Biochimica et Biophysica Acta 1814 (2011) 1127–1133

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbapap

Characterization of calcium ion sensitive region for β-Mannanase from *Streptomyces thermolilacinus*

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ARTICLE INFO

Article history:
Received 3 December 2010
Received in revised form 8 April 2011
Accepted 27 April 2011
Available online 12 May 2011

Keywords:
β-Mannanase
Streptomyces
Thermobifida
Calcium ion dependent activity
Chimera
Thermal stability

ABSTRACT

Despite the widespread industrial applications of β-mannanase, the relations between the enzymatic properties and metal ions remain poorly understood. To elucidate the effects of metal ions on β-mannanase, thermal stability and hydrolysis activity were characterized. The *stman* and *tfman* genes encoding β-mannanase (EC.3.2.1.78) from *Streptomyces thermolilacinus* NBRC14274 and *Thermobifida fusca* NBRC14071 were cloned and expressed in *Escherichia coli*. The thermal stability of each enzyme shifted to the 7–9 °C high temperature in the presence of Ca^{2+} compared with that in the absence of Ca^{2+}. These results show that the thermal stability of StMan and TfMan was enhanced by the presence of Ca^{2+}. StMan, but not TfMan, required Ca^{2+} for the hydrolysis activity. To identify the Ca^{2+} sensitive region of StMan, we prepared eight chimeric enzymes. Based on the results of the relationship between Ca^{2+} and hydrolysis activity, the region of amino-acid residues 244-349 of StMan was responsible for a Ca^{2+} sensitive site.

1. Introduction

Mannan, a polysaccharide composed mainly of galactomannans that are branched with galactosyl residues in β-1,4-linkage of mannosyl residues and glucomannans that often have glucosyl residues interspersed in the main chain of the linear polymer, is present in plant seeds and cell walls of the hemicellulosic fractions [1]. These degradation products, mannooligosaccharides, are known to possess bioactivity of various kinds. For example, some are used in domestic animal and marine organisms as a dietary supplement to improve growth performance, gut health, and immune responses [2–6]. Because mannooligosaccharides are usually produced by β-mannanase, this enzyme is an important tool for the production of bioactive mannooligosaccharides.

β-mannanase, known as hemicellulases, is widely distributed among bacteria, fungi, plant, and marine mollusks [7–11]. β-mannanase can bleach Kraft pulp and reduces the viscosity of coffee mannan [12,13]. Recently, woody biomass is anticipated as a new material instead of utilization of starch and sugarcane for carbon source as bioethanol. Therefore, hemicellulases have attracted scientific attention and industrial interest for such applications.

β-mannanase is known to belong to GHF 5, 26, and 113 from analysis of the hydrophobic cluster [14]. Several studies have been investigated in its mode of action toward mannan and mannooligosaccharides [15,16]. Although it is expected to apply β-mannanase for industrial utilization, there is little information that exists about the effects of protease, inhibitors, low-molecular-weight compounds, metal ions, etc. on enzymatic properties [17,18]. For example, α-amylase is a well-known polysaccharide-degrading enzyme that is applied to various industrial stages such as the food industry and bioethanol production [19]. Several studies of α-amylase have therefore been directed for industrial application. Among these studies, the effects of metal ion on α-amylase activities are noteworthy because Ca^{2+} enhances the thermal stability and the hydrolysis activity of α-amylase [20,21]. Therefore, divalent metal ions should be regarded as an important factor affecting the enzymatic properties of β-mannanase.

Here, we cloned and expressed β-mannanase genes from *Streptomyces thermolilacinus* NBRC14274 (*stman*) and *Thermobifida fusca* NBRC14071 (*tfman*) to assess the effects of metal ions on their enzymatic properties. According to these enzymatic characterizations, each enzyme required Ca^{2+} for the enhancement of thermal stability. Regarding their hydrolysis activity, StMan requires a bivalent ion for the activation of its activity, although TfMan expresses its activity constantly, irrespective of the presence or absence of bivalent ions.

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doi:10.1016/j.bbapap.2011.04.017

The control of hydrolysis activity by a bivalent ion is a remarkable mechanism in β-mannanase. In this study, we sought to determine the bivalent ion sensitive region of StMan by preparing chimeric enzymes.

2. Materials and methods

2.1. Materials

Locust bean gum and carboxymethyl cellulose (CMC) were purchased from Sigma-Aldrich Corp. (St. Louis, Mo. USA). Azo-Carob galactomannan, Azo-Barley glucan, and Azo-xylan were purchased from Megazyme International Ireland Ltd. (Bray, Ireland). Genome DNA of *S. thermolilacinus* NBRC14274 and *T. fusca* NBRC14071 were obtained from the NITE Biological Resource Center (NBRC). Other reagents were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

2.2. Cloning of StMan by inverse polymerase chain reaction (PCR)

The *stman* fragment was amplified from genomic DNA by PCR with the degenerated primers (sense (S1): 5′-ATGMGSGGSGTSAACCACGC-3′ and anti-sense (AS1): 5′-ACCTCSGCSGCSGTGTCGTA-3′), which were designed from the conserved amino-acid sequences (i.e. MRGVNH and YDTAAE) of GHF 5. Routinely, PCR was conducted using PrimeSTAR GXL DNA polymerase (TaKaRa Holginds Inc.) for 30 cycles of 20 s at 96 °C, 10 s at 60 °C, and 2 min at 68 °C, preceded by incubation for 1 min at 96 °C. The PCR product was cloned into the pCR®-Blunt II-TOPO vector (Invitrogen Corp.) and transformed into *E. coli* JM109 (Takara Bio Inc.) and sequenced. Then, the genome DNA was digested with MluI. Next, these fragments were recovered using agarose electrophoresis and were subsequently self-ligated. The ligation products were amplified using primer sets (sense (S2): 5′-GGGCTCGCGCACACCCTCATGATCG-3′ and anti-sense (AS2): 5′-ACCGCGTCTGCCCCGGGTACC-3′) designed from the partial DNA fragment of *stman*. The PCR products were cloned and sequenced. The open reading frame of *stman* was amplified using primer sets (sense (S3): 5′-CATATGCGGACCGCCCCGCCCG-3′ and anti-sense (AS3): 5′-AAGCTTTCAGCCGATGGCGCAGGG-3′) harboring the *Nde*I and *Hind*III site (shown as underlined).

2.3. Cloning of T. fusca β-mannanase TfMan

The *tfman* was amplified by PCR with the primer sets (sense (S4): 5′-CATATGGCCACCGGGCTCCACGTCAAG-3′ and anti-sense (AS4): 5′-AAGCTTTCAGCGAGCGG TGCAGCTCAG-3′), which were both designed from the *T. fusca* YX β-mannanase flanking sequence (Accession no. CP000088) harboring the *Nde*I and *Hind*III site (shown as underlined). The PCR product was cloned into the pCR®-Blunt II-TOPO vector and sequenced.

2.4. Preparation of chimeras and catalytic domain mutant β-mannanases

For analysis of bivalent metal ion sensitive region of StMan, the chimeric constructs were prepared by the digestion of common restriction enzyme sites between two β-mannanase genes. A schematic representation of the structure of these β-mannanases is portrayed in Fig. 1. Six chimeras were constructed by the ligation of those of two DNA fragments: STMan1, the *Nde*I-*Kpn*I region for *stman* (105–224 bp) and the *Kpn*I-*Hind*III region for *tfman* (173–1362 bp); STMan2 the *Nde*I-*Apa*LI region for *stman* (105–408 bp) and the *Apa*LI-*Hind*III region for *tfman* (337–1462 bp); STMan3, the *Nde*I-*Apa*LI region for *stman* (105–735 bp) and the *Pci*I-*Hind*III region for *tfman* (670–1362 bp); TSMan1, the *Nde*I-*Kpn*I region for (61–179 bp) and the *Kpn*I-*Hind*III region for *stman* (239–1686 bp); TSMan2, the *Nde*I-*Apa*LI region for *tfman* (61–342 bp) and the *Apa*LI-*Hind*III region for *stman* (403–1686 bp); TSMan3, the *Nde*I-*Pci*I region for *tfman* (61–675 bp) and the *Pci*I-*Hind*III region for *stmandc* (730–1047 bp) constructed as a StMan catalytic domain mutant (see below). Because the *stman* gene contains another undesirable *Pci*I site (1508–1513 bp) in the CBM, TSMan3 chimera was prepared as a catalytic domain mutant. Linker and CBM deletion mutant, *stmandc* and *tfmandc*, were prepared by PCR with specific primer sets of S3 and anti-sense 5′-AAGCTTTCAGGTGTCGCCGGGGGTTTCCCC-3′ for StMandC and of S4 and anti-sense 5′-AAGCTTTCACGGGCCCGGCTGGGAGCC-3′ for TfMandC (Underlined letters show the respective *Nde*I and *Hind*III sites).

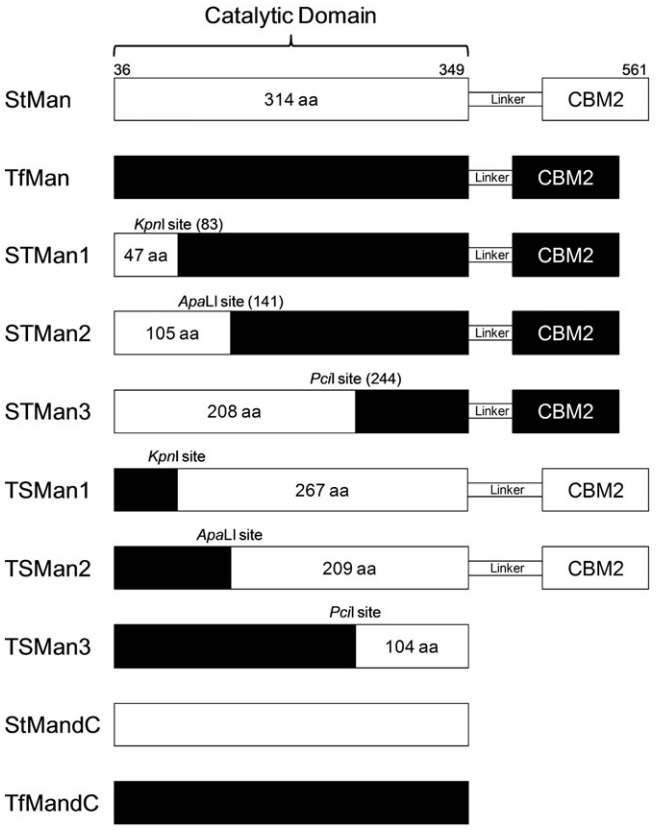

Fig. 1. Schematic representation of the structure of β-mannanases. The domains of StMan and TfMan are shown as white and dark bars, respectively. The number above the bars represents amino acid residues corresponding to the amino acid residues of StMan.

2.5. Expression and purification of β-mannanase in E. coli

We constructed expression plasmids to express a series of β-mannanases. These β-mannanases were amplified by PCR with the primers harboring the *Nde*I and *Hind*III sites; the PCR products were then cloned and confirmed through sequencing. The insert fragment was subcloned into the *Nde*I-*Hind*III gap of the pET28a vector, generating the expression plasmids pET (β-mannanase). Single colonies of *E. coli* BL21-Gold (DE3) harboring the expression plasmids were cultured in 50 ml of the overnight expression system 1 (Novagen Inc.) in 500 ml flasks. The colonies were cultured with shaking at 30 °C for 30 h. The cells were collected by centrifugation, freeze-thawed once, and suspended in 10 ml of buffer 1 (0.1 M Tris–HCl (pH 7.8), 300 mM NaCl, and 1 mM $CaCl_2$). Subsequently, they were disrupted by continuous 5 min sonication and 1 min standing for 45 min. After centrifugation to remove cell debris, the recombinants were purified from the supernatant using affinity chromatography (Talon; Clontech, Japan) as follows. The resin was incubated with the supernatant containing recombinants at 4 °C for 60 min and washed with buffer 1 containing 5 mM imidazole. Then protein