

# Peptidohydrolases of Soybean Root Nodules<sup>1,2</sup>

## IDENTIFICATION, SEPARATION, AND PARTIAL CHARACTERIZATION OF ENZYMES FROM BACTEROID-FREE EXTRACTS

Received for publication September 29, 1980 and in revised form February 10, 1981

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### ABSTRACT

Nodule extracts prepared from *Glycine max* var Woodworth possessed endopeptidase, aminopeptidase, and carboxypeptidase activities. Three distinct endopeptidase activities could be resolved by disc-gel electrophoresis at pH 8.8. According to their order of increasing electrophoretic mobility, the first of these enzymes hydrolyzed azocasein and *n*-benzoyl-L-Leu- $\beta$ -naphthylamide, while the second hydrolyzed *n*-benzoyl-L-Arg- $\beta$ -naphthylamine (Bz-L-Arg- $\beta$ NA), *n*-benzoyl-L-Arg-*p*-nitroanilide (Bz-L-Arg-*p*NA), and azocasein. The third endopeptidase hydrolyzed Bz-L-Arg- $\beta$ NA, Bz-L-Arg-*p*NA, and hemoglobin. Fractions of these enzymes extracted from electrophoresis gels were shown to have pH optima from 7.5 to 9.8. All of the endopeptidases were completely inhibited by diisopropylphosphorofluoridate, demonstrating that they were serine proteases.

Aminopeptidase activity was measured using amino acyl- $\beta$ -naphthylamides. Electrophoresis of nodule extracts at pH 6.8 resolved the aminopeptidase activity of nodule extracts into at least four fractions based on mobility and on activities toward amino acyl- $\beta$ -naphthylamides. The major activity of two of the aminopeptidases was directed toward L-Leu- and L-Met- $\beta$ -naphthylamide, while the other two aminopeptidases exhibited broader specificity and were capable of hydrolyzing a large number of amino acyl- $\beta$ -naphthylamides. Two of the aminopeptidases extracted from electrophoresis gels were classified as thiol type enzymes, and all four aminopeptidases had neutral to basic pH optima.

Senescence of soybean root nodules results in a decreased capacity to reduce atmospheric nitrogen, thus potentially limiting the supply of amino nitrogen to the plant. After the decline in nitrogenase activity, total soluble protein per unit fresh weight of nodule decreases rapidly (22), although the fresh weight per nodule shows little change. In addition, the total number of nodules per plant decreases sharply. Thus, nodule senescence can be characterized by a sharp decline in nitrogenase activity coupled with a degeneration of the nodule leading to loss of soluble protein and eventual abscission of the nodule from the root. The loss of soluble protein is a characteristic feature of all types of plant senescence, and is generally attributed to increased activity of proteolytic enzymes. The role of proteolytic enzymes in nodule senescence is not as well established as in leaf senescence (7, 8, 19,

35), although proteolytic activity in alfalfa nodules has been shown to increase as nitrogenase activity and soluble proteins are lost after harvest (32).

Studies with animal tissues have demonstrated that many proteolytic enzymes have extremely specialized regulatory roles in cellular processes such as translational polypeptide processing (14), hormone regulation (24), and blood coagulation (6). However, such specialized roles have not been elucidated in plant cells. In most cases, only total proteolytic activity, or activity of a single protease, has been correlated to a physiological change such as plant senescence. Differences in substrate specificity and in catalytic mechanism may reflect functional differences between the various proteolytic enzymes. Since individual proteases probably have distinct roles within the overall process of senescence, the characterization of these enzymes is important in understanding their role in senescence. The purpose of this investigation was to identify and characterize as many of the enzymes responsible for total proteolytic activity in soybean root nodules as possible, in order to investigate their roles in nodule senescence. Proteolytic enzymes unique to the bacteroid fraction will be characterized in a future report.

### MATERIALS AND METHODS

**Special Chemicals.** Ammonium persulfate and TEMED<sup>4</sup> were purchased from Bio-Rad Laboratories. Arcillite was obtained from IMC Chemical Corp. Hemoglobin substrate powder was the product of Worthington Biochemicals. All special reagents, chemicals, and substrates were obtained from Sigma. Acrylamide and bisacrylamide were recrystallized (18) prior to use, and other reagents were used without further purification.

**Seed Inoculation and Plant Growth.** Inoculum was prepared by growing *Rhizobium japonicum* (strain 311b110) in L-arabinose broth (33) for 5 to 7 days at 30 C with constant shaking. Soybean seeds (*Glycine max* var Woodworth) were immersed in inoculum for 30 min, then planted in Arcillite in 6-inch clay pots that had been sterilized for 1 h at 240 C. Four plants were grown in each pot. An additional 20 ml bacterial culture were added to the soil in each pot to ensure adequate nodulation. Plants were grown in a temperature-controlled greenhouse without artificial light from March to July of 1979, and supplied frequently with a nitrogen-free nutrient solution (9). Nodules were harvested during the pod-filling stage of plant development and were stored at -70 C until used.

**Bacteroid-Free Extracts.** Four g nodules were macerated in an ice-cold mortar and pestle with 5 ml 25 mM phosphate buffer (pH

<sup>1</sup> This work was supported by United States Department of Agriculture, Science and Education Administration-CRGO grant 509-0410-9-0233 and by a grant from the Nebraska Soybean Development, Utilization and Marketing Board.

<sup>2</sup> Published as Paper No. 6089, Journal Series, Nebraska Agricultural Experiment Station.

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<sup>4</sup> Abbreviations: TEMED, *N,N,N',N'*-tetramethylethylenediamine; DTE, dithioerythritol; Bz-, *n*- $\alpha$ -benzoyl-; -*p*NA, -*p*-nitroanilide; - $\beta$ NA, - $\beta$ -naphthylamide; Z-, benzoyloxycarbonyl-; PCMB, *p*-chloromercuribenzoate; DFP, diisopropylphosphorofluoridate; TEA, triethanolamine.

6.7–7.0). When extracts were prepared to assay aminopeptidases, buffers were made 1 mM with DTE. The homogenate was centrifuged at 12,000g for 15 min at 4 C. The supernatant (nodule extract) was assumed to contain soluble peptidohydrolases of legume origin, including soluble organelle proteins, because the bacteroids were found in the pellet, and they required passage through a French Press at 20,000 p.s.i. for adequate lysis. In addition, preliminary work with the proteolytic enzymes found in the bacteroids has indicated that they are present at much lower activity, so slight contamination with bacteroid enzymes would not be detectable in the nodule extracts (data not shown). The pelleted membranes were extracted with the nonionic detergent (1-*O*-*n*-octyl- $\beta$ -D-glucopyranoside, and additional peptidohydrolases were not identified (data not shown). The nodule extract was either used immediately or stored at -70 C. Preliminary experiments showed no loss of peptidohydrolase activities when nodules or extracts were stored up to 6 months under these conditions.

**Endopeptidase Assays.** Substrates used to determine endopeptidase activity included azocasein, hemoglobin, Bz-L-aminoacyl-pNAs (4), and Bz-L-aminoacyl- $\beta$ NAs (3).

Endopeptidase assays employing azocasein as the substrate (5, 25) were performed with the following modifications. Three ml freshly prepared substrate (5 mg/ml in 25 mM Na-phosphate [pH 7.0]) were allowed to react with 1 ml nodule extract or with 1 ml purified enzyme solution. After 1 h (nodule extract) or 18 h (purified enzyme) of incubation at 37 C, 0.75-ml aliquots of the reaction mixture were added to 3.0 ml 10% TCA, then heated for 5 min at 80 C. TCA was substituted for HClO<sub>4</sub> and the samples were heated to facilitate precipitation of the legume proteins in the nodule extract. After the solution had cooled, it was filtered through Whatman No. 1 paper and 2.5 ml 1.0 N NaOH was added to 2.5 ml filtrate, then the *A* was measured at 440 nm using a zero time sample as the reagent blank. One unit endopeptidase activity was the amount of enzyme required to produce a change in *A* of 1.0 in 1 h under the conditions of the assay.

Hydrolysis of hemoglobin (1.25 g/100 ml in either 0.1 M Na-citrate or 0.1 M Na-phosphate buffer at an appropriate pH) was measured by incubating 1.25 ml hemoglobin with 0.75 ml protein extracted from the slab gel for 18 h at 37 C. The reaction was terminated with 1 ml 10% TCA, allowed to precipitate for 15 min, and then filtered through Whatman No. 1 filter paper. The *A* of the filtrate was read at 280 nm. One unit activity was defined as the amount of enzyme required to increase the *A* at 280 nm by 1.0 in 1 h. In all assays employing either hemoglobin or azocasein, controls for spontaneous hydrolysis of substrate or for hydrolysis by microbial contaminants were performed by substituting an equivalent volume of extraction buffer for enzyme in the substrate incubation mixture. Controls were incubated for identical time periods. Under the conditions employed here, none of these controls possessed measurable levels of hydrolysis. When gel segments were extracted and assayed using either azocasein or hemoglobin, prolonged incubation times (15–18 h) were employed. For these assays, gel segments were extracted with freshly prepared buffers and then assayed immediately using freshly prepared substrate. Only gel segments containing proteases showed detectable activity, precluding the possibility of appreciable bacterial contamination. Finally, assays with protein substrates performed longer than 1 h were interpreted only qualitatively.

Hydrolysis of the synthetic substrate Bz-L-Arg-pNA was monitored spectrophotometrically as the increase in *A* at 380 nm. For the reaction, 10  $\mu$ l purified enzyme extract were added to a solution containing 0.1 ml 1 mM substrate and 0.89 ml Na-phosphate buffer (0.1 M) of the desired pH. Absorbance was recorded for 5 to 20 min at 25 C. One unit activity was defined as the amount of enzyme required to form 1  $\mu$ mol *p*-nitroaniline/h under the assay conditions. When Bz-L-Arg- $\beta$ NA was the substrate, the reaction was quantitated by measuring the liberated  $\beta$ -naphthylamine.

Substrate (0.5 ml 0.2 mg/ml in 50 mM Na-phosphate buffer) was added to 0.5 ml diluted cytosol. The mixture was incubated for 30 min at 25 C, then 0.5 ml 40% TCA were added. Zero time samples and substrate blanks were used as controls. The liberated  $\beta$ -naphthylamine was diazotized and quantitated by the method of Goldbarg and Rutenburg (12) as described by Blackwood and Mandl (3). One unit of activity was the amount of enzyme required to liberate 1  $\mu$ mol product/h under the conditions of the assay.

**Aminopeptidase Assays.** Aminopeptidase activity was measured using various L-amino acyl- $\beta$ NAs by the same procedure employed to measure endopeptidase activity using Bz-L-Arg- $\beta$ NA as the substrate (12).

**Carboxypeptidase Assay.** The presence of carboxypeptidase activity in nodule extract was detected by incubating 1 ml 10 mM Z-Gly-DL-Phe in 25 mM Na-phosphate (pH 7.0), with an equal volume of bacteroid-free extract at 37 C for 2 h. The reaction was terminated by adding 1 ml 24% TCA then centrifuged to remove precipitated proteins. Hydrolysis of the substrate liberated Phe, which was detected in the supernatant fluid by amino acid analysis (Hitachi Perkin Elmer, model KLA-3B amino acid analyzer). The resulting from autolysis of cytosolic proteins was determined by incubation of an equal volume of cytosol in 25 mM Na-phosphate (pH 7.0). One unit carboxypeptidase activity was defined as the amount of enzyme required to liberate 1  $\mu$ mol Phe from Z-Gly-DL-Phe/h under conditions of the assay.

In all assays synthetic substrates were incubated without enzymes under experimental conditions to determine levels of spontaneous hydrolysis. As expected, nonenzymatic hydrolysis was detectable only with the substrate Bz-L-Arg-pNA at pH values above 9.5.

**Electrophoresis.** Discontinuous polyacrylamide gel electrophoresis was performed either in cylindrical gels (0.3  $\times$  9 cm) or slab gels (14  $\times$  8  $\times$  0.5 cm). Different buffer systems were employed depending on whether separation and characterization of aminopeptidases or endopeptidases were desired.

Endopeptidase activities were separated by acrylamide gel electrophoresis by the method of Laemmli and Favre (16) except SDS was not used. Gels contained 7.5% acrylamide and 0.2% bisacrylamide in 0.375 M Tris-HCl (pH 8.8). The reservoir buffer was 0.025 M Tris-glycine (pH 8.3).

Aminopeptidase activities were separated in 9% acrylamide, 0.1% bisacrylamide gels using the method of Orr *et al.* (23), except that EDTA was omitted. The gel buffer was 0.0625 M HCl-TEA (pH 6.8). The anodic buffer was 0.038 M Tes-TEA (pH 6.3) and the cathodic buffer was 0.034 M H<sub>3</sub>PO<sub>4</sub>-TEA buffer (pH 5.8) (23).

Up to 50  $\mu$ l nodule extract in 10% sucrose were applied at the top of each disc gel. A current of 1.25 mamp per tube was used during stacking and was raised to 2.0 mamp per tube when the dye front entered the separating gel. Electrophoresis was terminated when the dye front had migrated 6 cm into the separating gel.

Either endopeptidases or aminopeptidases could be assayed in acrylamide gels after electrophoresis by qualitatively measuring the products,  $\beta$ -naphthylamine or *p*-nitroaniline, released from synthetic substrates (17, 30). When amino blocked L-aminoacyl-pNAs or L-aminoacyl-pNAs were used as substrates, they were dissolved to 1 mM in distilled water. Peptidase activity could be located within 15 min as the appearance of yellow bands due to the production of *p*-nitroaniline. Gels were scanned as soon as the bands were evident as the product diffused rapidly and the gels could not be stored. When  $\beta$ -naphthylamide derivatives of amino acids were used as substrates the gels were incubated in a solution of 0.04% substrate for 20 min, then transferred to a 0.1% solution of Fast Garnet GBC salt dissolved in water. The diazotized product of Fast Garnet GBC salt and  $\beta$ -naphthylamine was an insoluble red precipitate which did not diffuse from the gel. Using this technique peptidases were detected as dark red bands which

were stable for several days in water.

**Isolation of Purified Peptidases.** Preparative electrophoresis was performed in slab gels ( $14 \times 8 \times 0.5$  cm) of the same composition and pH as above for the separation and extraction of either aminopeptidases or endopeptidases. Nodule extract (2.5 ml, 20 mg protein/ml) in 10% sucrose was layered between the top of the slab gel and the buffer. Electrophoresis was performed at 50 mamp until the dye front entered the separating gel, then at 80 mamp until the tracking dye band migrated 6 cm. Five-mm-thick vertical strips from either side of preparative slabs were removed and stained for enzyme activity. Horizontal strips corresponding to enzyme bands were removed from the remaining gel and separately sliced into small pieces, then incubated overnight in 25 mM phosphate buffer (pH 7.0) at 4 C to extract the enzymes from the acrylamide gel. Peptidohydrolases partially purified in this manner were used to characterize each enzyme activity.

**Inhibition Experiments.** A few organic molecules known to inhibit certain classes of hydrolases (34) were tested on the purified nodule peptidohydrolases separated by slab gel electrophoresis. PCMB was prepared fresh daily in concentrations of 0.1 mM and 0.01 mM in 25 mM Na-phosphate buffer (pH 7.0). The reagent was diluted 2-fold with enzyme and the resulting solution was incubated at 25 C for 1 h prior to being assayed. 1,10-Phenanthroline (20 mM and 2 mM) was dissolved in 25 mM Na-phosphate (pH 7.0). Solutions were diluted-2 fold with enzyme and incubated 18 h at 4 C prior to being assayed. DFP was diluted to 10 mM or 2 mM in 50 mM Na-phosphate, 20 mM Cys (pH 7.0), and preincubated for 30 min at ambient temperature to reduce oxidants in DFP known to be inhibitory to enzymes (13). DFP solutions were then diluted 2-fold with enzyme solutions and incubated for 1 h at 25 C before assay. After being treated with inhibitors, enzyme solutions were diluted to an appropriate concentration with 0.1 M Na-phosphate having the pH of the test substrate solution.

**Isoelectric Focusing.** Isoelectric focusing was performed in 5% acrylamide gels (37) using a pH gradient of 3.5 to 10 for the purified enzyme fraction and from 4 to 6 for the nodule extract. The enzyme was detected by the staining procedure described above. The pH gradient of the gel was determined using a Bio-Rad micro pH electrode.

**Protein Determination.** Protein concentrations were determined by a modification of the Lowry procedure (2).

## RESULTS AND DISCUSSION

Bacteroid-free extracts of soybean root nodules possess activities toward substrates specific for all three classes of peptidohydrolases; *i.e.* endopeptidases, aminopeptidases, and carboxypeptidases (Table I). Endopeptidase activity was greatest toward the synthetic substrate Bz-L-Arg- $\beta$ NA. However, since endopeptidases which cannot hydrolyze this substrate may also be present in nodule extract, intact proteins were also used as substrates. Activity levels toward azocasein and Bz-L-Arg- $\beta$ NA may or may not reflect distinct endopeptidases. Inasmuch as pure proteases of known specificity hydrolyze intact proteins in numerous positions and since a protein offers many potentially susceptible bonds, activity levels toward azocasein or other proteins can only be given in arbitrary units; they can only indirectly reflect the number of susceptible bonds being hydrolyzed. Nodule extract also hydrolyzed a number of aminopeptidase substrates (Table I). Since aminopeptidases often have broad specificities, these data alone cannot be interpreted to infer the presence of multiple enzymes. Carboxypeptidase activity (0.5 unit/g fresh weight) was detected in the crude cytosol using Z-Gly-DL-Phe and was quantitated by amino acid analysis. Neither direct nor indirect spectrophotometric methods could be used to quantitate Phe in the cytosol substrate mixture due to strongly interfering substances in the crude cytosol and low levels of activity. Consequently, characterization of cytosolic extracts were restricted to endopeptidases and aminopep-

Table I. Activities of Peptidohydrolases in Nodule Extracts

All assays were performed as described under "Materials and Methods" The supernatant fraction of nodule extract was examined without purification, and also after removal of small molecular weight compounds by gel filtration through Sephadex G-25.

Substrates	Bond Broken	Specific Activity <sup>a</sup>	
		Crude extract	G-25 eluant
<i>units/g fresh wt nodule</i>			
Endopeptidase substrates			
Azocasein	Internal peptide	0.10	0.13
Benzoyl-Arg- $\beta$ NA	Internal peptide	1.70	1.49
Aminopeptidase substrates			
L-Leu- $\beta$ NA	Amino-terminal peptide	9.20	9.78
L-Ala- $\beta$ NA	Amino-terminal peptide	1.71	3.80
L-Val- $\beta$ NA	Amino-terminal peptide	0.03	0.04
Carboxypeptidase substrates			
Z-gly-DL-phe	Carboxy-terminal peptide	0.50	(not done)

<sup>a</sup> One unit of activity toward azocasein is expressed as the amount of enzyme which produced a  $\Delta A_{440}$  of 1.0/h. One unit of activity toward all the other substrates is defined as the number of  $\mu$ mol substrate cleaved/h.

tidases. Removal of small molecules and ions from the protein fraction of crude extracts by chromatography in Sephadex G-25 doubled the activity in the protein fraction toward L-Ala- $\beta$ NA, but had no effect on any other activities measured.

Endopeptidase activity in the nodule extract could be detected from pH 5.5 to pH 11 using both azocasein and Bz-L-Arg- $\beta$ NA as substrates. Aminopeptidase activity was measurable from pH 5 to pH 10. These broad pH ranges for both endopeptidase and aminopeptidase activities in the nodule extract suggested the presence of multiple enzymes.

**Separation of Peptidohydrolases.** Attempts were made to fractionate the peptidohydrolases of nodules by ammonium sulfate precipitation and chromatography in Sephadex G-100 and DEAE-cellulose; however, recovery of activity was usually low, and very little resolution of enzymatic activities was obtained. Superior separation was achieved by analytical and preparative disc gel electrophoresis. Separate experiments (see below) demonstrated optimum separation and detection of endopeptidases at pH 8.8, while aminopeptidases were separated most effectively at pH 6.8.

Synthetic substrates used to detect bands of endopeptidase activity in acrylamide gels included Bz-L-Arg- $\beta$ NA and Bz-L-Leu- $\beta$ NA. Analytical electrophoresis of crude cytosol at pH 8.8 resolved three endopeptidases (Fig. 1). Two bands of activity were seen when Bz-L-Arg- $\beta$ NA was used as the substrate; these endopeptidases, designated EP-Arg-1 and EP-Arg-2, did not hydrolyze Bz-L-Leu- $\beta$ NA or Bz-L-Tyr- $\beta$ NA. The position of the slowest migrating endopeptidase, indicated in Figure 1 by an arrow, was always too indistinct to record photographically. This endopeptidase possessed discernible activity toward Bz-L-Leu- $\beta$ NA but not toward Bz-L-Arg- $\beta$ NA when gels were incubated with substrate for 1 h. To locate endopeptidases which would not cleave synthetic substrates, slab gels were run at pH 8.8, cut in 0.5-cm horizontal sections, and extracted overnight in 25 mM phosphate buffer (pH 7.0). Aliquots of each fraction were then assayed with azocasein and hemoglobin for 15 h and with Bz-L-Arg- $\beta$ NA for 15 min. Prolonged incubation times were necessary when protein substrates were used because after the electrophoresis and extraction the enzyme fractions had been diluted considerably. Azocasein

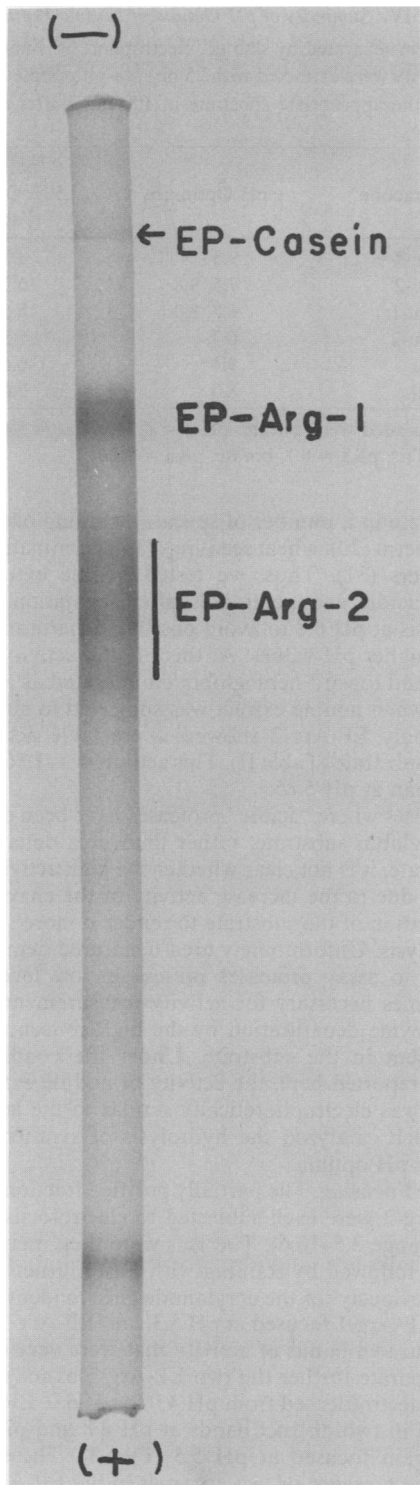


FIG. 1. Disc gel electrophoresis of cytosol extract. Samples of 50  $\mu$ l cytosol (4 g nodules/5 ml 25 mM Na-phosphate [pH 7.0]) were added to the gel tube. Electrophoresis was performed at a gel pH of 8.8 at 2.0 mamp/gel and at 4 C. After electrophoresis the gels were incubated in either Bz-L-Leu- $\beta$ NA (saturated in H<sub>2</sub>O) or Bz-L-Arg- $\beta$ NA (0.4 mg/ml H<sub>2</sub>O) for 1 h and for 15 min, respectively, then allowed to react with Fast Garnet GBC salt (0.1% in H<sub>2</sub>O). Positions of endopeptidases are indicated in the photograph.

hydrolysis was detected in a fraction which corresponded to the slow-migrating band that hydrolyzed Bz-L-Leu- $\beta$ NA (Fig. 1). Since azocasein hydrolysis was more pronounced than hydrolysis

of the synthetic substrate (Bz-L-Leu- $\beta$ NA), this band was designated as EP-casein. Azocasein hydrolysis was also detected in a fraction which corresponded to the band of activity designated as EP-Arg-1 in Figure 1. Hydrolysis of hemoglobin was detected in the same fraction that corresponded to the fast-migrating band designated as EP-Arg-2 in Figure 1. None of the endopeptidases detected in soybean nodule extract hydrolyzed all of the tested substrates. As shown in the summary in Table II, EP-casein hydrolyzed azocasein and the doubly blocked leucine substrate; EP-Arg-1 hydrolyzed azocasein and the doubly blocked arginine substrate; while EP-Arg-2 hydrolyzed hemoglobin and also the synthetic arginine substrate.

Aminopeptidase activity was detected in electrophoresis gels (pH 6.8) using a variety of L-amino acyl- $\beta$ NA representing different classes of amino acids (Fig. 2, Table III). Incubation of gels with L-Ala- $\beta$ NA showed the presence of two enzyme bands which migrated close together near the top of the gel. The slowest migrating band had a very high activity toward L-Ala- $\beta$ NA, as seen by the darkness of the band in Figure 2, and possessed no detectable activity toward L-Val- $\beta$ NA. It was designated as AP-Ala. The second band had less activity toward L-Ala- $\beta$ NA, and in addition hydrolyzed L-Val- $\beta$ NA, so it was designated AP-Val. Both of these enzymes also hydrolyzed arginine, glycine, and

Table II. Summary of Endopeptidases Detected in Soybean Nodule Extract

Enzyme	Position of Migration as Shown in Fig. 1	Substrates Hydrolyzed	Substrates NOT Hydrolyzed
EP-casein	Top	Azocasein Bz-L-Leu- $\beta$ NA	Hemoglobin Bz-L-Arg-pNA
EP-Arg-1	Middle	Azocasein Bz-L-Arg-pNA	Hemoglobin Bz-L-Leu- $\beta$ NA
EP-Arg-2	Bottom	Hemoglobin Bz-L-Arg-pNA	Azocasein Bz-L-Leu- $\beta$ NA

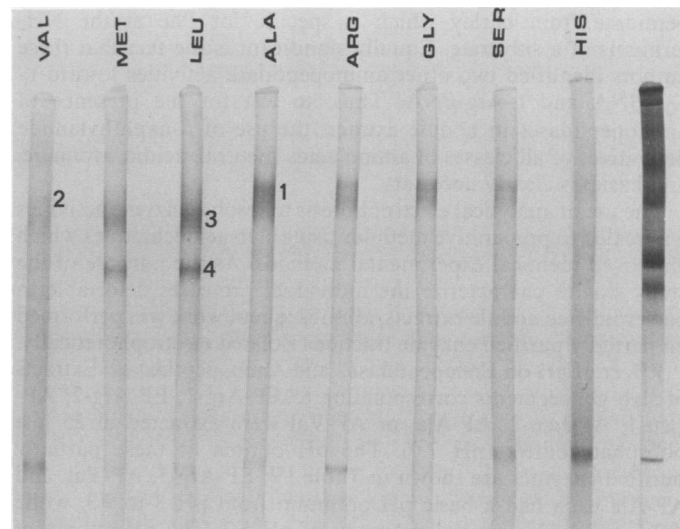


FIG. 2. Disc gel electrophoresis of cytosol extract. The procedure was the same as given in Fig. 1 except that the gel pH was 6.8. Gels were incubated with various L-aminoacyl- $\beta$ NA (0.4 mg/ml H<sub>2</sub>O) for 15 min. The gel labeled Val was incubated with L-Val- $\beta$ NA for 1 h at ambient temperature. After incubation the substrate gels were allowed to react with 0.1% Fast Garnet GBC salt for 10 min at ambient temperature. The identity of the amino acid is given above each gel. The gel at the extreme right of the photo was stained with Coomassie blue (0.02% in H<sub>2</sub>O). Numbers in the photograph indicate; (1) AP-Ala, (2) AP-Val, (3) AP-Leu-1, and (4) AP-Leu-2.

Table III. Activities of Aminopeptidase toward Test Substrates  
Data are condensed from Figure 2.

Substrate <sup>a</sup>	Enzyme and Its Relative Position of Migration as Shown in Fig. 2			
	AP-Ala (1st)	AP-Val (2nd)	AP-Leu-1 (3rd)	AP-Leu-2 (4th)
Alanine	++++	+	--	±
Valine	--	+	+	--
Leucine	--	±	++++	++++
Methionine	--	--	++	+++
Arginine	++	++	--	--
Glycine	++	++	--	--
Serine	++	++	--	--
Histidine	--	--	--	--
Proline	--	--	--	--
Glutamate	--	--	--	--

<sup>a</sup> All substrates were L-aminoacyl- $\beta$ -naphthylamides, and have been designated by the corresponding amino acid.

serine when they were used as the test amino acyl- $\beta$ NA substrates. The third and fourth bands migrated farther than AP-Ala and AP-Val, and demonstrated the highest activity toward L-Leu- $\beta$ NA, and also appreciable activity toward L-Met- $\beta$ NA. These two bands of activity were designated as AP-Leu-1 and AP-Leu-2, respectively. Histidine, proline, and glutamate substrates were not hydrolyzed by any of the enzymes (Table III). The band seen at the bottom of each gel in Figure 2 migrated with the dye front, and was detectable prior to substrate staining, therefore it was not an aminopeptidase.

L-Leu- $\beta$ NA and L-Leu-pNA are good substrates for classical leucine aminopeptidase (3.4.1.1). In many cases, these substrates have been used exclusively to test for the presence of aminopeptidases in plant tissues (1, 10, 24, 35). Others, however, have shown plant tissue to possess aminopeptidases of divergent specificity. Notably, Kolehmainen and Mikola (15) have purified an aminopeptidase from barley which is specific for Phe at the NH<sub>2</sub> terminus of a substrate. Equally significant is the fact that these authors identified two other aminopeptidase activities toward L-Lys- $\beta$ NA and L-Arg- $\beta$ NA. Thus, to test for the presence of aminopeptidases in nodule extracts the use of  $\beta$ -naphthylamide derivatives of all classes of amino acids (neutral, acidic, aromatic, and basic) is clearly necessary.

The use of analytical electrophoresis to resolve enzyme activities was scaled to preparative methods using slab gel techniques which employed identical experimental methods. As the purpose of the study was to characterize the individual proteases detectable in bacteroid-free nodule extracts, all subsequent work was performed on partially purified enzyme fractions isolated electrophoretically.

**Effect of pH on Endopeptidases and Aminopeptidases.** Extracts of slab gel segments corresponding to EP-Arg-1, EP-Arg-2, AP-Leu-1, AP-Leu-2, AP-Ala, or AP-Val were extracted in 25 mM phosphate buffer (pH 7.0). The pH optima of these partially purified enzymes are shown in Table IV; EP-Arg-1, AP-Val, and AP-Ala each had a basic pH optimum from pH 8 to 9.3, while AP-Leu-2 had a neutral optimum of pH 6.7. The pH optima of AP-Ala (pH 9.1) and AP-Val (pH 8.0) thus helped to differentiate these enzymes. The pH activity profiles of EP-Arg-2 and AP-Leu-1 each consisted of two optima, one near neutrality and the other more basic, suggesting the possibility of multiple enzymes in these fractions. In subsequent characterization work with enzyme inhibitors or activators, EP-Arg-2 and AP-Leu-1 were assayed at both pH optima in an attempt to detect any differences that might reflect the presence of more than one enzyme.

While proteases possessing acidic pH optima are not characteristic of all plant tissue (28), they have been detected using hemo-

Table IV. Summary of pH Optima of Nodule Peptidases

Enzymes were separated by slab gel electrophoresis. Bands of peptidohydrolase activity were extracted with 25 mM Na-phosphate (pH 7.0), then assayed using the appropriate substrate in 100 mM buffer of the desired pH.

Enzyme Fraction <sup>a</sup>	pH Optimum	pH Range ( $\pm$ 50% Optimum Activity)
EP-Arg-1	9.3	8.2-10.2
EP-Arg-2	7.5, 9.8	6.3-10.9
AP-Leu-1	6.7, 8.0	5.5-9.3
AP-Leu-2	6.7	5.5-7.4
AP-Ala	9.1	6.6-10
AP-Val	8.0	7.0-9.0

<sup>a</sup> Buffer salts used were: citrate, pK<sub>a2</sub> = 4.74, pK<sub>a3</sub> = 5.40; phosphate, pK<sub>a2</sub> = 7.21; Tris, pK<sub>a</sub> = 8.1; borate, pK<sub>a</sub> = 9.24.

globin substrate in a number of species including oat leaves (35), maize endosperm (20), wheat seedlings (36), germinating pea seeds (1), and others (31). Thus, we tested nodule extracts for the presence of acidic endopeptidases after separation by slab gel electrophoresis at pH 6.8 to avoid possible denaturation of acidic enzymes at higher pH values. At this pH the activity toward Bz-L-Arg-pNA and toward hemoglobin co-migrated as a single component, but when nodule extract was subjected to electrophoresis at pH 8.8, only EP-Arg-2 showed appreciable activity toward hemoglobin substrate (Table II). This activity was 1.86-fold greater at pH 4.85 than at pH 5.85.

In many cases where "acidic" proteases have been detected with native hemoglobin substrate, rather than urea-denatured hemoglobin substrate, it is not clear whether the high activity measured at low pH is due to the increase activity of the enzyme or to the acid denaturation of the substrate to render it more susceptible to enzyme catalysis. Unfortunately urea-denatured hemoglobin cannot be used to assay proteases present in low levels since the prolonged times necessary for activity measurements (>15 min) allow for enzyme denaturation by the high concentrations (4 M) of urea present in the substrate. Under the conditions of the experiments reported here, the activity of nodule extracts toward hemoglobin was electrophoretically similar to the activity of the enzymes which catalyzed the hydrolysis of synthetic substrates with alkaline pH optima.

**Isoelectric Focusing.** The partially purified fractions of EP-Arg-1 and EP-Arg-2 were each subjected to electrofocusing in acrylamide (pH range 3.5-10.0). The gels were then incubated in Bz-L-Arg- $\beta$ NA, followed by staining with Fast Garnet GBC salt, as described previously for the acrylamide gels, to identify the bands of activity. EP-Arg-1 focused at pH 5.3, and EP-Arg-2 was further separated into two bands of activity that were very close to each other. To separate further the two EP-Arg-2 bands crude nodule extract was electrofocused from pH 4.0 to pH 6.0. EP-Arg-2 could be separated in two distinct bands at pH 4.8 and pH 4.85, while EP-Arg-1 again focused at pH 5.3 (Fig. 3). These separations confirmed the presence of two enzymes in the EP-Arg-2 fraction; but they could not be separated by preparative slab gel electrophoresis for subsequent characterization.

Similar experiments to identify the isoelectric points of the aminopeptidases were unsuccessful because hydrolytic activity was not detectable in the isoelectrofocusing gels.

**Inhibition Experiments.** Compounds that are known to inhibit or stabilize certain types of peptidohydrolases (34) were used in an attempt to characterize the endopeptidases and aminopeptidases separated by preparative slab gel electrophoresis. As shown in Table V, DFP completely abolished the activity of EP-Arg-1 and EP-Arg-2, which is a characteristic of hydrolases possessing a functional serine in the catalytic mechanism. EP-casein could

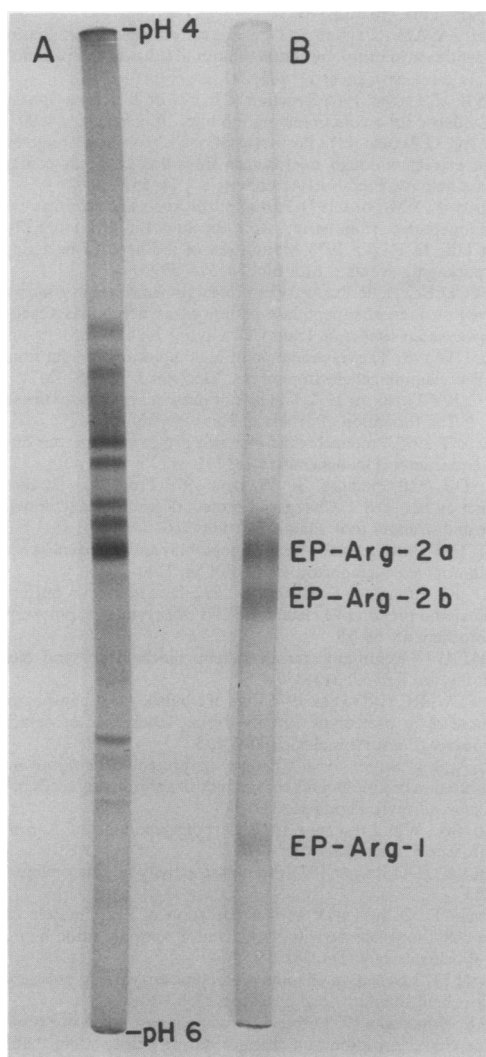


FIG. 3. Isoelectric focusing of crude cytosol in a pH gradient of 4.0 to 6.0 in 5% acrylamide gels. A: stained for total protein; B: stained for endopeptidase activity using Bz-L-Arg- $\beta$ NA and Fast Garnet GBC salt.

not be fractionated and tested in the same manner as EP-Arg-1 and EP-Arg-2 due to its extremely low activity toward synthetic substrates. However, when crude cytosol was incubated with DFP, complete inhibition of azocasein activity was obtained. Thus EP-casein also appears to be a serine-type enzyme. The activity of EP-Arg-1 and EP-Arg-2 was reduced slightly (66–89% of control activity) by PCMB, perhaps by complexing to a structurally important cysteine. Involvement of an active site cysteine would result in a considerably greater loss of activity. Reducing agents did not affect the activity of EP-Arg-2, and slightly enhanced the activity of EP-Arg-1. The chelating agent 1,10-phenanthroline reduced the activity of EP-Arg-1 and did not affect the activity of EP-Arg-2. At a concentration of 1 mM the inhibitor effect of 1,10-phenanthroline was about equal to that of PCMB. No difference in the effect of inhibitors on EP-Arg-2 was seen when assays were performed at either pH 7.5 or pH 9.8. Inasmuch as complete inhibition of all three endopeptidases was obtained with DFP, and since the other modifying agents had only slight effects, all three endopeptidases were classified as serine-type enzymes.

In contrast to the endopeptidases, 1.0 mM DFP did not significantly inhibit the activity of any of the aminopeptidases (Table VI). Partial inactivation was seen in all cases except AP-Ala where nearly 100% activity remained after 1 h incubation. Very low

Table V. Effect of Modifying Agents on Endopeptidase Activities Purified from Soybean Nodule Extracts

Nodule extracts were subjected to slab gel electrophoresis at pH 8.8. Bands corresponding to different enzymes were cut from the slab and extracted with 25 mM Na-phosphate buffer (pH 7.0), then treated with modifying agents. Activity was expressed relative to appropriate controls using Bz-Arg-pNA as the substrate.

Modifying Agent	Concn	Per cent Remaining Activity <sup>a</sup>		
		EP-Arg-1	EP-Arg-2 (pH 7.5)	EP-Arg-2 (pH 9.8)
	<i>mM</i>			
DFP <sup>b</sup>	1	0 <sup>c</sup>	0	0
PCMB <sup>d</sup>	0.025	89	66	73
Dithioerythritol	1.25	120	102	97
1,10-Phenanthroline	10	60	84	84
	1	77	99	93

<sup>a</sup> Bz-L-Leu- $\beta$ NA was only sparingly soluble, and therefore, quantitative assays were not feasible for the enzyme fraction EP-casein.

<sup>b</sup> DFP was preincubated for 30 min at ambient temperature with 20 mM cysteine to remove oxidizing contaminants in DFP (12). Control assay contained 20 mM cysteine.

<sup>c</sup> Standard deviations were less than 6% of the mean.

<sup>d</sup> Assays were performed with Bz-L-Arg- $\beta$ NA when PCMB was the test inhibitor.

Table VI. Effects of Modifying Agents on Aminopeptidase Activities Purified from Soybean Nodule Extract

Nodule extracts were subjected to slab gel electrophoresis (pH 6.8). Bands corresponding to different enzymes were cut from the slab and extracted with 25 mM Na-phosphate buffer (pH 7.0), then treated with modifying agents. Activity was expressed relative to appropriate controls using amino acyl- $\beta$ NA as substrate.

Modifying Agent	Concn	Per cent Remaining Activity				
		AP-Leu-1 (pH 6.7)	AP-Leu-1 (pH 8.0)	AP-Leu-2	AP-Ala <sup>a</sup>	AP-Val <sup>a</sup>
	<i>mM</i>					
DFP <sup>b</sup>	1	82 <sup>c</sup>	75	80	98	75
PCMB	0.05	0	17	287	64	0
Dithioerythritol	1.25	223	140	111	108	139
1,10-Phenanthroline	10	24	48	78	10	1
	1	23	25	87	157	36

<sup>a</sup> AP-Ala and AP-Val were contained in the same preparative electrophoretic fraction but were differentiated by assays employing L-Ala- $\beta$ NA (pH 9.1) or L-Val- $\beta$ NA (pH 8.0).

<sup>b</sup> DFP was preincubated for 30 min at ambient temperature with 20 mM cysteine to remove oxidizing contaminants in DFP (12).

<sup>c</sup> Standard deviations were less than 8% of the mean.

concentrations of PCMB (50  $\mu$ M and 5  $\mu$ M) completely abolished the activity of AP-Leu-1 at pH 6.7 and AP-Val, and severely inhibited AP-Leu-1 at pH 8.0, presumably by forming a mercaptide with catalytically essential sulfhydryl residues in the active site. These same enzymes were activated by DTE; thus, they appear to possess catalytically essential cysteine residues. In contrast, AP-Leu-2 was activated nearly 3-fold by PCMB. This activation by PCMB is difficult to account for based on preliminary results presented here; however, activating effects of this agent have been reported by others (29). The two most likely accounts of this effect would be modification of a structurally essential thiol group in the enzyme not essential for activity (11) or the modification of a thiol-dependent protein which regulates the activity of

AP-Leu-1. The activity of AP-Ala was only slightly reduced by PCMB. The decrease in activity of AP-Ala by PCMB was presumably due to the ability of AP-Val to hydrolyze L-Ala-βNA (Table III), since the two enzymes were not physically separated in the preparative gels. Although AP-Val and AP-Ala were electrophoretically similar, they are different enzymes since AP-Val requires a cysteine residue for activity and has a pH optima of 8.0, while AP-Ala is not sensitive to PCMB and has a pH optima of 9.3. The aminopeptidases that were classified as thiol-type enzymes (AP-Leu-1 and AP-Val) were also inhibited by 1,10-phenanthroline, suggesting a secondary role of metal ions, perhaps by influencing structural stability (27).

In summary, the total proteolytic activity found in extracts of soybean root nodules was composed of endopeptidase, aminopeptidase, and carboxypeptidase activities. Separation of at least three endopeptidases and at least four aminopeptidases was accomplished by preparative slab gel electrophoresis. None of the enzymes had an acidic pH optimum. The endopeptidases were all classified as serine enzymes, while two of the aminopeptidases were found to be thiol enzymes that also required a metal ion for activity. Differences in substrate specificity emphasize the importance of testing for proteolysis with a variety of substrates in order to detect enzymes of widely different specificity. The identification and characterization of these enzymes will make possible the study of changes in their activity levels during the life of the soybean plant and their possible regulating effect on nodule senescence. The appearance of proteolytic enzymes that may be unique to senescence will also be easier to detect, since the identity of pre-existing enzymes has been established.

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