Notes

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Studies on the Active Site of Papain. I. Inhibition by Barbituric Acid Derivatives with Active Methylene Group and Active Imide Group[1]

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Papain is one of the most conspicuous plant proteinase contained in the latex of the immature fruits of papaya, *Carica papaya* L. Numerous studies[3] has been carried out on the active site of papain [EC 3.4.4.10]. Smith proposed the thiol ester hypothesis[4] that the cysteinyl residue at position 25 and the aspartate at position 163 can form the active site. However, papain is inhibited by 1,3-dimethylbarbituric acid[5] which reacts readily with aldehyde but may not react with thiol ester in aqueous solution. Accoridng to this properties, Morihara proposed the thiohemiacetal hypothesis.[6] But the presence of the aldehyde group in papain has not been directly identified as yet, though Morihara[6] refered it in his inhibition studies that the indole ring of a tryptophanyl residue possesses some chemical properties as a carbonyl group.

Then, as one of the means of solving the active site of papain, inhibitory effects of papain with barbituric acid derivatives, which contain active methylene group and active imide group, were examined. Therefore, four barbituric acid derivatives, that is, 1,3-dimethylbarbituric acid (only active methylene group) (DMB), 5,5-diethylbarbituric acid (only active imide groups) (DEB), barbituric acid (active methylene group and active imide groups) (BA), and 1,3-dimethyl-5,5-diethylbarbituric acid (no active methylene group and no active imide group) (DMEB), were used for inhibitory studies of papain.

On 1 hour treatment with cyanide–activated papain, BA, which contains active methylene group and active imide groups, showed more stronger inhibitory effects than DMB, as seen from data in Fig. 1. Therefore, inhibitory effect with DEB, which contains only active imide groups, and DMEB were examined. On 1 hour treatment, DEB showed the similar inhibitory effect as DMB, but DMEB did not show inhibition, as shown in Fig. 2. In these inhibition, probably not only active methylene group but also active imide group must show inhibitory effect.

Okumura[5] reported that the activity of papain is progressively inhibited with time on treatment with DMB. Then, inhibitory effects of papain on 20 hours treatment were examined. On 20 hours treatment with cyanide–activated papain, DMB showed more stronger inhibitory effect than BA in contrast with on 1 hour treatment, as shown in Fig. 3. DEB

1) A part of this research was presented at the 89th Annual Meeting of the Pharmaceutical Society of Japan in Nagoya, April 1969.
2) Location: 5 *Nakauchicho, Misasagi, Yamashina, Higashiyama, Kyoto.*
3) E.L. Smith and J.R. Kimmel, "The Enzyme," ed. P.D. Boyer, H. Lardy and K. Myrbäck, Academic Press, New York and London, Vol. IV. 1960, p. 133.
4) E.L. Smith, *J. Biol. Chem.*, **233**, 1372 (1958); A. Light, R. Frater, J.R. Kimmel and E.L. Smith, *Proc. Natl. Acad. Sci. U.S.*, **52**, 1276 (1964).
5) S. Maeda, *Bull. Chem. Soc. Japan*, **12**, 319 (1937); S. Okumura, *Bull. Chem. Soc. Japan*, **13**, 534 (1938); *idem, ibid.*, **14**, 161 (1939).
6) K. Morihara, *J. Biochem.* (Tokyo), **62**, 250 (1967); K. Morihara and K. Nagami, *ibid.*, **65**, 321 (1969).

showed the similar inhibitory effect as BA, but DMEB did not show inhibition, as shown in Fig. 4.

On the other hand, on 1 and 20 hours treatment with cysteine-activated papain, DMB, BA, DEB and DMEB did not show inhibition, as shown in Fig. 5 and Fig. 6.

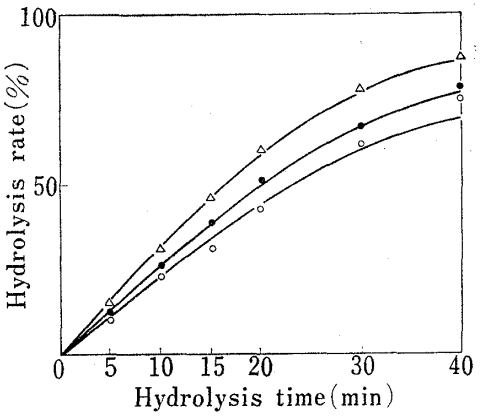

Fig. 1. Effect of BA and DNB on Native Papain

—○—: papain plus BA
—●—: papain plus DMB
—△—: papain plus water
inhibition: BA and DMB, aqueous 1×10^{-3}M, solution
activation: KCN (3×10^{-3}M) plus EDTA(1×10^{-3}M) solution (pH 6.0)
substrate: BAA(5×10^{-2}M)
papain solution: 6×10^{-6}M (C_1=1.10)
Mixture of enzyme and inhibitor solution were incubated for 1 hour at 40°, and after activation, activities were assayed by alkalimetric tirtation in alcohol.

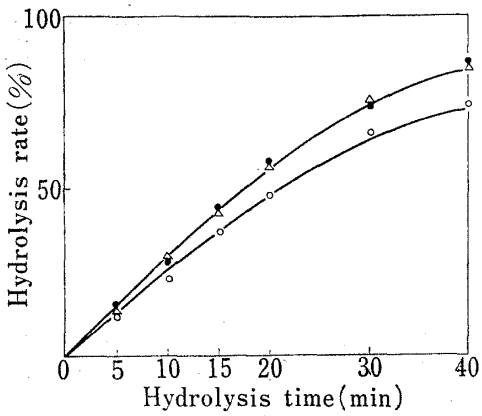

Fig. 2. Effect of DEB and DMEB on Native Papain

—○—: papain plus DEB
—●—: papain plus DMEB
—△—: papain plus ethanol
inhibition: DEB and DMEB, ethanolic 1×10^{-3}M, solution
activation: KCN (3×10^{-3}M) plus EDTA(1×10^{-3}M) solution (pH 6.0)
substrate: BAA(5×10^{-2}M)
papain solution; 6×10^{-6}M (C_1=1.10)
Mixture of enzyme and inhibitor solution were incubated for 1 hour at 40°, and after activation, activities were assayed by alkalimetric titration in alcohol.

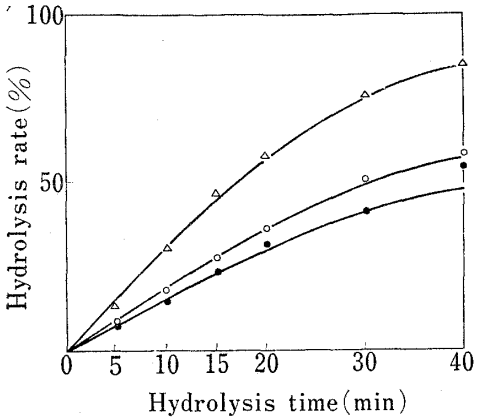

Fig. 3. Effect of BA and DMB on Native Papain

—○—: papain plus BA
—●—: papain plus DMB
—△—: papain plus water
inhibition: BA and DMB, aqueous 1×10^{-3}M, solution
activation: KCN (3×10^{-3}M) plus EDTA (1×10^{-3}M) solution (pH 6.0)
substrate: BAA(5×10^{-2}M)
papain solution: 6×10^{-6}M (C_1=1.10)
Mixture of enzyme and inhibitor solution were incubated for 20 hours at 40°, and after activation, activities were assayed by alkalimetric titration in alcohol.

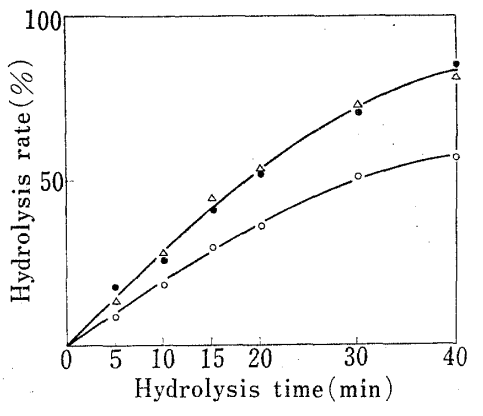

Fig. 4. Effect of DEB and DMEB on Native Papain

—○—: papain plus DEB
—●—: papain plus DMEB
—△—: papain plus ethanol
inhibition: DEB and DMEB, ethanolic 1×10^{-3}M, solution
activation: KCN (3×10^{-3}M) plus EDTA (1×10^{-3}M) solution (pH 6.0)
substrate: BAA(5×10^{-2}M)
papain solution: 6×10^{-6}M (C_1=1.10)
Mixture of enzyme and inhibitor solution were incubated for 20 hours at 40°, and after activation, activities were assayed by alkalimetric titration in alcohol.

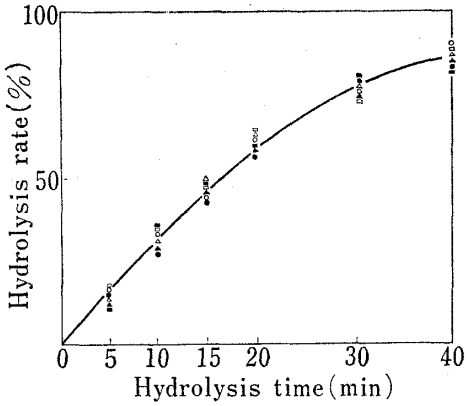

Fig. 5. Effect of BA, DMB, DEB and DMEB on Native Papain

—○—: papain plus BA
—□—: papain plus DMB
—●—: papain plus DEB
—■—: papain plus DMEB
—△—: papain plus water (control for BA and DMB)
—▲—: papain plus ethanol (control for DEB and DMEB)
inhibition: BA and DMB, aqueous 1×10^{-3}M, and DEB and DMEB, ethanolic 1×10^{-3}M, solution
activation: cysteine(5×10^{-3}M) plus EDTA(1×10^{-3}M) solution (pH 6.0)
substrate: BAA(5×10^{-2}M)
papain solution: 6×10^{-6}M (C_1=1.10)
Mixture of enzyme and inhibitor solution were incubated for 1 hour at 40°, and after activation, activities were assayed by alkalimetric titration in alcohol.

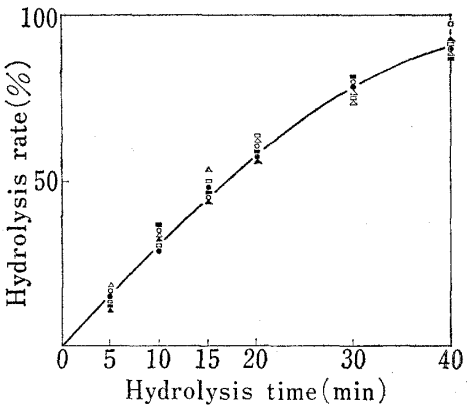

Fig. 6. Effect of BA, DMB, DEB and DMEB on Native Papain

—○—: papain plus BA
—□—: papain plus DMB
—●—: papain plus DEB
—■—: papain plus DMEB
—▲—: papain plus water (control for BA and DMB)
—△—: papain plus ethanol (control for DEB and DMEB)
inhibition: BA and DMB, aqueous 1×10^{-3}M, and DEB and DMEB, ethanolic 1×10^{-3}M, solution
activation: cysteine(5×10^{-3}M) plus EDTA(1×10^{-3}M) solution (pH 6.0)
substrate: BAA(5×10^{-2}M)
papain solution: 6×10^{-6}M (C_1=1.10)
Mixture of enzyme and inhibitor solution were incubated for 20 hours at 40°, and after activation, activities were assayed by alkalimetric titration in alcohol.

As cyanide–activated papain is apparently inhibited by active methylene group and active imide group, such as BA, DMB and DEB, then, the assays of the sulfhydryl group, which are thought to play an essential role in the enzymatic action,[7] were carried out by the method of Ellman[8] with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). The results obtained bythis method were rather semiquantitative, as shown in Table 1, but it is clear that the increase of sulfhydryl content took place in DMB–inhibition, the decrease of sulfhydryl content took place in DEB–inhibition, and no change of sulfhydryl content took place in BA– and DMEB–inhibition as compared with control.

Table I. Relationship of Sulfhydryl Content and Activity

Inhibitor	1 hr		20 hr	
	SH content (%)	Activity (%)	SH content (%)	Activity (%)
Control[a]	100	100	100	100
BA	100	74	105	62
DMB	169	84	161	52
Control[b]	100	100	100	100
DEB	62	84	52	66
DMEB	96	100	103	100

a) for BA and DMB b) for DEB and DMEB

7) T. Sanner and Pihl Alexander, *J. Biol. Chem.*, **238**, 165 (1963); M. Soejima, *J. Agr. Chem. Soc. Japan*, **36**, 743 (1962).
8) G.L. Ellman, *Arch. Biochem. Biophys.*, **74**, 443 (1958); *idem*, *ibid.*, **82**, 70 (1959).

According to the thiohemiacetal hypothesis, it is concluded that DMB was inhibitory because it condensed with the carbonyl group essential for the activity of papain, while DEB was inhibitory because it reacted with the sulfhydryl group essential for enzyme activity. But, it cannot be explained well that DMB, which contained only active methylene group, shows more stronger inhibitory effect than BA, which contains active methylene group and active imide groups, on 20 hours treatment in contrast with on 1 hour treatment. This phenomenon is necessary to investigate moreover.

Experimental

Crystalline Papain——Crystalline papain was prepared from dried papaya latex by the method of Kimmel and Smith.[9] The preparation had a C_1 value[10] of about 1.10 toward α-benzoyl-L-arginine amide according to the assay procedure by Kimmel and Smith.[9] The papain concentration was determined by the absorbance at 280 mμ.[11]

Barbituric Acid Derivatives——Barbituric Acid (BA): This reagent was purchased from Nakarai Chemicals, Ltd., Kyoto, Japan, and recrystallized from hot water. mp 247—247.5° (ref. 248°).

1,3-Dimethylbarbituric Acid (DMB): This reagent was prepared by the method of Biltz and Wittek.[12] mp 122—122.5° (ref. 123°).

5,5-Diethylbarbituric Acid (DEB): This reagent was purchased from Nakarai Chemicals, Ltd., Kyoto, Japan, and recrystallized from hot water. mp 188—189° (ref. 188—192°).

1,3-Dimethyl-5,5-diethylbarbituric Acid (DMEB): This reagent was prepared by the method of Dox.[13] mp 37° (ref. 37°).

α-Benzoyl-L-arginine Amide (BAA)——This substrate was prepared by the procedure of Kimmel and Smith.[9] mp 120—122° (ref. 120—123°).

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

Assay Procedure of Enzymatic Activity——The assay procedure described by Kimmel and Smith[9] was employed with slight modification, as follows: The mixture of 1 ml of 0.25M cysteine hydrochloride and 0.5 ml of 0.1M EDTA is adjusted to pH 6 with 0.5 ml of 0.5N sodium hydroxide and 1 ml of 0.1M citrate buffer (pH 6.0), and the solution was diluted to 9 ml with water. To 1.8 ml of this activating mixture, 0.2 ml of an enzyme preparation to be assayed was added, and the mixture was incubated at 40° for activation over 30 minutes. When cyanide was used as the activater, cysteine hydrochloride and sodium hydroxide was replaced by 1 ml of 0.15M potassium cyanide and 0.3 ml of 0.4M acetic acid, respectively. The substrate solution of 1.0 ml of 0.125M BAA, 0.3 ml of 0.2M citrate buffer (pH 6.0) and 0.7 ml of water was kept at 40° before starting assay. The reaction is started by addition of 0.5 ml of the enzyme activation mixture to the substrate solution. An aliquot of 0.2 ml was immediately removed to one of the titration vessels each contained about 2 ml of ethanol and a little thymolphthalein as the indicator. Additional 0.2 ml aliquots were also removed at appropriate intervals, and all aliquots were then titrated with 0.01N alcoholic potassium hydroxide which had been standardized with 0.02N standard biniodate (potassium hydrogen diiodate).

Procedure for Assay of Sulfhydryl Contents——The mixture of 0.2 ml of a papain preparation, 1.8 ml of water and 1 ml of 1×10^{-2}M barbituric acid derivative solution served as experimental sample. The mixture of 0.2 ml of a papain preparation, 1.8 ml of water and ethanol or water served as control sample. Both the experimental and control samples were incubated at 40° for 1 hour or 20 hours, and then the method of Ellman with DTNB[8] was applied, as follows: Three milliliter of sample, 2 ml of phosphate buffer (pH 8.0) and 5 ml of water were mixed, and 0.02 ml of DTNB (1×10^{-2}M aqueous solution) was added to 3.0 ml of this mixture in a photometer cell. The color developed rapidly (2 min), and the absorbance of 412 mμ was read.

9) J.R. Kimmel and E.L. Smith, "Biochemical Preparation," John Wiley and Sons, Inc., New York, Vol. VI, 1957, p. 61.
10) C_1 is the first order rate constant per mg protein nitrogen per ml of reaction mixture expressed decimal logarithms.
11) M. Ebata and K.T. Yasunobu, *J. Biol. Chem.*, **237**, 1086 (1962).
12) H. Biltz and H. Wittek, *Chem. Ber.*, **54**, 1035 (1921).
13) A.W. Dox, *J. Am. Chem. Soc.*, **58**, 1633 (1936).