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Binding of bivalent ions to actinomycete mannanase is accompanied by conformational change and is a key factor in its thermal stability

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ABSTRACT

The study aimed to define the key factors involved in the modulation of actinomycete mannanases. We focused on the roles of carbohydrate-binding modules (CBMs) and bivalent ions. To investigate the effects of these factors, two actinomycete mannanase genes were cloned from *Streptomyces thermoluteus* (StManII) and *Streptomyces lividans* (SlMan). CBMs fused to mannanase catalytic domains do not affect the thermal stability of the proteins. CBM2 of StManII increased the catalytic efficiency toward soluble-mannan and insoluble-mannan by 25%–36%, and CBM10 of SlMan increased the catalytic efficiency toward soluble-mannan by 40%–50%. Thermal stability of wild-type and mutant enzymes was enhanced by calcium and manganese. Thermal stability of SlMandC was also slightly enhanced by magnesium. These results indicated that bivalent ion-binding site responsible for thermal stability was in the catalytic domains. Thermal stability of mannanase differed in the kinds of bivalent ions. Isothermal titration calorimetry revealed that the catalytic domain of StManII bound bivalent ions with a K_a of $5.39 \pm 0.45 \times 10^3$–$7.56 \pm 1.47 \times 10^3$ M^{-1}, and the catalytic domain of SlMan bound bivalent ions with a K_a of $1.06 \pm 0.34 \times 10^3$–$3.86 \pm 0.94 \times 10^3$ M^{-1}. The stoichiometry of these bindings was consistent with one bivalent ion-binding site per molecule of enzyme. Circular dichroism spectrum revealed that the presence of bivalent ions induced changes in the secondary structures of the enzymes. The binding of certain bivalent ion responsible for thermal stability was accompanied by a different conformational change by each bivalent ion. Actinomycete mannanases belong to GHF5 which contained various hemicellulases; therefore, the information obtained from mannanases applies to the other enzymes.

1. Introduction

Mannans are the main component of lignocellulose that is present in the hemicellulosic material in plant cell walls [1]. Most mannans are contained as glucomannan or galactomannan in softwood or bean seed. Mannanase (EC 3.2.1.78) nonspecifically hydrolyzes β-1,4-mannosyl linkages in the main chain of mannan [2], and it is used to bleach kraft pulp with other enzymes [3]. The degradation products such as mannooligosaccharides have a prebiotic effect on the intestinal microflora of broilers [4,5]. In addition, the mannose produced by the hydrolysis reaction is also utilized as materials for fermentation [6]. Therefore, mannanase is an essential enzyme for degradation of biomass and production of bioactive products.

Most mannanases belong to the glycoside hydrolase (GH) family 5 or 26 (http://www.cazy.org/), and usually, have one or two non-catalytic domains, namely, carbohydrate-binding modules (CBM) [7]. It has been reported that the main function of CBMs is to attach to the substrate and increase the catalytic activity [8]. This is significant, as industrial applications require mannanase to show high activity and thermostability [9]. Most CBMs can bind to cellulose that is an abundant polysaccharide material. Except for cellulose, CBMs fused to mannanases also bound to insoluble mannan [10]. However, the information about the effect of CBMs on hydrolysis activity of mannanases is limited. Recently, some CBMs have been reported to enhance the thermal stability of proteins [11,12]. Therefore, it is important to evaluate the role of CBMs in the hydrolysis activity and thermal stability of actinomycetes mannanase. In addition, it has been reported that bivalent ions such as calcium, manganese, and zinc enhance the thermal stability of hemicellulases [13–15]. Indeed, we have demonstrated that calcium enhances the thermal stability of hemicellulases [16]; however, it is not clear whether other bivalent ions are capable of binding to mannanase.

Abbreviations: ANS, 1-anilinonaphthalene-8-sulfonic; CBM, carbohydrate-binding module; CD, circular dichroism; DNS, 3,5-dinitrosalicylic acid; GM, konjac glucomannan; ITC, isothermal titration calorimetry; IvMan, ivory nut mannan; LBG, locust bean gum; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); StManII, *Streptomyces thermoluteus* mannanase; StManIIdC, catalytic domain of StManII; SlMan, *Streptomyces lividans* mannanase; SlMandC, catalytic domain of SlMan

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In this study, we cloned and expressed two actinomycete mannanases that possess different CBMs. Although the catalytic domains of two mannanases showed high similarity, the linkers and CBMs showed low similarity. Previous study demonstrated that CBMs bound to insoluble mannan; however, its effect on hydrolysis activity and thermal stability of mannanases is not clear [10]. We revealed that CBM binding affected catalytic efficiency, but not thermal stability. In addition, we found that the thermal stability of mannanases was enhanced by the presence of certain bivalent ions. Furthermore, we confirmed that the binding of certain bivalent ion responsible for thermal stability was associated with a conformational change.

2. Materials and methods

2.1. Cloning of Streptomyces thermoluteus NBRC 14269 mannanase (StManII)

The *stmanII* fragment was amplified from genomic DNA by PCR using the degenerate primers StS1 (5′-ATGMGSGGSGTSAACCACGC-3′) and StAS1 (5′-ACCTCSGCSGCSGTGTCGTA-3′), which were designed from conserved amino acid sequences (i.e., MRGVNH and YDTAAE) of GH5 mannanase. PCR was performed using PrimeSTAR GXL DNA Polymerase (Takara Bio Inc., Otsu, Japan) for 30 cycles of 15 s at 96 °C, 10 s at 60 °C, and 1 min at 68 °C. The amplified fragment was cloned into the pCR®-Blunt II-TOPO vector (Invitrogen, Carlsbad, CA, USA), transformed into *Escherichia coli* JM109 cells (Takara Bio Inc., Otsu, Japan), and identified by sequencing. Then, the genomic DNA was digested with *Mlu*I. These fragments were recovered using agarose electrophoresis, and were subsequently self-ligated. The ligation products were amplified using the set of primers StS2 (5′-GTTCGGACACGACCACTCCGACGGCAAC-3′) and StAS2 (5′-CAGGTGGCGGGGTTCATGGGGATCAGCT-3′), which were designed from the partial DNA fragment of *stmanII*. The PCR product was cloned and sequenced. The *stmanII* gene was 1638-bp long, and encoded a 545-amino acid-long product. To construct the expression plasmid, the open reading frame of *stmanII* was amplified using the set of primers StS3 (5′-CATATGGCGCCCACCGGATTCCGG-3′) and StAS3 (5′-AAGCTTTCACGCCGCGGAGCACTCCG-3′; the underlined regions are the restriction sites). The amplified fragments were cloned into the pCR®-Blunt II-TOPO vector, transformed into *E. coli* JM109 cells, and identified by sequencing.

2.2. Cloning of the Streptomyces lividans mannanase gene

The DNA sequence of *S. lividans* mannanase (SlMan) was previously reported by Arcand et al. [17]. We utilized the sequence information (GenBank accession ID: M92297) to clone the *slman* gene by PCR using the set of primers SlFullFw (5′-ACTAAACCCGTAAACGCGCTGACC-3′) and SlFullRe (5′-TCCACATCCGCGACTCGAAGGC-3′). Subsequently, a second PCR was performed using the PCR product as a template and the set of primers SlFw (5′-CATATGGCGGCCGGTGGCATCCAC-3′) and SlRe (5′-AAGCTTTCAGTGGTCCGCCGCGCTG-3′; the underlined regions denote the restriction sites). The amplified fragments were cloned into the pCR®-Blunt II-TOPO vector, transformed into *E. coli* JM109 cells, and identified by sequencing.

2.3. Construction of catalytic domain mutants

To evaluate the role of CBMs, we generated mutants with deleted linkers and CBMs. The CBM-deleted mutants of StManII (StManIIdC) and SlMan (SlMandC) were prepared by PCR using the set of primers StS3 and StAS4 (5′-AAGCTTGCCCCGGTAGACCGCGG-3′) for StManIIdC, and SlFw and SlRe2 (5′-AAGCTTGCCGTACACGGTGGCCGT-3′) for SlMandC (the underlined regions denote the restriction sites). The amplified fragments were cloned into the pCR®-Blunt II-TOPO vector, transformed into *E. coli* JM109 cells, and identified by sequencing.

2.4. Expression and purification of the Streptomyces mannanases

To construct the pET28a (mannanase) expression vector, the plasmids containing the *SlMan*, *SlMandC*, *StManII*, and *StManIIdC* genes were digested with *Nde*I and *Hin*dIII, and ligated into the *Nde*I–*Hin*dIII sites of pET28a. Then, *E. coli* BL21-Gold (DE3) cells harboring the pET28a (mannanase: StManII, StManIIdC, SlMan and SlMandC) constructs were cultured by shaking at 30 °C for 30 h by using the overnight expression system 1 (Novagen Inc., Madison, WI, USA). The recombinant proteins were purified from the sonicated cell lysis using affinity chromatography (Talon; Clontech, Japan). The recombinant proteins were washed with 5 mM imidazole, and then eluted with 100 mM imidazole. The eluates were concentrated and dialyzed using a 10,000-MW cutoff Amicon® ultra membrane concentrator (Millipore, Billerica, MA, USA), and the concentrated enzymes were used in the following experiment. The purity of the recombinant proteins was confirmed by SDS-PAGE [18]. The protein concentrations were determined by the Bradford method [19] using bovine serum albumin (BSA) as the standard.

2.5. Mannanase activity assay

The mannanase activity was determined at 40 °C for 10 min in a reaction mixture (0.1 mL) containing an appropriate amount of enzyme, 1% (w/v) substrate, 5 mM calcium, and 50 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid; PIPES) at pH 7.0. The amount of reducing sugars produced by the reaction was determined by the 3,5-dinitrosalicylic acid (DNS) method [20]. One unit of mannanase activity was defined as the amount of enzyme that liberates reducing sugars equivalent to 1.0 μmol mannose per minute. The effect of the bivalent ions on hydrolysis activity was measured using locust bean gum (LBG) as the substrate. LBG contains various kinds of compounds such as bivalent ions. To reduce the effect of bivalent ion from the substrate, the LBG was pretreated with 1 mM EDTA, and then treated with 5 mM for all bivalent ion experiments, except those involving manganese. In the manganese experiments, LBG was pretreated with 0.02 mM EDTA, and then treated with 0.1 mM manganese because manganese affected color development during this assay. The kinetic parameters of mannanase with various mannan substrates were estimated by nonlinear regression ($v = k_{cat}[S]/K_m + [S]$); v, rate of catalysis; [S], concentration of substrate (Origin software; Lightstone Corp., Tokyo, Japan). The activity was assayed in the presence of 50 mM PIPES (pH 7.0), 5 mM calcium and 0.25–10 mg/mL of mannan at 40 °C. All activity assays were performed in quadruplicate.

2.6. Effect of bivalent ions on thermal stability

The effect of the bivalent ions on the thermal stability of mannanases was evaluated by heat treatment for 30 min in the presence of 50 mM PIPES (pH 7.0) and the bivalent ion (5 mM) or EDTA (5 mM). After heat treatment, the enzymes were cooled on ice, and the remaining activity of diluted heat treatment enzymes (contained 0.1 mM additive) was measured at 40 °C by using LBG containing 5 mM calcium as the substrate.

2.7. ITC assay

The binding of bivalent ions to mannanase was measured at 25 °C by using a MicroCal iTC_{200} calorimeter (MicroCal Inc., Northampton, MA, USA) following the manufacturer's recommended procedures. All samples were concentrated and dialyzed using a 10,000-MW cutoff Amicon® ultra membrane concentrator. The filtrate buffer (50 mM Tris–HCl buffer (pH 7.0) containing 150 mM NaCl) was used to dissolve the ligand (calcium, magnesium, and manganese). The protein sample (100–300 μM) was injected with a 0.4-μL aliquot and 18 successive 2-μL aliquots of ligand (1–5 mM) at 120-s intervals. Data were fitted by using the fitting algorithms of a single-site model ($\Delta Q(i) = Q(i) + dV_i/V_0$