APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1991, p. 879–881
0099-2240/91/030879-03$02.00/0

Vol. 57, No. 3

Analysis of Free Amino Acids in Microbially Colonized Sandstone by Precolumn Phenyl Isothiocyanate Derivatization and High-Performance Liquid Chromatography

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Received 27 August 1990/Accepted 13 December 1990

A fast, sensitive method for extraction and analysis of soluble free amino acids from microbially colonized sandstone is described. After precolumn phenyl isothiocyanate (PITC) derivatization, the PITC-amino acids were identified and quantified by reversed-phase high-performance liquid chromatography. This kind of analysis could be used to elucidate the role and function of amino acids in the nutrition of epi- and endolithic microorganisms active in biological weathering processes.

Amino acids play an important role in the nutrition of microorganisms in ecosystems (6, 7). It is well documented that algae and other microorganisms can excrete considerable amounts of these compounds into the environment during growth in culture and in situ (1, 10). Other sources of these low-molecular-weight compounds are autolysis of cells and exoenzymatic degradation of complex substrates (12). The low-molecular-weight fraction of dissolved organic matter is easily used by bacteria and algae because these substrates are recognized by several uptake systems and can be assimilated readily (11). Bacteria incorporate about 80 to 90% of assimilated amino acid carbon predominantly into proteins; 10 to 20% is respired (16, 26). In the absence of free amino acids, protein synthesis accounts for 60% of the energy budget (18). Therefore, the incorporation of amino acids directly into protein should result in substantial savings in the energy cost of growth. This energy conservation may be important for microorganisms in oligotrophic habitats.

Data on the role and dynamics of amino acids in nature have been collected primarily from marine and freshwater environments (e.g., see references 3 and 17). Dissolved free amino acids play a crucial role in aqueous environments (6). This is probably true in terrestrial systems as well, but evidence to support this assumption is lacking. Microbial activity (e.g., excretion of inorganic and organic acids) can contribute to biological weathering processes (16a, 27). Microbial activity is dependent on nutrition, and therefore nutritional conditions (concentrations and types of substrates such as amino acids) in stone should be investigated.

The analytical determination of amino acids is usually done by precolumn derivatization with *o*-phthaldialdehyde (14, 15). The derivatives are separated by high-performance liquid chromatography (HPLC) and detected by fluorescence. The sensitivity provided by *o*-phthaldialdehyde derivatization allows detection of dissolved free amino acids in the concentrations present in the free water column (30 to 720 nM) (13, 15). Disadvantages of the *o*-phthaldialdehyde method are that the derivatives are unstable and that secondary amines do not derivatize. Thus, reaction times during derivatization must be kept constant and the analysis must be done without delay. Normally this has been guaranteed by full automatization of the analytical system.

We used a phenyl isothiocyanate (PITC) precolumn derivatization procedure based on the method described by Heinrickson and Meredith (9). The derivatized amino acids are detected by UV absorbance at 245 nm. As little as 10 pmol of amino acid can be detected; this sensitivity is sufficient for investigations in oligotrophic media or colonized rock. The PITC derivatization procedure has two advantages over *o*-phthaldialdehyde: secondary amines such as proline can be detected and the derivatized amino acids are stable for weeks when stored frozen at pH 6.8 (9).

We used the PITC method on two types of microbially colonized sandstone. The first was a weathered crust from the Schleswig courthouse (Federal Republic of Germany) (sample L1 [16a]), and the second was Antarctic Beacon sandstone containing a cryptoendolithic microbial community (4) (sample 845/239 from the McMurdo Dry Valleys of South Victoria Land). The courthouse sample was processed within 2 h of sample collection; the Antarctic sample had been stored at −27°C since its sampling date (1985).

One to 2 g of colonized stone was crushed in a mortar. The soluble free amino acids (SFAA) were extracted with 0.15 M NaCl from 100 to 200 mg of crushed stone (1:1 [wt/vol]) at 4°C for 30 min on a rotary shaker. No significant increase in the amino acid concentration was seen with a longer extraction time (60 min). The samples were centrifuged, and 100 μl of supernatant was dried and used for derivatization. All drying steps were done in a Speed Vac concentrator (Savant). Hydroxyproline (HPr) was used as the internal standard (2.5 μl of a 10 mM solution). Prior to PITC derivatization, the dried samples were resuspended in 10 μl of redrying solution (ethanol-water-triethylamine, 2:2:1 [vol/vol/vol]) and redried. PITC derivatization was accomplished by the addition of 20 μl of coupling solution (ethanol-water-triethylamine-PITC, 7:1:1:1 [vol/vol/vol/vol]), followed by incubation for 20 min at room temperature. After a final drying step in which all PITC was removed, the samples were resuspended in 200 μl of 12.5 mM phosphate buffer (pH 6.4), vortexed, and centrifuged for 2 min at high speed. A 5-μl aliquot of the supernatant was used for HPLC analysis.

Analyses were performed with a Pharmacia-LKB HPLC system consisting of a model 2156 solvent conditioner, a model 2248 HPLC pump, a model 2157 autosampler, and a

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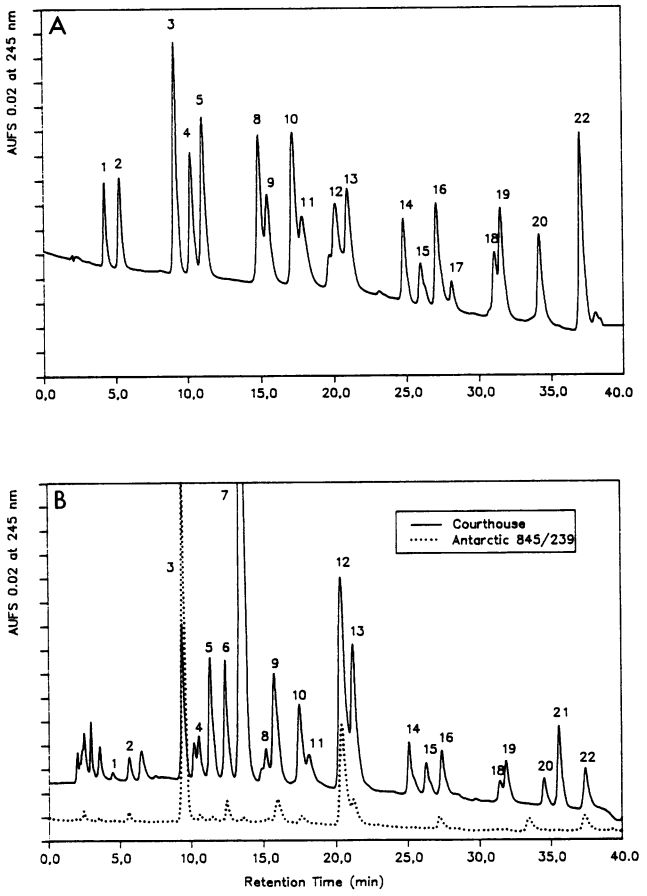

FIG. 1. Comparison of the dissolved free amino acid content of two colonized sandstones. PITC-amino acids were separated on a Spherisorb ODS II 5-μm column by HPLC. (A) Amino acid calibration standard AA-S-18 (Sigma), 250 pmol of each amino acid. (B) Samples. 1, Asp; 2, Glu; 3, Hpr (IS); 4, Ser; 5, Gly; 6, unknown; 7, unknown; 8, Thr; 9, Ala; 10, Pro; 11, His; 12, reagent peak; 13, Arg; 14, Tyr; 15, Val; 16, Met; 17, Cys; 18, Ile; 19, Leu; 20, Phe; 21, NH_3/Try; 22, Lys.

model 2141 variable wavelength monitor. The column was a Spherisorb octyldecylsilane II C_{18} (5 μm, 4.6 by 250 mm) (Bischoff, Leonberg, Federal Republic of Germany) preceded by a guard column (5 μm, 4.6 by 20 mm). The columns were held at 36°C. The binary gradient was composed of solvents A (12.5 mM phosphate buffer, pH 6.4) and B (acetonitrile in phosphate buffer, 50:50). The gradient conditions were 0 to 15% B for 15 min, 15 to 45% B for 20 min, 45 to 80% B for 1 min, and 80% B for 2 min. Washing was done with 100% B for 9 min. Equilibration was accomplished with 100% A for 8 min. The flow rate was 1.0 ml/min. Detection was at 245 nm and 0.02 absorption units full scale (AUFS). Peaks were integrated with model 2600 chromatography software (Perkin-Elmer Nelson Systems). Samples were normalized to HPr (internal standard) and quantified on the basis of peak area and retention time of external amino acid standards. In situ biomass of the samples was estimated on the basis of lipid-phosphate content. Lipids were extracted by the method of White et al. (24), and phospholipid-phosphate (PLP) was analyzed by the method of van Veldhoven and Mannaerts (19). Biomass in both sandstones was in the same order of magnitude: the courthouse sample contained 49 nmol of PLP g^{-1} (16a); the Antarctic sample contained 29 nmol of PLP g^{-1}. The Antarctic biomass value was in the range reported for similar sandstone samples (20). There is no simple correlation between biomass based on PLP and that based on cell numbers. White (23) has calculated that approximately 1 nmol of phosphate represents about 10^8 bacteria the size of *Escherichia coli*. The cryptoendolithic community analyzed in the present study consists of lichens, algae, and bacteria. Thus, our PLP results cannot be extrapolated to cell numbers.

Analysis of the two sandstone samples showed considerable differences in the types and quantities of SFAA (Fig. 1). Table 1 shows the concentrations of identified amino acids normalized to in situ biomass (nanomoles of PLP). The concentrations of SFAA were 2 to 46 times higher in the courthouse sample than in the Antarctic sample. Arginine, alanine, and glycine were the most abundant amino acids (48 mol%) in the courthouse sample, and an unidentified amino compound (peak 7) was present in large amounts. Aspartic acid, threonine, histidine, tyrosine, valine, isoleucine, leucine, and phenylalanine were absent in the Antarctic sample.

Differences between the Antarctic and the courthouse samples could be related to different microbial activities in the two ecosystems. Microorganisms could utilize different amino acids to different extents (25). For example, Hagström et al. (8) have demonstrated that marine bacteria preferentially utilize glutamine and serine. Also, primary producers can differ in their ability to excrete (10) or take up (22) amino acids; no phototrophic organisms were found in the courthouse stone (16a). Furthermore, autolysis or exoenzymatic activities in the communities could differ (12). Sorption reactions in the stone also play a role in amino acid availability (21), and in marine sediments, the dissolved free amino acid pool is balanced with an exchangeable, absorbed pool of amino acids (2).

Friedmann et al. (5) have calculated that a maximum of 29 days of metabolic activity per year would be possible for the Antarctic cryptoendolithic ecosystem. This low total activity could explain the low amino acid concentration in Antarctic samples. Investigations on other colonized Antarctic sandstone samples yielded similar results (4 to 11 nmol of SFAA/nmol of PLP [data not shown]). Thus, the low SFAA concentration was not the result of an atypical sample. Also, it is unlikely that the amino acids in the Antarctic sample were degraded during the long storage period because the PLP (known to degrade rapidly [24]) concentration in the

TABLE 1. Concentrations of free amino acids in two colonized sandstone samples

Sample	nmol/nmol of PLP															
	Asp	Glu	Ser	Gly	Thr	Ala	Pro	His	Arg	Tyr	Val	Met	Ile	Leu	Phe	Lys
Courthouse	0.2	0.8	1.4	4.6	1.4	5.7	2.8	1.8	6.5	2.0	1.3	1.8	0.6	1.7	1.0	0.9
Antarctic 845/239		0.3	0.2	0.1		1.2	0.1		1.2			0.5				0.4

stored sample was similar to that in freshly collected samples (20).

With the help of this fast, sensitive, and inexpensive precolumn PITC derivatization procedure, it may be possible to clarify the role of amino acids in the nutrition of microorganisms active in biological rock-weathering processes.

We thank E. I. Friedmann (Florida State University, Tallahassee) for providing the opportunity to collect the Antarctic rock sample.

E. I. Friedmann was supported by National Science Foundation grant DPP 83-14180. This research was supported by a grant to P.H. from the Deutsche Forschungsgemeinschaft. R.J.P. was supported by a Humboldt fellowship and a grant from the Schleswig-Holstein Kultusministerium.

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