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Rhizobins, a Group of Peptides in the Free-Amino-Acid Pool of the Soybean-*Rhizobium* System†

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Free-living *Rhizobium* (according to *Bergey's Manual of Systematic Bacteriology*, [1984, The Williams & Wilkins Co., Baltimore], *Bradyrhizobium*) *japonicum* was found to release a peptide into the nutrient media. Soybean nodules contained this peptide and exuded it into the soil. The name "rhizobin A" is suggested for this peptide. Nodules also contained another peptide, rhizobin B, as well as an unidentified, ninhydrin-positive compound, rhizobin C. The three peptides were confined to the free-amino-acid pool of the soluble fraction and eluted consecutively from a cation-exchange column. Rhizobin A was isolated in a highly purified form; its molecular mass was approximately 1,600 daltons as determined by Sephadex gel filtration and mass spectrometry. The amino-acid composition could be determined only approximately, because a long time was necessary for acid hydrolysis, possibly due to unusual linkages. The rhizobin concentration in soybean nodules continually increased during 50 days of growth, from 2 to approximately 400 μg/g (fresh weight). When combined nitrogen was added to nodulated soybean and subsequently removed, nitrogenase activity, nodulation, and nodule growth first decreased and then recovered. The relative amount of rhizobin A followed a similar pattern. Rhizobins were not detected in the roots, stems, and leaves of nodulated soybean plants. They were present in *Lupinus* nodules, but absent in alder nodules.

The vitally important functions of peptides as chemical messengers in animals are well known. Peptides communicate within and among the endocrine, nervous, reproductive, and circulatory systems, thus insuring homeostasis. Within plants, peptides with biologically critical functions have not yet been found, although there are increasing numbers of reports (9, 11, 12) which indicate the importance of peptides in both pathogenic and beneficial plant-microorganism interactions. Those reports which involve either legumes or nitrogen fixation are briefly noted. *Aspergillus* spp. produce a cyclic pentapeptide, malformin, which at very low concentrations causes curvature of bean roots (2). A toxic γ-glutamyl-peptide has been isolated from *Lathyrus* seeds (20); another toxic tripeptide causes the halo blight of bean (16). A yellow-green siderophore nonapeptide was isolated from *Azotobacter* spp. (3). Peptides were tentatively identified in plant tissues infected by *Agrobacterium tumefaciens* (5), as well as in root nodules of clover (4) and *Alnus* spp. (13). Bednarski and Reporter (1) and Storey and Reporter (21) claimed that *Rhizobium japonicum* produces an oxygen-sensitive peptidoglucan which induces soybean plant cells to synthesize compounds that eventually cause the expression of the *nif* gene.

We report here the discovery of a group of peptides in the free-amino-acid pool of the soybean-*Rhizobium* system.

MATERIALS AND METHODS

Organisms and growth. *Rhizobium japonicum* 110 from the USDA Beltsville collection and *Glycine max* (L.) Merr cv. Lee 74 were used throughout this study. Bacteria were incubated in nutrient broth (24) for 10 days; soybean seeds were soaked in the rhizobial suspension for 2 days, and then planted in a vermiculite-perlite (2:1 [vol/vol]) mix. Plants were irrigated with a half concentration of the nutrient solution of Machlis and Torrey (15), with 20 mM $CaCl_2$ substituted for 20 mM $Ca(NO_3)_2$. Plants were grown in a greenhouse with a diurnal temperature cycle from 15 to 30°C under natural day-length conditions for southeast Texas during April, May, and June. Flowering was delayed by removing the flower buds. Nodules of *Lupinus texensis* and *Alnus serrulata* were collected from wild plants in the College Station area.

Samples for analysis. All samples were collected between 10 and 11 a.m. Only the nodules located at the upper tap root region were selected and then detached, to insure maximum homogeneity with respect to both location and age. The third fully expanded leaf below the shoot apex, the internode of the stem above this leaf, and the upper part of the tap roots were analyzed immediately after sample collection. Nodules, however, were stored at −36 to −40°C and assayed for soyrhizins within 6 weeks of harvest. Control experiments demonstrated that 3 months of storage did not influence soyrhizin concentration. The vermiculite-perlite mix around the nodules, within a sphere with a diameter of ca. 1 cm, was analyzed. Two samples were assayed in each of three or more replications.

Suppression of nitrogen fixation by nitrate. The application of combined nitrogen was chosen as a method for study of the correlations between rhizobin production and nodulation (number of nodules per plant), nodule growth (milligrams [fresh weight] per nodule), and nitrogen fixation. Nodulation and nodule growth were allowed to progress in nitrate-free medium for 21 days. The nutrient solution was then replaced by a fresh one with 20.0 mM $Ca(NO_3)_2$ added to the treated group and a fresh application of the original solution (which

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contained 20.0 mM $CaCl_2$) added to the control. At day 14 after nitrate addition, both treated and control roots were rinsed twice with tap water and once with distilled water, and then nitrate-free solutions were applied. This procedure was repeated 2 days later to ensure that all nitrate was removed. During these 2 weeks, nitrate-dependent suppression of nodulation, nodule growth, and nitrogen fixation was monitored, and the concomitant rhizobin content was determined. Analysis continued during the recovery period after the removal of nitrate.

Peptide and acetylene assay. A Beckman 120B automatic amino-acid analyzer was used for quantitative analysis of peptides (21). With 0.5 g of fresh nodules or plant parts, well-defined peaks were obtained. Nitrogenase activity was determined by the acetylene reduction assay (8).

Localization of rhizobin. The nodule cortex was peeled from the medulla under a dissecting microscope, and then each tissue was analyzed. Bacteroids were separated from 1.5 g of fresh nodules by the method of Robertson et al. (17). The supernatant fraction from the bacteroid isolation was centrifuged in a Beckman model L-2 ultracentrifuge with an SW28.1 rotor at 100,000 × *g* for 1 h at 4°C to separate organelles from the soluble fraction. Both the bacteroid and particulate fractions were checked for purity by electron microscopy. The nutrient solution in which bacteria grew for 10 days was separated from the bacteria by centrifugation in a Sorval centrifuge with a GSA rotor at 16,000 × *g* for 20 min at 3°C. Of the supernatant fraction, 400 ml was reduced to 10 ml, by vacuum evaporation at 40°C, and assayed for soyrhizins.

Purification of soyrhizin A. Nodules (10 g) were homogenized in a Polytron homogenizer (Brinkman) at speed 7 for 3 min in 50 ml of 1% (wt/vol) picric acid to precipitate proteins and nucleic acids and then were centrifuged in a Sorval centrifuge with a GSA rotor for 20 min at 25,000 × *g* at 3°C. The excess picric acid was quantitatively removed by passage through Dowex AG 2x8 anion-exchange resin, prepared as described previously (21). The volume of the eluant was reduced to approximately 1 ml and adjusted to pH 4.0. A cation-exchange column was prepared by introducing a slurry of 10 g of Beckman PA-35 resin and 0.25 N NaOH in a column (16.5 by 0.9 cm), rinsed with 0.25 N NaOH, and then equilibrated with elution buffer-sodium citrate (pH 5.26). The concentrated extract (1 ml) ran through this column at 40°C with a flow rate of 40 ml/h. Three-ml fractions were collected, and 0.3 ml of each fraction was assayed with ninhydrin (19).

Rhizobins eluted after arginine in three distinct peaks (Fig. 1). The location of arginine (Calbiochem-Behring) was used as a marker. The retention time of rhizobins A, B, and C was approximately 4 h. Fractions which contained rhizobin A were pooled (approximately 25 ml) and passed through a Dowex-1 formate anion-exchange resin. To remove formate and water, the eluate was vacuum evaporated at 70°C until crystals formed and then further dried in an oven at 90°C for 1 h. To remove NaCl, the sample was suspended in 1 ml of water and eluted from Bio-Rad P-2 Bio-Gel (100/200 wet mesh). The purity of the sample was checked for amino acids on an amino-acid analyzer, for citrate (22), for Cl^- with 0.02 M $AgNO_3$, for phosphates (7), and for reducing carbohydrates (6). No contamination was present. This purified preparation was used to determine the molecular mass and amino-acid composition.

In the extraction procedure, 1% picric acid can be replaced by the same concentration of trichloroacetic acid, acetic acid, or HCl.

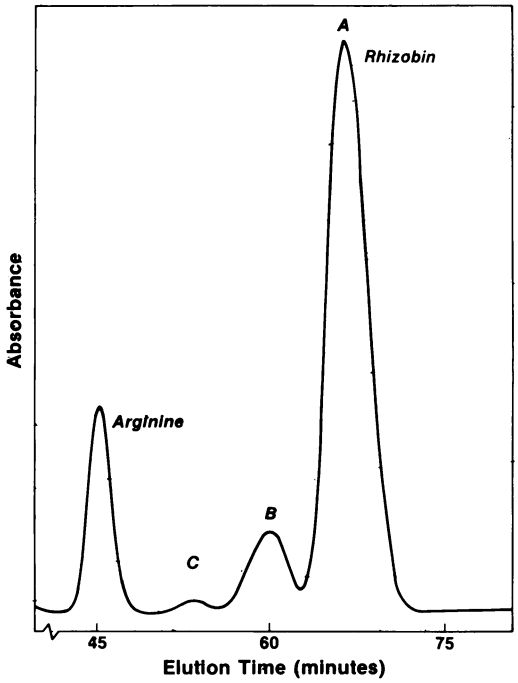

FIG. 1. Ion-exchange elution profile of rhizobins A, B, and C from 50-day-old nodules. Absorbance is in arbitrary units.

Molecular mass. Two different methods were used to determine the molecular mass of soyrhizin A. The first was the conventional method of Sephadex G-25 gel filtration chromatography. The column was calibrated with a series of peptides whose molecular masses were known, purchased from Serva Feinbiochemica, Heidelberg, Federal Republic of Germany. Rhizobin A was run on the column both separately and with the standards. Plasma desorption mass spectrometry (14) was the second method used to determine molecular mass.

Amino-acid composition. Samples of rhizobin A were hydrolyzed in 6 N HCl at 110°C for 6 to 48 h in the absence of oxygen. Hydrolysates sampled at various time intervals were analyzed to determine serine content by extrapolation (10). Surprisingly, hydrolysis was incomplete; even in 48 h only 70 to 85% of rhizobin A was disrupted. Thus, trifluoroacetic acid hydrolysis was performed by the method of Tsugita and Scheffler (23). The results were consistent with those for HCl hydrolysis, both with respect to the recovered amino acids and the difficulty of hydrolysis. The cause of this difficulty is unknown. In control experiments, bactracin A was quantitatively hydrolyzed by both methods, and the amino acids recovered were consistent with the known amino-acid composition. The lengthy (48 h) and incomplete hydrolysis caused an uncertainty in the quantitation. As a precaution samples of rhizobin A were sent to the protein laboratory of the University of Texas, Austin, for hydrolysis; the same difficulties were encountered.

RESULTS

Concentration and localization of rhizobins. The concentration and localization of rhizobins in approximately 50-day-old nodules and free-living bacteria are given in Table 1. The approximate age of the nodules was ensured by selecting

TABLE 1. Amount and localization of rhizobins A, B, and C in soybean nodules and in free-living *Rhizobium japonicum*[a]

Rhizobin type	Amt[b] of rhizobins in:			Nutrient solution[c]
	Cortex	Medulla	Soluble fraction (cytosol)	
A	483	589	405	147
B	43	45	38	—
C	4	4	2	—

[a] Nodules from 50-day-old plants were fractionated and tested for rhizobins A, B, and C. Free-living bacteria were cultured in liquid medium for 10 days and separated from the medium and then were tested for rhizobins. Rhizobins were detected by their characteristic elution profile from the basic column of the amino-acid analyzer. No rhizobins were detected in bacteroids, particulate fraction (obtained by centrifugation at 100,000 × *g* for 1 h), or free-living bacteria.

[b] Micrograms of rhizobins in arginine equivalents per gram (fresh weight) of the indicated nodule tissue, fraction, or solution. —, Not detected. The standard deviation (sigma) is ±14%.

[c] Solution in which free-living bacteria grew for 10 days.

only tap root nodules. All of the rhizobins were confined to the soluble fraction. They were present in both the cortex and medulla, and they could not be detected in either bacteria or bacteroids; however, rhizobin A, but not B and C, was present in the nutrient solution in which bacteria grew for 10 days. Similarly, 3 to 10 μg of rhizobin A could be identified in 1 g of vermiculite-perlite which surrounded the nodules.

The percentage of rhizobin B per total amount of rhizobins A plus B was 11.7 ± 4.2%. Rhizobin C was detectable only in tap root nodules older than 35 days. Rhizobins A, B, and C were found in *Lupinus texensis* nodules at concentrations of 152, 14, and 2 μg/g (fresh weight), respectively; thus, they were not confined to soybean nodules. In alder nodules, however, they were not detected. Soyrhizins were not detected in plant parts other than nodules (roots, stems, or leaves).

Chemical characterization. Purified rhizobin A was stable; neither boiling for 20 min in sodium citrate buffer (pH 5.2) nor evaporation to dryness at 90°C decreased the amount or damaged the integrity of the molecule as determined from the elution profile of the amino-acid analyzer. Based on the results of gel filtration on Sephadex G-25 (Fig. 2), the molecular mass of rhizobin A was around 1,600 daltons. In close agreement with this, the plasma desorption mass spectrum of soyrhizin A was centered at around 1,625 daltons.

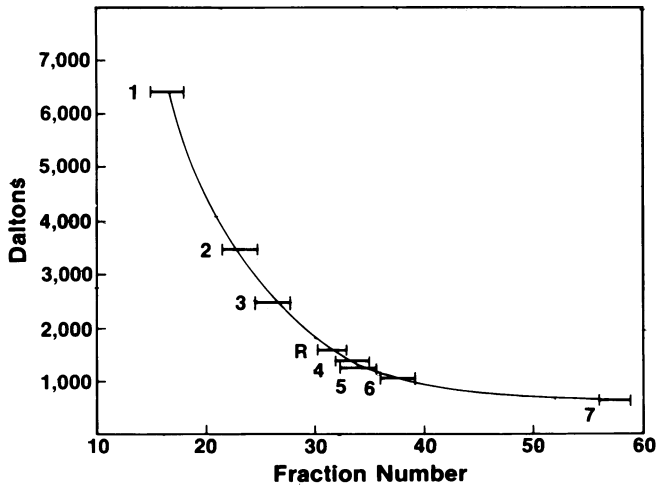

FIG. 2. Gel filtration pattern from Sephadex G-25 of rhizobin A (S) as compared with 7 peptides of known molecular mass. The estimated molecular mass of rhizobin A is 1,600 daltons.

The amino-acid composition is shown on Table 2. As a consequence of prolonged hydrolysis, the data were accompanied by large experimental errors; therefore, exact molecular-mass calculations are premature. Preliminary hydrolysis data indicate that rhizobin B is also a peptide. Rhizobin C has not yet been investigated because of limited amounts.

Effect of combined nitrogen. The effect of combined nitrogen was determined from an experiment in which nodulation and nodule growth were initially allowed to progress in a nitrate-free solution, followed by exposure to nitrate for 2 weeks. Nitrate caused a decrease in nodulation, nodule growth, and nitrogen fixation, as well as in soyrhizin concentration, compared with the control nodules (Fig. 3). Thus, all parameters decreased in response to nitrate addition; quantitatively, the decrease varied considerably.

The subsequent removal of nitrate resulted in an increase in both nitrogenase activity and rhizobin A content. Complete recovery of nitrogenase activity occurred. The presence of newly initiated nodules can not explain this recovery because all measurements were made from tap root nodules. Death, deterioration, and subsequent replacement by new nodules were impossible within the time course of this experiment. Rhizobin A content increased after nitrate removal, although it did not reach the levels attained by control nodules. Nodule growth followed a simlar trend, but only after a considerable lag phase. Nodulation, however, did not increase significantly after nitrate removal. In other words, after nitrate removal, all four parameters followed different patterns of recovery; however, similarities can be noted between the rhizobin content and acetylene reduction assay (Fig. 3).

It should be emphasized that Fig. 3 represents relative data, i.e., the percentage of the treated relative to control. The absolute concentration of rhizobins did not drop, but only their accumulation (synthesis) stopped due to nitrate addition on day 10 (Fig. 4). After nitrate removal on the day 24, the accumulation of rhizobins began again. In the control plants, which grew in nitrate-free solution, the content of rhizobins A and B steadily increased except for a large decrease on day 29. The cause of this decrease is unknown, although it may have been due to the several rinses at days 24 and 26. The amount of rhizobins per gram (fresh weight) increased during the 42 days of nodule growth from about 2 to 300 μg (Fig. 3).

TABLE 2. Amino-acid composition of rhizobin A.

Amino acid	No. of residues[b]
Ala	1.75 ± 0.71
Asx	3.01 ± 0.74
Glx	2.83 ± 0.99
Gly	2.74 ± 0.93
Ile	0.76 ± 0.25
Leu	1.26 ± 0.10
Ser	4.32 ± 0.39

[a] The amino-acid composition of rhizobin A was determined after hydrolysis in either 6 N HCl (4 samples) or concentrated HCl-triflouroacetic acid (2:1 mix, 2 samples).

[b] Number of residues per molecule and the variation from the mean.

DISCUSSION

Rhizobins appear by their chromatographic behavior to be basic (Fig. 1); thus, Asx and Glx are probably present as amides rather than acids. Additionally, it is likely that the chromatographic elution of rhizobins is not due entirely to ion-exchange chromatography but at least in part to hydrophobic interactions with the polystyrene support of the ion-exchange resin. The fixed ratios among rhizobins do not necessarily indicate a chemical relationship.

Rhizobin A is a rather stable peptide; prolonged hydrolysis by both the HCl and trifluoroacetic acid methods was necessary to cleave the molecule into its constituent amino acids. The cause of this difficulty is unknown, but unusual linkages, which have been observed among plant peptides (9), may be present in rhizobin A, as well as B and C.

Although rhizobins were not detected in free-living *Rhizobium japonicum*, rhizobin A was found in the nutrient media in which the bacteria had grown (Table 1). Small amounts of rhizobin A were also present in the vermiculite-perlite which surrounded the nodules. Apparently rhizobin A is synthesized by the bacteria or bacteroids and immediately released into the nutrient solution or into the surrounding plant part of the nodules. From the latter, rhizobin A is further exuded into the soil. Since rhizobins B and C could not be detected in saprophytic culture media, but in nodules only, they may be synthesized by either the plant part of the nodules or by the bacteroids as a response to some stimulant from the plant.

Rhizobin acumulation apparently depends on nitrogen fixation since the presence of nitrate inhibits both nitrogen fixation and rhizobin accumulation (Fig. 3). Experiments do not prove any direct correlation between nodulation or nodule growth and rhizobin A content. However, the continual accumulation of rhizobins with respect to the fresh weight of nodule tissue (Fig. 3) suggests a potentially important role in root nodule physiology.

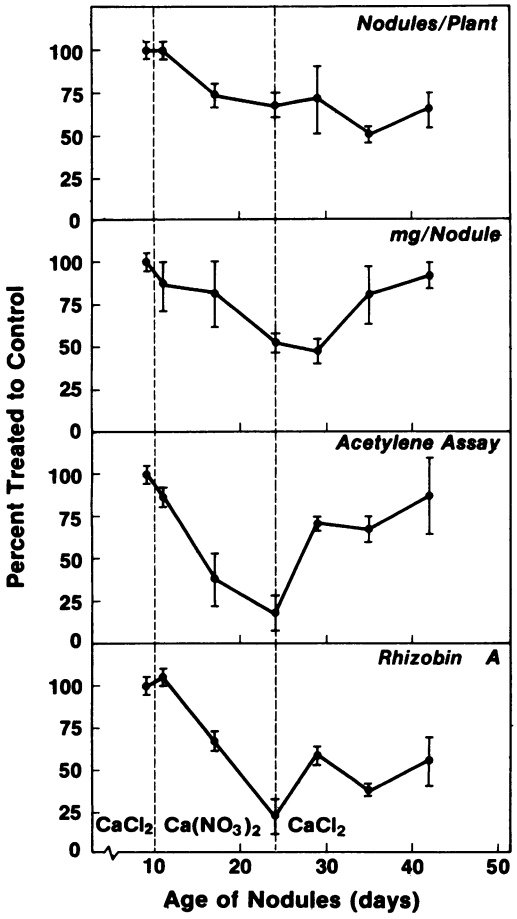

FIG. 3. Effect of combined nitrogen [$Ca(NO_3)_2$] on nodulation (number of nodules per plant), growth of nodules (milligrams [fresh weight] per nodule), nitrogenase activity (acetylene reduction assay), and rhizobin A content as expressed in arginine equivalents. Control plants grew without combined nitrogen ($CaCl_2$); combined nitrogen [$Ca(NO_3)_2$] was added to the nutrient solution of treated plants between days 10 and 24 as indicated by dashed lines. The following absolute data were observed during the time course for control plants: nodules per plant, 16 to 45 (includes all nodules); milligrams (fresh wt) per nodule, 7 to 34 (only root tap nodules); acetylene assay, 6 to 7 moles per gram (fresh weight; only tap root nodules); rhizobins, see Fig. 4.

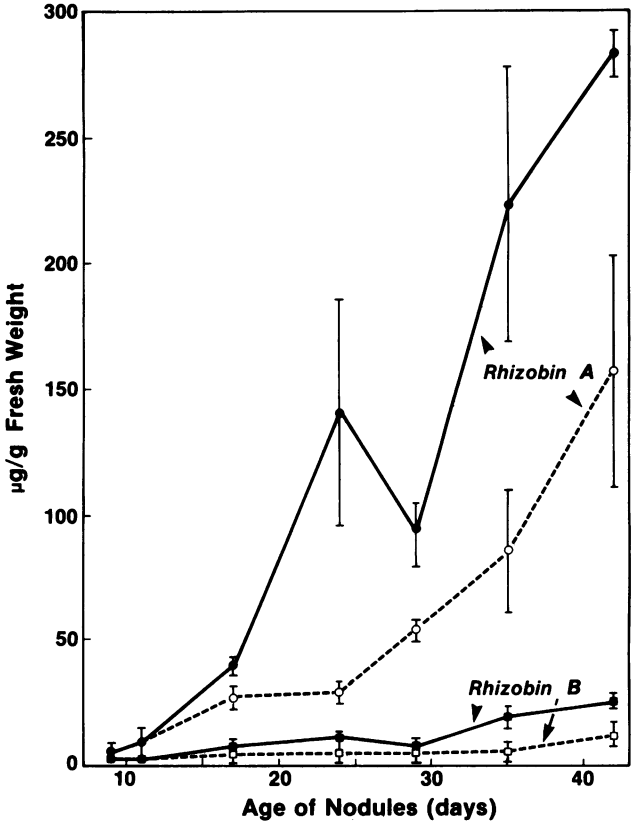

FIG. 4. Changes in the amount of rhizobin A (●, ○) and B (■, □), expressed in arginine equivalents, during the growth of nodules. Solid lines, Nodules grown with $CaCl_2$ in the nutrient solution; dashed lines, nodules grown with $Ca(NO_3)_2$ in the nutrient solution between days 10 and 24. These data are obtained from the same group of plants used to obtain the results shown in Fig. 3.

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