NORMAL APPARENT pK_a VALUE FOR THE IONIZATION OF THE HISTIDINE RESIDUE OF PAPAIN AND STEM BROMELAIN AS DETERMINED BY PHOTOOXIDATION REACTION

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1. Introduction

During the course of investigating photosensitized inactivation of stem bromelain, we found that the photooxidation of the enzyme in the presence of methylene blue yielded a modified enzyme which, upon reduction in 5 mM dithiothreitol, fully retained its essential sulfhydryl group, showed a markedly decreased catalytic activity toward casein, and had decreased hisitidine and methionine contents [1]. The reaction showed a pronounced pH dependence. It is well known that the unprotonated species of an imidazole is readily photooxidized while the protonated species is hardly oxidized under the conditions employed in our studies [2]. Thus the photooxidation method has permitted us to determine directly the apparent pK_a value of the single histidine residue in the stem bromelain molecule. The same technique was also applied to papain. We report here that the apparent pK_a values for the ionization of the histidine imidazole as determined by the pH dependence of the rate of the photooxidation in papain and stem bromelain are 6.7 and 6.4, respectively, which are in the normal range for an imidazole group.

2. Materials and methods

Papain was recrystallized according to the method described by Kimmel and Smith [3]. Stem bromelain, SB1, was purified by the method of Takahashi et al. [4]. In a jacketed beaker of a 25 ml capacity were mixed 15–62 μM enzyme protein, 6.85–16 μM methylene blue, and 0.1 M buffer in a total volume of 7 ml. The mixture was illuminated with an incandescent lamp (150 W) placed above the solution at a distance of 25 cm and at a temperature of 7°–15°C maintained by circulating cold water. Aliquots were withdrawn at desired time intervals and assayed for the caseinolytic activity in the presence of 5 mM dithiothreitol or cysteine [5]. The data obtained were expressed in per cent of the value for the unoxidized control. Histidine content of the oxidized enzyme protein was determined by an amino acid analyzer [6]. The modified protein for the amino acid analysis was prepared by filtration through a 1.7 × 15 cm column of Sephadex G-25 gel in 0.1 M ammonium hydrogencarbonate followed by lyophilization. The apparent first-order rate constants, k (sec^{-1}), were also calculated from the losses of enzymatic activity and a histidine residue.

3. Results and discussion

Fig. 1 shows that both papain and stem bromelain are progressively inactivated by photooxidation. However, the content of sulfhydryl group, as determined by the method of Ellman [7] upon reduction in 5 mM dithiothreitol, was found not to decrease as compared to the value before the oxidation. The amino acid analysis on the acid hydrolysate of the oxidized samples revealed significant changes in histidine content. Papain has two histidine residues per molecule, and 0.53 residue disappeared after oxidation for 120 min under the conditions employed.

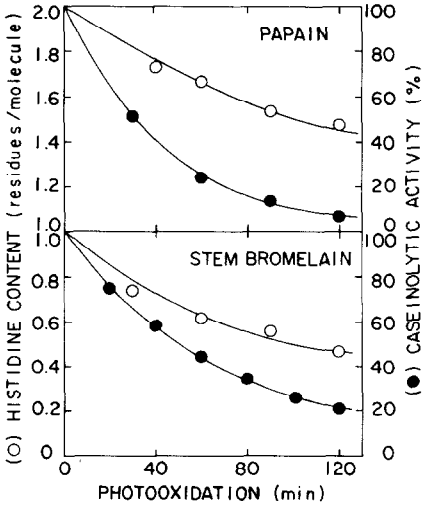

Fig. 1. Progressive losses of histidine content (○) and enzymatic activity (●) during the photooxidation. A 7.0 ml reaction mixture contained 15 μM papain and 16 μM methylene blue or 62 μM stem bromelain and 16 μM methylene blue in 0.1 M potassium phosphate buffer at pH 7.5. Temperature: 15°C for papain and 7°C for stem bromelain.

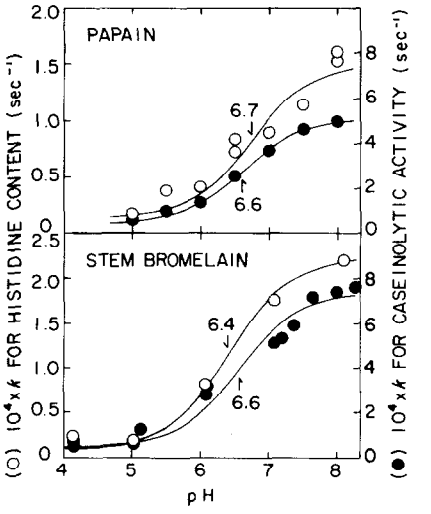

Fig. 2. pH dependence of decreases of histidine content (○) and enzymatic activity (●) by the photooxidation. At 7.0 ml reaction mixture contained 15 μM papain and 16 μM methylene blue or 16 μM stem bromelain and 6.85 μM methylene blue in 0.1 M buffer. Buffers used were: potassium citrate–phosphate at pH 4.2; sodium acetate at pH 5.0; and potassium phosphate at pH 6.0–8.3. The photooxidation was continued for 120 min at 15°C with papain or for 60 min at 7°C with stem bromelain. Solid lines in the figure are theoretical titration curves with pK_a values indicated.

Stem bromelain contains one histidine per molecule [5], and 0.53 residue disappeared after 120 min of the oxidation. Apparent discrepancies between the rates of the loss of caseinolytic activity and that of histidine residue can be seen in fig. 1 both with papain and with stem bromelain, suggesting some factor or factors other than oxidation of histidine is involved in the mechanism of inactivation. The loss of approximately one methionine residue in the case of stem bromelain will be discussed in greater detail elsewhere.

The most striking feature of the results of the present experiment is the pH-profile of the loss of histidine. As shown in fig. 2 the experimental data fit reasonably well with theoretical titration curves with apparent pK_a values of 6.7 for papain and 6.4 for stem bromelain. The loss of enzymatic activity was also pH-dependent with an apparent pK_a value of 6.6 for both enzymes. Similar results were also obtained with stem bromelain when the photooxidation was carried out at varying pH values in the presence of 0.57 mM sodium tetrathionate, an SH-blocking agent. The observed pK_a value for the pH-dependence of the loss of histidine was 7.1 and that of the inactivation was 7.0.

It has been known for a long time that thiol proteases of plant origin, including papain, bromelain, and ficin, show a common pH-profile of their kinetic parameters which is characteristic in that its acidic limb has a pK_a value near 4 [8,9]. This was frequently explained as reflecting an essential carboxyl group, but without concrete chemical evidence for it [10,11]. Three-dimensional structure of crystalline papain as revealed by X-ray diffraction analysis has shown that the N^{δ} atom of histidine at position 159 is only 3.4 Å away from the S atom of cysteine at position 25 [12]. The existence of an imidazole and a sulfhydryl within a 5 Å distance was also verified by the bifunctional reagent, 1,3-dibromoacetone with papain as well as with stem bromelain [13]. These findings, together with the characteristic pH-profile of the catalysis mentioned above, have led to the postulation that a histidine imidazole is in fact involved in catalysis, as it is the case in serine proteases, and that the pK_a of that imidazole group must be abnormal, being as high as 9.8–10 [12] or as low as 4 [14]. However, no direct chemical proof has ever

been made to demonstrate an abnormally high or low pK_a value for histidine. As far as we are aware, the present data are the first experimental demonstration that the apparent pK_a value of the imidazole group of a histidine residue in papain and in stem bromelain is *not* at all abnormal, but it remains in the normal range for an imidazole group. The present findings seem to cast a doubt on the hypothesis that the histidine residue in the active site is involved in a very intimate electronic interaction with the catalytically essential sulfhydrul group.

References

[1] Murachi, T., Tsudzuki, T. and Okumura, K. (1973) Abstracts, 9th International Congress of Biochemistry, July 1973, Stockholm, p. 95.
[2] Sluytermann, L.A.AE. (1962) Biochim. Biophys. Acta 60, 557.
[3] Kimmel, J.R. and Smith, E.L. (1954) J. Biol. Chem. 207, 515.
[4] Takahashi, N., Yasuda, Y., Goto, K., Miyake, T. and Murachi, T. (1973) J. Biochem. (Tokyo) 74, 355.
[5] Murachi, T. (1970) Methods in Enzymol. 19, 273.
[6] Spackman, D.H., Moore, S. and Stein, W.H. (1953) Anal. Chem. 30, 1190.
[7] Ellman, G.L. (1959) Arch. Biochem. Biophys. 82, 70.
[8] Inagami, T. and Murachi, T. (1963) Biochemistry 2, 1439.
[9] Whitaker, J.R. and Bender, M.L. (1965) J. Amer. Chem. Soc. 87, 2728.
[10] Smith, E.L. (1959) J. Biol. Chem. 233, 1392.
[11] Blow, D.M. and Steitz, T.A. (1970) Annu. Rev. Biochem. 39, 63.
[12] Drenth, J., Jansonius, J.N., Koekoek, R., Sluytermann, L.A.AE and Wolthers, B.G. (1970) Phol. Trans. Roy. Soc. London B 257, 231.
[13] Husain, S.S. and Lowe, G. (1968) Biochem. J. 108, 861; (1970) Biochem. 117, 341.
[14] Lowe, G. (1970) Phil. Trans. Roy. Soc. London B 257 237.