ORIGINAL

Novel *N*-Acyl Amino Acid Produced by *Deleya marina*

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The structure of a lipid produced by *Deleya marina* was identified as *N*-acyl amino acid, whose fatty acid component was linked by amide bond to the α -amino group of the amino acid. This component was determined as C₁₄, C₁₆, C_{16:1(n-7)}, and C_{18:1(n-7)} by GC-MS. The *N*-acyl amino acid possessed no amide-linked 3-hydroxy fatty acids, which are present in most *N*-acyl amino acids reported previously. The amino acid portion was determined as leusine and isoleusine by GC-MS and NMR spectrometry. The leusine or isoleusine containing *N*-acyl amino acid from microorganisms is presented here for the first time.

Introduction

N-acyl amino acids are widely distributed in microorganisms and were well reviewed by J. Asselineau⁴⁾. Serratamic acid and flavolipin were serine-containing lipid, isolated from *Serratia marcescens* and *Flavobacterium meningosepticum*, respectively^{1)~3)}. Ornithine-containing lipids were widely distributed in gram negative bacteria and has only been found in a small number of gram positive species⁴⁾. Cerilipin was ornithine and taurine containing lipid obtained from *Glucunobacter cerinus*⁵⁾. Lysine containing lipids were found in *Agrobacterium tumefaciens*⁸⁾ and *Streptomyces toyocaensis*⁹⁾. *Cytophaga johnsonae* produced glycine containing lipid¹⁰⁾. Most of these *N*-acyl amino acids had characteristic structure, where a 3-hydroxy fatty acid was linked by amide bond to the α -amino group of amino acids. A second fatty acid was esterified to the hydroxyl group of the first 3-hydroxy fatty acid.

Recently, we found four unknown spots on thin layer chromatograph separating the lipids from *Deleya marina* (ATCC 25374) in

the screening of functional compounds from marine microorganisms. We identified one of these spots as novel *N*-acyl amino acid. *N*-acyl amino acids reported so far had serine, ornithine, glycine or lysine in its amino acid portion^{4),8)~10)}. On the other hand, the *N*-acyl amino acid obtained from *Deleya marina* contained leusine or isoleusine. In amide-linked fatty acid portion, the *N*-acyl amino acid had C₁₄, C₁₆, C_{16:1}, or C_{18:1}. However, it did not have 3-hydroxy fatty acids which were found in most of other *N*-acyl amino acids. This paper describes a study of this new type *N*-acyl amino acid.

Materials and Methods

Bacterial strain and growth

Deleya marina (ATCC 25374) was grown at 25°C on a medium containing 10g glucose, 3 g pepton and 1g yeast extract in 1L of artificial sea water. Pepton was purchased from Nihon Seiyaku (Tokyo, Japan). As an artificial sea water, Tropic Marine produced by Aquarientecnik (Wertenberg, Germany) was used.

Isolation of *N*-acyl amino acids

Cells were collected after centrifugation and washed with a 0.8 % NaCl solution. The cells were extracted with CHCl₃-MeOH solution (1 : 2 vol/vol). The extract was parti-

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tioned between CHCl_3 and MeOH-water. The CHCl_3 -soluble portion was evaporated under reduced pressure, then the residue was dissolved into MeOH and filtrated with filter paper to remove polymer. The MeOH soluble portion was fractionated by CPC (centrifugal partition chromatography) in a solvent system hexane-methanol-water (10 : 9 : 1) with the higher layer stationary. The fractions containing *N*-acyl amino acid were combined and fractionated by flash chromatography on silica with CHCl_3 , $\text{CHCl}_3/\text{MeOH}$ (8/2) and MeOH. The fraction eluted with CHCl_3 was fractionated by flash chromatography on ODS with methanol. The fraction eluted with methanol was rechromatographed by HPLC on an ODS column by MeOH/MeCN/ H_2O (50/49/4) containing 0.1 % acetic acid, and several fractions were obtained.

The *N*-acyl amino acid was also isolated by preparative TLC on a Merck silica gel 60 plate. The solvent system was $\text{CHCl}_3/\text{MeOH}/\text{H}_2\text{O}$ (65/25/4, vol/vol). The lipid visualized with iodine vapor on TLC plate was scraped off and extracted with $\text{CHCl}_3/\text{MeOH}$ (2/1 vol/vol).

Analysis of *N*-acyl amino acid

FAB-MS spectra of *N*-acyl amino acid were obtained on VG 70-250 S mass spectrometer using triethanolamine as a matrix. Acids hydrolysis of the *N*-acyl amino acids was performed at 80°C for 20~22 h by 2M HCl in MeOH. After concentration, the hydrolysate was partitioned between hexane and water. The both phases were examined by GC-MS (HP 5971 A and HP 5890). Position of double bonds in fatty acid was determined by picoliny methods^{6),7)}. Fatty acid methyl esters were obtained from the acid hydrolysis of *N*-acyl amino acid isolated by preparative TLC. Those were hydrolyzed with 1M NaOH in MeOH for 45 min at 80°C to release the free acids. The acids were converted into its chloride by reaction with 100 μL of thionyl chloride for few min at room temperature. The thionyl chloride was evaporated under reduced pressure and the residue was reacted with a 10 % solution of a 3-pyridylcarbinol in acetonitrile (10 μL)

for few min at room temperature. The resulting solution was examined by GC-MS. The double bond position was determined by the 26 mass unit separation between ions. ^1H and ^{13}C NMR spectra of the water layer were recorded on JEOL EX-270 and compared with those of authentic methylesters of leusine and isoleusine. The authentic methylesters were prepared in our laboratory by the methylation of leusine and isoleusine at 80°C for 20 h by 2 M HCl in MeOH.

Results and Discussions

Fig.-1 shows TLC analysis of the lipids extracted from *Deleya marina*. The bacteria produced nutral lipids (NL), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and unidentified lipids 1-4. The lipid 1 was isolated as described in materials and methods. First, one of fractions by HPLC was examined.

Negative FAB-MS spectrum of the fraction by HPLC showed two molecular ion peaks, $m/z=368$ and 394 [Fig.-2 (a)]. The fraction showed negative response to ninhy-

The solvent system used was chloroform/methanol/water (65/25/4 vol/vol).

Fig.-1 TLC analysis of the lipids extracted from *Deleya marina*.

Fig.-2(a) Negative FAB-MS spectrum of one fraction by HPLC.

Fig.-2(b) Acid hydrolysis products of the *N*-acyl amino acid which showed peaks in Fig.-2 (a).

drin reagent, which indicated it did not have terminal NH_2 . Furthermore, it was not hydrolyzed by mild hydrolysis using 0.2 M methanolic HCl or 0.2M methanolic KOH, which indicated that lipid 1 did not have ester bond.

Then, the hydrolysis was performed with 2M methanolic HCl, and the hydrolysate was partitioned between hexane and water after concentration. The hexane layer was examined by GC-MS and two peaks appeared. The peaks showed typical mass spectra of fatty acid methyl ester and were determined as C_{16} and $\text{C}_{18:1}$ with mass fragmentation and molecular ions at m/z 270 and 296. The double bond position of $\text{C}_{18:1}$ was determined by picolinyl method as described in materials and methods. The double bond was located at $n-7$ position by the 26 mass unit separation between ions at m/z 262 and 288 (Fig.-3). Therefore, the hexane layer contained methylesters of C_{16} and $\text{C}_{18:1(n-7)}$. On the other hand, the water layer from the hydrolysate showed two spots on TLC and they showed positive response to ninhydrin reagent. Release of fatty acid and terminal NH_2 after hydrolysis indicated the presence of amide bond in the lipid 1. GC-MS analysis of the water layer implied the presence of leucine and isoleucine methylesters. To confirm the presence of leucine and isoleucine, ^1H and ^{13}C NMR spectra were recorded. The NMR spectra of

Fig.-3 Mass spectrum of the picolinyl derivative of $C_{18:1}$.

the water layer corresponded well with those of authentic methylesters of leusine and isoleusine. The lipid in the fraction was, consequently, determined as *N*-acyl amino acid, which consisted of C_{16} or $C_{18:1(n-7)}$ in its fatty acid portion and leusine or isoleusine in its amino acid portion [Fig.-2 (b)].

Fig.-4 shows Negative FAB-MS spectrum of the lipid 1 obtained by TLC. There were four molecular ion peaks, $m/z=340$, 366, 368 and 394. Hydrolysis of the lipid 1

gave the methylesters of C_{14} , $C_{16:1(n-7)}$, C_{16} , and $C_{18:1(n-7)}$ in hexane layer, and the methylesters of leusine and isoleusine in water layer. Therefore, the lipid 1 was *N*-acyl amino acid shown in Fig.-5.

Most *N*-acyl amino acids so far obtained from microorganisms had an amide linked 3-hydroxy fatty acid such as 3-hydroxydecanoic acid¹⁾, 3-hydroxy isoheptadecanoic acid^{3),10)}, 3-hydroxyhexadecanoic acid^{5),8)}, 3-hydroxyoctadecanoic acid, etc⁴⁾. In most of them, second fatty acid such as isopen-

Fig.-4 Negative FAB-MS spectrum of the lipid 1 obtained by TLC.

Fig.-5 *N*-acyl amino acid species contained in the lipid 1.

tadecanoic acid^{3),10)}, 2-hydroxyhexadecanoic acid⁵⁾, C₁₆, C₁₈ and so on⁴⁾ esterified the hydroxyl group of the first 3-hydroxy fatty acid. In amino acid portion of these *N*-acyl amino acids, serine, ornithine, glycine or lysine were found.

On the other hand, the *N*-acyl amino acid described in this paper is new type *N*-acyl amino acid, where a fatty acid other than 3-hydroxy fatty acid is linked by amide bond to α -amino group of amino acid. Furthermore, it contained leusine or isoleusine as amino acid. This is the first isolation of leusine or isoleusine containing *N*-acyl amino acid.

The lipids 2-4 are remained to be identified. Further analysis of these lipids is in progress.

(Received April 14, 1995)

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Delacy marina により生合成される 新規の *N*-アシルアミノ酸

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Deleya marina は、アミノ酸のアミノ基に脂肪酸がアミド結合した *N*-アシルアミノ酸を生合成した。GC-MS による分析から、脂肪酸部分は C₁₄, C₁₆, C_{16:1(n-7)} および C_{18:1(n-7)} と決定された。しかし、これまでに見いだされた大部分の *N*-アシルアミノ酸が含有しているアミド結合をした 3-ヒドロキシ脂肪酸を含んでいなかった。GC-MS と NMR の解析から、アミノ酸部分はロイシンとイソロイシンと決定された。これは、ロイシンとイソロイシンを含む *N*-アシルアミノ酸の微生物からの最初の単離である。