Structural snapshot of a glycoside hydrolase family 8 endo-β-1,4-glucanase capturing the state after cleavage of the scissile bond

Takaaki Fujiwara, a Ayumi Fujishima, b Yui Nakamura, b Kenji Tajima c and Min Yao a,b,*

aFaculty of Advanced Life Science, Hokkaido University, Kita-10, Nishi-8, Kita-ku, Sapporo 060-0180, Japan, bGraduate School of Life Science, Hokkaido University, Kita-10, Nishi-8, Kita-ku, Sapporo 060-0180, Japan, and cFaculty of Engineering, Hokkaido University, Kita-13, Nishi-8, Kita-ku, Sapporo 060-8628, Japan. *Correspondence e-mail: yao@castor.sci.hokudai.ac.jp

Bacterial cellulose (BC), which is produced by bacteria, is a biodegradable and biocompatible natural resource. Because of its remarkable physicochemical properties, BC has attracted attention for the development and manufacture of biomedical and industrial materials. In the BC production system, the enzyme endo-β-1,4-glucanase, which belongs to glycoside hydrolase family 8 (GH8), acts as a cleaner by trimming disordered cellulose fibers to produce high-quality BC. Understanding the molecular mechanism of the endo-β-1,4-glucanase would help in developing a reasonable biosynthesis of BC. Nevertheless, all of the steps in the reaction of this endo-β-1,4-glucanase are not clear. This study confirms the BC hydrolytic activity of the endo-β-1,4-glucanase from the BC-producing bacterium Enterobacter sp. CJF-002 (EbBcsZ) and reports crystal structures of EbBcsZ. Unlike in previously reported GH8 endo-β-1,4-glucanase structures, here the base catalyst was mutated (D242A) and the structure of this mutant bound to cellooligosaccharide [EbBcsZ(D242A)_CPT] was analyzed. The EbBcsZ (D242A)_CPT structure showed two cellooligosaccharides individually bound to the plus and minus subsites of EbBcsZ. The glucosyl unit in subsite −1 presented a distorted S1 conformation, a novel snapshot of a state immediately after scissile-bond cleavage. In combination with previous studies, the reaction process of endo-β-1,4-glucanase is described and the β-1,4-glucan-trimming mechanism of EbBcsZ is proposed. The EbBcsZ(D242A)_CPT structure also showed an additional β-1,4-glucan binding site on the EbBcsZ surface, which may help to accept the substrate.

1. Introduction

Worldwide, cellulose (β-1,4-glucan) production mainly relies on plants, but some bacteria can also produce a type of cellulose named bacterial cellulose (BC; Somerville, 2006; Ross et al., 1991). Cellulose-producing bacteria secrete BC as an exopolysaccharide, forming a hierarchical assembly outside the cell. Unlike the lignocellulosic matrix derived from plant cell walls, BC displays a naturally high purity and high crystallinity (Chundawat et al., 2011; Czaja et al., 2004). BC also has high mechanical strength (Yamanaka et al., 1989), biodegradability (Torgbo & Sukyai, 2020) and biocompatibility (Helenius et al., 2006). These unique characteristics make BC an attractive environmentally friendly resource for the development and manufacture of biomedical or industrial materials such as wound dressings (Czaja et al., 2006), artificial blood vessels (Lee & Park, 2017), electronic paper displays (Nogi & Yano, 2008; Shah & Brown, 2005) and speaker diaphragms (Nishi et al., 1990). Despite numerous efforts to
improve BC production (Lee et al., 2014), such as optimization of the composition of culture media, bioreactors and bacterial strains, bacteria do not satisfy the demand for BC. Understanding the mechanism of BC biosynthesis will therefore help to produce BC more efficiently, and has become a hot topic in BC research.

Cellulose-producing bacteria biosynthesize BC using a multi-protein complex (terminal complex; TC) localized on the outer and inner membranes. *Komagataeibacter xylinus* (formerly called *Acetobacter xylinum*) serves as a model bacterium for BC biosynthesis. In *K. xylinus*, the TC is composed of BC synthase subunits A, B, C and D (BcsA, BcsB, BcsC and BcsD; or alternatively BcsAB, BcsC and BcsD; Kawano et al., 2002; Wong et al., 1990). Analyzing the individual structures of the subunits clarified their roles in BC biosynthesis (Acheson et al., 2019; Nojima et al., 2017; Fujiwara et al., 2013; Morgan et al., 2013, 2016; Hu et al., 2010). The production of high-quality BC depends on both the TC and some accessory factors such as cleaner proteins. The *cmc* gene, which is located upstream of the BC synthase operon in *K. xylinus* (formerly called *Acetobacter xylinum*) encodes CMCAx, an endo-β-1,4-glucanase (EC 3.2.1.4) that hydrolyzes carboxymethyl cellulose (CMC) and cellobiose (Yasutake et al., 2006; Standal et al., 1994). Disruption of the *cmc* gene reduced the amount of BC produced (Nakai et al., 2013), while overexpression of CMCAx increased it (Kawano et al., 2013). Furthermore, adding a CMCAx antibody to the culture medium inhibited the formation of BC fibers (Koo et al., 1998). Considering the hydrolytic activity of CMCAx, endo-β-1,4-glucanase could act as a cleaner protein to produce high-quality BC by trimming disordered cellulose. Interestingly, BcsZ, an endo-β-1,4-glucanase encoded in the BC synthase operon, also exists in most bacterial lineages (Römling & Galperin, 2015). Recent studies showed that *Enterobacter* sp. CIJ-002, isolated from rocks in oil reservoirs, efficiently produces BC and is a promising bacterium for producing large amounts of BC (Sunagawa et al., 2012). In *Enterobacter* sp. CIJ-002, a single operon encodes BcsA, BcsB, BcsZ and BcsC. Although CMCAx and BcsZ have low sequence similarity (19% identity), the residues located in the catalytic site are highly conserved (Supplementary Fig. S1). BcsZ from *Enterobacter* sp. CIJ-002 (EcBcsZ; GenBank accession No. BAM44856) and cellulolytic *Escherichia coli* (EcBcsZ) also hydrolize cellobiose saccharides and CMC (Pang et al., 2019; Sunagawa et al., 2012; Mazur & Zimmer, 2011), indicating that BcsZ and CMCAx are functionally homologous. Therefore, a biological function of EcBcsZ could be to promote the formation of cellulose fibers by degrading β-1,4-glucan chains (Mazur & Zimmer, 2011).

Similar to endo-β-1,4-xylanases (EC 3.2.1.8), licheninases (EC 3.2.1.73), chitosanases (EC 3.2.1.132) and reducing-end xylose-releasing exo-oligoxylanases (EC 3.2.1.156), CMCAx and BcsZ belong to glycoside hydroylase family 8 (GH8; CAZY; http://www.cazy.org). The catalytic reaction of GH8 enzymes is a single-displacement mechanism accompanied by an inversion of the anomeric configuration of the sugar unit in subsite −1, which changes the conformation of pyranose from β-1C1 to α-4C1 (Koshland, 1953; Cremer & Pople, 1975; Fig. 1, Supplementary Fig. S1). The general acid catalyst donates a proton to the C1 atom to release the hemiacetal sugar. The general base catalyst activates a water molecule for a nucleophilic attack on the anomic center (C1) of the substrate. Displacement of the activated water molecule and liberation of the leaving group of the product occur on the C1 atom of a distorted oxocarbenium ion intermediate, forming an α-configuration at the C1 atom. To understand the reaction mechanism of GH8 enzymes, structures of GH8 enzymes and their acid catalyst mutants bound to oligosaccharides have been analyzed to date. In the structure of the E55Q mutant of EcBcsZ bound to cellopentaose (CPT) [EcBcsZ(E55Q)_{CP}; PDB entry 3qxq], the glucosyl unit in subsite −1 (G_−1) presented an α-4C1 conformation, showing that EcBcsZ (E55Q)_{CP} is the product-binding form (Mazur & Zimmer, 2011). In contrast, the structure of the E95Q mutant of CelA from *Clostridium thermocellum* bound to CPT [CelA(E95Q)_{CP}; PDB entry 1kwf] showed that G_−1 presented a 2,5B conformation and thus CelA(E95Q)_{CP} was an intermediate in the transition state (Guérin et al., 2002). Interestingly, the structure of wild-type endo-β-1,4-xylanase from *Teredinibacter turnerae* bound to xylotriose (XTO) (TGHXSTO; PDB entry 6g9b) confirmed the existence of a 2,5B conformation of the xylosyl unit in subsite −1 (X_−1; Fowler et al., 2018). Based on endo-β-1,4-glucanase.

**Figure 1**

General reaction mechanism of inverting glycoside hydrolases. The upper part shows the proposed conformational changes of the sugar in subsite −1 (G_−1). β-1C1, 2,5B and α-4C1 are the conformations of G_−1 in the substrate, oxocarbenium ion intermediate (transition state) and product, respectively. 2S_o and 5S_i are the conformations of the other intermediates. The lower part shows the proposed catalytic process of GH8 enzymes. The reaction follows the single-displacement mechanism through the oxocarbenium ion intermediate. The numbers in the left part represent the order of the displacement reaction, i.e. protonation of the glycosidic bond occurs followed by nucleophilic attack of an activated water molecule. G_−1 is shown as a six-membered sugar. The substrate, transition intermediate and product are shown in the left, middle and right panels, respectively. Before and after forming the oxocarbenium intermediate, G_−1 forms 2S_o and 5S_i conformations, respectively.
on these structures, a computational simulation predicted that in the reaction process of GH8 enzymes the $G_{-1}$ conformational change sequence was $\beta^+c_1 \rightarrow S_{0} \rightarrow ^{2}\cdot B \rightarrow S_{1} \rightarrow \alpha^+c_1$ (Ardévol & Rovira, 2015; Petersen et al., 2009; Fig. 1). However, the coordinates of $S_{0}$ and $S_{1}$ intermediates remain unclear since no structural data are available to prove the existence of such intermediates of GH8 enzymes.

In this study, we assessed the hydrolytic activity of EbBcsZ on BC produced by K. xylinus. Based on the idea that BcsZ hydrolizes cellulose through an inverting mechanism, we mutated the base catalyst Asp242 to alanine (D242A) and by adding 100 mM potassium chloride and 10% (v/v) glycerol in a linear gradient of glycerol from 50 to 1 M buffer [50 mM HEPES–KO buffer pH 8.0 containing 150 mM potassium chloride]. We introduced the D242A mutation using the designed primers by the QuikChange technique and confirmed it by sequencing. We prepared the EbBcsZ(D242A) mutant as described above for wild-type EbBcsZ. We concentrated the purified EbBcsZ and EbBcsZ(D242A) to a final concentration of 9 mg ml$^{-1}$ by ultrafiltration using Vivaspin 6 concentrators with a 10 kDa cutoff membrane (Cytiva).

2.2. Crystallization and X-ray diffraction data collection

We performed initial crystallization screening for both proteins using sparse-matrix crystallization kits from Qiagen, Hilden, Germany. We mixed 0.5 µl protein solution with an equal volume of reservoir solution and used the sitting-drop vapor-diffusion method. We obtained EbBcsZ and EbBcsZ(D242A) crystals using the same reservoir solution consisting of 0.2 M disodium tartrate, 20% (v/v) PEG 3350. Crystals grew to dimensions of 0.3 × 0.2 × 0.2 mm within three days at 20°C. We transferred the EbBcsZ(D242A) crystals into soaking solution [0.2 M disodium tartrate, 20% (v/v) PEG 3350, 20% (v/v) glycerol, 2 mM CPT] and incubated them for 20 h at 20°C. We soaked the EbBcsZ crystals in reservoir solution supplemented with 20% (v/v) glycerol and then flash-cooled both crystals under a stream of liquid nitrogen. We collected diffraction data on beamlines NW-12A at Photon Factory, Tsukuba, Japan and BL41XU at SPring-8, Hyogo, Japan and processed the data using XDS (Kabsch, 2010). Table 1 summarizes the data statistics.

2.3. Structure determination and refinement

To determine the structure of EbBcsZ in complex with glycerol (EbBcsZ$_{GOL}$), we used the molecular-replacement (MR) method with AutoMR from the Phenix package (Liebschner et al., 2019) using the EcBcsZ$_{apo}$ structure (PDB entry 3qxf; Mazur & Zimmer, 2011) as the search model. We calculated the rotation and translation functions using data in the resolution range 45.0–3.0 Å. Similarly, we determined the EbBcsZ(D242A)$_{CPT}$ structure by the MR method with AutoMR using the EbBcsZ$_{GOL}$ structure as the search model. We calculated rotation and translation functions using data in the resolution range 45.0–2.5 Å. We performed several rounds of refinement using phenix.refine from the Phenix suite, alternating with manual fitting and rebuilding based on $F_{o} - F_{c}$ and $F_{o} - F_{c}$ electron-density maps using Coot (Emsley et al., 2010). We confirmed the conformation of the β-1,4-glucan chains based on the omit map. Table 1 summarizes the final refinement statistics and geometry defined by MolProbity (Chen et al., 2010). We prepared the structural figures using PyMOL (http://www.pymol.org).
2.4. BC hydrolysis assay

To perform the BC hydrolysis assay, we cultured *K. xylinus* to obtain BC based on the method reported previously (Czaja et al., 2004). BC is porous and incorporates water molecules, forming a gelatinous structure. To reduce the variation in the produced BC, we collected BC from a cultured medium as follows. We placed *K. xylinus* cells into 5 ml Schramm–Hestrin (HS) medium (Schramm & Hestrin, 1954) and initially grew them at 30°C with stirring at 120 rev min⁻¹ for four days. We then inoculated 0.1 ml cultured medium into 3 ml fresh HS medium and grew the cells at 30°C with stirring at 120 rev min⁻¹ for three days. The dry mass of BC was referenced each time to control the amount of produced BC. We collected the gelatinous BC membrane and gently washed it with 3 ml buffer D (50 mM sodium citrate buffer pH 5.0 containing 200 mM sodium chloride). To measure BC hydrolysis, the solution containing the purified *EbBcsZ* and *EbBcsZ(D242A)* was changed to buffer D. We performed repetitive concentration and dilution of purified *EbBcsZ* and *EbBcsZ(D242A)* by ultrafiltration using Vivaspin 6 concentrators with a 10 kDa cutoff membrane, and finally adjusted the concentration of the samples to 10 mg ml⁻¹. We dissolved bovine serum albumin (BSA; Sigma–Aldrich) and cellulase ONOZUKA RS (Yakult Pharmaceutical Industry) in buffer containing solutions in the presence or absence of sodium chloride. To measure BC hydrolysis, we cultured BC hydrolysis assay for *EbBcsZ*. (a) The state of gelatinous BC after 20 h of incubation with cellulase ONOZUKA RS, bovine serum albumin (BSA), *EbBcsZ* and *EbBcsZ(D242A)*. (b) Turbidity of the assay solution measured after 20 h of incubation. Average and standard deviation values of the absorbance at 600 nm were calculated from four replicates (*N* = 4).

![Figure 2](image)

**Table 1**

| Data-collection and refinement statistics. | *EbBcsZ(D242A)* | *EbBcsZ(GOL)* |
|------------------------------------------|-----------------|----------------|
| Wavelength (Å)                           | 1.0000          | 1.0000         |
| Space group                              | P2₁             | P2₁            |
| a, b, c (Å)                              | 89.3, 92.9, 90.5 | 89.3, 91.5, 89.9 |
| α, β, γ (%)                              | 90, 98.4, 90     | 90, 98.2, 90   |
| Resolution (Å)                           | 50.0–1.30 (1.38–1.30) | 50.0–1.93 (2.05–1.93) |
| Rmerge†                                  | 0.030 (0.567)   | 0.063 (0.597)  |
| Multiplicity                             | 4.2 (3.5)       | 3.8 (3.8)      |
| Completeness (%)                         | 99.5 (98.5)     | 99.7 (99.3)    |
| No. of unique reflections                | 355775 (57751)  | 107156 (17177) |
| Mean I(0)/I                      | 21.4 (2.3)      | 18.3 (2.6)     |
| CC1/2                                   | 1.000 (0.873)   | 0.999 (0.837)  |

| Refinement |                      |                      |
|------------|-----------------------|-----------------------|
| Resolution (Å) | 42.51–1.30       | 38.34–1.93            |
| Rwork/Rfree | 0.1690/0.187        | 0.1620/0.197          |
| No. of atoms | Protein: 10990       | 10876                 |
|              | Water: 1179          | 957                   |
|              | Celloligosaccharide: 329 | 126                |
|              | Glycerol: —          | 20                    |
|              | Disodium tartrate: 20 | 20                    |
| R.m.s.d. (Å) | Bond lengths: 0.014  | 0.009                 |
|              | Bond angles (°): 1.573 | 0.952                |
| Ramachandran statistics (%) | Favored: 98.59   | 98.51                 |
|              | Allowed: 1.41        | 1.49                  |
|              | Outliers: 0          | 0                     |
| Mean B factors (Å²) | Protein: 24.57     | 30.69                 |
|              | Water: 33.01         | 38.22                 |
|              | Celloligosaccharide: 32.26 | —                 |
|              | Glycerol: —          | 38.99                 |
|              | Disodium tartrate: 22.11 | 26.78            |
| PDB code    | 7882                 | 7881                  |

† *Rmerge = ∑ |I(hkl)i|−|I(hkl)|/|I(hkl)|, where I is the number of observations of a given reflection and |I(hkl)| is the average intensity of the i observations. ‡ *Rwork = ∑ |Fcalc|−|Fobs|/|Fcalc|, where |Fcalc| was calculated using a randomly selected 5% of reflections that were excluded from refinement.

is hydrolyzed, we observed the turbidity of each enzyme solution to estimate the BC hydrolysis. Finally, we measured the absorbance of each sample at 600 nm. We performed four technical replicates for each condition.

3. Results

3.1. The hydrolytic activity of *EbBcsZ* towards BC

Like CMCax, *EbBcsZ* can hydrolyze celloligosaccharides, including cellopentaose, cellohexaose and CMC, an aqueous cellulose derivative (Nakai et al., 2013; Mazur & Zimmer, 2011). To investigate whether *EbBcsZ* acts on gelatinous BC, we performed a hydrolysis assay on BC produced by *K. xylinus*. To estimate the degree of BC hydrolysis, we measured the absorbance at 600 nm representing the turbidity of BC-containing solutions in the presence or absence of *EbBcsZ*. In the positive control, BC hydrolysis allowed the cells embedded in gelatinous BC to disperse into the solution, increasing the turbidity (Fig. 2, left). Incubating the BC-containing solution with wild-type *EbBcsZ* partly degraded
gelatinous BC (Fig. 2a). The turbidity of this solution was 40% of that of the positive control (Fig. 2b), indicating that *EbBcsZ* has a moderate hydrolytic activity on BC. In contrast, the base catalyst mutant *EbBcsZ*(D242A) barely changed the shape of gelatinous BC (Fig. 2a, right), and the turbidity of the BC solution incubated with *EbBcsZ*(D242A) was comparable to that of the negative control (Fig. 2b, right).

3.2. The structure of *EbBcsZ* in complex with celloligosaccharide

Similar to the homologous enzyme *EcBcsZ* (Mazur & Zimmer, 2011), we prepared *EbBcsZ* without the N-terminal signal peptide (Met1–Ala20) and with a mutation of the base catalyst (D242A) to elucidate the reaction mechanism of *EbBcsZ*. We determined the structure of *EbBcsZ*(D242A)CPT at 1.30 Å resolution by soaking *EbBcsZ*(D242A) crystals in a solution containing CPT. In this *EbBcsZ*(D242A)CPT structure, the residues Cys22–Trp359 were visible, while the N-terminal Ala21 and the eight C-terminal residues (Gly360–Gln367) could not be built due to poor electron density. The overall structure of *EbBcsZ*(D242A)CPT formed a classical (α/α)8 barrel composed of six inner and lateral antiparallel helices. Although *EbBcsZ* is a monomeric enzyme, the asymmetric unit contained four *EbBcsZ*(D242A)CPT monomers. These four monomers were essentially identical, with a root-mean-square deviation (r.m.s.d.) value of less than 0.41 Å for 335 Cα atoms. We selected monomer A for structural comparison and description of the active site. Structural comparison using PDBeFold (Krissinel & Henrick, 2004) showed that *EbBcsZ*(D242A)CPT was similar to the other GH8 endo-β-1,4-glucanases *EcBcsZ*(E55Q)CPT (PDB entry 3rq4), *CtCelA*(E95Q)CPT (PDB entry 1kwf) and CMCAx (PDB entry 1wzz), with r.m.s.d.s of 0.65 Å for 338 Cα atoms, 2.09 Å for 270 Cα atoms and 2.02 Å for 278 Cα atoms, respectively. Although we soaked the crystals in a CPT-containing solution, we did not obtain full-length CPT in the *EbBcsZ*(D242A)CPT structure, unlike in the *EcBcsZ*(E55Q)CPT and *CtCelA*(E95Q)CPT structures. In each monomer of the asymmetric unit, two celloligosaccharides occupied the plus and minus subsites (both sides of the cleavage position) of the active site (Fig. 3a, Supplementary Fig. S3), indicating an intermediate state that differs from those of reported GH8 endo-β-1,4-glucanase structures. In all four monomers, cellobiose binds to subsites +1 to +2 similarly, whereas the celloligosaccharide binds to the minus subsites differently. Cellotetraose bound to subsites −1 to −3 and extended to an additional subsite −4 in three monomers (monomers A–C), while cellobiose bound to subsites −2 to −4 in the remaining monomer (monomer D) (Fig. 3a).

We next compared the *EbBcsZ*(D242A)CPT structure with those of other GH8 endo-β-1,4-glucanases. The cellobiose orientation in subsites +1 to +2 of *EbBcsZ*(D242A)CPT was similar to that of *CtCelA*(E95Q)CPT (Guérin et al., 2002). G1 and G2 formed stacking interactions with the aromatic residues in the same manner. However, there were differences in the interaction between the hydroxy group of G1 and polar residues in the structures of both *EbBcsZ*(D242A)CPT and *CtCelA*(E55Q)CPT (Fig. 3b). Note that in the *EbBcsZ*(D242A)CPT structure the

---

**Figure 3**
The structure of the active site in *EbBcsZ*(D242A)CPT. (a) Electron densities of the β-1,4-glucan chains bound to the four monomers of the asymmetric unit. The electron densities are the omit map contoured at 3.0σ. (b) Interactions between the β-1,4-glucan chains and the residues in the active site. We selected monomer A as the representative active site. The water molecules and the residues interacting with the β-1,4-glucan chains are represented as red spheres and light blue lines, respectively. The hydrogen-bond network is represented as dotted lines. In (a) and (b), the β-1,4-glucan chains are shown as sticks and O, N and C atoms are shown in red, blue and dark cyan, respectively. (c) The conformation of G1 (dark cyan) and five other glucosyl units in subsites G−1, G−3, G+1, G+4, and G+2 (light gray) of *EbBcsZ*(D242A)CPT (d) Superposed views of glucosyl units. Left: superposition of G−1 (dark cyan) and G+2 (light gray) in *EbBcsZ*(D242A)CPT. Right: superposition of G−1 in *EbBcsZ*(D242A)CPT (dark cyan) and G+1 in *CtCelA*(E95Q)CPT (yellow).
Arg245 side chain presented two different conformations (Arg245$_{conf1}$ and Arg245$_{conf2}$). The Arg245$_{conf1}$ side chain interacts with the 4-OH of G$_{-1}$. Additionally, the side chain of the general acid catalyst Glu54, corresponding to E95Q in CrCelA and E55Q in EbBcsZ, interacted with the 3-OH and 4-OH of G$_{-1}$. Our structure also displayed an interaction network associated with Glu54. Glu54 interacts with Arg245 and Tyr330 through a salt bridge and a hydrogen bond, respectively. Except for G$_{-1}$, four glucosyl units interacted with the minus subsites in the same manner as those in EcBcsZ(E55Q)$_{CPT}$ and CrCelA(E95Q)$_{CPT}$ (Mazur & Zimmer, 2011; Guérin et al., 2002). In EbBcsZ(D242A)$_{CPT}$, the Asp115 side chains formed a bidentate interaction with G$_{-1}$, and the Glu54 side chain interacted with the 2-OH of G$_{-1}$. Furthermore, we observed an interaction between G$_{-1}$ and G$_{1}$ in the EbBcsZ(D242A)$_{CPT}$ structure. The 6-OH and sugar-ring O (O5) atoms of G$_{-1}$ interacted with the 3-OH and 4-OH of G$_{1}$, respectively, through hydrogen bonds.

3.3. The glucosyl unit conformation in subsite $-1$

In the EbBcsZ(D242A)$_{CPT}$ structure, G$_{-1}$ displayed a distorted $S_{1}$ conformation, whereas the glucosyl units in the other subsites (G$_{-2}$, G$_{-3}$, G$_{-4}$, G$_{1}$ and G$_{2}$) presented a stable $B^{4}$C$_{1}$ chair conformation (Fig. 3c, Supplementary Table S1). Moreover, considering the position of the 1-OH of G$_{-1}$, G$_{-2}$ has the $\alpha$-configuration at the C1 atom. Comparing the $S_{1}$ position of G$_{-1}$ with those of the other glucosyl units clearly shows this difference (Fig. 3d, left). G$_{-1}$ displayed a $2_{1}B^{3}$ conformation in the CrCelA(E95Q)$_{CPT}$ structure, and comparing the C1 positions of G$_{-1}$ in EbBcsZ(D242A)$_{CPT}$ and CrCelA(E95Q)$_{CPT}$ also shows the difference in the conformation of G$_{-1}$ (Fig. 3d, right). To confirm whether the alanine mutation of the general base catalyst Asp242 causes structural changes, we determined the structure of EbBcsZ in complex with glycerol (EbBcsZ$_{GOL}$). We obtained EbBcsZ crystals in the same crystal form as those of EbBcsZ(D242A)$_{CPT}$. Residues Cys22–Trp359 of each EbBcsZ$_{GOL}$ monomer were built in the asymmetric unit. EbBcsZ$_{GOL}$ and EbBcsZ (D242A)$_{CPT}$ had very similar structures, with an r.m.s.d. of 0.17 Å for 335 C$^\alpha$ atoms, demonstrating that the D242A mutation scarcely changed the overall EbBcsZ structure. In the EbBcsZ$_{GOL}$ structure, subsites $-1$, $-2$ and $+1$ were occupied by glycerol molecules used as a cryoprotectant for the diffraction experiment (Supplementary Fig. S4a). These glycerol molecules can be considered to be a mimic of the bound glucosyl unit of 1,4-glucan. In each EbBcsZ monomer the glycerol molecule in subsite $-1$ adopted a remarkably similar orientation. Superposing EbBcsZ$_{GOL}$ on EbBcsZ(D242A)$_{CPT}$ showed that three hydroxy groups of the glycerol and two water molecules were close to the O5 atom and 3-OH, 6-OH, 1-OH and 2-OH of G$_{-1}$, respectively (Supplementary Fig. S4b, Supplementary Table S2), supporting the plausibility of the formation of a distorted $S_{1}$ conformation of G$_{-1}$ during the hydrolytic reaction process. Besides, the existence of the $S_{1}$ conformation is consistent with the predicted result of a quantum mechanics/molecular mechanics (QM/ MM) dynamic simulation on the reaction process of GH8 enzymes (Ardévol & Rovira, 2015; Petersen et al., 2009).

3.4. The secondary binding site of the $\beta$-1,4-glucan chain

Among the GH8 endo-$\beta$-1,4-glucanases, the structure of EbBcsZ (D242A)$_{CPT}$ is the first to show that $\beta$-1,4-glucan binds to the surface away from the active site (Fig. 4a), which is called the secondary binding site (Cuyvers et al., 2012). Two of the four EbBcsZ(D242A)$_{CPT}$ monomers (monomers $A$ and $C$) bind to cellotriose through a tryptophan-rich cleft (Fig. 4a). However, electron density for the cellobiosecarboxylic acid did not appear in the corresponding region in monomers.
B and D, which can be considered to be an effect of crystal packing (Supplementary Fig. S5). This marked difference implies that the physical and chemical environment easily affects the binding of β-1,4-glucan at the secondary binding site. Cellotriose bound to monomers A and C in the same manner. The glucosyl units at the central part and the reducing end formed a T-shaped stacking with Trp97 and π–π stacking with Trp105, respectively. Stacking interactions with aromatic residues are common in secondary binding sites and seem to be essential for oligosaccharide binding (Cockburn et al., 2016; Cockburn & Svensson, 2013; Cuyvers et al., 2011). The 2-OH of the reducing-end sugar and the 6-OH of the central sugar interacted with the side chains of Arg40 and Trp78, respectively. The 4-OH of the non-reducing-end sugar interacted with the main-chain carbonyl of Asp81 through a hydrogen bond. These contacts are probably conserved among the other GH8 endo-β-1,4-gluconases, except for the stacking with Trp105 in a β-hairpin motif (Fig. 4b).

4. Discussion

BC, which is generated by cellulose-producing bacteria, is a biomaterial with remarkable characteristics that are superior to those of the cellulose fiber extracted from plants. It has potential applications in various fields. In BC-producing bacteria, the endo-β-1,4-gluconase serves as a cleaner protein that degrades disordered cellulose fibers, efficiently producing high-quality BC. Our work is focused on investigating the structural and functional properties of the endo-β-1,4-gluconase BcsZ in order to help to improve BC production.

A BC degradation assay showed that EbBcsZ has hydrolytic activity, although it is weaker than that of cellulase ONOZUKA RS, a multi-component cellulase. This weak activity may arise from limited degradation of the disordered region in gelatinous BC, which is in agreement with previous studies showing that EbBcsZ can degrade cellobiose- and CMC to noncrystalline cellulose (Koo et al., 1998). Considering that the endo-β-1,4-gluconase EbBcsZ needs to access the β-1,4-glucon chain to cleave internal bonds, secondary binding sites probably assist in the binding of the target substrate β-1,4-glucon chain. Moreover, immunostaining of CMCax (Standal et al., 1994) and digestion of BcsZ from Salmonella typhimurium by a protease (Ahmad et al., 2016) demonstrated that these endo-β-1,4-gluconases are located close to the outer membrane or periplasmic space, allowing them to degrade β-1,4-glucon chains before crystallization (Ahmad et al., 2016; Hu et al., 2010; Standal et al., 1994). A phylogenetic relationship shows that the gene encoding endo-β-1,4-gluconase appears with the bcsC gene in the bcs gene cluster, with some exceptions (Römling & Galperin, 2015). This little-studied BcsC subunit of TC localizes at the outer membrane and periplasmic space, and contributes to the export of BC to the extracellular matrix. Thus, endo-β-1,4-gluconase may be associated with BcsC to access noncrystalline β-1,4-glucon chains efficiently.

Several studies have reported structures of wild-type GH8 endo-β-1,4-gluconases and general acid catalyst mutants. These studies considered that the apo-form structures of these GH8 endo-β-1,4-gluconases are represented the ‘resting state’. Here, we select EcBcsZapo as representing the resting state (Fig. 5a, i) since it is most similar to EbBcsZ in both sequence and structure. In the structures of CtCelA(E95Q)crpt (Guérin et al., 2002) and TGiGH8XTO (Fowler et al., 2018), a single oligosaccharide was incorporated into the subsites, and G-1 and X-1 showed a distorted 2.5Β conformation. These complex structures represent the ‘reactive state’ that readily forms the oxocarbenium intermediate (Fig. 5a, ii). In the EcBcsZ(E55Q)crpt structure cellobiose-structure was only located in the minus subsites, meaning that EcBcsZ(E55Q)crpt represents the ‘post-hydrolysis state’ that occurs after release of the leaving group (Fig. 5a, iv). We employed a base-catalyst mutant EbBcsZ(D242A) and determined its cellobiose-saccharide-

---

**Figure 5**

Interactions between the bound β-1,4-glucan chains and the polar residues of GH8 endo-β-1,4-gluconases in each reaction state. (a) The structures of (i) the resting state EcBcsZapo, (ii) the reactive state CtCelA(E95Q)crpt, (iii) the post-cleavage state EbBcsZ(D242A)crpt and (iv) the post-hydrolysis state EcBcsZ(E55Q)crpt. The cellobiose-saccharides and the residues are represented as sticks and lines, respectively. (b) Schematic diagram of the motion of the GH8 endo-β-1,4-gluconase residues during the reaction process. The glutamate and arginine of interest are represented as orange and blue sticks, respectively. When the sugar units interact with the glutamate and/or arginine, the solid circles are in the same color as the glutamate and/or arginine.

---

**Takaaki Fujiwara et al. • Glycoside hydrolase family 8 endo-β-1,4-gluconase Acta Cryst. (2022). D78, 228–237**
bound structure, unlike previous studies. The $Eb$BcsZ(D242A)$_{CPT}$ structure provides a novel snapshot of the reaction state, in which two β-1,4-glucan chains lying in subsites $-1$ to $-4$ and $+1$ to $+2$ are separated. The distance between the C1 atom of G$_{-1}$ and the 4-OH of G$_{+1}$ is approximately 3 Å, which is longer than the typical length of a C−O single bond (1.43 Å). Thus, $Eb$BcsZ(D242A)$_{CPT}$ represents the ‘post-cleavage state’, with G$_{+1}$ in a distorted $^3S_1$ conformation, immediately after the scissile-bond cleavage (Fig. 5a, iii).

The residues Glu54 and Arg245 in $Eb$BcsZ(D242A)$_{CPT}$ differed in orientation compared with the corresponding residues in $Ec$BcsZ(E55Q)$_{CPT}$ ($E55Q$ and Arg246) and $Ct$CelA(E95Q)$_{CPT}$ (E95Q and Arg281). Similar to the reactive state [$Ct$CelA(E95Q)$_{CPT}$] and the post-hydrolysis state [$Ec$BcsZ(E55Q)$_{CPT}$], the side chain of the acid catalyst Glu54 was located between G$_{+1}$ and G$_{-1}$ in the post-cleavage state [$Eb$BcsZ(D242A)$_{CPT}$] (Fig. 5a, ii–iv). However, comparison between $Eb$BcsZ(D242A)$_{CPT}$ and $Ec$BcsZ$_{apo}$ indicated that when the substrate binds, the Glu54 side chain would simultaneously flip and interact with Tyr330 and Arg245 to avoid a clash with the 3-OH of G$_{-1}$ (Figs. 3b and 5a, i). Thus, upon substrate binding, the acid catalyst side chain flips and keeps this conformation, while the cellooligosaccharide occupies subsite $-1$. In the resting state ($Ec$BcsZ$_{apo}$), the N$^q1$ atom of Arg246 forms a salt bridge to the O$^21$ atom of Asp116 and was distant from the binding subsites (Fig. 5a, i). In the reactive state [$Ct$CelA(E95Q)$_{CPT}$], the corresponding Arg281 was distant from Asp152 and shifted to interact with G$_{-1}$ (Fig. 5a, ii). In the post-cleavage state [$Eb$BcsZ(D242A)$_{CPT}$], Arg245 presented two conformations: Arg245$_{conf1}$ was the same as that of the corresponding Arg281 of $Ct$CelA in the reactive state, whereas Arg245$_{conf2}$ shifted to interact with G$_{+1}$ (Figs. 3b and 5a, iii). In the post-hydrolysis state [$Ec$BcsZ(E55Q)$_{CPT}$], Arg246 returned to interact with Asp116 and was distant from subsite $+1$ (Fig. 5a, iv). Therefore, this arginine residue has an important role in tethering the cleaved β-1,4-glucan chains. Based on these findings, we summarized a series of conformational changes in the catalytic process of GH8 endo-β-1,4-glucanase to illustrate the whole catalytic mechanism (Fig. 5b). The flexibility of the arginine residue allows the post-cleavage state to be captured. The superimposed structures of $Eb$BcsZ(D242A)$_{CPT}$ and $Eb$BcsZ$_{GOL}$ clearly showed that the side chain of Asp242 does not sterically hinder the conformational changes of G$_{-1}$ and Arg245 (Supplementary Fig. S6).

Considering the reaction mechanism of an inverting glycoside hydrolase (Fig. 1), the acid catalyst mutation prevents the formation of the transition intermediate, and the $^3S_1$ conformation is invisible. The base catalyst activates a water molecule for a nucleophilic attack on the C1 atom of G$_{-1}$ and does not directly attack G$_{-1}$. Even though the base catalyst is mutated, the reaction may proceed by the very weak nucleophilicity of water. Indeed, previous studies demonstrated that mutating the base catalyst drastically reduced, but did not completely remove, the activity of GH8 enzymes (Fowler et al., 2018; De Vos et al., 2006; Collins et al., 2005). The structural comparison of G$_{-1}$ in the post-cleavage state $^3S_1$ [$Eb$BcsZ(D242A)$_{CPT}$] with the substrate state $^2S^2C_1$ [the other glucosyl unit in $Eb$BcsZ(D242A)$_{CPT}$] and the transition state $^2S^2B$ [$Ct$CelA(E95Q)$_{CPT}$] clearly showed the difference in planarity of G$_{-1}$ (Supplementary Fig. S3c). The boat $^2S^2B$ conformer changes to a skew-boat $^3S_1$ conformer, indicating that the hydrolysis reaction of GH8 enzymes precedes via an unstable conformation to meet the requirements of an antiperiplanar lone-pair hypothesis (Nerinckx et al., 2005).

In the structure of $Eb$BcsZ(D242A)$_{CPT}$, we obtained electron density for six glucosyl units in monomers A–C as shown in Fig. 3(a), although we utilized cellopentaose. Considering that no electron density appeared at subsite $-1$ in monomer D and the electron density for G$_{-1}$ in monomers A–C clearly showed the $^3S_1$ conformation, the electron density might be derived from a mixture of the post-cleavage state of cellopentaose in subsites $-3$ to $+2$ and cleaved cellobiose in subsites $-4$ to $-2$, but not noncleaved cellobiose in subsites $-4$ to $+1$. Combining the binding of oligosaccharides in the structure of $Eb$BcsZ(D242A)$_{CPT}$ with a previous thin-layer chromatography (TLC) analysis (Sunagawa et al., 2012), we proposed the binding patterns of oligosaccharides in the trimming mechanism of BcsZ (Fig. 6). The structure shows that $Eb$BcsZ has six subsites, $-3$ to $+2$ and additionally $-4$, for binding the β-1,4-glucan chain. The TLC analysis demonstrated

![Cellotetraose (G4)](image)

(4) $→$ 3 $→$ 2 $→$ 1 $→$ +2

![Cellopentaose (G5)](image)

(5) $→$ G3 + G2

![Cellohexaose (G6)](image)

(6) $→$ G3 + G3

(6) $→$ G4 + G2

**Longer cellooligosaccharide**

![Nonreducing end](image)

or

![Reducing end](image)

or

**Figure 6**

Schematic diagram of the proposed binding patterns of oligosaccharides in BcsZ. The β-1,4-glucan chains cellotetraose (G4), cellopentaose (G5), cellohexaose (G6) and longer cellooligosaccharides are represented as solid spheres connected by lines. The triangle indicates the scissile bond.
that \( \text{EbBcsZ} \) degrades pentaose into cellobiose and celiotriose but does not degrade cellotetraose. Thereby, five cellobiose glucosyl units should lie in subsites \(-3\) to \(+2\) and not in subsites \(-4\) to \(+1\) or in the four subsites \(-2\) to \(+2\) (Fig 6, G4 and G5). The TLC analysis also showed that \( \text{EbBcsZ} \) degrades cellobiohexaoxose into two celiotrioses or into cellobiose and cellotetraose. Cellobiohexaoxose needs to have five glucosyl units in subsites \(-3\) to \(+2\) and a free glucosyl unit to produce two celiotrioses. The six glucosyl units of cellobiohexaoxose need to be in subsites \(-4\) to \(+2\) (Fig. 6, G6) to obtain cellobiose and cellotetraose. Consequently, \( \text{EbBcsZ} \) hydrolyzes cellobiohexaoxose or longer cellooligosaccharides when the \( \beta,1,4\)-glucan chain occupies at least subsites \(-3\) to \(+2\) (Fig. 6, bottom). Moreover, considering that glycerol molecules preferentially bind to subsites \(+2\) to \(+3\) and cellotetraose. Cellohexaose needs to have five glucosyl units in subsites \(-3\) to \(+2\) and a free glucosyl unit to produce two celiotrioses. Therefore, subsites \(-3\) and \(+2\) might have a relatively lower affinity than those in subsites \(-2\) to \(+1\).

5. Conclusion
This work demonstrated that the GH8 endo-\( \beta,1,4\)-glucanase \( \text{EbBcsZ} \) partly degrades gelatinous BC, implying that it plays a potential role in the quality control of BCs. Unlike previous studies, we obtained the \( \text{EbBcsZ(D242A)}\) structure using a base-catalyst mutant, revealing a \( \delta_5 \) intermediate as the ‘post-cleavage state’. By comparing GH8 endo-\(\beta,1,4\)-glucanase \( \text{EbBcsZ} \) to enhance the understanding of the biosynthesis of high-quality cellulose by bacteria, and our findings open up a route to a deep understanding of the enzymology of glycoside hydrolases.

Acknowledgements
We thank the beamline staff of the Photon Factory and SPring-8 for their technical assistance during X-ray diffraction data collection (Proposal Nos. 2018B2508, 2019B2537 and 2021A2756).

Funding information
This work was supported in part by a Grant-in-Aid for Scientific Research (A) (No. 21H01754 to MY) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and also by Platform Project for Supporting Drug Discovery and Life Science Research [Basis for Supporting Innovative Drug Discovery and Life Science Research (BINDS)] from Japan Agency for Medical Research and Development (AMED) under Grant Numbers JP18am0101071 and JP19am0101083.

References
Acheson, J. F., Derewenda, Z. S. & Zimmer, J. (2019). Structure, 27, 1855–1861.
Ahmad, I., Rouf, S. F., Sun, L., Cimdins, A., Shafeeq, S., Le Guyon, S., Schottkowski, M., Rhen, M. & Römling, U. (2016). Microb. Cell Fact. 15, 177.
Arđêvöl, A. & Rovira, C. (2015). J. Am. Chem. Soc. 137, 7528–7547.
Chen, V. B., Arendall, W. B., Headd, J. J., Keedy, D. A., Immormino, R. M., Kapral, G. J., Murray, L. W., Richardson, J. S. & Richardson, D. C. (2010). Acta Cryst. D66, 12–21.
Chandwat, S. P. S., Beckham, G. T., Himmel, M. E. & Dale, B. E. (2011). Annu. Rev. Chem. Biomol. Eng. 2, 121–145.
Cockburn, D. & Svensson, B. (2013). Carbohydr. Chem. 39, 204–221.
Cockburn, D., Wilkens, C., Dilokpimol, A., Nakai, H., Lewi´ńska, A., Abou Hachem, M. & Svensson, B. (2016). PLoS One, 11, e0160112.
Collins, T., De Vos, D., Hoyoux, A., Savvides, S. N., Gerday, C., Van Beeumen, J. & Feller, G. (2005). J. Mol. Biol. 354, 425–435.
Cremer, D. & Pople, J. A. (1975). J. Am. Chem. Soc. 97, 1354–1358.
Cuyvers, S., Dornez, E., Delcour, J. A. & Courtin, C. M. (2016). Appl. Microbiol. Biotechnol. 92, 539–549.
Cuyvers, S., Dornez, E., Delcour, J. A. & Courtin, C. M. (2012). Crit. Rev. Biotechnol. 32, 93–107.
Czaja, W., Krystynowicz, A., Bielecki, S. & Brown, R. M. Jr (2006). Cellulose, 11, 403–411.
Czaja, W., Romanowicz, D. & Brown, R. M. Jr (2004). Cellulose, 11, 393–402.
De Vos, D., Collins, T., Nerinckx, W., Savvides, S. N., Claeyssens, M., Gerday, C., Feller, G. & Van Beeumen, J. (2006). Biochemistry, 45, 4797–4807.
Emsley, P., Lohkamp, B., Scott, W. G. & Cowtan, K. (2010). Acta Cryst. D66, 486–501.
Fowler, C. A., Hemsworth, G. R., Cuskin, F., Hart, S., Turkenburg, J., Gilbert, H. J., Walton, P. H. & Davies, G. J. (2018). Acta Cryst. D74, 946–955.
Fujitani, T., Komoda, K., Sakurai, N., Tajima, K., Tanaka, I. & Yao, M. (2013). Biochem. Biophys. Res. Commun. 431, 802–807.
Guérin, D. M., Lascombe, M. B., Costabel, M., Souchon, H., Lamzin, V., Béguin, P. & Alzari, P. M. (2002). J. Mol. Biol. 316, 1061–1069.
Helenius, G., Bäckdahl, H., Bodin, A., Nannmark, U., Gatenholm, P. & Risberg, B. (2006). J. Biomed. Mater. Res. 76A, 431–438.
Hu, S.-Q., Gao, Y.-G., Tajima, K., Uemori, Y., Yamashita, H., Erata, T., Munekata, M. & Takai, M. (2008). J. Biosci. 33, 802–807.
Kabsch, W. (2010). Acta Cryst. D66, 125–132.
Kawano, S., Tajima, K., Kono, H., Numata, Y., Yamashita, H., Satoh, Y. & Munekata, M. (2008). J. Biosci. 106, 88–94.
Kawano, S., Tajima, K., Uemori, Y., Yamashita, H., Erata, T., Munekata, M. & Takai, M. (2002). DNA Res. 9, 149–156.
Koo, H. M., Song, S. H., Pyun, Y. R. & Kim, Y. S. (1998). Biosci. Biotechnol. Biochem. 62, 2257–2259.
Koshland, D. E. (1953). Biol. Rev. 28, 416–436.
Krisinel, E. & Henrick, K. (2004). Acta Cryst. D60, 2256–2268.
Lee, K.-Y., Buldum, G., Mantalaris, A. & Bismarck, A. (2014). Macromol. Biosci. 14, 10–32.
Lee, S. E. & Park, Y. S. (2017). Mol. Cell. Toxicol. 13, 257–261.
Liebschner, D., Alonine, P. V., Baker, M. L., Bunkóczi, G., Chen, V. B., Croll, T. I., Hintze, B., Hung, L.-W., Jain, S., McCoy, A. J., Moriarty, N. W., Offenr, R. D., Poon, B. K., Prisant, M. G., Read, R. J., Richardson, J. S., Richardson, D. C., Sammito, M. D., Sobolev, O. V., Stockwell, D. H., Terwilliger, T. C., Urzhumtsev, A. G., Videau, L. L., Williams, C. J. & Adams, P. D. (2019). Acta Cryst. D75, 861–877.
Mazur, O. & Zimmer, J. (2011). J. Biol. Chem. 286, 17601–17606.
Morgan, J. L. W., McNamara, J. T., Fischer, M., Rich, J., Chen, H.-M., Withers, S. G. & Zimmer, J. (2016). Nature, 531, 329–334.
Morgan, J. L. W., Strumillo, J. & Zimmer, J. (2013). *Nature*, **493**, 181–186.
Nakai, T., Sugano, Y., Shoda, M., Sakakibara, H., Oiwa, K., Tuzi, S., Imai, T., Sugiyama, J., Takeuchi, M., Yamauchi, D. & Mineyuki, Y. (2013). *J. Bacteriol.* **195**, 958–964.
Nerinckx, W., Desmet, T., Piens, K. & Claeyssens, M. (2005). *FEBS Lett.* **579**, 302–312.
Nishi, Y., Uryu, M., Yamanaka, S., Watanabe, K., Kitamura, N., Iguchi, M. & Mitsuhashi, S. (1990). *J. Mater. Sci.* **25**, 2997–3001.
Nogi, M. & Yano, H. (2008). *Adv. Mater.* **20**, 1849–1852.
Nojima, S., Fujishima, A., Kato, K., Ohuchi, K., Shimizu, N., Yonezawa, K., Tajima, K. & Yao, M. (2017). *Sci. Rep.* **7**, 13018.
Pang, J., Wang, J., Liu, Z., Zhang, Q. & Qi, Q. (2019). *BMC Biotechnol.* **19**, 63.
Petersen, L., Ardèvol, A., Rovira, C. & Reilly, P. J. (2009). *J. Phys. Chem. B.*, **113**, 7331–7339.
Römling, U. & Galperin, M. Y. (2015). *Trends Microbiol.* **23**, 545–557.
Ross, P., Mayer, R. & Benziman, M. (1991). *Microbiol. Rev.* **55**, 35–58.
Schramm, M. & Hestren, S. (1954). *J. Gen. Microbiol.* **11**, 123–129.
Shah, J. & Brown, R. M. Jr (2005). *Appl. Microbiol. Biotechnol.* **66**, 352–355.
Somerville, C. (2006). *Annu. Rev. Cell Dev. Biol.* **22**, 53–78.
Standal, R., Iversen, T. G., Coucheron, D. H., Fjaervik, E., Blatny, J. M. & Valla, S. (1994). *J. Bacteriol.* **176**, 665–672.
Sunagawa, N., Tajima, K., Hosoda, M., Kawano, S., Kose, R., Satoh, Y., Yao, M. & Dairi, T. (2012). *Cellulose*, **19**, 1989–2001.
Torgbo, S. & Sukyai, P. (2020). *Polym. Degrad. Stabil.* **179**, 109232.
Wong, H. C., Fear, A. L., Calhoon, R. D., Eichinger, G. H., Mayer, R., Amikam, D., Benziman, M., Gelfand, D. H., Meade, J. H., Emerick, A. W., Bruner, R., Ben-Bassat, A. & Tal, R. (1990). *Proc. Natl Acad. Sci. USA*, **87**, 8130–8134.
Yamanaka, S., Watanabe, K., Kitamura, N., Iguchi, M., Mitsuhashi, S., Nishi, Y. & Uryu, M. (1989). *J. Mater. Sci.* **24**, 3141–3145.
Yasutake, Y., Kawano, S., Tajima, K., Yao, M., Satoh, Y., Munekata, M. & Tanaka, I. (2006). *Proteins*, **64**, 1069–1077.