Partial Purification and Characterization of Protease from Passion Fruit Juice

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Partial Purification and Characterization of Protease from Passion Fruit Juice

Fumio HASHINAGA, Fumiaki YAMATO and Saburo ITOO

(*Laboratory of Postharvest Physiology and Preservation of Fruits and Vegetables*)
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Introduction

Purple passion fruit (*Passiflora edulis* Sims) is a subtropical fruit and contains approximately 37% juice having quite a distinctive aroma and a high acidity[10].

In regard to the protease in fruits, sulfhydryl protease such as papain, ficin and bromelain is well known, but acid proteases observed are little[11]. Recently, two types of protease showing maximum activity at an acidic pH have been reported to be present in rice seeds[5,6] and in other plant seeds[13,16].

In the previous experiments we have described two types of protease (PFP-I; passion fruit protease I and PFP-II; passion fruit protease II) in passion fruit juice[7]. The present studies were carried out for the purpose of getting further purification and clarification of the properties of these enzymes.

Materials and Methods

1. Passion fruit juice and acetone powder preparation

Purple passion fruit was harvested at the beginning of August from Ibusuki in Kagoshima Prefecture. The juice was expressed from the pulp through a cotton cloth and centrifuged (3,000 rpm, 10 min) to separate starches and pulps. The supernatant was mixed with a 2-fold volume of a cold acetone (−20°C). The acetone powder was prepared by being washed with a stepwise increased concentration of acetone, and was dried in vacuo to obtain the crude enzyme powder.

2. Partial purification of protease

(1) Extraction of enzyme

The acetone powder (7 g) was mixed with 700 ml of 20 mM citrate buffer (pH 3.0), and was kept for 30 min at 4°C. After centrifugation at 10,000 rpm for 10 min, the supernatant was used as crude enzyme solution.

(2) Ammonium sulfate fractionation

To the supernatant (700 ml) ammonium sulfate was added gradually until reaching 70% saturation. After standing for 2 hr at 10°C, the precipitate was collected by centrifugation, and then was redissolved in the above buffer solution (200 ml). The precipitate obtained by the second salting-out was dialyzed against the citrate buffer. The dialyzate was centrifuged to remove minor insoluble materials.

(3) SP-Sephadex C-50 chromatography

The dialysate was placed on a column of SP-Sephadex C-50 (2.6 × 100 cm) and was equilibrated

with 50 mM citrate buffer, pH 3.0. The column was eluted with a linear gradient from 0.05 M to 0.5 M citrate buffer (pH 3.0) at a flow rate of 15 ml per hr. The effluent from the column was fractionated in 5-ml portions. Each of the fractions (PFP-I and PFP-II) showing a high specific activity was collected respectively, and was used subsequently to examine the characteristics.

3. Assay of protease activity

Protease activity was assayed by the method of Kunitz[8] with a slight modification. To 2.5 ml of a solution of 1.2% heat-denatured casein (Hammerstein) containing 20 mM citrate buffer at pH 2.3 or 5.7 was added 0.5 ml of enzyme solution. After incubation at 45°C for 60 min, the reaction was made to be stopped by the addition of 2.5 ml of 0.4 M trichloroacetic acid. The mixture was held for 30 min at room temperature and filtered through Toyo No. 5C filter paper. The absorbance at 275 nm of the filtrate was measured with a Shimadzu UV-200 double-beam spectrophotometer.

One unit of enzyme activity (U) was defined as the amount producing 1 μmole tyrosine per minute at 45°C. The specific activity was expressed as the number of enzyme units per mg of protein.

Protein concentration was determined by the method of Lowry at al.[9] or by the absorbance at 280 nm with bovine serum albumin as a standard.

Results

1. Partial purification of PFP-I and PFP-II

Fig. 1 shows the elution profile of passion fruit protease from SP-Sephadex C-50. PFP-I was separated from PFP-II and the two proteases were finally purified about 3-fold with 1% recovery.

The partial purification procedure and the results of a typical preparation are summarized in Table 1.

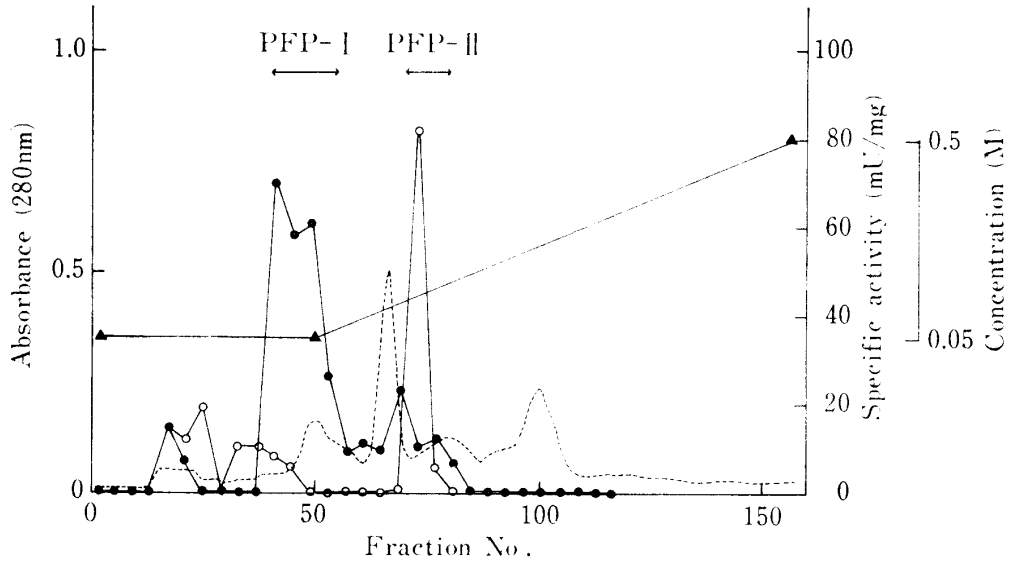

Fig. 1. Chromatography of the passion fruit protease on SP-Sephadex column. Column (2.6 × 100 cm) was eluted with a linear gradient from 0.05 M to 0.5 M sodium citrate buffer at a flow rate of 15 ml/hr and 5 ml fraction was collected.
····· Protein (OD_{280nm}), ●—● PFP-I activity, ○-○ PFP-II activity, ▲—▲ Buffer.

Table 1. Summary of the purification process of two proteases from passion fruit juice

Procedure	Enzyme	Volume (ml)	Protein (mg)	Total activity (U)	Specific activity (mU/mg)	Recovery (%)
Crude extract	PFP-I	750	968	83.7	87	100
	PFP-II	—	—	53.3	55	100
Ammonium sulfate fractionation (X2)	PFP-I	105	110	13.9	126	16
	PFP-II	—	—	11.3	102	22
SP-Sephadex	PFP-I	80	3.6	0.9	260	1
	PFP-II	25	5.3	0.8	142	1

2. Enzymatic properties

(1) pH optima

The activity of PFP-I and PFP-II against casein is presented in Fig. 2. The pH optimum of PFP-I and that of PFP-II were observed at 2.2–2.3 and at 5.7–5.8, respectively.

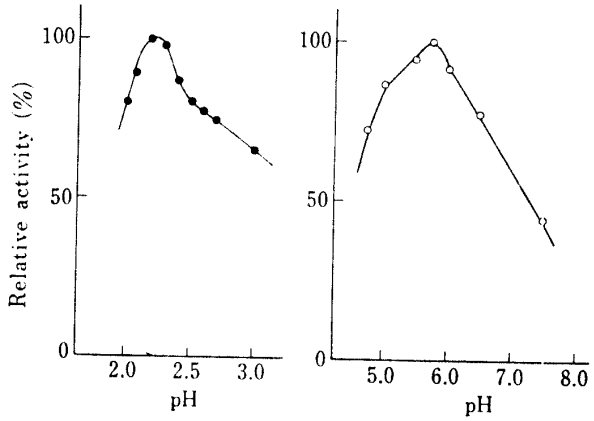

Fig. 2. Effect of pH on the PFP-I and PFP-II activities from passion fruit juice. The activity was measured with casein as a substrate in 20 mM citrate buffer. Left: PFP-I Right: PFP-II

(2) Temperature optima

The effect of temperature on the two protease activities is shown in Fig. 3. Temperature optima

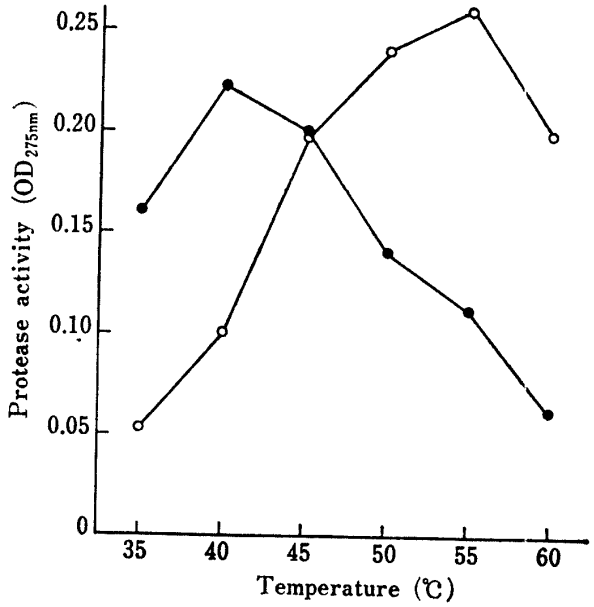

Fig. 3. Effect of temperature on the protease activities from passion fruit juice. The activities of PFP-I and PFP-II were measured at the respective pH optima for 60 min. ●—● PFP-I, ○—○ PFP-II.

for the enzyme reaction were 40°C for PFP-I and 55°C for PFP-II, respectively.

(3) pH stability

The enzyme was relatively stable in the range of pH 2 to 5 for 150 min at room temperature, being the most stable at pH 3.0 (Fig. 4).

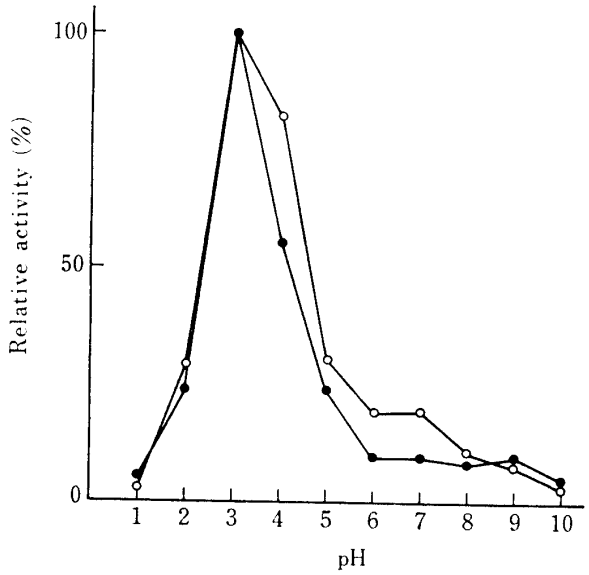

Fig. 4. Effect of pH on the stability of protease activities from passion fruit juice.
The substrate and enzyme mixture was left standing at the desired pH for 150 min. After it was adjusted to pH 2.3 and 5.7, the activity of enzymes was measured.
●—● PFP-I, ○—○ PFP-II.

(4) Thermostability

The remaining activity of the enzyme after exposure to various temperatures in the absence of

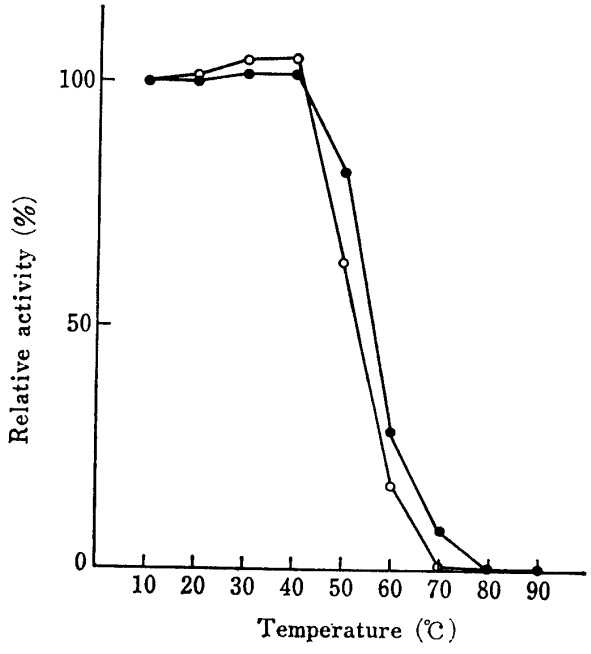

Fig. 5. Thermal stability of the proteases from passion fruit juice.
The enzyme solution (pH3) was preincubated at the desired temperature for 10 min. ●—● PFP-I, ○—○ PFP-II.

protein substrate for 10 min is shown in Fig. 5. The two enzymes were stable at 40°C, but almost completely inactive at more than 80°C for PFP-I and at more than 70°C for PFP-II, respectively.

(5) Effect of salts

As shown in Table 2, the activity of the PFP-I was inhibited by heavy metal ions such as Co^{++}, Pb^{++} and Ag^{+}, and that of the PFP-II was inhibited by Co^{++} and Pb^{++}.

Table 2. Effect of heavy metal salts on the protease activity of passion fruit juice

Salts (1 mM)	Residual activity (%)	
	PFP-I	PFP-II
None	100	100
$FeSO_4$	69	103
$MgSO_4$	92	97
$CuSO_4$	96	106
$Ni(NO_3)_2$	88	95
$Co(NO_3)_2$	46	46
$Pb(NO_3)_2$	49	57
$Mn(NO_3)_2$	90	71
$Cd(NO_3)_2$	74	88
$AgNO_3$	53	70
$CaCl_2$	89	103
$HgCl_2$	69	91
$ZnCl_2$	67	92
$SnCl_2$	88	98

One mM salt (final concentration) was added to the reaction mixture and the activity was assayed by the standard procedure.

(6) Effect of various reagents

The two enzymes were not inhibited by incubation with TPCK, TLCK, PMSF and EDTA (Table 3). The activity of the PFP-II was slightly inhibited by sulfhydryl reagents, and it was enhanced by cysteine.

Discussion

Plant protease, being active in an acid region, is widely distributed in seeds[1,2,13] and in germinating seeds[4,12,14,15], and functions in extracellular digestion and in the turnover of cellular proteins in the growing seedling[3,11]. In the passion fruit juice two types of protease increasing during maturation were described in our previous paper[7]. Therefore, these two proteases may play some physiological roles during maturation and senescence.

The attempt to purify the proteases was made by using Sephadex G-75 in stead of SP-Sephadex, but it gave only a poor recovery and reproducibility. The properties of partially purified proteases were similar to those of the crude enzymes, except for the increase of the temperature optima and thermal stability. PFP-I and PFP-II are estimated to be an acid protease and a sulfhydryl protease, respectively.

Further studies on the purification capacity and some other properties of these enzymes are in progress.

Table 3. Effect of various compounds on the proteolytic activities of passion fruit proteases

Chemicals	Final concentration (mM)	Residual activity (%)	
		PFP-I	PFP-II
None	—	100	100
EDTA	1	94	106
TPCK	0.01	104	94
TLCK	1	101	97
PMSF	1	104	105
Trypsin inhibitor	0.01 (%)	92	92
Monoiodoacetic acid	1	92	79
PCMB	0.01	105	88
2-Mercaptoethanol	1	108	98
Potassium cyanide	1	105	109
Ascorbic acid	1	75	100
L-Cysteine	1	106	140
Proline	1	105	105
Histidine	1	101	88
Serine	1	105	121
Aspartic acid	1	100	94
Glutamic acid	1	109	114
Tyrosine	1	95	110

EDTA: Ethylenediaminetetraacetic acid, TPCK: N-Tosyl-L-phenylalanine chloromethyl ketone, TLCK: N-Tosyl-L-lysine chloromethyl ketone, PMSF: Phenylmethylsulfonyl fluoride, PCMB: *p*-Chloromercuribenzoic acid.

Summary

Two types of protease from passion fruit juice were partially purified by acetone treatment, followed by ammonium sulfate fractionation and SP-Sephadex C-50 column chromatography.

The enzyme was purified 3-fold with 1 % recovery of the original activity. The PFP-I and PFP-II were fixed to have the following key-items; pH optima of 2.2–2.3 and 5.7–5.8 against casein, optimum temperatures of 40°C and 55°C, pH stability of 2–5 (most stable at pH 3.0) and thermal stability of 80°C and 70°C, respectively. The activity of the PFP-I was inhibited by Co^{++}, Pb^{++} and Ag^{+}, while that of PFP-II was inhibited by Co^{++} and Pb^{++}. Both proteases were hardly affected by common serine protease inhibitors and EDTA, however the activity of PFP-II was affected by sulfhydryl reagents, and was enhanced by cysteine.

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