Songklanakarin J. Sci. Technol.
42 (3), 549-556, May - Jun. 2020

http://www.sjst.psu.ac.th

Original Article

Characterization of a recombinant β-glucosidase of GH3 family from glucosinolate-metabolizing human gut bacterium *Enterococcus casseliflavus* CP1 for nitrile production

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Received: 6 September 2018; Revised: 29 December 2018; Accepted: 14 February 2019

Abstract

A recombinant β-glucosidase human gut bacterium capable of nitrile production from desulfo-glucosinolates was studied. The *bgl4* gene (2,151 bp) from *Enterococcus casseliflavus* CP1 was cloned and overexpressed in *Escherichia coli* BL21(DE3) at 25 °C for 16 h in LB medium using 0.5 mM isopropyl β-D-1-thiogalactopyranoside inducer. The recombinant bgl4 enzyme (79 kDa) was purified using Ni^{2+} affinity column chromatography. This recombinant bgl4 enzyme of the glycosyl hydrolase 3 family did not degrade glucosinolates; however, it transformed desulfo-glucosinolates, except for desulfo-glucoraphanin, to produce the corresponding pure nitriles in citrate phosphate buffer pH 7.0 and LB medium. The bgl4 enzyme activity toward *p*NPG in buffer was optimal at pH 7.0 and 37 °C at 23.4 U/mg, and promoted by Mn^{2+}; however, activity was slightly deactivated by Fe^{2+}. This provided a possible alternative metabolic route involving nitrile formation from desulfo-glucosinolates by β-glucosidase in certain bacteria.

Keywords: β-*O*-glucosidase, desulfo-glucosinolate, enterococcus, glycosyl hydrolase, nitrile

1. Introduction

Glucosinolates are natural compounds predominant in cruciferous vegetables. They can be hydrolyzed by myrosinase (EC 3.2.1.147) to generate mainly isothiocyanates or nitriles or both, depending on the glucosinolate type, pH, and the co-factors, e.g., epithiospecifier protein (ESP) or ferrous ion in the media (Bones & Rossiter, 2006). Current evidence suggests that isothiocyanates exhibit far greater anti-carcinogenic properties than nitriles (Mithen, Bennett, & Marquez, 2010). When cruciferous vegetables are cooked, the plant myrosinase is deactivated, and thus cannot hydrolyze glucosinolates; however, recent research suggests that certain bacterial strains of human origin including *Bacteroides*, *Bifidobacterium*, and *Lactobacilli* can metabolize glucosinolates to isothiocyanates or nitriles or both (Luang-In *et al.*, 2016). Therefore, the health benefits of hydrolysis products from glucosinolate metabolism depend on the putative myrosinase activity of human gut microbiota. Glucosinolates can also be hydrolyzed by the sulfatase (EC 3.1.6.1) isolated from the snail *Helix pomatia*. This results in their transformation into desulfo-glucosinolates that cannot be hydrolyzed through standard myrosinase-catalyzed reactions (Thies, 1979). The desulfation process of glucosinolate by sulfatase and transformation of desulfo-glucosinolate by β-*O*-glucosidase/β-*S*-glucosidase to pure nitrile is shown in

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Supplementary Figure 1. Sulfatase from human gut bacteria may play a role in glucosinolate degradation, either alone or in combination with bacterial myrosinase, although bacterial sulfatases do not generally accept glucosinolates as substrates (Lu, Hashimoto, & Uda, 2011). Here, a putative β-glucosidase gene (*bgl4*) from a glucosinolate-degrading bacterium *Enterococcus casseliflavus* CP1 (Luang-In *et al.,* 2016) was cloned and overexpressed in *Escherichia coli* BL21 (DE3). The gene was characterized for the first time and evaluated for its involvement in bacterial metabolism of desulfo-glucosinolate.

2. Materials and Methods

2.1 Materials, strain, vector, and media

Glucosinolates were prepared as substrates following Luang-In *et al.* (2016). The corresponding intact glucosinolates were desulfated according to the methodology used by Lu *et al.* (2011) to prepare desulfo-glucosinolates as substrates for β-glucosidase. The strain *E. casseliflavus* CP1 (GenBank: LC375241) with glucosinolate-metabolizing ability isolated from human feces (Luang-In *et al.,* 2016) was deposited at Quadram Institute Bioscience, Norwich, UK. *E. coli* DH5α (Promega) was used for gene cloning and BL21 (DE3) (Novagen) for gene expression in LB broth. The plasmid vector pET28b(+) (Novagen) was used with a size of 5.4 kb.

2.2 Construction of recombinant plasmid

Genomic DNA of *E. casseliflavus* CP1 was isolated using a Wizard Genomic DNA Purification Kit (Promega, UK). The gene *bgl4* (GenBank: LC375242) corresponding to β-glucosidase in the glycosyl hydrolase 3 family (GH3) was amplified by *Pfu* DNA polymerase (Promega) using a gene-specific pair of forward primer: 5′ GGTGCTAGCATGAA AAATCAAACACTGG 3′ (*NheI* site) and reverse primer: 5′ GTTGAGCTCTCATAGAAGTTCGAAAGTCG 3′ (*SacI* site) under a PCR program of initial denaturation at 95 °C for 1 min followed by 28 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, extension at 72 °C for 2 min/kb and final extension at 72 °C for 5 min. The PCR product was confirmed on a 0.8% agarose gel and purified according to the QIAquick PCR Purification Kit (Qiagen) manual. The purified gene fragment was digested with both *NheI* and *SacI* (Fermentas, UK), and inserted into a pET28b+ vector of approximately 5.4 kb previously digested with identical restriction enzymes. After ligation, the mixture was transformed into competent *E. coli* DH5α cells for gene cloning. The recombinant plasmid was extracted using QIAprep Spin Miniprep Kit (Qiagen) and then transformed into *E. coli* BL21(DE3).

2.3 Recombinant protein expression and purification

Positive colonies were selected for gene insertion by colony PCR screening using gene-specific primers under the PCR program detailed previously. The positive clone was grown at 37 °C, 200 rpm in 1 L of LB medium containing 50 μg/mL of kanamycin to OD_{600nm} of ~ 0.6. Isopropyl β-D-1-thiogalactopyranoside (IPTG) (at a final concentration of 0.5 mM) was then added to induce the T7/lac promoter for 16 h at 25 °C, 200 rpm. Induced cells were centrifuged at 10,000*g* for 20 min, then resuspended in 10 mL of 0.1 M citrate phosphate buffer pH 7.0, placed on ice, and disrupted by two shots of a 30k psi disruption cycle in a tissue disrupter (Constant Cell Disruption Systems). The soluble protein was recovered after centrifugation at 16,100*g* at 4 °C for 30 min. The supernatant was filtered through a 0.2 μm syringe filter and transferred into a Ni^{2+}-attached 4 mL Profinity IMAC column (Bio-rad) pre-equilibrated with 50 mM sodium phosphate pH 8.0 (300 mM NaCl), and washed with 50 mM sodium phosphate pH 8.0 (300 mM NaCl, 5 mM imidazole). The recombinant bgl4 enzyme was eluted using 50 mM sodium phosphate pH 8.0 (300 mM NaCl, 500 mM imidazole). Active fractions were pooled, concentrated, and desalted against 100 mM citrate phosphate pH 7.0 using Amicon Ultra-15 centrifugal filter units with 10K MWCO (Millipore). Protein quantification was performed using Bradford reagent (Sigma) with bovine serum albumin as a standard. Proteins were separated on a denatured discontinuous SDS-PAGE. Sizes of the purified enzymes were estimated by comparison with standard molecular weight markers. The partially purified protein fraction was also tested for myrosinase activity or sulfatase activity in the enzyme assays. Myrosinase activity was tested by incubating glucosinolate in buffer for 30 min and GOD-PERID was added. A green color was not developed due to absence of glucose product from glucosinolate which indicated that glucosinolate hydrolysis did not occur. Sulfatase activity was tested by incubating with the yellow substrate, *p*-nitrocatechol sulfate dipotassium in buffer for 30 min. Red-coloured *p*-nitrocatechol product was not detected which indicated that sulfate hydrolysis did not occur.

2.4 Characterization of recombinant bgl4 enzyme

The optimal pH for the purified enzyme was examined in 0.1 M citrate phosphate buffer (pH 3.0–8.0) at 37 °C using *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) as a substrate. The optimal temperature was also determined. Citrate phosphate buffer (0.1 M, pH 7.0) containing *p*NPG was preincubated at various temperatures (4–80 °C) for 5 min before adding the enzyme. Percentage activities were obtained under the optimal conditions. Metals ($CaCl_2$, $CoCl_2$, $MgCl_2$, $MnCl_2$, $NiCl_2$, $FeCl_3$, and $FeSO_4$, each at 1 mM) were tested to assess their effects on enzyme activity. Activities (%) of recombinant enzymes with metal ion co-factors were compared with the control without the addition of metal ions.

$$\text{Relative activity (\%)} = \frac{\text{Activity with ion addition} \times 100\%}{\text{Activity without ion addition}}$$

A standard reaction mixture containing 10 μL (1.5 mg/mL) of purified enzyme and 1 mM *p*NPG in 1 mL of 100 mM citrate phosphate buffer pH 7.0 was incubated at 37 °C for 5 min, and the reaction was stopped by adding 5 mL of 0.1 M NaOH. The A_{400nm} was measured and the amount of *p*-nitrophenol (*p*NP) product release was determined using a calibration curve of known *p*NP amounts. One unit (U) of enzyme is one μmol of *p*NP product formed per minute. The specific activity of the enzyme was also determined using several aryl glucosides and disaccharides as substrates.

2.5 Hydrolysis of glucosinolates and desulfo-glucosinolates by recombinant bgl4 enzyme

Sterile 2 mL Eppendorf tubes were used to mix 0.5 mM gluconasturtiin or desulfo-glucosinolates (desulfo-glucoraphanin, desulfo-erucin, desulfo-gluconasturtiin) with 1.5 mg of purified recombinant bgl4 enzyme in 1 mL of sterilized LB medium (pH 7.0), or citrate phosphate buffer pH 7.0 containing 1 mM $FeSO_4$, or $MnCl_2$ as co-factors aerobically incubated for 16 h at 37 °C without shaking. As a negative control, 0.5 mM gluconasturtiin or desulfo-glucosinolates (desulfo-glucoraphanin, desulfo-erucin, desulfo-gluconasturtiin) in 1 mL of sterilized LB medium or buffer containing metal ion without the recombinant bgl4 enzyme was incubated under the same operating conditions. After incubation, the reaction mixtures were extracted with 1 mL dichloromethane and the extract was analyzed by GC-MS (Luang-In *et al.*, 2016) to detect the degradation products of gluconasturtiin or desulfo-glucosinolates (desulfo-glucoraphanin, desulfo-erucin, desulfo-gluconasturtiin) by the recombinant bgl4 enzyme activity. The nitrile products detected in this work included erucin nitrile (129 (M+), 87, 61, 55) at 17.4 min and phenethyl nitrile (131 (M+), 91, 62) at 18.6 min. The product concentration was calculated using external standard curves of authentic standards of nitriles.

2.6 Statistical analysis

Triplicate measurements were conducted for each treatment with results expressed as means±standard deviation (SD). Significant differences between means were calculated by one-way analysis of variance (ANOVA) and Duncan's multiple range test at $P<0.05$ using the SPSS package for the social sciences.

3. Results and Discussion

3.1 Cloning of the *bgl4* gene

The well-characterized myrosinase from *Brevicoryne brassicae* (cabbage aphid) (GenBank: AF203780.1) was thought to be more closely related to bacterial myrosinases than plant myrosinase and used as a reference search for the putative β-glucosidase in *E. casseliflavus* CP1. The *bgl4* gene (GenBank:) corresponding to β-glucosidase in the GH3 family (Supplementary Table 1) with 30% identity to the cabbage aphid myrosinase gene was identified. Our preliminary results showed that the recombinant bgl4 enzyme did not exhibit myrosinase activity toward glucosinolate substrate (data not shown). Thus, it was evaluated for hydrolytic capacity toward desulfo-glucosinolates instead. Multiple sequence alignment and the phylogenetic tree among the seven putative β-glucosidases of the GH3 family in *E. casseliflavus* CP1 showed some conservation of residues as well as the aspartate (D) active site as opposed to the aphid myrosinase (GH1 family) (Supplementary Figure 2). The phylogenetic tree shows that aphid myrosinase is more closely related to the GH1 family, bgl1 (EEV30264.1), pBgl1 (WP_010748554.1), and 6pbg (EEV30191.1) than to the GH3 family, bgl2 (WP_123834560.1), bgl3 (WP_005231347.1), bgl4 (BBD17819.1), and a more distant GH1 enzyme bgl5 (WP_005228066.1).

3.2 Purification of recombinant bgl4 enzyme

The Ni^{2+}-affinity chromatography was used to purify a soluble recombinant bgl4 enzyme to homogeneity through a single purification step with a final yield at 49%, a 13-fold increase in purity (Table 1), and a molecular weight of approximately 79 kDa (Figure 1). The partially purified protein fraction was tested for myrosinase activity or sulfatase activity in the enzyme assays and neither activity was detected (data not shown). The enzyme fraction was then tested for β-*O*-glucosidase activity in the next experiment.

3.3 Optimum pH and temperature

The β-*O*-glucosidase activity of bgl4 enzyme for *p*NPG substrate was optimal at pH 7.0 and 37 °C (Figure 2) in 100 mM citrate phosphate buffer. The bgl4 enzyme activity at 23.4±0.31 U/mg was regarded as 100% enzyme activity. Residual activity of 40% was found at 4 °C, with 80–90% activity after 4–6 months storage in 100 mM citrate phosphate

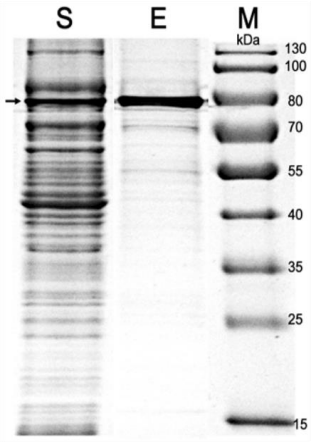

Figure 1. SDS-PAGE of recombinant bgl4 enzyme at purification steps. Crude extract and purified bgl4 were analyzed on a 12.5% SDS-PAGE gel: lane S, soluble crude extract; lane M, protein marker; lane E, bgl4 elution after purification using Ni^{2+}-affinity IMAC column chromatography. Arrow indicates the recombinant bgl4 enzyme.

Table 1. Purification of the bgl4 enzyme.

Purification Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purity (Fold)	Yield (%)
Crude extract	382	212	1.8	1	100
Ni^{2+} affinity column chromatography	187	8	23.0	13	49

One unit (U) of the purified bgl4 enzyme liberated 1 μmol min^{-1} of *p*NP product.

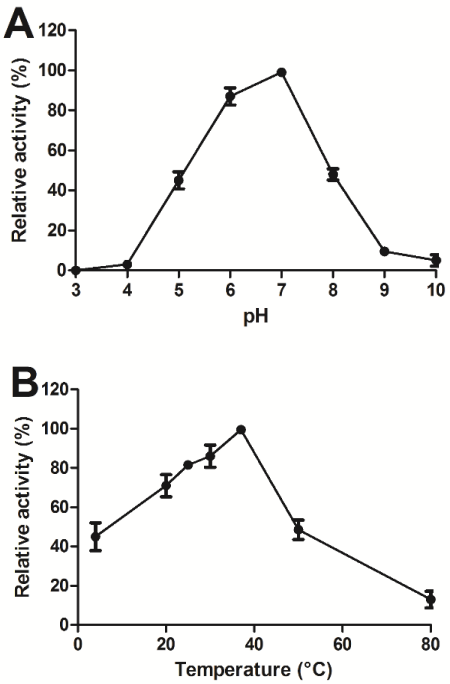

Figure 2. Effect of pH (A) and temperature (B) on catalytic activity of bgl4 enzyme. Enzyme activity was expressed as a percentage relative to pH 7.0 and 37 °C (100%, 23.4 U/mg) in 100 mM citrate phosphate buffer using 1 mM *p*NPG substrate. Values are means of triplicates.

buffer pH 7.0 at 4 °C (data not shown) which suggested high stability in the buffer. In comparison, β-glucosidase from *Caldicellulosiruptor saccharolyticus* DSM 8903 with a specific activity of 13 U/mg for *p*NPG substrate was identified as a single 54 kDa band on SDS-PAGE with optimal activity at pH 5.5 and 70 °C (Hong *et al.*, 2009).

3.4 Effect of metal ions on recombinant bgl4 enzyme

The recombinant bgl4 enzyme activity was slightly destabilized by Fe^{2+}, Mg^{2+}, Ca^{2+}, and Fe^{3+} and highly stimulated by Mn^{2+} (Table 2). In contrast, a recombinant β-glucosidase (EC 3.2.1.21) from the extremely thermophilic anaerobic bacterium Tp8 cloned and expressed in *E. coli* showed a broad specificity for β-D-glucosides, galactosides, fucosides, and xylosides (Plant, Oliver, Patchett, Daniel, & Morgan, 1988). Its β-glucosidase activity was destabilized by Sr^{2+}, Co^{2+}, Ca^{2+}, Mg^{2+}, and Mn^{2+}.

3.5 Substrate specific activity

The recombinant bgl4 enzyme was unable to hydrolyze α-glucosides because a β-*O*-glucosidase can only hydrolyze substrates with β-*O*-glucosidic bonds to release β-glucose (Table 3). Instead, bgl4 enzyme hydrolyzed both β-*O*-glucosidic bonds of β-glucosides and the β-*S*-glycosidic bonds of certain desulfo-glucosinolates obtained by sulfatase-assisted desulfation of intact glucosinolates. However, bgl4 enzyme was unable to hydrolyze β-*S*-glycosidic bonds of intact glucosinolates and octyl-β-D-1-thioglucopyranoside.

Table 2. Effect of metal ions on the activity of the recombinant bgl4 enzyme.

Metal ions (1 mM)	Relative activity (%)*
No ion	100 (23.4 U/mg)
Fe^{3+}	97
Fe^{2+}	84
Mn^{2+}	232
Mg^{2+}	95
Ca^{2+}	95
Co^{2+}	99
Ni^{2+}	99

*All reactions used 1 mM *p*NPG substrate in 0.1 M citrate phosphate buffer pH 7.0.
Relative activity (%) = $\frac{\text{Activity with ion addition x 100\%}}{\text{Activity without ion addition}}$

Table 3. Substrate specificities and kinetic values of the recombinant bgl4 enzyme.

Substrate (1 mM)	Specific activity (U/mg)*
p-Nitrophenyl-β-D-glucopyranoside	23.4±0.31 (100%)
Octyl-β-D-1-thioglucopyranoside	ND
Methyl-β-D-glucopyranoside	0.46±0.04 (2%)
Methyl-α-D-glucopyranoside	ND
Cellobiose	4.41±0.20 (19%)
Salicin	0.33±0.02 (1.4%)
Trehalose	ND
Sucrose	ND
Maltose	ND

*One unit (U) of purified bgl4 enzyme liberates 1 μmol min^{-1} of product. Numbers in brackets show specific activity in (%) relative to *p*NPG substrate defined as 100%. ND, not detected.

3.6 Nitrile production from desulfo-glucosinolate hydrolysis by bgl4 enzyme

In plants, specifier proteins, namely epithiospecifier protein (ESP) and nitrile specifier protein (NSP), are well known for the production of nitriles or epithionitriles in the myrosinase catalyzed hydrolysis of glucosinolates (Kuchernig, Burow & Wittstock, 2012). To date, no bacterial specifier proteins have been discovered that promote the formation of nitriles, and bacterial glucosinolate metabolism still remains much more complex than previously thought. In this work, myrosinase activity or sulfatase activity was not detected in the recombinant bgl4 enzyme using glucosinolates as substrates. Interestingly, desulfo-glucosinolates were previously shown as precursors of the recombinant β-*O*-glucosidase from *Caldicellulosiruptor saccharolyticum* for production of pure nitriles (Wathelet *et al.*, 2001). Therefore, desulfo-glucoraphanin, desulfo-glucoerucin, and desulfo-gluconaturtiin (0.5 mM) were used as substrates to test β-*O*-glucosidase activity of the recombinant bgl4 enzyme in 0.1 M citrate phosphate buffer pH 7.0 and also LB medium. Previously, increased nitrile production from glucosinolate degradation in the presence of Fe^{2+} was observed in the presence and absence of myrosinase (de Torres Zabala *et al.*, 2005) suggesting a Fe^{2+} dependency in nitrile production.

Likewise, we assumed there may be Fe^{2+} ions in the LB media which act as a co-factor of β-glucosidase in the catalysis of desulfo-glucosinolate to form nitriles. Thus, we tried using LB medium to test this hypothesis in this work.

The results showed that the recombinant bgl4 enzyme of the glycosyl hydrolase 3 family was able to transform desulfo-glucoerucin to produce erucin nitrile in citrate phosphate buffer pH 7.0 only when Fe^{2+} or Mn^{2+} was added and also in LB medium (Figure 3) (Table 4). Negative controls consisting of each desulfo-glucosinolate incubated with and without each metal ion without the recombinant bgl4 enzyme in the buffer at 16 h gave no nitrile production (data not shown) which indicated that nitrile production from desulfo-glucosinolate was due to the recombinant bgl4 enzyme activity. Its activity may also be promoted by Fe^{2+} or Mn^{2+} as a co-factor since no nitrile was detected without the metal ions. This finding supported the observation of epithionitrile production in one study with desulfo-sinigrin by rat intestinal microbiota (Lu *et al.,* 2011) with the hypothesis that the presence of Fe^{2+} is unlikely to be the only factor in the generation of this nitrile derivative. Interestingly, phenethyl nitrile was produced from desulfo-gluconasturtiin by the recombinant bgl4 enzyme in the buffer without metal ion addition (Table 4) which suggested that metal ion was not required for nitrile production during desulfo-gluconasturtiin metabolism, but possibly for promoting or maximum activity of the bgl4 enzyme. This was similar to the finding of Wathelet *et al.* (2001) which showed that the recombinant β-*O*-glucosidase Tp8 (from the GH1 family) was able to produce nitriles from desulfo-glucosinolates in 50 mM sodium phosphate buffer pH 6.0 without the requirement of metal ions. However, no nitrile from desulfo-glucoraphanin was detected in either the buffer or LB medium regardless of the presence or absence of metal ions (Table 4). In this respect, the reason why certain desulfo-glucosinolates were not hydrolyzed by the recombinant bgl4 enzyme may lie in the structure or property of the side chains of desulfo-glucosinolates, e.g., thio- and sulfinyl-groups in desulfo-glucoerucin and desulfo-glucoraphanin, respectively. These introduce steric effects, rendering hydrolysis by the recombinant bgl4 enzyme more difficult than desulfo-gluconasturtiin which has a benzene group. Thus, no nitrile was produced from desulfo-glucoraphanin in any of the conditions, and erucin nitrile was produced from desulfo-glucoerucin in the buffer only when facilitated by Fe^{2+} or Mn^{2+}. For a better understanding of nitrile production during desulfo-glucosinolate hydrolysis, further work is necessary, particularly the role of Fe^{2+} or other metal ion species.

Yields of erucin nitrile and phenethyl nitrile in LB medium were 13% and 16% respectively, relative to the initial dose of desulfo-glucosinolates, while yields of both nitriles in buffers were below 13% and 16%, respectively. This finding differed from a previous report that demonstrated good yields (>70%) of nitrile products from a series of desulfo-glucosinolates, including desulfo-sinigrin, desulfo-gluconasturtiin, desulfo-glucoerucin, and desulfo-glucoiberin by the recombinant β-*O*-glucosidase Tp8 (from the GH1 family), that occurred in 50 mM sodium phosphate buffer pH 6.0 without the requirement of any metal ion (Wathelet *et al.,* 2001). Differences in the structures and family origins of these two β-*O*-glucosidases may explain these disparate requirements for metal ion activity/mechanism of nitrile production from desulfo-glucosinolates. Desulfo-gluconasturtiin and desulfo-glucoerucin in LB medium were transformed into phenethyl nitrile and erucin nitrile, respectively, without the formation of isothiocyanates or other products by exploiting this glucosidase-catalyzed hydrolysis (Figure 3).

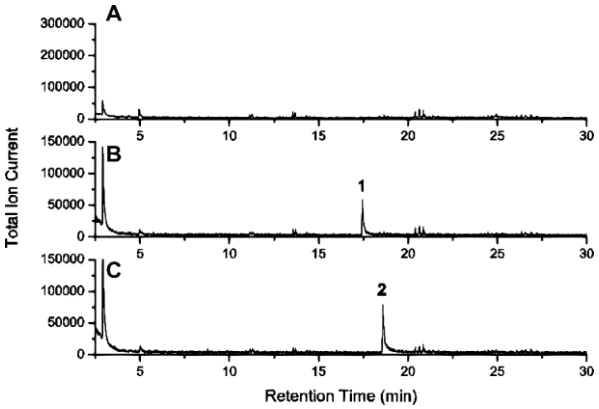

Figure 3. GC-MS chromatograms of nitrile products from hydrolysis of desulfo-glucosinolates catalyzed by bgl4 enzyme in LB medium. (A) No nitrile product from desulfo-glucoraphanin. (B) Erucin nitrile, **1** produced after 17.44 min from desulfo-glucoerucin. (C) Phenethyl nitrile, **2** produced after 18.57 min from desulfo-gluconasturtiin. All reactions were incubated with the recombinant bgl4 enzyme (50 µL) in 1 mL LB medium (pH 7.0) for 16 h at 37 °C. Figures represent triplicate averages.

Table 4. Nitrile production from desulfo-glucosinolates by recombinant bgl4 enzyme.

Substrate (500 µM)	Product	Product concentration (µM)			
		Buffer[a]			LB[b]
		No ion	Fe^{2+}	Mn^{2+}	No ion
DS-GRP	ND	ND	ND	ND	ND
DS-GER	ERN NIT	ND	47.1±4.06b	63.9±2.41a	66.1±3.56a
DS-GNT	PNIT	74.3±7.02a	n.d.	n.d.	80.5±1.57a

[a]0.1 M citrate phosphate buffer pH 7.0 in the absence or presence of 1 mM metal ions.
[b]LB medium pH 7.0 with no metal ion addition. Values are means±SD of triplicates.
Different small letters across rows represent statistically significant differences (P<0.05).
Abbreviations: DS-GRP, desulfo-glucoraphanin; DS-GER, desulfo-erucin; DS-GNT, desulfo-gluconasturtiin; ERN NIT, erucin nitrile; PNIT, phenethyl nitrile; ND, not detected; n.d., not determined.

Our previous study demonstrated activity of the native bgl4 enzyme existing in intact *E. casseliflavus* CP1 cells that metabolized 1 mM gluconasturtiin in LB medium for 24 h at 37 °C and yielded 180 µM of phenethyl nitrile (Luang-In *et al.*, 2016). Similarly, here, 500 µM desulfo-gluconasturtiin was hydrolyzed by the recombinant bgl4 enzyme for 24 h at 37 °C to yield 74 µM of phenethyl nitrile in citrate phosphate buffer and 81 µM in LB medium. Thus, the recombinant bgl4 enzyme was as effective as the native bgl4 enzyme in *E. casseliflavus* CP1.

In terms of industrial application, the gene sequence of β-*O*-glucosidase can be identified and knocked-out of the myrosinase-positive bacteria used to ferment glucosinolate-rich vegetables or glucosinolate extracts. This would leave only the chemopreventive isothiocyanate products via myrosinase-catalyzed hydrolysis without the less active glucosinolate-derived nitrile by-products. However, certain nitrile products such as allyl nitrile have been shown to exhibit antioxidant defense in the body and act as inducers of phase II enzymes glutathione S-transferase, quinone reductase, and glutathione in several tissues (Tanii, Higashi, Nishimura, Higuchi, & Saijoh, 2008). Crambene nitrile may also prevent tumor growth by inducing cell cycle arrest in the G2/M phase in mouse Hepa 1c1c7 cells, rat H4IIEC3 cells, and human HepG2 cells (Keck & Finley, 2004). The recombinant bgl4 enzyme would be useful for allyl nitrile and crambene production from glucosinolate-rich sources on an industrial scale for health-beneficial products. Moreover, the recombinant bgl4 enzyme could be used sequentially after a sulfatase from *H. pomatia* to generate thiohydroximates from glucosinolate substrates, which would be similar to a previous finding showing that a β-*O*-glucosidase from *C. saccharolyticus*, used with a sulfatase from *H. pomatia*, produced a diverse class of thiohydroximates important in both biological and industrial chemistry with possible pharmacological potential (Kopycki, Schmidt, Abel, & Grubb *et al.*, 2011).

4. Conclusions

Since the recombinant bgl4 enzyme cloned from intestinal bacteria exhibited activity towards desulfo-glucosinolates, we proposed that intact glucosinolates were transformed possibly by a bacterial sulfatase or a non-enzymatic chemical reaction to desulfo-glucosinolates which were then transformed to pure nitriles by a bacterial β-*O*-glucosidase or β-*S*-glucosidase. This report shows the transformation of desulfo-glucosinolates to pure nitriles by a recombinant bacterial β-*O*-glucosidase from GH3 family of human origin for the first time. Our findings contribute to a better understanding of bacterial glucosinolate metabolism which is deemed diverse and more complicated than previously thought.

Acknowledgements

This research was financially supported by Mahasarakham University, Thailand through Fast Track 2019. The authors declare no conflict of interest.

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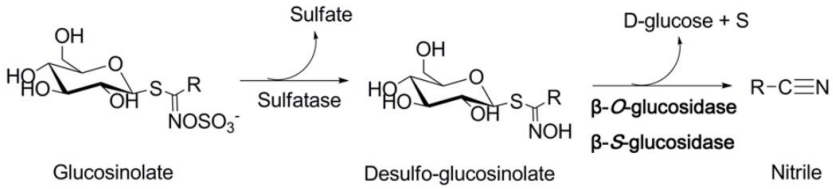
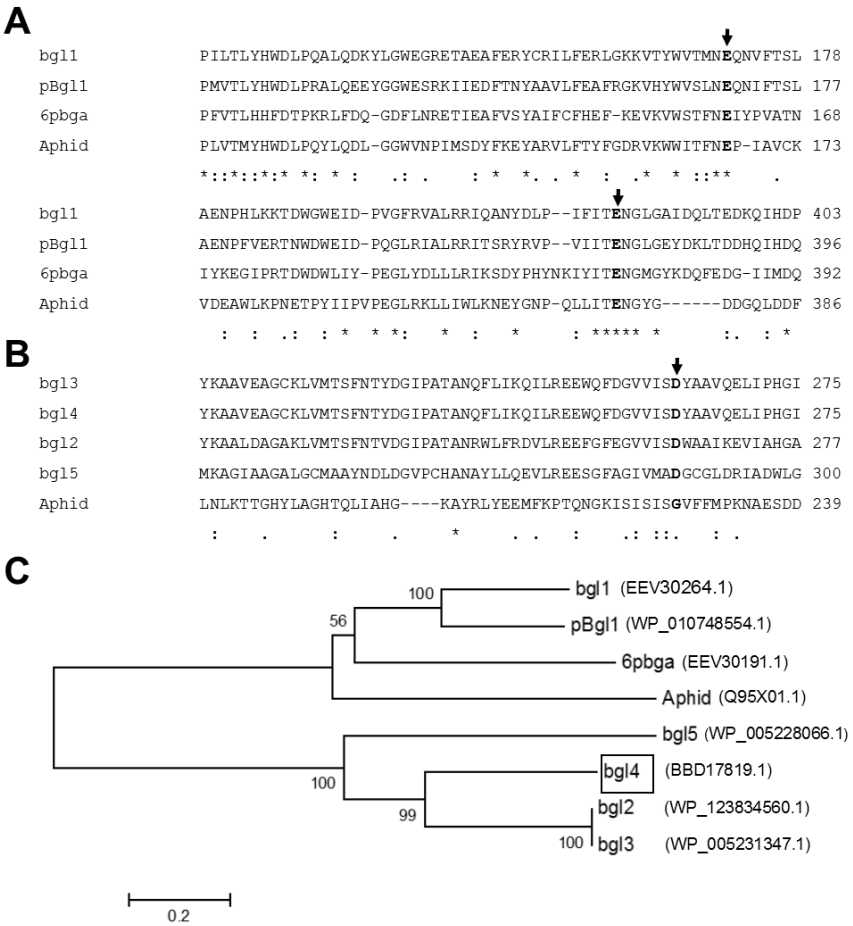
Appendix



Supplementary Figure 1. Proposed scheme of nitrile production for transformation of glucosinolate via sulfatase and β-*O*-glucosidase. Glucosinolate is desulfated by sulfatase to produce desulfo-glucosinolate which can then be transformed by β-*O*-glucosidase/β-*S*-glucosidase to produce D-glucose, sulfate ions and pure nitrile. Modified from Galletti *et al*.)2008(.



Supplementary Figure 2. Multiple sequence alignments and phylogenetic tree. (A) Multiple sequence alignment of aphid myrosinase with three β-glucosidases (GH1 family) from *E. casseliflavus* CP1. Arrows indicate active sites. (B) Multiple sequence alignment of aphid myrosinase (Aphid) with four β-glucosidases (bgl) from *E. casseliflavus* CP1. (C) Neighbor-joining phylogenetic tree derived from alignment of the above amino acid sequences by ClustalW using MEGA 7.0 software. Bgl = β-glucosidase; pBgl = phospho β-glucosidase; 6pbga = 6-phospho-β-glucosidase; Aphid = Aphid myrosinase.

Supplementary Table 1. 16S rRNA sequences of *E. casseliflavus* CP1 and its *bgl4* gene.

Gene	Length (bp)	Sequence
16S rRNA (partial sequence) GenBank Accession no. LC375241	1,447	atacatgcaagtcgaacgctttttctttcaccggagcttgctccaccgaaagaaaaagagtggcgaacgggtgagtaacacgtgggtaacctg cccatcagaaggggataacacttggaaacaggtgctaataccgtataacactattttccgcatggaagaaagttgaaaggcgcttttgcgtcac tgatggatggacccgcggtgcattagctagttggtgaggtaacggctcaccaaggcaacgatgcatagccgacctgagagggtgatcggcc acactgggactgagacacggcccagactcctacgggaggcagcagtagggaatcttcggcaatggacgaaagtctgaccgagcaacgcc gcgtgagtgaagaaggttttcggatcgtaaaactctgttgttagagaagaacaaggatgagagtaaaatgttcatcccttgacggtatctaacca gaaagccacggctaactacgtgccagcagccgcggtaatacgtaggtggcaagcgttgtccggatttattgggcgtaaagcgagcgcaggc ggtttcttaagtctgatgtgaaagccccgggctcaaccggggagggtcattggaaactgggagacttgagtgcagaagaggagagtggaatt ccatgtgtagcggtgaaatgcgtagatatatggaggaacaccagtggcgaaggcggctctctggtctgtaactgacgctgaggctcgaaagc gtggggagcgaacaggattagataccctggtagtccacgccgtaaacgatgagtgctaagtgttggagggtttccgcccttcagtgctgcagc aaacgcattaagcactccgcctggggagtacgaccgcaaggttgaaactcaaaggaattgacgggggcccgcacaagcggtggagcatgt ggtttaattcgaagcaacgcgaagaaccttaccaggtcttgacatcctttgaccactctagagatagagcttccccttcgggggcaaagtgaca ggtggtgcatggttgtcgtcagctcgtgtcgtgagatgttgggttaagtcccgcaacgagcgcaacccttattgttagttgccatcatttagttgg gcactctagcgagactgccggtgacaaaccggaggaaggtggggatgacgtcaaatcatcatgcccctatgacctgggctacacacgtgct acaatgggaagtacaacgagttgcgaagtcgcgaggctaagctaatctcttaaagcttctctcagttcggattgtaggctgcaactcgcctacat gaagccggaatcgctagtaatcgcggatcagcacgccgcggtgaatacgttcccgggccttgtacacaccgcccgtcacaccacgagagtt tgtaacacccgaagtcggtgaggtaacctttggagccagccgcctaaggtgggatag	
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