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Effects of Truncation at the Non-homologous Region of a Family 3 β-Glucosidase from *Agrobacterium tumefaciens*

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The function of the non-homologous region of a family 3 β-glucosidase from *Agrobacterium tumefaciens* (Cbg1) was studied by analyzing the properties of mutant enzymes that have internal truncated amino acid sequences in the region. Five truncated mutants named Cbg1-d4, Cbg1-d31, Cbg1-d62, Cbg1-d89, and Cbg1-d119 having deletions of 4, 31, 62, 89, and 119 amino acid residues starting from Phe417, respectively, were expressed in *Escherichia coli* and purified. All the mutants exhibited β-glucosidase activity, indicating that the non-homologous region was not essential for the activity. The truncation caused thermal instability, decrease in pK_a of the proton donor residue (Glu616), and deficient transglycosylation activity. The thermal stability and the pK_a of Glu616 were partially recovered with longer truncation, suggesting that the truncation perturbed the structure and that their presence in the region was not essential. The main role of the non-homologous region could be formation of a hydrophobic atmosphere at the acceptor site to make the enzyme suitable for hydrolyzing hydrophobic glucosides.

Key words: family 3 β-glucosidase; *Agrobacterium tumefaciens*; transglycosylation; truncated mutant

Agrobacterium tumefaciens causes crown gall disease in conifers. In this disease, β-glucosidase (Cbg1) in *A. tumefaciens* hydrolyzes coniferin to form coniferyl alcohol, which leads to a virulence cascade.[1,2] Based on its amino acid sequence, Cbg1 belongs to glycoside hydrolase family 3 (GH3, http://afmb.cnrs-mrs.fr/CAZY/).[2] It is a typical aryl β-glucosidase, exhibiting low K_m values (0.01–0.06 mM) on various aryl β-D-glucopyranosides and a high K_m value on cellobiose (360 mM).[3] Cbg1 possesses wide substrate specificity, being able to hydrolyze various aryl-glycosides of monosaccharides such as β-D-xylopyranoside, β-D-galactopyranoside, and α-L-arabinofuranoside, with comparable k_{cat} values to that of β-D-glucopyranoside. It should be noted that Cbg1 exhibits strong transglycosylation activity toward various primary alcohols.[3]

In GH3, barley β-D-glucan exohydrolase is the only enzyme whose three-dimensional structure is known.[4] The enzyme consists of an N-terminal $(\alpha/\beta)_8$ TIM barrel domain and a C-terminal six-stranded β sandwich, connected by a helix-like strand of 16 amino acid residues. The catalytic center is located in the pocket at the interface of the two domains. Asp285 in the N-terminal domain acts as a catalytic nucleophile, while Glu491 in the C-terminal domain acts as a proton donor.[4,5]

Alignment of the amino acid sequences of Cbg1 with several family 3 β-glucosidases has shown that these enzymes consist of three domains—an N-terminal homologous domain, a non-homologous domain, and a C-terminal homologous domain.[6] Hence the N-terminal and C-terminal homologous regions of Cbg1 might correspond to the N-terminal and C-terminal domains of barley β-D-glucan exohydrolase. Catalytic residues of Cbg1 were presumed to be Asp222 (nucleophile) and Glu616 (proton donor).[7] To investigate the role of the domains on the catalytic characters of the enzymes, chimeric enzymes were designed and constructed[6–9] from Cbg1, a family 3 β-glucosidase from *Cellvibrio gilvus* (CG-bgl),[10] and a family 3 β-glucosidase from *Thermotoga maritima* (TM-bglB).[11] Chimeric enzymes which had their junction in the C-terminal homologous region were expressed in *Escherichia coli* as active soluble enzymes. Most of the chimeric enzymes showed characteristics that were intermediate between those of the respective parental enzymes. On the other hand, none of the chimeric enzymes which had junctions in the N-terminal homologous region were expressed as inactive inclusion bodies. But chimeric enzymes with junctions in the non-homologous region were not investigated because of the difficulty in designing junction sites with completely difference sequences.

In the non-homologous region, the length varies with the enzyme. In particular, Cbg1 has a longer non-homologous region than do CG-bgl or TM-bgl.[6,7] To investigate the role of the long non-homologous region in Cbg1, mutant enzymes with truncated amino acid sequences in the non-homologous region were designed

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and expressed in *E. coli*. We found that the non-homologous region plays an important role in trans-glycosylation.

Materials and Methods

Bacterial strain and plasmid. An expression plasmid, pET28-AT, with an inserted *cbg1* gene (M59852), was prepared as described previously.[12] The schematic structure of pET28-AT is shown in Fig. 1. The plasmid codes for Cbg1 with an additional N-terminal sequence (23 amino acids) of His-tag and a thrombin cleavage site. *E. coli* BL21-Gold competent cells (Stratagene, La Jolla, California, USA) were used as the host for gene expression.

Construction of truncated mutants of Cbg1. Standard recombinant techniques and standard agarose gel electrophoresis were used.[13] As shown in Fig. 1, expression vectors for the truncated mutants of Cbg1 were constructed by swapping the *Eco*RI–*Hin*dIII fragment of pET28-AT with a 5′-end-truncated fragment prepared using a polymerase chain reaction (PCR). To produce the truncated fragments, the five forward primers were designed based on the sequences 12-357 base pairs (bp) downstream of the *Eco*RI site with additional *Eco*RI sequences at the 5′-end as shown below: d4fwd (5′-GG gaa ttc CTT CCG TCC GGC GAC CTT GA-3′), d31fwd (5′-GT gaa ttc ATC TTC GGC ATG ACC AAT G-3′), d62fwd (5′-GT gaa ttc TTT TTT GGA ACC GCG AAC AGC-3′), d89fwd (5′-TC gaa ttc GAG GCG CCG AAG GCC AG-3′), d119fwd (5′-CG gaa ttc GTC GAA ACC GCC CGC AAG T-3′). Additional *Eco*RI sequences are described in lower case letters. A reverse primer, 5′-GTG GCG AGA AAG GAA GGG AAG AAA G-3′, was designed based on the sequence of the pET28a vector (Novagen, Madison, Wisconsin, USA) at approximately 200 bp downstream of the stop codon of the *cbg1* gene. The plasmid pET28-AT was used as a template. KOD Dash polymerase (Toyobo, Osaka, Japan) was used in the PCR amplification. The PCR was carried out at 98 °C for 180 s followed by 30 cycles of 98 °C for 30 s and 72 °C for 90 s. The PCR products and pET28-AT were digested by *Eco*RI and *Hin*dIII, and ligated by a Ligation High Kit (Toyobo) at 16 °C for 16 h. The resultant plasmids were transformed into *E. coli* BL21 by electroporation (*E. coli* Pulser™, Bio-Rad, Hercules, California, USA). The plasmids in the positive colonies were purified with a QIAprep Spin Miniprep Kit (Qiagen GmbH, Hilden, Germany). To confirm the truncated genes, DNA sequencing was done on an Applied Biosystems 310 Genetic Analyzer with a Dye Terminator Cycle Sequencing Kit (Perkin-Elmer, Boston, Massachusetts, USA).

Preparation of the mutants of Cbg1. The positive transformants were grown in 2 ml of LB broth containing kanamycin (30 μg/ml) for 12 hr at 30 °C, then transferred into 100 ml of the same medium containing 0.1 mM (final concentration) isopropyl-thio-β-D-galactopyranoside and incubated for 16 h at 25 °C. Cells were harvested by centrifugation and disrupted in 20 mM phosphate buffer (pH 8.0) by sonication. The crude extract was obtained after centrifugation at 20,000 *g* for 15 min. The crude extracts were partially purified by a Ni-NTA agarose (Qiagen) column equilibrated with

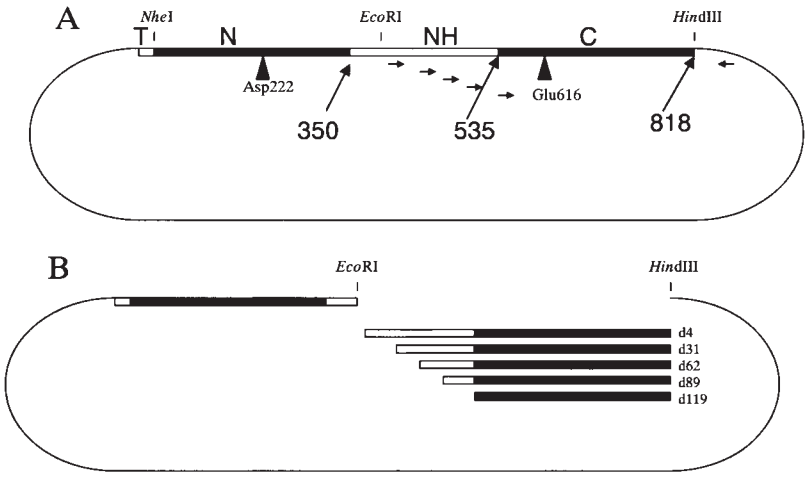

Fig. 1. Construction of the Plasmids Encoding the Truncated Mutants of Cbg1.

A: Schematic structure of pET28AT. T, N, NH, and C represent the regions encoding His-Tag, the N-terminal homologous region, the non-homologous region, and the C-terminal homologous region respectively. Sites for restriction endonucleases are indicated with the name of the enzyme. Right-sided arrows represent the forward primers, and the left-sided arrow indicates the reverse primer. Numbers indicate the positions of amino acid residues. Asp222 and Glu616 indicate the amino acid residues presumed to be a nucleophile and a proton donor respectively. B: pET28-AT and the PCR fragments digested with *Eco*RI and *Hin*dIII. These fragments were ligated to construct the expression vectors encoding the truncated mutants.

20 mM phosphate buffer (pH 8.0) and eluted with the same buffer containing 500 mM imidazole. The active fraction was further purified with a Q-Sepharose anion exchange column (Amersham Biosciences, Piscataway, New Jersey, USA) eluted with a linear gradient (0 to 500 mM) of NaCl in 20 mM MOPS buffer (pH 6.5). The purities of the enzymes were analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE),[14] followed by staining with Coomassie Brilliant Blue.

Measurement of β-glucosidase assay. In the standard assay, the enzymatic reaction was carried out in 25 mM MOPS buffer (pH 6.5) containing 2 mM *p*-nitrophenyl-β-D-glucopyranoside (pNP-Glc) and 0.02% bovine serum albumin (BSA) at 30 °C for 10 min. The reaction was stopped by adding an equal volume of 0.2 M glycine-NaOH buffer (pH 10.5). The concentration of the *p*-nitrophenol liberated was determined by its absorbance at 405 nm, with the molecular extinction coefficient taken to be 18,000 ($M^{-1}cm^{-1}$). One unit of β-glucosidase activity was defined as the amount of the enzyme that releases 1 μmol of *p*-nitrophenol per min under the above conditions.

To determine thermal stability, enzymes were incubated in 25 mM MOPS buffer (pH 6.5) containing 0.02% BSA at various temperatures for 30 min, followed by an activity assay under standard conditions. To determine optimum pH, the enzymatic reaction was carried out under standard conditions except that the following buffers were used: acetate buffer (pH 3.5–5.0), MES [2-(*N*-morpholino) ethanesulfonic acid] buffer (pH 5.1–6.1), MOPS buffer (pH 6.2–8.2), Tris–HCl buffer (pH 8.0–9.0), CHES [2-(*N*-cyclohexylamino) ethanesulfonic acid] buffer (pH 9.0–10.1), and CAPS [3-(cyclohexylamino)-1-propanesulfonic acid] buffer (pH 10.2–11.4).

To determine kinetic parameters, reactions at 6 concentrations of pNP-Glc ranging from 0.2 to 5 K_m were carried out in a spectrophotometer equipped with a sample changer and a temperature controller (DU640, Beckman Coulter, USA). The reactions were started by adding enzymes appropriately diluted with 25 mM MOPS buffer (pH 6.5) containing 0.02% BSA to reaction mixtures containing appropriate concentrations of substrate in the same buffer at 30 °C. Their absorbance at 405 nm was monitored every 30 s for 10 min. A molecular extinction coefficient of 3,290 ($M^{-1}cm^{-1}$) was used to quantify *p*-nitrophenol at pH 6.5. Values of K_m and k_{cat} were calculated using the nonlinear regression analysis program, GraFit Version 3 (Erithacus Software, Ltd., Surrey, UK).

Transglycosylation. Appropriately diluted enzymes were incubated in the reaction mixture in 25 mM MOPS buffer (pH 6.5) containing 0.02% BSA, 20 mM pNP-Glc, and 200 mM 1-propanol for 5 h at 30 °C. The reaction mixture (1 μl), in which approximately 20–40% of the pNP-Glc was cleaved, was spotted on a thin layer chromatography (TLC) plate of silica gel 60 (Merck, 7.5 cm L × 5 cm W). The plate was developed with acetonitrile/water (8/2 v/v), and the products on the plate were visualized by dipping the plate in 5% sulfuric acid in methanol and then heating it at 140 °C for 3 min.

Results

Construction, expression, and purification of truncated Cbg1

Five expression vectors coding truncated β-glucosidases were constructed by swapping the *Eco*RI-*Hin*dIII fragment of pET28-AT into truncated DNA fragments. The nucleotide sequence of each truncated β-glucosidase gene was confirmed by DNA sequencing. The deletions were the only mutations found. The enzymes with deletions of 4, 31, 62, 89, and 119 amino acid residues in the non-homologous region were named Cbg1-d4, Cbg1-d31, Cbg1-d62, Cbg1-d89, and Cbg1-d119 respectively. The mutant enzymes were expressed and purified. All five purified β-glucosidases gave single bands on SDS-PAGE with reasonable mobilities. The molecular masses of Cbg1-d4, Cbg1-d31, Cbg1-d62, Cbg1-d89, and Cbg1-d119 were 90.1, 87.3, 83.9, 80.8, and 77.8 kDa respectively (Fig. 2). The amino acid sequence of the truncated region starting from Phe417 is shown in Fig. 3.

Kinetic parameters

All the purified enzymes showed detectable activity. The kinetic parameters of the enzymes on pNP-Glc are summarized in Table 1. The increase in the K_m values and the decrease in the k_{cat} values corresponded with the increase in the size of the truncation. Drastic increases in K_m were observed, the K_m of Cbg1-d31 being 16-fold that of Cbg1-d4 and the K_m of Cbg1-d119 being 23-fold that of Cbg1-d89. On the other hand, drastic decreases in k_{cat} were observed, the k_{cat} of Cbg1-d31 and Cbg1-d89 being approximately 1/5 those of Cbg-d4 and Cbg1-d62 respectively.

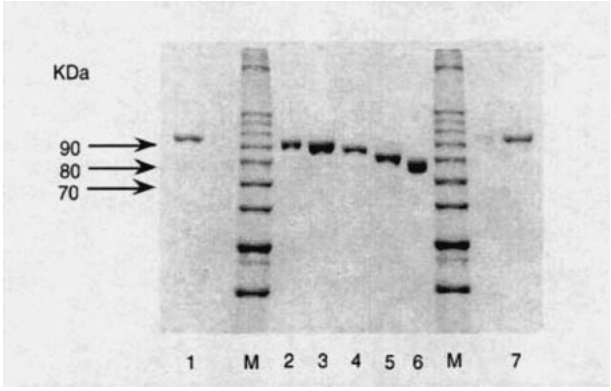

Fig. 2. SDS-PAGE of the Purified Enzymes.
M, Molecular mass standard; 1 and 7, Cbg1; 2, Cbg1-d4; 3, Cbg1-d31; 4, Cbg1-d62; 5, Cbg1-d89; 6, Cbg1-d119.

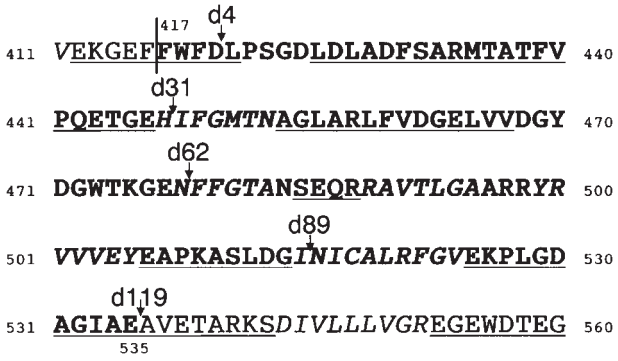

Fig. 3. Amino Acid Sequence of the Truncated Region.

The vertical line indicates the starting position of the truncation (Phe417). Bold characters indicate the truncated sequence in Cbg1-d119. Arrows indicate the end of the truncation in the mutants. Prediction of the secondary structures was performed by using Genetyx Win Ver. 5 software (Genetyx, Tokyo, Japan). Underlined and italic sequences are predicted to form an α-helix and a β-sheet structures respectively.

Table 1. Kinetic Parameters of the Mutants on pNP-Glc

Enzyme	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}mM^{-1}$)
Cbg1	0.013	95	7300
Cbg1-d4	0.030	44	1500
Cbg1-d31	0.49	9.0	18
Cbg1-d62	0.34	11	32
Cbg1-d89	0.39	2.1	5.5
Cbg1-d119	9.1	4.4	0.48

Thermal and pH properties

The thermal stabilities of the mutant enzymes were compared with that of the parental enzyme (Fig. 4). Deletion of the four amino acid residues caused a drastic decrease in thermal stability. The parental enzyme, Cbg1, was stable up to 55 °C, but Cbg1-d4 was stable only up to 25 °C for 30 min. But more deletion caused partial recoveries in thermal stability. Cbg1-d31 and Cbg1-d62 were stable up to 30 °C, and -d89 was stable up to 35 °C.

The pH properties of Cbg1 and the mutants are shown in Fig. 5. The optimum pH values were 7.7 (Cbg1), 7.5 (Cbg1-d4), 5.7 (Cbg1-d31), 5.2 (Cbg1-d62), 6.2 (Cbg1-d89), and 6.6 (Cbg1-d119).

Transglycosylation activity

The reaction products of the pNP-Glc reaction in the presence of 1-propanol were identified on TLC. As shown in Fig. 6, the reactions with Cbg1 and Cbg1-d4 formed propyl-glucoside but no detectable glucose. But Cbg1-d31, Cbg1-d62, Cbg1-d89, and Cbg1-d119 produced glucose and no detectable propyl-glucoside. This difference was not caused by secondary hydrolysis of the transglycosylation product, because each reaction mixture was analyzed before half of the starting substrate (pNP-Glc) was consumed. These results clearly indicate that deletion of more than 31 amino acid residues caused a deficiency in tranglycosylation activity.

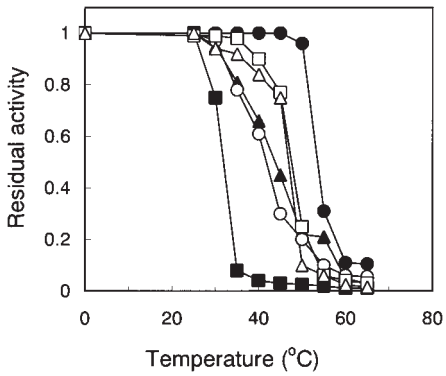

Fig. 4. Thermal Stability Curves.

The activity of each untreated enzyme was defined as 1. Solid circle, Cbg1; solid square, Cbg1-d4; solid triangle, Cbg1-d31; open circle, Cbg1-d62; open square, Cbg1-d89; open triangle, Cbg1-d119. For experimental details see the text.

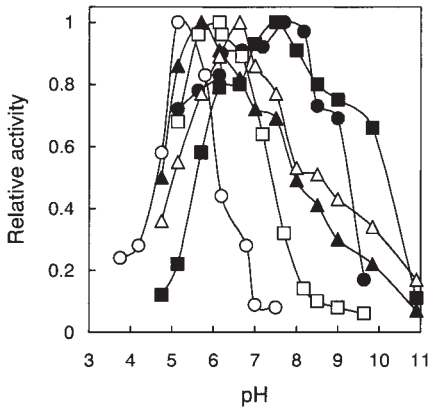

Fig. 5. pH-Activity Curves.

The highest activity of each enzyme was defined as 1. Solid circle, Cbg1; solid square, Cbg1-d4; solid triangle, Cbg1-d31; open circle, Cbg1-d62; open square, Cbg1-d89; open triangle, Cbg1-d119. For experimental details see the text.

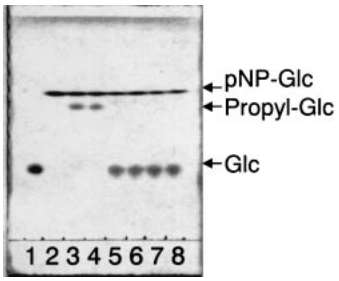

Fig. 6. TLC Analysis of the Reaction Products in the Presence of 1-Propanol.

1, Glc (standard); 2, pNP-Glc (standard), 3, Cbg1; 4, Cbg1-d4; 5, Cbg1-d31; 6, Cbg1-d62; 7, Cbg1-d89; 8, Cbg1-d119.

Discussion

The length of the non-homologous region of Family 3 β-glucosidase varies with the enzyme. But the effect of length on enzymatic characteristics is still unclear. Cbg1 possesses a longer non-homologous region than other enzymes. To investigate the role of the long non-homologous region located at the center of Cbg1, five truncated mutants were constructed and expressed. The non-homologous region was considered to be located between the N-terminal $(\alpha/\beta)_8$ TIM barrel domain and the C-terminal six-stranded β sandwich, with the catalytic center being at their interface.[4,5,7] All the truncated mutants prepared in this study were catalytically active, indicating that the non-homologous region is not essential for the expression of β-glucosidase. However, truncation resulted in shifts in optimum pH, decreases in thermal stability, and decreases in transglycosyl activity.

The truncated mutants showed lower optimum pH than did Cbg1. The pH-activity curves narrowed with truncation (Fig. 5). The narrow curve was most pronounced in the case of Cbg-d62. The widths at the midpoints of the curves were approximately 4.5 (Cbg1), 4.2 (Cbg1-d4), 3.2 (Cbg1-d31), 1.5 (Cbg1-d62), 2.5 (Cbg1-d89), and 3.3 (Cbg1-d119). These curves are narrow only on the basic side. There was no significant expansion on the acidic side. The acidic and basic sides of the pH-activity curve are regulated by dissociation/association of the nucleophile residue and the proton donor residue respectively. This explains the shift in optimum pH with decreases in the pK_a of the proton donor Glu616.

When the truncation was long, the thermal stability and pH optimum partially recovered. These results strongly suggest that the changes in the properties were due to structural perturbation caused by the truncation, rather than by a direct role of the non-homologous region.

Cbg1 exhibits a very low K_m value toward pNP-Glc. The high transglycosyl activity on hydrophobic alcohols suggests that the enzyme has a strong affinity toward hydrophobic compounds at the acceptor site. The truncation caused an increase in the K_m values of pNP-Glc and also a deficiency in transglycosylation. The transglycosylation was not recovered by the longer truncation, suggesting that this is the role of the non-homologous region.

A drastic increase in K_m toward pNP-Glc was observed, the K_m of Cbg1-d31 being 16-fold that of Cbg1-d4 (Table 1), where the transglycosyl activity disappeared (Fig. 6). The ratio of the hydrophobic amino acid residues in the truncated sequence of Cbg1-d31 from Cbg1-d4 (Leu421–His447) is 52% (14/27), and is not significantly different from that of the whole sequence of Cbg1 (56%, 461/818), indicating that the deficiency in the transglycosylation was not caused by the overall hydrophobicity of the region. The sequence analysis predicted that most of the region (Leu426–Glu446) formed an α-helix structure as shown in Fig. 3. Thus, it is suggested that the α-helix plays an important role in maintaining a strong affinity toward hydrophobic compounds at the acceptor site.

Acknowledgments

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