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A simplified method for bacterial nutritional status based on the simultaneous determination of phospholipid and endogenous storage lipid poly-β-hydroxyalkanoate

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Summary

The bacterial edogenous storage lipid poly-β-hydroxyalkanoate (PHA) can be recovered from environmental samples by extraction with boiling chloroform or after treatment with sodium hypochlorite. In this study the determination of PHA after extraction with a one-phase chloroform:methanol solvent is shown to be as effective as the boiling chloroform method which is quantitative for PHA added to environmental samples. The one-phase chloroform:methanol extraction also quantitatively recovers the ester-linked fatty acids of the phospholipids (PLFA). The lipid extract is then partitioned on a disposable silicic acid column with quantitative elution of neutral lipids with chloroform, glycolipids and PHA with acetone, and phospholipids with methanol for analysis of each component. This extraction and simple column fractionation method for determination of PHA and PLFA simplifies previous methods for the assessment of the ratio of rates of formation of PHA and PLFA after a brief exposure to [^{14}C]acetate which has been shown to be a sensitive measure of the nutritional status of bacteria in the environment.

Key words: Bacterial nutritional status; Poly-β-hydroxyalkanoate; Phospholipid fatty acid; Lipid extraction

Introduction

Limitations of direct microscopic methods for determination of microbial biomass and community structure has led to development of chemical methods based on measurement of cell components [1]. A suite of methods based on a one-

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phase lipid extraction with chloroform : methanol utilizing a detailed analysis of the phospholipid ester-linked fatty acids (PLFA) by gas chromatography/mass spectrometry provides a reproducible definition of the microbial community structure [2] in samples from a wide variety of situations [3]. The phospholipid content of various environmental samples has been shown to correspond to the 'viable, active or potentially active' biomass of the microbiota [4]. The rate of phospholipid synthesis has been shown to be an accurate and sensitive measure of bacterial growth in sediments [5]. [^{14}C]Acetate incorporated into phospholipids with short exposures is found primarily in the PLFA [6].

The endogenous storage lipid poly-β-hydroxyalkanoate (PHA) accumulates in sedimentary and detrital bacterial communities under conditions of unbalanced growth when cell division is not possible, whereas PLFA accumulates as the cellular membrane expands during growth [7–9]. The ratio of incorporation of ^{14}C into PHA to that in PLFA has been shown to be a sensitive measure of in situ community metabolic status [10]. The sensitivity and specificity of the detection of PHA was increased by the application of an analysis of the component monomers by gas chromatography (GC) [11]. With the analysis by GC, monomers other than β-hydroxybutyrate were demonstrated. The quantitative recovery of added PHA required an initial treatment with sodium hypochlorite or extraction with boiling chloroform during purification of PHA in the original studies [8, 11]. This was then combined with a separate one-phase chloroform : methanol (CM) extraction for the isolation of the PLFA. The study reported here establishes that both the PHA and PLFA can be recovered quantitatively by a single CM extraction and fractionated into neutral lipid, glycolipid (PHA) and phospholipid fractions with simple silicic acid column chromatography on disposable columns.

Materials and Methods

Materials

1-[^{14}C]Acetate (56 μmCi/μmol) was supplied by New England Nuclear, Boston, MA. Unisil (100–200 mesh) silicic acid was obtained from Clarkson Chemical Co., Inc., Williamsport, PA. Glass-distilled solvents (Burdick and Jackson, Muskegon, WI) or freshly redistilled analytical grade chloroform (Mallinckrodt, St. Louis, MO) were used. Derivatizing reagents and authentic standards were purchased from Pierce Chemical Co., Rockford, IL, Aldrich Chemical Co., Milwaukee, WI, U.S. Industrial Chemicals Co., Tuscola, IL and Sigma Chemical Co., St. Louis, MO. Fused silica capillary GLC Durabond columns (DB-1) were supplied by J&W Laboratories Inc., Rancho Cordova, CA.

Sampling sites

Sandy marine sediments were recovered from a tidal lagoon near the Florida State University Marine Laboratory, Franklin County, FL, U.S.A. (29°52′N, 84°21.5′W), and from the reef flat of the Davies Reef, Australia. Davies Reef is an open lagoon reef in the Central Great Barrier Reef approximately 70 km northeast of Cape Ferguson, Queensland, Australia (18°48′S, 147°39′E). Sediments were well sorted and had an average grain size of 506 μm (Davies Reef) and 177 μm (Franklin County).

Sampling

Ten sediment samples (Franklin County) were used to compare the hot chloroform and CM extraction methods. Samples were taken by hand while wading using 5 cm diameter polycarbonate cores. The cores were randomly assigned to one of the two extraction schemes.

Sediment samples (Davies Reef) were used to assess the recovery of radiolabeled PHA. Samples were taken by divers using SCUBA from the reef flat in 15 m of water utilizing cores made from 2 ml plastic syringes. Cores were constructed by cutting the syringe at the beginning of the barrel and beveling the edge. The diver gently inserted the beveled end of the syringe into the sand and partially withdrew the plunger as the barrel was inserted into the sediment. Syringes with undisturbed sediment were returned to the surface for incubation with radiolabeled acetate. Cores were randomly assigned to one of the two treatment groups.

[^{14}C]Acetate incorporations

The method utilized for introduction of the radiolabeled acetate produces minimal disturbance to the sediments and allows assessment of in situ microbial activity and community nutritional status [10]. Davies Reef sediment cores were inoculated by discharging 30 μl of filter sterilized seawater containing 0.2 μCi of 1-[^{14}C]acetate from a fine needle (50 μl syringe) as it was withdrawn through the center of the sediment core. Samples were incubated for 20 min at ambient seawater temperature (23 °C) in inverted sampling syringes. The incubation was terminated by extruding and discarding all the base of the core except for the top 2 cm of the original sediment and then discharging the top 2 cm into a test tube containing 4 ml of methanol:chloroform (2:1, v/v). The extractant and the sediment were mixed and held in the dark for at least 2 h. Controls were discharged into the test tubes immediately after addition of labeled acetate.

Extraction

Methanol:chloroform

Samples were assayed for PHA using the one-phase CM extraction. Samples were extracted in separatory funnels with 142.5 ml of chloroform:methanol:phosphate buffer, pH 7.4, 0.05 M (1.0:2.0:0.8, v/v/v) for 24 h. The extraction mixture was then separated into organic and aqueous phases by the addition of equal amounts of chloroform and water (final ratio; 1.0:1.0:0.9, chloroform:methanol:water, v/v/v). The organic phase was filtered through a Whatman 2V filter, recovered in round bottom flasks and the chloroform removed in a rotary evaporator. ^{14}C-Labeled lipid from sediment samples incubated with radiolabeled acetate was recovered in the laboratory by briefly centrifuging the extractant and sediment and decanting the lipid extract into a second test tube. The sediment was then washed with 2 ml portions of methanol:chloroform (2:1, v/v), chloroform, and of water with brief centrifugation between each wash. The decanted solvents were combined, mixed thoroughly, allowed to stand in the dark and centrifuged briefly. The upper phase was removed by suction and the lipid-containing chloroform phase recovered.

Hot chloroform

Samples to be assayed for PHA using the hot chloroform extraction were first lyophilized, then placed in a Soxhlet extractor in which the cellulose extraction thimble had been replaced by glass wool and the extraction chamber was heated by a strip heater to approximately 60 °C. Samples were refluxed for 2 h in chloroform, the solvent phase recovered, dried and the polymer transferred into test tubes using warm chloroform [11].

Lipid fractionation

Lipid from methanol:chloroform extraction was fractionated into neutral lipid, glycolipid and phospholipid fractions using silicic acid column chromatography. Radiolabeled lipid samples were partitioned on silicic acid columns made in a Pasteur pipette (0.5 × 2 cm) containing 0.2 g of Unisil that had been packed (using chloroform) after activation by heating to 120 °C for at least 2 h [10]. Neutral lipids were eluted with 2 ml of chloroform, glycolipids with 2 ml of acetone and polar lipids with 2 ml of methanol. The glycolipid was collected in scintillation vials and dried. Samples for chemical analysis of PHA were purified in a similar manner except that 1 g of Unisil was packed into a glass column (1 × 4 cm) and 10 ml of solvent were used to elute each fraction.

Purification

Test tube method

Lipid extract from the hot chloroform extraction was dried to the side of a test tube under a stream of nitrogen. Lipids other than PHA were removed by repeated extractions with 2 ml of absolute ethanol until the wash was colorless. This process was repeated with diethyl ether [11]. Glycolipid fractions from the silicic acid column were dried to the side of a test tube and extracted once with diethyl ether.

Filter paper method

Lipid was transferred to Whatman 3 mm filter paper by slowly pipetting the lipid solution onto the paper with removal of the solvent in a stream of warm air from a hair dryer. The PHA was fixed to the filter paper by heating at 80 °C for 20 min. Lipids other than PHA were removed from the filter paper by washing with ethanol and diethyl ether [8].

Quantification

Gas chromatography

Polymer samples were ethanolyzed and their constituent β-hydroxy acids were converted to ethyl esters. Polymer samples purified by the filter paper method were not removed from the paper before derivatization. The esters were separated and quantified by gas-liquid chromatography. The derivatization and chromatographic conditions were as previously described [11].

Liquid scintillation spectrometry

Samples that showed significant color were bleached with 1.0 N benzoylperoxide in chloroform by heating for 2 h at 60 °C [5]. Filter papers and glycolipid fractions

TABLE 1

EXTRACTION OF PHA FROM SEDIMENTS

Type of extraction	Hydroxybutyrate[a]	Hydroxyvalerate[a]	PHA[a]	Percent recovery[b]
Hot Chloroform[c]	3.83 ± 1.98	4.53 ± 0.99	8.36 ± 2.79	100
Methanol/Chloroform[d]	4.02 ± 1.06	3.51 ± 0.51	7.64 ± 1.61	91.4
Methanol/Chloroform[c]	4.00 ± 0.73	3.62 ± 0.46	7.81 ± 1.39	93.4

[a] μmol/g dry weight of sediment, mean ± S.D., $n = 5$.
[b] Calculated using amount of PHA recovered from Soxhlet extraction.
[c] Purified using filter paper method.
[d] Purified using test tube method.

from the silicic acid column were placed in scintillation vials, suspended in 15 ml of Aquasol and counted by liquid scintillation spectrometry. Quenching was controlled using commercially prepared standards and the channels ratio method.

Results

Extraction of PHA

Preliminary experiments indicated that PHA could be recovered in the 'glycolipid' eluate from a silicic acid column fractionation of a one-phase CM extract. In earlier methods the quantitative recovery of PHA added to environmental samples required either the initial hydrolysis in sodium hypochlorite or extraction with boiling chloroform followed by fixation to filter paper disks and subsequent purification by extractions [7, 8, 11]. The recovery of PHA from sediments by the single-phase CM extraction with purification of the PHA by either the filter paper fixation method or the test tube precipitation methods showed recoveries that were not significantly different (ANOVA, $P = 0.056$) in the recovery of β-hydroxybutyrate, β-hydroxyvalerate or the total PHA from the boiling chloroform method (Table 1). The monomers were assayed by capillary gas chromatography [11].

Recovery of PHA

Sediments labeled with [^{14}C]acetate were extracted with the single-phase CM extractant and the PHA purified by the filter paper method with multiple washes with ether and ethanol. A second portion of the lipid was fractionated using silicic

TABLE 2

RECOVERY OF [^{14}C]PHA FROM SEDIMENTS EXTRACTED WITH ONE-PHASE CHLOROFORM:METHANOL

Purification	DPM[a]
Paper fixation and extraction	13000 ± 2980
Silicic acid column – acetone fraction	13400 ± 3000

[a] DPM/g dry weight of sediment, mean ± S.D., $n = 5$.

acid chromatography and the acetone (glycolipid) fraction contained ^{14}C activity that was not significantly (ANOVA, $P = 0.05$) different from the filter paper fraction (Table 2). After addition of ^{14}C-labeled PHA to sedimentary samples, subsequent CM extraction and fractionation with silicic acid chromatography gave quantitative recovery of the radioactivity in the acetone (glycolipid) fraction.

Discussion

Recovery of PHA

The application of two improvements in the analysis of PHA have provided a method that will be particularly useful in field studies. The first improvement involves the ambient temperature, one-phase chloroform:methanol extraction that also quantitatively extracts the phospholipids. The second improvement is a simple fractionation on disposable silicic acid columns that yields both the PHA and PLFA.

Utilization of the CM extraction and simple silicic acid fractionation for the analysis of total PHA may require modification if samples contain high levels of other glycolipids. Glycolipids that are eluted from the silicic acid column with acetone can complicate the gas chromatographic analysis for the β-hydroxy acid monomers of PHA. Ethyl esters which are formed from the fatty acids in lipids such as the glycosyl glycerides have much longer retention times on the SE-30 capillary column than the ethyl β-hydroxyalkanoates or the ethyl malate internal standard. Removing these component esters requires that the temperature program be prolonged at a higher temperature which lengthens the procedure. These contaminating glycolipids can be removed prior to ethanolysis by fixing the PHA to filter paper with heat and then extracting at least twice with ethanol and diethyl ether. Subsequent ethanolysis of the PHA fixed to paper disks after extraction with ethanol and diethyl ether results in partial breakdown of the paper. This is detected as a single large component on the gas chromatographic trace that elutes before the β-hydroxyalkanoate esters. If complicating glycolipids are suspected to be present in the sample (such as samples enriched in photosynthetic microbes) and the PHA is to be recovered, then a separate extraction of the original sample with boiling chloroform can be utilized with the test tube purification method. The PHA recovered with boiling chloroform is very adherent to glass so care must be taken not to allow it to touch areas of the apparatus from which the polymer cannot be readily recovered for ethanolysis and analysis (unpublished data).

Analysis of microbial nutritional status

The improved method is particularly useful in the analysis of the rates of synthesis of PHA and PLFA after brief exposure to [^{14}C]acetate. This analysis has proved a simple and useful technique to determine the community nutritional status. Sediments can be recovered gently using syringe barrels with beveled ends, the acetate carefully injected into the center of the sediment plug by withdrawing the syringe needle as the radioactive precursor is expelled, the syringes incubated in inverted position in the field, and the incubation terminated by injecting the sediment plug into the CM extractant. Once the extraction is complete in the laboratory, the lipids are fractionated on disposable silicic acid microcolumns, the lipids recovered in

scintillation vials and the radioactivity determined. This is performed with disposable apparatus and allows the determination of the ^{14}C incorporation into the fatty acids of the triglycerides of the microeukaryotes found in the neutral lipids, the PHA of the bacteria and the fatty acids of the bacteria in the PLFA. Short exposure times (10–25 min) do not result in the formation of significant microeukaryote PLFA as evidenced by the lack of inhibition by cycloheximide [5].

Comparison of the relative rates of synthesis of PHA/PLFA have proved useful in the effects of amphipod grazing of the detrital microbiota [12], the disturbance by bioturbation or analytical artifact in stratified sediments [10], the effects of chelation of essential nutrients on the detrital microbiota [8], the effects of contamination on the subsurface aquifer microbiota [13], the effect of root exudate on the rhizosphere microbiota [14], and the effects of nutrients on the benthic spicule mat microbiota beneath Antarctic sea ice [15]. Measurements of the ratio of the rates of synthesis of triglyceride fatty acids to PLFA have been used to define the nutritional status of fungi and estuarine amphipods [16], and to follow the growth and senescence of sea ice diatoms in the Antarctic [17].

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