イチジク由来タンパク質分解酵素の品種特性

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Characterization of Proteases Activities in *Ficus carica* Cultivars

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In this study, we characterized protease activities of 23 *Ficus carica* cultivars. Extracts of fruit, branch, and leaf of Masui Dauphine, one of the most representative *F. carica* cultivars in Japan, exhibited gelatin-hydrolyzing activity, both in the absence and presence of a cysteine protease-specific inhibitor, E-64, suggesting that not only ficin (classified as cysteine protease) but also collagenase (classified as serine protease) were involved in the digestion of gelatin. In the hydrolysis of (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, all branch extracts of 23 *F. carica* cultivars exhibited the activity both in the absence and presence of cysteine protease-specific inhibitor E-64, indicating that they contain ficin and collagenase. During digestion of acid-solubilized type I collagen by the branch extract of Masui Dauphine at 40-55℃, collagen was completely digested in the absence of E-64, while it was partially digested in the presence of the inhibitor, indicating that the manner of digestion differed between ficin and collagenase contained in the extract. These results suggest that *F. carica* is attractive for industrial use to digest collagen.

Key words: collagen, collagenase, E-64, *Ficus carica*, ficin

Introduction

Some higher plants produce proteases in quantities more than 10% of the total protein. Such proteases include bromelain from *Ananas comosus* and papain from *Carica papaya*, and ficin from *Ficus carica*. Most plant-derived proteases, including bromelain, papain, and ficin, have been classified as cysteine proteases (González-Rábade and others 2011). Because they have broad substrate specificity and pH optima, they are widely used in the food industry, from the preparation of protein hydrolysates to tenderizing meat (Badgujar and others 2014).

F. carica, which is distributed in Asia, belongs to the mulberry family. It produces different isomers of ficin (Zare and others 2013) : in addition, certain *F. carica* cultivars produce collagenase (Raskovic and others 2014). Unlike the collagenase produced by microorganisms, which is classified as metalloprotease, this collagenase is classified as serine protease (Raskovic and others 2014). *F. carica* also contains various phytochemicals, and is therefore used in traditional medicine (Arvaniti and others 2019; Takahashi and others 2014; Takahashi and others 2017). Leaves of *F. carica* have possible medical application in the treatment of diabetes (Serraclara and others 1998), hyperlipidemia (Pérez and others 1999), and cancer (Ghanbari and others 2019). Recently, *F. carica* dried fruit extract, which is an excellent source of fiber and polyphenols, was used to produce iron oxide nanoparticles (Aksu Demirezen and others 2019).

To further expand the use of *F. carica* in the food industry, a better understanding of characteristics of ficin and collagenase in various *F. carica* cultivars is required. This would allow optimal selection of cultivars for industrial use. In this study, we selected 23 *F. carica* cultivars cultivated at the Toyo Institute

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of Food Technology and characterized their ficin- and collagenase-mediated protease activities using synthetic peptides. We also examined their collagen-hydrolyzing activity, focusing on the feasibility of its industrial use for collagen digestion.

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. 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℃ 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 (Teramura and others 2011: Takita and others 2018). The cysteine protease inhibitor E-64 was purchased from Sigma-Aldrich (Saint Louis, MO), and the serine protease inhibitor phenylmethanesulfonyl fluoride (PMSF) was purchased from Wako Pure Chemical Industries (Osaka, Japan). Acid-solubilized collagen (ASC), which was extracted from bovine skin and adjusted to 3 mg/ml, was purchased from Nippi Inc (Tokyo, Japan).

Preparation of F. carica extracts. Fruits, leaves and branches of 23 *F. carica* cultivars were collected at Toyo Institute of Food Technology, Kawanishi in Japan. They were frozen at -80℃ , smashed finely, broken, freeze-dried, and powdered with a food mill. The powdered leaves or branches (4.5 g) were stirred in water (45 ml) overnight at 4℃ followed by gauze filtration. The extract was centrifuged at 10,000 × *g* for 10 min at 4℃ , 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℃ . The solution collected from the dialysis tube was centrifuged at 17,300 × *g* for 10 min at 4℃ , 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 (Nacalai Tesque) as a standard.

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 (0.25 M 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 Coomassie Brilliant Blue R-250. The molecular mass marker kit consisting of myosin (230 kDa), β-galactosidase (140 kDa), phosphorylase-b (95 kDa), bovine serum albumin (70 kDa), ovalbumin (44 kDa), carbonic anhydrase (32 kDa), trypsin inhibitor (27 kDa), and lysozyme (17 kDa) was purchased from Funakoshi Co., Ltd. (Tokyo, Japan).

Hydrolysis of MOCAc-KPLGL(Dpa)-AR by ficin and collagenase. The reaction was carried out as described previously (Takita and others 2018: Hatanaka and others 2005). Briefly, a 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℃. 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 previously (Takita and others 2018). Briefly, 0.54 mM 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℃. The reaction was monitored by the 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 (0.12 M Tris-HCl buffer (pH 6.8), 48% glycerol, 2.4% SDS, 5 % 2-mercaptoethanol, 0.06% 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% Tween 20, 5 mM $CaCl_2$) at 37℃ for 1 h for gelatin hydrolysis. After the reaction, gels were stained with 0.25% Coomassie Brilliant Blue R-250, 50 % methanol, 7% acetic acid. The molecular mass marker kit consisting of rabbit muscle myosin (200 kDa), *E. coli* β-galactosidase (116 kDa), bovine serum albumin (66.2 kDa), chicken egg white ovalbumin (45 kDa), and bovine erythrocyte carbonic anhydrase (31 kDa) was purchased from Nacalai Tesque (Kyoto, Japan).

Digestion of collagen by ficin and F. carica extract. The reaction was initiated by mixing 81 µl of 1.11 mg/ml acid-solubilized collagen (ASC) in 250 mM sodium acetate buffer (pH 5.0) and 9 µl of 1 µM ficin or 0.9 mg/ml Masui Dauphin extract at predetermined temperatures (20–80 ℃) for indicated durations (30–120 min). 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 above.

Characterization of peptide hydrolysis products. 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℃ , followed by LC-MS analysis using LC-20A (Shimadzu, Kyoto, Japan) and micrOTOF-QⅡ (Burker Daltonics, Billerica, MA). Conditions of LC-MS are as follows: column, Poroshell 120 EC-C18 Column (particle size = 2.7 µm; 100 mm × 4.6 mm i.d., Agilent Technologies, Santa Clara, CA) ; column oven temperature, 40°C; 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 (180°C, 7 litre/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.

Results and Discussion

Hydrolytic activities of ficin and collagenase

First we analyzed the ficin and collagenase activities to hydrolyze MOCAc-KPLGL(Dpa)-AR (**Figure 1A**). This peptide has been used for the characterization of collagenase (Takita and others 2018; Hatanaka and others 2005). 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 (classified as cysteine protease) 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 (**Figure 1B**). This indicated that both ficin and collagenase cleaved MOCAc-KPLGL(Dpa)-AR.

We previously reported that the cleavage site of MOCAc-KPLGL(Dpa)-AR by *C. histolyticum* collagenase was Gly-Leu (Takita and others 2018). Here we analyzed the cleavage site by ficin. The peak corresponding to MOCAc-KPLG (629 Da) was obtained (**Figure S1a**), indicating that the peptide bond Gly-Leu was cleaved (**Figure 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 identifed Pyr-Phe-Leu-pNA as a synthetic substrate for characterizing cysteine protease activity (**Figure 1C**). 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 (**Figure 1D**). This result indicated that ficin cleaved Pyr-Phe-Leu-pNA while *C. histolyticum* collagenase did not. When the reaction products were analyzed by LC-MS, the peak corresponding to Pyr-Phe-Leu (389 Da) was obtained (**Figure S1b,c**), indicating that the peptide bond Leu-pNA was cleaved (**Figure 1C**).

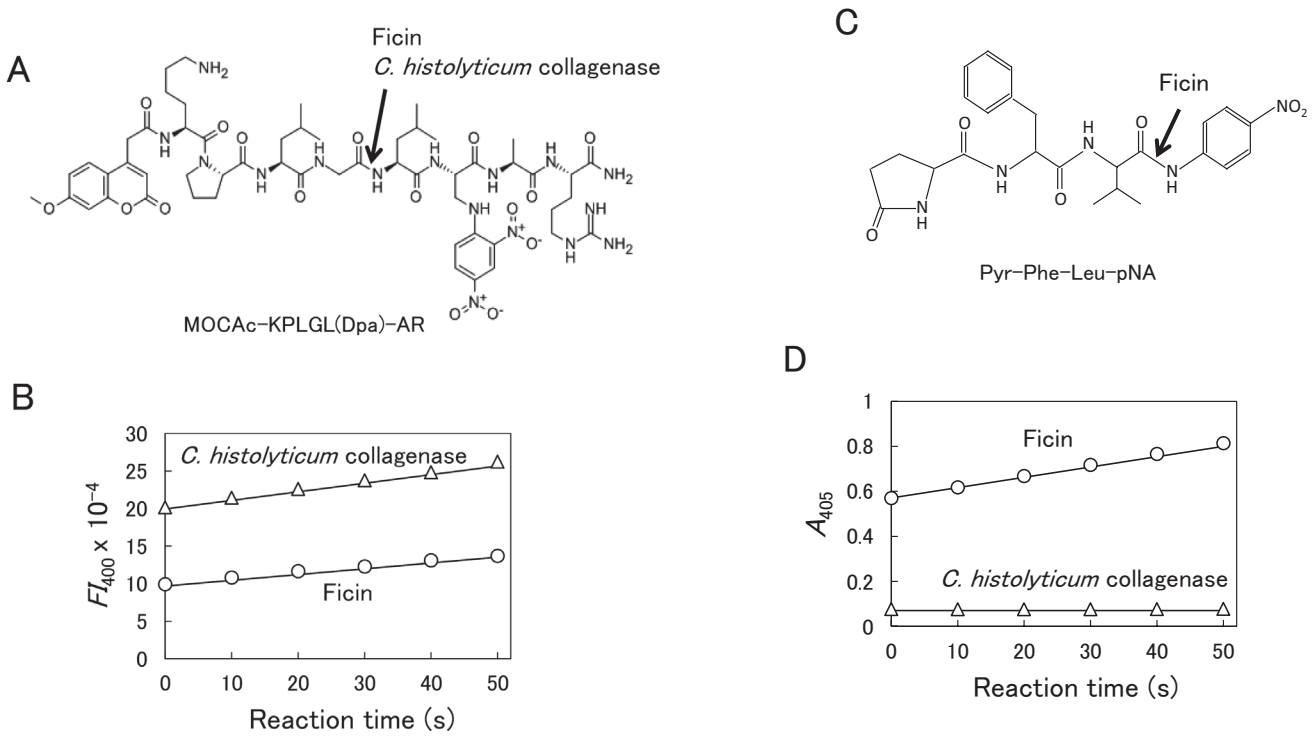

Figure 1 – Hydrolysis of synthetic peptides by ficin and collagenase. (A) Structure of MOCAc-KPLGL(Dpa)-AR. The arrow indicates the cleavage site by ficin and collagenase. (B) Hydrolysis of MOCAc-KPLGL(Dpa)-AR. The reaction was carried out with 1.0 nM ficin or 15 nM *C. histolyticum* collagenase in the presence of 1.4 μM MOCAc-KPLGL(Dpa)-AR at pH 7.5, at 37℃. (C) Structure of Pyr-Phe-Leu-pNA. The arrow indicates the cleavage site by ficin. (D) Hydrolysis of Pyr-Phe-Leu-pNA. The reaction was carried out with 1.0 μM ficin or 10 μM collagenase in the presence of 500 μM Pyr-Phe-Leu-pNA at pH 6.5, at 37℃.

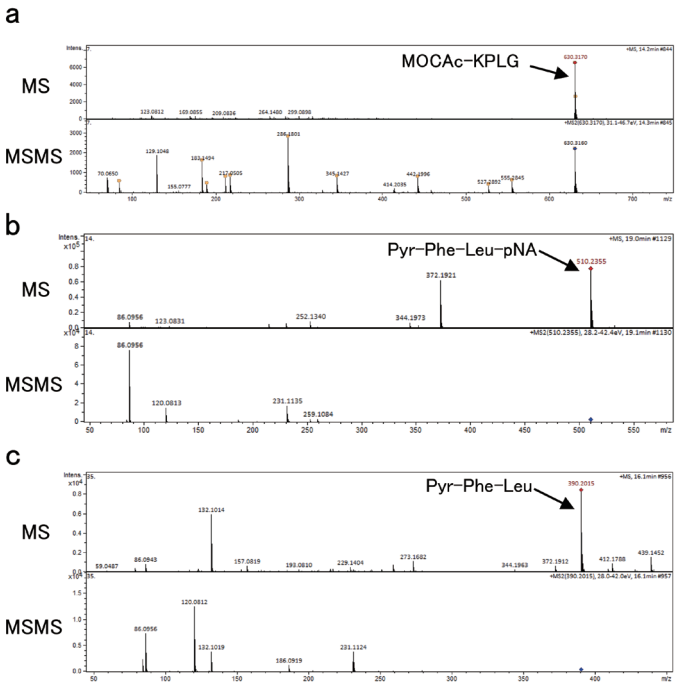

Figure S1. MS and MSMS spectra. The reaction products were analyzed by LC-MS using LC-20A (Shimazu, Kyoto, Japan) and micrOTOF-QII (Burker Daltonics, Billerica, MA). (a) MOCAc-KPLGL(Dpa)-AR (10 μM) was incubated in 2.2 mM HEPES-NaOH buffer (pH 7.5) with 100 nM ficin at 37℃. The peak corresponding to MOCAc-KPLGL(Dpa)-AR was not obtained under this condition. (b, c) Pyr-Phe-Leu-pNA (50 μM) was incubated in 2.2 mM HEPES-NaOH buffer (pH 7.5) with 0 (b) or 100 (c) nM ficin at 37℃.

Gelatin-hydrolyzing activities of the *F. carica* extracts

In order to see whether ficin or collagenase in *F. carica* is involved in the activity, the fruit, branch, and leaf extracts of Masui Dauphine, one of the most representative *F. carica* cultivars in Japan, were subjected to gelatin zymography in the absence and the presence of cysteine protease-specific inhibitor E-64 (**Figure 2**). 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 Dauphin, 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 collagenase (classified as serine protease). The appearance of smearing bands might be due to the possibility that ficin and/or collagenase were not fully denatured in the zymography sample buffer (Jelica and others 2019; Brankica and others 2015), and modified ficin and/or collagenase hydrolyzed gelatin during electrophoresis in the presence of SDS.

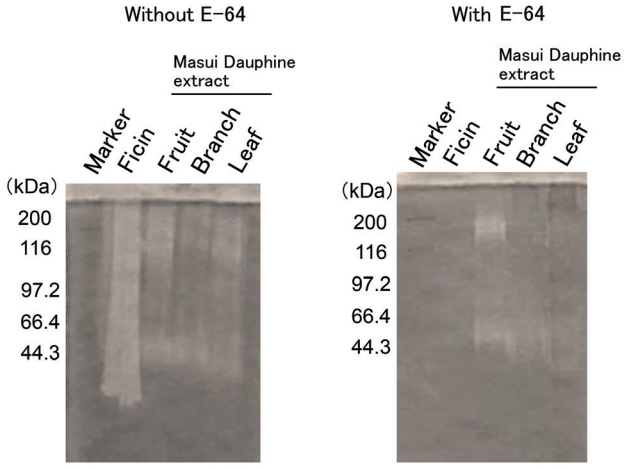

Figure 2 – Gelatin hydrolysis by ficin and the Masui Dauphine extract. Coomassie brilliant blue-stained 10% polyacrylamide gel containing 0.063% gelatin is shown. The concentrations of E-64 were 0 (left) or 280 μM (right) in the zymography sample buffer and 0 (left) or 2.8 μM (right) in the washing buffer and the reaction buffer.

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 (**Figure 2**), we selected branch extract for subsequent analysis because branch is the most easily available. In order to see whether ficin or collagenase 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 Dauphin extract were investigated (**Figure 3**). In the hydrolysis of MOCAc-KPLGL (Dpa) -AR, the relative activities decreased with increasing E-64 concentration and reached 5% at 1 μM, and were essentially constant over the range of 1-20 μM (**Figure 3A**). In the hydrolysis of Pyr-Phe-Leu-pNA, the relative activities reached almost zero at 5 μM E-64 (**Figure 3B**) . These results indicated that both ficin and collagenase 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 Dauphin extract. MOCAc-KPLGL (Dpa)-AR was treated by protease, and the reaction products were subjected to LC-MS (**Figure 3C**). In the reaction with ficin, one peak corresponding to MOCAc-KPLG (629 Da) appeared ((i) in **Figure 3C**). Because the cleavage sites by ficin and *C. histolyticum* collagenase are the same (**Figure 1**), we expected that the reaction with the Masui Dauphin extract would exhibit one peak corresponding to MOCAc-KPLG. However, it exhibited two peaks each corresponding to MOCAc-KPLG and MOCAc-KPL (572 Da), respectively ((ii) in **Figure 3C**). In the presence of E-64, the reaction with the extract exhibited one peak corresponding to MOCAc-KPL ((iii) in **Figure 3C**), while in the presence of E-64 and serine protease inhibitor PMSF, the peak was few ((iv) in **Figure 3C**). These results suggested that MOCAc-KPLG was produced by ficin while MOCAc-KPL was produced by collagenase, and the substrate specificity of the collagenase in the Masui Dauphin extract was different from that of *C. histolyticum* collagenase. Teramura et al. 2011, 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 (Teramura and others 2011). We 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 (Takita and others 2018). 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 **Figure 3C**). These results indicated that both ficin and collagenase were contained in the Masui Dauphine extract, and the former cleaved the peptide bond Gly-Leu while the latter cleaved Leu-Gly.

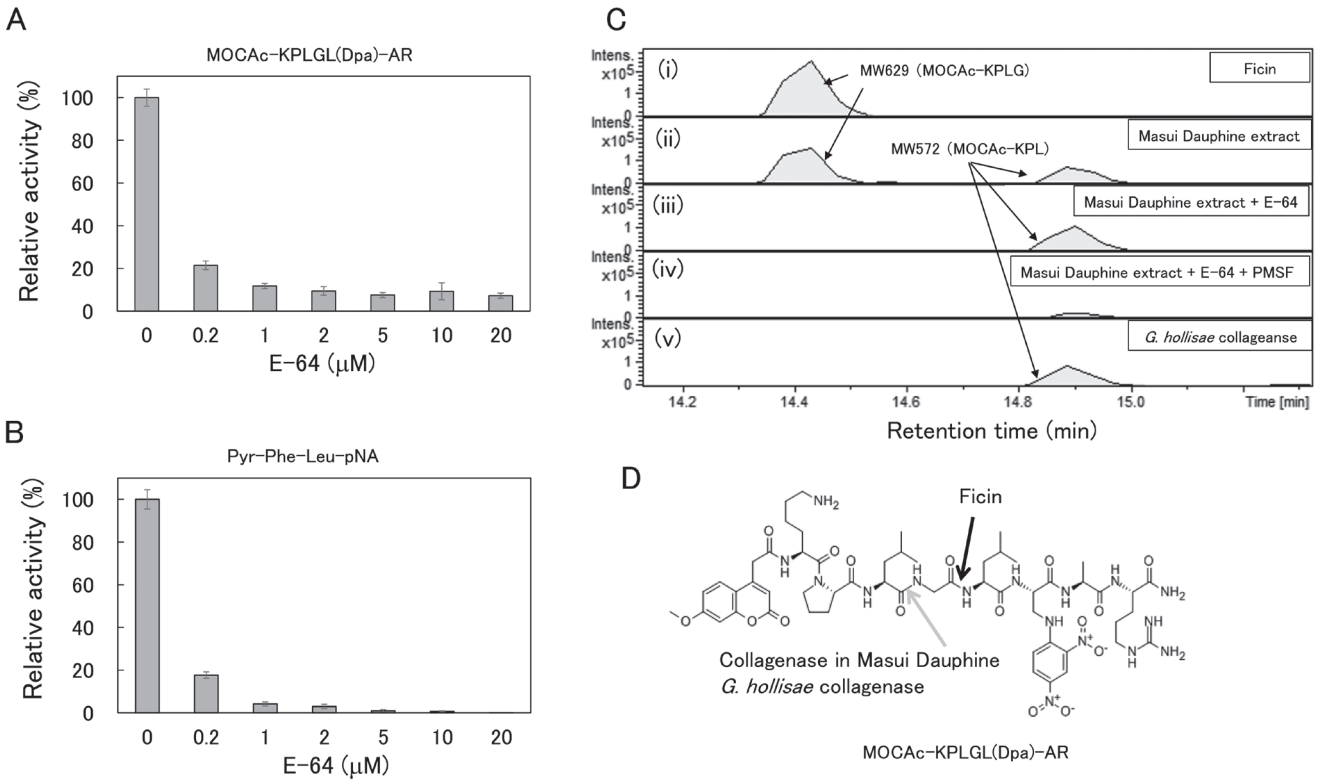

Figure 3 – Hydrolysis of synthetic peptides by the Masui Dauphine extract. (A) Hydrolysis of MOCAc-KPLGL(Dpa)-AR. The reaction was carried out with 15 μg/ml Masui Dauphine extract in the presence of 1.4 μM MOCAc-KPLGL(Dpa)-AR and 0–20 μM E-64 at pH 7.5, at 37℃. (B) Hydrolysis of Pyr-Phe-Leu-pNA. The reaction was carried out with 0.23 mg/ml Masui Dauphine extract in the presence of 375 μM Pyr-Phe-Leu-pNA and 0–20 μM E-64 at pH 7.5, at 37℃. 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 mg/ml Masui Dauphine 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℃, followed by LC-MS analysis. (D) Structure of MOCAc-KPLGL(Dpa)-AR. The black arrow indicates the cleavage sites by ficin, and the gray arrow indicates the cleavage site by collagenase contained in the Masui Dauphine extract and G. hollisae collagenase.

Characterization of enzyme extracts from *F. carica* branches

We prepared the extracts of 23 *F. carica* cultivars 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 (**Figure 4**). 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 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 (**Figure 5A**). The relative activity was defined as the ratio of the reaction rate with each extract to that of the Grise de Tarascon 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 uM E-64 were 2.4–7.8% . This suggested that all extracts contain both ficin and collagenase. **Figure 5B** 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 collagenase activity, and the horizontal line represents the total activities of ficin and collagenase. No correlation was observed for the relative activities at 0 and 20 μM E-64, suggesting that the ratio of the amounts of ficin and collagenase vary depending on species.

Proteases from *F. carica* are known under the general term ficin that belongs to cysteine protease class. However, Raskovic et al. reported that one of *F. carica* cultivars, Brown Turkey, produces collagenolytic

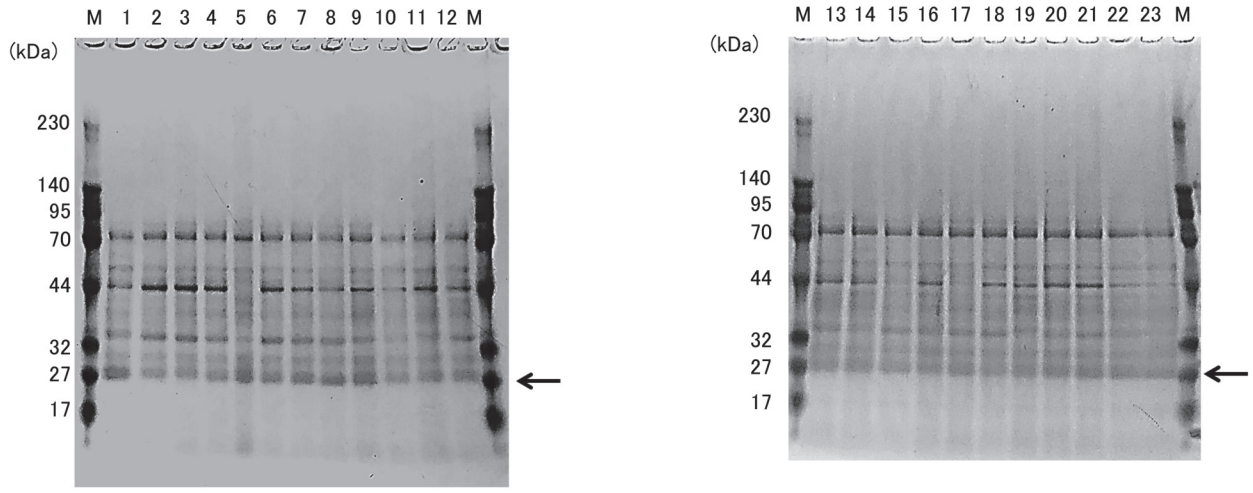

Figure 4 – SDS-PAGE of branch extracts of 23 *F. carica* cultivars under reducing conditions. Six μg protein was applied to each lane. Coomassie Brilliant Blue-stained gradient (5 to 20%) polyacrlamide gel is shown. Lane M, molecular-mass marker; and lanes 1-23, branch extracts of 23 *F. carica* cultivars. Numbers correspond to those of Fig. 5A and C. The arrow indicates the band corresponding to ficin.

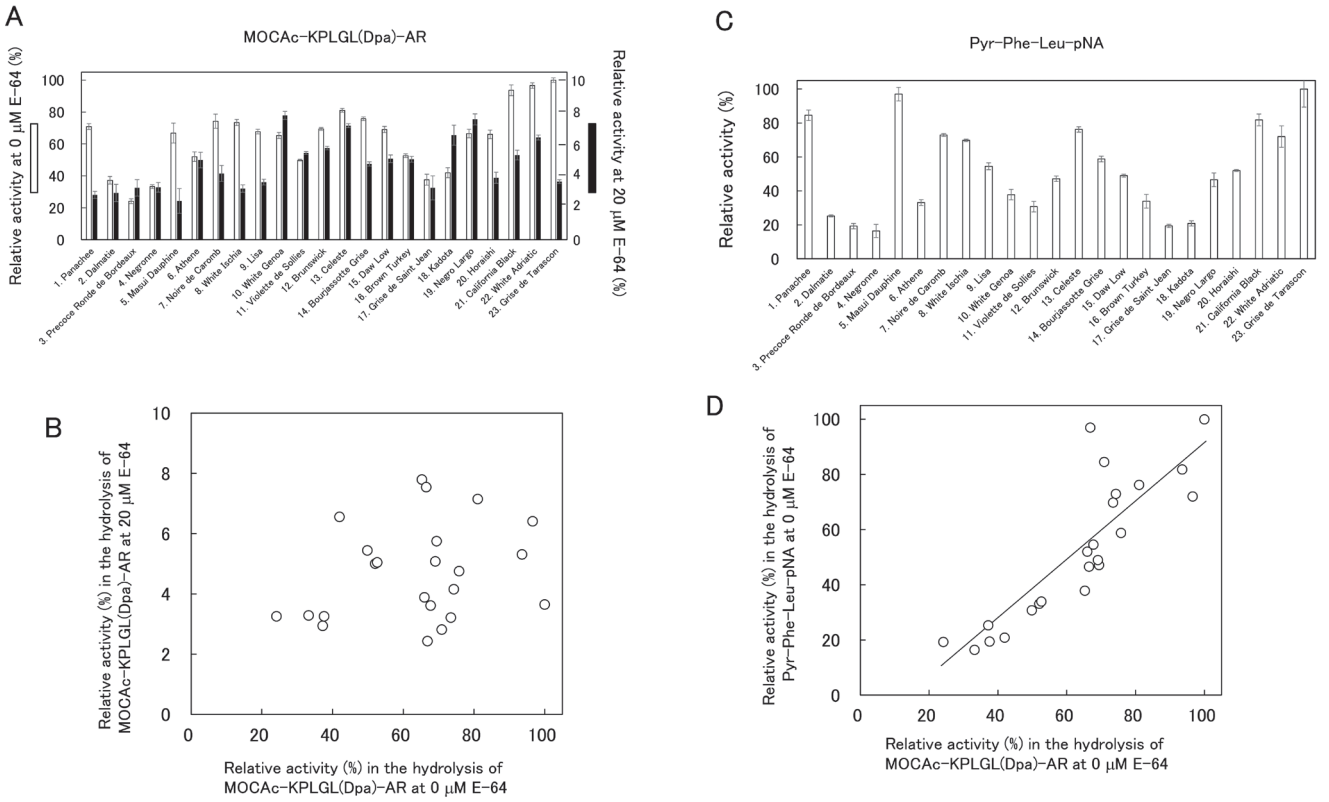

Figure 5 – Activities of branch extracts of 23 *F. carica* cultivars. (A) Hydrolysis of MOCAc-KPLGL(Dpa)-AR. The reaction was carried out with 1.4 μM MOCAc-KPLGL(Dpa)-AR in the presence of 3 μg/ml extracts and 0 μM E-64 (white bar) or 15 μg/ml extracts and 20 μM E-64 (filled bar) at pH 7.5, at 37℃. Relative activity is the activity compared to that of Grise de Tarascon extract. 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 0.23 mg/ml extracts with 375 μM Pyr-Phe-Leu-pNA at pH 7.5, at 37℃. Relative activity is the activity compared to that of Grise de Tarascon extract. (D) Comparison of the Pyr-Phe-Leu-pNA-hydrolytic activity at 0)M E-64 with the MOCAc-KPLGL(Dpa)-AR-hydrolytic activity at 0 μM E-64. The lines are drawn by the linear least-squares-regression. The regression coefficient, *r*, is 0.86.

serine protease (Raskovic and others 2014). Our results indicated that all 23 *F. carica* cultivars produce collagenolytic serine protease.

Next we examined the Pyr-Phe-Leu-pNA-hydrolyzing activities of the extracts of 23 *F. carica* cultivars (**Figure 5C**). The relative activity was defined as the ratio of the reaction rate with each extract to that with the Grise de Tarascon extract. The relative activities ranged 16.4–100 %, indicating that all extracts exhibited the activity with the Grise de Tarascon extract being highest. **Figure 5D** 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 collagenase does not have the Pyr-Phe-Leu-pNA-hydrolytic activity while the horizontal line represent the total activities of ficin and collagenase. 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 (γ = 0.86), suggesting that in all 23 extracts, the MOCAc-KPLGL(Dpa)-AR hydrolyzing activity derived mainly from ficin rather than collagenase.

Digestion of collagen by the *F. carica* extract

We characterized the digestion of acid-solubilized type I collagen (ASC) by the Masui Dauphine extract. **Figure 6A** shows the effect of reaction temperature on ASC. Untreated 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 (Sato and others 2000; Qian and others 2016). When ASC was incubated in the absence of protease for 2 h at 20, 37, and 55℃, all bands exhibited the same pattern to the untreated one. In the case of 80℃, all bands were of weaker intensity and, suggesting that some denatured ASC formed the aggregates at 80℃, which did not enter the gel.

Figure 6B show the effects of reaction temperature and E-64 on the digestion of ASC by ficin. When ASC was incubated with ficin and without E-64 at 20℃, all bands exhibited the same pattern to the untreated one. When ASC was incubated at 37℃, 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, 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 ASC at the N- and/or C-terminal telopeptide, but not at the helical region, at 37℃. When ASC was incubated at 55 or 80℃, all bands disappeared. This suggested that ficin completely degraded ASC at 55 ℃ and was involved in the degradation at 80℃. When 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 6C shows the effects of reaction temperature on the digestion of ASC by the Masui Dauphine extract. When ASC was incubated with the extract and without E-64 at 20, 37, 55, or 80℃, 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 ASC at 55 and 80 ℃. When 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℃. But they were different at 55℃: 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 collagenase contained in the extract partially cleaved ASC at the N- and/or C-terminal telopeptides and at the helical region at 55℃.

Figure 6D shows the effects of E-64 on the ficin- or the Masui Dauphine extract-mediated ASC digestion. Without E-64, all bands disappeared. With E-64, all bands were of stronger 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 the digestion with the Masui Dauphine extract, the γ, variant β11, β11, and β12 bands, but not the α1 and α2 bands, were of weaker intensity than those in the digestion with ficin. In addition, several bands appeared at 70–95 kDa, which were not observed in the digestion by ficin. These results suggested that the collagenase contained in the extract is involved in the difference between the digestion by the ficin preparation and that by the extract.

Figure 6E shows the effects of reaction temperature on the Masui Dauphine extract-mediated ASC digestion. Without E-64, at 37℃, all bands exhibited the same pattern to the untreated one, and at 40-55℃, all bands disappeared. With E-64, at 37℃, all bands exhibited the same pattern to the untreated one, and at 40-55℃, the γ, variant β11, β11, and β12 bands were of weaker intensity, and several bands appeared at 70–95 kDa. This indicated that the modes of digestion by ficin and

collagenase contained in the extract are different. This observation is in agreement with the previous reports that the melting temperature of bovine type I collagen is 40℃ (Komsa-Penkova and others 1996; Komsa-Penkova and others 1999; Duan and others 2009).

Commercial collagens are widely used in food, cosmetic, and medicine industries. Various food-derived collagen peptides have been found in blood (Iwai and others 2005). Interestingly, they show bioactive effects, including tissue repair, at damaged sites (Shibuya and others 2014; Kusubata and others 2015). It is widely accepted today that collagen and its digestion products could be useful as functional food. Our results suggest that *F. carica* is attractive for use in not only complete but also partial digestion of collagenase.

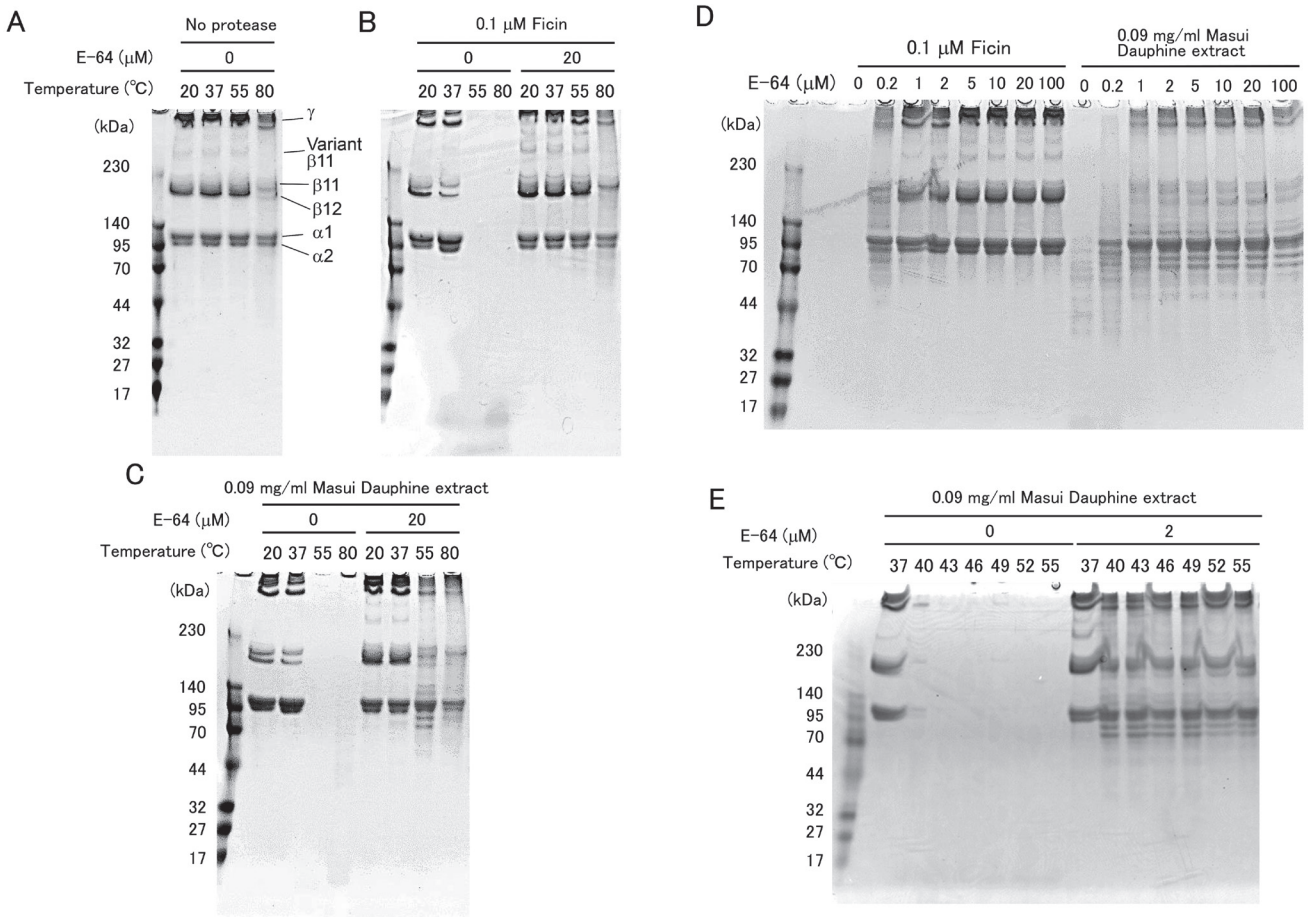

Figure 6 – Digestion of acid soluble collagen. Coomassie Brilliant Blue-stained gradient (5 to 20%) polyacrlamide gel is shown. (A–C) The reaction was carried out without protease (A), with 0.1 μM ficin (B), or with 0.09 mg/ml Masui Dauphine extracts (C), in the presence of 1.0 mg/ml acid soluble collagen (ASC) and 0 or 20 μM E-64, at pH 5.0, at 20–80℃ for 2 h. (D) The reaction was carried out with 0.1 μM ficin or 0.09 mg/ml Masui Dauphine extract in the presence of 1.0 mg/ml ASC and 0–100 μM E-64 at pH 5.0, at 55℃ for 2 h. (E) The reaction was carried out with 0.09 mg/ml Masui Dauphine extract, 1.0 mg/ml ASC, and 0 or 2 μM E-64 at pH 5.0, at 37–55℃ for 30 min.

Conclusion

We showed that both ficin (a cysteine protease) and collagenolytic serine protease are present in all 23 *F. carica* cultivars examined. We also showed that the former completely digests collagen while the latter partially digests it under certain reaction conditions. The industrial use of *F. carica* might be wider by utilizing efficiently these proteases.

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