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Original Article

Characterization of an extracellular β-glucosidase from *Dekkera bruxellensis* for resveratrol production

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ABSTRACT

Polygonum cuspidatum is a widely grown crop with a rich source of polydatin (also called piceid) for resveratrol production. Resveratrol is produced from piceid via enzymatic cleavage of the sugar moiety of piceid. In this study, *Dekkera bruxellensis* mutants were selected based on their high *p*-nitrophenyl-β-D-glucopyranoside and piceid conversion activities. The enzyme responsible for piceid conversion was a heterodimeric protein complex that was predominantly secreted to the extracellular medium and consisted of two subunits at an equal ratio with molecular masses of 30.5 kDa and 48.3 kDa. The two subunits were identified as SCW4p and glucan-β-glucosidase precursor in *D. bruxellensis*. Both proteins were individually expressed in *Saccharomyces cerevisiae exg1Δ* mutants, which lack extracellular β-glucosidase activity, to confirm each protein's enzymatic activities. Only the glucan-β-glucosidase precursor was shown to be a secretory protein with piceid deglycosylation activity. Our pilot experiments of piceid bioconversion demonstrate the possible industrial applications for this glucan-β-glucosidase precursor in the future.

1. Introduction

Resveratrol (3,5,4′-trihydroxystilbene), a polyphenolic phytoalexin primarily produced for the defense of certain plants against environmental stressors [1], was first isolated and identified by Takaoka [2] in 1940 from the root of white hellebore (*Veratrum grandiflorum* O. Loes). Later, resveratrol was also found in a traditional Chinese herb, *Polygonum cuspidatum*, and other plants, including grapes, peanuts, and cocoa [1,3–5]. Resveratrol has various bioactive properties, including

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antimicrobial, antiviral, antioxidant, and antiaging effects, and is also used to for relieving menopausal disorders [6–12]. However, the use of resveratrol is hindered as resveratrol is present in glycosylated form, also called piceid (polydatin; resveratrol-3-O-β-D-glucoside), in most plants [13,14]. The glycosylated resveratrol concentrations can be considerably influenced by environmental conditions, including exposure to fungal infections or planting conditions, which can limit the conversion of piceid to resveratrol. Recently, resveratrol was directly produced in yeast through integration of resveratrol biosynthesis genes. However, the production of resveratrol from bioengineered yeasts needs to be further optimized to generate sufficient levels of resveratrol for industrial uses [15]. Alternatively, identifying plants that contain high concentrations of piceid or microbes with efficient piceid conversion can be a valuable approach for the production of resveratrol.

Enzymes such as β-glucosidase can also be used to hydrolyze glucose from various aglycone structures to enhance the pharmacological effects of multiple chemical compounds, including resveratrol, norisoprenoids, terpenes, and volatile phenols [16–18]. Previously, extracellular protein concentrates from *Aspergillus oryzae* containing piceid-β-D-glucosidase showed exceptionally good piceid transformation properties by hydrolyzing the β-(1→3)-D-glucopyranoside of piceid to produce resveratrol under optimal conditions [19]. However, microbial enzyme reaction is different from microbial fermentation. Thus, an alternative way to produce resveratrol can be achieved through microbial fermentation.

P. cuspidatum has a high content of piceid (approximately 1–2% of the plants), making it a valuable source of resveratrol through the β-glucosidase reaction [20]. In this study, we obtained a wine yeast mutant derived from *Dekkera bruxellensis* that exhibited high β-glucosidase activity and was able to convert piceid from *P. cuspidatum* into resveratrol. We characterized the properties of the isolated extracellular heterodimeric β-glucosidase. Furthermore, we tested this identified mutant in a large-scale fermentation process to assess possible commercial resveratrol production.

2. Methods

2.1. Strains and chemicals

D. bruxellensis (BCRC920084) and other microbial strains were obtained from the Bioresource Collection and Research Center, Hsin-Chu, Taiwan. Standard compounds, including piceid, resveratrol, and β-glucosidase (almonds), were obtained from Sigma-Aldrich (BD, Sparks, MD, USA). Yeast extract peptone dextrose medium (YPD) and yeast extract medium (YE) broth and other culture media were purchased from Becton Dickinson.

2.2. Strain cultivation and culture medium

YPD, YE, and Vogel's minimum salt (VMS) media with/without additional nitrogen sources, including KNO_3, NH_4NO_3, NH_4Cl, or $(NH_4)_2SO_4$, were used to test the optimum growing conditions for high β-glucosidase activity. Overnight, yeast cultures were inoculated and incubated at 25°C with shaking at 100 rpm for 72 hours. Aliquots of the cell cultures were collected and centrifuged during fermentation to obtain the supernatants for β-glucosidase activity analysis. The medium that sustained the highest β-glucosidase activity during fermentation was selected as the base medium for piceid bioconversion.

2.3. Acidic, alkaline, and enzymatic hydrolysis of P. cuspidatum

P. cuspidatum roots were dried and ground into powder. *P. cuspidatum* powder (1 g) was mixed with 20 mL of H_2SO_4 (1% w/w) solution and autoclaved for 20 minutes for an acid hydrolysis test. Alkaline hydrolysis was performed by mixing 1 g *P. cuspidatum* powder with 20 mL of NaOH (0.2% w/w) with vigorous shaking at 100 rpm for 1 hour. Both acidic- and alkaline-treated samples were adjusted to pH 7 prior to extraction and high performance liquid chromatography analysis [20]. One hundred units of commercial almond β-glucosidase were added to 1 g *P. cuspidatum* powder dispersed in 20 mL of acetate buffer solution (20mM, pH 6) and incubated at 50°C for 12 hours. One volume of absolute ethanol was added to stop the reaction.

2.4. Piceid and resveratrol analysis

Authentic piceid and resveratrol were prepared at different concentrations (0.5 mg/L, 5 mg/L, 25 mg/L, 75 mg/L, 100 mg/L, 150 mg/L, and 175 mg/L) in 50% ethanol to generate a standard curve. Samples collected from fermentation were freeze-dried, and piceid and resveratrol were extracted with 50% ethanol at 70°C for 30 minute. The extracted samples were diluted and filtered with a 0.22-μm membrane. A C_{18} column (5 μm, 4.6 mm × 250 mm; Waters, Milford, MA, USA) was used to separate the compounds and detect signals at 307 nm. The resveratrol and piceid concentrations were calculated using the peak area relative to the standard curves. A multistep gradient protocol was applied using mixed mobile phases of water with 1% acetic acid (solvent A) and acetonitrile (solvent B) at a flow rate of 1.5 mL/min. The mobile phase started with 5% of B and gradually increased to 55% at 25.3 minutes and reached 100% from 25.3 minutes to 31 minutes [21].

2.5. β-Glucosidase activities

One milliliter of the supernatants was mixed with 1 mL *p*-nitrophenyl-β-D-glucoside (*p*NPG; 10mM in acetate buffer solution, pH 5) at 50°C for 30 minutes and deactivated at 100°C for 5 minutes. Equal amounts of supernatants without the addition of *p*NPG were used as a blank control. The glucosidase activity was determined by measuring the levels of *p*-nitrophenol (*p*NP) released from *p*NPG after the glucose was hydrolyzed by glucosidase. The released *p*NP was measured at a wavelength of 405 nm and calibrated with the *p*NP standard curve [22].

2.6. β-Glucosidase purification

Proteins from the *D. bruxellensis* supernatants were separated by centrifugation. Ammonium sulfate was slowly added to the supernatants until it reached a final concentration of 80%. The

supernatants were incubated on ice for 1 hour. Then, the precipitated proteins were collected after centrifugation at 8000 rpm at 4°C for 30 minutes and redissolved in 20mM acetate buffer (pH 6.0). Desalting was performed using 3-kDa molecular weight-cutoff (MWCO) dialysis membranes (Orange Scientific, Braine-l'Alleud, Belgium). After desalting, the proteins were purified using the Rotofor system (Bio-Rad, Hercules, CA, USA), which separated the proteins into 20 fractions based on isoelectrical point values. Then, each fraction was individually tested for β-glucosidase activity. Fractions with high β-glucosidase activity that were collected from the Rotofor system were further purified using DEAE Sepharose (GE, Marlborough, MA, USA) (1.6 × 10 cm, GE, HiPrep DEAE FF 16/10) ion exchange chromatography. The chromatography was conducted using 20mM of phosphate buffer (pH 6) and 2M of NaC1 dissolved in 20mM phosphate buffer (pH 6) with a linear gradient at a rate of 3 mL/min. After ion exchange chromatography, gel filtration was applied using a 16/600 Superdex 75 column (GE, Marlborough, MA, USA) with 0.15M NaCl (dissolved in 0.05M NaH_2PO_4/Na_2HPO_4, pH 7.2) at a rate of 0.3 mL/min. The purified proteins were desalted using dialysis membranes. Purified proteins were loaded to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Protein sequences were further analyzed by liquid chromatography tandem mass spectrometry [23]. Sequencing data were analyzed using MASCOT software (http://www.matrixscience.com/).

2.7. Effects of temperature and pH on β-glucosidase activities

Optimum temperature and thermal stability of the purified β-glucosidase activities were determined between 20°C and 90°C in 20mM of citrate phosphate buffer at pH 5 for 30 minutes and 60 minutes, respectively. Optimum pH and pH stability tests were performed at 60°C and 4°C between pH 3–8 for 1 hour. The release of *p*NP was measured at a wavelength of 405 nm [22].

2.8. β-Glucosidase kinetics and substrate specificity tests

Substrate specificities of the enzymes were measured using 10mM *p*NPG or piceid (Table S1). Kinetic parameters were tested using piceid (0.25–8mM) at pH 5 at 60°C. Michaelis constant (K_m) and maximum velocity (V_{max}) were calculated using Lineweaver–Burk double reciprocal plots (Table S1).

2.9. Construction of protein expression vectors and yeast expression

Gateway cloning technology (Invitrogen, Carlsbad, CA, USA) was used to construct the expression vectors according to the product manual. Briefly, genomic DNA from *D. bruxellensis* containing the gene sequences of the glucan-β-glucosidase precursor and Scw4p were used as templates for the first round of polymerase chain reaction (PCR). PCR amplification was conducted using the following primers: attB1-glucan-β-glucosidase precursor: 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCACCATGAAGTTTATTTTATTGTC-3′ (forward) and attB2-glucan-β-glucosidase precursor: 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCGAAGCTACACTGGTTAGG-3′ (reverse); attB1-Scw4p: 5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCAC CATGATGTTCACAGCTGTTC-3′ (forward) and attB2-Scw4p: 5′-GGGGACCACTTTGTACAAGAAAGCTGGGTCTAGCATTCCCCAGTACTGC-3′ (reverse). The PCR products were purified and mixed with the pDONR 221 vectors with BP clonase to generate the entry clone containing the target gene in the correct cloning sites. The resulting entry clone were transformed into DH5α *Escherichia coli* and then submitted for sequencing. The correct entry clone was then used, together with the pYES-DEST52 vector, to generate the expression clone as pEXPR-glucan-β-glucosidase precursor and pEXPR-Scw4p, which encoded proteins with a V5 antigen epitope and a 6× His tag. Finally, *Saccharomyces cerevisiae EXG1* defective (*exg1Δ*) strains were transformed with the pEXPR-glucan-β-glucosidase precursor or pEXPR-Scw4p using a standard lithium acetate protocol [24].

Cells containing protein expression vectors were inoculated overnight in SC-Ura^- media with 2% glucose. Overnight cultures were refreshed with 2% raffinose to deplete glucose in the cells. Galactose was then added to induce protein expression over a period of 9 hours. Total proteins were then extracted for Western blotting assays to confirm the expression of the proteins. His tag monoclonal antibodies from mouse (GE, USA) were used to identify the target proteins with a His_6 epitope by Western blotting.

3. Results

3.1. Microbial selection and piceid conversion analysis

We first screened and selected strains with high β-glucosidase activities to convert piceid in *P. cuspidatum* extracts into resveratrol from a pool of microbes, including bacteria, molds, and yeasts, from the Bioresouce Collection Research Center (No. 331, Shih-Pin Rd., Hsinchu, Taiwan). Interestingly, we found that *D. bruxellensis* exhibited the highest piceid conversion activity among all the selected microbes (data not shown). *D. bruxellensis* underwent *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG) mutagenesis. Twelve mutants out of 226 isolates had high β-glucosidase activities. Mutant no. 72 was finally selected for piceid conversion and for the following enzyme characterization studies (Figure S1).

Conversion can be achieved by acidic or alkaline hydrolysis. However, chemical reactions often result in low efficiency or yields. Thus, we compared various methods for piceid conversion using *P. cuspidatum* extracts. Our results demonstrated that acidic hydrolysis converted approximately 40% of the piceid into resveratrol (equal to 6 mg/g), whereas alkaline hydrolysis degraded resveratrol in the *P. cuspidatum* extracts (Figure 1A). Meanwhile, our *D. bruxellensis* mutant and the commercial β-glucosidase extracted from almonds converted substantial amounts of piceid and produced 10.8 mg/g and 10.5 mg/g of resveratrol, respectively (Figure 1A). The results indicate that acidic hydrolysis could remove the glucose moiety from piceid, but it had a low efficiency. By contrast, resveratrol was unstable under alkaline condition, which was consistent with previous studies showing that resveratrol started to degrade above pH 6.8 [25].

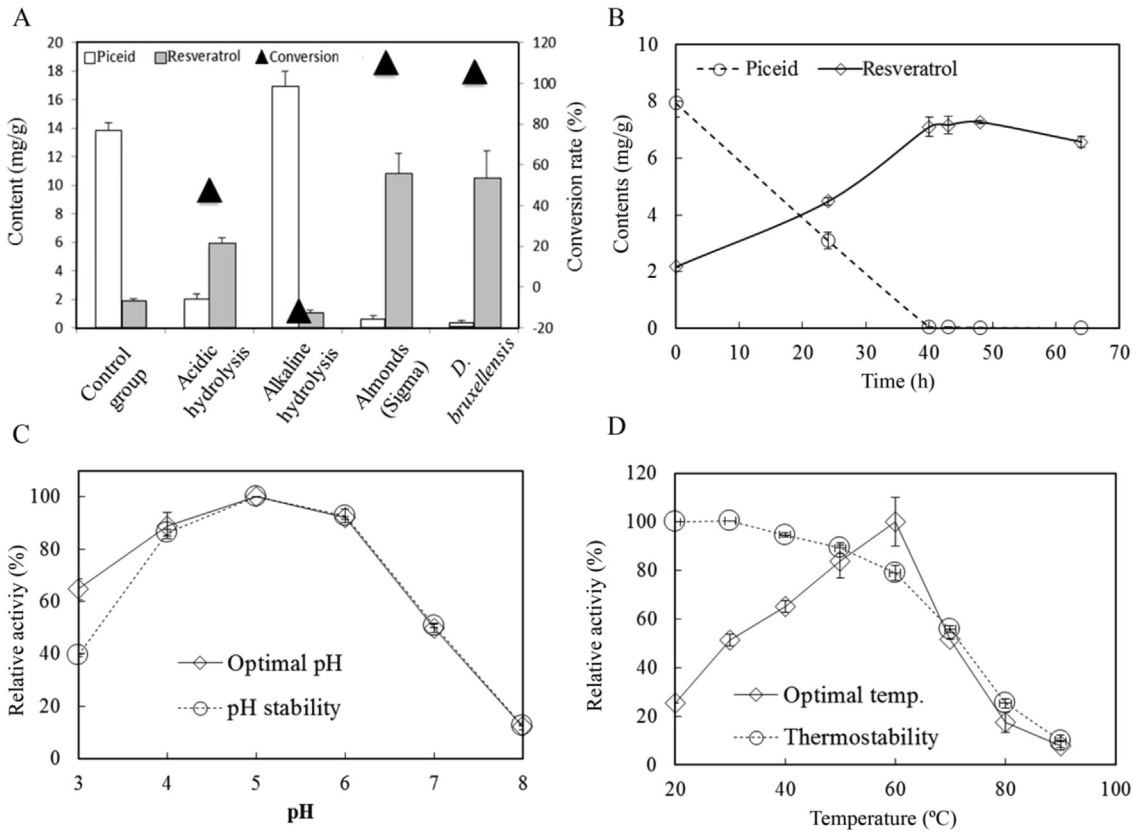

Figure 1 – Whole cell conversion of piceid from *Polygonum cuspidatum* extracts to resveratrol and the effects of pH and temperature on the activities of the purified enzyme. (A) Comparisons of different methods, including acidic, alkaline, and enzymatic hydrolysis, for piceid conversion and whole cell fermentation of *Dekkera bruxellensis*. White bars represent the piceid concentration; gray bars represent the resveratrol concentration. Black triangle represents the piceid conversion rates. (B) Changes in piceid and resveratrol concentrations during the fermentation of 5% (w/w) *P. cuspidatum* extracts and 1% (w/w) *D. bruxellensis* with shaking at 100 rpm at 25°C. The concentrations represent mg of compounds per gram of freeze-dried powder from the culture. (C) Effects of pH levels on the β-glucosidase activity of the purified enzymes. The solid line indicates the optimum pH level, and the dotted line indicates the pH stability. (D) Effects of temperature on the β-glucosidase activity of the purified enzymes. The solid line and the dotted line indicate the optimum temperature and the thermal stability of the purified enzyme, respectively.

To determine the optimal culture conditions for *D. bruxellensis* with high enzymatic activity, we tested various media, including YE and VMS, and YPD was used as a control. Media containing additional nitrogen sources, including KNO_3, NH_4NO_3, NH_4Cl, or $(NH_4)_2SO_4$ in VMS, were also tested. The results showed that inorganic nitrogen, NH_4C1 (0.3 g/L), in the VMS medium strongly increased β-glucosidase activity (22 U/ml) at the 72-hour cultivation time point (Figure S2). Therefore, we further cultured *D. bruxellensis* using VMS medium containing 0.3 g/L of NH_4C1 with the addition of 5% (w/w) of *P. cuspidatum* extracts. Our results showed that, under these culturing conditions, the cells completely converted piceid into resveratrol in 40 hours (Figure 1B).

3.2. *Purification and characterization of the enzyme responsible for piceid conversion*

To identify the specific β-glucosidase responsible for piceid bioconversion, we purified proteins from the supernatants of *D. bruxellensis* cultures. The concentrated supernatants were dialyzed and desalted following the separation of proteins based on their isoelectric points using a Rotofor protein separation system. Twenty fractions were collected, depending on the pH levels, and each fraction was assayed for β-glucosidase activity. Fractions 4, 5, and 6 (with pH values of 3.62, 3.95, and 4.22, respectively) showed significant β-glucosidase activity. This indicates that the target protein is present in these three fractions, with a protein p*I* ranging from 3.62 to 4.22 (Figure S3A). The three fractions were combined and further purified using ion exchange chromatography. Fractions 1–4, eluted with 0.8M salt, showed the highest β-glucosidase activity (Figure S3B). Therefore, these fractions were combined and loaded onto a size exclusion separation system to obtain the target protein. Three major peaks containing proteins were detected by a UV monitor at 280 nm. However, only the proteins in fractions 19–21, indicated as peak 3, showed *p*NPG conversion activity (Figure S3C, right panel). After the purification, the protein yield was 34.62% from the

starting crude extracts (Table S2), which resulted in a higher specific β-glucosidase activity (92.89 U/mg) compared to that of the crude extracts (1.05 U/mg). In addition, we also determined the optimal pH levels and the stability of the purified protein. The results showed that the maximal enzymatic activity and the most stable condition for the enzyme reaction was at pH 5 (Figure 1C). In addition, the enzyme had optimal activity at 60°C and still retained 90% of the original activity after 1 hour of incubation at 50°C (Figure 1D).

We further investigated the effects of metal ions and chemicals on the activity of the purified heterodimeric enzyme, as metal ions are often enzyme cofactors. Enzyme reactions were carried out under 60°C at pH 5 for 30 minutes using *p*NPG as a substrate. The release of *p*NP was measured as described in the Methods section. The activity in the presence of ions and chemicals was expressed as a percentage relative to the control. Results showed that enzyme activity was not affected by different concentrations (1mM or 10mM) of various metal ions, including Li^+, Na^+, K^+, Mg^{2+}, Ca^{2+}, and Mn^{2+} (Table 1); however, enzyme activity was significantly inhibited by the addition of 10mM of Fe^{3+} and Cu^{2+}. The presence of Hg^{2+}, which specifically binds to the sulfhydryl groups of enzymes, caused the complete loss of enzyme activity. The chelating agent EDTA did not affect the enzymatic activity, suggesting that the metal ions are not essential for the function of this enzyme. Furthermore, the SDS detergent (10mM) led to a marked inhibition (34%) of enzyme activity, but β-mercaptoethanol (β-ME), which reduces disulfide bonds, did not affect enzyme activity (Table 1).

The substrate preferences of the purified protein compared to those of the β-glucosidase from almond extract were tested using 10mM of *p*NP or *o*NP glycosides in the α- and β-linkage configurations. The enzymatic reactions were carried out at 60°C and pH 5 for 30 minutes. Relative activity was calculated based on the release of *p*NP from *p*-nitrophenol-β-D-glucopyranoside. Both enzymes did not react with α-glycosides, but showed extremely different substrate specificities, especially for *o*NP-β-D-glucopyranoside (Table 2). This was also observed in the specific piceid conversion activity (Table S1). Specifically, our purified protein had a 2-fold higher specific activity than β-glucosidase of almonds in piceid conversion. In addition, the purified proteins showed no activity toward α-glycosides, but most glucosidases from *S. cerevisiae* had at least 25% of the relative activity toward the β-glycosides [26].

Table 1 – Effects of metal ions and chemical agents on the relative activity of purified protein complex.

Metal ion & reagent	Relative activity ± SD (%)	
	1mM	10mM
Control	100.00 ± 7.72	100.00 ± 7.72
$LiCl_2$	92.93 ± 2.70	97.74 ± 1.69
NaCl	93.48 ± 4.36	96.41 ± 7.65
KCl	96.13 ± 2.45	99.01 ± 4.71
$MgCl_2$	98.35 ± 1.99	101.15 ± 8.80
$CaCl_2$	91.79 ± 1.88	100.12 ± 5.70
$MnCl_2$	95.20 ± 5.96	92.17 ± 3.66
$FeCl_3$	96.76 ± 5.60	78.71 ± 3.66*
$CuCl_2$	99.90 ± 1.59	89.10 ± 2.17*
$HgCl_2$	ND	ND
EDTA	86.96 ± 10.05	91.85 ± 1.60
SDS	73.10 ± 1.99 #	34.09 ± 1.90*
β-Mercaptoethanol	89.64 ± 6.97	96.63 ± 9.21

*Significant difference between metal ions/chemicals and control group analyzed by Student *t* test ($p < 0.05$).
ND = not determined; SD = standard deviation; SDS = sodium dodecyl sulfate.

Table 2 – Relative activity of purified protein complex and β-glucosidase (from almond) toward chromogenic substrates as measured by *o*NP or *p*NP release at 50°C at pH 5.

Substrate	Relative activity ± SD (%)	
	Purified protein	Almond β-glucosidase
*p*NP-α-D-galactopyranoside	ND	ND
*p*NP-β-D-galactopyranoside	5.74 ± 0.40	50.66 ± 1.98
*p*NP-α-D-mannopyranoside	ND	ND
*o*NP-β-D-glucopyranoside	260.57 ± 7.29	32.69 ± 3.56
*p*NP-α-D-glucopyranoside	ND	ND

ND = not determined; *o*NP = *o*-nitrophenol; *p*NP = *p*-nitrophenol; SD = standard deviation.

We further determined whether the isolated proteins formed a complex with other proteins, and thus SDS treatment may disrupt the protein complex and result in low β-glucosidase activities. Our purified proteins were subject to native- and SDS-PAGE analysis. The results showed a single band at approximately 83 kDa in the native gel, whereas 30 kDa and 50 kDa proteins were detected in SDS-PAGE gels (Figure 2A and Figure S3D). The results indicated that the proteins were composed of two different subunits. The amino acid sequences of the two subunits were further analyzed by matrix assisted laser deposition/ionization time-of-flight (MALDI-TOF)/TOF-mass spectrometry (MS). The results showed that the large and small subunits had protein sequence coverage of 42% and 43% relative to the glucan-β-glucosidase precursor (gi 385301784) and Scw4p (gi 385304065) in *D. bruxellensis*, respectively (Figure 2B).

3.3. *Overexpression and characterization of the two subunit proteins in S. cerevisiae exg1Δ mutants*

To determine which protein subunit had the deglycosylation activity, the DNA coding sequence of each subunit was analyzed and separately cloned into a yeast expression vector, pYES-DEST52, under the control of the galactose (GAL) promoter. The vectors were transformed into *S. cerevisiae exg1Δ* mutants, which had no extracellular β-glucosidase activity [22,27]. Western blotting analysis of the total proteins extracted from the transformants showed that both subunits were successfully expressed during the 9-hour galactose induction (Figure 3A). The glucan-β-glucosidase precursor with a His_6 tag had a molecular mass of 55 kDa and was predominantly distributed in the extracellular region, whereas Scw4p was mainly detected intracellularly with a molecular weight of 35 kDa (Figure 3A).

The expressed proteins were further purified by a Ni-agarose column and were verified by Western blotting using anti-His antibodies. Our results confirmed that the molecular

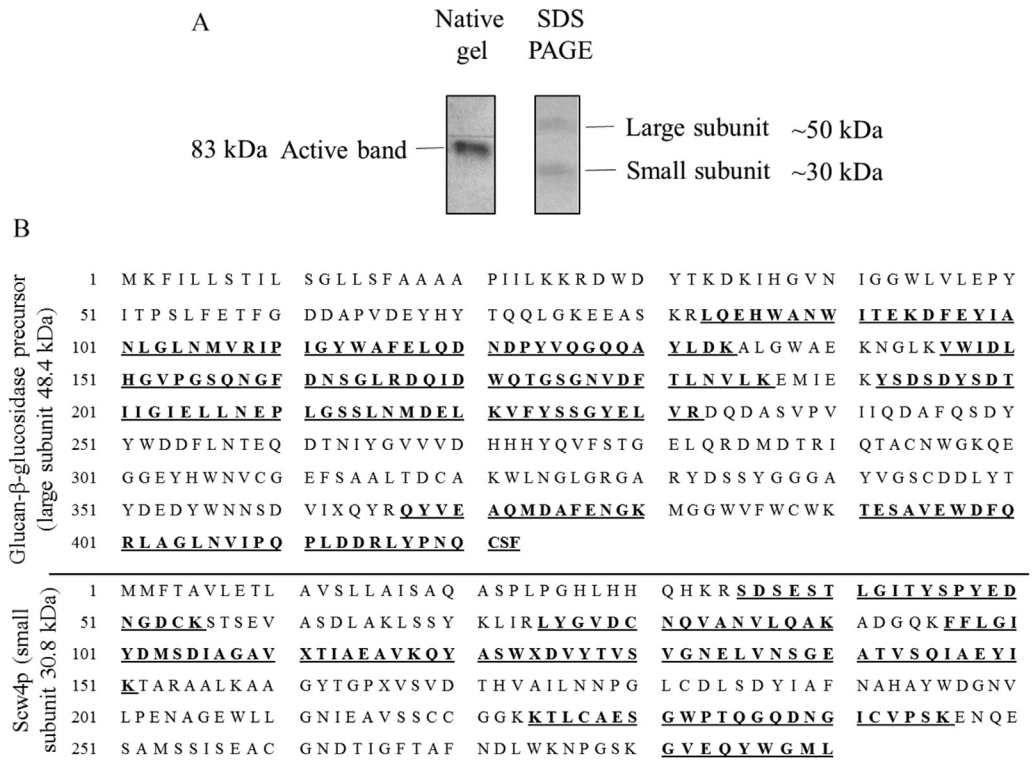

Figure 2 – Electrophoretic and protein sequence coverage analysis of the purified protein. (A) Native- and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) results. Purified proteins with β-glucosidase activity consist of large and small subunits. (B) Protein sequences analyzed by matrix assisted laser deposition/ionization time-of-flight (MALDI-TOF)/TOF-mass spectrometry (MS) revealed that the large and small subunits had high similarity to the glucan-β-glucosidase precursor (42%) and Scw4p (43%) sequences in *D. bruxellensis*.

mass of the recombinant glucan-β-glucosidase precursor was approximately 55 kDa and that of the recombinant Scw4p was 35 kDa (Figure 3B).

3.4. *Deglycosylation activity of the recombinant proteins*

Briefly, 1 mg/mL of purified recombinant glucan-β-glucosidase precursor and Scw4p were added to the piceid extract (1%) in citric-phosphate buffer to test their respective conversion activities under standard enzymatic conditions, which we determined (50°C at pH 5). The results demonstrated that the recombinant glucan-β-glucosidase precursor successfully converted piceid into resveratrol [Figure 4A(c)] after a 24-hour incubation. However, Scw4p did not show any deglycosylation activity or piceid conversion [Figure 4A(d)]. These results indicate that the glucan-β-glucosidase precursor is the major enzyme, instead of Scw4p, that is responsible for the pieced conversion. Nevertheless, the interaction between the glucan-β-glucosidase precursor and Scw4p currently remains unclear.

3.5. *Pilot test of piceid bioconversion*

To test the possibility of production scale-up of resveratrol from piceid, we performed 5- and 250-L fermentations consisting of 5% w/w of *P. cuspidatum* extracts and 1% of cells in bioreactors. The piceid and resveratrol concentrations were monitored throughout the fermentation process for 52 hours, and the conversion rates in each bioreactor were calculated (Figure 4B). Compared to small-scale flask fermentation, 40 hours of fermentation in both scale-up bioreactors was sufficient to complete piceid bioconversion and resveratrol production. In addition, for 1 g piceid ground powder prior to fermentation, the piceid and resveratrol concentrations were approximately 8.08 mg/g and 1.88 mg/g, respectively. Thus, we could obtain approximately 12.5 kg of the product with ~1% resveratrol purity in a 250-L fermentation. Moreover, for industrialization purposes, a 1200-L bioreactor loaded with 5% w/w of ground powder (60 kg/1200 L) was tested. Although a longer lag time was observed, piceid bioconversion could be completed after 40 hours. The final resveratrol concentration after fermentation was approximately 9.6 mg/g (Figure 4B). Therefore, we obtained a total of 576 g resveratrol.

4. Discussion

In this study, we identified a novel extracellular glucan-β-glucosidase precursor from *D. bruxellensis* that is responsible for the bioconversion of piceid from the plant extracts of *P. cuspidatum* into resveratrol. Extracellular enzymes are often soluble and stable and can be used to easily achieve efficient substrate conversion in food fermentation systems [28]. Therefore, the enzyme we identified in *D. bruxellensis* may have potential industrial applications, particularly in the production of resveratrol.

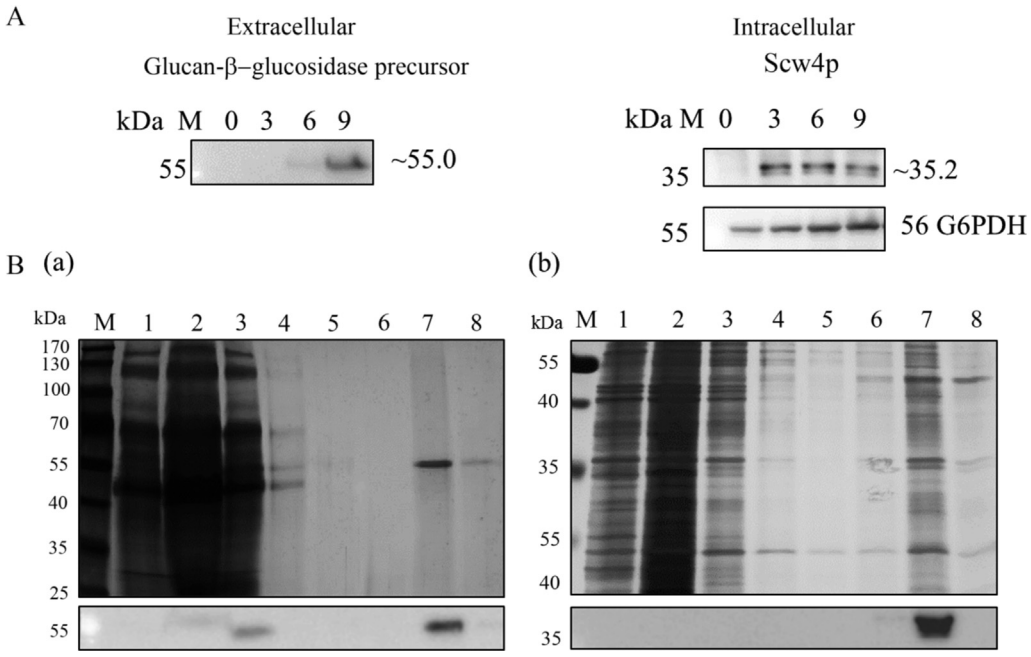

Figure 3 – Molecular cloning and purification of the glucan-β-glucosidase precursor and Scw4p. (A) Western blot analysis of the extracellular glucan-β-glucosidase precursor and intracellular Scw4p expressions in yeast transformants after 0 hour, 3 hours, 6 hours, and 9 hours of induction with galactose. (B) Purification of recombinant proteins with His tags by nickel columns. (a and b) The extracellular glucan-β-glucosidase precursor purification and the intracellular Scw4 purification, respectively. Each fraction collected from protein purification was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with silver staining (above) and Western blotting (bottom) using an anti-His antibody. Lane 1, flow through fraction; lanes 2–5, wash-step fractions; lanes 6–8, elution fractions from the protein purification process.

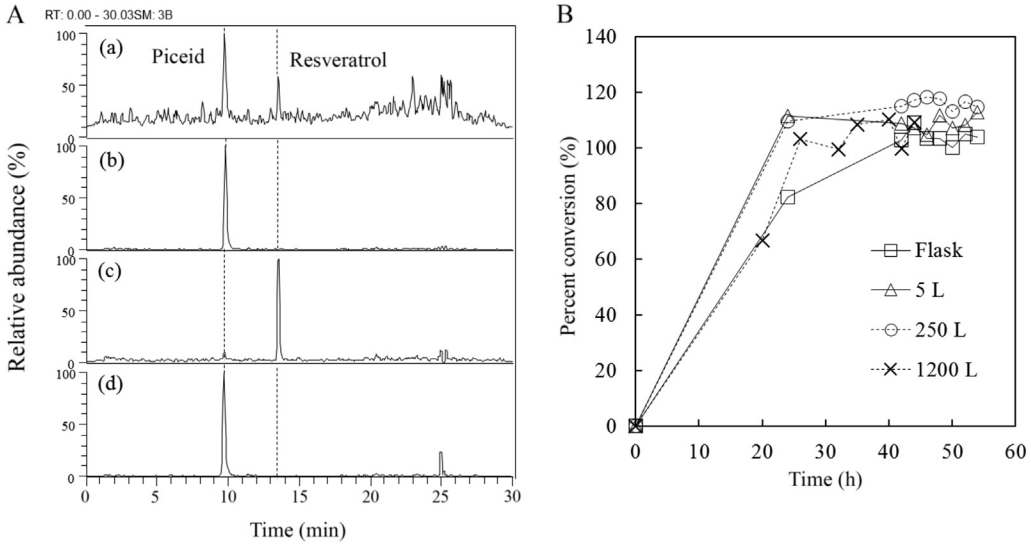

Figure 4 – Conversion of piceid by the purified recombinant glucan-β-glucosidase precursor and Scw4p and scale-up piceid biotransformation. (A) (a) The piceid and resveratrol standard peaks. (b) Nontransformed piceid. (c, d) Piceid and resveratrol concentrations after piceid was treated with the recombinant glucan-β-glucosidase precursor and recombinant Scw4p for 24 hours, respectively. (B) Conversion rates of piceid to resveratrol in flasks and in large fermentation bioreactors are shown at different time points.

Previously, Blondin et al [29] identified an intracellular β-glucosidase from *Dekkera intermedia* (today, it is called *D. bruxellensis* [30]) that could hydrolyze cellobiose into glucose. González-Pombo et al [18] isolated extracellular β-glucosidase from *Issatchenkia terricola* and showed that it can release monoterpenes and norisoprenoids and increase the aroma of wines [18]. Other research groups also demonstrated that yeasts isolated from grapes have β-glucosidase activities and

are able to transform glycosidic compounds for aroma enhancement during wine maturation [18,31]. Interestingly, our identified glucan-β-glucosidase precursor has higher piceid conversion activity than other β-glucosidases (Tables S1 and S3), and has a stronger preference for oNP-β-D-glucopyranoside than for pNP-β-D-glucopyranoside. We also demonstrated that the enzymatic activity of the glucan-β-glucosidase precursor was affected by Hg^{2+} but not by β-ME (Table 1), indicating that thiol groups, a covalent bond essential for the formation of heterodimeric proteins, are not involved in the catalytic site, but rather the sulfhydryl groups, which are essential for the catalytic activity.

Intriguingly, our purified β-glucosidase from *D. bruxellensis* is composed of two subunits, which were 48 kDa and 30 kDa in size (Figure 2A). However, several studies have indicated that β-glucosidases in other microbes typically exist as a monomer or a collection of identical monomers in dimer and tetramer complexes [32,33]. To find possible explanations and biological meanings for the binding of these two subunits, protein sequence analyses and sequence alignments were performed. The sequencing results indicated that the large and small subunits belonged to the GH1 and GH17 families. In addition, protein sequence alignments showed that the small subunit shared 59% and 54% similarities with the Scw4 and Scw10 of *S. cerevisiae*, respectively. The large subunit had 83% query coverage for Bgl2 of *S. cerevisiae*. Notably, Bgl2 is responsible for glucan degradation in the cell wall, whereas Scw4 and Scw10 encode transglycosylases and are responsible for cell wall assembly and remodeling [34]. Although the regulation of *SCW4*, *SCW10*, and *BGL2* expression and their levels are important for maintaining the cell wall stability, there is no indication that these proteins physically interact [34]. Thus, the functional protein complex containing two subunits in our initial discovery may warrant further investigation for a possible role in cell wall remodeling.

In this study, we isolated a *D. bruxellensis* mutant, and identified and characterized a novel β-glucosidase from *D. bruxellensis* for piceid bioconversion. Our pilot experiments of piceid bioconversion demonstrate possible industrial uses in the future. Thus, the mutant strain and the enzyme may be relevant for future applications in the production of bioactive compounds or in aroma development in the wine industry.

Conflicts of interest

All authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jfda.2016.12.016.

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