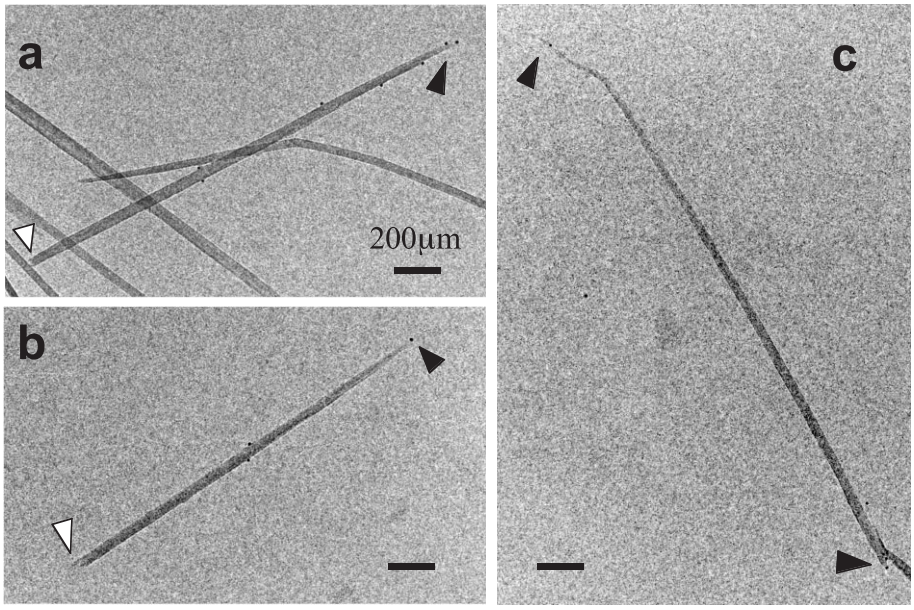

Fig. 3. a and b: β-Chitin microcrystals degraded by chitinase A1 and subsequently labelled by the BXH–SG method (black arrowheads: the labelled tips; white arrowheads: the non-labelled tips). Labels are observed only at the tapered tip, suggesting that chitinase A1 degrades a chitin molecule from its reducing end towards the non-reducing end. c: β-Chitin microcrystals degraded by chitinase A1, followed by IO_4^- treatment and finally labelled by BXH–SG. Both tapered and non-eroded tips are labelled.

end had the highest cleavage ratio. Thus, with such oligosaccharides, it seems that the degradation proceeds from the non-reducing end. There is therefore a discrepancy between the action of the enzyme on soluble and crystalline substrates. To our knowledge, such discrepancy has never been reported so far, but a rationale could be conceived to account for this phenomenon. It could be reasonable to think that a local arrangement of the catalytic residues would play an important role as a mold for the binding of soluble oligosaccharides. For the digestion of chitin microcrystals in a processive manner, a long-range structure including the Trp122 and Trp134 residues might be necessary to guide the chitin chains into the catalytic cleft. This difference between a short- and long-range interaction may explain the difference existing in the directionality of the enzymatic attack for the two substrates. A resolution of the structure of mutants such as E204Q co-crystal-

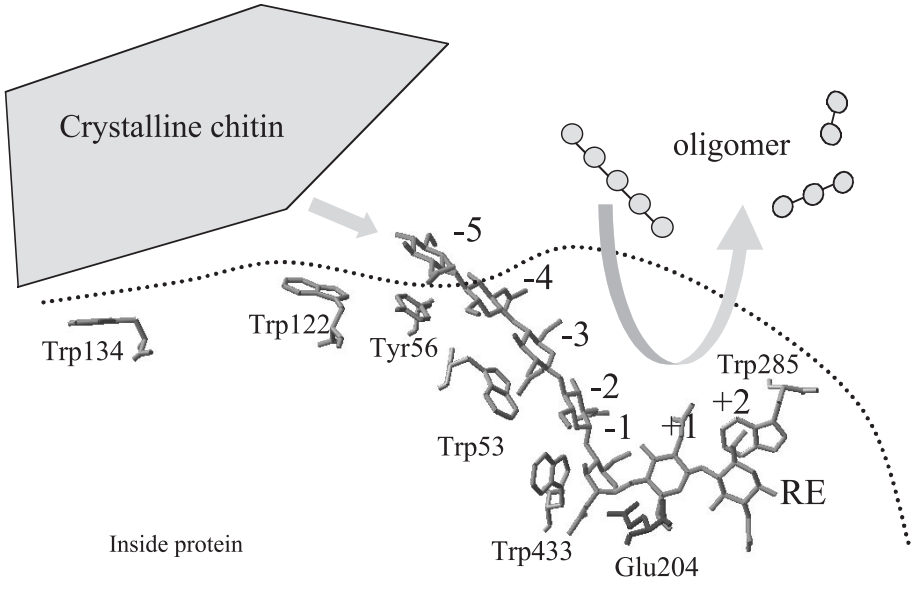

Fig. 4. 3D close-up of the catalytic domain of chitinase A1 [3] co-crystallized with chitoheptaose. A kink at the second glycosyl residue from the reducing ends is clearly demonstrated. Aromatic amino acids that may interact with chitin chains are indicated. Among them, Trp122 and Trp134 on the surface seem responsible for guiding the crystalline substrate into the catalytic cleft. This guiding may not be necessary for soluble oligomer catalysis [14].

lized with chitodextrins substituted by *p*-nitro-phenol residues needs to be attempted to infirm or confirm the mode of action of chitinase A1 on such substrates.

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