Studies on the activities of serine proteases from *Ficus carica*

Kosaku Nishimura

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Contents

Abbreviations

ASC	acid-solubilized collagen
BSA	bovine serum albumin
CBB	Coomassie Brilliant Blue
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate
Chcol	collagenase from *Clostridium histolyticum*
C. histolyticum	*Clostridium histolyticum*
cv.	cultivar
DTT	dithiothreitol
E-64	L-epoxysuccinylleucyl-amido(4-guanidino)butane
EDTA	ethylenediaminetetraacetic acid
F. carica	*Ficus carica*
FSP	serine protease from *Ficus carica*
Ghcol	collagenase from *Grimontia hollisae*
G. hollisae	*Grimontia hollisae*
HEPES	2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid
IEF	isoelectric focusing
LC-MS/MS	liquid chromatography-tandem mass spectrometry
MCU	milk clotting unit
MOCAc-KPLGL(Dpa)-AR	(7-methoxycoumarin-4-yl)acetyl-L-Lys-L-Pro-L-Leu-Gly-L-Leu-[N^3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl]-L-Ala-L-Arg-NH_2

PAGE	polyacrylamide gel electrophoresis
Pefabloc SC	4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride
PMSF	phenylmethylsulfonyl fluoride
PVDF	polyvinylidene fluoride
Pyr-Phe-Leu-pNA	L-pyroglutamyl-L-phenylalanyl-L-leucine *p*-nitroanilide
SDS	sodium dodecyl sulfate
T_d	complete denaturation temperature
TIMS	trapped ion mobility spectrometry
Tris	tris(hydroxymethyl)aminomethane

General introduction

Fig (*Ficus carica* L.) is a deciduous tree belonging to the Moraceae family and is considered one of the oldest known fruits along with apples and grapes (1, 2). Fig fruits can be eaten fresh, dried, canned, or preserved (3) and are reportedly rich in antioxidants (4). Fig leaves have long been used in traditional and Chinese medicines and have been reported to possess hypoglycemic (5–7) and anti-allergic properties (8, 9). *F. carica* exudes a milky fluid called latex (10) that is used as a milk coagulant (11, 12) or is administered to soften solid tumors and treat skin diseases (13, 14). There are many cultivars of *F. carica*, with variations in the appearance and flavor of fruits and phenylpropanoid composition of leaves (15, 16). Therefore, *F. carica* can serve as an interesting research target in various fields, including food science, life science, pharmacy, and medicine.

One of the most well-known enzymes produced by *F. carica* is ficin, a type of cysteine protease. Ficin has long been studied along with papain from papaya and bromelain from pineapple, which are also fruit-derived cysteine proteases. Ficin has a wide range of substrate specificity, optimal temperature, and optimal pH and is used in diverse industries, from meat tenderization to antibody fragmentation (17, 18). Furthermore, the presence of ficin has been reported in latex, and various *F. carica* cultivars have isozymes of ficin (19, 20).

In latex produced by *F. carica*, ficin is the major protein, and thus, limited attention has been paid to other enzymes. However, in the past decade, enzymes other than ficin present in *F. carica* latex have been studied. Kitajima *et al*. conducted a proteomic

analysis of *F. carica* latex and found the presence of ficin, subtilase, peroxidase, chitinase, and mandelonitrile lyase (21). Lazreg-Aref *et al*. reported that the latex of some cultivars of *F. carica* exhibited not only protease activity but also amylase and lipase activities (22). Raskovic *et al*. found a serine protease with collagenolytic activity in the latex extract of *F. carica* cultivar (cv.) Brown Turkey (23). Additionally, Hamed *et al*. reported that a serine protease in the latex extract of *F. carica* (unknown cultivar) demonstrated fibrinolytic activity (24). Because there are few reports of plant-derived serine proteases with collagenolytic and fibrinolytic activities, the serine protease from *F. carica* (FSP) is unique. However, to the best of our knowledge, there are only two reports on the activity of FSP (23, 24). Therefore, it is unclear whether FSP is universally present in *F. carica*. There is no report on its detailed substrate specificity, amino acid sequence, or role in the plant body.

The aim of this thesis was to characterize FSP. To elucidate the universality and basic characteristics of FSP, its synthetic substrate- and gelatin-degrading activities were examined and are described in Chapter 1. In Chapter 2, collagen degradation experiments were conducted to verify whether FSP has substrate specificity similar to collagenase and to determine the characteristics of the collagen-degrading activity of FSP. In Chapter 3, to determine the full-length amino acid sequence of FSP, FSP was purified from the latex of *F. carica* cv. Masui Dauphine using two-dimensional electrophoresis, and the partial amino acid sequences were determined using liquid chromatography-tandem mass spectrometry (LC-MS/MS).

The results of this study showed that FSP is universally present in several *F. carica* cultivars and that its substrate specificity is distinctly different from that of ficin; this indicates that not only ficin but also FSP may significantly contribute to food processing

and experiments using *F. carica* crude enzymes. We believe that the results of this study will be useful to determine the characteristics and roles of various proteases from *F. carica*, leading to the expansion of their industrial use.

Chapter 1

Characterization of serine proteases from *Ficus carica*

Introduction

As described in the General introduction, the latex of cv. Brown Turkey has been reported to contain ficin and FSP (23). However, it is unknown whether FSP is found in organs other than latex, such as fruits, branches, and leaves. It is also unknown whether FSP is present in various cultivars.

In this chapter, we attempted to detect the gelatinolytic activity of FSP in the extracts of fruits, branches, and leaves of cv. Masui Dauphine, one of the most representative *F. carica* cultivars in Japan, using gelatin zymography. We also examined the synthetic substrate hydrolysis activities of ficin and FSP in extracts prepared from 23 *F. carica* cultivars grown at the Toyo Institute of Food Technology, Kawanishi, Japan. As a synthetic substrate, we used (7-methoxycoumarin-4-yl)acetyl-L-Lys-L-Pro-L-Leu-Gly-L-Leu-[N^3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl]-L-Ala-L-Arg-NH_2, which has been used to measure the activity of collagenases and matrix metalloproteinases (25, 26). To specifically inhibit ficin and detect only FSP activity, E-64, a cysteine protease-specific inhibitor, was used. Additionally, to characterize the cleavage sites of the synthetic substrate by ficin and FSP, the reaction products were analyzed using LC-MS/MS.

Materials and Methods

Materials – (7-Methoxycoumarin-4-yl)acetyl-L-Lys-L-Pro-L-Leu-Gly-L-Leu-[N^3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl]-L-Ala-L-Arg-NH_2 [MOCAc-KPLGL(Dpa)-AR] (molecular mass 1221.3 Da) and L-pyroglutamyl-L-phenylalanyl-L-leucine *p*-nitroanilide (Pyr-Phe-Leu-pNA) (509.55 Da) were purchased from Peptide Institute (Osaka, Japan). Ficin from *F. carica* tree latex was purchased from Tokyo Chemical Industry (Tokyo, Japan). The titer of the ficin was 400–1,000 milk clotting unit (MCU)/mg where one MCU is defined as the amount which coagulates 25 mL milk at pH 6.0 at 40°C according to manufacturer's information. Collagenase from *Clostridium histolyticum* (Lot. M6M0414) was purchased from Nacalai Tesque (Kyoto, Japan). Collagenase from *Grimontia hollisae* was prepared as described previously (25, 27). The cysteine protease inhibitor L-epoxysuccinylleucyl-amido (4-guanidino) butane (E-64) was purchased from Sigma-Aldrich (Saint Louis, MO), and the serine protease inhibitor phenylmethanesulfonyl fluoride (PMSF) was purchased from Fujifilm Wako Pure Chemical (Osaka, Japan).

Preparation of F. carica *extracts* – Fruits, leaves and branches of 23 *F. carica* cultivars were collected at Toyo Institute of Food Technology. They were frozen at -80°C, smashed finely, broken, freeze-dried, and then powdered with a food mill. The powdered leaves or branches (4.5 g) were stirred in water (45 mL) overnight at 4°C followed by gauze filtration. The extract was centrifuged at 10,000 × *g* for 10 min at 4°C, and the supernatant was collected. The supernatant (45 mL) was put in the dialysis tube and dialyzed against saturated ammonium sulfate solution overnight at 4°C. The solution

collected from the dialysis tube was centrifuged at 17,300 × *g* for 10 min at 4°C, and the pellet was collected and dissolved in 200 mM sodium phosphate buffer (pH 6.5), 300 mM KCl, 0.1 mM EDTA, 3 mM dithiothreitol (DTT). Protein concentration was determined using Protein Assay CBB Solution (Nacalai Tesque) with bovine serum albumin (BSA) (Nacalai Tesque) as a standard.

Hydrolysis of MOCAc-KPLGL(Dpa)-AR – The reaction was carried out as described by Takita *et al.* (25) and Hatanaka *et al.* (26) with minor modifications. Briefly, 1.56 μM MOCAc-KPLGL(Dpa)-AR solution was prepared in 48.9 mM HEPES-NaOH buffer (pH 7.5), 0.1 mM EDTA, 3.3 mM DTT. The reaction was initiated by mixing 180 μL of the MOCAc-KPLGL(Dpa)-AR solution and 20 μL of enzyme solution. The reaction temperature was 37°C. The reaction was monitored by following the increase in fluorescence intensity at 400 nm with excitation at 324 nm (FI_{400}) with an EnSight multimodal plate reader (PerkinElmer, Waltham, MA) for 3 min.

Analysis of cysteine protease activity – The reaction was carried out as described by Baker *et al.* (28) with minor modifications. Briefly, 540 μM Pyr-Phe-Leu-pNA solution was prepared in 43.2 mM HEPES-NaOH buffer (pH 7.5), 0.1 mM EDTA, 3.2 mM DTT. The reaction was initiated by mixing 140 μL of the Pyr-Phe-Leu-pNA solution and 60 μL of enzyme solution. The reaction temperature was 37°C. The reaction was monitored by following the increase in absorbance at 405 nm (A_{405}) with an EnSight plate reader for 2 min.

Gelatin zymography – Samples were mixed with six volumes of the zymography

sample buffer [120 mM Tris-HCl buffer (pH 6.8), 48% (v/v) glycerol, 2.4% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 0.06% (w/v) bromophenol blue]. The solution (10 μL) was applied to 12.5% polyacrylamide gel that contained 0.063% (w/v) gelatin, and samples were subjected to electrophoresis at a constant current of 40 mA for 34 min.

After electrophoresis, gels were soaked first in the washing buffer [50 mM Tris-HCl buffer (pH 8.0), 0.1% (v/v) Tween 20] at room temperature for 10 min to remove SDS, secondly in the reaction buffer [50 mM Tris-HCl buffer (pH 8.0), 0.1% (v/v) Tween 20, 5 mM $CaCl_2$] at 37°C for 1 h for gelatin hydrolysis. After the reaction, gels were stained with 0.25% (w/v) Coomassie Brilliant Blue (CBB) R-250, 50% (v/v) methanol, 7% (v/v) acetic acid.

LC-MS/MS – MOCAc-KPLGL(Dpa)-AR (10 μM) was incubated in 2.2 mM HEPES-NaOH buffer (pH 7.5) with 100 nM ficin, 90 μg/mL Masui Dauphine extract or 100 nM *G. hollisae* collagenase and 0 or 2 mM E-64 at pH 7.5, at 37°C. The reaction solution was analyzed by LC-MS/MS using LC-20A (Shimadzu, Kyoto, Japan) and micrOTOF-Q II (Burker Daltonics, Billerica, MA). Conditions of LC-MS/MS are as follows: column, Poroshell 120 EC-C18 Column (100 mm × 4.6 μm, 2.7 μm; Agilent Technologies, Santa Clara, CA); column oven temperature, 40ºC; mobile phase, 0.1% formic acid (A) and 0.1% formic acid in acetonitrile (B); mobile phase flow rate, 0.4 mL/min; injection volume, 5 μL; ion source, electrospray ionization (positive mode); drying gas, nitrogen (180ºC, 7 L/min); nebulizing gas, nitrogen (1.6 bar); capillary voltage, -4500 V; hexapole RF, 100 Vpp; quadrupole ion energy, 5 eV; collision gas, nitrogen (1.6 bar); collision energy, 10 eV; collision RF, 100 Vpp; and mass range, *m/z* 50–1500. Elution gradients are as follows: 0–3 min, 2% B; 3-13 min, 2%–45% B; 13–15 min, 45%–65% B; 15-17

min, 90%; and 17-20 min, 2% B.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) – SDS-PAGE was performed in a gradient (5% to 20%) polyacrylamide gel under reducing conditions. Samples were mixed with four volumes of the SDS-PAGE sample buffer [250 mM Tris-HCl buffer (pH 6.8), 50% (v/v) glycerol, 10% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 0.05% (w/v) bromophenol blue] and were boiled for 10 min, and then applied onto the gel. A constant current of 25 mA was applied for 60 min. After electrophoresis, proteins were stained with CBB R-250.

Results and Discussion

Hydrolytic activities of ficin and collagenase – First we analyzed the ficin and collagenase activities to hydrolyze MOCAc-KPLGL(Dpa)-AR (Fig. 1A). This peptide has been used for the characterization of collagenase (25, 26). In this peptide, the fluorescent 7-methoxycoumarin group is quenched by energy transfer to the 2,4-dinitrophenyl group, and it emits fluorescence when the 7-methoxycoumarin group dissociates from the 2,4-dinitrophenyl group by the cleavage at the peptide bond between the two groups. When the substrate was incubated with a commercially available ficin from *F. carica* tree latex or a collagenase from *Clostridium histolyticum* (classified as metalloprotease and among all collagenases the most extensively studied), the fluorescence intensity at 400 nm (FI_{400}) increased with increasing reaction time (Fig. 1B). This indicated that both ficin and collagenase cleaved MOCAc-KPLGL(Dpa)-AR.

Takita *et al.* previously reported that the cleavage site of MOCAc-KPLGL(Dpa)-AR

by *C. histolyticum* collagenase was Gly-Leu (25). To confirm the cleavage site of this substrate by ficin, the substrate reaction products were analyzed by LC-MS/MS, and the peak and MS/MS spectrum corresponding to MOCAc-KPLG (629 Da) were obtained (Fig. 1C), indicating that the peptide bond Gly-Leu was cleaved (Fig. 1A). The peak corresponding to MOCAc-KPLGL(Dpa)-AR was not obtained under this condition. This indicated that the cleavage sites by ficin and *C. histolyticum* collagenase are the same.

Next we used Pyr-Phe-Leu-pNA as a synthetic substrate for characterizing cysteine protease activity (Fig. 2A). This peptide has been used for the characterization of cysteine protease. In this peptide, the absorbance at 405 nm (A_{405}) increases when it is cleaved at the bond Leu-pNA. A_{405} increased with increasing reaction time when the substrate was incubated with ficin, while it did not when incubated with *C. histolyticum* collagenase (Fig. 2B). When the Pyr-Phe-Leu-pNA that had been treated with ficin was analyzed by LC-MS/MS, and the peak and MS/MS spectrum corresponding to Pyr-Phe-Leu (389 Da) were obtained (Fig. 2C). These results showed that ficin cleaved Pyr-Phe-Leu-pNA but not *C. histolyticum* collagenase, suggesting that FSP does not degrade this substrate.

Gelatin-hydrolyzing activities of the F. carica *extracts* – In order to see whether ficin and FSP in *F. carica* are involved in the activity, the fruit, branch, and leaf extracts of cv. Masui Dauphine were subjected to gelatin zymography in the absence or the presence of cysteine protease-specific inhibitor E-64 (Fig. 3). In the reaction with ficin, the strong bands appeared in the absence of E-64, but they disappeared in the presence of E-64. This indicated that E-64 covalently bound the active-site cysteine of ficin and completely inhibited activity. In the reaction with the fruit, branch, or leaf extracts of Masui Dauphine, the bands appeared both in the absence and the presence of E-64. The bands were of

weaker intensity in the presence of E-64 than in its absence. This indicated that the gelatin-hydrolyzing activity of the extracts derive from not only ficin (classified as cysteine protease) but also from FSP (classified as serine protease). The appearance of smearing bands was presumably due to the fact that ficin was not reversibly denatured in the zymographic sample buffer (29, 30), and ficin hydrolyzed gelatin during electrophoresis.

Inhibitory effects of E-64 on peptide hydrolysis activities of F. carica *extracts* – Although the fruit extract of Masui Dauphine exhibited higher activity than the branch and leaf extracts (Fig. 3), we selected branch extract for subsequent analysis because branch is the most easily available. In order to see whether ficin or FSP in the *F. carica* extract is involved in the hydrolysis of MOCAc-KPLGL(Dpa)-AR and Pyr-Phe-Leu-pNA, the effects of E-64 on the activity of the Masui Dauphine branch extract were investigated (Fig. 4). In the hydrolysis of MOCAc-KPLGL(Dpa)-AR, the relative activities decreased with increasing E-64 concentration and reached 10% at 1 μM, and were essentially constant over the range of 1-20 μM (Fig. 4A). In the hydrolysis of Pyr-Phe-Leu-pNA, the relative activities reached almost zero at 5 μM E-64 (Fig. 4B). These results indicated that both ficin and FSP cleaved MOCAc-KPLGL(Dpa)-AR, while only ficin cleaved Pyr-Phe-Leu-pNA.

We analyzed the cleavage sites of MOCAc-KPLGL(Dpa)-AR by the Masui Dauphine branch extract. MOCAc-KPLGL(Dpa)-AR was treated by protease, and the reaction products were subjected to LC-MS/MS (Fig. 4C). In the reaction with ficin, one peak corresponding to MOCAc-KPLG (629 Da) appeared ((i) in Fig. 4C). Because the cleavage sites by ficin and *C. histolyticum* collagenase are the same (Fig. 1A), we

expected that the reaction with the Masui Dauphine branch extract would exhibit one peak corresponding to MOCAc-KPLG. However, it showed two peaks ((ii) in Fig. 4C), and the MS/MS spectrum of each corresponded to MOCAc-KPLG (Fig. 1C) and MOCAc-KPL (Fig. 4D). In the presence of E-64, the reaction with the extract exhibited one peak corresponding to MOCAc-KPL ((iii) in Fig. 4C), while in the presence of E-64 and serine protease inhibitor PMSF, the peak was few ((iv) in Fig. 4C). These results suggested that MOCAc-KPLG was produced by ficin while MOCAc-KPL was produced by FSP, and the substrate specificity of the FSP in the Masui Dauphine branch extract was different from that of *C. histolyticum* collagenase. Teramura *et al*. reported that the collagenase produced by a gram-negative bacterium *Grimontia hollisae* is classified as metalloprotease and degrades collagen more efficiently than *C. histolyticum* collagenase (27). Takita *et al*. reported that there was a striking difference between *G. hollisae* collagenase and *C. histolyticum* collagenase in the effect of buffers, $CaCl_2$ and NaCl on activity and pH-dependence of activity (25). Here we analyzed the cleavage sites of MOCAc-KPLGL(Dpa)-AR by *G. hollisae* collagenase. The reaction with *G. hollisae* collagenase exhibited the peak corresponding to MOCAc-KPL, but not the peak corresponding to MOCAc-KPLG ((v) in Fig. 4C). These results indicated that both ficin and FSP were contained in the Masui Dauphine branch extract, and the former cleaved the peptide bond Gly-Leu while the latter cleaved Leu-Gly (Fig. 4E).

Characterization of enzyme extracts from F. carica *branches* – We prepared the branch extracts of 23 *F. carica* cultivars (Table 1) by ammonium sulfate fractionation of the crushed branches. On SDS-PAGE under reducing conditions, all 23 extracts yielded several bands including the one with a molecular mass of 26 kDa corresponding to ficin

(Fig. 5). The yields of the crude enzyme extracts from 4.5 g of branches ranged from 12.6–46.8 mg of total protein.

Peptide hydrolysis activities of the branch extracts of 23 F. carica *cultivars* – In order to characterize the extracts of 23 *F. carica* cultivars, we investigated their MOCAc-KPLGL(Dpa)-AR-hydrolyzing activities in the absence and the presence of 20 μM E-64 (Fig. 6A). The relative activity was defined as the ratio of the reaction rate with each extract to that of the Grise de Tarascon (cv. No. 23) extract which displayed the highest activity. The relative activities were in the range of 24.1–100% at 0 μM E-64, indicating that all extracts exhibited activity, with the Grise de Tarascon extract being the highest activity. The relative activities in the presence of 20 μM E-64 were 2.4–7.8%. This suggested that all extracts contain both ficin and FSP. Figure 6B shows comparison of the MOCAc-KPLGL(Dpa)-AR-hydrolytic activity at 20 μM E-64 with that at 0 μM E-64, in which the vertical line represent the FSP activity, and the horizontal line represents the total activities of ficin and FSP. No correlation was observed for the relative activities at 0 and 20 μM E-64, suggesting that the ratio of the activities of ficin and FSP vary depending on cultivars.

Proteases from *F. carica* are known under the general term ficin that belongs to cysteine protease class. However, Raskovic *et al*. reported that *F. carica* cv. Brown Turkey produces FSP with collagenolytic activity (23). Our results indicated that all 23 *F. carica* cultivars produce FSP.

Next we examined the Pyr-Phe-Leu-pNA-hydrolyzing activities of the extracts of 23 *F. carica* cultivars (Fig. 6C). The relative activity was defined as the ratio of the reaction rate with each extract to that with the Grise de Tarascon (cv. No. 23) extract. The relative

activities ranged 16.4–100%, indicating that all extracts exhibited the activity with the Grise de Tarascon extract being highest. Figure 6D shows comparison of the Pyr-Phe-Leu-pNA-hydrolytic activity at 0 μM E-64 with the MOCAc-KPLGL(Dpa)-AR-hydrolytic activity at 0 μM E-64, in which the vertical line represent the ficin activity because FSP does not have the Pyr-Phe-Leu-pNA-hydrolytic activity while the horizontal line represent the total activities of ficin and FSP. High correlation was observed for the relative activities for the Pyr-Phe-Leu-pNA hydrolysis and for the MOCAc-KPLGL(Dpa)-AR hydrolysis at 0 μM E-64 ($\gamma = 0.86$), suggesting that in all 23 extracts, the MOCAc-KPLGL(Dpa)-AR hydrolyzing activity derived mainly from ficin rather than FSP.

Cleavage sites of MOCAc-KPLGL(Dpa)-AR by the branch extracts of 23 F. carica *cultivars* – Ficin and FSP in the branch extracts of Masui Dauphine cleaved the peptide bond Gly-Leu and Leu-Gly of MOCAc-KPLGL(Dpa)-AR, respectively (Fig. 4E). To investigate whether ficin and FSP of other cultivars also cleave MOCAc-KPLGL(Dpa)-AR at the same sites, we reacted branch extracts of 23 cultivars with MOCAc-KPLGL(Dpa)-AR and analyzed the reaction products by LC-MS/MS. As a result, the production of both MOCAc-KPLG and MOCAc-KPL was confirmed in all the cultivars (Fig. 7). This suggested that ficin cleaved the peptide bond Gly-Leu while FSP cleaved Leu-Gly in all cultivars of *F. carica*.

Conclusion – It was found that FSP and ficin are produced by several *F. carica* cultivars and that FSP is distributed not only in the latex but also in the fruits, branches, and leaves. We also found that during the hydrolysis of MOCAc-KPLGL(Dpa)-AR, ficin

cleaved the Gly–Leu bond, whereas serine protease cleaved the Leu–Gly bond, suggesting that the substrate specificities of the two enzymes and their roles in the plant body differ. Additionally, it has been reported that serine proteases are less likely to lose their activity in oxidizing conditions than cysteine proteases (31). Further, FSP may be more resistant to oxidation than ficin. These results suggest that FSP is an attractive enzyme for industrial use.

Table 1. Twenty-three *F. carica* cultivars (cv.).

cv. No.	cv. name	cv. No.	cv. name
1	Panachee	13	Celeste
2	Dalmatie	14	Bourjassotte Grise
3	Precoce Ronde de Bordeaux	15	Daw Low
4	Negronne	16	Brown Turkey
5	Masui Dauphine	17	Grise de Saint Jean
6	Athene	18	Kadota
7	Noire de Caromb	19	Negro Largo
8	White Ischia	20	Horaishi
9	Lisa	21	California Black
10	White Genoa	22	White Adriatic
11	Violette de Sollies	23	Grise de Tarascon
12	Brunswick		

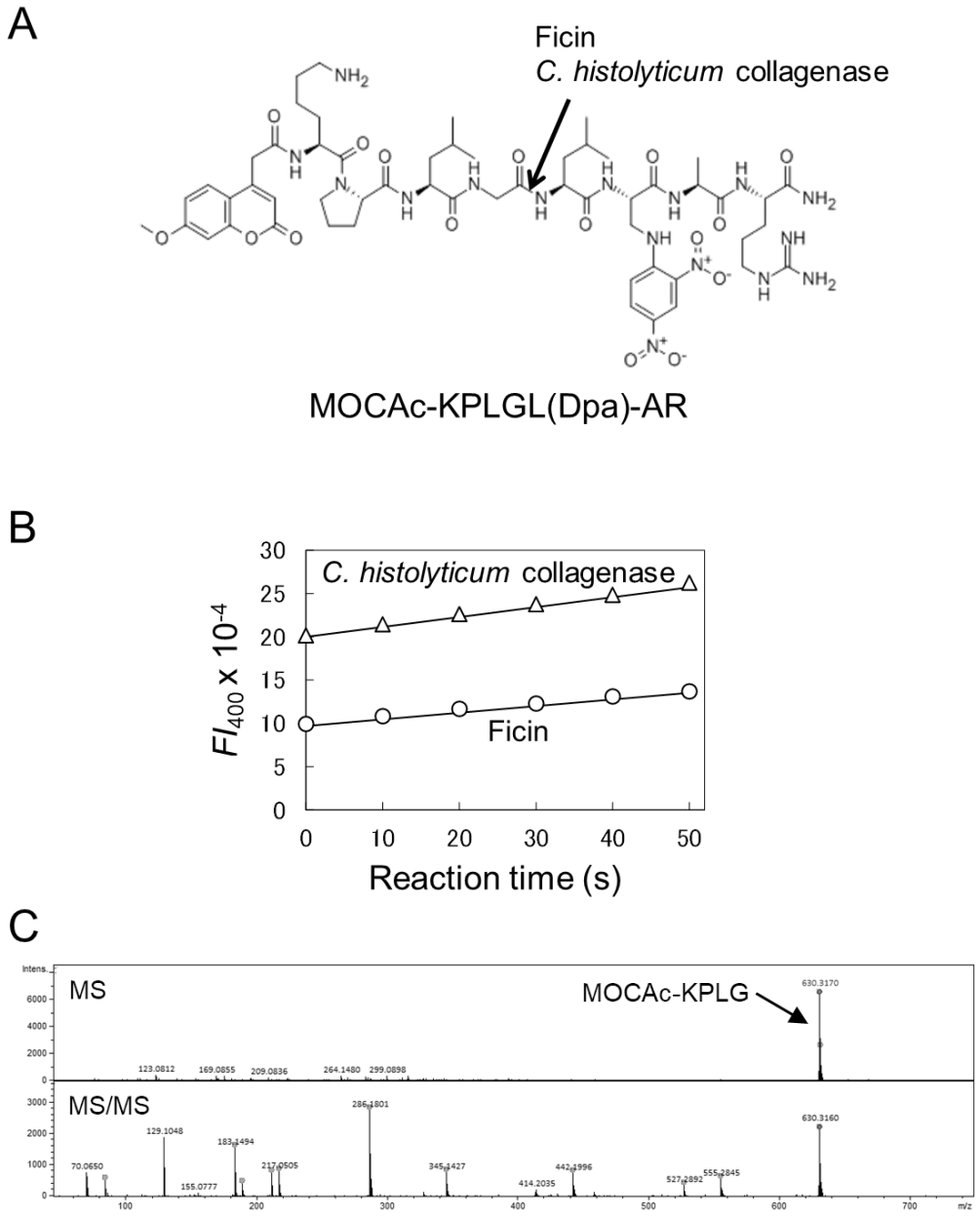


Fig. 1. Hydrolysis of MOCAc-KPLGL(Dpa)-AR by ficin and collagenase. (A) Structure of MOCAc-KPLGL(Dpa)-AR. The arrow indicates the cleavage site by ficin and *C. histolyticum* collagenase. (B) Hydrolysis of MOCAc-KPLGL(Dpa)-AR. The reaction was carried out with 1.0 nM ficin or 15 nM *C. histolyticum* collagenase in the presence of 1.4 μM MOCAc-KPLGL(Dpa)-AR at pH 7.5, at 37°C. (C) MS and MS/MS spectrum of MOCAc-KPLG. MOCAc-KPLGL(Dpa)-AR (10 μM) was incubated in 2.2 mM HEPES-NaOH buffer (pH 7.5) with 100 nM ficin at 37ºC.

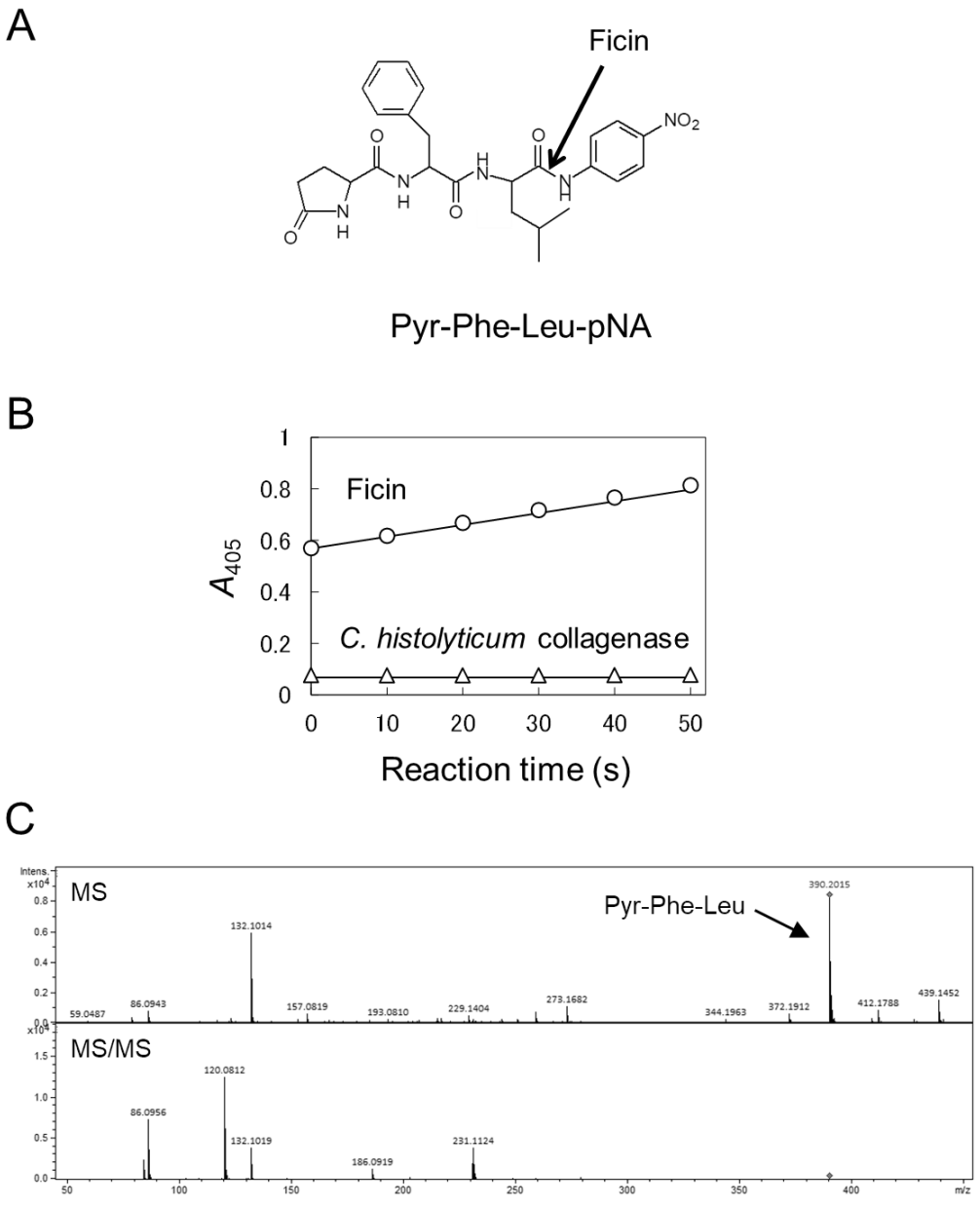


Fig. 2. Hydrolysis of Pyr-Phe-Leu-pNA by ficin and collagenase. (A) Structure of Pyr-Phe-Leu-pNA. The arrow indicates the cleavage site by ficin. (B) Hydrolysis of Pyr-Phe-Leu-pNA. The reaction was carried out with 1.0 μM ficin or 10 μM *C. histolyticum* collagenase in the presence of 500 μM Pyr-Phe-Leu-pNA at pH 6.5, at 37°C. (C) MS and MS/MS spectrum of Pyr-Phe-Leu. Pyr-Phe-Leu-pNA (50 μM) was incubated in 2.2 mM HEPES-NaOH buffer (pH 7.5) with 100 nM ficin at 37ºC.

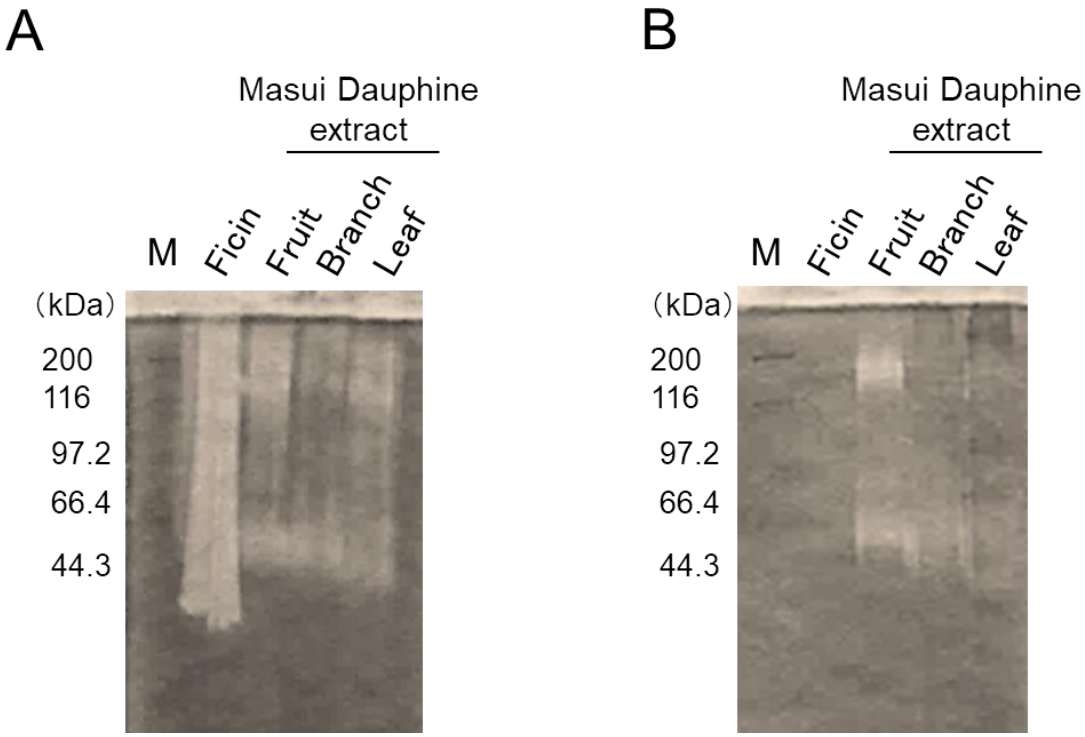
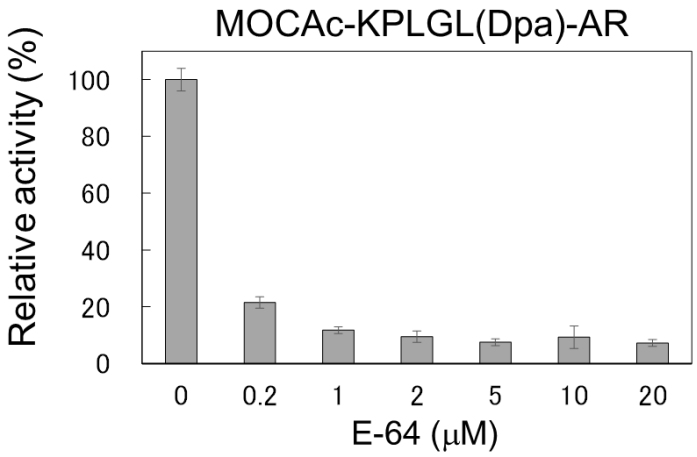
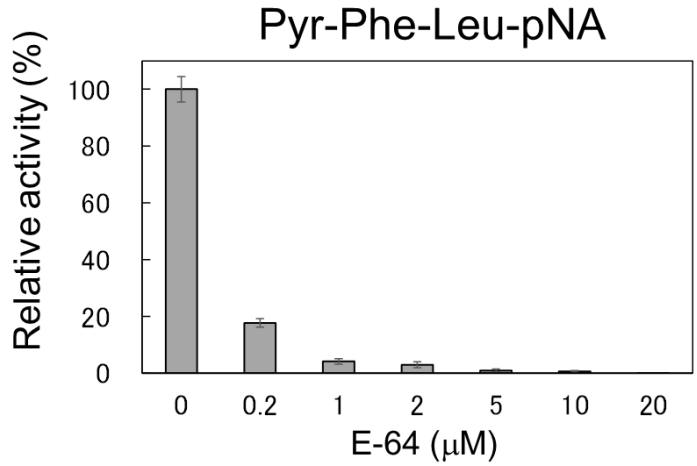


Fig. 3. Gelatin hydrolysis by ficin and the Masui Dauphine extracts. A CBB-stained 10% polyacrylamide gel containing 0.063% gelatin is shown. Lane M, molecular-mass marker. The concentrations of E-64 were 0 (A) or 280 μM (B) in the zymography sample buffer and 0 (A) or 2.8 μM (B) in the washing buffer and the reaction buffer.

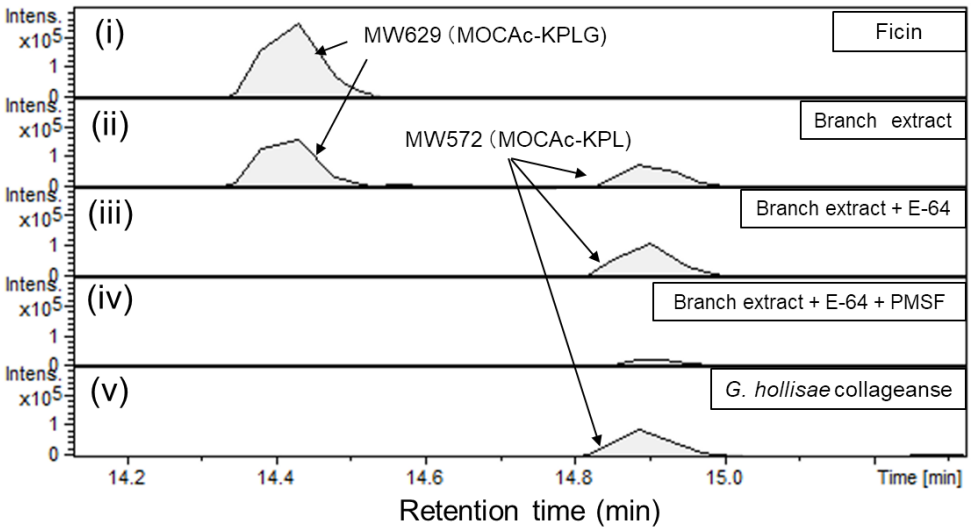
A


MOCAc-KPLGL(Dpa)-AR
Relative activity (%)
100
80
60
40
20
0
0
0.2
1
2
5
10
20
E-64 (μM)

B


Pyr-Phe-Leu-pNA
Relative activity (%)
100
80
60
40
20
0
0
0.2
1
2
5
10
20
E-64 (μM)

C


Intens.
x10^5
(i)
MW629 (MOCAc-KPLG)
Ficin
(ii)
MW572 (MOCAc-KPL)
Branch extract
(iii)
Branch extract + E-64
(iv)
Branch extract + E-64 + PMSF
(v)
G. hollisae collageanse
14.2
14.4
14.6
14.8
15.0
Time [min]
Retention time (min)

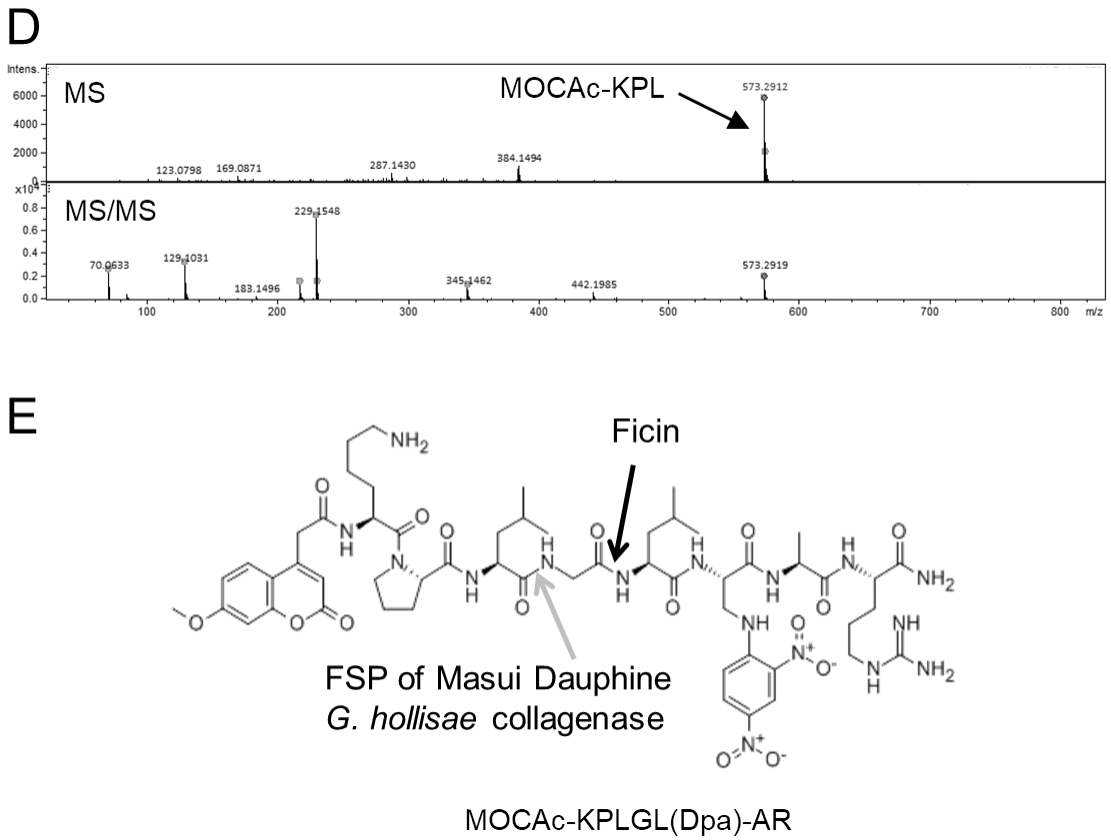


Fig. 4. Hydrolysis of synthetic peptides by the Masui Dauphine branch extract. (A) Hydrolysis of MOCAc-KPLGL(Dpa)-AR. The reaction was carried out with 15 μg/mL Masui Dauphine branch extract in the presence of 1.4 μM MOCAc-KPLGL(Dpa)-AR and 0–20 μM E-64 at pH 7.5, at 37°C. (B) Hydrolysis of Pyr-Phe-Leu-pNA. The reaction was carried out with 230 μg/mL Masui Dauphine branch extract in the presence of 375 μM Pyr-Phe-Leu-pNA and 0–20 μM E-64 at pH 7.5, at 37°C. Relative activity is the activity compared to that without E-64 (A, B). (C) Base peak ion chromatograms. MOCAc-KPLGL(Dpa)-AR (10 μM) was incubated in 2.2 mM HEPES-NaOH buffer (pH 7.5) with 100 nM ficin, 90 μg/mL Masui Dauphine branch extract or 100 nM *G. hollisae* collagenase in the absence or in the presence of 2 μM E-64 and 1 mM PMSF at pH 7.5, at 37°C, followed by LC-MS/MS analysis. (D) MS and MS/MS spectrum of MOCAc-KPL. (E) Structure of MOCAc-KPLGL(Dpa)-AR. The black arrow indicates the cleavage sites by ficin, and the gray arrow indicates the cleavage site by FSP contained in the Masui Dauphine branch extract and *G. hollisae* collagenase.

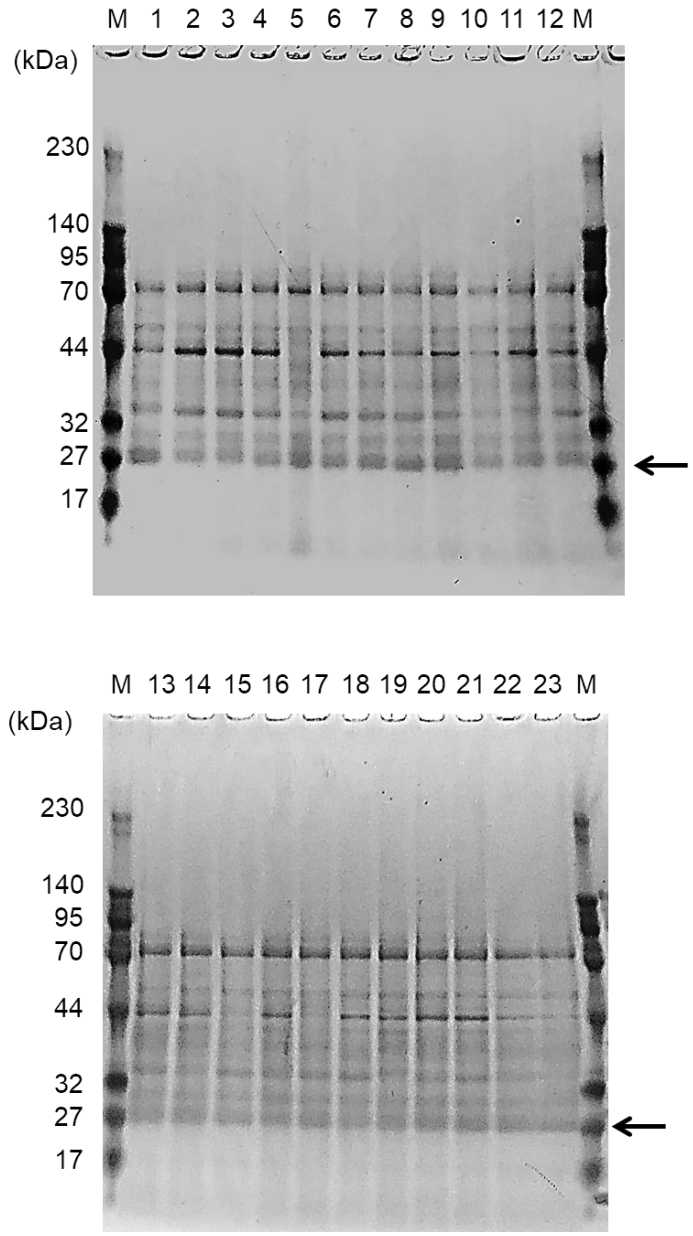
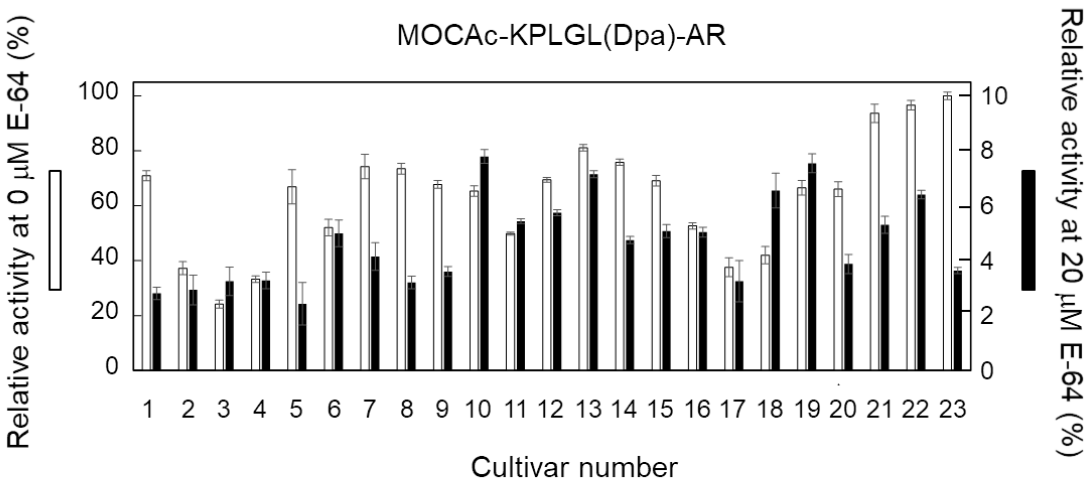
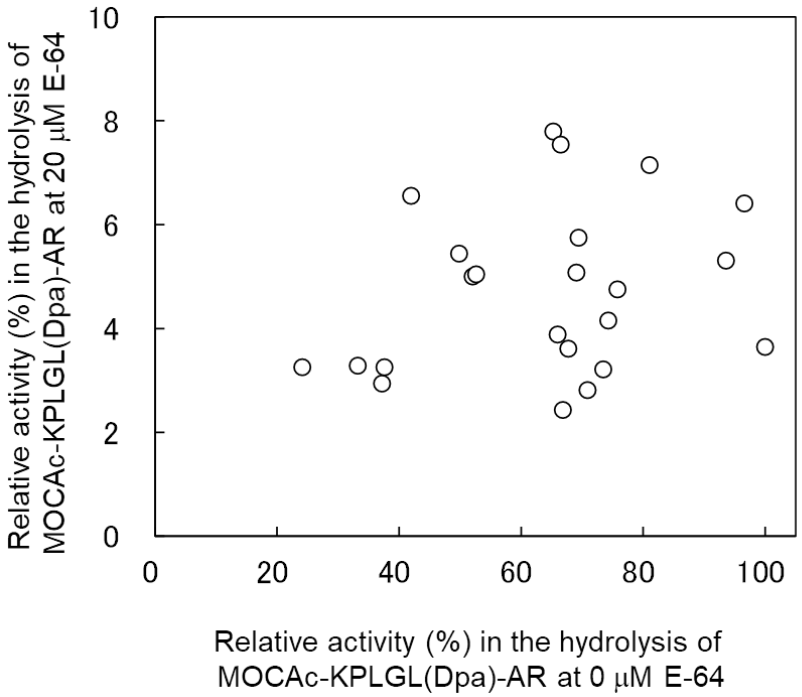


Fig. 5. SDS-PAGE of branch extracts of 23 *F. carica* cultivars under reducing conditions. A CBB-stained gradient (5 to 20%) polyacrylamide gel is shown. Lane M, molecular-mass marker; and lanes 1-23, branch extracts of 23 *F. carica* cultivars. Cultivar numbers correspond to those of Table 1. Six μg protein was applied to each lane. The arrow indicates the band corresponding to ficin.

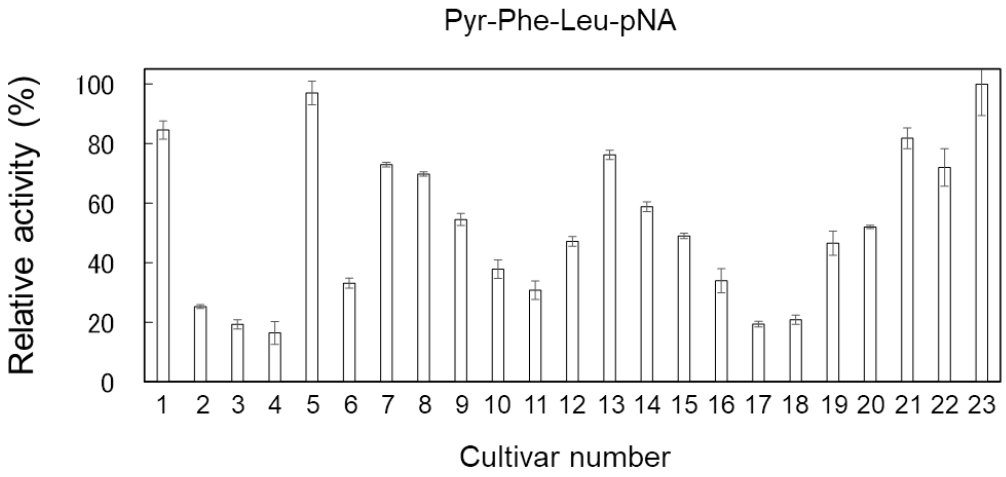
A


MOCAc-KPLGL(Dpa)-AR
Relative activity at 0 μM E-64 (%)
Relative activity at 20 μM E-64 (%)
Cultivar number
0
20
40
60
80
100
0
2
4
6
8
10
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23

B


Relative activity (%) in the hydrolysis of MOCAc-KPLGL(Dpa)-AR at 20 μM E-64
Relative activity (%) in the hydrolysis of MOCAc-KPLGL(Dpa)-AR at 0 μM E-64
0
2
4
6
8
10
0
20
40
60
80
100

C


Pyr-Phe-Leu-pNA
Relative activity (%)
0
20
40
60
80
100
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
Cultivar number

D

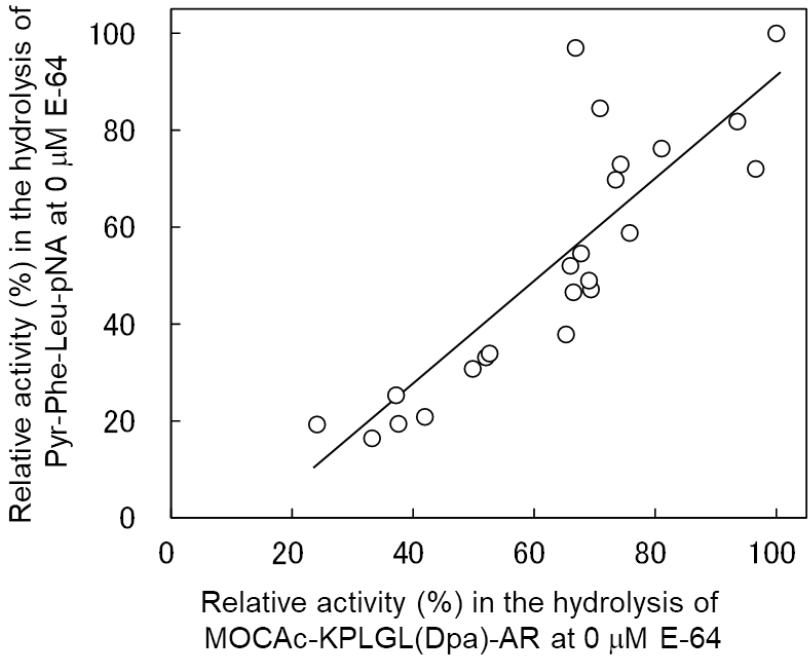

Relative activity (%) in the hydrolysis of Pyr-Phe-Leu-pNA at 0 μM E-64
0
20
40
60
80
100
0 20 40 60 80 100
Relative activity (%) in the hydrolysis of MOCAc-KPLGL(Dpa)-AR at 0 μM E-64

Fig. 6. Activities of branch extracts of 23 *F. carica* cultivars. (A) Hydrolysis of MOCAc-KPLGL(Dpa)-AR. The reaction was carried out with 1.4 μM MOCAc-KPLGL(Dpa)-AR in the presence of 3 μg/mL extracts and 0 μM E-64 (white bar) or 15 μg/mL extracts and 20 μM E-64 (filled bar) at pH 7.5, at 37°C. Cultivar numbers (cv. No.) correspond to those of Table 1. Relative activity is the activity compared to that of Grise de Tarascon extract (cv. No. 23). Error bars indicate SD values for three-times measurements. (B) Comparison of the MOCAc-KPLGL(Dpa)-AR-hydrolytic activity at 20 μM E-64 with that at 0 μM E-64. (C) Hydrolysis of Pyr-Phe-Leu-pNA. The reaction was carried out with 230 μg/mL extracts with 375 μM Pyr-Phe-Leu-pNA at pH 7.5, at 37°C. cv. No. correspond to those of Table 1. Relative activity is the activity compared to that of Grise de Tarascon extract (cv. No. 23). (D) Comparison of the Pyr-Phe-Leu-pNA-hydrolytic activity at 0 μM E-64 with the MOCAc-KPLGL(Dpa)-AR-hydrolytic activity at 0 μM E-64. The line is drawn by the linear least-squares-regression. The regression coefficient, *r*, is 0.86.

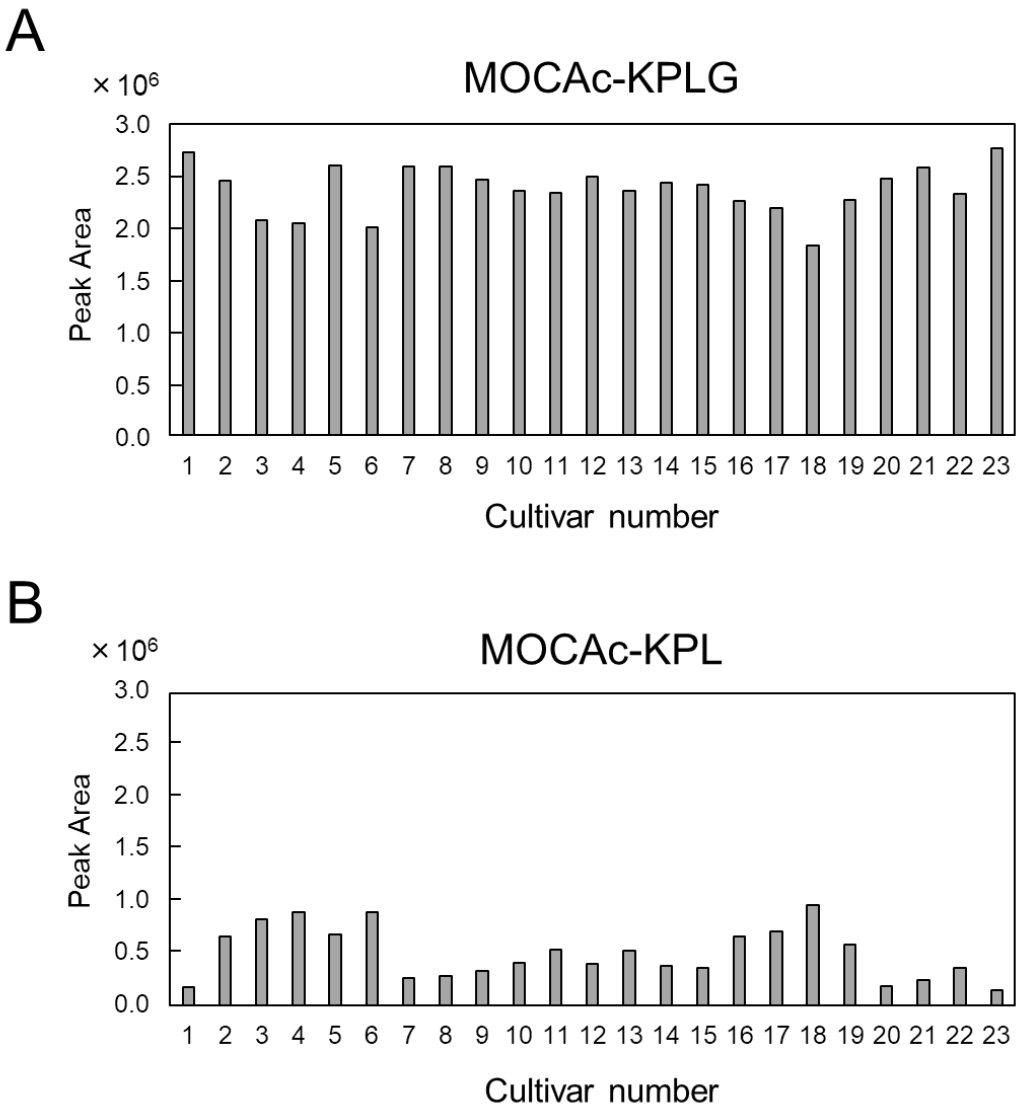


Fig. 7. Reaction products of MOCAc-KPLGL(Dpa)-AR by the branch extracts of 23 *F. carica* cultivars. MOCAc-KPLGL(Dpa)-AR (10 μM) was incubated in 2.2 mM HEPES-NaOH buffer (pH 7.5) with each 90 μg/mL *F. carica* branch extract at pH 7.5, at 37°C, followed by LC-MS/MS analysis. Peak areas of MOCAc-KPLG (A) and MOCAc-KPL (B) were obtained from base peak ion chromatograms. Cultivar numbers correspond to those of Table 1.

Chapter 2

Analysis of collagen degradation by a serine protease from *Ficus carica*

Introduction

Collagenase is a protease that specifically degrades collagen and is used for the treatment of Dupuytren's contracture (32), cell dispersion (33), and meat tenderization (34). Many collagenases currently used industrially are microbial metalloproteinases, such as collagenase from *Clostridium histolyticum* (Chcol). Collagenase from *Grimontia hollisae* (Ghcol) has been reported to possess high collagenolytic activity, and the zinc-binding motif HEXXH has been identified in the active sites of Chcol and Ghcol (35). Other than metalloproteases, serine proteases from marine microorganisms (36) and cysteine proteases from ginger (37) have been reported to possess collagenase-like properties. However, no plant-derived serine proteases with collagenase-like substrate specificity have been reported, except for FSP from cv. Brown Turkey (23).

Proteases that cleave the triple-helical structure of undenatured collagen are generally defined as collagenases, whereas proteases that degrade denatured collagen, such as gelatin, are often defined as collagenolytic proteases (38). The FSP present in the latex of cv. Brown Turkey may be classified as a collagenase because it degrades native collagen (23). However, additional research is needed to confirm this.

In this chapter, we conducted degradation experiments of acid-solubilized collagen (ASC) by FSP from branch or latex extract of *F. carica* to verify whether FSP cleaves the

stiff triple-helical structure of ASC and whether FSP is collagen-specific. Additionally, ASC degradation products by ficin or FSP were subjected to N-terminal amino acid sequence analysis to determine the ASC cleavage sites of both proteases.

Materials and Methods

Materials – The serine protease inhibitor Pefabloc SC [AEBSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride] was purchased from Sigma-Aldrich. Bovine skin acid-solubilized type I collagen (bovine ASC) was purchased from Nippi Inc. (Tokyo, Japan). Rat tail ASC (rat ASC) was purchased from Sigma-Aldrich. Bovine serum albumin (BSA) was purchased from Fujifilm Wako Pure Chemical. Polyvinylidene fluoride (PVDF) membranes were purchased from Sigma-Aldrich. Protein concentration was determined using Protein Assay CBB Solution with BSA as a standard.

Preparation of F. carica *branch extracts* – The *F. carica* branch extracts were prepared as described in Chapter 1.

Preparation of Masui Dauphine latex extract – Fresh latex from *F. carica* cv. Masui Dauphine was collected at Toyo Institute of Food Technology. The latex was centrifuged at 15,000 × *g* at 4°C for 15 min, and the supernatant was collected.

Digestion of ASC by F. carica *branch extracts* – The reaction was initiated by mixing 81 μL of 1.11 mg/mL bovine ASC in 250 mM sodium acetate buffer (pH 5.0) and 9 μL of 1 μM ficin or 900 μg/mL *F. carica* branch extract at predetermined temperatures (20–

80°C) for indicated durations (30 min–24 h). The reaction was stopped by adding 15 μL of SDS-PAGE sample buffer containing 20 μM E-64 followed by boiling for 10 min. The reaction products were analyzed by SDS-PAGE as described in Chapter 1.

Digestion of ASC by Masui Dauphine latex extract – The reaction was initiated by mixing 20 μL of 3 mg/mL ASC in 250 mM sodium acetate buffer (pH 5.5) and 160 μL of 33 μg/mL Masui Dauphine latex extract or Chcol at predetermined temperatures (32–42°C) for 24 h. The reaction was stopped by adding 126 μL of SDS-PAGE sample buffer containing 165 μM E-64 and 2.5 mM Pefabloc SC followed by boiling for 10 min. The reaction products were analyzed by SDS-PAGE as described in Chapter 1.

Digestion of BSA – The reaction was initiated by mixing 20 μL of 3 mg/mL BSA in 250 mM sodium acetate buffer (pH 5.5) or 150 mM Tris-HCl buffer (pH 8.0) and 160 μL of 33 μg/mL Masui Dauphine latex extract, ficin, or Chcol at 37°C for 24 h. The reaction was stopped by adding 126 μL of SDS-PAGE sample buffer containing 165 μM E-64 and 2.5 mM Pefabloc SC followed by boiling for 10 min. The reaction products were analyzed by SDS-PAGE as described in Chapter 1.

N-Terminal cleavage analysis – Samples were analyzed by SDS-PAGE as described in Chapter 1. Proteins were transferred to PVDF membranes by wet blotting using a Safety Blotting Cell STB-8 (TEFCO, Tokyo, Japan) as described in the manufacturer's instructions. Bands were visualized by staining with CBB R-250, and degradation products were excised and sequenced by Edman degradation (39).

Results and Discussion

ASC-degrading activities of the F. carica *branch extracts* – We characterized the digestion of ASC by the FSP from *F. carica* branch extracts. Figure 1A shows the effect of reaction temperature on bovine skin ASC (bovine ASC). Untreated bovine ASC yielded six protein bands corresponding to γ, variant β11, β11, β12, α1, and α2 chains, in the given order from the largest to the smallest size (40, 41). When bovine ASC was incubated in the absence of protease for 2 h at 20, 37, and 55°C, all bands exhibited the same pattern to the untreated one. In the case of 80°C, the intensity of all the bands was weak, suggesting that at 80°C, some of the denatured ASCs formed aggregates, which did not enter the gel.

Figure 1B show the effects of reaction temperature and E-64 on the digestion of bovine ASC by ficin. When bovine ASC was incubated with ficin and without E-64 at 20°C, all bands exhibited the same pattern to the untreated one. When bovine ASC was incubated at 37°C, the intensities of the β11 and β12 bands decreased while those of the α1 and α2 bands increased. Type I collagen consists of two α1 and one α2 chains. The α1 chain consists of a 16-amino acid (16-aa) N-terminal telopeptide, a 1,014-aa internal helical region, and a 26-aa C-terminal telopeptide. The α2 chain consists of a 9-aa N-terminal telopeptide, 1,014-aa internal helical region, and a 15-aa C-terminal telopeptide. Several Lys residues in the N- and C-terminal telopeptides are cross-linked as allysines (42, 43), and thus the cleavage of telopeptide decreases the β11 and β12 bands and decreases the α1 and α2 bands. Our results suggested that ficin cleaved bovine ASC at the N- and/or C-terminal telopeptide, but not at the helical region, at 37°C. When bovine ASC was incubated at 55 or 80°C, all bands disappeared. This suggested that ficin completely

degraded bovine ASC at 55°C and 80°C. When bovine ASC was incubated with ficin and E-64, the patterns were the same to those obtained when incubated in the absence of protease. This suggested that the ficin activity was completely inhibited by E-64.

Figure 1C shows the effects of reaction temperature on the digestion of bovine ASC by the Masui Dauphine branch extract. When bovine ASC was incubated with the extract and without E-64 at 20, 37, 55, or 80°C, the patterns were the same to those obtained when incubated with ficin and without E-64. This suggested that the ficin contained in the extract completely degraded bovine ASC at 55 and 80°C. When bovine ASC was incubated with the extract and E-64, the patterns were the same to the ones obtained when incubated with ficin and E-64 at 20, 37, or 80°C. But they were different at 55°C: the γ, variant β11, β11, and β12 bands, but not the α1 and α2 bands, were of weaker intensity, and several bands appeared at 70–95 kDa. This suggested that the FSP contained in the extract partially cleaved bovine ASC at the N- and/or C-terminal telopeptides and at the helical region at 55°C.

Figure 1D shows the effects of E-64 on the ficin- or the Masui Dauphine branch extract-mediated bovine ASC digestion. Without E-64, almost all bands disappeared. With E-64, all the bands showed strong intensity with increasing E-64 concentrations and reached the highest at 1 or 2 μM. This result indicated that E-64 exhibited the maximal inhibitory activity at 1 or 2 μM. In digestion with the Masui Dauphine branch extract, the bands of γ, variant β11, β11, and β12 were less intense than in digestion with ficin, but the bands of α1 and α2 were not. In addition, several bands appeared at 70–95 kDa, which were not observed in the digestion by ficin. These results suggested that the FSP contained in the extract is involved in the difference between the digestion by the ficin preparation and that by the extract.

Figure 1E shows the effects of reaction temperature on the Masui Dauphine branch extract-mediated bovine ASC digestion. Without E-64, at 37°C, all bands exhibited the same pattern to the untreated one, and at 40-55°C, all bands disappeared. With E-64, at 37°C, all the bands showed the same pattern as the untreated one, and at 40-55°C, the γ, variant β11, β11, and β12 bands were weaker in intensity, and some bands appeared at 70-95 kDa. This indicated that both ficin and FSP in the latex degrade bovine ASC only under conditions above 40°C.

Figure 1F shows the effect of different *F. carica* cultivars on the bovine ASC-degrading activity of FSP. The branch extracts of 23 *F. carica* cultivars (including Masui Dauphine and Brown Turkey) were reacted with bovine ASC in the presence of E-64 for 24 h at 37°C, but ASC was not degraded under any reaction conditions. This result suggests that FSPs of any of the cultivars does not degrade bovine ASC under 37°C conditions.

ASC-degrading activities of FSP from Masui Dauphine latex extract – The results of ASC degradation experiments with the branch extracts indicated that FSP in all *F. carica* cultivars, including Brown Tukey, may not degrade undenatured ASC. Therefore, we next evaluated the ASC-degrading activity of the FSP from Masui Dauphine latex. Bovine and rat ASCs were used as substrates to determine the effect of collagen denaturation on degradation. We also used Chcol for comparison.

On SDS-PAGE under reducing conditions, the latex extract of Masui Dauphine and the ficin preparation showed 26- and 28-kDa bands, respectively (Fig. 2A), each corresponding to an isoform of ficin, as previously reported (19, 44). The latex extract

showed a weak 41-kDa band, which corresponded to the FSP of Brown Turkey, as previously reported (23). Chcol was visualized as a single 66-kDa band.

Figure 2B shows the digestion of bovine ASC. The temperature was set between 32–42°C for ASC digestion, which is close to the complete denaturation temperature (T_d) of type I collagen [40°C for bovine type I collagen (45–47) and 36°C for rat type I collagen (48, 49)]. Since ASCs are insoluble in neutral to basic conditions, the reaction was performed in acidic conditions (pH 5.5). In the absence of inhibitors, where both ficin and the FSP of Masui Dauphine were active, bovine ASC incubated with the extract at 32°C or 37°C for 24 h was partially degraded. However, bovine ASC incubated at 42°C was completely degraded. In the presence of E-64 and in the absence of the serine protease inhibitor Pefabloc SC where ficin was inactive and the FSP of Masui Dauphine was active, bovine ASC incubated with the extract at 32°C or 37°C was hardly degraded at all, while it was partially degraded at 42°C. It may be that the triple-helical structure of bovine ASC became more flexible and more susceptible to enzymatic degradation at a temperature higher than the T_d of bovine ASC (40°C) (41). In the presence of E-64 and Pefabloc SC where both ficin and the FSP were inactive, bovine ASC incubated with the extract at 42°C was hardly degraded. Bovine ASC incubated with Chcol at 32–42°C was completely degraded, indicating that Chcol cleaved the stiff triple-helical structure of collagen.

Figure 2C shows the digestion of rat ASC. Like bovine ASC, untreated rat ASC yielded six protein bands. In the absence of inhibitors, rat ASC incubated with the extract at 37°C for 24 h was degraded completely, differing from the case with bovine ASC (Fig. 2B). This might be because the T_d of rat ASC (36°C) is lower than that of bovine ASC (40°C). In the presence of E-64 and in the absence of Pefabloc SC, rat ASC incubated at

32°C was hardly degraded at all; on incubation at 37 or 42°C, it was partially degraded. This result was consistent with a previous study reporting that the FSP of Brown Turkey degraded rat ASC at 37°C (23). In the presence of E-64 and Pefabloc SC, rat ASC incubated at 42°C was hardly degraded. Rat ASC incubated with Chcol at 32–42°C for 24 h was almost completely degraded. In summary, the FSP of Masui Dauphine degraded heat-denatured ASC, but not ASC with the stiff triple-helical structure.

BSA-degrading activities of FSP from Masui Dauphine latex extract – Results so far indicated that the FSP of Masui Dauphine has different characteristics from Chcol. To examine whether the FSP activity was specific to collagen, we examined BSA-degrading activity. Commercially-produced BSA was incubated with the latex extract of Masui Dauphine in the absence or presence of E-64 at pH 5.5 at 37°C for 24 h. As shown in Fig. 3, the untreated BSA showed a 58 kDa-band on SDS-PAGE. At pH 5.5, BSA that was incubated with the latex extract in the absence of inhibitors or incubated with ficin was almost completely degraded; BSA that was incubated with the latex extract in the presence of E-64 or incubated with Chcol was slightly degraded; and BSA that was incubated with the latex extract in the presence of E-64 and Pefabloc SC was hardly degraded at all.

We next examined the digestion of BSA at pH 8.0 because the optimal pH of the FSP of Brown Turkey is reported to be 8.0–8.5 (23). BSA that was incubated with the latex extract in the presence of E-64 was partially degraded, whereas BSA that was incubated with Chcol was hardly degraded, indicating that the FSP of Masui Dauphine degraded BSA more than Chcol did. The difference between the results at pHs 5.5 and 8.0 might be due to the difference in pH-dependence of the activity of the FSP of Masui Dauphine.

The results in Figs. 2B and C also suggested that the activity of the FSP of Masui Dauphine was not specific to collagen.

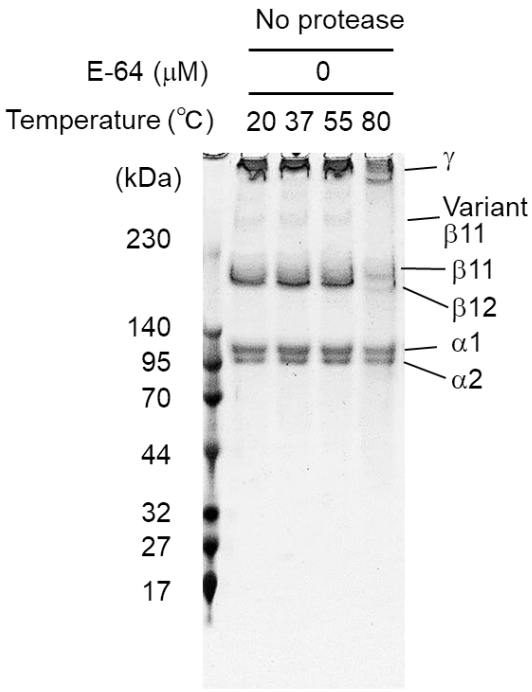
Cleavage sites of bovine ASC by FSP – Bovine ASC was incubated with the latex extract of Masui Dauphine plus E-64, ficin, Chcol, or Ghcol at 42°C for 24 h. Fig. 4 shows SDS-PAGE analysis of the resulting degradation products. Ficin-treated ASC exhibited 27-kDa and 18-kDa bands (named F1 and F2, respectively). The extract-treated ASC exhibited 60-kDa, 32-kDa, and 14-kDa bands (named L1, L2, and L3, respectively). These bands were not observed in Chcol- or Ghcol-treated ASC, indicating that the manner of digestion was different between enzymes.

F1, F2, L1, L2, and L3 were subjected to N-terminal amino acid sequence analysis. The N-terminal sequences of F1, F2, L1, L2, and L3 were IAGPPGARGP, ARGPAGPQGP, GDRGEPGPPG, GARGFPGTPG, and GARGFPGTPG, respectively. The N-terminal sequences of L2 and L3 were the same, indicating that L2 was produced first, and L3 was then produced from L2. Figures 5 and 6 show the amino acid sequences of the α1 and α2 chains, respectively, of bovine type I collagen in which the identified sequences are marked in bold and underlined. These results indicate that the FSP of Masui Dauphine in the latex cleaved at the N-terminal side of Gly, while ficin cleaved at the C-terminal side of Gly. These results agreed well with the result that in the hydrolysis of MOCAc-KPLGL(Dpa)-AR, MOCAc-KPLG was produced by ficin while MOCAc-KPL was produced by the FSP of Masui Dauphine (Chapter 1).

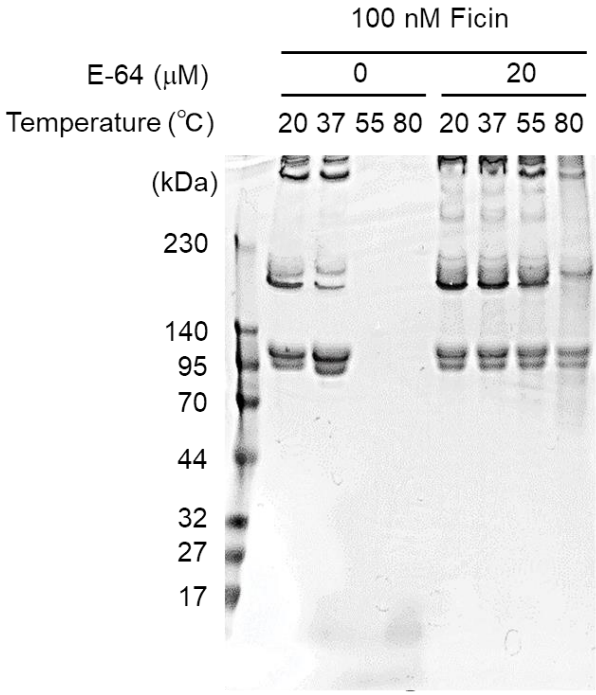
Conclusion – We evaluated the collagen-degrading activity of FSP from *F. carica* branch or latex extract. The results showed that FSP from cv. Masui Dauphine degraded

denatured collagen but not undenatured collagen with a stiff triple-helical structure, indicating that the FSP is not a collagenase. Furthermore, in contrast to a previous report (23), the activity of the FSP was not collagen-specific. We presume that the activity of FSPs in *F. carica* cultivars, including cv. Brown Turkey, might not be collagen-specific.

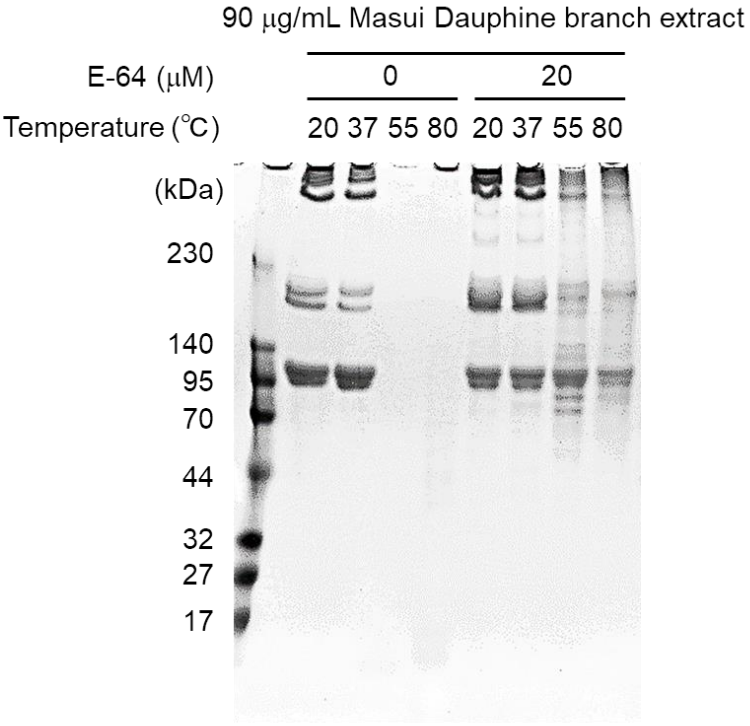
A


No protease
E-64 (μM)
0
Temperature (℃)
20 37 55 80
(kDa)
230
140
95
70
44
32
27
17
γ
Variant β11
β11
β12
α1
α2

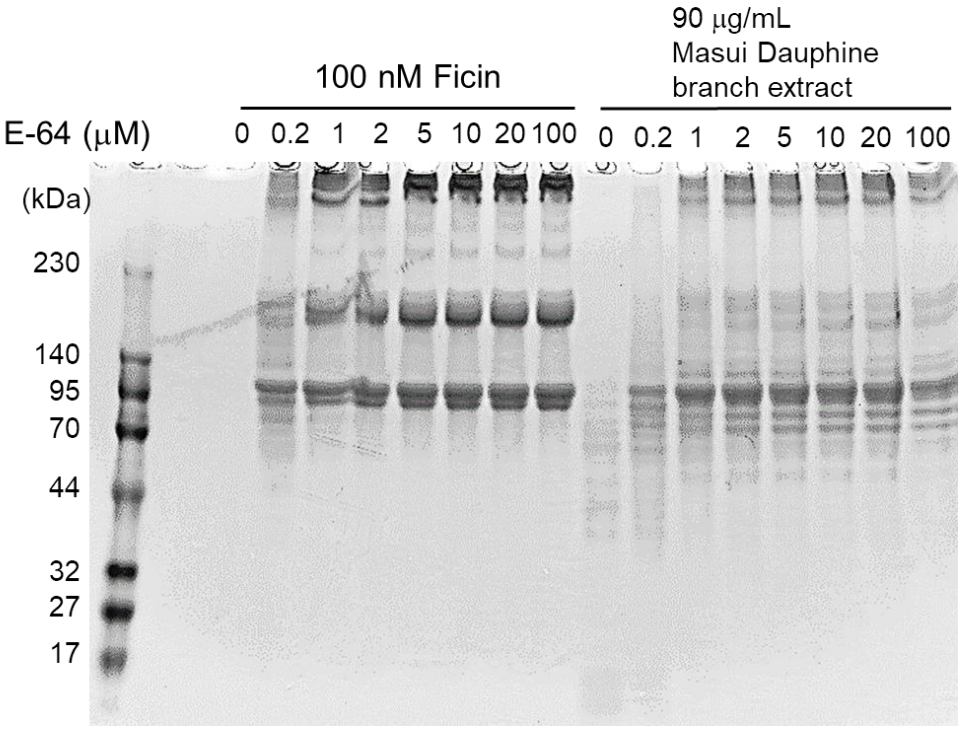
B


100 nM Ficin
E-64 (μM)
0
20
Temperature (℃)
20 37 55 80 20 37 55 80
(kDa)
230
140
95
70
44
32
27
17

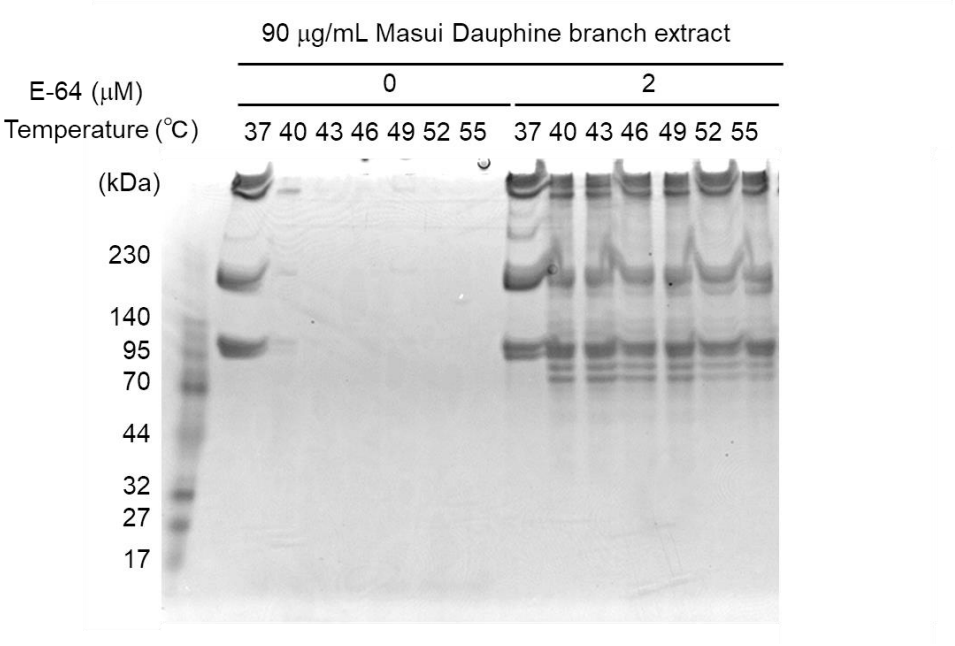
C


90 μg/mL Masui Dauphine branch extract
E-64 (μM)
0
20
Temperature (℃)
20 37 55 80 20 37 55 80
(kDa)
230
140
95
70
44
32
27
17

D


100 nM Ficin
90 μg/mL
Masui Dauphine
branch extract
E-64 (μM)
0 0.2 1 2 5 10 20 100 0 0.2 1 2 5 10 20 100
(kDa)
230
140
95
70
44
32
27
17

E


90 μg/mL Masui Dauphine branch extract
E-64 (μM)
0
2
Temperature (℃)
37 40 43 46 49 52 55 37 40 43 46 49 52 55
(kDa)
230
140
95
70
44
32
27
17

F

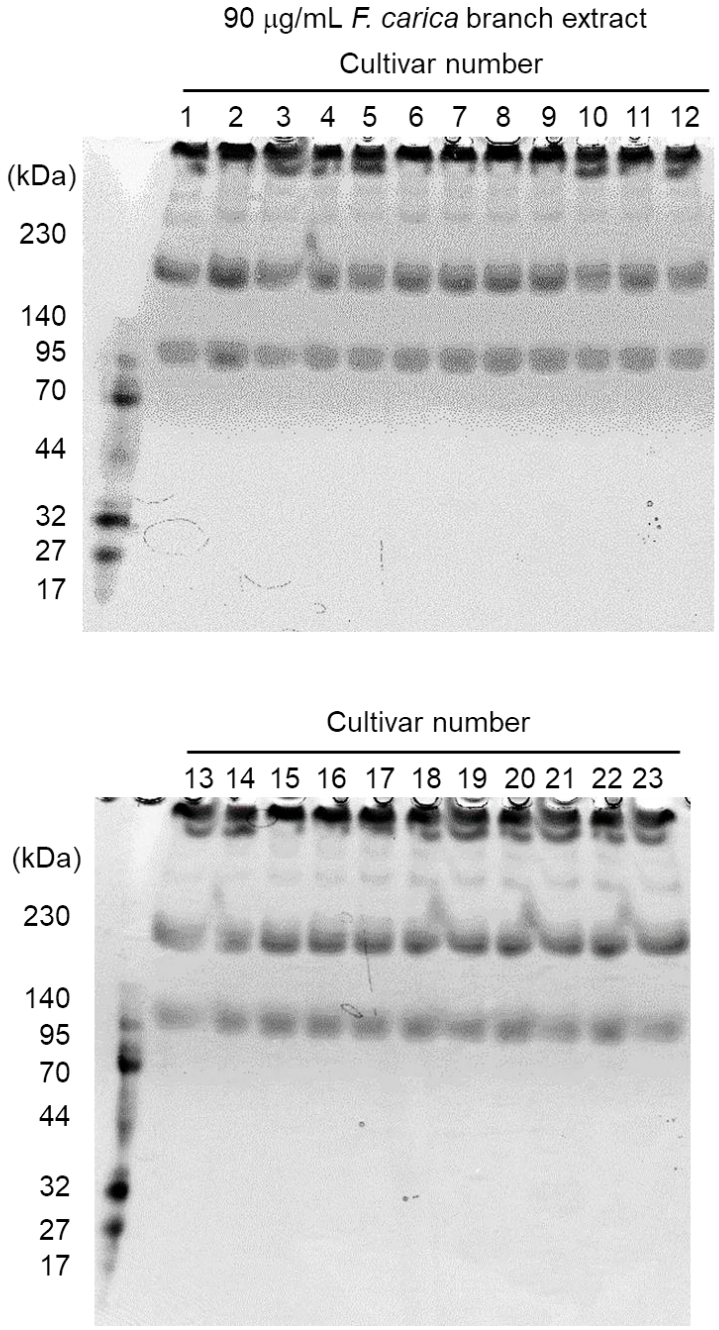
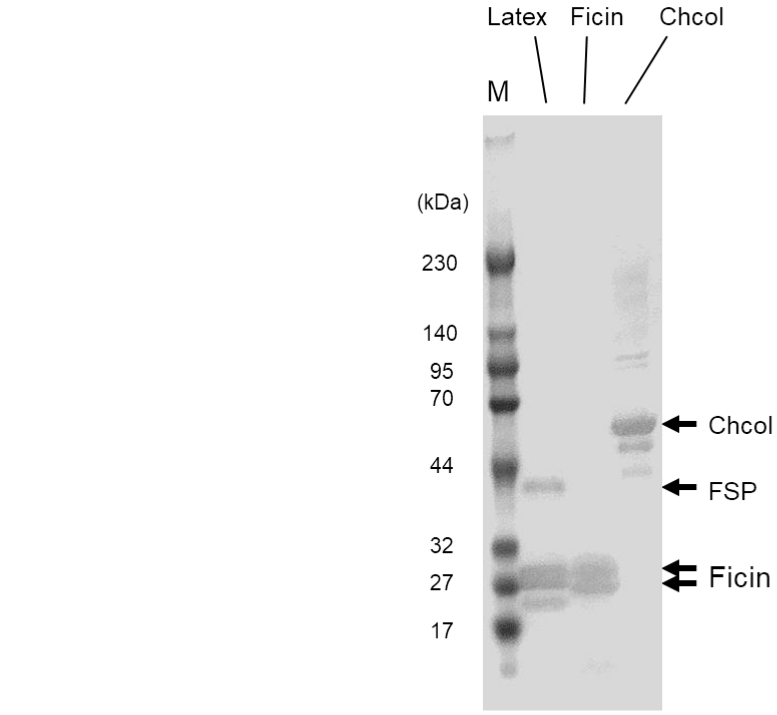

90 μg/mL F. carica branch extract
Cultivar number
1 2 3 4 5 6 7 8 9 10 11 12
(kDa)
230
140
95
70
44
32
27
17
Cultivar number
13 14 15 16 17 18 19 20 21 22 23
(kDa)
230
140
95
70
44
32
27
17

Fig. 1. Digestion of bovine acid-solubilized collagen (ASC) by FSP from *F. carica* branch extract. A CBB-stained gradient (5 to 20%) polyacrlamide gel is shown. Lane M, molecular-mass markers. (A–C) The reaction was carried out without protease (A), with 100 nM ficin (B), or with 90 μg/mL Masui Dauphine branch extract (C), in the presence of 1.0 mg/mL bovine ASC and 0 or 20 μM E-64, at pH 5.0, at 20–80°C for 2 h. (D) The reaction was carried out with 100 nM ficin or 90 μg/mL Masui Dauphine branch extract in the presence of 1.0 mg/mL bovine ASC and 0–100 μM E-64 at pH 5.0, at 55°C for 2 h. (E) The reaction was carried out with 90 μg/mL Masui Dauphine branch extract, 1.0 mg/mL bovine ASC, and 0 or 2 μM E-64 at pH 5.0, at 37–55°C for 30 min. (F) The reaction was carried out with 90 μg/mL branch extracts of 23 *F. carica* cultivars, 1.0 mg/mL bovine ASC, and 2 μM E-64 at pH 5.0, at 37°C for 24 h. Cultivar numbers correspond to those of Table 1 in Chapter 1.

A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Chcol
M
(kDa)
230
140
95
70
44
32
27
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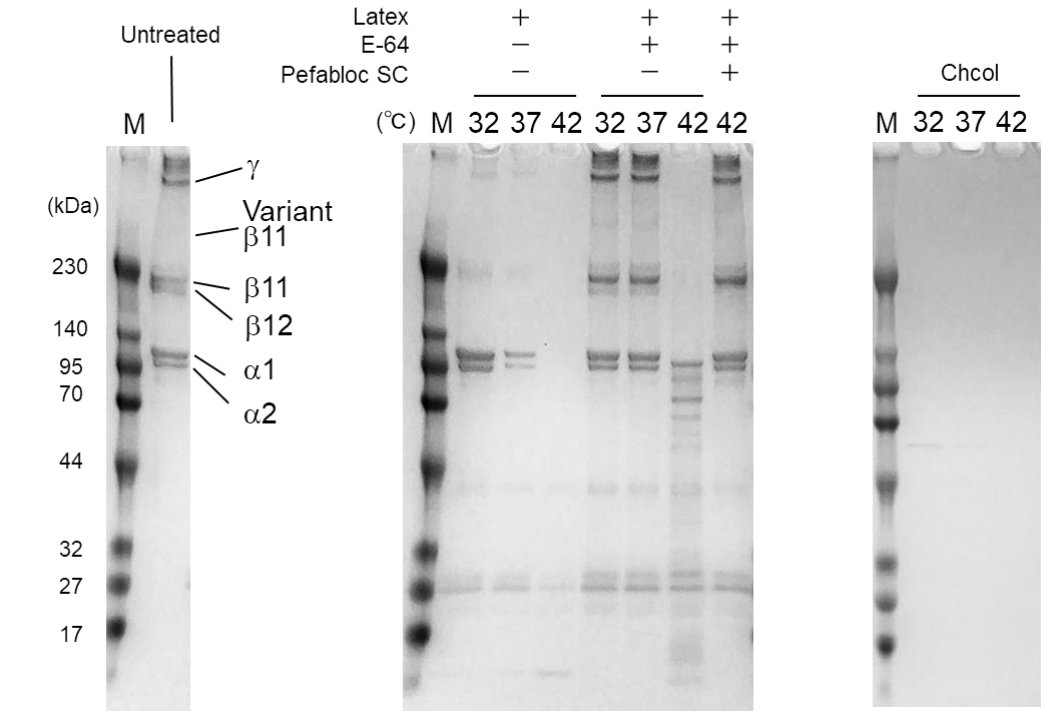
Chcol
FSP
Ficin

B


Untreated
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(kDa)
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140
95
70
44
32
27
17
γ
Variant
β11
β11
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α2
Latex
E-64
Pefabloc SC
(℃)
M 32 37 42 32 37 42 42
Chcol
M 32 37 42

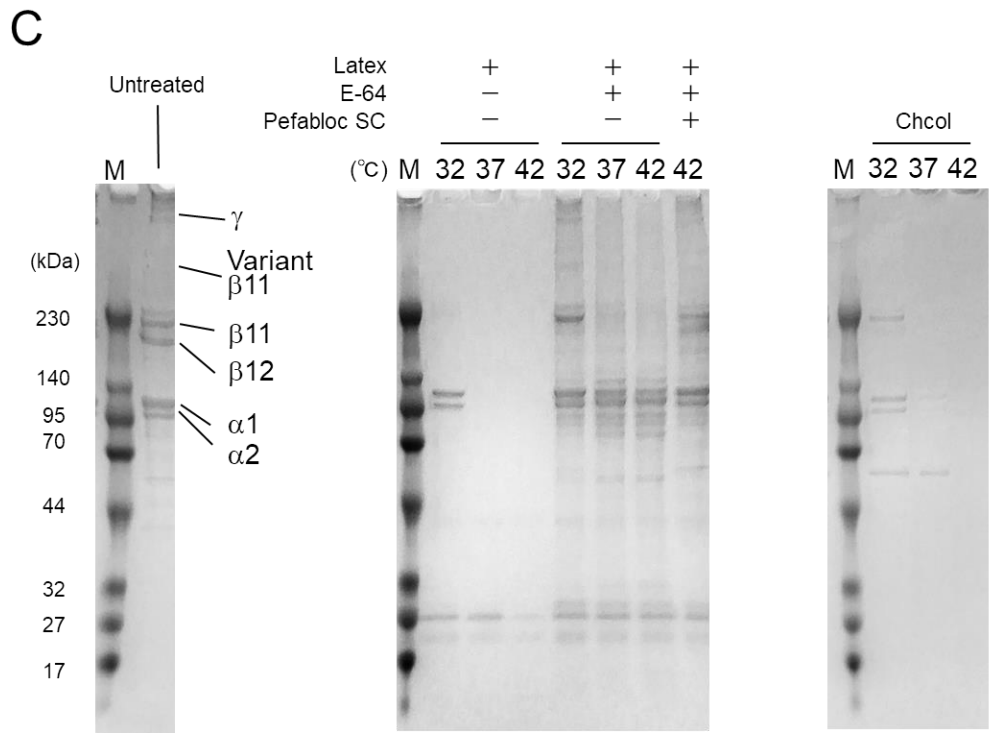


Fig. 2. Digestion of bovine and rat ASC. A CBB-stained gradient (5% to 20%) polyacrylamide gel is shown. Lane M, molecular-mass markers. (A) The Masui Dauphine latex extract we prepared, a commercial ficin preparation, and a commercial *Clostridium histolyticum* collagenase (Chcol) preparation; 1.6 μg of each protein were applied to the gel. (B, C) The reaction was carried out with 333 μg/mL bovine (B) or rat (C) ASC and 30 μg/mL Masui Dauphine latex extract or Chcol in the absence or presence of inhibitors (333 μM E-64 or 8.3 mM Pefabloc SC) at pH 5.5, at 32–42°C for 24 h.

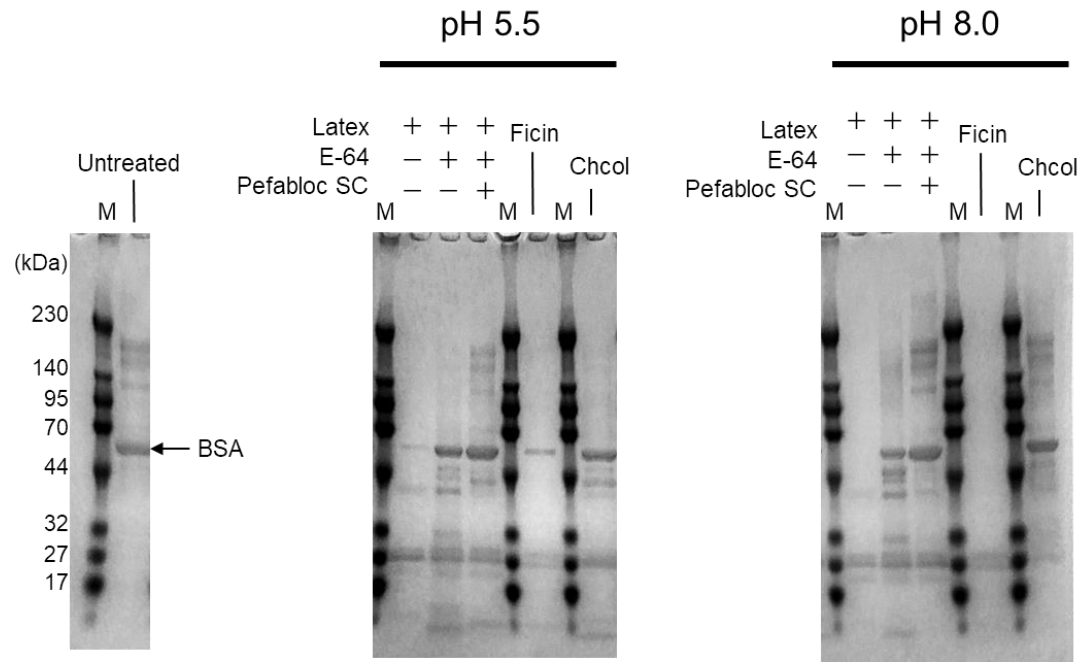


Fig. 3. Digestion of bovine serum albumin (BSA). A CBB-stained gradient (5% to 20%) polyacrylamide gel is shown. Lane M, molecular-mass markers. The reaction was carried out with 333 μg/mL BSA and 30 μg/mL Masui Dauphine latex extract, ficin, or Chcol in the absence or presence of inhibitors (333 μM E-64 or 8.3 mM Pefabloc SC) at pH 5.5 or 8.0, at 37°C for 24 h.

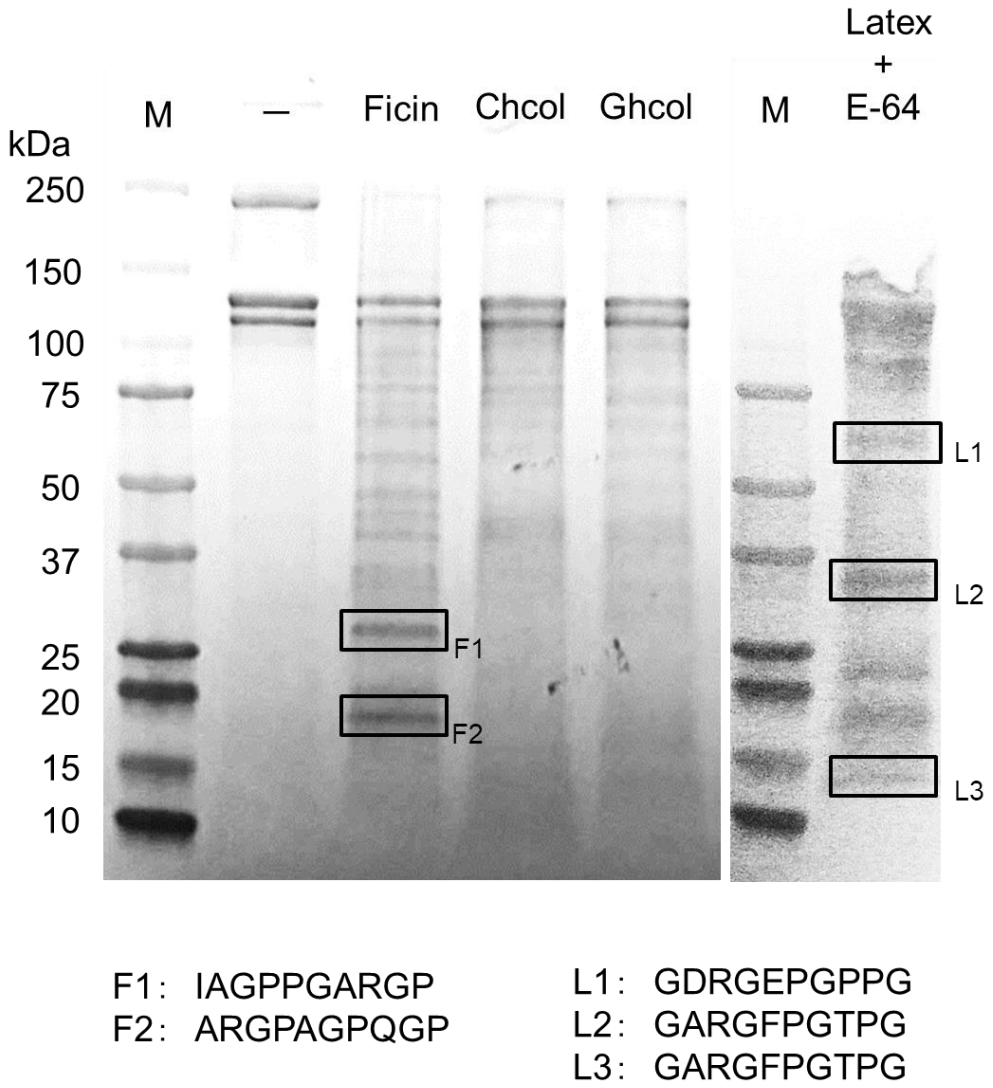


Fig. 4. Analysis of cleavage sites of bovine ASC. A CBB-stained gradient (5% to 20%) polyacrylamide gel is shown. Lane M, molecular-mass markers. The reaction was carried out with 15 μg/mL Masui Dauphine latex extract and 200 μM E-64, 7.8 ng/mL ficin, 200 ng/mL Chcol, or 600 ng/mL *Grimontia hollisae* collagenase (Ghcol), and 1 mg/mL bovine ASC, at pH 5.5, at 42°C for 24 h.

```
   1 MFSFVDLRLLLLLAATALLTHGQEEGQEEGQEEDIPPVTCVQNGLRYHDRDVWKPVPCQI
  61 CVCDNGNVLCDDVICDELKDCPNAKVPTDECCPVCPEGQESPTDQETTGVEGPKGDTGPR
 121 GPRGPAGPPGRDGIPGQPGLPGPPGPPGPPGPPGLGGNFAPQLSYGYDEKSTGISVPGPM
 181 GPSGPRGLPGPPGAPGPQGFQGPPGEPGEPGASGPMGPRGPPGPPGKNGDDGEAGKPGRP
 241 GERGPPGPQGARGLPGTAGLPGMKGHRGFSGLDGAKGDAGPAGPKGEPGSPGENGAPGQM
 301 GPRGLPGERGRPGAPGPAGARGNDGATGAAGPPGPTGPAGPPGFPGAVGAKGEGGPQGPR
 361 GSEGPQGVRGEPGPPGPAGAAGPAGNPGADGQPGAKGANGAPGIAGAPGFPGARGPSGPQ
 421 GPSGPPGPKGNSGEPGAPGSKGDTGAKGEPGPTGIQGPPGPAGEEGKRGARGEPGPAGLP
 481 GPPGERGGPGSRGFPGADGVAGPKGPAGERGAPGPAGPKGSPGEAGRPGEAGLPGAKGLT
 541 GSPGSPGPDGKTGPPGPAGQDGRPGPPGPPGARGQAGVMGFPGPKGAAGEPGKAGERGVP
 601 GPPGAVGPAGKDGEAGAQGPPGPAGPAGERGEQGPAGSPGFQGLPGPAGPPGEAGKPGEQ
 661 GVPGDLGAPGPSGARGERGFPGERGVQGPPGPAGPRGANGAPGNDGAKGDAGAPGAPGSQ
 721 GAPGLQGMPGERGAAGLPGPKGDRGDAGPKGADGAPGKDGVRGLTGPIGPPGPAGAPGDK
                                 L1
 781 GEAGPSGPAGPTGARGAPGDRGEPGPPGPAGFAGPPGADGQPGAKGEPGDAGAKGDAGPP
 841 GPAGPAGPPGPIGNVGAPGPKGARGSAGPPGATGFPGAAGRVGPPGPSGNAGPPGPPGPA
 901 GKEGSKGPRGETGPAGRPGEVGPPGPPGPAGEKGAPGADGPAGAPGTPGPQGIAGQRGVV
 961 GLPGQRGERGFPGLPGPSGEPGKQGPSGASGERGPPGPMGPPGLAGPPGESGREGAPGAE
1021 GSPGRDGSPGAKGDRGETGPAGPPGAPGAPGAPGPVGPAGKSGDRGETGPAGPAGPIGPV
        F2
1081 GARGPAGPQGPRGDKGETGEQGDRGIKGHRGFSGLQGPPGPPGSPGEQGPSGASGPAGPR
1141 GPPGSAGSPGKDGLNGLPGPIGPPGPRGRTGDAGPAGPPGPPGPPGPPGPPSGGYDLSFL
1201 PQPPQEKAHDGGRYYRADDANVVRDRDLEVDTTLKSLSQQIENIRSPEGSRKNPARTCRD
1261 LKMCHSDWKSGEYWIDPNQGCNLDAIKVFCNMETGETCVYPTQPSVAQKNWYISKNPKEK
1321 RHVWYGESMTGGFQFEYGGQGSDPADVAIQLTFLRLMSTEASQNITYHCKNSVAYMDQQT
1381 GNLKKALLLQGSNEIEIRAEGNSRFTYSVTYDGCTSHTGAWGKTVIEYKTTKTSRLPIID
1421 VAPLDVGAPDQEFGFDVGPACFL
```

Fig. 5. Amino acid sequence of α1 chain of bovine type I collagen (Accession No. P02453). Sequences identified by the N-terminal amino acid analysis are marked in bold and underlined.

```
   1 MLSFVDTRTLLLLAVTSCLATCQSLQEATARKGPSGDRGPRGERGPPGPPGRDGDDGIPG
  61 PPGPPGPPGPPGLGGNFAAQFDAKGGGPGPMGLMGPRGPPGASGAPGPQGFQGPPGEPGE
                                                    L2, L3
 121 PGQTGPAGARGPPGPPGKAGEDGHPGKPGRPGERGVVGPQGARGFPGTPGLPGFKGIRGH
 181 NGLDGLKGQPGAPGVKGEPGAPGENGTPGQTGARGLPGERGRVGAPGPAGARGSDGSVGP
 241 VGPAGPIGSAGPPGFPGAPGPKGELGPVGNPGPAGPAGPRGEVGLPGLSGPVGPPGNPGA
 301 NGLPGAKGAAGLPGVAGAPGLPGPRGIPGPVGAAGATGARGLVGEPGPAGSKGESGNKGE
 361 PGAVGQPGPPGPSGEEGKRGSTGEIGPAGPPGPPGLRGNPGSRGLPGADGRAGVMGPAGS
 421 RGATGPAGVRGPNGDSGRPGEPGLMGPRGFPGSPGNIGPAGKEGPVGLPGIDGRPGPIGP
 481 AGARGEPGNIGFPGPKGPSGDPGKAGEKGHAGLAGARGAPGPDGNNGAQGPPGLQGVQGG
 541 KGEQGPAGPPGFQGLPGPAGTAGEAGKPGERGIPGEFGLPGPAGARGERGPPGESGAAGP
 601 TGPIGSRGPSGPPGPDGNKGEPGVVGAPGTAGPSGPSGLPGERGAAGIPGGKGEKGETGL
 661 RGDIGSPGRDGARGAPGAIGAPGPAGANGDRGEAGPAGPAGPAGPRGSPGERGEVGPAGP
 721 NGFAGPAGAAGQPGAKGERGTKGPKGENGPVGPTGPVGAAGPSGPNGPPGPAGSRGDGGP
 781 PGATGFPGAAGRTGPPGPSGISGPPGPPGPAGKEGLRGPRGDQGPVGRSGETGASGPPGF
                                                            F1
 841 VGEKGPSGEPGTAGPPGTPGPQGLLGAPGFLGLPGSRGERGLPGVAGSVGEPGPLGIAGP
 901 PGARGPPGNVGNPGVNGAPGEAGRDGNPGNDGPPGRDGQPGHKGERGYPGNAGPVGAAGA
 961 PGPQGPVGPVGKHGNRGEPGPAGAVGPAGAVGPRGPSGPQGIRGDKGEPGDKGPRGLPGL
1021 KGHNGLQGLPGLAGHHGDQGAPGAVGPAGPRGPAGPSGPAGKDGRIGQPGAVGPAGIRGS
1081 QGSQGPAGPPGPPGPPGPPGPSGGGYEFGFDGDFYRADQPRSPTSLRPKDYEVDATLKSL
1141 NNQIETLLTPEGSRKNPARTCRDLRLSHPEWSSGYYWIDPNQGCTMDAIKVYCDFSTGET
1201 CIRAQPEDIPVKNWYRNSKAKKHVWVGETINGGTQFEYNVEGVTTKEMATQLAFMRLLAN
1261 HASQNITYHCKNSIAYMDEETGNLKKAVILQGSNDVELVAEGNSRFTYTVLVDGCSKKTN
1321 EWQKTIIEYKTNKPSRLPILDIAPLDIGGADQEIRLNIGPVCFK
```

Fig. 6. Amino acid sequence of α2 chain of bovine type I collagen (Accession No. P02465). Sequences identified by the N-terminal amino acid analysis are marked in bold and underlined.

Chapter 3

Analysis of partial amino acid sequences of a serine protease from the latex of *Ficus carica* cultivar Masui Dauphine

Introduction

Raskovic *et al*. reported that the molecular mass of FSP from the latex of *F. carica* cv. Brown Turkey was 41 ± 9 kDa and its isoelectric point was approximately 5.0 (23). Hamed *et al*. reported that the molecular mass of FSP from the latex of *F. carica* (unknown cultivar) was 48 kDa (24). However, they did not report the amino acid sequences of FSP. Therefore, we cannot discuss the characteristics and role of FSP based on the amino acid sequence.

Plant proteases are intricately involved in various aspects of plant physiology and development (50). For instance, cysteine proteases such as ficin and papain are toxic to insects, and thus, when E-64 was applied to leaves, cysteine proteases lost their toxicity and the leaves were damaged by insect feeding (51). It is unclear whether FSP is involved in such defense responses in *F. carica*. However, Hamed *et al.* indicated that ficin and FSP have procoagulant and anticoagulant effects, respectively, in latex, being involved in the control of latex coagulation (24).

Plant-derived proteases have attracted much attention in the medical and food industries because they have fewer problems in safety and religion than animal-derived proteases and are less likely to cause undesirable side reactions (52, 53). As mentioned in the General introduction, proteases present in the latex of plants such as *F. carica* have a

coagulant effect and are used in cheese production (11, 12, 54). Serine proteases present in the latex of Banyan (*Ficus benghalensis*) and sacred fig (*Ficus religiosa*) possess detergent as well as milk-clotting activities (55, 56). To expand the use of proteases from *F. carica* in industry, we plan to purify FSP from plant materials and express recombinant FSP in microorganisms or cultured cells.

This chapter examined which organ of *F. carica* is the best source and which method is the best for FSP preparation. We also attempted to purify FSP from cv. Masui Dauphine latex using two-dimensional SDS-PAGE to determine the partial amino acid sequences of FSP by LC-MS/MS analysis and estimate its full-length amino acid sequence via a BLAST search. For this purpose, two-dimensional gelatin zymography was performed in conjunction with SDS-PAGE.

Materials and Methods

Preparation of Masui Dauphine extracts – Fruit, leaves, and branches of *F. carica* cv. Masui Dauphine were collected at Toyo Institute of Food Technology. Extracts were prepared by two methods [Methods (i) and (ii)]. In Method (i), the fruit, leaves, and branches were frozen at −80°C, smashed finely, freeze-dried, and powdered with a food mill. The powder (9 g) was stirred in 90 mL of 100 mM Tris-HCl buffer (pH 8.3), 3 mM DTT, 0.1 mM EDTA, at 4°C overnight. In Method (ii), the fruit, leaves, and branches (each 45 g) were frozen at −80°C and smashed with a mixer in 54 mL of 100 mM Tris-HCl buffer (pH 8.3), 3 mM DTT, 0.1 mM EDTA, and stirred at 4°C overnight. The suspensions obtained in Methods (i) and (ii) were filtered with gauze. The filtrates were centrifuged at 15,000 × *g* at 4°C for 10 min, and the supernatants were collected. Proteins

in the extracts thus prepared were precipitated with ammonium sulfate or acetone. In the former, solid ammonium sulfate (19.6 g) was added to the supernatant (35 mL) to give 80% saturation and the mixture was left at 4°C overnight. In the latter, the supernatant (12 mL) was concentrated to 2 mL. To 300 μL of the concentrated supernatant, acetone (1.2 mL) was added, and the mixture was left at −30°C for 2 h. These solutions were centrifuged at 17,300 × *g* at 4°C for 10 min. The precipitates were dissolved in 1 mL of 100 mM Tris-HCl buffer (pH 8.3), 3 mM DTT, 100 μM E-64, 0.1 mM EDTA, and stored at 4°C.

Hydrolysis of MOCAc-KPLGL(Dpa)-AR. A 1.56 μM MOCAc-KPLGL(Dpa)-AR solution was prepared in 98 mM Tris-HCl buffer (pH 8.3), 98 μM EDTA, 3 mM DTT. The reaction was initiated by mixing 180 μL of the MOCAc-KPLGL(Dpa)-AR solution and 20 μL of enzyme solution containing 1 mM E-64. The reaction temperature was 37°C. The reaction was monitored by following the increase in fluorescence intensity at 400 nm with excitation at 324 nm using an EnSight multimodal plate reader for 3 min.

SDS-PAGE – SDS-PAGE was performed as described in Chapter 1.

Preparation of Masui Dauphine latex extract – The latex extract of Masui Dauphine was prepared as described in Chapter 2.

Two-dimensional gel electrophoresis – Isoelectric focusing (IEF) was performed with agarGel (Atto, Tokyo, Japan) containing carrier ampholytes (pH 3–10) and discRun (Atto). A constant voltage of 300 V was applied for 210 min. The latex extract of Masui

Dauphine [10 μL of 600 μg/mL in 60 mM Tris-HCl (pH 8.9), 5 M urea, 1 M thiourea, 1% (v/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS), 1% (w/v) Triton X-100, 1% (w/v) DTT] was applied to agarGel. After IEF, the gels were expelled from the tubes and equilibrated in 2.5% (w/v) trichloroacetic acid or water for 3 min, followed by immersion in SDS equilibration buffer [50mM Tris-HCl (pH 6.8), 2% (w/v) SDS、 0.01% (w/v) bromophenol blue] for 10 min. The gels were then placed on the top of a 12.5% polyacrylamide gel, and SDS-PAGE was performed as described above.

Gelatin zymography – Samples were applied to agarGel as described above. After IEF, the gels were expelled from the tubes and equilibrated in 2.5% (w/v) trichloroacetic acid or water for 3 min, followed by immersion in SDS equilibration buffer for 10 min. The gels were placed on the top of a 12.5% polyacrylamide gel that contained 0.063% (w/v) gelatin, and samples were subjected to electrophoresis at a constant current of 40 mA for 34 min. After electrophoresis, the gels were soaked in wash and reaction buffers and then stained as described in Chapter 1.

LC-MS/MS – Protein bands were reduced with 10 mM DTT, alkylated with 55 mM iodoacetamide, digested in-gel by treatment with trypsin, and purified using a C18 tip (C tip T300, AMR, Tokyo, Japan). The resultant peptides were subjected to nanocapillary reversed-phase LC-MS/MS analysis using a C18 column (10 cm × 75 μm, 1.9 μm; Bruker Daltoniks, Bremen, Germany) on a nanoLC system (Bruker Daltoniks, Yokohama, Japan) connected to a timsTOF Pro mass spectrometer (Bruker Daltoniks) and a modified nano-electrospray ion source (CaptiveSpray; Bruker Daltoniks). The mobile phase consisted of water containing 0.1% formic acid (solvent A) and acetonitrile containing 0.1% formic

acid (solvent B). Linear gradient elution was carried out from 2% to 35% solvent B over 18 min at a flow rate of 500 nL/min. The ion spray voltage was set at 1.6 kV in the positive ion mode. Ions were collected in a trapped ion mobility spectrometry (TIMS) device over 100 ms and MS and MS/MS data were acquired over an *m/z* range of 100–2,000. During the collection of MS/MS data, the TIMS cycle was adjusted to 0.53 s and included one MS plus four parallel accumulation serial fragmentation-MS/MS scans, each containing on average 12 MS/MS spectra (>100 Hz) (57, 58). Nitrogen was used as the collision gas. The resulting data were processed using DataAnalysis version 5.2 (Bruker Daltoniks), and proteins were identified using MASCOT version 2.6.2 (Matrix Science, London, UK) against the SwissProt and NCBI databases.

Results and Discussion

Comparison of the FSP activities of branch, leaf, and fruit extracts – In order to estimate the full-length amino acid sequence of the FSP of Masui Dauphine, the purification method of FSP from *F. carcia* was examined. The branch, leaf, fruit extracts and the latex extract were candidates for a sources of purification. To see which is the best source, we compared the protein amounts, the FSP activities, and the specific activities of FSP in the extracts of branches, leaves, and fruits of Masui Dauphine. We also compared two preparation methods of the extract. In one method, branch, leaf, or fruit was broken, freeze-dried, powdered, and then stirred in buffer. In the other method, each was smashed and directly stirred in buffer. Then, proteins in the extracts were precipitated with ammonium sulfate or acetone and redissolved in buffer.

We measured the MOCAc-KPLGL(Dpa)-AR-hydrolyzing activities of the extracts

in the presence of 100 μM E-64 (Table 1). The total activities of leaf extracts were in the range 140–210 U, followed by fruit extracts (61–130 U), and branch extracts (2.6–12 U). There was almost no difference in recovery of total enzyme between the two preparation methods, nor was there a difference between precipitation with ammonium sulfate and acetone. These results indicated that leaf was more suitable than branch and fruits as sources of FSP. We also tried to evaluate the specific activity of the Masui Dauphine latex extract, but the activity could not be measured accurately because the fluorescence intensity exceeded the upper limit of the measurement. This was thought to be due to impurities contained in the latex.

Amino acid sequence analysis of the FSP from Masui Dauphine latex – Based on the above results, it was inferred that the method of purifying FSP from Masui Dauphine leaf extracts was the most efficient. However, the results of SDS-PAGE of the leaf extracts prepared by the four different methods showed that all extracts contained many proteins other than FSP (Fig. 1), indicating that the purification of FSP by these methods was difficult. On the other hand, the impurities in the latex were estimated to be relatively small (Chapter 2, Fig. 2A). Therefore, we attempted to purify FSP from latex extracts of Masui Dauphine by two-dimensional electrophoresis.

We described in Chapter1 that in gelatin zymography in the presence of E-64, the branch extract of Masui Dauphine showed a band corresponding to 45–66 kDa. This result showed that the gelatin was degraded by the FSP in the extract. We performed two-dimensional gel electrophoresis of the latex extract of Masui Dauphine in the presence of E-64 (Fig. 2). When the gel was treated with trichloroacetic acid after IEF, two bands (named S-1 and S-2) appeared at the position corresponding to 45–66 kDa (Fig. 2A).

However, no band appeared in the gelatin zymography (Fig. 2B), possibly because the FSP was inactivated by trichloroacetic acid although some proteases were not completely inactivated by trichloroacetic acid (59). When the gel was not treated with trichloroacetic acid after IEF, one band (named S-3) corresponding to 45–66 kDa was observed (Fig. 2C). The band appeared at the same position in gelatin zymography (Fig. 2D).

S-1, S-2, and S-3 were excised from the gel and subjected to sequence analysis by trypsin digestion and LC-MS/MS. Six sequences were obtained from S-1 (Table 2). The same four sequences were obtained from both S-2 and S-3. BLAST searches of the sequences against the Viridiplantae (green plants) genome database of NCBI (Taxonomy ID: 33090) revealed that S-1 was derived from a mandelonitrile lyase of *F. carica* (Accession No. BBD74161), and S-2 and S-3 were derived from a subtilisin-like protease of *F. carica* (Accession No. BBD74156). Subtilisin is a protease initially obtained from *Bacillus subtilis* and is classified as serine protease. This strongly suggested that the FSP of Masui Dauphine was a subtilisin-like protease. Figure 3 shows the nucleotide and amino acid sequences of the subtilisin-like protease in the genome database of *F. carica* cv. Masui Dauphine. The number of amino acid residues was 743 and the predicted molecular mass was 79.8 kDa. However, the molecular mass estimated from gelatin zymography was 45–66 kDa. This difference might be due to removal of a propeptide region during the maturation of the enzyme (60).

Cucumisin from melon (*Cucumis melo* L.), a well-known plant-derived subtilisin-like protease, has been reported to have broad substrate specificity (61–63) and to produce peptides involved in plant defense responses (64). A comparison of the amino acid sequences of cucumisin and the subtilisin-like protease of *F. carica* showed high homology (Expect value: 2×10^{-157}), especially around the residues in the active site,

which were well conserved (Fig. 4). This is consistent with the fact that FSP showed broad substrate specificity in Chapter 2, indicating that FSP is likely to be indirectly involved in the defense response of *F. carica*.

Another subtilisin-like protease, deseasin MCP-01 from the deep-sea microbe *Pseudoalteromonas* sp. SM9913, cleaves the stiff triple-helical structure of collagen (65, 66). A comparison of the amino acid sequences of deseasin MCP-01 and the subtilisin-like protease of *F. carica* showed low homology (Expect value: 5×10^{-7}), and the collagen-binding domain, which is presumed to be essential for deseasin MCP-01 to degrade undenatured collagen, was not identified in the subtilisin-like protease of *F. carica*. This evidence supports our finding that FSP did not cleave the stiff triple-helical structure of collagen.

Conclusion – The branch, leaf, and fruit extracts of cv. Masui Dauphine were compared to see which organ is the best as a source for the production of FSP. The leaf extract showed the highest protein yield and FSP specific activity. Considering that many leaves are discarded in *F. carica* cultivation, leaf may be the best source of FSP. Amino acid sequence analysis identified FSP from the latex of cv. Masui Dauphine as a subtilisin-like protease. It has been reported that many subtilisin-like proteases from plants have broad substrate specificity (67), which is consistent with the findings in Chapters 1 and 2 that FSP acts on several substrates.

Table 1. Preparation of the extract from the branch, leaf, and fruit of Masui Dauphine.

Stage	Amount (g)	Volume (mL)	Protein (mg)	Activity (U)	Specific activity (U/mg)
Branch	45				
Extract A[a]		13	51	12 ± 1	0.23 ± 0.02
Extract B[b]		3.4	17	8.0 ± 0.4	0.47 ± 0.03
Extract C[c]		8.6	30	4.9 ± 0.3	0.16 ± 0.01
Extract D[d]		2.9	13	2.6 ± 0.5	0.20 ± 0.04
Leaf	45				
Extract A		15	140	210 ± 21	1.4 ± 0.1
Extract B		2.4	43	190 ± 18	4.4 ± 0.4
Extract C		17	83	180 ± 8	2.2 ± 0.1
Extract D		19	36	140 ± 8	3.8 ± 0.2
Fruit	45				
Extract A		5.1	49	83 ± 4	1.7 ± 0.1
Extract B		4.0	58	130 ± 10	2.3 ± 0.2
Extract C		6.0	45	61 ± 1	1.3 ± 0.02
Extract D		5.0	43	79 ± 6	1.8 ± 0.1

[a]Freeze-drying and powdering were conducted. Proteins were precipitated by ammonium sulfate.

[b]Freeze-drying and powdering were conducted. Proteins were precipitated by acetone.

[c]Freeze-drying and powdering were not conducted. Proteins were precipitated by ammonium sulfate.

[d]Freeze-drying and powdering were not conducted. Proteins were precipitated by acetone.

The reaction was carried out with 7.5 μg/mL Masui Dauphine extract in the presence of 1.4 μM MOCAc-KPLGL(Dpa)-AR and 100 μM E-64 at pH 8.3, at 37°C. One unit is defined as the amount which exhibits 1.0×10^5 fluorescence intensity in the reaction solution (200 μL) in 1 s. Average and SD values of triplicate determination are shown.

Table 2. Identification of proteins purified from the latex extract of Masui Dauphine.

Spot No.	Protein name	Accession No.	Number of matched peptides	Protein score	Matched peptide sequence	Peptide score
S-1	(R)-mandelonitrilelyase-like	BBD74161	21	402	TTGPLSHGSLR	60
	[*Ficus carica*]				TTGPLSHGSLR	61
					FTSEDGVANVR	57
					FTSEDGVANVR	61
					FTSEDGVANVR	75
					VSGSIFDGVGKR	59
					MGDMMNTNSMDR	51
					MGDMMNTNSMDR	66
					MGDMMNTNSMDR	50
					MGDMMNTNSMDR+Oxidation	57
					MGDMMNTNSMDR+Oxidation	45
					MGDMMNTNSMDR+Oxidation	61
					MGDMMNTNSMDR+2 Oxidation	52
					VLGGTGMINAGFYSR	58
					VLGGTGMINAGFYSR	63
					VLGGTGMINAGFYSR	75
					VLGGTGMINAGFYSR+Oxidation	75
					VLGGTGMINAGFYSR+Oxidation	62
					VLGGTGMINAGFYSR+Oxidation	60
					EALVEAGVVPDNGFR	67
					EALVEAGVVPDNGFR	64

Table 2. Identification of proteins purified from the latex extract of Masui Dauphine (continued).

Spot No.	Protein name	Accession No.	Number of matched peptides	Protein score	Matched peptide sequence	Peptide score
S-2	subtilisin-like protease [*Ficus carica*]	BBD74156	16	402	EFPADVVLGNSR	60
					EFPADVVLGNSR	64
					EFPADVVLGNSR	63
					YVVTFSSLSGAGVVPR	80
					YVVTFSSLSGAGVVPR	69
					YVVTFSSLSGAGVVPR	63
					SAIMTTTYNLDNSGQSIK	74
					SAIMTTTYNLDNSGQSIK	82
					SAIMTTTYNLDNSGQSIK	70
					SAIMTTTYNLDNSGQSIK	93
					SAIMTTTYNLDNSGQSIK+Oxidation	62
					SAIMTTTYNLDNSGQSIK+Oxidation	99
					SAIMTTTYNLDNSGQSIK+Oxidation	66
					SAIMTTTYNLDNSGQSIK+Oxidation	120
					DLSTGTASTPFAHGAGHVNPNR	108
					DLSTGTASTPFAHGAGHVNPNR	138

Table 2. Identification of proteins purified from the latex extract of Masui Dauphine (continued).

Spot No.	Protein name	Accession No.	Number of matched peptides	Protein score	Matched peptide sequence	Peptide score
S-3	subtilisin-like protease [*Ficus carica*]	BBD74156	13	372	EFPADVVLGNSR	68
					YVVTFSSLSGAGVVPR	74
					YVVTFSSLSGAGVVPR	59
					YVVTFSSLSGAGVVPR	69
					SAIMTTTYNLDNSGQSIK	59
					SAIMTTTYNLDNSGQSIK	77
					SAIMTTTYNLDNSGQSIK	71
					SAIMTTTYNLDNSGQSIK+Oxidation	84
					SAIMTTTYNLDNSGQSIK+Oxidation	100
					SAIMTTTYNLDNSGQSIK+Oxidation	70
					SAIMTTTYNLDNSGQSIK+Oxidation	69
					SAIMTTTYNLDNSGQSIK+Oxidation	69
					DLSTGTASTPFAHGAGHVNPNR	130

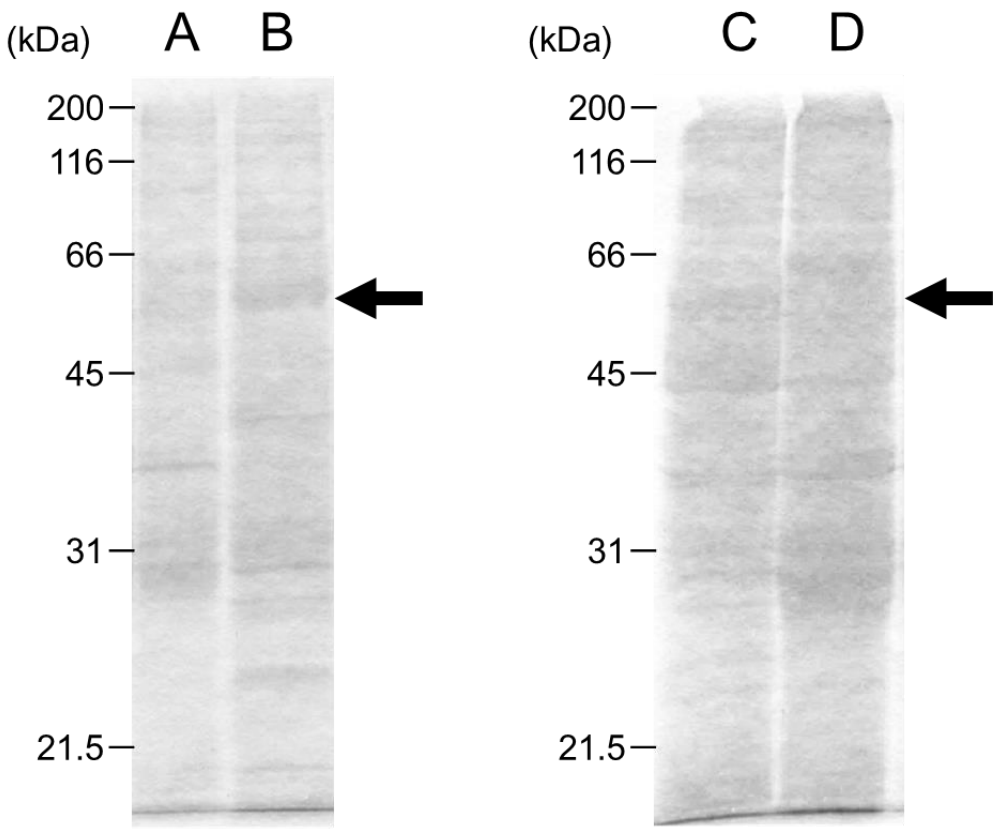
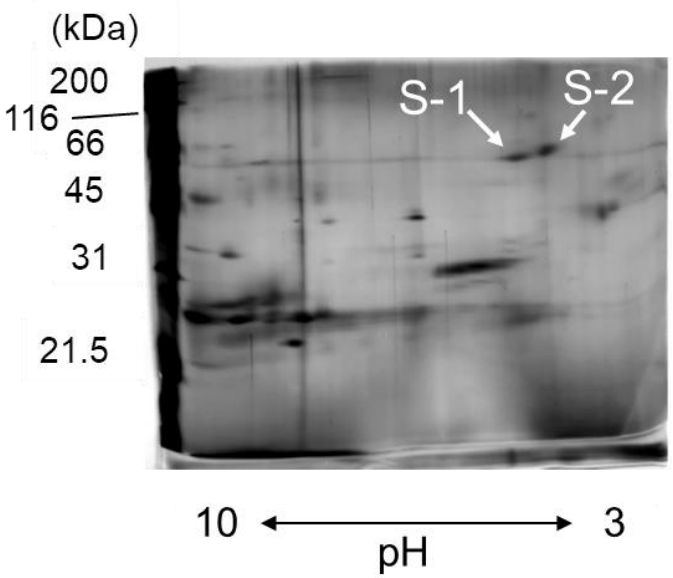
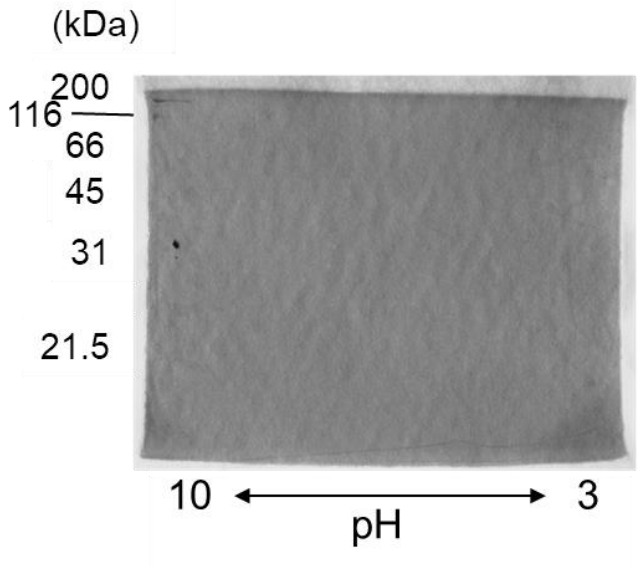


Fig. 1. SDS-PAGE of the leaf extracts of *F. carica* cv. Masui Dauphine. A CBB-stained 10% polyacrylamide gel is shown. Each extract (A−D) corresponds to Table 1. The arrow indicates the band corresponding to FSP.

A


(kDa)
200
116
66
45
31
21.5
S-1
S-2
10
3
pH

B


(kDa)
200
116
66
45
31
21.5
10
3
pH

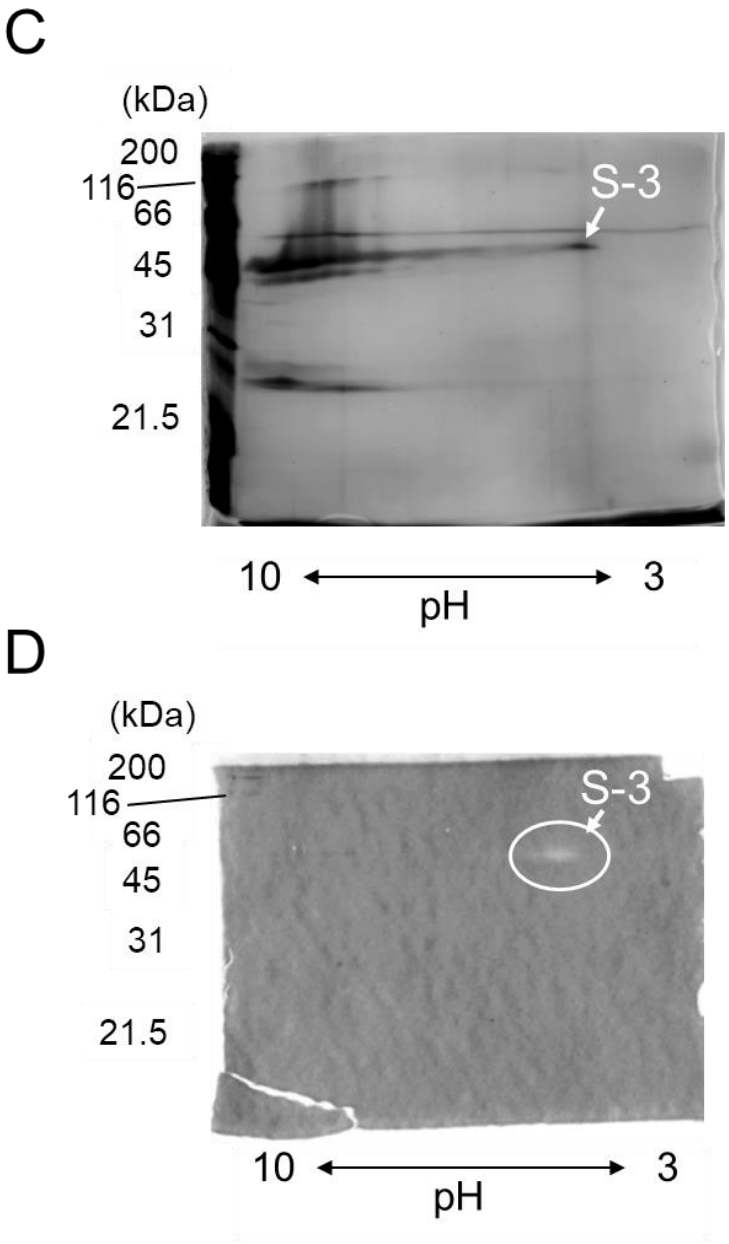


Fig. 2. Two-dimensional gel electrophoresis analysis of the latex extract of *F. carica* cv. Masui Dauphine. Isoelectric focusing (IEF) was conducted using an agar gel with pH range 3–10 in a capillary tube of 7.5 cm length and 2.5 mm I.D. After IEF, the gels were expelled from the tubes and equilibrated in 2.5% (w/v) trichloroacetic acid (A, B) or water (C, D). Electrophoresis was carried out with a 12.5% polyacrylamide gel. (A, C) Silver staining of the gel. (B, D) Gelatin zymography.

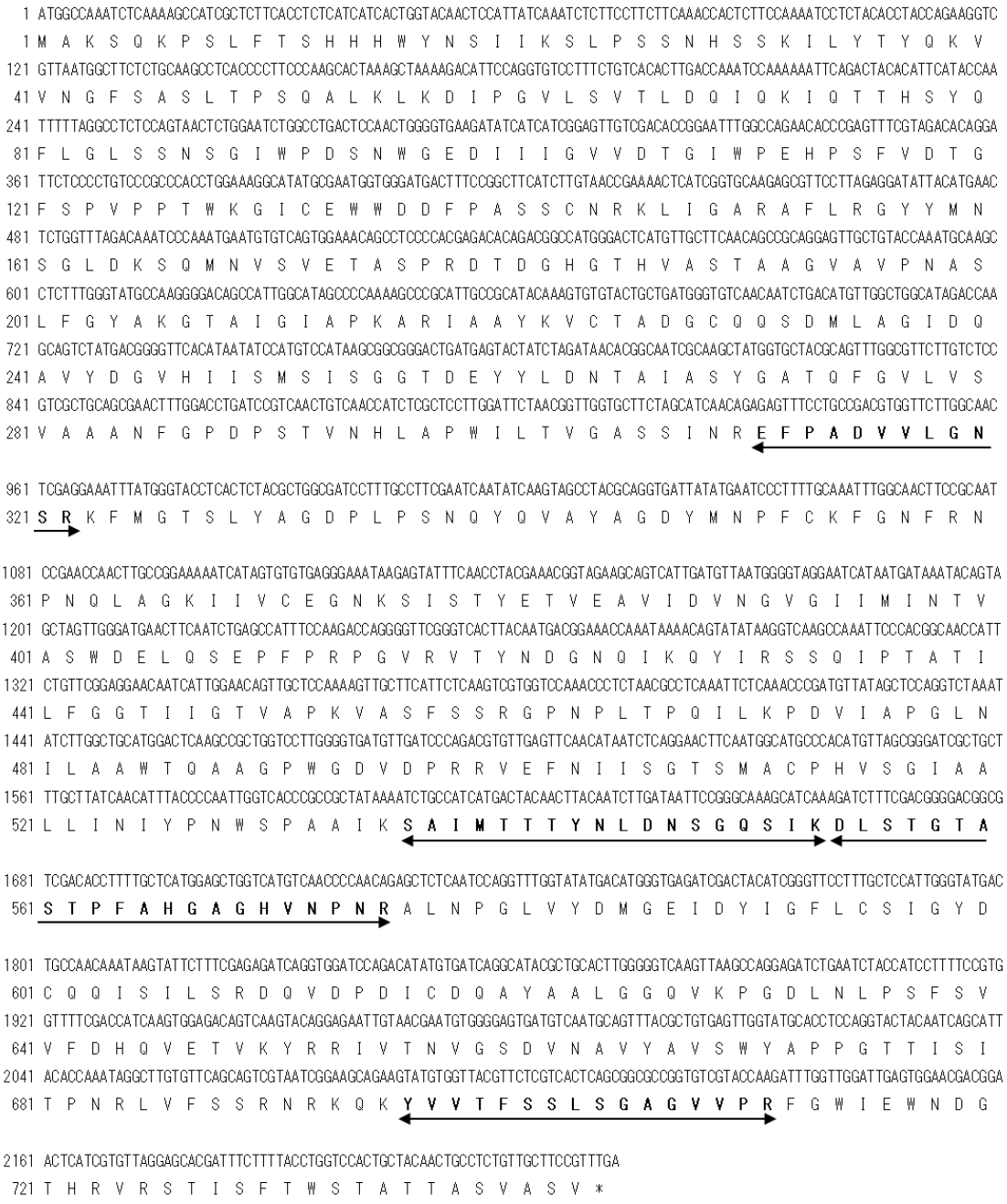


Fig. 3. Amino acid sequence of subtilisin-like serine protease from *F. carica* cv. Masui Dauphine (Accession No. BBD74156). Amino acids marked in bold indicate the regions matching the sequences determined by LC-MS/MS analysis.

Fig. 4. A comparison of the amino acid sequences of cucumisin and the subtilisin-like protease of *F. carica*. Query: cucumisin (Accession No. Q39547). Subject: subtilisin-like serine protease from *F. carica* cv. Masui Dauphine (Accession No. BBD74156). The putative catalytic triad amino acid residues (Asp, His, and Ser) are indicated by arrows.

Summary

Chapter 1

We investigated which organs and cultivars of *F. carica* contain FSP and analyzed the basic characteristics of FSP. The gelatin zymography results showed that the fruit, branch, and leaf extracts of cv. Masui Dauphine had gelatinolytic activity in the presence of the cysteine protease inhibitor E-64, indicating the presence of FSP. The hydrolytic activity of FSP to MOCAc-KPLGL(Dpa)-AR, a synthetic substrate widely used for the characterization of collagenases, was examined. LC-MS/MS analysis of MOCAc-KPLGL(Dpa)-AR reaction products revealed that ficin cleaved the Gly–Leu peptide bond and that FSP and *G. hollisae* collagenase cleaved the Leu–Gly bond. The MOCAc-KPLGL(Dpa)-AR hydrolytic activity of the branch extracts of 23 *F. carica* cultivars was determined. The results showed that the relative activities, which were defined as 100% for the activity of the cultivar showing the maximum activity, of the 23 *F. carica* cultivars were 24.1%–100% in the absence of E-64 (ficin and FSP are active) and 2.4%–7.8% in the presence of E-64 (ficin is inactive and FSP is active). These results indicate that FSP is universally produced in various organs in several *F. carica* cultivars, its substrate specificity is distinctly different from that of ficin, and its activity is weaker than that of ficin.

Chapter 2

We evaluated the ASC-degrading activity of FSP to investigate whether FSP has substrate specificity similar to that of collagenase. The reaction with bovine ASC and cv. Masui Dauphine branch extract showed that ASC was degraded by FSP at 55°C but not

at 37°C. Similar results were obtained for FSPs of other cultivars. To confirm whether the thermal denaturation of ASC affects the ASC-degrading activity of FSP, we used two ASCs (bovine and rat ASCs) with different complete denaturation temperatures (40°C and 36°C, respectively). The results showed that the FSP of cv. Masui Dauphine latex degraded bovine and rat ASCs only under temperatures higher than the respective complete denaturation temperatures. Therefore, FSP degraded only heat-denatured ASCs and not undenatured ASCs. In contrast, Chcol degraded both undenatured and denatured ASCs. The BSA-degrading activities of FSP and Chcol were examined. The results indicated that Chcol was collagen-specific whereas FSP was not. Analysis of the cleavage sites of bovine ASC revealed that ficin and FSP cleaved the C- and N-terminal sides of the Gly residues, respectively, consistent with the result for MOCAc-KPLGL(Dpa)-AR (Chapter 1).

Chapter 3

To determine the full-length amino acid sequence of the FSP from cv. Masui Dauphine latex, we purified the FSP and analyzed the partial amino acid sequence using LC-MS/MS. Branch, leaf, and fruit extracts of cv. Masui Dauphine were prepared using four different preparation methods. The results showed that the leaf extract had the highest protein yield and FSP specific activity, with a minor difference depending on the preparation method. However, all the leaf extracts contained many impurities. Considering that FSP purification by these methods was difficult, we attempted to purify FSP from the latex of cv. Masui Dauphine using two-dimensional electrophoresis. Two-dimensional gelatin zymography of the latex showed one spot when the gel was not treated with trichloroacetic acid, suggesting the presence of FSP at this position. Three

spots were excised from the SDS-PAGE gel and subjected to amino acid sequence analysis by trypsin digestion and LC-MS/MS. BLAST searches of the sequences against the Viridiplantae (green plants) genome database of NCBI revealed that some of the obtained sequences were identical to those of subtilisin-like protease of *F. carica*, suggesting that the FSP is a subtilisin-like protease. It showed high amino acid sequence homology with cucumisin, a representative plant-derived subtilisin-like protease, suggesting that the subtilisin-like proteases of *F. carica*, such as cucumisin and other subtilisin-like proteases from plants, are indirectly involved in plant defense responses.

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Kosaku Nishimura

List of publications

Original papers

1. Nishimura, K., Higashiya, K., Ueshima, N., Abe, T. and Yasukawa, K. (2020) Characterization of proteases activities in *Ficus carica* cultivars. *J. Food Sci.*, **85**, 535–544
2. Nishimura, K., Higashiya, K., Ueshima, N., Kojima, K., Takita, T., Abe, T., Takahashi, T. and Yasukawa, K. (2021) Insight into the collagen-degrading activity of a serine protease in the latex of *Ficus carica* cultivar Masui Dauphine. *Biosci. Biotechnol. Biochem.*, **85**, 1147–1156

Related papers

1. Nishimura, K., Shinomura, M., Konishi, A. and Yasukawa, K. (2013) Stabilization of human immunodeficiency virus type 1 reverse transcriptase by site-directed mutagenesis. *Biotechnol. Lett.*, **35**, 2165–2175
2. Nishimura, K., Yokokawa, K., Hisayoshi, T., Fukatsu, K., Kuze, I., Konishi, A., Mikami, B., Kojima, K. and Yasukawa, K. (2015) Preparation and characterization of the RNase H domain of Moloney murine leukemia virus reverse transcriptase. *Protein Expr. Purif.*, **113**, 44–50