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A Novel Cold-Active and Alkali-Stable β-Glucosidase Gene Isolated from the Marine Bacterium *Martelella mediterranea*

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Abstract A β-glucosidase gene designated *gluc3m* was cloned through construction of a genomic library of *Martelella mediterranea* 2928. The *gluc3m* consisted of 2,496 bp and encoded a peptide of 832 amino acids that shared the greatest amino acid similarity (59%) with a β-glucosidase of family 3 glycoside hydrolase from *Agrobacterium radiobacter* K84. The optimum reaction temperature and pH of Gluc3M were 45 °C and 8.0, respectively. The K_m and V_{max} for *p*-nitrophenyl-β-D-glucopyranoside were 0.18 mg/ml and 196.08 μmol/min/mg enzyme, respectively. Gluc3M was found to be highly alkali stable, retaining 80% of its maximum enzymatic activity after treatment with pH 11.0 buffers for 24 h. Furthermore, the activity of Gluc3M improved remarkably in the presence of univalent metal ions, whereas it was inhibited in the presence of divalent ions. Gluc3M also exhibited significant activities toward various substrates including pNPGlu, pNPGal, salicin, and konjac powder. It is important to note that Gluc3M is a cold-active enzyme that showed over 50% of the maximum enzymatic activity at 4 °C. SWISS-MODEL revealed that the amino acids near the conserved domain SDW of Gluc3M contributed to the cold-active ability. Based on these characteristics, Gluc3M has the potential for use in additional studies and for industrial applications.

Keywords β-Glucosidase · *Martelella mediterranea* · Glycosyl hydrolase family 3 · Cold active · Alkaline stability

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Introduction

β-Glucosidases (β-D-glucoside glucohydrolase; EC 3.2.1.21), which are widely distributed in bacteria, fungi, plants, and animal tissues, play an important role in carbohydrate hydrolysis due to their efficient catalysis of the selective cleavage of β-D-glucosidic bonds in oligosaccharide components [1]. For most cellulose degradation processes, the hydrolysis of cellulose requires the synergetic action of three major enzymes: endo-1,4-β-glucanase (EC3.2.1.4), exoglucanase (EC3.2.1.91), and β-glucosidase [2]. Exoglucanases catalyze the degradation of crystalline cellulose at the ends of the polysaccharide chains, whereas cellulose chains are attacked internally by endoglucanases. During the final step, β-glucosidases hydrolyze the β-1,4-glycosidic linkage, which leads to the conversion of cellobiose or cello-oligosaccharides to glucose [3].

The industrial application of β-glucosidases has been widely reported in the past few decades. Specifically, they have been used in ethanol production, agriculture, environmental protection, pharmaceutical production, foods, and textiles. Cellulases are also used to hydrolyze cellulose to increase the production of biofuels, which have the potential to decrease the current dependence on petroleum [4]. Moreover, β-glucosidases bioconvert cellulose-rich wastes into fermentable sugars, providing abundant energy resources for agricultural and manufacturing processes. Furthermore, β-glucosidase may be useful as food additives to increase the flavor of dairy products, beverages, wine, animal feed, and pulping [5]. Cold-active enzymes also have many economic values [6]. For example, the use of such enzymes to enable washing clothes under cold conditions could prevent fading and decrease the costs associated with heating the water. Additionally, these enzymes can enable food production at low temperatures to maintain the flavor of food. Most textile finishing procedures that use β-glucosidase in polymer structures are processed under alkaline conditions. In the above industrial processes, a microbial β-glucosidase is desirable for biorecycling, large-scale production, and reduced risk of contamination [7]. Accordingly, marine bacteria have attracted attention for their special characteristics, which include the production of an alkaline stable xylanase by *Demequina* sp. JK4 and a cellulase-free xylanase by deep-sea *Kocuria* sp. Mn22 [8, 9]. However, few studies of enzymes produced by marine bacteria have been conducted to date; therefore, these organisms have the potential to become an important global resource.

β-Glucosidases can be divided into two glycoside hydrolase families according to their amino acid sequences: family 1 (GHF1) and family 3 (GHF3) [10]. The majority of GHF1 enzymes possess significant β-galactosidase activity and β-glucosidase activity, while enzymes belonging to GHF3 possess β-glucosidase activity. We recently purified a β-glucosidase from *Martelella mediterranea* (designated *gluc3m*) that was found to be active against *p*-nitrophenyl-β-D-glucopyranoside and *p*-nitrophenyl-β-D-galactopyranoside. Interestingly, amino acid sequence analysis grouped Gluc3M into GHF3, which is a family that typically lacks appreciable β-galactosidase activity.

To the best of our knowledge, this is the first study to report isolation of the β-glucosidase gene from *M. mediterranea* encoding a protein that was cold-active, alkali-stable, and hydrolyzed *p*-nitrophenyl-β-D-galactopyranoside. Characterization of Gluc3M illustrated its potential for biotechnological applications.

Materials and Methods

Bacterial Strains, Plasmids, and Media

M. mediterranea that was originally isolated from deep sea water was supplied by the Marine Culture Collection of China (MCCC 1A02928). *M. mediterranea* was Gram-negative, halotolerant, and strictly aerobic strain. Colonies were mostly flat and smooth and white-colored on YED medium [11].

Escherichia coli DH5a was used for DNA manipulation, and *E. coli* BL21 (DE3) was used as a host for expression of the recombinant Gluc3M. Plasmid pUC19 was used for construction of the genomic library and gene subcloning, while the pGEX-6p-1 vector was used for the expression of β-glucosidase.

M. mediterranea was cultivated on YED medium (0.5% yeast extract, 0.7% glucose, 1.5% agar) at 28 °C. *E. coli* was grown in Luria–Bertani (LB) medium or on LB agar plates. Esculin medium (10 g of peptone per liter, 10 g of NaCl per liter, 5 g of yeast extract per liter, 2 g of ferric citrate amine per liter, and 2 g of esculin per liter) was used to screen the efficiency of β-glucosidase. The medium was supplemented with ampicillin (100 g/ml) as required.

Materials and Chemicals

p-Nitrophenyl-β-D-glucopyranoside (pNPGlu), *p*-nitrophenyl-β-D-galactopyranoside (pNPGal), *p*-nitropheny-α-L-arabinoside (pNPA), *p*-nitrophenyl-β-D-xyloside (pNPX), glucan, laminarin, carboxymethyl cellulose, locust bean gum, salicin, oat spelts xylan, birchwood xylan, konjac powder, esculin, cellobiose, cellotriose, cellotetraose, and cellohexaose were purchased from Sigma (USA). Restriction endonucleases, T4 DNA ligase, *pfu* DNA polymerase, and GC buffer were obtained from TAKARA (Japan). AxyPrep DNA purification kits were purchased from Axygen (USA). Bradford protein assay kits were obtained from Sangon (Shanghai, China). All other reagents used in this study were of analytical grade.

Construction of the Genomic Library and Screening for β-Glucosidase Genes

Genomic DNA from *M. mediterranea* was partially digested with *Sau*3AI. The DNA fragments of 4–9 kb were then purified using an AxyPrep DNA purification kit. Next, the fragments were collected, ligated into the *Bam*HI site of the dephosphorylated vector, pUC19, and subsequently transformed into *E. coli* DH5α through electrotransformation. The transformants were then plated on esculin medium plates containing 100 μg/ml ampicillin for screening of β-glucosidase. Media were incubated at 28 °C and then read for blackening at 24 and 48 h postinoculation. The colonies that turned brown-black were selected as β-glucosidase-positive recombinants.

Gene Sequencing and Analysis

DNA sequencing was conducted by the Shanghai Genomics Institute (SGI). Database similarity searches of DNA and protein sequences were then conducted using the BLAST program. Additionally, the open reading frame (ORF), promoter, and signal peptides were

predicted using the SoftberryGene Finding tool (http://linux1.softberry.com/berry.phtml). Multiple sequences were aligned using the Clustal W program. Protein functional analysis was conducted using InterProScan (http://www.ebi.ac.uk/Tools/InterProScan/). Structural classification of the sequences was conducted based on SCOP (http://scop.mrc-lmb.cam.ac.uk/scop-1.61/index.html). The crystal structure of Gluc3M was predicted using the Swiss-Model (http://swissmodel.expasy.org/).

Cloning the β-Glucosidase Gene in *E. coli*

To amplify the β-glucosidase gene of *M. mediterranea*, the following specific primers were designed: 5′-agcggatcc**atg**aacaatgatcagacg-3′ (sense, containing a *Bam*HI site as underlined and a start codon in bold) and 5′-acgctcgag**tca**ggccggttctgacca-3′ (antisense, containing a *Xho*I site as underlined and a stop codon in bold). The PCR conditions were as follows: one cycle of 4 min at 94 °C, followed by 25 cycles of 30 s at 94 °C, 45 s at 50 °C, and 2.5 min at 72 °C, with a final cycle of 10 min at 72 °C. The PCR product was then introduced to the *Bam*HI and *Xho*I sites on pGEX-6P-1, which resulted in the addition of a C-terminal GST tag. The absence of mutation within the coding region of β-glucosidases was verified by DNA sequencing. The resulting plasmid was designated pGEX-6p-Gluc3M.

Expression and Purification of β-Glucosidase

To enable large-scale production of the recombinant protein, the bacteria were cultured overnight in LB medium. Subsequently, the culture was transferred to fresh LB medium (1:100 dilution) supplemented with ampicillin (100 mg/ml). The samples were then cultured at 37 °C until they reached an OD_{600} log phase of 0.6–0.9. Next, expression was induced by the addition of isopropyl-1-thio-β-D-galactoside (IPTG) to a final concentration of 0.2 mM and incubation for 8 h at 18 °C.

The samples were then centrifuged, after which the cells were washed and resuspended in phosphate buffer (pH 7.4, 140.0 mM NaCl, 2.7 mM KCl, 10.0 mM Na_2HPO_4, and 1.8 mM KH_2PO_4). Next, the cells were subjected to high pressure at low temperatures, after which the lysates were centrifuged at 10,000×*g* for 30 min. The expressed Gluc3M in the soluble protein fraction was then purified using glutathione-*S*-transferase (GST) [12]. The GST tag was removed by digestion with 3C protease solution (10 U/μl, PreScission, Pharmacia), after which the purified protein was eluted with phosphate buffer [12]. All purification operations were conducted at 4 °C. After purification, the molecular weight of the enzyme was estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The protein concentration was determined by a Bradford assay using bovine serum albumin as a standard (0–300 μg/ml concentration range) [13]. The purified β-glucosidases were stored at −80 °C until subsequent analysis.

Enzyme Characterization

Effects of pH and Temperature on Enzyme Activity

The β-glucosidase activity was determined using standard procedures and pNPGlu as a substrate. Briefly, the reaction was conducted in a 200-μl reaction mixture that contained 10 μl of enzyme solution, 10 μl of substrate solution (20 mg/ml), and 180 μl of buffer. The reaction was conducted for 10 min at 55 °C, after which it was stopped by the addition of 100 μl of 1 M Na_2CO_3. The liberated *p*-nitrophenol was detected spectrophotometrically at 405 nm [14].

One unit of β-glucosidase activity was defined as the amount of enzyme required for the hydrolysis of 1 μmol of substrate (pNPG) per minute under assay conditions.

The optimal pH was determined by assaying the enzyme at 50 °C with the following buffer systems: 0.2 M Na_2HPO_4/0.1 M citric acid buffer at pH values ranging from 2.0 to 8.0 and 0.05 M glycine–NaOH buffer at pH values ranging from 8.0 to 13.6. The pH stability of Gluc3M was determined by measuring the residual activity after pre-incubation of the purified enzyme in buffer solutions of different pH for 24 h at 4 °C.

The optimum temperature was determined by incubating the enzyme for 10 min at temperatures ranging from 4 °C to 90 °C under optimum pH. To assess the thermal stability, the enzyme was incubated for 1 h at optimum pH without substrate at temperatures ranging from 4 °C to 90 °C, after which the remaining activities were determined under standard enzyme assay conditions. All of the above experiments were performed in triplicate.

Effects of Various Reagents on Enzyme Activity

The effects of various metal ions and reagents at 2 mM on β-glucosidase activity were determined by measuring the enzyme activity within the metal ions or reagents under standard enzyme assay conditions. The activity assayed in the absence of metal ions or reagents was taken to be 100%.

Substrate Specificity

The activities against pNPGlu, pNPGal, pNPA, and pNPX were tested under standard conditions [15]. The substrate specificity of the β-glucosidase was determined using a series of glucans at a concentration of 1% (*w*/*v*) as the substrates. Specifically, the specificity for cellobiose, glucan, laminarin, carboxymethyl cellulose, locust bean gum, salicin, oat spelts xylan, birchwood xylan, and konjac powder were evaluated. Additionally, the concentration of reducing sugars was determined using the 3,5-dinitrosalicylic acid (DNS) method as follows: 100 μl of reaction mixture containing 90 μl of 1% substrate and 10 μl of enzyme solution were treated with the sample at the optimum temperature for 0.5 h [16]. This mixture was then added to 100 μl of DNS solution and incubated at 100 °C for 10 min. Finally, the absorbance was measured at 540 nm after cooling and compared to that of glucose as a standard.

Kinetic Measurements

The kinetic parameters were determined by measuring the initial reaction velocity at various substrate (pNPGlu, cellohexaose, cellotetraose, cellotriose, and cellobiose) concentrations ranging from 0.1 to 1.0 mM in 50 mM glycine–NaOH buffer (pH 8.0) and 4 °C as described above. The values of the Michaelis constant (K_m) and the maximum velocity (V_{max}) were obtained from Lineweaver–Burk plots.

Results

Cloning, Expression, and Sequence Analysis

Approximately 4,500 clones from a library of *M. mediterranea* genomic DNA were screened for the production of β-glucosidases on esculin medium, and one positive recombinant containing an approximately 6-kb insert was obtained. This fragment was then

recovered and completely sequenced by SGI. Subsequent sequence analysis revealed that the fragment contained three ORFs: one that encoded a putative glyoxalase protein consisting of 80 amino acids, another which encoded a transcriptional regulator protein consisting of 369 bp, and one that encoded Gluc3M. In this paper, we focus on the third ORF, which encoded a β-glucosidase.

The β-glucosidase ORF started with an ATG codon and terminated in a TGA codon. Overall, this ORF consisted of 2,496 bp encoding an 832 residue polypeptide. The molecular weight of the Gluc3M was predicted to be 89.69 kDa by SDS-PAGE. Additionally, the ORF was GC rich, with an overall GC content of greater than 65%. Gluc3M was found to contain two catalytic domains belonging to GHF3. This result was predicted by blast alignment on NCBI CBD database. Based on the sequence analysis above, we conducted that Gluc3M belongs to the GHF3.

Gluc3M shared the greatest similarity (59%) with a hypothetical protein from *Agrobacterium vitis* S4 (ACM39228.1). Gluc3M was also 59% homologous with β-glucosidase from *Rhizobium etli* CFN 42 (ABC94363.1), 50% homologous with GHF3 protein from *Roseiflexus* sp. RS-1 (ABQ91838.1), 51% homologous with putative β-glucosidase from *Oceanicola granulosus* HTCC2516 (EAR50488.1), 48% homologous with GHF3 protein from *Leptothrix cholodnii* SP-6 (ACB36581.1), and 45% homologous with GHF3 domain protein from *Chloroflexus aggregans* (ACL24592.1). Further alignments indicated that Asp-261 is fully conserved within this family, which is consistent with its key role in the activity of GHF3.

Purification and Characterization

SDS-PAGE analysis of the purified Gluc3M showed the presence of a single band with a calculated molecular weight of approximately 89.69 kDa (Fig. 1). Gluc3M had no signal secretory sequence. The enzyme showed the optimum activity at pH 8.0, while over 60% of the maximal activity was achieved between pH 6.5 and 9.5 (Fig. 2a). In the absence of any stabilizer, the purified Gluc3M was apparently stable under alkaline conditions but was

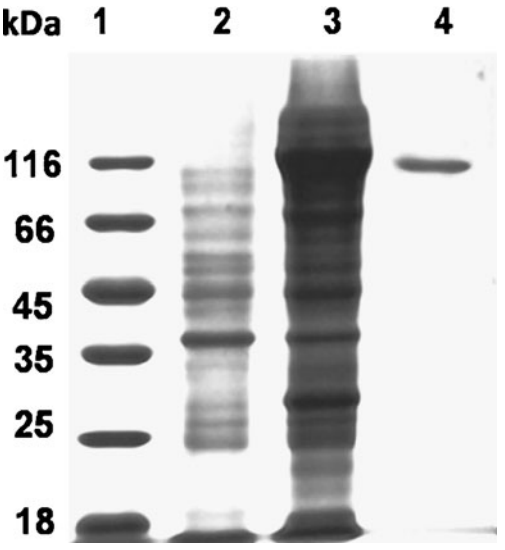

Fig. 1 Twelve percent SDS-PAGE analysis of the purified Gluc3M *lanes*: *1* protein marker, *2* recombinant bacterium (harboring pGEX-6P-Gluc3M) not-induced with IPTG, *5* recombinant bacterium (harboring pGEX-6P-Gluc3M) induced with IPTG, *6* purified β-glucosidase without GST (Gluc3M)

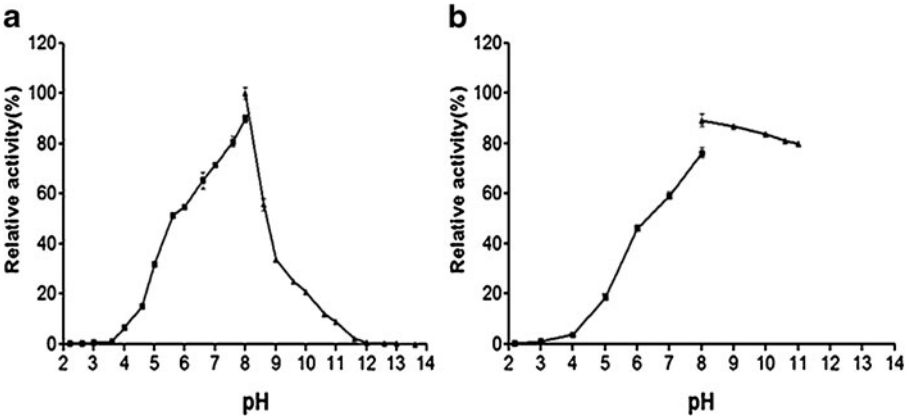

Fig. 2 Effect of pH on activity (**a**) and stability (**b**) of purified Gluc3M. **a** Effect of pH on the activity of purified Gluc3M. The activity was assayed at 50 °C at pHs ranging from 2.2 to 13.6 with the following buffers containing 1 mM pNPGlu: 0.2 M Na_2HPO_4/0.1 M citric acid for pH values 2.2–8.0 (*square*); 0.05 M glycine-NaOH buffer for pH values 8.0–13.6 (*triangle*). The activity at the optimum pH was defined as 100%. **b** Effect of pH on the stability of purified Gluc3M. After pre-incubation at 4 °C for 24 h in buffers ranging from pH 2.2 to 11.0, the activity was measured in 0.05 M glycine-NaOH buffer containing 1 mM pNPGlu (pH 8.0) at 45 °C. The activity without the pH treatment was defined as 100%. Error bars represent the standard deviation of the mean calculated for the three replicates

unstable under acidic conditions. Gluc3M was very stable at pH values ranging from 8.0 to 12.0, showing more than 90% of its maximal activity (the specific activity is 166.5 IU/mg) after incubation overnight at 4 °C. However, the enzyme was sensitive to low pH and displayed less than 20% of its maximal activity at pH 6.0 and nearly no activity below pH 5.5 (Fig. 2b).

The maximal activity of the purified Gluc3M was observed at 45 °C (Fig. 3a), and it was stable at less than 50 °C, retaining nearly 75% of its initial activity after 1 h of incubation at 45 °C. Furthermore, the enzyme still exhibited more than 30% and 15% of its maximum activity after incubation for 1 h at 55 °C and 60 °C, respectively. However, the activity decreased rapidly at temperatures above 60 °C, as indicated by only 16.2% of the original activity remaining after incubation for 1 h at this temperature. It should also be noted that Glu3M is also a cold-active enzyme, as indicated by 50–90% of its residual activity remaining after pre-incubation for 1 h at temperatures ranging from 4 °C to 40 °C. Indeed, the purified Gluc3M displayed 53% of its original activity (the specific activity is 101.8 IU/mg) at 4 °C and maintained 96% of its activity after incubation at this temperature for 1 h (Fig. 3b).

Substrate Specificity

The specific activities of purified Gluc3M against pNPGlu, pNPGal, and pNPX were 196.08, 200.00, and 36.43 IU/mg of protein, respectively. Additionally, the activity against pNPGal was almost two-fold greater than the activity against pNPGlu. Further examination of the substrate preference showed that the Glu3M enzyme also efficiently hydrolyzed a variety of cellulose materials, such as konjac powder. Gluc3M was found to have low activity against cellobiose, carboxymethyl cellulose, locust bean gum, and salicin. The activities of Gluc3M against glucan, laminarin, birchwood xylan, and oat spelts xylan were too low to be detected (Table 1).

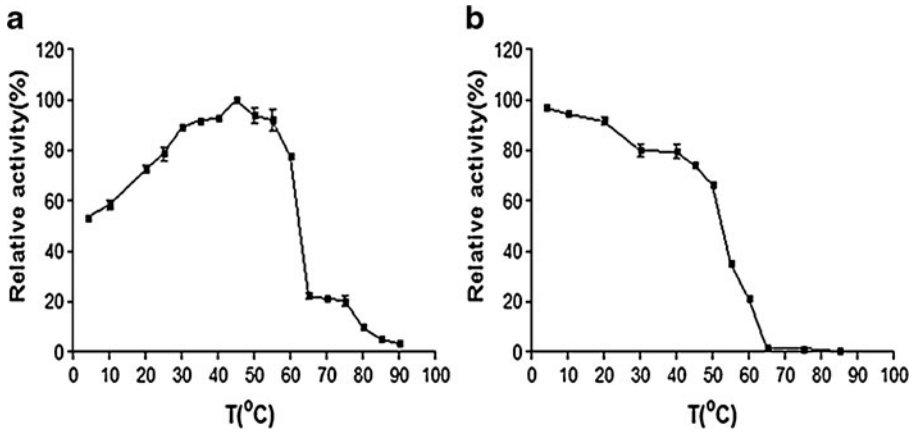

Fig. 3 Effect of temperature on the activity (**a**) and stability (**b**) of purified Gluc3M. **a** Effect of temperature on the stability of Gluc3M. The activity was evaluated in 0.05 M glycine-NaOH buffer containing 1 mM pNPGlu (pH 8.0) at various temperatures. **b** Effect of temperature on the stability of purified Gluc3M. After pre-incubation at a range of temperatures for 1 h, the activity of the enzyme measured in 0.05 M glycine-NaOH buffer containing 1 mM pNPGlu (pH 8.0) at 45 °C was determined. The activity without treatment was taken to be 100%. Error bars represent the standard deviation of the mean calculated for the three replicates

Effects of Metal Ions and Reagents on β-Glucosidase Activity

As shown in Table 1, the activity of Gluc3M was affected by most of the metal ions tested. Specifically, the activity was strongly inhibited by Zn^{2+}, Ca^{2+}, Cu^{2+}, Hg^{2+}, and Pb^{2+}, as indicated by less than 30% of the maximal activity remaining after incubation with these reagents. Co^{2+}, Mn^{2+}, Mg^{2+}, and DTT also slightly inhibited this activity, while SDS had no effect on the enzyme activity. Additionally, NH_4^+, Na^+, K^+, EDTA, and β-mercaptoethanol sharply increased the activity of Gluc3M (Table 2).

Table 1 Activity of purified β-glucosidase from *M. mediterranea* against different substrates.

Substrate	Activity[a] (IU/mg)
pNP β-D-glucopyranoside	196.08±0.23[b]
pNP β-D-galactoside	200.00±0. 32
pNP β-D-xyloside	36.43±0.006
pNP α-L-arabinoside	1.18
Glucan	0
Laminarin	0
Carboxymethyl cellulose	3.71±0.003
Locust bean gum	1.20±0.004
Salicin	9.12±0.001
Oat spelts xylan	0
Birchwood xylan	0
Konjac powder	15.69±0.001

[a] Assay was performed under optimum conditions

[b] Standard deviations were shown behind the specific activities

Table 2 Effect of metal ions and reagents on the activity of Gluc3M.

Reagent	Concentration (mM)	Relative activity (%)[a]
None	0	100
Mg^{2+}	1	31.9±0.6[b]
Zn^{2+}	1	21.3±0.6
Ca^{2+}	1	23.9±0.3
Mn^{2+}	1	75.9±1.1
NH_4^+	1	158.3±2.2
Na^+	1	145.2±1.1
K^+	1	164.2±3.2
Cu^{2+}	1	3.6±4.0
Hg^{2+}	1	3.7±0.03
Co^{2+}	1	67.9±5.1
Pb^{2+}	1	14.6±0.7
DTT	1	40.0±0.2
EDTA	1%	139.3±1.6
SDS	1	102.7±1.2
β-Mercaptoethanol	1%	161.2±0.3

None indicates that no chemicals were added to the reaction system

[a] Assay was performed under optimum conditions

[b] Specific activity ± the standard deviation

Kinetic Measurements

When pNPGlu was used as the substrate at pH8.0 (glycine–NaOH), the kinetic parameters K_m and V_{max} of Gluc3M were 0.18 mg/ml and 196.08 μmol/min/mg protein, respectively. Additionally, the K_m and V_{max} of Gluc3M toward pNPGal were calculated to be 0.02 mg/ml and 200 μmol/min/mg protein, respectively. When cellohexaose, cellotetraose, cellotriose, and cellobiose were used as the substrate, the kinetic values (K_m and k_{cat}) were shown in Table 3.

Nucleotide Sequence Accession Number

The nucleotide sequence of the β-glucosidase gene (*gluc3m*) from *M. mediterranea* has been deposited in the GenBank database under accession no. NCBI 383111.

Table 3 Kinetic measurements of Gluc3M.

Substrate	V_{max} (μmol/min/mg)	K_m (mM)	K_{cat} (1/s)
pNPGlu	196.08	0.59	40.1
pNPGal	200	0.06	41.7
Cellobiose	51.4	2.71	14.3
Cellotriose	92.9	1.68	25.8
Cellotetraose	205.5	0.57	57.1
Cellohexaose	132.7	0.12	36.9

Discussion

The gene designated *gluc3m* was isolated from *M. mediterranea*, which represents a separate line of descent within the order *Rhizobiales* of the class *Alphaproteobacteria* based on 16S rRNA gene sequence analysis. To the best of our knowledge, this is the first report of cloning a β-glucosidase gene from *M. mediterranea*. Gluc3M retained 59% similarity with a hypothetical protein from *A. vitis* S4. Gluc3M possesses three domains, as do most other members of this family. However, the amino acids analysis also predicted that Gluc3M, which belongs to cluster B, had a PA14 domain insertion in the C-terminal GHF3 domain. It is predicted that the PA14-like insertion sequence may represent a novel carbohydrate binding module [17].

A surprising feature of Gluc3M is that it is a cold-active enzyme. The purified Gluc3M displayed 53% of its activity (the specific activity is 101.8 IU/mg) at 4 °C and maintained over 90% activity (the specific activity is 176.6 IU/mg) after incubation at 4–20 °C for 1 h. This may have occurred because Gluc3M contained 45.97% random coil. Random coils increase the flexibility of the molecular structure [18]. Additionally, it has been reported that higher amounts of asparagine residues, higher percentages of glycine residues, lower arginine contents, and fewer prolines are responsible for adaptive ability under cold conditions [19]. Gluc3M has a percentage (23.6%) of arginines and pralines, which is lower than the percentage (33.7%) of asparagines and glycines. The three-dimensional structure of Gluc3M was determined using SWISS-MODEL (Fig. 4). Gluc3M possessed the conserved putative aspartate catalytic residue (position 285), which is conserved active site in the SDW (Ser-Asp-Trp) sequences of family 3 enzymes (shown in black) [20, 21]. It was observed that some amino acids such as aspartic acid, valine, serine, glycine, and alanine were around the active site (shown in purple). These amino acids may increase the flexibility of the molecular structure, as reported by Bauvois et al. and Huston et al. [22, 23]. Taken together, these characteristics may enable Gluc3M to adapt to cold environments. Under cold conditions, Gluc3M has a relatively higher activity than other members of the same family. Indeed, β-glucosidases from *Paenibacillus* sp. strain C7 displayed 20% activity at 4 °C, while β-glucosidases from *Pyrococcus furiosus* showed less than 5% activity at temperatures lower than 10 °C [24]. Recently, many studies have focused on cold adaptation enzymes due to their unique characteristics [25, 26]. Additionally, some

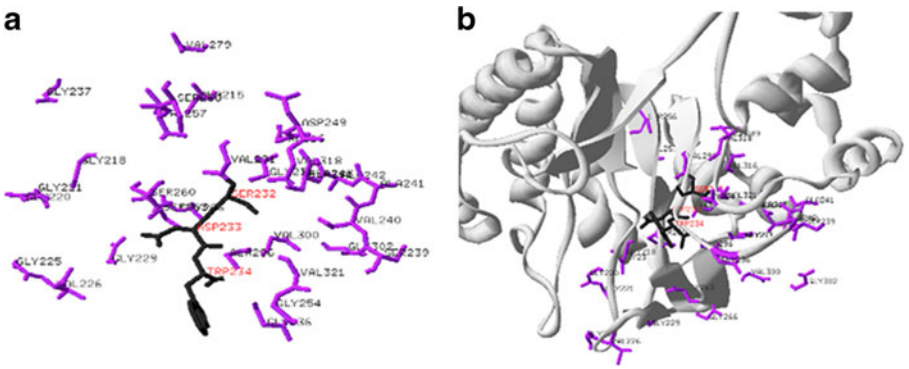

Fig. 4 Homology modeling of Gluc3M predicted using SWISS-MODEL. **a** Conserved domain "SDW" (Ser-Asp-Trp) is shown in *black*. The residues (aspartic acid, glycine, alanine, serine, valine) around the conserved domain are indicated in *purple*. **b** Three-dimensional structure of Gluc3M

industrial processes require low temperatures to maintain the flavor of the product, and reactions operated under low temperatures lead to considerable cost savings due to reduced energy use [27]. These advantages indicate the commercial importance of Gluc3M.

Another interesting feature of Gluc3M is that it is stable under alkaline conditions. Specifically, the activity of Gluc3M maintained over 166.5 IU/mg after incubation in buffers at pH values ranging from 7.0 to 11.0 for 24 h. The pH stabilities have been found to be in the range 4.0–7.0, 4.0–6.0, and 2.2–9.8 for β-glucosidase from *Candida peltata*, *Sclerotinia sclerotiorum*, and *Aureobasidium* sp. ATCC 20524, respectively [7, 28, 29], which indicates that Gluc3M is stable over a relatively high alkaline pH range. The enzymatic activity of the β-glucosidase expressed in *E. coli* BL21 was assayed, and the optimal pH was found to occur at pH 8.0 (glycine–NaOH buffer). These findings are the same as the results obtained when β-glucosidase produced by *Leuconostoc mesenteroides* strain DIP 20 were evaluated [30] and similar to the results obtained for β-glucosidase from *Agrobacterium tumefaciens*, which is most active at pH 7.2–7.4 [31]. A study conducted by Mosavi and Peng indicated that replacement of surface-exposed positively charged residues with hydrophobic residues can significantly improve protein stability at physiological pH [32]. The number of hydrophobic amino acids is almost two-fold greater than the number of polar amino acids in Gluc3M. It is possible that this property benefits Gluc3M activity under alkaline conditions. These findings indicate that Gluc3M can be kept under alkaline conditions for long time; therefore, it may be useful in the production of textiles and beverages.

The activity of Gluc3M improved remarkably in the presence of NH_4^+, Na^+, K^+, EDTA, and β-mercaptoethanol. However, Co^{2+}, Ca^{2+}, Mg^{2+}, and Zn^{2+} had a weakly negative effect on enzyme activity. Additionally, although SDS had no significant effect on enzyme activity, the enzyme activity decreased greatly in response to Hg^{2+}, Cu^{2+}, Mn^{2+}, and Pb^{2+}. This phenomenon may have been due to β-mercaptoethanol preventing the enzyme from being oxidized by a reducing agent, thereby acting as a reducing agent to prevent the formation of disulfide bonds. EDTA has a high capacity for chelating metal ions. These results indicated that the activity of Gluc3M is inhibited in the presence of divalent ions, but univalent metal ions are required for the activity.

Gluc3M displayed activities against both pNPGlu and pNPGal. β-Glucosidases from *Clostridium thermocellum* and *Paenibacillus* sp. strain C7 are unique in family 3 in that they have apparent activity against pNPGal; this may because Gluc3M could recognize aryl groups both of pNPGlu and pNPGal [33]. Although the mechanism that Gluc3M has remarkable activity on pNPGal is unclear and needed to further study, our results might supply useful information for researching on the glucosidases (GHF3) with high activity toward pNPGal. Moreover, the purified Gluc3M had a high activity against konjac powder. Conversely, the enzyme showed low hydrolysis efficiency toward, cellobiose, carboxymethyl cellulose, locust bean gum, and salicin and had no activity with glucan, laminarin, birchwood xylan, and oat spelts xylan. These results suggest that Gluc3M has no effect on β-1,3-glucosidic linkages and that it is slightly active against α-glucosidic bonds. Gluc3M efficiently hydrolyzed natural oligosaccharides with β-1,4-glycosidic bonds. Based on these findings, Glu3M may be useful in the candy industry as food sweetener due to its remarkable activity against konjac powder [34]. Gluc3M may also play a key role in wood pulping for cellulose-degrading function.

Gluc3M hydrolyzed cellulose to release glucose or cellobiose as the predominant product, which suggests that it attacks β-1,4-glycosidic bonds. Among four celluloses, the lowest K_m value indicated cellohexaose was optimum substrate of Gluc3M. These kinetic values demonstrate that Gluc3M can be a useful enzyme for the saccharification of cello-oligosaccharides.

In summary, Gluc3M is a β-glucosidase that is cold-active, alkaline-stable, and possesses significant pNPGal activity. This characterization suggests that Gluc3M will be useful in commercial processes.

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