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Artificial Hydrolase Using Modified Dimethyl-β-cyclodextrin

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Abstract. The first successful method for modification of dimethyl-β-cyclodextrin (β-DMCD) was demonstrated by the synthesis of a new artificial hydrolase (**2**) and the enzymatic activities of **2** were investigated. **2** caused an 1100-fold increase in the rate of hydrolysis of *p*-nitrophenyl acetate at pH 7.2, whereas unmodified β-DMCD depressed the reaction. The kinetic pK_a of **2** was 7.2, and the K_m of **2** was independent of pH values. **2** had *para*-selectivity for the hydrolysis of nitrophenyl acetate isomers.

Key words. Cyclodextrin, artificial enzyme, dimethyl-β-cyclodextrin, chymotrypsin.

1. Introduction

Cyclodextrins (CDs) can form inclusion complexes with a number of molecules. For this reason, biomimetic reactions using cyclodextrins and their derivatives have been actively studied [1–2]. We have also prepared successful artificial enzymes by modifications of cyclodextrins [6–7]. On the other hand, dimethylcyclodextrins (DMCDs) are a series of cyclic oligomers consisting of α-1,4-linked 2,6-di-*O*-methyl-D-glucopyranose units, and have quite unique and different properties from cyclodextrins [2–3]. β-DMCD has a deeper hydrophobic cavity than β-CD. Stezowski and his co-workers estimated the height of the torus of β-DMCD at 10–11 Å [4]. The most different character between them is solubility [3]. The solubility of β-DMCD in water is 55 g/100 ml water at 25°C, whereas that of β-CD is only 1.8 g/100 ml water. As temperature rises, the solubility of β-DMCD decreases, whereas that of β-CD increases (Figure 1). β-DMCD is highly soluble in many organic solvents, whereas β-CD is insoluble in most organic solvents. For example, β-DMCD is soluble in alcohol, acetone, chloroform, benzene, acetonitrile, THF, DMF, DMSO, and so on. β-CD is slightly soluble only in acetonitrile, DMF, DMSO and so on. Furthermore, the behavior of inclusion complex formation is different between CD and DMCD [3].

For these reasons, if β-DMCD were used as an enzyme model, a new unique enzyme model might be obtained. In this paper, we wish to provide the first successful method for modification of β-DMCD and to investigate the enzymatic activities of a new artificial hydrolase using modified β-DMCD.

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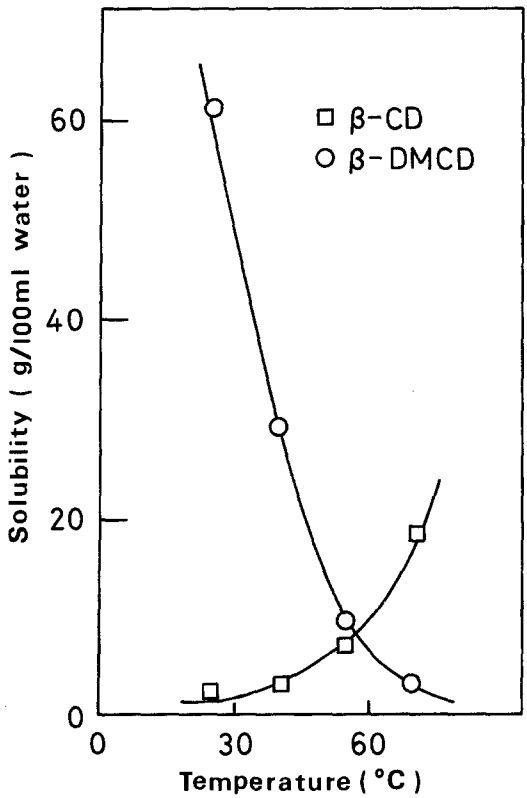


Fig. 1. Plots of the temperature dependence of solubilities of β-cyclodextrin and dimethyl-β-cyclodextrin.

2. Experimental

2.1. MATERIALS

Dimethyl-β-cyclodextrin was purchased from Toshin Chemical Co. Ltd., and recrystallized from water and a mixed solvent of chloroform and hexane several times. Its purity was checked by HPLC and NMR before use [5]. Nitrophenyl acetate isomers were prepared by a common method and purified by recrystallization from hexane.

3-[2-(4-imidazolyl)ethyl]dimethyl-β-cyclodextrin (**2**). A solution of dimethyl-β-cyclodextrin (5 g, 3.8 mmol) in dry THF (40 ml) was treated with NaH (631 mg, 13 mmol) at 0–40°C for 5 hours. To this reaction mixture was added a solution of 2-(4-imidazolyl)ethyl chloride (736 mg, 5.6 mmol) in dry THF (10 ml) at 0°C. The resulting mixture was stirred at room temperature for 10 h. The product was isolated by column chromatography on silica gel (Wakogel C-300) (elution with $CHCl_3$/MeOH = 35:1). (1.2 g, 20% yield)

Found: C, 48.58; H, 7.00; N, 1.75%, Calcd. for $C_{61}H_{104}O_{35}N_2 \cdot CHCl_3$: C, 48.20; H, 6.85; N, 1.80%. ^{1}H-NMR (500 MHz, C_6D_6) δ = 4.32 (1H, bs, C_3-H), 4.43 (6H,

bs, C_3-H), 4.99 (6H, bs, C_3-OH), 5.26 (1H, bs, $C_{1'}$-H), 5.41 (6H, bs, C_1-H), 6.59 (1H, s, $CHCl_3$), 7.08 (1H, s, imidazole), 7.69 (1H, s, imidazole).

2.2. KINETICS

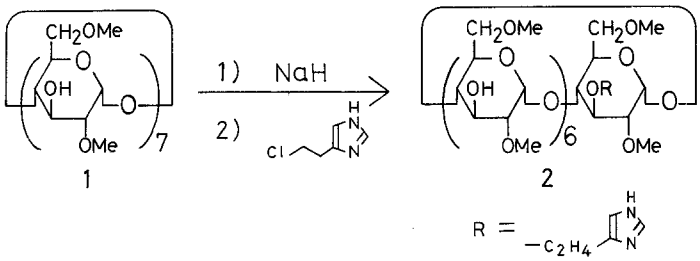
Hydrolysis reactions were followed by monitoring the appearance of nitrophenol spectrophotometrically using a HITACHI 220A spectrophotometer. The reaction was conducted in a quartz cell in the water-jacketed cell holder of the 220A. Temperature was maintained at 25°C by a HAAKE F3 circulating water bath. The reaction was initiated by adding a stock solution of ester in acetonitrile to a buffer solution in the quartz cell.

The pH of a reaction mixture did not change during the course of the reaction.

3. Results and Discussion

3.1. SYNTHESIS OF ARTIFICIAL HYDROLASE

β-DMCD (**1**) was treated with 3.5 equiv. of NaH at 0–40°C for 5 h and 1.5 equiv. of imidazolylethyl chloride at room temperature for 10 h in dry THF under argon (Scheme I). By chromatography on silica gel ($CHCl_3$/MeOH), the product (**2**) was isolated as a colorless solid in 20% yield. Elemental analysis data and estimation by peak area of the ^{1}H-NMR spectrum confirmed that **2** had only one imidazolylethyl group. The ^{1}H-NMR spectrum of **2** shows peaks at $\delta = 7.69$ and 7.08 ppm for the imidazolyl group, and small peaks at 5.26 and 4.32 for the up-field shift of $C_{1'}$-H and up-field shift of $C_{3'}$-H, respectively [8].



Scheme I.

3.2. HYDROLYSIS OF *p*-NITROPHENYL ACETATE BY 2

The initial rate of hydrolysis of *p*-nitrophenyl acetate (PNPA) was measured at 25°C in pH 7.2 phosphate buffer (1/15 M) in the presence of dimethyl-β-cyclodextrin bearing the imidazolylethyl group (**2**), dimethyl-β-cyclodextrin (β-DMCD) (**1**) or imidazole, and in the absence of them [8]. The conditions of large excess of PNPA were used and concentrations of PNPA were changed from 10-fold to 100-fold over those additives except **1**. The reaction was followed by monitoring the released *p*-nitrophenol at 400 nm.

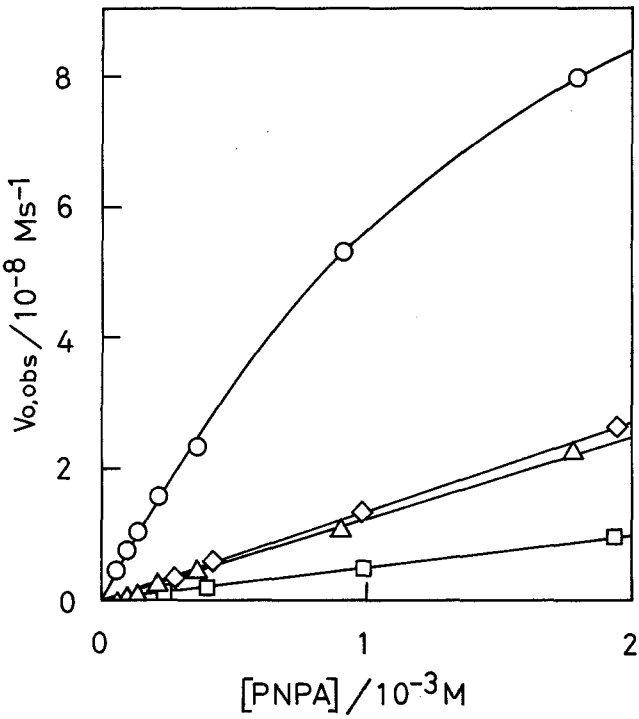


Fig. 2. Hydrolysis of *p*-nitrophenyl acetate in pH 7.2 phosphate buffer (1/15 M) at 25°C; ○ in the presence of **2** (1.10×10^{-5} M); ◇ in the presence of imidazole (1.10×10^{-5} M); □ in the presence of β-DMCD (9.67×10^{-3} M); and △ in the absence of them.

The result is shown in Figure 2. Only 1 mol% of **2** caused a 5-fold increase in the rate of hydrolysis of PNPA (10^{-3} M), compared with the condition of absence of **2**, whereas 5 equiv. of β-DMCD (**1**) caused 60% depression of the reaction. The reaction was scarcely accelerated by imidazole in the same concentration as **2**. Only the binding site (β-DMCD) or active site (imidazole) was not effective for the acceleration of the hydrolysis reaction. The combined action of the binding site and active site of **2** caused a large acceleration of the reaction.

By the plotting kinetic data for **2** in the form of $1/(V_{0,obs} - V_{un})$ vs. 1/[PNPA] (Lineweaver–Burk plot), a straight line was obtained (Figure 3). It suggests that this reaction by **2** proceeds by the Michaelis–Menten mechanism as do cyclodextrins and their derivatives [1, 2, 6, 9]. k_{cat} and K_m were calculated by a Michaelis–Menten treatment (Table I). k_{cat} is 1.44×10^{-2} s^{-1}, K_m is 2.6×10^{-3} M and k_{cat}/K_m is 5.54 M^{-1} s^{-1}. k_{cat} and k_{cat}/K_m of **2** are nine times larger than those of β-cyclodextrin bearing a histaminyl group at the C-6 position (**3**) [6]. K_m of **2** is nearly equal to that of **3** [6]. These indicate that the binding ability of **2** is nearly the same as that of **3**, whereas the ability to accelerate the reaction of **2** is much larger than that of **3**. The ability of rate acceleration of **2** can be estimated by the ratio k_{cat}/k_{un}. k_{cat}/k_{un} of **2** is over 1100.

Also, around the optimum pH of α-chymotrypsin or pH 8, the rate of the hydrolysis reaction was measured. k_{cat} of **2** is over twice that of α-chymotrypsin (Table I) [10].

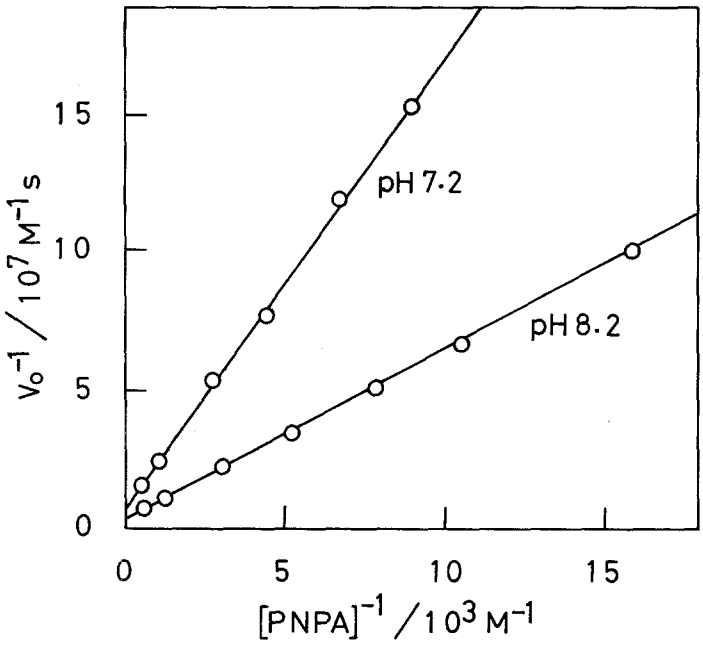


Fig. 3. Lineweaver–Burk plots for hydrolyses of *p*-nitrophenyl acetate by **2** (1.10×10^{-5} M) in pH 7.2 and by **2** (1.75×10^{-5} M) in pH 8.2 phosphate buffer (1/15 M) at 25°C.

Table I. Kinetic parameters for hydrolysis of *p*-nitrophenyl acetate.

	pH	k_{cat}	K_m	k_{cat}/K_m
		10^{-2} s^{-1}	10^{-3} M	M^{-1} s^{-1}
2	7.2	1.44	2.60	5.54
	8.2	2.67	2.90	9.21
3	7.2	0.165	2.63	0.627
α-chymotrypsin[a]	8.0	1.1	0.04	275

[a] Ref. 10.

3.3. pH DEPENDENCE OF ENZYMATIC ACTIVITY OF 2

The pH dependence of the enzymatic activity of **2** was examined. In phosphate buffers (1/15 M) of various pH values, the accelerations of the hydrolysis reaction of *p*-nitrophenyl acetate (PNPA) by **2** were measured at 25°C. Kinetic parameters were calculated by a Michaelis–Menten treatment. As **2** has only one ionizing group, i.e., the imidazolyl group, the pH dependence of k_{cat}, K_m and k_{cat}/K_m can be represented by Eqs. 1, 2, and 3, respectively [11].

$$k_{cat} = \frac{(k_{cat})_{max} K_{ES}}{K_{ES} + [H^+]} \quad (1)$$

$$K_m = \frac{K_S K_{ES} + [H^+] K_S K_{ES}/K_E}{K_{ES} + [H^+]} \tag{2}$$

$$k_{cat}/K_m = \frac{(k_{cat}/K_m)_{max} K_E}{K_E + [H^+]} \tag{3}$$

The pH dependence of k_{cat} of the hydrolysis reaction by **2** is shown in Figure 4a. pK_{ES} and $(k_{cat})_{max}$ of **2** were calculated by the nonlinear-least-squares fitting of

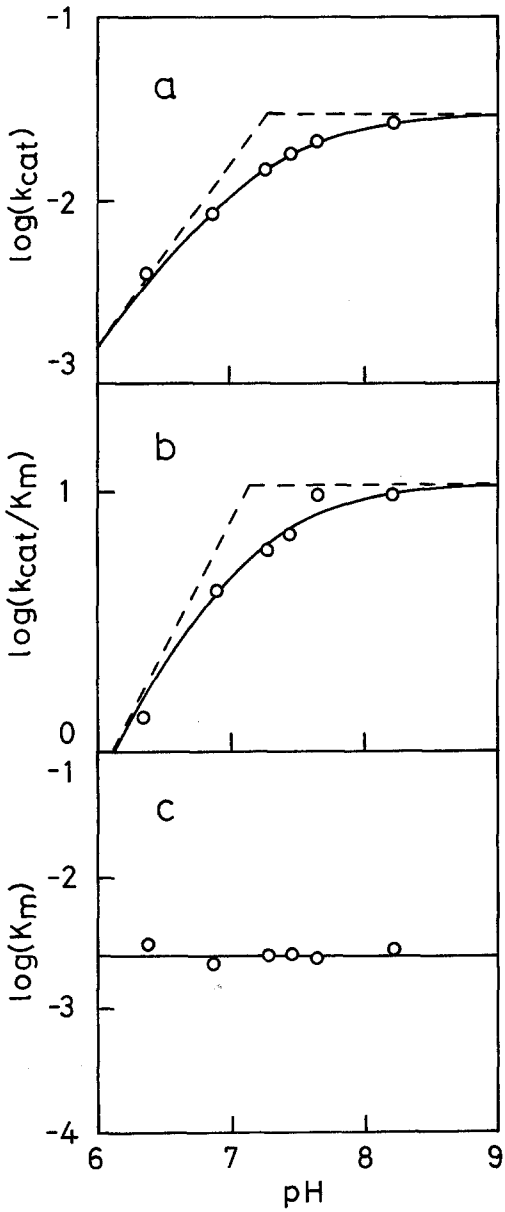


Fig. 4. Plots of the pH dependence of kinetic parameters for hydrolysis of *p*-nitrophenyl acetate by **2**.

kinetic data to Eq. 1. pK_{ES} is 7.28. It indicates that the imidazolyl group may play an important role in the rate determining step and that **2** can cause sufficient catalytic activity around a neutral pH condition. $(k_{cat})_{max}$ is $3 \times 10^{-2}\,s^{-1}$. It is larger than that of α-chymotrypsin. The pH dependence of k_{cat}/K_m is shown in Figure 4b. pK_E and $(k_{cat}/K_m)_{max}$ was calculated by the nonlinear-least-squares fitting of kinetic data to Eq. 3. pK_E is 7.12. $(k_{cat}/K_m)_{max}$ is $10.17\,M^{-1}\,s^{-1}$.

pK_{ES} is the ionization constant of **2** which is including a substrate. pK_E is the ionization constant of **2** which is not including a substrate. pK_{ES} of **2** is near to pK_E. It indicates that an included substrate might rarely influence the ionization equilibrium. If pK_{ES} is equal to pK_E, it is obvious from Eq. 2 that K_m is independent of pH value. The pH dependence of K_m of **2** is shown in Figure 4c. K_m is almost constant in this pH range and its average is 2.57×10^{-3} M. This is consistent with the relationship that pK_{ES} is nearly equal to pK_E.

3.4. SUBSTRATE SPECIFICITY OF HYDROLYSIS REACTION BY 2

The regioselectivity for the hydrolysis of nitrophenyl acetate isomers by **2** was demonstrated. The reactions were carried out under the conditions of excess of substrate in pH 8.2 phosphate buffer (1/15 M) at 25°C. Kinetic parameters are shown in Table II. Both k_{cat} and k_{cat}/K_m for PNPA are larger than for the others. k_{cat}/K_m for PNPA is 6 times larger than that for *o*-nitrophenyl acetate (ONPA). k_{cat}/k_{un} for PNPA is also largest (Table II). These are contrary to the case of unmodified cyclodextrins which have *meta*-selectivity [9]. This may be due to the difference of the geometry of the inclusion complex, especially the distance between reaction centers.

The K_m values indicate that **2** mostly tends to form an inclusion complex with *m*-nitrophenyl acetate (MNPA) and it is also different from unmodified cyclodextrins [9].

k_{cat} for ONPA is 1.1 times larger than that for MNPA and K_m for the former is 2.5 times larger than that for latter. Therefore k_{cat}/K_m for the latter is 2.3 times larger than that for the former. It shows that the ability to form an inclusion complex is important for accelerating the rate of the over-all reaction.

Table II. Kinetic parameters for hydrolysis of nitrophenyl acetate isomers at 25°C in pH 8.2 phosphate buffer by **2** (1.75×10^{-5} M)

Substrate	k_{cat}	K_m	k_{cat}/K_m	k_{un}	k_{cat}/k_{un}
	$10^{-3}\,s^{-1}$	10^{-3} M	$M^{-1}\,s^{-1}$	$10^{-5}\,s^{-1}$	—
ONPA	4.96	3.22	1.54	1.93	257
MNPA	4.48	1.27	3.54	1.40	320
PNPA	26.7	2.90	9.20	2.90	921

4. Conclusion

The first successful method for modification of β-DMCD was demonstrated as a synthesis of the new artificial hydrolase (**2**). This artificial hydrolase caused a

1100-fold increase in the rate of hydrolysis of *p*-nitrophenyl acetate (PNPA) at pH 7.2, whereas unmodified β-DMCD depressed the reaction. k_{cat} for the hydrolysis of PNPA of **2** is larger than that of the natural enzyme α-chymotrypsin. The kinetic pK_a of **2** was about 7.2 (that is, the average of pK_E and pK_{ES}) and K_m of **2** was independent of pH. **2** had *para*-selectivity for the hydrolysis of nitrophenyl acetate isomers.

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