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PHOTOCHEMICAL OXIDATION OF SNAKE GOURD PROTEINASE A_2, A SERINE PROTEINASE

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Key Word Index—*Trichosanthes cucumeroides*; Cucurbitaceae; snake gourd; serine plant proteinase; photooxidation.

Abstract—Snake gourd proteinase A_2 was rapidly inactivated by methylene blue catalysed photooxidation at pH 7.8 and 25°. The rate of inactivation was pH-dependent and became slower at lower pH values, suggesting the involvement of some histidine residues in the inactivation. Changes in amino acid composition occurred only with histidine residues. One mole or more of histidine residues in the molecule are of essential importance in the catalytic function of snake gourd proteinase A_2.

INTRODUCTION

Photooxidation of enzymes in the presence of dyes has often been used to obtain information on the amino acid residues essential for the catalytic activity of an enzyme. It is well known that the photooxidation of proteins in the presence of methylene blue causes a rapid destruction of histidine and tryptophan residues and a slower destruction of tyrosine, cysteine and methionine residues [1–4]. However, Martinez-Carrion studied a histidine-specific oxidation with aspartic aminotransferase [5]. Snake gourd proteinase A_2 isolated from the sarcocarp of the snake gourd, *Trichosanthes cucumeroides* Maxim, by Kaneda *et al.* is a serine proteinase [6]. Among proteases of plant derivation, snake gourd proteinase is unique because typical plant proteases so far isolated have belonged mainly to the thiol protease group. This report shows that snake gourd proteinase is sensitive to dye-sensitized photochemical oxidation, and suggests that destruction of histidine is responsible for the inactivation.

RESULTS AND DISCUSSION

As can be seen in Fig. 1, snake gourd proteinase A_2 was rapidly inactivated by methylene blue catalysed photooxidation. The inactivation was complete after 60 min at pH 7.8. The rate of inactivation became slower in the lower pH regions. After 100 min at pH 6.1, the enzyme retained nearly 50% of its original activity. The plot of the activity loss as a function of pH is of sigmoidal shape and has an inflection point near pH 6.5 as shown in Fig. 2. This pH dependency indicates that some amino acid residues which have a pK value of around neutrality are involved in the inactivation. Since only the photooxidation of histidine and its derivatives has been reported to show a pH-dependence similar to that observed here [5, 8–10], the result strongly suggests that photooxidation of histidyl residue(s) of the enzyme is responsible for the activity loss. In the dark, the enzyme was quite stable in the presence of methylene blue.

After removing methylene blue from the reaction mixture by gel filtration, the amino acid composition of photooxidized enzyme was analysed and compared with that for native enzyme (Table 1). The results showed that the amino acid residue which suffered a significant change on photooxidation was only histidine and that the other residues remained almost intact within the limit of experimental error. Figure 3 shows the relationship between the histidine residues lost and the enzymatic activity remaining when the photooxidation was carried out at pH 7.8. About three of the five histidine residues in the enzyme were lost when complete inactivation took place. The results show that the photooxidation of no more than three histidine residues is directly responsible for the inactivation of snake gourd proteinase A_2.

In conclusion, the results of photooxidation experiments described here indicate that histidyl residue(s) is

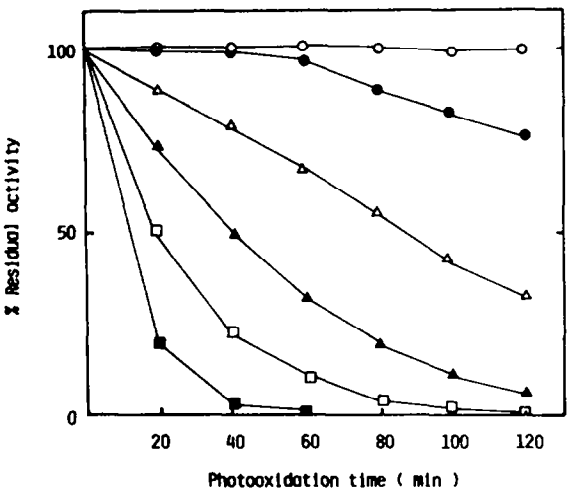

Fig. 1. Rates of inactivation of snake gourd proteinase A_2 by photooxidation at different pH values in the presence of methylene blue. Snake gourd proteinase A_2 (0.25% solution) was irradiated from a distance of 12 cm with a 100 W incandescent lamp in the presence of 0.0075% methylene blue at 25°. ○, pH 5.2; ●, pH 5.6; △, pH 6.1; ▲, pH 6.5; □, pH 6.8; ■, pH 7.8.

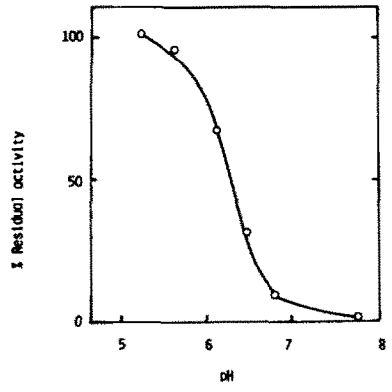

Fig. 2. Effect of pH on photooxidation of snake gourd proteinase A_2. The rate of photooxidative inactivation is expressed as the percentage of that at pH 7.8 after exactly 60 min.

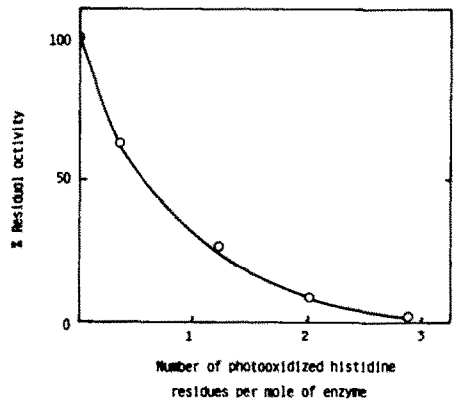

Fig. 3. Relationship of activity of snake gourd proteinase A_2 to the extent of photooxidation of histidine residues at pH 7.8.

Table 1. Amino acid composition of acid hydrolysates of snake gourd proteinase A_2 photooxidized in the presence of methylene blue at pH 7.8. The values are given in terms of the molar ratios of the amino acids assuming the number of aspartic acid residues to be 53.0. No correction has been made for losses resulting from decomposition during acid hydrolysis

Amino acid	Native enzyme	Photooxidized enzyme	
		20 min	60 min
Aspartic acid	53.0	53.0	53.0
Threonine	32.5	31.9	32.0
Serine	53.1	52.9	53.0
Glutamic acid	19.8	19.5	19.4
Proline	30.6	31.1	30.5
Glycine	47.2	47.0	46.9
Alanine	43.5	43.1	42.9
Half-cystine	4.2	4.1	3.9
Valine	38.6	38.4	37.8
Methionine	4.8	4.5	4.6
Isoleucine	28.6	28.7	28.0
Leucine	33.1	33.5	33.2
Tyrosine	15.2	15.3	15.0
Phenylalanine	18.8	19.2	19.0
Tryptophan	3.0	2.8	2.9
Lysine	15.0	15.3	14.8
Histidine	4.2	3.8	1.2
Arginine	24.1	24.0	24.3
Remaining activity (%)	100	20	0.5

involved in the catalytic centre of snake gourd proteinase A_2.

EXPERIMENTAL

Snake gourd proteinase A_2 was isolated from the sarcocarp of snake gourd according to the procedure described in ref. [6]. The proteinase activity of snake gourd proteinase was determined by a modified Kunitz method [6] using casein as substrate.

The pH dependence of the rate of inactivation of enzyme by methylene blue catalysed photooxidation was tested as follows. To 5 ml of a buffer soln (0.2 M phosphate buffer, pH 5.2–7.9) containing 25 mg of enzyme, 5 ml of a 0.015% methylene blue aq. soln was added and the mixture was irradiated from a distance of 12 cm with a 100 W incandescent lamp at 25°. Aliquots of 50 μl were withdrawn at appropriate times and used for assay of the enzymatic activity, and at the same time 1 ml aliquots were withdrawn for amino acid analysis. Photooxidized protein was freed from the reagents by passage through a column (1.5 × 32 cm) of Sephadex G-25 equilibrated and eluted with 0.1 M formic acid. The protein fractions were pooled and lyophilized. Native and photooxidized enzyme were hydrolysed with 6 M HCl at 110° for 24 hr in evacuated, sealed tubes and analysed with an amino acid analyser, model JLC-5AH (Japan Electron Optics Ltd.) Tryptophan was determined by the spectrophotometric method [7].

REFERENCES

1. Weil, L. and Buchert, A. R. (1951) *Arch. Biochem. Biophys.* **34**, 1.
2. Weil, L., Buchert, A. R. and Maher, J. (1952) *Arch. Biochem. Biophys.* **40**, 245.
3. Weil, L., James, S. and Buchert, A. R. (1953) *Arch. Biochem. Biophys.* **46**, 266.
4. Weil, L. and Seibles, T. S. (1955) *Arch. Biochem. Biophys.* **54**, 368.
5. Martinez-Carrion, M. (1967) *J. Biol. Chem.* **242**, 1426.
6. Kaneda, M., Sobue, A., Eida, S. and Tominaga, N. (1986) *J. Biochem.* **99**, 569.
7. Edelhoch, H. (1967) *Biochemistry* **6**, 1948.
8. Westhead, E. W. (1965) *Biochemistry* **4**, 2193.
9. Weil, L. (1965) *Arch. Biochem. Biophys.* **110**, 57.
10. Freude, K. A. (1968) *Biochim. Biophys. Acta* **167**, 485.