Biochemical Properties and Substrate Recognition Mechanism of GH31 α-Glucosidase from *Bacillus* sp. AHU 2001 with Broad Substrate Specificity

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Running title: GH31 α-Glucosidase from *Bacillus* sp. AHU 2001

Abbreviations: ABC, ATP-binding cassette; BspAG31A, *Bacillus* sp. AHU 2001 α-glucosidase; BSA, bovine serum albumin; BtAG, *B. thermoamyloliquefaciens* α-glucosidase II; CjAgd31B, *Cellvibrio japonicus* oligosaccharide α-1,4-glucosyltransferase; DP, degree of polymerization; GH, glycoside hydrolase family; MP, maltose phosphorylase; NCBI, National Center for Biotechnology Information; pNPG, *p*-nitrophenyl α-D-glucopyranoside

α-Glucosidases are ubiquitous enzymes that hydrolyze the α-glucosidic linkage at the non-reducing end of substrates. In this study, we characterized an α-glucosidase (BspAG31A) belonging to glycoside hydrolase family 31 from *Bacillus* sp. AHU 2001. Recombinant BspAG31A, produced in *Escherichia coli*, had high hydrolytic activity toward maltooligosaccharides, kojibiose, nigerose, and neotrehalose. This is the first report of an α-glucosidase with high activity toward neotrehalose. The transglucosylation products, nigerose, kojibiose, isomaltose, and neotrehalose, were generated from 440 mM maltose. Substitution of Tyr268, situated on the β→α loop 1 of BspAG31A, with Trp increased hydrolytic activity toward isomaltose. This mutation reduced the hydrolytic activity toward maltooligosaccharides more than toward kojibiose, nigerose, and neotrehalose. Analysis of the Y173A mutant of BspAG31A showed that Tyr173, situated on the N-terminal domain loop, is associated with the formation of subsite +2. In Y173A, the k_{cat}/K_m for maltooligosaccharides slightly decreased with an increasing degree of polymerization compared with wild type. Among the amino acid residues surrounding the substrate binding site, Val543 and Glu545 of BspAG31A were different from the corresponding residues of *Bacillus thermoamyloliquefaciens* α-glucosidase II, which has higher activity toward isomaltose than BspAG31A. The E545G mutation slightly enhanced isomaltase activity without a large reduction of hydrolytic activities toward other substrates. V543A showed 1.8–3.5-fold higher hydrolytic activities toward all substrates other than neotrehalose compared with wild type, although its preference for isomaltose was unchanged.

Keywords: α-Glucosidase; Glycoside hydrolase family 31; Substrate specificity; Neotrehalose; Transglucosylation

1 Introduction

α-Glucosidase (EC 3.2.1.20) catalyzes the hydrolysis of α-glucosidic linkages at the non-reducing end of substrates to liberate α-D-glucose. This enzyme is widely distributed in various organisms including bacteria, yeasts, fungi, archaea, plants, and animals. The substrate specificity of α-glucosidases varies depending on the enzyme origin. Chiba classified α-glucosidases into three groups based on substrate specificity: group I, enzymes which prefer heterogeneous substrates, represented by sucrose and synthetic glucosides, including *p*-nitrophenyl α-D-glucopyranoside (pNPG) and phenyl α-D-glucopyranoside, to homogeneous substrates such as maltose; group II, enzymes which prefer homogeneous substrates to heterogeneous substrates; group III, enzymes which are highly active toward homogeneous long-chain substrates such as starch [1]. In addition to hydrolysis, α-glucosidases catalyzes transglucosylation to form new glucosidic linkages, and thus it is used for the production of α-glucosides [2-5] and oligosaccharides such as isomaltooligosaccharide and nigerooligosaccharide [6,7].

According to sequence-based classification of glycoside hydrolases, α-glucosidases are mainly classified into glycoside hydrolase family (GH) 13 and 31 [8]. Most α-glucosidases from bacteria, yeast (*Saccharomyces cerevisiae*), and insects belong to GH13, and those from plants, animal, fungi, and yeast (*Schizosaccharomyces pombe*) to GH31 (characterized enzymes are listed in the CAZy database [9]: GH13, http://www.cazy.org/GH13_characterized.html; GH31, http://www.cazy.org/GH31_characterized.html). GH13 is a large family and contains various amylolytic enzymes, such as α-amylase (EC 3.2.1.1), cyclodextrin glucanotransferase (EC 2.4.1.19), and α-glucosidase. GH31 includes α-xylosidase (EC 3.2.1.177), α-glucan lyase (EC 4.2.2.13), isomaltosyltransferase, and oligosaccharide α-1,4-glucosyltransferase (EC 2.4.1.161). Only a few GH31 α-glucosidases have been found in bacteria thus far [10-13]. The catalytic domains of both GH13 and GH31 α-glucosidases are formed by $(\beta/\alpha)_8$ barrel-folds [14,15]. The catalytic nucleophiles of both family enzymes are located at the C-terminal end of the fourth β-strand of the catalytic domain. The general acid/base catalysts of GH13 and GH31 enzymes are at the C-termini of the fifth and sixth β-strands of the catalytic domains, respectively.

Bacterial α-glucosidase is generally involved in the intracellular metabolism of maltooligosaccharides. In the gram-positive soil bacterium, *Bacillus subtilis*, maltose inducible α-glucosidase (MalL) [16], belonging to GH13, is predicted to degrade maltooligosaccharides, which are taken up into the cell through the ATP-binding cassette (ABC) transporter, together with maltose phosphorylase YvdK (MP, EC 2.4.1.8) and neopullulanase YvdF (EC 3.2.1.135) [17]. Maltodextrin utilization machinery similar to that of *B. subtilis* was found in *Lactobacillus acidophilus* NCFM [18]. Although the genomic codes of many living things are available, it is very difficult to obtain new enzymes based on sequence information alone. Screening of enzymes with novel properties from nature and their biochemical and structural characterizations is still important because these analyses give us new valuable information on enzyme structure-function relationships. In the present study, we investigated the amino acid sequence, gene organization, and functions of amino acid residues in the substrate binding site of a GH31 α-glucosidase (BspAG31A) with novel substrate specificity (high hydrolytic activity toward neotrehalose) from the soil bacterium, *Bacillus* sp. AHU2001.

2 Materials and methods

2.1 Purification of BspAG31A from *Bacillus* sp. AHU 2001

Bacillus sp. AHU 2001 was grown in 2.5 L of culture medium, containing 10 g/L soluble starch (Nacalai Tesque, Kyoto, Japan), 5 g/L polypeptone, 5 g/L yeast extract, 1 g/L KH_2PO_4, 0.2 g/L $MgSO_4$, and 5 g/L NaCl, at 37°C for 24 h. Bacterial cells, harvested by centrifugation (12,000 ×g, 4°C, 10 min), were suspended in 200 mL of 10 mM sodium phosphate buffer (pH 6.5), and disrupted by sonication. The cell-free extract was applied to a DEAE Sepharose CL-6B column (2.7 cm I.D. × 26 cm, Amersham Biosciences, Uppsala, Sweden), equilibrated with 10 mM sodium phosphate buffer (pH 6.5). Adsorbed protein was eluted with a linear gradient of NaCl (from 0 to 0.5 M; elution volume, 1 L). Ammonium sulfate was added to the active fractions pooled up to 30% saturation, and the sample was applied to a Butyl Sepharose 4 Fast Flow column (2.7 cm I.D. × 13 cm, GE Healthcare, Uppsala, Sweden), equilibrated with 10 mM sodium phosphate buffer (pH 6.5)

containing 30% saturation ammonium sulfate. Adsorbed protein was eluted with a descending linear gradient of ammonium sulfate (30 to 0% saturation; elution volume, 0.5 L). Collected fractions were concentrated to 3 mL using a Vivaspin 20 (nominal molecular weight limit 30,000 Da; Sartorius, Göttingen, Germany), and subjected to Sephacryl S-300 HR column chromatography (1.6 cm I.D. × 60 cm, GE Healthcare) in 10 mM sodium phosphate buffer containing 0.1 M NaCl (pH 6.5). The sample collected was applied to a Resource Q column (1 mL, GE Healthcare), equilibrated with 10 mM sodium phosphate buffer (pH 6.5). Adsorbed protein was eluted with a linear gradient of NaCl (0 to 0.5 M; elution volume, 15 mL).

2.2 N-terminal amino acid sequence analysis

Purified BspAG31A (6 μg) was separated by SDS-PAGE, and transferred to a polyvinylidene difluoride membrane by electroblotting in a semidry blotting apparatus. The band of BspAG31A was cut off from the membrane and subjected to N-terminal sequence analysis with a Procise 492 protein sequencer (Perkin Elmer, Waltham, MA, USA).

2.3 Sequence analysis of BspAG31A

Partial *BspAG31A* was amplified by PCR. Primers, 5′-CARGAYACNWSNTTYGCNATHATGCC-3′ (sense) and 5′-GGYTCRTTCATRTCRTTCCA-3′ (antisense), designed based on the N-terminal amino acid sequence of BspAG31A and the consensus sequence of bacterial GH31 α-glucosidase, respectively, were used. Primester HS DNA polymerase (Takara Bio, Otsu, Japan) and the genomic DNA of *Bacillus* sp. AHU 2001 as template were used in the PCR. The amplified DNA fragment was cloned into the *Eco*RV site of pBluescript II SK (+) (Stratagene; La Jolla, CA, USA), and sequenced with an Applied Biosystems 3130 Genetic Analyzer (Life Technologies, Carlsbad, CA, USA). Upstream and downstream regions of the obtained region were obtained by inverse PCR. Five μg of genomic DNA was digested with *Bam*HI (Takara Bio). The resulting fragments were cyclized through self-ligation with T4 ligase (Takara Bio), and used as the template. The primer sequences were 5′-AAGTTCGTGAATGGTGGGGA-3′ (sense) and

5′-GCTGATGGTATGGGTATCTC-3′ (antisense). The amplified DNA fragment was directly sequenced as described above.

2.4 Preparation of recombinant BspAG31A

The *Nde*I and *Xho*I sites were introduced to the 5′- and 3′-termini of *BspAG31A* and an internal *Nde*I site was destroyed by the overlap extension PCR method [19], in which primers, 5′-CAAAGGGTGGAGCATATGTTGCAAG-3′ (sense, *Nde*I site underlined), 5′-CATTCCTTCATA**G**GTAGCTTTTCCC-3′ (antisense, substituted nucleotide in boldface), 5′-GGGAAAAGCTAC**C**TATGAAGGAATG-3′ (sense, substituted nucleotide in boldface), and 5′-AAAAGTTTCCTCGAGTACTATTAAAAATG-3′ (antisense, *Xho*I site underlined) were used. The genomic DNA of *Bacillus* sp. AHU 2001 was used as the template. The amplified DNA fragment was cloned into the *Nde*I and *Xho*I sites of pET23a (Novagen, Darmstadt, Germany).

The expression plasmid of BspAG31A was introduced into *E. coli* BL21 (DE3), and recombinant BspAG31A was produced. The *E. coli* transformant was cultured in 1 L of Luria–Bertani medium containing 100 μg/mL ampicillin at 37°C until the A_{600} reached 0.5. Production of the recombinant protein was induced by the addition of 1 mL of 0.1 M isopropyl β-D-thiogalactoside to the culture medium (final concentration, 0.1 mM), and the induction culture was carried out at 18°C for 24 h. Bacterial cells, harvested by centrifugation (12,000 ×g, 4°C, 10 min), were suspended in 150 mL of 10 mM sodium phosphate buffer (pH 6.0) containing 0.5 M NaCl and 50 mM imidazole, and disrupted by sonication. The cell-free extract, obtained by centrifugation (12,000 ×g, 4°C, 10 min), was applied to a Ni-chelating Sepharose column (2.5 cm I.D. × 3 cm, GE Healthcare). After thorough washing with the same buffer, the adsorbed protein was eluted with a linear gradient of imidazole from 50 to 500 mM (total elution volume, 300 mL). The pooled active fractions were dialyzed against 10 mM sodium phosphate buffer (pH 6.5).

2.5 Preparation of BspAG31A mutant enzymes

Expression plasmids of BspAG31A mutant enzymes were constructed using a Primestar

Mutagenesis Basal Kit (Takara Bio). The primers used were as follows: for Y173A, 5′-GATGTG**GC**TGCCCCTCATAATCCGGAA-3′ (sense, substituted nucleotides in boldface) and 5′-AGGGGCA**GC**CACATCCGTATTCCAGTT-3′ (antisense); for Y268W, 5′-TCTCGTT**GG**AGTTATAAAACAGAGCAA-3′ (sense) and 5′-ATAACT**CC**AACGAGATTGATGATATCC-3′ (antisense); for W370A, 5′-GATGTA**GC**GCCGGGTCGAAGTGCGTTT-3′ (sense) and 5′-ACCCGGC**GC**TACATCTCCAAAGAATAC-3′ (antisense); for V543A, 5′-CATAGCG**C**TATTGAGTCTGTACGTCAA-3′ (sense) and 5′-CTCAATA**G**CGCTATGGTTGCGGAAAAA-3′ (antisense); for E545G, 5′-GTTATTG**G**GTCTGTACGTCAAGAGCCA-3′ (sense) and 5′-TACAGAC**C**CAATAACGCTATGGTTGCG-3′ (antisense). The mutant enzymes were prepared from an *E. coli* transformant with the expression plasmid as described above.

2.6 Protein assay

During the purification procedures, the protein concentration was determined by the UV method [20], in which the A_{280} was measured. The extinction coefficient of 1.00 mg/mL purified BspAG31A was 2.20, which was calculated from the enzyme concentration of the purified enzyme, determined by amino acid analysis using the ninhydrin colorimetric method with a JLC-500/V (JEOL, Tokyo, Japan) [21].

2.7 Enzyme kinetics

2.7.1 Standard enzyme assay

A reaction mixture (50 μL), consisting of an appropriate concentration of enzyme, 4 mM maltose (Nihon Shokuhin Kako, Tokyo, Japan), 42 mM sodium phosphate buffer (pH 6.5), and 0.2 mg/mL bovine serum albumin (BSA), was incubated at 37°C for 10 min. The enzyme reaction was terminated by the addition of 100 μL of 2 M Tris-HCl buffer (pH 7.0), and liberated D-glucose was measured with the Glucose CII Test (Wako Pure Chemical Industries, Osaka, Japan). One U of

α-glucosidase activity was defined as the amount of enzyme able to hydrolyze 1 μmol of maltose in 1 min under these conditions.

2.7.2 Optimal pH

Enzyme activity was measured at various pH values. As the reaction buffer, 80 mM Britton–Robinson buffer (pH 4.6–9.0) was used. The other reaction conditions were the same as for the standard assay method for each enzyme.

2.7.3 Heat and pH stability

Residual activity was measured after heat and pH treatment to evaluate the stability ranges of the temperature and pH, respectively. In the heat treatment, 30 μL of a mixture containing enzyme, 67 mM sodium phosphate buffer (pH 6.5), and 0.33 mg/mL BSA was incubated at 30–70°C for 15 min. In the pH treatment, 20 μL of a mixture containing enzyme, 100 mM Britton–Robinson buffer (pH 3.4–11.1), and 0.5 mg/mL BSA was incubated at 4°C for 24 h.

2.7.4 Substrate specificity

Maltose, kojibiose (Wako Pure Chemical Industries), nigerose (Wako Pure Chemical Industries), neotrehalose (Hayashibara, Okayama, Japan), isomaltose (Tokyo Chemical Industry, Tokyo, Japan), trehalose (Hayashibara), and pNPG (Nacalai Tesque) were tested as substrates. Reaction rates towards 1 mM substrate other than pNPG were measured under the conditions of the standard enzyme assay. In the reaction with pNPG, the enzyme reaction was terminated by the addition of 100 μL of 1 M Na_2CO_3, and the *p*-nitrophenol liberated was determined from A_{400} using $\varepsilon_{1\ mM,\ 400\ nm}$ = 5.56, which was determined with the authentic *p*-nitrophenol (Kanto Chemical, Tokyo, Japan).

2.7.5 Kinetic parameters for the hydrolysis of various substrates

Kinetic parameters for a series of maltooligosaccharides, kojibiose, nigerose, isomaltose and neotrehalose were determined from reaction rates at various substrate concentrations.

Maltooligosaccharides other than maltose were purchased from Nacalai Tesque. A reaction mixture (50 μL), consisting of an appropriate concentration of enzyme, substrate, 42 mM sodium phosphate buffer (pH 6.5), and 0.2 mg/mL BSA, was incubated at 37°C for 10 min. Substrate concentrations were as follows: 0.2-2 mM for maltose, maltotriose, and maltotetraose; 1-10 mM for the other substrates. The enzyme reaction was terminated and liberated D-glucose was measured as described in the section 2.7.1. The Michaelis–Menten equation was fitted to the reaction rates obtained using Grafit version 7.0.2 (Erithacus Software, East Grinstead, UK).

2.8 Transglucosylation

A reaction mixture (1 mL) containing enzyme (0.75 U), 440 mM maltose, and 10 mM MES-NaOH buffer (pH 6.5) was incubated at 37°C. Two hundred microliters of the sample was taken at the indicated times, and the reaction was terminated by heating at 100°C for 10 min. The distribution of the degree of polymerization (DP) was analyzed by high performance liquid chromatography under the following conditions: injection volume, 10 μL (5-fold diluted sample); column, Aminex HPX42A (7.8 mm I.D. × 300 mm, Bio-Rad, Hercules, CA), column temperature, 75°C; elution, water; flow rate, 0.5 mL/min; detection, refractive index. High performance anion exchange column chromatography was used to analyze the transglucosylation product at 48 h. The analytical conditions were: column, CarboPac PA1 (4 mm I.D. × 250 mm, Dionex, Sunnyvale, CA, USA); elution, 100 mM NaOH; flow rate, 0.8 mL/min; detection, pulsed amperometry.

2.9 Comparison of amino acid sequence of BspAG31A with GH31 enzymes

To compare the amino acid sequences, a multiple-sequence alignment of BspAG31A and GH31 enzymes was constructed with a MAFFTash program [22].

2.10 Construction of a model structure of BspAG31A

Model structure of BspAG31A was constructed using a Phyre2 program [23], in which *Cellvibrio japonicus* oligosaccharide α-1,4-glucosyltransferase (EC 2.4.1.161; CjAgd31B; protein data bank

code, 4B9Z) [24] was selected as template. Acarbose, bound to CjAgd31B (4B9Z), was superimposed to the model structure of BspAG31A with the PyMOL Molecular Graphics System version 0.99rc6 (Schrödinger, New York, NY, USA).

3 Results and Discussion

3.1 Purification and sequence analysis of BspAG31A

Bacterial strain SW20, producing α-glucosidase (maltose-hydrolyzing enzyme), was isolated from soil sampled near a hot spring in Hokkaido, Japan. The partial sequence of the 16S rDNA of SW20 (1,389 bp) was 96.3% identical with that of *Bacillus cytotoxicus* NVH391-98, and thus the SW20 strain was designated as a *Bacillus* sp. This bacterial strain was deposited at Laboratory of Culture Collection of Microorganisms, Faculty of Agriculture, Hokkaido University (Sapporo, Japan) as AHU 2001. α-Glucosidase was purified to homogeneity from the cell-free extract of *Bacillus* sp. AHU 2001 by four steps of column chromatography, and 79.8 μg of purified enzyme (28.7 U/mg) was obtained (Fig. S1). The molecular mass of this enzyme was estimated at 91.3 kDa by SDS-PAGE.

The N-terminal amino acid sequence of this enzyme, determined by the Edman degradation, was Met-Leu-Gln-Asp-Thr-Ser-Phe-Ala-Ile-Met-Pro-Asp-Lys-Glu, which is similar to GH family 31 α-glucosidases. The partial BspAG31A gene (1,221 bp) was obtained by PCR using primers designed from this N-terminal sequence and region A of *Bacillus* GH31 α-glucosidases (Trp-Asn-Asp-Met-Asn-Glu-Pro) [25], and the genomic DNA as the template. Upstream and downstream regions of the obtained sequence were obtained by an inverse PCR method, and in total, 9,008 bp of nucleotide sequence, including full-length *BspAG31A* (2,373 bp), was determined.

Two (*malA* and *malB*) and three open reading frames (*malC*, *malD*, and *malE*) were found upstream and downstream of *BspAG31A*, respectively (Fig. 1). The obtained nucleotide sequence of *malA*, *malB*, *BspAG31A*, *malC*, *malD*, and *malE* were deposited in the DDBJ database under accession numbers AB971787, AB971788, AB971789, AB971790, AB971791, and AB971792, respectively. The amino acid sequence of MalA was 56% identical with *Brachybacterium faecium*

permeases of the ABC-type sugar transport system [National Center for Biotechnology Information (NCBI) code, YP_003153876.1], and that of MalB was 54% identical with the *B. faecium* ABC-type sugar transport system permease component (NCBI code, YP_003153875.1). Thus, these proteins are predicted to be involved in the uptake of maltooligosaccharides. The sequence identity of MalC with the *Bacillus cereus* maltose operon transcriptional repressor (NCBI code, YP_003793713) was 78%, and that of MalD with the *B. cereus* maltose operon transcriptional repressor (NCBI code, NP_980355.1) was 53%. The amino acid sequence of MalE was 46–52% identical with those of characterized MPs belonging to GH65. Although maltooligosaccharides can be hydrolyzed completely to D-glucose only by BspAG31A, the presence of MalE indicates that a part of maltose, resulting from the hydrolysis of maltooligosaccharides catalyzed by BspAG31A, is metabolized thorough phosphorolysis.

BspAG31A encoded 790 amino acid residues of the polypeptide. The N-terminal sequence of the deduced amino acid sequence was completely identical with the sequence obtained by the N-terminal sequence analysis of purified BspAG31A. The molecular mass of BspAG31A predicted from the amino acid sequence was 91251.24 Da, which coincided well with that of purified BspAG31A. The amino acid sequence of BspAG31A showed high sequence identity to bacterial GH31 α-glucosidases. The sequence identity of BspAG31A to characterized bacterial GH31 α-glucosidases was as follows: *Bacillus thermoamyloliquefaciens* α-glucosidase II (BtAG, GenBank code, BAA76396.1), 61.0%; *Lactobacillus johnsonii* α-1,3-glucosidase (GenBank code, ACO57638.1), 34.0%; *Ruminococcus obeum* α-glucosidase (UniProt code, A5ZY13), 26.7%; and *Thermoanaerobacter ethanolicus* α-glucosidase (GenBank code, ABR26230.1), 39.0%. The conserved regions A and B of GH31 [25], including catalytic nucleophile and general acid/base catalyst, respectively, were found in BspAG31A (Table 1). Structural analysis of GH31 enzymes demonstrated that the conserved regions A and B are located C-termini of β-strands 4 and 6 of the catalytic domain, respectively [13, 24, 26-33]. Asp406 and Asp483 of BspAG31A, included in the conserved regions A and B, were situated on the β→α loops 4 and 6, respectively, in the model structure of BspAG31A, which was constructed using CjAgd31B structure (sequence identity of

BspAG31A and CjAgd31B is 22%) [24], selected as the best template by a Phyre2 program [23] (Fig. 2). Thus Asp406 and Asp483 of BspAG31A are presumably the catalytic nucleophile and the general acid/base catalyst, respectively.

3.2 Production, purification, and basic properties of recombinant BspAG31A

Recombinant BspAG31A with a hexahistidine-tag at the C-terminus was produced in an *E. coli* transformant harboring the expression plasmid. From 1 L of culture broth, 380 U of the recombinant enzyme (13 mg, estimated from the specific activity of the purified enzyme) was obtained, and 9.45 mg of purified enzyme (29.3 U/mg) was obtained by Ni-chelating column chromatography. Recombinant BspAG31A showed the highest activity at pH 6.8 (activities close to the optimal activity were maintained between pH 6.3 to 6.8), similar to BtAG, which is most active at neutral pH [34]. This enzyme retained over 90% of its original activity between pH 4.2–9.9 (at 4°C for 24 h) and below 45°C (at pH 6.5 for 15 min).

3.3 Substrate specificity of recombinant BspAG31A

BspAG31A showed high regioselectivity for the α-(1→4)-glucosidic linkage, but had relatively high activity towards nigerose [α-(1→3)], kojibiose [α-(1→2)], and neotrehalose (α1↔β1): reaction rates for 1 mM maltose, kojibiose, nigerose, and neotrehalose were 30.7 ± 0.2 s^{-1} (100%), 3.83 ± 0.12 s^{-1} (12%), 7.63 ± 0.45 s^{-1} (25%), and 7.43 ± 0.21 s^{-1} (24%), respectively. The k_{cat}/K_m values for kojibiose, nigerose, and neotrehalose were 6.3%, 15%, and 13% of that for maltose, respectively (Table 2). The k_{cat} for neotrehalose was almost two-fold higher than kojibiose and nigerose. To the best of our knowledge, BspAG31A is the first α-glucosidase with hydrolytic activity toward neotrehalose. The reaction velocity for the hydrolysis of 1 mM isomaltose is very low, 0.157 ± 0.009 s^{-1} (0.51% of that for maltose). The k_{cat}/K_m for isomaltose, 0.185 $s^{-1}mM^{-1}$, was 0.26% of that for maltose. Trehalose and pNPG were not hydrolyzed at all. Compared with BtAG [34], which has high sequence identity with BspAG31A, BspAG31A has higher preference for the α-(1→2)- and α-(1→3)-glucosidic linkages and lower preference for the α-(1→6)-glucosidic linkage.

Recombinant BspAG31A had the highest k_{cat}/K_m value for maltotriose among the maltooligosaccharides. The k_{cat}/K_m value for maltooligosaccharides longer than maltotriose decreased with an increasing DP, mainly because of the increasing K_m value. In particular, the k_{cat}/K_m value for maltopentaose was 5.0-fold lower than that for maltotetraose.

3.4 Transglucosylation of recombinant BspAG31A

Transglucosylation products of recombinant BspAG31A were analyzed. Recombinant BspAG31A was incubated with 440 mM maltose, and the distribution of the reaction product DP was monitored (Fig. 3A). At the initial stage of the reaction, the transglucosylation products with DP of 3–5 were detected: at 3 h, the concentrations of products of DP3, DP4, and DP5 were 78, 13, and 0.2 mM, respectively. Transglucosylation products higher than DP2 were degraded at the late stage of the reaction, presumably because of hydrolysis, but the concentration of DP2 was only minimally changed. High performance anion exchange column chromatography analysis revealed that kojibiose, nigerose, isomaltose, and neotrehalose were generated over 48 h through transglucosylation, in which D-glucose served as the acceptor (Fig. 3B). Isomaltose, which was a poor substrate for the hydrolysis of BspAG31A, remained as the main product after further incubation (data not shown). In contrast to BspAG31A, CjAgd31B predominantly catalyzes transglucosylation to produce α-(1→4)-glucosidic linkage even at low substrate concentrations. Larsbrink *et al.* proposed that Glu417 and Arg463, binding O3 of the sugar at subsite +1, prevent binding of nucleophilic water, which accepts the glycosyl residue from the covalent bond intermediate in the hydrolysis, to the general acid/base catalyst [24]. BspAG31A possesses Ser411 at the position corresponding to Glu417 of CjAgd31B, while it has Arg467 corresponding to Arg463 of CjAgd31B (Fig. 2B). No interaction of Ser411 of BspAG31A with a substrate was predicted from the model structure, and most α-glucosidases also have small amino acid residue, Ser or Ala, at the corresponding position of Ser411 of BspAG31A (Table 1). Small amino acid residue at the Ser411 position of BspAG31A might be important for binding of nucleophilic water, and facilitate hydrolysis.

3.5 Site-directed mutagenesis of amino acid residues involved in the formation of the substrate binding site of BspAG31A

An aromatic amino acid residue (Tyr or Trp), situated on the β→α loop 1 of the catalytic $(\beta/\alpha)_8$-barrel domain, is suggested to be important for the specificity of α-glucosidases to the scissile glucosidic linkage [13, 31, 35]. In *R. obeum* α-glucosidase, which is more active toward isomaltose than toward maltose, substitution of Trp169, localized on the β→α loop 1, to Tyr enhances specificity to the α-(1→4)-glucosidic linkage [13]. Conversely, replacement of Tyr1251 of the human maltase glucoamylase complex, which is located on the β→α loop 1 of the C-terminal glucoamylase catalytic domain, with Trp increases preference for the α-(1→6)-glucosidic linkage [31]. Comparison of the aromatic amino acid residues of GH31 α-glucosidases indicates that many enzymes with high preference for the α-(1→4)-glucosidic linkage also have Trp at this position (Table 1). In *Schwanniomyces occidentalis* α-glucosidase, substitution of the equivalent Trp (Trp324) to Tyr reduced both the hydrolysis of the α-(1→6)-glucosidic linkage and formation of this linkage through transglucosylation [35]. As observed for α-(1→4)-linkage specific enzymes, BspAG31A was predicted to have Tyr268 on the β→α loop 1 (Table 1 and Fig. 2). Substitution of Tyr268 with Trp drastically enhanced hydrolytic activity towards isomaltose (Table 2). Y268W showed a 2.0-fold higher k_{cat} and 7.4-fold lower K_m for isomaltose. The Y268W mutation reduced the hydrolytic activity toward the other substrates tested. The decrease of hydrolytic activity toward maltooligosaccharides was more pronounced than those of kojibiose, nigerose, and neotrehalose, resulting in 5.8-, 4.7-, and 3.5-fold higher k_{cat}/K_m values (relative to maltose) for these substrates than for wild type, respectively. Conversely, for *S. occidentalis* α-glucosidase, the hydrolytic activities toward isomaltose, nigerose, and kojibiose were decreased to a greater extent by the W324Y mutation than that for maltose [35]. *L. johnsonii* NCC533 α-(1→3)-glucosidase, with hydrolytic activity toward α-(1→2)-, α-(1→4)-, and α-(1→6)-linkages in addition to the α-(1→3)-linkage [11], has Trp262 at the position corresponding to Tyr268 of BspAG31A (Table 1). These findings indicate that the Trp residue on the β→α loop 1 plays a role in the recognition of glucosidic linkages other than the α-(1→4)-linkage. In other words, Tyr on β→α loop 1 is important

for high specificity to α-(1→4)-linkage.

A long bulging loop from the N-terminal domain of GH31 α-glucosidases (N-loop) contributes to the formation of the substrate binding site. The sequence of this loop is diverse, but an aromatic residue important for high activity toward long-chain substrates has been identified in sugar beet α-glucosidase [36]. Substitution of Phe236 of sugar beet α-glucosidase with Ala or Ser results in reduction of hydrolytic activities toward maltooligosaccharides longer than maltose. Introduction of a Phe residue at the position of Thr228 of *Aspergillus niger* α-glucosidase enhances the preference for long-chain substrates through an increase in the affinity at subsite +3. From these kinetic experiments of mutant enzymes, the aromatic amino acid residue on the N-loop is considered to be important for the preference for long-chain substrates *via* interaction at subsites +2 and +3. In *Cj*Adg31B, Tyr179 on the N-loop forms the tyrosine clamp with Tyr376 on β→α loop 3 of the catalytic domain, and these aromatic residues form van der Waals contacts with glucosyl residue at subsite +2 [24]. Similarly, BspAG31A has aromatic residues, Tyr173 and Trp370, at the corresponding position of Tyr179 and Tyr376 of *Cj*Adg31B, respectively (Table 1 and Fig. 2). As shown in Table 1, Trp residue situated on the long β→α loop 3 of the catalytic domain is completely conserved in GH31 α-glucosidases. In several α-glucosidases, the Trp residue on β→α loop 3 contacts with substrates at the −1 and +1 subsites [29, 31, 32]. In this study, mutant BspAG31As, Y173A and W370A, were characterized. As observed for the mutant enzymes of sugar beet α-glucosidase, Y173A mutant of BspAG31A showed reduced preference for trisaccharides (Table 2), indicating that Tyr173 is involved in the substrate binding at subsite +2. This result is consistent well with the prediction from the model structure. In contrast to mutant enzymes of sugar beet α-glucosidase, for the Y173A mutant, the k_{cat}/K_m values for maltooligosaccharides longer than maltotriose decreased to a lesser extent with an increasing DP compared with wild type. Thus the substrate binding mode of maltooligosaccharides with ≥DP4 might be modified through elimination of an aromatic side chain on the N-loop. W370A mutation resulted in large loss of affinity for maltose (k_{cat}, 1.95 ± 0.16 s^{-1}; K_m, 21.5 ± 3.2 mM, which were 28-fold lower and 29-fold higher than those of wild type, respectively). This result indicates that the conserved Trp on β→α loop 3 is

fundamentally important for substrate binding.

Both BspAG31A and BtAG have high regioselectivity for the α-(1→4)-linkage in hydrolytic reactions, but these enzymes show different specificity for other glucosidic linkages. Upon comparison of their amino acid residues predicted to be located near the active sites, only Val543 and Glu545 of BspAG31A are different from the corresponding amino acid residues of BtAG (Ala and Gly are at the positions, respectively), although these amino acid residues do not appear to directly interact with the substrate (Fig. 2). The BspAG31A V543A mutant showed similar preference for the α-(1→6)-linkage to the wild type, but E545G showed slightly higher hydrolytic velocity for isomaltose relative to that for maltose than the wild type. The reaction velocities of wild type, V543A, and E545G for 1 mM maltose were 30.7 ± 0.2 s^{-1}, 56.5 ± 2.2 s^{-1}, and 27.2 ± 1.3 s^{-1}, respectively, while those for 1 mM isomaltose were 0.157 s^{-1} (0.51% of velocity for maltose), 0.244 ± 0.003 s^{-1} (0.43%), and 0.281 ± 0.014 s^{-1} (1.0%), respectively. Therefore, Gly546 of BtAG might partly contribute to the hydrolytic activity toward α-(1→6)-linked substrates. The E545G mutant showed similar kinetic parameters for maltooligosaccharides, kojibiose, and neotrehalose to those of the wild type (only k_{cat} and K_m of E545G for nigerose were approximately equal to twice those of wild type) (Table 2). V543A showed higher catalytic efficiencies (1.8–3.5-fold of those of wild type) for hydrolysis of all substrates tested except for neotrehalose compared with wild type. The k_{cat}/K_m of V543A for neotrehalose was 74% of that of the wild type, resulting in a large reduction of preference for this substrate. The predicted position of Val543 in AG31G was too distant from the substrates to interact directly with them (Fig. 2A). Thus a slight conformational change of the substrate binding site, induced by the V543A mutation, might be favorable for enhancement of catalytic activity.

4. Conclusion

In this study, we found a GH31 α-glucosidase, BspAG31A, from soil bacterium, *Bacillus* sp. AHU-2001. The BspAG31A gene was a member of a gene cluster containing genes encoding putative maltodextrin metabolizing proteins including ABC-type sugar transporters and maltose

phosphorylase. Recombinant BspAG31A produced in *E. coli* had high regioselectivity for α-(1→4)-glucosidic linkage at the non-reducing end of substrates, but it showed relatively high activity towards nigerose, kojibiose, and neotrehalose. Hydrolytic activity toward neotrehalose has been first found in BspAG31A. Mutational analysis of BspAG31A revealed that Tyr268 on β→α loop 1 is important for high specificity to α-(1→4)-linked substrate; Tyr173 on N-loop is involved in the formation of subsite +2 and regulates specificity for substrate chain-length; and Trp370 on β→α loop 3 is fundamentally important for substrate binding. BspAG31A showed structural similarity to CjAgd31B, predominantly catalyzing transglucosylation. This suggests that partial structural elements determine the reaction specificity (hydrolysis or transglucosylation). Further structural and mutational analyses of BspAG31A would reveal the molecular basis of the reaction specificity. Site-directed mutations, introduced based on comparison of the amino acid sequences between BspAG31A and BtAG, did not significantly change the specificity for glucosidic linkage, suggesting that indirect interactions are also important for substrate specificity. Thus precise comparison of three-dimensional structures of BspAG31A and BtAG is required to understand the difference of substrate specificity of these enzymes.

Acknowledgements

We are grateful to Tomohiro Hirose of the Instrumental Analysis Division, Equipment Management Center, Creative Research Institute, Hokkaido University for N-terminal amino acid sequence analysis and amino acid analysis. We also thank the staff of the DNA sequencing facility of the Research Faculty of Agriculture, Hokkaido University for assistance with DNA sequence analysis.

References

1. S. Chiba, α-Glucosidases, in: Amylase Research Society of Japan (ed.), Handbook of amylases and related enzymes, Pergamon Press, Oxford, United Kingdom, 1988, pp. 104-116.
2. H. Murase, R. Yamauchi, K. Kato, T. Kunieda, J. Terao, Synthesis of a novel vitamin E

derivative, 2-(α-D-glucopyranosyl)methyl-2,5,7,8-tetramethylchroman-6-ol, by α-glucosidase-catalyzed transglycosylation, Lipids 32 (1997) 73-78.

3. F. Takenaka, H. Uchiyama, Synthesis of α-D-glucosylglycerol by α-glucosidase and some its characteristics, Biosci. Biotechnol. Biochem. 64 (2000) 1821-1826.
4. T. Ojima, W. Saburi, T. Yamamoto, T. Kudo, Characterization of *Halomonas* sp. strain H11 α-glucosidase activated by monovalent cations and its application for efficient synthesis of α-D-glycosylglycerol, Appl. Environ. Microbiol. 78 (2012) 1836-1845.
5. T. Ojima, K. Aizawa, W. Saburi, T. Yamamoto, α-Glucosylated 6-gingerol: chemoenzymatic synthesis using α-glucosidase from *Halomonas* sp. H11, and its physical properties, Carbohydr. Res. 354 (2012) 59-64.
6. H. Takaku, Manufacture of oligosaccharides, in: Amylase Research Society of Japan (ed.), Handbook of amylases and related enzymes, Pergamon Press, Oxford, United Kingdom, 1988, pp. 212-222.
7. T. Yamamoto, T, Unno, M. Sugawara, T. Goda, Properties of a nigerose and nigerosylmaltooligosaccharides-supplemented syrup, J. Appl. Glycosci. 46 (1999) 475-482.
8. B. Henrrisat, G. Davies, Structural and sequence-based classification of glycoside hydrolases, Curr. Opin. Struct. Biol. 7 (1997) 637-644.
9. V. Lombard, H. Golaconda Ramulu, E. Drula, P.M. Coutinho, B. Henrissat, The carbohydrate-active enzymes database (CAZy) in 2013, Nucleic Acids Res. 42 (2014) D490-D495.
10. S. Kashiwabara, S. Azuma, M. Tsuduki, Y. Suzuki, The primary structure of the subunit in *Bacillus thermoamyloliquefaciens* KP1071 molecular weight 540,000 homohexameric α-glucosidase II belonging to the glycosyl hydrolase family 31, Biosci. Biotechnol. Biochem. 64 (2000) 1379-1393.
11. M.S. Kan, M. Okuyama, H. Mori, A. Kimura, The first α-1,3-glucosidase from bacterial origin belonging to glycoside hydrolase family 31, Biochimie 91 (2009) 1434-1442.
12. Y.H. Wang, Y. Jiang, Z.Y. Duan, W.L. Shao, H.Z. Li, Expression and characterization of an

α-glucosidase from *Thermoanaerobacter ethanolicus* JW200 with potential for industrial application, Biologia 64 (2009) 1053-1057.

13. K. Tan, C. Tesar, R. Wilton, L. Keigher, G. Babnigg, A. Joachimiak, Novel α-glucosidase from human gut microbiome: substrate specificities and their switch, FASEB J. 24 (2010) 3939-3949.
14. E.A. MacGregor, Š. Janeček, B. Svensson, Relationship of sequence and structure to specificity in the α-amylase family of enzymes, Biochim. Biophys. Acta 1546 (2001) 1-20.
15. M. Okuyama, Function and structure studies of GH family 31 and 97 α-glycosidases, Biosci. Biotechnol. Biochem. 75 (2011) 2269-2277.
16. S. Schönert, T. Buder, M.K. Dahl, Identification and enzymatic characterization of the maltose inducible α-glucosidase MalL (sucrase-isomaltase-maltase) of *Bacillus subtilis*, J. Bacteriol. 180 (1998) 2574-2578.
17. S. Schönert, S. Seitz, H. Krafft, E.A. Feuerbaum, I. Andernach, G. Witz, M.K. Dahl, Maltose and maltodextrin utilization by *Bacillus subtilis*, J. Bacteriol. 188 (2006) 3911-3922.
18. H. Nakai, M.J. Baumann, B.O. Petersen, Y. Westphal, H. Schols, A. Dilokpimol, M.A. Hachem, S.J. Lahtinen, J.Ø. Duus, B. Svensson, The maltodextrin transport system and metabolism in *Lactobacillus acidophilus* NCFM and production of novel α-glucosides through reverse phosphorolysis by maltose phosphorylase, FEBS J. 276 (2009) 7353-7365.
19. S.N. Ho, H.D. Hunt, R.M. Horton, J.K. Pullen, L.R. Pease, Site-directed mutagenesis by overlap extension using the polymerase chain reaction, Gene 77 (1989) 51-59.
20. M.O. Dayhoff, G.E. Perlmann, D.A. MacInnes, The partial specific volumes, in aqueous solution, of three proteins, J. Am. Chem. Soc. 74 (1952) 2515-2517.
21. S. Moore, W.H. Stein, Photometric ninhydrin method for use in the chromatography of amino acids, J. Biol. Chem. 176 (1948) 367-388.
22. D.M. Standley, H. Toh, H. Nakamura, ASH structure alignment package: sensitivity and selectivity in domain classification. BMC Bioinformatics 8 (2007) 116.
23. L.A. Kelley, M.J. Sternberg, Protein structure prediction on the Web: a case study using the Phyre server, Nat. Protoc. 4 (2009) 363-371.

24. J. Larsbrink, A. Izumi, G.R. Hemsworth, G.J. Davies, H. Brumer, Structural enzymology of *Cellvibrio japonicus* Agd31B protein reveals α-transglucosylase activity in glycoside hydrolase family 31, J. Biol. Chem. 287 (2012) 43288-43299.
25. M. Okuyama, A. Okuno, N. Shimizu, H. Mori, A. Kimura, S. Chiba, Carboxyl group of residue Asp647 as possible proton donor in catalytic reaction of α-glucosidase from *Schizosaccharomyces pombe*, Eur. J. Biochem. 268 (2001) 2270-2280.
26. A.L. Lovering, S.S. Lee, Y.W. Kim, S.G. Withers, N.C. Strynadka, Mechanistic and structural analysis of a family 31 α-glycosidase and its glycosyl-enzyme intermediate, J. Biol. Chem. 280 (2005) 2105-2115.
27. H.A. Ernst, L.L. Leggio, M.Willemoës, G. Leonard, P. Blum, S. Larsen, Structure of the *Sulfolobus solfataricus* α-glucosidase: implications for domain conservation and substrate recognition in GH31, J. Mol. Biol. 358 (2006) 1106-1124.
28. J. Larsbrink, A. Izumi, F.M. Ibatullin, A. Nakhai, H.J. Gilbert, G.J. Davies, H. Brumer, Structural and enzymatic characterization of a glycoside hydrolase family 31 α-xylosidase from *Cellvibrio japonicus* involved in xyloglucan saccharification. Biochem. J. 436 (2011) 567-580.
29. T. Tagami, K. Yamashita, M. Okuyama, H. Mori, M. Yao, A. Kimura, Molecular basis for the recognition of long-chain substrates by plant α-glucosidases. J. Biol. Chem. 288 (2013) 19296-19303.
30. H.J. Rozeboom, S. Yu, S. Madrid, K.H. Kalk, R. Zhang, B.W. Dijkstra, Crystal structure of α-1,4-glucan lyase, a unique glycoside hydrolase family member with a novel catalytic mechanism. J. Biol. Chem. 288 (2013) 26764-26774.
31. L. Ren, X. Qin, X. Cao, L. Wang, F. Bai, G. Bai, Y. Shen, Structural insight into substrate specificity of human intestinal maltase-glucoamylase, Protein Cell 2 (2011) 827-836.
32. L. Sim, R. Quezada-Calvillo, E.E. Sterchi, B.L. Nichols, D.R. Rose, Human intestinal maltase-glucoamylase: crystal structure of the N-terminal catalytic subunit and basis of inhibition and substrate specificity. J. Mol. Biol. 375 (2007) 782-792.
33. L. Sim, C. Willemsma, S. Mohan, H.Y. Naim, B.M, Pinto, D.R. Rose, Structural basis for

substrate selectivity in human maltase-glucoamylase and sucrase-isomaltase N-terminal domains. J. Biol. Chem. 285 (2010) 17763-17770.

34. Y. Suzuki, M. Nobiki, M. Matsuda, T. Sawai, *Bacillus thermoamyloliquefaciens* KP1071 α-glucosidase II is a thermostable M_r 540000 homohexameric α-glucosidase with both exo-α-1,4-glucosidase and oligo-1,6-glucosidase activities, Eur. J. Biochem. 245 (1997) 129-136.
35. K.M. Song, M. Okuyama, M. Nishimura, T. Tagami, H. Mori, A. Kimura, Aromatic residue on β→α loop 1 in the catalytic domain is important to the transglycosylation specificity of glycoside hydrolase family 31 α-glucosidase, Biosci. Biotechnol. Biochem. 77 (2013) 1759-1765.
36. T. Tagami, M. Okuyama, H. Nakai, Y.M. Kim, H. Mori, K. Taguchi, B. Svensson, A. Kimura, Key aromatic residues at subsites +2 and +3 of glycoside hydrolase family 31 α-glucosidase contribute to recognition of long-chain substrates, Biochim. Biophys. Acta, 1834, (2013) 329-335.
37. A. Kita, H. Matsui, A. Somoto, A. Kimura, M. Takata, S. Chiba, Substrate specificity and subsite affinities of crystalline α-glucosidase from *Aspergillus niger*, Agric. Biol. Chem. 55 (1991) 2327-2335.
38. F. Sato, M Okuyama, H. Nakai, H. Mori, A. Kimura, S. Chiba, Glucoamylase originating from *Schwanniomyces occidentalis* is a typical α-glucosidase, Biosci. Biotechnol. Biochem. 69 (2005) 1905-1913.
39. M. Rolfsmeier, C. Haseltine, E. Bini, A. Clark, P. Blum, Molecular characterization of the α-glucosidase gene (*mal*A) from the hyperthermophilic archaeon *Sulfolobus solfataricus*, J. Bacteriol. 180 (1998) 1287-1295.
40. S. Chiba, S. Inomata, H. Matsui, T. Shimomura, Purification and properties of an α-glucosidase (glucoamylase) in sugar beet seed, Agric. Biol. Chem. 42 (1978) 241-245.
41. K. Jones, L. Sim, S. Mohan, J. Kumarasamy, H. Liu, S. Avery, H.Y. Naim, R. Quezada-Calvillo, B.L. Nichols, B.M. Pinto, D.R. Rose, Mapping the intestinal alpha-glucogenic enzyme

specificities of starch digesting maltase-glucoamylase and sucrase-isomaltase, Bioorg. Med. Chem. 19 (2011) 3929-3934.

42. M. Okuyama, H. Mori, S. Chiba, A. Kimura, Overexpression and characterization of two unknown proteins, YicI and YihQ, originated from *Escherichia coli*, Protein Expr. Purif. 37 (2004) 170-179.

43. S. Yu, T. Ahmad, L. Kenne, M. Pedersén, α-1,4-Glucan lyase, a new class of starch/glycogen degrading enzyme. III. Substrate specificity, mode of action, and cleavage mechanism, Biochim. Biophys. Acta 1244 (1995) 1-9.

Figure legends

Fig. 1. Gene organization surrounding *BspAG31A*.

Genetic map of the *Bam*HI fragment of the genomic DNA of *Bacillus* sp. AHU 2001 including *BspAG31A*. Start and end positions of each gene are indicated.

Fig. 2. Model structure of BspAG31A and comparison of amino acid sequence of BspAG31A with BtAG and CjAgd31B.

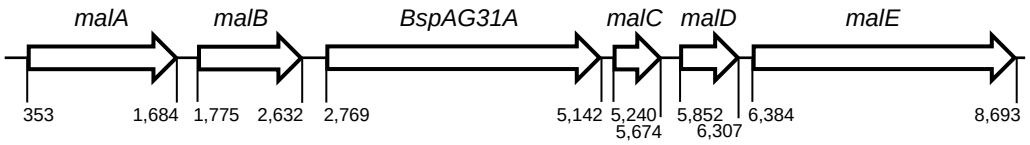
A, model structure of BspAG31A. B, sequence alignment. BspAG31A, *Bacillus* sp. AHU 2001 BtAG, α-glucosidase; *B. thermoamyloliquefaciens* α-glucosidase, BAA76396.1 (GenBank ID); CjAgd31B, *C. japonicas* oligosaccharide α-1,4-transglucosylase, ACE86259.1 (GenBank ID). Filled triangles below the sequence indicate amino acid residues involved in the substrate binding shown in panel A. Open triangles are amino acid residues important for transglucosylation predicted in CjAgd31B [23].

Fig. 3. Transglucosylation of recombinant BspAG31A with maltose as the substrate.

A, DP distribution analysis. Closed circle, DP1; open circle, DP2; closed triangle, DP3; open triangle, DP4; and closed square, DP5. B, high performance anion exchange column chromatography analysis. The reaction mixture after 48 h was subjected to analysis. 1, glucose; 2,

1 neotrehalose; 3, isomaltose; 4, kojibiose; 5, nigerose; and 6, maltose.

- Glycoside hydrolase family 31 α-glucosidase AG31A was found in *Bacillus* sp. AHU 2001.
- AG31A hydrolyzed maltooligosaccharides, nigerose, kojibiose, and neotrehalose.
- Isomaltase activity of AG31A was enhanced by substitution of Tyr268 with Trp.
- Y173A showed higher preference for long-chain substrate than wild type.


malA
malB
BspAG31A
malC
malD
malE
353
1,684
1,775
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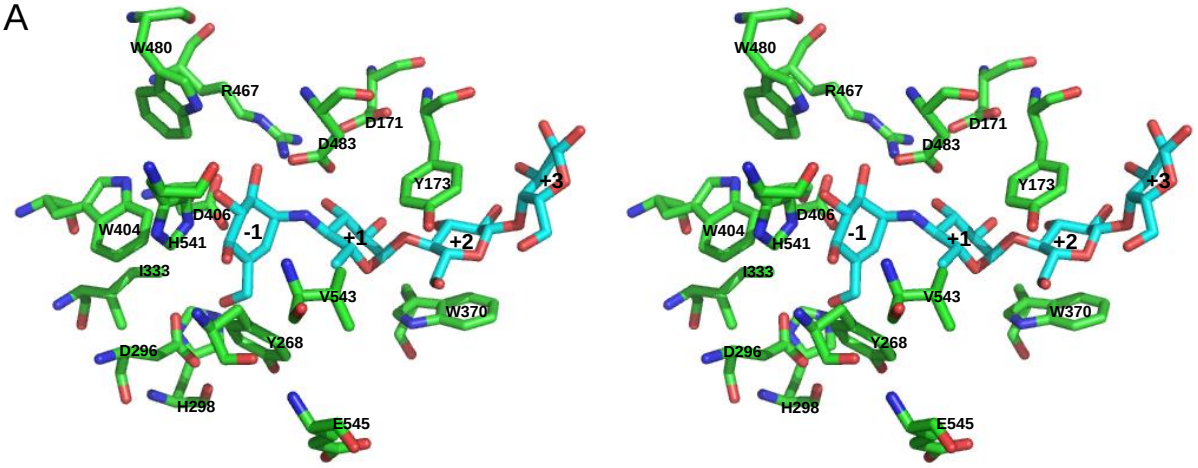
2,769
5,142
5,240
5,674
5,852
6,307
6,384
8,693

Fig. 1

A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Y268
H298
E545
W480
R467
D171
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Y173
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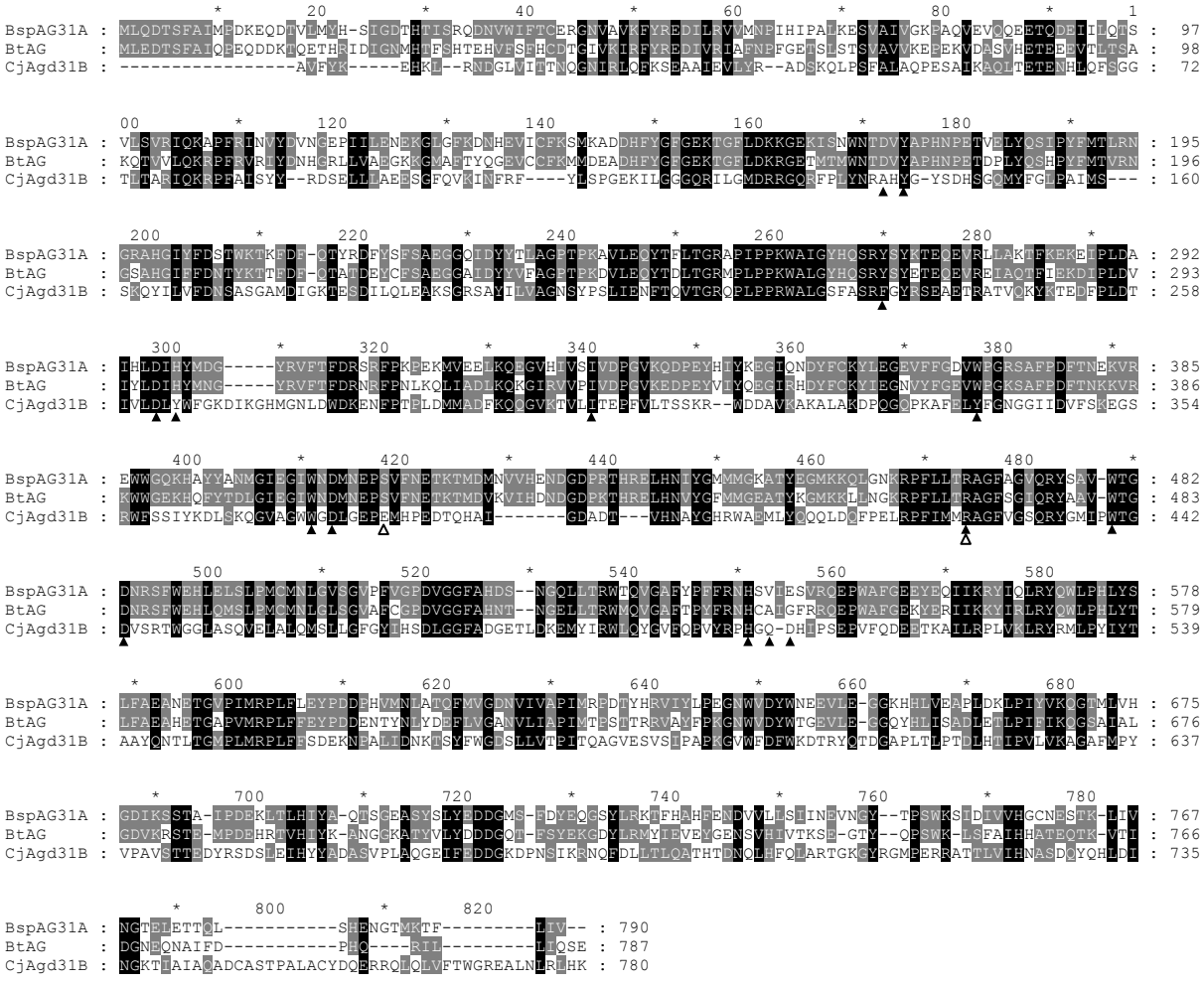
B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BtAG : MLEDTSFAIQPEQDDKIQETHRIDIGNMHTFSHTEHVFSIHCDTGIVKIRFYREDIVRIAFNPFGETSLSTSVAVVKEPEKVDASVHETEEEVTLTSA : 98
CjAgd31B : ----------AVFYK-----PHKL--RNDGLVILTNQGNIRLQFKSPAAIEVLYR--ADSKQLPSFALAQPESAIKAQLTETENHLQFSGG : 72
BspAG31A : VLSVRIQKAPFRINVYLVNCEPIILENEKGLGFKDNHEVICFKSVKADDHFVGFGEKTGFLDKKGEKISNWNTDVYAPHNPETVELYQSIPYFMTLRN : 195
BtAG : KQTVVLQKRPFVRLYLNHGRLLVAEGKKAGMAPTYQGEVCCFKMMDEADHFVGFGEKTGFLDKRGETMTMWNTDVYAPHNPETDPLYQSHPYFMTVRN : 196
CjAgd31B : TLTARIQKRPFAISYY--RDSELLLAEESGFQVKINFRF----YLSPGEKILGGGQRILGMDRRGQRFFLYNRAHYG-YSDHSGQMYFGLPAIMS--- : 160
BspAG31A : GRAHGIYFDSTWKTKFDF-QTYRDFYSFSAECGQIDYYTLAGPTPKAVLEQYTFLTGRAPIPPKWAIGYHQSRYSYKTEQEVRLLAKNFKEKEIPLDA : 292
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CjAgd31B : SKQYILVFDNSASGAMDIGKTESDILQLEAKSGRSAMILVAGNSYPSLIENFTQVTGRQPLPPRWALGSFASRFGYRSEAETRATVQKYKTEDFPLDT : 258
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CjAgd31B : IVLDLYWFGKDIKGHMGNLDWDKENFPTTLDMMADFKQQGVKTVLITEEFVLTSSKR--WDDAVKAKALAKDPQGQPKAFELYFGNGGIIDVFSKEGS : 354
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CjAgd31B : RWFSSIYKDLSKQGVASWWGDLGEEEMHPEDTQHAL-------GDADT---VHNAYGHRWAEMLYQQQLDQFPELRPFINMRAGFVGSQRYGMIPWTG : 442
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BtAG : LFAEAHETGAPVMRPLFFEYPDDENTYNIYDQFLVGANVLIAPIMTPSTTRRVAYFPKGNWVDYWTGEVLEGGQYHLISADLETLPIFIKQGSAIAL : 676
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BspAG31A : GDIKSSTA-IPDEKLTLHIYA-QSGEASYSLYEDDGMS-FDYEQGSYLRKTFHAHPENDVVLESLINEVNGY--TPSWKSLDLVVHGCNESTK-LIV : 767
BtAG : GDVKRSTE-MPDEHRTVHIYK-ANGKAIYVLYEDDGQT-FSYEKGDYIRMYIEVEMGENSVHLVTKSE-GTY--QPSWK-LSFAIHHATEQTK-VTI : 766
CjAgd31B : VPAVSTTETDYRSDSLELHIYPDASVPLAQGEIFEDDGKDPNSIKFNQFDLLTLQATHTDNQLHFQLARTGKGYRGMSERRATTLVIHNASDQYQHLDI : 735
BspAG31A : NGTEIETTQL----------SHENGTMKTF---------LIV-- : 790
BtAG : DGNEQNAIFD----------PHQ----RIL---------LIQSE : 787
CjAgd31B : NGKTIAIAQADCASTPALACYDQERRQLQLVFTWGREALNLRLHK : 780

Fig. 2

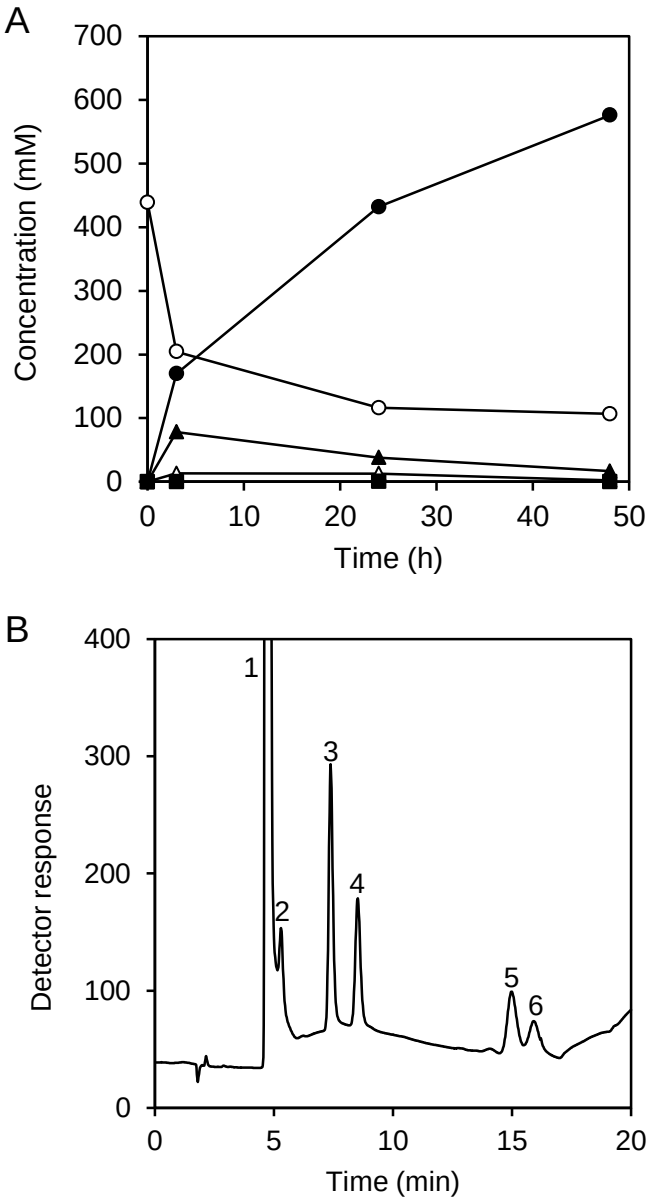
A
Concentration (mM)
700
600
500
400
300
200
100
0
0
10
20
30
40
50
Time (h)
B
Detector response
400
300
200
100
0
1
2
3
4
5
6
0
5
10
15
20
Time (min)

Fig. 3

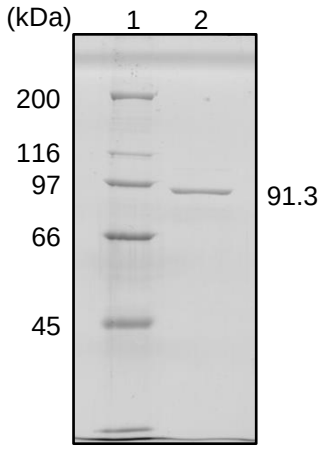

Fig. S1. SDS-PAGE analysis of BspAG31A purified from *Bacillus* sp. AHU 2001.

Purified BspAG31A (0.3 μg) was analyzed on SDS-PAGE. Lane 1, molecular size marker; lane 2, BspAG31A. Molecular masses of BspAG31A and marker proteins are indicated on the right and left of figure, respectively.

Table 1. Comparison of amino acid sequences of GH31 enzymes

Origin	N-loop	β→α loop 1	β→α loop 3	Region A	Region B	Reference
α-Glucosidase (EC 3.2.1.20)						
Bacillus sp. AHU2001	168: WNTDV<u>Y</u>APH----------NPE-TVELY	262: GYHQSR-<u>Y</u>SY	367: GDV<u>W</u>P	401: EGIWN**D**MNEPS	480: WTG**D**NRSFW	This study
B. thermoamyloliquefaciens	169: WNTDVYAPH----------NPE-TDPLY	263: GYHQSR-YSY	368: GEVWP	402: EGIWN**D**MNEPS	481: WTG**D**NRSFW	[34]
Lactobacillus johnsonii	161: WNTDNPEPQ----------VES-FTRLY	256: GYQQSR-WGY	362: NKVWP	396: DGIWD**D**MNEPA	472: WTG**D**NQSLW	[11]
Themoanaerobacter ethanolicus	138: WNTDEFMTH----------NQT-TKLLY	233: GNQQSR-YSY	338: GKVWP	372: DGIWN**D**MNEPA	452: WTG**D**NRSLY	[12]
Ruminococcus obeum	70: NCTDDPIH----------TED-KRSLY	163: GFGQSR-WGY	268: AAVWP	302: EGFWN**D**MNEPA	417: WMG**D**NKSWW	[13]
Aspergillus niger	222: YPSDDG----------TPI-DQNLY	337: GFHQCR-WGY	450: GAVWP	485: DGVWY**D**MSEVS	657: WGG**D**NYSKW	[37]
Schizosaccharomyces pombe	215: WANDEP----------SPV-DQNMY	321: GYHSCR-WGY	435: GAVWP	476: SGIWT**D**MNEPS	644: WLG**D**NHSLW	[25]
Schwanniomyces occidentalis	227: FANDVG----------DPI-DGNIY	318: GYHQCR-WGY	432: GAVWP	467: DGIWA**D**MNEVS	637: WGG**D**NTADW	[38]
Sulfolobus solfataricus	84: YNVDAGAY----------KKY-QDPLY	178: GYMISR-YSY	281: GKMWP	315: DGIWL**D**MNEPT	413: WTG**D**NTPSW	[39]
Sugar beet	229: WNADIA-S----------FNR--DLNLY	323: GFHQCR-WGY	429: GSVWP	464: DGIWI**D**MNEAS	565: WTG**D**NAARW	[40]
Human (isomaltase)	261: FTRDQL-P----------GDN-NNNLY	354: GFQLSR-WNY	465: GEVWP	500: DGLWI**D**MNEVS	601: WLG**D**NTASW	[41]
Human (sucrase)	1134: FTRDQP----------PGY-KLNSY	1225: GFQLCR-YGY	1232: AKVWP	1389: DGLWI**D**MNEPS	1497: WLG**D**NYARW	[33]
Human (maltase)	286: FNRDTT-P----------NGN-GTNLY	379: GFHLSR-YEY	489: GEVWP	524: DGIWI**D**MNEVS	625: WLG**D**NTATW	[41]
Human (glucoamylase)	1154: FSRDQP----------PGY-KLNSY	1245: GFQLCR-YGY	1352: GKVWP	1415: DGMWI**D**MNEPS	1523: WLG**D**NTAAW	[31]
α-Xylosidase (EC 3.2.1.177)						
Cellvibrio japonicus	203: TTYNL----------V-	265: GFWQSR-ERY	539: NLDWI	577: DAWWL**D**AVEPD	656: WSG**D**IVSRW	[28]
Escherichia coli	182: WNRDGGTS----------TEQAY	270: GLWLTTSFTT	377: WDKWQ	411: DCFKT**D**FGERI	479: WGG**D**CYANY	[42]
Oligosaccharide α-1,4-transglucosylase (EC. 2.4.1.161)						
Cellvibrio japonicus	174: YNRAHYGY----------SDH-SGQMY	265: GSFASR-FGY	373: FELYF	407: AGWWG**D**LGEPE	477: WTG**D**VSRTW	[24]
Glucan lyase (EC 4.2.2.13)						
Gracilariopsis lemaneiformis	286: YNYDNLNYNQWDLRPPHHDGALNPDYYIPMY	417: GFFQGV-FGT	559: GHLDY	598: DFVWQ**D**MTVPA	712: WVG**D**NSTTS	[43]

Catalytic nucleophile and general acid/base catalyst, conserved in Region A and B, respectively, are shown in boldface. Mutated amino acid residues are underlined. *B. thermoamyloliquefaciens* α-glucosidase, BAA76396.1 (GenBank ID); *L. johnsonii* α-glucosidase (α-1,3-glucosidase), ACO57638.1 (GenBank ID); *T ethanolicus* α-glucosidase, ABR26230.1 (GenBank ID); *R. obeum* α-glucosidase, ZP_01966167.1 (NCBI Reference Sequence ID); *A. niger* α-glucosidase, BAM72725.1 (GenBank ID); *S. pombe* α-glucosidase, CAC36906.1 (GenBank ID); *S. occidentalis* α-glucosidase, BAE20170.1 (GenBank ID); *S. solfataricus* α-glucosidase, AAC38215.1 (GenBank ID); sugar beet (*Beta vulgaris*) α-glucosidase, BAM74081.1 (GenBank ID); human (*Homo sapiens*) sucrose-isomaltase complex, AAT18166.1 (GenBank ID); human maltase-glucoamylase complex, AAC39568.2 (GenBank ID); *C. japonicus* α-xylosidase, ACE86259.1 (GenBank ID); *E. coli* α-xylosidase, AAC76680.1 (GenBank ID); *C. japonicus* oligosaccharide α-1,4-transglucosylase, ACE84782.1 (GenBank ID); and *G. lemaneiformis* glucan lyase, CAB51910.1 (GenBank ID).

Table 2. Kinetic parameters of wild-type and mutant BspAG31As.

	Wild type				Y173A				Y268W			
	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1}mM^{-1}$)	Relative k_{cat}/K_m (%)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1}mM^{-1}$)	Relative k_{cat}/K_m (%)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1}mM^{-1}$)	Relative k_{cat}/K_m (%)
Maltose	53.8 ± 0.2	0.748 ± 0.02	71.9	100	22.4 ± 1.2	1.26 ± 0.15	17.8	100	17.7 ± 1.0	2.18 ± 0.2	8.12	100
Maltotriose	50.7 ± 2.3	0.456 ± 0.06	111	155	25.8 ± 0.7	1.27 ± 0.05	20.3	114	8.60 ± 0.40	1.47 ± 0.13	5.85	72.1
Maltotetraose	49.8 ± 0.4	0.787 ± 0.01	63.3	88.0	27.4 ± 1.4	1.50 ± 0.15	18.3	103	10.8 ± 0.30	0.85 ± 0.070	12.6	156
Maltopentaose	42.2 ± 0.9	3.33 ± 0.14	12.7	17.6	27.3 ± 0.6	3.28 ± 0.25	8.32	46.8	5.83 ± 0.08	2.25 ± 0.09	2.59	31.9
Maltohexaose	47.0 ± 3.2	5.35 ± 0.7	8.79	12.2	27.4 ± 1.0	4.31 ± 0.34	6.36	35.8	3.80 ± 0.02	4.22 ± 0.06	0.900	11.1
Maltoheptaose	39.3 ± 0.8	5.31 ± 0.23	7.40	10.3	27.9 ± 1.0	5.15 ± 0.30	5.42	30.5	3.46 ± 0.08	4.93 ± 0.26	0.702	8.64
Kojibiose	19.2 ± 0.8	4.22 ± 0.29	4.55	6.33	20.8 ± 1.8	5.12 ± 1.01	4.06	22.9	3.58 ± 0.1	1.21 ± 0.12	2.96	36.4
Nigerose	23.5 ± 0.5	2.15 ± 0.12	10.9	15.2	35.8 ± 0.9	9.71 ± 0.46	3.69	20.7	11.2 ± 0.2	1.95 ± 0.08	5.74	70.7
Neotrehalose	40.9 ± 1.0	4.53 ± 0.26	9.03	12.6	37.5 ± 2.1	12.2 ± 1.20	3.07	17.3	8.96 ± 0.11	2.50 ± 0.12	3.58	44.1
Isomaltose	6.26 ± 0.41	33.8 ± 2.50	0.185	0.257	N.D.	N.D.	N.D.	N.D.	12.5 ± 0.5	4.57 ± 0.43	2.74	33.7

	V543A				E545G			
	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1}mM^{-1}$)	Relative k_{cat}/K_m (%)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($s^{-1}mM^{-1}$)	Relative k_{cat}/K_m (%)
Maltose	90.4 ± 1.6	0.532 ± 0.04	170	100	43.3 ± 1.7	0.551 ± 0.04	78.6	100
Maltotriose	75.2 ± 2.4	0.225 ± 0.03	334	197	40.1 ± 0.8	0.388 ± 0.02	103	132
Maltotetraose	77.7 ± 2.4	0.505 ± 0.05	154	90.5	42.3 ± 1.8	0.789 ± 0.1	53.6	68.2
Maltopentaose	64.2 ± 0.8	1.45 ± 0.12	44.3	26.1	44.3 ± 0.6	2.88 ± 0.11	15.4	19.6
Maltohexaose	59.6 ± 2.6	2.83 ± 0.36	21.1	12.4	35.7 ± 1.3	4.04 ± 0.25	8.84	11.2
Maltoheptaose	52.3 ± 0.3	2.88 ± 0.06	18.2	10.7	34.7 ± 1.1	4.62 ± 0.24	7.51	9.56
Kojibiose	39.9 ± 2.2	3.72 ± 0.60	10.7	6.31	16.5 ± 0.5	4.00 ± 0.39	4.13	5.25
Nigerose	78.6 ± 1.2	4.03 ± 0.19	19.5	11.5	38.5 ± 0.8	4.07 ± 0.20	9.46	12.0
Neotrehalose	35.5 ± 4.6	5.32 ± 2.24	6.67	3.93	36.1 ± 0.3	4.77 ± 0.20	7.57	9.63

N.D., not determined.