HPLC Analysis of Fatty Acyl-Glycine in the Aqueous Methanesulfonic Acid Hydrolysates of N-Terminally Fatty Acylated Peptides[1,2)]

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Acylation with long-chain fatty acids is a common modification at the N-terminal glycine residues of natural proteins. In this work, we performed HPLC analysis of myristoylglycine (Myr-Gly-OH), palmitoylglycine (Pal-Gly-OH) or lauroylglycine (Lau-Gly-OH), which were produced in the hydrolysates of synthetic Myr-Gly-, Pal-Gly-, or Lau-Gly-peptides, respectively, by means of a mild acid hydrolysis in methanesulfonic acid : dioxane : water (2 : 1 : 1) at 60 °C for 12 h. Myr-Gly-OH, Pal-Gly-OH and Lau-Gly-OH were quite stable under hydrolysis conditions. These fatty acyl-Gly-OH were conveniently detectable at a 20 nmol level by direct reversed-phase HPLC. Thus, mild acid hydrolysis, followed by HPLC analysis of the hydrolysate, provides a simple method of identification of the N-terminal structure of fatty acyl-Gly-peptides.

Key words myristoylglycine; N-terminal; modification; methanesulfonic acid; hydrolysis; HPLC analysis

Acylation at the N-terminus is a common modification in natural proteins.[3—6)] Myristic acid (Myr-OH) or other long-chain fatty acids, such as lauric acid (Lau-OH)[7—10)] and palmitic acid (Pal-OH),[11)] in rare cases, are found in amide linkage with the N-terminal Gly residue of proteins. The acylation by Myr-OH is catalyzed by *N*-myristoyl transferases, which have been identified and characterized. These enzymes catalyze transfer of Myr specifically to the N-terminal Gly of proteins, and require the consensus sequence Gly-X-X-X-Ser/Thr as a substrate.[3)] An N-terminal Gly residue is an absolute requirement for enzymatic myristoylation, and the resulting Myr moiety in the modified protein plays an important role in membrane binding.[3)] Determination of the structure of N-terminally modified proteins or peptides is laborious, because the blocking groups prevent straightforward application of amino acid sequence determination methods such as Edman degradation on the intact protein or peptides. To date, various methods have been reported to determine the structure of the Myr moiety at the N-terminally blocked peptides, namely, incorporation of [^{3}H]Myr-OH to obtain a labeled protein,[12—14)] enzymatic removal of Myr-OH from blocked peptides,[15)] identification of Myr-Gly-OH after mild acid hydrolysis followed by chemical derivatization[16)] and mass spectrometry.[17—20)]

We have already investigated the susceptibility and stability of the internal peptide linkage of model peptides Myr-Gly-X-Phe-OH (X=various amino acids) under mild acid hydrolysis conditions.[21)] Regioselective cleavage of the Gly-X peptide bond was shown in the presence of a high concentration of aqueous methanesulfonic acid (MSA) by HPLC analysis of H-X-Phe-OH as the major peak. Here, we report that Myr-Gly-OH, a counterpart of H-X-Phe-OH, produced from Myr-Gly-X-Phe-OH can be analyzed directly by reversed-phase HPLC (RP-HPLC) almost quantitatively, because Myr-Gly-OH is quite stable under the hydrolysis conditions examined here. Thus, the hydrolysis of Myr-Gly-peptides, as well as the other fatty acyl-Gly-peptides, followed by RP-HPLC analysis of fatty acyl-Gly-OH, provides a simple method to deduce the N-terminal structure of blocked peptides.

MATERIALS AND METHODS

Peptide Synthesis All reagents, solvents and protected amino acids were obtained from Watanabe Chemical Industries, Ltd., Japan, and chloromethylated polystyrene resin (1% divinylbenzene, 0.66 mmol/g) was from Peptide Institute Inc., Japan. Starting from Boc-amino acid-resin, protected peptides were constructed by a standard solid-phase method employing an ABI 433A peptide synthesizer (Applied Biosystems, U.S.A.). The side chain protecting groups of N^{α}-Boc amino acids were *p*-tosyl (Tos) for the guanidino group of Arg, N^{π}-benzyloxymethyl (Bom) for the imidazole ring of His, 2-chlorobenzyloxylcarbonyl (Cl-Z) for the ε-amino group of Lys, and *O*-benzyl (Bzl) for the hydroxy groups of Thr and Ser. Deprotection of the N^{α}-Boc group was accomplished using 33% trifluoroacetic acid (TFA) in dichloromethane (DCM) for 30 min. A coupling reaction was performed for 2 h employing N^{α}-Boc-amino acids (3 eq) and 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate[22)] (HBTU reagent, 3 eq) in the presence of 1-methylmorpholine (4.5 eq) in *N*-methylpyrrolidone (NMP). Boc-Gln was coupled exclusively by the dicyclohexylcarbodiimide (DCC)-1-hydroxybenzotriazole (HOBt) method.

The protected peptide resin was treated with anhydrous liquid hydrogen fluoride (HF) containing 10% anisole at −5 to 0 °C for 45 min in a Teflon HF apparatus (Peptide Institute Inc., Japan). After evaporation of HF *in vacuo* with ice-cooling, the residual mixture was washed with ether prior to extraction of the fatty acylated peptide with aqueous dioxane or 50% acetic acid to give the crude peptide.

Purification and Characterization The synthetic fatty acyl-peptides were highly purified by RP-HPLC on a column of YMC-pack D-ODS-5-ST S-5 120 Å (20×150 mm) with 0.1% TFA–acetonitrile (MeCN) solvent system in an isocratic manner, followed by gel-filtration on a column of Toyopearl HW-40 (super fine) (1.5×47 cm, Tosoh Co., Japan) with 12% acetic acid or 70—100% methyl alcohol as an eluent. Homogeneity of the purified peptides was confirmed by analytical RP-HPLC on a column of Puresil C18 (4.6×250 mm) with a 0.1% TFA–MeCN solvent system, and high-performance thin-layer chromatography (HP-TLC, Kieselgel 60, E. Merck,

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Table 1. FAB-MS Data of Synthetic Peptides

Peptide	FAB-MS	
	Found $[M+H]^+$	Formula
Myr-Gly-OH[a)]	286	$C_{16}H_{31}NO_3$
Pal-Gly-Ala-Phe-OH	532	$C_{30}H_{49}N_3O_5$
Pal-Gly-OH	314	$C_{18}H_{35}NO_3$
Lau-Gly-Ala-Phe-OH	476	$C_{26}H_{41}N_3O_5$
Lau-Gly-OH	258	$C_{14}H_{27}NO_3$
Peptide **1**	827	$C_{38}H_{70}N_{10}O_{10}$
Peptide **2**	1950	$C_{93}H_{153}N_{21}O_{24}$

a) Ref. 21.

Table 2. Characteristics of Synthetic Peptides

Peptide	$[\alpha]_D^{24}$	RP-HPLC[c)]	HP-TLC[d)]	
	(c=0.5)[a)]	t_R (min)	Rf^1	Rf^2
Pal-Gly-Ala-Phe-OH	−5.9	27.0	0.70	0.73
Pal-Gly-OH	—	26.7	0.68	0.75
Lau-Gly-Ala-Phe-OH	−3.7	16.4	0.70	0.73
Lau-Gly-OH	—	15.1	0.66	0.75
Peptide **1**	−39.6[b)]	9.7	0.50	0.40
Peptide **2**	−29.7	10.5	0.55	0.40

a) In DMF. *b*) c=0.1. *c*) HPLC conditions: column, Puresil C18 (4.6×250 mm); elution, 61.75—81.75% MeCN in 0.1% TFA; flow rate, 1 ml/min; detection, 210 nm; gradient. *d*) Rf^1, *n*-BuOH : pyridine : AcOH : H_2O (30 : 20 : 6 : 24); Rf^2, *n*-BuOH : AcOEt : AcOH : H_2O (1 : 1 : 1 : 1).

Germany) with two solvent systems. FAB-MS analysis was conducted on a JMS-DX300 mass spectrometer (JEOL, Ltd., Japan). Optical rotations of the peptides were measured with a 3.5×50 mm cell with a GIP-370 digital polarimater (Nippon Bunko Co., Ltd., Japan). The analytical data are shown in Tables 1 and 2.

HPLC Analysis of Fatty Acylglycine in MSA-hydrolysate MSA for hydrolysis of peptides was purchased from Wako, Japan and dioxane (analytical grade) was obtained from Nacalai Tesque, Japan. These reagents were employed without further purification. To analyze acid hydrolysates, 4×10^{-4} mol/l peptide solution (50 μl) in MSA : water : dioxane (2 : 1 : 1) was prepared in a glass tube with a tight cap on ice. These were maintained at 60 °C for 12 h. After hydrolysis, each tube was diluted with dioxane : water (1 : 1) (66.5 μl) and neutralized with 8 mol/l NaOH : dioxane (1 : 1) (50 μl) on ice, then stored at −40 °C until analysis. An aliquot of the solution was injected onto a column of YMC-Pack C4 (4.6×150 mm), which was eluted by a linear gradient of 28.5 to 95% MeCN in 0.1% TFA over a period of 15 min at a flow rate of 1 ml/min. The peak was monitored at 210 nm.

RESULTS

As reported previously,[21)] the major hydrolytic cleavage of Myr-Gly-X-Phe-OH in MSA : water : dioxane (2 : 1 : 1) at 60 °C for 3 h occurred at the Gly-X bond to give X-Phe-OH. In this study, direct RP-HPLC of Myr-Gly-OH, a counterpart of X-Phe-OH, in the hydrolysate was examined by measuring the eluate at 210 nm. After the large peaks of MSA and dioxane, the peak of Myr-Gly-OH appeared at 15.6 min from a

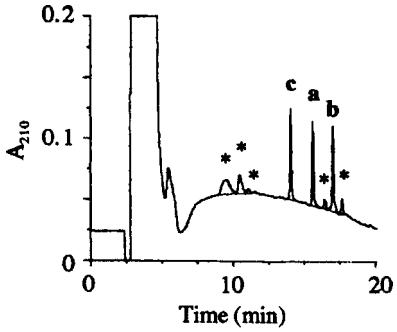

Fig. 1. RP-HPLC Profiles of 20 nmol Each of Standard Myr-Gly-OH (a), Pal-Gly-OH (b) and Lau-Gly-OH (c) in a MSA : Water : Dioxane (2 : 1 : 1) Solution after 12 h Incubation at 60 °C Followed by Neutralization

∗Marks are due to the reagents.

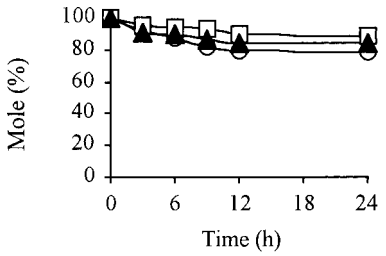

Fig. 2. Stability of Myr-Gly-OH (○), Lau-Gly-OH (□) or Pal-Gly-OH (▲) during Incubation in MSA : Water : Dioxane (2 : 1 : 1) at 60 °C for 24 h

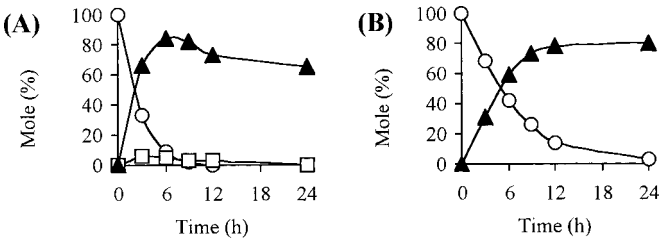

Fig. 3. Time Course of Changes in Concentration of Myr-Gly-X-Phe-OH (A; X=Ala, B; X=Ile) and Their Hydrolysates during Incubation in MSA : Water : Dioxane (2 : 1 : 1) at 60 °C for 24 h

(○), Myr-Gly-X-Phe-OH; (▲), Myr-Gly-OH; (□), Myr-Gly-Ala-OH.

C_4-column by linear gradient elution of 28.5 to 95% MeCN in 0.1% TFA over a period of 15 min (Fig. 1). Under the same HPLC conditions, Pal-Gly-OH (t_R 17.0 min) and Lau-Gly-OH (t_R 14.0 min) could be detected as seen in Fig. 1. Thus, it was found that these fatty acyl-Gly-OH could be analyzed at 20 nmol, although several unknown peaks appeared which were derived from the reagents.

Next, the stability of fatty acyl-Gly-OH was examined under mild hydrolysis conditions in MSA : water : dioxane (2 : 1 : 1) at 60 °C to determine when the highest concentration of fatty acyl-Gly-OH exists in the hydroysates of fatty acyl-peptides. Time courses of the degradation of Myr-Gly-OH, Pal-Gly-OH and Lau-Gly-OH in an incubation solution of MSA : water : dioxane (2 : 1 : 1) were examined at 60 °C. As shown in Fig. 2, around 80% of these fatty acyl-Gly-OH survived intact even after 24 h.

On the other hand, incubation of Myr-Gly-Ala-Phe-OH in MSA : water : dioxane (2 : 1 : 1) at 60 °C caused almost complete decomposition after 12 h, yielding about 70% of Myr-Gly-OH (Fig. 3A). When Myr-Gly-Ile-Phe-OH, containing

one of the most steric amino acids, Ile, was treated under the same conditions, 20% of the starting material remained intact after 12 h, most of which disappeared after 24 h as shown in Fig. 3B. Pal-Gly-Ala-Phe-OH and Lau-Gly-Ala-Phe-OH also almost completely decomposed after 12 h, yielding more than 60% of Pal-Gly-OH and 70% of Lau-Gly-OH, respectively (Fig. 4). Thus, fatty acyl-Gly-OH was released from fatty acyl-Gly-peptides in high yields by 12 h hydrolysis and was detectable by direct RP-HPLC. Yields of Myr-Gly-OH, Pal-Gly-OH and Lau-Gly-OH derived from various fatty acyl-Gly-peptides in 12 h-hydrolysis in MSA : water : dioxane (2 : 1 : 1) at 60 °C are summarized in Table 3. Synthetic peptides of Myr-Gly-Ala-Gln-Val-Ser-Arg-OH (**1**) and Myr-Gly-Gln-Thr-Val-Thr-Thr-Pro-Leu-Ser-Leu-Thr-Leu-Gly-His-Trp-Lys-OH (**2**) used in this study correspond to the N-terminal tryptic fragments of HRV(1B)VP4[23] and MuLVgag,[24] respectively. Peptides **1** and **2** decomposed very rapidly in MSA : water : dioxane (2 : 1 : 1) at 60 °C, and 20 nmol equivalent of Myr-Gly-OH in the incubation solution could be detected on HPLC chromatograms after 12 h-hydrolysis, as seen in Fig. 5.

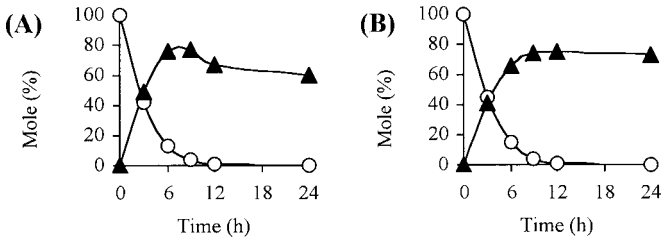

Fig. 4. Time Course of Changes in Concentration of Y-Gly-Ala-Phe-OH (A; Y=Pal, B; Y=Lau) and Their Hydrolysates during Incubation in MSA : Water : Dioxane (2 : 1 : 1) at 60 °C for 24 h

(○), Y-Gly-Ala-Phe-OH; (▲), Y-Gly-OH.

Table 3. Yields of Y-Gly-OH (Y=Myr, Pal or Lau) Derived from Y-Gly-Peptides at 60 °C after 12 h Hydrolysis in MSA : Water : Dioxane (2 : 1 : 1)

Peptide	Mole (%) of Y-Gly-OH
Myr-Gly-Ala-Phe-OH	83.1
Myr-Gly-Ile-Phe-OH	77.3
Peptide **1**	79.9
Peptide **2**	80.2
Pal-Gly-Ala-Phe-OH	67.4
Lau-Gly-Ala-Phe-OH	74.8

DISCUSSION

In our previous study,[21] a simple deblocking method for Myr-Gly-peptide (Myr-Gly-X-Phe-OH, X=various amino acids) was examined to obtain H-X-Phe-OH as a main product in the hydrolysate. Therefore, mild hydrolysis conditions were investigated for regioselective cleavage of the Gly-X bond, which was performed in a high concentration of aqueous MSA at 25 °C or 60 °C within 6 h. However, when X was Gln or Asn, many peaks were observed on HPLC examination of the hydrolysates and neither H-Gln-Phe-OH nor H-Asn-Phe-OH was the main product. In the case of peptide **1** and **2**, hydrolysis in MSA : water : dioxane (2 : 1 : 1) at 60 °C for 6 h produced numerous peaks, among which those of des[Myr-Gly]-peptides, namely, H-Ala-Gln-Val-Ser-Arg-OH and H-Gln-Thr-Val-Thr-Thr-Pro-Leu-Ser-Leu-Thr-Leu-Gly-His-Trp-Lys-OH, were not observed. Since both peptides **1** and **2** contain Ser and/or Thr, the internal peptide bonds might have been cleaved at the N-terminal of Ser and Thr *via* N to O acyl migration in MSA : water : dioxane (2 : 1 : 1) to give complex products. A similar degradation of peptide was reported in a short communication; hydrolysis of LH-RH, pGlu[1]-His-Trp-Ser[4]-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2, in 70% MSA, gave not only des-pGlu-peptide, but also LH-RH (4—10) by cleavage of the peptide bond at the N-terminal of Ser[4] of LH-RH.[25] Thus, aqueous high concentration MSA hydrolysis may not be applicable for obtaining des-[Myr-Gly]-peptide as the main product, which could be applied to Edman degradation. Nevertheless, the MSA hydrolysis could be applicable to selective removal of fatty acyl-Gly-OH such as Myr-Gly-OH, which was quite stable under the hydrolysis conditions, as demonstrated in this study. It was also reported by Goddard and Felsted[16] that the relative hydrolysis rate of Myr-Gly-OH to H-Gly-Gly-OH was 0.099 under mild acid hydrolysis conditions at 100 °C in 0.05 M HCl. Therefore, our attention focused only on analyzing fatty acyl-Gly-OH in the hydrolysate to obtain structural information on the modified peptides with long-chain fatty acids. For HPLC analysis of fatty acyl-Gly-OH, it was advantageous to decompose various peptides produced by hydrolysis to amino acids. Taking into account the stability of fatty acyl-Gly-OH, hydrolysis conditions in MSA : water : dioxane (2 : 1 : 1) at 60 °C for 12—24 h was recommended. Fatty acyl-Gly-OH was analyzed directly by RP-HPLC of the hydrolysate of blocked peptide and the peak measured at 210 nm was detectable at a 20 nmol level in this study. The detection limit of the peak of fatty acyl-Gly-OH may be improved by application of LC-

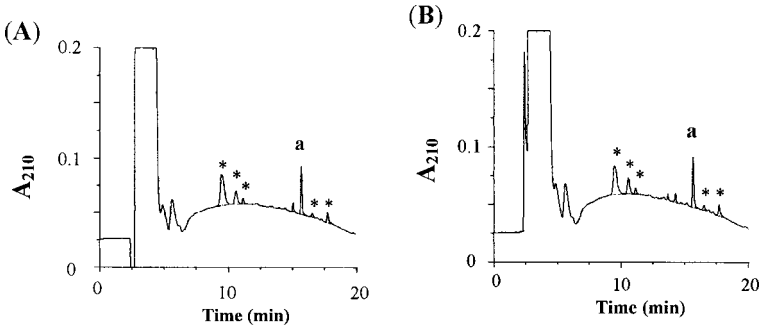

Fig. 5. RP-HPLC Profiles of Myr-Gly-OH (a) Obtained by Injection of 20 nmol of Peptide **1** (A) or **2** (B) Acid Hydrolysate Following 12 h Hydrolysis

* Marks are due to the reagents.

MS analysis, because in the literature[17—19] 0.8—10 nmol of peptides was used to identify N-terminal Myr-Gly-OH by mass spectrometry.

The results of the present study can be summarized as follows: 1) Myr-Gly-OH is cleaved out predominantly from Myr-Gly-peptides by mild hydrolysis in the presence of a high concentration of MSA; 2) Myr-Gly-OH is stable under hydrolysis conditions; and 3) Myr-Gly-OH, as well as Pal-Gly-OH and Lau-Gly-OH, can be identified by direct RP-HPLC at 20 nmol. This method could be applied to identify N-terminal fatty acylglycine directly from the hydrolysates of N-terminal blocked peptides by long-chain fatty acids, which could be prepared as enzymatic peptide fragments by the endopeptidases trypsin or chymotrypsin.

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1) With the exception of glycine, the amino acids and their derivatives mentioned in this paper were of the L-configuration. The abbreviations for amino acids and peptides are in accordance with the rules of the IUPAC-IUB Commission on Biochemical Nomenclature in *Eur. J. Biochem.*, **138**, 9—37 (1984).
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