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γ-Glutamylcysteinylglutamic acid – a new homologue of glutathione in maize seedlings exposed to cadmium**

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Exposure of plants to Cd induces the appearance of several thiols based on glutathione and known as class III metallothioneins (or phytochelatins). A new tripeptide with the structure γ-GluCysGlu accumulated in roots and shoots of Cd-exposed maize seedlings. This thiol was purified and identified by tandem mass spectrometry. The fragmentation pattern of the maize tripeptide was identical to that of the synthetic compound. Like glutathione, this new tripeptide may serve as a precursor for longer-chain peptides involved in metal detoxification through the formation of Cd-binding complexes.

γ-Glutamylcysteinylglutamic acid; Glutathione; Metal detoxification; Cd-binding peptide; Phytochelatin; MS–MS

1. INTRODUCTION

Plants exposed to excess metals such as Cd, Cu or Zn synthesize thiolate peptides typical of class III metallothioneins (also called phytochelatins or $(\gamma EC)_nG$) [1,2] that are widely distributed in the plant kingdom [3]. The γ-glutamylcysteine (γEC) portion of the peptides is repeated two to 11 times depending on the organism [4–7]. Glutathione (γECG) [8,9] and γEC [10] act as substrates for the synthesis of longer-chain thiolate peptides, which form a higher molecular weight complex containing Cd and often sulfide [11–18]. The complex binds Cd through thiolate groups [1,2] and may act as a carrier of Cd into the vacuole of the plant cell [19].

Some plants from the family Fabaceae do not contain γECG but a homologue tripeptide γ-glutamylcysteinyl-β-alanine (γECβA) [20–23]. When these legumes are exposed to Cd, γECβA substitutes for γECG as the precursor of the longer-chain homo-phytochelatins ($(\gamma EC)_n\beta A$) [24]. Another thiolate tripeptide, γ-glutamylcysteinylserine (γECS), was recently reported in several species of Poaceae but not in maize [25].

Based on tandem mass spectrometry (MS–MS) techniques, we show here evidence for the presence of a new thiolate tripeptide γ-glutamylcysteinylglutamic acid (γECE), induced in maize seedlings upon exposure to Cd. Its putative physiological role as a γECG analog for the synthesis of longer-chain thiolate peptides involved in metal detoxification is discussed.

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Abbreviations: $(\gamma EC)_nG$, phytochelatins; γEC, γ-glutamylcysteine; γECG, γ-glutamylcysteinylglycine (glutathione); γECβA, γ-glutamylcysteinyl-β-alanine; $(\gamma EC)_n\beta A$, homo-phytochelatins; γECS, γ-glutamylcysteinylserine; MS–MS, tandem mass spectrometry; γECE, γ-glutamylcysteinylglutamic acid; ACN, acetonitrile; TFA, trifluoroacetic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TNB, thiobis-(2-nitrobenzoic acid); DMF, dimethyl formamide.

2. MATERIALS AND METHODS

2.1. *Plant material*

Caryopses of *Zea mays* L. (hybrid 37701) were obtained from Cargill Hybrid Seeds Ltd. (Princeton, Ont.) and planted in moist paper towel scrolls as previously described [26]. After 3 d uniform seedlings were transplanted into aerated nutrient solution (50 seedlings in 4 L of 1/2 strength Hoagland solution) and grown at controlled temperature (22.5 ± 0.5°C) with a 16 h/d light period beginning at 07.00 h. The roots were exposed to 3 μM $CdSO_4$ in nutrient solution from 5 to 12 d after planting. Plant material was collected after 2, 4, 6, 9 and 12 h, and then daily up to 7 d as described [27] and stored at −80°C. Nutrient solutions of both control and treated plants were replaced daily.

2.2. *Measurements of thiolate peptides in acid extracts*

Frozen tissues were extracted in ice-cold 0.1 M HCl, 1 mM EDTA. The conditions for the quantification of thiolate peptides were previously described in detail [26–28]. Briefly, the polypeptides from a crude acid extract were first separated on a RP-18 column by eluting with 0 to 20% acetonitrile (ACN) in 0.1% trifluoroacetic acid (TFA) in 40 min at a flow rate of 1 ml/min and a temperature of 37°C. The column effluent was reacted with 5,5′-dithiobis(2-nitrobenzoic acid) (712.5 mg of DTNB/L of buffer; K phosphate 300 mM, K_2EDTA 15 mM; Ellman's reagent) delivered at a rate of 0.5 ml/min to a mixing device and allowed to react at 37°C for 1.2 min in a 1.8 ml postcolumn reactor. The absorbance was measured at 405 nm to quantitate the

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released thiobis(2-nitrobenzoic acid) (TNB) molecules produced by reaction of DTNB with thiolate groups. Fractions of TNB-derivatized peptides 1 and 8 (Fig. 1A) were collected and freeze-dried.

2.3. *Measurements of thiolate peptides in Cd-binding complex*

About 8 g of frozen roots were pulverized in liquid N_2 with a mortar and pestle and homogenized with 8 ml 100 mM Tris-HCl pH 8.6, 1 mM phenylmethylsulfonyl fluoride and 1% (v/v) Tween 20. The homogenate was centrifuged at 48,000×*g* for 6 min and the supernatant collected. The pellet was suspended in 12 ml 10 mM Tris-HCl pH 8.6, 1% Tween 20 followed by centrifugation as before. This step was repeated four times. The six supernatants were transferred to a 0.5 ml bed of the anion exchanger Q Sepharose and the column washed with 24 ml 10 mM Tris-HCl pH 8.6. The Cd-containing material eluted with 4 ml 10 mM HEPES pH 8.0, 1 M KCl was applied to a 16×505 mm column of Superose 12 operating in 10 mM HEPES pH 8.0, 300 mM KCl at a flow rate of 0.8 ml/min. Fractions high in Cd were pooled, diluted 1:3 with water and passed over a 0.25 ml bed of Q Sepharose. Cd-binding complex was eluted from the anion exchanger with 1.5 ml HEPES pH 8.0, 1 M KCl. Portions were acidified to pH 1.5 with 6 M HCl, clarified for 10 min in a microcentrifuge and thiolate peptides analyzed by HPLC as for the acid extracts above.

2.4. *Purification of the peptides*

Fractions of the TNB-derivatized peptides 1 and 8 (Fig. 1A) were further purified individually by reverse-phase HPLC using the same column, temperature, solvents and flow rate conditions as above with the following gradient elution conditions; 15% ACN in 0.1% TFA for 5 min, increased to 20.5% in 22 min, to 35% in 3 min and then to 50% in 0.5 min. Fractions were acidified with 1 M HCl, centrifuged and the supernatant injected onto the HPLC column. The retention times for peptide 1-TNB and 8-TNB derivatives at A_{220} were 16.7 min and 22.2 min, respectively. Due to the higher concentration of ACN in this gradient compared to the initial HPLC conditions (2.2), both TNB-derivatized peptides were completely resolved from other coeluting compounds present in earlier HPLC analyses of the crude extracts. Indeed, most of the contaminants of the biological extracts eluted close to the solvent front, whereas Ellman reagent peaks eluted after 30 min (data not shown).

2.5. *Amino acid composition*

The amino acid compositions of peptide 1-TNB and 8-TNB derivatives were determined after acid hydrolysis (HCl 6 M, phenol 0.01%, 24 h, 110°C). Single amino acids were separated by HPLC on a Na^+ anion-exchange column (Dionex) and quantitated by post-column reaction with ninhydrin (Dionex BioLC, Dionex, Mississauga, Ont). The Cys in the hydrolyzed peptide derivatives was quantitated as cysteic acid (the TNB group was released during the acid hydrolysis) using hydrolyzed TNB-derivatives of γECG and γEC as standards.

2.6. *Mass spectral analyses*

All mass spectrometric experiments were performed on a SCIEX API/III triple quadrupole mass spectrometer (Thornhill, Ont., Canada) equipped with an atmospheric pressure ionization (API) source operated in nebulizer-assisted electrospray mode. The mass spectrometer was equipped with a high pressure cell providing enhanced transmission efficiency and resolution in tandem mass spectrometric experiments. A MacIntosh Quadra 950 computer was used for instrument control, data acquisition and data processing. Conventional mass spectra were obtained by injecting approximately 1 nanomole of TNB-peptides previously purified by HPLC. The mass spectrometer was scanned from *m/z* 200 to 1200 with a dwell time of 5 ms per step of 1 Da. MS–MS analyses were obtained following mass selection of precursor ions using the first quadrupole while scanning (typically 50–600 Da) the third quadrupole mass analyzer with a dwell time of 1 ms per step of 0.1 Da. Collisional activation at energies of 20 eV (laboratory frame of reference) was achieved by introducing argon in the rf-only quadrupole corresponding to a target gas thickness of 3.5×10^{15} atoms/cm^{-2}.

2.7. *Chemical synthesis of ECE peptides*

Peptides were synthesized by solid phase methodology on a Millipore continuous flow synthesizer, model 9050 Plus using Novasyn PA500 resin (substitution 0.2–0.4 mM/g) as the base support. All peptides were synthesized using Fmoc chemistry [29,30]. Fmoc amino acid derivatives, Fmoc(Glu/OtBu) and FmocCys(Trt), were purchased from BACHEM California (Torrance, CA). Each addition consisted of the following steps: a solvent wash (dimethyl formamide, DMF, 2 min); a deblocking wash (piperidine/DMF, 1/5 v/v, 6 min); a solvent wash (DMF, 7 min); an acylation step (Fmoc amino acid, 2-(1*H*-benzotrazol-1-yl)-1,1,2,2-tetramethyluronium tetrafluoraborate, *n*-hydroxybenzotriazole, *N*-methylmorpholine in DMF, fourfold molar excess, 30 min); a solvent wash (DMF, 4 min). Peptides were cleaved from the support using reagent K (TFA/phenol (90%)/H_2O/thioanisole/1,2-dithioethane, 33:2:2:2:1, v/v/v/v/v) according to a procedure described by King et al. [31] After cleavage, peptides were recovered by evaporation of the cleavage mixture and precipitation with diethyl ether followed by dissolution in 0.1% TFA/H_2O and lyophilization. The material was then dissolved in 0.1% TFA, passed onto a Sep-Pak C18 cartridge, eluted with TFA (0.1%)/ACN (80:20, v/v), lyophilized, and the thiolate peptides separated by reverse-phase HPLC and collected after reaction with Ellman's reagent (section 2.2). The TNB-derivatives were then purified by HPLC prior to analysis by mass spectrometry.

3. RESULTS

A number of thiolate peptides were present in acid extracts (Fig. 1A) of roots from maize seedlings exposed to low concentrations of Cd [26,27]. However, only late eluting peptides were detected in the Cd-binding complex isolated from Cd-exposed roots (Fig. 1B) [32,33]. We were interested in identifying and quantifying peptide 1 which accumulated in roots as early as 2 h after Cd exposure (Fig. 3). The TNB-derivatives of peptides

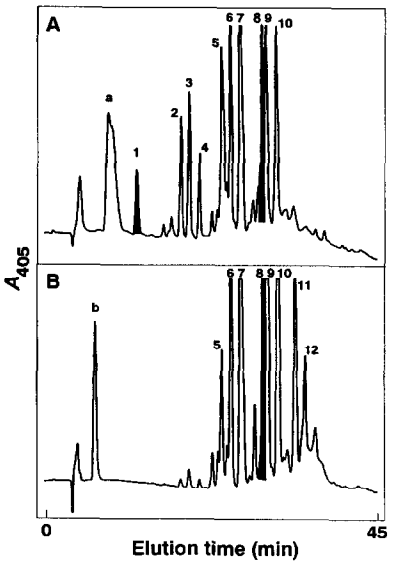

Fig. 1. HPLC chromatograms of thiols from roots of maize exposed to 3 μM $CdSO_4$ for 5 days. (A) Acid-soluble materials in an extract equivalent to 0.418 g FW of roots. (B) Materials from a Cd-binding complex equivalent to 1.081 g FW. γECG and γEC were eluted together in peak a, peak b was for sulfide. Peptide 1 contained 7.55 nmol thiol in the acid extract and was below the detection limit in the Cd-binding complex. Peptide 8 contained 23.74 and 45.63 nmol thiol in the acid extract and the Cd-binding complex respectively.

1 and 8 (Fig. 1A) were purified prior to amino acid analyses (Table I) and mass spectral analyses (Fig. 2).

The amino acid composition of peptide 1 (Table I) did not match the composition of the $(\gamma EC)_nG$ known to be induced upon metal exposure of plants [4–7]. No significant level of Gly was measured and only Glu and Cys were present in a ratio of two to one. Likewise, peptide 8 contained only Glu and Cys in a ratio close to three to two.

The mass spectra of the TNB peptides from fractions 1 and 8 gave abundant protonated molecular ions (MH^+) at *m/z* 577 and 1022, respectively. The peptide from fraction 1 was further investigated using tandem mass spectrometry and the spectrum obtained is presented in Fig. 2C. Standards of γECE and αECE were also synthesized, derivatized with DTNB and subjected to MS–MS analyses (Fig. 2A,B). Fragment ions observed for both synthetic derivatives are labelled according to a nomenclature described by Biemann [34]. For purposes of clarity only a few of these fragments could be labelled on the corresponding spectra. As observed, the MS–MS spectra of the MH^+ ion from the TNB derivatives of γECE (Fig. 2A) and αECE (Fig. 2B) gave distinct fragmentation behavior and provided characteristic features facilitating identification of these two isomers. It is noteworthy that similar observations were reported by Isobe et al. [35] for the MS–MS spectra of native α and γ cadystin A, although differentiation between the α and γ linkage was more subtle than for the present case. Of particular interest is the relatively strong abundance of ions at *m/z* 430 (b2) and 273 (immonium ion of Cys-TNB) for the γECE peptide and *m/z* 394 for αECE. The latter fragment arises from consecutive losses of two H_2O molecules from the fragment ion b2. This loss of H_2O is assumed to be facilitated through the formation of a pyroglutamate residue from the N-terminus amino acid and the expulsion of H_2O via a rearrangement reaction involving a proton transfer to the carboxylic acid of the TNB group. The facile formation of pyroglutamate in the case of αECE-TNB is also noted on Fig. 2B by the observation of a1 and (a1-H_2O)

Table I

Amino acid composition of TNB derivatives of peaks 1 and 8 (Fig. 1A) and of standards of γECG and γEC. No other amino acid residues were detected. Means of four replicates

Amino acid residue	Percentage of total residue			
	γECG	γEC	1	8
Cys[1]	33.6	52.9	32.8	40.9
Asx	0.2	2.4	0.7	1.1
Ser	0.1	–	0.2	1.3
Glx	33.5	49.2	63.8	55.1
Gly	32.6	0.1	0.7	1.6

[1]Cys measured as cysteic acid (recovery calculated from standards of TNB-derivatized γECG and γEC)

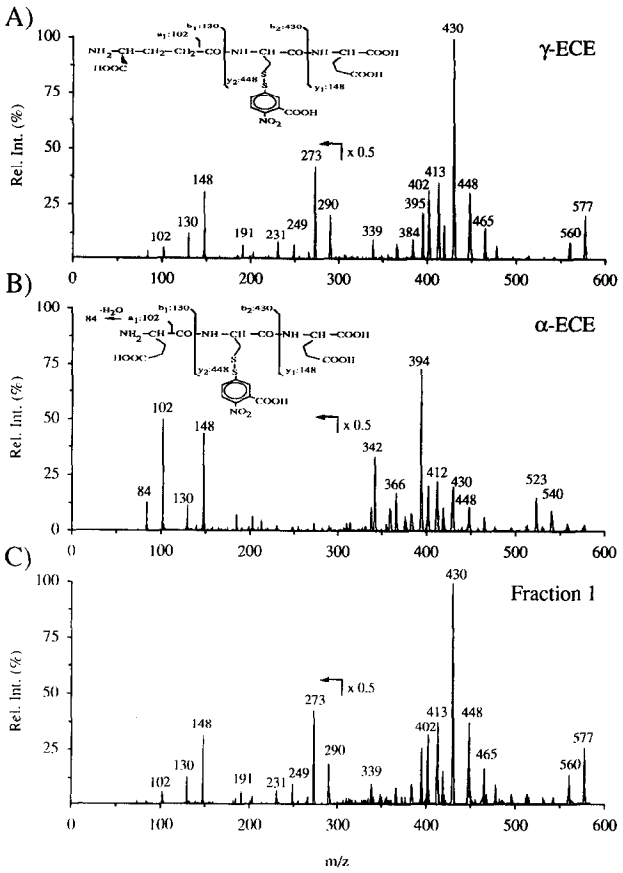

Fig. 2. Identification of TNB-derivatized γECE using the MS–MS technique. Tandem MS spectra of TNB derivatives of synthetic γECE (A), αECE (B), and TNB derivative of peptide of fraction 1 (Fig. 1A) isolated from Cd-exposed roots (C). Inserts correspond to major fragment ions observed in the MS–MS spectra.

fragment ions which are intense compared to the corresponding spectrum of the γECE-TNB isomer. The relative intensity ratio of fragments y1/a1, which is higher for the γE than for αE, thus offers a basis for distinguishing these two types of linkage. The characteristic features observed in the MS–MS spectra displayed in both Fig. 2A and B permitted unequivocal confirmation that peptide 1 was indeed γECE-TNB.

γECE accumulated rapidly in acid extracts of both roots and shoots of Cd-exposed maize seedlings (Fig. 3). In roots, its level was already higher than controls 2 h after exposure to Cd (to 0.31 nmol of thiol/g FW, not detectable in controls) reaching a maximal value 5 d post-treatment. In shoots, γECE accumulated significantly only 2 d after treatment.

4. DISCUSSION

γECG is the single most abundant thiol in plants [36,37] and its role as substrate for the synthesis of $(\gamma EC)_nG$ peptides after exposure to metals is well documented [1,2,8–10]. Only two other homologous thiolate tripeptides are known in plants, γECβA [20–23] in the

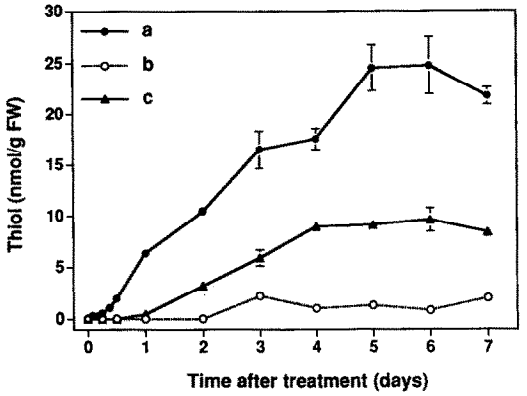

Fig. 3. Time course of γECE accumulation in Cd-exposed (a, roots; and c, shoots) and control (b, roots) maize seedlings. No γECE was measured in shoots of controls. Mean (± S.E.) of 3 replicates.

family Fabaceae and γECS recently found in several members of the family Poaceae [25], both playing a role as precursors for longer-chain peptides perhaps involved in metal detoxification [24,25]. γECE is thus a new thiolate tripeptide identified in plants. Whether other species accumulate γECE upon exposure to Cd is open to investigation. As γECE was almost absent from control plants and accumulated in Cd-exposed maize seedlings, its function is probably related to metal detoxification perhaps as a precursor for longer-chain peptides in a manner similar to γECG for $(\gamma EC)_nG$. Indeed, the Cd-binding complex isolated from Cd-exposed roots did not contain any γECE, γECG, γEC and few of the other early-eluting thiolate peptides measured in acid extracts (peptides 2–4, Fig. 1A) [32,33]. However, peptide 8 provided some thiol for sequestering Cd in Cd-binding complex (13.3% of the thiols shown in Fig. 1B). Formal identification of peptide 8 as $(\gamma EC)_2E$ has not yet been achieved but there is supportive evidence in this direction based on the detection of only Glu and Cys in the molar ratio 3:2 from amino acid analyses (Table I). If our hypothesis of precursor-product relationship between γECE and peptide 8 is correct, γECE should have a rapid turnover since we measured a 3-fold higher level of thiol in peptide 8 than in γECE in roots exposed to Cd for 6 h. Similarly, the dipeptide γEC also accumulated in maize roots upon exposure to Cd before the appearance of other thiolate peptides [27,38,39], thus supporting its role as a biochemical precursor [10].

The possibility that γECE is a degradation product of $(\gamma EC)_nG$ in vivo can not be discounted. Direct measurements of cellular turnover of the various peptides are required. The possibility that γECE arises artifactually through acid hydrolysis of polymers of γEC is deemed unlikely. The final concentration of HCl in our extracts was about 50 mM or 20-fold less than that used for limited hydrolysis of $(\gamma EC)_nG$ peptides at 100°C [40] and our extracts were kept on ice for a maximum of 20 min. Storage on ice for 2.5 h resulted in a loss of Ellman's reactive thiol for peaks 5 through 10 (Fig. 1A) without any change in γECE. Furthermore, no γECE was found in acid extracts of oat roots and the fission yeast *Schizosaccharomyces pombe* yet both produced $(\gamma EC)_2G$ (unpublished data of D.E. Salt and W.E. Rauser).

In addition, our report describes an efficient method for the purification of thiolate peptides from biological samples as their TNB derivatives. We also describe how tandem mass spectrometry can be used for distinguishing between α and γ linkages for relatively minute amounts of thiolate peptides (0.5–2 nmol) in root extracts.

In conclusion, we identified γECE as a new thiolate tripeptide in maize seedlings upon exposure to low amounts of Cd. This finding along with γECβA in the family Fabaceae and γECS in other species of the family Poaceae brings to three the number of γECG analogs in the plant kingdom putatively capable of acting as biochemical precursors for longer-chain peptides involved in metal detoxification.

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