Notes

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Studies on the Active Site of Papain. IV.[1] Influence of Dehydroascorbic Acid and Ascorbic Acid[2]

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Papain possesses a single reactive SH group which is thought to play an essential role in the enzymatic action. In the previous paper,[4] it was reported that inhibitory effect of alloxan on papain is due to poly-carbonyl group which reacts with SH group. Dehydroascorbic acid possesses the similar polycarbonyl group to alloxan. Papain is apparently inhibited by dehydroascorbic acid and ascorbic acid.[5] Although a possibility of interaction between dehydroascorbic acid and protein SH group has been suggested from the fact that dehydroascorbic acid affords inhibitory effect on several enzymes,[5,6] very little information is available about this reaction.

In order to get some knowledge on the active site of papain, the influence of dehydroascorbic acid and ascorbic acid on enzyme activity of papain, which possesses essential SH group for enzyme activity, and on SH group was studied.

Experimental

Materials——Crystalline papain and α-benzoyl-L-arginine amide (BAA) were prepared by the procedure of Kimmel and Smith.[7] 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB) and ascorbic acid (AA) were obtained from Nakarai Chemicals and dehydroascorbic acid (DHA) was obtained from Nutritial Biochemicals Corporation.

Assay Procedure of Enzyme Activity——The assay procedure described in the previous paper[8] was employed.

Assay Procedure of SH Contents——SH contents were analyzed essentially according to the procedure of Ellman[9] as follows: Sample (1.2 ml), 1 ml of 0.1M phosphate buffer (pH 8.0) and 1 ml of water were mixed, and 0.05 ml of DTNB (1×10^{-2}M aqueous solution) was added to the mixture. After incubation for 1 minute, optical density was read at 412 mμ.

Spectral Measurements——The ultraviolet (UV) absorption spectrum was measured in 2% metaphosphoric acid solution.

Kinetic Constants——The kinetic constants, *K*m, *V*max and *K*i, were obtained from Lineweaver and Burk's plot[10] and Dixon's plots.[11]

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Result and Discussion

Inhibitory effects of DHA and AA on papain were examined at pH 6.0 using BAA as substrate. The mode of the inhibitory effects of DHA and AA are found to be non-competitive (Fig. 1). The value of Ki was calculated from the equation;

$$K_i = i \times V_p/(V_{max} - V_p)$$

which may be readily derived from Lineweaver and Burk's method, where i is inhibitor concentration. The value of Ki was also obtained from Dixon's plot, as shown in Fig. 2. Kinetic constants, Km, V_{max} and Ki, of papain by DHA and AA are listed in Table I.

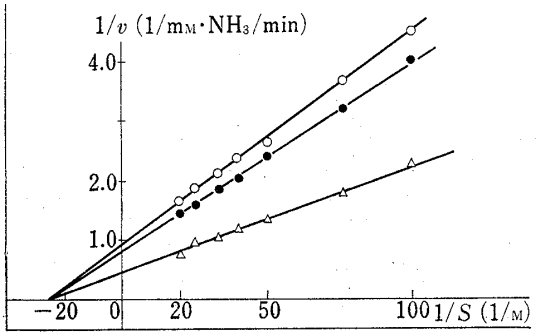

Fig. 1. Inhibitory Effects of DHA and AA on Papain

Mixture of enzyme and inhibitor (DHA and AA) were incubated for 1 hour at 39°, and after activation, activities were assayed by alkalimetric titration in alcohol. Reaction mixtures contained papain (5×10^{-6}M), DHA and AA (1×10^{-5}M), substrate (BAA) ($1—5\times10^{-2}$M), potassium cyanide (3×10^{-3}M) and EDTA (1×10^{-3}M) in a total volume of 2.5 ml.
○: papain+AA, ●: papain+DHA, △: papain

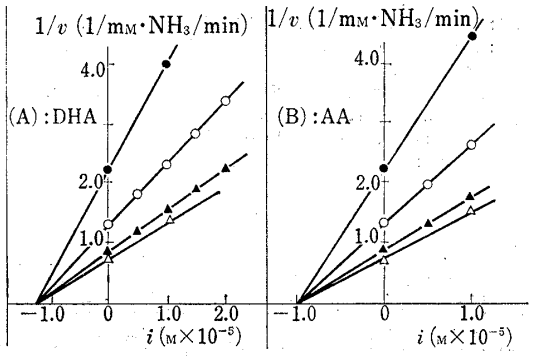

Fig. 2. Determination of Ki Value by Dixon's Plot

The inhibitory effects of DHA(A) and AA (B) on papain were expressed by Dixon's plot. The reaction conditions were the same as Fig. 1. The substrate (BAA) concentration was denoted as follows.
△: 0.05M, ▲: 0.04M, ○: 0.02M, ●: 0.01M

Table I. Kinetic Constants

Effector	Constants					
	K_m	K_p	V_{max}	V_p	K_i I	K_i II
	($M\times10^{-2}$)		(mM·NH_3/min)		($M\times10^{-5}$)	
None	3.85	—	2.22	—	—	—
DHA	—	3.85	—	1.25	1.29	1.25
AA	—	3.85	—	1.11	1.00	1.00

The kinetic constants, K_m, V_{max} and K_i (I), were obtained from Lineweaver and Burk's method. The value of K_i (II) was obtained from Dixon's method.

DHA and AA showed similar inhibitory effect on papain and was found to be non-competitive inhibitors, when papain was activated by cyanide. It is considerable that AA was oxidized to DHA during pre-incubation and so AA showed the similar inhibitory effect to DHA. Papain and AA have UV absorption, and glutathione (GSH) and DHA have little UV absorption, as shown in Chart 1 (a). DHA (or AA) and papain (or GSH) were treated for 1 hour, and the UV absorption spectrum was measured. The UV absorption spectra of these samples did not change on 1 hour treatment in contrast with 0 hour treatment, as shown in Chart 1 (b). This results may indicate that AA was not oxidized to DHA during pre-incubation and so both AA and DHA showed inhibitory effect on cyanide-activated papain.

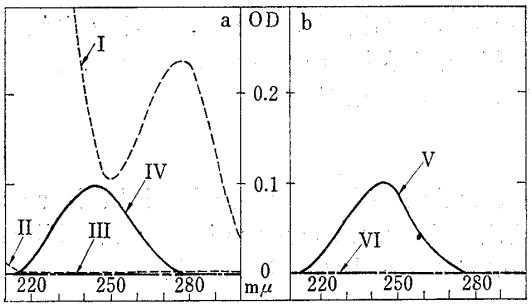

Chart 1. UV Absorption Spectrum

(a): Papain (I), GSH (II), DHA (III) and AA (IV).
(b): Papain (or GSH) and DHA (or AA) were treated on 1 hour.
Reaction mixture contained papain (5×10^{-6}M), GSH (1×10^{-5}M), DHA (1×10^{-5}M) and AA (1×10^{-5}M).
V: [I+IV]−I and [II+IV]−II
VI: [I+III]−I and [II+III]−II

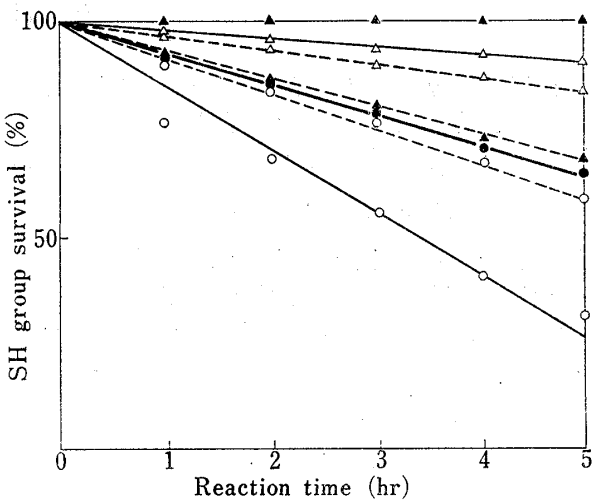

Fig. 3. Change of SH Group Contents in Glutathione

The effector (●; none, ▲; DHA, △; AA, ○; ALX) concentration was denoted as follows: a 1-fold excess of effector in relation to GSH concentration; ------, a 100-fold excess; ——

Papain possesses an essential SH group for enzyme activity, and so it seems to be very interesting to investigate the influence of DHA and AA on SH group. GSH was treated with DHA, AA and alloxan (ALX) (similar character to DHA) for 1 hour at 39°, and the SH contents were analyzed. As shown in Fig. 3, it is clear that the decrease of SH contents of GSH took place on ALX-treatment and was protected on DHA- and AA-treatment. On the other hand, the decrease of SH contents of papain took place on ALX- and AA-treatment and the increase of SH contents of papain took place in DHA-treatment, as shown in Table II (column 1). Furthermore, mixtures of papain and effector were incubated for 1 hour at 39°, and after activation by cyanide, their SH contents were analyzed. The decrease of SH contents of cyanide-activated papain took place on DHA-, AA- and ALX-treatment, as compared with control (column 2, Table II). Since AA is one of reductones, GSH was protected from oxidation by AA. However, the slight decrease of SH contents of non-activated papain

Table II. SH Contents in Papain

Effector		Papain; 5×10^{-6}M	
		Non-activated	Cyanide-activated
None		0.88×10^{-6}M (0.18)	2.60×10^{-6}M (0.52)
DHA	1×10^{-5}M	0.80×10^{-6}M (0.16)	2.18×10^{-6}M (0.44)
	1×10^{-3}M	1.06×10^{-6}M (0.21)	1.08×10^{-6}M (0.22)
AA	1×10^{-5}M	0.88×10^{-6}M (0.18)	2.18×10^{-6}M (0.44)
	1×10^{-3}M	0.72×10^{-6}M (0.15)	1.74×10^{-6}M (0.35)
ALX	1×10^{-5}M	0.82×10^{-6}M (0.16)	1.74×10^{-6}M (0.35)
	1×10^{-3}M	0.68×10^{-6}M (0.13)	0.76×10^{-6}M (0.15)

( ): mole of SH group per mole of papain

and cyanide-activated papain took place on AA-treatment. On the other hand, activity of papain was inhibited by ALX,[4] and SH contents in papain and GSH significantly decreased when papain and GSH were treated with ALX. These results suggested that the decrease of enzyme activity may be caused by the reaction of essential SH group with poly-carbonyl group in ALX. Since DHA possesses the poly-carbonyl group similar to that of ALX, it was expected that DHA shows inhibitory effect and the decrease of SH contents of GSH and papain take place in DHA-treatment. The activity of papain was apparently inhibited

by DHA. Nevertheless, on treatment of GSH with DHA, no decrease of SH contents was observed, and on treatment of papain (non-activated) with DHA, the increase of SH contents took place, as compared with control. From this results, it may be thought that inhibitory effect of ALX depends on the nature of polycarbonyl compound to react with SH group, but that the polycarbonyl group in DHA does not react with SH group.

DHA and AA for papain activated by cyanide was non-competitive inhibitors, and the mode of the inhibitory effects of DHA and AA was diverse from that of ALX (competitive inhibitor[12] for papain activated by cyanide). And non-activated papain had 0.18 mole of SH group per mole of papain and cyanide-activated papain had 0.52 mole of SH group (Table II). Morihara reffered it in his studies[13] that the active site is composed of both SH and aldehyde groups which act on each other (thiohemiacetal) in an equilibrium state and that the activators combine with the active site (thiohemiacetal) to convert it into an activated form. But the presence of the aldehyde group in papain has not been directly identified as yet. Recently, Klein, *et at.* also refered it in their studies[14] that the inactive form of papain prepared by the method of Kimmel and Smith[7] is that containing a mixed disulfide between the SH group at the active site of papain and free cysteine. The results in this paper and in the previous paper[8] support the idea that the active site is composed both SH and a certain groups which act on each other in an equilibrium state. A certain group in papain is now under investigation.

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Purines. XII.[1] Catalytic Hydrogenolysis of Alkoxyaminopurines and Related Derivatives

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In the course of our synthesis of N-alkoxyadenosines (type Ib,d)[1] and their 9-alkyl analogues (type Ia,c),[1,3] we became interested in studying the selective hydrogenolytic cleavage of the N-benzyloxy group at the C–O bond from which an alternative synthetic route to N-hydroxyadenosine (IIIb), an antileukemic substance[4] synthesized first by Giner-

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