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Biological origin for amino acids in a deep subterranean hydrothermal vent, Toyoha mine, Hokkaido, Japan.

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Abstract− Concentration of amino acids were determined for the investigation of subterranean microbiological activities in geothermal environments at Toyoha Mines, Hokkaido, Japan. Samples used were three specimens collected in hydrothermal water, and boring cores from a hydrothermal vein and a quartz vein. Total hydrolyzed amino acids (THAA) in the hydrothermal water were in the range of 2.5 μmol/l and 6.2 μmol/l. D/L ratios of aspartic acid, glutamic acid and alanine of the three samples were quite low, which was not more than 0.16. THAA in the hydrothermal vein rock and the quartz vein rock were 0.2 μmol/g-rock and 0.1 μmol/g-rock, respectively. D/L ratios of samples imply those amino acids were generated through microbiological activities, not abiotic hydrothermal chemical synthesis.

Keywords− amino acids, D/L ratio, hydrothermal environment, deep subterranean biosphere

INTRODUCTION

The deep subsurface is one of the major habitats for microorganisms (Gold, 1992). Subterranean microorganisms are recently detected in the course of ocean drilling experiments (Parkes *et al*., 1994; Craggs *et al*., 1994). These microbial populations are substantial (e.g. 10^7 cells/cm^3 at 500 m below sea floor) and likely to be widespread below sea floor. In order to evaluate microbial activities in extreme environments, several analytical chemical approaches have been proposed. Amino acids are common components of all organisms and constitute a major fraction of organic compounds in subsurface. Thus amino acid analysis is one of the most promising chemical techniques to search for the subterranean microbial activities.

The Toyoha mine is situated 40 kilometers southwest of Sapporo city, West Hokkaido, Japan. The mine is a hydrothermally active, polymetallic mineralized vein (Ohta, 1991) and well-known as one of most abundant indium mines in the world (Ohta, 1989; Ohta *et al*., 1998). It is also a rare geothermal site where not only sedimentary interstitial water but also magmatic water contributes to hydrothermal artesian spring water. The Toyoha deposit is at a very young age of mineralization, which have been formed 0.5-3 million years ago. The rock temperature is very high due to current geothermal activities (Urabe *et al*., 2001; Marumo, 2001; Mori *et al*., 2001, 2002).

One of our objectives is to survey biological activities in terrestrial subterranean hydrothermal environments in Toyoha mine. Besides, we aimed to evaluate hydrothermal stress on decomposition and racemization of amino acids in such extreme environments. Hydrothermal water and boring core samples were analyzed

for concentrations of total hydrolyzed amino acids (THAA) and their enantiomeric ratio. The concentrations of amino acids were determined by ion-exchange liquid chromatography (IE-HPLC), and D/L ratios of amino acids were measured by reversed-phase high performance liquid chromatography (RP-HPLC).

EXPERIMENTAL

Samples

The hydrothermal water and boring core samples used were obtained in the Archaean Park Project (Marumo, 2001). Hydrothermal water samples welling from the cracks were collected at three locations (referred to as A, B, C-site, respectively) of 550 m below the land surface in a pit of Toyoha Mines, Hokkaido, Japan. The highest temperature and pH of the hydrothermal water observed at the B-site were 71˚C and 5.8, respectively (Mori *et al*., 2002). The water seeped from the wall at the rate of 2.0 l/min. Two boring core samples (SN-101 and Toyoha 61) were analyzed: SN-101 was pyrite rich, and Toyoha 61 was quartz rich. The sample profile of geochemical aspect and biological survey have been preliminarily reported (Urabe *et al*., 2001; Kakegawa, 2001; Kuwabara *et al*., 2001; Mori *et al*., 2001, 2002).

Pre-treatment of water samples

An aliquot of the water sample were freeze-dried in test tubes which had been cleaned by soaking in 7 M HNO_3 over night and rinsed with pure water. Six molar HCl was added to the test tube. The test tube was sealed and at 110 ˚C in a block heater for 24 hours to obtain hydrolyzed amino acids. After evaporation to dryness,

the hydrolysates were dissolved in water, adjusted to pH 1, and applied to a Bio-Rad AG-50W-X8 cation-exchange resin column (200-400 mesh) for desalting. Before application of the sample, the resin had been washed by passing 1 M HCl, H_2O, 1 M NaOH and H_2O, successively. The resin was reactivated with 1 M HCl and rinsed with H_2O just before applying the sample. The amino acid fraction was eluted from the column with 10% NH_3 aqueous solution. The elute was freeze-dried and re-dissolved in 0.1 M HCl before amino acid analysis. To compare with blank level of amino acids during laboratory handling, blank analysis by using same ion-exchanged water, glass wares, HPLC, and RP-HPLC systems were performed.

Pre-treatment of rock samples

Rock core samples were carefully obtained and quickly sealed with dehydrating and deoxygenation agent package (AGELESS, Mitsubishi Gas Chemicals Co.). Interior portion of five hundred mg each of the freeze-dried rock samples were ground. In order to eliminate external contamination, sample vials were sealed by thin membrane filter (MILLI WRAP, Millipore Co.) in prior to dryness. Then, it was digested with 5 ml of 5 M HF - 0.1 M HCl mixture in a sealed Teflon vessel at 110 ˚C for 16 h. Then the mixtures were evaporated to dryness on a hot plate placed in a draft chamber. The organic residues were dissolved in pure water while applying ultrasonic waves. The aqueous solutions were filtered through GF/A glass filters, and then freeze-dried in test tubes. They were hydrolyzed in sealed test tubes with 6 M HCl at 110 ˚C for 2 hours. The hydrolysates were evaporated to dryness, and then desalted with AG-50W-X8 as previously described. Blank run was also performed

95 together with experimental samples.

Determination of total hydrolyzed amino acids (THAA)

The concentration of THAA was determined by ion-exchanged HPLC, which was composed of two high performance liquid chromatograph pumps (Shimadzu LC-6A), a
100 cation-exchange column (Shimpak ISC-07/S1504, 4 mm i.d.× 150 mm), a post column derivatization system, and a Shimadzu RF-535 fluorometric detector (Takano *et al,* 2001a). The derivatized reagents used were N-acetyl-L-cystein and *o*-phthalaldehyde (OPA) in borate buffer. Sodium hypochlorite solution was used as the second derivatization reagent for the detection of imino acids such as proline.

105

Measurement of enantiomeric ratio of amino acids

Separation of D- and L-amino acid enantiomers was achieved by a reversed-phase (RP) HPLC system, which was composed of a high performance liquid chromatograph pumps (TOSOH CCPM II), a reversed-phase column (YMC-pack Pro C18 4.6 mm i.d.
110 × 250 mm), and a TOSOH FS fluorometric 8020 detector (Excited wavelength: 355 nm and Emission wavelength: 435 nm). Gradient elution was applied by using the following eluents; A: 40 mM Sodium acetic acid buffer (pH 6.5), B: 100 % methanol which was ultra-pure HPLC grade. Aliquot of the pre-treated sample was mixed well with OPA and N-acetyl-L-cystein in a glass vial. Then the mixture was passed
115 through a solid phase extraction column (TOYOPACK-ODS) to eliminate hydrophobic compounds. The eluent was injected to the RP-HPLC system. Gradient elution was applied using the following eluents; A: 40 mM sodium acetic acid buffer (pH 6.5), B:

100% methanol (ultra-pure HPLC grade). Gradient program was performed as follows: 10 min (Eluent B: 0 %) – 25 min (Eluent B: 10 %) – 65 min (Eluent B: 20 %) – 80 min (Eluent B: 20 %) – 85 min (Eluent B: 40 %) – 115 min (Eluent B: 60 %) – 120 min (Eluent B: 80 %) – 135 min (Eluent B: 0 %).

All the glass wares were heated in a high temperature oven (Yamato DR-22) at 500 ˚C in prior to use in order to eliminate any possible contaminants. Water used was purified with a Milli-Q Labo and a Simpli Lab-UV (both Millipore Corporation) successively.

RESULT AND DISCUSSION

Amino acids in hydrothermal water samples

Figure 1 shows typical chromatograms of amino acids in hydrothermal water sample at the A-site and the B-site. Concentrations of amino acids in the hydrothermal water samples were summarized in Table 1. Glycine was the most abundant amino acid, and its concentration was ranged between 0.7 – 1.8 nmol/ml. Other major amino acids were among proteinous amino acids. Non-proteinous amino acids such as β-alanine, γ-aminobutyric were also detected as minor constituents. Blank analysis of amino acids during laboratory handling gave trace level amount of glycine (Takano *et al*., 2001b), which was less than ca. 10 pmol/ml. Consequently determination of amino acids here were derived from hydrothermal water samples in Toyoha mine.

Since the pioneering work of Miller (Miller, 1953), numerous experimental studies have been carried out concerning the formation of biologically interesting organic compounds simulated prebiotic conditions. Among these, amino acids could be

synthesized by hydrothermal abiotic processes such as laboratory experiment based on chemical evolution scenario (e.g. Yanagawa and Kobayashi, 1992; Islam *et al*., 2001). If the products of amino acids were abiotically formed, D/L ratio will converge nearly 1.0 (Yanagawa and Kobayashi, 1992). On the other hand, amino acids associated with life especially those form proteins are only L-form. Hence low D/L ratios seem to be good evidence of biological activities. As shown in Table 2, the D/L ratios of aspartic acid, glutamic acid and alanine showed that only slight racemization from L-form to D-form occurred. It was reported that *thermodesulfovibrio* and *Acetobacterium* were detected in hydrothermal vein at a depth of 550 m, Toyoha mine (Mori *et al*., 2002). As to biological population, microbial community analysis of subsurface hydrothermal water there were shown that microscopic observation indicated large population of subterranean microbe of *ca*. 10^5 cell/ml in hydrothermal water (Higashi and Maruyama, 2001). There is another organic source in hydrothermal water samples except microbial contents, that is, suspended particulate organic matter (POM) (e.g. Margaret *et al*., 2003) and dissolved organic matter (DOM) (e.g. David, 2001). POM and DOM contains multiple organics such as microbial corpse, hydrothermal altered peptides, sugars, nucleic acid bases and other wide variety of organic compounds. Number of organics in POM and DOM might be altered in some processes (e.g. Ratcliff *et al*., 1975) such as decarboxylation and deamination under hydrothermal condition, resulting formation of secondary products. At the early stage of proteinous diagenesis or thermal alteration in terrestrial environment, the degradation of polypeptides, oligopeptides to amino acid monomers might be occurred (Ogasawara *et al*., 2001). Hence it is difficult to mention the

consistency with amino acid species and bacterial or archaean origins. The deep subsurface is one of the major habitats for microorganisms (Gold, 1992). Indeed, procaryotic activities have been reported in environment at 120 ˚C or possibly even higher (Cragg *et al*., 1994). Those microbiological activities might contribute the production of organic compounds including amino acids under anaerobic environment and at high temperature in subterranean environments at Toyoha mine.

Amino acids in rock samples

Concentration of amino acids in the interior portion of rock samples were shown in Table 3. SN-101 was sulfur containing pyrite rich vein, but amino acids with sulfur such as methionine were not detected. Relative abundance of dicarboxylic amino acids such as aspartic acid and glutamic acid, and that of amino acids with hydroxylic group such as serine and threonine in the rock samples were much more than those in the hydrothermal water samples. Hydrothermal stress may cause decomposition of amino acid *via* decarboxylation (Ratcliff *et al*., 1974), i.e., aspartic acid will alter to β-alanine by decarboxylation at α-carbon (Schroeder, 1975; Cowie and Hedges, 1994). An interesting characterization of the ratio of β-ala/Asp in these samples were extracted: ratios of β-ala/Asp show 0.14, 0.15, and 0.20 in hydrothermal water sample A, B, and C, respectively. On the other hand, ratios of β-ala/Asp show 0.01 and 0.02 in rock sample SN-101 and 61, respectively. The minor constituent of β-alanine in rock samples are crucial that amino acids in interior rock seem to be more stable than dissolved in hydrothermal water.

Figure 2 shows a reversed-phase chromatogram of amino acid enantiomers in the

core sample SN-101. The process of amino acid racemizaion occurring in various geochemical samples in terrestrial and marine environments has been widely applied in geochemical research (e.g. Harada and Handa, 1995). Racemization of amino acids is primarily dependent on the age and temperature of the environment (Bada and Schroeder, 1975). The hydrothermal process may progress in kinetic control with environmental temperature (Bada, 1972), therefore, D/L ratios were determined and used as indicators of the extent of organic matter alteration and coincidence of subterranean microbial activities. Racemization rate constant was reported to be higher in hot geothermal condition (Bada, 1972), although, the observed D/L ratio of amino acids in the core samples may imply anaerobic microbiological activities as well as hydrothermal water samples. Since the temperature increases with depth, it has been suggested that hyperthermophiles, in particular chemiolithoautotrophs, are abundant in subterranean environments forming a deep hot biosphere (Gold, 1992). Possible existence of anaerobic subsurface lithoautotrophic microbial systems has been reported (Stevens, 1995). The present results showed the possibility of subterranean microbial activities in Toyoha mine. Additionally, isolation of a new thermophilic, strictly anaerobic, thiosulfate-reducing bacteria in hydrothermal vent at Toyoha mine gave an implication of the presence of deep subterranean microbial activities.

CONCLUSION

Total concentration of amino acids in the hydrothermal water ranged from 2.6 to 6.1 μmol/l, and that of the hydrothermal vein rocks was from 87 to 230 nmol/g-rock. Relative abundance of acidic amino acids such as aspartic acid and glutamic acid in the

hydrothermal waters was lower than that in the hydrothermal vein rocks. The D/L ratios of the samples implied the presence of subterranean microbiological activities, rather than the contribution of abiotic hydrothermal synthesis of amino acids.

It is widely believed that the hydrothermal systems in deep ocean have played important roles for the emergence of life under the primitive earth conditions (e.g. Holm, 1992; Yanagawa *et al,* 1988, 1992; Islam *et al*., 2001). From the point of view of chemical evolution and origins of life, it is of interest that distribution and stereo chemistry of amino acids in submarine hydrothermal sub-vent systems will have to be clarified (Takano *et al*., 2001b). In order to build up a consolidated model of extreme environmental geology, biology and chemistry in submarine hydrothermal vent, an integral research project on interaction between sub-vent biosphere, "Archaean Park Project" which is called, is now in progress (Urabe *et al*., 2001).

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Figure and Table caption

Fig.1 Ion-exchange chromatograms of hydrolyzed amino acids in the hydrothermal water sample of A-site sample. Abbreviations. Asp:aspartic acid, Ser: serine, Glu: glutamic acid, Pro: Proline, Gly: glycine, Ala: alanine, Val: valine, Met: Methionine, Ile: isoleucine, Leu: leucine, Tyr: tyrosine, Phe: phenylalanine, β–Ala: β-alanine, γ-ABA:γ-aminobutyric acid, δ-AVA: δ-aminovaleric acid.

Fig. 2 Reversed-phase chromatogram of amino acid enantiomers in the core sample SN-101. Abbreviations for amino acids: D, L-Asp: D, L-aspartic acid, Ser: serine, D, L-Glu: D, L-glutamic acid, D, L-Ala: D,L-alanine, β-Ala: β-Alanine. D- and L- serine peaks are not separated.

Table 1 Concentration of amino acids in the hydrothermal water at Toyoha mine, Hokkaido, Japan.

Table 2 D/L ratios of aspartic acid, glutamic acid and alanine in hydrothermal water and core samples in Toyoha mine, Hokkaido, Japan.

Table 3 Concentration of amino acids in the core samples at Toyoha mine, Hokkaido, Japan.

Figure 1

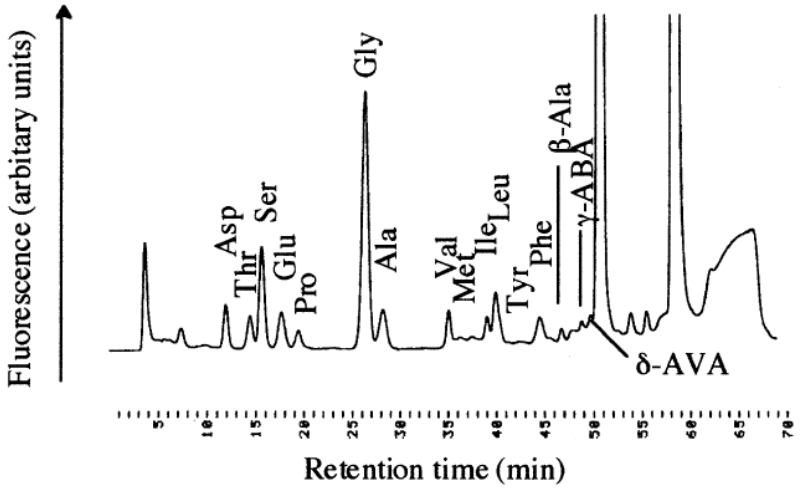
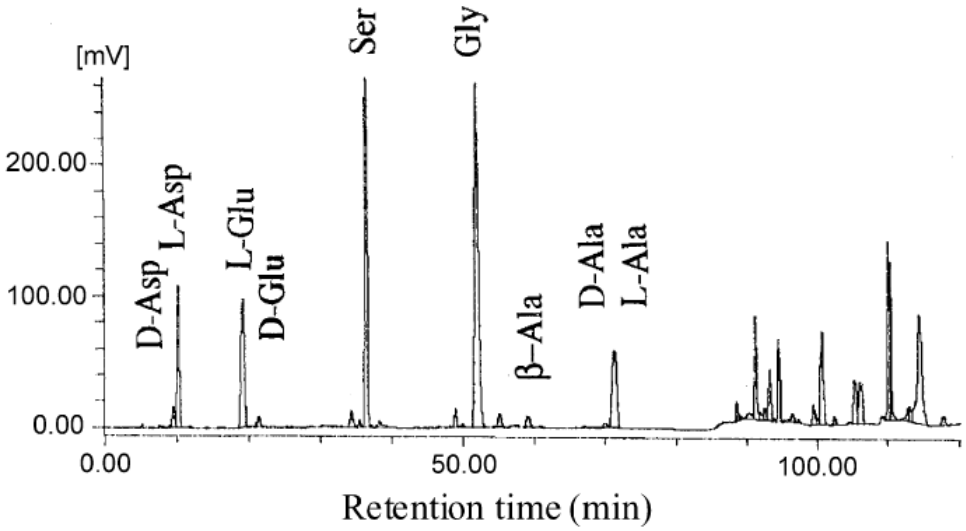


Figure 2



Takano *et al., Organic Geochemistry*, **34**, 1491-1496 (2003).

Table 1

	concentration			mole ratio		
Sampling site	A-site	B-site	C-site	A-site	B-site	C-site
temperature* / ˚C	63.5	71.3	48.3	-	-	-
pH*	6.6	5.8	6.6	-	-	-
unit		μmol/l			% mole	
Asp	0.36	0.26	0.30	5.93	10.30	11.60
Thr	0.24	0.14	0.13	3.92	3.03	5.00
Ser	0.75	0.38	0.38	12.37	8.23	14.74
Glu	0.19	0.13	0.20	3.18	2.80	7.60
α-AAA	n.d.	n.d.	n.d.	-	-	-
Gly	1.78	1.45	0.70	29.20	31.61	27.24
Ala	0.47	0.28	0.21	7.69	6.00	8.32
α-ABA	n.d.	n.d.	n.d.	-	-	-
Val	0.38	0.20	0.28	6.18	4.33	10.74
Cys	0.09	0.68	n.d.	1.47	14.84	-
Met	0.22	0.17	tr.	3.66	3.70	-
Ile	0.23	0.14	0.03	3.74	3.06	1.12
Leu	0.71	0.44	0.10	11.59	9.54	3.81
Tyr	tr.	n.d.	tr.	-	-	-
Phe	0.33	0.19	0.07	5.37	4.16	2.86
β-Ala	0.05	0.04	0.06	0.77	0.81	2.21
β-AiBA	n.d.	n.d.	n.d.	-	-	-
γ-ABA	0.14	0.02	tr.	2.34	0.54	-
δ-AVA	tr.	tr.	n.d.	-	-	-
Pro	0.16	0.11	0.12	2.54	2.49	4.71
Total	6.10	4.60	2.57	100	100	100

tr.: trace amount (detected but not quantified.), n.d.: not detected
* Mori et al., 2002.

Table 2

	hydrothermal water			core	
Sample	A-site	B-site	C-site	SN101-14	61
Asp	0.08	0.05	0.15	0.13	0.06
Glu	0.09	0.16	0.09	0.06	0.09
Ala	0.06	0.00	0.00	0.05	0.05

Table 3

	concentration		mole ratio	
core	SN101-14	61	SN101-14	61
unit	nmol/g-rock		% mole	
Asp	21.45	7.86	9.32	9.04
Thr	11.15	3.94	4.85	4.54
Ser	56.64	17.28	24.63	19.88
Glu	20.42	4.90	8.88	5.64
α-AAA	n.d.	n.d.	-	-
Gly	61.37	23.99	26.68	27.60
Ala	25.22	9.28	10.96	10.68
α-ABA	n.d.	n.d.	-	-
Val	9.19	4.50	4.00	5.18
Cys	n.d.	n.d.	-	-
Met	n.d.	n.d.	-	-
Ile	4.42	2.09	1.92	2.40
Leu	7.32	4.58	3.18	5.27
Tyr	0.54	tr.	0.24	-
Phe	2.93	3.55	1.27	4.09
β-Ala	0.35	0.20	0.15	0.24
β-AiBA	n.d.	n.d.	-	-
γ-ABA	0.50	0.32	0.22	0.36
δ-AVA	tr.	n.d.	-	-
Pro	9.78	4.43	4.25	5.10
Total	230.00	86.93	100	100

tr.: trace amount (detected but not quantified.), n.d.: not detected