Cloning and functional characterization of a homoglutathione synthetase from pea nodules

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The thiol tripeptide glutathione (GSH; γ Glu-Cys-Gly) is very abundant in legume nodules where it performs multiple functions that are critical for optimal nitrogen fixation. Some legume nodules contain another tripeptide, homoglutathione (hGSH; γ Glu-Cys- β Ala), in addition to or instead of GSH. We have isolated from a pea (*Pisum sativum* L.) nodule library a cDNA, *GSHS2*, that is expressed in nodules but not in leaves. This cDNA was overexpressed in insect cells and its protein product was identified as a highly active and specific hGSH synthetase. The enzyme, the first of this type to be completely purified, is predicted to be a homodimeric cytosolic protein. It shows a specific activity of 3400 nmol hGSH min⁻¹mg⁻¹ of protein with a standard substrate concentration (5 mM β -alanine) and K_m values of 1.9 mM for β -alanine and 104 mM for glycine. The specificity constant (V_{max}/K_m) shows that the pure enzyme is 57.3-fold more specific for β -alanine than for glycine. Southern blot analysis revealed that the gene is present as a single copy in the pea genome and that there are homologous genes in other legumes. We conclude that the synthesis of hGSH in pea nodules is catalyzed by a specific hGSH synthetase and not by a GSH synthetase with broad substrate specificity.

Abbreviations - γ EC, γ -glutamylcysteine; γ ECS, γ -glutamylcysteine synthetase; GSHS, glutathione synthetase; hGSH, homoglutathione; hGSHS, homoglutathione synthetase; ORF, open reading frame.

Introduction

The thiol tripeptide glutathione (GSH; γ Glu-Cys-Gly) is a major antioxidant metabolite in most procaryotic and eucaryotic cells. The synthesis of GSH involves two sequential reactions catalyzed by γ -glutamylcysteine (γ EC) synthetase (γ ECS; EC 6.3.2.2) and GSH synthetase (GSHS; EC 6.3.2.3) (Fig. 1). Both enzymes show a strict requirement for ATP and Mg^{2+} (Rennenberg 1997). However, plants may contain another thiol tripeptides (Fig. 1), such as hydroxymethylglutathione (γ Glu-Cys-Ser), found in cereals, and homoglutathione (hGSH; γ Glu-Cys- β Ala), found exclusively in legumes (Klapheck 1988, Rennenberg 1997, Matamoros et al. 1999). The pathway of hGSH synthesis is also thought to proceed through two steps, catalyzed respectively by γ ECS and either a specific hGSH synthetase (hGSHS) or a GSHS with broad substrate specificity (Macnicol 1987).

Thiol compounds are particularly abundant in nodules and this may be related to their critical role in the overall protection of nitrogen fixation (Dalton et al. 1986, Matamoros et al. 1999). In previous work on thiol metabolism in pea plants, we found GSHS activity in leaves and nodules, whereas hGSHS activity was only detected in nodules (Matamoros et al. 1999). We subsequently isolated two cDNA clones, *GSHS1* and *GSHS2*, from a pea nodule library. Based on the correlation between activity and expression data, we concluded that *GSHS1* and *GSHS2* code for GSHS and hGSHS, respectively (Moran et al. 2000). A similar correlative hypothesis was proposed for two partial GSHS clones obtained from a *Medicago truncatula* root cDNA library (Frendo et al. 1999). Sequence analysis revealed that, in pea, *GSHS1* encodes a protein bearing a mitochondrial signal peptide whereas *GSHS2* encodes a cytosolic protein (Moran et al. 2000). These data, although predictive, indicate that GSHS enzymes may be localized in at least two subcellular compartments of nodules.

In fact, we were able to detect GSHS activity in mitochondria of cowpea nodules (a GSH producing species) but not of bean nodules (a hGSH producing species), suggesting that hGSHS is not present in mitochondria.

Up to date a hGSHS enzyme has not been completely purified from any plant (Macnicol 1987) or from any heterologous organism (Frendo et al. 2001). This is probably due to the lability and low abundance of the enzyme in plant tissues (Macnicol 1987; Klapheck et al. 1988) and the low yield of conventional heterologous expression systems (Frendo et al. 2001). The availability of a cDNA that putatively encodes pea hGSHS, the absence of pure enzyme preparations for adequate kinetic analysis and thereby for function assignment, and the presence of *GSHS2* transcripts specifically in pea nodules, all prompted us to characterize the *GSHS2* cDNA and the corresponding protein product.

Materials and methods

Plant material

Nodulated plants of pea (*Pisum sativum* L. cv. Lincoln x *Rhizobium leguminosarum* biovar. *viciae* strain NLV8) and common bean (*Phaseolus vulgaris* L. cv. Contender x *Rhizobium leguminosarum* biovar. *phaseoli* strain 3622) were grown under controlled environment conditions as described by Gogorcena et al. (1997). Leaves and nodules to be used for extraction of genomic DNA or mRNA were harvested from plants at the vegetative growth period (approximately 30 days of age), immediately frozen in liquid N₂, and stored at -80°C.

Overproduction and purification of recombinant protein

The open reading frame (ORF) of *GSHS2* was PCR-amplified using cDNA from 3-week-old pea nodules as a template using gene-specific primers (*Nco*I and *Not*I sites are underlined in the respective primers): forward 5'-caccatggcta aatcatctcaacagc-3' and reverse 5'-CTAATCGCAGCGGCCGC AATGCTA-3'. The resulting 1.7 kb fragment was gel purified, subcloned into pCRIITOP0 (Invitrogen, Groningen, The Netherlands), and transformed into DH5 α competent cells. The inserted ORF of *GSHS2* was digested out with *Nco*I and *Not*I, gel purified, and ligated into pFastBac HTb. This procedure resulted in the *GSHS2* cDNA being placed under the transcriptional control of the strong polyhedrin promoter (*Autographa californica* nuclear polyhedrosis virus) and in the addition of a poly-His tag to the recombinant protein for further detection and purification. DH5 α competent cells were then transformed and positive colonies were identified by PCR using pFastBac specific primers. The pFastBac::*GSHS2* DNA was isolated from an overnight culture and used to transform DH10BAC competent cells following the BAC-to-BAC protocol (Life Technologies, Paisley, UK). White positive colonies were verified by colony PCR. High molecular mass recombinant bacmid DNA was produced overnight in *Escherichia coli* and used to transfect *Sf21 Spodoptera frugiperda* insect cells with CellFectin reagent (Life Technologies). Recombinant baculoviruses were harvested 72 h post-transfection and amplified by infecting monolayer cultures of insect cells. These cultures were grown at 27°C in TC-100 medium supplemented with 10% fetal calf serum and antibiotics, using media and chemicals from Sigma and protocols available from Life Technologies. Recombinant viruses were collected 48 h after infection from the culture supernatant and kept at 4°C or -20°C until subsequent infection of fresh cells.

To optimize infection conditions and protein yield, confluent *Sf21* cell cultures (5 ml of medium) were infected with different amounts of recombinant

viruses and cells were collected by centrifugation 24 to 96 h after infection. Cells were resuspended in lysis medium consisting of 10 mM Tris-HCl (pH 7.5), 130 mM NaCl, 10 mM NaF, 10 mM sodium phosphate buffer (pH 7.5), 10 mM sodium pyrophosphate, 1% Triton X-100, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, and protease inhibitor cocktail (Boehringer Mannheim, Mannheim, Germany). Cell-free extracts were loaded on a cobalt Talon affinity column (Clontech, Palo Alto, CA, USA) and protein was eluted with 50 mM sodium phosphate buffer (pH 7.0), 300 mM NaCl, and 150 mM imidazole.

Western blot analysis

Western blots were performed following protocols supplied by the manufacturer (Clontech). Briefly, protein samples were separated in SDS gels, blotted onto nitrocellulose membranes, incubated overnight at 4°C with blocking solution (0.1% Tween-20, 1% nonfat milk in phosphate-saline buffer), and then for 1 h with the antibodies diluted in blocking solution. The primary antibody (6xHis monoclonal antibody; Clontech) was used at a 1:5000 dilution and the secondary antibody (anti-mouse antibody-alkaline phosphatase conjugate; Sigma) at a 1:2500 dilution. Color was developed with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium chloride (Sigma).

Functional characterization of the enzyme

The assay of GSHS and hGSHS activities of the GSHS2 overexpressed protein was based on the amount of GSH and hGSH synthesized from γ EC and Gly or β -Ala, respectively (Matamoros et al. 1999). Thiol tripeptides were derivatized with monobromobimane and quantified by HPLC with fluorescence detection (Fahey and Newton 1987) with minor modifications (Matamoros et al. 1999). The K_m and V_m values were calculated from double-reciprocal plots using 0.5 mM γ EC and 10 to 150 mM Gly (for GSHS activity) or 0.5 mM γ EC and 0.4 to 5

mM β -Ala (for hGSHS activity). For comparison, the activity rates (V) of GSHS and hGSHS were also measured using fixed standard concentrations of γ EC (0.5 mM) and Gly or β -Ala (5 mM).

Southern blot analysis of GSHS2

Genomic DNA was isolated from pea and bean leaves, digested with the restriction enzymes stated in Figure 3, fractionated on agarose gels, and transferred to Hybond N⁺ membranes (Amersham) following standard protocols. Hybridizations were performed at high stringency with ³²P-labeled probes prepared from PCR products. For pea, the primers (forward 5'-GCAGTCGCAATCGTTTACTTCC-3', reverse 5'-CCCACCTTCATCAAATAATGATGG-3') amplified a 594-bp fragment within the ORF (GenBank accession no. AF258319). For bean, the primers (forward 5'-GAAAGTGGCTATATGGTGCG-3', reverse 5'-GACACCATTCAGTAGGAAAAGC-3') amplified a 233-bp fragment including part of the ORF and part of the 3'-untranslated region (GenBank accession no. AF258320).

Results and Discussion

We attempted initially to overproduce pea nodule GSHS2 using conventional *E. coli* expression systems but this approach proved unsuccessful. In contrast, we found that large amounts of virtually pure protein could be produced efficiently in insect cells. The yield of GSHS2 protein was optimized by monitoring the amount of baculovirus used to infect the insect cells and the time course of protein production. Western blot analysis demonstrated that the protein was correctly expressed (expected size of approximately 59 kD) in infected cells (Fig. 2). The protein yield was similar between 48 and 96 h after infection but after this time there were significant amounts of smaller degradation products. Therefore, protein production was scaled up by culturing

insect cells in 50 ml of medium and by harvesting cells 48 h after infection.

The baculovirus expression system allowed us to produce large amounts of highly pure active GSHS2 enzyme suitable for biochemical characterization. Thus, the GSHS and hGSHS activities of GSHS2 were first determined using identical standard concentrations (5 mM) of the substrates, Gly and β -Ala, respectively (Table 1). The specific activity of GSHS2 with β -Ala was 3433 nmol of hGSH produced $\text{min}^{-1} \text{mg}^{-1}$ protein, which is approximately between 100- and 1000-fold higher than the two putative hGSHS activities reported in the leaves of other legumes (Macnicol 1987, Klapheck et al. 1988). This is consistent with the highly purified enzyme preparation that we obtained using the insect expression system. Likewise, the hGSHS/GSHS ratio of activities was 21.7, thus suggesting a higher affinity of GSHS2 for β -Ala than for Gly. The catalytic constants of GSHS2 were then determined using a fixed saturating concentration of γ EC and a range of concentrations of Gly or β -Ala (see "Materials and methods"). The enzyme showed saturation kinetics and linear double-reciprocal plots with respect to both substrates. The K_m of GSHS2 for β -Ala was 55-fold lower than for Gly but, perhaps most relevant in terms of substrate specificity, the V_{max}/K_m ratio (specificity constant) for β -Ala was 57-fold higher (Table 1). These kinetic data using virtually pure, recombinant enzyme demonstrate that *GSHS2* encodes a genuine hGSHS.

Very recently, Frendo et al. (2001) reported the expression, in *E.coli*, of a cDNA from *Medicago truncatula*. The enzyme product in bacterial crude extracts showed a specific activity of $0.32 \text{ nmol min}^{-1} \text{mg}^{-1}$ protein as hGSHS and of $0.12 \text{ nmol min}^{-1} \text{mg}^{-1}$ protein as GSHS. These activities were therefore about 10000- and 1300-fold, respectively, lower than those of our enzyme preparation. These extremely large differences in activities are due to the use of crude extracts instead of purified enzyme and probably also to the fact that the pea GSHS2 protein has been expressed in an eucaryotic system, which can improve the folding and processing of the enzyme. Reliable kinetic analysis

requires enzyme purification. Clearly, our highly purified enzyme preparation is more appropriate for kinetic studies and also allows the subsequent structural analysis of the protein.

Genomic Southern blot analysis of *GSHS2* was performed in pea and common bean using gene-specific probes for each legume species (Fig. 3). Bean was included in this analysis because this plant has hGSHS (but not GSHS) activity and hence a functional *GSHS2* gene (Moran et al. 2000). In both legumes, restriction enzymes cutting inside (*Xba* I, *Hind* III) or outside (other enzymes) of the ORFs generated single fragments (Fig. 3). This observation, along with the high sequence identity (73%) between pea and bean *GSHS2* (Moran et al. 2000), allowed us to conclude that an homologous gene to pea nodule *GSHS2* is present in the bean genome, that both pea and bean *GSHS2* are present as single copies, and that the pea GSHS2 enzyme is responsible for the hGSH content and hGSHS activity found in nodule extracts (Matamoros et al. 1999).

Assuming that the molecular mass of native hGSHS is similar to that of GSHS (113-120 kD) of other plants (Rennenberg 1997), it follows that hGSHS is also present in the nodules as a homodimer. The derived amino acid sequence of hGSHS (*GSHS2*) is devoid of N-terminal signal peptides or C-terminal motifs, and the enzyme is predicted by several algorithms to be located to the cytosol (Moran et al. 2000). We conclude that thiol biosynthesis in pea nodules proceeds via two genuinely different enzymes (GSHS and hGSHS), rather than two GSHS isozymes. The enzymes are located in two nodule compartments known to generate toxic oxygen species at high rates (Becana et al. 2000). The synthesized GSH and hGSH may fulfil antioxidative and regulatory roles that are important during nodule initiation and senescence. Thus, GSH is involved in the osmotic and oxidative stress tolerance of bacteroids (Ricciolo et al. 2000), and both GSH and hGSH are involved in peroxide detoxification in the plant fraction of nodules via the Halliwell-Asada

pathway (Moran et al. 2000, Iturbe-Ormaetxe et al. 2001). The two thiols are generally assumed to be functionally interchangeable (Klapheck 1988). While this may be correct, our compartmentation results emphasize that there is at least the potential for specific different functions of GSH and hGSH. With the availability of hGSHS cDNAs (this work) and the use of antisense technology this question may be adequately addressed in future.

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Legends for Figures

Fig. 1. Proposed pathway for the synthesis of thiol tripeptides in plants. The synthesis of GSH proceeds through two steps catalyzed by γ ECS and GSHS. The synthesis of hGSH, a GSH homolog found exclusively in legumes, is thought to proceed through the same γ ECS enzyme and then by either a specific hGSHS or by a GSHS isozyme with broad substrate specificity. Hydroxymethyl-glutathione, found in cereals such as wheat and rice, could be synthesized by addition of a Ser residue to the C-terminus of γ EC or by hydroxymethylation of the C-terminal Gly of GSH (Klapheck et al. 1992), and γ Glu-Cys-Glu, detected in maize seedlings exposed to Cd, is thought to be synthesized from γ EC (Meuwly et al. 1995).

Fig. 2. Overproduction in insect cells and purification of pea nodule GSHS2. (A) Red Ponceau-stained SDS-gel of proteins from control (uninfected) and infected cells after 24, 48, 72, and 96 h. (B) Western blot of the same gel using 6xHis monoclonal antibody. (C) Coomassie-stained SDS-gel of proteins from cell free extracts prior to loading on the metal-affinity column (fraction 0) or subsequently eluted with 1 ml of imidazole elution buffer per fraction (fractions 1 and 2). (D) Western blot of a gel similar to (C) using the same antibody and conditions as in (B).

Fig. 3. Southern blot analysis of *GSHS2* in pea and bean. Genomic DNA was extracted from leaves, digested with restriction enzymes, electrophoresed (10 μ g of pea DNA per lane or 5 μ g of bean DNA per lane), blotted onto Hybond N⁺ membranes, and hybridized with ³²P-labeled probes.

Fig. 1

Fig. 2