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Separation and identification of free amino acid enantiomers in peat by capillary gas chromatography

A. V. Kunnas and T.-P. Jauhiainen

Department of Chemistry, University of Joensuu, P.O. Box 111, SF-80101 Joensuu (Finland)

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ABSTRACT

Racemization of free amino acids in a native peat bog at seven depths from 0 to 5.3 m was studied using glass capillary gas chromatography with a chiral liquid phase, Chirasil-L-Val. Fifteen L-enantiomers were identified: α-Ala, Val, Thr, Ile, Pro, Leu, Ser, Asp, Phe, Glu, Tyr, Orn, Met, Asn and Lys. Five D-enantiomers were also found: α-Ala, Ser, Asp, Glu and Phe. The D/L ratios at different depths were determined and are discussed in terms of racemization, plant and microbial degradation, and the physical and chemical conditions in the peat bog. In addition, three optically inactive amino acids were identified: β-Ala, Gly and γ-aminobutyric acid.

INTRODUCTION

The amino acids in active living tissues exist mostly as L-enantiomers, which is the only form used by animal enzyme systems. However, the cell walls of bacteria also contain some D-enantiomers, especially D-α-Ala, D-Glu and D-Asp [1]. After the tissue ceases to be metabolically active, both free and bonded amino acids eventually start to racemize in the soil. This process continues until the ratio of enantiomers is 1:1. The rate of the process has been used to date some fossil proteins in ocean sediments, archaeological bones and fossil shells [1–5].

Aspartic acid has been found to be a suitable indicator up to 80 000 years and the D-Allo/L-Ile ratio has been used for even older samples [2,3,6].

The rate of racemization depends on the structure of the amino acid, especially on the side chain at the α-carbon, and on the chemical and physical conditions of the environment, for example temperature, trace elements and acidity [1,5]. In aqueous solutions the rate of racemization decreases in the order Ser > Thr > Asp > Phe > α-Ala > Glu > Leu > Ile > Val [7].

This study is a sequel to our earlier work on the free amino acids in a typical Finnish peat bog [8]. The aim of the present study was to separate and identify the D- and L-enantiomers of the optically active free amino acids and to study the effect of the age of the peat on their D/L ratios. As far as we know, there have been no previous studies on the enantiomers of free amino acids in native peat bogs.

EXPERIMENTAL

Reagents and solutions

DL- and L-amino acids were obtained from Sigma (St. Louis, MO, USA) and pentafluoropropionic anhydride (PFP) from Fluka (Buchs, Schwitzerland). The other reagents were analytical grade and were obtained from Merck (Darmstadt, Germany). Aqueous solutions were prepared using deionized and distilled water.

Correspondence to: T.-P. Jauhiainen, Department of Chemistry, University of Joensuu, P.O. Box 111, SF-80101, Joensuu, Finland.

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Samples and isolation of amino acids

The peat samples were collected from various depths, 0–5.3 m, in the Kaitalamminsuo bog, at Eno, Finland (E30°07′, N63°01′). Characterization of the samples and the method used for separation of amino acids are presented elsewhere [8].

Gas chromatography

The gas chromatographic measurements were made using a Perkin-Elmer F22 capillary gas chromatograph with two flame ionization detectors. The structures of the amino acids were confirmed with a Jeol JMS-D300/JMA-3500 mass spectrometer.

Amino acids were separated on a silica glass capillary column (25 m × 0.22 mm I.D.) using a 0.12-μm Chirasil-L-Val liquid phase.

The carrier gas was nitrogen, which had a flow-rate of 1.8 ml/min and a split ratio of 1:32. Splitless injection was used, and the split was opened 40 s after injection of the sample. The temperatures of the injector and the detector were 220 and 230°C, respectively. The column oven was kept at 90°C for 4 min and then heated to 190°C at a rate of 4°C/min.

Derivatization of amino acids

The esters of amino acids were synthesized by mixing 2.0–3.5 mg of sample with 29 μl of acetyl chloride and 71 μl of isopropanol. The mixture was heated in a closed bottle at 110°C for 30 min, after which it was cooled to 50°C and the volatile reagents were evaporated carefully with nitrogen gas. The dry residue was dissolved in 50 μl of dichloromethane, and 50 μl of pentafluoropropionic anhydride were added. The mixture was heated for 10 min at 150°C in a closed bottle. After it was cooled to room temperature, volatile reagents were removed under a stream of nitrogen. To prevent the evaporation of α-Ala, Val and Thr, excessive drying was avoided. The residue was then dissolved in an appropriate volume of dichloromethane and the sample was chromatographed immediately [9,10].

RESULTS AND DISCUSSION

Kaitalamminsuo bog is an *Eriophorum–Sphagnum* mire. The samples for this study were taken from seven depths up to a depth of 5.3 m. The peat was classified and characterized by determining its water, element and ash contents as well as the pH and degree of humification on Von Post's scale (see ref. 8).

Because the chiral centres of the liquid phase, Chirasil-L-Val, had an L-configuration, the D-enantiomers eluted from the capillary column first. The net retention times, resolution factors (L/D) and mass spectrometric data for D,L-amino acid standards are presented in Table I. The resolution factors for all the amino acids were high enough to identify the enantiomers by their retention times.

Two typical gas chromatograms for amino acid extracts from peat are presented in Fig. 1A and B. Fig. 1A represents the surface layer, 20–40 cm, where the conditions were aerobic; Fig. 1B is from a depth of 3.0–3.3 m, at which the conditions were anaerobic. Eighteen different amino acids were separated and identified with the column used.

L-Enantiomers were found for all the optically active amino acids. For five of them, the D-enantiomer also occurred in detectable amounts. Of the epimers of isoleucine, only the *threo*-L-isomer was found. The method of derivatization used here was not suitable for arginine and histidine.

The D/L ratios for the enantiomers at various depths are presented in Table II as the means of five measurements. In the surface layer of the peat, where microbial degradation of plant and microbial material is most effective, the L-enantiomers were the most abundant enantiomers for all five amino acids. The living organisms and enzyme systems contain and use L-enantiomers of the amino acids almost exclusively. However, the microbial cell walls contain small amounts of D-α-Ala and some other D-enantiomers [1,11]; this together with racemization explains the low positive values of D/L ratios, which were also found in the surface layer of the bog.

At a depth of 40–100 cm there is a stepwise change in the properties of peat and in the total amount and qualitative composition of free amino acids [8]. Between these depths the D/L ratios for all five amino acids increase markedly, from 1.5 times for serine to 7.3 times for glutamic acid. An important reason for this may be changes in conditions in the bog from aerobic to anaerobic, where the microbial activity as well as the supplementary production of free amino acids is low.

Below 100 cm the proportions of D-enantiomers increase at different rates, but they seem to reach

TABLE I

NET RETENTION TIMES (t'_R), RESOLUTION FACTORS ($\alpha = t'_L/t'_D$), MOLECULAR MASSES (M_r) AND MASS SPECTROMETRIC DATA OF THE ISOPROPYL ESTERS OF N-(O,S)-PENTAFLUOROPROPIONYL-D,L-AMINO ACIDS

Amino acid	t'_R (min)	α	M_r	Main peaks in the mass spectra
D-α-Ala	1.28	1.390	277	190(100), 43(47), 191(43), 41(13),
L-α-Ala	1.79			119(11)
D-Val	2.62	1.237	305	218(100), 55(87), 43(54), 219(34),
L-Val	3.24			203(25), 41(14)
D-Thr	3.39	1.150	453	43(100), 203(70), 202(45), 57(26),
L-Thr	3.92			41(23), 119(16)
Gly	3.97	–	263	43(100), 177(63), 176(62), 41(24)
β-Ala	4.10	–	277	55(100), 218(67), 43(48), 189(24)
D-*threo*-Ile	3.98	1.128	319	69(100), 232(59), 42(31), 203(30),
L-*threo*-Ile	4.49			40(28), 233(21), 221(18), 57(11)
D-Pro	5.72	1.031	303	216(100), 217(12), 43(10), 41(10),
L-Pro	5.90			119(8), 71(7)
D-Leu	6.04	1.273	319	43(100), 69(85), 190(48), 232(41),
L-Leu	7.69			189(27), 41(26)
D-Ser	6.69	1.085	439	43(100), 189(68), 188(45), 41(20),
L-Ser	7.26			119(19), 230(16)
γ-Aminobutyric acid	10.39	–	291	232(100), 43(59), 41(44), 204(39)
D-Asp	11.99	1.019	363	43(100), 234(64), 262(46),
L-Asp	12.22			235(35), 189(33)
L-Asn	13.85		466	43(100), 215(51), 189(40), 216(35)
D-Met	13.67	1.081	337	61(100), 75(70), 221(34), 43(32),
L-Met	14.78			203(31), 263(20)
D-Phe	15.67	1.054	353	91(100), 190(75), 148(65),103(30),
L-Phe	16.52			43(26), 266(25)
D-Glu	16.14	1.051	377	248(100), 202(78), 230(72), 43(69),
L-Glu	16.97			85(55), 276(54)
D-Tyr	21.17	1.033	515	253(100), 310(91), 352(88), 43(48),
L-Tyr	21.86			248(30)
D-Orn	22.28	1.043	466	216(100), 43(27), 67(22), 231(11)
L-Orn	23.25			
D-Lys	25.45	1.024	480	230(100), 43(27), 67(22), 231(11),
L-Lys	26.07			110(11), 176(10)

TABLE II

D/L RATIOS OF FREE AMINO ACIDS AT VARIOUS DEPTHS IN THE KAITALAMMINSUO PEAT BOG

Peat types: 1 = *Eriophorum-Sphagnum*; 2 = *Bryales–Carex*; 3 = *Sphagnum–Carex*; 4 = *Carex*; 5 = *Carex–Bryales*; 6 = *Carex–Phragmites–Equisetum*

Sample	K1	K2	K3	K4	K5	K6	K7	K8
Peat type	1	2	1	3	3	4	5	6
Depth	0–20 cm	0–20 cm	20–40 cm	1.0–1.3 m	2.0–2.3 m	3.0–3.3 m	4.0–4.3 m	5.0–5.3 m
Degree of humification [8]	H 0–1	H 0–1	H 2–3	H 5–6	H 5	H 5	H 5	H 5
pH [8]	5.3	5.4	5.8	3.8	3.9	3.7	5.9	2.8
Amino acid	D/L ratio							
α-Ala	0.414	0.144	0.272	1.270	1.190	1.130	1.690	1.230
Ser	0.090	0.106	0.043	0.064	0.089	0.235	0.161	0.123
Asp	0.013	0.008	0.016	0.093	0.115	0.157	0.172	0.164
Phe	0.033	0.024	0.043	0.128	0.340	0.376	0.378	0.320
Glu	0.012	0.011	0.014	0.102	0.084	0.099	0.194	0.157

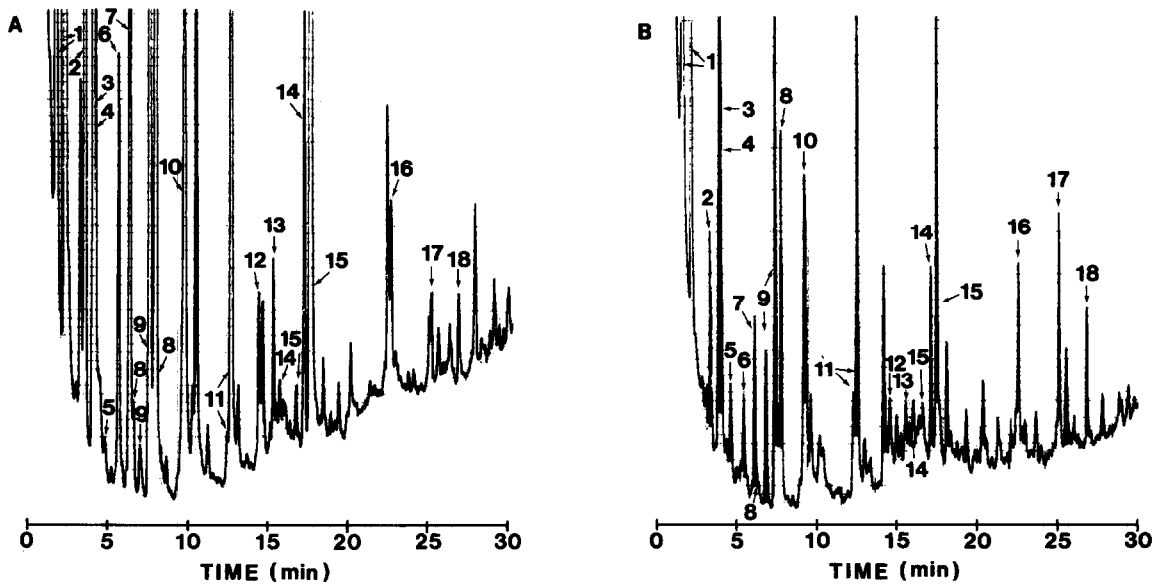

Fig. 1. Two typical gas chromatograms of isopropyl esters of N-(O,S)-pentafluoropropionyl-D,L-amino acids extracted from native peat. Depths of the samples were: A = 20–40 cm; B = 3.0–3.3 m. Peaks: 1 = α-Ala; 2 = Val; 3 = Gly; 4 = Thr; 5 = β-Ala; 6 = *threo*-Ile; 7 = Pro; 8 = Leu; 9 = Ser; 10 = γ-aminobutyric acid; 11 = Asp; 12 = Asn; 13 = Met; 14 = Phe; 15 = Glu; 16 = Tyr; 17 = Orn; 18 = Lys.

equilibrium values typical for each amino acid. Racemization probably continues at such a low rate that the process is impossible to follow owing to the large variation in results and the relatively young age of the peat, 9000 years [12].

The most rapid rate of racemization is that of α-alanine, for which equilibrium is reached already at a depth of 1 m and the D-enantiomer is predominant. Because the D/L ratio of α-alanine is greater than 1, the degradation of plants and microbes must produce not only the L-enantiomer but also the D-enantiomer.

The greatest changes in D/L ratios are for Asp, Phe and Glu. For these amino acids, at a depth of 5 m the proportion of D-enantiomer is 10–13 times higher than in the surface layer. However, the L-enantiomer is still clearly more abundant. On the other hand, the D/L ratio of serine does not change as much as that of the other four amino acids. It is impossible to say to what extent D-Asp, D-Phe and D-Glu are degradation products of plants and microbes or whether the racemization of these amino acids takes place in peat at an exceptionally high rate. The levelling off of the change at greater depths indicates that in the upper peat layer the degradation might provide an additional source of these D-enantiomers.

The type of peat has an obvious effect on the D/L ratios. The differences between Bryales (K2, K7) and *Sphagnum* types (K1, K3, K4, K5, K6, K8) are especially great. The same distribution was also found in the quantitative amounts of amino acids [8].

On the other hand, there are reports on the D/L ratios of bound amino acids in ocean sediments [1,4]. The order of the results for samples from different sources is for the most part the same, but there are some obvious differences. For example, in peat samples the D/L ratio of α-alanine is high compared with that in the ocean sediments, because D-α-Ala in peat is at least partly a degradation product of microbial material. In samples from the Antarctic Ocean sediments, ornithine razemices at a very high rate [1]. In peat, however, D-Orn was not found at all. The reason for this must be related to environmental factors. For example, the pH of the ocean sediments was 7–8, but of the peat bog was 2.8–5.9. The differences in acidity might change the

racemization rates by several orders of magnitude [5]. In addition, the peat bogs contain trace metals such as copper, zinc, manganese, magnesium, etc., and when chelated with amino acids they have the same effect on the racemization rates [5,13].

With the exception of threonine, the six amino acids that racemized at the highest rate in aqueous solutions also had the highest D/L ratios in peat [7]. D-Threonine was not found at all in these peat samples.

In light of these results, it seems unrealistic to estimate the age of peat from the D/L ratios of free amino acids. Obviously, too many environmental factors influence the D/L ratios in natural conditions, and the age of the peat in our samples was too low, at a depth of 5 m only about 9000 years, to allow us to obtain reliable results on pure racemization without an additional source of D-enantiomers from the destruction of plant and microbial material.

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