

Characterization of Water-Extractable Amino Acids in the Sub-Surface of Semi-Permafrost Environments

Yoshinori Takano,*[,1,2] Lallan P. Gupta,[2] Hodaka Kawahata,[2,3] Kensei Kobayashi,[4] and Katsumi Marumo[2]

[1]21st Century COE Program on "Neo-Science of Natural History," Division of Earth and Planetary Sciences, Graduate School of Science, Hokkaido University, N8W10, Kita-ku, Sapporo 060-0810

[2]Institute of Geology and Geoinformation (IGG), National Institute for Advanced Industrial Science and Technology (AIST), AIST Tsukuba Central 7, 1-1-1 Higashi, Tsukuba 305-8567

[3]Department of Ocean Floor Geoscience, Ocean Research Institute, The University of Tokyo, 1-15-1 Minamidai, Nakano-ku, Tokyo 164-8639

[4]Department of Chemistry and Biotechnology, Yokohama National University, 79-5 Tokiwadai, Hodogaya-ku, Yokohama 240-8501

Received February 4, 2005; E-mail: takano@nature.sci.hokudai.ac.jp

Terrestrial core samples of semi-permafrost sediment were analyzed for total free amino acids (TFAA). The TFAA content ranged from 12.0 to 460.2 nmol/g of sediment, and generally consisted of protein amino acids as the most abundant component of TFAA. The depth profiles in terms of the content drastically decreased with the depth, followed by the vertical distribution patterns of total hydrolyzed amino acids (THAA) within the same sediment. The abundance of THAA was 14 to 327 times larger than that of TFAA. Plots of TFAA versus THAA values yielded a straight line, as defined by the equation $THAA = 10.1 \times TFAA^{1.4}$ ($R^2 = 0.86$) based on the least-squares method. The molar ratio of β-alanine and γ-aminobutyric acid did not increase with the depth via diagenesis, as was the case in the THAA fraction. On the other hand, the molar ratio of basic amino acids such as histidine and ornithine gradually decreased with increasing depth. The present study determined that the major organic matter content in the sediment consisted not of free, but bound amino acid analogs.

The objectives of this study were to clarify the behavior of free amino acid analogs together with bound analogs in a subsurface environment. Permafrost environments consist of perennially frozen ground, and represent unique physical, chemical, and microbiological characteristics.[1,2] It has been determined that permafrost contains a large variety of ancient viable microorganisms.[3] The total number of microorganisms found in permafrost was as large as 10^8 cells/g,[4] and the number of viable microorganisms was in the range from 10^2 to 10^6 cells/g.[5] Organic matter in sediment is defined as consisting of a combination of (1) living biomass including animals, plants, and microbes, (2) recognizable dead and decaying biological matter, and (3) humic substances which are heterogeneous polymers formed during the process of decay and degradation of living biomass.[6] Hence, organic matter in the sediment is thought to consist of complex organics composed of biologically important organic compounds, rather than free analogs.

Investigations concerning amino acids in terrestrial sediments have also been reported in terms of TFAA in soil solutions and soil extracts,[7] the biodegradation of amino acids,[8,9] amino acids and nitrogen flux in taiga forest soils,[10] seasonal changes of amino acids in volcanic ash soils,[11] and TFAA in arctic tundra soils.[12] Amino acids held either in a polymeric state in soil organic matter or to a lesser extent free in the soil solution form one of the most widely and readily available sources of organic nitrogen present in the soil.[13] Hydrolyzed soil organic matter has been shown to be rich in organic-N with typically from 30 to 50% of the total organic-N present as amino acids, while the soil solution concentrations of free amino acids typically range from 1 μM to 50 μM.[14] Amino acids are a key component of the soil nitrogen cycle, and their turnover dominates the soil nitrogen flux in some high latitude ecosystems.[10] To date, we have investigated the carbon and total hydrolyzed amino acid content,[15] the chiral ratio of D- and L-amino acid forms,[15] total hydrolyzed amino sugars,[16] the enzymatic activities of phosphatase,[17] and the microbial distribution.[15] Since organic geochemical studies of water-extractable specimens such as terrestrial organic matter have been few, this paper describes the analysis of water-extractable amino acids in an effort to compare the results with those obtained for the analysis of hydrolyzed amino acids. The results represent one of the most important biogenic materials in semi-permafrost that exist under extreme terrestrial environments.

Experimental

Sampling. Boring core samples at Rikubetsu were obtained by the Ohbayashi Corporation in February, 1996. The site is located near the center of Hokkaido, one of the coldest cities in Japan (Fig. 1). The altitude of the boring site is 207 m, where the annual average temperature is 5.8 °C at 43° 28′ 0″ N, 143° 44′ 5″ E. The

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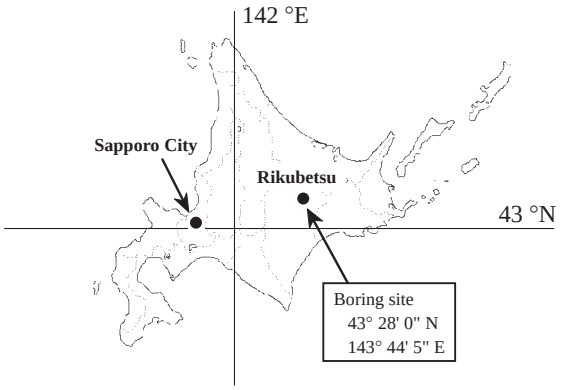

Fig. 1. Geological location of Rikubetsu, Hokkaido, Japan. The boring site was 143° 44′ 5″ E, 43° 28′ 0″ N.

monthly average precipitation and sunshine record at the site is 67 mm and 142.3 h, respectively.[15] The boring site is situated in a slightly marshy area, which is seasonally frozen to a depth of 80 cm, and is covered with ice during the winter. The boring was performed to a maximum depth of 300 cm. Hence, interactive studies[15–17] between the sub-surface biosphere and the organic geochemical aspect has been required to describe their linkage in the semi-permafrost environment.

Analytical Procedure. Approximately 2 g of powdered samples were extracted with 10 mL of pure water by sonication for 1 h, and then twice by sonication with 5 mL of water for 30 min. Visible polymer-like organic matter was observed in the extracted solution, which was precipitated by centrifugation for 20 min at 3000 rpm. The extracted solutions were combined and then freeze-dried. Subsequently, the test tube was gently washed with 1 mL of pure water by vibration for 1 min using a tube mixer, and was then again sonication for 5 min. The final portions were passed through a membrane filter (0.45 μm pore size) using a disposable syringe. Amino acids were detected and quantified by using the Waters AccQ-Tag method, which uses the AccQ-Fluor reagent (6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate) for the derivatization of amino acids.[18] In this method, a 20 μL sample is mixed with 60 μL Waters borate buffer and derivatized with 20 μL of a reagent at 55 °C for 10 min. The excess reagent decomposes to 6-aminoquinoline, which provides a control on the degree of derivatization of amino acids in the sample. Five μL of the derivatized solution is then injected into a Waters 2695 Separations Module consisting of an HPLC and an auto-sampler. The derivatized amino acids are detected by a fluorescence detector at 395 nm. The chromatograms were processed by an online data integrating system running the Empower software. Peak areas of individual amino acids were used to calculate the corresponding concentrations from a calibration curve of amino acid standards (0.1–10 pmol/μL). The reproducibility of results was controlled by analyzing one standard (5.0 pmol/μL) twice during the course of sample analysis. L-Norleucine was used as an internal standard, and its recovery was over 90%. The detection limit of this method is 0.1 pmol/μL, and standard deviation is less than ±5%.

Ion-exchanged water was further purified using a Millipore Milli-Q LaboSystem™ and Millipore Simpli Lab-UV (Japan Millipore Ltd.) to remove both inorganic ions and organic contaminants. All glassware was heated at 500 °C in a high-temperature oven (Shimadzu muffle furnaces, MPN-2N) for 2 h prior to use in an effort to eliminate any possible contaminants.

Results and Discussion

Distribution of Total Free Amino Acids. Figure 2 shows HPLC chromatograms of amino acids in core samples of 5–10 cm. As shown in Table 1, the concentration of total free amino acids (TFAA) varied from 12.0 to 460.2 nmol/g. The concentration of TFAA decreased significantly with the depth by one order of magnitude, as shown in Figs. 3a and 3b. The present data might be consistent with previous studies, where Pantoja and Lee (2003), for example, reported that the concentration of terrestrial origins of dissolved free amino acids (DFAA) in a costal surface sediment decreased significantly with increasing depth in the early diagenesis.[19]

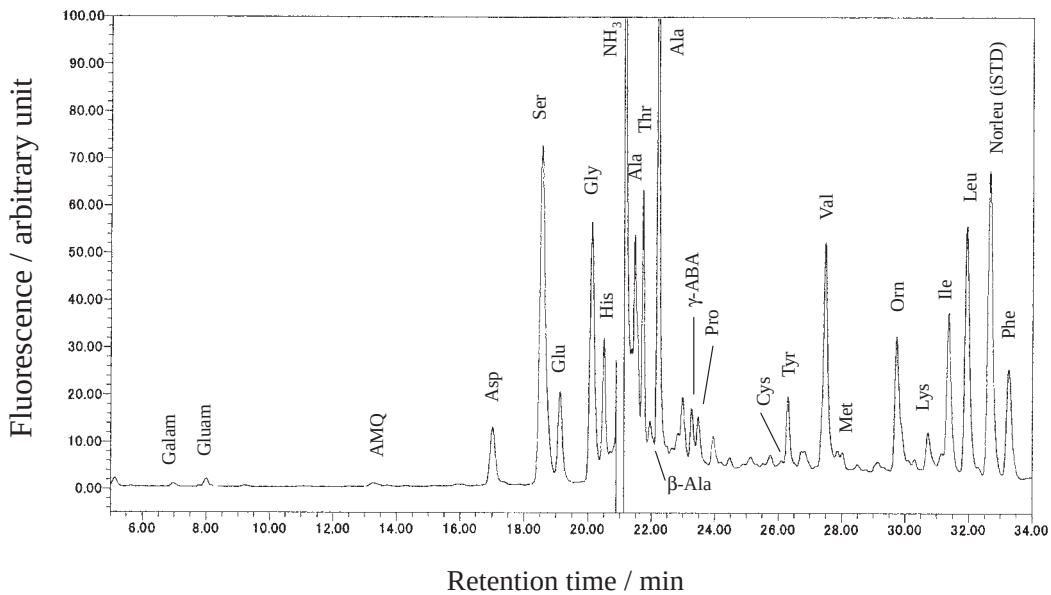

Fig. 2. The chromatogram of free amino acids in the sediment core sample at the depth of 5–10 cm.

Table 1. Concentration of Total Free Amino Acid (TFAA); Acidic, Basic, Hydroxy, Straight, Branched, Secondary, Aromatic, Sulfur-Containing, and Non-Protein at Rikubetsu, Hokkaido, Japan

Concentration/nmol g^{-1}	0–5 cm	5–10 cm	20–30 cm	30–40 cm	50–75 cm	75–100 cm	100–125 cm	125–150 cm	150–175 cm	175–200 cm	200–250 cm	250–300 cm
Acidic												
Aspartic acid (Asp)	26.0	4.7	8.1	2.9	0.5	2.0	1.7	2.0	1.6	2.9	1.9	1.1
Glutamic acid (Glu)	28.8	5.1	6.1	1.7	0.5	1.0	0.7	1.0	1.2	1.5	1.0	0.5
Basic												
Histidine (His)	13.8	2.4	5.0	0.9	0.1	0.4	0.4	0.4	0.3	0.6	0.3	0.2
Ornithine (Orn)	24.9	4.0	7.6	1.0	0.1	0.5	0.3	0.3	0.1	0.2	0.1	0.0
Lysine (Lys)	8.5	0.7	1.7	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Arginine (Arg)	25.8	2.1	2.9	0.9	0.1	0.4	0.3	0.3	0.3	0.5	0.3	0.1
Neutral												
Hydroxy												
Threonine (Thr)	21.2	4.4	6.8	3.0	0.6	1.6	1.4	1.6	0.9	2.0	1.0	0.5
Serine (Ser)	82.8	18.0	28.9	13.2	2.7	7.5	6.9	8.3	5.4	10.6	6.6	3.9
Straight												
Glycine (Gly)	69.0	15.2	24.5	12.1	2.1	6.0	5.6	7.1	4.9	9.1	5.3	2.9
Alanine (Ala)	50.6	10.0	14.5	7.6	1.6	4.4	3.6	4.5	3.3	5.9	3.4	1.9
Branched												
Valine (Val)	23.1	4.9	7.4	3.7	0.8	2.3	1.8	2.3	1.7	2.9	1.7	0.9
Isoleucine (Ile)	13.1	2.6	4.1	2.2	0.5	1.4	1.1	1.4	1.0	1.8	1.1	0.6
Leucine (Leu)	20.8	4.0	6.0	3.2	0.7	1.7	1.4	1.9	1.3	2.3	1.4	0.7
Secondary												
Proline (Pro)	12.5	3.6	5.2	2.3	0.5	1.2	1.0	1.4	1.1	2.0	1.1	0.6
Aromatic												
Tyrosine (Tyr)	9.8	2.3	3.5	2.0	0.5	1.1	1.0	1.5	0.9	1.5	0.9	0.5
Phenylalanine (Phe)	8.9	2.4	3.5	1.9	0.4	1.0	0.8	1.1	0.7	1.3	0.8	0.4
Sulfur-containing												
Cysteine (Cys)	10.8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Methionine (Met)	2.2	0.7	1.1	0.6	0.2	0.4	0.3	0.4	0.3	0.5	0.3	0.2
Non-protein												
β-Alanine (BALA)	2.7	0.7	0.5	0.2	0.1	0.1	0.1	0.0	0.0	0.0	0.0	0.0
γ-Amino butyric acid (GABA)	5.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.3	0.0	0.0
Acidic	*54.8*	*9.7*	*14.2*	*4.6*	*1.0*	*3.0*	*2.4*	*3.0*	*2.7*	*4.3*	*2.9*	*1.6*
Basic	*73.0*	*9.1*	*17.2*	*2.9*	*0.3*	*1.3*	*1.0*	*1.1*	*0.7*	*1.4*	*0.8*	*0.3*
Neutral	*293.1*	*62.8*	*97.6*	*47.3*	*9.4*	*26.3*	*22.6*	*28.5*	*19.7*	*36.7*	*21.7*	*12.1*
Hydroxy	*104.0*	*22.4*	*35.8*	*16.2*	*3.2*	*9.1*	*8.2*	*9.9*	*6.3*	*12.6*	*7.7*	*4.5*
Straight	*119.6*	*25.3*	*39.1*	*19.7*	*3.6*	*10.5*	*9.2*	*11.6*	*8.3*	*15.0*	*8.7*	*4.8*
Branched	*57.0*	*11.5*	*17.6*	*9.2*	*2.1*	*5.5*	*4.2*	*5.5*	*4.1*	*7.0*	*4.2*	*2.2*
Secondary	*12.5*	*3.6*	*5.2*	*2.3*	*0.5*	*1.2*	*1.0*	*1.4*	*1.1*	*2.0*	*1.1*	*0.6*
Aromatic	*18.6*	*4.7*	*7.0*	*3.9*	*0.9*	*2.1*	*1.8*	*2.6*	*1.6*	*2.8*	*1.7*	*0.9*
Sulfur-containing	*13.0*	*0.7*	*1.1*	*0.6*	*0.2*	*0.4*	*0.3*	*0.4*	*0.3*	*0.5*	*0.3*	*0.2*
Non-protein	*7.7*	*0.7*	*0.5*	*0.2*	*0.1*	*0.2*	*0.1*	*0.0*	*0.0*	*0.3*	*0.0*	*0.0*
Total free amino acids (TFAA)	**460.2**	**87.7**	**137.6**	**59.5**	**12.0**	**33.3**	**28.2**	**35.6**	**25.1**	**46.0**	**27.5**	**15.1**
Total hydrolyzed amino acids (THAA)*	**61836.0**	**28648.0**	**11822.0**	**2846.0**	**841.0**	**1857.0**	**1212.0**	**1298.0**	**539.0**	**659.0**	**714.0**	**649.0**
THAA/TFAA	**134.4**	**326.7**	**85.9**	**47.8**	**70.4**	**55.8**	**42.9**	**36.5**	**21.4**	**14.3**	**26.0**	**42.8**
%TFAA (vs THAA)	**0.74**	**0.31**	**1.16**	**2.09**	**1.42**	**1.79**	**2.33**	**2.74**	**4.66**	**6.98**	**3.85**	**2.33**

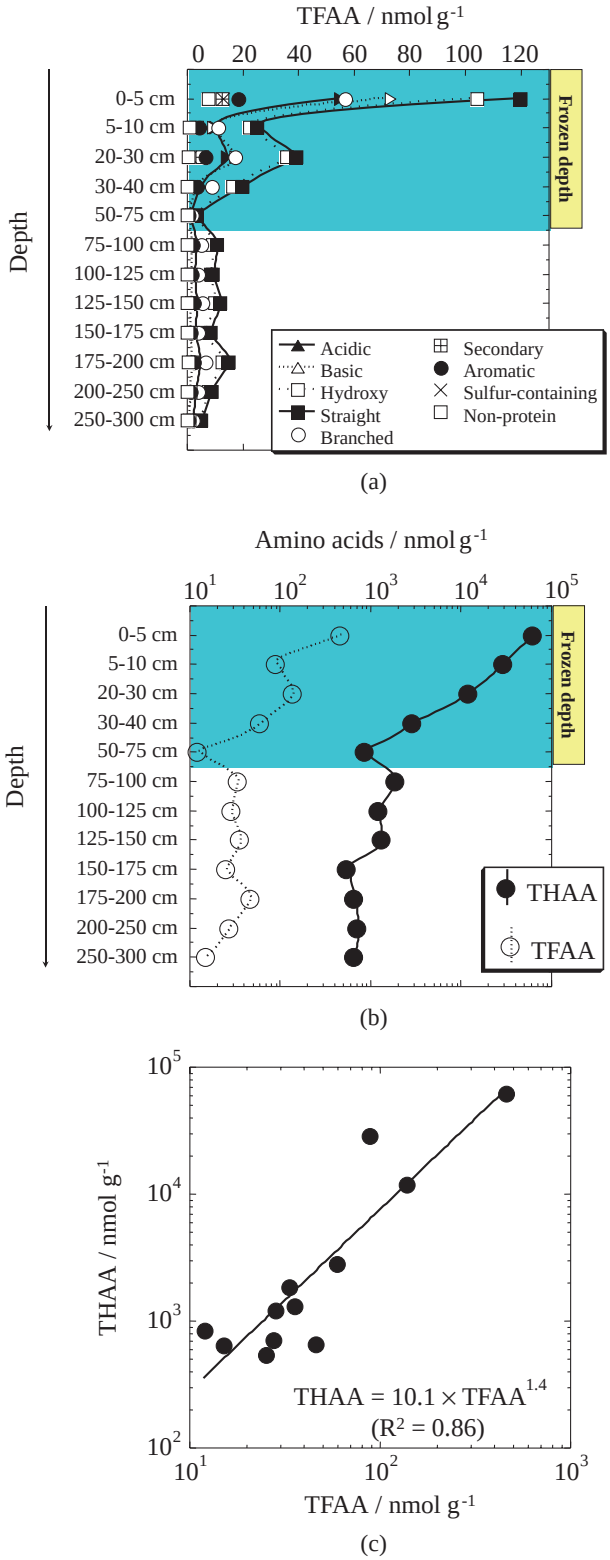

Fig. 3. (a) Vertical distribution of free amino acid analogs; acidic, basic, hydroxy, straight, branched, secondary, aromatic, sulfur-containing, and non-protein. (b) Concentration of total hydrolyzed amino acid (THAA) and total free amino acid (TFAA)[15] on the semi-logarithmic scale. (c) Straight line, as defined by a least-squares method in TFAA versus THAA. THAA data was referred by the preliminary report.[15]

Here, the concentration of total hydrolyzed amino acids (THAA) in the preliminary study[15] is positively correlated with TFAA and is far more predominant than the TFAA, as can be seen in Fig. 3b. The percentage of TFAA (%TFAA) versus THAA ranged from 0.3 to 6.9% (average 2.5%). The concentration of TFAA was minor constituent of terrestrial organic matter. Decreases in organic matter concentration with the depth in sediment have been frequently reported in studies of early diagenesis, whether measured as amino acids or total labile protein. These decreases are generally thought to be due to decomposition by sub-surface microorganisms and early diagenesis[20] as enzymatic decay and first-order degradation.[21] Actually, the vertical microbial population of the sediment is positively correlated with the TFAA and THAA trends over the last 10000 years.[15] Plots of TFAA versus THAA values of these samples are shown in Fig. 3c. These plots yielded a straight line, as defined by a least-squares method that could be expressed by the following equation:

$$\mathrm{THAA} = 10.1 \times \mathrm{TFAA}^{1.4} \ (R^2 = 0.86). \quad (1)$$

Protein amino acids, such as glycine (C_2), alanine (C_3), aspartic acid (C_4), and glutamic acid (C_5), were major constituents in TFAA as well as in THAA. Non-protein amino acids, such as β-alanine and γ-aminobutyric acid, were detected as minor constituents in certain sequences of the sediment.

Molar Ratio of Amino Acids in TFAA. Figure 4 shows the molar ratio of amino acids in the TFAA fraction. The assigned categories were as follows: acidic, basic, neutral (hydroxy, straight, branched, and secondary), aromatic, sulfur-containing, and non-protein amino acids. Among these, the sub-total in the molar ratio of basic amino acids, such as histidine and ornithine, gradually decreased with increasing depth. This trend might reflect the survivability of amino acids in sub-surface organic matter.

Mol % of β-alanine and γ-aminobutyric acid in TFAA did not increase with increasing depth, as shown in Fig. 4 and Table 2. This might be evidence to suggest that the decarboxylation of aspartic acid and glutamic acid does occur in an internal reaction in the bound matrix of organic matter, rather than as a monomer reaction. In our previous study of THAA, there was a notable increase in the relative abundances of β-

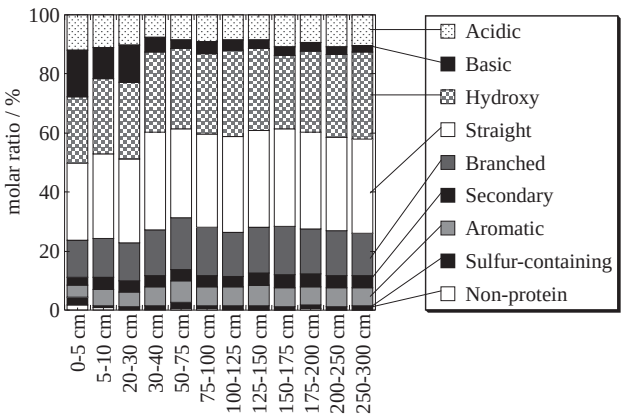

Fig. 4. Molar ratio of free amino acid analogs versus THAA. THAA data was referred by the preliminary report.[15]

Table 2. Molar Ratio of Free Amino Acid (TFAA) versus THAA at Rikubetsu, Hokkaido, Japan

Molar ratio of amino acids/mol %	0–5 cm	5–10 cm	20–30 cm	30–40 cm	50–75 cm	75–100 cm	100–125 cm	125–150 cm	150–175 cm	175–200 cm	200–250 cm	250–300 cm
Acidic												
Aspartic acid (Asp)	5.6	5.3	5.9	4.9	4.4	6.2	6.0	5.7	6.3	6.2	7.0	7.4
Glutamic acid (Glu)	6.3	5.8	4.4	2.8	4.1	2.9	2.5	2.7	4.6	3.2	3.7	3.2
Basic												
Histidine (His)	3.0	2.7	3.6	1.6	1.2	1.3	1.3	1.2	1.3	1.3	1.3	1.1
Ornithine (Orn)	5.4	4.5	5.6	1.7	1.0	1.6	1.2	0.8	0.4	0.5	0.5	0.3
Lysine (Lys)	1.8	0.8	1.2	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Arginine (Arg)	5.6	2.4	2.1	1.5	0.6	1.1	0.9	1.0	1.1	1.1	1.0	0.6
Neutral												
Hydroxy												
Threonine (Thr)	4.6	5.1	5.0	5.1	4.9	4.9	4.8	4.4	3.5	4.4	3.8	3.5
Serine (Ser)	18.0	20.5	21.0	22.2	22.3	22.5	24.3	23.4	21.4	23.0	24.1	26.0
Straight												
Glycine (Gly)	15.0	17.4	17.8	20.3	17.2	18.2	19.9	20.1	19.7	19.7	19.2	19.1
Alanine (Ala)	11.0	11.5	10.6	12.7	13.0	13.4	12.6	12.6	13.3	12.9	12.5	12.7
Branched												
Valine (Val)	5.0	5.5	5.4	6.3	7.0	7.1	6.3	6.4	6.9	6.3	6.3	5.9
Isoleucine (Ile)	2.9	3.0	3.0	3.7	4.4	4.1	3.8	3.9	4.1	3.9	3.9	3.7
Leucine (Leu)	4.5	4.6	4.4	5.4	6.1	5.3	4.9	5.3	5.3	5.1	5.1	4.8
Secondary												
Proline (Pro)	2.7	4.1	3.8	3.9	4.0	3.6	3.5	4.0	4.3	4.4	4.1	4.1
Aromatic												
Tyrosine (Tyr)	2.1	2.6	2.5	3.3	4.0	3.3	3.6	4.1	3.5	3.4	3.4	3.4
Phenylalanine (Phe)	1.9	2.8	2.5	3.3	3.4	3.1	2.9	3.2	2.9	2.8	2.8	2.7
Sulfur-containing												
Cysteine (Cys)	2.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Methionine (Met)	0.5	0.8	0.8	1.0	2.1	1.1	1.1	1.3	1.2	1.0	1.2	1.4
Non-protein												
β-Alanine (BALA)	0.6	0.7	0.4	0.3	0.5	0.3	0.2	0.1	0.1	0.1	0.1	0.1
γ-Amino butyric acid (GABA)	1.1	0.1	0.0	0.0	0.0	0.1	0.0	0.0	0.0	0.6	0.0	0.0
Total free amino acids (TFAA)	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**	**100.0**

alanine and γ-aminobutyric acid to THAA with increasing depth.[15] In samples from the upper part of the sediment column, the combined relative abundance of β-alanine and γ-aminobutyric acid was less than 4 mol % of all amino acids.[15] With increasing depth, the relative abundance of β-alanine and γ-aminobutyric acid increased and in the deepest sample they reached up to 28% of THAA.[15] Diagenesis in sediment results in the decomposition of amino acids via decarboxylation[22,23] where, for example, aspartic acid will be altered to β-alanine by decarboxylation at the α-carbon. Consequently, the present verification revealed that bound refractory organic matter consisting of β-alanine and γ-aminobutyric acid remains intact following the diagenetic decarboxylation of aspartic acid and glutamic acid.

Conclusion

The following characteristics with regard to water-extractable free amino acids in the sub-surface of semi-permafrost were found:

1) Core sediment of the semi-permafrost was analyzed for total free amino acid (TFAA) analogs in relation to protein and non-protein amino acids. The content of these amino compounds ranged from 12.0 to 460.2 nmol/g, indicating generally that protein amino acids are the most abundant.

2) The concentration of THAA is far more predominant than that of TFAA in all sequences of core samples investigated. The percentage of TFAA (%TFAA) versus THAA ranged from 0.3 to 6.9%. TFAA was positively correlated with THAA, and yielded a straight line, as defined by a least-squares method, and represented by the relation $\mathrm{THAA} = 10.1 \times \mathrm{TFAA}^{1.4}$ ($R^2 = 0.86$). Consequently, bound amino acid analogs form the major component in organic matter.

3) The vertical trend in the molar ratio of TFAA varied with increasing depth, in particular showing a decrease in basic amino acids. Decarboxylation, such as the conversion of aspartic acid and glutamic acid to β-alanine and γ-aminobutyric acid, respectively, was not observed in the free amino acid analogs, while β-alanine and γ-aminobutyric acid in THAA increased significantly with increasing depth.

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References

1 E. Rivkina, E. I. Friedmann, C. McKay, and D. Gilichinsky, *Appl. Environ. Microbiol.*, **66**, 3230 (2000).

2 E. Rivkina, K. Laurinavichius, J. McGrath, J. Tiedje, V. Shcherbakova, and D. Gilichinsky, *Adv. Space Res.*, **33**, 1215 (2004).

3 E. Vorobyova, V. Soina, M. Gorlenko, N. Minkovskaya, A. Mamukelashvili, N. Zalinova, D. Gilichinsky, E. Rivkina, and T. Vishnivetskaya, *FEMS Microbiol. Rev.*, **20**, 277 (1997).

4 E. Rivkina, D. Gilichinsky, S. Wagener, J. Tiedje, and J. McGrath, *Geomicrobiol. J.*, **15**, 187 (1998).

5 T. Vishnivetskaya, S. Kathariou, J. McGrath, D. Gilichinsky, and J. Tiedje, *Extremophiles*, **3**, 165 (2000).

6 M. R. Maier and L. I. Pepper, "Terrestrial Environment," in "Environmental Microbiology," ed by R. M. Maier, I. L. Pepper, and C. P. Gerda, Academic Press (2000).

7 L. D. Jones, A. G. Owen, and J. F. Farrar, *Soil Biol. Biochem.*, **34**, 1893 (2002).

8 L. D. Jones, *Soil Biol. Biochem.*, **31**, 613 (1999).

9 L. D. Jones and A. Hodge, *Soil Biol. Biochem.*, **31**, 1331 (1999).

10 L. D. Jones and K. Kielland, *Soil Biol. Biochem.*, **34**, 209 (2002).

11 M. Kawahigashi, H. Sumida, and K. Yamamoto, *Geoderma*, **113**, 381 (2003).

12 K. Kielland, *Biogeochemistry*, **31**, 85 (1995).

13 F. J. Stevenson, "Humus Chemistry: Genesis, Composition, and Reactions," John Wiley and Sons (1982).

14 C. M. Monreal and W. B. McGill, *Soil Biol. Biochem.*, **17**, 533 (1985).

15 Y. Takano, J. Kudo, T. Kaneko, K. Kobayashi, Y. Kawasaki, and Y. Ishikawa, *Geochem. J.*, **38**, 153 (2004).

16 Y. Takano, L. P. Gupta, H. Kawahata, K. Marumo, Y. Ishikawa, and K. Kobayashi, *Bull. Chem. Soc. Jpn.*, **77**, 729 (2004).

17 Y. Takano, H. Mori, T. Kaneko, K. Kobayashi, Y. Kawasaki, and Y. Ishikawa, Abstract for 16th International Symposium on Environmental Biogeochemistry (2003), Vol. 2, p. 242.

18 S. A. Cohen and D. P. Michaud, *Anal. Biochem.*, **211**, 279 (1993).

19 S. Pantoja and C. Lee, *Org. Geochem.*, **34**, 1047 (2003).

20 D. J. Burdige and C. S. Martens, *Geochim. Cosmochim. Acta*, **52**, 1571 (1988).

21 B. Dauwe, J. J. Middelburg, P. M. J. Herman, and C. H. R. Heip, *Limnol. Oceanogr.*, **44**, 1809 (1999).

22 M. A. Ratcliff, E. E. Medeley, and P. G. Simmonds, *J. Org. Chem.*, **39**, 1481 (1974).

23 E. Andersson, B. R. T. Simoneit, and N. G. Holm, *Appl. Geochem.*, **15**, 1169 (2000).