ORIGINAL

Glycolipid Enzyme Models. X. Catalysis of Glycolipids with Amino Acid Residue

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Abstract : Glycolipids classified into two categories were synthesized and applied, as hydrolase models, to the hydrolyses of *p*-nitro-anilides of amino acids ; one had a sugar group at the end (hydrophilic part) of a glycolipid molecule and the other had a histidine group bonded to ω -position of the sugar moiety. Both types of glycolipids could recognize the kinds of amino acids like α -chymotrypsin, but the combination system of both categories of glycolipids only recognized them in different modes. The combination system, however, distinguished the D,L-configuration of amino acids better than other glycolipids. In particular, it distinguished D,L-alanines perfectly like an enzyme. The recognition mechanism is discussed.

Key words : Hydrolase model, Complete D,L-stereorecognition, Alanine ester, Glycolipid, α -Chymotrypsin

1 Introduction

Enzymes are substrates that catalyze bio-reactions, and are characterized by such specificities as substrate-, stereo-, regio-, and reaction-specificities. If such specificities can be easily realized in organic chemical reactions, it will be highly useful. Recently, many chemists have developed and proposed enzyme models, although the realization of such specificities is only one of the reasons why they conduct model researches. Most of the models have been designed to act like enzymes in the stereochemistry of substrates and products, regardless of the kinds of reactions.

Enzymatic processes consist of the formation of a complex by taking-in of a substrate and the production of a product by decomposition of the complex as explained by Michaelis-Menten mechanism as follows :

In general, an enzyme includes a hydrophobic pocket to take up a hydrophobic substrate. Enzyme model systems on the basis of concept mentioned above are often constructed by utilizing micelle and vesicle-forming, "amphiphilic", substance(s), which can provide a reaction field mimicking a hydrophobic pocket.

α -Chymotrypsin, a hydrolase, whose structure and mechanism of action have been clarified relatively well, can hydrolyze peptide chains at the C-terminals of amino acids with an increased hydrophobicity, such as phenylalanine and thirosine. It hydrolyses ester substrates, too. Many researches of hydrolase models have been devoted to D,L-recognition of

substrates, especially D and L-phenylalanine esters. Almost all researches use alkylated amino acids, especially, histidine derivatives with a higher alkyl chain, as model catalysts, in the presence of amphiphilic substance(s). The D,L-recognition ability of such models is 80 at the highest. A model system consisting of special components, however, gives an enzyme-like recognition. For example^{1),2)}, a model system using a peptide catalyst

in the mixed vesicular system of

has been reported, in terms of the hydrolysis of a phenylalanine ester.

Simple glycolipids were also reported to hydrolyze esters^{3),4)} as well as amides⁵⁾ of amino acids, without any other amphiphiles, because the glycolipids can form micelles and vesicles spontaneously. In the hydrolysis of the *p*-nitro-phenyl ester of phenylalanine, the mixed system consisting of

gave a considerably high D,L-recognition ability (102)⁴⁾. Further, it has also been reported that glycolipids can distinguish the kinds of amino acids³⁾.

In this paper, the recognition abilities for the D,L-configuration and kinds of amino acids by glycolipid modified with histidine are examined and compared with those of a glycolipid without such an amino acid moiety. In addition, the complete recognition of D,L-alanine anilides is reported.

2 Experimental

2.1 Reagents

2.1.1 Substrates

p-Nitro-anilides of *N*-benzyloxycarbonyl-phenylalanine, -leucine, -valine, -methionine, -alanine, and -glycine were used as substrates. They were synthesized as described elsewhere⁶⁾.

2.1.2 Glycolipids

Glycolipids derived from hexoses such as glucose, galactose, mannose, L-rhamnose, and L-fucose, and pentoses such as xylose, ribose, and arabinose were also synthesized as described in previous papers^{5),6)}. They are represented, noting the presence or absence of a thiazolidine ring derived from cysteine. The case of mannose (Man) is shown below as an example :

In this study, the following glycolipids were also used as a model catalyst modified with histidine [Man (Lau)₂-HisCOOMe, called Glycolipid (A)], and as a reaction field (Man Lau).

The Man(Lau)₂-HisCOOMe was synthesized according to **Scheme 1**. First Man(Lau)₂ was synthesized by the trytyl method⁶⁾. Then Man(Lau)₂ (7×10^{-3} mol, 4.33 g) was dissolved in a small amount of methylene chloride, to which 2 mL of 61% nitric acid was added, and the mixture was stirred at 0°C for 6 h. The methylene chloride phase was extracted with neutralizing, followed by dehydration, and then was evaporated. The residue was dissolved in ethyl acetate, washed completely with water, dried, and then evaporated. Petroleum ether was added to the residue, and the resulting mixture was cooled to remove decomposed Man (Lau)₂ by filtration. The product Man(Lau)₂-COOH was obtained from the filtrate. Yield : 10.1%.

IR (cm⁻¹) : 3300, 1090, 1050 (-OH), 2920, 2850, 1460 (-CH₃, -CH₂-), 1640 (-CON).

The Man(Lau)₂-COOH (0.42 g) was dissolved in *N,N*-dimethyl formamide, to which the di-hydrogen chloride salt of L-histidine methyl ester (0.091 g), prepared previously, and triethyl amine (1.08×10^{-3} mol) were added. The mixture was stirred at 0°C for 3 h and, then, at room temperature for 24 h. After removal of unreacted materials, the residue was dis-

Scheme 1 Synthesis of Man(Lau)₂-HisCOOMe.

solved in methylene chloride, and washed with 10% citric acid, 4% sodium hydrogen carbonate, and saturated NaCl solution, successively. The product was recovered after purification through a silica gel column (eluent : chloroform : methanol, 17 : 3).

Yield : 10.0%.

IR (cm^{-1}) : 3300 (-OH), 2920, 2850, 1460 ($-\text{CH}_3$, $-\text{CH}_2-$), 1710 ($-\text{CONH}-$), 1630 ($-\text{C}=\text{N}-$)

$^1\text{H-NMR}$ (CDCl_3) (ppm) : 8.3 (1 H, $\text{N}=\text{CH}-\text{N}$), 7.5 (1 H, $\text{C}=\text{CH}-\text{N}$), 4.5-3.5 (6 H, -OH), 1.6 (46 H, $-\text{CH}_2-$), 1.2 (9 H, $-\text{CH}_3$)

2.2 Procedure

Hydrolyses of *p*-nitro-anilides with glycolipid(s) were carried out by the method described in a paper⁶⁾. In the combination system of Glycolipids (A) and Man Lau, the net concentration of the catalytically effective component, Glycolipid (A), was kept constant (2×10^{-6} mol of the glycolipid catalyst per 1.25×10^{-7} mol of a substrate) in 3 mL of tris buffer (pH 7.8-8.0). The reaction was consistent with the mechanism presented by Michaelis-Menten. Thus, the second-order rate constants (k_2) and Michaelis constants (K_m) were obtained by conventional methods.

3 Results and Discussion

3.1 Recongnition of the kinds of amino acids

It has been already reported that a glycolipid, synthesized by the authors, in hydrolyses of amides of amino acids, distinguishes the hydrophobic nature of side chains of amino acids, just as α -chymotrypsin does³⁾. **Figure 1** shows the results of substrate recognition obtained using $\text{Man(Lau)}_2\text{-HisCOOMe}$, and includes, for comparison, a part of the results reported so far. The Man(Lau)_2 showed the relative ability of substrate recognition of 0.35 (the value was calculated from the slope of linear part, taking the value of α -chymotrypsin as one). A micelle-forming glycolipid, Man Lau, could also recognize the kinds of amino acids and its ability was estimated as 0.20. Glycolipid (A), on the other hand, showed a very low ability of 0.14.

It was found, however, that the combination system of Glycolipid (A) and Man Lau did not behave like α -chymotrypsin in terms of the substrate recognition, although the individual component shows a similarity to α -chymotrypsin. Thus, the recognition ability for amino acids could not be calculated for such combination systems, because of the lack of linear relation of second-order rate constants to hydrophobic nature of amino acids. In addition, the curvature of the non-linear part depended on the mixing ratio of Glycolipid (A) and Man Lau. These results well suggest that both components are not in the same conformation, regardless of the mixing ratios, in the combination system, but one of them exists as islands (colonies) in the sea of the other, that is, they take different shapes depending on the mixing ratios. In such circumstances, hydrolysis is supposed to occur or proceed near the interface of the components, Glycolipid (A) and Man Lau, rather than in the inside of each component.

3.2 Recognition of the D,L-configuration of amino acids

Synthesized glycolipids hydrolyze amide bonds⁶⁾, which are not cleaved by the models proposed by others. In addition, they showed the D,L-recognition ability for amino acids, though it was remarkably low. The result is summarized in **Table 1**, which shows the ratios of second-order rate constants for hydrolyses of D and L-amino acids (k_{2D}/k_{2L}). As substrates, phenylalanine anilide and alanine anilide were chosen. The former is a representative amino acid having a side chain with a high hydrophobic nature, and the latter, having one with a low hydrophobic nature. As seen from **Table 1**, there is no remarkable correlation between the ratio and the configuration of sugar moieties of glycolipids, which is surprising, since the configuration of hydroxyls on 2,3-positions of a sugar moiety has been found to be responsible for the recognition of α and β -1,4-glycoside bonds⁷⁾ as well as to play

Fig. 1 Relationship between Second Order Rate Constants and Hydrophobicity Parameters.

Table 1 Ratios of Second-order Rate Constants (k_2D/k_2L) for Hydrolyses of D and L-amino acids.

		$\begin{array}{c} \text{X} \\ \\ \text{HO}-\text{C}-\text{OH} \\ \\ \text{HO}-\text{C}-\text{OH} \\ \\ \text{CH}_2\text{OH} \end{array}$	$\begin{array}{c} \text{X} \\ \\ \text{HO}-\text{C}-\text{OH} \\ \\ \text{HO}-\text{C}-\text{OH} \\ \\ \text{CH}_2\text{OH} \end{array}$	$\begin{array}{c} \text{X} \\ \\ \text{HO}-\text{C}-\text{OH} \\ \\ \text{HO}-\text{C}-\text{OH} \\ \\ \text{CH}_2\text{OH} \end{array}$	$\begin{array}{c} \text{X} \\ \\ \text{HO}-\text{C}-\text{OH} \\ \\ \text{HO}-\text{C}-\text{OH} \\ \\ \text{CH}_3 \end{array}$	$\begin{array}{c} \text{X} \\ \\ \text{HO}-\text{C}-\text{OH} \\ \\ \text{HO}-\text{C}-\text{OH} \\ \\ \text{CH}_3 \end{array}$
	Sugar	Gal	Glc	Man	L-Rha	L-Fuc
direct	Ala	0.84	0.76	1.09		
	Phe	1.18	1.02	1.10		
indirect	Ala	2.92	4.29	2.27	0.93	1.46
	Phe	1.10	2.18	1.12	1.05	1.00
		$\begin{array}{c} \text{X} \\ \\ \text{HO}-\text{C}-\text{OH} \\ \\ \text{HO}-\text{C}-\text{OH} \\ \\ \text{CH}_2\text{OH} \end{array}$	$\begin{array}{c} \text{X} \\ \\ \text{HO}-\text{C}-\text{OH} \\ \\ \text{HO}-\text{C}-\text{OH} \\ \\ \text{CH}_2\text{OH} \end{array}$	$\begin{array}{c} \text{X} \\ \\ \text{HO}-\text{C}-\text{OH} \\ \\ \text{HO}-\text{C}-\text{OH} \\ \\ \text{CH}_2\text{OH} \end{array}$		
	Sugar	Xyl	Rib	Ara	direct	
direct	Ala	0.98	1.19	0.63	X=	$\begin{array}{c} \text{CON}-\text{C}_{12}\text{H}_{25} \\ \\ \text{C}_{12}\text{H}_{25} \end{array}$
	Phe	1.20	1.07	1.33		
indirect	Ala	1.12	1.07	1.26	indirect	$\begin{array}{c} \text{CON}-\text{C}_{12}\text{H}_{25} \\ \\ \text{C}_{12}\text{H}_{25} \end{array}$
	Phe	1.43	1.39	1.01	X=	$\begin{array}{c} \text{CON}-\text{C}_{12}\text{H}_{25} \\ \\ \text{C}_{12}\text{H}_{25} \end{array}$

an important role in the recognition of the kinds of amino acids^{3),8)}. Considering that almost all the ratios are in the range from 0.6 to 1.5, which might be within the range of experimental errors, further discussion may have to be avoided about the recognition of D,L-configuration by glycolipids themselves.

It was found, however, that the introduction of amino acid residue into a glycolipid en-

hanced D,L-recognition as follows :

The development of the D,L-recognition ability of a glycolipid may need the presence of amino acid residue in a glycolipid. In the past, the authors reported a remarkably high D,L-recognition in the hydrolyses of phenylalanine *p*-nitro-phenyl esters. The glycolipids of

gave the recognition values of 61.05 and 0.88, respectively, but their mixed vesicular model system (mole ratio of 1 to 1) showed the D,L-recognition value of 96.20⁴⁾. Thus, the similar systems were also examined in the hydrolyses of D,L-amino acid amides.

Shown in Fig. 2 is the dependence of second-order rate constants on the composition of mixed vesicular systems in the hydrolyses of alanine anilides. As mentioned in the Experimental section, each system contains the same amount of Glycolipid (A). Nevertheless, a dramatic change in the rate constants was observed, depending on D and L anilides. L-Alanine anilide, for example, was hydrolyzed with a significantly increased rate by adding only 10 mol % of Glycolipid (A), but further addition did not remarkably change the rate constants. While D-alanine anilide, on the other hand, was hydrolyzed at a little higher rate by addition of a small amount of Glycolipid (A), the addition of Glycolipid (A) in the range of 20 to 30 mol % did not hydrolyze the anilide at all. Further added Glycolipid (A) served to recover some hydrolysis activity, in spite of a lower rate. These results show that a combination system, which contains 20 to 30 mol% of Glycolipid (A), can distinguish the D and L configurations of alanines perfectly, like enzymes.

The effect of the combination ratio of Glycolipid (A) and Man Lau on the recognition of D,L-configuration of four amino acids is shown in Fig. 3. The hydrophobicity of amino acids is in the order of Phe>Val $\geq$ Met>Ala. The recognition for each amino acid, on the other hand, was in the order of Ala>Val>Met $\geq$ Phe. Except for methionine which has a polar group in the side chain, the less the hydrophobicity, the higher the D,L-recognition activity. It is found by studying Fig. 3 in more detail that there are possibly two combination ratios

Fig. 2 Recognition of D,L-Alanines Using Catalyst System Consisting of $\text{Man(Lau)}_2\text{-HisCOOMe}$ and ManLau .

Fig. 3 Stereoselective Hydrolyses Using Catalyst System Consisting of $\text{Man(Lau)}_2\text{-HisCOOMe}$ and Man-Lau .

Table 2 Kinetic Parameters of Catalyst Systems Containing 10 or 15 mol% of Man(Lau)₂-HisCOOMe.

	10%		15%	
	L-	D-	L-	D-
K_m [M]	5.0×10^{-5}	25.0×10^{-5}	7.7×10^{-5}	33.3×10^{-5}
k_{cat} [s ⁻¹]	27.2×10^{-3}	49.9×10^{-3}	20.7×10^{-3}	5.0×10^{-3}
k_2 [mol ⁻¹ ·dm ³ ·s ⁻¹]	63.8	7.4	62.3	0.5
k_{2L}/k_{2D}	8.7		135.5	

which give higher recognition of D,L-configurations : that is, the presence of Glycolipid (A) at the concentration of 25 or 50 mol% seems to give a higher D,L-recognition. The former concentration, in particular, seems effective for the recognition of less hydrophobic D,L-amino acids, while the latter seems effective for the recognition of more hydrophobic D,L-amino acids.

These phenomena also suggest that the shape and size of aggregates of Glycolipid (A) dispersed in Man Lau, probably heterogeneously as mentioned above, are important in developing the ability for D,L-recognition. In other words, Glycolipid (A) is considered to function in the form of molecular aggregates rather than individual molecules.

The step was examined, in which the stereochemistry of a D,L-amino acid, especially D,L-alanine, was recognized. **Table 2** shows the kinetic parameters of the enzyme model systems containing 10 or 15 mol% of Glycolipid (A). These concentrations were chosen on the basis of the concentrations which showed greater increased abilities of hydrolysis and recognition of D,L-alanines, because the kinetic parameters at 20 to 30 mol% of Glycolipid (A) could not be determined due to the lack of data on the observed hydrolysis of D-alanine amide.

As seen in **Table 2**, k_{2L}/k_{2D} increases dramatically from 8.7 to 135.5, although the rate constants are approximately the same for L-alanine anilide and decrease from 7.4 to 0.5 for D-alanine anilide, when Glycolipid (A) is allowed to increase from 10 to 15 mol%. According to the K_m values, L-alanine anilide is taken up 4 to 5 times more easily than D-alanine anilide, but both D- and L-alanine anilide gives about the same values of K_m at 10 and 15 mol% of Glycolipid (A). That is to say, the degree of uptake looks unaffected by the concentrations of Glycolipid (A). On the contrary, the k_{cat} values for D-alanine anilide are quite different, depending on the concentration of Glycolipid (A), although those for L-alanine anilide are not appreciably different. This fact shows that overall rate constant k_2 is mainly concerned with rate constants k_{cat} rather than K_m value. Thus, the recognition of D,L-configuration is considered to occur mainly in the step in which the complex in Michaelis-Menten mechanism is decomposed into hydrolysates and the original enzyme model (A), in addition to a little recognition in the taking-in process.

The step of D,L-recognition clarified in this study cannot be explained satisfactorily by the "lock and key" theory, which says that an enzyme takes in only the substrate (key) having a chemical structure fitted in the hole (lock) of an enzyme. The molecular recognition performed in the decomposition step of the complex in Michaelis-Menten mechanism, as mentioned above does not seem peculiar only to an enzyme model system.

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[報文]

糖脂質酵素モデル (第9報)
糖残基の立体配置の影響

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ヘキソース, ペントース, およびテトロース型の糖脂質を用いてアミノ酸アミドを加水分解した。糖脂質としては, 親水性糖残基と疎水性残基の間に連結基としてチアゾリジン環を有するもの(間接型糖脂質)と有さないもの(直接型糖脂質)を使用した。一般に前者の糖脂質は後者のものよりも活性が高く, 基質識別能や加水分解活性のような活性は糖残基のC-2およびC-3位のヒドロキシル基の立体配置と関係のあることが判明した。すなわち, それらのヒドロキシル基がL-トレオ型の糖脂質は両活性共に高い活性を示し, 一方D-トレオ型のそれは共に低い活性を示した。またエリトロ型の糖脂質はそれらの中間的な活性を示した。

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[報文]

糖脂質酵素モデル (第10報)
アミノ酸残基を有する糖脂質の触媒活性

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二つの範疇に入る糖脂質を合成し, 加水分解酵素モデルとしてアミノ酸のp-ニトロアニリドの加水分解に適用した。一つの糖脂質は糖脂質分子の末端に糖残基を有し, そして他は糖残基の ω -位にヒスチジン基を有した。両方の種類の糖脂質は, α -キモトリプシンと同様にアミノ酸の種類を識別する事ができたが, 両範疇の糖脂質の組み合わせ系はそれと異なった具合にアミノ酸の種類を識別した。しかしながら, その組み合わせ系はアミノ酸のD, L-立体配置を他のモデルよりも良好に識別した。特に, それはD, L-アラニンを酵素のように完全に識別した。この識別機構が議論される。

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[報文] ニンジン汁液に植えた酵母による脂質生産
及びその組成変化 (第1報)

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ニンジン汁液に植えた酵母10株中より, *Phodotorula gracilis* 2及び*Trichosporon cutaneum* 1を選択した。Wickerham(▼)の方法で調製した培養液による予備試験では, 上記2株の油脂生産性が優れていた。 β -カロテン生産後のニンジン汁液廃水を油脂生産酵母の培養に用いた。培養した酵母菌体より得られる油脂のアシルグリセリン, 遊離脂肪酸, リン脂質, ステロール及び核酸を定量した。

(連絡者: 丸山武紀) Vol. 45, No. 6, 555 (1996).