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# Purification and Molecular Analysis of an Extracellular $\gamma$-Glutamyl Hydrolase Present in Young Tissues of the Soybean Plant

J. Huangpu, J. H. Pak, M. C. Graham, S. A. Rickle, and J. S. Graham[1]

*Department of Biological Sciences, Bowling Green State University, Bowling Green, Ohio 43403*

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A polypeptide present in intercellular wash fluids of young leaves of *Glycine max* has been purified to electrophoretic homogeneity. The protein has been identified as $\gamma$-glutamyl hydrolase (GGH) based on the shared homology with a recently cloned cDNA from rat. The enzyme is present within the extracellular space of young leaves and a portion is bound to the cell wall. Northern and Western analysis confirm that this polypeptide is expressed only in young (1-15 d old) leaf, stem and root tissue and is therefore expressed under a strict developmental program. The primary sequence of $\gamma$-glutamyl hydrolase shares amino acid identity with a cDNA clone from rat and two partially sequenced cDNAs from *Arabidopsis.* Although the complete *in vivo* function of $\gamma$-glutamyl hydrolase in plants is unclear, it is known that the protein plays a critical role in folate metabolism and therefore likely in meeting the physiological demands of growing plant tissues. 

It is becoming increasingly clear that the plant extracellular matrix is much more than a static compartment present only to provide organization for cells in organ systems of multicellular organisms. Recent experiments in animal systems have suggested molecules originating within the extracellular matrix (ECM) serve as regulatory signals that can migrate to the cell nucleus and affect gene transcription. In plants, much research has been devoted on polypeptides that comprise structural components of the cell wall. The extensins, proline-rich proteins and glycine-rich proteins are the most common (18). These are not the only proteins found within the extracellular compartment. For instance, our laboratory has shown that a wound-inducible peroxidase (5), a pathogenesis-related protein (7), and two proteinases termed SLAP and SMEP1 (8, 9, 13) reside within the extracellular compartment of soybean leaves. Other reports have demonstrated the presence of phosphatases, invertases, pectin methylesterase, polygalacturonases, mannosidases and a variety of glycosyltransferases reside extracellularly and probably, in part, within the cell wall (18).

In order to gain a further understanding of the function of components within the extracellular matrix of plant tissue, we have set about to purify and characterize molecules within this compartment to clarify the role of the ECM in higher plants. In this communication we report the isolation of a $\gamma$-glutamyl hydrolase and the developmental and tissue-specific expression of both the protein and its mRNA. The cDNA sequence is reported elsewhere (GenBank U63726; 10). The polypeptide is an important enzyme in folate metabolism (3, 4, 12, 21, 23) and therefore may play a key role in tissue growth. Indeed, we only find the enzyme present in rapidly growing stem, root and leaf tissue.

## MATERIALS AND METHODS

*Plant material.* Soybean plants (*Glycine max* var. Williams 82) were grown in environmental growth chambers under a 16 h photoperiod. Seeds were obtained from Mid-Wood Inc., Bowling Green, OH.

[1] Corresponding author. Fax: (419) 372-2024. E-mail: jgraham@opie.bgsu.edu.



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*Protein determination.* Protein concentrations were determined by the Bio-Rad assay system (2) according to the manufacturer's procedures. Bovine serum albumen and bovine gamma globulin were used separately as standards. The values obtained were averaged and used for construction of a standard curve.

*Enzyme isolation.* Five to ten day old leaves (50 g) were detached and cut into approximately 1 cm 2 pieces. Leaf pieces were rinsed three times with distilled $H_2O$ and placed into a 2 L vacuum flask and the intercellular wash fluid (IWF) was collected as previously described (9). The IWF solution was applied to a carboxymethyl (CM) cellulose (Sigma Chemical) column (2.5 cm $\times$ 8.0 cm) equilibrated with 10 mM $NaH_2PO_4$, pH 6.5, 10 mM $\beta$-mercaptoethanol. GGH present in the flow through and wash fractions was collected, adjusted to pH 8.5 with 1 M TrisHCl, and applied to a diethylaminoethyl (DEAE) cellulose (Sigma Chemical) column (2.5 cm $\times$ 8.0 cm) equilibrated with 10 mM Tris, pH 8.5, 10 mM $\beta$-mercaptoethanol. GGH bound to the column and was eluted with 0.05M NaCl, 10 mM Tris, pH 8.5. The eluate (100 mL) was concentrated in an Amicon concentrator to about 5 ml, and applied on a Cibacron Blue-3GA dye column (1 $\times$ 5 cm; Sigma) equilibrated in 10 mM Tris, pH 8.5. GGH bound to the column and was eluted with a step gradient of 0.5M, 1.0 M and 1.5M NaCl. The 1.5M fraction containing the homogeneous GGH preparation was dialyzed exhaustively against $ddH_2O$ and lyophilized to dryness.

*Cell wall protein isolation.* Five g of leaves were frozen in liquid $N_2$ and ground to a fine powder with a mortar and pestle. The powder was homogenized in a Polytron in 20 ml 0.25M sucrose, 40 mM Tris, pH 8.0, 10mM $\beta$-mercaptoethanol and centrifuged for 5 min at 1000g, The remaining portion of the procedure was as previously reported (1) except that cell walls were subjected to a 0.2 M $CaCl_2$ wash before the 0.5 M $CaCl_2$ extraction of cell wall proteins.

*Electrophoresis and Western analysis.* Denaturing gel electrophoresis (SDS-PAGE) was performed (11) on tissue samples followed either by transfer to nitrocellulose (MSI Laboratories) or by silver staining (15). Denaturing gels were blotted onto nitrocellulose (MSI Laboratories, Westboro, MA) for Western blot analysis (20) and probed with antiserum (1:1,000-3,000 dilution) to SMEP1 (8). This antiserum recognizes two polypeptides: an extracellular metalloproteinase termed SMEP1 (8) and GGH (see results).

*RNA isolation and Northern hybridization.* Total RNA from soybean leaf tissue was isolated using RNAgents Total RNA Isolation System as per the manufacturer's instructions (Promega, Madison, WI). Poly $A^+$ mRNA was isolated from total RNA by chromatography on oligo(dT)-cellulose (17). For Northern blot analysis, 10 $\mu$g total RNA was separated on a 1.2 % agarose/formaldehyde gel (17). The gel was dried on a gel dryer and hybridization performed *in situ* (6) with GGH DNA probes (10) labeled to a specific activity of $1\times10^8$ cpm/ug with [$\alpha$-$^{32}$P] CTP using the Amersham Multiprime Labelling System as per the manufacturer's instructions. Following hybridization at 60°C, the gel was washed with five changes of 2 $\times$ SSC/0.1%SDS at room temperature followed by two washings in 0.5 $\times$

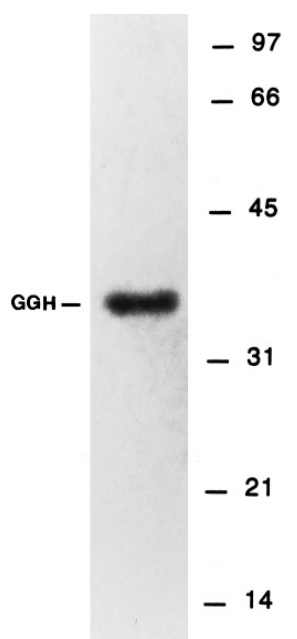


**FIG. 1.** Analysis of GGH purity. Silver-stained SDS−PAGE of Cibacron Blue 3GA purified material (2 $\mu$g). The position of GGH is shown in the left margin.

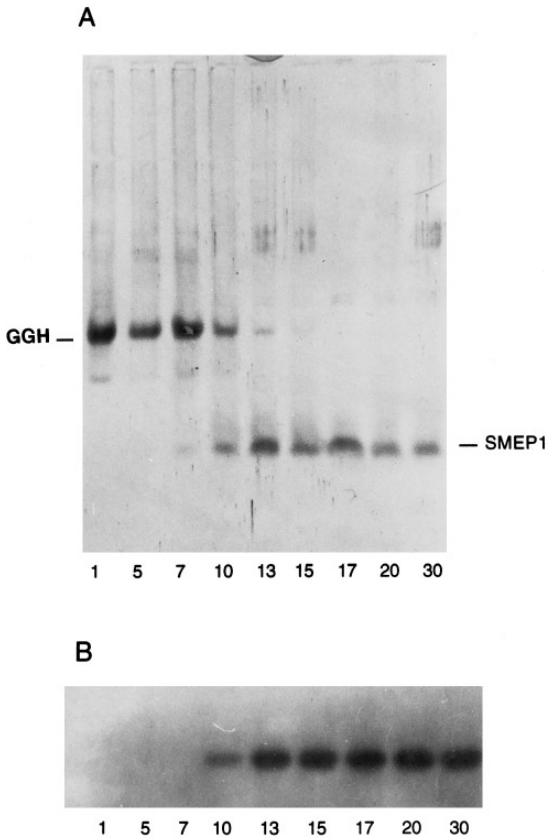


**FIG. 2.** (A) Western analysis of GGH accumulation in leaf extracts of indicated ages. (B) Time course of the accumulation of GGH mRNA during leaf development. Total RNA (10$\mu$g/lane) extracted from leaves (1-20 d post-emergence) was subjected to electrophoresis, blotted, and hybridized to radiolabeled GGH cDNA (10).

SSC/0.1% SDS for 30 minutes at 60°C. The moist gel was covered with Saran Wrap and exposed to Kodak Xomat X-ray film with enhancing screens at −70°C for 3-6 days.

## RESULTS AND DISCUSSION

Our initial observation was that a 35 kD polypeptide strongly cross-reacted with antibodies to the metalloproteinase SMEP1 during Western analysis of SMEP1 accumulation (8). We initially proposed that the 35 kD polypeptide might represent an inactive precursor of SMEP1, and therefore decided to isolate the polypeptide using anti SMEP1 antibody as an assay during the purification process. Indeed, most matrix metalloproteinases are secreted as inactive zymogens (19). We found that our hypothesis was incorrect as we cloned the cDNA (GenBank U63726) encoding the 35 kD polypeptide and found that the protein is $\gamma$-glutamyl hydrolase (GGH) based on amino acid homology with a recently published rat GGH cDNA clone (23). Henceforth, the 35 kD polypeptide will be referred to as GGH.

Our previous observation that GGH was present within intercellular wash fluids obtained from young leaves (9) resulted in choosing IWF from 3-5 d old leaves as the initial source for GGH isolation. The isolation of GGH was accomplished by employing a Western blot immunoassay due to the cross-reactivity of GGH to SMEP1 antisera. A straightforward and expeditious purification scheme was developed that employed CM and DEAE Cellulose chromatography followed by binding of the protein to a Cibacron Blue 3GA affinity column.

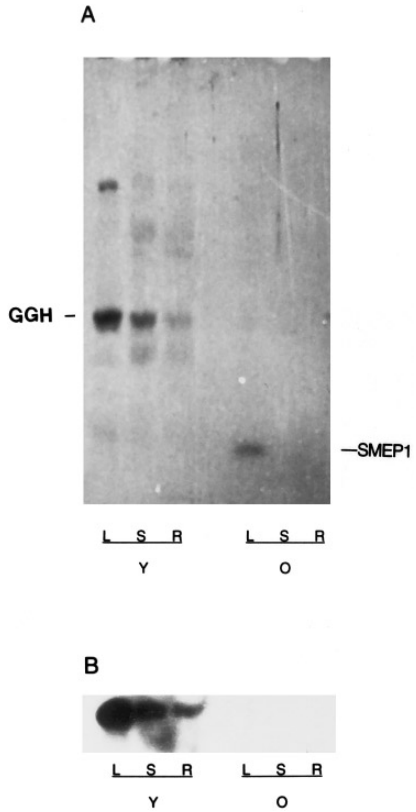


**FIG. 3.** Analysis of the tissue-specific and temporal expression of GGH. (A) Five $\mu$g of IWF were subjected to SDS-PAGE, transferred and probed with anti-SMEP1 (1:1,000 dilution). Y, young plant; O, old plant; L, leaf; S, stem; R, root. (B) Northern blot analysis of 10 $\mu$g of total RNA extracted from different tissues were hybridized with $^{32}$P-labeled GGH cDNA. Y, young plant; O, old plant; L, leaf; S, stem; R, root.

Although we do not understand the molecular basis of the interaction, we were able to obtain a homogeneous preparation of the protein (Fig. 1) by collecting a 1.0 M-1.5 M NaCl eluate from the column.

The temporal expression of GGH is restricted to young, rapidly expanding leaf tissue less than approximately 2 weeks old. The availability of a cDNA clone (10) allowed us to examine by Northern blot analysis levels of GGH mRNA in RNAs prepared from leaves of increasing age. Figure 2 clearly demonstrates a direct correlation between the steady state presence of GGH transcripts and protein in that the former remain fairly steady for the first 13 d post-emergence and abruptly decline to undetectable levels by 15 d. Indeed, the data show that GGH expression is tightly regulated, probably at the level of transcription or mRNA stabilily. Additionally we examined various soybean plant tissues for the expression of GGH transcripts. Figure 3A and B show a comparison of the presence of protein and transcript, respectively, in primary leaf, stem and root tissue from a 7 d old seedling and a 28 d old plant. GGH is expressed in all three tissues of the seedling, but is absent in the same tissues of a 28 d old plant. These data suggest that GGH expression is not limited to leaves but is present in all actively growing tissues.

Because GGH was isolated from the IWF during a period of rapid cell expansion requiring modification of the cell wall, we examined cell wall bound proteins for the presence of GGH

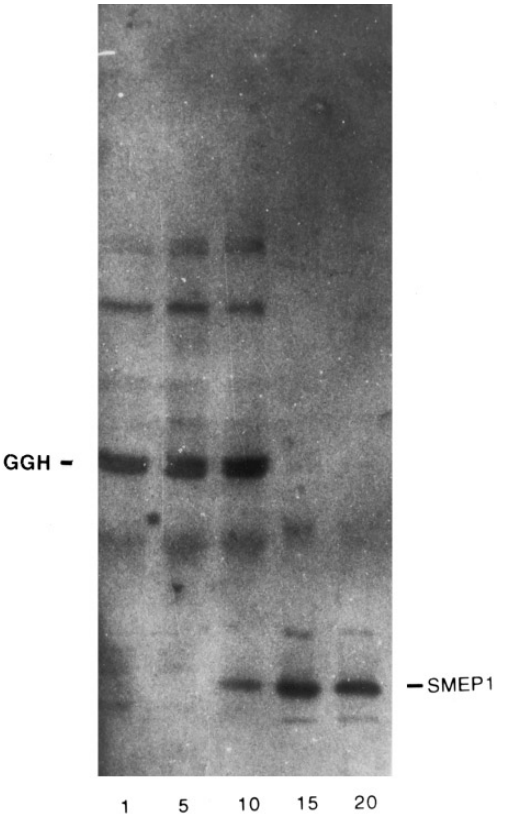


**FIG. 4.** Western analysis of cell wall bound polypeptides. One $\mu$g of a 0.2-0.5M wash of cell walls was subjected to SDS–PAGE, transferred and probed with anti-SMEP1 (1:3,000 dilution).

to determine if the polypeptide had any affinity for this cellular fraction in 1-10 d old leaves (Figure 4). These data show that at least a portion of GGH does bind strongly to cell walls as the protein is detectable in cell wall samples that were washed extensively with 0.2M $CaCl_2$ prior to extraction of tightly bound protein with 0.5M $CaCl_2$. We chose this method to minimize non-specific ionic interactions, and therefore those polypeptides remaining bound to cell walls during this stringent washing regime are likely to be cell wall associated *in vivo.*

Our investigation of the temporal expression of the soybean metalloendoproteinase (SMEP1) by western blot analysis consistently showed the presence of an cross-reactive 35 kD immunoreactive species that was detectable before the onset of SMEP1 expression (Pak et al., manuscript in preparation). To understand its biochemical nature, we undertook the isolation and characterization of this species. Molecular characterization to include chemical sequencing of internal peptides derived from the purified protein and cloning of the cDNA revealed that the protein we had suspected as possibly being related to SMEP1 instead represents $\gamma$-glutamyl hydrolase. It seems plausible that the cross-reactivity of the two unrelated proteins polypeptides to the same antisera may be explained by their sharing of a common immunogenic epitope, perhaps a structurally similar zinc binding site (23).

Information from the deduced amino acid sequence of the cDNA (10) showed that nascent $\gamma$-glutamyl hydrolase contains a typical hydrophobic signal peptide which we predict serves to direct export of the polypeptide to the extracellular compartment. Although GGH is thought to reside primarily in the lysosome, most of the GGH activity is secreted in neoplastic cells

(23). The enzyme is important in the dietary uptake of folate by the small intestine (14) and is involved in folylpolyglutamate turnover (16, 22) Additional studies are needed to establish the role of intracellular folylpolyglutamate metabolism and the function of GGH within the extracellular compartment of plants. Our data show that the expression of this protein is tightly regulated in a developmental program in that GGH mRNA and extractable are present only in young, rapidly growing tissue and the enzyme is quite possibly associated with the cell wall. The data contained in this report support the proposition that $\gamma$-glutamyl hydrolase may play an important role in growth processes. Indeed, further experimentation, including immunocytochemistry and the use of transgenic plants to examine the effects of inappropriate expression of GGH on cell growth are examples of future experiments. Although this research represents a preliminary study of an extracellular component, more studies of this type will provide a better foundation for plant scientists to better understand the complexity and importance of the extracellular matrix in plant biology. Furthermore, characterization of the enzyme may identify this as a potential target for an anti-folate herbicide.

## ACKNOWLEDGMENT

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