Notes

[Chem. Pharm. Bull. 18(9)1915—1917(1970)]

UDC 547.775.09 : 547.783.09 : 577.156.025.04

Studies on the Active Site of Papain. II.[1] Inhibition by Cyclic Imide Compounds[2]

Haruo Kanazawa, Akira Ohara and Masashichi Yoshioka

Kyoto College of Pharmacy[3]

(Received November 21, 1969)

In the preceding paper,[1] it was reported that the cyanide-activated papain is apparently inhibited and the cysteine-activated papain is hardly affected by 5,5-diethylbarbituric acid (barbital) with active imide group and that the sulfhydryl content of papain significantly decreases when papain was treated with this reagent.

Then, for the purpose of investigation of inhibitory effects of papain by active imide group moreover, cyclic imide compounds, such as succinimide, parabanic acid, allantoin, alloxan, hydantoin and 5,5-diethylbarbituric acid (barbital), were used for inhibitory studies of papain.

Experimental

Crystalline Papain——Crystalline papain was prepared from dried papaya latex by the method of Kimmel and Smith.[4] The preparation had a C_1 value[5] of about 1.10 toward α-benzoyl-L-arginine amide according to the assay procedure by Kimmel and Smith.[4] The papain concentration was determined by the absorbance at 280 mμ.[6]

Effector——Cyclic imide compounds used in this study were shown in Chart 1.

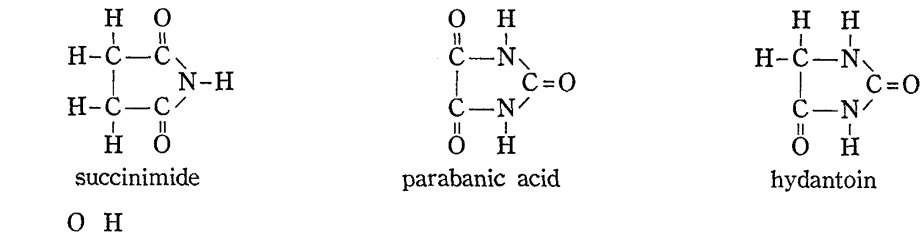

Chart 1

1) Part I: H. Kanazawa, S. Uchihara, A. Ohara and M. Yoshioka, *Chem. Pharm. Bull.* (Tokyo), **18**, 195 (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) J.R. Kimmel and E.L. Smith, "Biochemical Preparation," Vol. 6, John Willy and Sons, Inc., New York, 1957, p. 61.
5) C_1 is the first order rate constant per mg protein nitrogen per ml of reaction mixture expressed decimal logarithms.
6) M. Ebata and K.T. Yasunobu, *J. Biol. Chem.*, **237**, 1086 (1962).

α-Benzoyl-L-arginine Amide (BAA)——This substrate was prepared by the procedure of Kimmel and Smith.[4]

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

Assay Procedure of Enzymatic Activities——As described in the preceding paper,[1] the assay procedure described by Kimmel and Smith[4] was employed with slight modification.

Procedure for Assay of Sulfhydryl Contents——As described in the preceding paper,[1] the method of Ellman with DTNB[7] was applied.

Result

Inhibitory Effects of Succinimide, Parabanic acid, Allantoin and Alloxan

On 1 hour treatment with cyanide-activated papain, alloxan showed much stronger inhibitory effect than parabanic acid, but allantoin and succinimide did not show inhibition, as shown in Table I. In the previous paper,[1] it was reported that the activity of papain is progressively inhibited with time on treatment with barbital. Then, inhibitory effects of papain on 20 hours treatment were examined. On 20 hours treatment with cyanide-activated papain, alloxan showed the similar inhibitory effect and parabanic acid showed much stronger inhibitory effect in contrast with on 1 hour treatment, but allantoin and succinimide did not show inhibition, as shown in Table I.

On the other hand, on 1 and 20 hours treatment with cysteine-activated papain, alloxan showed the similar inhibitory effect as compared with cyanide–activated papain, but parabanic acid, allantoin and succinimide did not show inhibition, as shown in Table I.

Inhibitory Effects of Barbital and Hydantoin

On 1 hour treatment with cyanide–activated papain, hydantoin showed the similar inhibitory effect as barbital, as shown in Table I. On 20 hours treatment with cyanide-activated papain, barbital and hydantoin showed much stronger inhibitory effects in contrast with on 1 hour treatment, as shown in Table I.

On the other hand, on 1 and 20 hours treatment with cysteine–activated papain, barbital and hydantoin did not show inhibition, as shown in Table I.

Change of Sulfhydryl Content during Inhibitions

According to the thiohemiacetal hypothesis,[8] the sulfhydryl group in papain, which is thought to play an essential role in the enzymatic action, interacts with the aldehyde group. Then the assays of sulfhydryl group in papain and in glutathione, which have free sulfuhydryl group, were carried out by the method of Ellman with DTNB.[7] The results obtained by this method were rather semiquantitative, but it is clear that the decrease of sulfhydryl content took place in alloxan-, barbital- and hydantoin–inhibition, and no change of sulfhydryl content took place in parabanate-inhibition as compared with control, as shown in Table I.

Discussion

At present, it is recognized that a sulfhydryl group plays an essential role in enzymatic action of papain. Recently, Morihara proposed a new model of the active site of papain.[8] The most distinctive feature of this model is that the active site is composed of both sulfhydryl and aldehyde groups which form partially a thiohemiacetal, and at the equilibrium state, a thiohemiacetal form predominates. However, this model is based on the assumption that an aldehyde group readilly available for the enzymatic reaction is involved in papain as suggested by Bergmann and Ross.[9] But the presence of the aldehyde group in papain has not

7) G.L. Ellman, *Arch. Biochem. Biophys.*, **74**, 443 (1958); *idem*, *ibid.*, **82**, 70 (1959).
8) K. Morihara, *J. Biochem.* (Tokyo), **62**, 250 (1967); K. Morihara and K. Nagami, *ibid.*, **65**, 321 (1969).
9) M. Bergmann and W.F. Ross, *J. Biol. Chem.*, **111**, 659 (1935); *idem*, *ibid.*, **114**, 717 (1936).

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[a]	100	100	100	100	100	100	100	100
Allantoin	100	100	100	100	96	99	99	99
Alloxan	12	12	12	12	72	36	89	52
Parabanic acid	84	57	100	100	99	94	101	102
Succinimide	100	100	100	100	96	97	100	100
Control[b]	100	100	100	100	100	100	100	100
Barbital	86	68	100	100	62	52	88	48
Hydantoin	87	67	100	100	77	58	89	39

a) for allantoin, alloxan, parabanic acid and succinimide
b) for barbital and hydantoin
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

been directly identified as yet, though Morihara, *et al.* refered it in their inhibition studies that the indole ring of a tryptophanyl residue possesses some chemical properties as a carbonyl group.[8]

The results may indicate that alloxan, barbital and hydantoin were inhibitory because it reacted with the sulfhydryl group, as shown in Table I. But, on treatment with cysteine-activated papain, alloxan showed the similar inhibitory effect as compared with cyanide-activated papain, but barbital and hydantoin did not show inhibition. Soejima and Shimura reported that some poly-carbonyl compounds, such as alloxan, dehydroascorbic acid and quinones which are the vinylogs of diketone, exhibited the inhibitory effects on papain.[10] From this reason, it may be concluded that inhibitory effect with alloxan does not depend on the nature of active imide group, but of poly-carbonyl compound to react with sulfhydryl group, and, on the other hand, that with barbital and hydantoin depend on the nature of active imide group to react with sulfhydryl group. Then, it may be thought that the reactivity of poly-carbonyl compound for sulfhydryl group of papain is much greater than that for free sulfhydryl group, and, on the contrary, the reactivity of active imide group for free sulfhydryl group is much greater than that for sulfhydryl group of papain. Therefore, it can be explained that alloxan showed the inhibitory effect on treatment with cysteine-activated papain, but barbital and hydantoin did not show inhibition.

On the other hand, the cyanide-activated papain was apparently inhibited by parabanic acid, and the cysteine-activated papain was hardly affected by this reagent, such as barbital and hydantoin. But the sulfhydryl content of papain and glutathione did not change when papain and glutathione was treated with parabanic acid. In order to explain this result, it is necessary to investigate moreover.

10) M. Soejima and K. Shimura, "Symposia on Enzyme Chemistry," Vol. XVIII, Nankodo Inc., Tokyo, 1962, p. 76.