*Phytochemistry*, Vol 27, No 11, pp 3661–3662, 1988
Printed in Great Britain

0031-9422/88 $3.00+0.00


# SHORT REPORTS

## ISOLATION AND CHARACTERIZATION OF A PROTEASE FROM *PHYTOLACCA AMERICANA*

MAKOTO KANEDA, SHIGEMICHI IZUMI, TOYOKO FUKUDA, TETSUYA UCHIKOBA and NAOTOMO TOMINAGA

Department of Chemistry, Faculty of Science, Kagoshima University, Korimoto, Kagoshima 890, Japan

(*Received in revised form* 13 *April* 1988)

**Key Word Index**—*Phytolacca americana*; Phytolaccaceae; pokeweed, cysteine protease; plant protease; phytolacin.

**Abstract**—A cysteine protease of $M_r$ 26 000 has been isolated in homogeneous form from the fruit juice of *Phytolacca americana*. The enzyme, named phytolacin, has a maximum activity in the pH range 7.5–9.0 and contains carbohydrate moiety. The substrate specificity of phytolacin differs from that of papain.

### INTRODUCTION

The fruit juice of pokeweed, *Phytolacca americana* L. was examined during the course of a search for new plant proteases. The method of Kunitz [1] using caseinolysis revealed the presence of the endopeptidases which were completely inhibited by mono-iodoacetate. The pokeweed plants contain several active substances [2–4], but no proteinase activity has been reported previously This paper describes the isolation of pokeweed protease, we gave the protease the trivial name phytolacin, and its substrate specificity in comparison with that of papain.

### RESULTS AND DISCUSSION

All operations were performed at *ca* 10° except for the homogenization at the first step

Step 1. *Extraction.* Pokeweed fruits (30 kg) were homogenized and the homogenate was filtered through a cotton filter. The juice had a vol of 18 l, with a bright purple colour, pH 5.9.

Step 2. $(NH_4)_2SO_4$ *precipitation.* Solid $(NH_4)_2SO_4$ was added gradually to the filtrate from step 1 to 60% saturation After 24 hr the resulting precipitate was collected by centrifugation and then dialysed against the first CM-cellulose column chromatography buffer, 0.02 M phosphate buffer, pH 5.9, containing 2 mM cysteine and 1 mM EDTA The dialysate was centrifuged to remove insoluble materials.

Step 3. *CM-cellulose column chromatography.* Dialysate from step 2 was placed on a column of CM-cellulose (3.5 × 57 cm) equilibrated with the first buffer. The column was eluted with a linear gradient from the first buffer to the second buffer, 0.2 M phosphate buffer, pH 6.4, containing 2 mM cysteine, 1 mM EDTA, and 0.2 M sodium chloride. Proteolytic activity was measured against casein as a substrate. Active protein fractions were collected and precipitated by the addition of solid $(NH_4)_2SO_4$ to 60% saturation.

Step 4. *CM-Sepharose CL-6B column chromatography.* An aliquot of the precipitated enzyme from the major active fractions at step 3 was dialysed against the first buffer mentioned above. The dialysate was placed on a column of CM-Sepharose CL-6B (1.5 × 30 cm) equilibrated with the first buffer. The column was eluted with a linear gradient from the first buffer to a buffer, 0.2 M phosphate buffer, pH 6.4. The enzyme factions were collected and precipitated by solid $(NH_4)_2SO_4$ to 60% saturation, yielding 30 mg of the protease.

#### *Homogeneity and* $M_r$

Polyacrylamide-gel electrophoresis of the protease purified on the CM-Sepharose CL-6B column revealed a single protein band. Furthermore, after polyacrylamide-gel electrophoresis, the band corresponding to the enzyme was stained by Schiff's reagent. These results indicate that the enzyme is glycoprotein. Phytolacin was eluted in a position corresponding to a $M_r$ of 26 000 by gel filtration of Sephadex G-75 using 0.2 M phosphate buffer, pH 7.1 The $M_r$ found on SDS-gel electrophoresis was 25 500

#### *Effects of pH and temperature*

The pH optimum of phytolacin was about pH 8 with casein as a substrate. At pH 6.0 the activity was only 25% of the optimum, and at pH 2.0 the enzyme showed negligible activity. The temperature optimum of the enzyme for 20 min incubations was at least 55°, and at 90° the activity was negligible. The pH stability of the enzyme was examined by incubating at various pH values for 24 hr at 25°, prior to assay at pH 7.1. At least 90% of the activity remained after incubation between pH 4.0 and 7.5.

Abbreviations: DFP, diisopropyl fluorophosphate, TCA, trichloroacetic acid; TLCK, N-tosyl-L-lysine chloromethylketone; TPCK, N-tosyl-L-phenylalanine chloromethylketone; PCMB, *p*-chloromercuribenzoic acid

Table 1 Effects of various compounds on the proteolytic activity of phytolacin against casein as a substrate

| Addition | Concentration (mM) | Relative activity (%) |
|---|---|---|
| None | — | 100 |
| Mono-iodoacetate | 1 0 | 0 |
| PCMB | 1 0 | 0 |
| DFP | 1 0 | 98 |
| EDTA | 1 0 | 102 |
| TPCK | 0 1 | 0 |
| | 0 01 | 27 |
| TLCK | 0 1 | 0 |
| | 0 01 | 65 |
| Leupeptin | 0 1 | 1 |
| Pepstatin | 0 1 | 100 |
| $CaCl_2$ | 5 0 | 77 |
| $MnSO_4$ | 5 0 | 68 |
| $ZnSO_4$ | 5 0 | 22 |

The enzyme (5 $\mu$g) was preincubated in 0 2 ml of 0 5 M Tris–HCl buffer, pH 7 5, containing various compounds for 60 min at 30° After preincubation, 1 ml of 1% casein in 1/15 M Pi buffer, pH 7 1, was added to the mixture and the activity assayed by the standard procedure

*Effects of various compounds*

The effects of various compounds on the enzyme activity are presented in Table 1. Mono-iodoacetic acid and PCMB showed strong inhibition. DFP and EDTA did not show any appreciable effects. These results suggest that this enzyme is a cysteine protease [5].

*Substrate specificity*

The relative rates of hydrolysis of various *p*-nitroanilides by phytolacin were compared with those of papain (Table 2) It was most active on Bz-Tyr-*p*NA, but papain did not hydrolyse this substrate. However, Bz-Arg-*p*NA was a good substrate for papain, but phytolacin could not hydrolyse it

## EXPERIMENTAL

Pokeweed, *Phytolacca americana*, was obtained in the harvesting season from the July to October, in Kagoshima perfecture, Japan

Table 2 Relative rates of hydrolysis of *p*-nitroanilides by phytolacin and papain

| Substrate | Phytolacin | Papain |
|---|---|---|
| | ($\mu$mol/min) | ($\mu$mol/min) |
| Bz-Tyr-*p*NA | 0 02 | 0 |
| Suc-Ala-Ala-Ala-*p*NA | 0 004 | 0 01 |
| Bz-Arg-*p*NA | 0 | 0 5 |
| Suc-Ala-Pro-Ala-*p*NA | 0 | 0 2 |
| Glt-Ala-Ala-Pro-Leu-*p*NA | 0 | 0 1 |
| Suc-Ala-Ala-*p*NA | 0 | 0 01 |
| Suc-Ala-*p*NA | 0 | 0 |

The enzymic hydrolysis was carried out 0 2 M Tris–HCl buffer pH 7 5 at 25° Substrate concentration was 1 mM Hydrolytic activity ($\mu$mol/min) of each enzyme is expressed as the value per 1 PU (caseinolytic activity) of enzyme

*Proteolytic activity* was measured by the method of Kunitz [1], with casein Enzyme soln (1 ml) was added to 1 ml of a soln of 1% casein containing 1/15 M Pi buffer, pH 6 5, at 27° After incubation for 20 min the reaction was stopped by the addition of 3 ml of 5% TCA After standing 30 min at room temp the ppt was removed by filtration through Toyo filter paper No 5c and *A* at 280 nm the TCA-soluble peptides formed was determined A unit of activity was defined as that amount which yielded 0 001 $A_{280}$ unit of change per min under the above conditions

*Electrophoresis* in polyacrylamide gel was performed in 7 5% gel with acetate buffer, pH 4 0 SDS-gel electrophoresis was performed in 10% gels according to the method of ref [6]

The $M_r$ of the enzyme was estimated by gel filtration through a Sephadex G-75 column (2.0 × 126 cm) equilibrated with 0 2 M Pi buffer PH 7 1 Cytochrome c, chymotrypsinogen, ovalbumin and serum albumin were used for the calibration of the column

## REFERENCES

1 Kunitz, M (1947) *J Gen Physiol* **30**, 291
2 Barbara, E B and Patricia, F (1967) *Nature* **214** (5090), 787
3 Wyott, S D and Shepherd, R J (1969) *Phytopathology* **57**(12), 1787.
4. Nishio, K (1984) *Mukogawa Joshi Daigaku Kiyo, Shokumotsu-hen* **32**, 37
5 Ruth, A (1970) in *Methods in Enzymology* (Perlmann, G E and Lorand, L. eds) Vol 19, p 226 Academic Press, New York
6 Weber, K and Osborn, M (1969) *J Biol Chem* **244**, 4406