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Purification and Developmental Analysis of a Metalloendoproteinase from the Leaves of *Glycine max*[1]

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ABSTRACT

A metalloendoproteinase from leaves of soybean (*Glycine max*) has been purified 1160-fold to electrophoretic homogeneity. The native protein is monomeric with a molecular mass of 15 kilodaltons as estimated by gel filtration and 19 kilodaltons as estimated by denaturing gel electrophoresis. The enzyme has a pH optima of 8.0 to 9.0 using Azocoll as substrate. The proteolytic activity is susceptible to metal chelating agents and the inactivated enzyme can be restored to 69% of original activity by the addition of $ZnCl_2$. Western analysis shows that a fraction of the soybean metalloendoproteinase is present within the extracellular space of older leaves. Soybean metalloendoproteinase 1 is the Azocollase A activity first described by Ragster and Chrispeels (Plant Physiol 64: 857–862; 1979).

An understanding of proteolytic processes and the enzymes involved is paramount to a thorough knowledge of metabolism at both the cellular and tissue levels. Proteolytic enzymes are categorized into four classes based on catalytic mechanisms. These classes are serine, cysteine, aspartic acid, and metalloproteinases (1). Although the number of reports describing plant proteolytic activities has increased substantially over the last decade, few proteinases have been purified to homogeneity or studied in molecular detail (9, 15, 18). Most plant proteolytic activities examined appear to fall into the cysteine or aspartic acid classes. Those proteinases involved with the metabolism of seed storage proteins and senescence have received the most attention (8, 22). With the exception of a few reports (3, 19, 25), the literature contains little information on the presence of the metalloproteinase class of enzymes in plants. Indeed, to date, there have been no definitive reports of metalloproteinases in plants.

Type II metalloproteinases (EC 3.4.24) contain a zinc atom at the active site (24) and are widely distributed in prokaryotic and eukaryotic organisms. Thermolysin and collagenase are two representatives of this class (1). Ragster and Chrispeels (19) previously reported the presence of two Azocoll-digesting activities in soybean leaves. They demonstrated that these two activities differ in their susceptibility to EDTA and pCMB.[2] They referred to these activities, present in crude soybean leaf extracts, as Azocollase A (EDTA-sensitive) and Azocollase B (pCMB-sensitive) and suggested that these activities fall into the metallo and cysteine classes of proteinases, respectively. Their findings have aided us in further characterization of the Azocollase A activity, including the purification to homogeneity of this enzyme. We provide additional evidence that the enzyme is a metalloendoproteinase, which we have termed SMEP1. The proteinase is most abundant in maturing leaves and appears to be localized extracellularly. This is the first report of purification to homogeneity of a metalloendoproteinase from a plant.

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).

Enzymatic Assay

Determination of SMEP1 activity was accomplished by addition of 5 to 100 μL of protein sample to 1 mL of 25 mM Tris, pH 9.0, containing 1 mg (dry weight) of Azocoll (Calbiochem). The reaction mixtures were allowed to shake (250 rpm) for 1 h at 37°C in an orbital shaker. Mixtures contained a saturating amount of substrate and the amount of enzyme was adjusted so that rates of hydrolysis were linear with respect to time. Reaction mixtures were briefly centrifuged and the supernatant fluids monitored for an increase in absorbance at 520 nm. Units of activity are defined as Δ optical density 520 nm/h.

Protein Determination

Protein concentrations were determined by the Bio-Rad assay system (5) according to the manufacturer's procedures. BSA and bovine gamma globulin were used separately as standards. The values obtained were averaged and used for construction of a standard curve.

SMEP1 Isolation

Leaves (150 g) from 60-d-old plants were detached and rinsed in distilled water. All purification steps were performed at 4°C. Leaves were homogenized in a Waring blender with 3 L extraction buffer (25 mM K_2HPO_4, pH 7.5, 10 mM $Na_2S_2O_5$) followed by filtering through four layers of cheesecloth. The material retained by the cheesecloth was homogenized a

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[2] Abbreviations: pCMB, *p*-chloromercuribenzoic acid; SMEP1, soybean metalloendoproteinase 1; IWF, intercellular wash fluid.

second time in 2 L extraction buffer followed by filtration through cheesecloth. The extracts were combined, adjusted to pH 7.5, and centrifuged at 10,000*g* for 20 min. The clarified supernatant fluid was applied to a DEAE-Cellulose column (5 × 44 cm; Sigma Chemical Co.) equilibrated with 25 mM K_2HPO_4, pH 7.5, at a flow rate of 15 mL/min. SMEP1 activity contained in the flowthrough and wash fractions was precipitated by addition of solid ammonium sulfate to 60% saturation followed by stirring for 4 hr. The suspension was centrifuged at 10,000*g* for 20 min and the resulting pellet resuspended in 40 mL distilled water. Insoluble material was removed by centrifugation at 12,000*g* for 10 min. The supernatant fluid was loaded on a Bio-Gel P-60 column (5 × 144 cm; Bio-Rad) and equilibrated with water with a flow rate of 0.5 mL/min. Fractions (8 mL) containing Azocoll-digesting activity were pooled and applied to a hydroxylapatite column (1 × 10 cm; Bio-Rad) and equilibrated with water with a flow rate of 0.5 mL/min. After washing, proteins were eluted with 100 mL of a linear gradient of 0 to 200 mM Na_2HPO_4, pH 7.5 buffer. Fractions of 2 mL were collected and analyzed for SMEP1 activity. Fractions containing maximal activity were pooled and stored at −20°C. The enzyme activity is remarkably stable and can be stored frozen for at least 1 month with no appreciable loss of activity.

SDS-PAGE and Molecular Mass Determination

Denaturing gel electrophoresis (SDS-PAGE) was performed on 10% gels as described by Laemmli (10) and silver staining was carried out according to the methods employed by Morrissey (16). Molecular mass markers (Sigma Chemical Co.) run in parallel to samples were BSA (66 kD), egg albumin (45 kD), glyceraldehyde-3-phosphate dehydrogenase (36 kD), carbonic anhydrase (29 kD), trypsinogen (24 kD), trypsin inhibitor (20.1 kD), and α-lactalbumin (14.2 kD). Molecular mass markers (Sigma Chemical Co.) used for calibration of Bio-Gel P-60 (2.5 × 100 cm) gel filtration chromatography were pepsin (36 kD), carbonic anhydrase (29 kD), Cyt *c* (12.5 kD), and aprotinin (6.5 kD).

Amino Acid Analysis

Protein samples were hydrolyzed with either 6 N HCl at 110°C for 24 h or at 150°C for 1 h in sealed, evacuated tubes. Amino acids were analyzed by the method of Bidlingmeyer *et al.* (2) on an Applied Biosystems 420A derivatizer equipped with an on-line Applied Biosystems 130A separation system for the analysis of phenylthiocarbamyl-amino acids. Tryptophan and cysteine content were not determined.

Inhibitor Studies

Homogeneous preparations of SMEP1 (5 nM) in Azocoll assay buffer contained inhibitors at the concentrations indicated in Table III. Reactions were allowed to proceed for 1 h at 37°C and activity measured as stated above.

Reconstitution of SMEP1 Activity

Homogeneous preparations of SMEP1 (30 nM in 10 mM Na_2HPO_4) were inactivated by the addition of EDTA to 10 μM. After a 15 min incubation period at 4°C, Zn^{2+} or Ca^{2+} ions were added as their chloride salts over a 1000-fold concentration. The reconstituted samples were allowed to incubate for an additional 2 h at 4°C, followed by activity determination.

Localization of SMEP1 Activity

Proteinase activity directed toward Azocoll was determined in root, stem, seed, and leaf tissue. Plant material was frozen in liquid N_2 and ground to a fine powder. Samples were then extracted with 10 mL water/g of tissue (fresh weight) for 10 min. Extraction was performed in water because samples were adjusted for equal protein by lyophilization prior to loading onto SDS gels for Western analysis. Therefore, the adverse effect of high levels of salts on electrophoresis was minimized. We found no significant differences in enzyme recovery using water *versus* conventional buffers and no differential recovery of the enzyme between different tissues. After centrifugation at 12,000*g* for 10 min at 4°C, protein determinations were performed on the supernatant fluids. Leaf intercellular wash fluids were prepared as previously described (14) with the following modifications to the infiltration buffer. EDTA and PMSF were omitted and 10 mM Na_2HPO_4, pH 7.2, was added to the buffer. Protein extracts from leaves, after infiltration treatment, were prepared as described above. Samples were normalized for protein content and assayed in the presence and absence of pCMB or EDTA.

Antibody Production and Western Analysis

Antibodies to SMEP1 were raised in a rabbit with three subcutaneous injections of the purified SMEP1 (200 μg/injection) at 14 d intervals. The primary injection material was solubilized in Freund's complete adjuvant; subsequent injections were prepared in Freund's incomplete adjuvant. Serum was collected 14 d after the final booster by cardiac puncture. Partial purification of polyclonal antibodies was performed according to the method of Livingston (13). Western analysis was performed by the method of Towbin *et al.* (23). Denaturing gels were blotted onto nitrocellulose (MSI Laboratories) and probed with antiserum to SMEP1.

RESULTS

Purification of SMEP1

The proteinase activity directed against Azocoll was present in the unbound fraction obtained from DEAE-cellulose chromatography and represents a sixfold purification (Table I). This material was subjected to ammonium sulfate fractionation at 60% saturation, which resulted in a preparation purified 16-fold. The pellet was resuspended in H_2O and applied to a Bio-Gel P-60 gel filtration column to yield the elution profile shown in Figure 1A. This step separated the activity from the bulk of the protein. Fractions containing Azocoll-digesting activity were pooled and represented a 195-fold purified preparation. The pooled fractions were subjected to hydroxylapatite chromatography (Fig. 1B). This final purification step results in a preparation purified over 1100-fold and yielded 0.25 mg of protein with a recovery of 13%. Amino

Table I. *Purification of SMEP1 from Soybean Leaves*

Purification Step	Protein	Total Activity[a]	Recovery	Specific Activity[b]	Purification
	mg		%		*-fold*
Crude extract	2273	6682	100	3	1
DEAE-Cellulose	336	6048	91	18	6
$(NH_4)_2SO_4$	89	4139	62	47	16
Bio-Gel P-60	3.5	1988	30	568	195
Hydroxylapatite	0.25	849	13	3369	1160

[a] Activity is expressed as A_{520} units/1 h incubation with 1 mg/mL Azocoll in 25 mM Tris, pH 8.0, at 37°C. [b] A_{520} units/mg protein.

acid analysis of the polypeptide (Table II) provides for a calculated isoelectric point of 5.8 and a pH optimum of 8.0 to 9.0, which are in agreement with values reported by Ragster and Chrispeels (19).

Molecular Mass Determination

Silver-stained SDS-PAGE of the active hydroxylapatite eluate revealed a single band corresponding to a polypeptide of 19 kD (Fig 2A and B). This value is in agreement with the elution of SMEP1 on a calibrated Bio-Gel P-60 gel filtration column that shows a 15 kD species (Fig. 2C) and indicates that the protein exists as a monomer in its native state.

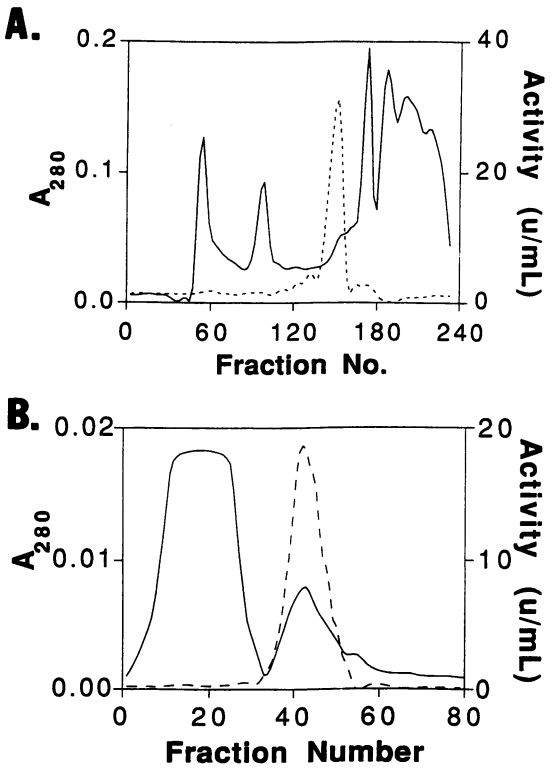

Figure 1. Fractionation of SMEP1 activity. A, Bio-Gel P-60 gel filtration chromatography of the preparation after ammonium sulfate precipitation. Protein, A_{280} (—); proteinase activity, units/mL (– – –); 8 mL fractions were collected. B, Hydroxylapatite chromatography of Bio-Gel P-60 material. Protein, A_{280} (—); proteinase activity, units/mL (– – –); 5 mL fractions were collected.

Determination of Mechanistic Class

The mechanistic class of the proteolytic activity was determined by treatment with inhibitors diagnostic for the different classes of proteolytic enzymes. The data in Table III reveal that the enzyme is inhibited by chelators such as EDTA, EGTA, and 1,10-phenanthroline, but not by inhibitors of serine, cysteine, or aspartyl proteinases. The enzymatic activity is also inhibited by thiols and CN^-, which bind metal ions and cause inhibition of metal-requiring enzymes (24). Further confirmation of the metal-requiring nature of this enzyme is provided by the observation that enzymatic activity can be restored to 69% of initial activity when the EDTA-inactivated enzyme is titrated with Zn^{2+} (Fig. 3). Concentrations of Zn^{2+} higher than 20 mM inhibit restoration of activity. This phenomenon has also been observed with other metalloproteinases (1). The data in Figure 3 also demonstrate that Ca^{2+} is capable of partially restoring proteolytic activity (48%), albeit

Table II. *Amino Acid Analysis of Purified SMEP1*

Amino acid analysis was performed as described in "Materials and Methods." The number of residues per molecule are based on the average of the two different hydrolysis conditions (see "Materials and Methods") and a molecular mass value of 19 kD.

Amino Acid	Residues per Molecule	Mole Percent
Asx	19.3	11.3
Glx	10.1	5.9
Ser	10.7	6.3
Gly	12.8	7.5
His	5.3	3.1
Arg	8.2	4.8
Thr	12.6	7.3
Ala	15.9	9.3
Pro	11.2	6.5
Tyr	7.1	4.1
Val	7.3	4.3
Met	1.6	0.9
Ile	9.8	5.8
Leu	10.4	6.1
Phe	10.2	6.0
Lys	6.3	3.7

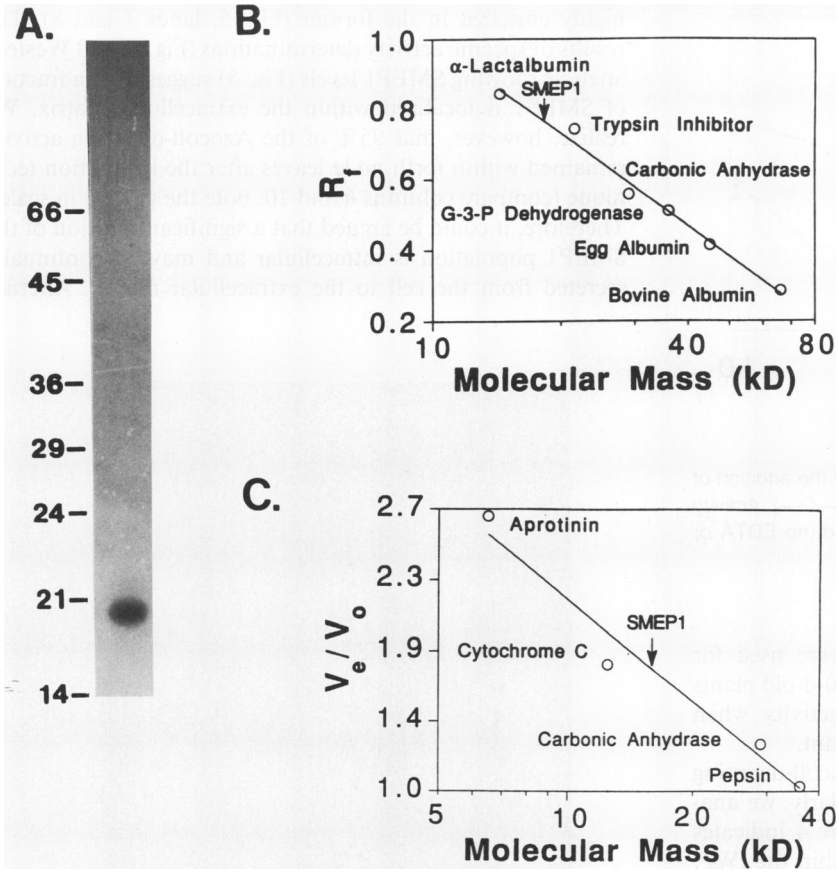

Figure 2. Determination of molecular mass of SMEP1 by SDS-PAGE and Bio-Gel P-60 gel filtration chromatography. A, Silver stained SDS-PAGE of purified (2 μg) SMEP1. Molecular mass markers are shown in left margin. B, Plot of data shown in A. C, Determination of native molecular mass by Bio-Gel P-60 gel chromatography. Size of molecular mass markers are listed in "Materials and Methods."

Table III. *Effects of Proteinase Inhibitors and Reducing Agents on the Azocoll-Digesting Activity of SMEP1*

Inhibitor	Concentration	Relative Activity
	mM	%[a]
None (control)	0.0	100
PMSF	10.0	136
Leupeptin	0.5	108
Benzamidine	10.0	97
Aprotinin	1.0[b]	90
pCMB	1.0	103
E-64[c]	0.02	74
Iodoacetamide	1.0	74
Iodoacetic acid	1.0	90
N-Ethylmaleimide	1.0	87
Cystatin	0.01	90
Pepstatin	5.0[d]	77
DANM[e]	1.0	101
EDTA	1.0	3
EGTA	1.0	2
1,10-Phenanthroline	1.0	1
Cysteine	1.0	10
DTT	1.0	62
KCN	1.0	6

[a] Relative activity to control; the protease activity is expressed as A_{520} units/1 h incubation with 1 mg/mL Azocoll in 25 mM Tris, pH 8.0, at 37°C. [b] Trypsin inhibitor units. [c] E-64 = *trans*-epoxysuccinyl-L-leucylamido (4-guanidine) butane. [d] μg/mL. [e] DANM = diazoacetyl DL-norleucine methyl ester.

at a 100-fold higher concentration than required for Zn^{2+}. Taken together, these data strongly suggest that SMEP1 be classified as a metalloendoproteinase.

Localization of SMEP1 Activity

We next sought to correlate the presence of EDTA-sensitive Azocoll-digesting activity in tissue extracts to the presence of SMEP1. Figure 4 shows Azocoll-digesting specific activity in various tissues of a 37-d-old soybean plant with eight trifoliate leaves. The highest specific activity is present in the leaves of the plant with stem, root, and dry seed tissue (commercially obtained) showing negligible activity. Closer examination of leaves shows that the highest specific activity is present in the primary leaves and decreases as the stage of leaf maturity decreases. The activity in older leaves is highly sensitive to EDTA as opposed to younger leaves, in which an appreciable portion of the Azocoll-digesting activity is EDTA insensitive, suggesting the presence of another activity distinct from SMEP1. This other activity probably represents Azocollase B, described by Ragster and Chrispeels (19). Azocoll-digesting activity in leaves of different developmental stages was correlated with the presence of SMEP1 by Western blot analysis of the leaf preparations used in Figure 4. As shown in Figure 5, relative levels of SMEP1, as a function of leaf position, reflect the activity measurements presented in Figure 4.

It should be noted that the specific activities of the lower leaves are much larger than those observed in the crude extracts for purification (Table I). The reason for this differ-

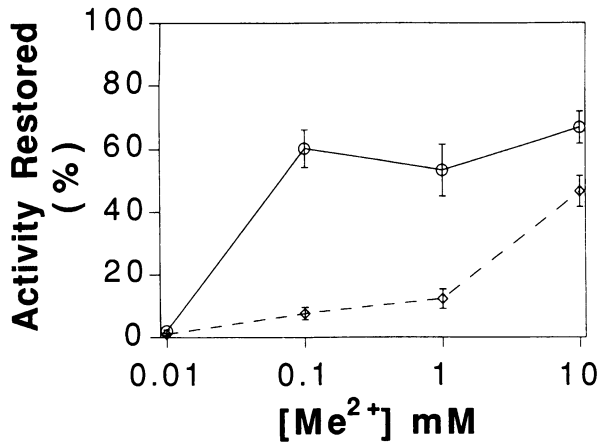

Figure 3. Restoration of Azocoll-digesting activity by the addition of the metals (Me^{2+}) Zn^{2+} or Ca^{2+}. Zn^{2+}, (—○—); Ca^{2+}, (—◇—). Activity restored represents activity compared with untreated (no EDTA or Me^{2+} addition) controls.

ence is that all of the leaves of the plant were used for purification purposes. Moreover, the leaves of 60-d-old plants (used for purification) contain less SMEP1 activity when compared with the same leaves of a 37-d-old plant.

As an initial step to determine if the Azocoll-digesting activity is localized intracellularly or extracellularly, we analyzed IWF of forth node trifoliate leaves. Figure 4 indicates that the specific activity of SMEP1, present within the IWF, is 50-fold higher than that observed in intact leaves and is completely inhibited in the presence of 3 mM EDTA (compare columns 6 and 9). A qualitative determination of the relative abundance of SMEP1 in the IWF is approximately 50-fold higher than that observed in intact leaf extracts (Fig. 5, lanes 4 and 7). Moreover, when equal protein from IWF and fourth node leaf material after treatment are examined, SMEP1 is highly enriched in the former (Fig. 5, lanes 7 and 8). The results of specific activity determinations (Fig. 4) and Western analysis showing SMEP1 levels (Fig. 5) suggest that a fraction of SMEP1 is localized within the extracellular matrix. We realize, however, that 95% of the Azocoll-digesting activity remained within forth node leaves after the infiltration technique (compare columns 6 and 10; note the change in scale). Therefore, it could be argued that a significant portion of the SMEP1 population is intracellular and may be continually secreted from the cell to the extracellular matrix. Alterna-

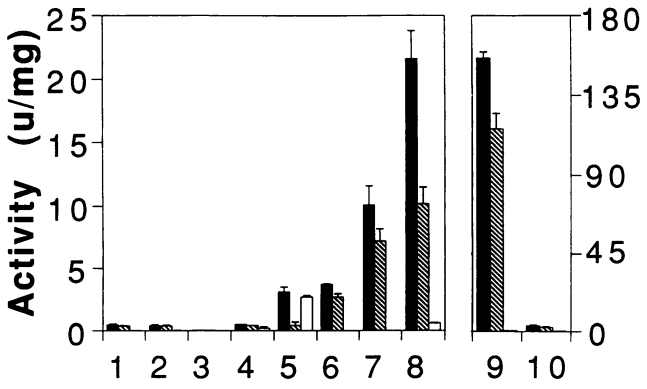

Figure 4. Localization of SMEP1 activity. Azocoll-digesting specific activity in the presence and absence of EDTA or pCMB was determined in total soluble protein extracts from root, stem, seed, leaves, and IWF. 1, root; 2, stem; 3, seed; 4, 8th node leaves; 5, 6th node leaves; 6, 4th node leaves; 7, 2nd node leaves; 8, primary leaves; 9, IWF from 4th node leaves; 10, 4th node leaves after removal of IWF. Control (■), pCMB (▨), EDTA (□). Error bars represent the standard error of the mean (± SE).

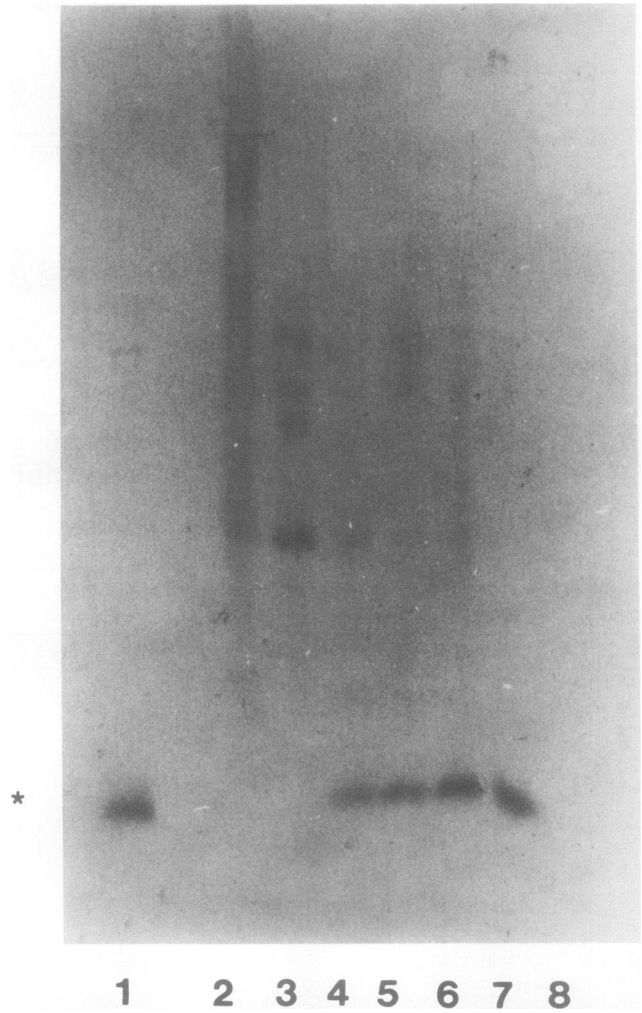

Figure 5. Western blot analysis of SMEP1. Total soluble proteins of leaf extracts were prepared for analysis from leaves of a 37-d-old plant. Proteins from each sample were electrophoresed on 10% SDS-polyacrylamide gels, transferred to nitrocellulose, and probed with 1:1000 dilution of anti-SMEP1 antisera. Visualization of antibody/antigen complexes was achieved by treating blots (1–5 min) with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (Promega). Lane 1, 30 ng of SMEP1; lane 2, 10 μg of 8th node leaf extract; lane 3, 10 μg of 6th node leaf extract; lane 4, 10 μg of 4th node leaf extract; lane 5, 10 μg of 2nd node leaf extract; lane 6, 10 μg of primary leaf extract; lane 7, 1 μg of IWF from 4th node leaves; lane 8, 1 μg of 4th node leaves after removal of IWF. Asterisk denotes position of SMEP1.

tively, a more trivial explanation is that the procedure does not quantitively extract total extracellular proteins.

It is noted that the polyclonal antibody recognizes several polypeptides in addition to SMEP1 in the blot of Figure 5. These bands are prevalent in younger leaves of the plant (lanes 2 and 3) and tend to decrease in intensity with increased leaf age. This may represent nonspecific binding, but we have not eliminated the possibility of a precursor/product relationship. In any case, the interpretation of the data in Figure 5 is unaffected by the presence of the larger cross-reacting proteins.

These data confirm that the levels of SMEP1 are primarily responsible for the observed pattern of Azocoll-digesting activity in developing soybean leaves and that a fraction of SMEP1 is localized extracellularly. Further experimentation is required to firmly establish the distribution of SMEP1 in leaf tissues.

DISCUSSION

We have shown that the EDTA-sensitive proteinase activity within soybean leaves is a 19-kD polypeptide by SDS-PAGE and a 15-kD species by gel filtration chromatography. These data suggest that the protein exists in a monomeric configuration. Hydroxylapatite column chromatography required that the sample be dissolved in H_2O prior to loading. For this reason, we ran the Bio-Gel P-60 column in H_2O to circumvent a dialysis step and provide a sample that could immediately be applied to the hydroxylapatite column. Many of our attempts to purify SMEP1 from plants reared under greenhouse conditions resulted in a preparation containing a major contaminant of 21 kD. The biochemical properties of the contaminant were such that separation from SMEP1 was extremely difficult. We were able to purify SMEP1 to homogeneity only when the 21-kD polypeptide was not expressed. This required using plants showing no signs of disease or environmental stress. We have characterized the 21-kD polypeptide (J.S. Graham, W. Burkhart, J. Xiong, J.W. Gillikin, submitted for publication) and found its amino acid sequence highly homologous to the stress-induced osmotin from tobacco (21).

Based on the sensitivity of SMEP1 to chelating agents, DTT, cysteine, and KCN, the restoration of SMEP1 activity by addition of Zn^{2+}, and an alkaline pH optimum, we feel these data support the previous suggestion by Ragster and Chrispeels (19) that this enzyme be classified as a metalloendoproteinase (20). Our data (Fig. 3) show that Ca^{2+} may play a role in SMEP1 activity, possibly at the level of stabilization or activation. However, addition of both Ca^{2+} and Zn^{2+} did not show increased restoration of activity compared with addition of Zn^{2+} alone. We are planning additional experiments to determine the role Ca^{2+} may perform because the ion has been implicated as an effector in previously studied metalloendoproteinases (1). Calcium has also been shown to be an absolute requirement for the calpains, a class of proteinases that have been observed in animal and fungal systems. These enzymes have yet to be described in higher plants (17).

Our findings show that SMEP1 accumulates in leaves as a function of leaf age. There exists a gradient of SMEP1 accumulation such that older (lower) leaves contain the greatest amount of the enzyme and younger (top) leaves contain the least. We have found that SMEP1 begins to accumulate in any leaf approximately 10 to 14 d after leaf emergence (data not shown). Western analysis confirms that the EDTA-sensitive Azocoll-digesting activity is largely, if not totally, represented by the SMEP1 enzyme. We are currently conducting further experiments using Western blot analysis to study the expression of this enzyme in response to environmental stress and as a course of normal development.

It should be noted that Bond and Bowles (3) reported on the presence of a metalloendoproteinase activity in fully mature soybean seed based on sensitivity to chelating agents. We were not able to detect any appreciable Azocoll-digesting activity in our specific activity measurements of Figure 4 (lane 3) and were unable to detect SMEP1 by Western analysis of seed extracts (data not shown). The low specific activity may be, as suggested in the report of Bond and Bowles, due to the presence of high levels of endogenous protein that might lead to a misinterpretation of the results presented in Figure 4. Also, the inability to detect SMEP1 in seed tissue by Western analysis (data not shown) may be the result of an insufficient amount of protein loaded onto gels. We certainly plan to examine, in more detail, whether SMEP1 is the metalloendoproteinase activity detected by Bond and Bowles (3).

SMEP1 appears to be an extracellular protein in soybean leaves because of its presence in IWF. This result is consistent with the findings of Van der Wilden *et al.* (25) describing a partially EDTA-sensitive activity in extracellular fluids from bean (*Phaseolus vulgaris*) leaves. Several clear distinctions can be made between SMEP1 and the bean enzyme. First, SMEP1 is completely inactivated at 1 mM EDTA and insensitive to pCMB. Second, SMEP1 exhibits a molecular mass of approximately 15 to 19 kD (Fig. 2) compared with 37 kD for the bean enzyme. Last, SMEP1 shows a different temporal expression in soybean leaves, as it is present in highest levels in mature (15 d) as opposed to immature (4 d) leaves in bean.

Van der Wilden *et al.* (25) speculated that the bean proteinase may be localized in cell walls and may possibly be involved in the metabolism of cell wall proteins. We find this speculation appealing in light of the growing list of proteins, such as hydroxyproline-rich proteins, proline-rich proteins, glycine-rich proteins, pathogenesis-related proteins, peroxidases, phosphatases, and glycosidases, residing outside the plasma membrane (4, 6, 11, 12, 26). However, the role played by proteolytic enzymes in this dynamic compartment is lacking. We would expect that the presence of extracellular proteolytic enzymes is a requirement for the proper functioning of the leaf. It is tempting to speculate that SMEP1 may be involved in tissue modeling, which must occur during leaf expansion. It is believed that the formation of intercellular spaces in the mature leaf requires the partial separation of cells following wall breakdown (7). We find it interesting that the accumulation of SMEP1 occurs during late stages of the expansion of the soybean leaf and thus may play a role in this process. Alternatively, the enzyme may play a defensive role in plant leaves.

We have obtained partial amino acid sequence information by chemically sequencing tryptic fragments of SMEP1. Partial sequence information reveals strong amino acid identity with the Zn^{2+} binding domain of type II metalloendoproteinases

(J. McGeehan, W. Burkhart, J.W. Gillikin, J.S. Graham, manuscript in preparation). We are using the amino acid sequence information to clone the SMEP1 gene, and immunocytochemistry to confirm the location of the gene product. Moreover, we are assaying purified endogenous leaf proteins as potential *in vivo* substrate(s) of the enzyme. This work should provide additional information about the regulation of this gene and possible insights in obtaining an understanding of the importance of SMEP1 in the physiology of the soybean leaf.

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