Expression and characterization of a lytic polysaccharide monooxygenase from *Bacillus thuringiensis*

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Abstract Lytic polysaccharide monooxygenases (LPMOs) are recently discovered oxidative enzymes that are capable of oxidative cleavage of recalcitrant polysaccharides such as chitin or cellulose. Despite the importance of LPMOs in biomass conversion and the large number of *lpmo* genes in microorganisms, only a few LPMOs have been well studied, and further characterization of these proteins is thus of interest. In this study, a chitin-active AA10 family LPMO from *Bacillus thuringiensis* subsp. *kurstaki*, BtLPMO10A, is described. This enzyme generates even-numbered oxidized oligosaccharides as the dominated products from crystalline chitin, however, interestingly, when colloidal chitin is used as the substrate, a ladder of oxidized oligosaccharides is observed. These results provide new insights into the action mode of LPMOs that may be affected by the substrates.

Keywords *Bacillus thuringiensis*; Lytic polysaccharide monooxygenases; Polysaccharides

1. Introduction

Chitin and cellulose are two most important and highly recalcitrant polysaccharides widely occurring in nature. For an efficient degradation of chitin or cellulose, bacteria generally utilize an enzyme system including nonprocessive endo-type glycoside hydrolases in tandem with processive exo-type glycoside hydrolases [1,2]. Previous studies pointed out that some bacteria produce polysaccharides binding proteins that were capable of increasing substrate accessibility and thus potentiating hydrolytic enzymes [3]. Recent studies highlighted that some of these proteins were capable of catalyzing the cleavage of glycosidic bonds of polysaccharides via an oxidative mechanism, generating a series of oxidized oligosaccharides [4,5]. These bacterial chitin- or cellulose-specific oxidative enzymes are now systematically classified as Auxiliary Activity 10 family (AA10) in the Carbohydrate Active Enzymes (CAZy) database (www.cazy.org) [6]. Functionally, AA10 LPMOs can be divided into two subclasses: chitin-oxidative (i.e. *SmCBP21* from *Serratia marcescens*) and cellulose-oxidative LPMOs (i.e. *CelS2* from *Streptomyces coelicolor* A3(2)) [7].

Based on genome information, the occurrence of *lpmo* genes has been confirmed in many chitinolytic and cellulolytic bacteria. It remains to be elucidated whether most of these putative LPMOs have oxidative activities, their large number, however, emphasizes the importance of their existence. What is more, we have no idea whether all bacterial LPMOs with confirmed activity have the same mode/catalytic mechanism when acting with chitin, cellulose or other

1 carbohydrates. To answer above questions, discovery of novel LPMOs is of major importance.

2 In the gram-positive bacterium *Bacillus thuringiensis*, a well-known biological pesticide,
3 three predicted genes coding for LPMOs are present based on sequence analysis. Their activities
4 have so far not been confirmed. In the present study, we performed the cloning, expression and
5 purification of *Bt*LPMO10A from *B. thuringiensis* subsp. *kurstaki*. The binding capacity and
6 oxidative activity of *Bt*LPMO10A toward different substrates were also determined, hopefully to
7 provide new clues for our understandings of LPMOs.

8 **2. Materials and methods**

9 2.1. Materials and methods

10 *Bacillus thuringiensis* subsp. *kurstaki* (ACCC 10066) was ordered from Agricultural Culture
11 Collection of China. Plasmid pET22b, *Escherichia coli* strains TOP10 and BL21 (DE3) were
12 purchased from Takara Biotechnolgy (Dalian). α -Chitin isolated from shrimp shells and Avicel
13 (Microcrystalline cellulose) were purchased from Sigma (St. Louis, MO, USA). β -Chitin isolated
14 from squid pen was purchased from Seikagaku (Tokyo, Japan). Colloidal chitin was prepared
15 according to the method reported previously [8]. Chitin beads were purchased from New England
16 Biolabs (NEB).

17 2.2. Cloning of *Btlpmo10A*

18 For constructing a recombinant vector containing *Btlpmo10A* from *B. thuringiensis* subsp.
19 *kurstaki*, a recently developed restriction-free (RF) cloning method was applied [9]. Genomic

1 DNA was isolated using a genomic DNA isolation kit (Qiagen). For the first amplification, the
2 gene of interest was amplified from genomic DNA using PrimeSTAR (NEB) according to the
3 manufacturers' instructions. Two primers (Forward primer: 5'-
4 CGCTGCCCAGCCGGCGATGGCCCATGGATATGTAGAATCACCAGCG-3'; Reverse primer:
5 5'-GCTTTGTTAGCAGCCGGATCTCAAAATAGTGTAGGAGTTTGCACTACG-3') were
6 designed using a gene encoding putative GlcNAc-binding protein A from *B. thuringiensis* Bt407
7 (GenBank ID: AFV18629.1) as a reference. For the second amplification, the PCR product was
8 used as the primer and the plasmid pET22b was used as the template. Once complete, the PCR
9 product was treated with *DpnI* (NEB) to digest the methylated parental plasmid. The final
10 construct (pET22b-*Btlpmo10A*) was transformed into *E. coli* Top10. DNA sequencing was
11 performed by Takara Biotechnology (Dalian).

12 2.3. Protein expression and purification

13 For protein expression, the plasmid containing *Btlpmo10A* was transformed into *E. coli* BL21
14 (DE3). Cultivation of *E. coli* BL21 (DE3) cells containing pET22b-*Btlpmo10A* was performed at
15 37 °C and 150 rpm in 100 ml of LB culture medium containing 0.1 mg/ml of ampicillin. When the
16 cell density reached OD₆₀₀=0.6, IPTG was added to a final concentration of 0.01 mM in order to
17 induce the production of *BtLPMO10A*. After cultivation at 30 °C and 150 rpm for 4 h, the cell
18 pellet was collected by centrifugation (6000 g 10 min at 4°C) and then washed twice by 10 ml
19 Tris-HCl buffer (20 mM, pH 8.0) containing sucrose and EDTA. After that, *BtLPMO10A* was

1 extracted from periplasmic space by washing the cell pellet with 10 ml MgCl_2 (1 mM). The
2 resultant *BtLPMO10A* was purified using chitin beads as the affinity chromatographic medium
3 according to a previously described method [4]. Protein purity was analyzed by SDS-PAGE.
4 Protein concentrations were determined using the Bio-Rad Protein Assay Kit (Bio-Rad, US).

5 2.4. Binding assays

6 Binding studies were performed using powdered α -chitin, β -chitin, Avicel, chitin beads and
7 colloidal chitin. Binding assays were conducted in 1 ml reactions in Eppendorf tubes containing
8 0.5% (w/v) of each substrate and 1 μM *BtLPMO10A* in 20 mM Tris-HCl buffer, pH 8.0.
9 Reactions were performed using a rotary mixer at room temperature for 24 h. Subsequently, the
10 substrates were pelleted by centrifugation at 14000 g for 10 min. For confirming of binding,
11 protein bound to the substrate was analyzed by SDS-PAGE after removal of nonspecifically
12 bound protein by washing with 2 ml of 20 mM Tris-HCl buffer, pH 8.0. The pellets were
13 resuspended in 50 μl SDS-PAGE sample buffer and boiled for 5 min in order to strip bound
14 protein of the substrate. Finally, 10 μl samples were analyzed by SDS-PAGE.

15 2.5. MS analysis of reaction products

16 To verify the reaction products of *BtLPMO10A*, several substrates were chosen, including α -
17 chitin, β -chitin, colloidal chitin and Avicel. The reactions were performed in 2 ml Eppendorf tubes
18 containing 0.2% (w/v) of substrates in 20 mM sodium phosphate buffer (pH 6.0), 1 mM ascorbic
19 acid, and 1 μM *BtLPMO10A*. After incubation at room temperature with rotation for 24 h,

1 remaining substrates were removed through centrifugation at 14000 g for 10 min and the
2 supernatant was used for analysis by mass spectrometry.

3 For MALDI-TOF/TOF MS analysis, 0.5 µl samples were mixed with an equal amount of 10
4 mg/ml solution of 2,5-dihydroxybenzoic acid (DHB) in 40% acetonitrile, 0.1% trifluoroacetic acid
5 on an Opti-TOF 384 target plate. The spotted samples were air-dried before being analyzed by
6 MALDI-TOF/TOF 5800 (AB SCIEX) instrument.

7 **3. Results and discussion**

8 3.1. Cloning and sequence analysis

9 In the present work, we performed the cloning, expression and purification of *BtLPMO10A*.
10 Since the *N*-terminal histidine is one of the fully conserved residues in mature AA10 LPMOs and
11 is essential for catalysis [7], obtaining of recombinant LPMOs with histidine as the *N*-terminus is
12 required. For this reason, genes coding for AA10 LPMOs were typically cloned into the pRSETB
13 expression vector containing the native signal sequence of a *S. marcescens* chitin-active LPMO
14 known as *SmCBP21* [10,11]. In this work, to complete this, we applied a recently described
15 restriction free cloning method that is simple and can be used to precisely insert a DNA fragment
16 into any desired location within a circular plasmid, such as pET22b, independent of restriction
17 sites, ligation, or alterations in either the vector or gene of interest. Based on this method, the gene
18 coding for mature *BtLPMO10A* with histidine as the *N*-terminus was successfully constructed into
19 the pET22b expression vector, containing a signal sequence that could lead the recombinant

1 protein secreted into the periplasmic space.

2 The gene (GenBank accession number KP096716.1), *Btlpmo10A*, encodes a mature protein
3 of 187 amino acids. Sequence alignments suggest that *BtLPMO10A* has a low level of sequence
4 identity with cellulose-oxidizing AA10 LPMOs (e.g. 26% with CelS2) [5] while a relatively high
5 level of sequence identity toward chitin-oxidizing AA10 family LPMOs (e.g. 63% identity with
6 *SmCBP21*; 46% identity with *EfCBM33* from *Enterococcus faecalis* V583) [10,12]. All
7 previously reported AA10 LPMOs have two well-conserved histidine residues that are believed to
8 be involved in metal binding and catalysis. Vaaje-Kolstad et al. indicated that *SmCBP21* interacts
9 with substrate by six surface-exposed hydrophilic residues (Tyr, Glu, Glu, His, Asp, and Asn) [10].
10 All these residues are fully conserved in *BtLPMO10A*, suggesting that *BtLPMO10A* might have
11 comparable substrate preferences and catalytic mechanism with *SmCBP21*.

12 3.2. Protein expression and purification

13 The gene *Btlpmo10A* was expressed in *E. coli* BL21 (DE3). The recombinant enzyme
14 *BtLPMO10A* was secreted into periplasmic spaces and further purified through an affinity
15 chromatography with chitin beads as the resin. SDS-PAGE analysis showed that *BtLPMO10A*
16 was purified to homogeneity, with a molecular mass approximately of 20 kDa which corresponds
17 to the estimated molecular mass (20.54 kDa) (Fig. 1).

18 **Fig. 1**

19 3.3. Binding assays

1 The binding studies of *BtLPMO10A* were performed by incubating *BtLPMO10A* with
2 various types of chitin substrates and Avicel. The data indicated that, at pH 8.0, *BtLPMO10A*
3 showed strong binding to crystalline α -chitin and β -chitin, weaker binding to chitin beads and
4 colloidal chitin, and little binding to Avicel (Fig. 2). The strong binding of *BtLPMO10A* towards
5 crystalline α -chitin and β -chitin was consistent with *EfCBM33A* [12]. Although *SmCBP21*
6 exhibits an overall structure comparable with that of *BtLPMO10A* and *EfCBM33A*, a different
7 substrate preference was observed. *SmCBP21* showed only strong binding to β -chitin [10]. The
8 weaker binding of *BtLPMO10A* towards chitin beads and colloidal chitin suggested that
9 *BtLPMO10A* prefers crystalline chitin to amorphous and noncrystalline chitin, which is
10 comparable to other AA10 LPMOs [7,10]. AA10 LPMOs have globular structures with a flat
11 binding surface that seems to be adapted to binding to crystalline parts of the chitin substrate
12 primarily through polar interactions [7,10]. Besides that, *BtLPMO10A* showed little bindings to
13 Avicel (Fig. 2), which was consistent with *EfCBM33A* and *SmCBP21* and expected, considering
14 all these AA10 LPMOs are active on chitin but not active on cellulose substrates.

15 Inconsistent with the above described chitin-active AA10 LPMOs, *CelS2* is a cellulose-active
16 AA10 LPMO and has no activity on chitin [5]. However, *CelS2* exhibits strong binding ability
17 towards chitin while weaker bindings to cellulose, which is of particularly interest and confused.
18 Moreover, the weak binding of *CelS2* to cellulose is unexpected either because the enzyme
19 contains a well-known 99-residue cellulose-binding domain (CBM2). Taken together, these results

suggested that there is no correlation between the substrate binding preferences and substrate specificity for AA10 LPMOs even though all enzymes have similar overall structures.

Fig. 2

3.4. Analysis of reaction products

For qualitative analysis of the oxidative activity of *Bt*LPMO10A on various substrates, MALDI-TOF/TOF MS was performed. As shown in Fig. 3, all tested substrates could be oxidatively hydrolyzed by *Bt*LPMO10A, producing a series of chitin oligosaccharides in aldonic acid or lactone form (Fig. 3). No identical peak was observed in the control experiments where no reducing agent or no enzyme was added in the reaction (Data not shown). *Bt*LPMO10A was active on chitin, producing a series of oxidized chitin oligosaccharides from DP_{ox3} to DP_{ox11} (Fig. 3). The relative intensities of DP_{ox4}, DP_{ox6}, DP_{ox8} and DP_{ox10} are remarkably higher than DP_{ox5}, DP_{ox7}, DP_{ox9} and DP_{ox11}, indicating that *Bt*LPMO10A prefers to generate even-numbered oxidized oligosaccharides from crystalline chitin (Fig. 3ab). Compared with crystalline α -chitin (Fig. 3a), a clearly longer oxidized oligosaccharide chain up to DP_{ox11} was observed from β -chitin (Fig. 3b), implying a different substrate specificity of *Bt*LPMO10A. When the crystalline α -chitin is modified to be colloidal chitin by treatment with phosphoric acid, the enzymatic reaction products by the same enzyme exhibited a completely different pattern. A ladder of oxidized oligosaccharides products was observed with the relative intensities of DP_{ox4} > DP_{ox5} > DP_{ox6} > DP_{ox7} > DP_{ox8} (Fig. 3c). The feature of dominant even-numbered oxidized oligosaccharides has

1 been discovered in several chitin- oxidative AA10 LPMOs, i.e. *Sm*CBP21 and *Ef*CBM33B, and in
2 cellulose-oxidative AA10 LPMOs such as CelS2 [5,12]. However, oxidative activity of AA10
3 LPMOs on colloidal chitin has not yet been reported. Colloidal chitin, an amorphous form of
4 chitin, is typically used as the substrates for chitinase activity assay because of its modified
5 structure with less crystallinity. Considering that AA10 LPMOs typically have a flat surface for
6 substrate-binding and their active sites are located in the center [4,12], it seems to be plausible to
7 predict that different oxidizing pattern of *Bt*LPMO10A on colloidal chitin compared to crystalline
8 chitin relates to the different substrate morphologies of these substrates.

9 **Fig. 3**

10 Combined with previous reports, the results in the present study lead to three important
11 conclusions: (1) AA10 LPMOs might exhibit diverse substrate specificity; (2) AA10 LPMOs
12 might have different action modes connected with the surface morphologies of the same
13 polysaccharides; (3) AA10 LPMOs are still poor and discovering novel LPMOs are required in
14 future studies.

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- 3

1 **Figure captions**

2 **Fig. 1.** SDS-PAGE analysis of the purified *BtLPMO10A*. The purified *BtLPMO10A* was
3 separated by 15% (w/v) SDS-PAGE followed by staining with Coomassie Blue R-250. Lane M:
4 protein marker; lane 1: proteins extracted from periplasmic spaces of recombinant *E. coli* BL21
5 (DE3) harboring pET22b-*Btlpmo10A* induced with 0.01 mM IPTG; lane 2: purified *BtLPMO10A*
6 by affinity chromatography.

7 **Fig. 2.** Substrate preferences for *BtLPMO10A*. (a) *BtLPMO10A* present in the supernatant after
8 24 h of incubation with α -chitin (lane 1), β -chitin (lane 2), Avicel (lane 3), colloidal chitin (lane 4)
9 and chitin beads (lane 5). (b) *BtLPMO10A* removed from the solid substrates by boiling in SDS-
10 PAGE sample buffer after the substrates had been washed to remove nonspecifically bound
11 protein. Note that the samples in (b) are approximately ten fold concentrated compared with the
12 corresponding samples in (a) (a shows 10 μ l of a 1 ml supernatant; b shows 10 μ l samples of
13 bound protein resolubilized in 100 μ l SDS-PAGE sample buffer). CK means control experiment
14 where 10 μ l purified *BtLPMO10A* (1 μ M) was analyzed as observed in a and b.

15 **Fig. 3.** MALDI-TOF/TOF MS analysis of reaction products after *BtLPMO10A* incubating with α -
16 chitin (a), β -chitin (b) and colloidal chitin (c) for 12 h. DP_{ox} indicates the degree of polymerization
17 of oxidized oligosaccharides produced during the reaction. Each labelled oxidized oligosaccharide
18 by arrow is observed as Na and K adducts of the oxidized oligosaccharide, as commonly seen for
19 carbohydrates containing carboxylic groups. The inset in panel a shows the different adducts

1 found in an ionic cluster starting with the sodium adduct of the δ -1,5 lactone, followed by the

2 sodium and potassium adducts of the aldonic acid, followed by the sodium adducts of the sodium

3 and potassium salts.

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