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Effect of the binding of bivalent ion to the calcium-binding site responsible for the thermal stability of actinomycete mannanase: Potential use in production of functional mannooligosaccharides

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ABSTRACT

In a previous study, we determined that the calcium-binding site in the catalytic domain of actinomycete mannanases is responsible for the thermal stability [18]. To evaluate whether the calcium-binding site could bind to other bivalent ions, we measured the ability of mannanase to bind bivalent ions by using isothermal titration calorimetry (ITC) by employing the catalytic domain mutants StMandC (from *Streptomyces thermolilacinus*) and TfMandC (from *Thermobifida fusca*) and the calcium-binding site deletion mutants StDEDAAAdC and TfDEDAAAdC. The calcium-binding site in StMandC and TfMandC bound bivalent ions with a K_a of 0.10×10^4 to $3.02 \times 10^4\ M^{-1}$ and 0.21×10^4 to $1.52 \times 10^4\ M^{-1}$, respectively. Among the tested bivalent ions, thermal stability was enhanced in the following order: magnesium, manganese, and calcium. Magnesium barely enhanced the thermal stability in mannanases. On the other hand, StDEDAAAdC and TfDEDAAAdC did not bind to the tested bivalent ions. From these results, we showed that the calcium-binding site is involved in the binding of the other bivalent ions. The association constant comprised of negative enthalpy and low entropy was suitable for bivalent ion binding in actinomycete mannanases.

1. Introduction

Lignocellulose is recognized as a potential sustainable biomaterial comprised of various kinds of sugars for bioactive materials and for fermentation into bioenergy [1]. Mannan is one of the major components of the lignocellulose found in plant cell walls [2]. Most mannans exist as glucomannan or galactomannan in softwood or bean seed. Mannans are hydrolyzed using a combination of several glycoside hydrolase enzymes, namely, endo-β-1,4-mannanase (EC 3.2.1.78), β-mannanosidase (EC 3.2.1.25), and α-galactosidase (EC 3.2.1.22), resulting in the production of mannose, galactose, and mannooligosaccharides. Among them, mannanase is a primary enzyme for the nonspecific hydrolysis of β-1,4-mannosyl linkages in the main chain of mannan [3]. Degradation products such as mannooligosaccharides have a prebiotic effect on intestinal microfloral broiler performance [4–7]. Therefore, mannanase is an essential enzyme for biomass degradation and production of bioactive products.

For industrial applications, thermostability and high activity are essential factors [8]. From the hydrophobic cluster analysis of the primary structure, most mannanases belong to the glucoside hydrolase (GH) family 5 or 26 (http://www.cazy.org/). The catalytic domains usually possess 1 or more additional modules such as carbohydrate-binding modules (CBMs) and linker modules [9]. A gene from a thermophilic bacterium is a good candidate to obtain the stable enzyme [10]. On the other hand, there are many factors that stabilize the lignocellulosic enzymes, such as molecular architecture. The main function of the additional modules in CBM is to increase the catalytic efficiency by attaching the substrate to the catalytic domain. On the other hand, the CBM that is attached to the catalytic domain enhances the thermal stability of the proteins in addition to enhancing the hydrolytic activity [11–13]. The addition of non-glycoside hydrolase, as well as the interaction with cofactors, also stabilizes the proteins [14–17]. Therefore, the evaluation of additional cofactors to the protein is necessary for thermostability and high activity.

We have been studying the interaction between bivalent ions and mannanases. In a previous study, we determined that the calcium-binding site in the catalytic domain of actinomycete

Abbreviations: DNS, 3,5-dinitrosalicylic acid; ITC, isothermal titration calorimetry; LBG, locust bean gum; PIPES, piperazine-*N*,*N*′-bis-(2-ethanesulfonic acid); StMan, *Streptomyces thermolilacinus* mannanase; StMandC, catalytic domain of StMan; StDEDAAAdC, D285A, E286A, and D287A triple mutant of StMandC; TfMan, *Thermobifida fusca* mannanase; TfMandC, catalytic domain of TfMan; TfDEDAAAdC, D264A, E265A, and D266A triple mutant of TfMandC.

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mannanases (StMan from *Streptomyces thermolilacinus* and TfMan from *Thermobifida fusca*) is responsible for the enhancement of thermal stability [18]. The calcium-binding site in StMan is also involved in catalytic efficiency. The thermal stability of actinomycete mannanase (StManII from *Streptomyces thermoluteus* and SlMan from *Streptomyces lividans*) was enhanced by specific bivalent ions [19]; however, we did not determine the binding site of the bivalent ion. Therefore, whether the calcium-binding site participated in the binding of the other bivalent ions was not clear.

In this study, we investigated the specificity of the calcium-binding site in actinomycete mannanases, and evaluated the effect of bivalent ion binding on their activities and thermal stabilities. These results indicated that the calcium-binding site in actinomycete mannanase was also involved in the binding of magnesium and manganese. The effect of bivalent ions on mannanases was dependent on the identity of the bivalent ion. Our data provide essential information on the relationship between the binding sites for the bivalent ions in GH5 mannanases.

2. Experimental

2.1. Construction, expression, and purification of actinomycete mannanases

To evaluate whether the calcium-binding site binds other bivalent ions, we employed an expression plasmid with the catalytic domain of StMan (StMandC) and TfMan (TfMandC), and the non-calcium ion-binding mutants of StMandC (StDEDAAAdC) and TfMandC (TfDEDAAAdC). The construction of these plasmids was described previously [17,18]. The recombinant proteins were expressed in *Escherichia coli* BL21-Gold (DE3) cells (Agilent Technologies, Palo Alto, CA, USA) harboring the pET28a construct (mannanase: StMandC, StDEDAAAdC, TfMandC, and TfDEDAAAdC). The recombinant proteins were purified from the cells as previously described [18]. The purified proteins were concentrated using a 10,000-MW cutoff Amicon® ultra membrane (Millipore, Billerica, MA, USA) for use in subsequent experiments. The purity of the recombinant proteins was confirmed by using SDS-PAGE [20]. The protein concentrations were determined by the Bradford method [21] using bovine serum albumin (BSA) as the standard.

2.2. Mannanase activity assay

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, 1 mM $CaCl_2$, and 50 mM piperazine-*N*,*N*′-bis-(2-ethanesulfonic acid) (PIPES, pH 7.0). The amount of reducing sugars produced by the reaction was determined by the 3,5-dinitrosalicylic acid (DNS) method [22]. 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 bivalent ions on hydrolysis activity was measured using a locust bean gum (LBG) as 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. All the activity assays were performed in triplicate. The hydrolysis products were analyzed by using high-performance anion-exchange chromatography with pulsed amperometric detection (HPEAC-PAD) (Dionex, Sunnyvale, CA, USA). A Carbopac PA1 column (4 mm × 250 mm) (Dionex) was used for the separation with an isocratic flow of 100 mM NaOH. 10 mg/mL of LBG was degraded by 50 μg/mL of mannanase at 40 °C.

2.3. Effect of bivalent ions on thermal stability

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

2.4. Isothermal titration calorimetry (ITC)

Bivalent ions were bound to mannanase at 25 °C by using a MicroCal iTC$_{200}$ calorimeter (MicroCal Inc., Northampton, MA, USA) following the manufacturer's instructions. All samples were dialyzed using a 10,000-MW cutoff Amicon® ultra membrane, and the filtrate buffer (50 mM Tris–HCl buffer, pH 7.0, containing 150 mM NaCl) was used to dissolve the ligand ($CaCl_2$, $MgCl_2$, and $MnCl_2$). The protein sample (150–300 μM) was injected with a 0.4-μL aliquot and 18 successive 2-μL aliquots of ligand (2–5 mM) at 120-s intervals. Data were fitted by nonlinear regression using a single-site model (MicroCal Origin), and thermodynamic parameters were calculated using the Gibbs free energy equation ($\Delta G = \Delta H - T\Delta S$) and the relation $-RT \ln K_a = \Delta G$.

3. Results

3.1. Effect of bivalent ions on the hydrolytic activity of mannanases

By using the mannanase mutants (StMandC, TfMandC, StDEDAAAdC, and TfDEDAAAdC), we investigated the effect of bivalent ions on the hydrolytic activity of catalytic domain mutants (Table 1). Here, we did not show the non-ion additive hydrolytic activity compared to the tested bivalent ions, because mannan prepared from natural materials contains various contaminants. To reduce these effects, we used mannan that was pretreated with a small amount of EDTA. Among the tested bivalent ions (calcium, magnesium, manganese, and zinc), the addition of calcium induced the greatest hydrolytic activity. The hydrolytic activity of both enzymes was strongly inhibited by zinc. The effect of NaCl and bivalent ions on hydrolytic activity was almost the same between catalytic domain mutants and wild-type enzymes [17]. The hydrolysis activity of StMandC was slightly enhanced by manganese compared with that of the EDTA additive. The hydrolytic activity of StDEDAAAdC by manganese and magnesium was almost the same in the presence of calcium, indicating that the calcium-binding site contained key amino acid residues that are necessary for binding bivalent ions that enhanced hydrolytic activity of StMandC. The hydrolytic activity of TfMandC and TfDEDAAAdC did not change with addition of bivalent ions compared to that

Table 1
The effects of bivalent ion on mannanase activity.

Metal ion[a]	Relative activities (%)			
	StMandC	StDEDAAAdC	TfMandC	TfDEDAAAdC
EDTA	29 ± 4	75 ± 5	99 ± 2	96 ± 5
NaCl	25 ± 2	80 ± 4	101 ± 3	98 ± 2
Calcium	100 ± 1	100 ± 3	100 ± 6	100 ± 6
Magnesium	96 ± 9	90 ± 3	100 ± 1	98 ± 2
Manganese	58 ± 4	94 ± 5	96 ± 5	91 ± 3
Zinc	0 ± 1	5 ± 2	12 ± 1	8 ± 1

The enzyme reaction was performed at 40 °C for 10 min at pH 7.0 containing 1% (w/v) substrate pre-treated with 1 mM EDTA and 5 mM bivalent ion.

[a] The effect of manganese ion on the hydrolysis activity was measured at 0.1 mM because manganese ion affected the background in the DNS method.