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Essential role of a family-32 carbohydrate-binding module in substrate recognition by *Clostridium thermocellum* mannanase *Ct*Man5A

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ABSTRACT

The family-5 glycoside hydrolase domain (GH5) and the family-32 carbohydrate-binding module (CBM32) of *Clostridium thermocellum* mannanase *Ct*Man5A, along with their genetically inactivated derivatives, were collectively or separately expressed. Their catalytic and substrate-binding abilities were measured to investigate importance of CBM32 in substrate recognition by *Ct*Man5A. Characterization of the truncated derivatives of *Ct*Man5A and isothermal calorimetry analysis of the interaction between the inactivated proteins and mannooligosaccharides suggested that GH5 and CBM32 collectively formed a substrate-binding site capable of accommodating a mannotetraose unit in *Ct*Man5A. This suggested that CBM32 directly participated in the substrate recognition required for catalytic action.

1. Introduction

Glycoside hydrolases (GHs) such as cellulases, xylanases, and mannanases generally consist of catalytic domain(s), carbohydrate-binding module(s) (CBM), and additional functional domain(s), including a dockerin domain and a SLH domain. The catalytic domains of GHs are classified into more than 130 families and the CBMs are grouped into more than 60 families on the basis of amino acid sequence similarities (CAZy database, http://www.cazy.org/) [1]. The catalytic domains consist of the catalytic residues while the CBMs possess the carbohydrate-binding activities but not the catalytic activities. Because both domains form discrete protein folds, the individual domains are usually functional when they are expressed separately.

Possible functions of CBMs include: (i) increasing the substrate concentration in the catalytic domain region, (ii) targeting specific polysaccharides in native biomass such as plant cell walls, and (iii) disrupting or modifying polysaccharide structures [2]. It is rational that the removal of CBM(s) from native enzymes reduces their hydrolytic activities toward insoluble substrates but not soluble substrates. The removal of CBM(s) from modular glycoside hydrolases such as cellulases and xylanases [3–5] or the artificial connection of a CBM and a catalytic domain [6,7] sometimes affects enzyme activity towards insoluble and even soluble substrates. In addition, more drastic effects of the removal of CBMs from native enzymes are sometimes observed, e.g., the removal of a family-3 CBM (CBM3c) from a family-9 GH (GH9) cellulase resulted in an almost complete loss of the original activity against even soluble substrates [8–10]. A similar phenomenon was observed for the family-43 β-xylosidase [11]. In another example, a *Clostridium stercorarium* Xyn10B that is highly active toward barley β-glucan (β-1,3-1,4 mixed glucan) as well as β-1,4-xylan lost only β-1,3-1,4 glucanase activity but not xylanase activity upon removal of its CBM22s [12,13]. These observations suggest the possibility that the presence of CBMs is important for not only orientating GH domains on the surface of insoluble substrates but also for ensuring catalytic activity and specificity in some modular enzymes.

Clostridium thermocellum mannanase *Ct*Man5A is a modular enzyme consisting of a GH5 domain, CBM32 and a dockerin domain from its N terminus (Fig. 1). When a truncated derivative consisting of GH5 and CBM32 (GH5–CBM32) was incubated with mannopentaose, it produced mainly mannotetraose and mannose in the early stage of the reaction. Conversely, a recombinant GH5 polypeptide (GH5) released mainly mannotriose and mannobiose from mannopentaose under the same condition [14]. Differences in the hydrolysis products by GH5–CBM32 and GH5 were observed for other substrates including mannooligosaccharides and ivory nut mannan. These results suggested that CBM32 was directly involved

Abbreviations: *Ct*Man5A, *Clostridium thermocellum* mannanase Man5A; GH, glycoside hydrolase; ITC, isothermal calorimetry

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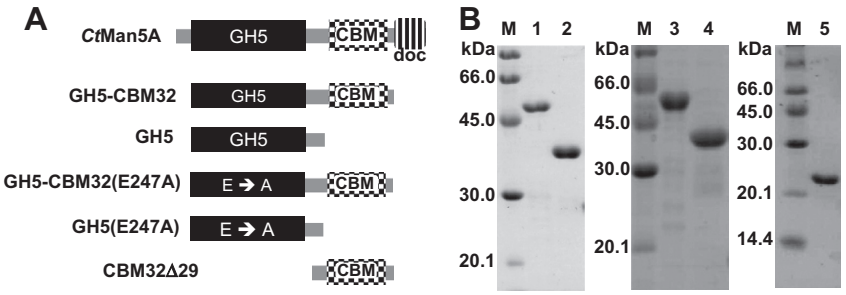

Fig. 1. Schematic diagrams of *Ct*Man5A and its truncated derivatives (A) and SDS–PAGE of the purified recombinant proteins (B). In (A), GH5–CBM32 and GH5 are catalytically active enzyme, GH5–CBM32(E247A) and GH5(E247A) are inactivated enzymes constructed by site-directed mutagenesis, and CBM32Δ29 is a CBM32Δ29 polypeptide. In (B), lane M, protein molecular mass standard (molecular masses shown at the left); lane 1, GH5–CBM32; lane 2, GH5; lane 3, GH5–CBM32(E247A); lane 4, GH5(E247A); lane 5, CBM32Δ29.

in the substrate recognition of *Ct*Man5A [14]. To confirm this hypothesis, we constructed inactive derivatives of GH5–CBM32 and GH5 and determined the binding affinities between these inactive proteins and mannooligosaccharides by isothermal titration calorimetry (ITC).

2. Materials and methods

2.1. Bacterial strain, plasmids, and culture medium

Plasmids for the expression of GH5–CBM32 and GH5 (Fig. 1) were derived from pET-28a(+) (Novagen, Madison, WI, USA) as described previously [14]. *Escherichia coli* BL21-CodonPlus(DE3)-RIPL (Stratagene, La Jolla, CA, USA) carrying a recombinant plasmid was cultivated in Super Broth (3.5% Bacto™ tryptone [BD Diagnostic, Sparks, MD, USA], 2% Bacto™ yeast extract [BD Diagnostic], 0.5% NaCl pH 7.5) supplemented with chloramphenicol (34 μg/mL) and kanamycin (50 μg/mL) at 37 °C for protein expression.

2.2. Expression and purification of CBM32Δ29, GH5–CBM32(E247A), and GH5(E247A)

The gene encoding CBM32 was amplified by PCR from *C. thermocellum* ATCC27405 genomic DNA with KOD-Plus DNA polymerase (Toyobo, Osaka, Japan) and the PCR primer set, 5′-gggg ccatggcggttcgtccggcttcaat-3′ and 5′-gggggtcgacttccgcaattccaccctt tgg-3′ (the *Nco*I and *Sal*I sites are underlined). The resulting PCR fragment was digested with *Nco*I or *Sal*I, ligated into similarly restricted pET-28a(+), yielding the plasmid pET28a(+)–CBM32Δ29. The translated product CBM32Δ29 contained the *Ct*Man5A residues from 321 to 475. The catalytic nucleophile (Glu247) of GH5–CBM32 and GH5 was changed to Ala to obtain clearer data in qualitative and quantitative binding assays. The site-directed mutants of a codon encoding the nucleophile Glu of GH5–CBM32 and GH5 were constructed by using the overlap extension method [15] with pET28a(+)–GH5–CBM32 or pET28a(+)–GH5 as a template and the primers, 5′-ggtgtccgaatGctcccaccac-3′ and 5′-gtggtgg gagCattcggacacc-3′ for mutation (uppercase letters G and C are mutagenized nucleotides). After PCR, the amplified fragments were cloned into pET-28a(+), yielding pGH5–CBM32(E247A) and pGH5 (E247A). These plasmids produce inactivated proteins GH5–CB M32(E247A) and GH5(E247A) in which the catalytic nucleophile Glu are replaced with Ala. All the recombinant proteins contained a 6 × His-tag at the C-termini. The wild-type and mutant protein produced by the recombinant *E. coli* BL21-CodonPlus(DE3)-RIPL carrying a recombinant plasmid were purified by a HiTrap chelating HP column (GE Healthcare Japan, Tokyo, Japan) according to the supplier's protocol. The purity of each fraction was analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) [16]. Protein concentration was determined with bovine serum albumin (BSA) as the standard using a Bio-Rad protein assay kit (Bio-Rad Laboratories, K.K., Tokyo, Japan).

2.3. Preparation of chemically reduced mannopentaose

The chemical reduction of mannopentaose was carried out by converting it to the corresponding non-reducing sugar, mannotetraosylmannitol, as described for cellooligosaccharides [17]. In brief, 20 μL of mannopentaose (10 mg/mL) was mixed with 3 μL of 0.2 mM $NaBH_4$ and incubated at 100 °C for 30 min. Excess $NaBH_4$ was destroyed by addition of 1 μL of 50% acetic acid.

2.4. Analysis of hydrolysis products

Mannopentaose and mannotetraosylmannitol (about 20 μg) were incubated with 0.18 units (determined with konjac glucomannan) of the purified enzymes, GH5–CBM32 or GH5, in 10 μL of 50 mM MES buffer (pH 6.0) at 60 °C. Thin-layer chromatography (TLC) of the hydrolysis products was performed on a Silica gel 60 plate (Merck, Darmstadt, Germany), using a solvent of water:acetic acid:n-butanol (1:1:2). Hydrolysis products were visualized by spraying the plate with 10% sulfuric acid in ethanol followed by incubation at 140 °C.

2.5. Native affinity PAGE

The affinity of GH5–CBM32(E247A), GH5(E247A), and CBM32 for soluble mannans, including konjac glucomannan, carob galactomannan, and guar gum, was examined by native affinity PAGE as described previously [18].

2.6. ITC analysis

The thermodynamic parameters of the binding of GH5–CB M32(E247A), GH5(E247A), and CBM32 to mannooligosaccharides were determined by ITC using a VP-ITC calorimeter (MicroCal, Northampton, MA, USA). Briefly, titrations were performed at 25 °C by injecting 2–10 μL aliquots of 5–20 mM ligand in 50 mM Na-HEPES buffer, pH 7.5, containing 5 mM $CaCl_2$, into the cell containing 100 μM of each protein dialyzed into the Na-HEPES buffer, and the release of heat was recorded. The stoichiometry of binding (n), the association constant K_a, and the binding enthalpy ΔH were evaluated by using MicroCal Origin 7.0 software. The standard Gibbs energy change ΔG^0 and the standard entropy change ΔS^0 were calculated from $\Delta G^0 = -RT \ln K_a$ and $\Delta G^0 = \Delta H^0 - T\Delta S^0$, where R is the gas constant and T is the absolute temperature.