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Purification and cloning of a γ-glutamyl transpeptidase from onion (*Allium cepa*)

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Abstract

γ-Glutamyl transpeptidase (E.C. 2.3.2.2; GGT) catalyses hydrolysis of γ-glutamyl linkages in γ-glutamyl peptides and transfer of the γ-glutamyl group to amino acids and peptides. Although plant γ-glutamyl peptide metabolism is important in biosynthesis and metabolism of secondary products and xenobiotics, plant GGTs are poorly characterised. We purified a membrane-associated GGT from sprouting onion bulbs that catalyses transpeptidation of methionine by the synthetic substrate γ-glutamyl-*p*-nitroanilide (GGPNA) and obtained N-terminal peptide sequence. We also cloned the full-length coding region of an onion GGT by homology with the *Arabidopsis* enzyme and confirmed that this shared the same N-terminal sequence. Enzyme kinetic studies show that the enzyme has high affinity for glutathione and glutathione conjugates, and that affinity for S-substituted glutathione analogs decreases as the substituted chain length increases. The major onion γ-glutamyl peptide, γ-glutamyl *trans*-*S*-1-propenyl cysteine sulfoxide (GGPrCSO) exhibited uncompetitive inhibition of transpeptidation by GGPNA. This suggests that GGPrCSO is a poor glutamyl donor and therefore unlikely to be an in vivo substrate for peptidase activity by this enzyme.

Keywords: *Allium cepa*; Alliaceae; Onion; Enzymology; Cloning; γ-Glutamyl peptides; γ-Glutamyl transpeptidase; Glutathione

1. Introduction

A significant proportion of onion bulb *S*-alk(en)yl cysteine sulfoxide (ACSO) flavour precursors occur as γ-glutamyl peptides, the most abundant being γ-glutamyl *trans*-*S*-1-propenyl cysteine sulfoxide (GGPrCSO) and 2-carboxypropylglutathione (2CPropGSH; Fig. 1). γ-Glutamyl peptides may also represent a significant pool of bioactive organoselenium compounds in *Allium* (Dong et al., 2001) and *Brassica* species (Dong et al., 2001; Ellis et al., 2004). Substantial hydrolysis of γ-glutamyl ACSOs occurs in germinating onion seeds and sprouting bulbs (Lancaster and Shaw, 1991). Although γ-glutamyl ACSOs are not substrates for hydrolysis by alliinase to volatile flavour bioactives, substantial hydrolysis of γ-glutamyl peptides can occur in crushed tissues (Lancaster et al., 1998). Radiolabelling studies have suggested that biosynthesis of ACSOs (Lancaster and Shaw, 1989) and metabolism of the fungicide PCNB (Lamoureux and Rusness, 1980) proceed in onion via γ-glutamyl peptide intermediates.

γ-Glutamyl transpeptidase [E.C. 2.3.2.2], (5-L-glutamyl)-peptide: amino-acid 5-glutamyltransferase; GGT) catalyses the hydrolysis of the γ-glutamyl linkages in γ-glutamyl peptides and transfer of the glutamate moiety to amino acids, peptide acceptors or water (Connell and Adamson, 1970). The catalytic cycle is thought to proceed via transient acylation of the enzyme by a γ-glutamyl donor substrate followed by reaction of the acyl-enzyme intermediate with water or an acceptor substrate containing a free amino group (Tate

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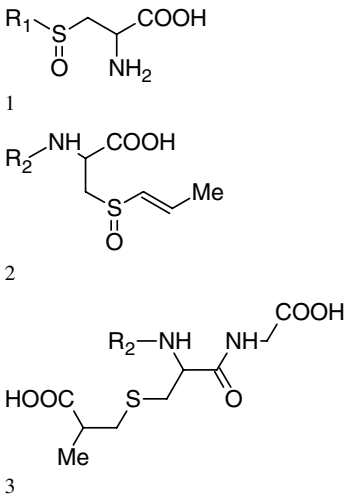

Fig. 1. Structures of major onion flavour precursors and γ-glutamyl-peptides. **1** = *S*-alk(en)yl cysteine sulfoxide, R_1 = Me or propyl or *trans*-*S*-1-propenyl;. **2** = γ-glutamyl *trans*-*S*-1-propenyl cysteine sulfoxide. **3** = 2-carboxypropylglutathione. R_2 = γ-glutamyl–.

and Meister, 1985). The enzyme is well characterised in mammalian systems, where it initiates the degradation of glutathione (γ-L-glutamyl–L-cysteinyl–L-glycine) and glutathione-*S*-conjugates of xenobiotics and pharmaceuticals resulting from detoxification processes.

The role and properties of plant GGTs are less clearly understood (Leustek et al., 2000). Recent in vivo functional characterisation in tobacco of an *Arabidopsis* GGT homolog suggested that it can catabolise glutathione conjugates in a manner similar to the animal systems (Storozhenko et al., 2002). Crude extracts of ripening tomato fruit GGT can catalyse conjugation of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) to form γ-glutamyl-ACC, but it is not clear if this is the in vivo activity of the enzyme (Martin and Slovin, 2000). Other plant GGTs have been purified and characterised from ackee plant *Blighia sapida*, (Kean and Hare, 1980); kidney bean fruit (Goore and Thompson, 1967) and onion (Lancaster and Shaw, 1994).

Bacterial and mammalian GGTs are heterodimers consisting of a large and small subunit generated by proteolytic cleavage of a single precursor, which proceeds in *Escherichia coli* K-12 GGT via an intramolecular autocatalytic event (Suzuki and Kumagai, 2002). Purified plant GGTs characterised to date (summarised in Table 1) have been reported to consist of a single polypeptide chain, though an *Arabidopsis* GGT homolog expressed in tobacco exhibited a large and a small subunit with molecular weights similar to those reported in the non-plant species (Storozhenko et al., 2002). Mammalian and plant GGTs are heterologously glycosylated (Tate and Khadse, 1986) and this can account for the wide variation in reported peptide sizes. No studies to date have reported peptide sequence from a purified plant GGT.

To better understand the properties and biosynthetic role of onion GGTs, we purified an isoform to homogeneity from sprouting onion bulbs and characterised its catalytic properties and N-terminal peptide sequence. We also cloned a partial cDNA and genomic sequence of GGT by homology with *Arabidopsis* and demonstrated that it was a precursor of the mature peptide.

2. Results and discussion

2.1. Purification of onion bulb GGT

We successfully used approaches previously used in tomato (Martin and Slovin, 2000) to extract and purify GGT activity from sprouting onion bulbs. Acetone precipitation greatly improved handling of crude onion extracts compared to ammonium sulfate precipitations we previously employed (Lancaster and Shaw, 1994). Following a hydrophobic interaction chromatography step, ConA Sepharose chromatography resolved activity into a major fraction (isoform I) that eluted with 5 mM α-methyl-D-glucopyranoside (MGP) and a minor fraction (isoform II), which required 200 mM α-methyl-D-mannopyranoside (MMP) for elution (Fig. 2). Isoform I was further purified further by Dye-Matrix Green affinity chromatography to achieve a 266-fold purification (Table 2). SDS–PAGE analysis of the purified isoform I fraction showed only major bands of 36 and 39

Table 1
Structural properties of selected plant, fungal, microbial and animal γ-glutamyl transpeptidases

Species	M_r	Subunit M_r: large, small	Glycosylated	Cellular location	Reference
Arabidopsis thaliana	70,000	41,000, 29,000	Yes	Plasma membrane-bound outside the cell	Storozhenko et al. (2002)
Tomato	43,000	Monopeptide	Yes	Peripheral membrane protein	Martin and Slovin (2000)
Onion	56,700	Monopeptide	Yes	ND	Lancaster and Shaw (1994)
Blighia sapida	12,500	Monopeptide	Yes	ND	Kean and Hare (1980)
Kidney bean	180,000	ND	ND	ND	Goore and Thompson (1967)
Penicillum roquefortii	120,000	66,000, 55,000	Yes	ND	Tomita et al. (1990)
Escherichia coli	58,000	39,200, 22,000	No	Soluble periplasmic enzyme	Suzuki et al. (1986)
Human (kidney)	94,000	63,000, 21,000	Yes	Membrane-bound	Tate and Khadse (1986)

ND, not determined.

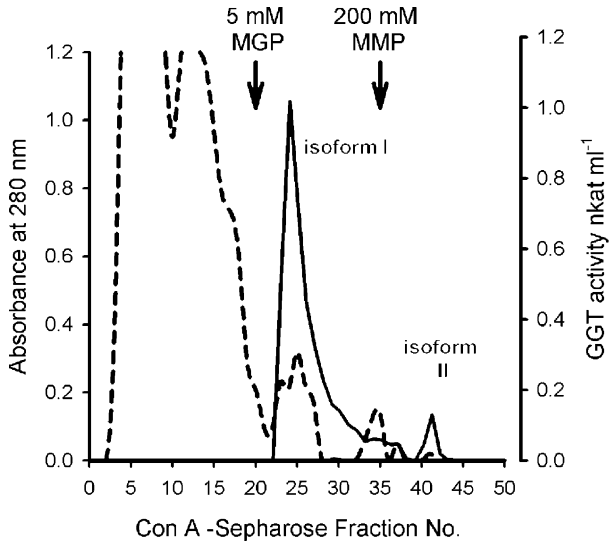

Fig. 2. Concanavalin A-Sepharose affinity chromatography of onion GGT isoforms I and II. Arrows indicate the application of 5 mM α-methyl-D-glucopyranoside to elute isoform I and 200 mM α-methyl-D-mannopyranoside to elute isoform II. Elution of protein is indicated by the dashed line and GGT activity by a solid line.

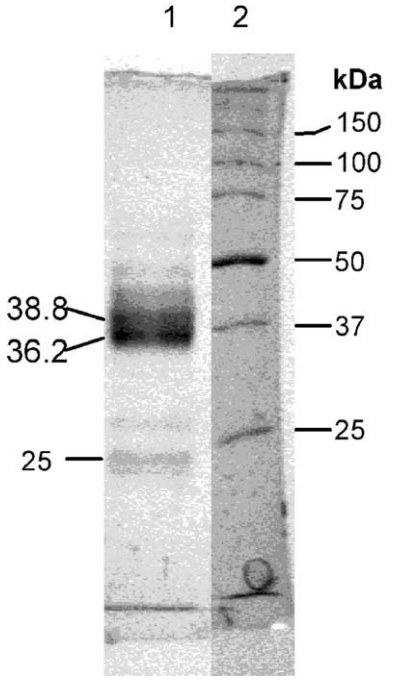

Fig. 3. SDS–polyacrylamide gel electrophoresis of onion GGT. Lane 1: Coomassie Blue stained GGT isoform I after ultrafiltration of Dye-Matrix Green eluate. Lane 2: Protein molecular size markers.

kDa and a minor band of 25 kDa (Fig. 3). This suggests that although purification to the same level of specific activity as in our previous report (Lancaster and Shaw, 1994) was achieved, this enzyme preparation was significantly more homogeneous. N-terminal peptide sequencing of the 39-kDa band revealed the sequence (QG)–D–A–I–K–H–R–R–E–V–I–T–(DST)–K–N–G–A–(MV). Partial sequencing of the 36-kDa band revealed the sequence (QG)–D–A–I–K–H–R–R–E–V, suggesting that it shared the same sequence. The small quantities of the 25 kDa band were not sufficient to permit sequencing.

2.2. Characterisation of purified GGT

Analysis of the pH optima of the purified enzyme for hydrolysis and transpeptidation revealed activity over a broad range with optima of 9 and 7, respectively (Fig. 4), similar to the recombinant enzyme from *Arabidopsis* (Storozhenko et al., 2002). Qualitative analysis of reaction products showed that the enzyme catalysed transpeptidation of methionine by γ-glutamyl-*p*-nitroanilide (GGNA) to form γ-glutamyl methionine (Fig. 5). The kinetic properties of the enzyme were studied by determining the K_i for ability of γ-glutamyl peptides to inhibit transpeptidation of GGNA in the presence of the acceptor substrate methionine. If γ-glutamyl derivatives serve as a donor substrate participating in a non-colorimetric enzyme reaction, the background colorimetric reaction will appear to be inhibited and the inhibition constant K_i is equal to the kinetic constant K_m (Castonguay et al., 2002; Storozhenko et al., 2002). The enzyme exhibited apparent K_m values of 12.0 mM for hydrolysis of GGNA and 4.33 mM for transpeptidation of methionine with GGNA. Glutathione (GSH), *S*-methyl glutathione (MeGSH), *S*-propyl glutathione (PropGSH), and 2CPropGSH competitively inhibited GGNA as γ-glutamyl donors in this assay system, with K_i values of 16.4, 23.3, 67.9 and 113.7 μM, respectively. These low micromolar K_m values for glutathione conju-

Table 2
Purification scheme of onion bulb GGT isoform I

Procedure	Total activity (nKat)	Total protein (mg)	Specific activity (nKat/mg)	Purification (fold)	Recovery (%)
Crude extract					
Low salt	0	308	0	–	–
High salt	118	167	0.71	1.00	100
Acetone precipitation	57.6	14	4.1	5.83	48.8
Phenyl-Sepharose	52.6	1.25	42.1	59.39	44.5
Con A-Sepharose	22.8	0.342	66.6	94.2	19.3
Dye-Matrix Green A	16.2	0.086	188.2	266.3	13.7

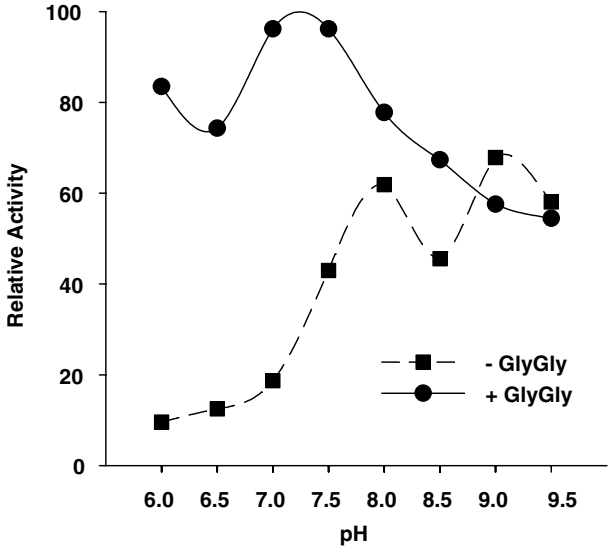

Fig. 4. pH Dependence of transpeptidation and hydrolysis by onion GGT (isoform I). MES (2-*N*-morpholino ethanesulfonic acid) and Tris buffers were used to cover the pH range 6.0–9.5. Transpeptidation activity in the MES buffer was 2.5 times lower and has been scaled to match levels in Tris and provide a continuous curve.

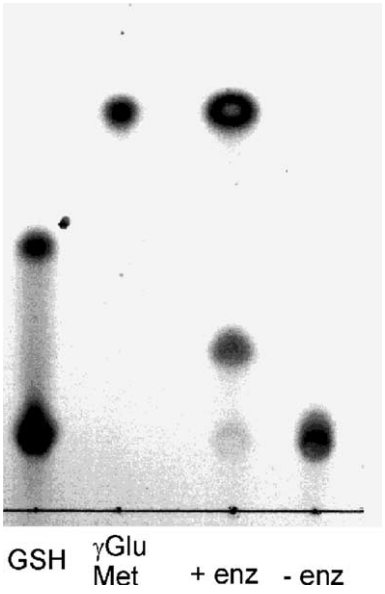

Fig. 5. Qualitative TLC of products of transpeptidation catalysed by onion GGT (isoform I). Glutathione (GSH) and methionine were incubated with (+enz) or without (−enz) purified onion GGT. Reaction products were fractionated by anion exchange on Dowex 1 and the 1 M acetic acid fraction containing γ-glutamyl peptides was resolved by TLC with authentic standards of substrates and putative product (γ-glutamyl methionine; γ Glu Met).

gates are in contrast to the much higher values we reported earlier for a partially purified onion bulb GGT (Lancaster and Shaw, 1994). By contrast, GGPrCSO exhibited uncompetitive inhibition. The secondary plot of $K_{m\ app}/V_{max\ app}$ against GGPrCSO concentration (Fig. 6) shows the zero slope for that is diagnostic of

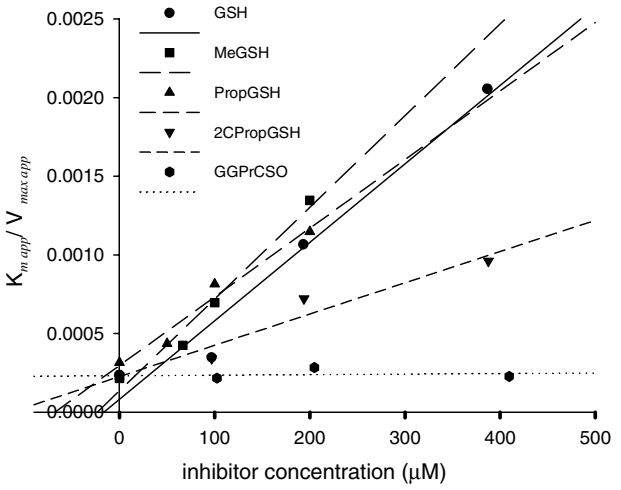

Fig. 6. Secondary plot of apparent K_m/apparent V_{max} versus peptide inhibitor concentrations for inhibition of onion (isoform I) GGT-catalysed transpeptidation of methionine by GGNA.

uncompetitive inhibition (Cornish-Bowden, 1995). This observation suggests that GGPrCSO is not competing for the acyl donor substrate binding site but rather for the acyl acceptor substrate binding site (Castonguay et al., 2002).

2.3. Molecular cloning of GGT

RT-PCR was used to amplify an 886 bp product (Genbank Accession No. AF401622) whose deduced amino acid sequence showed 66% amino acid identity with *Arabidopsis* GGTs. This clone detected a 2.4-kb transcript in Northern blot analysis of root, leaf and bulb tissues (Fig. 7). Initial attempts to clone downstream regions of the gene by 3′ RACE were unsuccessful and we therefore used TAIL-PCR from genomic DNA to isolate downstream regions (Genbank Accession No. AY517547). Subsequent use of the primary TAIL primer enabled 3′ RACE amplification of the corresponding mRNA sequence (Genbank Accession No. AY517548). Alignment of the cloned genomic and 3′ RACE sequences revealed conservation of positions of

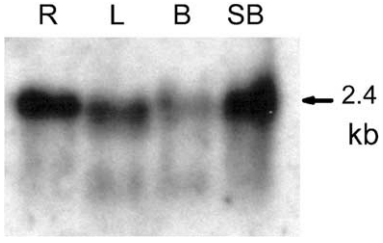

Fig. 7. Northern Blot analysis of GGT expression in onion tissues. Lanes contained 5 μg total RNA isolated from root (R), leaf (L), bulb (B) and sprouting bulb (SB) tissue and were probed with ^{32}P-labelled GGT cDNA clone AF401622.

introns 3 and 4 compared to *Arabidopsis* GGT (At4g39640). 5′ RACE was then used to amplify most of the 5′ coding region (Genbank Accession No. AY517549). Efforts to amplify and clone a full-length cDNA by RACE were not successful. Analysis of the inferred peptide sequence of the 5′ RACE product using PSORT (www.psort.nibb.ac.jp/form.html; (Nakai and Kanehisa, 1992) identified a 31-amino acid hydrophobic signal peptide sequence, a predicted cleavage site and putative transmembrane domain (position 70–86) consistent with plasma membrane localisation (Fig. 8). The N-terminal peptide sequence resulting from signal peptide cleavage at position 32 corresponded with the sequence we determined from the purified protein. The predicted mass of the protein after signal peptide cleavage, but before processing into large and small subunits, is 59.9 kDa. The 37.8 kDa predicted size of the large subunit, resulting from post-translational cleavage at the conserved cleavage site G_{381} T_{382}, is similar to the value of 41 kDa reported in *Arabidopsis* (Storozhenko et al., 2002). Our calculation from SDS–PAGE of a molecular weight of 38.9 kDa possibly results from glycosylation of 1 of the 5 putative glycosylation sites present on the large subunit at either N_{185}, N_{204}, N_{236}, N_{338} or N_{362}, by a small complex-type sugar chain. This is consistent with the lower level of MGP required to elute

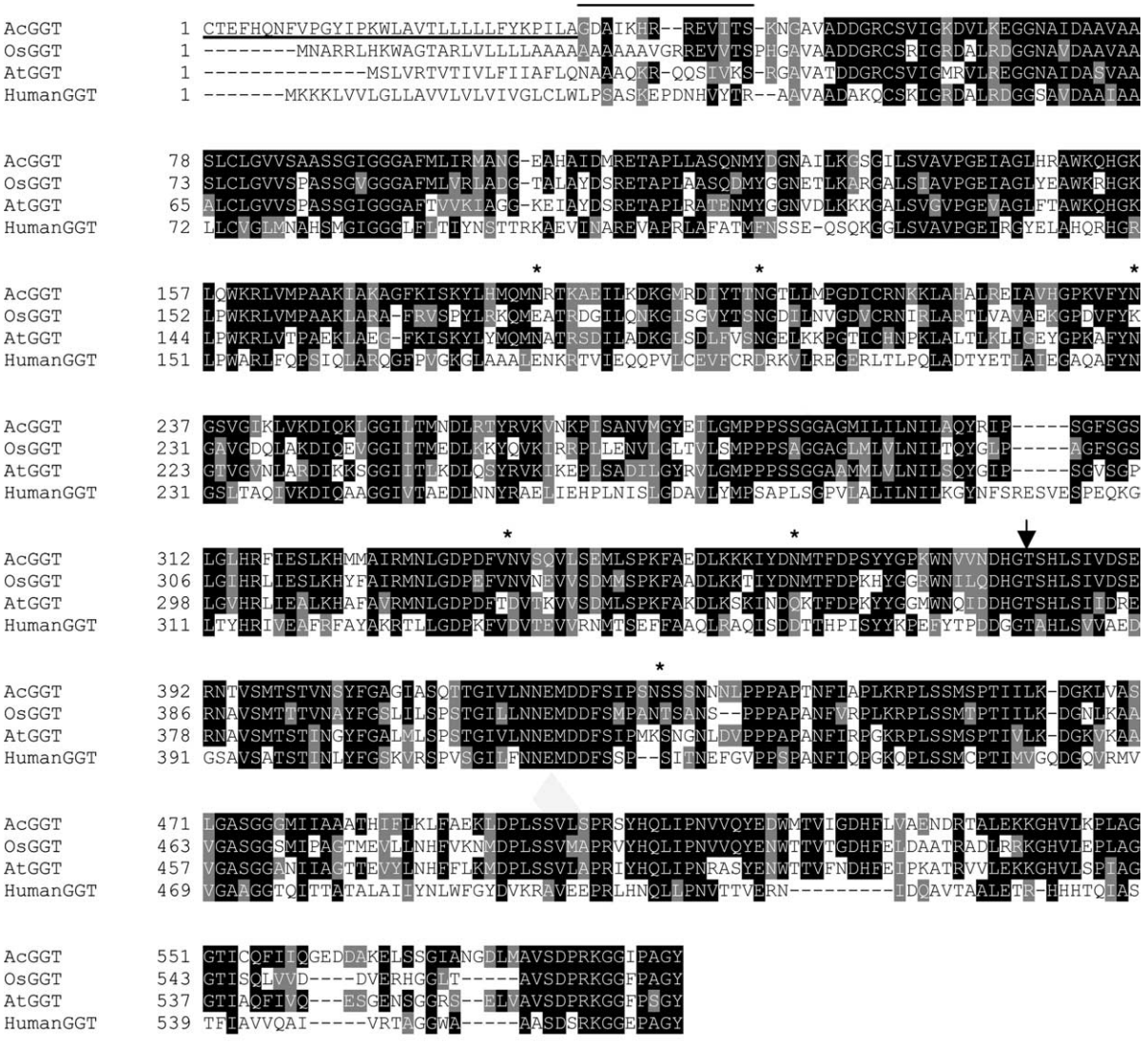

Fig. 8. Alignment of deduced amino acid sequence onion γ-glutamyl transpeptidase (AcGGT) with rice (OsGGT; CAD40892.2), *Arabidopsis* (AtGGT; AAL47428.1), and human (HumanGGT; NP_005256.1). The GGT sequences were compared using Clustal W (Thompson et al., 1997), and formatted with BOXSHADE 3.21 (www.ch.embnet.org/software/BOX_form.html). Black background represents identities, grey background represents similarities, and dashes represent gaps, all relative to the onion sequence from the deduced N-terminus. The putative signal peptide domain of the onion sequence is underlined, and the N-terminal peptide sequence verified by protein sequencing is overlined. Putative *N*-linked glycosylation sites are marked by asterisks and the conserved protease cleavage site between the large and small subunits by an arrow.

it from the ConA-Sepharose glycoprotein affinity column during purification. While the second sequenced band from the blotted SDS–PAGE gel also has a sequence indicating that it is a large subunit of the protein, the lower molecular weight indicates that some truncation at the C-terminal end may have occurred. The small subunit is calculated to be around 22.1 kDa prior to glycosylation. One putative *N*-glycosylation site is indicated from the sequence at N_{430}. Other prominent bands on the gel include a band at 25 kDa. As the active site on other GGTs is known to reside on the small subunit (Ikeda et al., 1995) we would expect to find a band of about that molecular weight if the glycosylation site was utilised. Martin and Slovin (2000) reported a single peptide of 43 kDa in tomato GGT but did not present protein sequence data.

3. Conclusion

We initially attempted to purify GGT activity from hydroponically grown onion roots and purified GGT activity 235-fold (data not shown). We obtained partial N-terminal peptide sequence from a prominent 48-kDa peptide on SDS–PAGE gels of a purified fraction, which had the sequence S–K–L–V–P–L–E–A–L–P–A–K–X–G–Y–L–P–V–X–R. BLAST homology searches showed weak homology to *Arabidopsis* serine carboxypeptidase-like (SCPL) proteins of identical size. SCPL proteins have been identified as catalysts in transacylation reactions of secondary metabolism such as *Arabidopsis* sinapoyl glucose metabolism (Lehfeldt et al., 2000; Shirley et al., 2001). It has been suggested that successive action of carboxypeptidase and GGT is involved in the pathways of ACSO biosynthesis (Lancaster and Shaw, 1989) and PCNB fungicide metabolism (Lamoureux and Rusness, 1980) in onion. Due to the practical difficulties in producing sufficient enzyme for detailed study we returned to the examination of bulb enzymes.

This is the first report of the purification to homogeneity and peptide sequence characterisation of a plant GGT. Although the extent of our characterisation was limited by the quantities of enzyme obtained, our kinetic studies conclusively demonstrate that this enzyme acts as a transpeptidase, utilising glutathione and glutathione derivatives at low K_m values. This very high affinity for GSH is similar to that reported for the *Arabidopsis* GGT homolog characterised by Storozhenko et al. (2002) but in contrast to the properties of the partially purified onion GGT we characterised previously (Lancaster and Shaw, 1994). This evidence supports an in vivo role for this enzyme as a transpeptidase in glutathione conjugate metabolism. Our observation of uncompetitive inhibition by GGPrCSO of methionine transpeptidation, using the artificial donor GGNA, indicates that GGPrCSO is not a good acyl donor substrate for this enzyme. Because acylation is a prerequisite for peptidase activity it is unlikely this enzyme functions as a peptidase in γ-glutamyl alk(en)yl cysteine sulfoxide hydrolysis.

4. Experimental

4.1. Materials

GGPrCSO and 2CPropGSH were isolated from onion bulbs by ion exchange chromatography (Shaw and Lancaster, 1989). GSH, MeGSH and PropGSH, were obtained from Sigma Chemicals (St Louis, MO, USA). γ-Glutamyl methionine was obtained from Bachem Feinchemikalien AG (Bubendorf, Switzerland). *Allium cepa* L. *cv.* 'Canterbury Longkeeper' (Crop & Food Research) was grown in the field (McCallum et al., 2001a, 2002) or hydroponically (McCallum et al., 2002) as described previously. Genomic DNA (McCallum et al., 2001b) and total RNA (McCallum et al., 2002) were isolated as described previously.

4.2. Homology cloning of onion GGT

RT-PCR, 3′ RACE, cloning of PCR products and Northern blot analysis were performed as described earlier (McCallum et al., 2002).

Initially a core 886-bp fragment of the GGT coding region was amplified by RT-PCR from root cDNA with primers GGT5′ #2 and GGT3′ #2 (Table 3), which were based on conserved regions of plant GGTs (Genbank Accession Nos. AW203399, AI823204, Z49240).

Because initial attempts based on 3′ RACE were unsuccessful, sequence of the 3′ region of the gene was first cloned by TAIL-PCR ((Liu et al., 1995) using primers designed from the core RT-PCR fragment. Genomic DNA isolated from leaf of field-grown plants (*cv.* 'Canterbury Longkeeper') was used as template for the primary PCR. The arbitrary primer AD1 was used in combination with nested primers GGT3′ TAIL1, GGT3′ TAIL2 and GGT3′ TAIL3 for primary, secondary and tertiary PCR reactions respectively. The remaining 3′ coding region and UTR were subsequently

Table 3
PCR primers used in this study

Primer name	Primer sequence
GGT5′ #2	GAGCTGTTGCTACNGAYGAY
GGT3′ #2	ATCAGGATCTCCNARRTTCAT
AD1	NTCGASTWTSGWGTT
GGT3′ TAIL1	ACATGCATTGCGTGAAATTG
GGT3′ TAIL2	GCCGATATCTGCAAATGTTATG
GGT3′ TAIL3	TTCTGGTTCACTTGGGCTTC
GGT RACE1	TTAATTCCTACTGAACC
GGT RACE2	CACTTTTGGTCCATGAACTGC

amplified and cloned by 3′ RACE using the primary TAIL-PCR primer GGT3′ TAIL1.

The 5′ coding region of the gene was amplified and cloned by 5′ RACE. Reverse transcription was carried on total RNA from leaf tissue of field-grown *A. cepa cv.* 'Pukekohe Longkeeper' using the gene-specific primer GGT RACE1, followed by tailing of the first strand product with dATP by terminal transferase. First round PCR was performed with the gene-specific primer GGT RACE2 and OligodT adaptor primer (McCallum et al., 2002). Second round PCR was performed with GGT RACE 3 and the general primer PCR Anchor (McCallum et al., 2002).

4.3. Purification of onion bulb GGT

Assays contained 0.033 M Tris–HCl pH 9.0, 1.53 mM GGNA, 33 mM methionine plus enzyme in a total volume of 1.5 ml. Reactions were incubated at 37 °C and terminated by the addition of 2 ml of 5 M acetic acid. Sequential addition of 1 ml 0.1% $NaNO_2$, 1 ml of 1% ammonium sulfamate and 1 ml of 2 mM napthylethylenediamine · diHCl at 3-min intervals resulted in the formation of a pink azo dye that was recorded spectrophotometrically at 540 nm (Lancaster and Shaw, 1994). Bulb tissue from sprouting onions was frozen in liquid N_2 after removal of dry outer scales and stored at −80 °C until required. Approximately 1 kg of material was blended in 1 l of low salt buffer (50 mM Tris, pH 7.5 + 5 mM 6-amino hexanoic acid + 1 mM PMSF), filtered through cheesecloth and the residue collected. This material was re-extracted with 500 ml high salt buffer (50 mM Tris, 1 M NaCl, pH 7.5 + 5 mM 6-amino hexanoic acid + 1 mM PMSF), filtered through cheesecloth and spun 10,000*g* for 10 min. The supernatant was adjusted to 75% v/v acetone and centrifuged for 10 min at 10,000*g* to collect precipitate. The precipitate was resuspended in 50 ml of 50 mM Tris, 0.5 M NaCl, 1 M ammonium sulfate, pH 7.5 and applied to a Phenyl-Sepharose column (2.8 × 11 cm) equilibrated in the same buffer. The column was eluted at 0.5 ml min^{-1} until baseline $A_{280\ nm}$ was observed and then with a linear gradient of 1 to 0 M ammonium sulfate in 50 mM Tris, 0.5 M NaCl pH 7.5 buffer over 100 ml. Active fractions were pooled, adjusted to 1 mM Mn^{2+} and Ca^{2+} and applied to a Concanavalin A-Sepharose column (1.6 × 7 cm) equilibrated with 50 mM Tris, 0.5 M NaCl, 1 mM Mn^{2+} and Ca^{2+} pH 7.5 at 0.4 ml min^{-1}. Active fractions were eluted in a stepwise gradient using 5 mM MGP and 200 mM MMP. Active fractions from the 5 mM MGP fraction (isoform I) were concentrated using a stirred cell with a 30000 MWCO membrane (Amicon, typeYM 30) and diluted 1:1 with 50 mM Tris pH 7.5. This was applied to a Dye-Matrix Green column (1 × 3.5 cm) equilibrated in 50 mM Tris, 0.25 M NaCl, pH 7.5 at 0.2 ml min^{-1}. Active fractions were eluted with 50 mM Tris, 1.2 M NaCl, pH 7.5 and concentrated as before. For N-terminal microsequencing, samples were blotted to PVDF membrane (Millipore) from 10% SDS polyacrylamide gels using a semi-dry transfer apparatus (NovaBlot, LKB) and analysed by narrowbore HPLC of PTC derivatives as described by (Hubbard et al., 2000).

4.4. Enzyme kinetics

Kinetic studies were performed in 96-well microtitre plates by monitoring *p*-nitroaniline release with a SpectraMAX 190 microplate spectrophotometer at 405 nm. Incubations were performed in 300 µl total volume, in 0.1 M Tris buffer, pH 8 at 30 °C with 40 mM methionine as acceptor. GGNA was varied between 1.93 and 6.70 mM, while competing γ-glutamyl derivatives varied between 0 and 0.41 mM. Replicate velocity measurements were made for each substrate concentration. K_i values were calculated from secondary plots of the slopes from Lineweaver–Burk plots (Cornish-Bowden, 1995) using the exploratory enzyme statistics package in Sigmaplot (SPSS, 2002).

4.5. Qualitative analysis of transpeptidation products

Reactions contained 1 mM GSH, 40 mM methionine, 0.1 M Tris–HCl, pH 8.0 with or without 0.43 µg purified enzyme in a total volume of 1 ml. After 1 h incubation at 30 °C the reaction was stopped by application to a column of Dowex 1 × 8 ion-exchange resin in the acetate form. Columns were eluted stepwise with increasing concentrations of acetic acid (Lancaster and Shaw, 1989) and lyophilised fractions were re-dissolved in 50 µl of water and resolved on 0.2 mm silica gel TLC plates (Merck) as previously described (Shaw and Lancaster, 1989).

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