0273-2289/96/5603—0325$06.50

# Milk-Clotting Activity of Cucumisin, a Plant Serine Protease from Melon Fruit

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Received February 14, 1995; Accepted March 29, 1995

## ABSTRACT

Cucumisin (EC 3.4.21.25) isolated from prince melon fruit is a plant serine protease. Its milk-clotting activity was compared with plant cysteine proteases such as papain (EC 3.4.22.2) and ficain (EC 3.4.22.3). Cucumisin was more stable than papain under the condition of pH 7.1, 37°C for 24 h. The milk-clotting activity of cucumisin was the same to that of papain and was half value of that of ficain.

**Index Entries:** *Cucumis melo*; serine protease; plant protease; cucumisin; milk-clotting.

## INTRODUCTION

Chymosin is a milk-clotting enzyme from the fourth stomach of the calf and is recently scarce. Therefore, the dairy industry has required a new milk clotting enzyme having the chymosin action. The gene for chymosin has been cloned and expressed in microorganisms such as yeasts or *E. coli* (*1*). Some plant materials having milk-clotting activity have been used in the manufacturing of cheese. Plant proteases employed for the cheese production in some areas of the world include ficain from fig, *Ficus carica*, a papain like protease from young seeds of *Albizia julibrissin* (*2*), and juice of leaves of the sodom apple, *Calotropis procera* (3).

Cucumisin, isolated from the sarcocarp of prince melon (*Cucumis melo* ssp. melo "Prince Melon") is a rare plant serine type endopeptidase (*4*).

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Typical plant proteases belong mainly to the cysteine protease group (*5*). Cucumisin has broad substrate specificity analogous to subtilisin (*4,6*). Its amino acid sequence around the reactive serine residue is similar to those of subtilisin BPN' (*7*). This report describes the milk-clotting activity of cucumisin, compared with the typical plant cysteine protease such as papain and ficain.

## MATERIALS AND METHODS

### Material and Reagents

Cucumisin was purified from the sarcocarp of melon, *Cucumis melo* ssp. melo "Prince Melon," according to the method of Kaneda and Tominaga (*4*). Pepsin and trypsin were obtained from Nakarai Chemicals, Japan. Casein was purchased from E. Merck. Skim-milk powder was a product of Snow Milk Products, Japan. All other materials were purchased from Wako Pure Chemicals, Japan.

### Assay of Enzyme Stability

Each protease solution in 67 m*M* phosphate buffer at pH 7.0 was incubated at 37°C. Aliquots (0.1 mL) of protease solutions were withdrawn at regular time intervals and were assayed for proteolytic activity using casein, according to the method of Kunitz (*8*). One milliliter of 2% (w/v) casein (Hammersten casein) solution in 67 m*M* phosphate buffer pH 7.0 was added to 0.1 mL of enzyme solution at 37°C. After incubation for 20 min, the reaction was terminated by the addition of 3 mL of 5% trichloroacetic acid. After standing for 30 min at room temperature, the precipitate was removed by filtration through Advantec filter paper No. 5C and the absorbance of the trichloroacetic acid-soluble peptides formed was determined with a Hitachi model 100-60 spectrophotometer at 280 nm.

### Turbidimetry of Casein Solution

Three milliliters of 2% (w/v) casein in 67 m*M* phosphate buffer, pH 7.3, was added to 0.5 mL of cucumisin solution at 30°C. The turbidity of the reaction mixture was measured at regular time intervals using a Hitachi Model 100-60 spectrophotometer at 550 nm.

### Determination of Milk-Clotting Activity and Proteolytic Activity

Milk-clotting activity (MCA) and Proteolytic Activity (PA) were measured by the method of Arima et al. (*9*).

#### *MCA*

The enzyme solution (0.5 mL) was added to 10% solution of skim-milk powder containing 10 m*M* calcium chloride (2.5 mL) at 35°C. The

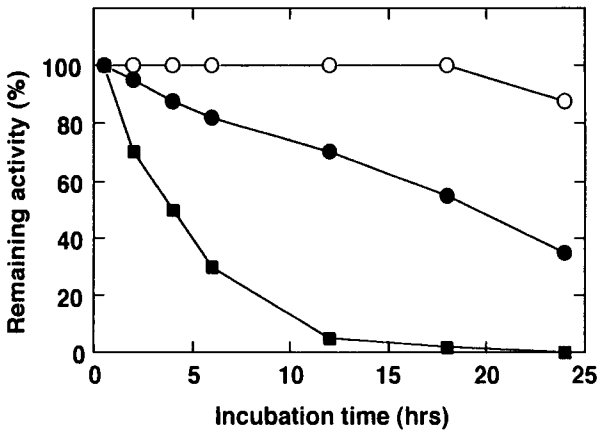


Fig. 1. Stability of papain and cucumisin. The activity at 30 min of incubation was taken as 100%. ○, Cucumisin; ●, papain with 10 m*M* cysteine; ■, papain without cysteine.

time elapsing between the mixing of reagents and the first appearance of solid material against the background was measured.

*PA*

The enzyme solution (0.5 mL) was added to 1% casein solution (2.5 mL) in 20 m*M* phosphate buffer at pH 6.5 and was incubated at 35°C for 10 min. The reaction was terminated by addition of 2.5 mL of 0.44*M* trichloroacetic acid solution. After standing for 30 min at room temperature, the precipitate was removed by filtration through Advantec No. 5C filter paper. To an aliquot (1 mL) of filtrate, Folin reagent (1 mL) and 0.55M sodium carbonate (2.5 mL) were added. Color was developed at 35°C for 20 min, and optical density was measured at 660 nm.

## RESULTS AND DISCUSSION

### Comparison of Stability Between Papain and Cucumisin

Papain and ficain are plant cysteine proteases, and so these enzymes require a reductant and/or a chelate compound for the reactivation and stabilization of the enzyme. Cucumisin is a plant serine protease that does not require a reductant or a chelate compound.

The stability of papain and cucumisin was compared under the same conditions. The activity of papain without cysteine as the reductant completely disappeared at 24 h of incubation. In the presence of 10 m*M* cysteine, the remaining activity of papain was about 35% as shown in Fig. 1. However, under no addition of reductant or chelator, the activity of cucumisin was maintained at 90%, even at 24 h of incubation. It seems that the higher stability of cucumisin originates in the characteristics of the serine protease.

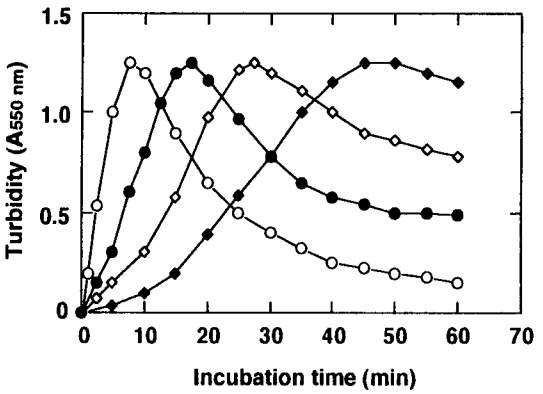


Fig. 2. Turbidimetry of casein solution by cucumisin action. Cucumisin was used at 0.11 $\mu M$ (◆), 0.17 $\mu M$ (□), 0.43 $\mu M$ (●), and 0.85 $\mu M$ (○).

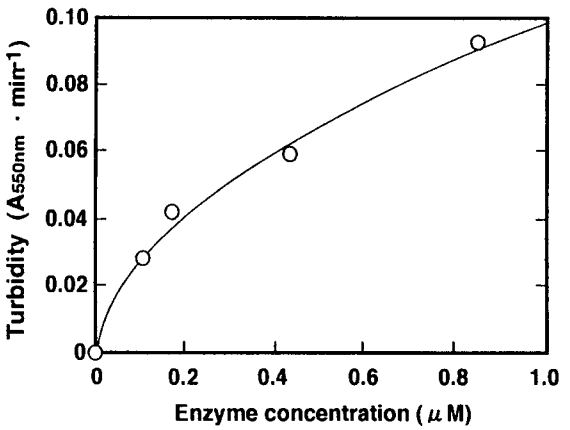


Fig. 3. Plot of the initial rates of turbidity vs cucumisin concentrations.

## Turbidimetry of Casein Solution

The required time of the maximum turbidity depended on the concentration of cucumisin as shown in Fig. 2. However, the reaction did not show first-order rate plots as shown in Fig. 3. All the values of the maximum turbidity were closely constant in the range of the present enzyme concentrations. After attaining to the maximum, the turbidity decreased gradually in all cases. The rate of decreasing of the turbidity increased in proportion to the concentration of the enzyme. The coagulated casein was finally dissolved by cucumisin.

## Milk-Clotting Activity of Cucumisin

MCA of cucumisin was increased in dependence with the enzyme concentration as shown in Table 1. PA of cucumisin was also increased in the similar manner. The values of MCA/PA of cucumisin were consequently alike over all the concentration of the enzyme.

Table 1
Milk-Clotting Activity of Cucumisin

| Enzyme concentration, ($\mu M$) | MCA (U) | PA ($A_{660nm}$) | MCA/PA ($U/A_{660nm}$) |
|---|---|---|---|
| 1.5 | 25 | 0.26 | 96 |
| 3.0 | 54 | 0.52 | 104 |
| 4.5 | 80 | 0.82 | 98 |
| 7.5 | 117 | 1.07 | 109 |

Table 2
Milk-Clotting Activity of Various Plant Proteases

| Protease | MCA (U) | PA ($A_{660nm}$) | MCA/PA ($U/A_{660nm}$) |
|---|---|---|---|
| Cucumisin | 54 | 0.52 | 104 |
| Ficain | 170 | 0.67 | 254 |
| Papain | 67 | 0.65 | 103 |

## Comparison of Milk-Clotting Activity Between Cysteine and Serine of Plant Protease

Three enzyme concentrations were prepared in such a manner so each enzyme could have approximately the same value for proteolytic activity. Two cysteine proteases, papain and ficain, held the activity of 90% or above without a reductant within about 1 h.

The value of MCA/PA of ficain was greater than that of papain as shown in Table 2. The values of PA of both enzymes were analogous, but the MCA of ficain was 2.5-fold that of papain. Each value of cucumisin was similar to those of papain. The bitter taste had not been found in a cucumisin hydrolysate. The stable immobilized cucumisin was reported by Kaneda et al. (*10*). We think that cucumisin will be available for the continuous hydrolysis of milk or milk-clotting production setting up the optimum conditions.

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