Identification of glycinin *in vivo* as a polyamine-conjugated protein via a γ -glutamyl linkage

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To identify a polyamine-conjugated protein by the action of transglutaminase in the absence of radiolabelled polyamine, extracts prepared from the leaves and developing soybean seeds were investigated for the specific activity of transglutaminase and the content of free polyamines. We identified the major storage protein, glycinin, as a polyamine-conjugated protein. This was established by the following procedures: (1) immunolocalization with antibody against putrescine prepared in rabbit against

putrescine–BSA conjugate; (2) immunocross-reactivity on nitrocellulose transblot of the purified glycinin subunits by using antibody against putrescine; (3) identification of polyamines in acid hydrolysates of purified glycinin; (4) release of polyamines in proteolytic digests through the catalytic action of γ -glutamylamine cyclotransferase, an enzyme specific for the disassembly of γ -glutamylamines. The activity of γ -glutamylamine cyclotransferase was also identified in soybean seeds.

INTRODUCTION

The transglutaminases (TGs) catalyse an acyl-transfer reaction between glutamyl amino acid side chains in substrate proteins and primary amines [1]. This element of specificity provides these enzymes with the potential of mediating post-translational modifications in specific protein molecules. These enzymes have been purified from various animal sources and their biological functions have been studied extensively [2–6].

Until recently the presence of most plant TG activities was suggested only by the recovery of trichloroacetic acid-pelletable polyamines whose linkage to protein was not broken by treatment with surfactants [7–9]. These findings were supported by the recovery of labelled mono-(γ -glutamyl)putrescine after incubation of *N,N'*-dimethylcasein with labelled putrescine in an assay mixture in *Beta vulgaris* [10]. It was also found in the chloroplast of *Helianthus tuberosus*, where bisglutamylputrescine and bisglutamylspermidine in addition to monoglutamylputrescine were detected in proteolytic digests of chloroplast proteins [11]. The formation of these glutamyl derivatives confirmed that TG activity was also present in plants. Moreover some plant TGs cross-react with antibodies against TGs of animal sources [12]. In contrast with their wide specificity for amine substrates, TGs possess a limited specificity for glutamine substrate. The specificity seems to be dependent, at least in part, on amino acid residues surrounding a given glutamine residue. Thus only certain protein substrates for TGs were labelled and separated by SDS/PAGE after incubation of crude extracts with labelled polyamines [8]. Some of these labelled proteins were identified as apoproteins of the chlorophyll *a/b* antenna complex, actin, tubulin and the large subunit of ribulose biphosphate carboxylase/oxygenase (Rubisco) [12–15]. In view of the biological functions of these substrate proteins, it can be inferred that TGs are involved in cell-wall organization, in the transfer of excitation energy between the photosystems and in the assembly of the oligomeric protein [13,16].

γ -Glutamyl derivatives of polyamines have been identified in proteolytic digests of various tissues and cells including mitogen-stimulated lymphocytes [17], amniotic fluid [18], fibroblasts [19]

and stimulated mast cells from mouse [20]. γ -Glutamylamine cyclotransferase acts on γ -glutamylamines to form oxoproline and free amines. The presence of this enzyme has been reported in a number of mammalian tissues; the rabbit kidney proved to be the richest source [21]. However, there have been no reports of this enzyme in plants.

Our purification and characterization of a TG from soybean [22] raises interesting questions of whether soybean contains polyamine-bound proteins. These proteins are important for understanding the physiological functions of TGs in plants. Generally, proteases have been known to be unable to cleave the γ -glutamylamine bond, which γ -glutamylamine cyclotransferase can attack. Therefore the enzyme is of interest in view of its metabolic function involving the glutamyl derivatives of plant polyamine. We report here the identification of a polyamine-conjugated protein *in situ* in the absence of exogenous polyamine. We identified the polyamine-conjugated protein as glycinin, the major storage protein in soybean seeds. We also report that γ -glutamylamine cyclotransferase is present in soybean.

MATERIALS AND METHODS

Plant materials

Seeds containing pods and mature leaves grown on soybean plants (*Glycine max*) in the greenhouse were harvested at a middle stage of development and stored at -70°C if not used immediately. Mature seeds were also harvested under the same conditions.

Assay of TG activity

Enzyme activity was measured by the method of Kang and Cho [22] by detecting the incorporation of [^{14}C]putrescine into a piece of *N,N'*-dimethylcasein bound to nitrocellulose.

Preparation of putrescine–BSA conjugate

This was synthesized by the method of Fujiwara et al. [23]. Putrescine (70 μmol) in 750 μl of water was adjusted to pH 7.1 and reacted with 2 mg (7.1 μmol) *N*-(γ -maleimidobutyloxy)-

Abbreviations used: GMBS, *N*-(γ -maleimidobutyloxy)succinimide; Rubisco, ribulose biphosphate carboxylase/oxygenase; TG, transglutaminase.

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succinimide (GMBS) in 0.5 ml of tetrahydrofuran at 25 °C for 100 min. The incubation mixture was maintained at near neutral pH. The mixture was concentrated to about half the volume in a desiccator and the aqueous solution was washed twice with 3 ml of diethyl ether/dichloromethane (2:1, v/v) to remove any remaining free GMBS. At the same time acetylmercaptosuccinyl-BSA prepared by the method of Klotz and Heiney [24] was incubated in 0.2 ml of 0.1 M hydroxylamine (pH 7.9) at 25 °C for 30 min to remove the protecting acetyl groups. The resulting mercaptosuccinyl-BSA was diluted with 2 ml of 0.1 M Tris/HCl buffer, pH 7.2, and added immediately to the GMBS-acylated putrescine solution, followed by incubation for 30 min. The preparation was chromatographed on a column of Sephadex G-75 (2 cm × 50 cm) with 20 mM Tris/HCl buffer, pH 7.9, containing 3 M urea as an eluent solvent.

Generation of antisera against putrescine-BSA conjugate

Antiserum was prepared by injecting each rabbit intradermally with 2.0 mg of putrescine-BSA conjugate in Freund's complete adjuvant. Booster injections were then given three times at 2-week intervals with half the dose of the first immunization. Anti-putrescine antibodies were purified on a Protein A-Sepharose CL-4B column with the low-salt method. These antibodies were further purified by using an ω -aminoethylagarose column as an antigen column ([25], pp. 313–315).

Immunocytochemical localization

Immunoelectron microscopy was performed on cotyledons, previously imbibed with distilled water for 12 h, by the method of Craig and Miller [26]. The cotyledons were fixed with 3% (w/v) glutaraldehyde in 25 mM sodium phosphate buffer, pH 7.1, for 2 h. After dehydration in a graded ethanol series the cells were embedded in Spurr solution. Polymerized blocks were sectioned on a ultramicrotome. Sections 1 μ m thick were picked up on Formvar-coated 200-mesh nickel grids, blocked with 1% BSA in PBS-T [15 mM potassium phosphate buffer (pH 7.1)/20 mM NaCl/0.2% (v/v) Tween 20] and then incubated in PBS-T and 1% BSA for 1 h at 37 °C with purified anti-putrescine antibody (dilution 1:1000); they were then washed twice with PBS-T and incubated with 30 nm gold-conjugated goat anti-rabbit antibodies (GAR-G-30; Janssen Pharmaceuticals) (dilution 1:20) for 30 min at 37 °C. The samples were washed three times with PBS-T and twice with distilled water. The sections were stained with 5% (w/v) uranyl acetate for 10 min. The specimens were viewed on a JEOL JEM 100X-II transmission electron microscope at 80 kV.

Purification of glycine

Glycine was purified by a modified procedure of Kitamura et al. [27]. Frozen seeds were ground with a chilled electric blender and extracted with cold acetone (−20 °C) until the extract was pale yellow to colourless. The extract was washed with diethyl ether, dried and stored in a desiccator at −20 °C. Acetone-dried powder was extracted with 0.4 M sodium chloride/0.02% sodium azide/0.01 M 2-mercaptoethanol in 0.035 M potassium phosphate, pH 7.6 (buffer A). After removal of the insoluble residue by centrifugation, the soluble extract was dialysed against 0.02% sodium azide/0.01 M 2-mercaptoethanol in 0.035 M potassium phosphate, pH 6.6, in ice to precipitate glycine. The precipitate was sedimented by centrifugation, dissolved in buffer A and applied to a Sephacryl S-300 column (2 cm × 120 cm). A small peak of aggregated material passed through the column in

the void volume, followed by a major peak (glycine) and finally a smaller peak (conglycin).

Acid hydrolysis and analysis of polyamines by TLC and HPLC

An equal volume of 20% (w/v) trichloroacetic acid was added to the purified glycine solution and the mixture was vortex-mixed vigorously and centrifuged at 13000 rev./min for 10 min. The precipitate was washed three times with 1 ml of 5% (w/v) trichloroacetic acid and three times with 1 ml portions of diethyl ether. The residual ether was allowed to evaporate in N₂. The dried protein was suspended in 0.1 M NaOH. Hydrolysis of glycine was performed in sealed glass tubes, which were repeatedly evacuated and filled with oxygen-free N₂ before hydrolysis. Acid hydrolysis was performed with 6 M HCl at 110 °C for 24 h. Polyamines in the hydrolysate were purified by the method of Fujihara and Harada [28], with some modifications as follows. The acid hydrolysate was neutralized with the same volume of 6 M NaOH and applied to a Dowex 50W-X8 column (H⁺ form, 200–400 mesh, 1.5 cm × 2 cm). After the column had been washed with 50 ml of 1.5 M HCl, the polyamines were eluted with 20 ml of 6 M HCl then evaporated to dryness with a vacuum evaporator. Polyamines were qualitatively and quantitatively determined by TLC and HPLC systems as described previously [29]. Polyamines were converted to dansyl derivatives by reaction with dansyl chloride and separated on silica gel 60 plates with a chloroform/triethylamine (25:2, v/v) solvent system. Polyamines, after derivatization with benzoyl chloride, were also analysed by HPLC (Waters 600) equipped with a reverse-phase column (μ Bondapak C₁₈; 3.9 mm × 300 mm). The solvent system consisted of methanol:water, run isocratically at 53% methanol, at a flow rate of 1 ml/min.

Assay of γ -glutamylamine cyclotransferase activity

Soybean seeds were soaked in distilled water and harvested 2 h after imbibition. The seeds were homogenized in a chilled mortar and pestle with 2–3 vol. of 10 mM potassium phosphate buffer, pH 7.2. The homogenate was centrifuged at 15000 rev./min at 4 °C for 15 min and the supernatant was assayed for enzyme activity. The γ -glutamylamine cyclotransferase activity was measured by a modified method of Cariello et al. [30]. The assay mixture, containing 50 μ l of plant extract and 5 μ l of 1 mM γ -glutamyl-dansylcadaverine (5 nmol), was brought to a volume of 100 μ l with 10 mM potassium phosphate buffer, pH 7.2, containing 10 mM dithiothreitol and incubated for 30 min at 37 °C. The reaction was stopped with 500 μ l of cold acetone, and the mixture was stored at −70 °C for 1 h and then centrifuged at 15000 rev./min for 30 min. The resulting supernatant was concentrated by heating at 65 °C and separated on silica gel 60 plates with a chloroform/methanol/acetic acid (10:2:1, by vol.) solvent system. γ -Glutamyl-dansylcadaverine was kindly provided by Dr. L. Lorand (Northwestern University, Chicago, IL, U.S.A.).

Proteolytic digestion of purified glycine and identification of γ -glutamyl-polyamines

Enzymic digestion was performed by the method of Cariello et al. [30], with some modifications as follows. Purified glycine was precipitated and dried with the method described above. The dried proteins were suspended in 0.1 M NH₄HCO₃, pH 8.0. Protein digestion was performed by the sequential addition of pronase (5 mg/ml) (twice), leucine aminopeptidase (1 mg/ml), thermolysin (1 mg/ml), carboxypeptidase A (0.6 mg/ml), carboxypeptidase B (0.4 mg/ml) and carboxypeptidase. Before each

addition of enzyme the sample was boiled for 5 min to destroy the proteases added previously.

Proteolytic digests were incubated with purified γ -glutamylamine cyclotransferase from soybean (details available from the authors) for 2 h at 37 °C. The reaction was stopped by the addition of the same volume of 1.5 M HCl. The precipitate was removed by centrifugation. The supernatant was loaded on a Dowex 50W-X8 column and the polyamines were purified and dansylated, then analysed by TLC. Spots corresponding to polyamines on the TLC plate were scraped and eluted in 2 ml of ethyl acetate. The eluted samples were mixed and evaporated completely; the residue was dissolved in 200 μ l of 57% (v/v) methanol. The samples were then separated by reverse-phase HPLC with a C_{18} column (μ Bondapak, 3.9 mm $\times$ 300 mm). Elution was performed by a linear gradient of methanol from 57% to 100% (v/v) for 45 min at a flow rate of 1 ml/min. Fluorescence of the effluent was monitored (λ_{exc} 334 nm, λ_{em} 530 nm) with a Waters Model 420 AC detector at the lowest sensitivity.

RESULTS

Following our first report of TG in plants [22], we investigated the cellular localization of polyamine–protein conjugates by the action of TG in the absence of exogenous polyamines. We assayed the transglutaminase activity and quantitatively determined the content of free polyamine in leaves and seeds from soybean (Table 1). The specific activity of TG in the seeds was 37-fold that in the leaves; the polyamine content in the seeds was 2–3-fold that in the leaves. This suggests that protein–polyamine conjugates are produced preferentially in the seeds.

To identify the intracellular polyamine–protein conjugates in the seeds, we synthesized putrescine–BSA conjugates for the production of an antibody against the putrescine moiety by immunization of rabbits. To minimize any cross-reactivity with other proteins, anti-putrescine antisera were purified on a Protein A–Sepharose CL-4B column followed by a polyamine analogue ω -aminohexylagarose column. The specificity of anti-putrescine antibody was determined by measuring the displacement of [^{14} C]putrescine by unlabelled putrescine, other polyamines and lysine with a competitive radioimmunoassay. The relationships between concentrations of unlabelled compounds and the percentage of [^{14} C]putrescine are presented in Figure 1. The anti-putrescine antibody showed 5.3% cross-reaction with cadaverine, 3.5% with spermine and 2.3% with spermidine in terms of the amount of each compound required for 50% inhibition of binding. 1,3-Diaminopropane and L-lysine did not cross-react.

To study the localization of these protein–polyamine conjugates in the seeds, we performed immunoelectron microscopy. Soybean seeds were imbibed for 12 h, fixed and processed for immunoelectron microscope staining with purified anti-putres-

Figure 1 Standard curve for putrescine and the cross-reactivity of other polyamines and lysine with anti-putrescine antibody

The curves show the amount of radioactive bound putrescine for various doses of putrescine or other agents (B) expressed as a percentage of that bound with putrescine alone (B°).

cine antibody, followed by staining with 30 nm gold-conjugated goat anti-rabbit antibodies. The polyamine conjugates were mainly localized in the protein body (Figure 2).

Storage proteins aggregate within the vacuolar protein body [31]. The major storage protein glycinin constitutes approx. 45% of total seed protein, with β -conglycinin being the next most abundant. Finding conjugated polyamines in the protein body strongly suggests that polyamines could be conjugated with the storage proteins. To verify this, glycinin was purified as described in the Materials and methods section. Native pore-gradient electrophoresis yielded a molecular mass of 320 kDa for the protein; SDS/PAGE analysis of the purified glycinin in the presence of dithiothreitol showed major bands at 38 and 19 kDa (results not shown). These results coincided with those previously reported [32]. Because no band was apparent at 54 kDa, the size of the conglycinin subunits, the glycinin was used without further purification.

On the basis of these results, glycinin was hydrolysed with acid to determine whether the polyamines were covalently linked to glycinin. The acid hydrolysates were passed through a Dowex

Table 1 Comparison of specific activity of TG and polyamine level in developing seeds and mature leaves from soybean

The specific activity of TG in extracts of the leaves and the developing seeds was determined as described in the Materials and methods section. For the analysis of polyamine content, the fresh tissue derived from leaves and developing seeds (1 g fresh weight for each) was homogenized in 2 ml of cold 5% (w/v) HClO₄. The extracts were centrifuged for 20 min at 13 000 rev./min and the supernatant fraction was used for the analysis of free polyamine. Quantitative analysis of polyamine was performed by TLC and HPLC as described in the Materials and methods section.

Source	Specific activity of TG (nmol/h per mg of protein)	Polyamine concentration (μ mol/g fresh weight)			
		Putrescine	Cadaverine	Spermidine	Spermine
Mature leaves	1.96	0.284 $\pm$ 0.002	0.228 $\pm$ 0.006	0.849 $\pm$ 0.004	0.124 $\pm$ 0.001
Developing seeds	71.27	0.675 $\pm$ 0.005	0.238 $\pm$ 0.008	2.604 $\pm$ 0.025	0.235 $\pm$ 0.003

Figure 2 Immunocytochemical localization of putrescine in cotyledons of soybean

(A) Sections from soybean cotyledon after imbibition in distilled water for 6 h. (B) Sections from soybean cotyledon after imbibition in distilled water for 24 h. Abbreviations: PB, protein body, CW, cell wall. Scale bar, 1.0 μ m.

50W-X8 resin cation-exchange column. The samples were concentrated with a vacuum evaporator, then analysed by reverse-phase HPLC and TLC (Figure 3). These results suggest that polyamines were covalently bound to glycinin *in vivo*.

If polyamines are covalently conjugated with glycinin via a γ -glutamyl linkage, an enzyme for the degradation of such an isopeptide bond might exist in the soybean seeds. The presence of a γ -glutamyl linkage can be indicated by the occurrence of the γ -glutamylamine cyclotransferase activity, which acts on γ -glutamylamines to form free amines. It is also known that the isopeptide bond is resistant to proteinase action [33]. Assaying extracts for the presence of this enzyme revealed a pronounced activity in germinating seeds. When the enzyme activity was assayed by TLC analysis as a function of the duration of incubation, the amount of reaction product increased in exact proportion to the incubation time. The time course of the product formation is shown in Figure 4.

We then identified γ -glutamylpolyamines indirectly by detecting free polyamines released from proteolytic digests of glycinin by the action of purified γ -glutamylamine cyclotransferase. Released polyamines were purified, derivatized and subjected to

Figure 3 Detection of polyamines in acid hydrolysates of purified glycinin by HPLC and TLC

Polyamines were extracted from acid hydrolysates of purified glycinin and purified on a Dowex 50W-X8 column. In (A) and (B) polyamines were derivatized with benzoyl chloride and subjected to HPLC analysis: (A) standard polyamines; (B) polyamines derived from acid hydrolysates. (C) Polyamines were derivatized with dansyl chloride and subjected to TLC analysis. Lane 1, arginine standard; lane 2, lysine standard; lane 3, putrescine (PUT) standard; lane 4, cadaverine (CAD) standard; lane 5, spermidine (SPD) standard; lane 6, spermine (SPN) standard; lane 7, acid hydrolysates of glycinin (2 mg); lane 8, acid hydrolysates of glycinin (0.5 mg); lane 9, mixture of polyamine standards.

TLC analysis. Figure 5(A) shows that the proteolytic digests pretreated with γ -glutamylamine cyclotransferase revealed several spots, which could be assigned to the standard polyamines.

Figure 4 Identification of γ -glutamylamine cyclotransferase activity in soybean seeds

Seeds were collected 2 h after imbibition in distilled water, then homogenized in 10 mM potassium phosphate buffer, pH 7.2. The homogenate was centrifuged at 15000 rev./min at 4 °C for 15 min and the supernatant was used as the enzyme source. The time course of monodansylcadaverine formation is shown. In the inset the incubation times were as follows: 15 min (lane 2), 30 min (lane 3), 1 h (lane 4), 2 h (lane 5), 3 h (lane 6) and 6 h (lane 7). Lane 1, standard γ -glutamylcadaverine (3 nmol); lane 8, standard monodansylcadaverine (3 nmol in TLC).

Control samples not treated with the enzyme did not show the typical spots of polyamines on the TLC plate. For further identification, the spots corresponding to the polyamines were eluted in ethyl acetate solution and analysed by HPLC equipped with a fluorescence detector. In Figures 5(B) and 5(C) the chromatographic separation of a mixture of polyamines eluted from lanes 1–4 is compared with samples eluted from lane 6 in the TLC plate of Figure 5(A). The results shown are consistent with polyamines' being covalently linked to glycinin via a γ -glutamyl bond by the catalytic action of TG.

DISCUSSION

Until recently, studies on TG activity and its physiological roles focused on animal cells, where it post-translationally modifies several proteins. Although such proteins were frequently related to the formation of bridges between extracellular or cytoskeletal proteins and involved in the regulation of cell growth and differentiation, the direct role of intracellular TG in these biological events is still unknown.

Indirect evidence has been reported for the presence and several characteristics of TGs in various organs of higher plants [10,12,17,34]. Despite extensive work, the existence of the enzyme activity in plant has been verified only recently by the presence of the monoglutamylpolyamines and bisglutamylpolyamines [10,11]. The presence of γ -glutamyl derivatives represents a direct indication of the formation of cross-links via TG. Confirmation of the identity of the plant TGs is also provided by the fact that the substrate for mammalian TGs, benzoyloxycarbonyl-L-Gln-L-Leu, is a good substrate for TG from etiolated apices of the sprouts growing from tubers of *H. tuberosus* [14]. Previously we purified TG from soybean leaves [22]. Here we identified glycinin subunits as the substrate of TG *in vivo* in the absence of an exogenous polyamine.

Figure 5 Identification by TLC and HPLC of the released polyamines through the catalytic action of γ -glutamylamine cyclotransferase in proteolytic digests of glycinin

Proteolytic digestion was performed by the procedure as described in the Materials and methods section. (A) The release of polyamines in a digest of glycinin, before (lanes 5 and 8) and after (lanes 6 and 9) its incubation with γ -glutamylamine cyclotransferase in TLC plate. Lane 1, putrescine (PUT) standard; lane 2, cadaverine (CAD) standard; lane 3, spermidine (SPD) standard; lane 4, spermine (SPN) standard; lane 5, proteolytic digest (50 μ l) untreated with γ -glutamylamine cyclotransferase; lane 6, proteolytic digest (50 μ l) treated with γ -glutamylamine cyclotransferase; lane 7, polyamine standard mixture; lane 8, proteolytic digest (150 μ l) untreated with γ -glutamylamine cyclotransferase; lane 9, proteolytic digest (150 μ l) treated with γ -glutamylamine cyclotransferase. (B,C) Elution profiles of polyamines eluted from standard polyamines from lanes 1–4 in panel A (B) and from lane 6 (C). HPLC analysis was conducted with a Waters instrument as described in the Materials and methods section.

The TG activity was identified in both soybean leaves and developing seeds. The enzyme in both tissues seems to be identical, judging from its chromatographic profiles of activity. In animal tissues, tissue-type II TG (type C) is present as a ubiquitous monomer, having several functions such as the regulation of cell growth and differentiation, tumour metastasis and programmed cell death. As the enzyme identified in leaves and developing

seeds has similar characteristics to those of animal tissue-type TG including molecular mass, solubility and inhibition pattern by GTP, this enzyme might also be ubiquitous with several functions in plants. Comparative studies of the results described here suggest that the covalent linkage between endogenous polyamines and proteins is formed by the preferential action of TG during seed germination. The oligomeric form of the major light-harvesting complex (LHC II) and the large subunit of Rubisco in leaves are better substrates for plant TGs [12,15]. However, the possible substrate for TGs has only rarely been examined in plant seeds. Pea legumin, the storage protein of pea seeds, has been shown to be a substrate of guinea-pig liver TG [35].

Immunolocalization with the use of antibody against putrescine revealed that the polyamine conjugates are mainly localized in the protein bodies. Soybean seeds contain approx. 45 % protein at maturity, of which approx. 70 % is in the form of the storage proteins β -conglycinin (7 S protein) and glycinin (11 S protein) [36]; these proteins tend to aggregate within the vacuolar protein body [31]. We also suggest that glycinin is one of the polyamine-conjugated proteins in soybean seed.

Western blot analysis of the purified glycinin after SDS/PAGE revealed two major immunoreactive bands of apparent molecular masses 19 and 38 kDa, corresponding to glycinin subunits (results not shown). Evidence for the existence of covalent polyamine–glycinin conjugates is shown in Figure 3. Covalent binding to nucleic acid and phospholipid is unlikely, because only electrostatic bonds have been demonstrated between these compounds and polyamines [37]. However, it is possible that polyamines are covalently bound to the proteins, because these amines are substrates for TG, the enzyme that conjugates polyamines to proteins through a γ -glutamyl linkage [17]. Our results raise the possibility that the polyamines are covalently linked to glycinin by the action of TG.

The activity of γ -glutamylamine cyclotransferase was detected in a number of mammalian tissues, and partial purification of the enzyme was accomplished in rabbit kidney homogenates [21]. Information on the production and distribution of ϵ -(γ -glutamyl)-lysine cross-links and protein-bound γ -glutamylamines is accumulating. For example, it is known that this cross-linking is essential for the functional or structural integrity of a number of mammalian proteins [38]. However, little attention has been directed towards the metabolic fate of these products of TG, especially in plants. γ -Glutamylamine cyclotransferase has not previously been observed in plants. We have now identified the enzyme activity by using γ -glutamyl dansylcadaverine as a substrate in soybean seeds. The occurrence of this enzyme activity implies the existence of γ -glutamyl cross-links in plant tissues.

Until now there have been fairly extensive studies on TG activity and endogenous substrates for TG in various plant sources. The subcellular distribution of this activity seems to be organ-specific. In green leaves of *Beta vulgaris* and *H. tuberosus* and in pollen of *Malus domestica*, most of the activity is found in the membrane fractions [10,14,34]. In contrast, in etiolated apices of *H. tuberosus* and of *Pisum sativum*, most of the activity is localized in the supernatant fractions [9]. The polyamine conjugates formed with endogenous proteins and separated by SDS/PAGE are apparently heterogeneous. In sprout apices and in leaves of *H. tuberosus*, labelled proteins having a low molecular mass were found in addition to the high-molecular-mass proteins [14,34]. In germinating pollen of *M. domestica*, incubation-purified actin and tubulin with labelled polyamines revealed that both are substrates for pollen TG. In *H. tuberosus* leaves, proteins conjugated to radiolabelled polyamine have been also identified as apoproteins of the chlorophyll *a/b* antenna complex

as well as the large subunit of Rubisco [12,15]. The exclusive ability of TG to form monoglutamylpolyamines and bisglutamylpolyamines has been checked only rarely. Mono-(γ -glutamyl)-putrescine formation has been reported on incubation of leaf homogenates from *B. vulgaris* with *N,N'*-dimethylcasein and radiolabelled putrescine exogenously supplied in the assay mixture [10]. Del Duca et al. [11] first showed that the glutamyl and acetyl derivatives of polyamine were identified in chloroplasts from the leaves of *H. tuberosus*. However, the identities of polyamine-bound proteins have not been determined *in vivo* without the exogenous addition of a polyamine probe. The occurrence of γ -glutamylamine cyclotransferase in soybean seeds and the release of polyamines through the catalytic action of γ -glutamylamine cyclotransferase in proteolytic digests of glycinin (Figures 4 and 5) strongly suggest that polyamines are covalently linked to glycinin subunits via a γ -glutamyl linkage.

As to the role of post-translational modification, it has been observed that the large subunits of Rubisco are polymerized and stabilized by the action of TG [39]. In the post-translational modification of light-harvesting centre II, the added positive charges of polyamines might have the opposite effect to that of phosphorylation and could thus favour the transfer of excitation energy between the photosystems [16]. Actin and tubulin are known to have an important role in the cytoskeleton organization, being closely involved in pollen tube elongation [14]. Glycinin contains six acidic subunits and probably three kinds of basic subunit [40]. In terms of such a function for TG, which might be responsible for the polymerization of glycinin subunits such as that of Rubisco subunits, this proposal agrees with the model of TG's stabilizing effects by cross-linkage formation.

As well as the functions proposed above, it is possible that TG-modified proteins in cells are subject to rapid and efficient protein degradation to the amino acid level by the cellular system [41]. The formation of mono-glutamylpolyamines is regulated by the polyamine concentration because high levels of polyamines might saturate the acyl donor residues of substrate proteins. This addition of positive charges could affect the conformation and function of substrate proteins. All storage proteins including glycinin are degraded more rapidly within several days of the initiation of imbibition than other proteins in the cotyledons [42]. Post-translational modifications by TG might be a signal for this rapid degradation. Thus the nature of polyamine-conjugated glycinin becomes particularly attractive in view of the fact that post-translationally modified proteins and those with abnormal structures are degraded especially rapidly within cells [43,44].

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