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Proteolytic Activity in Soybean Root Nodules[1]

ACTIVITY IN HOST CELL CYTOSOL AND BACTEROIDS THROUGHOUT PHYSIOLOGICAL DEVELOPMENT AND SENESCENCE

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ABSTRACT

Root nodules were harvested from chamber-grown soybean (*Glycine max* L. Merrill cv Woodworth) plants throughout development. Apparent nitrogenase activity (acetylene reduction) peaked before seeds began to develop, but a significant amount of activity remained as the seeds matured. Nodule senescence was defined as the period in which residual nitrogenase activity was lost. During this time, soluble protein and leghemoglobin levels in the host cell cytosol decreased, and proteolytic activity against azocasein increased. Degradative changes were not detected in bacteroids during nodule senescence. Total soluble bacteroid protein per gram of nodule remained constant, and an increase in proteolytic activity in bacteroid extracts was not observed. These results are consistent with the view that soybean nodule bacteroids are capable of redifferentiation into free-living bacteria upon deterioration of the legume-rhizobia symbiosis.

Nitrogenase activity profiles of nodules have been followed throughout the developmental cycle of soybean plants by a number of investigators (9, 10, 13, 20). Total nitrogenase activity per plant, measured by the acetylene reduction assay, generally peaks at full flowering or as pod formation begins, and then declines as seeds develop (13, 20). However, the extent of this decline appears to vary considerably and is dependent upon such factors as plant cultivar (13), row spacing (25), environmental conditions such as temperature and photoperiod, and whether plants are field- or chamber-grown. In some cases, nitrogenase activity exhibited an initial decline during pod formation, but then was maintained at a significant level throughout the period of seed fill (9, 10, 17). Some investigators have used the initial decline in nitrogenase activity as an indication that nodule senescence has begun (12), even though significant activity may remain for quite some time. Others (19) have defined nodule senescence as the period of rapid nodule decay which occurred at physiological maturity (leaf yellowing). Additional problems arise when investigators fail to give any indication of the developmental stage of the plant when monitoring nitrogenase activity. Since the number of days from planting to harvest maturity depends upon plant cultivar and growth conditions, in many cases it is difficult to relate nodule senescence, as defined by a decline in nitrogenase activity, with a particular stage of plant development.

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Declines in total soluble protein (15) and total leghemoglobin (12) contents have been observed in senescing soybean nodules, although these losses generally occurred later than the initial decline in nitrogenase activity. Similar declines in soluble protein and leghemoglobin contents occurred in senescing alfalfa (*Medicago sativa* L.) nodules following foliage harvest (24) which were correlated with an increase in proteolytic activity. It has been postulated (2) that autolytic processes in the bacteroids and host legume cells were probably responsible for the senescence of legume nodules, and Truchet and Coulomb (21) suggest that 'phytolysosomes' *i.e.* vacuoles, may be involved in the cellular disorganization that occurs at senescence in pea (*Pisum sativum* L.) nodules.

Previous work in this laboratory has identified and partially characterized the proteolytic enzymes of soybean nodule cytosol (14). This report describes the peptidases found in bacteroid extracts, monitors these activities in both host cells and bacteroids throughout the developmental cycle of soybean plants, and relates these activities to changes in nitrogenase activity that occur throughout the period of pod formation, seed development, and maturation.

MATERIALS AND METHODS

Seed Inoculation and Plant Growth. The methods used to prepare inoculum, to infect soybean seeds (*Glycine max* L. Merrill cv Woodworth) and to grow soybean plants have been reported previously (14), except that *Rhizobium japonicum* (strain 61A89) was used and each 15-cm pot contained only one plant. After plants had nodulated (at about the time when the first trifoliate leaf was fully expanded), they were repotted in sterile Arcil lite (IMC Chemical Corp.), an inert root support. They were grown at 26 ± 2°C in an environmentally controlled chamber on a 16-h photoperiod until flowering commenced, and thereafter on a 14-h photoperiod. Light intensity averaged 700 $\mu E \cdot m^{-2} \cdot s^{-1}$. Plants were watered twice daily and given nitrogen-free Hoagland solution twice weekly.

Acetylene Reduction. Nitrogen fixation was measured on intact plants using the acetylene reduction assay (7) as reported previously (16).

Nodule Harvest. Root nodules were harvested throughout the developmental cycle of the soybean plants at 1- to 2-week intervals. The first nodules were collected during early vegetative growth, as soon as nodules were large enough to be picked from the root, and the last nodules were collected when the plants had reached harvest maturity. At each harvest date, the physiological development of the plants was classified according to the descriptions suggested by Fehr *et al.* (5) for developmental stages of soybean growth. As many nodules as possible were harvested from the plants on the day following acetylene reduction assays. All nodules from a single plant were pooled, weighed, frozen, and stored at −80°C until used. A 1-g aliquot of nodules was counted

to obtain an estimation of nodule number. Percent dry weight was determined on a 1-g sample of nodules by drying at 100°C for 24 h. The foliage (stems and leaves) of each plant was severed from the root system and weighed. After pod development began, all pods were removed from the plant, weighed, and frozen at −20°C. At a later time, both seeds and pods were dried at 60°C for 48 h and then total nitrogen of seeds plus pods was determined by Kjeldahl analysis.

Bacteroid extracts used to identify and characterize the bacteroid peptidases were obtained from nodules collected during the pod-filling stage of plant development (R-5 and R-6) (5).

Nodule Cytosol. Nodules were crushed thoroughly with an ice-cold mortar and pestle (4 g nodules/8 ml 25 mM Na-phosphate [pH 7.0], 1 mM $MgCl_2$, 1 mM dithioerythritol), and the homogenate centrifuged at 12,000*g* for 20 min. The crude supernatant fluid (nodule cytosol) was assumed to contain soluble proteins of legume (*i.e.* host cell) origin, including soluble organellar proteins. The supernatant fraction was passed through Sephadex G-25 equilibrated with extraction buffer to remove small mol wt compounds, including phenols, which might interfere with enzymic analyses.

Bacteroid Extracts. The pellet obtained after centrifugation of the nodule homogenate (above) was resuspended in 15 ml of the same buffer and filtered through a polypropylene filtration cloth, mesh 105 (Spectrum Medical Industries, Inc., Los Angeles, CA), and the filtrate was centrifuged at 300*g* for 5 min to remove most of the starch granules and cell debris. The supernatant fluid was then centrifuged at 8,000*g* for 5 min and the pellet suspended in 4 ml of 16% sucrose (w/v) prepared in the same buffer. The suspension was layered on a sucrose density gradient made of equal volumes of 55% (w/v) and 35% (w/v) sucrose solutions also prepared in the extraction buffer. Centrifugation was done at 16,000*g* for 15 min using a SV-288 vertical rotor in a Sorvall RC-5B Refrigerated Centrifuge equipped with an automatic rate controller. After centrifugation, two bands were visually detected: the heaviest one located at the 35% and 55% (w/v) sucrose interphase and a much more dilute band located in the upper part of the 35% (w/v) sucrose solution. A predominantly Gram-negative population of bacteroids, morphologically resembling the bacteria seen in broth cultures of *Rhizobium japonicum* was observed by microscopic examination in the fraction collected at the interphase of the 35% and 55% (w/v) sucrose solutions, while mainly membranes and cell debris were observed in the minor band seen at the upper part of the 35% sucrose solution. The activity of βOHB[3] dehydrogenase, a 'marker' enzyme for bacteroids (26), was determined in the extract from the two visible fractions by the method of Werner *et al.* (26). βOHB dehydrogenase activity was 0.19 μmol of NADH/min·mg protein in the extract resulting from the main band located at the interphase between the two sucrose solutions. Activity was not detected in the extract resulting from the minor band. The bacteroid preparation collected from the major band described above contained rhizobial cells isolated from nodules regardless of size or state of bacteroid development. The collected bacteroid suspension was rinsed at least three times with extraction buffer and then lysed by passage through a French Pressure Cell at 23,000 p.s.i. Whole cells and cell debris were removed by centrifugation at 30,000*g* for 1 h. The supernatant fluid was designated the crude bacteroid extract and contained an average of 5.85 mg protein/g fresh wt of nodule. The extract was either used immediately or stored at −70°C.

Protein and Leghemoglobin. Total soluble protein in the cytosol and bacteroid extracts was determined by the Bradford dye-binding assay using Bio-Rad dye reagent (3). BSA was used as the protein standard. Total leghemoglobin in the nodule cytosol fraction was determined by the pyridine hemochrome assay (1) as described previously (16). The average mol wt of leghemoglobin was assumed to be 16,375 (4).

Proteolytic Enzyme Assays. The nodule cytosol fraction was analyzed for endopeptidase and aminopeptidase activities using synthetic substrates as described previously (14). Bacteroid extracts were assayed in a similar fashion, except that the incubation time was 1 h and the incubation mixtures were centrifuged in a Beckman Microfuge B to remove turbidity after the reactions were stopped with TCA. One unit of activity was defined as the amount of enzyme required to produce 1 μmol βNA/h under the conditions of the assay. In all assays, the enzyme activity was proportional to time throughout the period of incubation. The azocasein-digesting activity of nodule cytosol was determined as reported previously (16). The proteinase activity of the crude bacteroid extracts was measured using azocasein and hemoglobin as substrates. In addition, autolysis of endogenous proteins was measured. Autolytic activity was defined as the ability of the soluble bacteroid peptidohydrolases to digest any of the proteins present in the extract, and was measured as release of acid-soluble, ninhydrin-positive materials after incubation of the extracts without exogenous substrate.

To determine the autolytic and hemoglobin-digesting activities, aliquots (150 μl) of the crude bacteroid extract were diluted with an equal volume of 100 mM Na-phosphate buffer (pH 7.0) in Beckman microfuge tubes. When the autolytic activity was measured, the extract was incubated with buffer only. For hemoglobin-digesting activity assays, 250 μl hemoglobin (40 mg/ml in 100 mM Na-phosphate buffer [pH 7.0]) were added before incubation began. Before use, the hemoglobin solution was passed through a 0.5- × 5-cm Sephadex G-25 column (Pharmacia) equilibrated with 100 mM Na-phosphate buffer (pH 7.0) to remove TCA-soluble material. The reaction mixtures were incubated for 3 h at 37°C in a shaking water bath. The reaction was stopped with TCA at a final concentration of 5%, and in the case of the autolytic reaction mixtures, 250 μl of hemoglobin (40 mg/ml) were added just before terminating the reactions. After standing at room temperature for 15 min, precipitates were removed by centrifugation in a Beckman Microfuge B. Aliquots (250 μl) of the supernatant fluid were used to measure the soluble α-amino groups released by the enzymic reaction, using the ninhydrin procedure reported by Spies (18) with leucine as the standard. Controls without bacteroid extract were run to determine the possible effect of denaturation of the hemoglobin during the assay. The autolytic and hemoglobin-digesting activities assays were always performed simultaneously. One unit of activity was defined as the amount of enzyme required to release 1 μmol of α-amino nitrogen/h under the conditions of the assay.

The azocasein-digesting activity was determined by incubating aliquots (250 μl) of crude bacteroid extracts with an equal volume of azocasein (10 mg/ml in 100 mM Na-phosphate buffer [pH 6.8]) at 37°C for 18 h. Streptomycin (160 μg/ml) was added to the reaction mixture to eliminate bacterial growth. An aliquot of each assay mixture was plated on nutrient agar at the end of the incubation period to test the effectiveness of the antibiotic. The addition of streptomycin was found to inhibit bacterial growth satisfactorily. After incubation, 1.2 ml of 10% TCA were added to 350 μl of the reaction mixture. The precipitated proteins were removed by centrifugation in a Beckman Microfuge B and 1.4 ml of 1 N NaOH were added to an equal volume of the supernatant fluid. Absorbance of the azo-dye released was measured colorimetrically at 440 nm. One unit of activity was expressed as the amount of enzyme which produced a ΔA_{440} of 1.0/h. Table I gives the specific activities, pH optima, and sensitivity to some modifying agents for all these proteolytic activities.

Statistical Analysis. Root nodules were collected at 1- to 2-week

[3] Abbreviations: βOHB, β-hydroxybutyrate; -βNA, -β-naphthylamide; Bz-, N-α-benzoyl.

Table I. *Characteristics of Peptidases from Bacteroid Extracts*

Modifying agents were used as described previously (14).

Proteolytic Activity	pH Optimum	Specific Activity	Relative Inhibition		IAc (0.5 mM)
			DFP[a] (5 mM)	OP (10 mM)	
		units × 10^2/ mg protein		%	
Synthetic substrates					
L-Leu-βNA	9.5	2.29	74	93	50
Bz-L-Arg-βNA	6.5	3.13	100	83	34
	9.5	3.01	100	40	58
Hemoglobin	7.0	8.48	32	50	0
Autolysis	7.0	6.56	67	95	0
Azocasein	6.8	1.58	44	57	0

[a] DFP, diisopropylphosphorofluoridate; OP, 1,10-phenanthroline; IAc, iodo-acetamide.

intervals throughout the developmental cycle of the plants, and nodules were obtained from two or three plants at each harvest date. Nodules were grouped into eight categories according to the developmental stage of the plant from which they were harvested: vegetative growth (V), flowering (R-2), early pod development (R-3), full pod development (R-4), early seed development (R-5), full seed development (R-6), physiological maturity (R-7), and harvest maturity (R-8) (5). Thus, each developmental grouping contained nodules from several harvest dates representing a continuum of physiological development within a particular stage. A total of 40 plants were harvested during the course of the experiment. The results from enzymic analyses of nodule cytosol and bacteroid extracts were analyzed by an approximation of Duncan's multiple range test to detect significant differences in activity between each developmental stage.

RESULTS

Physiological Stages of Plant Growth. Physiological development of the soybean plants was monitored according to the method of Fehr *et al.* (5) throughout the growth cycle. The validity of the visual classification system used was determined after harvest by measuring the fresh weight of foliage, and the fresh weight and total nitrogen of the seeds plus pods (Fig. 1). The fresh weight of foliage (leaves and stems) increased steadily throughout vegetative growth and flowering until pod development began, remained fairly constant throughout seed development, and then declined as leaf yellowing accelerated leading to physiological and harvest maturity (Fig. 1A). The fresh weight of pods plus seeds increased steadily through full seed development, and then declined as the pods began to yellow and desiccate (Fig. 1A). Total nitrogen in the seeds plus pods increased from the time of beginning seed development until physiological maturity was reached, and then remained constant as plants reached harvest maturity (Fig. 1B).

Acetylene Reduction. Apparent nitrogenase activity was measured on intact potted soybean plants using the acetylene reduction assay (Fig. 1B). Acetylene reduction activity increased steadily during vegetative growth, flowering, and early pod development. Nitrogenase activity peaked during full pod development, and then decreased during seed development to about the same level as seen during flowering and early pod development. Acetylene reduction activity remained quite high throughout the period of seed development, and did not decline to low levels until the plants had reached physiological maturity.

Nodule Weight and Number. Nodules were collected from the roots of soybean plants at intervals from early vegetative growth until harvest maturity. The total fresh weight and number of root nodules increased steadily during early vegetative growth, flowering, and pod development (Table II). Significant changes in total nodule weight or numbers did not occur from full pod development through physiological maturity, but then a significant decrease in both parameters was evident as the plants reached harvest maturity. Nodule greening, which is indicative of leghemoglobin loss, occurred when the plants reached physiological maturity. Since the percent dry weight of nodules did not vary significantly throughout the developmental cycle (Table II), the decrease in total nodule fresh weight was probably a result of nodule senescence and abscission from the root system.

Bacteroid-Free Nodule Cytosol. Soluble protein measured per fresh weight of nodule remained fairly constant in nodule cytosol from early vegetative growth through full seed development, but then dropped significantly as the plants attained physiological maturity (Table III). Leghemoglobin content of the cytosol measured as mg per g fresh weight of nodule peaked when the plants were flowering, and then declined steadily as pods and seeds developed. A significant decline in leghemoglobin content occurred at physiological maturity, concomitant with the decline in soluble protein (Table III). When leghemoglobin content was expressed as a percent of the total soluble protein content, the highest percentage of leghemoglobin was found during early pod development, when nearly 50% of the soluble protein in bacteroid-free nodule extracts was leghemoglobin. Similar leghemoglobin concentrations in soybean nodules have been reported by others (15). The percent leghemoglobin declined steadily thereafter, and accounted for only 28% of the total soluble protein by the time the plants reached harvest maturity (Table III).

Proteolytic enzyme activity of bacteroid-free nodule cytosol was measured with azocasein and the synthetic substrate Bz-L-Arg-βNA. Specific activity toward azocasein exhibited two peaks of activity (Table IV). The first peak of activity occurred as pod development commenced, which was just before the initial decline in nitrogenase activity (Fig. 1). The second peak of activity occurred between the time of full seed development and physiological maturity (Table IV), which correlated with the decrease in soluble protein and leghemoglobin that occurred at this time (Table III). An increase in azocasein-digesting activity has also been correlated to protein and leghemoglobin loss during dark-induced senescence of soybean nodules (16) and in senescing alfalfa nodules following foliage removal (24). Previous work (14) demonstrated that proteolytic activity toward the synthetic substrate Bz-L-Arg-βNA was most active at pH 7.5 and pH 9.8, thus activity in crude extracts was measured at both pH values. Specific activity at both neutral and alkaline pH was highest as pod

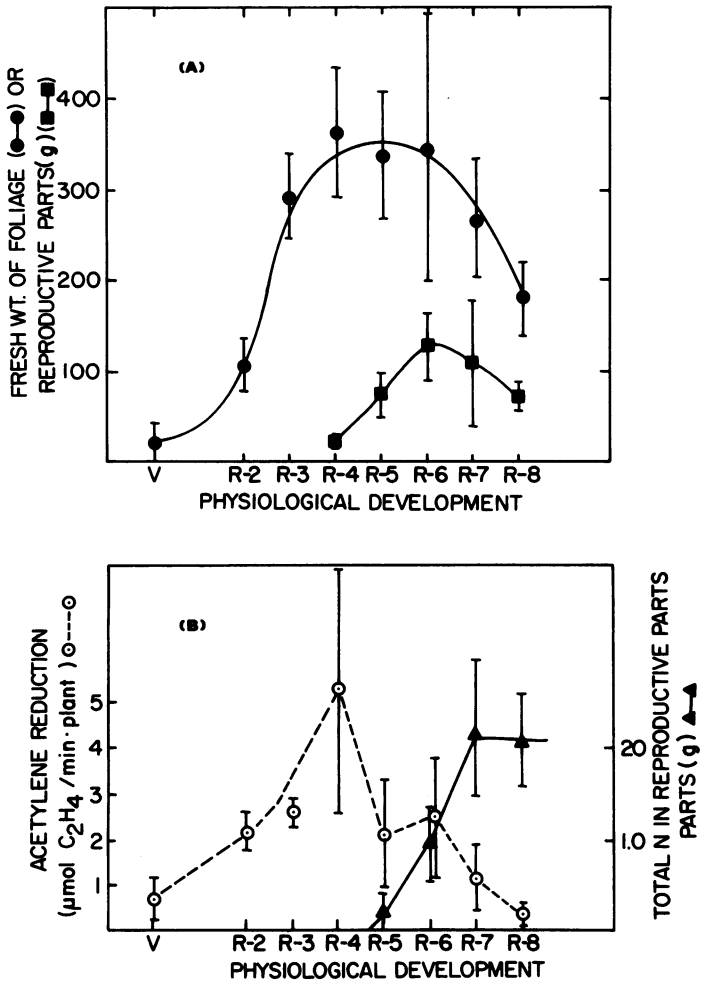

FIG. 1. A, Fresh weight of foliage (leaves and stems) (●) and reproductive parts (pods and seeds) (■). Total N in reproductive parts (▲) and apparent nitrogenase activity (μmol C_2H_4 formed/min·plant) (○) throughout physiological development of soybean plants. Vegetative growth (V), flowering (R-2), early pod development (R-3), full pod development (R-4), early seed development (R-5), full seed development (R-6), physiological maturity (R-7) (pod and leaves yellowing), harvest maturity (R-8) (pods 95% brown). Bars represent SD.

development commenced, but then declined as seeds began developing. In contrast to azocasein-digesting activity, proteolytic activities toward Bz-L-Arg-βNA did not increase during the period of nodule senescence (Table IV).

Previous work has shown that at least four distinct enzymes present in bacteroid-free nodule cytosol hydrolyze various aminopeptidase substrates (14). Although each electrophoretically distinct enzyme hydrolyzed a number of substrates representing various amino acid specificities, two enzymes were responsible for the majority of activity against L-Leu-βNA, while another was most active against L-Ala-βNA and L-Arg-βNA. A fourth enzyme hydrolyzed L-Val-βNA (14). In the present study, no attempt was made to quantitate the activity of the individual aminopeptidases due to their overlapping specificities in crude nodule extracts, but a survey of activity was made using three different aminopeptidase substrates, L-Leu-βNA, L-Arg-βNA, and L-Val-βNA. The specific activity of bacteroid-free nodule cytosol against all three aminopeptidase substrates tended to be higher during the early stages of plant growth, and then to drop off slightly when seeds began developing on the plants (data not shown). There was no correlation between aminopeptidase activity and the loss of soluble

Table II. *Total Fresh Weight and Number of Soybean Root Nodules Collected from Early Vegetative Growth through Harvest Maturity*

Plants were grown in an environmentally controlled growth chamber at 26 ± 2°C on a 14-h photoperiod.

Physiological Stage	Nodule Fresh Wt[a]	Nodule No.[a]	Dry Wt of Nodules[a]
	g		%
Vegetative	2.3 C	164 B	
Full flower	5.9 C	312 B	17.4 A
Early pod	13.8 BC	471 AB	17.1 A
Full pod	19.0 AB	609 A	18.3 A
Early seed	18.7 AB	583 A	18.4 A
Full seed	19.5 AB	656 A	18.3 A
Physiological maturity	26.1 A	712 A	16.7 A
Harvest maturity	9.1 C	315 B	15.9 A

[a] Data were analyzed using Duncan's multiple range test. Means denoted by the same letter are not significantly different at the 5% level of probability.

Table III. *Soluble Protein and Leghemoglobin Contents in Bacteroid-Free Nodule Cytosol from Soybeans Harvested throughout the Growing Season*

Physiological Stage	Soluble Protein[a]	Leghemoglobin[a]	Leghemoglobin[a,b]
	mg/g fresh wt of nodule		%
Vegetative	7.53 AB	2.95 AB	39.2 BCD
Full flower	7.62 A	3.03 A	39.8 AB
Early pod	5.26 BC	2.54 ABC	48.3 A
Full pod	6.51 AB	2.29 BC	35.2 BC
Early seed	6.82 AB	2.02 C	29.6 CDE
Full seed	7.10 AB	1.99 C	28.0 DEF
Physiological maturity	4.71 C	1.24 D	26.3 F
Harvest maturity	4.68 C	1.30 D	27.8 EF

[a] Data were analyzed using Duncan's multiple range test. Means denoted by the same letter are not significantly different at the 5% level of probability.

[b] Leghemoglobin content expressed as a percent of soluble protein in the extract.

protein and leghemoglobin that occurred at physiological maturity. Thus, the peptidohydrolase activities of host cell cytosol measured by aminopeptidase substrates probably are not functionally significant to the process of nodule senescence.

Bacteroid Extracts. Purified bacteroids were isolated from root nodules collected from soybean plants from early vegetative growth through harvest maturity and extracts were prepared and assayed for soluble protein and peptidohydrolase activities. In contrast to the decline in soluble protein observed in the host cell cytosol at physiological maturity (Table III), soluble protein content of bacteroid extracts did not change significantly throughout the growth cycle of the plants (Table V) and remained unchanged even when nodule senescence was well advanced. Thus, it appears that the bacteroid proteins are much more stable during nodule senescence than are host cell proteins.

The proteolytic activity of bacteroid extracts was measured using hemoglobin and azocasein as substrates. In addition, the autolytic activity of the extracts was determined by monitoring the release of acid-soluble, ninhydrin-positive materials after incubation of the extracts without exogenous substrate. Thus, autolytic activity represents hydrolysis of endogeneous proteins by bacteroid peptidases. Proteinase activity against hemoglobin and azocasein did not exhibit any significant increases as bacteroids aged,

Table IV. *Endopeptidase Activities of Bacteroid-Free Nodule Cytosol from Soybeans Harvested throughout the Growing Season*

Physiological Stage	Azocasein[a]	Bz-L-Arg-βNA[a] (pH 9.8)	Bz-L-Arg-βNA[a] (pH 7.5)
		units/mg protein[b]	
Vegetative	0.23 C	0.54 ABC	0.28 BC
Full flower	0.25 C	0.63 A	0.33 B
Early pod	0.43 AB	0.73 A	0.43 A
Full pod	0.35 B	0.59 AB	0.30 BC
Early seed	0.33 BC	0.51 BC	0.29 BC
Full seed	0.33 BC	0.45 C	0.24 C
Physiological maturity	0.51 A	0.46 C	0.30 BC
Harvest maturity	0.54 A	0.46 BC	0.29 BC

[a] Data were analyzed using Duncan's multiple range test. Means denoted by the same letter were not statistically different at the 5% level of probability.

[b] One unit of activity against azocasein is expressed as the amount of enzyme which produced a ΔA_{440} of 1.0/h, while 1 unit of activity against Bz-L-Arg-βNA is expressed as the amount of enzyme required to liberate 1 μmol product/h.

while autolytic activity against endogenous proteins actually decreased in bacteroids from plants that had reached harvest maturity (Table V). Endopeptidase activity against the synthetic substrate Bz-L-Arg-βNA was measured in bacteroid extracts at pH 6.5 and 9.5 since previous work has demonstrated the existence of multiple activities in bacteroid extracts having these pH optima (unpublished data). Activities at both pH values were an order of magnitude lower than the corresponding activities found in the cytosol, and highest activities were found in the youngest nodules. Activities in the bacteroid extract ranged from 0.040 units/mg protein in nodules from very young plants to 0.027 units/mg protein in nodules from plants at harvest maturity. The lack of any significant increase in endopeptidase activity in bacteroids during nodule senescence is probably responsible for the observed stability of bacteroid proteins, and suggests that bacteroid integrity is maintained during nodule senescence as the host cell deteriorates.

A single aminopeptidase having broad specificity against a large number of amino acids has been identified by us in soybean nodule bacteroids (unpublished data). This enzyme was surveyed in bacteroid extracts using L-Leu-βNA as the substrate, and no significant change in its specific activity was observed throughout the developmental cycle of the plants. Aminopeptidase activity averaged 0.02 units/mg protein throughout the life of the nodules.

DISCUSSION

The nitrogenase activity profile observed in this study for chamber-grown 'Woodworth' soybeans exhibited a peak before seed development began, but a very significant amount of activity remained during the period of seed development. The final decline in activity began after approximately 50% of the total seed plus pod nitrogen had already accumulated. The initial decline in nitrogenase activity that occurred as seeds began developing probably reflects a change in partitioning of photosynthate away from nodules and into seeds (6, 13). Although nitrogenase activity does decline at this stage in development, there is no evidence of any nodule deterioration at this time. The significant amount of nitrogenase activity that was observed during seed development in this study indicates that although nodules may not be functioning at full capacity during the period of seed growth, they are still quite viable, and the possibility remains for increasing seed yield through genetic manipulations which might influence partitioning of photosynthate at this critical time. In fact, increasing available photosynthate by plant thinning before seed development began has been shown to delay the onset of nodule greening (*i.e.* senescence) by several weeks (25).

The final decline in nitrogenase activity that began in the later stages of seed development in this study was correlated with degradative changes in the host cell cytosol. Nodule greening, the loss of soluble protein and leghemoglobin, and the increase in proteolytic activity against azocasein observed in the host cell cytosol at physiological maturity are perhaps better indicators of nodule senescence than the initial decline in nitrogenase activity observed at early seed development.

Studies on the ultrastructure of senescing nodule tissue have generally not been correlated with nitrogenase activity, so it is difficult to relate biochemical events occurring as nodules age to morphological changes. However, Tu (23) has suggested that the host cell and bacteroid envelope membranes of soybean nodules

Table V. *Soluble Protein Content and Proteinase Activity of Bacteroid Extracts from Soybean Nodules Harvested throughout the Growing Season*

Physiological Stage	Soluble Protein	Protein-Digesting Activity[a]		
		Hemoglobin[b]	Autolysis[b]	Azocasein[c]
mg/g fresh wt of nodule	*units × 10^2/mg protein*			
Vegetative	5.14 A	11.08 A	5.17 AB	1.63 AB
Full flower	5.78 A	8.39 A	5.66 A	1.49 AB
Early pod	6.08 A	5.99 A	6.89 A	1.45 B
Full pod	5.84 A	7.64 A	7.19 A	1.60 AB
Early seed	6.06 A	7.30 A	6.56 A	1.46 B
Full seed	5.78 A	8.00 A	6.71 A	1.62 AB
Physiological maturity	6.10 A	7.76 A	7.76 A	1.75 A
Harvest maturity	6.06 A	11.73 A	1.35 B	1.70 AB

[a] Data were analyzed using Duncan's multiple range test. Means denoted by the same letter were not significantly different at the 5% level of probability.

[b] One unit of hemoglobin digesting or autolytic activity is defined as the amount of enzyme which released 1 μmol amino-N/h.

[c] One unit of activity against azocasein is expressed as the amount of enzyme which produced a ΔA_{440} of 1.0/h.

deteriorate in the advanced stages of infection (*i.e.* the older sections of the nodule) leaving rhizobia in a ghost cell, but nodules in advanced stages of senescence were not examined. Kijne and Planque (11) examined the development of the endomembrane system of soybean nodules during infection, maturation, and senescence. They identified vacuoles containing cytosolic debris-like material which they assumed might result from autophagic activity, but no evidence was ever seen of any bacterial uptake into a vacuole. Tsien *et al.* (22) found an increase in the rhizobia population in soil at the end of the growing season, and was able to isolate viable bacteroids from nodules of mature plants. Gresshoff and Rolfe (8) isolated and cultured bacteroids from soybean nodule protoplasts from both young and old plants. The protoplasts from senescing nodules were fragile and unstable, indicating significant deterioration of the host cell, but the bacteroids from these protoplasts were still viable. Data presented in the present report fully support the view that as the soybean-rhizobia symbiosis breaks down during nodule senescence, the host cell deteriorates while the bacteroid does not. Soluble proteins in the host cell cytosol are degraded, probably as a result of increased proteolytic activity, while bacteroid proteins remain intact and autolytic activity actually decreases.

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