Cloning, purification, and characterization of the organic solvent tolerant \( \beta \)-glucosidase, \( \text{OaBGL84} \), from \( \text{Olleya aquimaris DAU311} \)

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Abstract A marine bacterium, \( \text{Olleya aquimaris DAU311} \), was isolated from Goraebul beach in the Republic of Korea. This strain had \( \beta \)-glucosidase activity on Luria–Bertani esculin plates. The \( \beta \)-glucosidase, \( \text{oabgl84} \), was isolated, cloned, and sequenced, based on fosmid library. The gene encoded novel \( \beta \)-glucosidase and consisted of an open reading frame of 2304 bp, which encodes 768 amino acids. The deduced amino acid sequence had 99% identity to \( \text{Olleya sp. VCSM12} \), 84% identity to \( \text{Olleya marilimosa} \), and 78% similarity to \( \text{Lacinutrix sp. Hel_I_90} \). \( \text{OaBGL84} \) belongs to the glycoside hydrolase family 3, and it was visualized using SDS-PAGE, approximately 84 kDa. The optimal temperature and pH of \( \text{OaBGL84} \) were analyzed as 40°C and 6.0, respectively, using \( p\)-NPG as substrate. The \( K_m \) and \( V_{\text{max}} \) values for \( \text{OaBGL84} \) were 1.35 mM and 25.3 \( \mu \)M/s, respectively. Furthermore, \( \text{OaBGL84} \) activity was completely inhibited by \( \text{Cu}^{2+} \) and \( \text{Hg}^{2+} \) ions. \( \text{OaBGL84} \) demonstrated extraordinary stability until 50% \((\text{v/v})\) benzene, \( n \)-hexane, or toluene. These results indicate that \( \text{OaBGL84} \) is useful candidate to degrade cellulose or soy isoflavone in the organic solvents for various biotechnological applications.

Keywords \( \beta \)-Glucosidase · Glycoside hydrolase family 3 · \( \text{Olleya aquimaris} \) · Organic solvent tolerance · \( \text{OaBGL84} \)

Introduction

\( \beta \)-Glucosidases (EC 3.2.1.21) hydrolyze the \( \beta \)-glucosidic bond of various oligosaccharides and glycosides [1, 2]. In general, \( \beta \)-glucosidases are classified using two methods, which are based on substrate specificity and amino acid sequences. On the basis of substrate specificity, \( \beta \)-glucosidases are categorized as (1) aryl \( \beta \)-glucosidases (2) true cellobiases, and (3) wide range substrate specificity enzymes [1]. On the basis of amino acid sequences, these enzymes are categorized into six glycoside hydrolase (GH) families: GH 1, GH 3, GH 5, GH 9, GH 30, and GH 116 [2, 3]. Most \( \beta \)-glucosidases are categorized as either GH 1 or GH 3. Furthermore, most of bacterial, archaeal, plant, and animal \( \beta \)-glucosidases belong to the GH 1. In contrast, some bacterial enzymes, as well as all yeast and mold enzymes belong to GH 3 [4]. The catalytic activity of GH 3 members depends on aspartic acid (Asp) and glutamic acid/histidine (Glu/His) residues. Asp residues as nucleophiles and Glu/His residues may function as proton donors [1, 5].

Recently, \( \beta \)-glucosidases have attracted increased interest in sectors such as biofuel, food, and medicine. For instance, \( \beta \)-glucosidases transform phenolic compounds or phyto-estrogen to aglycones, promote the flavor of tea and wine, reduce viscosity of gellan gum, remove bitterness of citrus fruit juices, and provide regenerative energy [1, 6, 7]. Furthermore, numerous studies have reported that \( \beta \)-glucosidase is known to be involved in the hydrolysis of cellulose to glucose and converts isoflavone glycosides to their aglycone form. The conversion of cellulose to glucose is an important industrial process. The hydrolytic system of cellulose to glucose conversion requires a minimum of three groups of cellulase. First, endo-1,4-\( \beta \)-glucanase (EC 3.2.1.4) cleaves cellulose to produce cello-oligosaccharides.
without rules. Second, cellubiohydrolase (EC 3.2.1.91) cleaves cellulose to produce cellobiose from the end of cellulose. Finally, \( \beta \)-glucosidase cleaves cellobiose to produce glucose. Cellulose hydrolysis is inhibited by cellubiose and cello-oligosaccharides [5, 8]. However, \( \beta \)-glucosidase can overcome the inhibitory action in order to remove the cellobiose and cello-oligosaccharides. Thus, \( \beta \)-glucosidase both produces glucose and reduces inhibitory actions [9]. Soy isoflavone has a similar structure to estrogen and presents in glycoside form (genistin and daidzin) and aglycone form (genistein and daidzein) which are found in soybeans. Most isoflavones have biological effects such as prevention of cancer, osteoporosis, and cardiovascular diseases. These effects result from the aglycone form, but not from the glycoside form. Isoflavone aglycones have high biological activity due to unimpeded intestinal absorption [7, 10]. Therefore, conversion of isoflavones to aglycones is necessary in order to obtain optimal pharmacological effects. Thus, \( \beta \)-glucosidase is an important contributor to cellulose and isoflavone hydrolysis.

Organic solvent tolerant enzymes have attracted interest in research, due to advantages related to biocatalysis in organic liquid. The enzyme utilizations in organic liquid are limited because predominantly enzymes lose active and stable in organic solvents [11, 12]. Numerous experiments to increase enzyme activity in organic media have involved methods such as protein engineering, lyophilization in the presence of additives, chemical modification, and immobilization of novel supports [13]. Enzymes, that naturally retain stability in organic solvents, may be very practical for enterprise applications that require organic solvents [14]. The goal of this study was to assess the biochemical properties of an organic solvent tolerant \( \beta \)-glucosidase (OaBGL84) from Olleya aquimaris. O. aquimaris was isolated from the seawater in Korea. We assessed parameters of OaBGL84 including pH, temperature, and the influence of effectors such as glucose, metal ions, and organic solvents. We also studied the catalytic properties and stability of OaBGL84 which may be relevant to industrial or biotechnological applications.

**Materials and methods**

**Chemicals and reagents**

Esculin, ammonium iron (III) citrate, \( p \)-nitrophenyl (\( p \)NP), \( p \)-nitrophenyl-\( \beta \)-\( d \)-glucopyranoside (\( p \)NPG), \( p \)-nitrophenyl-\( \beta \)-\( d \)-xylopyranoside (\( p \)NP\( \beta \)X), \( p \)-nitrophenyl-\( \alpha \)-\( d \)-glucopyranoside (\( p \)NP\( \alpha \)G), isoflavone glycoside (genistin and daidzin), isoflavone aglycone (genistein and daidzein), celllobiose, cellobiose, and cellophenoase were purchased from Sigma (St. Louis, MO, USA). All chemicals and reagents were of analytical grade.

**Microbial strains, plasmids, and culture conditions**

Strain DAU311 was isolated from seawater collected at Goraebul Beach in the East Sea, Republic of Korea, using marine agar (Difco, Detroit, MI, USA) plates supplemented with 0.1% esculin and 0.25% ammonium iron (III) citrate [15]. This strain was incubated at 30 °C for 3 days, and exhibited a dark brown zone from which a colony was selected. *Escherichia coli* (E. coli) JM109, EPI300-T1, and BL21 (DE3) were used as hosts. *E. coli* EPI300-T1 transformants were cultured in LB medium with ampicillin (50 \( \mu \)g/mL) or chloramphenicol (12.5 \( \mu \)g/mL) at 37 °C. Plasmids pUC118 and pCC1FOS (Epicentre, Madison, WI, USA) were used as vectors to make the genome library, and the pCold I vector (TaKaRa, Kyoto, Japan) was used for protein expression.

**Phylogenetic analysis with 16S rDNA**

16S rDNA was amplified by polymerase chain reaction (PCR) using two oligonucleotides (forward: 5'-GAGTTTG ATCTTGGCTCAG-3' and reverse: 5'-AGAAAGGAGGT GATCCAGGCC-3'). The amplified 1.5 kb fragment was cloned into the pMD20-T vector (TaKaRa). The phylogenetic analysis was inferred using MEGA 6.0.5 software by the neighbor-joining method with 1000 bootstraps [16].

**Construction and screening of genome library**

The genomic DNA of *Olleya aquimaris* DAU311 was purified and sheared by pipetting 500 numbers. End-repair was conducted on sheared DNA to make blunt end, and the repaired DNA was then electrophoresed in 1% low melting point agarose gel at 35 V for 12 h. GELase (Epicentre) was then used to extract 25–40 kb fragments from the gel. The fragments were then ligated into the pCC1FOS vector and packaged by Lambda Packaging Extracts (Epicentre). The packaged DNA was infected with EPI300-T1, spread on the LB agar plate containing 0.1% esculin, 0.25% ammonium iron (III) citrate, and chloramphenicol, and then incubated at 30 °C for 3 days. The positive fosmid clone, FosBG4 had a dark zone of colonies, from which the experimental colony was selected. FosBG4 was partially digested with *XbaI*. Next, 2–8 kb DNA fragments were ligated into the *XbaI*-digested pUC118 and transformed into JM109. The first subclone of BG4Xb1 that produced a dark zone around the colonies was selected and spread on an LB agar plate containing ampicillin, esculin, and ammonium iron (III) citrate. Then, the first subclone of BG4Xb1 was digested by *EcoRI* and ligated into an *EcoRI*...
digested pUC118, in order to produce the 2nd subclone. The positive clone, BG4Xb1E1, was confirmed. Homology of the nucleotide sequences was assessed using NCBI BLAST program, and alignment was performed using the ClustalW [17] and MacVector 6.5 (Oxford Molecular Group, Apex, NC, USA). The 3D structure of OaBGL84 was organized by the Phyre \(^2\) server [18]. The 3D structure was realized by PyMOL.

**Overexpression and purification of the recombinant OaBGL84**

The oabgl84 was amplified by PCR with two nucleotides, as follows (forward: \(5'\)-GATTATATAAGACTTTATCA \(\text{AATTC}\) AACTGAGAAATTT-3' and reverse: \(5'\)-AGTCACTAC \(\text{AAATTTAATTTTAAACTCTTC-3'}\)). EcoRI and SalI restriction sites (italics and underlined) were attached at the end of the forward/reverse primers, respectively. PCR was conducted with ExPrime Taq DNA polymerase (GeNetBio, Daejeon, Republic of Korea). Approximately 2.3 kb PCR products of oabgl84 were double digested with EcoRI and SalI, and were then ligated into pCold I, which was digested with EcoRI and SalI. Finally, pCold-OaBGL84 was constructed and transformed into BL21 (DE3) for OaBGL84 expression. The transformant was cultivated in LB broth containing ampicillin for 3 h at 37 °C. When the cell cultures reached 0.4–0.5 of OD \(600\), protein was overexpressed by addition of 0.2 mM isopropyl-\(\beta\)-d-thiogalactoside (IPTG) and incubated at 15 °C for 1 day. The cells were centrifuged to harvest for 30 min at 4 °C and 3980×g. The cell pellets were re-suspended in binding buffer (20 mM sodium phosphate [pH 7.4], 0.5 M NaCl, and 5 mM imidazole) and sonicated (on 15 s, off 15 s, 10 times) in the same buffer. His-Trap HP column (Amersham Biosciences) was equilibrated by binding buffer and the crude OaBGL84 was loaded. The purified OaBGL84 was eluted in elution buffer (20 mM sodium phosphate [pH 7.4], 0.5 M NaCl, and 5 mM imidazole). The active fractions were collected and the buffer was replaced with 20 mM sodium phosphate (pH 6.0), using an Amicon Ultra-4 filter (Millipore, Bedford, MA, USA). The collected fractions were used to characterize the OaBGL84.

**Enzyme assay and characterization**

Enzyme activity was performed with pNPG as substrate. Diluted and purified OaBGL84 in 20 mM sodium phosphate buffer (pH 6.0) was incubated in 200 \(\mu\)L of reaction mixture containing 1.25 mM pNPG (final concentration), at 40 °C for 15 min. The reaction was terminated by adding 800 \(\mu\)L of 0.5 M \(\text{Na}_2\text{CO}_3\), followed by measurement of the liberated pNP at an absorbance of 420 nm. One unit of \(\beta\)-glucosidase activity was defined as the amount of enzyme that produces 1 \(\mu\)mol of pNP per min.

To reveal the optimal temperature conditions, the OaBGL84 was assayed in 20 mM sodium phosphate buffer (pH 6.0) at several temperatures (10–80 °C) for 15 min. Thermal stability was accomplished by a pre-incubated OaBGL84 for 15 min without pNPG at a range of temperatures (10–80 °C) and residual activity was analyzed in the optimal temperature, for 15 min. The optimal pH was analyzed at 40 °C, using buffers such as, citrate (pH 4.0–6.0), sodium phosphate (pH 6.0–8.0), and Tris-HCl (pH 8.0–9.0). Stability of pH was performed by analyzing the residual activity at an optimal temperature. For pre-incubation, the OaBGL84 was kept on ice for 30 min, in several buffers without pNPG.

The influences of metal ions and chemical reagents on OaBGL84 activity were assessed by pre-incubation on ice for 1 h with 1, 5, or 10 mM metal ions. Residual activity was investigated in standard conditions, using pNPG as substrate. \(\text{Ba}^{2+}, \text{Ca}^{2+}, \text{Co}^{2+}, \text{Cs}^+, \text{Cu}^{2+}, \text{Hg}^{2+}, \text{K}^+, \text{Li}^+, \text{Mg}^{2+}, \text{Na}^+, \text{Ni}^{2+}, \text{Zn}^{2+}, \text{DTT}, \text{and EDTA were used for the test of metal ions and chemical reagents.}

To analyze the influences of organic solvents, the purified OaBGL84 was pre-incubated with several organic solvents, such as acetone, acetonitrile, benzene, dimethyl formamide (DMF), dimethyl sulfoxide (DMSO), ethanol, \(n\)-hexane, isopropanol, methanol, tetrahydrofuran (THF), and toluene, at 20 °C. Samples were centrifuged at 3×g for 30 min without pNPG, and residual OaBGL84 activity was assayed in standard conditions [13].

The substrate specificity of OaBGL84 was analyzed using several substrates like 1.25 mM of pNPG, pNPaG, or pNP\(\beta\)X, in standard assay conditions.

**Thin-layer chromatography analysis**

The hydrolysis patterns were investigated by thin-layer chromatography (TLC). Reaction mixtures of cello-oligosaccharides, genistin, and daidzin (25 \(\mu\)g) were incubated with diluted purified OaBGL84 in 20 mM sodium phosphate (pH 6.0) at 40 °C for 15 min. Hydrolys products of cello-oligosaccharides were dropped on silica gel plate (Dieselgel 60, Merck, Berlin, Germany), developed with \(n\)-butanol/acetic acid/water (6:3:2), and detected by aniline-diphenylamine reagent (4 mL of aniline, 4 g of diphenylamine, 200 mL of acetone, and 30 mL of 85% phosphoric acid) before baking it at 180 °C for 10 min [15]. The hydrolysis products of isoflavone glycosides were developed with ethyl acetate/methanol/water (8:1:1) and detected using UV light [7]. Genistein and daidzein were used as standards for isoflavone aglycones.
HPLC analysis and degradation of isoflavone glycosides

Waters 1500 series HPLC system with ZORBAX Eclipse XDB-C18 column (4.6 × 150 mm, 5.0 μm) was used to monitor isoflavone glycosides and aglycones. Solution A consisted of water plus 0.1% acetic acid and solution B consisted of acetonitrile plus 0.1% acetic acid. Genistein and daidzein standards were diluted in ethyl alcohol. Sample volumes of 10 μL were injected, and solution B was run at 5% for 5 min before being increased from 5 to 35% for an additional 15 min. Finally, solution B was run at 35% for 20 min at a flow rate of 1 mL/min [7, 19]. The chromatograms were measured at 260 nm.

Kinetic parameters and glucose tolerance

Enzyme kinetics were performed using various concentrations (0.5–1.5 mM) of pNPG at 40 °C. The $K_m$ and $V_{max}$ values of OaBGL84 were measured using Lineweaver-Burk plots.

To investigate glucose inhibition on OaBGL84 activity, the enzyme reaction was conducted in various glucose concentrations. The purified OaBGL84 was pre-incubated with glucose concentrations ranging from 0 to 200 mM (final concentration) for 30 min on ice, and each pre-incubation mixture was combined with pNPG. The residual OaBGL84 activity was measured in standard conditions.

Nucleotide sequence accession numbers

The nucleotide sequences of the 16S rDNA and β-glucosidase gene reported in this study have been deposited in GenBank under the accession number KU882051 and KU882052.

Results and discussion

Isolation and identification of the DAU311 strain

Approximately 100 distinct microorganisms were screened from the coastal seawaters of the East Sea, Korea. DAU311 showed β-glucosidase activity (as indicated by a dark brown zone) on MB-esculin plates. Consequently, DAU311 was isolated as an esculin-degrading enzyme-producing bacterium. This strain is gram-negative and produces a yellow pigment after incubation at 37 °C for 2 days. Additionally, DAU311 hydrolyzes carboxymethyl cellulose, soluble starch, and skim milk (data not shown). The phylogenetic position was confirmed by comparing with each of the 16S rDNA sequences. The sequence identity of DAU311 was 98% (1417/1443) with Olleya aquimaris L-4, 98% (1417/1443) with Olleya namhaensis WT-MY15, and 97% (1424/1462) with Olleya marilimosa CAM030. Figure 1 shows the phylogenetic tree, based on investigation of 16S rDNA sequences. O. namhaensis and O. marilimosa in Olleya genus have been reported that those can grow at 4 and 30 °C, but not at 37 °C, respectively. Contrastively, O. aquimaris has been reported that can grow at 37 °C [20–22]. Based on these data, we suggest naming this strain Olleya aquimaris DAU311.

Gene cloning and sequence analysis of oabgl84

Approximately 3000 fosmid clones were obtained using a Fosmid Library Production Kit. Many transformants showed hydrolytic activity, such as dark brown zones on LB-esculin-chloramphenicol plates, indicating that β-glucosidase can convert esculin to esculetin and glucose. Esculetin then interacts with iron to develop dark brown zone [23]. Some of these transformants were labeled as FosBG1–FosBG9. One of the positive clones, FosBG4 was selected and digested sequentially with XbaI and EcoRI. The recombinant plasmid of BG4Xb1E1 was sequenced and analyzed. BG4Xb1E1 contained one open reading frame of 2304 bp, which encodes 768 amino acids that are 84,512 Da. This gene was given the name oabgl84. The signal peptide sequence was not found at the N-terminus of the OaBGL84 of DAU311 by SignalP 4.1 [24]. The amino acid sequence of oabgl84 was measured in the bacterial β-
Expression and purification of OaBGL84

The pCold-OaBGL84 was transformed into BL21 (DE3) for OaBGL84 expression, and incubated at 15 °C during 12 h under 0.2 mM IPTG. The recombinant protein, OaBGL84, was overexpressed as soluble proteins. Next, cells were sonicated and crude OaBGL84 were purified using His-Trap HP column. The purified OaBGL84 was investigated by SDS-PAGE, and a single band was detected. This single band indicating OaBGL84 was between 70 and 100 kDa and was similar to the expected molecular weight of 84,000 dalton (Fig. 4). The specific activity of OaBGL84 was 0.7 U/mg.

Influences of temperature and pH on OaBGL84

The influences of temperature on OaBGL84 activity are presented in Fig. 5. The optimal temperature of OaBGL84 activity was assessed within temperature limits, 10–80 °C, with pNPG as substrate. OaBGL84 showed maximal activity at 40 °C, which is similar to NpaBGS from Neocallmastix patriciarum W5 [25] and UeBgl3A from Ustilago esculenta (NBRC 9887), which are also GH 3 family members [26]. The thermostability of purified OaBGL84 was assessed with incubated at temperatures ranging from 10 to 80 °C and residual activities were detected under standard conditions. OaBGL84 has high thermostability at temperature < 40 °C; however, stability was significantly decreased at temperatures > 40 °C. The residual activity was 53% of maximal activity after pre-incubation of OaBGL84 for 15 min at 40 °C.

The influences of pH on OaBGL84 activity are presented in Fig. 6. The optimal pH of OaBGL84 was assessed at various pHs limits, 4.0–9.0, with pNPG as substrate. The activities of OaBGL84 were 12% (pH 5.0), 64% (pH 7.0), 45% (pH 8.0), and 7% (pH 9.0), and OaBGL84 activity was greatest at pH 6.0. These results indicate that OaBGL84 prefers weak acid conditions. In addition, OaBGL84 displayed 100% in 20 mM sodium phosphate buffer and 85% activity in 20 mM citrate buffer at pH 6.0. These results demonstrate similar properties of β-glucosidase to those reported in a previous study [7]. Furthermore, OaBGL84 has similar optimal pH 6.0 to nBGL3 and rBGL3 of Aspergillus fumigatus ZS [27], BGL of Aspergillus unguis NII 08123 [28], β-glucosidase of Stachybotrys sp. [29], and NpaBGS of Neocallmaistix patriciarum W5 [25]. The pH stability of OaBGL84 was maintained 95% after pre-incubation in sodium phosphate buffer (pH 6.0) for 30 min at 4 °C. Moreover, maximum activity of OaBGL84 was 6% (pH 5.0), 55% (pH 7.0), 38% (pH 8.0), and 7% (pH 9.0), with pH variations.

Influences of metal ions and chemical reagents on OaBGL84

The influences of metal ions and chemical reagents on OaBGL84 activity were determined (Table 1). K+, Ca2+, Li+, Na+, Mg2+, Ca2+, and DTT had no significant influences on OaBGL84 activity, at all tested ranges (1, 5, and 10 mM). The effects of Co2+, Ni2+, and EDTA on OaBGL84 activity were not significant at low ion concentrations; however, enzyme inhibition occurred at high concentrations. Bgl1D and Bgl1E, from the metagenomics library of uncultured soil microorganisms, were inhibited by EDTA. The remaining enzymatic activities of Bgl1D and Bgl1E were 56.6 and 53.9%, respectively, at 2 mM [30]. Furthermore, OaBGL84 activity was completely inhibited by Ba2+ and Zn2+ at high concentrations, in addition by Cu2+ and Hg2+ at all concentrations. In comparison with other β-glucosidases, BglA of Paenibacillus xylanilyticus KJ-03 [7], BglA94 of Serratia sp. TN49 [31], and HGT-BG of Aspergillus oryzae CBS 12559 [32] are inhibited by Zn2+. BglA of Paenibacillus xylanilyticus KJ-03 [7], nBgl3 and rBgl3 of Aspergillus fumigatus ZS [27], HGT-BG of Aspergillus oryzae CBS 12559 [32], and BGL of Fomitopsis pinicola KMJ812 [8] are also inhibited by Hg2+ and Cu2+. Bgl1D and Bgl1E, from the metagenomics library, are inhibited by Hg2+, but are not inhibited by Cu2+ [30]. The β-glucosidases of Penicillium citrinum...
YS40-5 [33] and NpaBGS of Neocallimastix particiarum W5 [25] are also considerably inhibited by 10 mM Cu²⁺.

Influences of organic solvents on OaBGL84

Organic solvents differentially affected OaBGL84 performance. The influences of several organic solvents on OaBGL84 activity at concentrations of 10, 20, 30, 40, and 50% (v/v) are summarized in Table 2. OaBGL84 was highly stable at all concentrations of water-immiscible organsics (high log P), like benzene, n-hexane, and toluene. In contrast, activity decreased at high concentrations of water-miscible organsics (low log P), like acetone, acetonitrile, DMF, DMSO, ethanol, isopropanol, methanol, and THF. However, at 10% concentrations of water-miscible solvents including acetone, DMF, DMSO, ethanol, isopropanol, and methanol, OaBGL84 retained 72–106% of its original activity. Although β-glycosidases are sensitive to organic solvents, organic solvent tolerant β-glucosidases from Pichia etchellsii [13] and Bacillus subtilis [34] have been reported. Purified WT-BGL1 from Pichia etchellsii retained approximately 115% of its original activity in 15% toluene, 53.3% in 30% DMSO, 11.2% in 30% methanol, 10.2% in 30% DMF, 9.2% in THF, and 8.2% in isopropanol, after incubation in organic solvents at 40°C for 3 h [13]. BGL from Bacillus subtilis PS retained approximately 106% and 128% of its original activity in 10% and 15% toluene, respectively, after incubation in organic solvents at 37°C for 2 h [34]. In addition, BGL from Bacillus subtilis PS retained approximately 66–94% of its original activity in DMF, DMSO, ethanol, glycerol, n-hexane, isopropanol, methanol, and THF, at 10 and 30% concentrations. Based on these results, water-immiscible

Fig. 2 Alignment of an OaBGL84 sequence from Ollely aquimarisis DAU311 with bacterial glycosyl hydrolase family 3 β-glucosidase. Sequence alignments were performed using the CLUSTAL W and ESPript 3.0 programs. Similar residues are indicated by transparent boxes and identical residues are indicated by colored backgrounds. The essential residues, H224, and D302, are represented by red triangles. The secondary structure elements (alpha helixes [α], beta sheets [β], random coils [η], and beta turns [T]) are shown above the alignment for OaBGL84, O.a, OaBGL84 from Ollely aquimarisis DAU311 (KU882052); L.s, β-glucosidase from Lacinutrix sp. Hel_1_90 (WP_044397560); M.z, β-glucosidase from Mesoflavibacter zeaxanthinifaciens (WP_027879812); P.s, β-glucosidase from Psychroserpens sp. Hel_1_66 (WP_047545772); and A.l, β-glucosidase from Algibacter lecutis (WP_042504054).

Fig. 3 Three-dimensional structural feature of OaBGL84 of Ollely aquimarisis DAU311. (A) Cartoon representation of OaBGL84 (green). The conserved amino acids of OaBGL84 are shown in magenta. (B) Detailed schematic of the conserved amino acid residues. The proton donor, H224, of the KHF motif, as well as the nucleophilic residue, D302, of the active site are indicated by red lettering.
solvents, such as toluene, may stabilize OaBGL84 without competition in the hydration shell of OaBGL84 [35].

Substrate specificity of OaBGL84

Substrate specificity for OaBGL84 was assayed with aryl-glycosides, such as pNPG, pNPβX, and pNPzG. Maximal
OaBGL84 activity was analyzed using pNPG. No activity was analyzed using pNPβX or pNPζG (data not shown). The hydrolysis patterns of cello-oligosaccharides were observed using TLC and hydrolysis products of isoflavone glycosides were observed using TLC and HPLC. Cellobiose was converted to glucose; cellotriose was converted to cellobiose and glucose; cellotetraose was converted to cellotriose, cellobiose, and glucose by OaBGL84 (Fig. 7A). After 15 min incubation at 40 °C, isoflavone glycosides (genistin and daidzin) were completely converted to isoflavone aglycones (genistein and daidzein) by OaBGL84 (Fig. 7B, C). The retention times of isoflavone glycoside and aglycone standards were detected by HPLC: genistin (17 min), genistein (24.3 min), daidzin (14.8 min), and daidzein (20.8 min) (Fig. 8). The hydrolysis products of isoflavone glycosides were

| Organic solvent | log P | Residual activity (%) |
|-----------------|-------|-----------------------|
|                 |       | 10        | 20        | 30        | 40        | 50        |
| Control         | 0.00  | 100       | 100       | 100       | 100       | 100       |
| DMSO            | 2.03  | 106 ± 1.7  | 108 ± 2.6  | 53 ± 2.2  | 4 ± 0.6   | N.D.      |
| Methanol        | 0.74  | 89 ± 1.8   | 39 ± 1.9   | N.D.      | N.D.      | N.D.      |
| DMF             | 0.72  | 73 ± 1.6   | 15 ± 1.9   | N.D.      | N.D.      | N.D.      |
| Acetonitrile    | 0.34  | 20 ± 0.7   | N.D.      | N.D.      | N.D.      | N.D.      |
| Ethanol         | 0.30  | 91 ± 1.2   | 23 ± 2.0   | N.D.      | N.D.      | N.D.      |
| Acetone         | 0.23  | 102 ± 2.0  | 24 ± 3.1   | N.D.      | N.D.      | N.D.      |
| Isopropanol     | 0.05  | 72 ± 3.5   | 4 ± 1.1   | N.D.      | N.D.      | N.D.      |
| THF             | 0.22  | 7 ± 1.6    | N.D.      | N.D.      | N.D.      | N.D.      |
| Benzene         | 2.0   | 119 ± 3.0  | 113 ± 1.6  | 111 ± 4.1 | 109 ± 4.3 | 103 ± 4.3 |
| Toluene         | 2.5   | 133 ± 2.1  | 135 ± 2.6  | 126 ± 4.5 | 120 ± 1.7 | 121 ± 3.6 |
| n-Hexane        | 3.1   | 119 ± 2.6  | 110 ± 2.0  | 101 ± 2.0 | 101 ± 4.5 | 95 ± 1.2  |

Data represent the means ± SD from three independent assays

N.D. not detected
compared to the retention times of the standards. Based on these results, OaBGL84 hydrolyzed pNPG, esculin, cellobiose, cellobiobiose, cellotriose, cellotetraose, genistin, and daidzin, but could not hydrolyze pNpXo r or pNPzG. These results demonstrate that OaBGL84 has broad substrate specificity.

The kinetics of OaBGL84 were analyzed through Lineweaver-Burk plots in different concentrations of pNPG, as substrate. The $K_m$ and $V_{max}$ values were 1.35 mM and 25.3 $\mu$M/s, respectively (data not shown).

Inhibitory influences of glucose on OaBGL84

$\beta$-Glucosidases have a general characteristic, such as competitive inhibition by glucose, thus limiting their activity [10]. Glucose inhibition of OaBGL84 was investigated using various concentrations of glucose (0–200 mM). OaBGL84 retained 40% and 17% of its initial activity, in 100 and 200 mM glucose, respectively (Fig. 9), similarly to the effects described with $\beta$-glucosidases from many other microorganisms. $\beta$-Glucosidase from Flammulina velutipes CFK 3111 retains 38.5 and 20% of its initial activity following addition of 50 and 200 mM glucose, respectively [36]. $\beta$-Glucosidase from Fusarium proliferatum NBRC109045 retains approximately 20 and 15% of its initial activity, with 100 and 200 mM glucose addition, respectively [37].

In the present study, we elucidated characteristics of the organic solvent tolerant $\beta$-glucosidase, OaBGL84, from Olleya aquimaris DAU311. This is the initial report characterizing $\beta$-glucosidase from the Olleya genus. Because OaBGL84 is greatly stable in the high log $P$ value organic solvents, OaBGL84 have potential applications to enterprise processes.

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