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Cyclodextrin as a Biomimetic Model Enzyme- the Catalysis of Aspirin Hydrolysis Included by Cyclodextrins

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생체효소 유사물질로서의 시클로덱스트린의 작용- 시클로덱스트린으로 포접된 아스피린의 가수분해 촉매작용

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The molecular nature of aspirin hydrolysis was studied using cyclodextrin as a biomimetic model for esterase. Cyclodextrin was selected for this purpose because it meets the necessary requirements for the hydrolysis study. Dissociation constants and catalytic rates were obtained under alkaline conditions by the kinetic method.

Keywords—Cyclodextrin, aspirin, enzyme

The mechanism of enzyme-substrate complexation[1,2] is very complicated. Therefore a well defined and simplified model, the cyclodextrin inclusion complex system, has been chosen as an enzyme substrate model to study the activity of the esterase enzymes. The hydrophobic cavity acts as the hydrophobic binding site of the esterase and the hydroxyl groups located on the broad end of the cyclodextrin act as the hydrogen donors or acceptors. The most important aspect is that the hydroxyl group can also mimic the catalytic function of the serine, and act as proton acceptors/donors of histidine, and the carboxyl residue on the active sites of the esterase. Therefore, cyclodextrin is proposed as a biomimetic model for elaborating the molecular mechanism of esterase catalysis in the stereospecific hydrolysis of aspirin. A proposed mechanism for the esterase hydrolysis of aspirn is shown in Fig. 1.

Substrate specificity in the cyclodextrin-catalyzed cleavage of aspirin in alkaline solution was studied using a spectrophotometric method by Machida *et al.* in 1976.[3] The cleavage of aspirin by α- and β-cyclodextrin in basic aqueous solution was also studied by Tee *et al.* in 1985.[4] Dissociation constants(K_D) and catalytic rate constants(k_c) were obtained from saturation kinetics.[3,4] Since the K_D value reported in literatures[3,4] are not consistent, a more accurate determination of this constant is needed. Neither of these groups has established detailed molecular mechanisms for the catalytic cleavage of aspirin. For these purposes,

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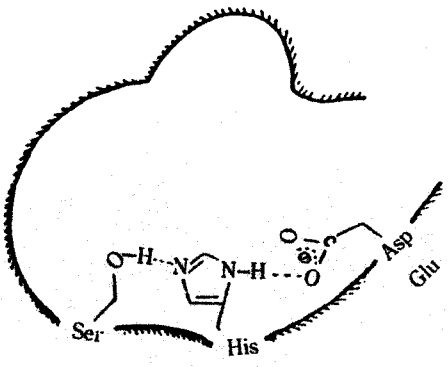
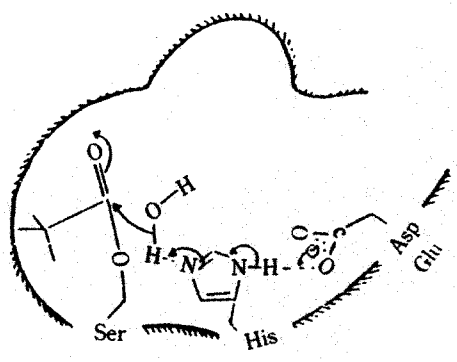
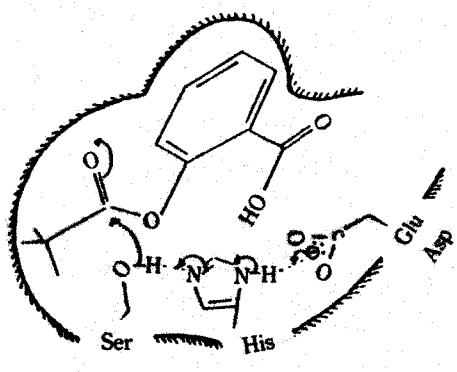


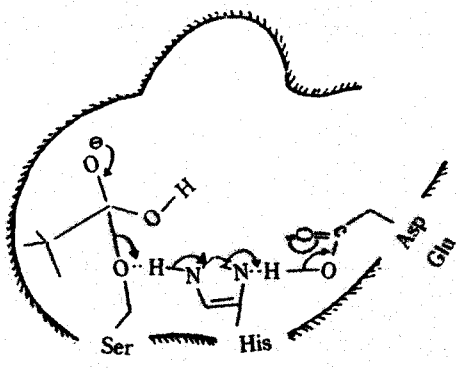
enzyme active-site



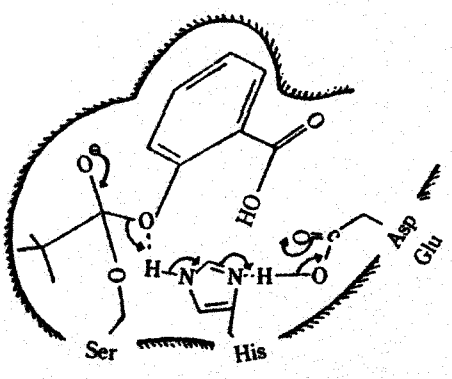
III. Acyl enzyme



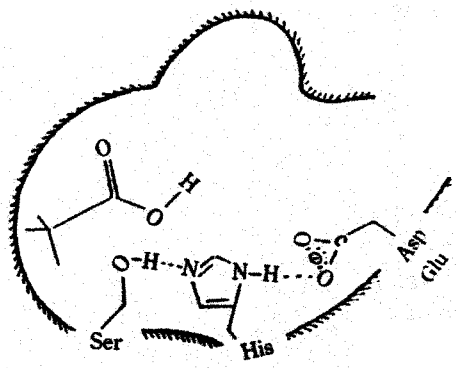
I. enzyme-substrate complex



IV. Tetrahedral intermediate



II. Tetrahedral intermediate



V. Enzyme-product

Figure 1—A proposed mechanism for the esterase hydrolysis of aspirin.

two different pH (11, 12.25) conditions were adopted. Also these kinetic parameters are correlated with structural specificity and hydrolysis mechanism.

The mechanistic approach to the real enzyme structure at the atomic level is important to understanding the enzyme catalytic mechanism. The results of the basic mechanistic study will be used

to design a better drug-carrier system for aspirin and its derivatives.

Materials and Methods

Materials

α-Cyclodextrin was obtained from Anspec in U. S.A. and β-Cyclodextrin was obtained from Chemical Dynamic Corp. in U.S.A. Aspirin was obtained from Sigma Chemical Co. in U.S.A.

UV/VIS Spectroscopy

UV/VIS measurements were made on a Beckman DU/7HS spectrophotometer controlled by a built-in high speed microprocessor.

NMR Spectroscopy

Intermediate studies of complexes by ^1H NMR were recorded on Varian XL-200 spectrometer with 16K computer memory. DMSO-d_6 was used as external reference.

Mass Spectrometry

Fast atom bombardment (FAB) mass spectra were obtained with a Kratos MS-50 sector mass spectrometer utilizing 3;1 dithiothreitol/dithioerythritol as the matrix. An accelerating voltage of 8 kV and a 100 sec/dec scanning rate were used for the experiments.

Determination of the Catalytic Rate Constants and Dissociation Constants of the Complexation Reaction Under Alkaline Condition

The effect of increasing α- or β- cyclodextrin in catalyzing the aspirin hydrolysis was monitored by the UV/VIS spectrophotometry time domain kinetic mode. Fixed concentration of aspirin was 0.25 mM and varying molar concentration of cyclodextrin(s) (α, β) ranged from 0 mM to 10 mM in pH 11, 0.2 M phosphate buffer in 2 mM increments. Kinetics were measured at 25℃ by monitoring salicylate ion appearance every 10 seconds at 302 nm until almost all aspirin were hydrolyzed. Substrate solutions were prepared by diluting an aliquot of a stock 2.5 mM aspirin solution into a cyclodextrin at 0 kinetic time.

Half-lives ($t_{1/2}$) were calculated by plotting the percentage of remaining aspirin versus time. Dissociation constants (K_D) and catalytic rate constants (k_c) were calculated from a linear regression of (k_{obs}-k_u) versus (k_{obs}-k_u)/[CDX], an Eadie-Hofstee approach.[4]

Table I—*The summary of dissociation constants (K_D) in mM of α-cyclodextrin-aspirin and β-cyclodextrin aspirin complexes in alkaline solution*

pH	α-cyclodextrin	β-cyclodextrin	Glucose[a]
11	6.4	11.3	
12.3	10.2	20.8	21.3

a: Molar ratio (glucose/aspirin)=6

Identification of the Aspirin-β-Cyclodextrin Intermediate

Equal moles of β-cyclodextrin (0.5 mmole) and aspirin were dissolved in 150 m*l* of 0.2 M, phosphate buffer pH 11.0 at room temperature. The hydrolysis was monitored by ^1H NMR. The hydrolysis was quenched by neutralizing the solution to pH 7.0 with 4 N HCl solution when the intermediate component was at the maximum concentration with minimum amount of degradation products produced. This solution was extracted with 3-15 m*l* ether then lyophilized to dryness under high vaccum. Finally, the acyl-β-cyclodextrin intermediate was obtained. Structural elucidation of this intermediate was carried by FAB mass spectrometry.

Results and Discussion

Catalytic Effect of Cyclodextrin on the Hydrolysis of Aspirin

The hydrolyses of aspirin with and without equimolar α- and β-cyclodextrin under alkaline condition were followed by UV spectroscopy. From saturation kinetics, the dissociation constants and catalytic rate constants were obtained.

Six moles of glucose was used as the basis of comparison for α-cyclodextrin. These data are summarized in Table I and II. The anion of aspirin bound better to α-cyclodextrin (K_D=6.4 mM) than β-cyclodextrin(K_D=11.18 mM). Also larger catalytic effect was observed in α-cyclodextrin (k_c=0.47 m^{-1}) than β-cyclodextrin (k_c=0.28 m^{-1}) pH 11. At pH 12.3, the rate of aspirin cleavage by α-cyclodextrin (k_c=4.81 m^{-1}) was faster than by β-cycloextrin (k_c=2.41 m^{-1}). The lowest rate constant

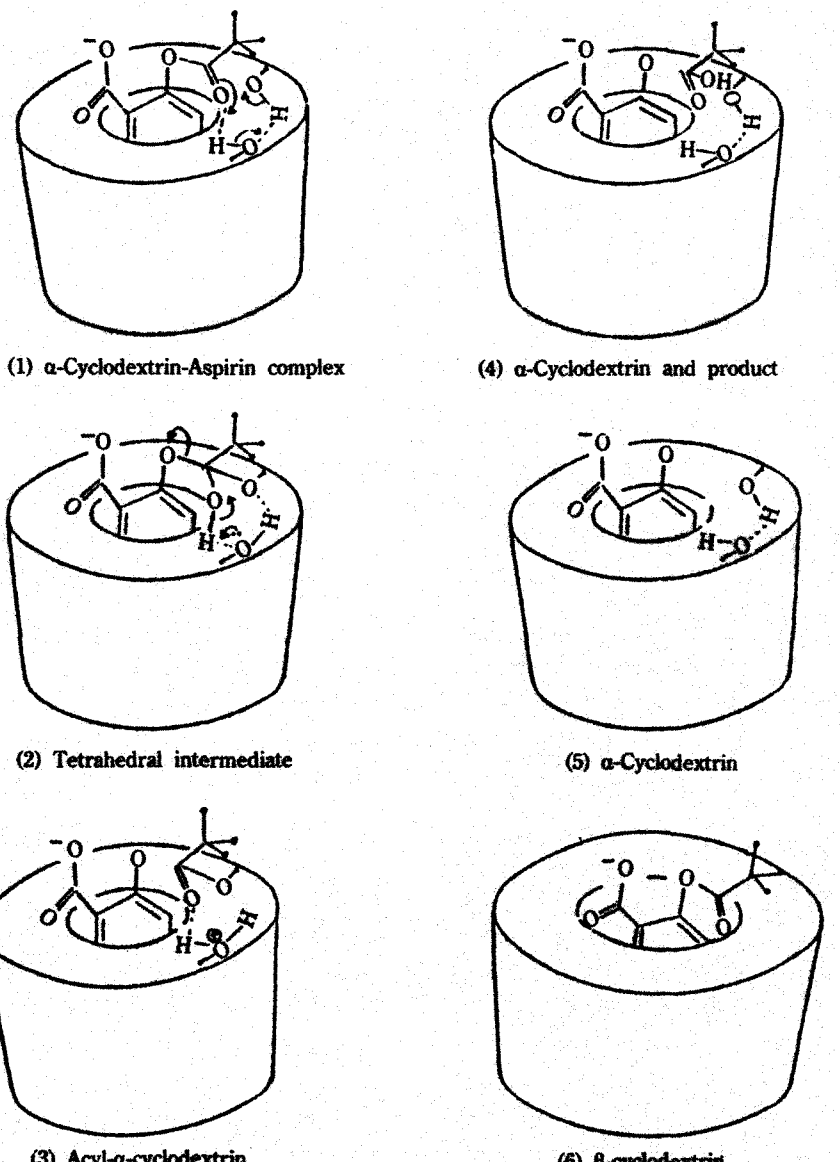
Table II—*The summary of α-cyclodextrin and β-cyclodextrin hydrolytic rate constants (k_c) in alkaline solution*

	pH	$k_{un}(min^{-1})$	$k_c(min^{-1})$	k_c/k_{un}
α-CDX	11	0.04	0.47	11.75
β-CDX	11	0.04	0.28	7.00
α-CDX	12.3	0.33	4.81	14.58
β-CDX	12.3	0.33	2.41	7.30
Glucose[a]	12.3	0.33	0.88	2.67

a: Molar ratio (glucose/aspirin)=6

was obtained with glucose (k_c=0.88 m^{-1}). Substrate specificity in the cyclodextrin-catalyzed cleavage of aspirin at pH 12 was studied by a spectrophotometric method by Mochida *et al.* in 1976.[3] The cleavage of aspirin by cyclodextrin at pH 12.3 was also reported by Tee *et al.* in 1985.[4] Mochida reported that β-cyclodextrin-aspirin complex (K_D =12 mM) is more stable than α-cyclodextrin-aspirin complex (K_D=23 mM).

On the other hand, Tee's results suggested the



(1) α-Cyclodextrin-Aspirin complex

(2) Tetrahedral intermediate

(3) Acyl-α-cyclodextrin

(4) α-Cyclodextrin and product

(5) α-Cyclodextrin

(6) β-cyclodextrin

Figure 2—Proposed mechanism for α-cyclodextrin-catalyzed hydrolysis of aspirin.

aspirin with α-cyclodextrin complex (K_D=12 mM) was more stable than that of the β-cyclodextrin complex (K_D=20 mM). Tee's results are similar to our data at pH 11 and pH 12.3. All three groups showed that the catalytic rate of the α-cyclodextrin-aspirin complex was faster than that of the β-cyclodextrin-aspirin complex and all three works showed same conclusion for the catalytic rate constants.

Mechanism for β-Cyclodextrin-Catalyzed Hydrolysis of Aspirin

On the basis of the ^{1}H NMR spectral data in solution, and the catalytic rate and dissociation constants, a plausible mechanism may be proposed (Fig. 2). The initial molecular recognition lies in the specific inclusion interaction between the phenyl group of aspirin and the hydrophobic cavity of the α-cyclodextrin. According to the catalytic rate results, and proposed structure by space filling model, α-cyclodextrin appeared to be a better enzyme model for aspirin catalysis than β-cyclodextrin. The molecular complexation with α-cyclodextrin provides an appropriate intermolecular hydrogen-bonding between acetoxyl carbonyl oxygen and the secondary hydroxyl groups of α-cyclodextrin. First secondary hydroxyl group acts as a hydrogen donor/acceptor and the second one acts as nucleophile to attack the carbonyl carbon of the acetoxyl group through a six-membered ring transition state which formed a tetrahedral intermediate. This labile tetrahedral intermediate is rapidly degraded by the cleavage of the ester bond of aspirin through a series of intramolecular electron transfers yeilding an acetyl-cyclodextrin intermediate. This acetyl intermediate is further hydrolyzed into acetic acid and regenerates α-cyclodextrin.

Identification of an Acyl-β-Cyclodextrin Intermediate

In order to prove the existence of the acetyl cyclodextrin, the crude intermediate was isolated from reaction mixture. As we discussed earlier, the α-cyclodextrin-aspirin inclusion complex did not form in solid state therefore the β-cyclodextrin-aspirin complex was used to isolate the intermediate. This intermediate was analyzed by the

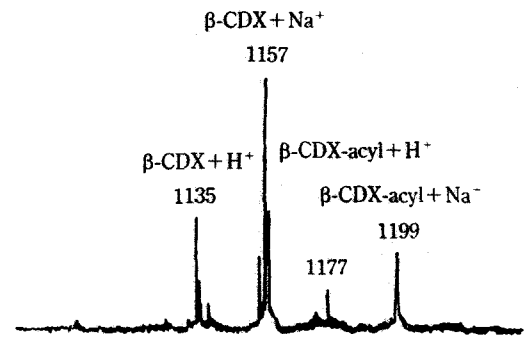


Figure 3—FAB mass spectrum of β-cyclodextrin acyl intermediate.

FAB mass spectrometry (Fig. 3). The m/z of 1177 and 1199 could be assigned to the pseudomolecular ion of the acyl-β-cyclodextrin intermediate adduct ($M^+ + H^+$) and its sodium adduct, respectively. Cleavage of the ester linkage will regenerate the β-cyclodextrin with a proton and a sodium ion adduct which gives the m/z of 1135 and 1157, respectively.

The 500 MHz ^{1}H NMR spectrum acetyl cyclodextrin showed the acetyl group at 2.2 ppm and as a degradation product acetate was assigned at 1.5 ppm. Our results were similar to the Bender's [5] results of 2.3 ppm. From the kinetic experiment [5] of deacylation of cyclohexaamylose acetate they found the methyl group of the acetyl moiety of cyclodextrin was assigned at 2.3 ppm. These spectral results provided strong evidence for the existence of the acetyl cyclodextrin intermediate.

In conclusion, this research shows that the dissociation constant of the β-cyclodextrin-aspirin complex is larger than that of the α-cyclodextrin-aspirin complex. This finding means that the α-cyclodextrin-aspirin complex is more stable than β-cyclodextrin-aspirin complex in solution.

The catalytic rate of the α-cyclodextrin-aspirin complex is faster than the rate of β-cyclodextrin-aspirin complex in alkaline solution.

The mechanism for the cyclodextrin-catalyzed aspirin hydrolysis involves an acyl cyclodextrin intermediate; reminiscent of the aspirin-esterase intermediate.

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