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Studies on the Active Site of Papain. III.[1] Inhibition by Dibasic Acids[2]

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In the previous paper,[4] it was reported that the cyanide-activated papain is apparently inhibited by active methylene group and active imide group in barbituric acid. The active methylene group in barbituric acid is derived from malonic acid.

Morgan and Friedmann have been shown that maleic acid and SH-compounds interact with the formation of stable addition compounds.[5] They have applied this reaction to the study of some SH-enzyme reaction.

The present research has been planed to clarify relation between activity of papain and influence of the dibasic acids.

Experimental

Crystalline Papain——Crystalline papain was prepared from dried papaya latex by the method of Kimmel and Smith.[6] The preparation had a C_1 value[7] of about 1.10 toward α-benzoyl-L-arginine amide according to the assay procedure by Kimmel and Smith.[6] The papain concentration was determined by the absorbance at 280 mμ.[8]

Effector——Dibasic acids used in this study were shown in Chart 1.

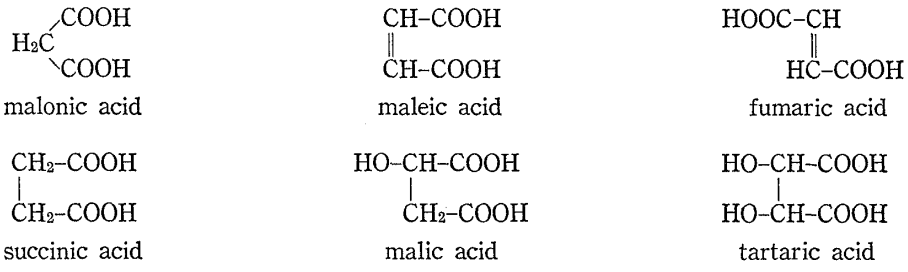

Chart 1

α-Benzoyl-L-arginine Amide (BAA)——This substrate was prepared by the procedure of Kimmel and Smith.[6]

5,5′-Dithiobis(2-Nitrobenzoic Acid) (DTNB)——This reagent was purchased from Nakarai Chemicals, LTD., Kyoto, Japan.

1) Part II: H. Kanazawa, A. Ohara and M. Yoshioka, *Chem. Pharm. Bull.* (Tokyo), **18**, 1904 (1970).
2) A part of this research was presented at the 19th Meeting of Kinki Branch, Pharmaceutical Society of Japan, Osaka, Oct. 1969.
3) Location: 5 *Nakauchicho, Misasagi, Yamashina, Higashiyama, Kyoto.*
4) H. Kanazawa, S. Uchihara, A. Ohara and M. Yoshioka, *Chem. Pharm. Bull.* (Tokyo), **18**, 195 (1970).
5) E.J. Morgan and E. Friedmann, *Biochem. J.*, **32**, 733 (1938); *idem, ibid.*, **32**, 862 (1938).
6) J.R. Kimmel and E.L. Smith, "Biochemical Preparation," Vol. VI, John Willy and Sons, Inc., New York, 1957, p. 61.
7) C_1 is the first order rate constant per mg protein nitrogen per ml of reaction mixture expressed decimal logarithms.
8) M. Ebata and K.T. Yasunobu, *J. Biol. Chem.*, **237**, 1086 (1962).

Assay Procedure of Enzymatic Activities——As described in the preceding paper,[4] the assay procedure described by Kimmel and Smith[6] was employed with slight modification.

Procedure for Assay of Sulfhydryl Contents——As described in the preceding paper,[4] the method of Ellman with DTNB[9] was applied.

Result

Inhibitory Effrcts of Saturated Dibasic Acids

On 1 hour treatment with cyanide–activated papain, malonic acid showed inhibitory effect, but succinic acid, malic acid and tartaric acid did not show inhibition, as shown in Table I. Okumura[10] reported that the activity of papain is progressively inhibited with time on treatment with 1,3-dimethylbarbituric acid, which contains active methylene group as malonic acid. Then, inhibitory effects of papain on 20 hours treatment were examined. On 20 hours treatment with cyanide–activated papain, malonic acid showed much stronger inhibitory effect in contrast with on 1 hour treatment, but other saturated dibasic acid did not show inhibition, as shown in Table I.

On the other hand, on 1 and 20 hours treatment with cysteine–activated papain, malonic acid, succinic acid, malic acid and tartaric acid did not show in Table I.

Inhibitory Effects of Unsaturated Dibasic Acid

On 1 hour treatment with cyanide–activated papain, maleic acid showed the similar inhibitory effect as fumaric acid, as shown in Table I. On 20 hours treatment with cyanide–activated papain, maleic acid showed much stronger inhibitory effect than fumaric acid, as shown in Table I.

Table I. Relationship of Activity and Sulfhydryl Content

Effector	Activity (%)				SH content (%)			
	Cyanide activation		Cysteine activation		In papain		In glutathion	
	1 hour	20 hours	1 hour	20 hours	1 hour	20 hours	1 hour	20 hours
Control	100	100	100	100	100	100	100	100
Malonic acid	84	75	100	100	98	99	92	100
Succinic acid	100	100	100	100	96	101	100	99
Malic acid	100	100	100	100	88	97	99	98
Tartaric acid	100	100	100	100	100	99	100	99
Maleic acid	74	42	86	82	77	69	84	16
Fumaric acid	73	56	88	81	77	64	84	17

Mixture of enzyme and inhibitor solution was incubated for 1 or 20 hours at 40°, and after activation, activities were assayed by alkalimetric titration in alcohol.
final concentration: papain; 6×10^{-6}M
substrate (BAA); 5×10^{-2}M
effector; 1×10^{-3}M
activator
potassium cyanide; 3×10^{-3}M
cystein hydrochloride; 5×10^{-3}M
EDTA; 1×10^{-3}M

On the other hand, on 1 hour treatment with cysteine–activated papain, maleic acid and fumaric acid showed the faint inhibitory effects as compared with cyanide–activated papain, as shown in Table I. On 20 hours treatment with cysteine–activated papain, maleic acid and

9) G.L. Ellman, *Arch. Biochem. Biophys.*, **74**, 443 (1958); *idem*, *ibid.*, **82**, 70 (1959).
10) S. Maeda (Okumura), *Bull. Chem. Soc. Japan*, **12**, 319 (1937); S. Okumura, *ibid.*, **13**, 534 (1938); *idem*, *ibid.*, **14**, 161 (1939).

fumaric acid showed much stronger inhibitory effects in contrast with on 1 hour treatment, as shown in Table I.

Change of Sulfhydryl Content during Inhibitions

According to the thiohemiacetal hypothesis,[11] the functional group of native papain which reacts with 1,3-dimethylbarbituric acid[10,11] is assumed to be an aldehyde group which interacts with the sulfhydryl group essential for the activity. Then, the assays of sulfhydryl group in papain and in glutathion, which have free sulfhydryl group, were carried out by the method of Ellman with DTNB.[9] The results obtained by this method were rather semiquantitative, but it is clear that the decrease of sulfhydryl content took place in maleate- and fumarate-inhibition, and no change of sulfhydryl content took place in malonate-inhibition as compared with control, as shown in Table I.

Discussion

Thiohemiacetal hypothesis was proposed for a new moel of the active site of papain by Morihara.[11] This hypothesis is based on the assumption that an aldehyde group readily available for the enzymatic reaction is involved in papain. According to this assumption, the increase of the sulfhydryl content in papain is expected when papain is treated with carbonyl reagents. In previous paper,[4] it was reported that papain is inhibited by 1,3-dimethylbarbituric acid with active methylene group which reacts readily with aldehyde[10,11] and the increase of sulfhydryl contents in papain take place in 1,3-dimethylbarbiturate-inhibition. The active methylene group in 1,3-dimethylbarbituric acid is derived from malonic acid. Therefore, it is expected that malonic acid shows inhibitory effect on papain and the increase of sulfhydryl contents in papain take place in malonate-inhibition. Papain was inhibited by malonic acid but both sulfhydryl contents in papain and in glutathione did not change in malonate-inhibition. Therefore, it is necessary to investigate moreover in order to explain fully the inhibition of active methylene group, such as barbituric acid and malonic acid.

On the other hand, Morgan and Friedmann[5] reported that maleic acid reacted with, but fumaric acid did not react with the sulfhydryl group. Nevertheless not only maleic acid but also fumaric acid reacted with the sulfhydryl group and showed the inhibitory effect on native papain, as shown in Table I. The results indicate that maleic acid and fumaric acid were inhibitory because it reacted with the sulfhydryl group essential for enzyme activity.

11) K. Morihara, *J. Biochem.* (Tokyo), **62**, 250 (1967); K. Morihara and K. Nagami, *ibid.*, **65**, 321 (1969).