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Discovery of a bacterial glycoside hydrolase family 3 (GH3) β-glucosidase with myrosinase activity from a *Citrobacter* strain isolated from soil

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ABSTRACT

A *Citrobacter* strain (WYE1) was isolated from a UK soil by enrichment using the glucosinolate sinigrin as a sole carbon source. The enzyme myrosinase was purified using a combination of ion exchange and gel filtration to give a pure protein of approximately 66 kDa. The *N*-terminal amino acid and internal peptide sequence of the purified protein were determined and used to identify the gene which, based on InterPro sequence analysis, belongs to the family GH3, contains a signal peptide and is a periplasmic protein with a predicted molecular mass of 71.8 kDa. A preliminary characterization was carried out using protein extracts from cell free preparations. The apparent $K_M$ and $V_{\text{max}}$ were 0.46 mM and 4.91 mmol dm$^{-3}$ min$^{-1}$ mg$^{-1}$ respectively with sinigrin as substrate. The optimum temperature and pH for enzyme activity were 25°C and 6.0 respectively. The enzyme was marginally activated with ascorbate by a factor of 1.67.

Keywords: myrosinase, bacteria, isothiocyanate, sequence
INTRODUCTION

Bacterial myrosinases are not well understood and there has been little work on this topic. Bacterial myrosinases were first purified some years ago as an active enzyme but without any characterisation in terms of sequence.\textsuperscript{1,2} Since then there have been various reports examining the metabolism of glucosinolates by bacteria and attempts have been made to characterize the enzyme but with limited success.\textsuperscript{3-7} We have set out to isolate a bacterium that can grow on sinigrin, purify the myrosinase and obtain a sequence. β-Glucosidases (EC 3.2.1.21) catalyse the hydrolysis of the glucosidic linkages of a variety of β-glucosides and, depending on sequence and structure, can be placed in the glycoside hydrolase (GH) families GH1, GH3, GH9, GH 30 and GH116.\textsuperscript{8-10} With the exception of GH9, the remainder utilize a two-step mechanism that involves a catalytic nucleophile that displaces the aglycone and is followed by protonation of the nucleofuge and attack by a water molecule to give a glycosyl residue. Plant myrosinases (thioglucoside glucohydrolase EC 3.2.3.147) belong to the family GH1 and their structure and mechanisms have been well studied.\textsuperscript{11-14} Myrosinases hydrolyse glucosinolates, a group of plant secondary metabolites, to give a variety of products (Figure 1) depending on the presence of specifier proteins and type of glucosinolate, but the default product is an isothiocyanate (ITC).\textsuperscript{15-18} The mechanism of plant myrosinase does not utilize the typical catalytic acid/base employed by β-glucosidases but instead uses ascorbate as a catalytic base. The \textit{Sinapis alba} myrosinase is a dimer linked by a zinc atom and has a characteristic (β/α)\textsubscript{8} barrel structure in common with GH1 enzymes. The insect \textit{Brevicoryne brassicae} (cabbage aphid) contains a myrosinase that has been studied in some detail and also belongs to the glucose hydrolase family GH1\textsuperscript{19-21} and its structure has been determined.\textsuperscript{22} In contrast to the \textit{S. alba} myrosinase, the aphid myrosinase does not require ascorbate for activity and its mechanism is the same as for β-glucosidases \textit{i.e.} a nucleophile (glutamic acid residue) that forms a glycosyl-enzyme intermediate together with a glutamic acid residue that acts as a catalytic base/acid typical of most GH1 glucosidases.
There is, however, increasing interest in bacteria that can metabolise glucosinolates, particularly those of
the human gut, where the chemoprotective nature of glucosinolate hydrolysis products is of dietary
importance. In addition, soil bacteria are also of importance in terms of soil ecology and in the
application of biofumigation, where plants belonging to the Brassicaceae, which are high in glucosinolate
content, are used to fumigate soil. In this work we describe the isolation of a soil bacterium that
possesses an inducible β-glucosidase that transforms glucosinolates into isothiocyanates. The properties
and sequence of the enzyme are described.
METHODS

Glucosinolates

Glucosinolates were isolated from seed sources as previously described.\(^5\)

Isolation of a glucosinolate-metabolizing bacterial strain by enrichment

Soil was taken from a field in Wye (Kent, UK), which had previously been used for growing oilseed rape, and 1 gram was placed into 10 ml of M9 medium with sinigrin (10 mg) as the sole carbon source and placed in the dark at room temperature for 2 weeks. After gentle agitation, 1 ml of solution was removed, placed into fresh M9 media with sinigrin as the sole carbon source and incubated for a further 2 weeks. This process was repeated a further 5 times. Finally, dilutions were made and a small amount of the enrichment culture was streaked onto a nutrient agar plate for each dilution. The plates were incubated overnight at 30 °C. Colonies were added to fresh M9 media containing sinigrin as the sole carbon source and incubated at 30 °C, and the OD\(_{600\text{nm}}\) (optical density at 600nm) was monitored. The culture was centrifuged at 10000g for 5 min and stored at -20 °C until analysis. Sinigrin-metabolizing colonies were grown overnight in M9 media supplemented with sinigrin as the sole carbon source, and the following day glycerol stocks (40%) were made and stored at -80 °C.

HPLC analysis of sinigrin fermentation

The fermentation of sinigrin was monitored by removing 1 mL of culture into an Eppendorf (1.5 mL) tube and centrifuging at 10000g for 10 min. The supernatant was removed and stored at -20 °C until analysis. Samples were thawed out (1 mL), 100 µL of a barium/lead acetate solution (equimolar, 1M) were added, and samples were thoroughly mixed and allowed to stand for a few minutes. The samples were centrifuged at 10000g for 5 min and the supernatant was removed and transferred to a fresh Eppendorf tube. DEAE Sephadex-A25 (Sigma, UK) was mixed with an excess of NaOAc buffer (1M, pH 5), which was changed several times over 6 h and finally left overnight to equilibrate. The excess buffer was decanted and the ion exchanger was mixed with deionized water, decanted (x 2) and finally stored in 20
% EtOH to give an approximately 50% settled gel. One mL of suspended DEAE Sephadex-A25 slurry was pipetted into Poly-Prep Chromatography Columns (BioRad, UK) and allowed to drain. The ion exchange material was washed twice with 1 mL de-ionised water, and the sample was applied and allowed to drain. The column was washed with 2 x 1 mL de-ionised water, followed by two washes of 0.5 mL sodium acetate buffer (0.05 M, pH 5.0). Sulfatase (Sigma, UK) was added (75 µL, 0.3U/mL) carefully to the top of the gel and the column was left overnight at room temperature. The desulfo-glucosinolate (DS-GSL) was eluted with 3 x 0.5 mL of deionized water and stored at -20 °C until HPLC analysis. The DS-GSLs were analysed by HPLC on a Synergi Hydro-RP column (4 µm, 15 x 0.2 cm, Phenomenex Inc. Torrance, CA). The following conditions were used for the HPLC analysis: Water (solvent A), ACN (solvent B); 2% B (15 min), 2-25% B (2 min), 25-70% B (2 min), 70% (2 min), 70-2% B (2 min), 70-2%B (15 min); flow rate 0.2 mL/min, column temperature 35 °C. HPLC was carried out using an Agilent HP1100 system with a Waters UV detector (model 486) at a wavelength of 229 nm.

**GC-MS analysis of glucosinolate metabolites**

One mL of culture or cell free extract was removed and centrifuged at 10000g for 10 min. The supernatant was removed and transferred to a 2 mL Eppendorf tube, 0.5 mL DCM was added and the tube was vortexed for 30 s. The tubes were centrifuged at 10000g for 5 min and the DCM was carefully removed with a glass syringe. The sample was re-extracted with a further 0.5 mL DCM and combined with the first extraction. A small amount of dried MgSO₄ was added to the DCM extract to remove any water and briefly vortexed. The extract was centrifuged and the DCM was carefully removed, transferred to an Agilent 2 mL screw cap vial and stored at 4 °C until analysis.

An Agilent HP 6890 series system connected to an Agilent HP 5973 mass selective detector was used for the GC-MS analysis. A capillary column, Agilent HP-5MS (5% Phenylmethylsiloxane, 30 m × 0.25 mm i.d.; film thickness, 0.25 µm), was used with helium as the carrier gas (split mode, 25:1; splitter inlet pressure, 40 kPa). The temperature was kept at 40 °C for 10 min and ramped up to 150 °C at the rate of 4°C/min for 25 min and then to 250°C at the rate of 4°C/min for 15 min, with the flow at 1 mL/min,
average velocity of 36 cm/s, pressure at 7.56 kPa and injection volume of 1 µL. Mass spectra were obtained by electron ionization (EI) over a range of 50−550 atomic mass units. Ion source temperature was 230 °C and the electron multiplier voltage was 70.1 eV. The analysis of allyl amine, allyl nitrile and 2-propenoic acid (Sigma UK) were carried out on a Machery-Nagel (UK) Optima Delta-6 column (30 m × 0.25 mm i.d.; film thickness, 0.25 µm) using the same programme as previously described, with the exception that the final oven temperature was 200 °C. The limit of detection for pure ITC/NIT standards by GC-MS analysis was 10 ng/mL.

**16S rRNA Sequence determination, genomic DNA assembly and preparation of phylogenetic tree**

An overnight culture was centrifuged at 13000 g for 5 min, and the pellet was washed with milliQ water (x1), resuspended in 15 µL milliQ water and boiled at 95 °C for 5 min. The sample was cooled on ice, spun for 5 min at 13,000 g and the supernatant was collected. The gene was amplified by PCR using the primers AMP_F: 5': GAG AGT TTG ATY CTG GCT CAG, Tm: 60.5 and AMP_R: 5': AAG GAG GTG ATC CAR CCG CA, Tm: 68.9, GoTaq DNA polymerase, PCR buffer (Promega) and dNTPs (200 µM each). The PCR conditions were the following: initial denaturation 95 °C, 2 min (1 cycle); denaturation 95°C, 30 s (20 cycles); annealing 55 °C, 30 s (20 cycles); extension 72 °C, 1 min 30 s (20 cycles); final extension 72 °C, 1 min (1 cycle). The PCR product (1500 bp) was purified by QIAquick PCR purification kit (Qiagen) and quantified by a Nanodrop 2000 spectrometer (Thermo scientific). The product was sequenced with both primers (AMP_F and AMP_R; Eurofins Sequencing). The sequences were assembled using Seqman to create a contig and this contig was searched on the RDP database ([https://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp](https://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp)). The genomic DNA of the *Citrobacter WYE1* was sequenced at The Genome Analysis Centre, Norwich, UK, using the Illumina MiSeq platform as previously reported (29). The 251bp length of DNA reads from Genomic DNA sequencing was analysed and quality-trimmed extended contigs were produced by using SPAdes program version 3.6.1.29 These contigs of *Citrobacter WYE1* were used in construction of a phylogenetic tree. Draft genomes of different *Citrobacter* species were obtained from Genbank whole genome shotgun projects. Core genome single
nucleotide polymorphisms (SNPs) in draft genomes and *Citrobacter WYE1* were used to prepare the phylogenetic tree using parSNP program Version 1.2.30

**Myrosinase assay**

Myrosinase activity determinations were based on the glucose oxidase/peroxidase enzyme assay of glucose.31 Preparation of the GOD-PERID (glucose oxidase-peroxidase) reagent (all reagents purchased from Sigma, UK): 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) 250 mg, glucose oxidase 12.7 mg (157.5 U/mg) and peroxidase 4.7 mg (148U/mg) were dissolved in Tris buffer (3 g Tris, final concentration 0.1M, pH 7.2) to a volume of 250 mL. The reagent was mixed well, wrapped with aluminium foil and kept at 4°C. A calibration curve was obtained using glucose. Samples were assayed in a total volume of 300 µL containing the myrosinase extract (maximum volume 20 µL), 2 mM sinigrin, CPB (citrate phosphate buffer, 20 mM, pH 6.0), and incubated at 25 °C for 2 h or less depending on the activity. The reaction was stopped by placing in a heating block (95 °C) for 5 min and cooled on ice. 1 mL of GOD-PERID reagent was added to the sample and incubated for 15 min at 37 °C. The absorbance at 420 nm was measured and the glucose concentration was determined from a calibration graph.

**General assay conditions for myrosinase activity**

As there was insufficient pure protein for characterization studies, preliminary studies were carried out using cell-free protein extracts. An overnight culture of *Citrobacter WYE1* was grown on nutrient broth (NB), inoculated into M9 medium (50 mL) with 50 mg of sinigrin and incubated aerobically overnight at 30 °C in a shaking incubator at 180 rpm. The culture was centrifuged, the pellet was washed as described in the purification methods and the cells re-suspended in 5 ml of extraction buffer (CPB, 50 µL 100 xs protease inhibitor cocktail (Melford Ltd) and 1 mM DTT) and lysed using a one shot cell disruptor (CONSTANT SYSTEMS Ltd, Northants, UK). The lysate was centrifuged at 10,000 g for 15 min at 4°C. The cell-free protein extract was desalted against CPB using Econo-Pac 10DG Desalting Columns (BIORAD, UK) and stored at 4°C. The protein concentration was determined using Bradford reagent
The activity of the myrosinase was initially determined to ensure linearity and an appropriate amount of protein used for each assay. As the activity of the myrosinase markedly diminished over time, the cell-free protein extracts were used within 72 h of preparation.

**pH Optimisation**

Citrate phosphate buffer (pH range of 3.6-7.6, 20 mM) was used to determine the optimum pH for *Citrobacter WYE1* myrosinase. Crude protein extract (5 µg) was added to a mixture of sinigrin (2 mM) in CPB to a final volume of 300 µL at specific pH values over the range 3.6-7.6 and incubated at 37 °C for 2 h. The reaction was stopped by immersing the tubes in boiling water for 5 min. Glucose release measurements were then carried out using the GOD-PERID assay.

**Temperature optimization**

The effect of temperature on the activity of the *Citrobacter WYE1* myrosinase was measured over a range of temperatures (5-70 °C). A total volume (300 µL) with protein (5 µg), sinigrin (2 mM) and CPB was incubated at the specified temperature for 30 min and glucose release was assayed by the GOD Perid assay.

**Substrate specificity**

Myrosinase assays were carried out using CPB, 2 mM glucosinolate substrate, 60 min incubation and 20 µg protein. Samples for qualitative GC-MS analysis were incubated overnight and extracted with DCM as previously described for glucosinolate metabolites.

**Activation by ascorbate**

A mixture of the protein (16 µg) and CPB was incubated (30 °C, 1h) with ascorbate concentrations in the range 0.025-20 mM and gluconasturtiin (0.5 mM) to a volume of 300 µL. The reaction was stopped by boiling (5 min). ITCs were extracted with dichloromethane and quantitated by GC-MS using an Agilent...
HP-5MS column as previously described. Quantitation was carried out using a calibration graph of known phenethylisothiocyanate concentrations versus total ion current.

**Determination of apparent $K_m$ & $V_{max}$ for sinigrin**

A range of sinigrin concentrations (0.1-5 mM) was incubated (25 °C, 10 min) with protein (2 µg) and CPB in a total volume of 300 µL. The reaction was stopped by boiling (5 min) and cooled before the GOD Perid reagent (1 mL) was added and incubated (15 min, 37 °C), followed by absorbance measurement (420 nm). Assays were carried out in triplicate and the kinetic parameters evaluated using GraphPad version 6.

**Estimation of pI**

A range of buffers at different pH (N-methyl piperazine pH 4.5 and 5; L- histidine pH 5.5 and 6.0; bis-Tris pH 6.6; bis-Tris propane pH 7; triethanolamine pH 7.6; Tris pH 8) was used to determine the approximate pI of the *Citrobacter* myrosinase. Mini anion Q columns (Thermo scientific) were equilibrated with the appropriate buffer (400 µL) and centrifuged on an Eppendorf 5425 centrifuge (2000g, 4 °C, 5 min). The protein extract was desalted using Zebra spin desalting columns 0.5 mL (Thermo scientific) against the appropriate pH buffer and applied to the mini anion Q column and then centrifuged (2000 x g, 4 °C, 5 min). The column was washed twice with the appropriate buffer (400µL). Finally the protein was eluted with buffer that contained NaCl (1 M) and desalted against CPB before being assayed for activity.

**Inducibility of myrosinase**

*Citrobacter* WYE1 was grown overnight in M9 media and separately supplemented with sinigrin (10 mM) or glucose (10 mM). Cell protein extracts were prepared as previously described (general assay conditions) and assayed for myrosinase activity using 5 µg of crude desalted protein extract/assay.

**Purification of Citrobacter WYE1 myrosinase**
An overnight culture of *Citrobacter* WYE1 was grown on nutrient broth (NB), inoculated into M9 medium (500 mL) with 0.5 g of sinigrin and incubated aerobically overnight at 30 °C in a shaking incubator at 180 rpm. The bacteria were harvested by centrifugation (Avanti J26 XP, Beckman coulter, USA) (8000g, 15 min, 4 °C) and washed twice with CPB. The cell pellet was re-suspended in extraction buffer (CPB, 250 µL 100 Xs protease inhibitor cocktail (Melford Ltd), 1 mM DTT) and lysed using a one shot cell disruption system. The cell debris was pelleted by centrifugation (20,000 x g, 20 min, 4°C) and the supernatant was desalted against CPB using a desalting column (Econo-Pac 10DG) and concentrated to 11 mL using an ultrafiltration membrane tube (molecular weight cut-off 10 KDa, 4 mL UFC801024, Amicon ultra, Millipore, USA). The protein concentration was measured using Bradford’s reagent (BioRad, UK) and the activity of the extract using the GOD-PERID reagent.

Proteins were separated on a Mono Q HR 515 column (50 x 5 mm I.D., Pharmacia, Uppsala, Sweden). The column was attached to a WATERS HPLC purification system (650 E, Millipore, USA) equipped with a UV detector (ABI 757 absorbance detector, Applied Biosystems) and protein elution monitored at A280 nm. Both the initial starting buffer (Tris 20 mM, pH 8.0) and elution buffer (Tris 20 mM, plus NaCl 1M pH 8.0) were filtered using a Millipore membrane filter. The column was pre-equilibrated with starting buffer. The sample (40 mg of protein) was desalted against the initial starting buffer using Econo-Pac 10DG desalting columns then filtered through a 0.2 µm mini-Sart filter prior to injection. Fractions (1.5 mL) were collected every 2 min at flow rate of 0.75 mL/min. The bound protein was eluted by using a linear buffer concentration gradient from 0-1M NaCl (Supplementary information S1).

Fractions with myrosinase activity were combined and desalted against starting buffer using Econo-Pac 10DG desalting columns and concentrated using an ultrafiltration membrane tube. The sample (0.77 mg) was applied to an ion exchange column (Mono Q column) equilibrated with the same buffer. The separation was carried out exactly as previously outlined except that 0.75mL fractions were collected.

The active fractions following the ion exchange purification second step were combined, desalted against CPB (50 mM, pH 6.0) using Econo-Pac 10DG desalting columns and concentrated by ultrafiltration.
(molecular weight cut-off 10 KDa, 4 mL UFC801024, Amicon ultra, Millipore, USA). The sample (50 µg) was loaded onto a Superdex 75 gel filtration column (Superdex 75, 75, 10/300 GL, GE Healthcare Life Sciences, Sweden) equilibrated with CPB buffer containing NaCl (150 mM) and sodium azide (0.02%). Fractions (0.35 mL) were collected every 1 min at flow rate of 0.35 mL/min and assayed for myrosinase activity. Molecular weight markers 12 - 79 kDa (GE Healthcare Ltd) were used to calibrate the column: cytochrome c (12.4 kDa), carbonic anhydrase (29 kDa), albumin (45 kDa), albumin bovine serum (66 kDa) and transferrin (79 kDa).

**SDS PAGE**

Gels (13%) were prepared as previously described and stained with SimplyBlue™ SafeStain (Life Technologies).

**N-Terminal sequencing and internal peptide sequencing**

Pure protein was used for N-terminal sequencing (Edman degradation) which was carried out at the Protein Sequence Analysis Facility, Department of Biochemistry, Cambridge (UK). Internal peptide analysis was carried out by slicing out the pure protein from an SDS-PAGE gel stained with SimplyBlue™ SafeStain. The gel bands were processed and analysed by LC-MS as previously described.

The full-length DNA coding sequence was obtained using Geneious Pro 5.6.6. Software.

**GenBank accession numbers**

*Citrobacter_WYE1_Myr_KT821094; Citrobacter_WYE1_16sRNA_KT825141*
RESULTS

Strain isolation and identification

Serial dilutions were made from the enrichment culture and 100 µL spread onto nutrient agar plates followed by incubation at 30 °C. A single colony was picked and cultured in M9 medium with sinigrin as the sole carbon source overnight. The pure isolate was identified by 16S rRNA sequence analysis. S_ab scores of > 0.95 for twenty matches with known *Citrobacter* species were obtained and it was deduced that the isolated organism belongs to the family Enterobacteriaceae and the genus *Citrobacter* and was named *Citrobacter* WYE1 to indicate the origin of the isolate. It was not possible to assign *Citrobacter* WYE1 to a specific species based on the 16S rRNA sequence. A phylogenetic tree was constructed to show its relationship among selected *Citrobacter* strains (Figure 2). It is likely that *Citrobacter* WYE1 is a new species.

Fermentation of sinigrin

The metabolism of sinigrin was monitored by HPLC and OD values were recorded over a 24 h period (Figure 3). Sinigrin was completely utilized although no product could be identified. The potential products allylthiocyanate, or corresponding nitrile, carboxylic acid or amine could not be detected by GC-MS.

Properties of the myrosinase activity
Fresh cell-free protein extracts were used for all assays and were tested for activity prior to use. The testing of activity was necessary as there were differences between preparations. An experiment was carried out to determine whether the myrosinase was constitutive or inducible. Growth of Citrobacter WYE1 in M9 media supplemented with glucose gave only low levels of myrosinase activity as did nutrient broth. It was clear, however, that in the presence of sinigrin, myrosinase activity was substantially induced (Figure 4). Sinigrin was stable over the 24 h period in M9 medium alone. The approximate pI of the protein was between pH 6-7, based on binding of the enzyme to miniQ ion exchange columns. Thus, for purification purposes, ion exchange buffers of pH 8 were used. The temperature optimum was 25 °C for the myrosinase (Figure 5), which is unlike most plant and aphid myrosinases where temperature optima are generally higher. The pH optimum of the enzyme was found to be 6, while at pH 7.6 the activity was markedly diminished (Figure 6). With this in mind, it was necessary to desalt all purified fractions against CPB in order to obtain the full myrosinase activity. The myrosinase was active towards three other glucosinolates tested: glucoerucin had similar specific activity to sinigrin, while glucotropaeolin and glucoraphanin had lower values, suggesting that the nature of the side chain is important (Figure 7). In addition to the GOD-PERID assays, the ITCs were identified by GC-MS. All of the substrates gave the expected ITCs and no other products such as nitriles were observed (Supplementary information S2). GC-MS analysis of the hydrolysis products was used to determine the activation of myrosinase by ascorbate, as this compound interferes with the GOD-PERID assay. In addition, gluconasturtiin, which results in phenethylisothiocyanate on hydrolysis, was used as the substrate, since allylisothiocyanate produced by sinigrin is very volatile and can result in significant losses during work up. The ascorbate-treated myrosinase assays resulted in an activation of 1.67 (Figure 8) which is very low in comparison to the plant enzymes. The apparent Km and Vmax were 0.46 mM and 4.91 mmol dm⁻³ min⁻¹ mg⁻¹ respectively (Figure 9). The Km value of the Citrobacter WYE1 myrosinase was similar to those obtained for the aphid myrosinase and some plant myrosinases.

Purification of Citrobacter WYE1 myrosinase
The enzyme was purified by ion exchange chromatography (Supplementary information S1) on a high resolution monoQ ion exchange column, followed by gel filtration. The procedure gave a pure single band with an approximate molecular mass of 66 kDa on analysis by SDS-PAGE (Figure 10). The gel filtration step using a high resolution Superdex 75 column gave a native molecular size of approximately 66 kDa, based on GE-Health Care molecular weight markers. Thus it would appear that the bacterial myrosinase is a monomeric protein. The myrosinase is however of low abundance, with a yield of 4.5 µg pure protein from a fermentation of 500 ml (Table 1).

**Sequence analysis**

The N-terminal sequence (*SIQSAQQPELGYDTV*) of the purified protein was determined (Figure 11a) as well as peptides from tryptic digestion. The N-terminal peptide was used as a query to identify the respective gene by tBLASTn against the genomic sequence reads obtained by Illumina. The DNA sequences found were then assembled; the resulting consensus sequence was then used in iterative searches of the non-assembled genome reads to extend the locus encoding the myrosinase gene, until a full-length coding sequence corresponding to a plausible ORF for myrosinase was obtained. No other genes could be identified using the sequence data obtained, suggesting that there is only one myrosinase gene. The validity of the DNA sequence was confirmed by carrying out PCR of the genomic DNA (unpublished data).

BLAST analysis of the translated sequence of *Citrobacter* WYE1 myrosinase showed a high degree of homology with a number of bacterial β-O-glucosidases (Figure 11b), the greatest being with a hypothetical protein sequence from *Citrobacter sp. 30_2*. This bacterium was made available (Dr. Emma Allen-Vercoe, University of Guelph) to us but showed no activity towards glucosinolates. There was no significant homology with either plant or aphid myrosinases. Using InterProt, the protein was shown to belong to the GH 3 family of glucosidases. This is in contrast to known myrosinases which so far all belong to GH 1.
The full length gene appears to code for an N-terminal signal peptide which presumably targets the enzyme to the periplasm. This observation fits with the purified protein which appears to have lost a peptide as evident from the actual N-terminal sequencing of the protein (Figure 11a).

DISCUSSION

The bacterial myrosinase is of low abundance in comparison with plant and aphid myrosinases but nonetheless is an active inducible enzyme (Figure 4). In contrast to both plant and insect myrosinases, which appear to have a function in defence, the bacterial myrosinases are more likely to be involved in scavenging glucose from glucosides. *Citrobacter* WYE1 is a soil microorganism which was isolated from a field where oilseed rape had previously grown. The stubble and roots left in the field were ploughed back into the soil which would consequently contain a significant amount of glucosinolate that would be available for bacterial growth. It would seem therefore that bacteria can adapt to changes in the environment, and it is likely that other soil bacteria will also have the ability of the *Citrobacter* WYE1 to metabolise glucosinolates. This is of importance in the field of biofumigation with Brassicaceae crops where glucosinolate metabolism can yield the bioactive isothiocyanates, and is key to the success of this technology. Thus bacteria that degrade glucosinolates can potentially counter the effects of biofumigation by decreasing the available glucosinolate, although at this stage more work is required to establish the
products of hydrolysis in the soil.\textsuperscript{25, 36, 37} On the other hand, bacteria that can metabolise glucosinolates in the human gut play a key role in the generation of the chemoprotective isothiocyanates.\textsuperscript{5, 28, 38}

The \textit{Citrobacter} WYE1 myrosinase belongs to the GH3 family of the glucosidases\textsuperscript{7} and INTERPRO analysis shows that the full length gene codes for a signal peptide, which presumably targets it to the periplasm. In terms of sequence homology there is no resemblance to either plant or aphid myrosinases, although there is high homology with other bacterial β-O-glucosidases (Figure 11b). A feature of the GH3 glucosidases is the conserved motif ‘SDW’ which contains aspartate as the nucleophile, as part of the mechanism for hydrolysis.\textsuperscript{8, 39} The SWISS-MODEL webpage tools\textsuperscript{40} were used to generate models based on available templates. The scores, however, were too low to obtain a meaningful model. With the GH3 glucosidases, the general acid/base catalytic component of the mechanism is much more variable and will require structure determination to fully elucidate the mechanism, work that is currently underway. Recent reports\textsuperscript{41} have suggested that \textit{E. coli} 0157:H7 possesses 6-phospho-β-glucosidases (\textit{bglA} and \textit{ascbB}) and, following gene disruption, the sinigrin degradation capacity of the bacteria is substantially diminished. This result\textsuperscript{41} is supported by previous work\textsuperscript{7} where it was not possible to isolate myrosinase activity in cell free extracts of bacteria, despite the production of isothiocyanates and nitriles during fermentation. A differential proteomic study of \textit{E. coli} VL8 with or without sinigrin did not reveal any glucosidase associated with glucosinolate hydrolysis.\textsuperscript{33} A glucose-specific phosphotransferase system was however induced, which suggests that glucosinolates may undergo phosphorylation at the 6-hydroxyl group of the sugar residue. If this is the case, then it may well be that 6-phospho-β-glucosidases in cell free extracts cannot recognize glucosinolates without prior phosphorylation or that they are membrane-bound and loss of integrity results in loss of activity. This might explain why it has proven so difficult to show myrosinase activity in cell free extracts of bacteria\textsuperscript{4} 28 that can metabolise glucosinolates, although enzyme instability cannot be ruled out. Thus a synthesis, either chemical or chemoenzymatic\textsuperscript{42}, of a 6-phospho-glucosinolate would go some way to probing the mechanism of glucosinolate metabolism if 6-phospho-β-glucosidases are involved. Clearly at this time there are several potential distinct mechanisms.
for glucosinolate hydrolysis by bacteria that may involve phosphorylation, membrane-bound enzyme systems and a more typical hydrolysis by a glucosidase as shown in our work.

It was not possible to identify the fermentation products at this time, although they were not any of the usual glucosinolate hydrolysis products, *i.e.* nitrile, carboxylic acid, amine or thiocyanate (unpublished data) that have been previously described.\textsuperscript{17, 18} Previous work\textsuperscript{43} has shown isothiocyanates to be unstable over a period of 24 h in phosphate buffers, although cell free extracts of the *Citrobacter* WYE1 produce isothiocyanates in citrate phosphate buffer. Of course, it may also be possible that *Citrobacter* WYE1 possesses a detoxification pathway. M9 media contain not just phosphate but also ammonium ions which likely add to the possible interactions with ITCs. Thus at this stage it is not clear what the actual degradation metabolites are *in vivo* and more work is required on this topic. In conclusion, this is the first report to describe a fully characterized bacterial myrosinase in terms of its properties and sequence.

**ACKNOWLEDGEMENTS**

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**SUPPORTING INFORMATION**

A table describing the FPLC programme for the ion exchange chromatography is shown in S1.

Figures for the qualitative GC-MS analysis of cell free extracts with sinigrin, glucoerucin, glucoraphanin, glucotropaeolin, glucoraphanin and gluconasturtiin are shown in S2. This material is available free of charge via the internet at http://pubs.acs.org.
REFERENCES


Figure 1. Generalised scheme of the hydrolysis of glucosinolates by myrosinase. RNCS, isothiocyanate; RCN, nitrile; RSCN, thiocyanate; ETN, epithionitriles.

Figure 2. Phylogenetic tree of *Citrobacter* WYE1 compared with related *Citrobacter* species. Draft genomes of *Citrobacter* species were obtained from Genbank WGS projects (accession prefix supplied). Core genome single nucleotide polymorphisms (SNPs) in draft genomes were used to prepare the phylogenetic tree using the parSNP program.

Figure 3. The metabolism of sinigrin (10 mM) in M9 media by *Citrobacter* WYE1 over 24 h. The metabolism of sinigrin was monitored by HPLC and OD$_{600nm}$ recorded.
Figure 4. The induction of myrosinase activity in cultures grown with sinigrin. Cell free protein extracts were prepared as described in the methods and assayed for myrosinase activity with 5 µg of crude desalted protein extract/assay.

Figure 5. The effect of temperature on the activity of the Citrobacter WYE1 myrosinase was measured over a range of temperatures (5-70 °C). Assays were carried out as described in the methods with 5µg crude cell free protein.

Figure 6. The pH optimisation of the enzymatic degradation of sinigrin. Crude protein extract (5 µg) was added to a mixture of sinigrin (2 mM) in CPB to a final volume of 300 µL at specific pH values over the range 3.6 -7.6 and incubated at 37 °C for 2 h. Activity was measured by determining the release of glucose using the GOD-PERID assay.

Figure 7. The enzymatic degradation of a range of glucosinolates (10 mM) in CPB. Activity was measured by determining the release of glucose using the GOD-PERID assay.

Figure 8. The effect of differing concentrations of ascorbate on the enzymatic activity of myrosinase. Activity was determined by monitoring the production of phenethylisothiocyanate using GC-MS. ● = ascorbate, ■ = control.

Figure 9. The determination of the apparent Km and Vmax of the myrosinase.

Figure 10. SDS PAGE analysis of the purification steps of Citrobacter WYE1 myrosinase; M= marker; IE1= Ion exchange first run; IE2= Ion exchange second run; GF= gel filtration fraction of pure myrosinase at 66 kDa. Gels were washed three times with MilliQ H2O (100 mL) and stained with SimplyBlue SafeStain (Invitrogen LC6060) for an hour and destained in MilliQ H2O.

Figure 11a. Full length protein based on the first ORF derived from the annotated gene. Putative signal peptide is underlined, N-terminal sequence in bold and italics and internal peptide sequence in bold type.

Figure 11b. BLAST search results of the Citrobacter WYE1 showing the homology with a range of bacterial β-O-glucosidases. The signature ‘SDW’ of the GH3 glucosidases is highlighted.
<table>
<thead>
<tr>
<th>Purification step</th>
<th>Volume (mL)</th>
<th>Protein (mg)</th>
<th>Specific activity (nmole/mg protein/min)</th>
<th>Total activity</th>
</tr>
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<tbody>
<tr>
<td>Crude extract</td>
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<td>40</td>
<td>611</td>
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<tr>
<td>Ion exchange 1st (Mono Q column)</td>
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<td>0.775</td>
<td>1111</td>
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<tr>
<td>Ion exchange 2nd (Mono Q column)</td>
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<td>593</td>
<td>29.6</td>
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<tr>
<td>Gel filtration (Superdex 75 column)</td>
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<td>0.0045</td>
<td>2686</td>
<td>12.1</td>
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</tbody>
</table>

**Table 1** Summary of the purification steps of *Citrobacter* myrosinase. Activity assays were carried out with sinigrin (10 mM) at 25°C (pH 6.0, CPB 20 mM) and are based on glucose release (GOD-Perid assay).
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 8
Figure 9
<table>
<thead>
<tr>
<th>kDa</th>
<th>M</th>
<th>IE1</th>
<th>IE2</th>
<th>GF</th>
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</tbody>
</table>

Figure 10
MLTAFKMNTVSLAVCLPLSVSASIQAQQPELGDTYPLLHLSGLSFKDLNDRGKLNPYEDWRLSPQTRAADLVKRMSVAEKAGVM
MHGTAPAEGSTFGNGSVYDSEATRKMVDAHVNSLITRLNgeeParLaEAEQNNMIQTKAETTRGPVITIPRNSYQALVGISNPAGK
FTQWPEAIGLGAAGSEALAQEYADHRREYRAVGITEALSPQADITTEPRWARISGTFGEDPANKLVRGYITGMQKGGQGLNPQSV
AAVVKHWGVYGAEDGWDGHNAAYGKHTVLSNESLQKHIPFRGAEANVAAMPTYSVVKMGTWNGRETEQVAAGFSHFLTDL
RKQNNFSGVIIISSDLITNDCDDECVNGSAPKKPVGMPWGVYESLKRFFVKAENVAGIDQFGGVTDASAFLVTAVEVGLITQRALDA
SVERILQQKFLGLFEQPYVDAGKAEKIVGAPDTSKCAADDAQFRTLVLLQKNILPLKPTKVWLYGADKSAAEKAGLWVEPENegal
VALMRTSAPFEQPQNYFGRHHSLEYREDNKFAVLKRVSHTPVMTMYERPAVLTVNTDTSFNGLSDEVFSSRLTSD
TPYTARLPFALPSSMASLKVKSDEPDSDLTPLFQRGFGLTR

Figure 11a

Citrobacter WYE1
Enterobacter cloacae β-glucosidase (70%)
Citrobacter sp.30_2 hypothetical protein (71%)
Escherichia vulneris β-glucosidase (72%)
Rahnella aquatilis β-glucosidase
Pectobacterium carotovorum β-glucosidase (60%)
Serratia sp 1,4- β-xylosidase (61%)
Dickeya solani β-xylosidase periplasmic (61%)

Figure 11b