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# Rapid and Sensitive Assay for the Phytotoxin Rhizobitoxine†

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**Rhizobitoxine is a phytotoxin synthesized by some strains of the legume symbiont genus *Bradyrhizobium* and the plant pathogen *Pseudomonas andropogonis*. We demonstrate here a new enzymatic assay which is 100-fold more sensitive than previous assays and can detect as little as 1.0 pmol of rhizobitoxine. The assay is based on the inhibition of *Salmonella typhimurium* β-cystathionase by rhizobitoxine. Interestingly, β-cystathionase from *Bradyrhizobium japonicum* is insensitive to rhizobitoxine at concentrations lower than 75 μM.**

Rhizobitoxine, a phytotoxin, is synthesized by some strains of the legume symbionts in the genus *Bradyrhizobium* (1, 13, 14) and by the plant pathogen *Pseudomonas andropogonis* (9, 10). This bacterial toxin induces foliar chlorosis of the host plant. The role of the toxin in the symbiosis between the genus *Bradyrhizobium* and its plant hosts is not understood. Rhizobitoxine production is common among bradyrhizobia, with over 50% of strains surveyed from diverse geographical areas testing positive for rhizobitoxine production (5). Several recent studies have tested strains of *Bradyrhizobium japonicum* for rhizobitoxine production, hydrogenase activity, and nodulation effectiveness (2, 6, 7). These studies used time-consuming and insensitive methods based on the induction of chlorosis symptoms in plants or on large-scale purification procedures followed by thin-layer chromatography. Another method to detect rhizobitoxine uses an amino acid analyzer and is more sensitive, but it requires expensive equipment and is time consuming (8).

Rhizobitoxine is a structural analog of cystathionine (Fig. 1B) and irreversibly inactivates β-cystathionase (3). Its $K_i$ for *Salmonella* β-cystathionase is $2.2 \times 10^{-8}$ M (11). We developed an enzyme assay, based on the inhibition of β-cystathionase by rhizobitoxine, which is both rapid and sensitive to concentrations as low as 10 nM.

The rhizobitoxine assay is schematically presented in Fig. 1A. β-Cystathionase was isolated from a 2-liter culture of *Salmonella typhimurium* 757 grown overnight in M9 medium at 37°C. The purification method was essentially that described by Kaplan and Flavin (4). Two liters of *S. typhimurium* were cultured in M9 medium for 20 h at 37°C, and cells were harvested by centrifugation at 10,000 rpm in a Beckman JA10 rotor for 15 min at 4°C. Cells were resuspended in cold extraction buffer (50 mM potassium phosphate [pH 7.3]–0.1 mM β-mercaptoethanol–0.05 mM pyridoxal phosphate) at about 3 ml per 1 g of cells. Resuspended cells were lysed by sonication for 3 to 5 min with a double-step microtip at 10% duty cycle with an output of about 20 W. The cell lysate was cleared by centrifugation in a Beckman SW28.1 rotor at 20,000 rpm for 30 min at 4°C. The supernatant was collected, and the protein concentration was determined by use of the Bio-Rad protein assay. The protein concentration was adjusted to 10 mg/ml by dilution with extraction buffer and supplemented with pyridoxal phosphate to a final concentration of 0.2 mM. The pH was adjusted to 6.5 with acetic acid. The extract was quickly heated in a glass beaker to 60°C by being placed in a 95°C water bath. The extract was allowed to cool by standing at room temperature for 3 min. The extract was further cooled to 20°C in an ice-water bath. The cloudy white precipitate was removed by centrifugation at 22,000 rpm for 1 h at 4°C in an SW28.1 rotor. The protein concentration of the supernatant (faintly yellow cloudy solution) was determined as described above. Protein concentrations ranged between 2.0 and 3.0 mg/ml, and the specific activity of β-cystathionase was 30 to 60 U/mg of protein. The enzyme extract was diluted with an equal volume of 0.2 mM pyridoxal-P–0.1 mM β-mercaptoethanol–50 mM $K_2HPO_4$ (pH 7.3), divided into aliquots of 1 ml, and stored at −80°C. Ninety-five percent of the enzyme activity remained after 2 years of storage.

The enzyme assay was performed conveniently in 96-well microtiter plates. To each well was added a 50-μl aliquot of a test sample, such as bacterial culture, culture supernatant, rhizobitoxine standard, or plant tissue extract. The β-cystathionase was added in 25-μl aliquots. The enzyme was prepared by dilution of β-cystathionase enzyme stock with reaction buffer (100 mM Tris HCl [pH 8.3]) such that there were about 0.3 to 0.4 enzyme units per 25-μl aliquot. The sample and enzyme were mixed by carefully tapping the side of the microtiter plate, and the reaction mixture was incubated for 10 to 15 min at room temperature. This procedure allows rhizobitoxine the time necessary for it to inactivate the enzyme. A 25-μl aliquot of 10 mM L-(+)-cystathionine–0.1 M Tris HCl (pH 8.6) was added and mixed as described above. The reaction mixture was covered and incubated for 1 h at 37°C.

The products of the β-cystathionase reaction are homocysteine, ammonia, and pyruvate (Fig. 1A). Pyruvate was detected by adding 50 μl of 2,4-dinitrophenylhydrazine (color reagent from Sigma Chemical Co.) to each microtiter well and incubating them for 10 to 15 min at room temperature. Color developed upon addition of 50 μl of 5 N NaOH, and $A_{450}$ was determined with a microtiter plate reader. Since rhizobitoxine inhibits pyruvate formation, the absence of color is a positive indicator of rhizobitoxine. To determine the background reactivity of the color reagent with the sample, controls without β-cystathionase or without cystathionine were included. The control without β-cysthathionase gives a background level of reaction of the color reagent with the sample. This value is subtracted from the assay value to determine the change in absorbance caused by the production of pyruvate. The control without cystathionine

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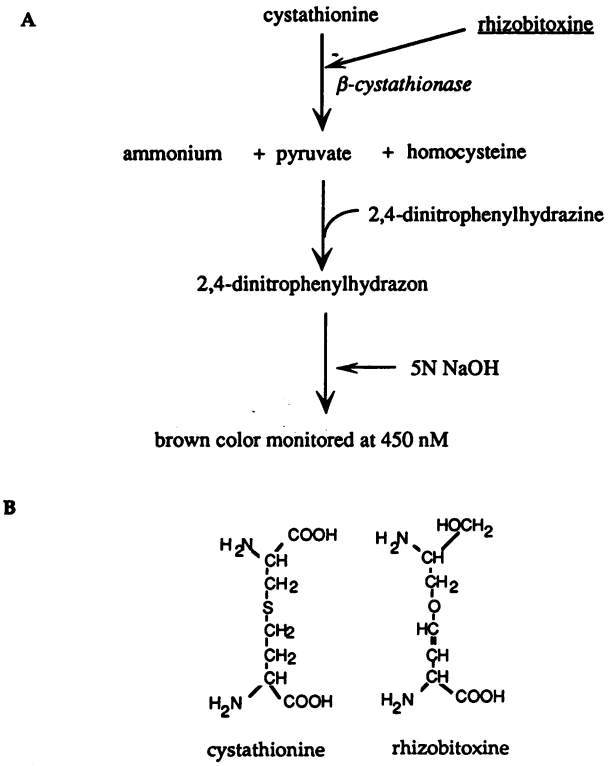


FIG. 1. Enzyme assay flow diagram and molecular structures. (A) β-Cystathionase converts cystathionine into three products. One of these products, pyruvate, can be quantitatively determined spectrophotometrically by measuring the color change caused by 2,4-dinitrophenylhydrazine. However, in the presence of the toxin, the β-cystathionase is quantitatively inactivated, preventing the conversion of cystathionine to pyruvate. (B) Structures of cystathionine and rhizobitoxine [2-amino-4-(2-amino-3-hydroxypropoxy)-*trans*-but-3-enoic acid].

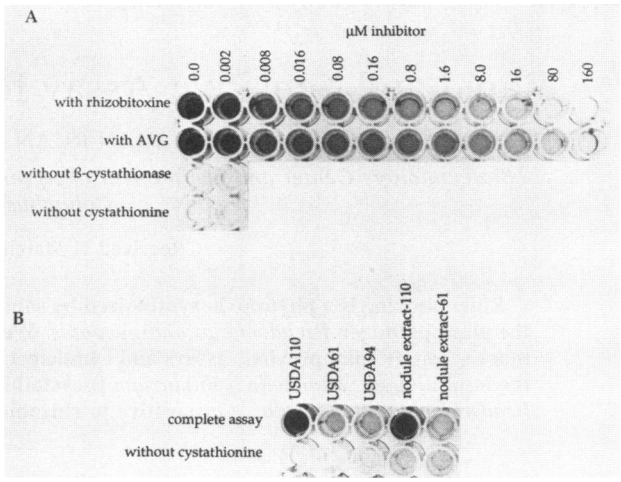


FIG. 2. Enzyme assay in microtiter plate. (A) A dilution series of authentic rhizobitoxine was used to create a standard inhibition curve of β-cystathionase along the top row. The second row shows a similar dilution series with AVG. The concentrations of rhizobitoxine and AVG are indicated above the wells. Controls without β-cystathionase and without cystathionine are included in the third and fourth rows, respectively. (B) The concentration of rhizobitoxine was determined in the bacterial culture supernatants of strains USDA 110, USDA 61, and USDA 94, and from cultivar Lee nodule extracts formed by strains USDA 110 and USDA 61.

ensures that any production of pyruvate is dependent upon addition of the substrate and not on some other extraneous enzyme activity. The absorbance values obtained ranged from 1.2 for the complete reaction mixture to 0.1 for the control with no cystathionine. For each measurement, one standard deviation was less than 10% of the mean.

The amount of rhizobitoxine in a given sample can be determined by comparing the color densities with a standard inhibition curve generated by using purified rhizobitoxine (Fig. 2A and 3). Authentic rhizobitoxine isolated from *P. andropogonis* 7076 by ion-exchange chromotography was kindly provided by R. E. Mitchell (9, 10). As shown in Fig. 3, a sample containing as little as 0.01 μM rhizobitoxine can be distinguished from background. In a sample volume of 50 μl, this is equal to 1.0 pmol of rhizobitoxine. This level of detection is 100-fold more sensitive than the detection limit of 110 pmol per 50-μl sample as previously described for the amino acid analyzer method of Minamisawa and Kume (8). To achieve this sensitivity, it is important to use the amount of enzyme specified and not more, because more will swamp out the rhizobitoxine.

In addition to rhizobitoxine, there are other antimetabolites produced by microorganisms which have similar structures and biochemical activities. Aminoethoxyvinylglycine (AVG) has a structure similar to that of rhizobitoxine, and the ability of AVG to inhibit β-cystathionase was determined for comparison (Fig. 2A). AVG is approximately 10 times less effective than rhizobitoxine as an inhibitor of β-cystathionase.

The usefulness of this assay is illustrated by detection of rhizobitoxine in cultures of *Bradyrhizobium* strains such as USDA 61, which make too little rhizobitoxine in culture to have been detected by previous methods (14). *Bradyrhizobium* strain USDA 61 was cultured in YEMG medium (15) and in YEMG supplemented with 1 g of Casamino Acids per liter (YEMG+CA) (Fig. 4). Samples of cultures were taken at the indicated times, cells were removed by centrifugation, and the supernatants were stored frozen at −80°C for processing later. Culture samples (50 μl) were assayed

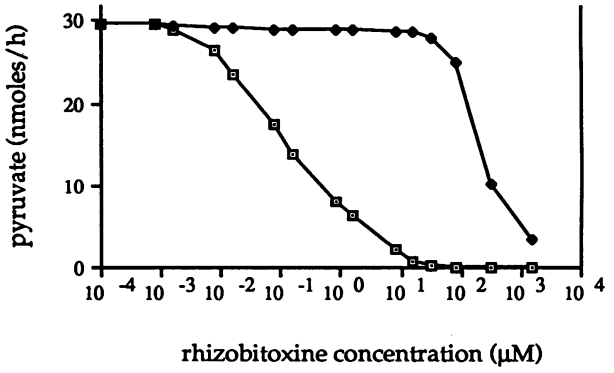


FIG. 3. Inhibition of *Salmonella* (□) and *Bradyrhizobium* β-cystathionases (◆) by rhizobitoxine. Data from an experiment performed as described in the legend to Fig. 2A are presented in graph form for both the inhibition of *Salmonella* and *Bradyrhizobium* β-cysthathionases. The enzyme activity is reported as nanomoles of pyruvate produced in 1 h.

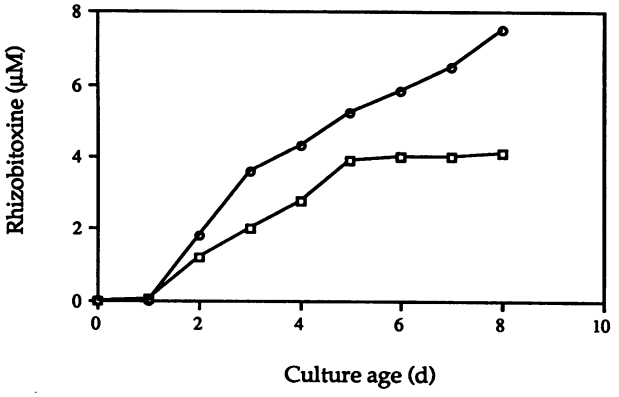


FIG. 4. Accumulation of rhizobitoxine in cultures of *B. japonicum* USDA 61. *B. japonicum* USDA 61 was cultured in YEMG (□) or YEMG+CA (○) medium, and rhizobitoxine was quantified in culture supernatant.

directly without being concentrated or undergoing any other sample preparation. Significant amounts of rhizobitoxine were produced in both cultures, but the rhizobitoxine concentration in YEMG+CA (7.6 μM) was almost double the concentration in YEMG. The greater amount of rhizobitoxine in YEMG+CA versus that in YEMG is associated with a parallel increase in the growth of the culture and not a specific enhancement of rhizobitoxine production. As a negative control, strain USDA 110 showed no production of rhizobitoxine from 8-day-old cultures in either YEMG or YEMG+CA (Fig. 2B).

The culture or culture supernatant can often be assayed directly without sample preparation. However, some bacteria, such as *P. andropogonis*, produce metabolites in culture which react with the color reagent, causing a high background. In these cases, it is possible to clean up the sample by using cation-exchange chromatography as described below for nodule extracts.

Because of the low $K_i$ of rhizobitoxine for the *Salmonella* β-cystathionase, we also isolated β-cystathionase from strain USDA 61 as described above to determine its sensitivity to rhizobitoxine. Interestingly, we found that rhizobitoxine has no effect on the activity of the USDA 61 β-cystathionase at concentrations lower than 75 μM, a concentration at which the *Salmonella* enzyme activity is 99% inhibited (Fig. 3). However, at higher concentrations of rhizobitoxine (1,000 to 1,500 μM), the activity of USDA 61 β-cystathionase also can be inhibited.

Soybean cultivars show differential reactions to the rhizobitoxine-synthesizing bradyrhizobial strains such that many cultivars do not show chlorosis and are considered resistant. Previously, rhizobitoxine had not been found in the nodules of resistant plants (13). To examine this situation more closely with our assay, a sensitive soybean cultivar, Lee, and a resistant cultivar, Williams, were inoculated with a rhizobitoxine-producing strain (USDA 61) and a nonproducing strain (USDA 110) of *B. japonicum*. Plants were grown in modified Leonard jars constructed from Magenta boxes (Magenta Co., Chicago, Ill.) containing vermiculite. Plants were watered with sterile Jensen's nitrogen-free solution (16) and were grown in a plant growth chamber with a cycle of 16 h of light (28°C)–8 h of dark (18°C). Each root was inoculated with 1 ml of *Bradyrhizobium* cells which had been grown in YEMG to log phase and diluted with sterile water to a density of about $10^8$ cells per ml. After 30 days of growth, 1 g of fresh nodule tissue was ground in liquid $N_2$ and extracted with 5 ml of 0.1 M Tris HCl (pH 7.5). The homogenates were heat treated at 95°C for 10 min and cleared by centrifugation at 14,000 rpm for 5 min. The resulting supernatants had a high reactivity with the color reagent, generating a significant background. To reduce background, supernatants were passed over an ammonium-charged Dowex 50W column (5-ml bed volume). The column was washed with 10 column volumes of distilled water, and rhizobitoxine was eluted with 4 column volumes of 0.1 M $NH_4OH$ (11). The eluted materials were dried by vacuum distillation, and the pellet was resuspended in 2 ml of distilled water for use in the enzyme assay. Rhizobitoxine was found only in nodules formed by the rhizobitoxine-producing strain USDA 61 (Fig. 2B): 142 nmol of rhizobitoxine per g (fresh weight) was found in the cultivar Lee and 5 nmol/g was found in the cultivar Williams. The amount of rhizobitoxine found in soybean cultivar Lee nodules using our assay is similar to the value measured by amino acid analysis in nodules of cultivar Norin-2 and *B. japonicum* USDA 94 (8). Furthermore, rhizobitoxine was 30-fold less abundant in the nodules of the resistant cultivar Williams than in the root nodules of the sensitive cultivar Lee.

With the high specificity and sensitivity of this enzyme assay, rhizobitoxine production can be detected directly in bacterial culture or in tissue samples after minor sample preparation. The usefulness and importance of this assay are illustrated by the detection of the toxin under conditions in which it was previously not detected. This assay will facilitate a broad survey of rhizobitoxine production among bacterial species and provides a useful tool for further study of the regulatory mechanism of rhizobitoxine production and the isolation of rhizobitoxine mutants of *Bradyrhizobium* strains.

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