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ISOLATION AND CHARACTERIZATION OF A PROTEINASE FROM WHITE GOURD

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Key Word Index—*Benincasa cerifera*; Cucurbitaceae; white gourd; serine-type proteinase.

Abstract—A proteinase from the sarcocarp of *Benincasa cerifera* was purified. ItsMW was estimated by two different methods to be about 50000. The maximum activity was found in the alkaline pH region against casein as a substrate. The enzyme was strongly inhibited by di-isopropyl fluorophosphate and not inhibited by EDTA and *p*-chloromercuribenzoic acid.

INTRODUCTION

Recently we described the complete purification of a proteinase from the sarcocarp of melon fruit (*Cucumis melo* L. var. Prince) [1]. The purified melon proteinase is strongly inhibited by di-isopropyl fluorophosphate (DFP) but is unaffected by reducing compounds such as cysteine and β-mercaptoethanol. Therefore, the enzyme seems to be a serine-type proteinase. Deb-Sarma [22] reported that the proteolytic enzyme of white gourd is not affected by reducing agent such as KCN, H_2S, glutathione, and ascorbic acid. This enzyme showed a pH optimum of 8.6 against gelatin as a substrate and a temperature optimum of 60° [2]. These properties are similar to those of the above melon proteinase.

The present paper describes the purification of a proteinase from white gourd and some properties.

RESULTS AND DISCUSSION

All the operations of the purification of a proteinase were performed at about 10° except for the homogenization at the first step.

Step 1. Extraction. White gourd (40.3 kg) was homogenized and the homogenate was packed into a cotton bag and centrifuged in a domestic spin drier. The debris was washed with 4 l. water and the washings were added to the above supernatant. The final supernatant had a vol of 37 l., with a pale greenish colour, pH 5.1

Step 2. CM-cellulose Treatment. Activated CM-cellulose fibres (350 g, wet wt) were added to the juice from step 1 and the mixture was stirred for 30 min. The CM-cellulose fibres were collected by filtration through a cotton bag. After this extraction had been repeated 2×, the activity remaining in the juice was less than 10% of the original. The white gourd proteinase was eluted from the CM-cellulose fibres with 1 M NaCl.

Step 3. $(NH_4)_2SO_4$ precipitation. Solid $(NH_4)_2SO_4$ was added gradually to the eluate from step 2 to 60% saturation and the soln was kept 18 hr. The resulting ppt. was collected by centrifugation at 6000 *g* for 25 min and then dialyzed against the first CM-cellulose column chromatography buffer, 0.02 M Pi buffer, pH 6. The dialysate was centrifuged to remove minor insoluble materials.

Step 4. CM-cellulose column chromatography. Dialysate from step 3 was placed on a column of CM-cellulose (5.3 × 75 cm) equilibrated with 0.02 M Pi buffer, pH 6. The column was eluted with a linear gradient from 0.02 M Pi buffer (pH 6) to 0.1 M Pi buffer (pH 6.5) containing 0.3 M NaCl at a flow rate of 170 ml/hr and 18.5 ml fractions were collected. 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 5. Gel-filtration on Sephadex G-75. An aliquot of the precipitated enzyme was dissolved in 0.1 M HOAc and applied to a Sephadex G-75 column (3.8 × 100 cm) previously equilibrated with 0.1 M HOAc. A single component having constant specific proteolytic activity across the peak was obtained. The enzyme fractions were pooled and dialyzed exhaustively against H_2O and lyophilized. The purification and yield of the enzyme are summarized in Table 1.

Homogeneity and MW. Polyacrylamide-gel electrophoresis of the proteinase purified by Sephadex G-75 gel filtration revealed a single protein band. The MW was determined by two methods. The white gourd proteinase was eluted in a position corresponding to a MW of 51000 by gel filtration on Sephadex G-75. Another estimation of the MW was performed by SDS polyacrylamide gel electrophoresis. A plot of the logarithm of the MW's of the reference proteins against mobility gave a straight line, from which the MW of the proteinase was calculated to be around 50000. This value of MW is in good agreement with that estimated by gel filtration.

Effects of pH and temperature. The pH optimum of the proteinase was about pH 9.2 with casein as a substrate. At pH 5.2 the activity was only 25% of the optimum, and at pH 2.3 the enzyme showed negligible activity. The temperature optimum of the proteinase for 20 min incubations was at least 70°, and at 94° the activity was negligible. The pH stability of the proteinase was examined by incubating at various pH values for 20 hr at 25°, prior to assay at pH 7.1. At least 80% of the activity remained after incubation between pH 4.5 and 9.5. The enzyme

Table 1. Purification of white gourd proteinase

Purification step	Total protein (mg)	Total activity (units $\times 10^{-6}$)	Specific activity (units/mg $\times 10^{-3}$)	Recovery (%)
Juice	12500	4.5	0.36	100
Ammonium sulfate	920	1.8	2.0	41
CM-cellulose	322	1.4	4.3	32
Sephadex G-75	132	0.9	6.8	21

after exposure to various temperatures up to 65° in the absence of protein substrate was fairly stable.

Effects of various compounds on the activity. The enzyme was completely inactivated by incubation with 0.5 mM DFP for 20 min, but under similar condition iodoacetamide, *p*-chloromercuribenzoic acid and EDTA had no effect on the activity of the proteinase. The enzyme does not require any bivalent cations such as Ca^{2+}, Cd^{2+}, Mn^{2+}, Co^{2+}, and Zn^{2+}, and its activity is not affected by them. Reducing agents such as β-mercaptoethanol and cysteine had no effect on the activity.

The proteinases of white gourd and melon [1] are remarkably similar to one another. No significant difference could be detected between the two in such criteria as MW, pH and temperature optima, pH and temperature stabilities and the sensitivity to various compounds. The presence of an active serine in the white gourd proteinase is indicated by the inhibition by DFP [3].

EXPERIMENTAL

White gourd (*Benincasa cerifera*) was obtained in the harvesting season from the end of July to the beginning of September, in Kagoshima prefecture, Japan. CM-cellulose fibres for batch-wise treatment were prepared in this laboratory by carboxymethylation of filter paper.

Proteolytic activity was measured by the method of ref [4], with casein as a substrate. Enzyme soln (1 ml) was added to 1 ml of a soln of 2% (w/w) casein containing 0.02 M Pi buffer, pH 7.8, at 37°. After incubation for 20 min the reaction was stopped by the addition of 2 ml of 5% TCA. After standing for 30 min at room temp., the ppt. was removed by filtration through Toyo filter paper No. 5C and *A* at 280 nm of the TCA-soluble peptides formed was determined. A unit of activity was defined as that amount which yielded 0.001 $A_{280\,nm}$ unit of change per min under conditions mentioned above. The sp. act. is expressed as the number of enzyme units per mg of protein. Protein was determined by the method of ref. [5] with BSA as a standard.

Electrophoresis in polyacrylamide gel was performed in 7.5% gel with acetate buffer, pH 4. SDS polyacrylamide gel electrophoresis for estimation of the MW of the proteinase was performed by the method of ref. [6], as described in ref [7]. The following reference proteins were used; cytochrome *c*, chymotrypsinogen, ovalbumin, and BSA.

The MW of the proteinase was estimated by gel filtration through a Sephadex G-75 column (1.5 × 95 cm) equilibrated with 0.1 M HOAc. The void vol. of the column was measured with Blue Dextran 2000. Cytochrome *c*, chymotrypsinogen, ovalbumin, and BSA were used for the calibration of the column. Fractions of 3.5 ml were collected at a flow rate of 20 ml per hr, and protein was monitored by measuring the A at 280 nm, or at 410 nm for cytochrome *c*.

To test the effects of various compounds the enzyme (12 μg) was preincubated in 0.5 ml of 0.05 M acetate buffer, pH 7.1, containing the compounds at 0.5 mM for 60 min at 37°. Casein (0.5 ml of 2%) in 0.05 M acetate buffer, pH 7.1, was then added to the mixture and the activity assayed by the standard procedure.

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