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# CUCUMISIN LIKE PROTEASE FROM THE SARCOCARP OF *BENINCASA HISPIDA* VAR. *RYUKYU*

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**Key Word Index**—*Benincasa hispida*; Cucurbitaceae; white gourd; serine protease; plant protease.

**Abstract**—A protease has been purified from the sarcocarp of *Benincasa hispida* (Thunb.) Cogn. var. *Ryukyu* by two steps of chromatography. Its $M_r$ was estimated by SDS–PAGE to be about 67,000. The enzyme was strongly inhibited by diisopropyl fluorophosphate, but not by EDTA and cysteine protease inhibitors. The substrate having alanine at the position of $P_1$ was the best among the Ala-Ala-Pro-X-*p*NAs (X = Ala, Lys, Phe, Glu, and diaminopropionic acid (Dap)). The N-terminal sequence of the first 33 residues was determined and 25 of the residues agreed with that of cucumisin [EC 3.4.21.25], a protease from the sarcocarp of melon fruit (*Cucumis melo* L. var. *Prince*). The results indicated that the *B. hispida* protease is a cucumisin like serine protease. 

## INTRODUCTION

Several useful endopeptidases have been isolated from plant sources, for instance, papain and bromelain, which have been used extensively in the food industry. It has become apparent that most of the isolated plant endopeptidases have been classified as cysteine endopeptidases. Usually, the activity of cysteine endopeptidase is readily reduced by air oxidation or metal ions. The application of cysteine endopeptidases requires reductants and chelating agents, whereas serine endopeptidases do not require these, and for this reason, serine protease is useful in the food industry.

Whereas the cysteine proteases have been studied extensively, relatively little is known about other types of plant proteases [1–3]. Previously, we found cucumisin [EC 3.4.21.25] from melon fruit, *Cucumis melo* L. var. *Prince* [4, 5]. The protease is strongly inhibited by diisopropyl fluorophosphate (DFP) but is unaffected by reducing compounds such as cysteine and 2-mercaptoethanol. Therefore, the enzyme seems to be a serine protease. The amino acid sequence around the reactive serine and histidine of cucumisin has been identified [6, 7], and the primary structure of cucumisin has been predicted from the cDNA [8]. The structure was homologous to those of proprotein processing proteases such as Kex2 from yeast [9] and human furin [10].

In the course of the screening of protease from edible plants, we found that milk casein was rapidly hydrolysed by juice of *Benincasa hispida* (Thunb.) Cogn. var. *Ryukyu*, 'Ryukyu white gourd' at neutral pH. This fruit is readily available in East Asia. The protease is present at a high concentration in the fruit, and large scale preparation is easier than with the common variety of white gourd, *Benincasa hispida* (*Benincasa cerifera*) [11]. *Benincasa hispida* var. *Ryukyu* protease appears to be suitable for food technology. First however, it is necessary to elucidate the enzymatic properties of *B. hispida* var. *Ryukyu* protease.

## RESULTS AND DISCUSSION

A protease from this fruit was purified by a simple procedure (see Table 1 for a summary of the procedure) 57-fold, with 49% recovery. From 725 g of fruit of *B. hispida* var. *Ryukyu*, 17 mg of the purified enzyme was obtained. The elution profile of the CM-Sepharose column chromatography showed a single active peak (Figure 1).

SDS-PAGE of the purified enzyme revealed a single protein band as shown in Fig. 2. Purified protein migrated as a single band having a $M_r$ of *ca* 67,000. This value is in agreement with the $M_r$ of the enzyme isolated from prince melons (*Cucumis melo* var. *Prince*) [5].

The pH stability of the enzyme was examined by incubating at various pH values for 24 hr at 25° prior

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Table 1. Purification of *B. Hispida* var. *Ryukyu* protease

| Purification step | Total protein (mg) | Total activity (units) | Specific activity (units/mg) | Recovery (%) |
|---|---|---|---|---|
| Juice | 1970 | 128,000 | 65.1 | 100 |
| CM-Sepharose | 17 | 63,200 | 3720 | 49 |

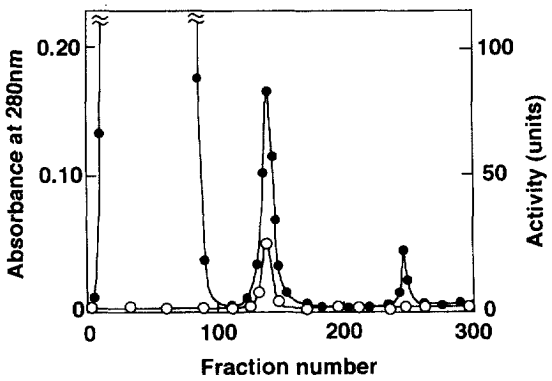


Fig. 1. Elution profiles of protein (●) and protease activity (○) from a CM-Sepharose column. The unadsorbed fraction on a DEAE-cellulose column of *B. hispida* var. *Ryukyu* fruits juice was applied to CM-Sepharose. The flow rate of the column was 50 ml/hr and an 8 ml fraction was collected. The protease activity of 100 $\mu$l aliquots of each fraction was measured for 20 min at 37°.

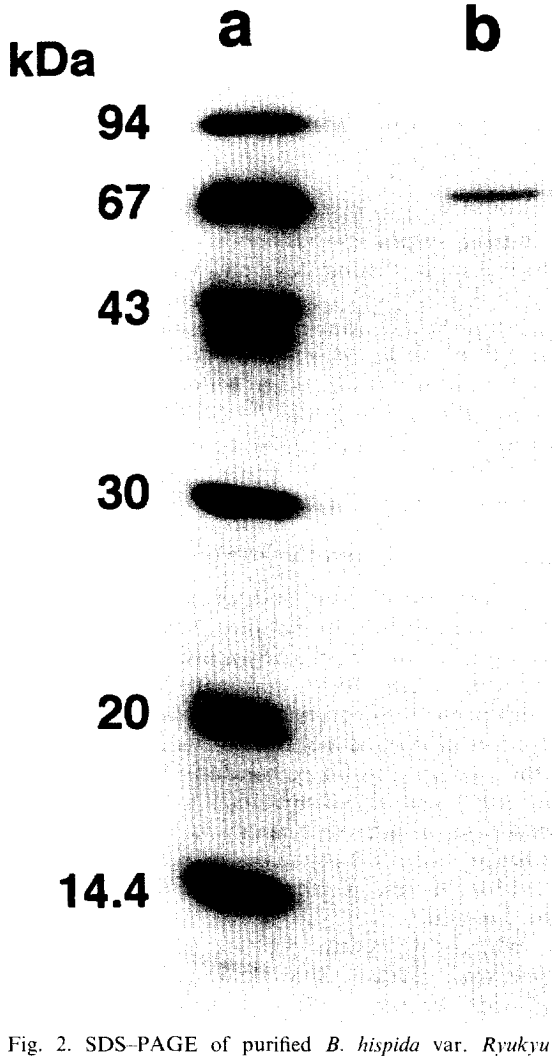


Fig. 2. SDS-PAGE of purified *B. hispida* var. *Ryukyu* protease. (a) Protein markers; (b) the purified *B. hispida* var. *Ryukyu* protease (10 $\mu$g).

to assay at pH 7.5. The enzyme is relatively stable at alkaline pH. At least 90% of the activity remained after incubation at between pH 7 and 11. The enzyme had optimal activity at about 60°. Holwerda and Rogers stated that the temperature-activity profiles for plant cysteine proteases showed some variations [12]. The optimum temperature of *B. hispida* var. *Ryukyu* protease seems to be higher than that of the plant cysteine proteases.

The activity of *B. hispida* var. *Ryukyu* protease maintained the initial level till 2 hr at pH 7.5 and 60° (not shown), but the enzyme was inactivated at 90°, pH 7.5, after 15 min. Some proteases form an active and stable intermediate in the course of autolysis. Cucumisin ($M_r$ 67 k) gradually generated the active protease (54 k) during incubation at 60° [13]. It was proved that the protease (54 k) was derived on limited autolysis of the cucumisin [13]. The 54 k protease is identical to the 67 k protease in activity and stability of enzyme, and both proteases had the same specific activity for Suc-Ala-Ala-Pro-Phe-*p*NA. Cucumisin (67 k) was present in prince melon from both unripe and ripe fruit. The 54 k protease was negligible in the fruits, and was produced during the process of purification. On the other hand the cleavage site of *B. hispida* var. *Ryukyu* protease was not limited in autolysis after incubation at 50° or 60°. In the case of incubation above 60°, the enzyme (67 k) only gradually decayed without forming the stable intermediate. The autolysis profile of *B. hispida* var. *Ryukyu* protease apparently differs from that of cucumisin.

The effects of various compounds on the enzymatic activity are presented in Table 2. The enzyme was completely inactivated by incubation with 3.8 mM DFP for 1 hr at 37°. The inhibitory activity of chymostatin was strong for the enzyme, but pefabloc SC and PMSF were weak. APMSF, TLCK, TPCK, and STI had no effect on the enzymatic activity. Iodoacetic acid and EDTA, also had no effect. The cysteine pro-

Table 2. Effects of various compounds on the proteolytic activity of *B. Hispida* var. *Ryukyu* protease

| Compounds | Conc.* (mM) | Relative activity (%) |
|---|---|---|
| None | — | 100† |
| DFP | 3.8 | 0 |
| Pefabloc Sc | 3.8 | 52 |
| PMSF | 3.8 | 77 |
| APMSF | 3.8 | 95 |
| TLCK | 0.5 | 109 |
| TPCK | 0.5 | 115 |
| Chymostatin | 0.2 | 4 |
| STI | 0.4 | 91 |
| MIA | 3.8 | 91 |
| E-64 | 0.6 | 89 |
| EDTA | 3.8 | 105 |

*Concentrations are those in the preincubation mixture.

† Activity of a control with no addition was taken as 100%.

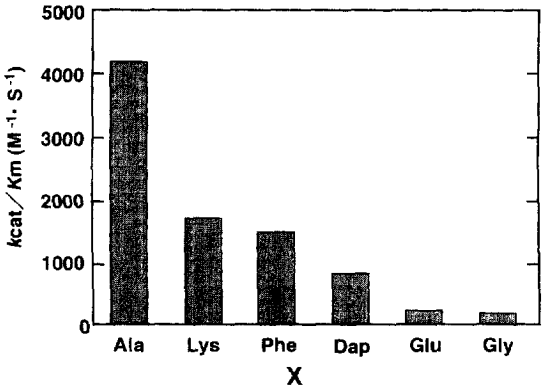


Fig. 3. Kinetic parameters for hydrolysis of Ala-Ala-Pro-X-*p*NAs by *B. hispida* var. *Ryukyu* protease. X; Ala, Lys, Phe, Glu, Gly, and diaminopropionic acid (Dap).

tease inhibitors E-64 caused no inhibition at the concentrations employed. The results indicated that the *B. hispida* var. *Ryukyu* protease is a serine protease. The results with these protease inhibitors are consistent with those for cucumisin [4, 5] and white gourd protease [11].

The kinetic constants ($k_{cat}/K_m$) for the hydrolysis of several Ala-Ala-Pro-X-*p*NAs substrates by *B. hispida* var. *Ryukyu* protease are shown in Fig. 3. The most favorable substrates for the protease were Ala-Ala-Pro-Ala-*p*NA. This is a good substrate for pancreatic elastase. It was shown that the $k_{cat}/K_m$ for *B. hispida* var. *Ryukyu* protease for this substrate was of almost the same order as that of elastase (not shown). The substrates Ala-Ala-Pro-Lys-*p*NA and Ala-Ala-Pro-Phe-*p*NA, and Ala-Ala-Pro-Dap-*p*NA were also hydrolysed by the enzyme, but the enzyme showed little activity with the substrates Ala-Ala-Pro-Glu-*p*NA and Ala-Ala-Pro-Gly-*p*NA in the same conditions. The substrates that contained Val and Pro at the $P_1$ position were not hydrolysed by the protease (data not shown). Aminopeptidase substrates, X-*p*NA (X = Leu, Tyr, Lys and Glu), were not hydrolysed (data not shown). From the digestion of the peptidyl-*p*NAs, the specificity of the protease was determined to be broad, but the preferential cleavage sites were small hydrophobic amino acid residues at the $P_1$ position. *B. hispida* var. *Ryukyu* protease also preferred the positively charged residues at $P_1$.

It was proved that the enzymatic properties of *B. hispida* var. *Ryukyu* protease are similar to those of cucumisin.

The N-terminal sequence of the first 33 residues of *B. hispida* var. *Ryukyu* protease was determined. The sequence of the enzyme was aligned with that of cucumisin for maximum homology (Fig. 4).

The N-terminal sequence of *B. hispida* var. *Ryukyu* protease is comparable with those of other plant serine endopeptidases, such as cucumisin, expressed in prince melon fruit [8], PR–P69; a pathogen-induced in tomato [14], and ag12; expressed in the early stages of actinorhizal nodule formation in *Alnus glutinosa* [15] (Fig. 4). The N-terminal 8 and no. 19–30 residues of *B. hispida* var. *Ryukyu* protease were identical to those of cucumisin. The Asp30 residue of cucumisin, one constituent of the catalytic triad, was predictable from the homology with other serine proteases [16]. The catalytic site of the serine peptidases is made up of three amino acid residues, forming a catalytic triad; histidine, aspartic acid, and serine. The constituent three amino acid residues in chymotrypsin are typical of serine protease and distributed in the order H-D-S. On the other hand, those of subtilisin from microbe and cucumisin from plant are in the order D-H-S, respectively. Enzymes having D-H-S can be divided further into the subtilisin family and cucumisin family by the criteria of the chain length of D-H-S. The residues numbers of the three amino acids are D(32)-H(67)-S(224) for subtilisin BPN′ and D(30)-H(97)-S(415) for cucumisin. The distances of D-H and H-S in cucumisin are about twice as long as those of subtilisin BPN′. The size of D-H-S in the cucumisin family goes up to over 385 residues, for example, cucumisin(385 residues) [8], and PR-69 (386 residues) [14], ag12 (392 residues) [15], SBTI of tomato (385 residues, EMBL accession number X85974), serine protease induced in meiotic prophase in lily microsporocytes (429 residues) [17], and *Arabidopsis thaliana* protease (401 residues, EMBL accession number X85974), whereas those of the subtilisin family are around 200 residues, such as Kex2 (210 residues) and subtilisin BPN′ (130 residues). Although the amino acid sequences around the catalytic amino acid residues are homologous with those of subtilisin, e.g. the four amino acids, Gly-Thr-Ser-Met in sequence around the reactive serine of cucumisin [6] are identical with those of subtilisin BPN′ [18], it is difficult to classify cucumisin into the subtilisin family for the following reason. The molec-

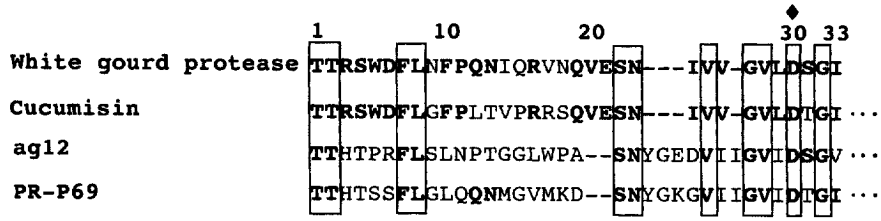


Fig. 4. N-terminal sequences of plant serine proteases. ◆ shows predicted reactive Asp residues of each serine protease. White gourd protease, *B. hispida* var. *Ryukyu* protease of the present study; cucumisin [9]; ag12, *Alnus glutinosa* protease [15]; PR–P69, tomato pathogen-induced protease [14].

ular size, the chain length of D-H-S, and the enzymatic properties are all distinct. The thermal stability of cucumisin (60°) is higher than that of subtilisin BPN′ (40°) at pH 11 [19], and cucumisin has high stability at acidic pH at which subtilisin is unstable. Cucumisin cleaved preferentially a peptide bond on the carboxyl side of negatively charged amino acid residues [5] which were not hydrolysed by subtilisin. *B. hispida* var. *Ryukyu* protease is classified as a member of the cucumisin family on the basis of its enzymatic properties, $M_r$ and amino acid sequence.

## EXPERIMENTAL

### *Materials*

*B. hispida* var. *Ryukyu* was obtained in Kagoshima prefecture, Japan. DEAE-cellulose was purchased from Whatman. CM-Sepharose was a product of Pharmacia. Casein was obtained from MERCK. TLCK was a product of Sigma. TPCK was from Seikagaku Kogyo. Other materials were purchased from Wako Pure Chemical.

The peptidyl-*p*-nitroanilide (*p*NA) substrates, Ala-Ala-Pro-X-*p*NA (X = Phe, Ala, Gly, Lys, Glu, and Dap) were prepared by the soln method [20] and purified by HPLC on a reverse-phase octadecyl silica column. Homogeneity of the synthetic substrates was demonstrated by HPLC, amino acid analysis and elemental analysis.

### *Assay of Protease*

Proteolytic activity was measured by the modified method of ref. [21] with casein as a substrate. Enzyme soln (10 μl) was added to 1 ml of 2% (w/v) casein in 67 mM Na, K-Pi buffer, pH 7.5. After incubation at 35° for 20 min, the reaction was stopped by the addition of 3 ml of 5% $CCl_3$ COOH. After standing for 30 min at room temp, the ppt was removed by filtration through Advantec Toyo filter paper No. 5C (Tokyo, Japan) and $A$ of the filtrate was determined at 280 nm. A unit of activity was defined as the activity giving 0.001 $A_{280}$ unit of change per min under the above conditions.

### *Hydrolysis of peptidyl-pNAs*

Enzymatic hydrolysis was done in 0.2 M Tris-HCl buffer, pH 7.5 at 25°. The enzyme soln (0.1 ml) was added to 0.8 ml of 0.5 mM substrate soln. The rate of enzymatic hydrolysis for peptidyl-*p*NA substrates was followed spectrophotometrically with a Hitachi U-1100 spectrophotometer. An extinction coefficient of 8800 $M^{-1} \cdot cm^{-1}$ at 410 nm was used for the yield of the hydrolysis product, *p*-nitroaniline [22]. A unit of activity was defined as the activity giving 1 μmol of the product per min under the above conditions.

### *Electrophoresis*

SDS–PAGE–The method of Ref. [23] was followed, using a 12.5% polyacrylamide gel. The purified enzyme was treated to avoid autolysis, with 67 mM DFP for 30 min at 30°. The diisopropyl-phosphorylated enzyme was subjected to SDS–PAGE. The proteins were stained with Coomassie Brilliant Blue.

The $M_r$ standards used were phosphorylase b (94 k), bovine serum albumin (67 k), ovalbumin (43 k), carbonic anhydrase (30 k), soybean trypsin inhibitor (20 k), and α-lactoalbumin (14.4 k) (Pharmacia).

### *Enzyme purification*

All procedures for purification of the enzyme were carried out at 7°. Frozen *B. hispida* var. *Ryukyu* fruits (725 g) were homogenized in an appropriate vol. of buffer A (50 mM Na OAc buffer, pH 5.0). The homogenate was filtered through a cotton cloth and then centrifuged (3000*g*, 15 min). The supernatant (1050 ml) was placed on a column of DEAE-cellulose (8 × 20 cm) equilibrated with buffer A to remove the undesired proteins. The column was washed with buffer A (400 ml). The protease passed through the column. The fraction passed through the DEAE-cellulose column was chromatographed with a linear gradient of 0–0.16 M NaCl in buffer A on a CM-Sepharose column (1.5 × 12 cm). Proteolytic activity in each fraction was measured against casein as a substrate. The active fractions were collected and kept at −20°.

*Effect of pH*

The effect of pH on the activity of the enzyme towards Ala-Ala-Pro-Ala-*p*NA as a substrate was determined using the following buffer; 0.2 M Na,K-Pi (pH 6.0–8.5), 0.2 M glycine-NaCl-NaOH (pH 8.0–12.0). The assay was done as for protease as described above.

*Effect of temp.*

The enzyme was incubated in Ala-Ala-Pro-Ala-*p*NA containing 67 mM Tris-HCl buffer at pH 7.5 for 15 min at different temp. (5–80°). The assay was done as for protease.

*Effect of compounds*

The stock soln of protease inhibitors was prepared in 67 mM Tris-HCl buffer at pH 7.5 and contained various compounds. The enzyme soln (0.1 ml) was added to 0.1 ml of inhibitor soln in 67 mM Tris-HCl buffer at pH 7.5 and incubated at 37° for 60 min. Ala-Ala-Pro-Ala-*p*NA (0.8 ml) in 67 mM Na,K-Pi buffer, pH 7.2 was added to 0.1 ml of the above reaction mixture, and remaining activity was assayed. Control assay was done with enzyme soln without the compounds.

*N-terminal sequencing analysis*

Automated Edman degradation of *B. hispida* var. *Ryukyu* protease was performed with an Applied Biosystems 477A protein sequencer. The phenylthiohydantoin derivatives were identified by an Applied Biosystems 120A analyzer.

## REFERENCES


1. Arnon, R., in *Methods in Enzymology*, Vol. 19, ed. G. E. Perlmann and L. Lorand. Academic Press, New York, 1970, pp. 226–244.
2. Liener, I. E. and Friedenson, B., in *Methods in Enzymology*, Vol. 19, ed. G. E. Perlmann and L. Lorand. Academic Press, New York, 1970, pp. 261–273.
3. Murachi, T., in *Methods in Enzymology*, Vol. 19, ed. G. E. Perlmann and L. Lorand. Academic Press, New York, 1970, pp. 273–284.
4. Kaneda, M. and Tominaga, N., *J. Biochem.*, 1975, **78**, 1287.
5. Uchikoba, T., Yonezawa, H. and Kaneda, M., *J. Biochem.*, 1995, **117**, 1126.
6. Kaneda, M., Omine, H., Yonezawa, H. and Tominaga, N., *J. Biochem.*, 1984, **95**, 825.
7. Yonezawa, H., Uchikoba, T. and Kaneda, M., *J.Biochem.*, 1995, **118**, 917.
8. Yamagata, H., Matsuzawa, T., Nagaoka, Y., Onishi, T. and Iwasaki, T., *J. Biol. Chem.*, 1994, **269**, 32725.
9. Mizuno, K., Nakamura, T., Oshima, T., Tanaka, S. and Matsuo, H., *Biochem. Biophys. Res. Commun.*, 1988, **156**, 246.
10. Van den Ouweland, A. M. W., Van Duijinhoven, H. L. P., Keizer, G. D., Dorssers, L. C. J., Van de Ven, W. J. M., *Nucleic Acids Res.*, 1990, **18**, 664.
11. Kaneda, M. and Tominaga, N., *Phytochemistry*, 1977, **16**, 345.
12. Holwerda, B. C. and Rogers, J. C., *Plant Physiol.*, 1992, **99**, 848.
13. Yamagata, H., Ueno, S. and Iwasaki, T., *Agric. Biol. Chem.*, 1989, **53**, 1009.
14. Tornero, P., Conejero, V. and Vera, P., *Proc. Natl. Acad. Sci. USA*, 1996, **93**, 6332.
15. Ribeiro, A., Akkermans, A. D. L., van Kammen, A., Bisseling, T. and Pawlowski, K., *Plant Cell*, 1995, **7**, 785.
16. Rowlings, N. D. and Barrett, A. J., in *Methods in Enzymology*, Vol. 244, ed. A. J. Barrett. Academic Press, New York, 1994, pp. 19–61.
17. Kobayashi, T., Kobayashi, E., Sato, S., Hotta, Y., Miyajima, N., Tanaka, A. and Tabata, S., *DNA Res.*, 1994, **1**, 15.
18. Hill, R. L., Swenson, R. T. and Schwartz, H. C., *J. Biol. Chem.*, 1960, **235**, 3182.
19. Kaneda, M., Yonezawa, H. and Uchikoba, T., *Biotechnol. Appl. Biochem.*, 1995, **22**, 215.
20. Delmar, E. G., Largman, C., Brodrick, J. W. and Geokas, M. C., *Anal. Biochem.*, 1979, **99**, 316.
21. Kunitz, M., *J. Gen. Physiol.*, 1947, **30**, 291.
22. Erlanger, B. F., Kokowsky, N. and Cohen, W., *Arch. Biochem. Biophys.*, 1961, **95**, 271.
23. Laemmli, U. K., *Nature*, 1970, **227**, 680.