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Recognition mechanism of triple-helical β-1,3-glucan by a β-1,3-glucanase

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TABLE OF CONTENTS

1 SUPPORTING FIGURES

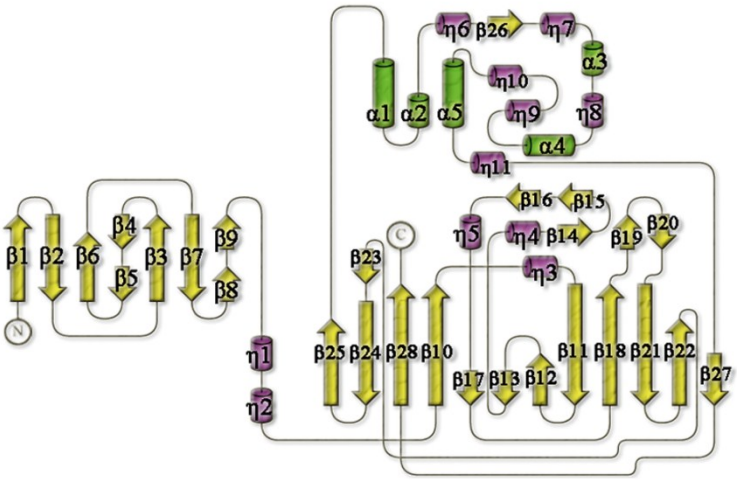

Figure S1. Topology diagram of PbBgl64A. Color code: yellow, β-strand; green, α-helix; purple, 3_{10}-helix.

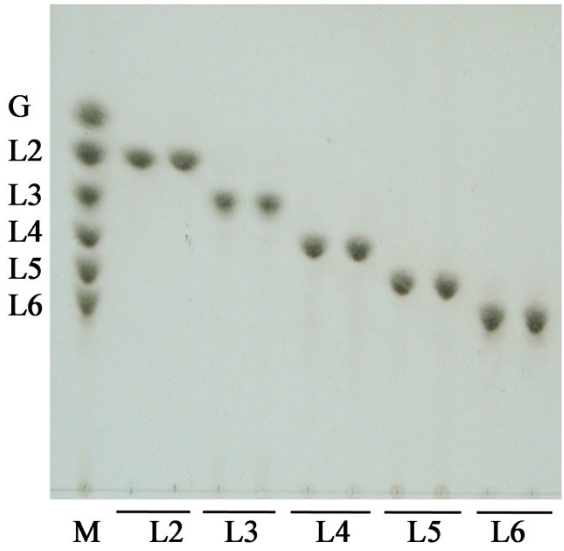

Figure S2. TLC analysis of the hydrolysis products of β-1,3-oligosaccharides by PbBgl64A. M, marker sugars; G, glucose; L2–L6, laminaribiose, laminaritriose, laminaritetraose, laminaripentaose and laminarihexaose, respectively. For each oligosaccharide, the left lane is the reaction mixture before hydrolysis, and the right lane is the reaction mixture after hydrolysis.

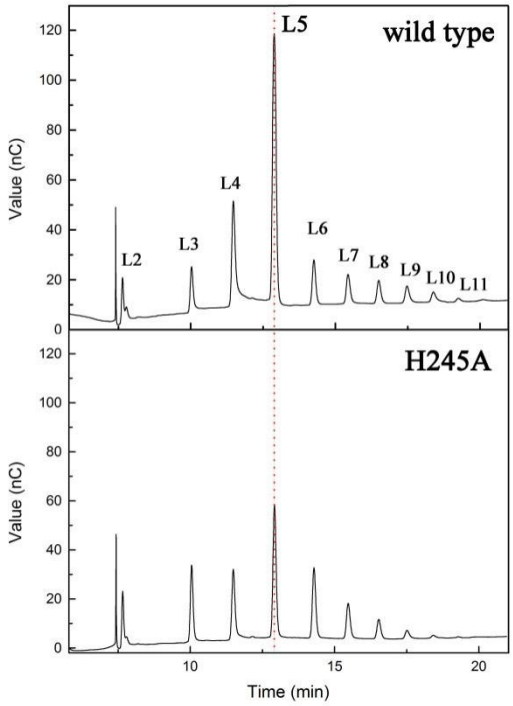

Figure S3. Comparison of the hydrolysis products of curdlan by wild-type and H245A PbBgl64A. The hydrolysis products were analyzed by HPAEC. L2–L11 indicate the different β-1,3-oligosaccharides, and their numbers indicate the degree of polymerization of the β-1,3-oligosaccharides.

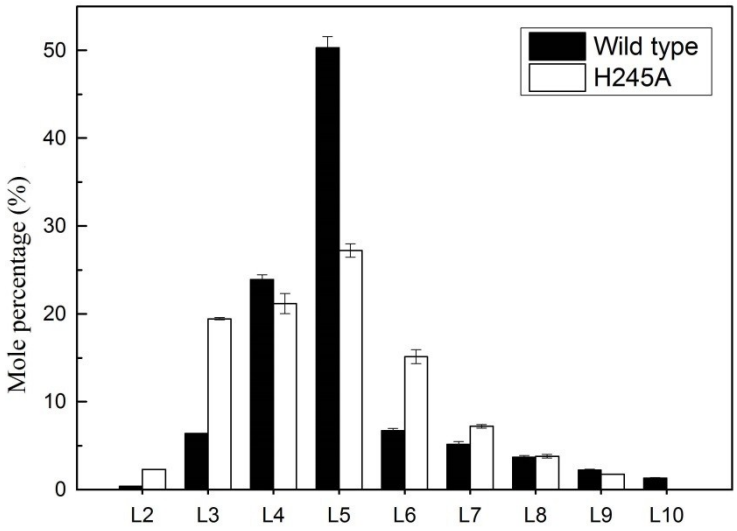

Figure S4. Quantification of hydrolysis products of curdlan by wild-type and H245A PbBgl64A. L2–L10 indicate the different β-1,3-oligosaccharides, and their numbers indicate the degree of polymerization of the β-1,3-oligosaccharides.

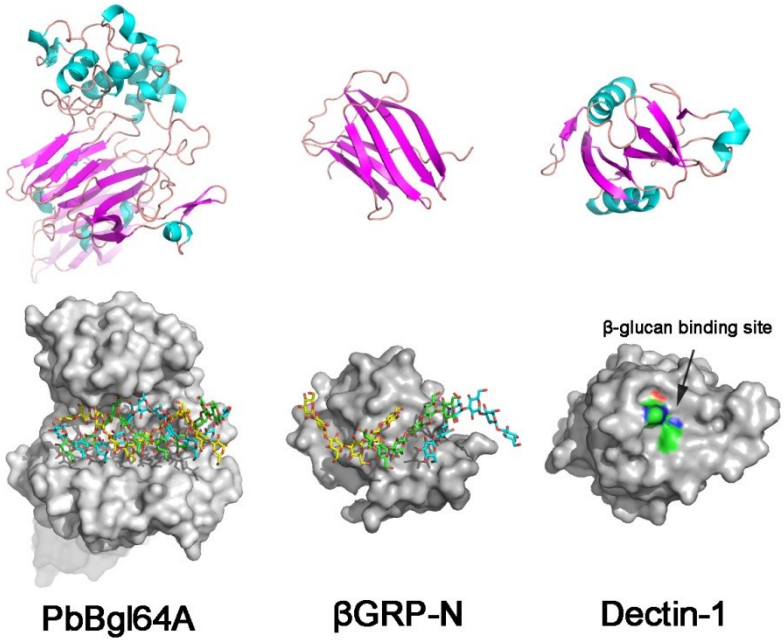

Figure S5. Structures of three typical pattern β-glucan recognition receptors. βGRP-N: Insects β-1,3-glucan recognition proteins N-terminal domain, PDB: 3AQZ; Dectin-1: mammalian β-glucan receptor Dectin-1 C-type lectin-like domain, PDB: 2CL8.

2 SUPPORTING TABLES

Table S1. Primers used in this study.

Primers	Primer sequence (5'→3')	Bases (bp)
PbBgl64A-up[a]	TGACTGCTAGCGCTGATTTCACTCAAGGAGCGG	33
PbBgl64A-down	TGACTCTCGAGTTACCAGCCCACTCTGACGATG	33
PbBgl64A-N-up	ATTCATGCCATGGCTGATTTCACTCAAGGAGCGG	34
PbBgl64A-N-down	ATTCCGCTCGAGTCGATAAGTGAATGTTGTGGTGTC	36
PbBgl64A-C-up	ATTCATGCCATGGGAACCAACTCCATTTATTCGATTCCAG	40
PbBgl64A-C-down	ATTCCGCTCGAGCCAGCCCACTCTGACGATG	31
E236A-up[b]	TTTTTTGACTTTGTCGCGTTCACTGTGG	28
E236A-down	GCGACAAAGTCAAAAATGATATTCAGATTCG	31
D252A-up	ATACGACTCGCGTCGCTCAGTTTGGT	26
D252A-down	GCGACGCGAGTCGTATTTCCGTGATAG	27
R197A-up	AAGATCGAATCCGGCGCGTTGTTCCTT	27
R197A-down	GCGCCGGATTCGATCTTCGGAAGGTT	26
N221A-up	CCGGTCCGGATCTGGCCAATCCCAC	25
N221A-down	GCCAGATCCGGACCGGCAAAGCCGTC	26
H245A-up	GTGGATAAGGACGGCTATGCCGGAAATACGACT	33
H245A-down	GCATAGCCGTCCTTATCCACAGTGAACTCGACAAAG	36

[a] The restriction enzyme sites incorporated into the primers are underlined.

[b] Mutations are indicated by boxes.

Table S2. X-ray data-collection and refinement statistics.

	SeMet-PbBgl64A	Apo-PbBgl64A	PbBgl64A-L6
Data-collection statistics			
Radiation source	KEK-BL1A	KEK-BL1A	SSRF-BL17U
Wavelength (Å)	0.9787	1.1000	1.0093
Temperature of measurements (K)	100	100	100
Resolution (Å)	44.60–2.29 (2.37–2.29)	32.32–1.95 (2.02–1.95)	28.27–1.99 (2.06–1.99)
Space group	*C*2	*C*2	*C*2
Unit cell parameters			
a, *b*, *c* (Å)	133.6, 65.5, 62.2	134.1, 65.8, 61.6	132.6, 65.2, 61.4
α, β, γ(°)	90, 105.4, 90	90, 105.4, 90	90, 104.0, 90
Protein molecules in asymmetric unit	1	1	1
Unique reflections	23291 (2044)	34633 (3692)	33130 (2447)
Completeness (%)	98.55 (86.65)	91.47 (97.44)	94.67(70.60)
R_{merge}[a] (%)	10.9 (59.4)	9.5 (46.7)	9.0 (67.5)
Mean I/sigma(I)	28.85 (3.20)	14.37 (3.31)	11.36 (3.60)
Wilson B-factor (Å^2)	43.22	25.38	23.74
Refinement statistics			
Resolution (Å)		1.95	1.99
R_{work}[b] (%)		17.70 (21.31)	16.90 (17.90)
R_{free}[b] (%)		21.76 (27.14)	21.31 (25.22)
No. residues		412	412
No. ligands		0	111
No. water molecules		302	455
No. atoms		3538	3805
RMSD			
Bond lengths (Å)		0.007	0.010
Bond angles (°)		1.11	1.29
Average B-factors (Å^2)		25.90	20.30
Macromolecules		25.40	18.90
Ligands		-	26.80
Solvent		31.70	27.40
Ramachandran			
Most favored regions (%)		96.57	97.29
Additional allowed regions (%)		3.43	2.71
Disallowed regions (%)		0	0
Clashscore		2.39	2.01
PDB code		5H9X	5H9Y

[a] $R_{\mathrm{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where *Ii*(*hkl*) is the *i*th observation of reflection *hkl* and $\langle I(hkl)\rangle$ is the weighted average intensity for all observations *i* of reflection *hkl*.

[b] $R_{\mathrm{work/free}} = \sum_{\mathrm{hkl}} ||F_{\mathrm{obs}}| - k|F_{\mathrm{calc}}|| / \sum_{\mathrm{hkl}} |F_{\mathrm{obs}}|$; R_{work} is the *R* value for the reflections used in the refinement, whereas R_{free} is the *R* value for 5% of the reflections selected randomly and not included in the refinement

3 SUPPORTING MATERIAL AND METHODS

3.1 Cloning, expression and purification

The marine bacterium *Paenibacillus barengoltzii* CAU904 used in this study has been deposited in the China General Microbiological Culture Center (CGMCC) under accession number CGMCC9530 [1]. From the genome information of *P. barengoltzii* [2], a putative GH family 64 β-1,3-glucanase gene (WP_016313499) was identified and amplified with the primers PbBgl64A-up and PbBgl64A-down (Table S1). The PbBgl64A cDNA sequence was deposited in the GenBank under accession No. KU363233 and subcloned into the pET-28a(+) vector (Novagen, USA) with *Nhe*I and *Xho*I restrictive digestion sites. Mutants E236A, D252A, R197A, N221A and H245A were generated using the Fast Mutagenesis System site-directed mutagenesis kit (TransGen Biotech, China) with primers shown in Table S1. All recombinant plasmids encoding these mutations were sequenced and verified.

The recombinant plasmids were transformed into *E. coli* BL21 (DE3) competent cells and cultured in LB medium with 50μg/mL kanamycin. After IPTG induction, the recombinant proteins were purified using a Ni-IDA column (1×5 cm) and Sephacryl S-100 HR column (GE Life Sciences, USA) as described previously [3]. The purified protein fractions were combined and concentrated for subsequent experiments. All mutants were expressed and purified in an identical manner. Selenomethionine-derivatized PbBgl64A was produced using a metabolic inhibition protocol and M9 medium supplemented with 50 μg/mL L-selenomethionine (Se). The derivatized protein was also purified using the same protocol.

3.2 Crystallization and data collection

PbBgl64A was concentrated to 20 mg/mL in crystallography buffer (20 mM Tris-HCl pH 8.0, 100 mM NaCl). Crystallization experiments were performed in 48-well plates by the sitting-drop vapor diffusion method at 20 °C, and each sitting drop was prepared by mixing 1 μL each of protein solution and reservoir solution. Optimized crystals suitable for diffraction were grown in drops containing 2 μL of protein solution and 0.5 μL of reservoir solution (1.2 M di-ammonium tartrate pH 7.0) at 20 °C. The crystals were obtained 20 days later. Se-derivatized PbBgl64A was prepared and concentrated to 15 mg/mL in crystallography buffer contain 5 mM DL-dithiothreitol (DTT), and optimized crystals were grown in drops containing 2 μL of protein solution and 0.5 μL of reservoir solution (14 mM sodium cholate, 1.1 M di-ammonium tartrate pH 7.0) at 20 °C. The Se-derivatized crystals were obtained 20 days later. To obtain complex crystals, we first attempted to co-crystallize the E236A and D252A mutant with different oligosaccharides, but this was not successful. Complex crystals were ultimately obtained by oligosaccharide-soaking experiments. A laminarihexaose complex crystal (PbBgl64A-L6) was obtained by adding 50 mM laminarihexaose to the reservoir solution (1.2 M di-ammonium tartrate pH 7.0) and soaking the E236A mutant crystals in the solution for 12 h.

Crystals were soaked in reservoir solution supplemented with 20% glycerol and then vitrified in liquid nitrogen. Diffraction data for SeMet-PbBgl64A and apo-PbBgl64A were collected at 100 K using beamline BL1A at KEK (Tsukuba, Japan), and diffraction data for PbBgl64A-L6 were collected at 100 K using beamline BL17U at SSRF (Shanghai, China). All diffraction data were indexed, integrated and scaled using the program HKL-2000 [4]. The X-ray data-collection statistics are presented in Table S2.

3.3 Phase determination, model building and refinement

The crystal structure of Se-derivatized PbBgl64A was determined using the single-wavelength anomalous diffraction (SAD) method. The phase calculations and the initial model building were performed using the Phenix.autosol [5]. Thereafter, model building and refinement were performed using COOT [6] and Phenix.refine [5], respectively. The resulting PbBgl64A model was determined using the molecular replacement (MR) method with the Se-derivatized PbBgl64A model as the search model. Structural homologs of PbBgl64A were identified by the DALI server [7]. The refinement statistics are shown in Table S2. The secondary structural elements were identified employing DSSP [8]. The cartoon depictions of the structures were prepared in PyMOL (v.1.3; Schrödinger LLC). The sequence alignments were created with T-coffee [9] and ESPript [10].

3.4 Substrate preparation and enzyme assay

As curdlan suspension is prone to form low-set-gel by heat treatment at 50–60 °C [11], the curdlan beads were prepared as

follows: 1.2 g curdlan (C7821, Sigma, USA; purity≥98%; insoluble) were suspended in 30 mL of 50 mM sodium acetate buffer pH 5.0, 100 mM NaCl to form a 4% (w/v) curdlan suspension. The suspension was then suspended using magnetic stirrers at 55 °C for 4 h to form an aqueous slurry of beads.

Enzymatic activity was assayed using the dinitrosalicylic acid (DNS) method [12] with minor modifications. The curdlan beads were used as the substrate. Briefly, the standard assay mixture contained 50 μL of a properly diluted enzyme solution and 350 μL of curdlan beads. The reactions were performed at 55 °C for 20 min and terminated by adding 0.6 mL of DNS reagent. The resulting mixture was then boiled for 10 min, chilled and centrifuged to remove the insoluble curdlan. The resulting reducing sugars were analyzed and measured spectrophotometrically at 540 nm using glucose as the standard. One unit of enzymatic activity was defined as the amount of enzyme required to produce 1 μmol of glucose per minute under the above conditions.

Substrate specificity of PbBgl64A towards different natural β-1,3-glucan was determined by measuring the enzyme's activity in the presence of different substrates (1%, w/v) such as curdlan (C7821, Sigma, USA), laminarin (L9634, Sigma, USA) and yeast β-D-glucan (Angel Yeast, China; beta-1,3/1,6-linkage ratio ≈ 85:15).

3.5 Hydrolysis properties of PbBgl64A

The hydrolysis properties of PbBgl64A were investigated by analyzing the hydrolysis products from the substrates including curdlan (Sigma, USA) and laminari-oligosaccharides: laminaribiose, laminaritriose, laminaritetraose and laminaripentaose (Megazyme, Ireland). Purified PbBgl64A (5 U/mL) was added to a 1% (w/v) solution of the substrates and then incubated at 55 °C for 2 h in 50 mM sodium acetate buffer pH 5.0. Samples were withdrawn at different times and immediately boiled for 5 min, followed by TLC analysis. The samples were spotted on a TLC plate, developed in butan-1-ol:acetic acid:water (2:1:1, v/v) as the solvent, and sprayed with a methanol-sulfuric acid mixture (95:5, v/v). The hydrolysis products were visualized by heating the plate at 130 °C in an oven for a few minutes. Quantitative hydrolysate analysis was performed using high-performance anion-exchange chromatography coupled with pulsed ampere detection (HPAEC-PAD; ICS-5000$^+$ system and CarboaPac^{TM} PA1 column, Thermo, USA) [3].

3.6 Mass spectrometric analysis

Hydrolytic products derived from curdlan by PbBgl64A were analyzed by matrix-assisted laser desorption ionization/time of flight mass spectrometry (MALDI-TOF MS). Appropriately diluted enzyme was incubated with 2% (w/v) curdlan in 50 mM sodium acetate buffer pH 5.0 at 55 °C for 2 h (500 μL assay volume). The sample was diluted 100-fold prior to mixing with an equal volume of matrix (2,5-dihydroxybenzoic acid, 10 mg/mL in water). Sample (1 μL) was then spotted onto the MALDI plate and analyzed in an AB SCIEX TOF/TOF^{TM} 5800 System operated in positive-ion mode.

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