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Studies on Inactivation of Papain by Ascorbic Acid in the Presence of Cupric Ions[1)]

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The mechanism of inactivation of papain by ascorbic acid in the presence of cupric ions was investigated.

Dehydroascorbic acid and hydrogen peroxide which were oxidative product of ascorbic acid, were much less effective. In addition, bubbling nitrogen gas and radical scavengers prevented the inactivation by ascorbic acid and cupric ions. The results indicated that the inactivating effect of ascorbic acid was oxygen dependent and caused by free radicals formed during the autoxidation of ascorbic acid.

The N-terminal amino acid and polyacrylamide disc gel electrophoretical patterns of the inactivated papain did not so differ from those of the native papain. On the other hand, the amino acids composition and fluorescence spectrum were changed in the inactivated papain as compared with the intact papain. These facts suggest that the cleavage of the S-S bond of papain molecule produced by the treatment with ascorbic acid and cupric ions resulted in inactivation.

Keywords——papain; inactivation; ascorbic acid; cupric ion; radical scavenger; N-terminal amino acid; fluorescence

Although the inactivation effect of ascorbic acid in the presence of cupric ions on enzyme activity has been recognized by many investigators with various enzymes,[3)] the mechanisms of the inactivation have not yet been clear.

Free amino acids including histidine,[4)] tryptophan,[5)] methionine[6)] and phenylalanine[7)] were found to be decomposed by ascorbic acid and cupric ions. In addition, Homma *et al.*[8)] have recently reported that the release of peptide and amino acid was occurred in ovalbumin in coupled with autoxidation of ascorbic acid. However, very little definite information is available about the structural change of the inactivated enzyme by the treatment with ascorbic acid and cupric ions.

From the above point of view, our interest is focused on the examination of the relationship between structural change and inactivation of enzyme by ascorbic acid and cupric ions. The choice of papain as an enzyme in this study was made from the reasons that papain was extensively studied on the catalytic properties and amino acid composition, and easily obtainable in pure form.

The present paper describes the factors affecting the inactivation and structural change of papain by the treatment with ascorbic acid and cupric ions.

1) A part of this research was presented at the 27th Meeting of Kinki Branch, Pharmaceutical Society of Japan, Kobe, Nov., 1977.
2) Location: 5 *Nakauchicho, Misasagi, Yamashina-ku, Kyoto, 607, Japan.*
3) L.W. Mapson, *Biochem. J.*, **40**, 240 (1946); A.W. Rowe and C.E. Weill, *J. Am. Chem. Soc.*, **81**, 921 (1959); A.W. Rowe and C.E. Weill, *Biochim. Biophys. Acta*, **65**, 245 (1962); C.W.M. Orr, *Biochem. Biophys. Res. Commun.*, **23**, 854 (1966); C.W.M. Orr, *Biochemistry*, **6**, 3000 (1967).
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Experimental

Enzyme——Papain was prepared by the procedure of Kimmel and Smith[9] and its concentration was determined spectrophotometrically using a value of $E_{1\%}^{1.0cm}=24.0$ at 280 nm[10] and a molecular weight of 23400.[10] Mercuripapain was purchased from Sigma Chemical Co., St. Louis.

Materials——α-N-Benzoyl-DL-arginine-*p*-nitroanilide (BAPA), L-ascorbic acid and cupric sulfate were purchased from Wako Pure Chemicals. Dehydroascorbic acid was obtained from ICN Pharmaceuticals, Inc. U.S.A. All other chemicals were of the highest purity available and were used without further purification.

Enzyme Assay——The enzyme activity was routinely assayed using BAPA as substrate, as follows; To 0.4 ml of the activating mixture,[11] 0.2 ml of an enzyme solution and 0.4 ml of citrate buffer (pH 6.0) were added and the mixture was incubated at 37° for 30 min. To 1.4 ml of citrate buffer (pH 6.0) containing 5×10^{-4} M BAPA was added 0.1 ml of the activated enzyme soultion described above. After incubation for 15 min, the reaction was stopped by the addition of 1.5 ml of 0.1 M Na_2CO_3. The enzyme activity was determined by measuring the increase in absorbance at 410 nm.[12]

Inactivation Procedure——A typical reaction mixture contained 1.2×10^{-4} M papain, 5×10^{-3} M ascorbic acid and 5×10^{-5} M cupric sulfate in 1.0 ml of 0.2 M acetate buffer, pH 6.0. At various times, aliquots were withdrawn and assayed for the enzyme activity described above.

Polyacrylamide Disc Gel Electrophoresis——Disc gel electrophoresis was carried out with 7.5% polyacrylamide gel at pH 4.0 of glycine-Tris buffer according to the method of Davis,[13] at a constant current of 3 mA/tube for 90 min. SDS-Polyacrylamide gel electrophoresis was performed by the method of Weber and Osborn[14] under a constant current of 8 mA/tube for 4 hr. The gels were stained with 0.25% Coomassie brilliant blue R. The protein samples for SDS-polyacrylamide gel electrophoresis were prepared as follows. The reaction mixture containing ascorbic acid, cupric ions and papain was fractionated by passing through a Sephadex G-25 column equilibrated with 0.1 M acetic acid. The protein fractions free from ascorbic acid and cupric ions were lyophilized. The lyophilized material was subjected to performic acid oxidation by the method of Sanger.[15]

Fluorescence Measurement——Fluorescence was measured at a constant temperature of 37° using a Shimadzu RF-502 spectrofluorometer equipped with a Shimadzu U-125 MO recorder in a quartz cell of 1×1 cm light path.

Amino Acid Analysis——Samples for amino acid analysis were hydrolyzed with 6 N HCl at 110° for 24 hr in evacuated sealed tubes. Amino acid analysis was performed with a Hitachi 034 liquid chromatograph by the procedure of Spackman *et al.*[16] Tryptophan content was determined spectrophotometrically[17] and also by the method of Spies and Chambers with PDAB.[18]

N-Terminal Amino Acid——The reaction mixture incubated for 60 min at 37° was adjusted to pH 3.0 with acetic acid. The reaction mixture was fractionated by passing through a Sephadex G-25 column equilibrated with 0.1 M acetic acid. The fractions containing ascorbic acid were again applied on a Dowex 50×8 column (1.3×10 cm), and the column was washed with water enough to remove ascorbic acid. Adsorbed peptide was eluted with 2 M pyridine acetate buffer, pH 7.0. The eluate without ascorbic acid from the Sephadex column and the peptide adsorbed on the Dowex column were combined and then lyophilized. The lyophilized material was converted into 2,4-dinitrophenyl (DNP) derivatives by Sanger's method.[19] DNP-Derivative was hydrolyzed with 2 ml of 6 N HCl for 24 hr at 110°, and the released DNP-amino acid was extracted with ether and ethyl acetate, successively. Amino acid was released after heating DNP-amino acid with 33% ammonia in a sealed tube for 3 hr at 100° and the resultant solution was dried up under reduced pressure. The released amino acid was analyzed using a Hitachi 034 liquid chromatograph.

Results

Inactivation of Papain by Ascorbic Acid and Cupric Ions

When papain was incubated in acetate buffer (pH 6.0) containing ascorbic acid and cupric ions, inactivation of papain was observed. No significant inactivation was, however,

9) J.K. Kimmel and E.L. Smith, *J. Biol. Chem.*, **207**, 515 (1954).
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14) K. Weber and M. Osborn, *J. Biol. Chem.*, **244**, 4406 (1969).
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16) D.H. Spackman, W.H. Stein, and S. Moore, *Anal. Chem.*, **30**, 1190 (1958).
17) W.L. Bencze and K. Schmid, *Anal. Chem.*, **29**, 1193 (1957).
18) J.S. Spies and D.C. Chambers, *Anal. Chem.*, **21**, 1249 (1949).
19) F. Sanger, *Biochem. J.*, **39**, 507 (1945).

observed when ascorbic acid or cupric ions was omitted from the reaction mixture. The results are shown in Fig. 1. To examine the effect of the other metal ions, transition matel ions were added to the reaction mixture instead of cupric ions and the inactivation of papain by ascorbic acid was measured. The results were summarized in Table I. Although the inactivation of papain by ascorbic acid was markedly accelerated by the addition of cupric ions, all of the other cations tested had no effect. Therefore, the requirement for cupric ions for such inactivation is highly specific.

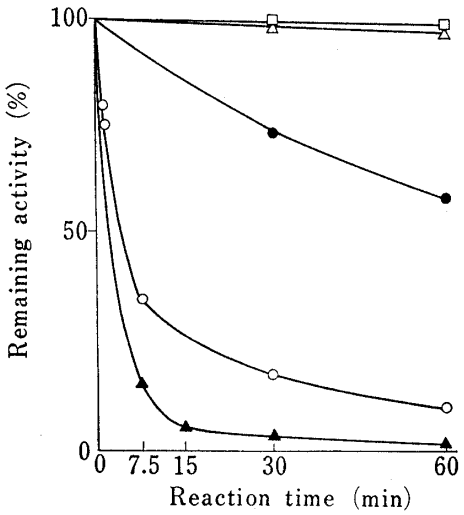

Fig. 1. Inactivation of Papain in the Reaction with Ascorbic Acid and Cupric Ions

Papain (1.2×10^{-4} M) was incubated with ascorbic acid (5×10^{-3} M) and cupric ions in 0.2 M acetate buffer (pH 6.0) at 37°.
—□—: papain+ascorbic acid,
—△—: papain+cupric ions (5×10^{-4} M),
—●—: papain+ascorbic acid+cupric ions (5×10^{-6} M),
—○—: papain+ascorbic acid+cupric ions (5×10^{-5} M),
—▲—: papain+ascorbic acid+cupric ions (5×10^{-4} M).

Effectors on the Inactivation of Papain by Ascorbic Acid and Cupric Ions

To obtain some information about the factors involved in the inactivation of papain by ascorbic acid and cupric ions, the following experiments were carried out.

Table I. Effect of Metal Ions on Inactivation of Papain in the Reaction with Ascorbic Acid

Metal ion	Remaining activity (%)
None	97
Cu^{2+}	11
Fe^{2+}	85
Fe^{3+}	87
Zn^{2+}	89
Ni^{2+}	97
Mn^{2+}	93
Co^{2+}	93

Papain (1.2×10^{-4} M) was incubated with ascorbic acid (5×10^{-3} M) and metal ions (5×10^{-5} M) in 0.2 M acetate buffer (pH 6.0) for 60 min at 37°.

a) Effect of pH——Papain was incubated with ascorbic acid in the presence of cupric ions, which were dissolved in buffer solutions having various pH values, at 37°. As shown

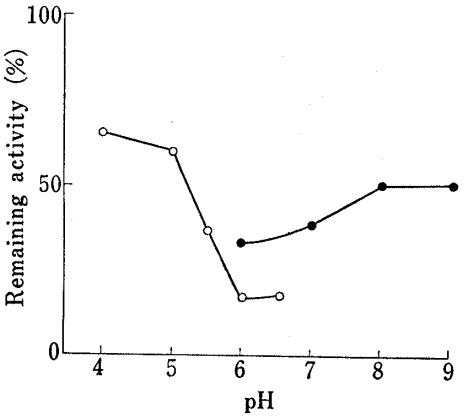

Fig. 2. Effect of pH on Inactivation of Papain in the Reaction with Ascorbic Acid and Cupric Ions

Papain (1.2×10^{-4} M) was incubated with ascorbic acid (5×10^{-3} M), and cupric ions (5×10^{-5} M) in 0.2 M acetate buffer (—○—) or 0.2 M Tris-acetate buffer (—●—) for 30 min at 37°.

Table II. Effect of Radical Scavengers on Inactivation of Papain in the Reaction with Ascorbic Acid and Cupric Ions

Scavenger	Concentration (M)	Remaining activity (%)
None	0	34
Potassium iodide	10^{-2}	72
Potassium iodide	10^{-3}	40
Dimethyl sulfoxide	10^{-2}	40
Ethyl alcohol	10^{-2}	33
Sodium formate	10^{-2}	32

Papain (1.2×10^{-4} M) was incubated with ascorbic acid (5×10^{-3} M), cupric ions (5×10^{-5} M) and scavengers in 0.2 M acetate buffer (pH 6.0) for 10 min at 37°.

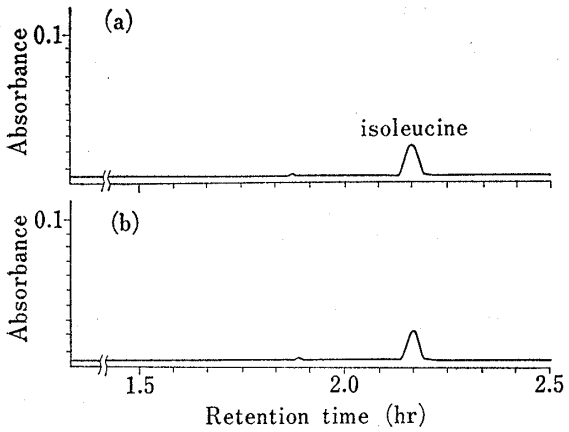

Fig. 3. Determination of N-Terminal Amino Acid of Ascorbic Acid Treated Papain in the Presence of Cupric Ions

(a) inactivated papain.
(b) intact papain.
Papain (1.2×10^{-4} M) was incubated with ascorbic acid (5×10^{-3} M) and cupric ions (5×10^{-5} M) in 0.2 M acetate buffer (pH 6.0) for 60 min at 37°. The detail was described in "Experimental".
Absorbance was determined at 570 nm after ninhydrin reaction.

in Fig. 2, the inactivation was dependent on pH. The rate was most rapid at pH 6.0 and ascorbic acid in the presence of cupric ions in acetate buffer was more effective than that in Tris-acetate buffer.

b) Effect of Oxygen——Under anaerobic condition where nitrogen gas was bubbled through the reaction mixture, inactivation of papain by ascorbic acid and cupric ions was not observed.

c) Effect of Radical Scavengers——Radical scavengers were added to the reaction mixture. As shown in Table II, dimethyl sulfoxide and potassium iodide prevented the inactivation of papain but ethyl alcohol and sodium formate did not prevent the inactivation of papain by ascorbic acid and cupric ions.

d) Effect of Dehydroascorbic Acid and Hydrogen Peroxide——The mechanism of the

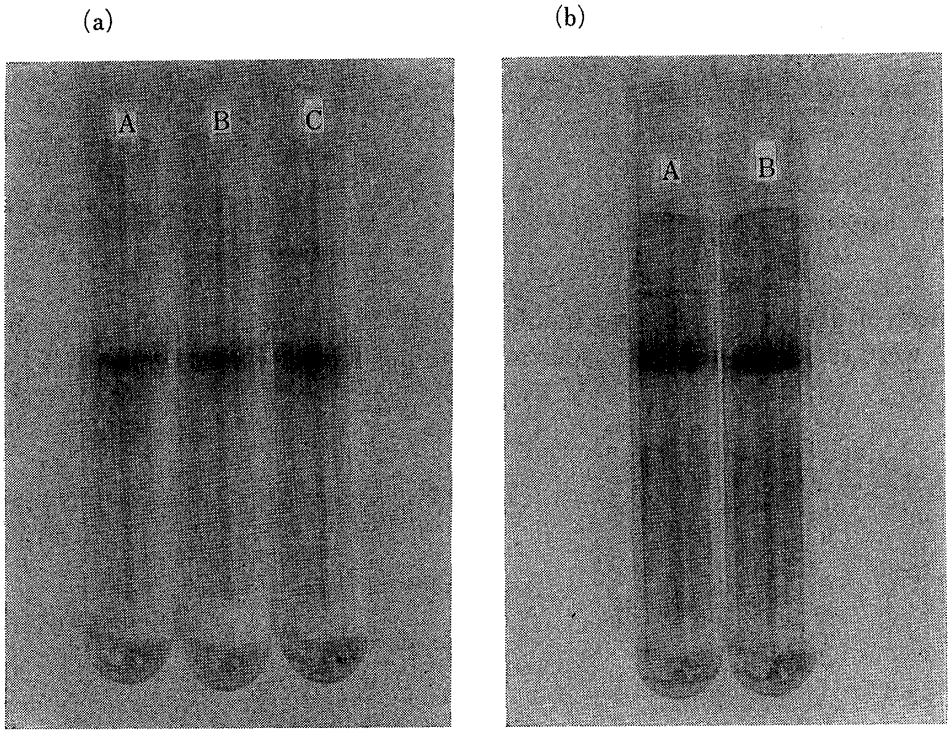

Fig. 4. Polyacrylamide Gel Electrophoretical Patterns

(a) SDS disc gel electrophoresis.
A: intact papain,
B: inactivated papain,
C: the equivalent weight mixed of A and B.
(b) polyacrylamide gel electrophoresis.
A: intact papain,
B: inactivated papain.
Papain (1.2×10^{-4} M) was incubated with ascorbic acid (5×10^{-3} M) and cupric ions (5×10^{-5} M) in 0.2 M acetate buffer (pH 6.0) for 60 min at 37°. The protein samples were subjected to electropholesis on 10% polyacrylamide gel containing 0.1% SDS. The enzyme (15 μg as protein) was applied to a tube. Conditions of electrophoresis are described in the text.

oxidation of ascorbic acid in the presence of cupric ions may be described by the following steps at around pH 6.0[20];

$$AH_2 \rightleftarrows AH^- + H^+ \quad (I)$$

$$Cu^{2+} + AH^- \rightleftarrows CuAH^+ \quad (II)$$

$$CuAH^+ \longrightarrow Cu^+ + AH\cdot \quad (III)$$

$$AH\cdot + Cu^{2+} \longrightarrow Cu^+ + A + H^+ \quad (IV)$$

$$2Cu^+ + 2H^+ + O_2 \longrightarrow 2Cu^{2+} + H_2O_2 \quad (V)$$

where AH^- represents the monoionic ascorbic acid, $CuAH^+$ the cupper-ascorbic acid complex, $AH\cdot$ the monodehydroascorbic acid radicals and A the dehydroascorbic acid. When dehydroascorbic acid and hydrogen peroxide which were oxidative product of ascorbic acid were added to the reaction mixture, inactivation of papain was not observed.

Structural Changes of Inactivated Papain

To obtain some information about the mechanism of inactivation of papain by ascorbic acid and cupric ions, the structural change of the inactivated papain by ascorbic acid and cupric ions was examined.

a) Determination of N-Terminal Amino Acid——N-Terminal amino acids of the inactivated papain were determined as described in "Experimental". As shown in Fig. 3, the N-terminal residue of inactivated papain was identified as isoleucine, which was the same as that in intact papain.

Table III. Amino Acid Composition of Ascorbic Acid Treated Papain in the Presence of Cupric Ions

Amino acid[a]	Papain	
	Intact	Treated
Tryptophan[b]	4.3	4.3
Lysine	9.6	9.7
Histidine	1.6	1.8
Arginine	12.0	12.0
Cysteic acid	0	0.6
Aspartic acid	19.3	19.1
Threonine	8.2	8.4
Serine	12.3	12.4
Glutamic acid	21.8	21.6
Proline	10.5	10.6
Glycine	29.3	29.2
Alanine	14.2	14.4
Cystine (half)	6.9	5.8
Valine	17.4	17.3
Methionine	0	0
Isoleucine	11.3	11.2
Leucine	11.0	11.0
Tyrosine	18.4	18.5
Phenylalanine	4.4	4.4

Papain (1.2×10^{-4} M) was incubated with ascorbic acid (5×10^{-3} M) and cupric ions (5×10^{-5} M) in 0.2 M acetate buffer (pH 6.0) for 60 min at 37°.

a) The values in the table denote number of residues per protein molecule, assuming the number of leucine residue to be 11.0 and the number of arginine residue to be 12.0. No correction was made for decomposition during acid hydrolysis.

b) Tryptophan content was determined spectrophotometrically[17] and also by the method of Spies and Chambers.[18]

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b) Polyacrylamide Disc Gel Electrophoresis——SDS-Polyacrylamide disc gel electrophoresis was performed with the inactivated papain as compared with the intact papain. As shown in Fig. 4(a), one protein band is revealed in common in the gels. Moreover, polyacrylamide gel electrophoresis without SDS was carried out with the same sample and the results are represented in Fig. 4(b). The electrophoretical patterns of the gels for the inactivated papain did not so differ from that of the intact papain.

c) Amino Acid Analysis——The amino acid compositions of intact and inactivated papain, are summarized in Table III. It was found that cystine was converted to cysteic acid by the prolongation of reaction time of inactivation. The other amino acids were not affected by the inactivation in this system. Furthermore, inactivation of mercuripapain in which the reactive SH-group is blocked by mercuric ion was examined. Mercuripapain was also inactivated, and cystine was converted to cysteic acid. These data indicate that cleavage of the S–S bond of papain molecule occurs by the ascorbic acid–cupric ions system.

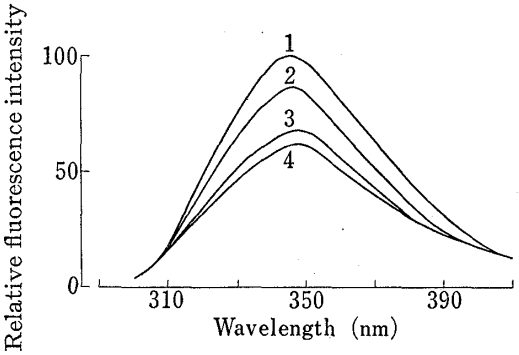

Fig. 5. Emission Spectra of Ascorbic Acid Treated Papain in the Presence of Cupric Ions

Papain (1.2×10^{-4} M) was incubated with ascorbic acid (5×10^{-3} M) and cupric ions (5×10^{-5} M) in 0.2 M acetate buffer (pH 6.0) at 37°.
1: 0 min, 2: 2 min, 3: 10 min, 4: 20 min.

d) Fluorometric Measurement——The fluorescence spectra of the reaction mixture containing papain were measured at 295 nm. The results are shown in Fig. 5. The fluorescence spectrum showed an emission maximum at 345 nm. The fluorescence emission spectra of the inactivated papain were shifted with a slight displacement to longer wevelengths and the fluorescence intensity decreased with elongation of the reaction time. On the other hand, tryptophan content was not changed in the inactivated papain as compared with the intact papain as described in the former section. Therefore, these changes are understandable if ascorbic acid in the presence of cupric ions deforms the protein conformation which provides the specific quenching environment of tryptophan residue.

Discussion

The following is an analysis of the results on the reaction mechanism involved in the inactivation of papain by ascorbic acid in the presence of cupric ions.

It has been shown that the inactivation was dependent on pH and the addition of cupric ions, which is known to exert a catalytic effect upon the autoxidation of ascorbic acid. In contrast, removal of oxygen by bubbling nitrogen gas through the reaction mixture prevented the inactivation. The formation of free radical is known to be generated during the reaction of hydrogen peroxide and cupric ions.[20] In addition, both dehydroascorbic acid and hydrogen peroxide, products formed from ascorbic acid in the presence of cupric ions and oxygen, were much less effective. Radical scavengers such as dimethyl sulfoxide and potassium iodide prevented the inactivation of papain. These results indicate that the inactivation of papain by ascorbic acid in the presence of cupric ions is due to free radical transients, known to be generated during the oxidation of ascorbic acid, and that the most likely candidates are hydroxyradical, hydroperoxyradical and monodehydroascorbic acid radicals. Recently, Lin *et al.*[21] reported that the oxidation of SH-group of papain by superoxide radical has been postulated during radiolysis under aerobic condition. However, the efficiency of papain

21) W.S. Lin, J.R. Clement, G.M. Gaucher, and D.A. Armstrong, *Radia. Res.*, **62**, 438 (1975).

inactivation by superoxide radical was much less effective in the ascorbic acid–cupric ions system.

The next problem is to elucidate the target in papain molecules attacked by ascorbic acid and cupric ions. The papain inactivated by ascorbic acid in the presence of cupric ions was not altered in N-terminal amino acid as compared with the intact papain. In addition, SDS–polyacrylamide disc gel electrophoretic and disc electrophoretic patterns on the gels of the inactivated papain did not so differ from those of the intact papain. On the other hand, the stage of about 90% inactivation, 0.6 residue of cysteic acid was detected per molecule of papain and the other amino acid composition was not affected by the inactivation in this system. Mercuripapain was also inactivated by ascorbic acid in the presence of cupric ions and about 0.5 residue of cysteic acid was detected per molecule of mercuripapain. Therefore, it is suggested that the inactivation of papain was not due to the modification of the essential SH-group of papain. In addition, the fluorescence spectrum of the native papain at pH 6.0 has a peak at 345 nm and the peak shifted to longer wavelength by the reaction with ascorbic acid in the presence of cupric ions. From the results described above, one of the most possible illustration on the inactivation of papain by ascorbic acid in the presence of cupric ions is found in the cleavage of the S–S bond of papain molecule and not in the cleavage of the peptide bond.

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