Regioselective Hydrolytic Cleavage of *N*-Terminal Myristoyl-peptide[1,2)]

Keiko OKIMURA, Kaori SUGIMOTO, Kazuhiro OHKI, Naoki SAKURA,* and Tadashi HASHIMOTO

Faculty of Pharmaceutical Sciences, Hokuriku University, Kanagawa-machi, Kanazawa 920–1181, Japan.
Received March 25, 1999; accepted June 24, 1999

To develop a simple chemical deblocking method for Myr–Gly–peptide, we investigated the susceptibility and stability of the internal peptide linkages of model peptides, Myr–Gly–X–Phe–OH (X=Gly, Ala, Val, Lys, His, Arg, Ile, Glu, Gln, Asp, Asn), to HCl, H_2SO_4 and methanesulfonic acid (MSA) at 25 and 60 °C, and analyzed the hydrolysates by HPLC. The results indicated that acid hydrolysis in concentrated acids was superior for selective cleavage of the Gly–X linkage. Here, we describe a simple method for regioselective cleavage of the Gly–X bond of Myr–Gly–X–Phe–OH by acids and determination of the decomposition products by RP-HPLC.

Key words myristoyl; regioselective cleavage; methanesulfonic acid; hydrochloric acid; sulfuric acid

Myristoic acid (Myr–OH) and other long-chain fatty acids are found in amide linkages with the *N*-terminal Gly residues of proteins. The fatty acid moiety has been identified in electron density maps obtained by high-resolution X-ray crystallographic studies.[3)] The acid moiety has also been elucidated by gas chromatography of Myr–peptide and confirmed by FAB-MS.[4)] The fatty acids released by hydrolysis of the protein were identified as fatty acid phenacyl esters by HPLC.[5)] The *N*-terminal blocking groups prevented straightforward application of amino acid sequence determination such as Edman degradation on the intact protein and peptides. Enzymatic deblocking was developed for removal of Myr–OH from peptides,[6)] but no simple chemical means have been reported.

To develop a simple chemical method for deblocking of Myr–Gly–peptide, we investigated the susceptibility and stability of the internal peptide linkages of model peptides, Myr–Gly–X–Phe–OH (X=Gly, Ala, Val, Lys, His, Arg, Ile, Glu, Gln, Asn, Asp), to HCl, H_2SO_4 and MSA at 25 and 60 °C, and analyzed the hydrolysates by HPLC. Here, we describe a simple method for regioselective cleavage of the Gly–X bond of Myr–Gly–X–Phe–OH by acids and determination of the decomposition products by RP-HPLC.

Results and Discussion

Hydrolysis of Myr–Gly–Ala–Phe–OH in HCl To examine whether the regioselective cleavage of a peptide linkage of Myr–Gly–X–Phe–OH is possible, the susceptibilities of the Myr–Gly, Gly–Ala and Ala–Phe bonds of My–Gly–Ala–Phe–OH in dilute acid were examined. When a solution of Myr–Gly–Ala–Phe–OH [4×10^{-4} mol/l in 6% HCl : dioxane (1 : 1)] was incubated at 60 °C for 6 h, the hydrolysis resulted in the formation of three hydrolysates. The three peaks and starting material peak in the HPLC were quantified and isolated by HPLC, and assigned on the basis of amino acid analysis and direct comparison with authentic samples. The main reactions were cleavage of the Myr–Gly and Gly–Ala linkages resulting in the production of H–Gly–Ala–Phe–OH and H–Ala–Phe–OH, with H–Phe–OH as a minor product (Fig. 1). The yield of H–Ala–Phe–OH was higher than that of H–Gly–Ala–Phe–OH. Thus, the Gly–Ala linkage was more susceptible than the Myr–Gly linkage in dilute acid. Next, the susceptibilities of the Gly–Ala and Ala–Phe bonds of *N*-terminal free peptide H–Gly–Ala–Phe–OH were examined under the same conditions to test the further decomposition of the hydrolysate. H–Gly–Ala–Phe–OH was quite stable under these conditions, that is, 90% of the starting material remained intact after 6 h incubation. The yields of H–Ala–Phe–OH and H–Phe–OH were less than 4% (Fig. 2). These results indicated that the hydrolysates of Myr–Gly–Ala–Phe–OH were derived from the direct decomposition of the starting material.

Next, to examine whether more selective cleavage of the three amide linkages of Myr–Gly–X–Phe–OH is possible, the susceptibilities of the Myr–Gly, Gly–Ala and Ala–Phe bonds of Myr–Gly–Ala–Phe–OH to several concentrations of 6—36% HCl : dioxane (1 : 1) were examined at 25 °C. Our earlier study revealed that acid hydrolysis at low temperature

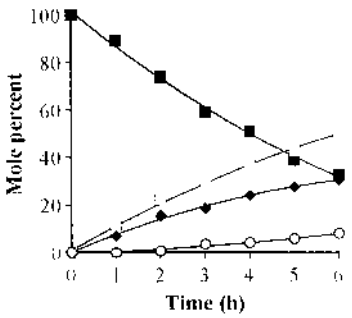

Fig. 1. Time Course of Changes in Concentration of Myr–Gly–Ala–Phe–OH and Its Hydrolysates during Incubation in 6% Hydrochloric Acid in Water : Dioxane (1 : 1) at 60 °C for 6 h

(■), Myr–Gly–Ala–Phe–OH; (◆), H–Gly–Ala–Phe–OH; (□), H–Ala–Phe–OH; (○), H–Phe–OH.

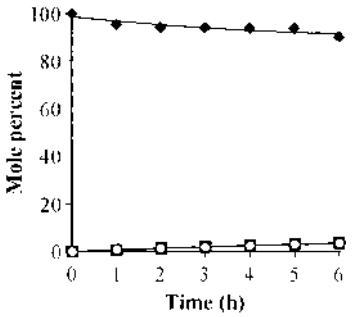

Fig. 2. Time Course of Changes in Concentration of H–Gly–Ala–Phe–OH and Its Hydrolysates during Incubation in 6% Hydrochloric Acid in Water : Dioxane (1 : 1) at 60 °C for 6 h

(◆), H–Gly–Ala–Phe–OH; (□), H–Ala–Phe–OH; (○), H–Phe–OH.

* To whom correspondence should be addressed.

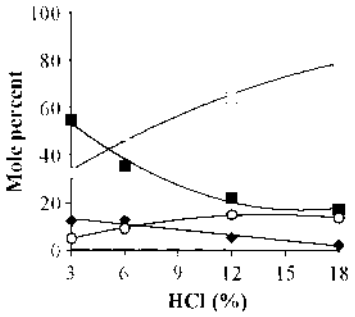

Fig. 3. Relationship between the Concentration of Hydrochloric Acid, and Myr–Gly–Ala–Phe–OH and Its Hydrolysates after Incubation at 25 °C for 5 d

(■), Myr–Gly–Ala–Phe–OH; (◆), H–Gly–Ala–Phe–OH; (□), H–Ala–Phe–OH; (○), H–Phe–OH.

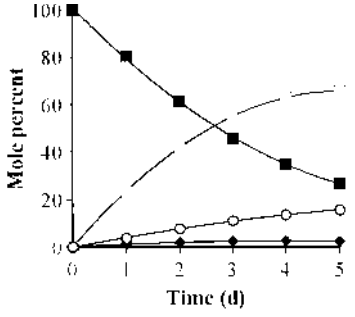

Fig. 4. Time Course of Changes in Concentration of Myr–Gly–Ala–Phe–OH and Its Hydrolysates during Incubation in 36% Hydrochloric Acid in Water : Dioxane (1 : 1) at 25 °C for 5 d

(■), Myr–Gly–Ala–Phe–OH; (◆), H–Gly–Ala–Phe–OH; (□), H–Ala–Phe–OH; (○), H–Phe–OH.

Table 1. Myr–Gly–X–Phe–OH and Its Hydrolysates during Incubation in 36% HCl in Water : Dioxane (1 : 1) at 25 °C for 5 d

Peptide and amino acid	X (mole percent)				
	Gly	Val	Glu	His	Lys
Myr–Gly–X–Phe–OH	5.5	41.5	30.4	43.0	33.3
H–Gly–X–Phe–OH	6.3	6.1	4.5	1.7	10.4
H–X–Phe–OH	65.1	42.0	62.7	43.2	51.8
H–Phe–OH	15.2	0.8	10.3	7.5	4.0

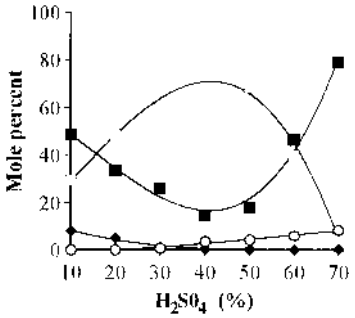

Fig. 5. Relationship between the Concentration of Sulfuric Acid, Myr–Gly–Ala–Phe–OH and Its Hydrolysates after Incubation at 25 °C for 5 d

(■), Myr–Gly–Ala–Phe–OH; (◆), H–Gly–Ala–Phe–OH; (□), H–Ala–Phe–OH; (○), H–Phe–OH.

was superior to that at 60 °C for selective cleavage of the pGlu–peptide linkage of pGlu–peptide.[7] When a solution of Myr–Gly–Ala–Phe–OH [4×10^{-4} mol/l in 6—36% HCl : dioxane (1 : 1)] was incubated at 25 °C for 5 d, hydrolysis resulted in the formation of one major and two minor hydrolysates. The main reaction was the cleavage of the Gly–Ala linkage resulting in the production of H–Ala–Phe–OH, and the minor products were H–Gly–Ala–Phe–OH and H–Phe–OH (Fig. 3). On 6% HCl hydrolysis, the yield of H–Ala–Phe–OH was twice that of H–Gly–Ala–Phe–OH at 60 °C. Therefore, acid hydrolysis at low temperature was superior to that at 60 °C for the selective cleavage of the Gly–Ala linkage. The results also indicated that a higher concentration of HCl during hydrolysis gave a lower yield of the product H–Gly–Ala–Phe–OH and a higher yield of H–Ala–Phe–OH. The yield of the cleavage product H–Ala–Phe–OH was highest after hydrolysis in 18% HCl. The optimal concentration of HCl to achieve selective and rapid cleavage of the Gly–Ala linkage was 36% HCl : dioxane (1 : 1). The yield ratios for cleavage of the Gly–Ala bond relative to the Myr–Gly and Ala–Phe bonds were about 35 and 6, respectively. Hydrolysis of Myr–Gly–X–Phe–OH (X＝Gly, Val, Glu, His, Lys) under the same conditions gave similar results (Table 1).

The time course of the Myr–Gly–Ala–Phe–OH hydrolysis was next examined in 36% HCl : dioxane (1 : 1) at 25 °C. HPLC analysis (Fig. 4) revealed that the major product was H–Ala–Phe–OH (66%) and the minor products were H–Gly–Ala–Phe–OH (3%) and H–Phe–OH (16%) after 5 d. The yield ratios for cleavage of the Gly–Ala bond relative to the Myr–Gly and Ala–Phe bonds were about 26 and 4, respectively. These results showed that the rate of cleavage of the Gly–Ala bond increased greatly in comparison with hydrolysis (data not shown) in 3% HCl : dioxane (1 : 1) at 60 °C, whereas the cleavage rates of other bonds were reduced. Since H–Gly–Ala–Phe–OH was stable and 90% of the starting material remained after incubation as described above in 3% HCl at 60 °C for 6 h, most H–Ala–Phe–OH was produced by direct cleavage of the Gly–Ala linkage of Myr–Gly–Ala–Phe–OH (data not shown) in 36% HCl : dioxane (1 : 1) at 25 °C.

Hydrolysis of Myr–Gly–Ala–Phe–OH in H_2SO_4 As a high concentration of HCl was superior for regioselective hydrolysis of Myr–Gly–Ala–Phe–OH, use of H_2SO_4 was examined to obtain a higher acid concentration for hydrolysis. The susceptibilities of the Myr–Gly, Gly–Ala and Ala–Phe bonds of Myr–Gly–Ala–Phe–OH to several concentrations of 10—70% H_2SO_4 in water : dioxane (1 : 1) were examined at 25 °C. Hydrolysis resulted in the formation of H–Ala–Phe–OH as the major product and H–Gly–Ala–Phe–OH and H–Phe–OH as minor products. The yields of H–Ala–Phe–OH were highest after hydrolysis in 40% H_2SO_4. As the concentration of H_2SO_4 was increased to over 50%, the rate of the hydrolysis reaction decreased markedly (Fig. 5). The optimal concentration of H_2SO_4 to selectively and rapidly cleave the Gly–Ala linkage was 40%.

The time course of the selective hydrolysis of Myr–Gly–Ala–Phe–OH was examined in H_2SO_4 : water : dioxane (4 : 3 : 3) at 25 °C for 5 d (Fig. 6A) and at 60 °C for 6 h (Fig. 6B). Under the latter conditions, H–Ala–Phe–OH (69%) was obtained as the major product, with H–Gly–Ala–Phe–OH (0.4%) and H–Phe–OH (24%) as minor products. The yield ratios of H–Ala–Phe–OH to H–Gly–Ala–Phe–OH and H–

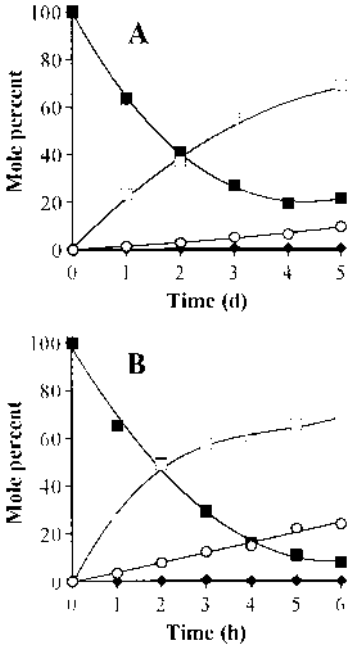

Fig. 6. Time Course of Changes in Concentration of Myr–Gly–Ala–Phe–OH and Its Hydrolysates during Incubation in Sulfuric Acid : Water : Dioxane (4 : 3 : 3) at 25 °C for 5 d (A) and at 60 °C for 6 h (B)

(■), Myr–Gly–Ala–Phe–OH; (◆), H–Gly–Ala–Phe–OH; (□), H–Ala–Phe–OH; (○), H–Phe–OH.

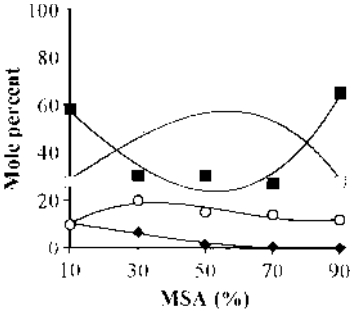

Fig. 7. Relationship between the Concentration of MSA, Myr–Gly–Ala–Phe–OH and Its Hydrolysates after Incubation at 60 °C for 3 h

(■), Myr–Gly–Ala–Phe–OH; (◆), H–Gly–Ala–Phe–OH; (□), H–Ala–Phe–OH; (○), H–Phe–OH.

Phe–OH were about 172 and 3, respectively. These results showed that the Gly–Ala linkage of Myr–Gly–Ala–Phe–OH was highly susceptible to 40% H_2SO_4 compared with other peptide bonds, and was selectively hydrolyzed at 25 °C rather than at 60 °C to predominantly yield the cleavage product.

Hydrolysis of Myr–Gly–X–Phe–OH (X=Ala, Gly, His, Arg, Lys, Val, Ile, Glu) in MSA Our earlier study revealed that acid hydrolysis in aqueous MSA was superior for selective cleavage of the pGlu–peptide linkage of pGlu–peptide[7] and for selective removal of acetyl (Ac)-amino acid from Ac–peptide.[8] Thus, we examined the susceptibility of the Myr–Gly, Gly–Ala and Ala–Phe bonds of Myr–Gly–Ala–Phe–OH to several concentrations of 10—90% MSA in water : dioxane (1 : 1) at 60 °C for 3 h (Fig. 7). The hydrolysate contained H–Ala–Phe–OH as the major product and H–Gly–Ala–Phe–OH and H–Phe–OH as the minor products. The yields of H–Ala–Phe–OH were highest after hydrolysis in 50—70% MSA. As the concentration of MSA

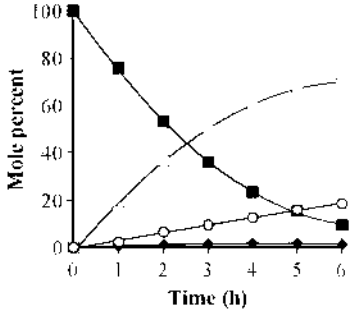

Fig. 8. Time Course of Changes in Concentration of Myr–Gly–Ala–Phe–OH and Its Hydrolysates during Incubation in MSA : Water : Dioxane (2 : 1 : 1) at 60 °C for 6 h

(■), Myr–Gly–Ala–Phe–OH; (◆), H–Gly–Ala–Phe–OH; (□), H–Ala–Phe–OH; (○), H–Phe–OH.

Table 2. Myr–Gly–X–Phe–OH and Its Hydrolysates during Incubation in MSA : Water : Dioxane (2 : 1 : 1) at 60 °C for 6 h

Peptide and amino acid	X (mole percent)						
	Gly	His	Arg	Lys	Val	Glu	Ile
Myr–Gly–X–Phe–OH	1.1	33.2	2.9	24.7	39.5	66.7	44.6
H–Gly–X–Phe–OH	5.0	2.9	6.2	2.9	6.1	0.7	6.0
H–X–Phe–OH	68.2	51.5	81.9	66.3	46.4	29.2	46.0
H–Phe–OH	29.6	12.6	15.4	8.6	5.5	7.6	6.4
pGlu–Phe–OH	—	—	—	—	—	1.7	—

was increased to over 70%, the rate of the hydrolysis reaction gradually decreased. The optimal concentration of MSA to selectively and rapidly cleave the Gly–Ala linkage was 50%. These results were similar to those of the hydrolysis of Myr–Gly–Ala–Phe–OH in 10—70% H_2SO_4.

Thus, we next examined the acid hydrolysis (Fig. 8) of Myr–Gly–Ala–Phe–OH in MSA : H_2O : dioxane (2 : 1 : 1) at 60 °C for 6 h. The results indicated that the major hydrolytic cleavage occurred at the Gly–Ala bond, and that the Myr–Gly and Ala–Phe bonds were highly resistant to acid hydrolysis. The acid hydrolyses of Myr–Gly–X–Phe–OH (X=Gly, His, Arg, Lys, Val, Glu, Ile) in MSA : H_2O : dioxane (2 : 1 : 1) at 60 °C for 6 h gave similar results (Table 2).

Hydrolysis of Myr–Gly–X–Phe–OH (X=Gln, Asp, Asn) in MSA The hydrolysate of Myr–Gly–Gln–Phe–OH in MSA : H_2O : dioxane (2 : 1 : 1) contained complex products. Myr–Gly–Gln–Phe–OH was rapidly degraded to give six hydrolysates (Fig. 9). After 1 h, the hydrolysate contained H–Glu–Phe–OH, H–Gly–Glu–Phe–OH, H–Phe–OH, almost the same amounts of H–Gln–Phe–OH and pGlu–Phe–OH, and Myr–Gly–Glu–Phe–OH, in which the γ-carboxamide of the Gln residue of Myr–Gly–Gln–Phe–OH was hydrolyzed. The production of Myr–Gly–Glu–Phe–OH reached a maximum at 2 h of hydrolysis, and then gradually decreased. The yields of H–Gln–Phe–OH and pGlu–Phe–OH also reached maximum levels at 2 h incubation, and then gradually decreased. The former was converted to the latter, which was hydrolyzed to give pGlu–OH and H–Phe–OH. After incubation of Myr–Gly–Gln–Phe–OH for 6 h, the hydrolysate contained no H–Gln–Phe–OH and small amounts of pGlu–Phe–OH, and the major product was H–Glu–Phe–OH. H–Gln–Phe–OH was readily converted to pGlu–Phe–OH. The hydrolysate contained a higher yield of H–Phe–OH than in those obtained by

acid hydrolysis of Myr–Gly–X–Phe–OH (X=Ala, His, Gly, Arg, Lys, Val, Glu, Ile), due to the production of pGlu–Phe–OH, following hydrolysis to pGlu–OH and H–Phe–OH (Chart 1). These results might be explained as the hydrolysis of pGlu–Phe–OH to give pGlu–OH and H–Phe–OH as previously reported.[7]

Myr–Gly–Asp–Phe–OH in MSA : H_2O : dioxane (2 : 1 : 1) at 60 °C was rapidly degraded and converted into Myr–Gly–aminosuccinyl (Asu)–Phe–OH (Fig. 10). About 80% of Myr–Gly–Asp–Phe–OH was converted to Myr–Gly–Asu–Phe–OH within 1 h. The Asu derivative was then gradually hydrolyzed probably to produce H–Asu–Phe–OH. The hydrolysate of Myr–Gly–Asp–Phe–OH in MSA : H_2O : dioxane (2 : 1 : 1) at 60 °C for 6 h contained H–Asu–Phe–OH as the main product, and Myr–Gly–Asu–Phe–OH, H–Asp–Phe–OH, H–β-Asp–Phe–OH, H–Phe–OH and H–Gly–Asp–Phe–OH as by-products (Fig. 10). Myr–Gly–Asu–Phe–OH and H–Asu–Phe–OH were isolated and treated with 0.1 N NaOH at 25 °C for 10 min to give a mixture of Myr–Gly–Asp–Phe–OH and Myr–Gly–β-Asp–Phe–OH, and H–Asp–Phe–OH and H–β-Asp–Phe–OH, respectively (data not shown). The incubation of H–Asp–Phe–OH in MSA : H_2O : dioxane (2 : 1 : 1) at 60 °C gave H–Asu–Phe–OH as the major product and H–β-Asp–Phe–OH as the minor product, and H–β-Asp–Phe–OH gave H–Asu–Phe–OH and H–Asp–Phe–OH as major and minor products, respectively (data not shown). Also, the incubation of H–Asu–Phe–OH under the same conditions produced mainly H–β-Asp–Phe–OH and H–Asp–Phe–OH as a by-product (data not shown). As the yields of H–Asp–Phe–OH and H–β-Asp–Phe–OH did not increase after 2—3 h of hydrolysis and that of H–Asu–Phe–OH increased almost linearly, a part of H–Asu–Phe–OH was hydrolyzed to give H–Asp–Phe–OH and H–β-Asp–Phe–OH (Chart 2), most of which were probably converted again into H–Asu–Phe–OH. The β-Asp derivatives were confirmed by coelution with the respective authentic samples.

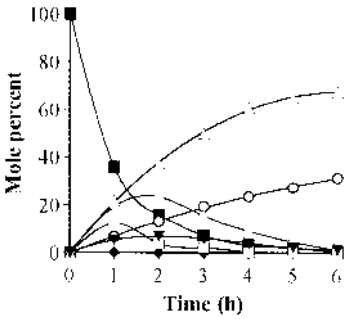

Fig. 9. Time Course of Changes in Concentration of Myr–Gly–Gln–Phe–OH and Its Hydrolysates during Incubation in MSA : Water : Dioxane (2 : 1 : 1) at 60 °C for 6 h

(■), Myr–Gly–Gln–Phe–OH; (◆), H–Gly–Gln–Phe–OH; (□), H–Gln–Phe–OH; (○), H–Phe–OH; (◇), Myr–Gly–Glu–Phe–OH; (△), H–Glu–Phe–OH; (▼), pGlu–Phe–OH.

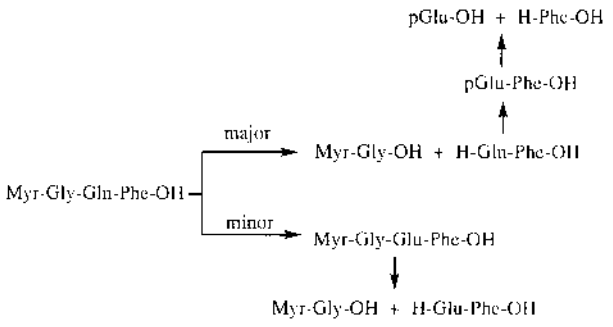

Chart 1

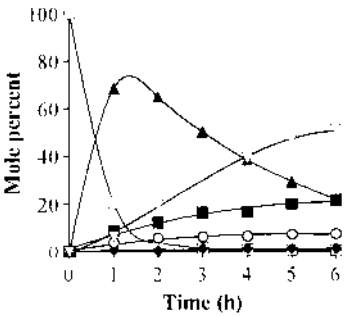

Fig. 10. Time Course of Changes in Concentration of Myr–Gly–Asp–Phe–OH and Its Hydrolysates during Incubation in MSA : Water : Dioxane (2 : 1 : 1) at 60 °C for 6 h

(□), Myr–Gly–Asp–Phe–OH; (◆), H–Gly–Asp–Phe–OH; (■), H–Asp–Phe–OH; (○), H–Phe–OH; (▲), Myr–Gly–Asu–Phe–OH; (▽), H–Asu–Phe–OH.

A similar decomposition pattern for an Asp-containing peptide was also observed in the acid hydrolysis of Ac–Asp–Ala–Phe–OH in 70% MSA at 60 °C.[9] This peptide decomposed within 2 h to produce Ac–Asu–Ala–Phe–OH, which was hardly hydrolyzed to the selective cleavage product. After 2—6 h of incubation, the hydrolysis of Ac–Asu–Ala–Phe–OH was unexpectedly slow and the increase in the yield of H–Ala–Phe–OH was extremely small. The dehydrated product decomposed very slowly. The yield of H–Phe–OH increased reasonably. The intramolecular dehydration of the Ac–Asp–peptide and concomitant formation of the Ac–aminosuccinyl–peptide occurred.

The incubation of Myr–Gly–Asn–Phe–OH in MSA : H_2O : dioxane (2 : 1 : 1) at 60 °C gave a complex hydrolysate mixture. The initial decomposition mainly formed the Asu derivative, Myr–Gly–Asu–Phe–OH, and direct hydrolysis of the Gly–Asn linkage gave H–Asn–Phe–OH, which were gradually decomposed after 2 h (Fig. 11). The maximum yield of

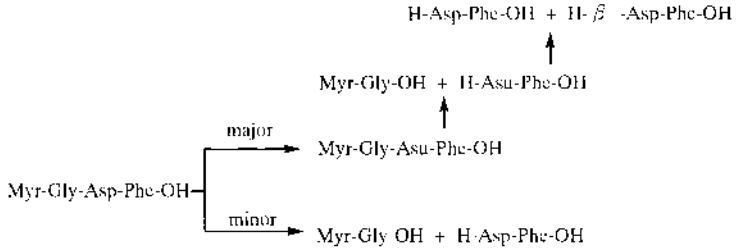

Chart 2

Myr–Gly–Asp–Phe–OH in MSA : H_2O : dioxane (2 : 1 : 1) at 60 °C was about 2% at 1 h of incubation. After 6 h hydrolysis, the hydrolysate contained H–Asu–Phe–OH as the main product, Myr–Gly–Asu–Phe–OH, H–Asp–Phe–OH, H–Phe–OH, H–Asn–Phe–OH, H–β-Asp–Phe–OH and H–Gly–Asp–Phe–OH. The yields of H–Asu–Phe–OH and H–β-Asp–Phe–OH increased almost linearly. The incubation of H–Asn–Phe–OH in MSA : H_2O : dioxane (2 : 1 : 1) at 60 °C yielded H–Asp–Phe–OH as the main product, H–Asu–Phe–OH and a trace of H–β-Asp–Phe–OH (data not shown). The yield of H–Asu–Phe–OH was less than one tenth of that of H–Asp–Phe–OH. The possible degradation pathway of Myr–Gly–Asn–Phe–OH in MSA : H_2O : dioxane (2 : 1 : 1) at 60 °C is shown in Chart 3.

This study revealed that the Gly–peptide linkage of Myr–Gly–peptide is highly labile in MSA : H_2O : dioxane (2 : 1 : 1) compared with other amide bonds, and is selectively hydrolyzed to give predominantly the cleavage products Myr–Gly–OH and H–X–peptide, when X is Gly, Ala, Val, Lys, His, Arg, Ile or Glu. These reaction patterns were similar to the selective removal of pGlu–OH from pGlu–peptide[7] and of the Ac–amino acid residue from Ac–amino acid–peptide[9] in aqueous MSA. When X was Gln in Myr–Gly–X–Phe–OH, the *N*-terminus of the main cleavage product was Glu. When X was Asp or Asn, the main decomposition product was H–Asu–Phe–OH in MSA : H_2O : dioxane (2 : 1 : 1), which cannot be subjected directly to Edman degradation. It is well-known that Asu–X bond formation occurs occasionally in diluted acid from Asp–X or Asn–X bonds in proteins, when X is Gly or Ser. This succinimide formation reaction seemed to take place very easily at a high concentration of aqueous MSA, even if X is Phe in the Asp–X or Asn–X–peptide.

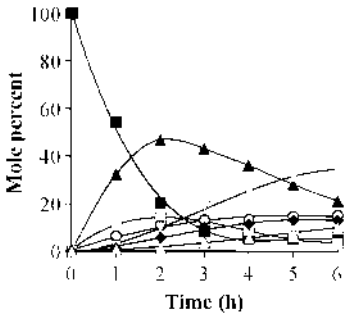

Fig. 11. Time Course of Changes in Concentration of Myr–Gly–Asn–Phe–OH and Its Hydrolysates during Incubation in MSA : Water : Dioxane (2 : 1 : 1) at 60 °C for 6 h

(■), Myr–Gly–Asn–Phe–OH; (□), H–Asn–Phe–OH; (○), H–Phe–OH; (▲), Myr–Gly–Asu–Phe–OH; (▽), H–Asu–Phe–OH; (◆), H–Asp–Phe–OH; (△), H–β-Asp–Phe–OH.

These results should contribute to the development of a simple means of deblocking Myr–Gly–OH from Myr–Gly–peptide. Qualitative and quantitative analyses of Myr–Gly–OH, as well as other fatty acid–Gly–OH derivatives, in the MSA hydrolysate are currently underway in our laboratory.

Experimental

General Synthesis of peptides used in this study was carried out on an ABI 433A peptide synthesizer (Applied Biosystems, Perkin-Elmer, U.S.A.). Semi-preparative RP-HPLC was performed on an apparatus equipped with 590 and 510 pumps (Waters, U.S.A.), a Rheodyne 7125 injector (Rheodyne Inc., U.S.A.), an UV 8011 detector (Tosoh Co., Japan), a 680 automated gradient controller (Waters) and a chromatocorder 21 integrator (System Instruments Co., Ltd., Japan). Analytical RP-HPLC was accomplished on a system comprising a gradient controller PX-8010 (Tosoh), two CCPD pumps (Tosoh), a Rheodyne 7125 injector (Rheodyne) or an automatic sample injector AS-8020 (Tosoh), a UV 8000 detector (Tosoh) and an 805 data station (Waters). Gel chromatography was performed on a Toyopearl HW-40 (super fine) column. Amino acid analysis of the acid hydrolysate was conducted on a 7300 amino acid analyzer (Beckman, U.S.A.). HF cleavage reactions were carried out in a Teflon HF apparatus (Peptide Institute Inc., Japan). FAB-MS were obtained on a JMS-DX300 mass spectrometer (JEOL Ltd., Japan). Optical rotations of peptides were measured with a DIP-370 digital polarimeter (Nippon Bunko Co., Ltd., Japan). HP-TLC was performed on precoated silica gel plates (Kieselgel 60, E. Merck, Germany).

Reagents Unless otherwise stated, all reagents and solvents were obtained as reagent grade products from Watanabe Chem. Ind. Ltd. or Wako Pure Chem. Ind. Ltd., Japan, and used without further purification. Boc-protected amino acids were purchased from Peptide Institute. Boc-amino acid Merrifield resins were purchased from Watanabe Chem. Ind. Ltd.

Peptide Synthesis Peptides were prepared by a standard solid-phase method. All amino acids were protected as α-Boc derivatives. The protecting groups for the amino acid side chains were *p*-tosyl for the guanidino group of Arg, N^{π}-benzyloxymethyl (Bom) for the imidazole ring of His, chlorobenzylcarbonyl (Cl-Z) for the ε-amino group of Lys, cyclohexyl (cHex) for β-carboxyl group of Asp and benzyl (Bzl) for γ-carboxyl group of Glu and hydroxy group of Ser. Solid-phase peptide synthesis was performed starting from Boc-amino acid Merrifield resin. Elongation of the peptide chain was carried out using Boc-amino acids with DCC (2.5 eq)–HOBt (2.5 eq) in DMF. Incorporation of an amino acid and Myr-OH was repeated if necessary until the Kaiser ninhydrin test became negative. Peptides were deblocked and cleaved from the resin with anhydrous liquid HF containing 10% anisole. The reaction mixture was kept at 0 °C for 45 min. After evaporation of HF *in vacuo* with ice-cooling, the residual mixture was washed with ether prior to extraction of the crude peptide with 12—50% AcOH. In the case of Myr-peptide, the residual mixture was extracted with dioxane. The combined extracts were lyophilized. The crude peptide was revealed as a main peak on analytical RP-HPLC.

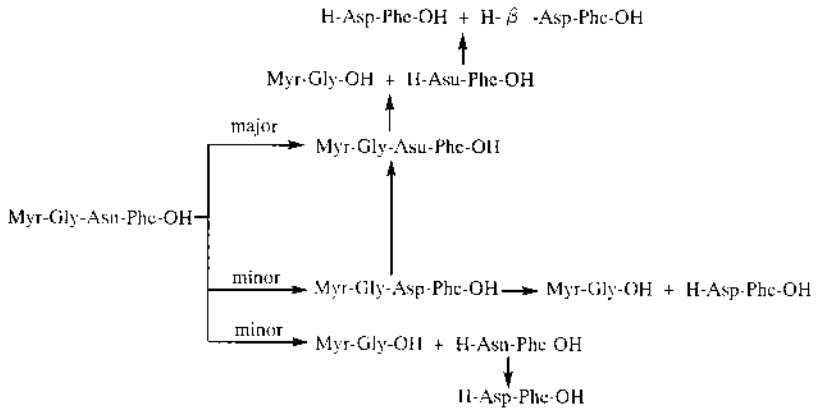

Chart 3

Peptide Purification The synthetic peptides and Myr–peptides were highly purified by semi-preparative RP-HPLC on a column of μ-Bondasphere C_{18} 5—100 A (19×150 mm) or YMC-pack D-ODS-5-ST S-5 120 A (20×150 mm) with 0.1% TFA–MeCN in an isocratic system, followed by gel-filtration on a column of Toyopearl HW-40 (super fine) (1.5×47 cm) with 12% AcOH or 70—100% MeOH as an eluent.

Peptide Characterization Homogeneity of the purified peptides was confirmed by analytical RP-HPLC with a 0.1% TFA–MeCN system, HP-TLC with two solvent systems, amino acid analysis of acid hydrolysates and FAB-MS. HPLC analysis of the purified peptides was performed with a Puresil^{TM} C_{18} (4.6×250 mm) with isocratic elution of 6.4 or 8% MeCN in 0.1% TFA or linear gradient elution of 8—36.8% MeCN over a period of 30 min in 0.1% TFA (flow rate, 1 ml/min; UV detection, 210 nm). HPLC analysis of the purified Myr-peptides was carried out using a YMC-Pack C_4 (4.6×150 mm) with a linear gradient elution of 0—52% MeCN over a period of 30 min in 0.1% TFA (flow rate, 1 ml/min; UV detection, 210 nm). For

Table 3. FAB-MS Data and Amino Acid Analyses of Myr–Gly–X–Phe–OH and Related Peptides

Peptide	FAB-MS[a]		Amino acid analysis[b]			
	Found	Formula	Gly	Phe	X	Recovery (%)
Myr–Gly–Ala–Phe–OH	504	$C_{28}H_{45}N_3O_5$	0.93 (1)	1.15 (1)	Ala 0.92 (1)	98.6
Myr–Gly–Gly–Phe–OH	490	$C_{27}H_{43}N_3O_5$	1.82 (2)	1.18 (1)	—	95.4
Myr–Gly–Val–Phe–OH	532	$C_{30}H_{49}N_3O_5$	1.14 (1)	0.83 (1)	Val 1.03 (1)	99.7
Myr–Gly–Glu–Phe–OH	562	$C_{30}H_{47}N_3O_7$	0.94 (1)	1.20 (1)	Glu 0.86 (1)	90.0
H–Gly–Glu–Phe–OH	—	—	0.91 (1)	1.11 (1)	Glu 0.98 (1)	90.7
Myr–Gly–His–Phe–OH	570	$C_{31}H_{47}N_5O_5$	0.97 (1)	1.15 (1)	His 0.88 (1)	93.1
Myr–Gly–Lys–Phe–OH	561	$C_{31}H_{52}N_4O_5$	1.02 (1)	0.95 (1)	Lys 1.03 (1)	94.8
H–Gly–Lys–Phe–OH	—	—	0.91 (1)	1.19 (1)	Lys 0.90 (1)	96.2
Myr–Gly–Ile–Phe–OH	546	$C_{31}H_{51}N_3O_5$	1.12 (1)	0.94 (1)	Ile 0.94 (1)	89.4
H–Gly–Ile–Phe–OH	—	—	1.17 (1)	0.93 (1)	Ile 0.90 (1)	90.2
Myr–Gly–Asp–Phe–OH	548	$C_{29}H_{45}N_3O_7$	0.92 (1)	1.15 (1)	Asp 0.93 (1)	91.6
H–Gly–Asp–Phe–OH	—	—	0.92 (1)	1.16 (1)	Asp 0.92 (1)	89.9
Myr–Gly–Asn–Phe–OH	547	$C_{29}H_{46}N_4O_6$	1.01 (1)	1.04 (1)	Asp 0.95 (1)	99.8
H–Gly–Asn–Phe–OH	—	—	1.00 (1)	1.00 (1)	Asp 1.00 (1)	92.2
Myr–Gly–Gln–Phe–OH	561	$C_{30}H_{48}N_4O_6$	1.10 (1)	0.98 (1)	Glu 0.99 (1)	96.3
H–Gly–Gln–Phe–OH	—	—	1.05 (1)	0.95 (1)	Glu 1.00 (1)	90.9
Myr–Gly–Arg–Phe–OH	589	$C_{31}H_{52}N_6O_5$	1.04 (1)	0.96 (1)	Arg 1.00 (1)	96.1
H–Gly–Arg–Phe–OH	—	—	0.95 (1)	0.93 (1)	Arg 1.12 (1)	91.7
Myr–Gly–OH	286	$C_{16}H_{31}N_1O_3$	1.00 (1)	—	—	94.2
Myr–Gly–Asu–Phe–OH	530	$C_{29}H_{43}N_3O_6$	—	—	—	—
H–Asu–Phe–OH	263	$C_{13}H_{14}N_2O_4$	—	—	—	—
Myr–Gly–β-Asp–Phe–OH	548	$C_{29}H_{45}N_3O_7$	1.01 (1)	0.99 (1)	Asp 1.00 (1)	91.1
H–β-Asp–Phe–OH	281	$C_{13}H_{16}N_2O_5$	—	1.02 (1)	Asp 0.98 (1)	98.5

a) For $[M+H]^+$. *b*) Numbers in parentheses are theoretical values.

Table 4. Characteristics of Myr–Gly–X–Phe–OH and Related Peptides

Peptide	$[\alpha]_D^{26}$	RP-HPLC[d]	HP-TLC[f]	
	(c=0.5)[a]	t_R[e] (min)	Rf^1	Rf^2
Myr–Gly–Ala–Phe–OH	14.0	17.4	0.73	0.74
Myr–Gly–Gly–Phe–OH	14.1	16.9	0.71	0.71
Myr–Gly–Val–Phe–OH	14.5	19.1	0.73	0.79
Myr–Gly–Glu–Phe–OH	10.6	17.7	0.68	0.72
H–Gly–Glu–Phe–OH	−3.2	18.8	0.31	0.38
Myr–Gly–His–Phe–OH	6.2	15.5	0.68	0.55
Myr–Gly–Lys–Phe–OH	−16.0[b]	15.6	0.61	0.54
H–Gly–Lys–Phe–OH	17.2	16.3	0.32	0.26
Myr–Gly–Ile–Phe–OH	−5.9	20.1	0.74	0.81
H–Gly–Ile–Phe–OH	−18.3	26.9	0.55	0.49
Myr–Gly–Asp–Phe–OH	−24.0	18.0	0.58	0.66
H–Gly–Asp–Phe–OH	−0.1	18.0	0.32	0.35
Myr–Gly–Asn–Phe–OH	−3.1	18.0	0.65	0.64
H–Gly–Asn–Phe–OH	−5.1	16.6	0.36	0.34
Myr–Gly–Gln–Phe–OH	−4.9	17.7	0.65	0.65
H–Gly–Gln–Phe–OH	−2.9	17.2	0.35	0.35
Myr–Gly–Arg–Phe–OH	0.7[c]	16.1	0.63	0.56
H–Gly–Arg–Phe–OH	−2.1	17.3	0.32	0.31
Myr–Gly–OH	—	13.9	0.67	0.72
Myr–Gly–β-Asp–Phe–OH	−10.7	17.2	0.55	0.62
H–β-Asp–Phe–OH	44.1	17.6	0.35	0.39

a) In DMF for Myr–peptide. In 50% AcOH for H-peptide. *b*) In 50% AcOH, c=0.1. *c*) In AcOH, c=0.1. *d*) HPLC conditions for Myr–peptide: column, YMC-Pack C_4 (4.6×150 mm); elution, 12—64% MeCN in 0.1% TFA; flow rate, 1 ml/min; detection, 210 nm; gradient HPLC conditions for H-peptide: column, Puresil^{TM} C_{18} (4.6×250 mm); elution, 4—44% MeCN in 0.1% TFA; flow rate, 1 ml/min; detection, 210 nm; gradient. *e*) Retention time. *f*) Rf^1, n-BuOH : pyridine : AcOH : H_2O (30 : 20 : 6 : 24); Rf^2, n-BuOH : AcOEt : AcOH : H_2O (1 : 1 : 1 : 1).

amino acid composition analyses, peptides were hydrolyzed with 6 N HCl vapor at 130 °C for 3 h as previously described.[7] The amino acid compositions of the acid hydrolysates were consistent with theoretical values. These analytical data are shown in Table 3.

Optical rotations of peptides were measured with a 3.5×50 mm cell. Myr–peptides were dissolved in DMF and other peptides in 50% AcOH at a concentration of 0.50%. Values shown in Table 4 were obtained by calculation from the means of 3 successive 30-s integrations. *Rf* values in HP-TLC refer to the following solvent systems: Rf^1, *n*-BuOH–pyridine–AcOH–H_2O (30 : 20 : 6 : 24) and Rf^2, *n*-BuOH–AcOEt–AcOH–H_2O (1 : 1 : 1 : 1). These data are shown in Table 4.

When single peaks on HPLC and single spots on HP-TLC in two solvent systems were observed for a peptide, and amino acid composition and FAB-MS were consistent with the calculated values, the peptide was subjected to the acid hydrolysis study. Quasi-molecular ion $(M+H)^+$ peaks of peptides by FAB-MS are shown in Table 3.

HPLC Analysis of Acid Hydrolysate To analyze acid hydrolysates, solutions of peptide at a concentration of 4×10^{-4} or 10^{-4} mol/l in 6—36% HCl in water : dioxane (1 : 1), 10—70% (v/v) H_2SO_4 in water : dioxane (1 : 1), 10—50% (v/v) MSA in water : dioxane (1 : 1), or 70—90% (v/v) MSA in water were prepared in polypropylene tubes (2 ml) under ice-cooling and divided into seven to twelve aliquots (100 μl each) in polypropylene tubes (2 ml) with tight caps. These were maintained at an appropriate temperature in a thermostatically regulated apparatus. Each tube was removed from the apparatus at 0 h or an appropriate time point, and after neutralization by 8 N NaOH, stored at −40 °C until analysis. To analyze acid hydrolysates, the solution (37.5 μl) was examined by RP-HPLC [column, YMC-Pack C_4 (4.6×150 mm); elution, 0—52% MeCN in 0.1% TFA; flow rate, 1 ml/min; detection, 210 nm] to determine the amount of starting material remaining. To analyze the amounts of deblocked peptides and amino acid, the remaining neutralized solution was lyophilized and then dissolved in the same amount of 1 N HCl, and the amounts of water-soluble hydrolysates were determined by RP-HPLC [column, Puresil™ C_{18} (4.6×250 mm); elution, 6.4 or 8% MeCN in 0.1% TFA; flow rate, 1 ml/min; detection, 210 nm]. To identify the hydrolysates, each peak on HPLC was collected, analyzed for amino acid composition, identified and confirmed by coelution with authentic samples on HPLC. The peak areas of the starting material and the hydrolysates were compared with those of standard samples. The acid hydrolysates of each peptide were examined by HPLC in triplicate.

References and Notes

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). Other abbreviations used are: Myr, myristoyl; Z, carbobenzoxy; Boc, *tert*-butoxycarbonyl; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DCM, dichloromethane; HOBt, 1-hydroxybenzotriazole; DMF, *N*,*N*-dimethylformamide; TEA, triethylamine; BOP, benzotriazol-1-yl-oxy-tris(dimethylamino) phosphonium hexafluorophosphate; MeCN, acetonitrile; MSA, methanesulfonic acid; AcOH, acetic acid; TFA, trifluoroacetic acid; EtOH, ethyl alcohol; MeOH, methyl alcohol; BuOH, butyl alcohol; AcOEt, ethyl acetate; RP-HPLC, reversed-phase high-performance liquid chromatography; HP-TLC, high-performance thin-layer chromatography.
2) A part of this work was reported previously as a preliminary communication; Okimura K., Sugimoto K., Sakura N., Hashimoto T., "Peptide Chemistry 1996," ed. by Kitada C., Protein Research Foundation, Osaka, 1997, pp. 89—92; Sugimoto K., Okimura K., Sakura N., Hashimoto T., Proceedings of 1st International Peptide Symposium; Okimura K., Sugimoto K., Ohki K., Sakura N., Hashimoto T., Proceedings of the 25th European Peptide Symposium.
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8) A part of this work was reported previously as a preliminary communication; Ohki K., Sakura N., Hashimoto T., Proceedings of the 1st International Peptide Symposium; Ohki K., Okimura K., Sakura N., Hashimoto T., Proceedings of the 25th European Peptide Symposium.
9) In preparation.