SYNTHESIS AND REACTIVITY OF β-CYCLODEXTRIN-HISTAMINE AS ENZYME MODEL

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ABSTRACT. Cyclodextrin (CD) has a hydrophobic cavity which acts like a binding site of an actual enzyme. But enzymatic turnover reaction did not occur in CD-catalyzed reactions. β-CD was modified by a histamine group to attach a reactive functional group. β-CD-histamine accelerates the hydrolysis of p-nitrophenyl acetate. Catalytic rate constant of this reaction is close to an actual enzyme, α-chymotrypsin. Enzymatic turnover reaction is realized with this compound at around neutral pH value.

1. INTRODUCTION

CD has been extensively studied for hydrolysis of phenyl ester as a model of serine protease, because CD has a hydrophobic cavity which acts like a binding site of enzyme [1]. These studies show that CD-catalyzed hydrolysis proceeds quite similarly to enzyme hydrolysis. However, there are three differences between CD-catalyzed hydrolysis and chymotrypsin-catalyzed hydrolysis.

a) Maximum rate constant for CD-catalyzed reaction was determind at pH 13, while that for chymotrypsin is at pH 8.
b) Enzymatic turnover reaction did not occur in CD-catalyzed reaction, resulting in its inefficiency as a catalyst.
c) Lower activity compared with chymotrypsin.

For improvement of these faults, many modified CD derivatives were synthesized in which catalytic or reactive functional groups were present to attack the bound substrate [2]. To attach a reactive functional group, we modified α-CD by a histamine group and this compound catalyzed the hydrolysis of phenyl ester 15 times more than CD itself at pH 8.0 [3].

This paper reports that we modified β-CD by a histamine group and we realized enzymatic turnover reaction in the hydrolysis of p-nitrophenyl acetate with this compound at around neutral pH value for the first time.

2. MATERIALS AND METHODS

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Mono-tosylated-β-CD was prepared in an alkaline aqueous solution in the same manner as described in reference 4. This compound is identical in all respects with C-6 mono-tosylated-β-CD [5]. Iodination of mono-tosylated-β-CD (20 g) with sodium iodide (24 g) was carried out in methanol (300 ml) at 70 °C for 50 hours. After reprecipitation with acetone, β-CD-iodide was purified by a column of highly porous polystyrene gel (DIAION HP-20). The purity was confirmed by HPLC. β-CD-iodide (10 g) and free histamine (9.1 g) were added to DMF (400 ml), and the solution was kept at 100 °C for 24 hours. The reaction mixture was condensed and dissolved in water and applied to a column of HP-20. The column was eluted with water and 20% aqueous methanol. The methanolic eluate was evaporated to dryness and the dried material was dissolved in water and applied to a column of silica gel (WAKOGEL C-100). The column was eluted with water and 5% aqueous sodium carbonate. The sodium carbonate eluate was again applied to a column of HP-20. Water and 20% aqueous methanol were used to eluate. The methanolic eluate was evaporated to dryness to give the β-CD-histamine. The purity of the product was confirmed by HPLC (Waters, Radial-PAK Cartridge C-8, solvent; acetonitrile-water system). Yield; 8∿10%. Anal.Calc.for $C_{47}H_{77}O_{34}N_3 \cdot H_2O$: C,45.26; H,6.34; N,3.37. Found: C,45.13; H,6.20; N,3.26. All reagents were purchased from commercial suppliers and were used without further purification.

The reaction was followed by the appearance of p-nitrophenol spectrometrically at 400 nm using a HITACHI model 220A. The reaction medium was pH 6.8 phosphate buffer and kept at 25 °C.

3. RESULTS AND DISCUSSION

To confirm the turnover of a catalyst, substrate must be added in large excess to the catalyst. Variation of p-nitrophenol concentration with time in the hydrolysis of p-nitrophenyl acetate under this condition ([substrate] >> [catalyst]) is shown in Figure 1. It shows that after reaction for 5 hours β-CD hydrolyzed p-nitrophenyl acetate only 1/4 molar quantity of the catalyst, but β-CD-histamine acted more than 4 times. After the reaction for 24 hours, β-CD-histamine was separated from the substrate and products by HPLC, and this recovered catalyst reacted at the same rate as the original catalyst. This result reveals that the catalyst is not modified by p-nitrophenol or p-nitrophenyl acetate. Under this condition : [S] >> [E], the deacylation of acetyl-catalyst is the rate-determing step for hydrolysis of p-nitrophenyl acetate (scheme 1). In the case of β-CD, the rate constant of deacylation (k_3)

[Scheme 1]

$$E + S \underset{}{\overset{K_s}{\rightleftharpoons}} ES \xrightarrow{k_2} ES' \xrightarrow{k_3} E + P_2$$
$$\qquad\qquad\qquad\qquad\quad + P_1$$

E : β-CD or β-CD-histamine P_1 : p-Nitrophenol
S : p-Nitrophenyl acetate P_2 : Acetic acid

is very small, so, acetyl-β-CD is stable and turnover reaction has not

occured. However, in the case of β-CD-histamine, deacylation quickly occurs and the molar quantity of hydrolyzed p-nitrophenyl acetate is more than 4 times molar quantity of the catalyst.

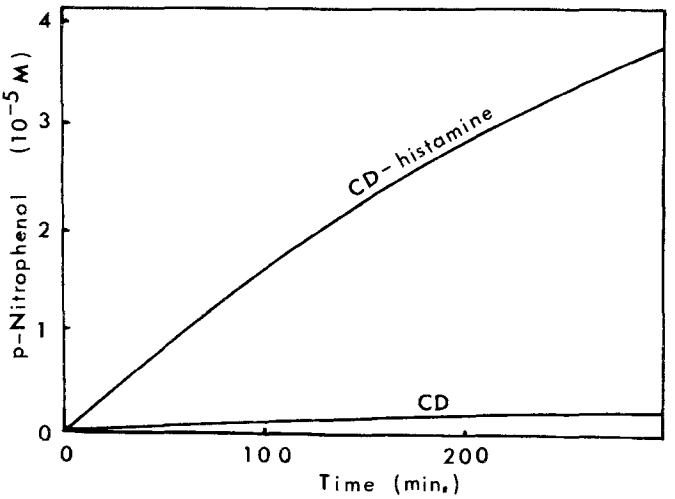


Figure 1. Variation of p-nitrophenol concentration with time in the hydrolysis of p-nitrophenyl acetate. [E] = 1.0 x 10^{-5} M, [S] = 1.0 x 10^{-3} M.

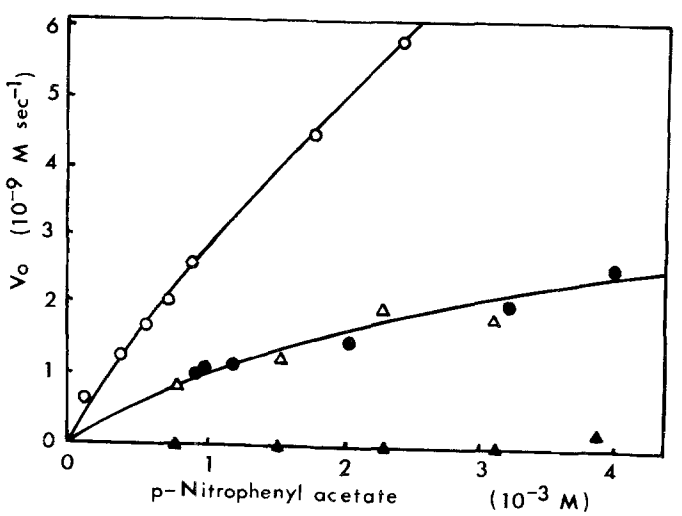


Figure 2. Variation of rates of hydrolysis of p-nitrophenyl acetate in the presence of various catalysts. β-CD-histamine (○), histamine (△), β-CD (▲), β-CD + histamine (1:1 mixture) (●). [E] = 2.0 x 10^{-5} M.

Figure 2 shows variation of rates of hydrolysis of p-nitrophenyl acetate in the presence of various catalysts. It shows that β-CD does not accelerate p-nitrophenyl acetate hydrolysis, but β-CD-histamine enhances the rate significantly. A mixture of β-CD and histamine accelerrates the hydrolysis, but it is nearly equal to the case of histamine only, and lower than the case of β-CD-histamine. This result reveals that the ability of β-CD-histamine in hydrolysis is caused jointly by a histamine group and a hydrophobic cavity of β-CD.

Figure 3 shows a Lineweaver-Burk plot of this result. The data give a straight line, so this reaction is a type of the Michaelis- Menten mechanism. Catalytic rate constants in the hydrolysis of p-nitrophenyl acetate are shown in Table 1. Compared with α-chymotrypsin, the value of $k_2/K_s(k_{cat}/k_m)$ is about 1/4, close to an actual enzyme.

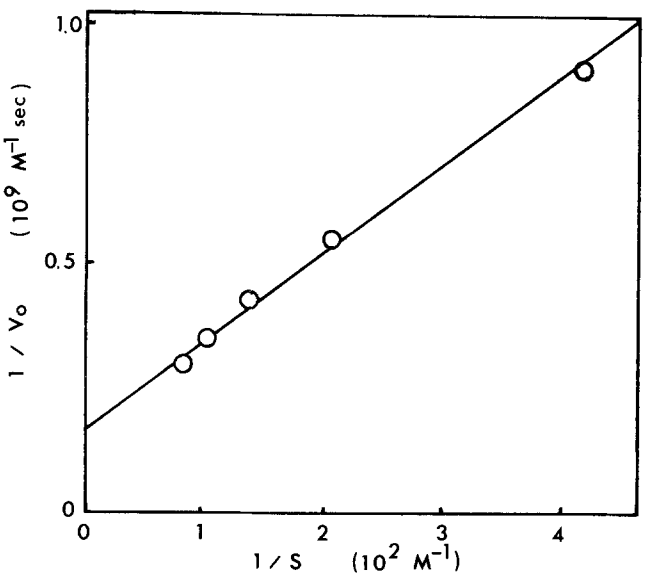


Figure 3. Lineweaver-Burk plot of hydrolysis of p-nitrophenyl acetate with β-CD-histamine.

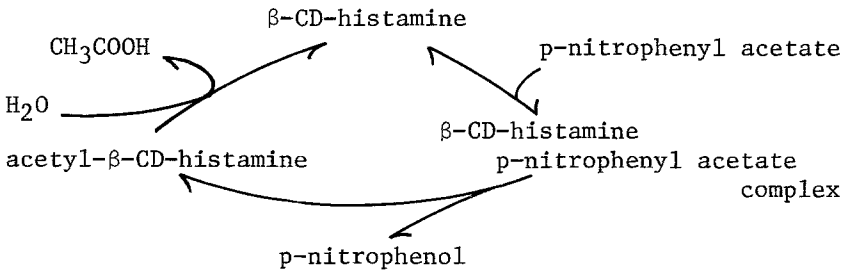
Table 1. Catalytic rate constants in the hydrolysis of p-nitrophenyl acetate.

Catalyst	$k_{cat}(10^{-3}$ sec$^{-1})$	$K_m(10^{-3}$ M)	$k_{cat}/K_m(M^{-1}$ sec)
β-CD-histamine	0.82	4.4	0.19
β-CD	≃ 0	-	-
α-chymotrypsin	6.5	7.7	0.85

The reaction of hydrolysis of p-nitrophenyl acetate with β-CD-histamine is a cyclic mechanism as follows. β-CD-histamine binds p-

nitrophenyl acetate, hydrolyzes it, is acylated and then is regenerated by H_2O (Scheme 2). Enzymatic turnover reaction was realized.

[Scheme 2]



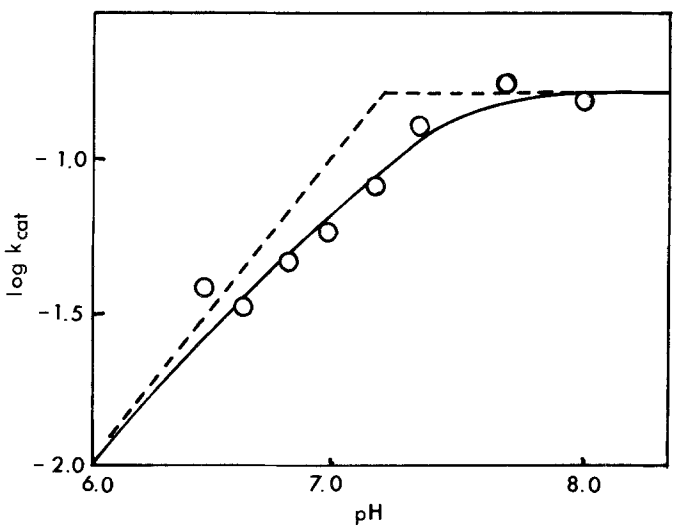


Figure 4. pH dependence of k_{cat} in the hydrolysis of p-nitrophenyl acetate with β-CD-histamine.

Figure 4 shows the pH dependence of k_{cat} in the hydrolysis of p-nitrophenyl acetate with β-CD-histamine. The maximum rate constant is determined at around neutral pH value.

These results suggest that β-CD-histamine is an excellent artificial enzyme and acts similarly to an actual enzyme in the points of pH, activity and turnover.

4. ACKNOWLEDGMENT

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