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PURIFICATION AND PROPERTIES OF PROTEINASE FROM RICE-BRAN

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Abstract

A proteinase from rice-bran was purified. Its molecular weight was estimated by gel-filtration to be about 46,000. The maximum activity was found in about pH 6.5 against casein as a substrate. The enzyme was not inhibited by di-isopropyl fluorophosphate, iodoacetoamide and EDTA.

Introduction

In the course of work to obtain proteinase inhibitor from rice-bran, caseinolytic activity was found in a break-through fraction on CM-cellulose chromatography.

A number of proteinase have already been purified from plant sources (1). Most of these enzymes are affected by various reagents which show pronounced reactivity toward the free sulfhydryl group. However, the rice-bran proteinase seemed not to be thiol-dependent proteinase.

This paper reports the isolation of proteinase from commercially available rice-bran and the characterization of the enzyme.

Materials and Methods

Rice-bran was purchased from commercial source in Kagoshima City.

Casein was a product of E. Merk. Darmstadt, West Germany. Phenylmethanesulfonyl fluoride (PMSF) was a product of Sigma Chemical Co., U.S.A. Di-isopropyl fluorophosphate (DFP) was a product of BDH Chemicals, Ltd., England. L-Cysteine, trichloroacetic acid and iodoacetoamide were purchased from Wako Pure Chemical Industries, Ltd., Osaka.

Assay of Proteinase – Proteolytic activity was measured by the method of Kunitz (2), with casein as a substrate. One ml of sample solution was added to 1 ml of a solution of 1% (W/W) casein containing 0.02 M phosphate buffer, pH 7.3, at 30°. After incubation for 20 min the reaction was terminated by the addition of 3 ml of 5%

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trichloroacetic acid. After standing for 30 min at room temperature, the precipitate was removed by filtration through Toyo filter paper No. 5C and the absorbancy at 280 nm of the trichloroacetic acid-soluble peptides formed was determined with a Hitachi spectrophotometer 102. A blank was run with each assay.

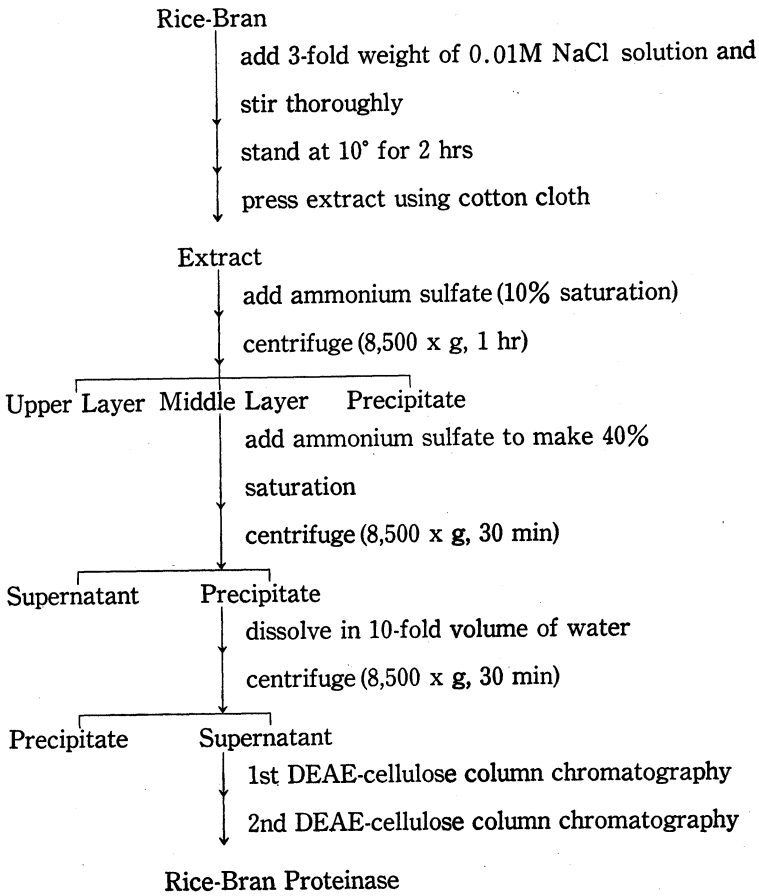
A unit of activity was defined as that amount which yielded 0.001 A_{280nm} unit of change per min under the conditions mentioned above.

Results and Discussion

Purification of enzyme – Purification was carried out mainly in two steps as follows; (1) removal of lipids and precipitation of enzyme fraction by ammonium sulfate fractionation, (2) column chromatography on DEAE-cellulose. All the operations were performed at about 10°. The purification procedure is summarized in the flow diagram shown in Table 1.

Step 1. Extraction – In a typical experiment, 1.0 kg of commercial rice-bran was suspended in 3 liters of 0.01 M NaCl. After standing at 10° for 2 hrs, the suspension was packed into a cotton bag and then pressed. The extract obtained had a volume of 2 liters, with a light yellow color. The removal of lipids from the extract was

Table 1. Purification of Proteinase from Rice-Bran



critical step in this preparation, because the emulsified lipid was present in large amount. Finally this step was successfully accomplished through centrifugation following salting-out of ammonium sulfate.

Solid ammonium sulfate was slowly added to the extract with gently stirring to 10% saturation and was stand for 2 hrs after final addition of salt. By centrifugation for 1 hr, three layers were formed. The yellowish upper layer appered to be the lipids. Proteolytic activity was contained predominantly in the middle layer. The bottom layer was constituted almost insoluble material. The upper and bottom layer were discarded. The middle layer supernatant was brought to 40% saturation by the further addition of solid ammonium sulfate. The precipitate was collected by centrifugation at 8,500 ×g for 30 min and dissolved in 10-fold volume of water.

Step 2. DEAE-cellulose column chromatography – The enzyme solution obtained in Step 1 was applied to a column of DEAE-cellulose equilibrated with 0.02 M phosphate buffer, pH 7.0. After washing sufficiently with starting buffer, the active enzyme was eluted by a linear salt gradient from starting buffer to 0.1 M phosphate buffer, pH 7.0, containing 1.0 M NaCl, as shown in Figure 1. The active fraction was pooled and precipitated by the addition of solid ammonium sulfate to 50% saturation. The resulting precipitate was collected by centrifugation at 8,500× g for 30 min, dissolved in above starting buffer and applied to a second DEAE-cellulose column. The active material was eluted by applying an exponential gradient to 0.1 M phosphate buffer, pH 7.0, containing 1.5 M NaCl. The protease fraction was pooled and concentrated

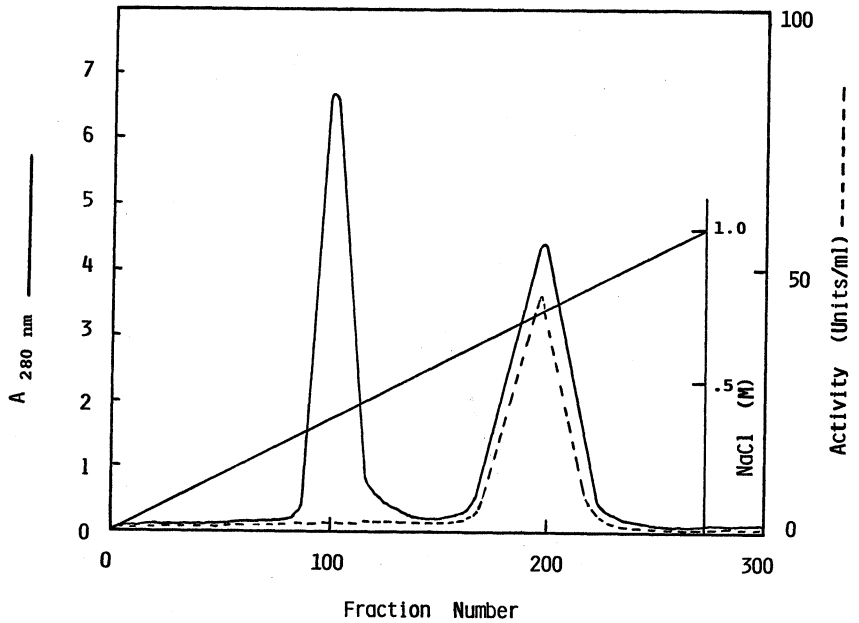


Fig. 1. Chromatography of crude rice-bran proteinase on DEAE-cellulose.
The column (4.5×52 cm) was eluted with a linear gradient from 0.02 M phosphate buffer (pH 7.0) containing 1.0 M NaCl. Fractions of 17 ml were collected. Proteolytic activity was measured against casein as described in "Materials and Methods".

by precipitation with ammonium sulfate as above, dialyzed exhaustively against distilled water, and lyophilized.

Homogeneity and Molecular Weight – Electrophoresis in polyacrylamide gel was performed in 7.0% gel with Tris-HCl buffer, pH 8.0. Gel-electrophoresis of the proteinase purified revealed a single protein band. The molecular weight of the proteinase was estimated by gel-filtration through a Sephadex G–75 column (1.3×92 cm) equilibrated with 1.0 M acetic acid. The following reference proteins, viz. cytochrome c, chymotrypsinogen, serum albumin and aldolase were used for the calibration of the column. Fractions of 4.0 ml were collected at a flow rate of 20 ml per hour, and protein was monitored by measuring the absorbance at 280 nm. Cytochrome c was located in the effluent by determining the absorption at 410 nm. The rice-bran proteinase was eluted in a position corresponding to a molecular weight of 46,000.

Effect of pH – The effect of pH on the proteolytic activity was studied with casein as a substrate. The enzyme showed optimum activity at pH 6.5. It was not possible to performed the assay in the pH range shown by a dotted line in Figure 2, because casein tends to precipitate in this pH range. For this reason, the precise optimum pH of the enzyme activity may be acidic rather than the apparent optimum pH.

Effect of pH on the stability of the enzyme was determined by incubation at several

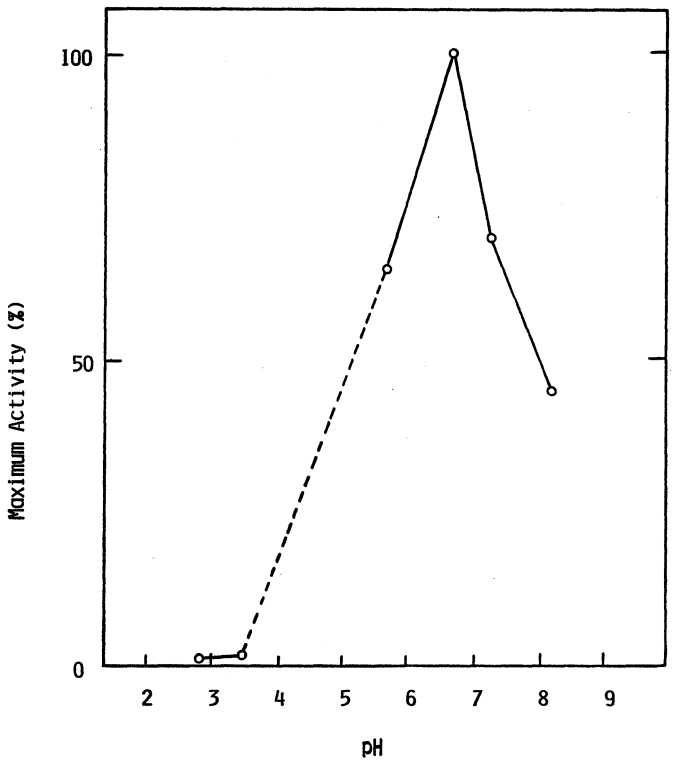


Fig. 2. Effect of pH on the proteolytic activity of rice-bran proteinase.
The activity was assayed by the method of Kunitz (2) at various pH values. The buffer used was citric acid-sodium phosphate.

Table 2. Effects of Various Compounds on the Proteolytic Activity of Rice-Bran Proteinase Against Casein as a Substrate

Addition	Concentration[a] (mM)	Relative Activity[b] (%)
PMSF	0.8	99
DFP	0.36	100
Cysteine	0.8	107
MIA	0.8	97
EDTA	0.8	107

The enzyme was preincubated in 0.5 ml of 0.02 M phosphate buffer, pH 7.3, containing various compounds for 20 min at 30°. After preincubation, 0.5 ml of 1% casein in 0.02 M phosphate buffer, pH 7.3 was added to the mixture and the activity assayed by the standard procedure.
[a] Concentrations are those in the preincubation mixture.
[b] Activity of a control with no addition was taken as 100%.
PMSF; phenylmethanesulfonyl fluoride
DFP; di-isopropyl fluorophosphate
MIA; monoiodoacetoamide

pH values from 4 to 12. The enzyme was incubated for periods of 20 min at 50° and then assayed at pH 6.5 with casein. The enzyme was found to be most stable at pH 7.0–8.5.

Effects of Various Compounds on the Activity – Requirement of metal ions for the activity of the protease was tested by assaying the enzyme activity in presence of metal chelator. The enzyme activity was not affected by the addition of EDTA. Preincubation of the enzyme with reducing compound such as cysteine did not influence proteolytic activity of the rice-bran. A sulfhydryl blocking reagent, iodoacetoamide, did not inhibit the enzyme. Hence, free sulfhydryl group(s) of the enzyme do not participate in catalysis. A specific serine protease inhibitor such as di-isopropyl fluorophosphate and phenylmethanesulfonyl fluoride had no effect on the enzyme activity.

The protease that have been studied in detail are classified into four class (3). These include: (I) the serine proteases such as trypsin, chymotrypsin and others which have serine at the active site (4); (II) the cysteine proteases which require a free sulfhydryl group in their active center, exemplified by papain, ficin and bromelain; (III) the metal proteases like aminopeptidase and carboxypeptidase which are inhibited by metal chelators; and (IV) the acid proteases such as pepsin and rennin which have acidic amino acid at the active site.

Studies with various types of inhibitors have shown that rice-bran proteinase belong to the class of acidic protease.

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