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Analysis of Poly-β-Hydroxybutyrate in *Rhizobium japonicum* Bacteroids by Ion-Exclusion High-Pressure Liquid Chromatography and UV Detection†

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Ion-exclusion high-pressure liquid chromatography (HPLC) was used to measure poly-β-hydroxybutyrate (PHB) in *Rhizobium japonicum* bacteroids. The products in the acid digest of PHB-containing material were fractionated by HPLC on Aminex HPX-87H ion-exclusion resin for organic acid analysis. Crotonic acid formed from PHB during acid digestion was detected by its intense absorbance at 210 nm. The Aminex-HPLC method provides a rapid and simple chromatographic technique for routine analysis of organic acids. Results of PHB analysis by Aminex-HPLC were confirmed by gas chromatography and spectrophotometric analysis.

The symbiotic fixation of atmospheric nitrogen by rhizobial species and leguminous plants requires considerable energy (5, 12). This energy is derived from the metabolism of photosynthetically produced carbohydrates that are transported to the root nodules, the site of symbiotic nitrogen fixation. It is generally assumed that during the most active period of dinitrogen reduction, the fixation process is limited by availability of carbon metabolites (6, 19). However, it is during this period that the carbon storage polymer, poly-β-hydroxybutyrate (PHB), rapidly accumulates. To determine the interaction of carbon and nitrogen metabolism in the *Rhizobium japonicum*-soybean symbiosis, we have focused on the metabolism and regulation of PHB and its relationship to nitrogen fixation.

PHB analysis relies on measuring crotonic acid which is formed by acid-catalyzed elimination during chemical depolymerization of PHB. Recently, several papers have appeared describing qualitative methods for monitoring PHB (2, 10, 11). Findlay and White (4) have reported a quantitative method that uses gas chromatographic mass spectral analysis to measure polymeric beta-hydroxyalkanoates. However, only a few alternative methods, gas chromatographic (GC) (13) and infrared (7) analyses, for routine selective quantitation of PHB have been reported since the spectrophotometric method of Law and Slepecky appeared in 1961 (8). Although the latter method is suitable for surveys and qualitative comparisons, it lacks accurate quantitation and the specificity that is required to determine the interaction between PHB metabolism and nitrogen fixation. Since many common biological compounds will absorb in the 200- to 230-nm region after sulfuric acid digestion, the use of the procedure of Law and Slepecky (8) requires that samples be highly purified. The purification procedure may result in losses of short-chain-length PHB polymers and involves a considerable amount of time to process and analyze samples. This report describes ion-exclusion high-pressure liquid chromatography (HPLC) for the rapid assay of PHB. The method uses Aminex HPX-87H for organic acid analysis and UV detection and can measure crotonic acid in samples containing 0.01 to 14 μg of PHB. Since the HPLC method can be used to measure PHB from sulfuric acid digestion of *R. japonicum* bacteroids, the method avoids problems associated with, and time required for, sample purification. Data are presented comparing the HPLC method with the spectrophotometric and GC methods.

MATERIALS AND METHODS

Source of materials. *R. japonicum* 3I1B-143, was kindly supplied by Harold Evans, Laboratory for Nitrogen Fixation Research, Oregon State University, Corvallis. To obtain purified PHB, strain 3I1B-143 was grown to early-stationary phase (4 days) in 20 liters of Vincent's liquid medium (17) which had been inoculated with 500 ml of a suspension containing 10^9 cells per ml. The cells were harvested with the aid of a Sharples centrifuge. After digestion of the bacterial cells in Chlorox (5.25% sodium hypochlorite; The Chlorox Co., Oakland, Calif.) (20), the insoluble material was collected by centrifugation. Final purification of PHB

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was accomplished by extraction into and then precipitation from chloroform following the procedure of Law and Slepecky (8) as modified by Tanaka et al. (16). Buffers and chemicals were purchased from Sigma Chemical Co., St. Louis, Mo. Crotonic acid was recrystallized from freshly distilled *n*-butanol before use. Reagent-grade sulfuric acid was purchased from Fisher Scientific Co., Pittsburgh, Pa., or American Scientific Products. Polyvinyl polypyrrolidone (Polyclar AT) was purchased from GAF Corp., New York, N.Y. Before use, the polyvinyl polypyrrolidone was washed with 10% hydrochloric acid, neutralized, washed with distilled water, and dried. High-purity gases (N_2, H_2, O_2) were obtained from local commercial sources. Soybean seeds (cv. Williams) coated with a Bactosoil-based inoculum (Michigan Peat Co., Houston, Tex.) of strain 3I1B-143 were planted in 8-in. (ca. 20.32-cm) pots containing Perlite as the growth support. Plants were supplied daily with a nitrogen-free nutrient solution (1) for 5 days and flushed with tap water on days 6 and 7. They were provided with supplemental light (250 microeinsteins $\cdot m^{-2} \cdot s^{-1}$) for 14 h each day. Nodules were harvested from 2- to 6-week-old plants and frozen at −80°C until use.

Nitrogenase activity. Nitrogenase activity (micromoles of ethylene produced per hour per gram of fresh nodule weight) was determined by the acetylene reduction method on intact root nodule segments (14). Ethylene production was measured at 4-min intervals over a 20-min period.

Isolation of bacteroids. Nodules were disrupted (3) with a Waring blender in MEP buffer (5 mM $MgCl_2$, 1 mM EDTA, 50 mM potassium phosphate [pH 7.0]) containing 17% sucrose (wt/vol) and polyvinyl polypyrrolidone (1 g of nodules, 0.33 g of polyvinyl polypyrrolidone, and 10 ml of MEP containing sucrose). The preparation was filtered through four layers of cheesecloth and centrifuged at 400 × *g* for 10 min at 5°C. The supernatant fraction was centrifuged at 8,000 × *g* for 15 min at 5°C. The pellet was dispersed into MEP buffer containing sucrose (2 ml/g of original weight of nodules) and was layered onto a gradient consisting of 30% (10 ml), 40% (5 ml), and 57% (6 ml) (wt/wt) sucrose in MEP. The gradient tubes were centrifuged in an SW28 rotor at 72,000 × *g* for 35 min at 5°C in a Beckman L8-55 ultracentrifuge. The bacteroid layer at the 40 to 57% sucrose interface was collected, diluted with 2 volumes of MEP, and applied to a second gradient composed of 30% (15 ml) and 40% (10 ml) (wt/wt) sucrose in MEP. The tubes were centrifuged as described above. The pellet was suspended in 30 ml of deionized, distilled water, collected by centrifugation at 8,000 × *g* for 15 min at 5°C, and resuspended in deionized, distilled water (1 ml/0.5 g of nodules). Samples were removed for dry weight determination and for PHB analysis. To obtain partially purified PHB, the suspension (0.5 to 1.5 ml), placed in 15-ml Corex tubes (prewashed in sulfuric acid; Corning Glass Works, Corning, N.Y.), was digested in 12 ml of Chlorox (5.25% sodium hypochlorite) for 2 h at 40°C. The insoluble material was collected by centrifugation at 9,000 × *g* for 10 min at 5°C, washed with water and then ethanol, and dried 30 min at 100°C. A possible option with samples of limited quantity is to omit the Chlorox treatment. The isolated bacteroid pellet was dried 1 h at 100°C.

Digestion of PHB and Aminex-HPLC analysis of crotonic acid. Samples ranging from 0.01 to 500 mg of PHB-containing material were digested in 1 ml of concentrated sulfuric acid at 90°C for 30 min. The tubes were cooled on ice, after which, a 4-ml volume of 0.014 N H_2SO_4 was added with rapid mixing. Before analysis by HPLC, samples were diluted an additional 5- to 100-fold with 0.014 N H_2SO_4 containing 0.8 mg of adipic acid per ml as an internal standard and filtered through a 0.45-μm HAWP membrane filter (Millipore Corp., Bedford, Mass.) to remove particulate material. The injection volumes ranged from 10 to 50 μl or sample concentrations from 0.2 to 560 μg/ml.

Samples were eluted with 0.014 N H_2SO_4 at a flow rate of 0.7 ml/min from an Aminex HPX-87H ion-exclusion organic acid analysis column (300 by 7.8 mm) (Bio-Rad Laboratories, Richmond, Calif.) preceded by an ion-exclusion guard column of Aminex HPX-85X. HPLC was performed with either a Waters Associates 6000 A solvent delivery system with U6K injector or a series 3 chromatograph (The Perkin-Elmer Corp., Norwalk, Conn.) with a variable loop injector (Rheodyne, Inc., Berkeley, Calif.). Absorbance of crotonic acid was measured at 214 nm (Waters 441 absorbance detector) or 210 nm (Perkin-Elmer LC-55 B detector). The amount of crotonic acid produced from PHB was calculated from the regression equation derived from known crotonic acid standards.

Spectrophotometric analysis. Samples containing PHB were digested in concentrated sulfuric acid and subsequently diluted with 0.014 N H_2SO_4 (0.3 to 12 μg of sample per ml of final concentration). The UV absorbance spectrum was measured with a model 250 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio). The concentration of crotonic acid was calculated from a set of standards or its molar extinction coefficient, log $\epsilon = 4.15$ at 208 nm (9).

GC analysis. Samples of digested PHB, either diluted to 0.35 N H_2SO_4 or extracted into *n*-butanol (freshly distilled and saturated with deionized, distilled water), were analyzed with a model 900 gas chromatograph (The Perkin-Elmer Corp.) equipped with a glass column (2 m by 4 mm) containing 10% SP 1200 on a Chromosorb W, 80 to 100 mesh poisoned with 1% H_3PO_4 (18). Compounds were eluted with a temperature program of 2°C increases per min from 130 to 150°C and detected with a flame ionization detector. Crotonic acid eluted at 142°C.

RESULTS

Conversion of PHB to crotonic acid. The conversion of PHB to crotonic acid in concentrated sulfuric acid at 85°C was complete within 20 min (Fig. 1). The maximum conversion was 82.4 ± 3.0%. Digestion at 100°C produced the same extent of conversion, 84.2 ± 3.2%. The recrystallized crotonic acid standard was stable for 60 min at either 85 or 100°C (Fig. 1), and under either digestion condition, 97.3 ± 1.8% was recovered. Loss of crotonic acid, however, did occur at high temperatures. At 160°C only 8.5 ± 1.8% was recovered after a 10-min digestion. For routine analysis, digestion was carried out at 90°C.

Analysis of crotonic acid by HPLC. Analysis of recrystallized crotonic acid in 0.014 N H_2SO_4 showed one peak with a retention time of 29 min

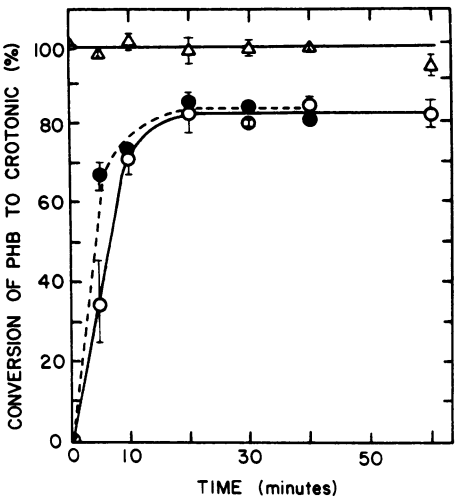

FIG. 1. Sulfuric acid digestion of PHB. Samples of recrystallized crotonic acid (5.85 mg, △) and PHB from Chlorox-treated bacteroids (5.1 mg, ○) were digested in 1-ml of concentrated H_2SO_4 at 85°C. PHB was digested also at 100°C (●). Samples were removed at the indicated time, and the crotonic acid content was measured by Aminex-HPLC (see text), 0.2 AU full scale (AUFS).

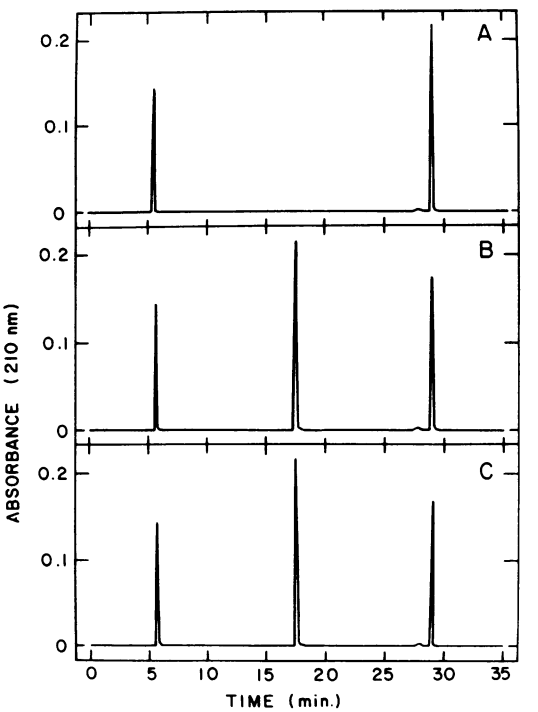

FIG. 2. Aminex-HPLC analysis of crotonic acid. (A) Crotonic acid, at 29 min, recrystallized from freshly distilled *n*-butanol. (B) PHB from Chlorox-treated bacteroids digested in concentrated H_2SO_4. (C) PHB-containing bacteroids digested directly in concentrated sulfuric acid. Chromatographic conditions are given in the text, 0.5 AUFS. The peaks at 6 and 17.5 min represent the solvent front and the internal standard, adipic acid, respectively.

(Fig. 2A). The treatment of crotonic acid with concentrated sulfuric acid (30 min, 85°C) caused no change in the elution pattern or signal intensity. The analysis of purified PHB after conversion to crotonic acid by sulfuric acid is displayed in Fig. 2B, and PHB from isolated bacteroids treated directly with sulfuric acid is presented in Fig. 2C. A comparison of the three chromatograms showed that each contained one major peak whose retention time was identical to that of crotonic acid. The total dilution of the samples in Fig. 2B and C was 50-fold. The elution profiles from digested PHB samples was identical regardless of the developmental stage at which the bacteroids were isolated. The omission of Chlorox did not affect PHB recovery. In duplicate bacteroid samples the PHB measured from untreated cells was 94.6 ± 4.0% of that from Chlorox-treated cells. Samples were retained in their original Corex tubes throughout the procedure. Their total dilution was 50-fold.

The relationship between the absorbance units (AU) and micrograms of digested PHB per injection is presented in Fig. 3. It was described by the regression equation $y = 0.116x - 0.005$, where y is absorbance at 210 nm and x is micrograms per injection. The square of the regression coefficient (r^2) was 0.995. The relationship between AU and micrograms of sample injected for recrystallized crotonic acid is compared to that for PHB purified from bacteroids or cultured strain 3I1B-143 (Fig. 4). The slope

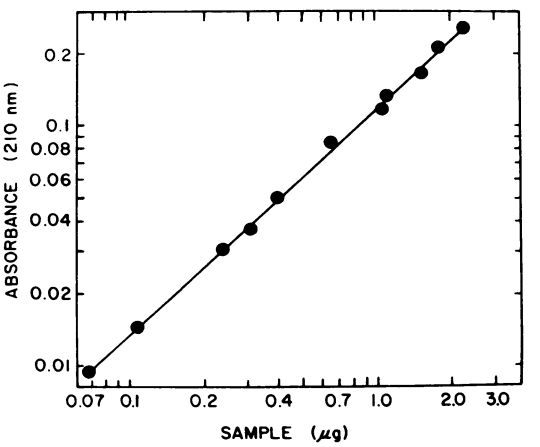

FIG. 3. The relationship between absorbance and PHB content. Samples from purified PHB digested in concentrated sulfuric acid were analyzed by Aminex-HPLC (see text), 0.5 AUFS. The standard deviation averaged 2.3% of the mean of duplicate injections, and the r^2 was 0.995.

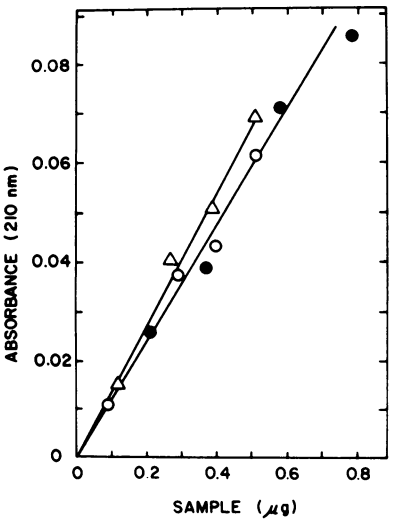

FIG. 4. Comparison of recrystallized crotonic acid and purified PHB. Samples of recrystallized crotonic acid (△) and PHB isolated by Chlorox-treated bacteria (○) or bacteroids (●) were digested in concentrated sulfuric acid and analyzed by Aminex-HPLC (see text), 0.05 AUFS. For recrystallized crotonic acid, the standard deviation averaged 3.3% of the mean of triplicate injections; the r^2 value was 0.984. For PHB (●, ○), the standard deviation averaged 3.3% and 1.2% for duplicate injections with r^2 values of 0.987 and 0.976, respectively.

from purified PHB-derived crotonic acid, 0.125 AU/μg, was 88% of that for recrystallized crotonic acid, 0.142 AU/μg. The lower slope may reflect impurities retained in the PHB.

GC analysis. GC analysis verified that the H_2SO_4 digest from Chlorox-digested material contained a single, major product which chromatographed as crotonic acid. Recrystallized crotonic acid produced a single peak, eluting at 142°C. The chromatograph of digested PHB was identical to the scan from crotonic acid. The conversion of Chlorox-insoluble material to crotonic acid was 88 ± 2%. This result was consistent with those obtained from HPLC analysis. The relationship between peak height (centimeters) and the amount of crotonic acid injected (2 to 6 μg) was described by the regression equation $y = 2.7x - 0.38$, where y is centimeters and x is micrograms per injection. The r^2 was 0.980.

UV analysis of crotonic acid in dilute acid. The absorbance spectra of recrystallized crotonic acid and H_2SO_4-digested PHB showed an intense absorbance maximum at 210 nm. The molar extinction coefficients calculated for recrystallized crotonic acid and PHB-derived crotonic acid were 1.545×10^4 (5.45 μg/ml) and 1.436×10^4 (4.68 μg/ml), respectively. These results agreed with those reported by Luskin (9); log ε was 4.15 in aqueous solutions at 208 nm. The data indicated a 93% conversion of PHB to crotonic acid and were linear over a range of 1.5 to 15 μg/ml. However, overestimation of the actual PHB content may be expected since absorbance measurement at 210 nm is not selective.

PHB accumulation in bacteroids from soybean nodules. The accumulation of PHB in bacteroids of soybean nodules inoculated with *R. japonicum* 3I1B-143 is shown in Fig. 5. PHB levels were determined by Aminex-HPLC analysis. The maximum levels of PHB, 40 to 45% of the bacteroid dry weight, were similar to those reported by Wong and Evans (20). Also, 80% of the PHB had been accumulated during the peak period of nitrogenase activity. Use of HPLC analysis, however, enabled measurement of PHB content of bacteroids from nodules in early development. The PHB content represented 6% of bacteroid dry weight from 14-day-old soybeans. Because prior purification is unnecessary, levels of PHB amounting to 0.5% of dry weight would have been detectable. The lowest level Wong and Evans (20) measured was 15% of the bacteroid dry weight from 18-day-old soybeans. This would approach the limit of the spectrophotometric method because of the required purification and restricted amounts of starting material.

DISCUSSION

This paper presents a method for measuring PHB content in bacterial samples by Aminex HPX-87H-HPLC analysis. In general, Aminex-HPLC methods are in common use for analysis of organic acids, sugars, and heterocyclic compounds. The major advantage of these methods is the separation and resolution of compounds before their detection and measurement. In particular, Aminex-HPLC analysis is especially applicable to experiments following PHB accumulation in physiologically developing material or verifying that mutants lack PHB. Such experiments may involve limited amounts of biological material with rapidly changing or vastly differing quantities of PHB. Methods requiring multiple steps in the isolation and purification of PHB often result in losses of material. In addition, measurement of PHB-derived crotonic acid at nonselective wavelengths without prior fractionation could introduce further errors. This may account for the differences in the percentage of PHB recovered by methods which incorporate fractionation of PHB before its measurement (HPLC, 84%, and GC analysis, 88%) and methods which do not (spectrophotometry, 93%). Slepecky and Law noted that certain compounds can interfere with the assay of crotonic acid at 235 nm (15). Many organic acids and

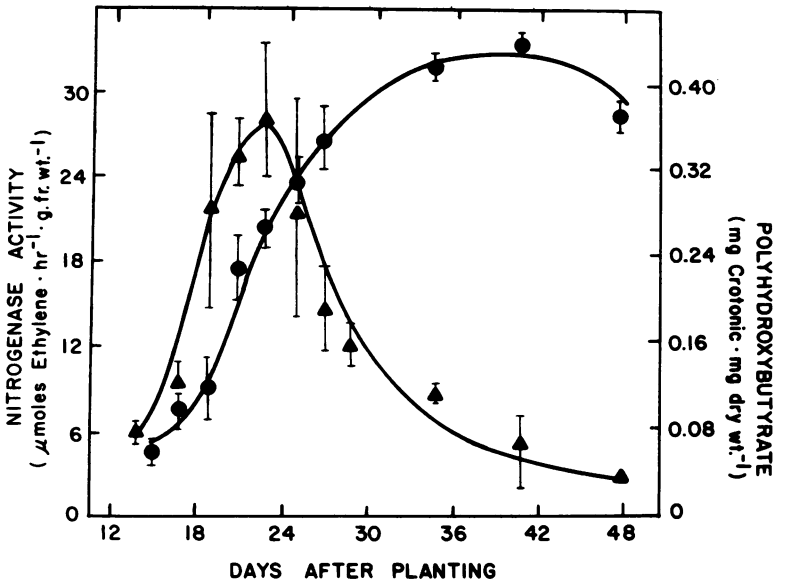

FIG. 5. PHB accumulation in soybeans. PHB (●) from Chlorox-treated bacteroids was digested in sulfuric acid and analyzed by Aminex-HPLC (see text), 0.05 AUFS. Values of nitrogenase activity (▲) and PHB content were the average of four replicates ± standard deviation.

bases as well as heterocyclic compounds absorb near 210 nm, and many of these compounds are common metabolites. Variation in their concentrations is not unexpected in experimental samples. Thus, an analytical method like HPLC, which includes fractionation before measurement, is required.

For routine analysis of PHB, the Aminex-HPLC method provides a wide range of detection (0.01 to 14 μg of crotonic acid). Its sensitivity is an order of magnitude greater than that of Law and Slepecky (8). This enhancement is because of a highly sensitive detection system and because the absorbance maximum (λ_{max}) of crotonic acid is near the wavelength used in these detectors. A series of multiple injections can be used to increase the number of samples analyzed per unit time.

Routinely, bacteroid samples are treated with Chlorox to remove extraneous material before their exposure to concentrated sulfuric acid and subsequent injection onto the Aminex column. However, the Chlorox treatment was omitted and the total dilution was reduced to 20-fold when handling small quantities of bacteroids or samples with low PHB content. The column material appeared to be stable to the concentration of sulfuric acid (1.7 N) in such samples, and interference from other metabolites was not observed.

GC analysis of the product(s) from sulfuric acid digestion of Chlorox-treated samples from bacteroids confirmed the presence of a single peak whose retention time was identical to that of crotonic acid. The UV spectrum of this product was identical to that of crotonic acid. Recrystallized crotonic acid is the preferred standard, since the purity of PHB may vary with the purification procedure used. The formula weight of crotonic acid corresponds to the digestion product of PHB, and their molar extinction coefficients are identical.

HPLC, with Aminex HPX-87H (ion-exclusion column for organic acids, isocratic mode) and UV detection, provides a simple technique for routine analysis of PHB. It provides the advantages of (i) fractionation of materials in the digest before detection of crotonic acid, (ii) greater sensitivity and accuracy across a wide concentration range, and (iii) easy and rapid sample analysis.

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