Crystallization and crystallographic analysis of branching enzymes from *Cyanothece* sp. ATCC 51142

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Several cyanobacterial species, including *Cyanothece* sp. ATCC 51142, remarkably have four isoforms of α -glucan branching enzymes (BEs). Based on their primary structures, they are classified into glycoside hydrolase (GH) family 13 (BE1, BE2 and BE3) or family 57 (GH57 BE). In the present study, GH13-type BEs from *Cyanothece* sp. ATCC 51142 (BE1, BE2 and BE3) have been overexpressed in *Escherichia coli* and biochemically characterized. The recombinant BE1 was crystallized by the hanging-drop vapour-diffusion method. Crystals of BE1 were obtained at 293 K in the presence of 0.2 M Mg^{2+} , 7–10% (w/v) ethanol, 0.1 M HEPES–NaOH pH 7.2–7.9. The crystals belonged to the tetragonal space group $P4_12_12$, with unit-cell parameters $a = b = 133.75$, $c = 185.90$ Å, and diffracted to beyond 1.85 Å resolution. Matthews coefficient calculations suggested that the crystals of BE1 contained two molecules in the asymmetric unit.

1. Introduction

Branching enzyme (BE; EC 2.4.1.18) catalyzes the intra-molecular or intermolecular transglucosylation of α -1,4-glucan to produce an α -1,6 branch point (Tetlow & Emes, 2014) and therefore serves as an important determinant for the molecular structure of α -glucans such as glycogen and amylopectin. Animals and fungi have a single BE, while plants have two (or three in cereal plants) BE isoforms. The BEs in eukaryotes are closely related in their amino-acid sequences and are classified into glycoside hydrolase (GH) family 13 subfamily 8 (GH13_8; Stam *et al.*, 2006; Cantarel *et al.*, 2009; <http://www.cazy.org/>). In contrast, two types of BE have been reported in prokaryotes. These prokaryotic BEs belong to GH13_9 (moderately related to the GH13_8 proteins in eukaryotes) and GH57, and their primary structures are quite different from each other (Murakami *et al.*, 2006). Most cyanobacteria have one GH13_9 BE (the principal BE or BE1) and one GH57 BE (Colleoni & Suzuki, 2012), although the latter has not been characterized. Furthermore, several cyanobacteria have two additional isoforms of GH13_9 BEs (BE2 and BE3; Suzuki *et al.*, 2013). The occurrence of multiple GH13_9 BEs is intimately correlated with the production of amylopectin-like α -glucans rather than glycogen in the latter type of cyanobacteria (Suzuki *et al.*, 2013, 2015).

The catalytic specificities (the preferred lengths of the glucan chains produced) have been examined for three BE

Table 1

Macromolecule-production information.

Source organism	<i>Cyanotheca</i> sp. ATCC 51142
DNA source	Genomic DNA
Forward primers†	
BE1	5'-CATATGACCACCACCATTTCCGCCGATC-3'
BE2	5'-CATATGAACGACAACGCAACTGC-3'
BE3	5'-CATATGAAAGAACCGTCCCTCGAAA-3'
Reverse primers	
BE1	5'-TTACTCTTCTTCGTCGGCAGC-3'
BE2	5'-CTATCGCTTTAATTATACAGTACAGCAC-3'
BE3	5'-CTAAGGATGATTTTTTAATTT-3'
Cloning vector	pET-15b
Expression vector	pET-15b
Expression host	<i>E. coli</i>
Complete amino-acid sequence of the construct produced	
BE1	MGSSHHHHHSSGLVPRGSHMTTISADQVNQIIYNLHDPFEILGCHLLEEGKNTKKWVVRAYLPAKAAAWVIRPTERKEDPMNSVHHPNFFEC-I IETPELNHYQLKVKEGEHEKVIYDPAFSSPYLTDEIYLFSEGNHHRIYEKLAHVGEINGVKGVYFAVWAPNARNVSVIGDFNNWDGREH- QMRKRNYTIWELFVPEIGSGTVYKYEIKNSEGHIYEKSDPYGFYREVRPNTASIVVDIDNIYQWHDDEWLEKRRNSDPLKQPVSVYEVHLGWS- LHGSSAEKMPLLNGEADPVIWSEWNPGRFLSYELAEKLIPYVKDMGYTHIELPIAEHPFDGSGWGYQVTGFYSPTSRFRPEDFMYFVDDKC- HENGIGVILDWVPGHFKDGLAYFDGTHLYEHADPRIGEHEKWTGLVFNYGRHEVRNFLVANVLFWFDKYHVDGIRVDVAVSMLYRNYLRK- EGEWIANEYGGDEHIEAVSFIREVNTLLFEYFPGILSIAEESTEWKVSVPYDGGGLGNLKWDMGWMHMDLDYFNIDPYFRQYHQNNVTFSM- LYYYNENFMLALSHDEIVHGKSNMLGKMPGDEWQKYANVRALFTYMYTHPGKKTMFMSMEFGQWSEWNVWGDLEWHLLQYEPHQQLKQFFTDL- NALYQQEPALYTHDFEYHGFIEWIDCNDNTHSVVSLRRSDPNDSLVRVNCNFTPQPHSHYRIGVPEAGYYVELFNSDAKQYGSNMGNLGGKW- ADEWSFHNKPYSLDCLPLPLAVLILKLDPTKVPEGTTIKEIAADEEE
BE2	MGSSHHHHHSSGLVPRGSHMNDNATAKNRLLGEFDYYLFGQGDHRIYEKMGHAHLVEIDGVSQVYFATWAPNAYEVAVVGDFNNWNPEANKMER- TEVGIWELFIPGMKVGDLKYSVKNNHHHCYKTDPPYGYQGEVRPATASIVADLSTYTWNDQGWMDKRETNPEKQPMVSVEVHLGWSLHDSI- DNPPKEGSLPVEQKPGARYLNYRELAELIPYVKDMGYTHIELLPVTEYPPFDGSGWGYQVVGFYAPTSTRYGPQDFMYFVDDKCHEAGIGVILD- WVPGHFKDQGLAYFDGGLYAYADPRKGEIKTWGLTFVDFYGRNEVRNFLIASAMFWFDKYHIDGIRVDVAVYMIELDKGREGNWWPNKYGD- NGHLEAISFLQHLNNTIAHNPYGVVTIAEDSTAWGNVSKPASEGGLGFTFKWNGMWMNDTLKYLKEHPNHRSNCHNKLTFSIWYAFDEKFLA- LSHDEVVHLKGHLVNKMPGDEWQKMANVRTLLTYMFAPGKKTFLMGMEFGQTSEWQEFIDWYLLQYEPHKGLKRLVQDLNKMVYREPALY- MEDFSREGFEWIEANDAPRGLISFIRRDPAASGDTIVAVCNFKPYVYEEYVWGLHQPGYVELINSDAQVYWGSGVSNPSVIQARQWNPAPWY- ALEITVPPLGAVLYKLKR
BE3	MGSSHHHHHSSGLVPRGSHVKEPSLETIKQDPYQYFGAHPNETETTFRVPYAPRATGVNLMREGTGWDCFAEPMTKNEDGVWEITIPENLTWSE- YKYYIENSDDYLNQPYGMARIDPFSPQLAVTWGNDGTRNFNSIVCDRNYFQWTHKHIDLPHPEMSIYEMHLSTFFHDGNYRDI AHKIVDHMSYM- GFTHVQIMPPFQTPIHQSWGLVGCPIAYIERHGTVDVDFKYLNVHCHAHGIGVIVDVPVLFQVQDWDCCGLANYDGTDLHYHSSGRGWNQWNS- RIYNVGDYVKNYLIGLCTLYLHELIGIDGARIDAVASQIFFDYDRGFWDWPRNDREQVELENWELFNNLGGDRYFEERYWLSAEDVAFGLRF- LRDLHARLQHTAPKFITIAEESRRVFPKLACPVDEGGLGFTYAQNMGMHRIRSYLQIPIEHRLIEHIELIHNNSAEKMVNAMNTHDECANG- KVRLITELGNHILIGLAALCWFRPGAMIFQGDEFGEEGYFDVFGHVDWSKTGPYAALHQQISNNFHDNLQLLRHEPALARHGINSMTIRNG- SNNERKWFSEIRWGDVGFESSDPKDKDDIIFVRNETPYPECHAEIYVPVAAEYRVIYNSIDQRYIGSQHYNQHPDYWTIHSAGQLFYFDL- HPYQNIALKLNHP

† NdeI restriction sites are underlined.

isoforms (BE1, BE2 and BE3) from *Cyanobacterium* sp. NBRC 102756 (abbreviated as NBRC 102756; Suzuki *et al.*, 2015). BE1 and BE2 predominantly produced short glucan chains with a degree of polymerization (DP) of 6, 7 and 10, whereas BE3 produced short glucan chains (DP 6–12) as well as long glucans (DP 30–37). These specificities coincided with those of the rice BE isoforms OsBEIIb (for BE1 and BE2) and OsBEI (for BE3), indicating the functional differentiation for the synthesis of amylopectin and related polysaccharides in different organisms.

Enzymatic specificities have been determined for BEs from various organisms (humans, yeast, microalgae and *Escherichia coli*) and classified into three types corresponding to the three BE isoforms of rice: OsBEI, OsBEIIa and OsBEIIb (Sawada *et al.*, 2013, 2014). However, no correlation was readily obvious between the primary structure and catalytic specificity of BE. In this respect, the crystal structures of the enzyme, especially in complex with the substrate or product, will greatly improve our understanding of the catalytic specificity. In this study, we report the crystallization and preliminary X-ray studies of BE1 from *Cyanotheca* sp. ATCC 51142 (abbreviated as ATCC 51142). In addition, biochemical characterization of BE1, BE2 and BE3 is reported.

2. Materials and methods

2.1. Macromolecule production

The *cce_2248* gene encoding BE1 (2322 bp), the *cce_4595* gene encoding BE2 (1956 bp) and the *cce_1806* gene encoding BE3 (1944 bp) were amplified by PCR using genomic DNA of ATCC 51142 as the template. The sequences of all primers used in this study are listed in Table 1. The amplified DNA fragments were inserted into the pGEM-T Easy vector (Promega, Madison, Wisconsin, USA). The plasmids were digested at two NdeI sites (one is in the multicloning site of the pGEM-T Easy vector and the other is introduced by PCR amplification) and then inserted into the NdeI site of the expression vector pET-15b (Merck Millipore, Billerica, Massachusetts, USA). The expression plasmids have a sequence encoding a His₆ tag at the N-terminus to facilitate purification of the recombinant proteins using an Ni²⁺-affinity column.

Expression of the genes in *E. coli* and purification of the recombinant enzymes were performed as described previously (Suzuki *et al.*, 2015). The purity and homogeneity of the recombinant enzymes were checked by SDS-PAGE (Fig. 1).

Table 2
Crystallization.

Method	Hanging-drop vapour diffusion
Plate type	24-well plate
Temperature (K)	293
Protein concentration (mg ml ⁻¹)	15
Buffer composition of protein solution	5 mM HEPES–NaOH pH 7.0
Composition of reservoir solution	0.2 M MgCl ₂ , 7–10%(w/v) ethanol, 0.1 M HEPES–NaOH pH 7.2–7.9
Volume and ratio of drop	6 µl, 1:1
Volume of reservoir (µl)	500

Amylose type III from potato (Sigma, St Louis, Missouri, USA) was treated with *Pseudomonas amyloclavata* isomylase (Hayashibara, Okayama, Japan) and *Klebsiella pneumoniae* pullulanase (Wako Pure Chemical Industries Ltd) and used as a substrate (Suzuki *et al.*, 2015). Amylopectin from potato starch was purchased from Sigma. The specific activities of recombinant BEs were determined by iodine-staining assay (Suzuki *et al.*, 2015). Chain-length distribution analyses of the BE reaction products using capillary electrophoresis were carried out as described previously (Suzuki *et al.*, 2015). Detailed procedures are described in the caption to Supplementary Fig. S1.

2.2. Crystallization

Preliminary screening of crystallization conditions for BE1, BE2 and BE3 was performed using the commercial Crystal Screen and Crystal Screen 2 kits (Hampton Research, Aliso Viejo, California, USA) and Wizard Classics 1–4 (Emerald Bio, Bainbridge Island, Washington, USA). Crystallization experiments were performed manually using the sitting-drop vapour-diffusion method at 288 and 293 K. Initial crystals of BE1 were obtained using condition No. 23 of the Wizard Classic 2 kit [0.2 M MgCl₂, 15%(w/v) ethanol, 0.1 M HEPES–NaOH pH 7.5], but no crystals of BE2 and BE3 were obtained. The crystallization conditions of BE1 were optimized manu-

Figure 1
SDS–PAGE analysis of purified recombinant preparations (BE1, BE2 and BE3). Lane 1, BE1; lane 2, BE2; lane 3, BE3; lane M, molecular-weight markers (labelled in kDa).

Table 3
Data-collection and processing statistics.

Values in parentheses are for the highest resolution shell.

Diffraction source	BL-5A, PF, KEK
Wavelength (Å)	1.00000
Temperature (K)	100
Detector	ADSC Quantum 315r
Crystal-to-detector distance (mm)	231.7
Rotation range per image (°)	0.5
Total rotation range (°)	180
Exposure time per image (s)	1
Space group	<i>P</i> 4 ₁ 2 ₁ 2
<i>a</i> , <i>b</i> , <i>c</i> (Å)	133.75, 133.75, 185.90
α , β , γ (°)	90.0, 90.0, 90.0
Mosaicity (°)	0.14–0.29
Resolution range (Å)	50.0–1.85 (1.88–1.85)
Total No. of reflections	2096822
No. of unique reflections	143511
Completeness (%)	100.0 (100.0)
Multiplicity	14.6 (14.8)
$\langle I/\sigma(I) \rangle$	61.0 (10.7)
<i>R</i> _{meas}	0.082 (0.313)
Overall <i>B</i> factor from Wilson plot (Å ²)	19.2

ally. Under the optimized conditions using the hanging-drop vapour-diffusion method, 3 µl protein solution (15 mg ml⁻¹) was mixed with an equal volume of reservoir solution [0.2 M MgCl₂, 7–10%(w/v) ethanol, 0.1 M HEPES–NaOH pH 7.2–7.9] and equilibrated against 500 µl reservoir solution at 293 K. Crystals of BE1 suitable for X-ray analysis were obtained within a few weeks (Fig. 2). Crystallization information is summarized in Table 2.

2.3. Data collection and processing

The crystals were quickly soaked in a cryoprotectant solution consisting of 25%(v/v) glycerol in the reservoir solution. The crystals were mounted in nylon-fibre loops (Hampton Research) and flash-cooled in liquid nitrogen at 100 K. Diffraction experiments were conducted on beamline BL-5A at the Photon Factory (PF), High Energy Accelerator Research Organization (KEK), Tsukuba, Japan (λ = 1.00000 Å). Diffraction data were collected using a Quantum

Figure 2
A crystal of BE1 grown by the hanging-drop method.

315r CCD X-ray detector (Area Detector Systems Corporation, Poway, California, USA) with an oscillation angle of 0.5° over a range of 180° with an exposure time of 1 s per frame. The images were processed and scaled using *DENZO* and *SCALEPACK* from the *HKL-2000* package (Otwinowski & Minor, 1997). The data-collection statistics are shown in Table 3.

3. Results and discussion

Recombinant BE1, BE2 and BE3 were overexpressed in *E. coli* and purified to homogeneity by Ni^{2+} -affinity and gel-filtration chromatography. The predicted molecular masses of BE1, BE2 and BE3 with an N-terminal His₆-tag sequence (MGSSHHHHHSSGLVPRGSH) calculated from the amino-acid sequences were 92 573.6, 77 380.3 and 77 486.5 Da, respectively. As judged from the molecular masses of BE1 (50 kDa), BE2 (95 kDa) and BE3 (63 kDa) estimated by gel-filtration chromatography (Supplementary Fig. S2), each recombinant preparation was a monomer in solution. The molecular mass of BE1 estimated by gel-filtration chromatography was much lower than the predicted value. Purified BE1 was detected as a single band at 80.0 kDa by SDS-PAGE (Fig. 1), indicating that the polypeptide chain was not degraded. This discrepancy may be owing to the molecular shape of BE1, which may deviate from a globular form, and/or interaction of BE1 with the Superdex resin, as reported for BE from rabbit (Caudwell & Cohen, 1980).

The activities of recombinant preparations of BE1, BE2 and BE3 were measured by an iodine-staining assay using 1 mg ml^{-1} amylose and 5 mg ml^{-1} potato amylopectin as the substrates. The specific activities of BE1, BE2 and BE3 for potato amylopectin were 973 ± 56 , 1350 ± 43 and $30 \pm 4 \text{ U mg}^{-1}$, respectively. The specific activities of BE1, BE2 and BE3 for amylose were 2030 ± 22 , 2270 ± 29 and $32 \pm 2 \text{ U mg}^{-1}$, respectively. The specific activities were similar to the values observed for the corresponding BE isoforms from NBRC 102756 (Suzuki *et al.*, 2015). The result may reflect the high similarities of the amino-acid sequences of BE1 (71.3%), BE2 (63.7%) and BE3 (81.3%) between strains ATCC 51142 and NBRC 102756.

The chain-length distributions of the products formed from the BE1, BE2 and BE3 reactions were analyzed using *ae*-amylopectin (Nakamura *et al.*, 2010; Sawada *et al.*, 2013; Suzuki *et al.*, 2015) as a substrate. The chain-length distribution of the *ae*-amylopectin substrate before reaction with BE showed a major peak at DP 14, as reported previously (Supplementary Figs. S1a, S1b and S1c; Nakamura *et al.*, 2010; Sawada *et al.*, 2013). In the reaction products of BE1 and BE2, large peaks of DP 6–7 and a minor peak at DP 10 were observed (Supplementary Figs. S1a and S1b). These chain-length distribution patterns were similar to that of OsBEIIb (Nakamura *et al.*, 2010; Sawada *et al.*, 2013). In contrast, BE3 did not produce large peaks with specific chain lengths, but led to increases in short (DP 5–11) and long (DP 30–35) chains (Supplementary Fig. S1c). The chain-length distribution shown for BE3 was similar to that of OsBEI in terms of

producing long chains (Nakamura *et al.*, 2010; Sawada *et al.*, 2013). The characteristics of the BE isoforms in ATCC 51142 were essentially the same as those of the counterpart isoforms in NBRC 102756 (Suzuki *et al.*, 2015).

BE1, BE2 and BE3 showed higher specific activities against amylose than amylopectin, indicating that cyanobacterial BEs prefer linear substrates to branched substrates. The substrate preferences of other bacterial GH13_9 BEs from *E. coli* (EcBE; Guan *et al.*, 1997), *Deinococcus geothermalis* and *D. radiodurans* (Palomo *et al.*, 2009) are not uniform (Supplementary Table S1). Plant type I BEs (ZmBEI from *Zea mays* and OsBEI) and type II BEs (ZmBEIIb from *Z. mays*, OsBEIIa and OsBEIIb) preferred amylose and amylopectin, respectively (Supplementary Table S1; Guan *et al.*, 1997; Nakamura *et al.*, 2010). GH13_8 BEs from plants therefore showed a clearer correlation between chain-length distribution of the reaction product and substrate preference than GH13_9 BEs from bacteria.

We next attempted to crystallize BE1, BE2 and BE3. Good-quality single crystals of BE1 were obtained (Fig. 2), whereas BE2 and BE3 have not been crystallized. The crystals of BE1 diffracted to beyond $1.85 \text{ \AA}$ resolution and were suitable for structural analysis. As shown from the data-collection statistics, the crystals belonged to the tetragonal space group $P4_12_12$, with unit-cell parameters $a = b = 133.75$, $c = 185.90 \text{ \AA}$ (Table 3). The V_M value of the crystal was calculated to be $2.3 \text{ \AA}^3 \text{ Da}^{-1}$, assuming that two molecules were located in the asymmetric unit, and this corresponded to an estimated solvent content of 46.5% (Matthews, 1968).

The crystal structure of a BE with BEIIb-type catalytic specificity remains to be elucidated. Moreover, no information is available for the structure of a BE from a starch-producing prokaryote. Therefore, it will be of interest to compare the structure of BE1 from ATCC 51142 (BEIIb-type) with those from glycogen-producing bacteria (EcBE, BEI-type; Abad *et al.*, 2002) and starch-producing plants (OsBEI, BEI-type; Noguchi *et al.*, 2011; Chaen *et al.*, 2012). Attempts to determine the structure of BE1 by the molecular-replacement method using EcBE (47.9% identity; Abad *et al.*, 2002), BE from *Mycobacterium tuberculosis* (44.5% identity; Pal *et al.*, 2010), BE from *Homo sapiens* (26.4% identity; PDB entry 4bzy; Structural Genomics Consortium, unpublished work) and OsBEI (24.0% identity; Noguchi *et al.*, 2011) as search models are currently under way.

4. Related literature

The following references are cited in the Supporting Information for this article: Nakamura (2002), Nishi *et al.* (2001), O'Shea *et al.* (1998) and Utsumi & Nakamura (2006).

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