Cloning, expression, and characterization of the β-glucosidase hydrolyzing secoisolariciresinol diglucoside to secoisolariciresinol from *Bacteroides uniformis* ZL1

Y.-L. Tao · D.-H. Yang · Y.-T. Zhang · Y. Zhang · Z.-Q. Wang · Y.-S. Wang · S.-Q. Cai · S.-L. Liu

Abstract Previously, from the human intestinal flora we isolated the bacterial strain *Bacteroides uniformis* ZL1, which could convert secoisolariciresinol diglucoside (SDG) to its aglycone secoisolariciresinol (SECO) in vivo. In this study, 24 putative β-glucosidase genes were screened from the genome of *B. uniformis* ATCC 8492, which were used as templates to design PCR primers for the target genes in *B. uniformis* ZL1. Fifteen genes (*bgl1–bgl15*) were amplified from strain ZL1, and among them we identified *bgl8* as the gene encoding the SDG-hydrolyzing β-glucosidase. We sequenced the *bgl8* gene, cloned it into the expression vector and then transformed *Escherichia coli* to construct the recombinant bacteria that could synthesize the target β-glucosidase (BuBGL8). We purified and characterized BuBGL8, which showed maximal activity and stability under the culture conditions of pH 6.0 and 30 °C. SDG (2.0 mg/ml) was converted to SECO by both the purified BuBGL8 (0.035 mg/ml) and crude enzyme extract (0.23 mg crude protein/ml) with the efficiency of more than 90 % after 90 min at the reaction conditions. This is, to our knowledge, the first report of using recombinant bacteria to synthesize the SDG-hydrolyzing β-glucosidase, which could be used to produce SECO from SDG conveniently and highly efficiently.

Keywords *Bacteroides uniformis* · β-glucosidase · Secoisolariciresinol diglucoside · Secoisolariciresinol · Recombinant bacteria

Y.-L. Tao · D.-H. Yang (✉) · Y.-T. Zhang · Y. Zhang · S.-Q. Cai
State Key Laboratory of Natural and Biomimetic Drugs, School of Pharmaceutical Sciences, Peking University, Beijing 100191, China
e-mail: ydhui@bjmu.edu.cn

Z.-Q. Wang · Y.-S. Wang
National Key Laboratory of Biochemical Engineering, Chinese Academy of Sciences, Institute of Process Engineering, Beijing 100190, China

S.-L. Liu (✉)
Genomics Research Center, Harbin Medical University, Harbin 150081, China
e-mail: slliu@ucalgary.ca

S.-L. Liu
Department of Microbiology and Infectious Diseases, University of Calgary, Calgary, AB T2N 4N1, Canada

Introduction

Enterodiol (END) and enterolactone (ENL), known as mammalian lignans, are phytoestrogens that may be used for the prevention of menopausal syndrome, cardiovascular diseases, and cancers of the colon, breast, and prostate (Kitts et al. 1999; Meagher and Beecher 2000; Wang 2002). The human intestines contain bacteria that are capable of converting plant lignans, such as secoisolariciresinol diglucoside (SDG), to END and ENL (Clavel et al. 2006; Wang et al. 2000; 2010).

Secoisolariciresinol (SECO) was found to be the first intermediate in the biotransformation pathways from SDG to END and ENL (Raffaelli et al. 2002). To date, several bacterial strains capable of converting SDG to SECO have been reported, including *Bacteroides distasonis* DSM 20701[T], *Bacteroides fragilis* DIfE-05, *Clostridium cocleatum* DSM 1551[T], *Clostridium ramosum* DSM 1402[T] (Clavel et al. 2006), *Clostridium saccharogumia* (Clavel et al. 2007), and *Klebsiella* sp. (Wang et al. 2010). In our previous work, we isolated an active bacterial strain, *Bacteroides uniformis* ZL1, from human intestinal flora, which could effectively transform SDG in flaxseed to SECO, with a transformation efficiency of more than 80 % (Li et al. 2012). However, several factors limit the application of bacterial biotransformation in large scale, such as the low content of functional enzyme in the bacteria, which leads to the need of a huge system to meet the desired production. The anaerobic culture condition was also a serious

challenge as it will significantly increase the cost of production. Furthermore, the repeated subculture processes may reduce the activity of the bacteria and may even lead to their genetic changes (Iguchi et al. 2002).

To solve these problems, one effective way would be to identify, clone and express at high level the functional gene(s) involved in the hydrolysis of SDG in a bacterial host. Unlike the wild-type bacterial strain that can carry out the biotransformation but tend to lose the capability following repeated sub-culturing, the enzymes expressed and isolated through the gene engineering process would be able to convert the SDG to SECO more stably and effectively (Imanaka and Aiba 1981; Kirk et al. 2002). In addition, biotransformation with the cloned enzymes will be more efficient due to much higher substrate concentration tolerance, greater catalytic rate, and easier post-processing (de Carvalho 2011; Faber 2011).

Although there are reports about the isolation and characterization of SDG-β-D-glucosidases, such as that purified from *Aspergillus oryzae* (Zhang et al. 2009), the sequence of the enzyme has not been determined. The purpose of this study is to identify the β-glucosidase gene of *B. uniformis* ZL1 that is involved in SDG hydrolysis. We cloned and sequenced the SDG-hydrolyzing β-glucosidase gene and characterized it after expressing it in a prokaryotic host.

Materials and methods

Materials

The *pEASY*-E1 Expression Vector and PCR-related reagents were purchased from TransGen Biotech CO., Ltd (Beijing, China). TIANAmp bacteria DNA kit, TIANprep Mini Plasmid Kit and isopropyl β-D-1-thiogalactopyranoside (IPTG) were purchased from TianGen Biotech CO., Ltd (Beijing, China). Primers for PCR reaction were synthesized by Sangon Biotech Co., Ltd (Shanghai, China). 4-Nitrophenyl β-D-glucopyranoside (*p*NPG) and *p*-nitrophenol were purchased from Sigma-Aldrich (St. Louis, MO, USA). 5-bromo-4-chloro-3-indolyl glucopyranoside (X-Glu) was purchased from BioDee BioTech Co., Ltd (Beijing, China). SDG was prepared in our laboratory from defatted flaxseed with the purity of 96 % by HPLC analysis (Li et al. 2012). Crude SDG (50 %) was purchased from Baoji FangSheng Biotechnology Co., Ltd (Shanxi, China). The HPLC-grade acetonitrile and methanol were purchased from Merck KGaA Co., Ltd (Darmstadt, German), and the purified water was purchased from Hangzhou Wahaha Co., Ltd (Zhejiang, China).

Bacterial strains and culture condition

B. uniformis ZL1 (CGMCC No.4897) was isolated from the human intestine in our previous study (Li et al. 2012).

Escherichia coli strains *Trans*1-T1 and BL21(DE3)pLysS and ArtMedia Protein Expression medium were purchased from TransGen Biotech Co., Ltd (Beijing, China). *E. coli* strain Rosetta(DE3) was purchased from CoWin Bioscience Co., Ltd (Beijing, China). Anaerobic Broth medium base, Luria-Bertani (LB) nutrient agar and LB Broth medium base were purchased from Beijing Land Bridge Technology Co., Ltd (Beijing, China). Anaerobic Broth (g/L) consisted of peptone (15.0), yeast (5.0), soybean peptone (5.0), beef powder (5.0), glucose (5.0), sodium chloride (5.0), soluble starch (3.0), cysteine (0.5), potassium dihydrogen phosphate (2.5), hemin (0.005), and vitamin K1 (0.001).

B. uniformis ZL1 was cultured in Anaerobic Broth medium at 37 °C. *E. coli* BL21(DE3)pLysS cells were cultured in LB Broth media supplemented with 100 μg/ml ampicillin and 34 μg/ml chloramphenicol. *E. coli* *Trans*1-T1 and *E. coli* Rosetta(DE3) cells were cultured in LB Broth media containing 100 μg/ml ampicillin.

Cloning of the β-glucosidase gene from *B. uniformis* ZL1

The genome DNA of *B. uniformis* ZL1 was extracted using the TianAmp bacteria DNA kit according to the manufacturer's protocol. Primers were designed based on the sequences of 24 putative β-glucosidase genes screened in the genome of *B. uniformis* ATCC 8492. The PCR reaction mixture contained 12.5 μl 2× *TransTaq*™ High Fidelity (HiFi) PCR SuperMix, 1 μl genome DNA as template, 1 μl of each PCR primer and 9.5 μl deionized water. Thermal cycles included initial denaturation at 94 °C for 5 min, 30 cycles of 94 °C for 30 s, appropriate annealing temperature for 30 s and 72 °C for 2 min, and a final extension for 10 min at 72 °C. The amplified PCR products of gene *bgl1–bgl15* were cloned into the *pEASY*-E1 Expression vector according to the manufacturer's protocol, and then were used to transform *E. coli Trans*1-T1. The positive colonies were verified by colony PCR, by using the T7 promoter primer and each of the reverse primers of *bgl1–bgl15*. The recombinant plasmids (*pEASY*-BuBGL1–*pEASY*-BuBGL15) derived from these positive colonies were extracted using the TIANprep Mini Plasmid Kit and then sequenced by Beijing Genomics Institute (China).

Screening for the SDG-hydrolyzing β-glucosidase

The recombinant plasmids *pEASY*-BuBGL1–*pEASY*-BuBGL15 were used to transform *E. coli* BL21(DE3)pLysS according to the manufacturer's protocol. The selection of the positive recombinant colonies was performed by plate assays with X-Glu as indicator substrates (Bhatia et al. 2002; Love and Streiff 1987). The blue colonies were cultured in 3 ml ArtMedia Protein Expression medium (a kind of commercial automatic induction medium) at 37 °C overnight (Studier 2005). The induced cells were lysed according to established protocol of

molecular cloning (Sambrook and Russell 2001). Then, the lysate was centrifuged, and 10 µl supernatant (crude enzyme extract) was incubated with 190 µl SDG solution (1.0 mg crude SDG/ml) in 20 mM sodium phosphate buffer (pH 7.0) at 37 °C for 1 h, then the reaction mixture was analyzed by HPLC.

Sequence analysis of recombinant β-glucosidase BuBGL8

The BLAST program at NCBI server (http://www.ncbi.nlm.nih.gov/BLAST/) was used to perform DNA/Protein sequence similarity search (Altschul et al. 1990). Motif was identified using PROSITE program (http://prosite.expasy.org/) (Sigrist et al. 2010), and conserved domain analysis was retrieved form SMART server (http://smart.embl-heidelberg.de/) (Letunic et al. 2012). Multiple sequence alignment was performed using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). ExPASy ProtParam tool was used to calculate the molecular weight and isoelectric point (pI) (http://web.expasy.org/protparam/) (Gasteiger et al. 2011). Signal sequence prediction was performed using SignalP 4.0 server (http://www.cbs.dtu.dk/services/SignalP/) (Petersen et al. 2011).

Homology modeling and substrate docking analysis of recombinant β-glucosidase BuBGL8

The 3-D structure model of BuBGL8 was generated using Swiss-Model server (http://swissmodel.expasy.org/) (Schwede et al. 2003). Then, the predicted structure model was optimized by using NAMD 2.6 software. According to the NAMD tutorials, the model was subjected to 5,000 steps of conjugate gradient minimization, followed by 100 ps of constrained molecular dynamics simulation with temperature at 310 K with a time step of 1.0 fs. Afterwards, the model was minimized again for 5,000 steps and equilibrated for 100 ps again with the protein moving freely. Finally, the model was simulated and equilibrated via 1 ns with a time step of 2.0 fs (Graciano et al. 2012).

The minimized and equilibrated model was used for the docking study. pNPG and SDG were docked into the active site of BuBGL8 model by using AutoDock 4.2 program (Morris et al. 1998; Morris et al. 2009).

Expression and Purification of the recombinant β-glucosidase BuBGL8

Plasmid pEASY-BuBGL8 was used to transform E. coli Rosetta(DE3) to construct the recombinant bacteria pEASY-BuBGL8/Rosetta(DE3) according to the manufacturer's protocol. pEASY-BuBGL8/Rosetta(DE3) cells were grown in 4 L LB medium until the optical density of the culture at 600 nm (OD$_{600}$) reached 0.5–0.6 (Jabbour et al. 2012). Afterwards,

the expression of recombinant BuBGL8 was performed by adding IPTG (0.48 g) to a final concentration of 0.5 mM and subsequent incubation at 20 °C overnight (Ferrer et al. 2004; Graslund et al. 2008; Rabhi-Essafi et al. 2007). The induced cells were harvested by centrifugation (5,000×g, 4 °C, 30 min) and resuspended in 100 ml PBS (NaCl 137 mM, KCl 2.7 mM, Na$_2$HPO$_4$ 10 mM, KH$_2$PO$_4$ 2 mM, pH 7.4). Then, the cells were lysed by sonication till sample was no longer viscous, and the lysate was centrifuged (10,000×g, 4 °C, 30 min) and the supernatant (crude enzyme extract) was collected. BuBGL8 was purified from crude enzyme extract with His-tag Protein Purification Kit (CoWin Bioscience Co., Ltd, Beijing, China). The purified BuBGL8 was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie blue R-250 staining.

Enzyme assay

The activity of recombinant β-glucosidase BuBGL8 was determined by using 1 mM pNPG as substrate. For each assay, 100 µl enzyme solution was incubated with 1.9 ml sample solution in 20 mM sodium phosphate buffer (pH 6.0) at 40 °C for 5 min. The reaction was stopped by adding 2 ml Na$_2$CO$_3$ (0.2M), the amount of released pNPG aglycone (p-nitrophenol) was measured at OD$_{400}$ by TU-1810 spectrophotometer (Purkinje General Instrument, Beijing, China). The activity of BuBGL8 (in units per milliliter) was calculated as $(C×V)'/(V×T)$, where C (in micromole per liter) represents the concentration of p-nitrophenol, V' (in liter) represents the total volume of the reaction system, V (in milliliter) represents the volume of BuBGL8 added, and T (in minute) represents the reaction time. The amount of enzyme required to release 1 µmol of p-nitrophenol per minute was defined as one unit (U) (Yan et al. 2008).

Effects of pH and temperature on the activity of recombinant β-glucosidase BuBGL8

To investigate the effect of pH on the activity of recombinant β-glucosidase BuBGL8, it was incubated at 37 °C for 5 min in varying buffers with pH range of 3.0–9.0 (20 mM citric acid–sodium citrate, pH 3.0–5.0; 20 mM sodium phosphate, pH 6.0–7.0; 20 mM Tris–HCl, pH 8.0–9.0), respectively. The effect of temperature on the activity of BuBGL8 was determined by incubating the enzyme at temperatures ranging from 20 to 70 °C for 5 min in 20 mM sodium phosphate buffer (pH 6.0) (Quan et al. 2012).

Effects of pH and temperature on the stability of recombinant β-glucosidase BuBGL8

The effect of pH on the stability of recombinant β-glucosidase BuBGL8 was studied by pre-incubating BuBGL8 solution in

the buffers described above (pH 3.0–9.0) at 20 °C for 60 min, and then the activity of BuBGL8 was measured by the standard assay method. The thermal stability of recombinant BuBGL8 was investigated by pre-incubating the enzyme in 20 mM phosphate buffer (pH 6.0) at different temperatures (30, 37, 40, and 50 °C) for 0–120 min, respectively. The activity of BuBGL8 in different time point was determined as described above.

Comparison of SDG-hydrolyzing effects of purified recombinant β-glucosidase BuBGL8 and crude enzyme extract

One milliliter BuBGL8 solution (0.035 mg/ml) and 1 ml crude enzyme extracts (0.14, 0.18, and 0.23 mg crude protein/ml) of induced *pEASY*-BuBGL8/Rosetta(DE3) cells in 20 mM sodium phosphate buffer (pH 6.0) were reacted with equal volume of SDG solution (2.0 mg/ml) at 30 °C, respectively. The concentration of SDG-hydrolyzing product SECO in the reaction mixture at different time points was determined by HPLC.

The effect of substrate concentration on the activity of crude enzyme extract

One milliliter SDG solutions, each at different concentrations of 1.0, 2.0, and 3.0 mg/ml, was incubated with 1-ml crude enzyme extract (0.2 mg crude protein/ml) in 20 mM sodium phosphate buffer (pH 6.0) at 30 °C, respectively. The concentration of SECO in the reaction mixture was determined by HPLC.

Kinetic analysis of recombinant β-glucosidase BuBGL8 acting on *p*NPG

The initial velocities of BuBGL8 using *p*NPG as substrate with various concentrations of 32–320 μM were measured at 40 °C and pH 6.0 for 5 min. The maximum reaction rate V_{max} and Michaelis constant K_m were determined using Lineweaver–Burk plotting method based on the Michaelis–Menten equation : $1/v = K_m/V_{max}[S] + 1/V_{max}$, where v is the initial velocity and [S] is the substrate concentration (Chen et al. 2010; Michaelis et al. 2011).

HPLC analysis

The above SDG-hydrolyzing reaction was stopped by adding an equal volume of methanol. After centrifugation at $10,000 \times g$ for 10 min, the supernatant was used for HPLC analysis. The HPLC system consisted of Agilent 1260 series HPLC apparatus (Agilent Technologies, USA), including binary pump, standard autosampler, thermostatted column compartment, diode array detector and Agilent Chemstation (D.

04. 03). The BDS HYPERSIL C_{18} (4.6 mm×250 mm, 5 μm) (Thermo Fisher Scientific Inc., USA) was used to analyze the samples. The temperature of the column oven was 25 °C. The mobile phase consisted of water (A) and acetonitrile (B) in a linear gradient change from 100 % A and 0 % B to 50 % A and 50 % B in 30 min, with flow rate of 1.0 ml/min. The samples were detected at UV 280 nm.

Preparation of the SECO standard curve

A SECO stock solution of 2.36 mg/ml was prepared by exactly weighting dissolving SECO (4.71 mg) in methanol. The working solutions (0.03–2.36 mg/ml) were prepared by diluting the stock solutions with methanol. Twenty microliters of each working solution was analyzed by HPLC. The SECO standard curve was obtained by plotting HPLC peak areas on the *y*-axis versus the concentration (in milligram per milliliter) of SECO on *x*-axis, which was resulted as follows: $y=14798x-10.74(R^2=0.9999)$, with a linearity over the concentration range of 0.03 to 2.36 mg/ml.

Nucleotide sequence accession numbers

The nucleotide sequences of *bgl1–bgl15* from *B. uniformis* ZL1 have been deposited in GenBank nucleotide sequence database. The accession numbers of *bgl1–bgl15* are from KF110993 to KF111007, respectively.

Results

Cloning of putative β-glucosidase genes and screening of the SDG-hydrolyzing β-glucosidase

Although the genome of *B. uniformis* ZL1 was unknown, the genome sequence data of *B. uniformis* ATCC 8492 was available in NCBI database, and 24 putative β-glucosidases (BuBGL1-BuBGL24), as well as their encoding genes, were annotated in the genome of strain ATCC 8492 (Table 1). Based on the fact that the strains belong to same genus may have high genetic similarities (Kuhnert and Korczak 2006), the primers for the target genes in strain ZL1 were designed by using the 24 encoding genes in strain ATCC 8492 as templates. And 15 *bgl* genes (*bgl1-bgl15*) from *B. uniformis* ZL1 were amplified by using the 24 pairs of primers successfully. We cloned them into the *pEASY*-E1 expression vector and then transformed the *E. coli* expression strain BL21(DE3)pLysS with the constructs. As shown in Fig. 1, the colonies of recombinant bacteria harboring *pEASY*-BuBGL8 displayed blue color on LB medium plate with X-Glu as indicator, while others (without *pEASY*-BuBGL8) formed white transparent colonies, indicating that the recombinant BuBGL8 played the roles of β-glucosidase. To further

Table 1 Putative β-glucosidases retrieved in genome of *Bacteroides uniformis* ATCC 8492 in NCBI database and primers for the target genes in strain ZL1

Protein	Accession number in GenBank	Putative function	Primer sequence (5′–3′)	Appealing temperature (°C)
BuBGL1	EDO56275.1	Bile acid β-glucosidase	GCGTCAACTATGGTAAGATCAAC (forward) CTATCCTAATTGACCTCAATCCACA (reverse)	55
BuBGL2	EDO56172.1	β-Glucosidase-related glycosidase	CGCAGGACGGACAGTTACA (forward) CGTTGTTGTGGCATAACTCGCT (reverse)	59
BuBGL3	EDO56126.1	β-Glucosidase-related glycosidase	CGCAAATCTGACTAGGCTATG (forward) CACCTAAGCACTACAAATCAACCT (reverse)	56
BuBGL4	EDO56038.1	β-Glucosidase-related glycosidase	CTGGCACATTAGCATATTAACCA (forward) GCAGTGGTTACGCACTGATTATAAG (reverse)	57.6
BuBGL5	EDO55919.1	β-Lactamase class C and other penicillin binding proteins	GAAAGAGTGGCATCTACGT (forward) TCAGAAGCGGACAAAGACAAC (reverse)	58
BuBGL6	EDO55918.1	β-Lactamase class C and other penicillin binding proteins	ATGAAGAGAATATACCTGCTTGCCT (forward) AAGAGCCTTTCCTGCGTGAG (reverse)	58
BuBGL7	EDO55370.1	β-Glucosidase-related glycosidase	GTACGGACTGATTATGATTGCT (forward) ACCCTCCATCTACACTATCTTTG (reverse)	55
BuBGL8	EDO55247.1	β-Glucosidase-related glycosidase	CCGGGTGTGCCGCAGCTTGGAAAGT (forward) CAGACAATCCTACTCTCATCTCTTC (reverse)	58
BuBGL9	EDO53124.1	β-Glucosidase-related glycosidase	GACCTTTGTGTAGGAGCAGATTC (forward) ATAGAAATTGCAGGCCATCTGTC (reverse)	57
BuBGL10	EDO52638.1	β-Glucosidase-related glycosidase	GAATGCGTGATACAGACCATC (forward) CATTACAAGGTAACGTACCGAGA (reverse)	54
BuBGL11	EDO52739.1	β-Glucosidase-related glycosidase	GCAACCATGATGAAACAGAAAC (forward) AGTGATGTATCAAGGCTGAATGAC (reverse)	57
BuBGL12	EDO52360.1	β-Glucosidase-related glycosidase	GTACGGGATGTGGAGATTGTC (forward) TATTGCCGCCTTGGGAATCA (reverse)	56
BuBGL13	EDO52217.1	β-Glucosidase-related glycosidase	GCAATAGAGTGACGGCAATCT (forward) AGCTGCCAAGGAGCAGAATG (reverse)	59
BuBGL14	EDO52212.1	β-Glucosidase-related glycosidase	ACATGATGGAGCAGCTAAGCT (forward) GCGGACTGCCCTCTTACCTA (reverse)	58
BuBGL15	EDO51978.1	β-Glucosidase-related glycosidase	TGCCCGGCAATATAACAAACAC (forward) GCTTCCGCAGCATCCATCT (reverse)	57
BuBGL16	EDO55251.1	β-Glucosidase-related glycosidase	CCCGTGGGAATCAGCTAATTGTAC (forward) CCTCATCTCTTCACCATCGGATAC (reverse)	58
BuBGL17	EDO53061.1	β-Glucosidase-related glycosidase	CGACACGCTTTCGCCCAATAAG (forward) GCTAAAGGTCTGTTCCGTTTCTGA (reverse)	59
BuBGL18	EDO52636.1	β-Glucosidase-related glycosidase	CTGTTTATACAGTTGTAGCTCCTGA (forward) GACGCAGAAAGATGAAGTTTGGT (reverse)	55
BuBGL19	EDO52644.1	Bile acid β-glucosidase	TGCTCCAGTATGGAAGAAGTACGT (forward) CGATGCATATACACTTCCCACACT (reverse)	55
BuBGL20	EDO52403.1	β-Glucosidase-related glycosidase	AAGCCTGATGCGGAGCGAT (forward) AATTGTAGAGGATTCACCACTCCA (reverse)	55
BuBGL21	EDO52404.1	β-Glucosidase-related glycosidase	TGTGGAGTGGTGAATCCTCTAC (forward) ACCAGCAACGCTATCGAGAG (reverse)	55
BuBGL22	EDO52348.1	β-Glucosidase-related glycosidase	TCATGCTCCCGTAATGGCTATC (forward) GAACAGCAGGAACCAATGGAAG (reverse)	57
BuBGL23	EDO52406.1	β-Glucosidase-related glycosidase	AAAGTATTCAGGCGCTGACCA (forward) CGTGGCAACCAATCCATACAG (reverse)	58
BuBGL24	EDO51976.1	β-Glucosidase-related glycosidase	TACCATTGAAGGCAACTGTTG (forward) GGATATGATTCCAGACAGCCT (reverse)	56

validate the activity of BuBGL8, we cultured the blue colonies in automatic induction medium and extracted the enzyme. When incubated with the crude enzyme for 60 min, SDG was completely transformed into its aglycone SECO (Fig. 2), indicating that BuBGL8 was the SDG-hydrolyzing β-glucosidase.

Sequence analysis of the recombinant β-glucosidase BuBGL8

BuBGL8 is composed of three domains as revealed by analysis through the SMART server. Domains 1 (residues 37 to 326) and 2 (residues 359 to 638) are conserved domains of

Fig. 1 Plate assays with X-Glu as indicator. The colonies harboring *pEASY*-BuBGL8 (**a**) and the colonies without *pEASY*-BuBGL8 (**b**) were grown on LB agar plate with X-Glu at 37 °C overnight
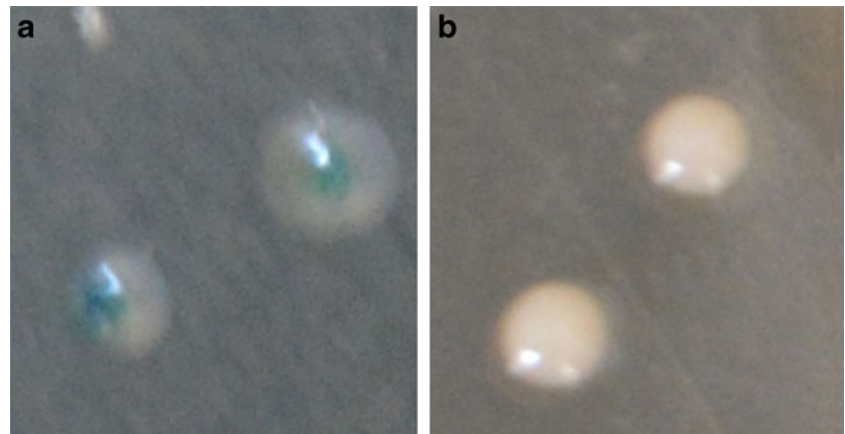
glucoside hydrolase family 3, and domain 3 (residues 669 to740) has a fibronectin type III fold with unknown function. When multiple sequence alignments of BuBGL8 with six similar known proteins were made by ClustalW2 (Fig. 3), a conserved region (S/TDW, residues 254–256) was found; the central aspartate (ASP255) was thought to be catalytic nucleophile residue (Dan et al. 2000; Li et al. 2001; Pozzo et al. 2010) and GLU462 was predicted to be catalytic acid/base residue (Bhatia et al. 2002; Pozzo et al. 2010). The molecular weight of BuBGL8 was 81.5 kDa and p*I* was 5.89 calculated by ProtParam tool.

A signal peptide was found in BuBGL8 with a cleavage site between residues 19 and 20 when analyzed by SignalP 4.0 server. To heterogeneously express BuBGL8 in *E. coli*, the signal peptide had to be cut-off because the signal peptidase from *B. uniformis* did not exist in *E. coli*. Thus, the design of primers was based on the partial sequence of the encoding gene *bgl8* without signal peptide sequence.

Homology modeling and substrate docking analysis of recombinant β-glucosidase BuBGL8

The sequence similarity search for BuBGL8 was performed in PDB archive by BLAST program, and the highest identity of 48.7 % was found with the β-glucosidase 3B from *Thermotoga neapolitana* (TnBgl3B, PDB ID 2X40). Thus, the 3-D structure model of the recombinant β-glucosidase BuBGL8 was built by using the crystal structure of TnBgl3B as template by Swiss-Model server, and energetically optimized by the NAMD software. The refined structure was validated using the PROCHECK and ERRAT programs. The Ramachandran plot generated by PROCHECK showed that more than 99 % of the residues were at the core region (89.0 %) or allowed region (10.8 %), indicating that the model has good stereochemical quality (Kalyani et al. 2012; Almeida et al. 2002). The overall quality factor obtained by ERRAT was 88.853, which also confirmed a good quality of the structure (accepted range for
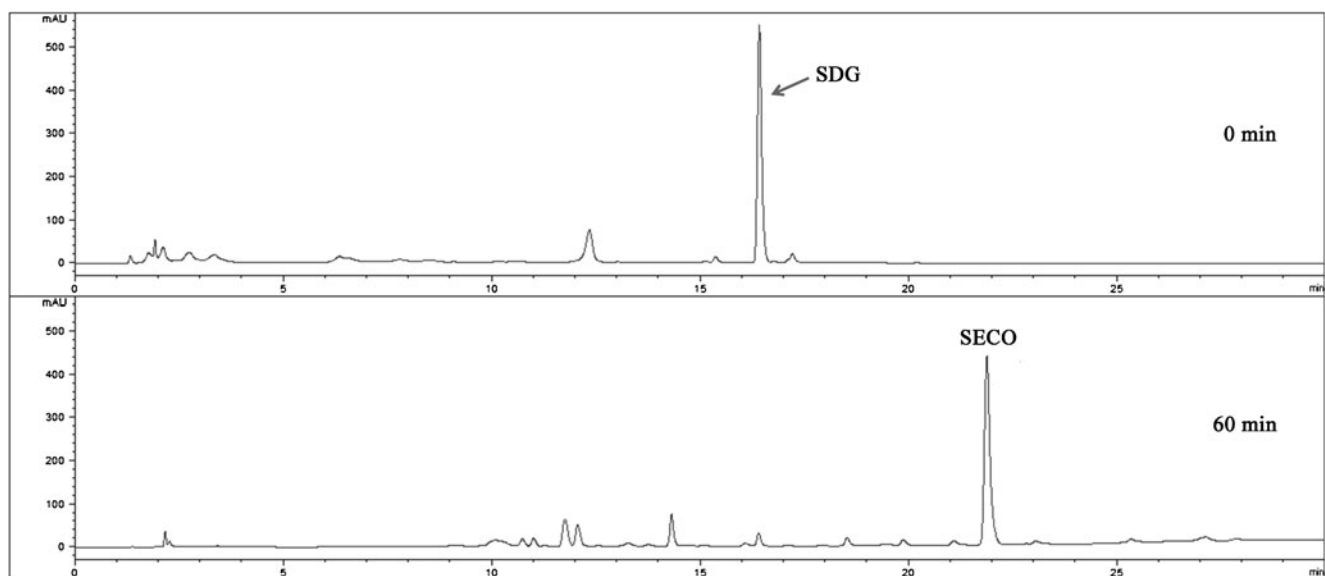

Fig. 2 HPLC profile of crude enzyme transformation products of SDG after incubating at 37 °C for 0 min and 60 min

```
B. uniformis    TMLRDEWGFKGMVMTDWFGGK------------DAVAQMVAGNDMLQPG 276
P. bergensis    TALRTEWGYKGMVMTDWFGGK------------DAVAQMKAGNDMLQPG 295
P. ruminicola   TILRDEWGYEGTVMTDWFGGK------------DGAKQMWAGNDMLQPG 296
B. fluxus       TVLRDEWGFDGIVMTDWIGQR------------NTAAQVHAGNDLMEPG 297
F. frigoris     TVLRDEWGFKGLVMTDWFGGYNGFGAIRDKDAVSDVVKQLTAGNDLLMPG 350
T. neapolitana  KVLREEWGFEGFVMSDWYAGD------------NPVEQLKAGNDLIMPG 265
F. nodosum      KVLRDEWQFDGFVMTDWFAGD------------SAAKQLMAGNDLIMPG 266
                 · ** **  :.*  **:**  .          . . *: ****:: **
                                    ▲
                                  ASP255


B. uniformis    TLGRTSGEFLDRMVAD--FNLTKEEQNMLKAVSDAFHAAGKKVVVVLNIG 502
P. bergensis    TIGRNSGEFIDRSLAD--FSLSASELALIRQTTAAFHAAGKKVVVVLNIG 524
P. ruminicola   TLGRISGEFMDRKAAD--FNLSDSERQLITQVCDVYHKAGKQVVVLLNIG 533
B. fluxus       TVGRQGGEAWDRKVKDD-FNLTAQERQLLDDLCAAFHAEGKKVVVVINAG 525
F. frigoris     TIGRNAGEGDDRKVDED-FNLAQDEVNLINTVSTAFHAQGKKVVVVLNIG 579
T. neapolitana  VISRISGEGYDRKPVKGDFYLSDDETDLIKTVSREFHEQGKKVIVLLNIG 502
F. nodosum      IITRISGEGYDRKAEKGDYYLSDDEFELLKSVSEIFRKHNKKVIVVLNIG 504
                 : * .** **   . : *:.* ::      ::  .*:*:*::* *
                                    ▲
                                  GLU462
```

* = the residues that are identical in all sequences in the alignment

: = conservative substitutions

. = semiconservative substitutions

Fig. 3 Identification of the catalytic nucleophile (ASP255) and the acid/base catalyst (GLU462) of recombinant β-glucosidase BuBGL8. Multiple sequence alignment of BuBGL8 was performed with other six known proteins using ClustalW2. These proteins include: β-glucosidase from *Prevotella bergensis* DSM 17361 (accession number ZP_06005092.1, identity 74 %), glucan 1,4-β-glucosidase from *Prevotella ruminicola* 23 (accession number YP_003575940.1, identity 69 %), glycosyl hydrolase family 3 protein from *Bacteroides fluxus* YIT 12057 (accession number ZP_08300624.1, identity 61 %), β-glucosidase from *Flavobacterium frigoris* PS1 (accession number ZP_09895978.1, identity 57 %), β-glucosidase from *Thermotoga neapolitana* DSM 4359 (accession number YP_002534212.1, identity 50 %), glycoside hydrolase family 3 protein from *Fervidobacterium nodosum* Rt17-B1 (accession number YP_001409810.1, identity 49 %). The catalytic nucleophile and acid/base catalyst sites were indicated by *filled triangles*

a high quality model is higher than 50) (Banerjee et al. 2009; Colovos and Yeates 1993). Therefore, the structure model of BuBGL8 was reliable for further docking studies.

Two substrates, pNPG and SDG, were taken to proceed the molecular docking with the 3-D structure model of BuBGL8. By using AutoDock 4.2 program, the structure of pNPG was docked with that of BuBGL8 (Fig. 4a). H_3 and H_1 of pNPG could form hydrogen bonds with O atom in carboxyl groups of D255 (2.18 Å) and E462 (2.00 and 2.15 Å), respectively (Fig. 4b). Similar interactions formed between BuBGL8 and SDG, as shown in Fig. 4c, d. For example, the O atoms in carboxyl groups of D255 made hydrogen bonding interactions with H_2 (2.23 Å) and H_3 (1.65 Å and 2.23 Å) of one glucose moiety in SDG, and O atom in E462 made hydrogen bonds with H_1 of the glucose moiety (2.05 and 2.26 Å). Moreover, the carboxyl groups of D255 and E462 pointed to the C_1 atoms of pNPG and SDG (Fig. 4a–c). Based on these findings, we predicted that D255 made nucleophilic attack on the C_1 atom and E462 played the role of catalytic proton donor (Ketudat Cairns and Esen 2010). Besides, another six residues

of BuBGL8 including ASP71 (only with pNPG), ARG143, LYS176, ARG187 (only with SDG), TRP256, and SER377 also participated in the formation of H-bond interactions with pNPG and SDG, which were also identified as active sites to take part in the catalysis process of BuBGL8.

Expression and purification of the recombinant β-glucosidase BuBGL8

The plasmid *pEASY*-BuBGL8 containing the encoding gene of BuBGL8 was propagated in *E. coli* Rosetta(DE3), with the recombinant strain being designated *pEASY*-BuBGL8/Rosetta(DE3). The soluble protein and insoluble protein extracts from *pEASY*-BuBGL8/Rosetta(DE3) cells induced by IPTG, as well as that from uninduced *pEASY*-BuBGL8/Rosetta(DE3) cells, were analyzed by SDS-PAGE. As shown in Fig. 5a, there was a band of about 80 kDa in the soluble protein extract from the induced cells (*lane 2*), which was in accordance with the calculated weight of BuBGL8 (81.5 kDa). There was no similar band in the insoluble
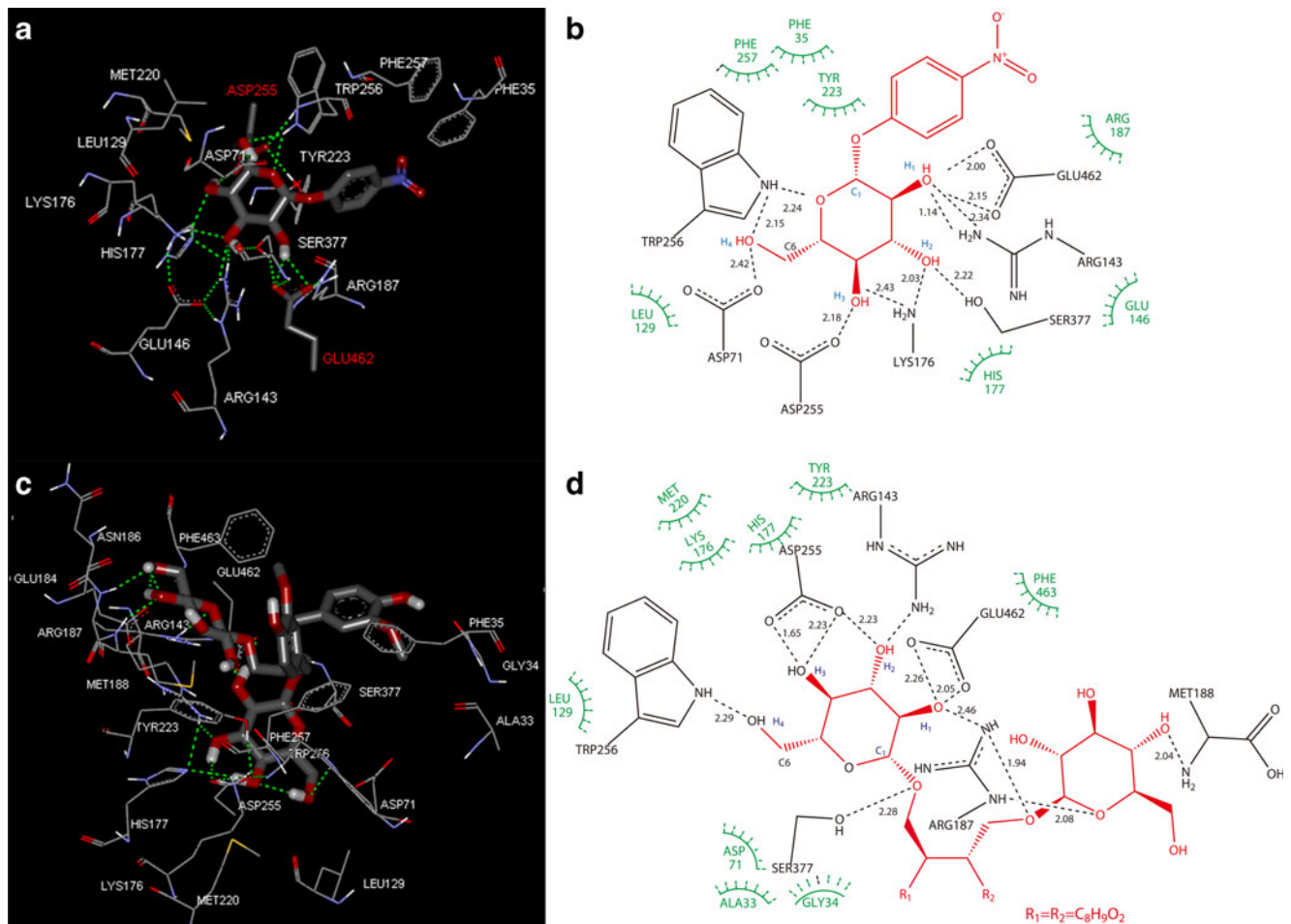
Fig. 4 Substrate docking study of recombinant β-glucosidase BuBGL8 with *p*NPG or SDG as ligand. **a** Stereo view of active-site pocket of BuBGL8 docked with *p*NPG, hydrogen bonds between *p*NPG and the contact residues are shown as dashed lines (*green color*), D255 and E462 are located closely with C1 atom and O1 atom of *p*NPG (3.93 and 4.66 Å). **b** Schematic representation of hydrogen bond network between *p*NPG and active site of BuBGL8, C and H atoms of glucose moiety of *p*NPG were numbered. H-bond interactions were shown as *dashed line* (distances are in angstrom), the intermolecular H-bonds of BuBGL8 itself are not shown but the residues are represented on an arc. **c** Stereo view of active-site pocket of BuBGL8 bonded with SDG. **d** Schematic representation of hydrogen bond network between SDG and active site of BuBGL8. The bonding interactions were represented using Discovery Studio 2.5 and ChemBioDraw Ultra 11.0

fraction of the induced cells (*lane 1*). The content of BuBGL8 in the total soluble protein extract was about 20 % calculated by Quantity One v4.62. Thus, the recombinant β-glucosidase BuBGL8 was expressed in a soluble form in *pEASY*-BuBGL8/ Rosetta(DE3) cells induced by IPTG. A volume of 1 ml BuBGL8 (2.8 mg/ml) was purified from the soluble protein extracts of induced *pEASY*-BuBGL8/Rosetta(DE3) cells from 4 L culture medium by affinity chromatography on Ni-Agarose Resin, which was detected as a single band (81.5 kDa) by SDS-PAGE analysis (Fig. 5b).

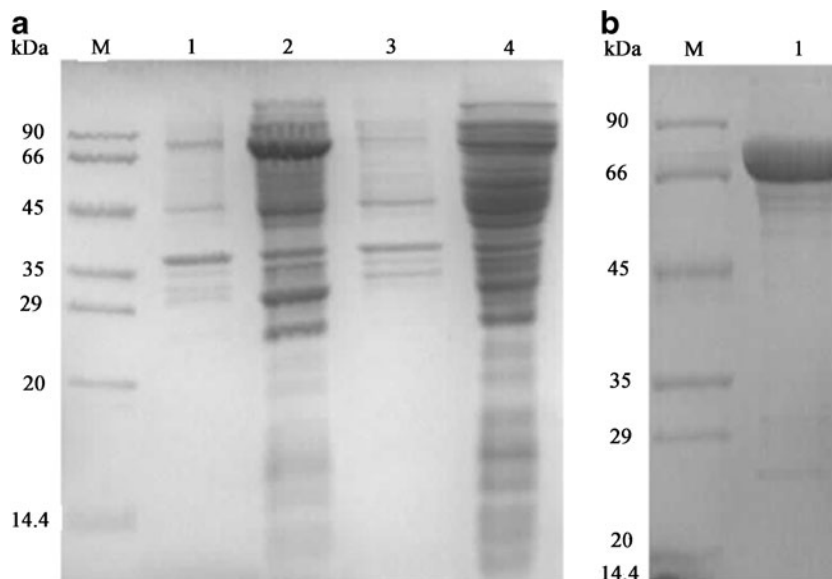**Effects of pH and temperature on the activity of recombinant β-glucosidase BuBGL8**

It is well known that pH and temperature are the most two important factors that may affect the activity of an enzyme. Thus, the effects of pH and temperature on the activity of the

recombinant β-glucosidase BuBGL8 were investigated with *p*NPG (1 mM) as the substrate. As shown in Fig. 6a, BuBGL8 could transform *p*NPG to *p*-nitrophenol at the pH range of 5.0–9.0, with the best activity at pH 6.0. The effect of temperature was shown in Fig. 6b: the activity of BuBGL8 increased gradually with temperature varying from 20 °C to 40 °C under the condition of pH 6.0; when the temperature was above 40 °C, the activity of BuBGL8 begun to decrease significantly, and there was almost no activity detected when the temperature was higher than 60 °C.

Effects of pH and temperature on the stability of recombinant β-glucosidase BuBGL8

To make sure that the enzymatic reaction was successfully developed, BuBGL8 should remain stable during the reaction period. Although BuBGL8 had optimal activity at pH 6.0 and

Fig. 5 SDS-PAGE analysis of recombinant β-glucosidase BuBGL8 expression (**a**) and purification (**b**). **a** *lane M* molecular weight standard; *lanes 1,2* insoluble and soluble extracts of *pEASY*-BuBGL8/Rosetta(DE3) cells induced by IPTG, respectively; *lanes 3, 4* insoluble and soluble extracts of *pEASY*-BuBGL8/Rosetta(DE3) cells without induction. **b** *lane M* molecular weight standard, *lane 1* purified BuBGL8

40 °C, the stability of BuBGL8 under this condition was unknown. Thus, the effects of pH and temperature on the stability of BuBGL8 were investigated. After pre-incubating
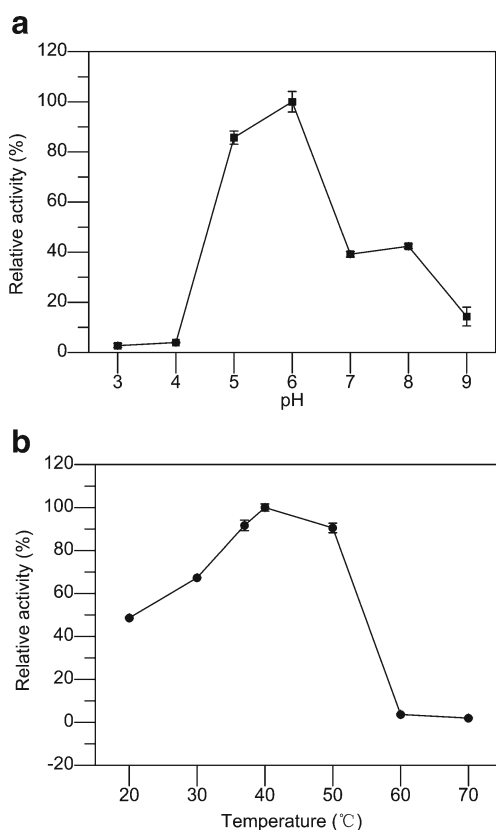
Fig. 6 Effects of pH (**a**) and temperature (**b**) on the activity of recombinant β-glucosidase BuBGL8. Values are means calculating from triplicate determinations±SD. Relative activity (%) was calculated by assuming that activities of BuBGL8 at pH 6.0 (**a**) or 40 °C (**b**) was 100 %. The analysis of data acquired was performed using Origin Pro 8.5

BuBGL8 at pH range of 3.0-9.0 and 20 °C for 60 min, we measured its activity for transforming *p*NPG (1 mM) to *p*-nitrophenol at 40 °C for 5 min. As shown in Fig. 7a, BuBGL8 showed best stability at pH 6.0, with 93 % of initial activity left after incubating for 60 min. In other conditions BuBGL8 retained 86 % (pH 5.0), 60 % (pH 7.0), and 67 % (pH 8.0) of initial activity, and almost no activity was detected at pH 4.0. Similarly, BuBGL8 was pre-incubated at temperatures of 30–50 °C and pH 6.0 for 0–120 min, and then its activity was measured at 40 °C for 5 min. As shown in Fig. 7b, the temperature of 30 °C was most suitable for the stability of BuBGL8, more than 90 % initial activity was retained after 120 min. When the temperature was above 37 °C, the activity of BuBGL8 decreased quickly, with only 50 and 31 % of the initial activity remaining at 37 °C and 40 °C in 120 min, respectively; and almost no activity was detected when pre-incubated at 50 °C for just 20 min.

Comparison of the SDG-hydrolyzing effects of purified recombinant β-glucosidase BuBGL8 and crude enzyme extract

In large-scale production technology, the crude enzyme extract would be more economical than the purified enzyme for avoiding the expensive and complex protein purification process (Hou et al. 2012; Zhang et al. 2009). Therefore, the SDG-hydrolyzing activities of purified BuBGL8 protein (0.035 mg/ml) and crude enzyme extracts at three different concentration of crude protein at 0.14 mg (I), 0.18 mg (II), 0.23 mg (III), were compared, all of them were incubated with 4.0 mg/ml SDG at pH 6.0 and 30 °C. As shown in Fig. 8, among the three crude enzyme extracts, III showed similar activity to that of BuBGL8, with the SDG transformation efficiency of about 90 % after 90 min, while the activities of I and II were much
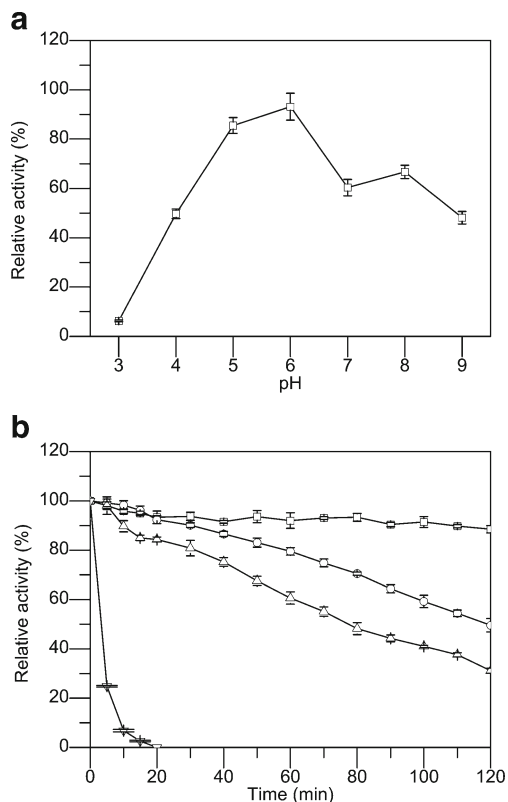
Fig. 7 Effects of pH (**a**) and temperature (**b**) on the stability of the recombinant β-glucosidase BuBGL8. **a**. BuBGL8 was pre-incubated at various pH (3.0–9.0) at 20 °C for 60 min, then the remaining enzyme activity was detected as the standard assay method. **b**. Thermal stability of BuBGL8 at temperatures of 30 °C (*open square*), 37 °C (*open circle*), 40 °C (*open upright triangle*), and 50 °C (*open inverse triangle*). The enzyme activity was measured as the standard assay method. Relative activity (%) was calculated by assuming that the initial activity of the BuBGL8 was 100 %
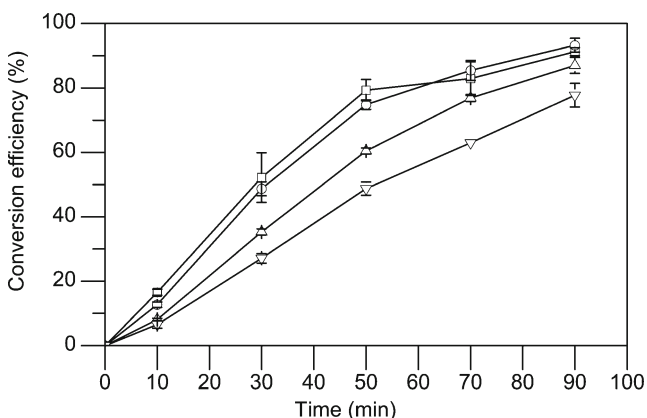

Fig. 8 Conversion efficiency-time courses of SDG to SECO by purified BuBGL8 and crude enzyme extract of induced *pEASY*-BuBGL8/ Rosetta(DE3) cells. SDG solutions (2.0 mg/ml) were incubated with 0.035 mg/ml BuBGL8 (*open square*) and crude enzyme extracts (0.23 mg crude protein/ml, *open circle*; 0.18 mg crude protein/ml, *open upright triangle*; 0.14 mg crude protein/ml, *open inverse triangle*). The conversion efficiency of SDG to SECO was calculated as $C_{SECO}/C_{SECO}' \times 100\%$: C_{SECO} was the concentration of SECO in the reaction solution, C_{SECO}' was the concentration of SECO which was transformed completely by the original pure SDG

lower. Hence, we could replace purified glucosidase BuBGL8 with the crude enzyme extract of induced *pEASY*-BuBGL8/ Rosetta(DE3) cells to complete the large-scale enzymatic catalysis, the ratio of protein concentration in BuBGL8 versus that in crude enzyme extract was 1:7.

The effect of substrate concentration on the activity of crude enzyme extract

Theoretically, if the amount of an enzyme remains constant, a lower concentration of substrate means fewer molecules will get attached to the enzyme active sites and leads to a slower reaction rate. On the contrary, the substrate may be an inhibitor at improper high level (Hou et al. 2012; Reed et al. 2010). Thus, the SDG-hydrolyzing activities of crude enzyme extract by taking three substrate concentrations of SDG at 1.0, 2.0, and 3.0 mg/ml were investigated. As shown in Fig. 9, the optimal reaction rate for SECO production was observed at 2.0 mg/ml SDG within a 90-min period, which was two times more than that by taking 1.0 mg/ml SDG as substrate. And when SDG concentration increased to 3.0 mg/ml, the reaction rate was slightly reduced, which means that excess substrate will show inhibitory action on the SDG-hydrolyzing activity. Thus, 2.0 mg/ml was opted as the substrate concentration for SDG in the present system, i.e., the amount ratio of crude enzyme extract (0.2 mg) versus substrate SDG (2.0 mg) was appropriately 1:10.

Kinetic parameters of recombinant β-glucosidase BuBGL8 acting on *p*NPG

The kinetic parameters of BuBGL8 by using *p*NPG as substrate were determined by using the Lineweaver–Burk plotting method. The equation was created by plotting the inverse of initial velocity as a function of the inverse of the substrate concentration, which resulted as $y = 1.3905x +$
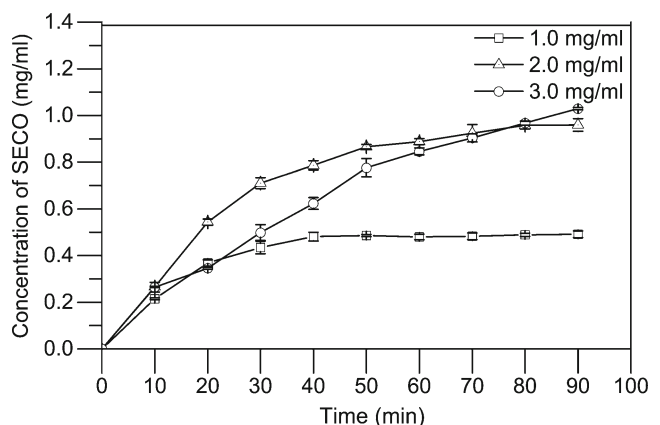

Fig. 9 Content-time courses of SECO produced from SDG (1.0, 2.0 and 3.0 mg/ml) by crude enzyme extract (0.2 mg crude protein/ml) of induced *pEASY*-BuBGL8/Rosetta(DE3) cells

$0.0317(R^2=0.9985)$. The substrate concentration for half-maximum reaction rate (K_m) was calculated to be 44 μM and V_{max} was 31.5 μmol min^{-1} mg-protein^{-1}.

Discussion

In this study, the β-glucosidase BuBGL8 involved in SDG hydrolysis was identified from *B. uniformis* ZL1, its encoding gene was cloned into the *pEASY*-E1 expression vector and heterogeneously expressed in *E. coli* Rosetta(DE3). To the best of our knowledge, the sequence of SDG-hydrolyzing β-glucosidase was first identified and this is the first report using the genetic recombination method to produce the SDG-hydrolyzing β-glucosidase.

To date, several methods have commonly been used to identify functional gene or protein from microorganism, such as DNA/cDNA library screening, protein extraction and purification, bioinformatics analysis, and so on (Bhatia et al. 2002; Bork et al. 1998; Kaul and Asano 2012). Library screening may be the first choice when there was little information about the functional gene, but the library need contain a huge number of clones for being screened to find the functional gene. And even if the high-throughput screening method was developed, the procedure was still very time-consuming (Wahler and Reymond 2001). With the development of chromatography technology and protein purification equipment, the purification strategy was adopted to obtain the pure active protein from microorganism, its sequence will be determined on the basis of mass spectroscopy analysis (Domon and Aebersold 2006; Smith 2005). However, the functional protein is usually produced in low-level in natural host strains, which leads to the requirement for large abundant sources of strain cells to enrich the proteins. Furthermore, after several protein purification steps, the activity of the target protein will be lost in the procedure if it had low stability (Linn 2009). With the increasing biological data and developed bioinformatics software in recent years, bioinformatics analysis was used to identify functional genes/proteins more and more, the computational method of bioinformatics could predict the protein function prior to the screening experiments, which will significantly shorten and streamline the functional gene/protein selection progress (Lee et al. 2007; von der Lieth et al. 2006). Nonetheless, the known genome sequence of microorganism as well as rich research on the related functional gene/protein was prerequisites. In this study, both the whole genome sequencing of *B. uniformis* and the sequence information of known β-glucosidases are available (Bhatia et al. 2002; Opassiri et. al. 2006; Rojas and Romeu 1996), the identification of the sequence of β-glucosidase in *B. uniformis* was smoothly identified via bioinformatics analysis.

The stability of an enzyme is one of the influencing factors for an enzymatic biocatalysis, β-glucosidases are usually unstable at high temperature above 40 °C (Ketudat Cairns and Esen 2010; Nakajima et al. 2012; Woodley 2013). In our research, the activity and stability of purified recombinant β-glucosidase BuBGL8 were investigated simultaneously, it was found that BuBGL8 had the highest activity at the condition of pH 6.0 and 40 °C, while being unstable at 40 °C with 60 % of the initial activity left after 60 min and 30 % left after 120 min. The thermal stability study of BuBGL8 indicated that the temperature being lower than 30 °C could keep it highly active for at least 120 min, with 90 % of initial activity remained. As the enzymatic conversion of SDG to SECO in our present work need to take about 90 min, it is suggested that the enzymatic reaction could be performed at the condition of pH 6.0 and 30 °C in which both the enzyme activity and stability are better.

In this study, the enzymatic reaction could be finished within 90 min with transformation efficiency of SDG to SECO being more than 90 %, which was at least 48-fold faster than that by bacteria transformation of *B. uniformis* (72–120 h). And in our previous work, the reaction time of 7 days was needed to reach the maximum transformation efficiency of about 80 % for producing SECO from defatted flaxseed (containing 6.7 mg SDG/g) (Li et al. 2012). It is obvious that the enzymatic reaction rate and transformation efficiency were both improved in comparison to that of bacteria transformation. Besides that, the recombinant bacteria *E. coli* could grow rapidly and easily, which solved the difficulties for culturing *B. uniformis* in scale-up, such as the complex anaerobic culture condition and slower grown rate.

It has been confirmed that the recombinant β-glucosidase BuBGL8 also exhibited the deglycosylation activities for other natural glucosides, such as astragalin, hyperoside, isoquercetin, and rutin (data not shown). Therefore, BuBGL8 could be applied to obtain active aglycones from corresponding glucosides which are ubiquity in plant resources.

References

Almeida MS, Cabral KMS, Kurtenbach E, Almeida FCL, Valente AP (2002) Solution structure of *Pisum sativum* defensin 1 by high resolution NMR: plant defensins, identical backbone with different mechanisms of action. J Mol Biol 315(4):749–757

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Banerjee A, Arora N, Murty U (2009) Structural model of the *Plasmodium falciparum* thioredoxin reductase: a novel target for antimalarial drugs. J Vector Borne Dis 46(3):171–183

Bhatia Y, Mishra S, Bisaria VS (2002) Microbial β-glucosidases: cloning, properties, and applications. Crit Rev Biotechnol 22(4):375–407

Bork P, Dandekar T, Diaz-Lazcoz Y, Eisenhaber F, Huynen M, Yuan Y (1998) Predicting function: from genes to genomes and back. J Mol Biol 283(4):707–725

Chen WW, Niepel M, Sorger PK (2010) Classic and contemporary approaches to modeling biochemical reactions. Genes Dev 24(17):1861–1875

Clavel T, Henderson G, Engst W, Doré J, Blaut M (2006) Phylogeny of human intestinal bacteria that activate the dietary lignan secoisolariciresinol diglucoside. FEMS Microbiol Ecol 55(3):471–478

Clavel T, Lippman R, Gavini F, Doré J, Blaut M (2007) *Clostridium saccharogumia* sp. nov. and *Lactonifactor longoviformis* gen. nov., sp. nov., two novel human faecal bacteria involved in the conversion of the dietary phytoestrogen secoisolariciresinol diglucoside. Syst Appl Microbiol 30(1):16–26

Colovos C, Yeates TO (1993) Verification of protein structures: patterns of nonbonded atomic interactions. Protein Sci 2(9):1511–1519

Dan S, Marton I, Dekel M, Bravdo B-A, He S, Withers SG, Shoseyov O (2000) Cloning, expression, characterization, and nucleophile identification of family 3, *Aspergillus niger* β-glucosidase. J Biol Chem 275(7):4973–4980

de Carvalho CCCR (2011) Enzymatic and whole cell catalysis: finding new strategies for old processes. Biotechnol Adv 29(1):75–83

Domon B, Aebersold R (2006) Mass spectrometry and protein analysis. Science 312(5771):212–217

Faber K (2011) Introduction and background information. In: Faber K (ed) Biotransformations in organic chemistry, 6th edn. Springer, Berlin, pp 3–10

Ferrer M, Chernikova TN, Timmis KN, Golyshin PN (2004) Expression of a temperature-sensitive esterase in a novel chaperone-based *Escherichia coli* strain. Appl Environ Microbiol 70(8):4499–4504

Gasteiger E, Hoogland C, Gattiker A, Se D, Wilkins MR, Appel RD, Bairoch A (2011) Protein identification and analysis tools on the ExPASy server. In: Walker JM (ed) The proteomics protocols handbook. Humana Press, Totowa, pp 571–607

Graciano L, Corrêa J, Gandra R, Seixas F, Kadowaki M, Sampaio S, da Conceição SJ, Osaku C, Rd S (2012) The cloning, expression, purification, characterization and modeled structure of *Caulobacter crescentus* β-Xylosidase I. World J Microbiol Biotechnol 28(9):2879–2888

Graslund S, Nordlund P, Weigelt J, Hallberg BM, Bray J, Gileadi O, Knapp S, Oppermann U, Arrowsmith C, Hui R, Ming J, Dhe-Paganon S, Park HW, Savchenko A, Yee A, Edwards A, Vincentelli R, Cambillau C, Kim R, Kim SH, Rao Z, Shi Y, Terwilliger TC, Kim CY, Hung LW, Waldo GS, Peleg Y, Albeck S, Unger T, Dym O, Prilusky J, Sussman JL, Stevens RC, Lesley SA, Wilson IA, Joachimiak A, Collart F, Dementieva I, Donnelly MI, Eschenfeldt WH, Kim Y, Stols L, Wu R, Zhou M, Burley SK, Emtage JS, Sauder JM, Thompson D, Bain K, Luz J, Gheyi T, Zhang F, Atwell S, Almo SC, Bonanno JB, Fiser A, Swaminathan S, Studier FW, Chance MR, Sali A, Acton TB, Xiao R, Zhao L, Ma LC, Hunt JF, Tong L, Cunningham K, Inouye M, Anderson S, Janjua H, Shastry R, Ho CK, Wang D, Wang H, Jiang M, Montelione GT, Stuart DI, Owens RJ, Daenke S, Schutz A, Heinemann U, Yokoyama S, Bussow K, Gunsalus KC (2008) Protein production and purification. Nat Meth 5(2):135–146

Hou J, Xue J, Wang C, Liu L, Zhang D, Wang Z, Li W, Zheng Y, Sung C (2012) Microbial transformation of ginsenoside Rg3 to ginsenoside Rh2 by *Esteya vermicola* CNU 120806. World J Microbiol Biotechnol 28(4):1807–1811

Iguchi A, Osawa R, Kawano J, Shimizu A, Terajima J, Watanabe H (2002) Effects of repeated subculturing and prolonged storage at room temperature of enterohemorrhagic *Escherichia coli* O157:H7

on pulsed-field gel electrophoresis profiles. J Clin Microbiol 40(8):3079–3081

Imanaka T, Aiba S (1981) A perspective on the application of genetic engineering: stability of recombinant plasmid. Ann N Y Acad Sci 369(1):1–14

Jabbour D, Klippel B, Antranikian G (2012) A novel thermostable and glucose-tolerant β-glucosidase from *Fervidobacterium islandicum*. Appl Microbiol Biotechnol 93(5):1947–1956

Kalyani D, Lee K-M, Tiwari M, Ramachandran P, Kim H, Kim I-W, Jeya M, Lee J-K (2012) Characterization of a recombinant aryl β-glucosidase from *Neosartorya fischeri* NRRL181. Appl Microbiol Biotechnol 94(2):413–423

Kaul P, Asano Y (2012) Strategies for discovery and improvement of enzyme function: state of the art and opportunities. Microb Biotechnol 5(1):18–33

Ketudat Cairns J, Esen A (2010) β-Glucosidases. Cell Mol Life Sci 67(20):3389–3405

Kirk O, Borchert TV, Fuglsang CC (2002) Industrial enzyme applications. Curr Opin Biotechnol 13(4):345–351

Kitts DD, Yuan YV, Wijewickreme AN, Thompson LU (1999) Antioxidant activity of the flaxseed lignan secoisolariciresinol diglycoside and its mammalian lignan metabolites enterodiol and enterolactone. Mol Cell Biochem 202(1):91–100

Kuhnert P, Korczak BM (2006) Prediction of whole-genome DNA–DNA similarity, determination of G+C content and phylogenetic analysis within the family *Pasteurellaceae* by multilocus sequence analysis (MLSA). Microbiology 152(9):2537–2548

Lee D, Redfern O, Orengo C (2007) Predicting protein function from sequence and structure. Nat Rev Mol Cell Biol 8(12):995–1005

Letunic I, Doerks T, Bork P (2012) SMART 7: recent updates to the protein domain annotation resource. Nucleic Acids Res 40(D1):D302–D305

Li MX, Zhu HY, Yang DH, Ma XQ, Wang CZ, Cai SQ, Liu GR, Ku BS, Liu SL (2012) Production of secoisolariciresinol from defatted flaxseed by bacterial biotransformation. J Appl Microbiol 113(6):1352–1361

Li YK, Chir J, Chen FY (2001) Catalytic mechanism of a family 3 beta-glucosidase and mutagenesis study on residue Asp-247. Biochem J 355(3):835–840

Linn S (2009) Strategies and considerations for protein purifications. Methods Enzymol 463:9–19

Love DR, Streiff MB (1987) Molecular cloning of a β-glucosidase gene from an extremely thermophilic anaerobe in *E. coli* and *B. subtilis*. Nat Biotech 5(4):384–387

Meagher LP, Beecher GR (2000) Assessment of data on the lignan content of foods. J Food Compos Anal 13(6):935–947

Michaelis L, Menten ML, Johnson KA, Goody RS (2011) The original Michaelis constant: translation of the 1913 Michaelis–Menten paper. Biochemistry 50(39):8264–8269

Morris GM, Goodsell DS, Halliday RS, Huey R, Hart WE, Belew RK, Olson AJ (1998) Automated docking using a Lamarckian genetic algorithm and an empirical binding free energy function. J Comput Chem 19(14):1639–1662

Morris GM, Huey R, Lindstrom W, Sanner MF, Belew RK, Goodsell DS, Olson AJ (2009) AutoDock4 and AutoDockTools4: automated docking with selective receptor flexibility. J Comput Chem 30(16):2785–2791

Nakajima M, Yamashita T, Takahashi M, Nakano Y, Takeda T (2012) Identification, cloning, and characterization of β-glucosidase from *Ustilago esculenta*. Appl Microbiol Biotechnol 93(5):1989–1998

Opassiri R, Pomthong B, Onkoksoong T, Akiyama T, Esen A, Cairns JRK (2006) Analysis of rice glycosyl hydrolase family 1 and expression of Os4bglu12 β-glucosidase. BMC Plant Biol 6:33

Petersen TN, Brunak S, von Heijne G, Nielsen H (2011) SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat Meth 8(10):785–786

Pozzo T, Pasten JL, Karlsson EN, Logan DT (2010) Structural and functional analyses of β-glucosidase 3B from *Thermotoga neapolitana*: a thermostable three-domain representative of glycoside hydrolase 3. J Mol Biol 397(3):724–739

Quan L-H, Min J-W, Yang D-U, Kim Y-J, Yang D-C (2012) Enzymatic biotransformation of ginsenoside Rb1 to 20(S)-Rg3 by recombinant β-glucosidase from *Microbacterium esteraromaticum*. Appl Microbiol Biotechnol 94(2):377–384

Rabhi-Essafi I, Sadok A, Khalaf N, Fathallah DM (2007) A strategy for high-level expression of soluble and functional human interferon α as a GST-fusion protein in *E. coli*. Protein Eng Des Sel 20(5):201–209

Raffaelli B, Hoikkala A, Leppälä E, Wähälä K (2002) Enterolignans. J Chromatogr B 777(1–2):29–43

Reed MC, Lieb A, Nijhout HF (2010) The biological significance of substrate inhibition: a mechanism with diverse functions. BioEssays 32(5):422–429

Rojas A, Romeu A (1996) A sequence analysis of the β-glucosidase subfamily B. FEBS Lett 378(1):93–97

Sambrook J, Russell DW (2001) Purification of maltose-binding fusion proteins by affinity chromatography on amylose resin. In: Sambrook J (ed) Molecular cloning: a laboratory manual, 3rd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp 15.40–15.43

Schwede T, Kopp J, Guex N, Peitsch MC (2003) Swiss-Model: an automated protein homology-modeling server. Nucleic Acids Res 31(13):3381–3385

Sigrist CJA, Cerutti L, de Castro E, Langendijk-Genevaux PS, Bulliard V, Bairoch A, Hulo N (2010) PROSITE, a protein domain database for functional characterization and annotation. Nucleic Acids Res 38(suppl 1):D161–D166

Smith C (2005) Striving for purity: advances in protein purification. Nat Meth 2(1):71–77

Studier FW (2005) Protein production by auto-induction in high density shaking cultures. Protein Expr Purif 41(1):207–234

von der Lieth CW, Lutteke T, Frank M (2006) The role of informatics in glycobiology research with special emphasis on automatic interpretation of MS spectra. Biochim Biophys Acta 1760(4):568–577

Wahler D, Reymond J-L (2001) High-throughput screening for biocatalysts. Curr Opin Biotechnol 12(6):535–544

Wang CZ, Ma XQ, Yang DH, Guo ZR, Liu GR, Zhao GX, Tang J, Zhang YN, Ma M, Cai SQ, Ku BS, Liu SL (2010) Production of enterodiol from defatted flaxseeds through biotransformation by human intestinal bacteria. BMC Microbiol 10:115

Wang L-Q (2002) Mammalian phytoestrogens: enterodiol and enterolactone. J Chromatogr B 777(1–2):289–309

Wang LQ, Meselhy MR, Li Y, Qin GW, Hattori M (2000) Human intestinal bacteria capable of transforming secoisolariciresinol diglucoside to mammalian lignans, enterodiol and enterolactone. Chem Pharm Bull 48(11):1606–1610

Woodley JM (2013) Protein engineering of enzymes for process applications. Curr Opin Chem Biol 17(2):310–316

Yan Q, Zhou W, Li X, Feng M, Zhou P (2008) Purification method improvement and characterization of a novel ginsenoside-hydrolyzing beta-glucosidase from *Paecilomyces Bainier* sp. 229. Biosci Biotechnol Biochem 72(2):352–359

Zhang C, Zhong Y, Sun X, Ma Y (2009) Purification and characterization of SDG-β-D-glucosidase hydrolyzing secoisolariciresinol diglucoside to secoisolariciresinol from *Aspergillus oryzae*. Process Biochem 44(6):607–611