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Purified γ-Glutamyl Transpeptidases from Tomato Exhibit High Affinity for Glutathione and Glutathione *S*-Conjugates[1]

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γ-Glutamyl transpeptidases (γGTases) are the only enzymes known to hydrolyze the unique N-terminal amide bonds of reduced glutathione (γ-L-glutamyl-cysteinyl-glycine), oxidized glutathione, and glutathione *S*-conjugates. Two γGTases (I and II) with K_m values for glutathione of 110 and 90 μM were purified 2,977-fold and 2,152-fold, respectively, from ripe tomato (*Lycopersicon esculentum*) pericarp. Both enzymes also hydrolyze dipeptides and other tripeptides with N-terminal, γ-linked Glu and the artificial substrates γ-L-glutamyl-*p*-nitroanilide and γ-L-glutamyl(7-amido-4-methylcoumarin). They transfer the glutamyl moiety to water or acceptor amino acids, including L-Met, L-Phe, L-Trp, L-Ala, or the ethylene precursor 1-aminocyclopropane-1-carboxylic acid. γGTase I and II were released from a wall and membrane fraction of a tomato fruit extract with 1.0 M NaCl, suggesting that they are peripheral membrane proteins. They were further purified by acetone precipitation, Dye Matrex Green A affinity chromatography, and hydrophobic interaction chromatography. The two γGTases were resolved by concanavalin A (Con A) affinity chromatography, indicating that they are differentially glycosylated. The native and SDS-denatured forms of both enzymes showed molecular masses of 43 kD.

γ-Glutamyl transpeptidases (γGTases) ([5-L-glutamyl]-peptide:amino acid 5-glutamyl transferase; EC 2.3.2.2) catalyze the hydrolysis of the uniquely linked N-terminal Glu from the reduced glutathione (GSH) (γ-L-glutamyl-L-cysteinyl-glycine), oxidized glutathione (GSSG), and glutathione *S*-conjugates, as well as from a number of dipeptides and other tripeptides having an N-terminal γ-linked Glu. The Glu moiety is transferred to either water (hydrolysis) or to an acceptor amino acid, dipeptide, or tripeptide, including GSH (transpeptidation), resulting in a new amide bond with an N-terminal, γ-linked Glu.

In mammals, γGTases are well characterized and have several physiologically and pharmaco-toxicologically important functions. First, they initiate degradation of GSH to release Cys, which is the primary stored and transported form of sulfur. GSH hydrolysis occurs primarily on the outer surface of cell membranes in organs that secrete a large amount of GSH; the component amino acids are salvaged and transported back into the cells (Meister, 1988, 1989). Knock-out mice lacking a functional γGTase died of Cys starvation unless their diets were supplemented with Cys (Lieberman et al., 1996). The γ-glutamyl peptides resulting from the transpeptidation reaction may enter the "γ-glutamyl cycle," where they are also hydrolyzed and the amino acids are salvaged (Meister, 1988, 1989). Cys-Gly dipeptidases complete the hydrolysis of GSH (Habib et al., 1998). Second, γGTases are part of the pathway for detoxification and elimination of many xenobiotics and pharmaceuticals (Meister, 1988; Ishikawa, 1992). These compounds are first conjugated to GSH by glutathione *S*-transferases, which reduces their reactivity, increases their polarity, and tags them for transport or excretion by the ATP-binding cassette class of transporters (Ishikawa, 1992). γGTases initiate degradation of the excreted conjugates by hydrolyzing the γ-Glu moiety (Meister, 1988). Finally, γGTases are part of a pathway for sequestering, transporting, and modulating the activity of hormones, neurotransmitters, and other biologically active compounds, including hepoxilins, leukotrienes, and prostaglandins (Meister, 1988; Pace-Asciak et al., 1990; Ishikawa, 1992; Carter et al., 1997). As with xenobiotics, the pathway involves glutathione *S*-conjugation, transport, and, finally, further metabolism of the conjugate. For conjugates of some hormones, removal of the N-terminal Glu by a highly specific γGTase results in 100-fold activation, while for other hormone conjugates it results in inactivation (Ishikawa, 1992; Carter et al., 1997).

Plant γGTases are poorly characterized, and it is not known whether they serve the same functions as in animals. GSH is reported to be the major form in which reduced sulfur (Cys) is stored and transported in plants, but there are conflicting data as to the sequence of reactions leading to Cys release from GSH. γ-Glu-Cys was identified as an intermediate in the degradation of [^{35}S]GSH by tobacco (Steinkamp and Rennenberg, 1985). The authors proposed that a carboxypeptidase initiates hydrolysis of GSH at the C terminus, followed by hydrolysis of γ-Glu-Cys by a γGTase or the "γ-glutamyl cycle" enzymes (γ-glutamylcyclotransferase and oxo-prolinase). Cys-Gly is found in soybeans, suggesting that GSH hydrolysis is catalyzed by the sequential action of a γGTase and a Cys-Gly dipeptidase (Bergmann and Rennenberg, 1993). The sequence of reactions leading to hydrolysis of GSH in plants

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may be species specific. Only Cys-Gly has been identified as an intermediate in the degradation of GSH and GSSG by animals (Meister, 1988).

Plants also form glutathione *S*-conjugates of many herbicides and pesticides (Marrs, 1996). Some of these conjugates are transported to the vacuole by ATP-binding cassette transporters (Martinoia et al., 1993; Gaillard et al., 1994; Li et al., 1995; Rea et al., 1998). In the vacuole, conjugates undergo modification, which may begin with the cleavage of Glu and Gly (Lamoureux and Rusness, 1981, 1983, 1993; Lamoureux et al., 1991). At least one endogenous plant compound, the anthocyanin cyanidin 3-glucoside, appears to be targeted to the vacuole by the same mechanism (Marrs et al., 1995; Marrs 1996; Alfenito et al., 1998). The glutathione *S*-transferases (Marrs, 1996) and glutathione *S*-conjugate transporters (Rea et al., 1998) have been at least partially characterized. The enzymes responsible for subsequent modifications of these conjugates have not been characterized.

γGTases may have an additional role in plants for which there is no analogy in animals. Numerous dipeptides with an N-terminal γ-linked Glu have been isolated from plant tissues, most often from storage tissues such as seeds or bulbs. Examples include Glu γ-linked to Met, methionine sulfoxide, Leu, Tyr, Asp, and Phe in legumes (Thompson et al., 1962a); Phe, Asp, and Tyr in soybeans (Ishikawa et al., 1967); Asp, Glu, and Tyr in asparagus (Kasai et al., 1982); 2-methylenecyclopropyl-alanine in ackee fruit (Kean and Hare, 1980); D-Ala and homoserine in pea seedlings (Kawasaki et al., 1982); and β-cyanoalanine in vetch seeds (Ressler et al., 1969). In *Allium* species, at least 18 γ-glutamyl peptides of sulfur compounds such as alk(en)yl-cysteine sulfoxides have been identified (Lancaster et al., 1989; Lancaster and Shaw, 1989, 1991). γGTases are the only enzymes known to catalyze the formation of the N-terminal γ-linked amide bond with Glu. One exception is γ-glutamyl cysteine synthetase, which catalyzes the first step in GSH synthesis and uses only Cys as the acceptor amino acid. Likewise, γGTase and γ-glutamyl cyclotransferase (operating in the γ-glutamyl cycle) are the only enzymes known to hydrolyze the unique N-terminal amide bond in these dipeptides.

γGTases are widely distributed in monocots and dicots, and both constitutive and developmentally regulated activities have been reported (Thompson et al., 1962b; Goore and Thompson, 1967; Kean and Hare, 1980; Kasai et al., 1982; Kawasaki et al., 1982; Steinkamp and Rennenberg, 1984; Lancaster and Shaw, 1994; Martin et al., 1995; M. Martin, unpublished results). Activity is particularly high in seeds and storage tissues (Lancaster and Shaw, 1994; Martin et al., 1995; Martin and Slovin, 1996; M. Martin, unpublished results). In contrast, other activities that might have a role in GSH and glutathione *S*-conjugate hydrolysis (GSH carboxypeptidase, Cys-Gly dipeptidase, and the "γ-glutamyl cycle" enzymes γ-glutamylcyclotransferase and oxo-prolinase) have been reported in only one or two plant tissues (Rennenberg et al., 1981; Steinkamp et al., 1984, 1985). In plants, a γ-glutamyl cycle has not been established. A vacuolar carboxypeptidase capable of initiating hydrolysis of glutathione *S*-conjugates from the carboxy terminus was recently isolated from barley (Wolf et al., 1996).

We have previously reported the identification of a γGTase in tomato (*Lycopersicon esculentum*) fruit and seeds. This enzyme uses the precursor to the plant hormone ethylene, 1-aminocyclopropane-1-carboxylic acid [ACC], as an acceptor amino acid (Martin et al., 1995). We measured a high level of this activity in tomato seeds throughout development and dehydration, and an increase in the activity in the pericarp and other maternal tissues during the course of fruit development and ripening (Martin et al., 1995). We report the isolation from the pericarp of ripe tomato fruit of several γGTases that differ in γ-glutamyl donor specificity. We also describe the purification and characterization of two of these enzymes, both of which exhibit high affinity for GSH, GSSG, and glutathione *S*-conjugates.

MATERIALS AND METHODS

Plant Material

Red ripe fruit from tomato (*Lycopersicon esculentum* Mill cv Ailsa Craig) were used. Plants were grown in a greenhouse and received supplemental lighting during winter.

Radiometric Measurement of Transpeptidase Activity

The assays were performed in 0.5-mL microcentrifuge tubes and contained in a volume of 50 μL, enzyme, 100 mM Tris-Cl, pH 8.0, 2 mM GSH, 5 mM ACC, and 3.75 kBq [2,3-^{14}C]ACC per assay. [2,3-^{14}C]ACC with a specific activity of 1.87 GBq/mmol was synthesized by the Commissariat a L'Energie Atomique, France. The reactions were initiated with GSH, incubated for 15 to 60 min at 30°C, and terminated by the addition of 50 μL of absolute ethanol or by boiling. Five microliters of each assay mix was spotted on a lane of a 10 × 20 cm HPTLC-GHLF[3] normal phase silica TLC plate (Analtech, Newark, DE). [^{14}C]ACC and the product, [^{14}C]-1-(γ-glutamyl)cyclopropane-1-carboxylic acid ([^{14}C]-GACC), were resolved using a solvent system of 1-propanol:ammonium hydroxide (6:4, v/v). Prior to chromatography, assays containing crude protein extracts were centrifuged for 5 min at 13,000*g* to pellet protein and other debris. Products and substrate were detected and quantified using a radioisotope image acquisition and analysis system (Ambis model 1000, Scanalytics, Billericka, MA). Where indicated, other γ-glutamyl donors were substituted for GSH, and other amino acids were substituted for ACC or added as competitive acceptors. One unit of enzyme activity was defined as the amount catalyzing the formation of 1 nmol of GACC per minute.

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Spectrophotometric Measurement of γ-Glutamyl-*p*-Nitroanilide (γ-GPNA) Hydrolysis

The assays were performed at 30°C in 96-well microtiter plates. Each well contained in a volume of 100 μL, 100 mM Tris-Cl, pH 8.0, enzyme, 5 mM γ-GPNA, and 5 mM ACC. Assays were initiated with γ-GPNA and activity was measured spectrophotometrically as the formation of *p*-nitroaniline at 405 nm using a microplate reader (Thermomax, Molecular Devices, Menlo Park, CA). One unit of enzyme activity was defined as the amount catalyzing the formation of 1 nmol *p*-nitroanilide per minute.

Purification of the γGTase

Six kilograms of pericarp and epidermis was obtained from tomato fruit harvested at the red-ripe stage. All subsequent steps were performed at 5°C. The tissue was ground for 30 s in a blender in aliquots of 200 g with 1 g of Polyclar AT powder (GAF, New York) and 400 mL of buffer A, which contained 100 mM Tris-Cl, pH 8.0, 1 mM benzamindine, 1 mM 6-amino-*n*-hexanoic acid, 1 mM phenylmethylsulfonyl fluoride (PMSF), 250 μM *N*-α-*p*-tosyl-L-arginine methyl ester, 250 μM *N*-tosyl-L-phenylalanine chloromethyl ketone, 3 μM pepstatin, and 1 μM leupeptin. The crude extract was centrifuged for 20 min at 7,000*g*, and the pellet was recovered and reextracted with 2 L of buffer A. The pellet was again recovered and extracted twice with 3 L of buffer A plus 1.0 M NaCl and 5 mM EDTA. The supernatants from the two extractions with NaCl were filtered through Miracloth (Calbiochem, La Jolla, CA) to remove any remaining debris. Acetone (chilled to −20°C) was added to 75% (v/v).

After 6 h, precipitated proteins were pelleted by centrifugation for 15 min at 7,000*g*. The pellet was dissolved in buffer containing 100 mM Tris-Cl, pH 8.0, 5 mM EDTA, 1 M NaCl, 1 mM benzamidine, 1 mM 6-amino-*n*-hexanoic acid, 3 μM pepstatin, and 1 μM leupeptin, and centrifuged for 1 h at 150,000*g*. The 150,000*g* supernatant was diluted 1:4 with 100 mM Tris-Cl, pH 8.0, to a final NaCl concentration of 0.25 M, and applied at a flow rate of 0.5 mL min^{-1} to a Dye-Matrex Green A column (1.0 × 30 cm, Amicon, Beverly, MA) that had been equilibrated in 100 mM Tris-Cl, pH 8.0 plus 0.25 M NaCl (buffer B). This and subsequent columns were interfaced with a FPLC system (Pharmacia, Piscataway, NJ). The column was washed with buffer B, followed by a gradient of 0.25 to 0.4 M NaCl in 100 mM Tris-Cl, pH 8.0, which removed most of the bound protein. The γGTase activity was then eluted with a 0.4 to 2.0 M gradient of NaCl. Active fractions were pooled, ammonium sulfate was added to a concentration of 1.7 M, and the protein was loaded at a flow rate of 0.5 mL min^{-1} onto a Phenyl Superose HR 5/5 column (Pharmacia) equilibrated with 100 mM Tris-Cl, pH 8.0, containing 1.7 M ammonium sulfate. The enzyme was eluted with a 1.7 to 1.0 M gradient of ammonium sulfate in 100 mM Tris-Cl, pH 8.0. Fractions with activity were pooled, concentrated, and desalted using concentrators (Centricon 10, Amicon). They were then loaded at a flow rate of 0.25 mL min^{-1} onto a 0.5- × 19-cm Con A column (Pharmacia) equilibrated in buffer C (100 mM Tris-Cl, pH 8.0, plus 0.5 M NaCl). After washing with buffer C, the column was washed with a gradient of 0 to 15 mM Glc in buffer C followed by a gradient of 0 to 50 mM methyl-α-D-glucopyranoside in buffer C. After elution from this column, active fractions were used immediately or stored at −80°C. The γGTases eluted from the Con A column were used for all further characterization.

All stages in the purification were monitored by both the hydrolysis of γ-GPNA and transpeptidation to form GACC. Protein concentrations were determined using a Bio-Rad (Richmond, CA) protein assay kit or a protein-gold reagent (Integrated Separation Systems, Natick, MA). Bovine serum albumin (BSA) was used as a standard for both assays. Protein purity was determined by one-dimensional SDS-PAGE (8%–25% gradient of acrylamide) using the Pharmacia Phast System. The 10-kD protein ladder from Gibco-BRL (Gaithersburg, MD) was used as standard. Proteins were detected by silver staining using a modified Phast System protocol. Identical gels or halves of gels were blotted to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad) using the Phast System, probed with a Con A-horseradish peroxidase conjugate (Sigma, St. Louis), and visualized using an ImmunoPure Metal Enhanced DAB Substrate Kit (Pierce, Rockford, IL). Molecular masses were estimated by gel filtration on two Pharmacia Superdex 75 HR 10/10 columns connected in series. The columns were calibrated with proteins of known molecular mass.

RESULTS

Purification of γGTases

Red-ripe pericarp was used for the purification of γGTases because we previously showed that γGTase activity increased in all maternal tissues as the tomato fruit developed and ripened (Martin et al., 1995). Because mammals have some γGTases with very broad specificity and others with very narrow specificity for their γ-Glu donor, we monitored the progress of the purification by two methods using two different γ-Glu donors. The spectrophotometric assay measures *p*-nitroaniline formed by the hydrolysis of the artificial substrate γ-GPNA (Pennickx and Jasper, 1985). The radiometric transpeptidase assay measures transfer of the γ-glutamyl moiety from the donor (GSH) to [^{14}C]ACC to form [^{14}C]GACC. Representative results are summarized in Table I for purification of two γGTases from 6 kg of pericarp. γGTase I was purified 2,977-fold to a specific activity of 24,028, and γGTase II was purified 2,152-fold to a specific activity of 17,366, as measured by the hydrolysis of γ-GPNA.

After centrifugation of the crude tomato extract at low speed, 100% of the activity measured by both methods was recovered in the pellet. Activities measured by both methods were quantitatively released from the pellet by washing with buffer containing either 1.0 M NaCl or 1.0 KCl. Lower concentrations of salt failed to release as much as 50% of both activities from the pellet. The γGTases released by NaCl were concentrated by precipitation with 75% (v/v) acetone. Ultracentrifugation was used to remove remaining membranes and particulate material from the re-

Table I. *Purification of two γGTases with different affinities for Con A from ripe tomato pericarp*

Purification Step	Total Protein	γ-GPNA Hydrolysis				Transpeptidase				Ratio Hydrolase to Transpeptidase
		Total activity	Specific activity	Purification	Recovery	Total activity	Specific activity	Purification	Recovery	
	mg	*units*[a]	*units/mg*	*-fold*[b]	*%*	*units*[c]	*units/mg*	*-fold*[b]	*%*	
Crude extract	8,749	70,600	8.069	1	100	105,901	12.10	1	100	0.67
Crude pellet	2,345	70,550	30.09	3.7	100	110,636	47.180	3.9	104	0.64
NaCl extract of pellet	1,497	74,001	49.43	6.1	105	92,325	76.238	6.3	87	0.80
Acetone precipitation	210	68,806	327.6	41	97	91,620	436.3	36	86	0.75
High speed supernatant	150	55,710	371	46	78	21,420	142.8	12	19	2.6
Dye-Matrex Green A	2.44	36,923	15,132	1,875	52	14,137	5,794	479	13	2.6
Phenyl Superose HR 5/5	1.07	21,831	20,403	2,528	31	8,480	7,927	655	8.0	2.6
Concanavalin A										
γGTase I	0.141	3,388	24,028	2,977	4.8	1,891	13,411	1,108	1.8	1.8
γGTase II	0.527	9,152	17,366	2,152	13	4,308	8,175	675	4.1	2.1

[a] One unit of activity is defined as the amount of enzyme catalyzing the formation of 1 nmol *p*-nitroanilide min^{-1}. [b] Purification values are based on total activity in a crude extract prepared from 6 kg of red ripe pericarp. [c] One unit of activity is defined as the amount of enzyme catalyzing the formation of 1 nmol GACC min^{-1}.

suspended acetone pellet. Nearly 70% of the activity measured by the transpeptidase assay, but less than 20% of the γ-GPNA-hydrolyzing activity, pelleted at this stage. As a result, the ratio of γ-GPNA-hydrolyzing to transpeptidase activities in the soluble fraction increased from 0.75 to 2.6. Only the soluble γGTase activity was applied to a Dye-Matrex Green A column and further purified; elution from this column with 0.8 to 1.2 M NaCl resulted in a 1,875-fold overall purification, as measured by hydrolysis of γ-GPNA. The γGTase exhibited no requirement for ATP/ADP, pyridine nucleotides, or coenzyme A as cofactors, substrates, or activators (Martin et al., 1995). Thus, we have no explanation for the high-affinity binding of the γGTase to Dye-Matrex Green A and other dye-ligand columns, including Amicon Dye-Matrex Red A and Blue A and Cibacron Blue 3GA-agarose (Sigma; M. Martin, unpublished results).

The final step in the purification protocol, chromatography on a Con A affinity matrix, resolved the γGTase activity into three fractions. One fraction, containing about 30% of the γ-GPNA-hydrolyzing activity, failed to bind to Con A. This γGTase was not further characterized. Two fractions, designated γGTase I and II, bound to the column and were eluted as sharp peaks with Glc and methyl-α-D-glucopyranoside, respectively. Overall, recovery of activity (γGTase I plus II) as measured by hydrolysis of γ-GPNA was 18% and by the transpeptidase assay was 4%.

Properties of the Purified γGTases

γGTase I and II and the γGTase that did not bind Con A appeared as single 43-kD bands when visualized by silver staining after SDS-PAGE (Fig. 1, lanes 1–3). Further manipulation of the purified γGTases often resulted in the appearance of a 27-kD band (Fig. 1, lanes 4 and 5), that may be the result of proteolysis. Both γGTase I and II and the 27-kD peptide reacted with the Con A-horseradish peroxidase conjugate, confirming that they are glycosylated. γGTase I and II exhibited identical native molecular masses of 43 kD by gel filtration chromatography on Superdex 75 columns, indicating that each is monomeric.

γGTase I and II exhibited almost identical pH profiles (Fig. 2). Both enzymes had very broad pH optima between 7.0 and 9.0 as measured by either the hydrolysis of γ-GPNA or the transfer of Glu from GSH to ACC. However, the effect of pH on the activity measured by the two methods was notable. The rate of transpeptidation by both γGTase I and II was near zero between pH 5.5 and 6.5. In contrast, the rate of hydrolysis of γ-GPNA by both γGTase I and II was only 2- to 3-fold lower between pH 5.5 and 6.5 than at the optima. At pH 10, the rates of transpeptidation of Glu to form GACC and the rate of hydrolysis of γ-GPNA were nearly equal.

Substrate Specificity

γGTase I and II exhibited very broad and similar specificities for the γ-glutamyl donor substrate in the transpeptidation reaction. For both activities, the rates of transpeptidation were very similar for GSH, GSSG, and several *S*-substituted analogs of GSH. Activities, expressed as a

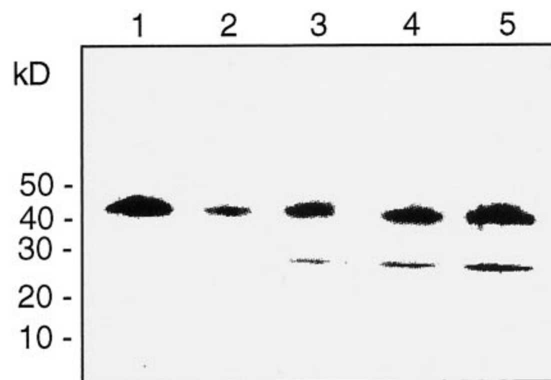

Figure 1. SDS-PAGE of the purified γGTases visualized by silver staining. Lane 1, Purified γGTase I after Con A; lane 2, purified γGTase II after Con A; lane 3, the γGTase activity not binding to Con A; lane 4, purified γGTase I after Superdex 75; and lane 5, purified γGTase II after Superdex 75. Molecular masses in kD of protein standards are designated at the left.

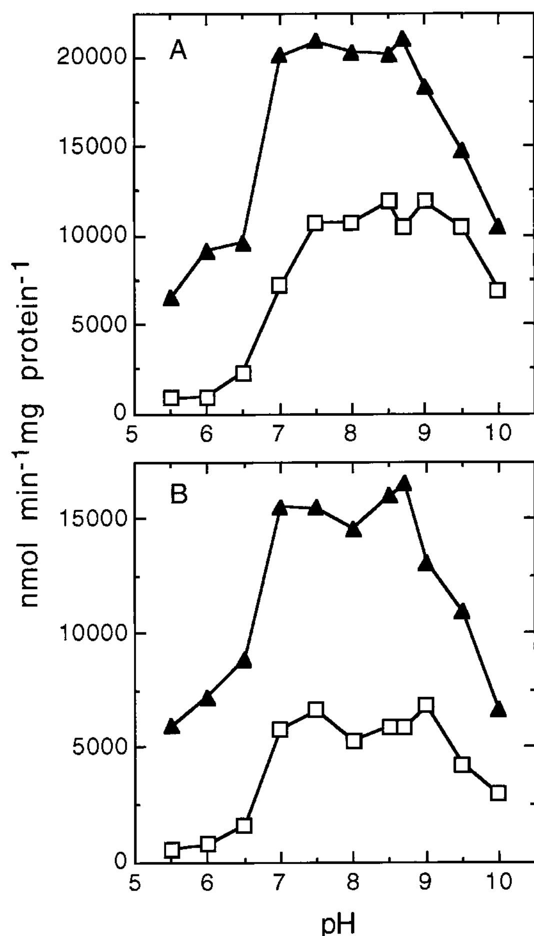

Figure 2. γGTase I (A) and γGTase II (B) have similar pH profiles for hydrolysis of γ-GPNA (▲) and for transpeptidation using GSH as the donor (□). Assays were performed as described in "Materials and Methods" in several buffers over their operating pH range. These included MES, MOPS, POPSO, Tris, AMPSO, and CAPSO. Data using only MES, Tris, AMPSO, and CAPSO are shown because activities were 2-fold lower in MOPS or POPSO than in Tris at the same pH values.

percentage of the rate with GSH, are shown using 0.4 and 2 mM donor peptide (Table II). γGTase II, but not γGTase I, was inhibited by *S*-substituted analogs of GSH at 2 mM. Several dipeptides with N-terminal γ-linked Glu, including γ-Glu-Cys, also served as donors for both γGTase I and II (Table III). The rates of reaction for all dipeptides tested except γ-Glu-Cys and γ-Glu-Trp were lower than for GSH, and the K_m values were higher (data not shown). Several of the dipeptides were less effective donors for γGTase II than for γGTase I. For both γGTase I and II, the reaction rates were consistently lower with the artificial substrates routinely employed in spectrophotometric, fluorimetric, or histochemical assays of animal γGTases than with GSH (Table III). 4-Methoxy-β-napthylamide exhibited a low rate of reaction with γGTase I and II when γ-linked to Glu, and no reaction when α-linked. Dipeptides and tripeptides lacking the N-terminal γ-linked Glu failed to function as donors (data not shown).

ACC, the acceptor in the transpeptidase assay, is a neutral, optically inactive amino acid. Some L-amino acids also serve as acceptors for γGTase I and II in the transpeptidation reaction. In transpeptidation reactions containing [^{14}C]L-amino acids instead of [^{14}C]ACC as the acceptor, radiolabeled products were formed from Met, Trp, Phe, Ala, and Asp, but not from the ACC analog α-aminoisobutyric acid (Table IV). In every case, the percentage of L-amino acid converted to the γ-Glu-amino acid was lower than the percent of ACC converted to GACC. [^{14}C]Dipeptides, tripeptides, and D-amino acids are not commercially available. However, two lines of evidence indicate that both γGTase I and II use several other L-amino acids, dipeptides, and tripeptides as acceptors, but use the corresponding D-isomer poorly or nor at all. First, L-amino acids and the L-isomers of several dipeptides and tripeptides inhibited the [^{14}C]ACC-dependent transpeptidation reaction (Table V and Table VI). L-Amino acids at concentrations equal to the ACC concentration (1 mM) inhibited both γGTase I and II between 7% and 57% (Table V), suggesting that they compete with ACC as acceptors. In contrast, D-amino acids at concentrations 4-fold higher than the ACC concentration were poor inhibitors; the ACC-dependent γGTase I reactions were inhibited between 0% to 22%, and the γGTase II reactions between 0% and 33% (Table V). Both dipeptides and tripeptides at a concentration equal to the ACC concentration inhibited γGTase II between 20% and 95%, but only dipeptides significantly inhibited γGTase I (Table VI). Greater than 50% inhibition of the ACC-dependent reac-

Table II. *S-Substituted analogs of GSH serve as donors for both γGTase I and II*

Radiometric transpeptidase assays were performed as described in "Materials and Methods" using GSH or other donor peptide concentrations of 0.4 or 2 mM. The acceptor was 5 mM [^{14}C]ACC. The specific activity of γGTase I was 13,200 nmol min^{-1} mg^{-1} with both 0.4 and 2 mM GSH. The specific activity of γGTase II was 8080 nmol min^{-1} mg^{-1} with both 0.4 and 2 mM GSH. Values are the mean ± SD of three to six measurements and are representative of results obtained in three independent experiments.

Donor Peptide	γGTase I	γGTase II
	% activity with GSH	
GSH		
0.4 mM	100	100
2.0 mM	100	100
S-Methylglutathione		
0.4 mM	88 ± 5	95 ± 3
2.0 mM	103 ± 7	84 ± 5
S-Propylglutathione		
0.4 mM	96 ± 3	84 ± 5
2.0 mM	99 ± 5	66 ± 1
S-Pentylglutathione		
0.4 mM	85 ± 2	93 ± 3
2.0 mM	104 ± 5	64 ± 6
S-*p*-Nitrobenzylglutathione		
0.4 mM	37 ± 4	65 ± 5
2.0 mm	—	—
GSSG		
0.4 mM	130 ± 2	115 ± 3
2.0 mM	108 ± 5	72 ± 7

Table III. *Several dipeptides with N-terminal γ-L-glutamic acid serve as donors for γGTase I and II*

Radiometric transpeptidase assays were performed as described in "Materials and Methods" using 0.4 or 2 mM GSH or other donor. [14C]ACC was the acceptor. Concentrations of 2 mM but not 0.4 mM were saturating for all dipeptides but not for the artificial substrates. All amino acids are L-isomers except where otherwise indicated. Values are the mean ± SD of three to six data points and are representative of results obtained in three independent experiments. The specific activity of γGTase I was 13,100 nmol min^{-1} mg^{-1} with both 0.4 and 2 mM GSH. The specific activity of γGTase II was 7,990 nmol min^{-1} mg^{-1} with both 0.4 and 2 mM GSH.

Donor Peptide	γGTase I Percent of Activity with GSH	γGTase II Percent of Activity with GSH
GSH		
0.4 mM	100	100
2.0 mM	100	100
γ-Glu-Cys		
0.4 mM	71 ± 10	60 ± 5
2.0 mM	119 ± 5	95 ± 4
γ-Glu-His		
0.4 mM	59 ± 4	41 ± 7
2.0 mM	89 ± 6	92 ± 3
γ-Glu-Phe		
0.4 mM	46 ± 5	37 ± 7
2.0 mM	93 ± 8	50 ± 3
γ-Glu-Trp		
0.4 mM	95 ± 6	61 ± 8
2.0 mM	156 ± 10	98 ± 7
γ-Glu-Gln		
0.4 mM	77 ± 5	29 ± 8
2.0 mM	92 ± 3	51 ± 10
γ-Glu-Leu		
0.4 mM	68 ± 10	37 ± 6
2.0 mM	95 ± 5	54 ± 8
γ-Glu-Glu		
0.4 mM	60 ± 3	37 ± 8
2.0 mM	49 ± 7	32 ± 10
γ-L-Glu-Gly		
0.4 mM	9 ± 2	3 ± 3
2.0 mM	11 ± 3	4 ± 2
γ-D-Glu-Gly		
0.4 mM	5 ± 2	2 ± 1
2.0 mM	5 ± 2	4 ± 3
γ-Glu-*p*-nitroanilide		
0.4 mM	–	–
2.0 mM	30 ± 5	34 ± 6
γ-Glu-(7-amino-4-methyl coumarin)		
0.4 mM	–	–
2.0 mM	23 ± 7	22 ± 5
γ-Glu-(β-naphthylamide)		
0.4 mM	–	–
2.0 mM	3 ± 2	3 ± 2
γ-Glu-(4-methoxy-β-naphthylamide)		
0.4 mM	–	–
2.0 mM	7 ± 3	6 ± 3
α-Glu-(4-methoxy-β-naphthylamide)		
0.4 mM	–	–
2.0 mM	0	0

tion suggests that Cys-Gly and Glu-Ala may be better acceptors than ACC.

The [14C]dipeptide product of the transpeptidation reaction, GACC (γGlu-ACC), also serves as an acceptor for a second transpeptidation reaction. The product of the second transpeptidation reaction, γGlu-γGlu-ACC, can then serve as acceptor for a third transpeptidation, resulting in polyglutamated ACC (γglu-γglu-γglu-ACC). Polyglutamation was observed when GSH was saturating, ACC became limiting, and the concentration of GACC approached the concentration of ACC (Fig. 3, lane 2). When both donor and acceptor were saturating, a single product was formed (Fig. 3, lane 1). The identity of these products was confirmed by analyzing the products of acid hydrolysis by TLC and by GC-MS (data not shown). Multiple radiolabeled products were also synthesized from [14C]L-amino acids (data not shown). On the other hand, when ACC was saturating and GSH was limiting in the assay, polyglutamation was not observed. Instead, the reaction product, GACC, was used as donor in subsequent reactions. GACC first increased (Fig. 3, lane 3) and then decreased during the course of an assay (Fig. 3, lane 4). The reversibility of the reaction was best demonstrated by following the rapid disappearance of GACC after the addition of excess unlabeled ACC to the reaction.

γGTase I and II, at varied GSH concentrations and a fixed ACC concentration of 5 mM, exhibited Michaelis-Menten kinetics with K_m values for GSH of 110 and 90 μM, respectively. In the hydrolase assay, γGTase I and II exhibited K_m values for γ-GPNA of 1.7 and 2.1 mM, respectively, indicating that γ-GPNA is a poor substrate for both enzymes (Table VII). In the transpeptidase assay, γGTase I and II activities exhibited biphasic kinetics with respect to ACC concentration. K_m values for ACC were 0.130 and 3.1 mM for γGTase I and 0.210 and 2.0 mM for γGTase II (Table VII). Although care was taken to use only initial reaction rates, the kinetic constants for ACC should be viewed as estimates, since the reaction is reversible. GACC can serve as a donor or acceptor for subsequent reactions. Similarly, GSH can serve as either a donor or acceptor.

Table IV. *Other L-amino acids serve as acceptors for both γGTase I and II*

Radiometric transpeptidase assays were run for 2 h as described in "Materials and Methods" with 20 μM [14C]acceptor amino acid and 2 mM GSH. Values are the mean ± SD of three data points.

Amino Acid (20 μM)	Percent of Amino Acid Converted	
	γGTase I	γGTase II
ACC	50 ± 5	50 ± 7
L-Phe	20 ± 4	18 ± 2
L-Trp	25 ± 2	15 ± 3
L-Met	45 ± 8	47 ± 10
L-Ala	10 ± 2	9 ± 1
L-Asp	11 ± 2	8 ± 1
α-Aminoisobutyric acid	0	0

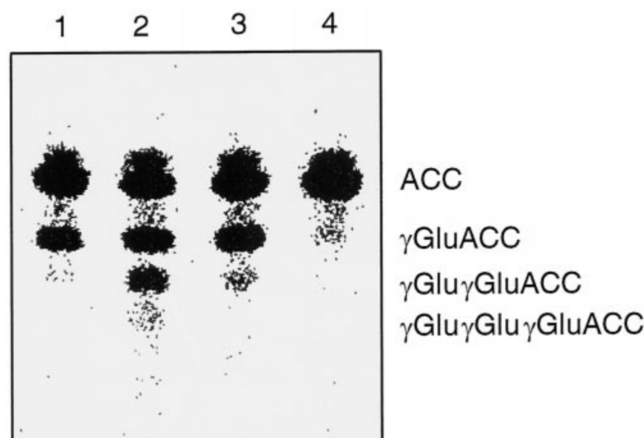

Figure 3. Radioisotope image showing the use of the reaction product GACC as a donor or acceptor in subsequent transpeptidation reactions by γGTase I. Assays were chromatographed on a normal phase high performance silica gel thin-layer plate to resolve [^{14}C]ACC and [^{14}C]GACC and were imaged and quantified using an Ambis Radioisotope Imaging System. Lane 1, Both donor GSH and acceptor [^{14}C]ACC were saturating; lane 2, GSH was 2 mM and [^{14}C]ACC was 40 μM (ACC became limiting and GACC was used as an acceptor for the subsequent transpeptidation reaction resulting in polyglutamation of ACC); lane 3, [^{14}C]ACC was 0.1 mM and GSH was 80 μM; and lane 4, the same reaction as in lane 3 was incubated 1 h longer following the addition of 2 mM unlabeleled ACC. Because GSH was limiting, [^{14}C]GACC was used as a donor.

Table V. *ACC-dependent γGTase I and II activities are inhibited more by L-amino acids than by D-amino acids*

Radiometric transpeptidase assays were performed as described in "Materials and Methods," except the unlabeled amino acids (4 mM D-amino acids or 1 mM L-amino acids) were added 5 min prior to the addition of 1 mM [^{14}C]ACC. Values are the mean ± SD of three to six data points and are representative of results obtained in two independent experiments. The specific activities of γGTase I and II in the control assay were 5,300 and 2,700 nmol min^{-1} mg^{-1}, respectively, in the control assays.

Amino Acid 4 mM D-isomers and 1 mM L-isomers	γGTase I	γGTase II
	% of control	
None (control)	100	100
D-Phe	108 ± 5	106 ± 8
D-Trp	82 ± 10	72 ± 5
D-Met	127 ± 10	100 ± 6
D-Ala	94 ± 4	71 ± 6
D-His	93 ± 5	67 ± 8
D-Glu	92 ± 4	78 ± 5
D-Asp	90 ± 6	82 ± 4
D-Asn	78 ± 2	80 ± 8
L-Phe	63 ± 3	61 ± 6
L-Trp	77 ± 5	55 ± 8
L-Met	93 ± 5	70 ± 5
L-Ala	75 ± 8	55 ± 2
L-His	61 ± 3	72 ± 2
L-Glu	64 ± 5	55 ± 8
L-Asp	71 ± 5	78 ± 6
L-Asn	72 ± 8	64 ± 6
L-Cys	43 ± 3	50 ± 8

Other Requirements for Activity

Salts (KCl, KNO_3, KBr, NaCl, and $CaCl_2$) had no effect on the activity of the purified enzymes at concentrations of 0.1 and 1.0 M. Several metal chlorides (10 mM $MgCl_2$, 1 mM, $MnCl_2$ or $CoCl_2$, or 0.1 mM $ZnCl_2$) also had no effect on either activity. The presence of DTT or EDTA had no effect on either the activity or stability of the enzyme, suggesting that reduced sulfhydryl groups are not required for activity.

DISCUSSION

Little is known about the properties, in vivo substrates, and functions of γGTases in plants. As a first step toward elucidating the function(s) of these enzymes, we have identified several γGTases in the pericarp of ripe tomato fruit. We purified and determined the catalytic properties of two of these enzymes.

The purification and characterization of γGTases from tomato were monitored using two assay methods. The standard spectrophotometric assay, developed for measuring the activity of γGTases from mammals, is facile but it does not detect all γGTases. Many γGTases from mammals have very broad specificity for their γ-Glu donor (Meister, 1989). Others, such as γ-glutamyl leukotrienase, have very narrow specificity and are unable to use γ-GPNA, GSSG, or even GSH as a glutamyl donor (Heisterkamp et al., 1991; Carter et al., 1997). A γGTase from yeast catalyzes hydrolysis but not the transpeptidation reaction (Pennickx and Jasper, 1985). In addition, the spectrophotometric assay measures only hydrolysis. It does not address the fate of Glu, which may be particularly important in plants in which many γ-glutamyl-linked peptides are found. We also used a radiometric assay, which measures transpeptidation reactions in which the Glu is transferred to a radiolabeled acceptor following hydrolysis. This assay can accommodate a broad spectrum of γ-Glu donors, including

Table VI. *ACC dependent γGTase I and II activities are inhibited by several peptides*

Radiometric transpeptidase assays were performed as described in "Materials and Methods" except that 2 mM of the indicated peptide was added 5 min prior to addition of 2 mM [^{14}C]ACC. The GSH concentration was 2 mM. Values are the mean ± SD of three to six data points and are representative of results obtained in two independent experiments. The specific activities of γGTase I and II in the control assay were 7,200 and 4,300 nmol min^{-1} mg^{-1}, respectively, in the control assays.

Peptide (2 mM)	γGTase I	γGTase II
	% of control	
None (control)	100	100
Gly-Gly	58 ± 4	56 ± 3
Cys-Gly	41 ± 5	39 ± 7
Glu-Ala	9 ± 3	5 ± 2
Glu-Glu	84 ± 6	41 ± 5
Glu-Val-Phe	92 ± 7	69 ± 10
Glu-Gly-Phe	108 ± 10	80 ± 8
Cyclopro-Gly	112 ± 5	56 ± 8

Table VII. *Kinetic parameters for γGTase I and II*

Substrate	K_m	
	γGTase I	γGTase II
	mM	
GSH[a]	0.11	0.09
γ-Glutamyl-*p*-nitroanilide[b]	1.7	2.1
ACC[c]	0.13	0.21
	3.1	2.0

[a] Radiometric transpeptidase assays were performed as described in "Materials and Methods" with 5 mM ACC and varying concentrations of GSH. [b] Spectrophotometric assays to measure *p*-nitroanilide formation were performed as described in "Materials and Methods" with 5 mM ACC and varying concentrations of γ-GPNA. [c] Radiometric transpeptidase assays were performed as described in "Materials and Methods" with 2 mM GSH and varying concentrations of ACC.

GSH, γ-GPNA, and glutathione *S*-conjugates (Tables II and III), and an equally broad spectrum of acceptors (Tables IV–VI). The only requirement is that either the donor or acceptor be appropriately radiolabeled. Furthermore, the spectrophotometric and radiometric methods can be coupled to measure hydrolysis and transpeptidation in the same assay. Using these assays, we detected γGTases that (like animal γGTases) differ in the their specificity for γ-GPNA, GSH, and other γ-Glu donors (Tables I and II). In fact, γGTases differing in specific activity in our two assays were resolved by ultracentrifugation at 150,000*g*.

In mammals, γGTases are highly expressed in tissues with secretory or absorptive functions (Meister et al., 1981; Meister, 1988, 1989). Both soluble and membrane-bound γGTases have been identified. The membrane-bound enzymes are heterodimeric glycoproteins. They have a small subunit with a M_r between 21,000 and 28,000 that contains the catalytic site. A large subunit, with a M_r between 38,000 and 72,000, contains a short hydrophobic domain, which anchors the enzyme in the membrane (Meister et al., 1981; Meister, 1989). γGTases were first purified from mammals as soluble proteolytic fragments lacking the short membrane-anchoring region. The two subunits of γGTases are synthesized as a single, nonglycosylated, precursor peptide, which is then translocated, auto-cleaved, glycosylated, and inserted into the membrane with the catalytic site most often on the external surface of the membrane. Eighteen γGTase isozymes from humans have been identified by isoelectric focusing, and many were shown to result from differing degrees of glycosylation (Meister, 1989).

We have presented evidence that tomato fruit also contains several γGTases that differ in membrane association, degree of glycosylation, and substrate specificity. Three γGTases purified from ripe tomato exhibited native and subunit molecular masses of 43 kD. Based on their binding to and differential elution from Con A, two of these enzymes may be differently glycosylated. A third 43-kD γGTase did not bind to Con A, or react with a Con A-horseradish peroxidase conjugate, indicating that it is not glycosylated. These three γGTases were released from either 7,000 or 100,000*g* wall and membrane pellets of tomato fruit with 1.0 M NaCl, suggesting that they are peripheral membrane proteins. Even with 1.0 M NaCl, nearly 70% of the transpeptidase activity but only 20% of the γ-GPNA-hydrolyzing activity pelleted upon ultracentrifugation, resulting in a dramatic change in the ratio of hydrolase to transpeptidase activity (Table I). Work is under way to further characterize the pelleted γGTase. It is possible that tomato contains both peripheral and integral membrane γGTases. A second possibility is that high salt dissociates one catalytically active subunit of a dimeric or multimeric protein. Alternatively, we may have extracted a catalytically active proteolytic fragment of a membrane-bound γGTase such as was initially isolated from animal tissues. Cognizant of this possibility, we included a cocktail of several protease inhibitors during the early stages of the purification to reduce the likelihood of proteolysis. In fact, the purified and partially purified γGTases were very susceptible to proteolysis. Continued handling and even SDS denaturation often resulted in the appearance of a 27-kD fragment in preparations initially having a single 43-kD band. Additionally, some plant tissues, notably leaves of Arabidopsis and *Brassica juncea*, contain soluble γGTases that exhibit activity only in the spectrophotometric assay, and membrane-associated γGTases that exhibit activity in both assays (M. Martin, unpublished results). Work is under way to establish the subcellular localization of these activities in Arabidopsis.

Several earlier reports described the partial purification and characterization from other plants of soluble γGTases that use γ-GPNA as a donor, but there is no consensus regarding protein size. A γGTase from kidney bean was reported to have a native molecular mass of 180 kD (Goore and Thompson, 1967). A γGTase from *Blighis sapida* (ackee fruit) was reported to have a native and subunit molecular mass of 12.5 kD (Kean and Hare, 1980). A γGTase from onion scale was reported to have a native and subunit molecular mass of 56.7 kD (Lancaster and Shaw, 1994). A γGTase from soybean seeds was reported to have a native and subunit molecular mass of 27 kD (Martin and Slovin, 1996).

The catalytic properties of the two purified γGTases from tomato are consistent with broad in vivo functions, including the hydrolysis of GSH, GSSG, and/or glutathione *S*-conjugates. In fact, the two γGTases from tomato fruit, like some γGTases from animals and the γGTase from soybean seeds, have very broad donor specificity and a high affinity for GSH (with K_m values of 90 and 110 μM for the tomato enzymes compared with 5–10 μM for some animal enzymes and 80 μM for the soybean enzyme; Meister et al., 1981; Meister, 1989; Martin and Slovin, 1996). In contrast, the enzymes isolated from bean and onion have K_m values for GSH between 1 and 5 mM (Goore and Thompson, 1967; Lancaster and Shaw, 1994). Both γGTase I and II exhibited higher affinity for GSH, GSSG, and glutathione *S*-conjugates than for most dipeptides and for several artificial substrates with an N-terminal, γ-linked Glu. In vitro, both γGTase I and II can initiate hydrolysis of GSH and glutathione *S*-conjugates from the N terminus or can hydrolyze the dipeptide resulting from initial hydrolysis of GSH and its conjugates at the C terminus. Both

activities function only as hydrolases at low pH, and thus might be able to hydrolyze glutathione *S*-conjugates in the vacuole.

Like γGTases from animals, the acceptor specificity of γGTase I and II is broad and includes the L-isomers of several amino acids, dipeptides, and tripeptide. We have also shown that both γGTase I and II catalyze a reversible reaction by using the product, GACC, as a donor for subsequent reactions. In vitro, both γGTase I and II can synthesize as well as hydrolyze the numerous peptides with N-terminal, γ-linked Glu that occur in various plant tissues. In crude extracts of tomato pericarp, γGTases catalyzed the formation of a novel conjugate of the ethylene precursor, ACC (Martin et al., 1995). γGTase activity increased through the course of tomato fruit maturation and ripening (Martin et al., 1995) and paralleled the increase in conjugated ACC reported by other researchers (Su et al., 1984). Similarly, levels of γGTase activity in onion paralleled the hydrolysis of γ-glutamyl-peptides of sulfur compounds such as γ-glutamyl alk(en)yl-cysteine sulfoxide, making them available for defensive purposes at key developmental points and, incidentally, as the flavor of onion (Lancaster et al., 1989; Lancaster and Shaw, 1989, 1991, 1994). The in vivo functioning of γGTases in these reactions has not been demonstrated.

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