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Note

Isolation and Some Properties of a Serine Protease from the Fruits of *Cudrania cochinchinensis* (Lour.) Kudo et Masam.

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An endopeptidase (*Cudrania* protease) with a molecular mass of 76 kDa has been purified from the fruits of *Cudrania cochinchinensis* (Lour.) Kudo et Masam. The enzyme was stable between pH 6 and 10 at 30°C for 60 min. The enzyme activity was inhibited by diisopropyl fluorophosphate, chymostatin, and aprotinin, but not by EDTA or pepstatin. These results indicated that the enzyme was a serine protease.

Key words: *Cudrania cochinchinensis*; fruits; *Moraceae*; serine protease

Although the cysteine proteases have been studied extensively, little is known about other types of plant proteases.[1] In the course of protease screening from plants, we found that milk casein was rapidly hydrolyzed by juice from the fruits of *Cudrania cochinchinensis* (Lour.) Kudo et Masam. In this paper, we describe about the isolation, general properties, and substrate specificity of a new serine protease from the fruits of *Cudrania cochinchinensis*.

Fruits of *Cudrania cochinchinensis* were collected in Kagoshima City, Japan. For enzymatic hydrolysis of peptidyl-*p*NA substrates, peptidyl-*p*NA stock solution (10 mM in dimethylsulfoxide, 10 μl) was added to 0.2 M Tris-HCl buffer (pH 7.0, 0.6 ml). The enzyme solution (0.2 ml) was then added to the substrate solution at 25°C. The rate of enzymatic hydrolysis for the substrates was followed spectrophotometrically with a Hitachi U-1100 spectrophotometer. An extinction coefficient of 9,920 $M^{-1}\,m^{-1}$ at 405 nm[2] was used for the yield of the hydrolysis product, *p*-nitroaniline. One unit of activity was defined as the activity producing 1 μmol of the product per min, under these conditions.

Proteolytic activity was measured with casein as a substrate by the method described previously.[3] One unit of activity was defined as the activity giving 0.001 A_{280} per min under the stated conditions (pH 7.5, at 35°C).

Cudrania protease was purified as follows. Ripened fruits (300 g) of *Cudrania cochinchinensis* were homogenized with equal weights of buffer A (17 mM sodium, potassium-phosphate buffer, pH 7.0). After centrifugation (10,000 × *g*, 8 min), the supernatant was placed on a column of DEAE-cellulose (8 × 20 cm) equilibrated with buffer A to remove any undesired proteins. The enzyme was then eluted with buffer B (0.2 M sodium, potassium-phosphate buffer, pH 7.0). The active fraction was dialyzed for 15 h against buffer A, the dialysate was put on a column of DEAE-Sepharose (1.5 × 11 cm) equilibrated with buffer A. The enzyme was eluted with a linear gradient from buffer A (1.0 l) to buffer B (1.0 l) at a flow rate of 1.0 ml/min. The active fractions were collected and concentrated with an Advantec-UHP-76 ultra filtering unit. The concentrated solution was put on a Sephacryl S-300 (1.5 × 120 cm) column equilibrated with buffer A. The active fraction was collected and kept at −20°C. From 300 g of the fruits, 1.4 mg of the purified enzyme was obtained in a final yield of 40%. The final preparation shown as a single band on SDS-PAGE, having a molecular mass of about 76 kDa, as shown in Fig. 1.

The optimum pH for caseinolytic activity was found at 11. The enzyme was fairly stable at alkaline pH region, and 90% of the enzyme activity was retained as measured at 7.0 after incubation of the enzyme solution (60 μg/ml) at various pHs (pH 6–10) at 30°C for 60 min.

The optimum temperature of the protease was at 60°C with casein as a substrate. To examine the effects of temperatures on the stability of the en-

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Abbreviations: DFP, diisopropyl fluorophosphate; MIA, monoiodoacetic acid; *p*-APMSF, *p*-amidinophenylmethanesulfonylfluoride; PCMPS, *p*-chloromercuriphenylsulfonic acid; Pefabloc SC, 4-(2-Aminoethyl)-benzenesulfonyl fluoride; *p*NA, *p*-nitroanilide; Tos-Lys-CH_2Cl, *N*-Tosyl-L-lysine chloromethylketone; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis

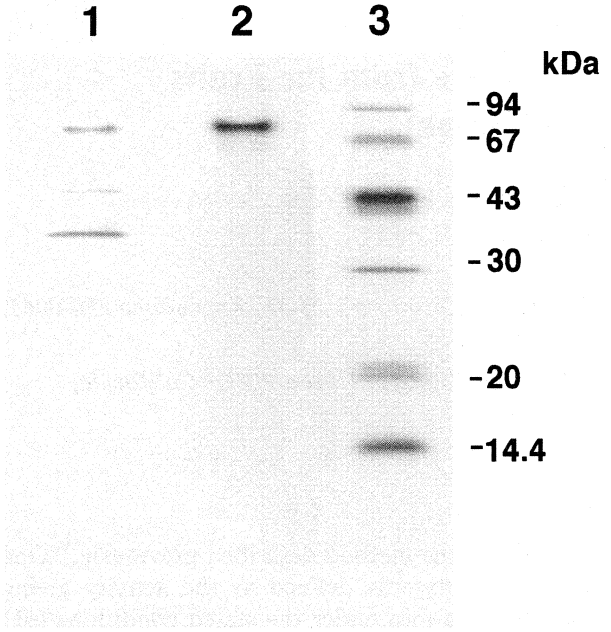

Fig. 1. SDS-PAGE of Purified *Cudrania* Protease.
To avoid autolysis, the purified enzyme was inactivated by treatment with 10 mM DFP. The inactivated enzyme was electrophoresed on SDS-PAGE by the method of Laemmli[4] in a 15% polyacrylamide gel. The gel was stained in Coomassie Brilliant Blue R-250 for 15 min and then destained. Lane 1, 10 μg of pooled fractions from DEAE-Sepharose chromatography; Lane 2, 10 μg of purified *Cudrania* protease from Sephacryl S-300 gel-filtration. Lane 3, molecular mass markers.

zyme, the residual activities of the enzyme were measured after the enzyme (0.5 ml) was incubated in buffer B at temperatures between 20–70°C for 30 min. At least 80% of the caseinolytic activity remained after incubation at 55°C.

The enzyme (0.5 ml, 60 μg/ml) was incubated with 0.5 ml of 67 mM sodium, potassium-phosphate buffer, pH 7.0, containing various inhibitors at 30°C. After 120 min, 1 ml of 2% (w/v) casein in the same buffer was added to the reaction mixture, and the remaining caseinolytic activity was measured. *Cudrania* protease was completely inactivated by incubation with 2.0 mM DFP. The activity was strongly inhibited by aprotinin and chymostatin. Inhibitory activity of *p*-APMSF, Pefabloc SC, Tos-Lys-CH_2Cl, and leupeptin proved weak for the enzyme. Under similar conditions, PCMPS, MIA, and antipain had a definite effect on the over-all activity, and STI caused no inhibition. Almost full enzyme activity of the enzyme was retained by incubation with EDTA or pepstatin. The results indicated that the protease was a serine protease.

In Table 1 the hydrolysis of several peptidyl-*p*NA substrates by *Cudrania* protease was compared. The best substrate for the protease was Glt-Ala-Ala-Pro-Leu-*p*NA. Boc-Ala-Ala-Pro-Ala-*p*NA, Suc-Ala-Pro-Ala-*p*NA, Suc-Ala-Ala-Ala-*p*NA, and Suc-Ala-Ala-*p*NA, which are all substrates for elastase, were

Table 1. Hydrolysis of Peptidyl-*p*NA with *Cudrania* Protease

Substrates	Hydrolysis rate (units/min/μmol protein)	Relative activity (%)
Glt-Ala-Ala-Pro-Leu-*p*NA	390	100[a]
Boc-Ala-Ala-Pro-Ala-*p*NA	89	23
Suc-Ala-Ala-Pro-Phe-*p*NA	65	16
Suc-Ala-Pro-Ala-*p*NA	57	15
Bz-Arg-*p*NA	57	15
Pyr-Phe-Leu-*p*NA	41	11
Suc-Ala-Ala-Ala-*p*NA	33	8
Suc-Ala-Ala-*p*NA	8	2
Z-Gly-Gly-Leu-*p*NA	0	0
Z-Ala-Ala-Leu-*p*NA	0	0

[a] The enzyme activity toward Glt-Ala-Ala-Pro-Leu-*p*NA was taken as 100%.

also available for *Cudrania* protease. Z-Gly-Gly-Leu-*p*NA, a substrate for neutral endopeptidase, was not hydrolyzed by *Cudrania* protease. On the other hand, Bz-Arg-*p*NA, a suitable substrate for trypsin, was hydrolyzed by *Cudrania* protease. Bz-Arg-*p*NA was not hydrolyzed by cucumisin.[5] These results showed that the specificity of *Cudrania* protease toward peptidyl-*p*NAs was similar to that of cucumisin except for Bz-Arg-*p*NA.

Cudrania cochinchinensis is a plant of the mulberry family. Macluralisin, a serine protease from another plant of the mulberry family, was already isolated from the fruits of *Cudrania tricuspidata* (Carr.) Lav. (*Maclura pomifera* (Raf.) Schneid).[6] The optimum temperature for *Cudrania* protease was similar to that of macluralisin, however the molecular mass of *Cudrania* protease (76 kDa) was slightly larger than that of macluralisin (65 kDa).[6]

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