Homo-Phytochelatins Are Synthesized in Response to Cadmium in Azuki Beans[1]

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In a recent report, it was claimed that azuki beans (*Vigna angularis*) do not synthesize phytochelatins (PCs) upon exposure to cadmium, although glutathione (GSH), the substrate for PC synthesis, is present in this plant. This legume species thus would be the first exception in the plant kingdom that would fail to complex heavy metals by PCs. Here, we report that not GSH, but only homoglutathione can be detected in this plant and that homo-phytochelatins are formed when azuki beans are challenged with heavy metals such as cadmium. We also show that the 5,5′-dithiobis(2-nitrobenzoic acid)-oxidized GSH reductase recycling assay, used for GSH quantification in the recent study of heavy metal tolerance in azuki beans, reacts both with GSH and homoglutathione and therefore cannot be used when biological samples should be analyzed exclusively for GSH.

There is by now overwhelming evidence that plants detoxify intracellular heavy metal ions by complexing them with phytochelatins (PCs) through thiolate coordination. PCs are small peptides (5 to 23 amino acids) that are synthesized from glutathione (GSH) by a heavy metal-activated γ-glutamyl-Cys dipeptidyl transpeptidase (PC synthase, EC 2.3.2.15; Grill et al., 1989; Zenk, 1996).

In some plants, PCs ($[\gamma\text{-Glu-Cys}]_n$-Gly) are replaced by γ-Glu-Cys oligomers carrying a terminal Ser (iso-PC[Ser]; Klapheck et al., 1994), Glu (iso-PC[Glu]; Meuwly et al., 1995), or Gln (iso-PC[Gln]; Kubota et al., 2000). These compounds are collectively termed iso-PCs. In the order Fabales, especially in the tribe Phaseoleae, GSH is replaced in several species by homoglutathione (hGSH), a homolog of GSH with the terminal amino acid Gly replaced by β-Ala (Carnegie, 1963; Klapheck, 1988). As a result, upon heavy metal exposure, these plants produce homo-phytochelatins (hPCs; iso-PC[β-Ala]) instead of PCs (Grill et al., 1986). Some plants of this tribe, however, synthesize upon exposure to Cd^{2+} both sets of metal-complexing peptides, PCs and hPCs, simultaneously (Gekeler et al., 1989). These results have been fully corroborated by Klapheck et al. (1995). The endogenous occurrence of hGSH in a given plant species thus would determine the subsequent formation of hPCs when plants or cell cultures are challenged by heavy metal ions.

A thorough survey of the plant kingdom (Gekeler et al., 1989) for the ability to bind heavy metals through PC or iso-PC formation revealed that over 200 plant species investigated ranging from algae to orchids produce these metal-complexing peptides.

A report was published recently that roots and cell cultures of azuki beans (*Vigna angularis*), a species of the order Fabales, tribe Phaseoleae, did not contain PC peptides when challenged with Cd^{2+} and had a hypersensitivity to the metal ions (Inouhe et al., 2000). Azuki beans, furthermore, are claimed to contain reduced GSH when grown in the absence of Cd^{2+}. Externally applied GSH to the azuki beans resulted neither in Cd^{2+} tolerance nor in PC synthesis. And finally, protein extracts of azuki bean cells had no enzyme activity to convert GSH to PCs, unlike tomato preparations, which were used as a positive control.

Azuki beans thus would be the first observed example in the plant kingdom not to contain this important detoxification mechanism for heavy metals. This finding contradicts our previous hypothesis of the ubiquitous presence of the PC/iso-PC detoxification/homeostasis system and therefore we decided to reexamine the experiment published by Inouhe et al. by rigorously applying mass spectroscopy (MS) to identify the compounds involved and thus clarify these results.

RESULTS

Identification of hGSH in Azuki Beans

An HPLC chromatogram of the control azuki bean root extracts (not treated with Cd^{2+}) is shown in Figure 1A. No GSH could be detected in the azuki beans (expected retention time [Rt] 3.1 min). In contrast, another -SH-containing compound is present (retention time 4.1 min). This compound was isolated

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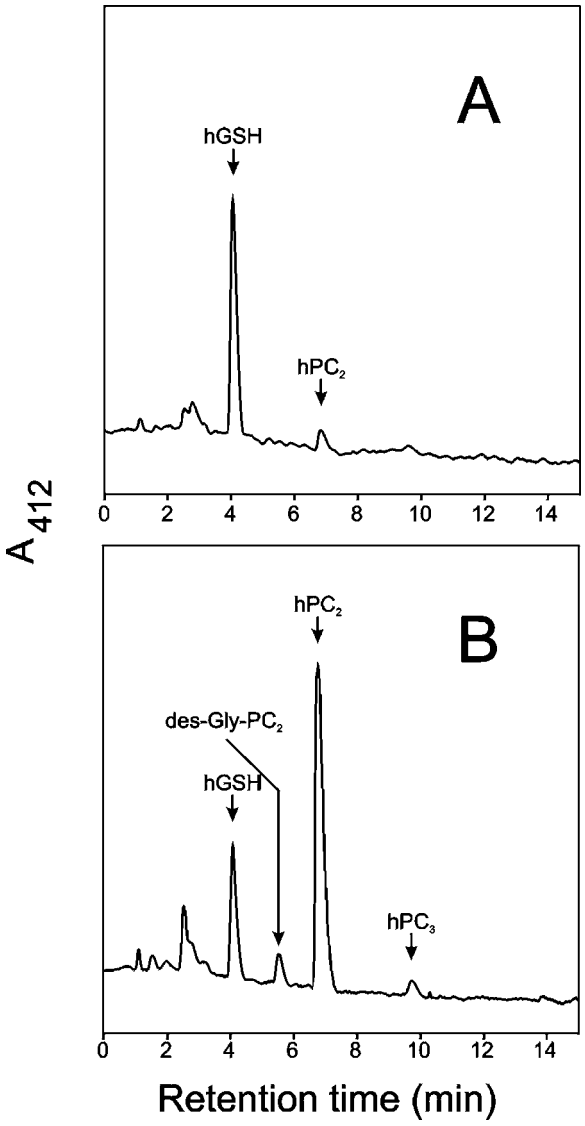

Figure 1. HPLC analysis of a root extract of azuki beans. A, Extract from control plants shows the presence of hGSH (Rt 4.1 min), whereas GSH (Rt 3.1 min) is not present in these plants. B, Plants grown for 7 d in the presence of 10 μM $CdCl_2$ are synthesizing des-Gly-PC_2 (Rt 5.5 min), hPC_2 (Rt 6.8 min), and hPC_3 (Rt 9.7 min). In each case, 100 μL of acidified extract corresponding to 30 mg (fresh weight) azuki bean roots was injected on the RP-HPLC column.

by HPLC and subjected to mass spectrometric analysis (Fig. 2). Electrospray mass spectrometry revealed a mass of 322 $[M + H]^+$, which would correspond to hGSH. The tandem mass spectrometry (MS/MS) fragmentation pattern gave the final evidence that the major -SH-containing component in azuki beans is hGSH. Hereby, the characteristic peptide fragments, particularly of the y and b series, have been found (Fig. 2). Both y and b fragments result from a fragmentation at a peptide bond, with y fragments representing the C terminus and b fragments coming from the N terminus. The subscript numbers indicate the number of amino acids in each peptide fragment.

The results obtained using the Q-Tof instrument are in accordance with those obtained with an ion trap system in MS/MS and multiple stage mass spectrometry (MS^3 and MS^4). Q-Tof has the more effective fragmentation in MS/MS and superior mass accuracy, while the stepwise fragmentation using MS^n in the ion trap provides additional evidence for correct structural identification.

The tandem mass spectrum of hGSH obtained using the ion trap system is very similar to the Q-Tof spectrum (data not shown). The same fragments can be observed at m/z = 305, 233, 215, 193, 176, and 158. MS^3 of m/z = 193 results in m/z = 176 and 158. MS^3 of m/z = 176 and m/z = 233 gives rise to ions at m/z = 158 and 215, respectively, which supports the assumption that a simple water loss in that molecule occurs.

Roots of control plants contain 1.65 μmol hGSH per gram dry weight. Mass spectrometry confirmed that no GSH can be detected in the azuki beans. The characteristic m/z = 308 for the $[M + H]^+$ of GSH has not been detected.

Azuki Beans Synthesize hPCs upon Exposure to Cadmium

The HPLC profile of roots of azuki beans, exposed for 1 week to 10 μM Cd^{2+}, shows the presence of hGSH (Rt 4.1 min) and of three additional thiol-containing compounds with retention times at 5.5, 6.8, and 9.7 min (Fig. 1B). The compound eluting at 6.8 min was isolated and submitted to mass spectrometry, which revealed clearly the presence of hPC_2, indicated by the $[M + H]^+$ ion at m/z = 554. The tandem mass spectrum of this ion (Fig. 3) is dominated by the y_4 peak at m/z = 425 generated by the loss of γ-Glu. However, m/z = 322, representing the fragment γ-Glu-Cys-β-Ala, was also found. In MS^3 of this fragment (obtained using ion trap), the same fragments result as described above for MS/MS of hGSH. This analysis establishes unequivocally this compound to be hPC_2 ([γ-Glu-Cys]$_2$-β-Ala).

Cadmium-induced compounds eluting from the HPLC column at 5.5 and 9.7 min were identified by comparing them with the elution of purified standards. The first compound (Rt 5.5 min) was identified as des-Gly-PC_2 ([γ-Glu-Cys]$_2$) and the second (Rt 9.7 min) as hPC_3 ([γ-Glu-Cys]$_3$-β-Ala; Fig. 1B). The concentration of hGSH (Rt 4.1 min) in roots of plants exposed to cadmium is reduced from 1.65 μmol per gram dry weight (control) to 0.95 μmol per gram dry weight (Cd-treated plants). hPC_2 is also present in low concentration in the control plants (92 nmol per gram dry weight; Fig. 1A), but its concentration increases 15-fold (1.35 μmol per gram dry weight) upon exposure to cadmium ions.

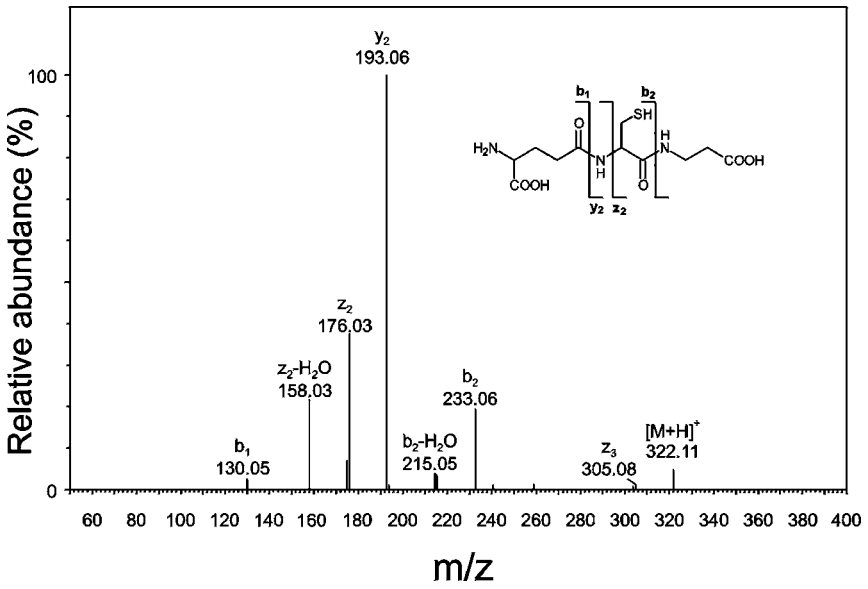

Figure 2. Electrospray tandem mass spectrum of hGSH ($[M + H]^+$, $m/z = 322$) from azuki beans. The extract of azuki bean roots was separated by HPLC. The fraction containing hGSH was submitted to Q-Tof-MS. Inset, Formula showing the peptide fragmentation.

The 5,5′-Dithiobis(2-Nitrobenzoic Acid)-Oxidized GSH (DTNB-GSSG)-Reductase Recycling Assay Cannot Discriminate between GSH and hGSH

To test the specificity of the GSH determination system used by Inouhe et al. (2000), we analyzed both GSH and hGSH under the experimental conditions as described by Anderson (1985). The DTNB-GSSG reductase recycling assay is a two-step reaction:

$$2GSH + DTNB \rightarrow GSSG + TNB \quad (1)$$

$$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+ \quad (2)$$

Step 2 only occurs when the enzyme GSSG reductase is present in the assay. It is obvious that GSH produced in the second step of this reaction may reenter into the whole reaction (hence "recycling assay"). The rate-limiting factor in this reaction therefore is not GSH, but rather NADPH or GSSG reductase. Calculation of GSH concentration in the assay is based on the kinetics of 5′-thiobis(2-nitrobenzoate) (TNB) formation, which depends on the GSH concentration in the assay and can be easily monitored photometrically at 412 nm.

Different amounts (0.5–5 nmol) of GSH and hGSH were assayed in 1 mL DTNB-GSSG reductase recycling assay. Figure 4 shows a standard curve for GSH and hGSH obtained by DTNB-GSSG reductase recycling assay. From this figure, it is clear that GSSG reductase shows no preference for GSH when compared with hGSH in the assay. The kinetics of this reaction are practically identical when either GSH or hGSH is present in the assay. These results clearly show that the DTNB-GSSG reductase recycling assay

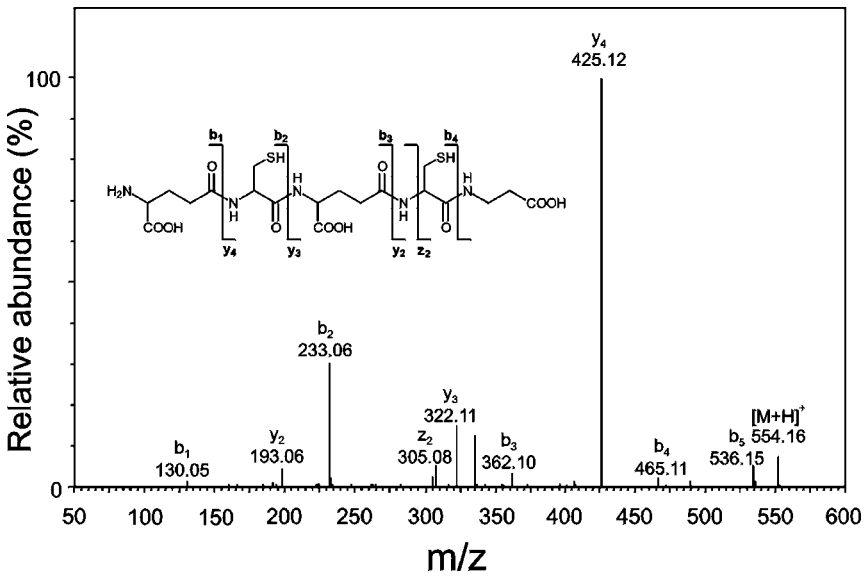

Figure 3. Electrospray tandem mass spectrum of hPC_2 ($[M + H]^+$, $m/z = 554$) from azuki beans. The extract of azuki bean roots was separated by HPLC. The fraction containing hPC_2 was submitted to Q-Tof-MS. Inset, Formula showing the peptide fragmentation.

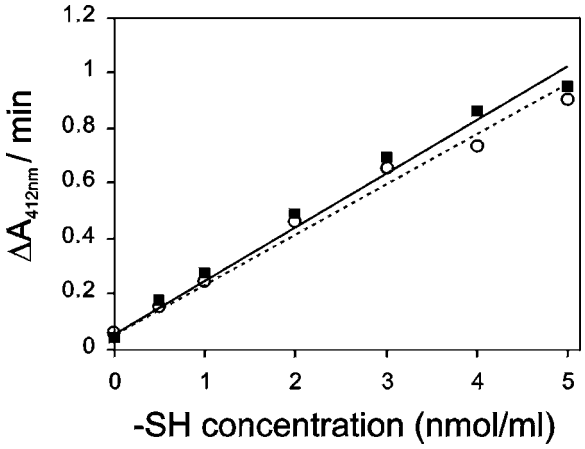

Figure 4. Standard curve for GSH (—) and hGSH (- - - -) in the DTNB-GSSG reductase recycling assay. Either GSH or hGSH (0.5–5 nmol) was added to 1 mL of prewarmed assay buffer. Each reaction was initiated by addition of 2.5 units of yeast GSSG reductase. Kinetics of TNB formation was monitored photometrically at 412 nm. ΔA, Change in absorbance.

used by Inouhe et al. (2000) cannot discriminate between GSH and hGSH. When either of the two substrates was used in the assay, the same kinetics of the TNB formation was observed ($\Delta A_{412\ nm}$ = 0.29 units $nmol^{-1}$ min^{-1}).

DISCUSSION

All living cells are confronted with the dilemma that on one side they need a certain amount of free heavy metal ions (such as Zn^{2+}, Cu^{2+}, etc.) for their normal metabolic function, and on the other side, they have to protect themselves from an intracellular excess of heavy metal ions that would lead to cell death. This dilemma can be overcome only by a stringent regulation of free metal ion concentration within the cell. Plant cells have developed one general mechanism to achieve this goal. They synthesize small, Cys-rich peptides capable of binding heavy metal ions through thiolate coordination. The general structure of this set of peptides is $(\gamma\text{-Glu-Cys})_n$-R ($n$ = 2–11) and R = Gly, β-Ala, L-Ser, L-Glu, and L-Gln. In addition, $[\gamma\text{-Glu-Cys}]_n$ are also ubiquitous in plants.

This unique heavy metal-complexing mechanism is obviously of acute importance for all higher plants. Until now, all plants thus far investigated (>200 species) have been shown to contain this heavy metal-complexing mechanism forming either PCs or iso-PCs (Zenk, 1996).

The report of Inouhe et al. (2000) that azuki beans do not synthesize PCs upon treatment with Cd^{2+} ions would have represented the first exception in the plant kingdom and would point to a different mechanism of heavy metal detoxification and homeostasis in this plant species. This published report therefore deserved an unambiguous check. In contrast to the previous claim, the analysis of the azuki bean root system here showed that there is no GSH present in detectable amounts in this plant. The DTNB-GSSG reductase recycling assay that these authors used is not specific for GSH as was previously claimed. The major -SH-positive compound found in unchallenged azuki bean tissue, which reacted fully with the DTNB-GSSG reductase recycling assay, but had a consistent, marginally longer retention time than GSH upon HPLC analysis, is a compound with a mass of 321. This is 14 mass units higher than the mass of GSH, which in turn corresponds to an additional -CH_2-group. This SH-containing metabolite was subjected to MS/MS analysis. The resulting fragmentation pattern unequivocally allowed the structural assignment as hGSH. The azuki bean is a legume, belonging to the tribe Phaseoleae, for which the presence of hGSH has often been reported (Grill et al., 1986; Klapheck, 1988; Gekeler et al., 1989).

The azuki bean root system was next exposed to an aqueous 10-μM $CdSO_4$ solution for a period of 7 d. The root system of the exposed plants was excised, extracted, and subjected to HPLC. It could be shown that the major -SH-containing compound was no longer hGSH (which has decreased 40% compared with control), but rather a compound with a retention time of 6.8 min. Rigorous structure identification by MS/MS showed this compound to be hPC_2 ($[\gamma\text{-Glu-Cys}]_2$-β-Ala). Two other minor compounds with retention times of 5.5 and 9.7 min were also induced by cadmium treatment. They were identified as des-Gly-PC_2 ($[\gamma\text{-Glu-Cys}]_2$) and hPC_3 ($[\gamma\text{-Glu-Cys}]_3$-β-Ala), respectively. Therefore, azuki beans contain, as expected for a species belonging to the Phaseoleae, a hPC-based heavy metal-complexing system (Grill et al., 1986).

Careful reading of the report from Inouhe et al. (2000) reveals that GSH could not be detected in azuki beans as determined also by their analytical system. Based on Figures 7 and 10 in their report, we conclude that GSH elutes at 9.5 min in their HPLC system. Figure 7 of their report confirms that GSH is clearly present as an endogenous molecule in tomato cells (Inouhe et al., Fig. 7a), but absent from both azuki bean cells (Inouhe et al., Fig. 7b) and roots (Inouhe et al., Fig. 7c).

Inouhe et al. (2000) observed that azuki bean cells were more sensitive to cadmium when compared with control tomato cells. This observation, however, may be based upon other processes involved in detoxification of metals rather than upon biosynthesis of PCs. Although synthesis of PCs was clearly shown to be indispensable for heavy metal tolerance in Arabidopsis (Howden et al., 1995), other mechanisms such as vacuolar compartmentalization of low molecular weight PC metal complexes (Ortiz et al., 1992) and incorporation of acid-labile S^{2-} into these complexes (Verkleij et al., 1990; Mehra et al., 1994) were shown to significantly supplement metal tolerance.

It is satisfying that the fundamental role of the PC/iso-PC heavy metal-complexing system has been demonstrated in this study also in the azuki bean. Until now, there is no exception found in higher plants that would equivocate the role of these peptides in heavy metal detoxification and homeostasis. The respective presence of GSH and its isoforms in higher plants determines the formation of the corresponding heavy metal-complexing PCs after exposure to physiological or excessive heavy metal concentration. Azuki beans are no exception in this rule.

MATERIALS AND METHODS

Plant Material

Common azuki beans (*Vigna angularis* [Willd.] Ohwi & Ohashi; Reformhaus, Germany) were grown under greenhouse conditions. Beans were allowed to imbibe for 24 h in tap water and were afterward sown on tissue paper. Seven days after germination, the seedlings were treated with 10 μM $CdCl_2$ (Sigma, Taufkirchen, Germany). The plants were harvested 7 d later. The plant roots were washed, excised from the aerial parts of the plant, frozen in liquid nitrogen, and ground with a pestle and mortar. Ground roots were extracted as described by Grill et al. (1989). Azuki bean plants without Cd^{2+} challenge were used as controls.

HPLC Procedure

HPLC chromatography with post-column derivatization with DTNB was performed as described in Grill et al. (1989; Detector K-2600 and Pump K-1001 for the HPLC, Knauer, Berlin; Pump 64 for the DTNB derivatization, Knauer). For MS analysis, hGSH and hPC_2 from the azuki beans were isolated after HPLC resolution without post-column derivatization.

DTNB-GSSG Reductase Recycling Assay (Anderson, 1985)

Reaction tubes containing 0.6 mM DTNB (Sigma), 4.4 mM EDTA (Sigma), and 0.25 mM NADPH (Sigma) in 100 mM sodium phosphate buffer (pH 7.5) were incubated at 30°C for 15 min. Known concentrations (0.5–5 nmol) of either GSH (Biomol, Hamburg, Germany) or hGSH (Bachem, Heidelberg) were added to the reaction tubes. Reactions were initiated by addition of 2.5 units of yeast GSSG reductase (Roche, Mannheim, Germany). Kinetics of TNB formation was monitored photometrically at 412 nm (Ultrospec 3000, Pharmacia, Piscataway, NJ). A sample blank lacking GSH or hGSH was used to determine the background reaction rate.

Electrospray Mass Spectrometry

All experiments have been carried out in positive ionization mode. An ion trap mass spectrometer Finnigan LCQ (ThermoQuest, San Jose, CA) has been used for MS, MS/MS, and MS^n. A quadrupole time-of-flight hybrid mass spectrometer Q-Tof 2 (Micromass, Manchester, UK) has been applied for MS and MS/MS. The PepSeq software included in MassLynx (Micromass) has been used to confirm the fragmentation pattern of hGSH.

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