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Koga, Daizo
Laboratory of Biochemistry, Faculty of Agriculture, Kyushu University

Aso, Yoichi
Laboratory of Biochemistry, Faculty of Agriculture, Kyushu University

Hayashi, Katsuya
Laboratory of Sericultural Chemistry, Faculty of Agriculture, Kyushu University

Funatsu, Masaru
Laboratory of Biochemistry, Faculty of Agriculture, Kyushu University

https://doi.org/10.5109/22843

出版情報：九州大学大学院農学研究院紀要. 18 (1), pp.11-20, 1973-12. Kyushu University

J. Fac. Agr., Kyushu Univ., 18, 11-20 (1973)

On the Mechanism of Lysozyme Catalysis
II. Hydrolysis of Sugar Oxazoline

Daizo Koga, Yoichi Aso, Katsuya Hayashi* and Masaru Funatsu

Laboratory of Biochemistry and Laboratory of Sericultural Chemistry",
Faculty of Agriculture, Kyushu University, Fukuoka

(Received September 17, 1973)

The hydrolysis of 2-phenyl-4,5-(D-glucopyrano)-Δ^{2}- oxazoline was studied at various pHs. The pH-log k_{obs} profile showed that the apparent pK value of the oxazoline was 3. It was found that the hydrolyzed products of oxazoline in acid and neutral region were the amide. In addition to lyate species, catalytic activities of imidazole, acetic acid and Tris(hydroxymethyl) aminomethane for hydrolysis of the sugar oxazoline were examined. However, no effects were observed with these compounds.
The mechanism of lysozyme catalysis was discussed on the basis of the expermiental results.

INTRODUTION

Lysozyme is the first enzyme to have its three-dimensional structure determined by X-ray crystallographic method. It functions catalytically for the cleavage of β-1,4-glycoside linkage and following hydrolysis or transglycosylation. It was well known that carboxyl group of Glu 35 acts as a general acid to donate hydrogen ion to the oxygen atom of glycoside linkage, resulting in the cleavage of the bond between the C, of atom of sugar residue and the glycoside oxygen atom followed by the formation of the C, carbonium ion as an intermediate (Blake et al., 1967; Raftery and Rand-Meir, 1968). This intermediate must be stabilized for a moment for occurring the further reaction, the attack of hydroxide ion (hydrolysis) or the attack of other nucleophiles (transglycosylation). The stabilization of the intermediate may be attained in four ways: (1) The intermediate carbonium ion changes its structure to more stable one. (2) The intermediate cation reacts with some anions to form covalent bond. (3) The positive charge of the carbonium ion interacts electrostatically with some negative charges. (4) The intermediate is stabilized by hydrophobic or nonaqueous environment.

Many discussions, speculations or proposals have been done on the real intermediate structure, but clear-cut conclusion seems not to be obtained. Piszkiewicz and Bruice (1967, 1968a 1968b) studied the hydrolysis of some model compounds and suggested that the 2-acetamido group plays an important role as an intramolecular nucleophile in the cleavage of β-1,4-glucosaminide linkage, forming the sugar oxazoline as a second intermediate. They assumed that the

hydrolysis of the sugar oxazoline would occur by spontaneous reaction, though there were no clear evidences for the rapid spontaneous hydrolysis of the sugar oxazoline.

In general, it has been well known that oxazolines are very labile for the hydrolysis and the mechanism of their hydrolytic reaction shows a duality according to the experimental conditions; for instance, oxazolines hydrolyze to the corresponding esters in acid medium and to the amide in alkaline medium (Bruice and Benkovic, 1966).

In a lysozyme catalyzed reaction, there is only the hydrolyzed or transglycosylated product with β-anomeric structure and no ester product was found. Therefore, the hydrolysis of sugar oxazoline in a lysozyme catalyzed reaction, if it was formed as an intermediate in the reaction process, must be restricted and specific one. The purpose of the present experiment is to examine the possibility of the sugar oxazoline as the intermediate in a lysozyme catalyzed reaction, by studying the hydrolysis of model compound, an analog of the assumed sugar oxazoline intermediate.

EXPERIMENTAL

Materials

2-Phenyl-4,5-(D-glucopyrano)-Δ^2-oxazoline was prepared according to the method of Pravdic *et al.* (1967), and the method of Micheel and Koechling (1960).

2-Phenyl-4,5-(3',4',6'-triacetyl-D-glucopyrano)-Δ^2-oxazoline: The solution of N-benzoyl-D-glucosamine (2-benzamido-2-deoxy-D-glucopyranose) (10.5 g) dissolved in 40 ml of acetic anhydride containing 12.5 g of freshly fused zinc chloride was kept at 85-90°C for 25 min under stirring. After cooling the solution, it was diluted by 100 ml of dichloromethane, washed with cold water and saturated sodium bicarbonate solution until acetic acid disappeared. The solvent was removed under reduced pressure after the solution was dried on sodium sulfate overnight. Ether was added to the resulted syrup and the solution was filtered to remove a small amount of insoluble materials. To the ether solution was added petroleum ether until no more turbity appeared. Then, a small amount of colored precipitate was removed by filtration. Needle crystals were appeared after standing the solution for 24 hr in a cold room. mp: 56°C.

2-Phenyl-4,5-(D-glucopyrano)-Δ^2-oxazoline (sugar oxazoline): One gram of 2-phenyl-4,5-(3',4',6'-triacetyl-D-glucopyrano)-Δ^2-oxazoline was dissolved in absolute methanol containing 0.1 % sodium and it was kept at room temperature for 12 hr. After the yellow solution was evaporated under reduced pressure, hot acetone was added and decolorized by active carbon. To the solution were added ether and petroleum ether, and the solution was kept in ice box. The crystalline product was filtered and dried in *vacuo*. mp: 140°C, λ_{max}=239 nm.

Methods

Paper-chromatography was carried out at room temperature using Toyo filter paper No. 51. The developing solvent systems were butanol: acetic acid: water: pyridine (15: 3: 12: 10), butanol: ethanol: water (4: 1 : 5) and bu-

tanol : acetic acid : water (3 : 1: 1).

All kinetic experiments were followed difference spectrophotometrically using a Cary 14 spectrophotmeter. The rate constants for the hydrolysis of the sugar oxazoline (final concentration : 3.77×10^{-5} M) were measured by reading the optical density of the peak in difference spectrum A OD recorded in the range from 200 to 300 nm, shown in Fig. 1. In sample cell of the spectrophotometer, an aqueous solution of the sugar oxazoline stored in ice bath was filled, and in reference cell, the sugar oxazoline solution at desired pH value was filled in order to read blue-shift difference spectrum as positive value. The temperature of the solution in the cell of reference compartment was thermostatted by circulating the water from a Haake type constant-temperature bath at 5" or 50°C. The aqueous solution of the sugar oxazoline in the cell of sample compartment was held at low temperature as possible as. The apparent pseudo-first-order rate constant k_{obs} of the hydrolysis was obtained as a slope in the plotting of log $(A\ OD_{\infty}/\Delta\ OD,)$ vs reaction time t in second, where A OD, is the A OD value of the completely hydrolyzed reaction mixture and A OD, the value at reaction time t. A OD, was estimated by extrapolating A OD, value to infinite time.

RESULTS

Identification of hydrolyzed products

The sugar oxazoline was hydrolyzed by 1 N hydrochloric acid and 1 N sodium hydroxide at 50°C for 1 hr. The hydrolyzate was evaporated to dryness and the products were chromatographed. The products in acid hydrolyzate were identified as 2-amino-2-deoxy-D-glucopyranose, 2-benzamido-2-deoxy-D-glucopyranose and a trace of unknown compound. In alkaline hydrolyzate, 2-amino-2-deoxy-D-glucopyranose and unknown compound which seems to be a condensed product of 2-amino-2-deoxy-D-glucopyranose were found. Only 2-benzamido-2-deoxy-D-glucopyranose was, however, found in the hydrolyzates obtained in the region from pH 3 to pH *10.*

Hydrolytic rate constant

The hydrolysis of the sugar oxazoline were carried out at 50°C (above pH 6) and at 5°C (below pH 6). During the hydrolytic reaction, the position of the peak of absorption spectrum of the reaction mixture (239 nm) shifted to lower wavelength, attained finally to 226 nm. Correspondingly, the difference spectrum was appeared with increase in height of a main peak at 243 nm as shown in Fig. 1.

Examples of pseudo-first-order rate constant calculated are listed in Table 1. The pH$-\log k_{obs}$ profile is shown in Fig. 2. The values above pH 6 at 5°C were obtained from corresponding values at 50°C by parallel shift. The curve may be divided to three parts ; below pH 2, there is no pH-dependence of the hydrolytic rate constant; around pH 4.5, curve shows the slope of -1; above pH 7, nearly the same rate constants are observed.

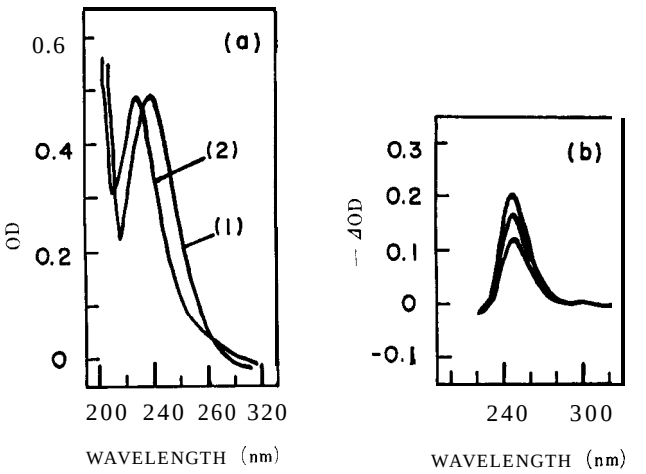


Fig. 1. (a) Absorption spectra of sugar oxazoline and its hydrolyzed product. (b) Difference spectra of reaction mixture referred to hydrolyzed product. (a)-(l) : Sugar oxazoline recorded immediately after dissolved in medium. (a)-(2) : Hydrolyzed product of sugar oxazoline. Concentration of solution was 3.77×10^{-5} M.

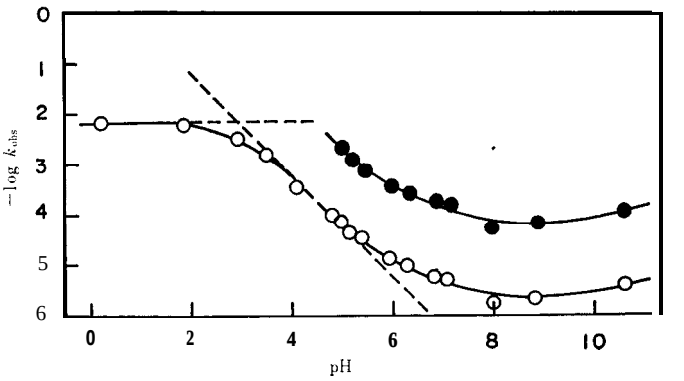


Fig. 2. Plotting of log k_{obs} vs pH in hydrolysis of sugar oxazoline. ● : Measured at 50°C, ○ : Measured at 5°C. The values above pH 6 at 5°C were obtained by parallel shift of values at 50°C.

Effect of additive

The k_{obs} measured in the presence of some additives are shown in Table 2. Neither of them was found to be catalytically effective for the hydrolysis of the sugar oxazoline.

DISCUSSION

The hydrolysis of 2-methyl-Δ^2-oxazoline was studied by Martin and Parcell (1961). Their results indicated that 2-methyl-Δ^2-oxazoline hydrolyzed rapidly

Table 1. Rate constants for hydrolysis of the sugar oxazoline.

Buffer	Temp. (°C)	pH	k_{obs} (sec^{-1})
HCl	5	0.20	7.46×10^{-3}
HCl	5	1.80	5.84×10^{-3}
HCl	5	2.90	3.52×10^{-3}
0.1 M acetate	5	3.22	2.60×10^{-3}
0.1 M acetate	5	4.03	3.54×10^{-4}
0.1 M acetate	5	4.95	8.01×10^{-5}
0.067 M phosphate	50	5.00	2.77×10^{-3}
0.067 M phosphate	50	5.20	1.31×10^{-3}
0.067 M phosphate	50	6.32	2.95×10^{-4}
0.067 M phosphate	50	6.85	2.08×10^{-4}
0.067 M phosphate	50	8.00	7.92×10^{-5}
0.067 M phosphate	50	8.90	9.89×10^{-5}
0.2 M carbonate	50	30.65	1.60×10^{-4}

Table 2. Effects of various additives on hydrolysis of sugar oxazoline.

Additive, Conc. (M)		Temp. (°C)	pH	k_{obs} (sec^{-1})
acetate	0.01	5	3.5	1.29×10^{-3}
acetate	0.02	5	3.5	1.62×10^{-3}
acetate	0.05	5	3.5	1.54×10^{-3}
imidazole	0.043	50	5.3	0.99×10^{-3}
imidazole	0.215	50	5.3	1.00×10^{-3}
imidazole	0.430	50	5.3	1.02×10^{-3}
imidazole	0.125	50	5.9	3.52×10^{-3}
imidazole	0.375	50	5.9	3.60×10^{-3}
imidazole	0.500	50	5.9	3.40×10^{-3}
Tris	0.038	50	7.0	1.78×10^{-3}
Tris	0.380	50	7.0	1.02×10^{-3}

in acid region through an orthooxazoline and an amide intermediates to corresponding ester product. de Jersey et *al.* (1969a, 1969b and 1969c) reported that the hydrolysis of p-nitrophenyl hippurate in alkaline region above pH 7 occurred very rapidly and 2-phenyl-Δ^2-oxazoline-5-one may be formed as an intermediate in the formation of amide product. This result indicated that the C_5 carbonyl carbon of 2-phenyl-Δ^2-oxazoline-5-on emay be subjected to the attack of hydroxide ion vertically to sp^2 orbital. Thus, it has been believed that oxazolines in general gave the ester product in acid hydrolysis and the amide product in alkaline hydrolysis.

Contrary to above, it was confirmed that the hydrolysis of the sugar oxazoline produced only the amide product independently of the reaction medium below pH 10. This may be caused from the carbonyl nature of C_5 carbon in the sugar oxazoline ring.

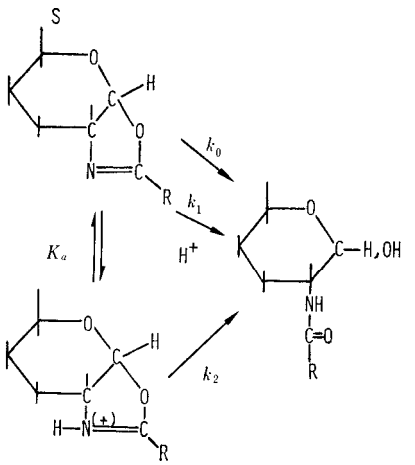
The hydrolytic mechanism of the sugar oxazoline in acid medium may be represented by Scheme I.

The hydrolytic rate of the sugar oxazoline may be represented by equation :

$$v = \left\{k_0 + k_1(H^+)\right\}(S) + k_2(SH) \qquad (1)$$

where v is the reaction rate, k is the rate constant, k_0 the rate constant for

Scheme I



spontaneous hydrolysis, S and SH the neutral and protonated sugar oxazoline, respectively. K_a is the dissociation contsant of the system of $S+H^+=SH$. Then the following equations are obtained:

$$\left.\begin{aligned} (S) &= \frac{K_a}{K_a+(H^+)}(S)_0, \quad (SH) = \frac{(H^+)}{K_a+(H^+)}(S)_0, \\ K_a &= \frac{(S)(H^+)}{(SH)} \qquad (S)+(SH)=(S), \end{aligned}\right\} \tag{2}$$

Substituting the equation (2) to (1) the reaction rate v is written as follows :

$$v=\frac{k_0K_a+(k_1K_a+k_2)(H^+)}{K_a+(H^+)}(S)_0 \tag{3}$$

and k_{obs} is written as

$$k_{obs}=\frac{k_0K_a+(k_1K_a+k_2)(H^+)}{K_a+(H^+)} \tag{4}$$

When the condition for kinetic measurement matches to the limit of $(H^+)\ll K_a$, $(pH\gg pK)$, the k_{obs} is reduced to

$$\left.\begin{aligned} k_{obs} &= k_0+\left(k_1+\frac{k_2}{K_a}\right)(H^+) \\ \log(k_{obs}-k_0) &= \log\left(k_1-\frac{k_2}{K_a}\right)-pH \end{aligned}\right\} \tag{5}$$

When the condition matches to $(H^+)\gg K_a$, $(pH\ll pK)$, the equation (4) is reduced to the following equations and k_{obs} becomes to be independent of pH value.

$$\left.\begin{aligned} k_{obs} &= k_1K_a+k_2 \\ \log k_{obs} &= \log(k_1K_a+k_2)=\text{constant} \end{aligned}\right\} \tag{6}$$

The terms k,(H+)(S) and k_2(SH) are not able to be distinguished kinetically. The pK value of the sugar oxazoline was found to be 3.0 from Fig. 2 and the spontaneous rate constant k_0 and k_1+k_2/K_a were calculated as shown in Table 3.

Table 3. Kinetic parameters of hydrolysis of sugar oxazoline.

$K_a = 1.0 \times 10^{-3}$ M

p$K = 3.0$

$k_s = 6.0 \times 10^{-6}$ sec^{-1}

$k_1 + \frac{k_2}{K_a} = 7.8$ M^{-1}sec^{-1}

$k_1 = 7.8$ M^{-1}sec^{-1} (reaction species : neutral)

$k_2 = 7.8 \times 10^{-3}$ sec^{-1} (reaction species: protonated)

$k_{obs} = (6.0 \times 10^{-9} + 7.8 \times 10^{-3}(H^+))/(1.0 \times 10^{-3} + (H^+))$

If neutral sugar oxazoline denoted by S is assumed to be reactive, k_2 could be considered to be zero and k_1 can be calculated. Reversely, if protonated sugar oxazoline SH is assumed to be reactive, k_2 can be calculated from experimental result. The calculated values of k_1 and k_2 under the above assumptions are listed in Table 3. Thus the sugar oxazoline is rapidly hydrolyzed by specific acid hydrolysis with bond fission between C_5 and 0, atoms of the oxazoline ring.

If it is assumed that 2-methyl-Δ^2-4,5-(glucopyrano) oxazoline is the true intermediate of the lysozyme catalyzed reaction, the roles of Glu 35 and Asp *52* in lysozyme catalysis may be speculated as follows on the basis of the result of the present experiment.

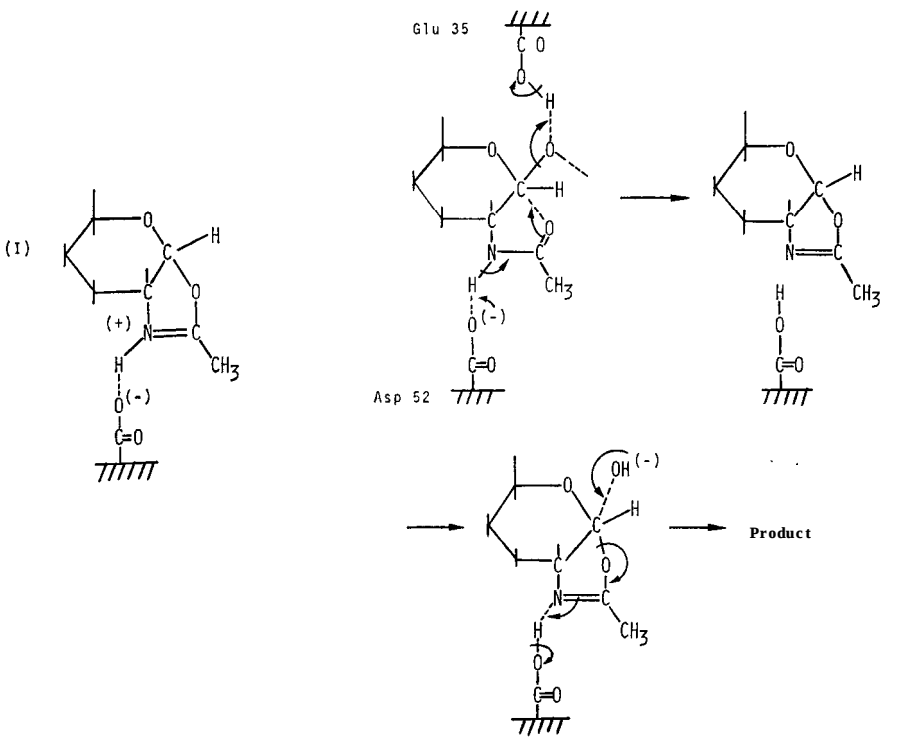
(1) The hydrolysis of the sugar oxazoline showed the fission type of C_5-O_1, forming the amide product in acid media, which is different from that of ordinary oxazolines that show the N_3-C_2 fission, forming the ester product. The C_5– 0, fission in acid media may be commonly seen on the hydrolysis of oxazolines with carbonyl or with carbon of carbonyl-nature at C_5-position. In the lysozyme catalyzed reaction, the fission was cnosidered to occur at C_5-O_1 bond. This means that in the course of its catalytic action, lysozyme needs not alter the fission type in the hydrolysis of sugar oxazoline intermediate.

(2) 2-Phenyl oxazoline derivative of sugar showed a remarkable resistance for the attack of hydroxide ion. This fact suggests thet C-position in oxazoline ring or C_1-position of glucopyranose ring is resistant for S_N 2 type attack of hydroxide ion. On the other hand, it has been assumed that conjugate base of carboxyl group of Glu 35 which was formed as consequence of its catalytic action at the first step as general acid, may act as general base on the C_1 carbon of pyranose ring of the intermediate. Mechanistically, it has been believed that the carboxylate anion (conjugate base) of side chain of Glu 35 withdraws first the hydrogen ion from water and the formed hydroxide ion attacks on the C, of pyranose ring of the intermediate in the manner of S_N B-like substitution. If the alignment of the carboxylate anion of Glu 35-water molecule-C, of pyranose ring is fixed in proper direction, the retention of β-anomeric structure of the product will be easily explained without any extra consideration. In general, it is considered that the S_N 2 substitution of hydroxide ion on sp^3 carbon is

not effectively catalyzed by general base *via* water molecule (Bruice and Benkovic, 1966). Therefore, the catalytic action of Gul 35 as general base to accelerate the S_N 2-like substitution of hydroxide ion originated from water molecule should be further assisted by other factors which interact with the oxazoline ring and promote the increase in the reactivity of C, carbon of pyranose ring of the intermediate.

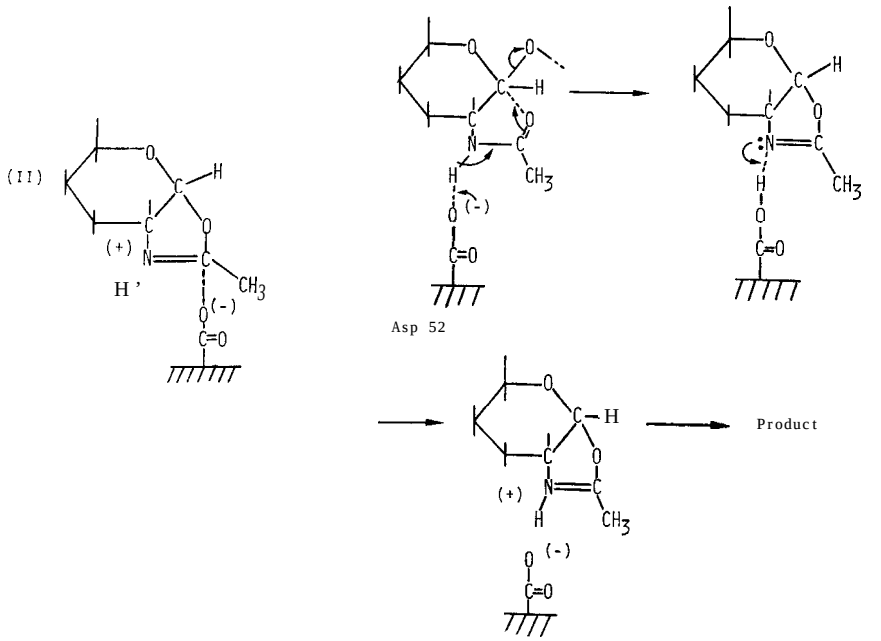
(3) If the neutral species of the oxazoline intermediate is reactive toward the attack of hydroxide ion (hydrolysis) or other nucleophiles (transglycosylation), the carboxylate anion of Asp 52 may act in the formation of oxazoline ring as general base withdrawing proton from NH group of 2-acetamido group and the resulted carboxyl group may accelerate the cleavage of C_5–O_1 bond of oxazoline ring by protonating the ring nitrogen (see Scheme II). Another possibility of the action of carboxylate anion of Asp 52 is to shift the p*K* value of the oxazoline by attracting the proton from the oxazoline ring (I). This type of interaction may lower the p*K* value of the oxazoline, and assist the oxonium ion formation of the ring O_1, resulting in the acceleration of the cleavage of C_5–O_1 bond of oxazoline. Orthooxazonline or ortho-ester of oxazoline has been considered to be important intermediate of the reaction of oxazoline (Bruice and Benkovic, 1966). The interaction of carboxylate anion of Asp 52 and C_2 of the ring to form an orhto-ester intermediate may also be account for the role of

Scheme II



Asp *52* carboxylate anion in the lysozyme catalysis (II).
(4) If the protonated oxazoline intermediate is assumed to be reactive in the hydrolysis or transglycosylation, the role of Asp *52* will be speculated as shown in the Scheme III for both the formation and cleavage of the oxazoline ring.

Scheme III



In the present experiment, 2-benzamido derivative of D-glucopyranose was used in place of 2-acetamido derivative. Therefore, some data obtained, such as p*K* value, seem to be slightly different from those for true substance 2-acetamido derivative in the enzymatic catalysis.

The authors are now studying the relations between p*K* value or fission type and the substituent of C_2-position of the oxazoline ring using derivatives of oxazoline-5-ones.

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