Photosensitized Inactivation of Stem Bromelain. Oxidation of Histidine, Methionine, and Tryptophan Residues†

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ABSTRACT: Pineapple stem bromelain was photooxidized in the presence of Methylene Blue used as a sensitizer. The essential sulfhydryl group of the enzyme protein rapidly became inaccessible to react with 5,5′-dithiobis(nitrobenzoic acid), but the reactivity was readily regained to the original level upon treatment with dithiothreitol. Even after such reduction, the photooxidized enzyme showed a markedly decreased hydrolytic activity on casein. Spectral examination revealed that the oxidized enzyme had tyrosine residues intact. Amino acid analysis showed significant decreases in histidine, methionine, and tryptophan residues. Photoinactivation occurred in a similar manner also in the presence of tetrathionate which reversibly blocked the essential sulfhydryl group. It is concluded that the irreversible photoinactivation of stem bromelain must be related to the oxidation of histidine, methionine, and tryptophan residues. When the photooxidation was carried out at different pH values ranging from 4.0 to 8.3, the inactivation and the decrease in histidine content were found to be markedly pH dependent. Thus, the photooxidation experiment provided a method for directly measuring the apparent pK_a of the ionization of the single histidine residue in stem bromelain. Apparent pK_a values of 6.4 and 7.1 were obtained for the histidine imidazole in the absence and in presence of tetrathionate, respectively. In view of these *normal* pK_a values for an imidazole, a mechanism of ionization of the active-site group in a plant thiol proteinase is proposed, in which the validity of mechanism involving a close electronic interaction between histidine and cysteine residues is seriously questioned.

Pineapple stem bromelain has one reactive cysteine residue and one histidine residue per molecule of mol wt 28,000 (Takahashi *et al.*, 1973). This unique composition makes stem bromelain of much interest for studying the roles of these residues in catalysis. Unlike papain, stem bromelain is a glycoprotein (Yasuda *et al.*, 1970). We have demonstrated that its neutral sugar residues are not directly involved in the catalytic mechanism, since they could be oxidized by sodium periodate without appreciable change in the proteinase and esterase activities (Yasuda *et al.*, 1971). When periodate was used as an oxidant, it was necessary to protect the sulfhydryl group of the enzyme protein from oxidation. For this purpose the addition of an excess of sodium tetrathionate to the oxidation reaction mixture was found to be satisfactory, because after an oxidation under such conditions a full amount of the sulfhydryl was recovered upon treatment with dithiothreitol. Periodate oxidized, besides the neutral sugars, a methionine residue but not the histidine residue.

Based on these lines of experience an attempt was made to carry out a photooxidation of the histidine residue of stem bromelain in the presence of tetrathionate in the hope that the latter reagent could also protect the sulfhydryl from photooxidation. It was soon found that tetrathionate did protect the sulfhydryl, while the histidine, methionine, and tryptophan residues were oxidized, causing a marked inactivation of the enzyme. Furthermore, even in the absence of tetrathionate, the photosensitized oxidation of the sulfhydryl group was found to be reversed by a reducing agent like dithiothreitol, whereas the irreversible oxidation took place on histidine, methionine, and tryptophan residues. The latter findings seemed to be contrary to the prevailing concepts that the reactive sulfhydryl must be the first to be photooxidized and that such an oxidation is primarily responsible for the irreversible inactivation of the enzyme. The present paper describes the details of experiments which show that such a concept was not correct.

When the photooxidation of stem bromelain was carried out at different pH values ranging from 4.0 to 8.3, the inactivation as well as the loss of histidine content were found to be markedly pH dependent. Thus, this technique has permitted us to determine directly the apparent pK_a value for the ionization of the single histidine residue in the stem bromelain molecule. No previous reports have described an experimentally determined pK_a value for histidine imidazole group in thiol proteinase, although some abnormally high or low values were speculated (Drenth *et al.*, 1970; Lowe, 1970). The present paper also describes our experimental results demonstrating that the apparent pK_a value for the histidine residue in stem bromelain falls in a normal range pertaining to an imidazole group. A part of the latter findings together with some results on papain have been published in a preliminary form (Murachi and Okumura, 1974).

Materials and Methods

Materials. Stem bromelain, SB1, was purified from a crude preparation[1] (Bromelain, lot N-VI-1) by the method of Takahashi *et al.* (1973). Since the latter method involved blocking the essential sulfhydryl group of the enzyme with sodium tetrathionate, the final product had no proteolytic activity unless it was activated by, for example,

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[1] We are indebted to Dr. T. Ito, Jintan-Dole Co., Ltd., Osaka, for a generous supply of this material.

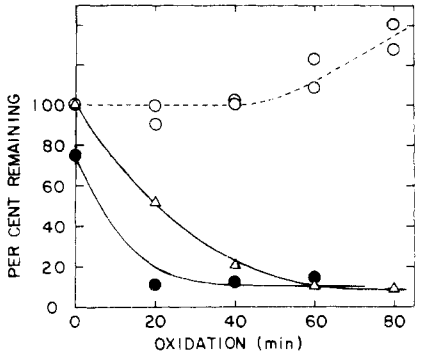

FIGURE 1: Changes in enzymatic activity and SH content of stem bromelain by photooxidation. Stem bromelain (160 μM) in potassium phosphate buffer (pH 8.0) was photooxidized with 16 μM Methylene Blue at 7°: (△) caseinolytic activity; (●) SH content determined directly; (○) SH content determined on the sample reduced by 5 mM dithiothreitol.

5 mM L-cysteine. When stem bromelain having an unblocked sulfhydryl group was needed, the tetrathionate-blocked enzyme preparation was first treated with 0.01 M cysteine and then the activator was removed by gel filtration under anaerobic conditions as described previously (Takahashi *et al.*, 1973). The protein concentration of the enzyme was determined by either measuring absorbance at 280 nm, $A_{1cm}^{1\%}$ = 20.1 (Murachi *et al.*, 1965), or by performing amino acid analyses (Takahashi *et al.*, 1973). α-*N*-Benzoyl-L-arginine ethyl ester (Bz-L-Arg-OEt) used as a substrate was obtained from the Protein Research Foundation, Minoh.

Assay of Enzymatic Activity. Proteinase activity was determined by a modified Kunitz method using casein as a substrate (Murachi, 1970). Esterase activity on 0.025 M Bz-L-Arg-OEt was determined, using a pH-Stat (Radiometer Model SBU2/ABU11/TTT1) at pH 6.5 and at 30° (Murachi, 1970).

Photooxidation. Photosensitized oxidation of stem bromelain was performed in essentially the same way as that of ribonuclease T_1 (Takahashi, 1970). The reaction mixture contained 15.8 or 160 μM stem bromelain, 6.85 or 16 μM Methylene Blue, respectively, and 0.1 M buffer of varying pH values in a total volume of 7.0 ml. The buffers used were: sodium acetate or sodium citrate–phosphate for pH 4.0–6.0, and potassium phosphate for pH 6.0–8.3. When needed, sodium tetrathionate was added to make a final concentration of 0.57 mM. In an experiment, the photooxidation was carried out in the presence of 5 mM Bz-L-Arg-OEt at pH 6.0. The reaction mixture was constantly stirred in a jacketed beaker, the temperature of the circulating water being kept at 7° unless otherwise noted. An incandescent lamp of 150 W was placed 25 cm above the surface of the reaction mixture which was exposed to open air. Control experiments were performed under identical conditions without illumination.

Amino Acid Analysis. The reaction mixture after photooxidation was filtered through a Sephadex G-25 column (1.7 × 17 cm; 40 ml) which had been equilibrated with 0.1 M ammonium bicarbonate, and the protein fractions were pooled and lyophilized. The protein sample thus obtained was hydrolyzed in an evacuated sealed tube with twice distilled hydrochloric acid at 105–110° for 20 hr. Alkali hydrolysis in 5 N KOH at 110° for 24 hr was also performed to obtain information concerning methionine, methionine sulfoxide, and tryptophan. For determination of tryptophan the following methods were also employed which involved enzymatic hydrolysis (Matsumura *et al.*, 1975). The sample protein (5–10 mg) in 1 ml of 0.05 M Tris-HCl buffer at pH 8.2 containing 0.1 mM $CaCl_2$ was heated at 100° for 5 min. Commercial thermolysin (Daiwa Kasei K.K., Osaka, Japan, Lot T2EB61, 8780 units/mg of protein) was added to make an enzyme:substrate ratio of 1:100 (w/w), and the mixture was incubated at 65° for 8 hr. After heating at 100° for 5 min, the digest was further incubated at 40° for 23 hr with $^1/_{100}$ weight of the aminopeptidase preparation obtained from *Bacillus subtilis* and with an addition of 1 mM $CoCl_2$. The aminopeptidase (14.7 units/mg of protein) was a gift from Dr. T. Yamamoto, Osaka City University, Faculty of Science, Osaka.[2] The enzymatic digestion was terminated by lowering the pH below 4.0. An aliquot was used for the direct amino acid analysis, and another aliquot was subjected to further hydrolysis by 3 N *p*-toluenesulfonic acid at 105–110° for 3 hr (Liu and Chang, 1971). Amino acid analysis was carried out by the method of Spackman *et al.* (1958) using a Hitachi Model KLA-3B amino acid analyzer.

Determination of Sulfhydryl Groups. The content of sulfhydryl groups of proteins was determined by the method of Ellman (1959). The sample protein was allowed to react with 5,5′-dithiobis(nitrobenzoic acid) in 0.1 M Tris-HCl buffer at pH 8.1 for 50–55 min at room temperature. The determination was conducted either directly on a 2-ml aliquot of the photooxidation reaction mixture (*i.e.*, the unactivated sample), or on the activated sample that was obtained by first treating the reaction mixture with 5 mM dithiothreitol or cysteine and then passing a 1.1-ml aliquot of the mixture through a 1.7 × 17 cm column of Sephadex G-25 in 0.1 M Tris-HCl buffer at pH 8.1 under anaerobic conditions.

Ultraviolet Absorption Spectrum. Aliquots (0.5–1.0 ml) were withdrawn from the reaction mixture before and after the photooxidation. To each aliquot a small portion of air-dried Dowex 50-X2 (H^+ form) resin was added. By shaking and then centrifuging, Methylene Blue in the reaction mixture was effectively removed. Using the colorless supernatant fluid, the absorption spectra were compared. Ionization of phenolic hydroxyl groups in the sample protein was spectrophotometrically determined by the method of Tachibana and Murachi (1966). A Hitachi Model UV-VIS-139 spectrophotometer or a Shimadzu Model MSP-50L recording spectrophotometer was used.

Other Methods. Sedimentation velocity experiments were carried out with a Hitachi Model UCA-1A ultracentrifuge equipped with a Schlieren optical system. Carbohydrate contents were determined by the orcinol–sulfuric acid method (Winzler, 1955). Approximately 0.05 μmol of the sample was used for the carbohydrate analysis.

Results

Progressive Changes in Enzymatic Activity and SH Content by Photooxidation. Figure 1 shows a typical example of the experimental results obtained. After an oxidation for 80 min at pH 8.0 and at 7° the caseinolytic activity was decreased to less than 10% of the original activity. There was no change in activity over a period of 80 min when the control experiment was run under identical conditions but without irradiation. As shown in Figure 1, the single SH

[2] We wish to express our appreciation to Dr. T. Yamamoto, for the gift of purified aminopeptidase.

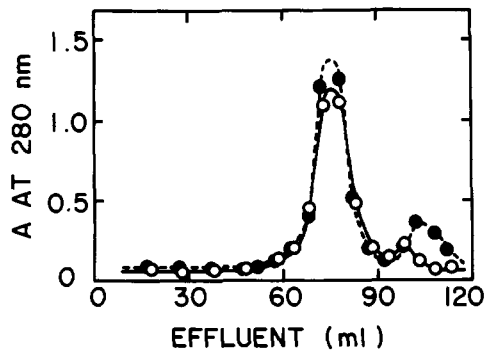

FIGURE 2: Gel filtration of stem bromelain on Sephadex G-100 before and after photooxidation. An aliquot of reaction mixture (10 mg of protein in 2 ml) was filtered through a Sephadex column (1.6 × 49 cm) with 0.1 M potassium phosphate buffer (pH 7.5) at room temperature: (○) after oxidation for 80 min, pH 7.5; (●) before oxidation.

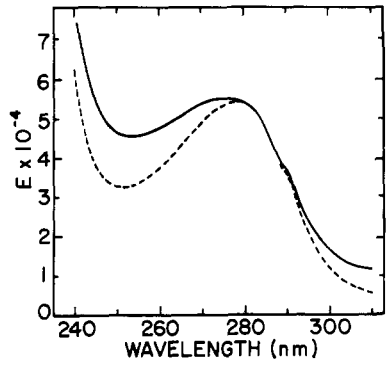

FIGURE 3: Ultraviolet absorption spectra before and after photooxidation. The sample before oxidation (- - -) and after oxidation for 90 min at pH 8.0 (—) had been treated with Dowex 50-X2 to remove Methylene Blue as described in the text; *E*, molar extinction coefficient.

group of bromelain molecule became inaccessible to Ellman reagent very rapidly when the assay was made directly, or without prior reduction, on aliquots of the irradiated reaction mixture. By contrast, when the determination of SH content was made on the sample which had first been treated with 5 mM dithiothreitol and then was filtered anaerobically through a Sephadex G-25 column, the photooxidized stem bromelain gave a recovery close to 100% of the SH group even after 40 min of irradiation. The latter irradiation, as shown in Figure 1, caused approximately 80% loss in activity which was always assayed in the presence of 5 mM dithiothreitol. This would imply that the 40-min oxidized sample retained 100% SH content in the presence of 5 mM dithiothreitol while it had only 20% activity. The question then arose as to which residue or residues other than the SH group of bromelain molecule were modified by photooxidation so as to cause a marked decrease in enzymatic activity.

The reason for recoveries of SH content over 100% as were observed after oxidation for 60 min (Figure 1) was unknown, but such higher recoveries seemed to represent cleavage of a small fraction of four disulfide bridges present in the bromelain molecule (Takahashi *et al.*, 1973).

Molecular Size and Integrity of Photooxidized Products. Sedimentation analyses in ultracentrifuge were carried out on the bromelain samples (0.5%) before and after a photooxidation for 60 min at pH 7.5. Both samples gave a single, symmetrical peak in Schlieren optics and there were no signs of dimerization or fragmentation. The sedimentation coefficient, s_{20}, was calculated to be 2.60 S for the sample before oxidation and 2.56 S for the sample after oxidation. A similar result was also obtained by gel filtration of the photooxidized preparation. Thus, a portion of the 60-min oxidized sample was applied to a Sephadex G-100 column in 0.1 M potassium phosphate buffer at pH 7.5 and the elution was done with the same buffer at room temperature. The elution profile obtained was compared with the results of an identical experiment on the unoxidized sample (Figure 2). It is clear from Figure 2 that stem bromelain molecule did not undergo a change in molecular size during the photooxidation. It is significant that these results have disproved dimerization of the bromelain molecule by an intermolecular disulfide linkage, since such a linkage was found to be very readily formed when reduced glutathione was photooxidized under identical conditions (K. Okumura and T. Murachi, unpublished observation).

Ultraviolet Absorption before and after Photooxidation. Figure 3 shows that a change in absorption spectra after oxidation for 90 min at pH 8.0 was noted on either side of wavelength 280 nm. This pattern did not simply accord with that observed when tryptophan was photooxidized under identical conditions. The oxidation of tryptophan caused a marked decrease in absorption at 280 nm. The difference spectrum over the range 260–320 nm of the sample protein in 1 N KOH *vs.* 1 N KCl (pH 7.0) did not change before and after an oxidation at pH 7.5 for 60 min, indicating that tyrosine residues of the oxidized sample could be ionized in alkali in the same way as in the case of the unoxidized control. Hence, no oxidative destruction of tyrosine residues should have taken place.

Decreases in Contents of Histidine, Methionine, and Tryptophan by Photooxidation. Aliquots of the photooxidation reaction mixture were withdrawn at given time intervals and subjected to amino acid analysis according to the procedures described under Materials and Methods. Detectable changes were noted only in histidine, methionine, and tryptophan contents, while the contents of other amino acids remained practically unchanged. For example, 0.50 residue of histidine, 0.87 residue of methionine, and 1.45 residues of tryptophan per molecule were lost after an oxidation for 80 min at pH 7.5. A native stem bromelain molecule contains one histidine, three methionine, and seven tryptophan residues (Takahashi *et al.*, 1973).

Table I shows analytical data pertaining to methionine content. A decrease in methionine content can be seen only after alkali hydrolysis. The oxidation must lead to the formation of methionine sulfoxide, not methionine sulfone, because methionine sulfoxide (1.03 residues per molecule) was actually detected on the amino acid analysis chart for the alkali hydrolysate of the 90-min oxidized sample, while no methionine sulfone was found after acid hydrolysis.

The tryptophan contents of the unoxidized sample found after enzymatic hydrolysis with and without further hydrolysis by *p*-toluenesulfonic acid were 5.69 and 5.86 residues per molecule, respectively. Decreased figures, 4.57 and 3.83 tryptophan residues per molecule, were obtained by the same two methods as above for the sample that had been oxidized at pH 7.5 for 80 min. The alkali hydrolysis of the unoxidized and oxidized samples gave values of 5.03 and 3.73 tryptophan residues per molecule, respectively. In view of a considerable wide range of experimental errors, it is uncertain whether one or two tryptophan residues per molecule were oxidized.

Kinetics of the Photooxidation Reaction. Figure 4 shows

Table I: Amino Acid Analysis of Photooxidized Stem Bromelain by Different Methods of Hydrolysis.

Hydrolysis	Photooxidation Condition	Residues/mol of Protein[a]				
		Asp	Val	Met	Leu	Phe
Acid	Control	19.8	16.0	3.52	7.00	6.68
	pH 8.0, 80 min	18.2	15.1	2.99	7.00	7.20
	pH 7.0, 80 min	20.3		3.23	7.00	7.71
Alkali[b]	Control	19.0	17.3	3.46	7.00	7.87
	pH 8.0, 80 min	21.6	16.7	2.23	7.00	7.31
	pH 7.5, 60 min	20.4	16.8	2.19	7.00	8.06

[a] The values are given in terms of molar ratio of amino acids, assuming the number of leucine residues to be 7.00. [b] Alkali hydrolysis was performed with 5 N KOH at 105–110°, 24 hr.

semilogarithmic plots of the experimental data for the inactivation and the loss of histidine during the photooxidation at pH 7.5. The progress curve for the loss of methionine is shown in the inset. It is evident that both the inactivation and the loss of histidine conform to the first-order kinetics with different rates. The inactivation was always faster than the rate of the destruction of histidine; a practically complete loss of the proteinase activity took place after an oxidation for 150 min, while 0.39 histidine residue per molecule still remained. In Figure 4 are also included data for the loss of esterase activity (▲) after oxidation for 40 and 60 min. An apparent parallel decrease in the proteinase and esterase activities has been previously shown to occur in the case of periodate oxidation of stem bromelain (Yasuda *et al.*, 1971). Since a 50× amount of stem bromelain is usually necessary for the esterase assay as compared to the amount required for the proteinase assay (Murachi, 1970), most of the enzyme assays in the present study were performed with casein as the substrate.

Since the loss of methionine apparently deviates from the first-order kinetics, it is shown only in terms of per cent remaining. It amounted to approximately one-third of the original content after 90 min of oxidation and seemed to level off thereafter. Since stem bromelain contains three methionine residues per molecule (Takahashi *et al.*, 1973), the present data imply that one methionine was readily oxidized. The oxidizability of at least one methionine residue in stem bromelain was earlier reported from this laboratory (Yasuda *et al.*, 1971) using periodate as an oxidant. The time course of the loss of tryptophan was not studied.

pH Dependence of Photoinactivation Reaction. The reaction was found to be profoundly sensitive to the pH of the medium. In Figure 5 are summarized the data of pH dependence of several parameters obtained in the presence of 0.57 mM sodium tetrathionate (△), in the presence of 5 mM Bz-L-Arg-OEt (●), or in the absence of such additives (○). Solid lines in Figures 5A and 5B are theoretical titration curves with pK_a values indicated by arrows, which best fit with the experimental points obtained. Broken lines in Figures 5C and 5D simply connect the experimental points for methionine and tryptophan, since these data could not deserve theoretical treatment. The results shown in Figure 5 indicate (1) that the decreases in enzymatic activity and in histidine content are markedly pH dependent in the range from pH 5.5 to 7.5, (2) that the apparent pK_a values for the inactivation are reasonably close to those for the decrease in histidine, (3) that the addition of an SH-blocking agent (tetrathionate) during the course of the oxidation has resulted in shifting the pH profile curves toward alkaline pH by 0.4–0.7 pH unit, (4) that the photooxidation occurs even in the presence of a synthetic substrate (Bz-L-Arg-OEt) with a pH profile not very much different from that obtained in its absence, and (5) that the decreases in methionine and tryptophan contents are also pH dependent. From the theoretical titration curves in Figures 5A and 5B, the apparent pK_a values for the rates of decreases in enzymatic activity and histidine content were calculated and the obtained values are summarized in Table II. It should be noted that the values for histidine are in the normal range for an imidazole group.

Although the photoinactivation reaction was retarded in the presence of tetrathionate when compared with the reaction in its absence at the same pH value (Figure 5), the temperature dependence of the reaction constants was found to be almost identical in both cases. Thus, the activation energy calculated from the rate constants for the inactivation reaction (pH 7.5) at 3 and 19° was 6.11 kcal/mol in the presence of 0.57 mM sodium tetrathionate or 6.15 kcal/mol in its absence. The almost identical values of activation energy imply that identical amino acid residue or residues in the stem bromelain molecule were oxidized to cause inactivation regardless of whether the essential sulfhydryl group was reversibly blocked or unblocked.

Photoinactivation in the Presence of Rose Bengal. When Rose Bengal was used as a photosensitizer in place of Methylene Blue, a very similar result of photoinactivation was obtained. For example, after 15 min of oxidation at pH 7.5 in the presence of 7.14 μM Rose Bengal and 0.57 mM sodium tetrathionate, the enzyme lost 45% of the original activity toward casein. However, a part of this dye tended to become firmly bound to the enzyme protein as the photooxidation progressed. The separation of the enzyme protein free from the dye by gel filtration or by dialysis was impossible and hence chemical and spectrophotometric analyses on the modified enzyme became more difficult to pursue.

Discussion

It is well known that amino acid residues of a protein, such as cysteine, histidine, methionine, tyrosine, and tryptophan, are liable to be oxidized by photochemical reaction when Methylene Blue or Rose Bengal is used as a sensitizer under the conditions employed in this study (Ray, 1970). We have demonstrated, in the case of stem bromelain with Methylene Blue, that histidine, methionine, and tryptophan residues were oxidized, but not tyrosine residues (Tables I and II, and Figures 3 and 4). The situation of the cysteine

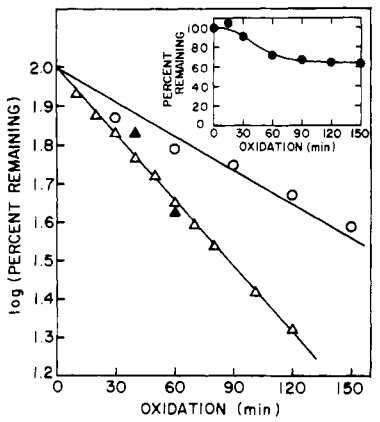

FIGURE 4: Rates of decreases in enzymatic activity, and histidine and methionine contents. Photooxidation was performed at pH 7.5 at 7°, with 62 μM enzyme and 16 μM Methylene Blue. For methionine content (inset), 160 μM enzyme was photooxidized: (△) proteinase activity on casein; (▲) esterase activity on Bz-L-Arg-OEt; (○) histidine content; (●) methionine content.

residue was somewhat complex; upon photooxidation the sulfhydryl gooup very rapidly became inaccessible to Ellman reagent but the reactivity to the latter reagent could be readily reversed by the addition of an excess of a reducing agent, like dithiothreitol or cysteine (Figure 1). This indicated that the cysteine residue had not been transformed into a cysteic acid residue. We were not able to elucidate how the cysteine residue was *reversibly* oxidized, although we could eliminate the possibility of an intermolecular disulfide bond being formed (Figure 2).

Now we wish to focus our attention on a molecular species of stem bromelain which had been photooxidized and then treated with a reducing agent. Such a molecular species fully retained its essential sulfhydryl group, but lost parts of histidine, methionine, and tryptophan residues, while it showed a decreased caseinolytic activity. It follows that the decrease in enzymatic activity could be related to the oxidation of histidine, methionine, and tryptophan residues, but not to that of the cysteine residue. The loss of histidine could not entirely account for the inactivation (Figure 4); hence the mechanism of inactivation must not be as simple as a single hit on a certain catalytic group, *e.g.*, a histidine residue. The complexity of chemical changes caused by a photoinactivation was well established, for example, in the case of phosphoglucomutase (Ray and Koshland, 1962). There is ample reason to believe that the oxidation of methionine has contributed at least partly to the inactivation, since the earlier study in this laboratory demonstrated that periodate oxidized a methionine residue causing some 20% decrease in caseinolytic activity (Yasuda *et al.*, 1971). One must also consider a conformational change which might occur in association with the oxidation of histidine, methionine, and tryptophan residues. The increase in sulfhydryl content over the original value, as observed upon reduction of a 60- or an 80-min oxidized sample (Figure 1), could be explained as the result of a conformational change that has effectively loosened some of the intramolecular disulfide bonds.

As was expected, the photooxidation reaction of stem bromelain was actually found to be dependent on the pH value of the reaction medium (Figure 5). We are unable to explain why a methionine residue became liable to be oxidized between pH 4 and 5. Sluytermann (1962) demonstrated that Methylene Blue sensitized photooxidation of

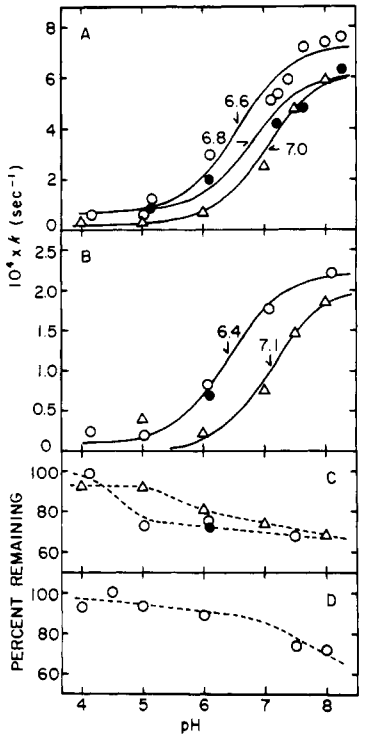

FIGURE 5: pH dependence of photoinactivation reaction. The rate of inactivation (A) and that of the loss of histidine (B) are presented in terms of the first-order rate constant, k. Arrows show the apparent pK_a values of the theoretical titration curves. Changes in the contents of methionine (C) and tryptophan (D) are shown in terms of per cent remaining. Photooxidation was performed with 16–38 μM enzyme and 6.85 μM Methylene Blue at 7°: (△) in the presence of 0.57 mM sodium tetrathionate; (●) in the presence of 5 mM Bz-L-Arg-OEt; (○) in the absence of additives.

Table II: Apparent pK_a Values for the Rates of Decreases in Enzymatic Activity and Histidine Content.

Additive	Apparent pK_a Value[a]	
	Inactivation	Loss of His
None	6.6	6.4
Tetrathionate	7.0	7.1
Bz-L-Arg-OEt	6.8	

[a] Calculated from the theoretical titration curves in Figures 5A and 5B.

amino acid methionine was rather insensitive to pH over a range from pH 4 to 7. On the other hand, it is a well established fact that the pH dependence of photooxidation of histidine reflects the ionization of its imidazole group; the unprotonated species is readily oxidized while the protonated species is hardly oxidized (Sluytermann, 1962; Takahashi, 1970). We could therefore determine the apparent pK_a value for the ionization of the single histidine imidazole from the titration curve that should best fit with the experimental points. Thus, values of 6.4 and 7.1 for that imidazole group were obtained when the experiments were made in the absence and in the presence of tetrathionate, respectively (Table II). These values fall within the normal range for an imidazole of histidine. It is interesting to note that the pK_a value for the ionization of a histidine residue in thiol proteinases, including papain, stem bromelain, ficin, etc.,

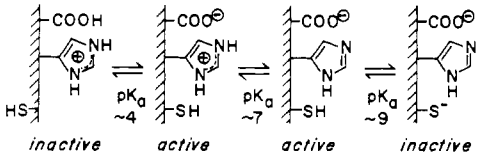

FIGURE 6: Proposed situation describing the catalytically active and inactive species of a plant thiol proteinase.

has never been determined directly by any chemical means. This was probably because the sulfhydryl group is far more reactive than a histidine imidazole and hence every modifying reagent would have coupled with the sulfhydryl before it could attack the imidazole. Even a bifunctional reagent, 1,3-dibromoacetone, which successfully coupled both with the sulfhydryl S and with the imidazole N, was reported to react first with the sulfur atom and then with the nearby nitrogen atom (Lowe, 1970).

What can a normal pK_a value for the histidine residue in stem bromelain imply with reference to the mechanism of catalysis? According to Husain and Lowe (1970), the histidine imidazole must be located within a 5-Å distance from the essential sulfhydryl group because these two residues could be linked together by reacting with 1,3-dibromoacetone. The latter reagent also cross-links the cysteine residue at position 25 and the histidine residue at position 159 of papain (Husain and Lowe, 1968), which are believed to be only 3.4 Å apart, based on an X-ray crystallographic analysis (Drenth *et al.*, 1970). Extensive homology in amino acid sequence exists among papain, stem bromelain, and ficin not only around the catalytic cysteine residue but also around the histidine residue that could be cross-linked (Husain and Lowe, 1970). These facts have led to the postulation that the imidazole group is in fact involved in catalysis as it is in the case of serine proteinases (Drenth *et al.*, 1970; Lowe, 1970). The participation of an imidazole in the catalysis by thiol proteinases has been postulated in two different ways: the one assigning the imidazole proton-donating function so that the imidazole must be a protonated form in a catalytically active enzyme protein (Drenth *et al.*, 1970), and the other assuming an unprotonated imidazole that is hydrogen bonded with the sulfhydryl and functions as a proton-withdrawing group in the acylation step of the catalysis (Lowe, 1970). In both of these mechanisms the pK_a of the imidazole group in question has to be abnormal, being claimed to be as high as pH 9.8–10 (Drenth *et al.*, 1970) or as low as pH 4 (Lowe, 1970). As an experimental fact, however, we have demonstrated that the apparent pK_a value for the imidazole group of the histidine residue in stem bromelain is *not* at all abnormal. The same was found to be true also in the case of papain (Murachi and Okumura, 1974). Consequently, the above-mentioned two mechanisms will have to be modified. We propose a mechanism as shown in Figure 6 whereby one can describe the catalytically active and inactive species of a plant thiol proteinase. Participation of a carboxyl group is postulated to account for the well-known fact that thiol proteinases of plant origin show a common pH profile of their kinetic parameters, which is characteristic in that its acidic limb has a pK_a value near 4 while its basic limb has a pK_a value near 9 (Kimmel and Smith, 1954; Inagami and Murachi, 1963; Whitaker and Bender, 1965). So far as the three-dimensional structure of crystalline papain is concerned, there is no carboxyl group close enough to interact with the sulfhydryl group. Therefore, we place one carboxyl group rather away from the sulfhydryl, but it is assumed that the ionization of this carboxyl, with a pK_a value near 4, induces the conformational change to expose the catalytic group, the sulfhydryl. A very similar but somewhat reversed situation has been known to hold for the isoleucine residue at position 16 in chymotrypsin which interacts with the aspartic acid residue at position 194 to conform to the proper active-site geometry (Mathews *et al.*, 1967).

We admit the fact that a particular histidine residue is located spatially very close to the sulfhydryl, but we are doubtful of the hypothesis of assigning a very intimate electronic interaction between these two residues. Instead, we propose that the catalysis can be accomplished by the sulfhydryl group alone if all the other environmental conditions were to be favorable for the substrate to be properly oriented toward it. The latter was shown to be actually the case using a number of model sulfhydryl compounds that exerted intramolecular catalysis of hydrolysis of different amides (Koshland, 1973). Not being involved in electronic interaction with the catalytic sulfhydryl, the imidazole at the active site can be ionized as normally as is a free amino acid histidine. This means that the enzyme molecule is equally active whether its histidine residue is protonated or unprotonated; the actual pH profile of the kinetic parameters is usually very flat between pH 5 and 8 (Kimmel and Smith, 1954; Inagami and Murachi, 1963; Whitaker and Bender, 1965). Finally, the basic limb of the pH profile can be explained assuming a pK_a value near 9 for the ionization of the catalytic sulfhydryl group itself. Thus, the proposed mechanism can explain most of the experimental findings obtained in this and other laboratories. A drawback still inherent to this mechanism is that a carboxyl group essential to the mechanism remains only a speculation. An essential carboxyl group was frequently postulated in the past, but without concrete chemical evidence for it (Smith, 1959; Blow and Steitz, 1970).

In the light of the proposed mechanism, the results of the present experiments on the photosensitized inactivation may be interpreted as follows. When the pH of the medium was raised from 4 to 5, some methionine residues became oxidized resulting in a partial loss of the enzymatic activity. As the pH was increased further, the oxidation of unprotonated species of the histidine residue progressively ensued, with the oxidation of tryptophan residues at higher pH values. In a pH range from 5 to 7, the oxidation of the histidine residue resulted in another and larger partial loss of the activity. This was because the oxidized form or forms of imidazole might hinder the proper orientation of the substrate toward the very closely located sulfhydryl, for the oxidized form(s) must have been more bulky than the unoxidized one, and/or because the oxidation of histidine, as well as that of methionine and tryptophan, might induce an unfavorable conformational change in the active site. That the oxidation of a residue, which is not a catalytic group by itself but is located very closely to the catalytic groups, causes a profound inactivating effect was reported in the case of the periodate oxidation of methionine residue at position 192 in chymotrypsin (Knowles, 1965). The results with chymotrypsin bear comparison with those obtained in the photooxidation of histidine in stem bromelain.

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A Study of Subtilisin Types Novo and Carlsberg by Circular Polarization of Fluorescence†

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ABSTRACT: The circular polarization of the luminescence of a chromophore, in addition to its circular dichroism and optical rotatory dispersion, is a manifestation of its asymmetry. In the study of proteins, the circular polarization of luminescence yields more specific information than circular dichroism or optical rotatory dispersion since nonfluorescent chromophores do not contribute, and the spectra of the tyrosine and the tryptophan residues are much better resolved in emission than in absorption. The circular polarization of the fluorescence of the tyrosine and tryptophan residues in derivatives of subtilisin Carlsberg and subtilisin Novo were indeed resolved in this study. The tyrosine residues in the Carlsberg protein, and both tyrosine and tryptophan residues in the Novo protein, were found to be heterogeneous with respect to their optical activity and emission spectra. Changes in the environment of the emitting tyrosine residues in both proteins and in the tryptophan residues in the Novo protein were found on changing the pH from 5.0 to 8.3. The pH dependence of the enzymatic activity of these proteins may thus be due, at least in part, to conformational changes in the molecules. Fluorescence circular polarization also revealed that covalently bound inhibitors at the active site of subtilisin Novo affect the environment of the emitting aromatic side chains, presumably *via* changes in conformation.

A wide variety of spectroscopic techniques have contributed to our growing understanding of the role played by macromolecular conformation in the molecular biology of proteins. Among these techniques, circular dichroism, CD, has helped to elucidate some of the details of the secondary and tertiary structure of proteins. CD is an absorption phenomenon arising from the preferential absorption of right- or left-handed circularly polarized light by an optically active chromophore which may be either intrinsically asymmetric or perturbed by an asymmetric environment. In the case of proteins several chromophores, each involving one or more electronic transitions in the experimentally accessible spectral range, can contribute to the observed CD spectrum.

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