APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1979, p. 349-350
0099-2240/79/08-0349/02$02.00/0

Vol. 38, No. 2

Muramic Acid Assay in Sediments

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Received for publication 15 May 1979

An improved chromatographic assay for muramic acid which is sufficiently sensitive for marine sandy sediments is described; it involves acid hydrolysis, thin-layer chromatography, and gas-liquid chromatography.

Muramic acid (MA) is a unique component of the bacterial or cyanophyte cell wall (11) and has proved to be a useful measure of bacterial biomass in a number of environmental studies (1-3, 5-9, 12). Our modification (3) of a chromatographic method (5) allowed determination of turnover rates in estuarine detritus but proved time-consuming (12) and too insensitive for use in marine sands of low microbial content.

Sand sediments (40 g, wet weight) are thawed and extracted with a single-phase extractant (18.75 ml of chloroform, 37.5 ml of methanol, 15 ml of water, including the water in the sand), which is shaken and allowed to stand for 2 h at room temperature. Chloroform-water (1:1; 37.5 ml) is then added, the suspension is mixed, and the phases are separated after 4 h. The aqueous upper phase is removed, the chloroform is recovered for lipid analysis (4, 13; D. C. White, W. M. Davis, J. S. Nickels, J. D. King, and R. J. Bobbie, Oecologia [Berlin], in press), and the sand residue is transferred with several portions of 6 N HCl (up to a final volume of 25 ml) into a 250-ml round-bottom flask and refluxed for 4.5 h. After cooling, the mixture is transferred to a glass column (13-mm inside diameter by 24 cm) plugged with glass wool with a Teflon stopcock and a 200-ml reservoir. The eluate is collected in a test tube with periodic evaporation of the dilute acid in vacuo with a Buchler Rotary Evapomix. The evaporation takes fewer than 5 min if a heat lamp is used to warm the traps and ice water is circulated in the condenser. In this way, the total hydrolysis volume, plus 1.5 times the hydrolysis volume of water eluate, can be recovered in a single test tube. The recovery of authentic MA from a 40-g sand sample in a column with the elution volumes was 99.6 ± 2% (mean ± standard deviation) for 62.5 ml of eluate. The residue containing MA was dissolved in 50 μl of water and streaked onto the thin-layer chromatography (TLC) plate in a thin uniform line parallel and 3 cm from the bottom of the plate. The most variable part of this assay is in the application of the hydrolysate to the TLC plate; the greater the evenness of the application, the less irregular the final solvent front. A semimechanical applicator such as the Brinkmann Desaga template is very helpful in laying down thin-layer applications. Three applications of about 10 μl/cm of TLC plates with gentle drying with a hair dryer at 50°C were satisfactory.

TLC is performed at 25°C in a glass brick (8 cm in width by 27 cm in length by 22 cm in height) lined with paper containing 220 ml of isopropyl alcohol-acetic acid-water (9:1:1, vol/vol), using a nonactivated Polygram Sil G (20 by 20 cm; Brinkmann Instruments Inc., Westbury, N.Y.) plate without gypsum binder, 0.25 mm thick. The chromatography takes about 6 h. When the solvent front is about 1 cm from the top, the plate is removed and allowed to dry in the air, and an area ±1 cm from the R_f 0.38 is loosened with a razor blade and picked up in a glass wool-plugged, disposable Pasteur pipette with vacuum.

TLC results in a clear separation from the heavily ninhydrin-staining origin and the solvent front. MA runs consistently with an R_f of 0.38 ± 0.001. Commercial Silica Gel G plates without binder give a slower development, but narrower bands and more satisfactory elution, than do laboratory-prepared plates.

The MA is eluted from the Silica Gel G in the Pasteur pipette by adding 5 ml of 0.1 N acetic acid in portions and collecting the eluate in a test tube. Elution is essentially quantitative after 2.0 ml is collected, but 5 ml is routinely eluted. It is particularly important that no Silica Gel G gets into the eluate as it seriously decreases the yield of the derivatization procedures.

At this point, 50 μl of inositol internal standard (2.49 mg/ml) is added. The acetic acid is removed in vacuo, 0.4 ml of 0.22 N hydroxylamine hydrochloride (0.301 g/20 ml) in pyridine is added, and the reagents are mixed. The reaction is heated for 2 h at 55°C after mixing, with a

glass marble used to close the top. After cooling, the reagents are removed, and 0.5 ml of acetic anhydride-pyridine (1:1, vol/vol) is added, mixed, and heated for 2 h at 55°C. The reagents are removed in vacuo, dissolved in 50 μl of chloroform, and separated by gas-liquid chromatography (10).

A significant purification from residual pyridine results if equal volumes of chloroform in 1 N aqueous HCl are added, mixed, and allowed to separate in tubes suspended in an ice bath. The syringe is then put into the chloroform and purged with air, and the gas-liquid chromatography sample is drawn up and injected.

The peracetylated aldonitriles are applied to a glass column (3.2 m by 2 mm) packed with 3% SP-2330 on 100/120-mesh Supelcoport (Supelco, Inc., Bellefonte, Pa.) and chromatographed isothermally at 225°C with a nitrogen carrier gas flow of 18 ml/min. The detector is held at 245°C and the injector is held at 205°C in a Varian Aerograph model 2100 chromatograph with flame ionization detectors. Under these conditions, the peracetylated inositol eluted in 6 min, and the peracetylated aldonitrile of MA eluted in 16 min. The molecular response of MA to inositol in 34 measurements was 1 to 3.34 (±0.22). With this derivatization procedure 0.04 μmol of MA could be detected in 1 μl.

The peracetylated aldonitrile of MA showed a molecular ion at 416, with major fragmentation peaks at M−59, M−89−57, M−18−145, and M−186 in the electron impact mass spectra and major responses at M−18+37, M−18+35, and M−18−73 in the chemical ionization mass spectra performed in an AEI 902 mass spectrometer.

The modifications in the MA assay reported in this note have resulted in a significant shortening of the time per assay when compared with our previous method (3). The total time required for the assay is about 17 h, of which only 3 require the continuous attention of the analyst. Multiple analyses can be performed simultaneously. Up to 10 samples can be easily processed simultaneously. The reproducibility of the entire assay measured with 33 samples of the same sand suspension containing added authentic MA was 206.7 ± 19.4 μmol/40 g (mean ± standard deviation), for a reproducibility of ±9.5%. The recovery of added MA to sediments based on six measurements in each triplicate was 99.8 ± 10.5%.

We have reproducibly detected 11.3×10^{-12} ± 1.2 mol of muramic acid per g (wet weight) of marine sand. Since the assay involves concentration of large volumes of hydrolysate from the sediment and concentration of the derivatized material before gas-liquid chromatography, the sensitivity can be increased 10- to 80-fold without greatly increasing the time and inconvenience of the assay.

This work was supported by NOAA Office of Sea Grant, Department of Commerce, under grant 04-7-158-4406, grant 76-19671 from the Biological Oceanography Program of the National Science Foundation, and grant R80614301 from the U.S. Environmental Protection Agency.

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