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Glucobrassicin hydrolysis by human gut bacteria and putative glycosyl hydrolases involved in the process

Abdulhadi Ali Albaser[1], John T. Rossiter[2], Vijitra Luang-In[3*]

[1] *Department of Microbiology, Sebha University, Sebha, Libya*

[2] *Faculty of Life Sciences, Imperial College London, London, United Kingdom*

[3] *Natural Antioxidant Innovation Research Unit, Department of Biotechnology, Faculty of Technology, Mahasarakham University, Mahasarakham 44150, Thailand*

[*] *Corresponding author: vijitra.l@msu.ac.th*

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Abstract - Hydrolysis of glucobrassicin by plant or bacterial myrosinase produces multiple indoles predominantly indole-3-carbinol (I3C) and 3,3'-diindolylmethane (DIM), which show promise in clinical trials as effective cancer chemopreventive agents. This work aimed to study the capacity of two human gut bacteria; *Escherichia coli* VL8 and *Enterococcus casseliflavus* CP1 to hydrolyze glucobrassicin from yellow mustard (*Sinapis alba*) seeds. Myrosinase-positive bacteria from enrichment culture were cultured in media containing 0.1 mM glucobrassicin for 16 h. The HPLC results showed that *E. casseliflavus* CP1 was able to degrade glucobrassicin by 73% at 16 h, whilst *E. coli* VL8 gave 47% degradation. The putative glycosyl hydrolase (GH) enzymes from *E. casseliflavus* CP1 involved in the hydrolysis were predicted based on the UniProt database, cloned, inserted into the pET28b(+) vector and expressed in *E. coli* BL21(DE3) as recombinant enzymes using IPTG inducer. All the tested recombinant GH enzymes did not exhibit myrosinase activity towards glucosinolate substrate; however, some displayed GH and/or β-glucosidase activity towards various sugars using GOD-PERID and β-glucosidase assays. These enzymes may be inactive in the pH buffers used or activity only occurs in intact cells where the integrity of transport/phosphorylation system is intact. In spite of some caveats in this work, the findings would still be useful to better understand glucosinolate metabolism by human gut bacteria which is linked with chemopreventive benefits.

Keywords: β-glucosidase, glucobrassicin, glucosinolate, glycosyl hydrolase, recombinant enzyme

1. Introduction

Cruciferous vegetables are the best sources of glucosinolates (GSL) (Hayes *et al.*, 2008). The indole-3-carbinol (I3C) produced when glucobrassicin is broken down. Cruciferous vegetables contain high concentrations of glucosinolate glucobrassicin. Hydrolysis of glucobrassicin by myrosinase (β-thioglucoside glucohydrolase, EC 3.2.3.1), an enzyme found in plant cells (and some gut bacteria), produces the reactive intermediate indole-3-methylisothiocyanate, which then largely decomposes into indole-3-acetonitrile and I3C. The yield of I3C from glucobrassicin in Brussels sprouts is around 20%, which equates to 0.11 to 0.18 mg/g dry weight (Kushad *et al.*, 1999).

Human gut bacteria can metabolize GSL, leading to the generation of bioactive chemicals, according to a number of studies (Luang-In *et al.*, 2014; Luang-In *et al.*, 2016). Myrosinase-like activity and glucoraphanin hydrolysis, both *in vitro* and *in vivo*, to bioactive sulforaphane (SFN) by certain microbial strains, such as gut microbiota, have been demonstrated in a number of investigations. However, only a small number of research have focused on the individual bacterial myrosinases.

Initially, a bacterial myrosinase with a molecular weight of 71.8 kDa was identified from *Enterococcus cloacae* strain 506 (Tani *et al.*, 1974). Myrosinase was purified from the bacteria *Citrobacter* sp. strain Wye1 with a molecular weight of 66 kDa. This enzyme belongs to the glycoside hydrolase family 3 (GH3). β-O-glucosidases and was shown to have the ability to generate isothiocyanates (ITCs) (Albaser *et al.*, 2016).

Another pathogenic *E. coli* 0157: H7 strain was shown to have the ability to metabolize GSL due to the presence of two genes encoding for 6-phospho-β-glucosidases; however, the activity of this enzyme has not been thoroughly characterized (Cordeiro *et al.*, 2015). The non-gut *Bacillus thuringensis* myrosinase was partially isolated and characterized by El Shora *et al.* (2016) (El-Shora *et al.*, 2016).

Although there have been numerous reports of myrosinase-like bacterial activity, there have been surprisingly few investigations characterizing these enzymes, and the GSL metabolites that are produced have been shown to be highly variable. High interindividual variability among the species involved in ITC generation has been hypothesized as a possible explanation for the lack of specific taxonomic correlations previously described (Weir *et al.*, 2021). There is some debate as to whether or not ITCs are the primary byproduct of hydrolysis by microbiota, with some writers arguing that bacterial myrosinases, most of which belong to GH family 3, do not support complete hydrolysis (Hayes *et al.*, 2008). Myrosinase is a glucoside hydrolase belonging to the GH family, which is found in plants and aphids. Many studies have confirmed the conversion of GSL, despite our poor understanding of the specific enzymatic action of myrosinases or myrosinase-like bacterial enzymes.

However, data on glucobrassicin metabolism by human gut bacteria is still scarce. Thus, this work aimed to determine the capacity of two human gut bacteria; *E. coli* VL8 and *E. casseliflavus* CP1 whether they can hydrolyze glucobrassicin and to study the putative hydrolase enzymes involved in the hydrolysis using

recombinant DNA technology. The results from this study would be useful to better understand glucosinolate metabolism by human gut bacteria which is linked with chemopreventive benefits.

2. Materials and methods

2.1 Materials

Sinigrin was purchased from Sigma-Aldrich Co. (UK). Glucobrassicin (ca. 97% purity) from yellow mustard (*S. alba*) was extracted from powdered seed using a previously reported method (Luang-In *et al.*, 2014). Allyl isothiocyanate (AITC) was purchased from Sigma-Aldrich Co. (UK). The *Lactobacillus agilis* R16 strain used in this work was obtained from Llanos-Palop *et al.* (1995).

2.2 Enrichment culture technique to isolate mysosinase-positive human gut bacteria

Using a Stomacher 400 homogenizer (Seward, UK) at 180 g for 45 s, a healthy volunteer's fresh feces was homogenized in Phosphate Buffer Saline (PBS), pH 7.0. In 900 µL of culture medium with 1 mM sinigrin, 100 µL of feces homogenate was inoculated. Two liquid broths without glucose were used: Wilkins Chalgren (WC) and nutritional broth (NB). In an anaerobic cabinet (MACS-MG-1000-anaerobic workstation, DW Scientific) with 5% CO_2, 10% H_2, and 85% N_2, aliquots were serially diluted (tenfold) in the same media every two days until the 16th day. Each culture media (100 µL) was plated onto the matching selective agar (1.5% agar w/v added to liquid broth) containing 1 mM sinigrin at day 16 and cultured in an anaerobic cabinet at 37°C until colonies were visible. Each selective medium's colonies with varied morphologies were subcultured overnight in broths containing 1 mM sinigrin. To identify myrosinase-positive colonies, GC-MS analysis was performed on each bacterial supernatant for sinigrin hydrolysis products as previously studied (Luang-In *et al.*, 2014). The selected bacteria were identified using 16S rRNA gene analysis (Luang-In *et al.*, 2014).

2.3 Bacterial cultivation and HPLC analysis for GSL hydrolysis

Strains of *E. casseliflavus* CP1 and *E. coli* VL8 were subcultured in 5 mL WC and NB (containing 0.1 mM glucobrassicin) overnight at 37°C in a 2.5 L Anaerobic jar with an AnaeroGen (Oxoid, UK) sachet to maintain anaerobic conditions. The next day, 100 µL of the overnight culture was inoculated into 900 µL of fresh media containing 0.1 mM glucobrassicin. For each time interval, biological triplicates were incubated at 37°C for 16 h under the same circumstances. The cultures were then centrifuged for 5 min at 16,000×g. As previously investigated, aliquots of the clear supernatants were submitted to HPLC examination for GSL hydrolysis (Luang-In *et al.*, 2016).

2.4 Protein extractions

Each bacterium was anaerobically cultivated overnight at 37°C in 100 mL of matching media supplemented with 1 mM sinigrin (induced cells) or without it (controlled cells). The bacterial culture was centrifuged at 4,000×g for 15 min at 4°C,

and the pellets were washed twice with 0.1 M citrate phosphate buffer, pH 7.0, to eliminate any residues of GSL or ITC products. The bacterial suspensions in the same buffer (with 20 µL protease inhibitor cocktails (Melford, UK) added) were then disrupted with two 30k psi shoots from a cell disruption machine (Constant Systems, UK). Whole cell lysates were centrifuged at 16,000×g for 30 min at 4°C, and the clear supernatant, known as cell-free extracts, was collected and sterilely filtered. Using Bradford's reagent, the amount of protein was quantified (Bio-Rad, UK). Protein analysis was performed using both native gel and SDS-PAGE on whole cell lysates, cell-free extracts, and broths.

2.5 Protein analysis by native gel electrophoresis and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The native gels were prepared in the same manner as the denatured SDS-PAGE gels (12.5%), but no SDS was added to any of the solutions. Protein samples (20 µg) were thoroughly combined with twofold native gel loading buffer. The native gel was electrophoresed for 2 h at 100 V. One portion of the gel was then stained with Coomassie Brilliant Blue R-250 solution, while the other was incubated at 37°C for 1 h with substrate solution (sufficient to cover the gel). The solution of 50 mM sodium acetate buffer (pH 7.0), 10 mM sinigrin, 0.5 mM ascorbic acid, and 50 mM barium chloride were included in the substrate solution (Sigma-Aldrich, UK). On the gel, GSL degradation was indicated by a white precipitate of barium sulfate. Positive control, purified myrosinase from *S. alba* (5 µg), was also put on the native gel to evaluate the validity of native gels for detecting GSL-degrading activity.

Twenty micrograms of protein samples were combined with 2×SDS PAGE loading buffer. Using a Mini-PROTEAN Tetra Cell equipment, the mixture was resolved on a 12.5% SDS-PAGE gel in a 1×SDS running buffer after being heated at 100°C for 2 min (Bio-Rad, UK). Fifty minutes were spent electrophoresizing the gel at 180 V. The gel was then stained with Coomassie Brilliant Blue R-250 staining solution for 15 min, washed once with Milli-Q water, then destained with the destaining solution until the gel background became transparent and the protein bands were visible.

2.6 UniProt Search for putative glycosyl hydrolases involved in GSL hydrolysis

Bacterial proteins with some degrees of similarity to the existing well-characterised aphid myrosinase from *Brevicoryne brassicae* (UniProt accession no. Q95X01) were searched for. This is because aphid myrosinase is assumingly more closely related to bacterial myrosinases than plant myrosinase (Jones *et al.*, 2002). The genome/proteome database of *E. casseliflavus* CP1 is not available. However, the close relative bacteria *E. casseliflavus* strain EC10/20/30 is accessible. There are seven candidate GSL-degrading enzymes (with the highest 25-50% similarity to aphid myrosinase with different Max Scores) of *E. casseliflavus* EC20 to be cloned from genomic DNA of *E. casseliflavus* CP1 (Table 1). The genes with the highest similarity to aphid myrosinase chosen to be cloned from *E. casseliflavus* CP1 (Table 1) have met the selection criteria; (i) Based on BLASTp

searches, these candidate proteins showed at least 25% sequence similarity to aphid myrosinase, (ii) The chosen proteins are ranked within top 10 according to Max score (BLASTp search) from GH1 and GH3 families that have similar sequences to aphid myrosinase. The rationale was that the putative bacterial GSL-degrading enzymes may come from any GH families than GH1 or they may be periplasmic enzyme or 6-phospho-β-glucosidase.

Primers with designated restriction sites for PCR experiments were synthesized by Sigma-Aldrich primer synthesis service. These primers are listed in Table 2.

Table 1. List of putative bacterial GSL-degrading enzymes of *E. casseliflavus* strain EC10/20/30 from Uniprot with high similarity to aphid myrosinase

UniProt assession no.	Gene name	Assigned name	Gene family	Gene length (bp)	Protein length (amino acid)	Protein size (kDa)	pI	% Sequence identity*
C9CJJ3	Glycoside Hydrolase	*GH3#1*	GH3	2211	736	81	5.51	50 (16.9)
C9CMM9	Periplasmic β-glucosidase	*pBgl*	GH3	2256	751	83	5.93	33 (18.1)
C9CKZ9	Glycoside Hydrolase	*GH3#3*	GH3	2151	716	79	4.73	32 (17.7)
A0A8D9WHF4	Glycoside Hydrolase	*GH3#2*	GH3	1488	495	54	5.15	25 (12.3)
C9ABS9	β-glucosidase	*bgl*	GH1	1470	488	56	5.21	34 (244)
C9A961	Glycoside Hydrolase	*GH1*	GH1	1437	478	55	5.18	33 (234)
C9CMX3	6-phospho-βgalactosidase	*6pbg1*	GH1	1407	468	54	5.18	30 (172)

* Amino acid sequence identity (%) was from BLASTp search. Numbers in brackets are Max Score.

Table 2. Primers used to clone putative glycosyl hydrolase genes from *E. casseliflavus* CP1

No.	Primer name*	Primer sequence (5'-3')	Restriction site
1	bgl-F	GGTTTGCCATATGTTTCACACAAACT	*NdeI*
2	bgl-R	AGGGAGCTCTCATGTTTCACTTGTC	*SacI*
3	GH3#1-F	GGTCATATGGAACAGCAGAAATTAACCGA	*NdeI*
4	GH3#1-R	GTTGAGCTCTTACCTAACTAATTGCAGGG	*SacI*
5	GH1-F	GGTTTGCCATATGGATCATAAACAACT	*NdeI*
6	GH1-R	GTTGAGCTCCTAGCACTCTTGC	*SacI*
7	GH3#2-F	GGTCATATGAAAAATCAAACACTGGTA	*NdeI*
8	GH3#2-R	GTTGAGCTCTTACGTTCGACTGCC	*SacI*
9	GH3#3-F	GGTGCTAGCATGAAAAATCAAACACTGG	*NheI*
10	GH3#3-R	GTTGAGCTCTCATAGAAGTTCGAAAGTCG	*SacI*

Table 2. Primers used to clone putative glycosyl hydrolase genes from *E. casseliflavus* CP1 (cont.)

No.	Primer name*	Primer sequence (5'-3')	Restriction site
11	6pbg1-F	GGTTTGCCATATGTACATGCTTAAATTACC	*NdeI*
12	6pbg1-R	CCAGAGCTCTTAAATAATCGTTTTGGTT	*SacI*
13	pBgl-F	GGTCATATGGAGAAGCATATGATTGAG	*NdeI*
14	pBgl-R	GTTGAGCTCTCATTCTTTTGCTCCTTT	*SacI*

* Referred to Table 1 for gene assigned names. Restriction sites are underlined. F: Forward primer; R: Reverse primer.

2.7 Recombinant plasmid construction

Genomic DNA from *E. casseliflavus* CP1 was obtained using a Wizard Genomic DNA Purification Kit (Promega, UK). Seven genes (Table 1) were cloned by *Pfu* DNA polymerase (Promega, UK) using a gene-specific pair of forward primer and reverse primer (Table 2) for PCR (95°C for 1 min, followed by 28 three-step cycles of 95°C for 30 s, 58°C for 30 s and 72°C for 2 min/kb and final extension at 72°C for 5 min). The PCR products were confirmed on 0.8% agarose gel and obtained according to the QIAquick PCR Purification Kit (Qiagen, UK) manual. The purified gene fragments were cut with respective restriction enzymes (Table 2)(Fermentas, UK), and inserted into a pET28b(+) vector (Novagen, UK) of approximately 5.4 kb previously digested with identical restriction enzymes. Ligation of a recombinant DNA and a pET28b(+) vector was performed using T4 DNA Ligase (Promega, UK) according to the producers' specifications. A 3:1 ratio of desired PCR insert to pET28b(+) vector (50 ng) was well-mixed by pipetting in a microcentrifuge tube and left at 4°C overnight. The combination was then introduced to competent *E. coli* DH5 cells for gene cloning. QIAprep Spin Miniprep Kit (Qiagen, UK) was used to extract the recombinant plasmid, which was subsequently transformed into *E. coli* BL21 (DE3) cells via heat-shock technique.

2.8 Expression and purification of recombinant enzymes

Positive colonies harboring the genes of interest were screened and selected using gene-specific primers under the thermocycle program detailed above. The pET28b(+) - gene recombinant plasmids were extracted from *E. coli* BL21 (DE3) and sent for DNA sequencing to confirm the DNA sequence of the seven genes of *E. casseliflavus* CP1. The positive clone was cultured (1 L of LB medium mixed with 50 μg/mL of kanamycin) at 37°C and 200 rpm to reach an OD_{600} of ~ 0.6. The inducer, 0.5 mM of isopropyl β-D-1-thiogalactopyranoside (IPTG), was used to induce the T7/lac promoter for 16 h at 25°C and 200 rpm. Induced cells were pelleted at 10,000g for 20 min, resuspended in 0.1 M citrate phosphate buffer pH 7.0 (10 mL), placed on ice, and disrupted by two shots of a 30k psi disruption cycle in a tissue disrupter (Constant Cell Disruption Systems, UK). After spinning down at 16,100g for 30 min at 4°C, the soluble protein was restored. The supernatant was obtained after filtration through a 0.2 μm syringe filter and then added to a Ni^{2+}-attached 4 mL Profinity IMAC column

(Bio-Rad, UK) pre-equilibrated with 50 mM sodium phosphate pH 8.0 containing 300 mM NaCl, and washed with 50 mM sodium phosphate pH 8.0 containing 300 mM NaCl and 5 mM imidazole. Next, 50 mM sodium phosphate pH 8.0 containing 300 mM NaCl and 500 mM imidazole was used to elute the ARS enzyme. Active fractions were pooled and desalted against 100 mM citrate phosphate pH 7.0 using Amicon Ultra-15 Centrifugal Filter Units with 10K MWCO (Millipore, UK). Protein concentrations were measured using the Bradford reagent (Sigma, UK), with bovine serum albumin acting as a standard. The proteins were analyzed using a denatured, discontinuous SDS-PAGE.

2.9 GOD-PERID Assay to Determine Myrosinase/β-O-glucosidase Activity

This assay was used to determine glucose release upon GSL or β-glucoside breakdown by myrosinase activity or β-thioglucosidase/β-O-glucosidase activity, respectively (Bones, 1990). The GOD-PERID reagent consists of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) or ABTS (1 mg/mL), glucose oxidase (8 U/mL) and peroxidase (0.35 U/mL) dissolved in Tris buffer (1.2 g/100 mL) pH 7.2. To make 250 mL GOD-PERID reagent, 3 g of Tris was dissolved in distilled water with adjustment to pH 7.2 by using HCl, 12.7 mg of glucose oxidase (157.5 U/mg) was dissolved in 10 mL of Milli-Q water and then added to the Tris buffer, 4.7 mg peroxidase (148 U/mg) was dissolved in 20 mL Milli-Q water, and a 2.5 mL aliquot was added to Tris buffer, 250 mg ABTS was added and stirred to dissolve, and finally the mixture was made up to 250 mL with Milli-Q water. This reagent was stored in dark cold place until use. All the chemicals were purchased from Sigma-Aldrich, UK. GSL was added to GOD-PERID reagent as a substrate for myrosinase, and if present, the breakdown of GSL would produce AITC and/or NIT and D-glucose. This D-glucose acts as a substrate for glucose oxidase in the GOD-PERID reagent which in turn leads to the formation of a green, soluble end-product catalyzed by peroxidase. The green dye absorbance maximum of 420 nm light ($\varepsilon = 3.6 \times 10^4\ M^{-1}\ cm^{-1}$) can easily be followed with a spectrophotometer, and therefore one can easily determine whether an enzyme has myrosinase activity.

2.10 β-O-glucosidase activity assay

The reaction mixture (1 mL) contained 10 µL of protein solution (~ 20 µg), 1 mM *p*NPG (100 µL of 10 mM *p*NPG stock solution) and 100 mM citrate phosphate pH 7.0 (890 µL). After incubation at 37°C for 5 min, the reaction was stopped by adding 5 mL of 0.1 M NaOH. The absorbance was measured at 400 nm by LKB Novaspec II spectrophotometer (Pharmacia, UK). The amount of *p*NP product from the reaction can be determined by using a calibration curve of known *p*NP amounts versus A400. To make a calibration curve, different dilutions of *p*NP ranging from 0.02 µmol to 0.6 µmol from a 10 mM *p*NP stock solution dissolved in 100 mM citrate phosphate pH 7.0 were prepared in 1 mL and 5 mL of 0.1 M NaOH was added. The corresponding A400 values of the total volume of 6 mL reaction mixtures were plotted against those dilutions. All chemicals were purchased from Sigma-Aldrich, UK. One unit of β-*O*-glucosidase activity was defined as the amount of enzyme liberating 1 µmol of *p*NP per min.

2.11 Statistical analysis

Data analysis including calculation of average values, percentage products, linear regression and standard deviations were performed using Microsoft Excel 2010 or GraphPad Prism 6. The data were analyzed by one-way analysis of variance (ANOVA) with Duncan's Multiple Range Test. Differences were considered significant if $p < 0.05$.

3. Results

3.1 Screening for GSL-metabolizing human gut bacteria

It is well-known that plant myrosinase can metabolize GSL to ITC product depending upon the conditions of the hydrolysis. Accumulating evidence suggests that certain bacteria may exhibit GSL-degrading activity (or myrosinase-like activity) as ITC products were detected upon GSL incubation with bacterial culture *in vitro*. Thus, the hypothesis of this experiment is that certain human gut bacteria may be able to metabolize GSL to ITC product like plant myrosinase.

It was found that six bacteria including three Gram-positive *Enterococcus*, two Gram-negative *Escherichia coli*, and one clone SEW-E-011 were able to hydrolyze sinigrin. Most of them produced both AITC and allyl nitrile (ANIT) from sinigrin degradation except *Enterococcus* sp. C213 and *Enterococcus faecium* KT4S13 that only produced ANIT without AITC (Table 3).

E. casseliflavus CP1 and *E. coli* VL8 were chosen for further experiments as they all produced both AITC and ANIT as degradation products with high degradation rates. Importantly, accessibility to the genome/proteome database of relative *E. casseliflavus* and *E. coli* would facilitate molecular cloning work.

Table 3. Bacterial isolates exhibiting > 50% degradation of 1 mM sinigrin in 24 h anaerobic incubation at 37˚C

Broth	Bacterial strain	Type	OD_{600}	Degradation (%)	Degradation products
WC	Clone SEW-E-011	Gram +	0.557^{b}	82.3 ± 1.05^{a}	AITC and ANIT
WC	*Enterococcus casseliflavus* CP1	Gram +	0.382^{d}	78.7 ± 2.13^{a}	AITC and ANIT
WC	*Enterococcus* sp. C213	Gram +	0.487^{c}	75.1 ± 1.12^{b}	ANIT
MRS	*Lactobacillus agilis* R16	Gram +	0.771^{a}	71.7 ± 0.98^{b}	AITC and ANIT
NB	*Escherichia coli* VL8	Gram -	0.511^{b}	57.8 ± 3.11^{c}	AITC and ANIT
NB	*Escherichia coli* UMNF18	Gram -	0.474^{c}	57.4 ± 2.45^{c}	AITC and ANIT
MRS	*Enterococcus faecium* KT4S13	Gram +	0.524^{b}	50.1 ± 1.89^{d}	ANIT

Values are mean ± SD, n = 3, but only means are shown for OD_{600} values. AITC : Allyl isothiocyanate; ANIT : Allyl nitrile. The results are presented as means (n=3) standard deviation (SD). The means with the different letters ($^{a,b,c,\cdots}$) within the columns are significantly different at $p < 0.05$.

Although clone SEW-E-011 yielded the highest degradation of sinigrin, it was not chosen for further study due to unavailability of its genome/proteome database.

Table 4. Bacterial growth and glucobrassicin degradation of *E. coli* O83:H1 NRG 857C and *E. casseliflavus* NCCP-53 over a time course of 16 h

Time (h)	ECO			EC		
	OD_{600}	GBS (μM)[A]	Degradation (%)[B]	OD_{600}	GBS (μM)[A]	Degradation (%)[B]
0	0.12 ± 0.01^{e}	100 ± 2^{a}	0 ± 0^{e}	0.10 ± 0.02^{f}	100 ± 4^{a}	0 ± 0^{e}
2	0.21 ± 0.02^{d}	76 ± 3^{b}	24 ± 5^{d}	0.18 ± 0.02^{e}	65 ± 5^{b}	35 ± 7^{c}
4	0.32 ± 0.02^{c}	61 ± 5^{c}	39 ± 6^{c}	0.35 ± 0.03^{d}	62 ± 4^{b}	38 ± 4^{c}
6	0.44 ± 0.03^{b}	58 ± 2^{c}	42 ± 3^{b}	0.42 ± 0.02^{c}	58 ± 3^{b}	42 ± 5^{c}
8	0.48 ± 0.02^{a}	55 ± 5^{d}	45 ± 6^{a}	0.51 ± 0.01^{b}	35 ± 2^{c}	65 ± 3^{b}
16	0.51 ± 0.01^{a}	53 ± 4^{d}	47 ± 5^{a}	0.56 ± 0.02^{a}	27 ± 4^{d}	73 ± 5^{a}

[A] Remaining glucobrassicin (GBS) in the reaction solution, 100 μM was an initial concentration.
[B] Degradation (%) of GBS = the amount of GSL degraded in (%) relative to the initial amount.
Values are mean ± SD, n = 3. ECO : *E. coli* VL8; EC : *E. casseliflavus* CP1. The results are presented as means (n=3) standard deviation (SD). The means with the different letters ($^{a,b,c,\cdots}$) within the columns are significantly different at $p < 0.05$.

3.2 Hydrolysis of glucobrassicin

Only two bacteria, *E. coli* VL8 and *E. casseliflavus* CP1, were tested on glucobrassicin. Bacterial growth and glucobrassicin degradation in the two bacteria were monitored over a time course (Table 4). Both bacteria were able to degrade glucobrassicin with 47% degradation at 16 h by *E. coli* VL8 and 73% degradation by *E. casseliflavus* CP-1. The degradation products of glucobrassicin by these bacteria were not detected under current GC-MS conditions. The products may be extremely volatile, and thus LC-MS analysis instead of GC-MS analysis may be required for further analysis. The HPLC chromatograms of glucobrassicin metabolism by the two bacteria over a time course are shown in Figure 1.

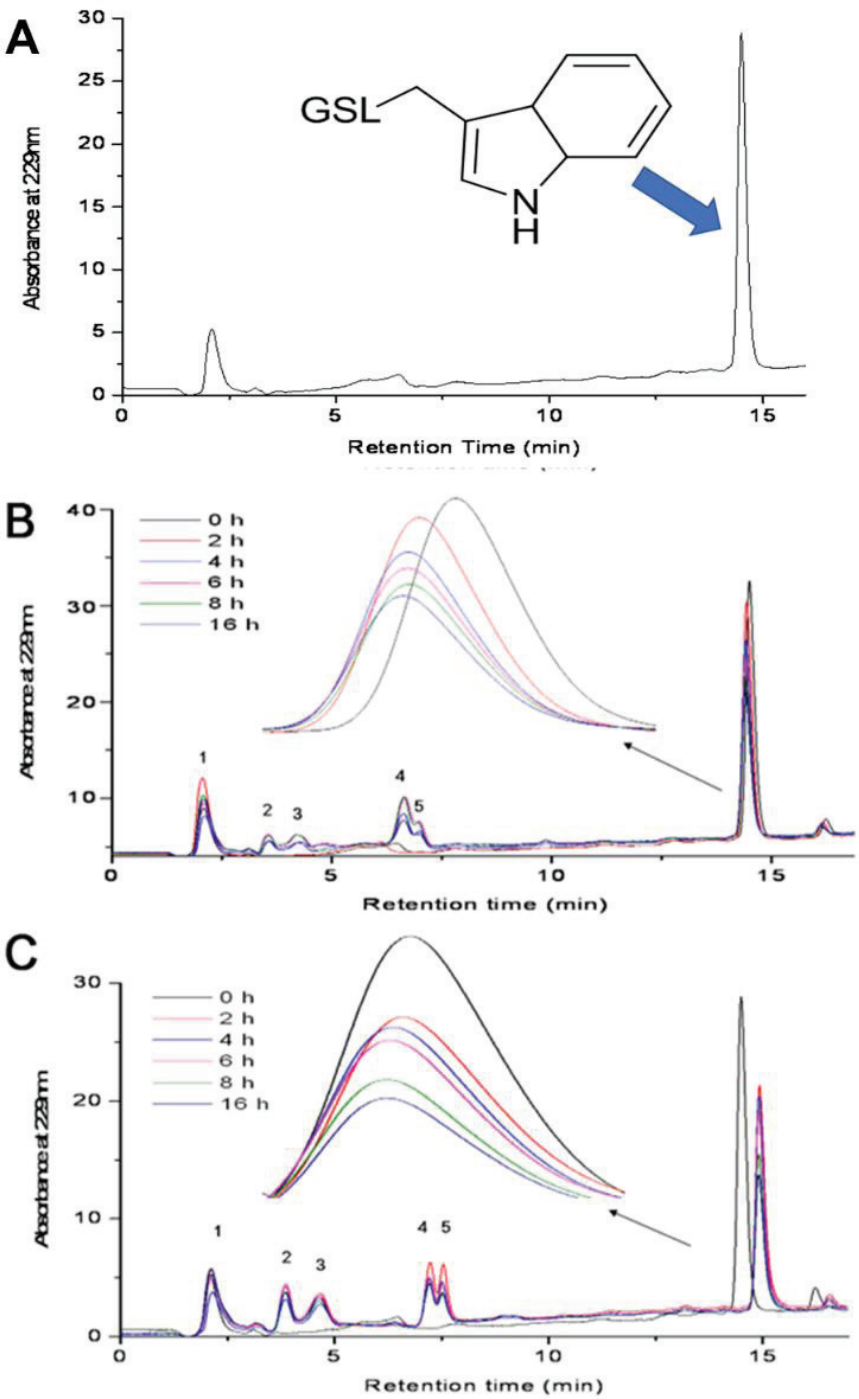


Figure 1. HPLC chromatograms of bacterial degradation of glucobrassicin over a time course. (A) Glucobrassicin (14.6 min) before fermentation. (B) *E. coli* VL8. (C) *E. casseliflavus* CP1. The arrows indicate the insets showing enlarged peaks of glucobrassicin. Peaks no. 1 to 3 found in all figures are unknown residues, no. 4 and 5 are probably polar GSLs emerged during the metabolism. These figures are representatives of triplicates with similar results.

3.3 Protein analysis of whole cell lysate, cell-free extract and broths

Native PAGE gels have been a successful assay for GSL-degrading enzyme activity. Myrosinase can be located after native polyacrylamide gel electrophoresis by incubating the gel with sinigrin. The sulfate released from sinigrin by myrosinase action reacts with barium ion in the incubation solution to produce white precipitate of barium sulfate. This activity gel assay was used to determine whether there is bacterial GSL-degrading enzyme activity in the whole cell lysates. No difference in protein band patterns was observed in native gel between non-induced and induced samples (Figure 2A).

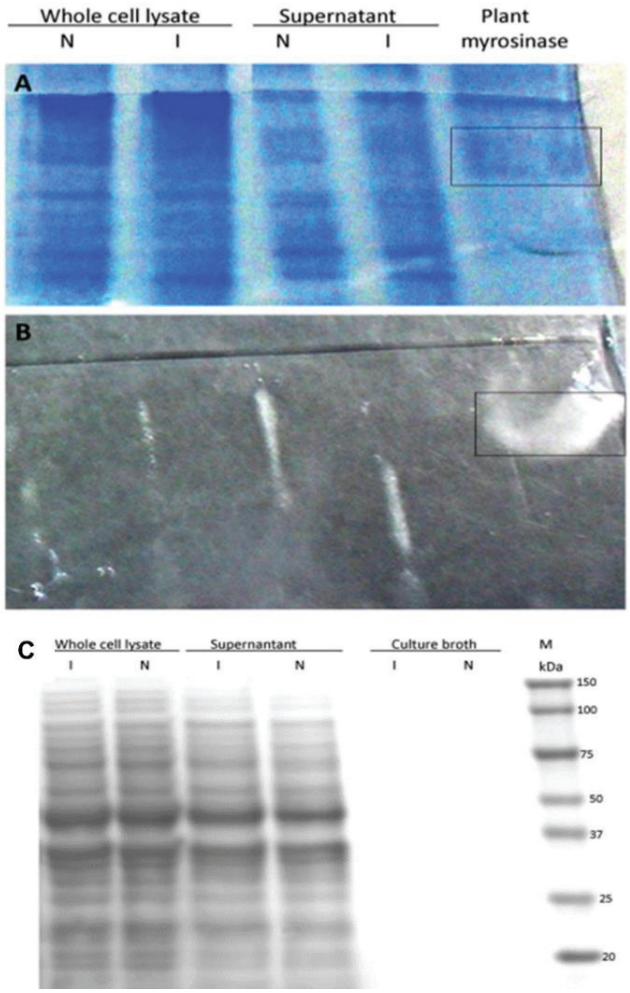


Figure 2. Gel electrophoresis results. (A) Native gel. (B) Native gel with myrosinase activity tested. (C) 12% SDS-PAGE. The whole cell lysates and supernatants of non-induced (N) and induced (I) samples of *E. coli* VL8 were analyzed on the native gels**.** Coomassie-stained native gel showing the band of purified plant myrosinase (boxed). Activity native gel showed white precipitate of barium sulfate (boxed) from the activity of plant myrosinase indictaing the presence of GSL-degrading enzyme activity towards sinigrin. Lane M is PageRuler prestained protein marker (ThermoScientific, UK).

The positive control, purified plant myrosinase from white mustard (*S. alba*), was also included to test the validity of the assay. Only the positive control displayed GSL-degrading enzyme activity as indicated by white precipitate. However, no GSL-degrading enzyme activity was detected from bacterial whole cell lysates or supernatants (Figure 2B). Other white precipitates between the lanes are false positive because once the bands excised and incubated with a solution of gluconasturtiin (aromatic GSL), phenethyl isothiocyanate (PITC) product was not detected while the plant myrosinase produced this product (data not shown). This result confirmed that putative bacterial GSL-degrading enzyme activity was not detected in cell-free extracts.

Since no bacterial GSL-degrading enzyme activity were detected from cell-free extracts of these bacteria, it was hypothesized that bacterial GSL-degrading enzyme might be extracellular (i.e. secreted into the culture broths). Interestingly, extracellular β-glucosidase secreted by fungi such as *A. fumigatus* Z5 was reported (Liu *et al.*, 2012). To test this hypothesis, the overnight culture broths from both sinigrin-induced and non-induced *E. coli* VL8 cultures were centrifuged, and the clear broths were analyzed on SDS-PAGE to see whether there were any proteins secreted into the culture broths. No protein bands were found (Figure 2C). This indicates that bacterial GSL-degrading enzyme was still in the whole cell lysate. However, no difference in protein band patterns was observed in SDS-PAGE between non-induced and induced samples.

3.4 Expressions of Recombinant Enzymes by IPTG Inducer

Recombinant proteins with the expected sizes were shown in boxes; bgl (56 kDa), GH1 (55 kDa), GH3#2 (54 kDa), 6pbg1 (54 kDa), GH3#3 (79 kDa), pBgl (83 kDa) and GH3#1 (81 kDa)(Figure 3) which are similar to the predicted molecular weight (Table 1).

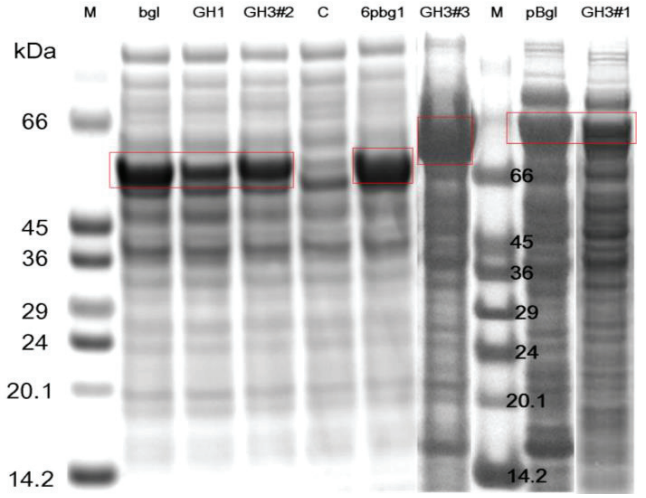
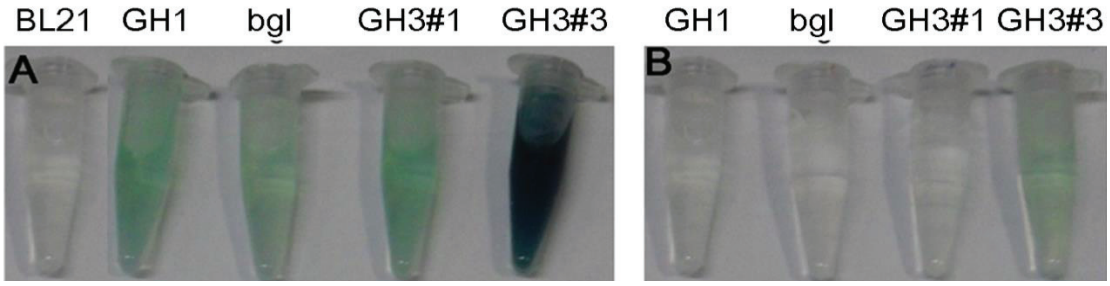


Figure 3. Recombinant protein expressions on SDS-PAGE.
Lane C = Control non-recombinant cells ;
Lane M = Low range unstained marker (Sigma-Aldrich).

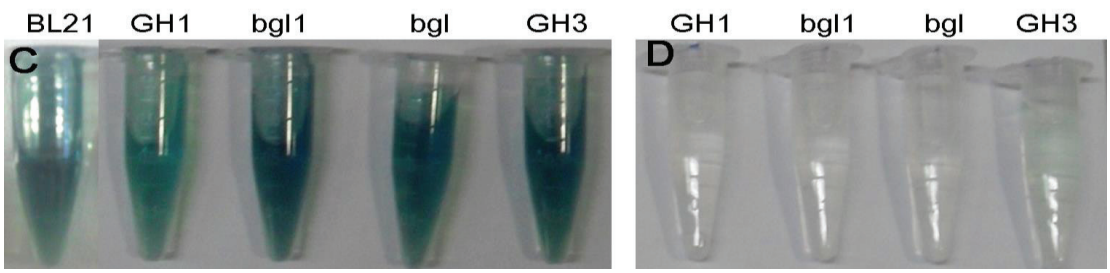
The negative control, non-recombinant BL21(DE3) culture was induced by IPTG in the same manner as other recombinant cultures. However, there was no protein band of interest expressed in this control sample as expected. These cell-free extracts were desalted to be ready for the following assays; GOD-PERID assay and β-*O*-glucosidase activity assay.

3.5 Enzyme activity of recombinant enzymes

No myrosinase activity was detected in any of cell-free extracts of the recombinant enzymes. AITC or/and ANIT were not detected by GC-MS analysis either (data not shown). The negative control containing cell-free extracts from BL21 (DE3) cells without recombinant protein expression showed negative result. The positive control (with *S. alba* myrosinase) showed development of green-coloured product from sinigrin substrate in GOD-PERID assay. This is an indication of myrosinase activity with specific activity of 28.3 ± 0.12 μmol/min/mg. This positive result from the positive control proves the validity of GOD-PERID assay for testing myrosinase activity. From this result, it was hypothesized that the current pH 7.0 might not be optimal for the myrosinase to function. Therefore, different pHs of 4.0, 5.0, 6.0 and 8.0 of 0.1 M citrate phosphate buffer and also different buffers such as 0.1 M Tris-Cl, 0.1 M PBS, and 0.1 M sodium phosphate buffer were used with the same sinigrin concentration under the same experimental conditions. In spite of several trials, green product was still not detected in any recombinant enzyme or in any buffer conditions (data not shown). Since most of the recombinant proteins come from GH family which are supposed to hydrolyze β-*O*-glucosides such as cellobiose, salicin, trehalose and methyl β-D-glucopyranoside, GOD-PERID assay was carried out with these substrates to determine whether any of the recombinant enzymes can hydrolyze them. It was found that GH1, GH3#1, bgl and GH3#3 enzymes all were able to hydrolyze cellobiose and trehalose with GH3#3 having the highest specific activity (Figure 4A and 4C). In contrast, only GH3#3 was able to hydrolyze salicin and methyl β-D-glucopyranoside (Figure 4B and 4D). The specific activity of GH3#3 on substrates in descending order is cellobiose > trehalose > salicin > methyl β-D-glucopyranoside. The negative controls (cell-free extracts of BL21(DE3) cells without any recombinant protein expression) showed no activity on most substrates except for trehalose. These results indicate that recombinant enzymes exhibit β-*O*-glucosidase or glycosyl hydrolase activity upon natural β-*O*-glucoside substrates.



(A) Specific activity (μmol/min/mg)					(B) Specific activity (μmol/min/mg)			
BL21	**GH1**	**bgl**	**GH3#1**	**GH3#3**	**GH1**	**bgl**	**GH3#1**	**GH3#3**
ND	1.43 ± 0.12[b]	0.87 ± 0.06[c]	1.47 ± 0.10[b]	6.48 ± 0.11[a]	ND	ND	ND	0.79 ± 0.05[c]

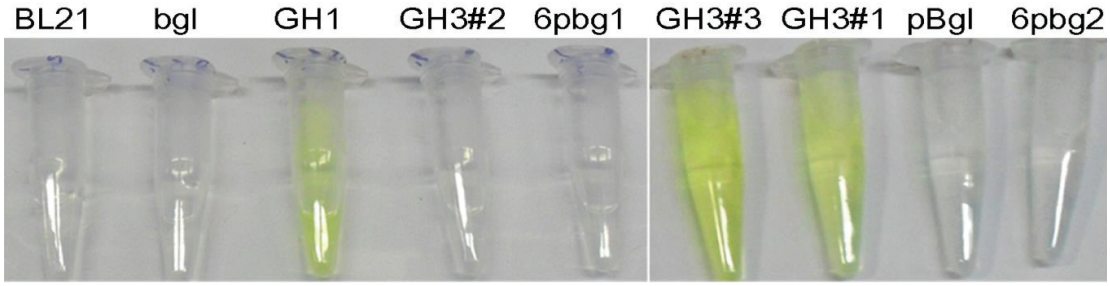


(C) Specific activity (μmol/min/mg)					(D) Specific activity (μmol/min/mg)			
BL21	**GH1**	**bgl**	**GH3#1**	**GH3#3**	**GH1**	**bgl**	**GH3#1**	**GH3#3**
2.02 ± 0.07[e]	2.67 ± 0.12[d]	3.70 ± 0.09[a]	3.41 ± 0.13[c]	3.59 ± 0.08[b]	ND	ND	ND	0.09 ± 0.02[f]

Figure 4. β-*O*-glucosidase activity using different substrates in GOD-PERID assay. (A) cellobiose (B) salicin (C) trehalose and (D) methyl β-D-glucopyranoside. BL21 is a negative control. Data of specific activity of each enzyme on each substrate was shown below the pictures. Values are means ± SD of triplicates. ND, Not detected. The means with the different letters [(a,b,c,...)] within the rows are significantly different at $p < 0.05$.

Since GOD-PERID assay only produced positive results when β-*O*-glucosides were used as substrates, it was thought that these enzymes may not exhibit β-thioglucosidase (i.e. myrosinase) activity to hydrolyze GSLs, but they may only have β-*O*-glucosidase activity. Therefore, another experiment to reassure the existence of β-*O*-glucosidase activity of these enzymes was carried out using *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) as a synthetic substrate in 0.1 M citrate phosphate buffer pH 7. If β-*O*-glucosidase activity is present, the breakdown of *p*NPG would produce D-glucose and a yellow-coloured *p*-nitrophenol (*p*NP) product that can be determined spectrophotometrically at A400nm. It is important to note that plant myrosinase from GH1 can also hydrolyze *p*NPG in addition to GSLs. It was found that three recombinant enzymes GH1, bgl and GH3#3 produced yellow-coloured products

suggesting β-*O*-glucosidase activity of these enzymes on *p*NPG substrate (Figure 5). Since the GH3#3 enzyme showed the highest specific activity on all β-*O*-glucosides tested, this enzyme was further studied in the recent work (Luang-In *et al.*, 2020) and reassigned as bgl4 enzyme.



Specific activity (μmol/min/mg)

BL21	bgl	GH1	GH3#2	6pbg1	GH3#3	GH3#1	pBgl	6pbg2
ND	ND	0.43 ± 0.11^{b}	ND	ND	1.54 ± 0.06^{a}	0.39 ± 0.08^{b}	ND	ND

Figure 5. β-*O*-glucosidase activity assay. BL21 is a negative control without recombinant enzyme. Data on specific activity of each enzyme on each substrate was shown below the pictures. Values are means ± SD of triplicates. ND, Not detected. The means with the different letters $^{(a,b,c,\ldots)}$ within the rows are significantly different at $p < 0.05$.

4. Discussion and conclusion

Since knowledge on glucobrassicin degradation by gut bacteria is still lacking, this work aimed to find out if the selected human gut bacteria were able to metabolize glucobrassicin. The HPLC results showed that *E. casseliflavus* CP1 was able to degrade glucobrassicin by 73% at 16 h indicating high capacity of glucobrassicin degradation. Previously, it was found that sauerkraut consumed soon after fermentation contains the most health-promoting ascorbigen and indole-3-carbinol from glucobrassicin metabolism by lactic acid bacteria in sauerkraut (Palani *et al.*, 2016). This implied chemopreventive benefits conferred by microbial metabolism of glucobrassicin.

A reverse genetics approach was used to identify and express the bacterial putative genes for myrosinase activity in this work. Seven genes from *E. casseliflavus* CP1 were cloned and expressed. No GSL-degrading activity was detected in any recombinant enzymes tested. These enzymes may be inactive in the pH buffers used or GSL substrate may need to be modified e.g. phosphorylation of the 6-OH group before being hydrolyzed by these enzymes and only occur in intact cells where the integrity of transport/phosphorylation system is intact. The previous study has shown that 6-phosphoryl-β-D-glucopyranosyl hydrolase (P-β-glucosidase, EC 3.2.1.86) purified from *Fusobacterium mortiferum* hydrolyzed several P-β-glucosides, including the isomeric disaccharide phosphates cellobiose-6-phosphate, gentiobiose-6-phosphate, sophorose-6-phosphate, and laminaribiose-6-phosphate, to yield glucose-6-phosphate and appropriate aglycons

(Thompson, 2002). These substrates had to be phosphorylated by a β-glucoside kinase (BglK) of *K. pneumonia* bacteria prior to the hydrolysis by 6-phospho-β-glucosidases (Thompson *et al.*, 1997). Since GSLs are β-glucosides, it is thought that they may need to be phosphorylated by β-glucoside kinase prior to being hydrolyzed by myrosinase which recognizes the phosphorylation on the GSL structure. However, this hypothesis still remains untested.

Our results showed that crude extracts of some recombinant enzymes showed β-*O*-glucosidase activity on some β-*O*-glucosides, but not GSL. This indicates that these recombinant enzymes are at least active upon these substrates with different specific activities. Information on β-*O*-glucosidases is currently limited to relatively few species of bacteria from the human colonic ecosystem (Dabek *et al.*, 2008). With this work, new data on β-*O*-glucosidases from *E. casseliflavus* NCCP-53 has been provided. Among broad substrate specificity β-glucosidases reported to date, a number of β-glucosidases have higher activity for aryl-glucosides e.g. methyl β-D-glucopyranoside and *p*NPG than cellobiose (González-Pombo *et al.*, 2008; Matsui *et al.*, 2000; Parry *et al.*, 2001). These enzymes have only 20-50% enzyme activity against cellobiose, comparing to that for the *p*NPG. Certain enzymes have only 20-50% enzyme activity against cellobiose, comparing (Hashimoto *et al.*, 1998; Marques *et al.*, 2003). In contrast, the recombinant GH3#1, GH1, GH3#3 and bgl enzymes from *E. casseliflavus* CP1 in this study have higher activity on cellobiose than aryl glucosides. Interestingly, all crude extracts of these recombinant enzymes and also the extracts from the control BL21(DE3) without recombinant enzyme expression exhibited activity for trehalose containing an α,α-1,1-glucosidic bond indicating that BL21(DE3) host expressed endogeneous trehalase enzyme to hydrolyze trehalose. Therefore, the recombinant enzymes need to be purified in order to determine whether they can hydrolyze trehalose. However, no activity was observed for salicin and and methyl β-D-glucopyranoside (except for GH3#3).

The basis of the vast diversity in biological function of β-glucosidases from different GH families is the substrate aglycone specificity differences that determine their natural substrates (Cairns & Esen, 2010). There is also a difference in substrate specificity within the GH1 and GH3 groups (Cairns & Esen, 2010). Thus, different members from different GH families and even members in the same GH family can have different activity towards the same substrate. As demonstrated in the results, the β-*O*-glucosidase activity towards *p*NPG was detected from GH1, GH3#3 and GH3#1 whereas there was no activity from other members of the same families. Thus far, bacterial GSL-degrading activity has not been found despite several experiments including cell-free extract experiment, native activity gel analysis, and reverse proteomics approaches were carried out. It was speculated that bacterial GSL-degrading enzyme system may be more complicated than previously thought.

To summarize, β-*O*-glucosidase activity with highest specific activity was found in the recombinant GH3#3 enzyme. This enzyme may be of importance in GSL metabolism in human gut bacteria. Despite several limitations of this study, the findings might still be valuable for a better understanding of glucosinolate

metabolism by human gut bacteria, which has chemopreventive benefits.

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