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Characterization of a novel cold-adapted GH1 β-glucosidase from *Psychrobacillus glaciei* and its application in the hydrolysis of soybean isoflavone glycosides

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ABSTRACT

The novel β-glucosidase gene (*pgbgl1*) of glycoside hydrolase (GH) family 1 from the psychrotrophic bacterium *Psychrobacillus glaciei* sp. PB01 was successfully expressed in *Escherichia coli* BL21 (DE3). The deduced PgBgl1 contained 447 amino acid residues with a calculated molecular mass of 51.4 kDa. PgBgl1 showed its maximum activity at pH 7.0 and 40 °C, and still retained over 10% activity at 0 °C, suggesting that the recombinant PgBgl1 is a cold-adapted enzyme. The substrate specificity, K_m, V_{max}, and K_{cat}/K_m for the *p*-Nitrophenyl-β-D-glucopyranoside (*p*NPG) as the substrate were 1063.89 U/mg, 0.36 mM, 1208.31 U/mg and 3871.92/s, respectively. Furthermore, PgBgl1 demonstrated remarkable stimulation of monosaccharides such as glucose, xylose, and galactose, as well as NaCl. PgBgl1 also demonstrated a high capacity to convert the primary soybean isoflavone glycosides (daidzin, genistin, and glycitin) into their respective aglycones. Overall, PgBgl1 exhibited high catalytic activity towards aryl glycosides, suggesting promising application prospects in the food, animal feed, and pharmaceutical industries.

1. Introduction

β-Glucosidases (EC 3.2.1.21, BGs), also known as β-D-glucopyranoside hydrolases, are an important component of the cellulose decomposition enzyme system. They can hydrolyze β-D-glucose bonds, releasing β-D-glucose and corresponding ligands (Bhatia et al., 2002; Srivastava et al., 2019; Singhania et al., 2017). It is generally present in almost all organisms and is an essential class of enzymes in the pathway of sugar metabolism in living organisms (Ketudat Cairns and Esen, 2010). Based on substrate specificity, BGs are mainly classified into three distinct groups: aryl-glucosidases act on aryl glucoside substrates, cellobiases exhibit specificity towards cellobiose, and broad-spectrum glucosidases have a wide range of substrates (Zhang et al., 2021; Godse et al., 2021). In glycoside hydrolase (GH) families, BGs have been classified into GH1, GH2, GH3, GH5, GH9, GH30, GH39, and GH116 (Drula et al., 2022; http://www.cazy.org/accessed on 6 June 2023). Among them, most of those identified so far belong to the GH1 and GH3 families. BGs from two families exhibit structural variations, but the catalytic domain of the $(\beta/\alpha)_8$-barrel is identical. GH1 BGs contain two conserved carboxylic acid residues, while GH3 BGs have an additional $(\alpha/\beta)_6$-sandwich domain, with each domain contributing one catalytic residue to the active site (Ketudat Cairns and Esen, 2010). Furthermore, GH1 BGs are 10- to 1000-fold more tolerant to glucose inhibition compared to the GH3 family (Godse et al., 2021). Therefore, many studies have been conducted to enhance enzyme properties by investigating the correlation between structure and function to advance

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industrial production (Cota et al., 2015).

As one of the most extensively studied enzymes, BGs have a wide range of applications in many fields. In the biofuel industry, BGs have been considered a rate-limiting enzyme that determines the efficiency of cellulase and biomass hydrolysis. They act as a potent commercial cellulase supplement to enhance the conversion efficiency of lignocellulosic biomass into fermentable sugars (Qi et al., 2021; Li et al., 2022). In the food fermentation industry, BGs have the ability to release bioactive aglycons in food products. These compounds can be absorbed by animals and humans, providing additional health benefits. Many BGs have been studied for the hydrolysis of isoflavone glycosides and major ginsenosides to produce soybean isoflavones and minor ginsenosides (Lee and Paik, 2017; Shin and Oh, 2016). Moreover, BG plays an important role in enhancing the quality of wine and fruit juice by influencing the aroma and flavor of the product (Godse et al., 2021; Zhang et al., 2021; Liang et al., 2022). In the pharmaceutical industry, several BGs have been reported that not only catalyze the hydrolysis of glycosidic bonds but also can be used for glycoside formation with transglycosylation activity, aiding in the synthesis of mono-, oligo-, and polysaccharide acceptor moieties (Godse et al., 2021; Wang et al., 2011; Méndez-Líter et al., 2020).

In recent decades, the discovery and biochemical characterization of numerous novel microbial BGs have been the focus of intensive research efforts. Among them, cold-adapted BGs have higher catalytic efficiency at low temperatures, reduced activation energy, and production costs, minimizing the risk of microbial contamination compared to mesophilic or thermophilic counterparts (Mangiagalli et al., 2020; Hamid et al., 2022). In the coldest environments on Earth, such as the Antarctic, Arctic, and deep seas, extremely low temperatures are a defining characteristic. Following genomic and metagenomic sequencing, only a few cold-active enzymes from Antarctic bacteria have been characterized (Parvizpour et al., 2021; Miao et al., 2016; Crespim et al., 2016). Recently, a previously unknown psychrotolerant species of *Psychrobacillus*, designated *P. glaciei* sp. PB01 was isolated from an Antarctic iceberg. It can grow at temperatures ranging from −2 to 30 °C (optimum at 15 °C) and in the presence of 0–8.0% (w/v) NaCl (Choi and Lee, 2020). In this study, the novel gene *pgbgl1* was successfully overexpressed and characterized. The enzymatic properties of PgBgl1 were comprehensively characterized to assess their suitability for use at low temperatures.

2. Materials and methods

2.1. Strains, plasmids and chemicals

The expression vector pET-30a(+), *Escherichia* coli DH5α strain, and the expression host strain *E. coli* BL21 (DE3) were purchased from Novagen (Madison, WI, USA), while the recombinant plasmid pET-30a (+)-*pgbgl1* was synthesized at General Biosystems (Anhui, China). 4-Nitrophenyl-β-D-xylopyranoside (*p*NPX) and 4-Nitrophenyl-β-D-mannopyranoside (*p*NPM) were purchased from Megazyme (Bray, Ireland). *p*-Nitrophenyl-β-D-galactopyranoside (*p*NPgal), *p*-Nitrophenyl-β-D-glucopyranoside (*p*NPG), *o*-Nitrophenyl-β-D-galactopyranoside (*o*NPG), daidzin, genistin, glycitin, daidzein, genistein, and glycitein were purchased from Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade and commercially available.

2.2. Bioinformatic analysis

The sequence identity and coding gene were predicted through BLAST analysis against the National Center for Biotechnology Information (NCBI) non-redundant database (https://www.ncbi.nlm.nih.gov/; accessed on 6 June 2023). The protein functions were further annotated using Pfam (http://pfam-legacy.xfam.org/search#tabview=tab1; accessed on 6 June 2023). Signal peptide was predicted using the SignalP 5.0 Server (https://services.healthtech.dtu.dk/services/SignalP-5.0/6 June 2023). Multiple alignments of PgBgl1 and other GH1 β-glucosidases from various bacteria were obtained using ClustalW. Homology modeling of PgBgl1 was performed using the Swiss-Model server (http://swissmodel.expasy.org/; accessed on 6 June 2023). The homologous structure of the GH1 β-glucosidase, with 61% sequence identity, was derived from *Niallia circulans* subsp. alkalophilus (PDB code: 1QOX).

2.3. Expression and purification of recombinant PgBgl1

Transformation of recombinant plasmids was carried out following the transformation protocol for *E. coli* BL21(DE3) recipient strain. Overnight-cultured pET-30a(+)-*pgbgl1* was incubated in 300 of mL liquid Luria–Bertani (LB) medium containing 50 mg/mL kanamycin at 37 °C with shaking at 220 rpm until the OD_{600} reached 0.4–0.8. Subsequently, 180 μL of 1 mol/L isopropyl-β-D-thiogalactopyranoside (IPTG) was added, and the culture was further incubated at 16 °C with shaking for 6 h. After the completion of induction, the cells were centrifuged at 10,000 rpm for 10 min, and the resulting pellet was resuspended in the His-tagged protein purification buffer (NTA) (20 mM Tris-HCl, 500 mM NaCl, 0 mM imidazole, pH 7.0). Ultrasonic fragmentation was performed under the following working parameters: 25 min, 6 cycles of 10 s each, 35% amplitude, in an ice bath. The supernatant and fragmented precipitate were collected. The recombinant protein in this supernatant was purified using the Ni-NTA column following the manufacturer's handbook (Hilden, Germany). The purity of the recombinant protein was assessed using 12% SDS-PAGE, and the protein concentration was quantified with the BCA Protein Assay Kit, using bovine serum albumin (BSA) as the standard.

2.4. Activity assay

For the artificial substrate, the enzymatic activity of the enzyme was determined by adding 250 μL of 4 mmol/L *p*-Nitrophenyl-β-D-glucoside (*p*NPG) substrate in a test tube in a warm bath at 40 °C for 5 min. Subsequently, 250 μL of enzyme solution diluted with 100 mM Na_2HPO_4-citric acid buffer at pH 7.0 and the mixture was allowed to react in a water bath at 40 °C for 10 min. At the end of the reaction, 1.5 mL of Na_2CO_3 solution (1 mol/L) was added to terminate the reaction. After cooling to room temperature, the reaction was measured using a spectrophotometer. The OD_{405} values were measured using a spectrophotometer, and three replicates and one control were included.

To measure the PgBgl1 activities towards cellobiose (4 mM), gentiobiose (4 mM), and sophorose (4 mM), the GOD-POD method was used to determine the amount of reducing glucose with a commercial kit (Biosino, Beijing, China). The specific reaction steps were as follows: 70 μL of reducing oligosaccharides or glycoside substrate and 70 μL of properly diluted enzyme solution were reacted at 40 °C for 10 min. The mixture was then boiled for 5 min to terminate the reaction, followed by the addition of 2.1 mL of GOD-POD coloring solution for 10 min. The amount of released glucose was estimated by measuring the absorbance at 520 nm.

Definition of enzyme activity unit (U): One enzyme activity unit (U) is defined as the amount of enzyme needed to hydrolyze the substrate to produce 1 μmol of *p*NP or to hydrolyze the glycosidic substrate to produce 1 μmol of glucose per minute under specific reaction conditions.

2.5. Temperatures and pH

The optimal temperature was determined by investigating the ideal reaction temperature of PgBgl1 at pH 7.0 within the temperature interval range of 0–50 °C. The optimal pH was determined by measuring the relative enzyme activity in a Na_2HPO_4-citric acid buffer (pH 5.0, 6.0, 7.0, 8.0) at the established optimal temperature to ascertain the ideal reaction pH of the enzyme.

The enzymes were kept at 30 °C, 40 °C and 50 °C for a certain period,

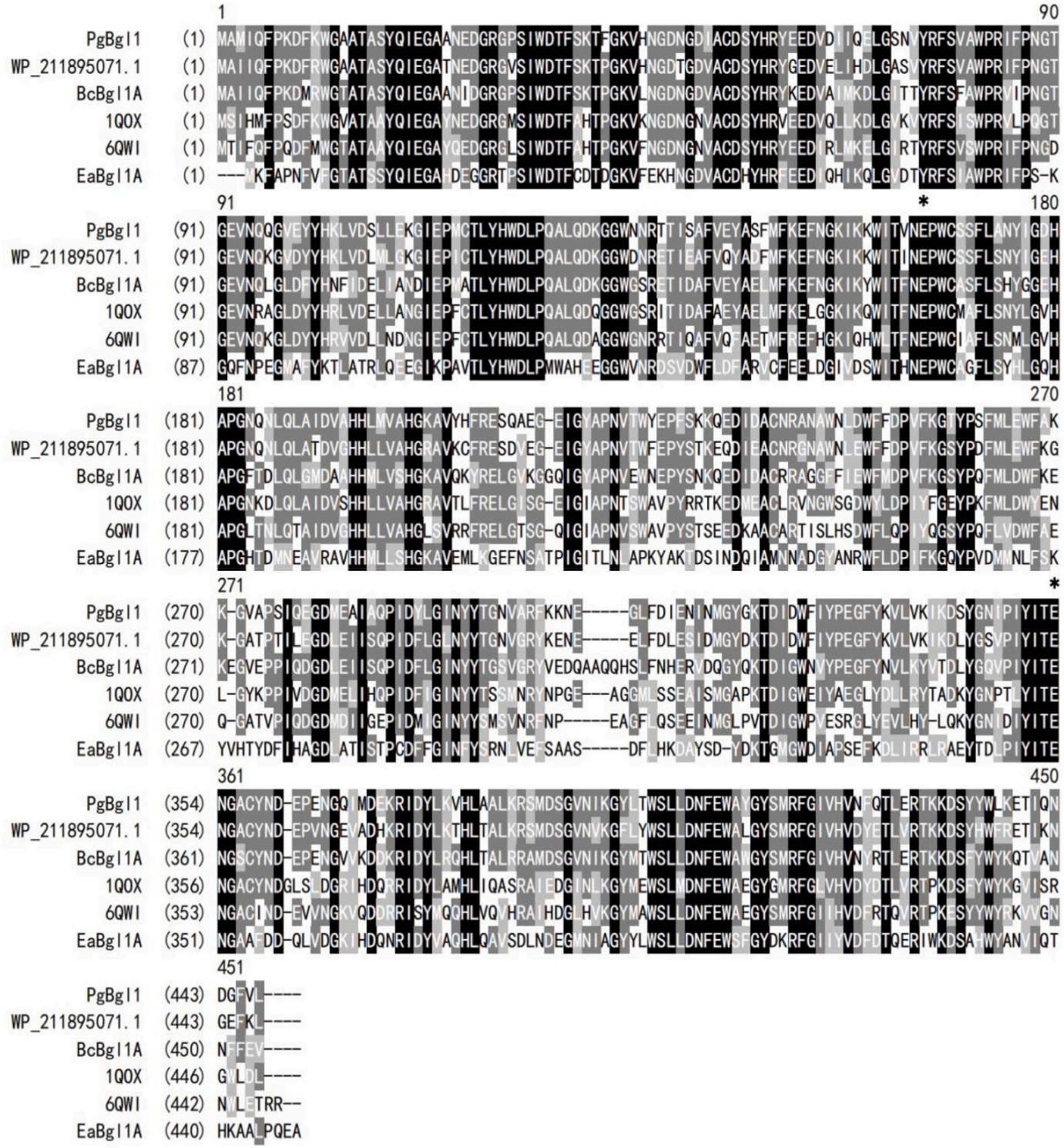

Fig. 1. Multiple sequence alignment of PgBgl1 with available protein sequences from GenBank. The aligned sequences originated from the following organisms: WP_211895071.1, *Psychrobacillus* sp. INOP01; BcBgl1A, *Evansella cellulosilytica* DSM 2522; 1QOX, *Niallia circulans* subsp. alkalophilus; 6QWI, *Paenibacillus polymyxa*; EaBgl1A, *Exiguobacterium antarcticum*; The alignment was performed by the Clustal W method and annotated by GeneDoc. *: The catalytic site of the enzyme. Identical amino acids are shown on a black background, similar amino acids are shown on a gray background, and different amino acids are shown on a white background.

and then their residual activity was measured to determine the thermal stability of the enzymes. The enzyme was placed in a Na_2HPO_4-citric acid buffer at different pH levels (pH 4.0, 5.0, 6.0, 7.0, 8.0) for 1 h at 37 °C. Subsequently, the residual activity of the enzyme was measured to assess its pH stability.

2.6. Metal ions and chemical reagents

To assay the effects of metal ions and chemical reagents on enzyme activity, thirteen metal ions (K^+, Ca^{2+}, Zn^{2+}, Mg^{2+}, Fe^{2+}, Fe^{3+}, Cu^{2+}, Na^+, Pb^{2+}, Ni^{2+}, Li^+, Cd^{2+}, and Co^{2+}) and six chemical reagents (SDS,

EDTA, methanol, ethanol, isopropanol, and β-mercaptoethanol) were prepared with Na_2HPO_4-citric acid buffer (pH 7.0) at a final concentration of 5 mmol/L or 5% (v/v). In the control group (CK), metal ions and chemical reagents were replaced with equivalent amounts of Na^2HPO^4-citric acid buffer (pH 7.0). Enzyme activity was determined using the *p*NP method under the optimal conditions (pH 7.0, 40 °C).

2.7. Determination of substrate specificity and kinetic parameters

To investigate the substrate (4 mM) specificity of PgBgl1, the artificial substrates (*p*NPG, *p*NPX, *p*NPM, *p*NPgal, *o*NPG) and natural substrates (cellobiose, gentiobiose, sophorose) were utilized to assess enzymatic activity under optimal conditions. For the artificial substrate, the relative enzyme activity was determined using the *p*NPG method. The release of *p*NP (at 405 nm) was measured using a UV spectrophotometer. The specific activities towards substrates cellobiose, sophorose and gentiobiose were assayed using the glucose oxidase-peroxidase (GOD-POD) method under the respective optimal conditions (pH 7.0, 40 °C). The absorbances at 520 nm were measured to determine the quantity of glucose released. Each experiment was performed in triplicate.

Under the optimum reaction conditions, the enzyme activities were measured at final concentrations of 0.1, 0.2, 0.3, 0.5, 1.0, and 2.0 mmol/L of *p*NPG substrate for 5 min of reaction. The Lineweaver-Burk double inverse curves were plotted to calculate the Michaelis constant (K_m), the maximum reaction rate (V_{max}), and catalytic efficiency (K_{cat}/K_m).

2.8. Different concentrations of sugars and NaCl on the enzyme activity

Under the optimum conditions, no sugars or NaCl were added as a control. A final volume ratio of 0–2 mol/L of glucose, cellobiose, D-xylose, D-galactose, and D-sucrose or NaCl was added to the reaction. The relative enzyme activities were determined using the method of enzyme activity determination to analyze the effect of sugars or NaCl on enzyme activity.

2.9. HPLC analysis of soybean isoflavone glycosides hydrolyzed by PgBgl1

To evaluate the potential application of PgBgl1, soybean isoflavone glycosides (daidzin, glycitin, and genistin) were used as substrates for biotransformation. The enzymatic hydrolysis reaction was 800 μL, 200 μL of purified enzyme solution with final concentration of 17.10 U, 200 μL of 0.25 mg/mL solutions of soybean isoflavone glycosides substrate and 400 μL of sterile water were taken. The reaction was carried out at 40 °C and pH 7.0 for 15 min. It was terminated on ice immediately after completion. Among them, the purified enzyme solution in the control group was replaced with sterile water, and three controls were conducted for each treatment. Finally, the reaction system was transferred to the ultrafiltration tube and centrifuged 4 °C, 12,000 rpm for 30 min to ultrafiltration. After ultrafiltration, the filtrate was transferred to the sample bottle for analysis using HPLC detection.

The hydrolysis products of daidzin, genistin, and glycitin by PgBgl1 β-glucosidase were assayed using a Shimadzu VP-ODS C18 column (250 mm × 4.6 mm, 5 μm) on an HPLC system equipped with a UV detector set at 254 nm. The column was eluted with a gradient of solvent A (0.1% acetic acid in water) and solvent B (0.1% acetic acid in acetonitrile) at 25 °C. And 5 μL of the sample was injected, and then solvent B was run at 20% for 5 min, increased from 20% to 60% over 25 min, and then continuously increased from 60% to 95% over 10 min at a flow rate of 1.0 mL/min. Then the peaks of daidzin, glycitin, and genistin, as well as their corresponding isoflavone aglycones, were identified by comparing their elution profile with those of the commercial standards (Fang et al., 2014; Park et al., 2013).

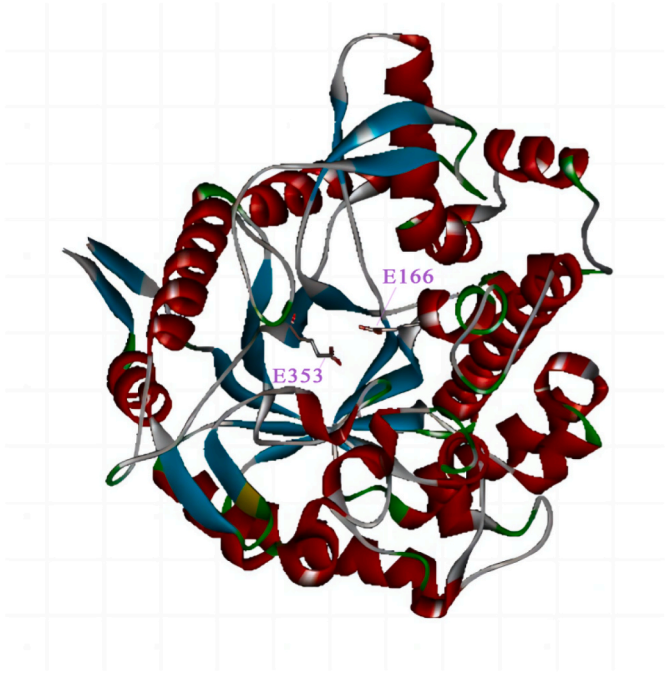

Fig. 2. The homology modeling of the recombinant protein PgBgl1. The catalytic sites of PgBgl1 have been labeled as E166 and E353. The 3D structure of PgBgl1 was predicted using the Swiss-Model server (https://swissmodel.expasy.org/on 6 June 2023).

3. Results and discussion

3.1. Sequence analysis of PgBgl1

The predicted BG PgBgl1 (GenBank No: QFG00756.1) from *P. glaciei* sp. PB01, belonging to the GH1 family, was examined. The open reading frame (ORF) of the PgBgl1 contains 1443 bp and encodes 447 amino acids. The deduced PgBgl1 does not have an apparent signal peptide sequence according to the SignalP 5.0. It is a mature protein with a calculated molecular weight (*MW*) of 51.43 kDa and an isoelectric point (*p*I) value of 5.08, respectively. Multiple sequence alignment of PgBgl1 with available protein sequences from GenBank (Fig. 1) indicated that it shares the highest identity of 82% with a putative GH1 family BG from *P. psychrotolerans* (GenBank No: WP_093537721.1), followed by the characterized GH1 BcBgl1A from *Bacillus cellulosilyticus* (GenBank No: ADU28569.1, 69%) (Wu et al., 2018), and a structurally resolved counterpart BG from *Niallia circulans* subsp. alkalophilus (PDB:1QOX, 61%).

Based on the sequence and homology analysis (Fig. 2), the putative tertiary structure of PgBgl1 adopts a typical $(\alpha/\beta)_8$ "TIM barrel fold," common to other GH1 family members (Hakulinen et al., 2000). The acidic catalytic residues are Glu353, which acts as a nucleophile, and Glu166, which serves as the acid/base catalyst. Other residues, including His121, Trp122, Asn165, Tyr296, Trp399, Glu406, Trp407, and Phe415, which are essential for substrate recognition and binding, are also conserved (de Giuseppe et al., 2014). However, due to limitations of homology modeling methods with low sequence identity (approximately 60%), the actual crystallographic structure of PgBgl1 is necessary for a comprehensive structural comparative analysis. Meanwhile, no information about the characteristics of BGs from the genus *Psychrobacillus* was reported (Choi and Lee, 2020). This analysis showed that PgBgl1 may be considered a novel enzyme with some distinct properties.

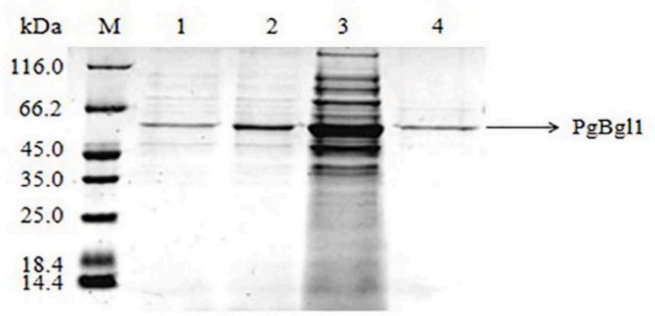

Fig. 3. SDS-PAGE of recombinant protein PgBgl1. M: marker; lane1: IPTG; lane2: +IPTG; lane 3: crude enzyme; lane4: purified protein.

3.2. Expression and purification of recombinant PgBgl1

The recombinant plasmid pET-30a(+)-*pgbgl1* was transferred into *E. coli* BL21(DE3) host cells for induced expression. The soluble protein was purified to homogeneity using successive Ni^{2+}-affinity chromatography and confirmed by SDS-PAGE (Fig. 3). The yield of the recombinant protein PgBgl1 was 43.7 μg/mL. The apparent molecular mass of purified recombinant PgBgl1 was found to be about 52.0 kDa, which was consistent with the theoretical molecular mass of 51.4 kDa.

3.3. Enzyme characterization of purified enzyme

The enzyme activities of PgBgl1 were determined using *p*NPG as a substrate in various pH buffers and temperatures. The recombinant enzyme retained 70–100% of its activity in the pH range from 6.0 to 9.0, with the maximum activity observed at pH 7.0. The enzyme's activity was rapidly lost at pH levels below 5.0. In pH stability studies, it exhibited good stability within a pH range of 6.0–8.0. However, its activity noticeably decreased outside of this pH range. Thus, PgBgl1 is active and stable at a neutral pH. As shown in Fig. 4, the optimum temperature of PgBgl1 was 40 °C, and it exhibited over 70% residual activity in the temperature range of 30–45 °C. It is worth noting that at temperatures of 0 °C and 20 °C, the enzyme activity still retained 11.1% and 37.2% of the maximum value, respectively. However, at temperatures exceeding 50 °C, the enzyme was rapidly inactivated. For the thermostability assay, PgBgl1 was more stable at 30 °C or lower, and retaining 77.4% of its original activity after pre-incubation for 1 h at 30 °C. However, almost complete loss of activity was observed after 10 min at 50 °C. So, the temperature results indicated that PgBgl1 may be a novel cold-adapted enzyme. Moreover, the typical cold-active psychrophilic GH1 β-glucosidases sensitive to heat, such as Bgl1629, BglMKg, MeBglD2, and Bgl66 from the metagenomic library (Li et al., 2012; Wierzbicka-Woś et al., 2013; Matsuzawa and Yaoi, 2017; Mai et al., 2021); Bgl1C, EaBgl1A, and BglG from several *Exiguobacterium* genus strains (Crespim et al., 2016; Chen et al., 2010; Yin et al., 2019), BcBgl1A from *Bacillus cellulosilyticus* (Wu et al., 2018), and BglU from *Micrococcus antarcticus* (Miao et al., 2016). The most psychrophilic GH1 β-glucosidases from cold-adapted organisms are characterized by high catalytic efficiencies at low temperatures compared to other mesophilic or thermophilic counterparts. In general, the neutral and low-temperature properties of PgBgl1 make it advantageous in industrial processes. It saves energy costs, protects thermolabile substrates and/or products from degradation, decreases the rate of nonspecific chemical reaction, prevents the loss of volatile compounds, and reduces the risk of infection by mesophilic pathogenic microorganisms, especially in food processing.

3.4. Metal ions and chemical reagents on enzyme stability

The effects of various metal ions at a concentration of 5 mM on the enzyme activity were investigated under optimal conditions (Table 1). Among the metals tested, the presence of Cu^{2+} and Cd^{2+} showed moderately negative effects on the enzyme activity, with the relative activity decreasing by 30.7% and 61.9%, respectively. However, the enzymatic activity was slightly affected by the addition of other metal ions. The addition of Cu^{2+} strongly or almost completely inhibited the

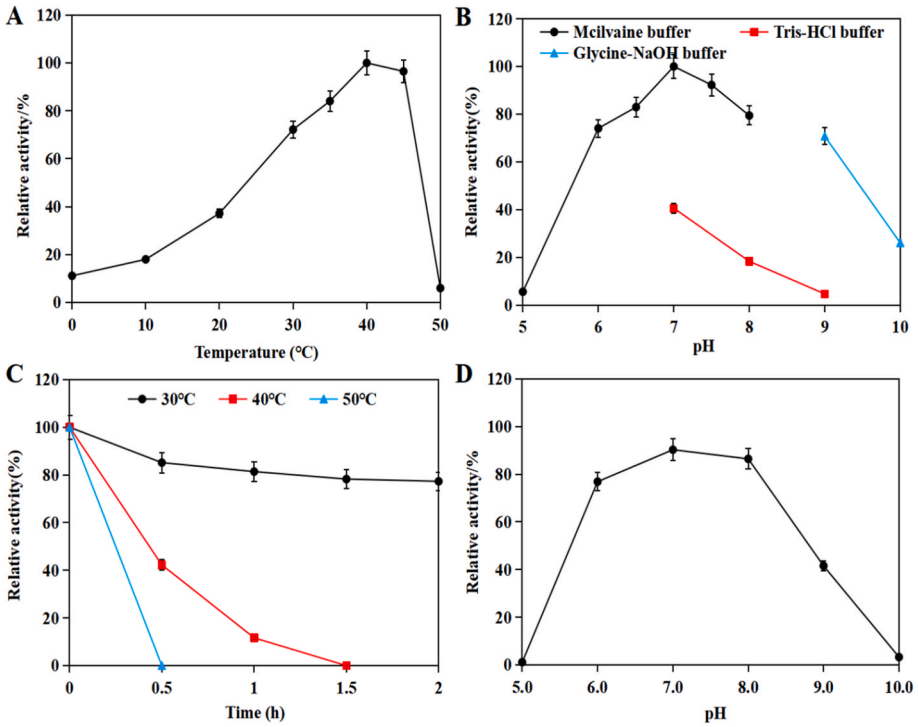

Fig. 4. Characterization of recombinant PgBgl1. (A) Optimum temperature. (B) Optimum pH. (C) Thermal stability (30 °C, 40 °C and 50 °C). (D) pH stability. The optimum activity was set as 100%. Data represent the means of triplicate measurements and error bars represent standard deviation.

Table 1
Effects of various metal ions and chemicals on the activity of PgBgl1.

Reagent (5 mM)	Relative activity (%)	Reagent (5 mM)	Relative activity (%)
CK	100.00 ± 2.02	Ni^{2+}	108.88 ± 2.67
K^{+}	97.66 ± 1.04	Li^{+}	90.21 ± 2.06
Na^{+}	98.31 ± 0.62	Co^{2+}	98.10 ± 0.54
Cu^{2+}	69.34 ± 2.74	Cd^{2+}	38.10 ± 0.39
Fe^{2+}	84.36 ± 1.64	SDS	ND
Fe^{3+}	95.73 ± 1.08	EDTA	105.24 ± 1.45
Mg^{2+}	95.12 ± 0.51	β-ME (5%)	18.27 ± 0.56
Zn^{2+}	87.64 ± 2.03	Ethanol (5%)	93.48 ± 0.38
Ca^{2+}	86.92 ± 0.65	Methanol (5%)	104.85 ± 2.37
Pb^{2+}	92.83 ± 1.41	Isopropanol (5%)	96.60 ± 1.87

Data represent the means of three separate replications. CK: In the control group, metal ions and chemical reagents were substituted with equal amounts of Na_2HPO_4-citric acid buffer. ND means no activity was detected by the method used in this study.

enzymatic activity, which was also observed with other psychrophilic GH1 BGs, including EaBgl1A from *Exiguobacterium antarcticum* B7 (Crespim et al., 2016), BcBgl1A from *Bacillus cellulosilyticus* (Wu et al., 2018), and BglU from *Micrococcus antarcticus* (Miao et al., 2016). Thus, almost cold-adapted GH1 BGs were very sensitive to Cu^{2+}, while PgBgl1 exhibited higher tolerance to Cu^{2+} than the others.

The effects of detergents and other chemicals on PgBgl1 are also shown in Table 1. The chelating agent EDTA had no influence on enzyme activity, indicating that PgBgl1 is not a metalloprotein. In contrast, β-mercaptoethanol (β-ME) showed a significant effect on the enzymatic activity, retaining 18.3% activity at the tested concentrations, while SDS almost completely inhibited the activity of PgBgl1. This behavior is similar to that observed in BglMKg from the marine metagenomic library and BglU from *M. antarcticus* (Miao et al., 2016; Wierzbicka-Woś et al., 2013). However, SDS showed slightly inhibitory effects on the enzyme activity of BcBgl1A from *B. cellulosilyticus* (Wu et al., 2018), EaBgl1A from *E. antarcticum* B7 (Crespim et al., 2016), and MaGlu1A from *Microbulbifer* sp. ALW1 (Jiang et al., 2021). In addition, the impact of methanol and ethanol, common organic solvents, on PgBgl1 enzyme activity exhibited a general decrease (Fig. 5).

3.5. Determination of substrate specificities and kinetic parameters

The substrate specificity of PgBgl1 was examined by measuring its activity with synthetic and natural substrates (Table 2). Surprisingly, the substrate specificity of PgBgl1 towards *p*NPG was 1063.89 U/mg, which was higher than that of most reported bacterial GH1 BGs. It exhibited the highest relative activity for *p*NPG, and weak activity on *o*NPG, *p*NPX, and cellobiose, but no activity towards *p*NPgal, *p*NPM, and gentiobiose was observed. These results show that PgBgl1 has a preference for highly degrading aryl β-glycosidic compounds. Therefore, PgBgl1 can be classified as an aryl-glucosidase (Godse et al., 2021).

Using Lineweaver–Burk plots, the kinetic parameters of PgBgl1 were calculated using *p*NPG as the substrate. The K_m, V_{max}, and K_{cat}/K_m values of PgBgl1 were 0.36 mM, 1208.31 U/mg, and 3871.92/s. PgBgl1 exhibited a high affinity for *p*NPG compared to most psychrophilic GH1 BGs, which have the K_m range of 0.1–3.29 mM (Bhatia et al., 2002). In addition, the K_{cat} of PgBgl1 was higher than that of most other GH1 BGs. These results indicate that PgBgl1 has high substrate affinity and catalytic efficiency in the hydrolysis of substrates.

3.6. Sugars and NaCl on PgBgl1 activity

To test the effect of sugars, including glucose, cellobiose, D-xylose, D-galactose, and D-sucrose, enzyme activity was analyzed by measuring it at different concentrations up to the solubility limits of the sugars. Fig. 5 shows that even at a concentration of 1000 mM, not all sugars will degrade the enzyme activity. PgBgl1 retained 96.3% and 88.7% of its original activity in the presence of cellobiose and D-sucrose, respectively. While glucose, D-xylose, and D-galactose moderately stimulated PgBgl1 activity, a remarkable improvement (38.4%, 64.8% and 61.0%, respectively) in enzymatic activity was observed. Furthermore, in the presence of 500 mM glucose and D-xylose, the enzyme activity was activated 1.8- and 2.0-fold, respectively. These results indicate that glucose and D-xylose were the stronger activator, while all test sugars slightly inhibited the enzyme activity at high concentrations. Now,

Table 2
Substrate specificity of recombinant PgBgl1.

Substrate	Specific activity (U/mg)
Disaccharide	
Cellobiose	0.16 ± 0.0039
Sophorise	ND
Gentiobiose	ND
Aryl β-glycoside	
*p*NPG	1063.89 ± 20.17
*o*NPG	130.71 ± 4.12
*p*NPX	45.27 ± 12.79
*p*NPM	ND
*p*NPgal	ND

Data represent the means of three separate replications. ND means no activity was detected by the method used in this study.

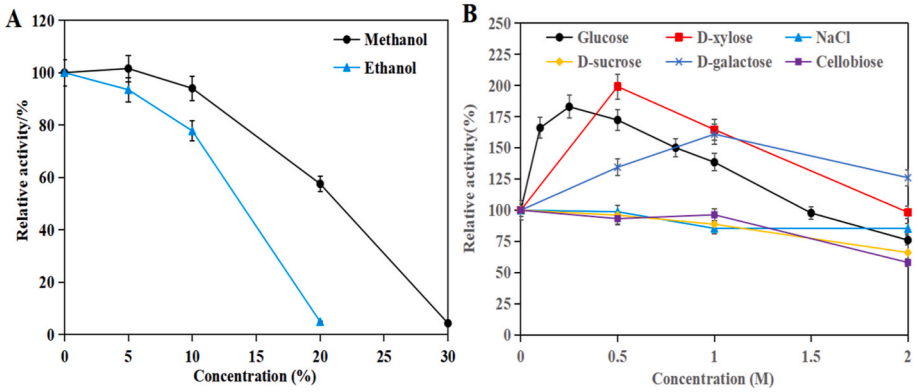

Fig. 5. Different concentrations of methanol, ethanol, NaCl, and sugars on the activity of PgBgl1. (A) Methanol and ethanol tolerance of recombinant PgBgl1. Activity was measured in the presence of 0–20% ethanol and 0–30% methanol. Activity with 0% ethanol or methanol was set as 100%. (B) NaCl and various sugars (glucose, cellobiose, D-xylose, D-galactose and D-sucrose) tolerance of recombinant PgBgl1. Activity was measured in the presence of 0–2 M NaCl or sugars. Activity with 0 M NaCl or sugars was set as 100%. Data represent the means of triplicate measurements and error bars represent standard deviation.

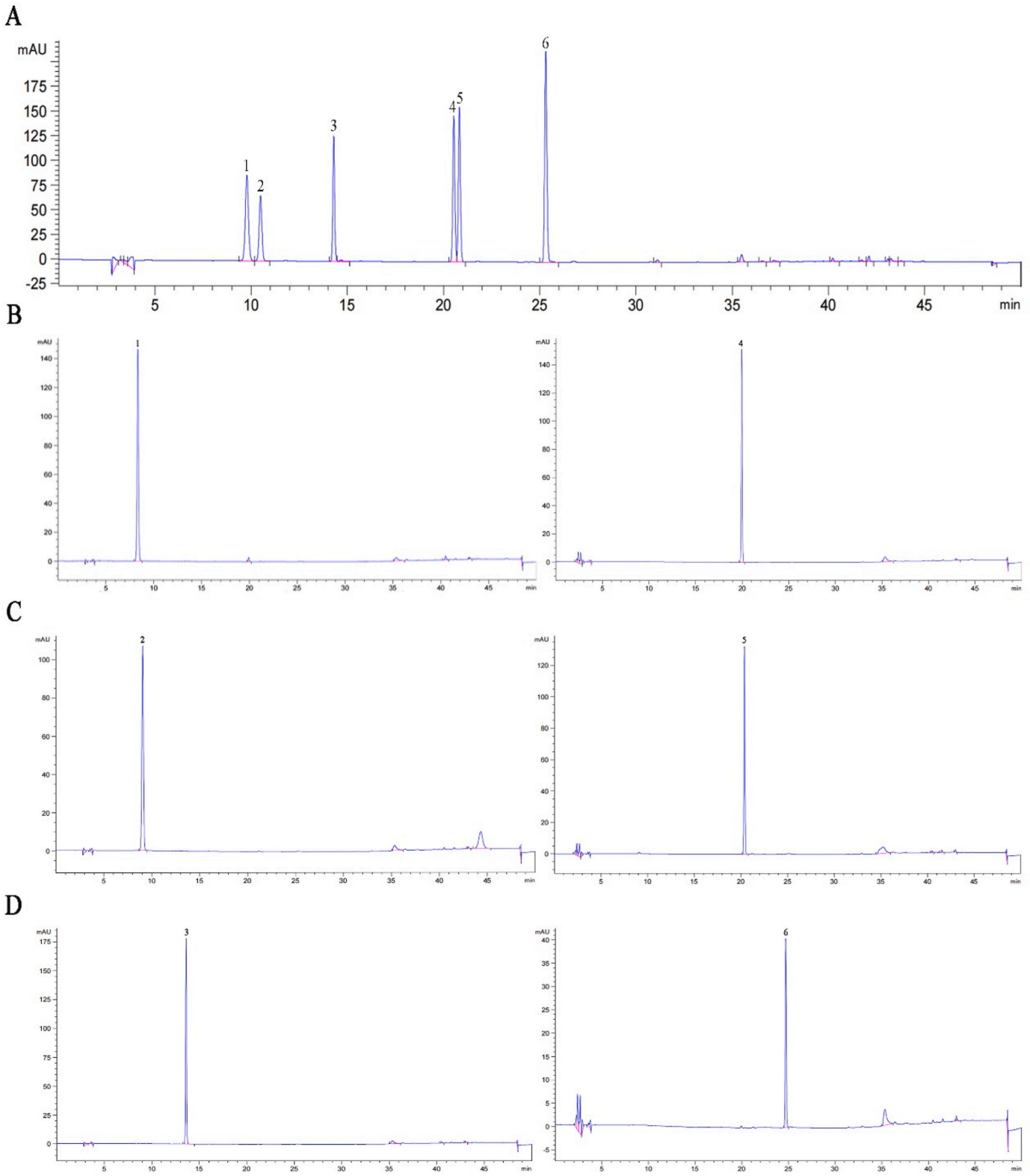

Fig. 6. HPLC of soybean isoflavone standards and PgBgl1 hydrolyzed soybean isoflavone glycosides. (A) HPLC profiles of daidzin, glycitin, genistin, daidzein, glycitein, and genistein. (B) HPLC detection of soybean isoflavone glycoside daidzin hydrolyzed by PsBgl1. (C) HPLC detection of soybean isoflavone glycoside glycitin hydrolyzed by PsBgl1. (D) HPLC detection of soybean isoflavone glycoside genistin hydrolyzed by PsBgl1. Notes: 1, daidzin (9.781 min); 2, glycitin (10.490 min); 3, genistin (14.298 min); 4, daidzein (20.531 min); 5, glycitein (20.821 min); 6, genistein (25.311 min).

several reports have examined β-glucosidase's tolerance and stimulation by glucose or xylose in the past decade (Godse et al., 2021; Meleiro et al., 2015; Xu et al., 2011). For example, GH1-1 (Meleiro et al., 2015) from *Neurospora crassa* showed a 1.8-fold and 2.0-fold stimulation in response to glucose (100 mM) and xylose (150 mM), respectively. The β-glucosidase Bhbgl (Xu et al., 2011) from *Bacillus halodurans* C-125 is also stimulated by sucrose, D-galactose, xylose, and glucose at concentrations ranging from 50 to 800 mM. The molecular mechanism of BGs stimulation involves an adjacent loop region assuming an extended conformation after interacting with sugars at substrate binding regions. This process increases the entrance to the active site, thereby enhancing product formation (Corrêa et al., 2021).

The effect of NaCl on various concentrations (0–2000 mM) was further investigated. As shown in Fig. 5, the enzyme retained up to 85% of its activity across all concentrations. Such salt tolerance of BGs has been reported to be based on the interplay of hydrophobic and electrostatic interactions between the protein and the salt solution (Godse et al., 2021; Qi et al., 2021). For example, the cold-adapted β-glucosidase Bgl (Sun et al., 2018) belonging to the GH1 family can tolerate 2 M NaCl. The enzyme activity of the halophilic β-glucosidase BGL2 from *Aspergillus niger* is increased by 44% in the presence of 4 M NaCl (Cai et al., 2019). The result, according to PgBgl1, was cloned from *P. glaciei* sp. PB01, which was isolated from an Antarctic iceberg in a high-salt environment. Thus, the simultaneous enhancement of sugar and high NaCl tolerance in PgBgl1 makes it a promising candidate for a biocatalyst in sugar fermentation.

3.7. Hydrolysis products of soybean isoflavone glycosides generated by PgBgl1

Soybean isoflavones, as a class of bioactive compounds, are abundant secondary metabolites produced during soybean growth. They play an important role in improving animal production performance and human health (Kim, 2021; Fujita et al., 2015; Abdella et al., 2018). Soy isoflavones exist in the form of aglycones (daidzein, genistein and glycitein) and β-glycoside conjugates, which include glycosides (daidzin, genistin, and glycitin), malonylglycosides, and acetylglycosides. The content of glycosides is high in soybeans, while the aglycones are found in trace quantities. Soybean isoflavone aglycones are more readily absorbed into the cells and have higher biological activity than glycosides. β-Glucosidase catalyzes the hydrolysis of the β-glycosidic linkage from the non-reducing end of isoflavone glucosides, disaccharides, oligosaccharides, aryl-glucosides, and alkyl-glucosides (Singh et al., 2016). In addition, the enzymatic hydrolysis of β-glucosidase to prepare soybean isoflavone glycosides is an important application with high commercial value (Li et al., 2012). This process has been investigated by directly using of β-glucosidases from leguminous plants and microorganisms (Kaya et al., 2008; Cao et al., 2018; Li et al., 2018; Abdella et al., 2018). However, in the industrial production of isoflavones, the low solubility of isoflavones and the poor stability of β-glucosidase, product inhibition, limited enzymatic hydrolysis efficiency, and other scientific problems reduce the conversion efficiency of isoflavone aglycones (Godse et al., 2021; Ouyang et al., 2023; Jin et al., 2023). Therefore, the hydrolysis rate of soybean isoflavones by recombinant β-glucosidase PgBgl1 was determined using soybean isoflavone glycosides as substrates. As shown in Fig. 6, PgBgl1 exhibited the highest hydrolysis activity for soybean isoflavone glycosides after treatment at 40 °C for 15 min, with the hydrolysis rate could reaching nearly 100%. It was found that low-temperature enzymes were more sensitive to the organic solvent methanol reaction than high-temperature enzymes under low-temperature conditions (Li et al., 2020; Huang et al., 2020). By reducing the methanol concentration to 25% or less in the PgBgl1 hydrolysis system, the negative effects of methanol on PgBgl1 were quickly minimized. The high-efficiency hydrolysis ability of PgBgl1 at low temperatures and short durations against soybean isoflavone glycosides could enhance the health benefits of soybean products.

4. Conclusions

This study demonstrated the expression and characterization of a GH1 β-glucosidase (PgBgl1) from *P. glaciei*. The enzyme was robust under both cold and neutral conditions. PgBgl1 was found to be a salt-tolerant and monosaccharide-stimulated β-glucosidase. For all we know, PgBgl1 was the first GH1 β-glucosidase from the genus *Psychrobacillus* that functions with high specific activity and catalytic efficiency for aryl β-glycosides, which had a high ability to hydrolyze isoflavone glucosides. Moreover, PgBgl1 can be considered a promising candidate for enhancing conventional commercial enzymes in the food industry.

Ethical approval

This article does not involve any studies with human participants or animals conducted by any of the authors.

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CRediT authorship contribution statement

Jinjian He: Conceptualization, Investigation, Methodology, Software, Visualization, and, Writing – original draft, preparation, and. **Jiajing Duan:** Conceptualization, Investigation, Methodology, Software, Visualization, and, Writing – original draft, preparation. **Pinglian Yu:** Investigation, Formal analysis, and, Visualization. **Yuying Li:** Conceptualization, Validation, Writing – review & editing, Supervision, and, Funding acquisition, and. **Mansheng Wang:** Investigation, Formal analysis, and, Visualization, and. **Xiu Zhang:** Investigation, Formal analysis, and, Visualization. **Zishu Chen:** Writing – review & editing, All authors have read and agreed to the published version of the manuscript. **Pengjun Shi:** Conceptualization, Validation, Writing – review & editing, Supervision, and, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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