ORIGINAL

Glycolipid Enzyme Models. V.

Hydrolysis of Amide Bonds

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Glycolipid catalysts, with sugar residues as active sites were used to hydrolyses of several amino acid derivatives possessing amide bonds. The glycolipid hydrolyzed amide bond under mild conditions and recognized substrates similarly to α -chymotrypsin. A comparison of the hydrolytic activity of the catalysts indicated the terminal part of sugar residues (methanol or carboxyl group) and structure of the linkage between a sugar residue and double-hydrocarbon chain to determine substrate selectivity to some extent.

1 Introduction

Enzymes in a biobody show excellent catalytic activities and molecular-recognizing ability under mild conditions. Their functions are realized by electrostatic interactions, hydrophobic interactions, hydrogen bonds between enzyme and substrate, and others. Now, it is important, in the understanding of biological phenomena, to clarify the mechanism of such interactions. Recently, artificial compounds which show molecular-recognizing functions like enzymes called, "enzyme models", have been proposed and studied widely. In the field of hydrolyses, several models which exhibit molecular-recognizing ability have been reported^{1)~5)}, but almost all of them can only recognize D,L-configurations of substrates.

Biomembranes work to take-in essential substrates and release unnecessary or consumed substances for vital actions, as well as to give reaction fields for enzymes. In model reactions for hydrolases, micellar and vesicular systems are used as reaction fields⁶⁾. Ueoka *et al.*^{7)~8)} reported enantioselective hydrolyses of amino acid esters using a compound system of a tripeptide type model and a surfactant. We also reported

that L/D recognition ratio of a phenylalanine ester reached 102 when the vesicular system consisting of compounds (A) and (B) (1 : 1) shown in Fig.-1 was used as catalyst⁹⁾. Furthermore, several glycolipid catalysts synthesized by the authors were found for the first time to hydrolyze amino acid esters while recognizing the kinds of amino acids esters^{10)~12)}. Such ability of hydrolase models like α -chymotrypsin has not been reported except for glycolipids. No hydrolase models, including micelle and vesicle type catalysts that cleave peptide or amide bonds under mild conditions, however, have been reported, though a hydrolase in nature performs hydrolyses of such bonds of proteins.

In this research, several amides of amino acids were synthesized (Table-1) and, as a challenge, these were used in hydrolyses of amide bonds using glycolipid catalysts (C)~(H) as shown in Fig.-1. The ability of glycolipids to hydrolyze amide bonds will be reported. Further, the structural effect of glycolipids on the hydrolyzing ability and accompanying substrate recognition will be discussed.

2 Experiments

2.1 Syntheses of substrates

2.1.1 Protection of an amino group

L-Phenylalanine [L-Phe] (0.01 mol) was

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Fig.-1 Structure of glycolipids

dissolved in 200 mL of 0.1 M NaOH, a mixture of benzyloxycarbonyl chloride [Z-Cl] (7.0 mL) and diethyl ether (15.0 mL) was added in drops, and the resulting mixture was stirred at temperature in the range of 0~5°C for 2 h, after which 1 M HCl was added until the solution turned into white color. After extraction with chloroform (100 mL), the solvent was evaporated. The desired product (*N*-benzyloxycarbonyl-L-phenylalanine) [Z-L-Phe] was obtained as colorless crystal by recrystallization from *n*-hexane.

2.1.2 Syntheses of amino acid amides

The Z-L-Phe (0.05 mol) was dissolved in chloroform (100 mL), and *N,N'*-dicyclohexylcarbodiimide [DCC] (0.05 mol) was added over a lapse of 20 min, keeping the temperature within a range of 0~5°C. After 30 min, *p*-nitroaniline [*p*-NA] (0.05 mol) in ethyl acetate was introduced, and the mixture was stirred at room temperature for 24 h. The resulting mixture was washed successively with 5 % sodium hydrogen carbon-

ate, water, and 5 % citric acid. After the organic layer was dried over anhydrous sodium sulfate, the solvent was removed under vacuum. The residue was recrystallized from ethanol to give *N*-benzyloxycarbonyl-L-phenylalanyl-*p*-nitroanilide [Z-L-Phe-*p*-NA].

All of other substrates were synthesized according to similar methods and were characterized by elemental analyses, melting points and specific rotations. (Table-1)

2.2 Catalysts

Self-vesicle forming catalysts used in this study were shown in Fig.-1. These catalysts have sugar residues as hydrophilic segment and di-*n*-dodecyl amino group as hydrophobic one. Catalysts (C), (D), (F), (G), and (H) were synthesized as described in previous papers¹³⁾.

The glycolipid (E) Man-*d*-(Lau)₂, was synthesized as follows :

D-Mannose (1.80 g, 0.01 mol) and di-*n*-dodecylamine (3.53 g, 0.01 mol) were dissolved in methanol and refluxed for a week

Table-1 Analyses of substrates

Symbol	R	Calc. (%)			Found. (%)			mp (°C)	[α] _D ²⁰
		C	H	N	C	H	N		
L-Phe	CH ₂ -	65.85	5.06	10.02	65.58	4.97	9.75	161.8	+7.2
L-Leu	CH ₂ -	62.34	5.97	10.91	62.12	6.03	10.69	142.3	-29.0
L-Val	CH-	61.46	5.66	11.32	60.25	5.70	11.13	190.8	-23.5
L-Met	CH ₃ SCH ₂ CH ₂ -	56.56	5.26	10.42	56.63	5.27	10.07	158.9	-22.0
L-Ala	CH ₃ -	59.46	5.00	12.24	59.36	4.97	12.05	105.2	-54.9
Gly	H-	58.36	4.56	12.76	57.65	4.31	13.07	174.2	0.0
L-Gln	H ₂ NCOCH ₂ CH ₂ -	57.00	5.05	14.00	57.36	4.72	14.22	181.3	—

under atmosphere. After the reaction, the solvent was removed in vacuum, and the residue was treated with ethyl acetate to separate by filter undissolved materials. The solvent, then, was evaporated and the oily residue was purified by means of a column chromatography using silica gel (Wako-gel C-300, development solvent, chloroform : methanol=9 : 1) to obtain the oily product (**E**) ; yield 0.82 g (15.4 %), [α]_D²² = +3.4° (c=0.5, chloroform).

2.3 Procedure

The solution of a glycolipid (0.01 M) in methanol (120 μ L) was added to tris(2-amino-2-hydroxymethyl-1,3-propanediol) buffer (90 mL), and sonicated by means of SUC 2820 (Shibuya Electric Mfg. Co., Ltd.; 28 kHz, 200 W) for 10 min, to prepare a vesicle solution. The solution (3 mL) was put in a quartz cell having width of 1 cm, into which an amino acid amide (1.25 μ L in acetone) was syringed as substrate. The hydrolysis was pursued by measuring the amount of *p*-nitroaniline liberated at 400 nm using a Hitachi UV-124 spectrometer.

2.4 Kinetics

The catalytic activity of a glycolipid was evaluated as shown in the previous paper¹²). It should be pointed out, in this study, that any spontaneous hydrolysis was not observed, because amide type substrate have strong bonds, as compared with ester type ones.

3 Results and discussion

Self-vesicle forming catalysts, with sugar residues, hydrolyzed amide bonds. These are rather stable, under mild conditions suitable for enzymes at work, for example : at room temperature under atmospheric pressure. Shown in Table-2 are second order rate constants (k_2), when glycolipids were used as catalysts. In previous study¹²), it was reported that several glycolipid catalysts hydrolyzed amino acid esters, at the same time recognizing the kind of substrates on the taking-in, as suggested by Michaelis constant (K_m) calculated by Lineweaver-Burk plots. This process also could be reasonably applied to hydrolyses of amino acid amides. It was observed, further, that the catalyst hydrolyzed substrates in the order of hydrophobic nature of side chains of amino acids. These results suggest that a catalyst acts on a substrate by hydrophobic interaction and hydrolyzes the amide bond with its sugar moiety as active site.

The catalytic activity of D-Xyl-(Lau)₂ having a pentose (D-xylose) residue, was higher than that of D-Glc-(Lau)₂ or D-Gal-(Lau)₂, derived from D-glucose or D-galactose, respectively. In general, the catalytic activity was not influenced by the number of hydroxyl groups of a glycolipid. In other

Table-2 Second order rate constants on hydrolyses.

Catalyst	$k_2 \times 10^{-1} \text{ (s}^{-1} \cdot \text{M}^{-1}\text{)}$						
	L-Phe	L-Leu	L-Val	L-Met	L-Ala	Gly	L-Gln
Man (Lau) ₂	117	41.1	23.6	29.7	12.0	4.91	5.75
Man (Lau) ₂ -COOH	158	78.8	24.5	38.3	16.7	1.56	1.33
Man- <i>d</i> -(Lau) ₂	82.6	44.3	29.9	25.2	9.76	7.19	3.51
Gal(Lau) ₂	108	39.2	29.0	11.7	10.2	3.26	1.03
Glc(Lau) ₂	74.3	36.0	18.4	14.7	2.23	1.30	0.66
D-Xyl(Lau) ₂	114	56.3	16.2	24.3	7.66	3.35	2.66

words, the catalytic activity did not depend on the bulkiness of hydrophilic domain.

α -Chymotrypsin, one of the proteases *in vivo*, is known preferentially to hydrolyze peptide bonds of a protein chain at the C-terminal of hydrophobic amino acids, such as tyrosin and phenylalanine. To compare glycolipids with α -chymotrypsin, illustrated in Fig.-2 are substrate-recognizing abilities of mannose type glycolipid (C), (D), (E) and of α -chymotrypsin. The ordinate of the figure shows the second order rate constant (k_2) of hydrolysis, and the abscissa shows hydrophobicity parameters¹⁴⁾, which mean the hydrophobic degree of side chains of amino acids [the hydrophobicity increases with increase in the value (ΔG_t); Gln (-0.1), Gly (0.0), Ala (+0.5), Met (+1.3), Val (+1.5), Leu (+1.8), and Phe (+2.5)]. The straight line (—) can be drawn among hydrophobic substrates such as Phe, Leu, Val, and Met, depending on catalysts, except for the case of hydrophilic substrates such as Ala, Gly and Gln. The slopes of lines may correspond to substrate recognizing abilities of catalysts. On the assumption that the slope for α -chymotrypsin is a unit, relative values of slopes can be calculated: 0.35 for (C), 0.48 for (D), and 0.24 for (E). Table-3 also summarized Michaelis constants K_m , which shows the affinity of Z-L-Phe-*p*-NA with mannose type glycolipid catalyst. The values obtained by (C), (D) and (E) were 2.44×10^{-2} , 2.27×10^{-2} , and 8.33×10^{-2} , respectively. The smaller the K_m value obtained, the higher the affinity with the glycolipid. Thus it was found that glycolipid (D) exhibited both the best catalytic activity

1) ΔG_t for transfer of amino acid side chain from 100 % organic solvent to water at 25°C.

Fig.-2 Relationship between second order rate constants and hydrophobicity parameters.

and substrate recognizing ability.

As a matter of interest, the previous paper reported that the glycolipid (D) well hydrolyzed carbohydrates, such as cellulose and chitin¹⁵⁾. In this study, the glycolipid also hydrolyzed, within 1 min, 70 % of the phenylalanine type substrate injected in quartz cell. Now, it cannot be understood clearly why such neutral glycolipids hydrolyze comparatively strong bonds. The co-existence of a substrate such as phosphatidyl glycerol as a component of vesicles, however, resulted in a certain degree of retardation or complete inhibition of hydroly-

Table-3 Substrates recognition.

Catalysts	Relative ratio	$k_2 \times 10^{-1} \text{ (s}^{-1} \cdot \text{M}^{-1})$	$Km \text{ (} 10^{-2} \text{) M}$
α -Chymotrypsin	1.00	—	31.0
Man(Lau) ₂	0.35	117	2.44
Man(Lau) ₂ -COOH	0.48	158	2.27
Man- <i>d</i> -(Lau) ₂	0.24	82.6	8.33

Substrate=Z-L-Phe-*p*-NA* *N*-acetyl-L-phenylalanineamide

ses¹⁶⁾. These facts may suggest that phosphatidyl glycerol, which can form hydrogen bonds with a glycolipid, shields the active site of the glycolipid, or prevents the sugar moiety of a glycolipid from polarizing or dissociating, which are attributed to the hydrogen bonds. Considering the possible existence of hydrogen bonds among glycolipids themselves, the nature of bonds formed in vesicles seems to be related with the activity of glycolipids.

In Table-3, it is found also that the hydrolysis was influenced by linkages between hydrophobic moiety and sugar moiety of glycolipids. The glycolipid (C) was superior to (E) in terms of the substrate recognizing ability. The fact that *Km* value of (C) was about a quarter of that of (E), means that the bulkier thiazolidine ring, which the glycolipid (C) possesses, prevents the inactivation of catalysts or, on the contrary, enhances the intramolecular hydrogen bond mentioned above, and/or that the ring, derived from cysteine, is effective to act on substrates by an amino acid interaction. It was observed, however, that the glycolipid (E), without any linkage member, also hydrolyzed amide bonds in substrate recognizing manners. This fact demonstrates that a sugar residue itself is active site for hydrolyses.

While vaguely, it is known that sugar chains, contained in glycolipids or glycoproteins *in vivo*, are connected with information communication among cells. It has not been clarified yet, however, the role of the sugar chain in such communications, from the standpoint of biomolecular chemistry. In this research, it was found that diverse gly-

colipids, having simple structures, hydrolyzed amide bonds under mild conditions. This fact suggests that the sugar chains of glycolipids or glycoproteins *in vivo* may exhibit mystic powers, which has not been taken up so far. Furthermore, many kinds of glycolipids, having different sugar moieties, are synthesized and applied to hydrolyses. The effects of these sugar moieties on catalytic activity and substrate-recognizing ability presented by the authors are believed to be helpful in assuming at least one of the functions of sugar segments in a bio-body.

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糖脂質酵素モデル (第5報)

アミドの加水分解

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活性部位を糖残基とした糖脂質を用い、アミド結合を有するアミノ酸誘導体の加水分解を行った。糖脂質は温和な条件下でアミド結合を加水分解し、同時に α -キモトリプシン類似の基質選択性を示した。さらに糖残基末端(メチロールまたはカルボキシル基)と糖と二本鎖との連結構造が異なる糖脂質触媒の加水分解活性を比較すると、それが基質選択性に大きく影響していることが判明した。

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