On the Mechanism of Inactivation of Papain by Hydroxylamine

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The mechanism of inactivation of papain (EC 3.4.4.10) by hydroxylamine was investigated. The inactivation was pH-dependent and the rate was rapid at alkaline pH. Nitrogen gas bubbling through the reaction mixture or addition of radical scavengers to the reaction mixture largely prevented the inactivation. The inactivation was accompanied by a decrease of the essential sulfhydryl group of papain. Treatment of inactivated papain with 2-mercaptoethanol led to partial reactivation. The inactivated papain was judged not to be dimeric on the basis of molecular weight determination.

Based on these results, it is likely that hydroxylamine inactivates papain indirectly by reacting with O_2 to produce some reactive species which modifies the essential sulfhydryl group of the enzyme.

Keywords papain; hydroxylamine; radical; radical scavenger; autooxidation; inactivation

Papain (EC 3.4.4.10) is a well known thiol protease and posesses a single essential sulfhydryl (SH) group.[1] It has been reported that papain is inactivated by aldehyde reagents including (bi)sulfite and hydroxylamine[2] and that the inactivation is due to reaction with an aldehyde-like group in the protein molecule.[3]

We have previously reported that the inactivation of papain by (bi)sulfite involves modification of the essential SH group by reactive species produced during the aerobic oxidation of (bi)sulfite.[4] The mechanism of inactivation by hydroxylamine, however, has not yet been clarified.

It has been reported that hydroxylamine inactivates deoxyribonucleic acid (DNA),[5] cleaves the backbone of DNA,[6] and releases bases from DNA.[7] These actions were proposed to be mediated by reactive species formed by reaction between hydroxylamine and O_2.

The present paper deals with the inactivation mechanism of papain by hydroxylamine.

Materials and Methods

Hydroxylamine hydrochloride was purchased from Nakarai Chemicals Ltd., Japan. Catalase from bovine liver (44000 unit/mg protein) and superoxide dismutase (SOD) from bovine erythrocytes (3050 unit/mg protein) were obtained from Sigma Chemical Co., U.S.A. All other chemicals used were as described previously.[4]

Crystalline papain was prepared by the method of Kimmel and Smith.[8] It is well known that the enzyme preparation obtained by this method contains an enzyme fraction in unreactived form. In the present study, therefore, papain fully activated by treatment with cysteine was used. The cysteine-activated papain was prepared as described previously.[4]

Protein concentration was determined spectrophotometrically using a value of $E^{1\,cm}_{1\%}=24.0$ at 280 nm and a molecular weight of 23400.[9] Papain activity was determined by measuring the rate of liberation of *p*-nitroaniline from *N*-α-benzoyl-DL-arginine-*p*-nitroaniline as described previously.[4]

Molecular weight determinations were made by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) and urea,[10] without reducing agent, and by molecular sieve chromatography on Sephadex G-100.[11]

Samples for amino acid analysis were hydrolyzed with 6 N HCl at 110 °C for 24 h in evacuated sealed tubes. For tryptophan analysis, protein samples were hydrolyzed with $Ba(OH)_2 \cdot 8H_2O$.[12] Amino acid analysis was performed with a Hitachi 835 amino acid analyzer. SH content was determined by the method of Boyer with *p*-chloromercuribenzoic acid.[13]

Results and Discussion

Inactivation of Papain by Hydroxylamine When papain was incubated with hydroxylamine in phosphate buffer (pH 8.0) at 37 °C, the enzyme was inactivated progressively as shown in Fig. 1. The inactivation was dependent on pH. The rate of inactivation was rapid at alkaline pH: when the reaction rate at pH 8.5 was taken as 1.0, the reaction rates at pH 8.0, 7.6, 7.1, 6.6 and 6.1 were 0.99, 0.79, 0.45, 0.14 and 0.07, respectively. The following experiments were carried out at pH 8.0 in 50 mM phosphate buffer.

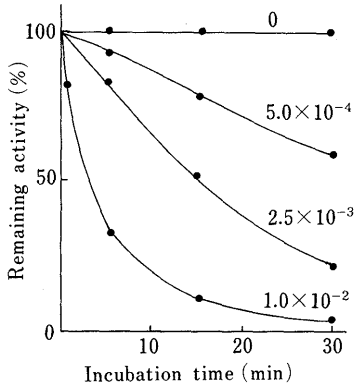

Fig. 1. Inactivation of Papain by Hydroxylamine

The enzyme (400 μg/ml) was incubated at 37 °C with the indicated concentrations of hydroxylamine (M) in 50 mM phosphate buffer (pH 8.0). Aliquots were withdrawn at the indicated time intervals and assayed as described in Materials and Methods.

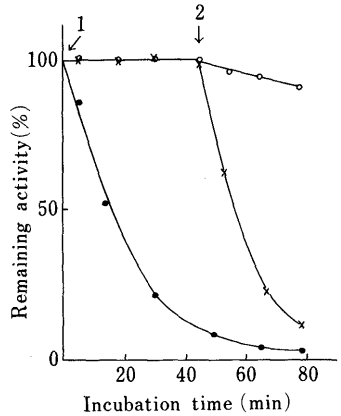

Fig. 2. Effect of Oxygen on the Inactivation of Papain by Hydroxylamine

The enzyme (400 μg/ml) was incubated at 37 °C with hydroxylamine (2.5×10^{-3} M) in 50 mM phosphate buffer (pH 8.0). Nitrogen gas and air bubbling were carried out through the incubation mixture at the times indicated by arrows 1 and 2, respectively. Aliquots were withdrawn at the indicated intervals and assayed as described in Materials and Methods.

○, control (papain) (with bubbling of N_2 gas and air); ×, papain + hydroxylamine (with bubbling of N_2 gas and air); ●, papain + hydroxylamine (without bubbling of N_2 gas and air).

Effectors Modifying the Inactivation of Papain by Hydroxylamine The role of molecular oxygen was tested by performing the reaction under a nitrogen atmosphere. As shown in Fig. 2, significant inactivation by hydroxylamine was not observed under anaerobic conditions with nitrogen gas bubbling through the reaction mixture. The enzyme, however, was greatly inactivated when the reaction mixture was made aerobic by bubbling air through it. The results suggest the participation of oxygen in the inactivation on papain by hydroxylamine. The inactivation under aerobic conditions with air bubbling through the reaction mixture was faster than that under aerobic conditions without air bubbling (Fig. 2). This finding also suggests the participation of oxygen in the inactivation.

The actions of various substances on the inactivating effect of hydroxylamine are summarized in Table I. Among radical scavengers tested, sulfhydryl compounds and catalase significantly prevented the inactivation of papain by hydroxylamine. The inhibitory effect of catalase may suggest that the inactivation mechanism involves H_2O_2. It has been reported that the oxidation of hydroxylamine apparently proceeds according to the following overall reaction.[5,14]

$$NH_2OH + O_2 \rightarrow HNO + H_2O_2$$

In addition, *N*-methyl-hydroxylamine also gives rise to peroxide, whereas *O*-methyl-hydroxylamine does not.[5] The inactivating effects of *N*-methyl-hydroxylamine and *O*-methyl-hydroxylamine on papain were examined. When papain was incubated with 2.5 mM hydroxylamine and its derivatives in phosphate buffer (pH 8.0) for 30 min at 37 °C, the activity of papain was reduced by hydroxylamine and *N*-methyl-hydroxylamine to 20% and 65% of the initial activity, respectively, but was not reduced by *O*-methyl-hydroxylamine. These findings also suggest that the free-NOH group is necessary and production of hydrogen peroxide is essential for the inactivation of papain.

Amino Acid Analysis of Hydroxylamine-Treated Papain To obtain some information about the mechanism of inactivation of papain by hydroxylamine, amino acid analyses of hydroxylamine-treated papain were performed. The amino acid compositions of native and hydroxylamine-treated papains with an activity of only 2.5% of that of native enzyme are summarized in Table II. The most significant change was found in the content of SH group. No appreciable change was found in the contents of the other amino acids, which are not listed in the table. In addition, the correlation between disappearance of SH group and inactivation of papain by treatment with hydroxylamine was examined. As shown in Fig. 3, the extent of the inactivation and the disappearance of SH group were in parallel. The above results suggest that the inactivation is a consequence of modification of the essential SH group of papain.

There is a possibility that the SH group of papain was oxidized directly by reactive species produced in the reaction medium, such as H_2O_2 and $OH\cdot$,[15] to -S-S-, -SOH, $-SO_2H$, or $-SO_3H$. The reactivation of hydroxylamine-inactivated papain by reduction under mild conditions was therefore examined. Papain (400 μg/ml) was incubated with hydroxylamine (10 mM) in phosphate buffer (50 mM, pH 8.0) for 10 min at 37 °C. Then, the hydroxylamine-treated papain with 30% of the initial activity was treated with 2-mercaptoethanol (0.1 M) at 37 °C for 30 min. The treatment resulted in restoration of the activity to 60% of

TABLE I. Effect of Various Substances on the Inactivation of Papain by Hydroxylamine

Substance added	Conc. (M)	Relative activity (%)
None		100
NH_2OH	2.5×10^{-3}	18
+cysteine	5.0×10^{-5}	50
	1.0×10^{-4}	90
	8.0×10^{-4}	103
+*S*-carboxymethyl cysteine	12.5×10^{-2}	22
+dithiothreitol	5.0×10^{-5}	71
	1.0×10^{-4}	102
+catalase	10 μg/ml	62
	100 μg/ml	93
+boiled catalase	100 μg/ml	23

KBr (2.5×10^{-2} M), mannitol (2.5×10^{-2} M), guanosine (4×10^{-3} M), mannose (1×10^{-2} M), Tiron (2.5×10^{-3} M), SOD (25 μg/ml), methionine (2.5×10^{-2} M), histidine (2.5×10^{-2} M), and NaSCN (1×10^{-2} M) had no protective effect against the inactivation of papain by hydroxylamine. Boiled catalase: catalase heat-treated in boiling water for 10 min.

TABLE II. Amino Acid Composition and SH Group Content of Hydroxylamine-Treated papain

Amino acid[a] and SH	Amino acid residues and SH (mol/mol of protein)	
	Native	Hydroxylamine-treated
Trp	5.2	4.9
Tyr	18.9	18.6
His	2.2	2.2
Phe	4.6	4.5
Met	0	0
SH	1.0	0.07
Remaining activity	100	2.5

Papain (400 μg/ml) was incubated with hydroxylamine (2.5×10^{-3} M) in phosphate buffer (50 mM, pH 8.0) at 37 °C for 60 min. After removal of the reagents by passage of the reaction mixture through a Sephadex G-25 column, protein fractions were analyzed for amino acid composition, SH group and remaining activity as described in Materials and Methods. *a*) The values in the table denote the numbers of residues per protein molecule, assuming the number of leucine residues to be 11.0 and the number of arginine residues to be 12.0. No correction was made for decomposition during hydrolysis.

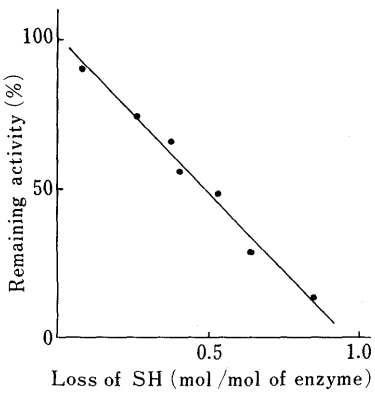

Fig. 3. Relationship between Inactivation and the Loss of SH Group of Papain Caused by Hydroxylamine

The enzyme (533 μg/ml) was incubated with hydroxylamine (6.6 mM) at 37 °C. After appropriate intervals of incubation, aliquots were withdrawn and SH contents and enzyme activity were assayed as described in Materials and Methods.

the initial activity. It is thought that -SOH can reduced to -SH by 2-mercaptoethanol treatment, but $-SO_2H$ and $-SO_3H$ can not. When acid-hydrolyzed samples of native and hydroxylamine-treated papains were applied to an amino acid analyzer, a small peak at the retention time corresponding to cysteic acid was observed on chromatograms from both papain samples. However, the peak observed in the case of hydroxylamine-treated papain was larger than that observed in the case of native papain. In addition, the peak observed with the papain sample inactivated to 2.5% of the initial activity by hydroxylamine was larger than that observed with the enzyme sample inactivated to 50%. It was not certain that the above small peak truly represented cysteic acid, since some unknown substances formed by acid hydrolysis of protein were also eluted at the same retention time as that of cysteic acid under the analytical conditions employed in this study. The above findings, however, raise the possibility that a part of the SH group of papain may be oxidized to $-SO_3H$. In fact, when cysteine (1 mM) was incubated with hydroxylamine (10 mM) in phosphate buffer (50 mM, pH 8.0) at 37 °C for 2 d, formation of a significant quantity of cysteic acid was observed by using an amino acid analyzer.[16] If the decrease of SH group was due to the formation of disulfide bond, one would expect an increase in molecular weight, because the enzyme contains only a single SH group per molecule. Molecular weight determination for hydroxylamine-inactivated papain with 2.5% of the initial activity was done by both the SDS-polyacrylamide gel electrophoresis method without reducing agent and the molecular sieve method. The gel filtration and electrophoretic patterns of native and hydroxylamine-treated papains showed that there was no significant difference in molecular weight between the two proteins (data not shown). This indicates that the inactivation does not involve disulfide bond formation.

It has been postulated that peroxide formation probably involves radicals which catalyze chain reactions similar to the autooxidation of aldehyde.[15] Possible radical intermediates would be $OH\cdot$, $NH_2O\cdot$, $OHNHOO\cdot$, $N\cdot OH^-$ and $O\text{-}N\cdot HOO^-$. There may also be a possibility of formation of adduct between the SH group of papain and the above radical intermediates.

In conclusion, the inactivation of papain by hydroxylamine is indirectly caused by modification of the essential SH group in papain by some products of the reaction between hydroxylamine and O_2.

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