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COMPARISON OF PHYTOLACAIN R, A CYSTEINE PROTEASE FROM *PHYTOLACCA AMERICANA*, WITH PAPAIN

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Key Word Index—*Phytolacca americana*; Phytolaccaceae; pokeweed; cysteine protease; phytolacain R.

Abstract—Nine sites of oxidized insulin B-chain were cleaved by phytolacain R, isolated from pokeweed, after 20 hr of hydrolysis. Five cleavage sites resembled those of papain. Substrate specificity of phytolacain R was similar to that of papain, preferring hydrophobic P_2 residues. The activities of fibrin formation and elastin hydrolysis of phytolacain R were higher than those of papain. The amino terminal sequence of phytolacain R, although similar was not identical with that of papain.

INTRODUCTION

Phytolacain R is a cysteine protease isolated from the reddish fruit of pokeweed, *Phytolacca americana*, formerly called 'phytolacin' [1]. Since a homologous protease was isolated from an unripe greenish fruit of pokeweed, we changed the name to phytolacain R for the protease from the ripe reddish fruit. The M_r of the enzyme was estimated to be 26 000. The enzyme strongly hydrolysed casein having a maximum activity in the pH range 7.5–8 and was completely inhibited by iodoacetic acid [1]. A different substrate specificity for synthetic substrates was observed between phytolacain R and papain. A good substrate of papain, Bz-Arg-*p*NA, was not hydrolysed by phytolacain R. In contrast, a susceptible substrate of phytolacain R, Bz-Tyr-*p*NA, was not hydrolysed by papain [1–3]. We report a discrepancy of the substrate specificity and N-terminal sequence between phytolacain R and papain.

RESULTS AND DISCUSSION

The cleavage sites and the extent of hydrolysis by phytolacain R or papain were determined after 20 hr digestion of the oxidized insulin B-chain (Fig. 1). Comparison of the amino acid composition of the recovered peptides with the published insulin B-chain sequence permitted unequivocal identification of the cleavage sites by the enzymes. Thirteen peptides were recovered from the oxidized insulin B-chain digest by phytolacain R, and nine peptides were recovered from the digest by papain. Only two common peptides were formed between the two digests. One was the decapeptide at residues 4–13, and the other was the dodecapeptide at residues 14–25. The decapeptide from the phytolacain R digestion was obtained in low yield (3%), but in high yield (63%) from

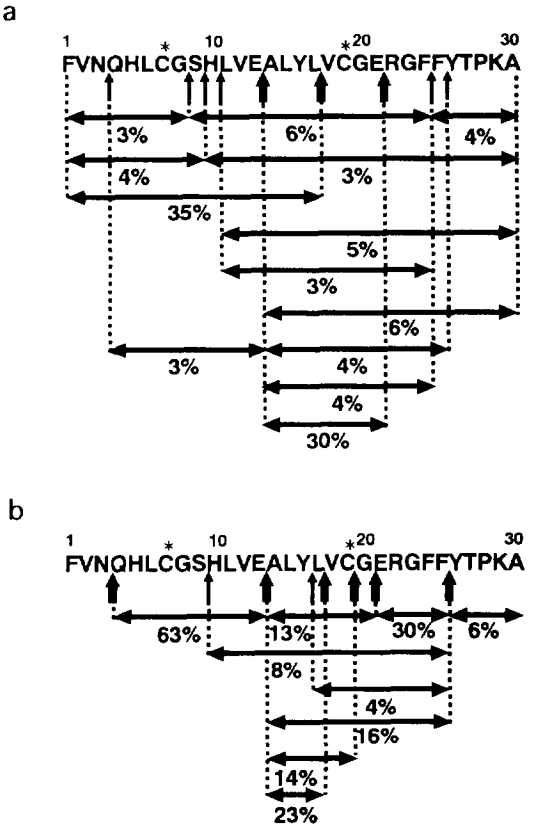

Fig. 1. Cleavage sites of oxidized insulin B-chain by phytolacain R (a) and papain (b). The cleavage sites were identified from amino acid analysis of the recovered peptides. C* is cysteic acid. The thin arrows mark the cleaved sites with less than 10% of recovery and the thick arrows with more than 10%. The peptides produced by the digestion are indicated by horizontal bars with both arrow-heads accompanying the recovery.

Table 1. Comparison of various protease activities of phytolacain R and papain

Enzyme	Caseinolytic activity (Units)	Milk clotting activity (Units)	Fibrin formation (Sec)	Congo-Red elastin (Units)
Phytolacain R	156	21	210	79
Papain	151	75	90	32

the papain digestion. The recoveries of dodecapeptides were 4% from phytolacain R and 16% from papain. The generation of the deca- and dodecapeptide, respectively, were caused by the cleavage of the Glu–Ala bond at residues 13–14. The Leu–Val bond at residues 17–18 was hydrolysed at a greater rate by these enzymes. The nine cleavage sites were detected in the phytolacain R digestion, while the eight cleavage sites were detected in the papain digestion. Five common cleavage sites existed between them. The substrate specificities of two enzymes were consequently classified as broad type, judging by the P_1 position of the cleavage sites. On the other hand, the P_2 position of the cleavage sites by phytolacain R were mainly occupied by the hydrophobic amino acid residues such as Val_2, Val_{12}, Tyr_{16} and Phe_{24} in four out of nine cleavage sites. Furthermore, the P_2 position was occupied by the smallest amino acid residue glycine at residue numbers 8, 20 and 23. All three glycines in the insulin B-chain were linked to the hydrolysis of phytolacain R at the P_2 position of the cleavage sites. However, the glycine at residue 8 was only linked to papain hydrolysis. The P_2 position of the papain cleavage sites was mainly occupied by the hydrophobic amino acid residues such as Val_2, Val_{12}, Leu_{15}, Tyr_{16}, Val_{18} and Phe_{24} in six out of eight cleavage sites. It has been previously stated that papain preferred hydrophobic amino acid residues at the P_2 position of the cleavage sites [4].

The hydrolytic activities of phytolacain R and papain against the proteins were compared by using substrates such as casein, elastin and fibrinogen. Enzyme solutions of phytolacain R and papain were prepared and were used in the following assay. Both enzyme solns had almost the same caseinolytic activity (*ca* 150 units, Table 1). The hydrolytic activity of phytolacain R against Congo-Red elastin was 2.5-fold higher than that of papain. The milk clotting activity of phytolacain R exhibited 28% of the papain value. This activity corresponds to 4% of the chymosin value, the milk-clotting enzyme from the fourth stomach of the calf (data not shown). The time of fibrin formation by phytolacain R was slower than that of papain. The two fibrin clottings were gradually digested by the enzymes and disappeared the next day.

The N-terminal sequence of the first 36 residues of phytolacain R was determined. When this sequence of phytolacain R is compared with that of papain (Fig. 2), high homology is observed. However, phytolacain R has asparagine at the N-terminal in comparison with papain. This phenomenon also exists in the case of the stem bromelain, changing to alanine instead of asparagine [5]. The reactive cysteine at residue 25 of phytolacain R had been identified by using ^{3}H-tosyl-L-phenylalanine chloromethyl ketone (unpublished data).

EXPERIMENTAL

Materials. Fruits of pokeweed were collected in July at Kagoshima Prefecture, Japan. Purification of phytolacain R was carried out by the method described previously [1].

Digestion of the insulin B-chain. Oxidized bovine insulin B-chain was prepared by the method of ref. [6] and purified by reversed phase HPLC. The oxidized B-chain (70 μg, 20 nmol) was digested with 0.2 nmol phytolacain R in 0.1 ml of 0.1 M Tris–HCl buffer, pH 7.5 at 35° for 20 hr. The reaction was stopped by addition of 10 μl of 70% HCO_2H. The digested peptides were sepd by reversed phase HPLC using an Applied Biosystems 150A HPLC system on a Dynamax-150A C_8, Rainin (250 × 4.6 mm) column with a linear gradient of 0–60% MeCN containing 0.1% TFA. The eluate was monitored by measuring the *A* at 230 nm. The purified peptides were hydrolysed in 6 M HCl containing 0.1% phenol at 110° for 24 hr. The samples were derivatized with phenylisothiocyanate using a Pico·Tag Work Station (Waters). Phenylthiocarbamyl derivatives of amino acid were analysed on a Pico·Tag HPLC system. The location of each peptide on the sequence of insulin B-chain were fixed from each amino acid composition.

Protease assay. Proteolytic activity for casein was measured in accordance with the principles of ref. [7].

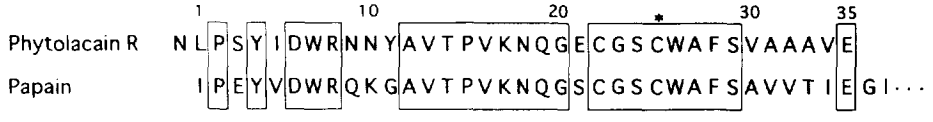

Fig. 2. Comparison of the N-terminal amino acid sequences of phytolacain R and papain [11]. The two sequences were aligned for maximum homology. Numbering in accord with that of papain. The asterisk indicates the active site cysteine.

One mililitre of enzyme soln was added to 1 ml of 2% (w/v) casein containing 67 mM K-Pi buffer, pH 7.2. After incubation for 20 min at 35° the reaction was stopped by the addition of 3 ml of 5% TCA. After standing for 30 min at room temp, the ppt. was removed by filtration through a Toyo filter paper No. 5C and A_{280} of the TCA-soluble peptides formed was determined. A unit of activity was defined as 0.001 A_{280} unit of change min^{-1} under the above conditions.

Assay of elastinolytic activity. Congo-Red-impregnated elastin (1 mg ml^{-1}) was dissolved in 20 mM borate buffer, pH 8.9 and homogenized with a Teflon homogenizer. Enzyme soln (1 ml) was added to 7 ml of the above suspension of the Congo-Red-impregnated elastin and incubated with stirring at 25° for 5 min, and then centrifuged at 3000 *g* for 1 min. The *A* of the supernatant was measured at 495 nm. A unit of activity was defined as 0.001 A_{495} unit of change min^{-1} under the above conditions.

Milk clotting activity assay. Milk clotting activity was measured by the method of ref. [8]. After skimmed milk (10%) soln containing 10 mM $CaCl_2$ was preincubated at 35° for 10 min, 0.5 ml of the enzyme soln was added and was gently stirred. The time of the first appearance of solid coagulant was measured. The amount of enzyme that clotted the milk soln in 1 min was defined as containing 400 units of activity [8].

Fibrinogen clotting activity assay. Fibrin clotting activity was measured by the modified method of ref. [9]. After fibrinogen (Sigma, St. Louis) soln (2.6 mg/0.1 ml of 67 mM K-Pi buffer, pH 7.2) was preincubated, at 25° for 10 min, the enzyme soln (50 μl) was added and gently agitated periodically. The time of the first appearance of fibrin threads was measured.

N-Terminal sequencing analysis. *S*-Pyridylethylation of phytolacain R was performed by the method of ref. [10]. Automated Edman degradation was performed with an Applied Biosystems 477A protein sequencer. The phenylthiohydantoin derivatives were identified using an Applied Biosystems 120A analyser.

REFERENCES

1. Kaneda, M., Izumi, S., Fukuda, T., Uchikoba, T. and Tominaga, N. (1988) *Phytochemistry* **27**, 3661.
2. Boller, T. (1986) in *Plant Proteolytic Enzymes* (Dalling, M. D., ed.), Vol. 1, pp. 67–96. CRC Press, Florida.
3. Kaneda, M. (1993) in *Tanpakushitsu-bunkaikouso* (Tsuru, D. and Funatsu, M., ed.), Vol. 2, pp. 145–180. Gakkai Shuppan, Tokyo.
4. Asboth, B., Majer, Z. and Polgar, L. (1988) *FEBS Letters* **233**, 339.
5. Ritonja, A., Rowan, A. D., Buttle, D. J., Rawlings, N. D., Turk, V. and Barrett, A. J. (1988) *FEBS Letters* **247**, 419.
6. Hirs, C. H. W. (1967) in *Methods in Enzymology* (Perlmann, G. E. and Lorand, L., eds), Vol. 11, pp. 197–199. Academic Press, New York.
7. Kunitz, M. (1947) *J. Gen. Physiol.* **30**, 291.
8. Arima, K., Yu, J. and Iwasaki, S. (1970) in *Methods in Enzymology* (Perlmann, G. E. and Lorand, L., eds), Vol. 19, pp. 446–459. Academic Press, New York.
9. Lundblad, R. L., Kingdon, H. S. and Mann, K. G. (1976) in *Methods in Enzymology* (Lorand, L., ed.), Vol. 45, pp. 156–161. Academic Press, New York.
10. Ruegg, U. T. and Rudinger, J. (1977) in *Methods in Enzymology* (Hirs, C. H. W. and Timacheff, S. N., ed.), Vol. 47, pp. 111–116. Academic Press, New York.
11. Mitchell, R. E. J., Chaiken, I. M. and Smith E. L. (1970) *J. Biol. Chem.* **245**, 3485.