Note Plant Biotechnology, 21 (3), 243-246 (2004)

Occurrence of Two Forms of γ-Glutamyltransferases in Radish Plant

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Received 1 April 2004; accepted 1 June 2004

Abstract

In radish (*Raphanus sativus* L.) cotyledons, γ-glutamyltransferase (GGT, EC 2.3.2.2) activity was detected in both fractions extracted with grinding buffer alone (low ionic strength buffer) and the same buffer supplemented with 1 M NaCl (high ionic strength buffer). The former was referred to as soluble GGT and the latter, as bound GGT. Bound GGT was not solubilized by 1% Triton X-100. The activities of both soluble and bound GGTs were inhibited by acivicin, an inhibitor of mammalian and *Escherichia coli* GGTs. The activity of GGT extracted with the high ionic strength buffer disappeared when protoplasts were prepared, indicating the localization of bound GGT to cell walls. These results strongly indicate the occurrence of two forms of GGTs, soluble and bound ones. The two forms of GGTs were also found in *Arabidopsis thaliana*, cabbage, pea, soybean, maize and rice among the eight plants tested, in addition to radish.

Key words: γ-glutamyltransferase; soluble form; bound form; *Raphanus sativus* L.; radish

γ-Glutamyltransferase (GGT, EC 2. 3. 2. 2) catalyzes the transfer of the γ-glutamyl residue of γ-glutamyl peptides to appropriate acceptors and is involved in the first step of glutathione (GSH) catabolism in mammals (Meister and Anderson, 1983; Taniguchi and Ikeda, 1998), *Saccharomyces cerevisiae* (Jaspers *et al.*, 1985) and *Escherichia coli* (Suzuki *et al.*, 1993). In higher plants, GGT is presumed to catalyze the synthesis and degradation of numerous γ-glutamyl peptides (Kean and Hare, 1980; Lancaster and Shaw, 1994; Kasai *et al.*, 1982; Kawasaki *et al.*, 1982; Kasai *et al.*, 1986). It has also been reported that GGT is involved in GSH catabolism in higher plants (Schneider and Rennenberg, 1992; Bergmann and Rennenberg, 1993).

GGT is a membrane-bound enzyme in mammals (Meister and Anderson 1983) and *S. cerevisiae* (Payne and Payne, 1984; Jaspers and Penninckx, 1984), while it occurs in the periplasmic space in *E. coli* (Suzuki *et al.*, 1993). Complete purification of plant GGT was achieved from onion bulb scales (Lancaster and Shaw, 1994) and tomato fruits (Martin and Slovin, 2000). Onion and tomato GGTs that were extracted with 50-100 mM Tris-HCl buffer containing 0.5-1.0 M NaCl were composed of a single polypeptide, unlike heterodimeric GGTs in mammals (Tate and Meister, 1976; Taniguchi and Ikeda, 1998), *S. cerevisiae* (Jaspers *et al.*, 1985) and *E. coli* (Suzuki *et al.*, 1986). On the other hand, *Arabidopsis thaliana* GGT expressed in tobacco plant is a heterodimeric enzyme (Storozhenko *et al.*, 2002). GGT activity was detected in the soluble fraction extracted with 20 mM HEPES buffer from tobacco suspension culture (Steinkamp and Rennenberg, 1984). These findings prompted us to hypothesize that plant GGTs occur in multiple forms. To confirm this, we attempted to fractionate plant GGTs by differential extraction. Here we describe the occurrence of soluble and bound GGTs in radish plant (*Raphanus sativus* L.).

GGT activity was measured according to the method of Naftalin *et al.*, (1969) with some modifications. The standard reaction mixture (150 μl) contained 3 mM γ-L-glutamyl-*p*-nitroanilide (γ-Glu*p*NA), 50 mM glycylglycine, 100 mM Tris-HCl buffer, pH 8.0, and 5 to 20 μl of the enzyme solution. After 20 min incubation at 37℃, the reaction was terminated by the addition of 500 μl of 10% acetic acid. Then, 250 μl of 0.1% $NaNO_2$ was added and the mixture was incubated for 3 min at room temperature. To this, 250 μl of 1% ammonium amidosulfate was added. After 1 min incubation at room temperature, 250 μl of 0.05% *N*-naphthylethylenediamine was added and the absorbance at 540 nm was measured. The amount of *p*-nitroaniline released was calculated from the stan-

dard curve obtained using *p*-nitroaniline. Under the conditions used, the amounts of *p*-nitroaniline released by the enzyme reaction increased linearly up to 30 min of the incubation time.

The crude enzyme solution was prepared at 4℃. Cotyledons of 8-day-old radish green seedlings were homogenized with 3 volumes of grinding buffer (50 mM Tris-HCl buffer, pH 8.0, containing 10 mM 2-mercaptoethanol, 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride; low ionic strength buffer) and 5% (w/w) insoluble polyvinylpyrrolidone. The homogenate was centrifuged at 10,000*g* for 10 min. The supernatant and the precipitate obtained were referred to as 10,000*g* supernatant and 10,000*g* precipitate, respectively. The 10,000*g* supernatant was further centrifuged at 170,000*g* for 60 min and the supernatant obtained was referred to as 170,000*g* supernatant. The precipitate was dissolved in buffer A (20 mM Tris-HCl buffer, pH 8.0, containing 10 mM 2-mercaptoethanol) and referred to as 170,000*g* precipitate. The 10,000*g* precipitate, after washing twice with the grinding buffer, was homogenized gently for 30 min with buffer A supplemented with 1 M NaCl (high ionic strength buffer), 1% Triton X-100, or both, and the homogenate was centrifuged at 10,000 *g* for 20 min. The supernatants thus obtained were referred to as 1 M NaCl extract, 1% Triton X-100 extract and 1 M NaCl plus 1% Triton X-100 extract, respectively. Proteins were measured according to the method of Bradford (1976) with bovine serum albumin as the standard.

Protoplasts were prepared as follows. Cotyledons (4 g) cut into small pieces were placed in 40 ml of the isolation mixture (0.5 M sorbitol, 2.0% Cellulase Onozuka RS, 0.5% Macerozyme R-10, pH 6.0) in a Petri dish, subjected to vacuum infiltration for 5 min, and then incubated at 25℃ for 2 h. Protoplasts passing through a nylon mesh (100 μm) were centrifuged at 100*g* for 5 min. The resulting protoplast pellet was resuspended in 0.5 M sorbitol solution gently and centrifuged at 100*g* for 5 min. The washing procedures were repeated once more. Crude protoplasts were purified as described by Larkin (1976). Chlorophyll contents in cotyledons and protoplasts were measured according to the method of Wintermans and de Mots (1965).

When radish cotyledons were homogenized with the grinding buffer, GGT activity was detected in the 10,000*g* supernatant. The GGT activity in the 10,000*g* supernatant was recovered in the 170,000*g* supernatant when the 10,000*g* supernatant was further centrifuged, but not in the 170,000*g* precipitate (**Table 1**). Thus, the GGT detected in the 10,000*g* supernatant was soluble GGT. Another GGT activity was detected in the 1 M NaCl extract obtained by solubilizing the 10,000*g* precipitate with buffer A supplemented with 1 M NaCl (**Table 1**). GGT activity was also detected in the 1 M NaCl plus 1% Triton X-100 extract, but not in the 1% Triton X-100 extract. Thus, GGT in the 10,000*g* precipitate was solubilized by the high ionic strength buffer and this GGT was referred to as bound GGT. Acivicin, an inhibitor of mammalian and *E. coli* GGTs (Gardell and Tate, 1980; Suzuki and Kumagai, 2002), strongly inhibited both soluble and bound radish cotyledon GGTs. The concentrations of acivicin inhibiting 50% of soluble and bound GGT activities were 0.012 and 0.26 mM under the conditions used, respectively. Soluble and bound GGTs were also detected in radish leaves and edible roots (**Table 2**). Hereafter, the 10,000*g* supernatant and the 1 M NaCl extract of the 10,000*g* precipitate were used as the enzyme sources for soluble and bound GGTs, respectively.

To confirm the occurrence of two forms of GGT in other plants, we measured GGT activity in eight plants other than radish. GGT activity was detected in the soluble fraction in six of the eight plants tested (**Table 2**). Tobacco and onion did not exhibit any significant GGT activity in the soluble fraction, as reported previously (Lancaster and Shaw, 1994; Storozhenko *et al.*, 2002). On the other hand, all plants tested exhibited GGT activity in the bound fraction, although the activity level varied (**Table 2**).

Table 1 Localization of GGT activity in radish cotyledons

Fraction	GGT activity (nkat/g fresh wt)
10,000*g* supernatant (soluble)	0.284 ± 0.026
170,000*g* supernatant	0.255 ± 0.032
170,000*g* precipitate	0.011 ± 0.003
1 M NaCl extract of 10,000*g* precipitate (bound)	3.271 ± 0.085
1 M NaCl plus 1% Triton X-100 extract of 10,000*g* precipitate	2.258 ± 0.085
1% Triton X-100 extract of 10,000*g* precipitate	nd

Radish cotyledons were fractionated according to the methods described in the text and GGT activity in each fraction was measured. Values are means ± SD (n=3). nd: not detected.

Table 2 GGT activities in soluble and bound fractions of various plants

Plant	Tissue	Fraction	Protein (mg/g fresh wt)	GGT activity (nkat/g fresh wt)
Radish (*Raphanus sativus*)	Cotyledon	Soluble	7.18 ± 0.37	0.486 ± 0.027
		Bound	1.03 ± 0.06	2.583 ± 0.122
	Leaf	Soluble	0.96 ± 0.01	0.614 ± 0.028
		Bound	0.31 ± 0.04	2.131 ± 0.172
	Root	Soluble	0.16 ± 0.01	0.073 ± 0.003
		Bound	0.05 ± 0.01	0.266 ± 0.054
Arabidopsis thaliana	Leaf	Soluble	3.37 ± 0.04	0.388 ± 0.086
		Bound	0.43 ± 0.11	1.500 ± 0.034
Cabbage (*Brassica oleraceae*)	Leaf	Soluble	1.25 ± 0.12	0.052 ± 0.002
		Bound	0.27 ± 0.04	0.453 ± 0.022
Pea (*Pisum sativum*)	Leaf	Soluble	9.60 ± 0.22	0.120 ± 0.002
		Bound	1.17 ± 0.19	0.083 ± 0.005
Soybean (*Glycine max*)	Leaf	Soluble	7.07 ± 0.12	1.300 ± 0.020
		Bound	1.16 ± 0.05	1.803 ± 0.043
Tobacco (*Nicotiana tabacum*)	Leaf	Soluble	3.22 ± 0.25	nd
		Bound	0.67 ± 0.01	0.218 ± 0.083
Onion (*Allium cepa*)	Bulb scale	Soluble	0.81 ± 0.03	nd
		Bound	0.24 ± 0.03	0.070 ± 0.007
Maize (*Zea mays*)	Leaf	Soluble	4.51 ± 0.75	0.122 ± 0.004
		Bound	0.54 ± 0.15	0.257 ± 0.015
Rice (*Oryza sativa*)	Leaf	Soluble	5.58 ± 1.00	0.277 ± 0.013
		Bound	0.82 ± 0.10	0.432 ± 0.028

Plant materials except radish cotyledons were obtained from local producers or the university's experimental farm. Soluble and bound fractions represent 10,000*g* supernatant and 1 M NaCl extract of the 10,000*g* precipitate, respectively. Values are means ± SD (n=3). nd: not detected.

Radish bound GGT was not a membrane-bound enzyme as it was not solubilized by 1% Triton X-100. To examine whether bound GGT is localized outside the cells, protoplasts were prepared from the radish cotyledons. We assumed that the chlorophyll content per unit cotyledon cell is the same as that per unit protoplast. When proteins were extracted from protoplasts with the grinding buffer supplemented with 1 M NaCl, GGT activity was lowered to a level similar to that of GGT extracted with the grinding buffer alone (**Table 3**). This result strongly indicates that bound GGT localizes to the cell walls.

Table 3 GGT activities in radish cotyledons and protoplasts

Enzyme source	NaCl (1 M)	GGT activity (nkat/mg chlorophyll)
Cotyledon	-	0.279 ± 0.002
	+	2.825 ± 0.260
Protoplast	-	0.272 ± 0.004
	+	0.313 ± 0.004

Plant materials were homogenized with 5% insoluble PVP and the grinding buffer with or without 1 M NaCl. The homogenate was centrifuged at 10,000*g* for 10 min at 4℃ and GGT activity in the supernatant was measured. Values are means ± SD (n=3).

Our results indicate that most plants have two forms of GGTs, soluble and cell wall bound ones, in contrast to mammals, *S. cerevisiae* and *E. coli* (Jaspers and Penninckx, 1984; Payne and Payne, 1984; Suzuki *et al.*, 1993 Taniguchi and Ikeda, 1998). The occurrence of bound GGT as shown in this study is supported by the results obtained with onion and tomato GGTs (Lancaster and Shaw 1994; Martin and Slovin, 2000). *A. thaliana* has three putative GGT genes, At4g39640, At4g39650 and At4g29210. The DNA sequences of these genes, which are homologous to those of mammals, *S. cerevisiae* and *E. coli*, suggest that the genes encode heterodimeric GGTs. Indeed, tobacco plants transformed with an *A. thaliana* cDNA (D22), that is highly homologous to At4g39640, expressed a heterodimeric GGT (Storozhenko *et al.*, 2002). This result is contrary to those obtained with onion and tomato regarding subunit composition. Our

preliminary results on the purification of radish GGTs suggest that the subunit compositions of soluble and bound GGTs are different. Storozhenko *et al.* (2002) reported that *A. thaliana* GGT expressed in tobacco plants was membrane bound. However, the heterodimeric GGTs detected in radish cotyledons were not membrane bound (data not shown). The complete purification of radish soluble and bound GGTs is in progress and the results will be described soon.

Acknowledgements

This work was partially supported by a JSPS-RFTF97R16001 grant and Grants-in-Aid for Scientific Research (C) (12660059 and 14560052) from the Japan Society for the Promotion of Science.

References

Bergmann, L., Rennenberg, H. 1993. Glutathione metabolism in plants. In: de Kok L. A. et al. (Eds): Sulfur Nutrition and Assimilation in Higher Plants, 109-123. SPB Academic Publishing, The Hague.

Bradford, M. M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., **72**: 248-254.

Gardell, S. J., Tate, S. S., 1980. Affinity labeling of γ-glutamyl transpeptidase by glutamine antagonists. FEBS Lett., **122**: 171-174.

Jaspers, C. J., Gigot, D., Penninckx, M. J., 1985. Pathways of glutathione degradation in the yeast *Saccharomyces cerevisiae*. Phytochem., **24**: 703-707.

Jaspers, C. J., Penninckx, M. J., 1984. Glutathione metabolism in yeast *Saccharomyces cerevisiae*. Evidence that γ-glutamyltranspeptidase is a vacuolar enzyme. Biochimie, **66**: 71-74.

Kasai, T., Ohmiya, A., Sakamura, S., 1982. γ-Glutamyltranspeptidases in the metabolism of γ-glutamyl peptides in plants. Phytochem., **21**: 1233-1239.

Kasai, T., Shiroshita, Y., Sakamura, S., 1986. γ-Glutamyl peptides of *Vigna radiata* seeds. Phytochem., **25**: 679-682.

Kawasaki, Y., Ogawa, T., Sasaoka, K., 1982. Occurrence and some properties of a novel γ-glutamyltransferase responsible for the synthesis of γ-glutamyl-D-alanine in pea seedlings. Biochim. Biophys. Acta, **716**: 194-200.

Kean, E. A., Hare, E. R., 1980. γ-Glutamyl transpeptidase of the ackee plant. Phytochem., **19**: 199-203.

Lancaster, J. E., Shaw, M. L., 1994. Characterization of purified γ-glutamyl transpeptidase in onions: evidence for in vivo role as a peptidase. Phytochem., **36**: 1351-1358.

Larkin, P. J., 1976. Purification and viability determinations of plant protoplasts. Planta, **128**: 213-216.

Martin, M. L., Slovin, J.P., 2000. Purified γ-glutamyl transpeptidases from tomato exhibit high affinity for glutathione and glutathione *S*-conjugates. Plant Physiol., **122**: 1417-1426.

Meister, A., Anderson, M. E., 1983. Glutathione. Annu. Rev. Biochem., **52**: 711-760.

Naftalin, L., Sexton, M., Whitaker, J. F., Tracey, D., 1969. A routine procedure for estimating serum γ-glutamyltranspeptidase activity. Clin. Chim. Acta, **26**: 293-296.

Payne, G. M., Payne, J. W., 1984. γ-Glutamyltransferase is not involved in the bulk uptake of amino acids, peptides or γ-glutamyl-amino acids in yeast (*Saccharomyces cerevisiae*). Biochem. J., **218**: 147-155.

Schneider, A., Rennenberg, H., 1992. Degradation of glutathione (GSH) in heterotrophic tobacco cells. Phyton, **32**: 113-117.

Steinkamp, R., Rennenberg, H., 1984. γ-Glutamyl transpeptidase in tobacco suspension cultures: catalytic properties and subcellular localization. Physiol. Plant., **61**: 251-256.

Storozhenko, S., Belles-Boix, E., Babiychuk, E., Herouart, D., Davey, M. W., Slooten, L., Montagu, M. V., Inze D., Kushnir, S., 2002. γ-Glutamyl transpeptidase in transgenic tobacco plants. Cellular localization, processing, and biochemical properties. Plant Physiol., **128**: 1109-1119.

Suzuki,. H., Hashimoto, W., Kumagai, H., 1993. *Escherichia coli* K-12 can utilize an exogenous γ-glutamyl peptide as an amino acid source, for which γ-glutamyltranspeptidase is essential. J. Bacteriol., **175**: 6038-6040.

Suzuki, H., Kumagai, H., 2002. Autocatalytic processing of γ-glutamyltranspeptidase. J. Biol. Chem., **277**: 43536-43543.

Suzuki, H., Kumagai, H., Tochikura, T., 1986. γ-Glutamyltranspeptidase from *Escherichia coli* K-12: purification and properties. J. Bacteriol., **168**: 1325-1331.

Taniguchi, N., Ikeda Y., 1998. γ-Glutamyl transpeptidase: catalytic mechanism and gene expression. In: Purich, D. L. (Ed): Advances in Enzymology, Vol. **72**: 239-278. John Wiley & Sons, New York.

Tate, S. S., Meister, A., 1976. Subunit structure and isoenzymic forms of γ-glutamyltranspeptidase. Proc. Natl. Acad. Sci., USA, **73**: 2599-2603.

Wintermans, J. F. G. I., de Mots, A., 1965. Spectrophotometric characteristics of chlorophylls a and b and their pheophytins in ethanol. Biochim. Biophys. Acta, **109**: 448-453.