

Isolation and partial characterization of a protease from *Cucurbita ficifolia*

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A protease from the pulp of *Cucurbita ficifolia* was purified. Its molecular mass was estimated to be about 60 kDa. Its maximum activity is in the alkaline region against azocollagen as substrate. The enzyme is inhibited by phenylmethylsulphonyl fluoride but not by EDTA and iodoacetic acid.

Protease; Alkaline protease; Enzyme purification; (*Cucurbitaceae*, *Cucurbita ficifolia*)

1. INTRODUCTION

Some cucurbits are known to contain large amounts of proteases in their ripe fruits. A proteinase has been isolated from the sarcocarp of melon fruit (*Cucumis melo*) and shown to be a serine proteinase [1]. Fruit of the white gourd (*Benincasa cerifera*) is also a rich source of protease; this enzyme accounts for 15% of the total extractable fruit pulp protein and is also a serine proteinase [2]. This paper presents the isolation and purification to homogeneity of a protease from the fruit pulp of *Cucurbita ficifolia* and describes some properties of that enzyme.

2. EXPERIMENTAL

C. ficifolia was obtained in the harvesting season from April to September in the province of Valparaíso, Chile.

Proteolytic activity was measured as described in [3] with azocollagen as substrate. 10 mg Azocoll (Calbiochem) were placed in a test tube and to this was added 0.5 ml of a suitable diluted protease solution in 0.067 M phosphate buffer (pH 8.0) at 37°C. After incubation for 10 min, and with manual stirring

every 5 min, the reaction was stopped by the addition of 3 ml of 4°C water and further filtration through Whatman 1 filter paper. No difference in the filtrate absorbance was found when the reaction was stopped by centrifugation. The absorbance of the coloured solution was measured at 520 nm. Considering the nature of the substrate azocollagen, the product of its hydrolysis [4] and the assay conditions, a relative unit of activity has been defined as follows: $1 \text{ U} = 0.35 \times A$. The specific activity is expressed as the number of enzyme units per mg protein. Protein was determined as in [5] with bovine serum albumin as a standard.

SDS-polyacrylamide gel electrophoresis for estimation of the molecular mass of the protease was performed according to [6]. The following standard proteins were used: carbonic anhydrase, ovalbumin, bovine serum albumin, and alcohol dehydrogenase. The molecular mass of the protease was estimated by HPLC through a Bondapak 300 column (Waters) equilibrated with 0.067 M phosphate buffer, pH 6.0 at a flow rate of 0.7 ml/min. Lactate dehydrogenase, ovotransferrin, ovalbumin, carbonic anhydrase and myoglobin were used for the calibration of the column. Protein was monitored by measuring the absorbance at 254 nm.

Amino acid analyses were as follows: Hydrolysates were prepared in 6 M HCl under vacuum at 110°C for 24, 48 and 72 h. Tryptophan content was measured after hydrolysis in 5% KOH at 110°C for 16 h. Amino acid analyses were made on a Beckman 119 CI automatic analyser. The independently determined molecular mass was employed in the calculation of the amino acid residues.

To test the effect of various compounds the enzyme was preincubated in 1.0 ml of 0.067 M phosphate buffer, pH 8.0, containing the compound at 0.5 mM for 60 min at 37°C. 0.5 ml of this solution was then added to azocollagen (10 mg) and the activity assayed by the standard procedure.

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3. RESULTS AND DISCUSSION

All the operations of the purification were performed at room temperature. After separating peel and seeds the pulp was homogenized in food processor. The turbid extract was filtered through cotton and centrifuged. The final supernatant had a pale greenish colour, pH 7.0.

3.1. Ammonium sulphate precipitation

To the clear supernatant was added gradually solid ammonium sulphate to 30% saturation. The solution was kept 18 h. The resulting precipitate was collected by centrifugation at $5000 \times g$ for 20 min and dissolved in 0.067 M phosphate buffer, pH 6.0.

3.2. Gel filtration on Sephacryl S-300

The solution from the previous step was placed in a column of Sephacryl S-300 (2.0×95 cm) previously equilibrated with 0.067 M phosphate buffer (pH 6.0). The active peak was collected and then applied to an ion-exchange column.

3.3. CM-Sepharose column chromatography

The solution from the previous step was applied to a column of CM-Sepharose (2.5×10 cm) equilibrated with 0.067 M phosphate buffer (pH 6.0). The column was washed with a linear gradient of 50–100 mM NaCl in phosphate buffer. Active protein fractions were pooled and dialyzed exhaustively against water and lyophilized. The purification and yield of the enzyme are summarized in table 1.

3.4. Molecular mass

Molecular mass was determined by two methods: HPLC on a gel filtration column showed that the protease eluted in a position corresponding to around 60 kDa SDS-polyacrylamide gel electrophoresis revealed a single band. A plot of the logarithm of the M_r of the reference proteins against mobility gave a straight line, from which the M_r of the protein was calculated to be also around 60000.

3.5. Amino acid composition

In table 2 the amino acid composition of the protease is given. Analysis of the data suggests that the protease is related to cucumisin, a serine pro-

Table 1

Purification of *Cucurbita ficifolia* protease

Purification step	Total protein (mg)	Total activity ($U' \times 10^4$)	Specific activity (U'/mg)	Recovery (%)
Juice	6600	17	25.8	100
Ammonium sulphate	1580	7.2	45.6	42
Sephacryl S-300	916	5.6	61.7	33
CM-Sepharose	116	3.2	279	19

teinase from *Cm. melo* whose amino acid composition has also been demonstrated [7]. Both enzymes are from the Cucurbitaceae family.

3.6. Effects of pH and temperature

The pH of maximum enzyme activity was 9.2 with azocollagen as substrate, though 90% activity is observed at pH 8.0 and 11.0. At pH 6.0 the activity was 20% of the maximum. The pH stability of the enzyme was examined by incubation at various pH values for 70 h at 25°C prior to assay at pH 9.2. Full activity remained after incubation at pH 5.0, 9.0 and 12.0. The temperature of maximum activity was 55°C under the assay condi-

Table 2

Amino acid compositions as molar ratios assuming the number of aspartic acid residues to be 54.0 for *Cucurbita ficifolia* protease

Amino acid	<i>Cucurbita ficifolia</i>	<i>Cucumis melo</i>
Aspartic acid	54.0	54.0
Threonine	32.6	32.1
Serine	57.6	53.9
Glutamic acid	17.7	18.3
Proline	43.1	32.3
Glycine	51.6	47.6
Alanine	59.2	40.3
Half-cystine	0.0	3.8
Valine	39.5	40.6
Methionine	0.5	3.9
Isoleucine	28.6	29.6
Leucine	32.6	29.8
Tyrosine	14.5	14.8
Phenylalanine	17.3	22.4
Tryptophan	15.3	2.8
Lysine	10.5	16.3
Histidine	4.4	5.7
Arginine	24.6	25.8

tions. The enzyme was fairly stable after exposure to various temperatures up to 65°C. In the presence of protein substrate (0.4% casein), 50% of the activity was retained at 55°C for 30 min.

Considering that azocollagen is a protein substrate it will also be sensitive to environmental conditions, influencing its rate of proteolysis. For this reason proper controls have been performed and pH and temperature effects are expressed in terms of maximum activity rather than optimal values.

3.7. Effects of various compounds on the activity

The enzyme was 95% inactivated by incubation with 1.0 mM phenylmethylsulphonyl fluoride (PMSF) for 10 min prior to the assay, but under similar conditions of concentration and time of incubation iodoacetate and EDTA had no effect on the activity of the enzyme. The protease does not require cations such as Ca^{2+} , Mn^{2+} , Mg^{2+} , Co^{2+} and Zn^{2+} . Reducing agents tested under the same conditions as PMSF such as dithioerythritol and cysteine had no effect on the activity. The proteases from *C. ficifolia* and from the sarcocarp of *Cm. melo* are remarkably similar to one another. No significant difference could be detected between the two in such criteria as molecular mass, pH and temperature of maximum activity and

stability and the sensitivity to various compounds. The probable presence of an active serine in the *C. ficifolia* protease is indicated by the inhibition by PMSF [8]. Experiments are presently being undertaken with synthetic substrates to determine the specificity of the protease, and to proceed then to assess its kinetic and mechanistic characteristics.

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