# ‘桝井ドーフィン’のイチジク樹液由来セリンプロテアーゼのコラーゲン分解活性に関する考察

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# Insight into the collagen-degrading activity of a serine protease from *Ficus carica* cultivar Masui Dauphine

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*Abbreviations*: ASC, acid-solubilized collagen; BSA, bovine serum albumin; DTT, dithiothreitol; 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-$NH_2$.

*Ficus carica* produces, in addition to the cysteine protease ficin, a serine protease. Earlier study on a serine protease from *F. carica* cultivar Brown Turkey showed that it specifically degraded collagen. In this study, we characterized the substrate specificity of a serine protease from *F. carica* cultivar Masui Dauphine. The serine protease degraded denatured, but not undenatured, acid-solubilized type I collagen. It also degraded bovine serum albumin, while the collagenase from *Clostridium histolyticum*, which is widely used in industry, did not. These results indicated that the serine protease is not collagen-specific. The protease was purified to homogeneity by two-dimensional gel electrophoresis, and its partial amino acid sequence was determined by liquid chromatography-MS/MS. BLAST searches against the Viridiplantae (green plants) genome database revealed that the serine protease was a subtilisin-like protease. Our results contrast with the results of the earlier study stating that the serine protease from *F. carica* is collagen-specific.

**Keywords:** collagen, collagenase, *Ficus carica*, serine protease

## Introduction

Fig (*Ficus carica*) produces a cysteine protease called ficin (EC 3.4.22.3). Ficin has broad substrate specificity and is active across a wide pH range, and it is therefore widely used in the food industry, for purposes from the preparation of protein hydrolysates to tenderizing meat (Badgujar *et al*. 2014). In addition to ficin, *F. carica* contains many other enzymes. Kitajima *et al*. conducted a proteomic analysis of *F. carica* latex and found ficin, subtilase, peroxidase, chitinase, and mandelonitrile lyase (Kitajima *et al*. 2018). Raskovic *et al*. found a serine protease with collagenolytic activity in the latex extract of *F. carica* cultivar (cv.) Brown Turkey (Raskovic *et al*. 2014). Additionally, Hamed *et al*. reported that a serine protease in the latex extract of *F. carica* (unknown cultivar) demonstrated fibrinolytic activity (Hamed *et al*. 2020). We previously prepared extracts from branches of 23 *F. carica* cultivars and found that both ficin and serine protease were present in all the extracts (Nishimura *et al*. 2020). We also found that during the hydrolysis of a synthetic peptide substrate of collagenase, (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-NH2 (MOCAc-KPLGL(DPA)-AR), ficin cleaved the Gly–Leu peptide bond, while serine protease cleaved Leu–Gly bonds, suggesting that substrate specificity was different between the two proteases (Nishimura *et al*. 2020). In addition, it is generally reported that serine proteases are less likely to lose activity in oxidizing conditions than cysteine proteases (Li *et al*. 2018), and the serine protease in *F. carica* may be more resistant to oxidation than ficin. These results suggest that the serine

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protease from *F. carica* may be an attractive enzyme for industrial use.

Proteases that cleave the triple-helical structure of undenatured collagen are generally defined as collagenases, while proteases that degrade denatured collagen, such as gelatin, are often defined as collagenolytic proteases (Zhang *et al*. 2015). The serine protease in the latex extract of *F. carica* cv. Brown Turkey may be classified as a collagenase because it degraded native collagen (Raskovic *et al*. 2014). However, additional work is needed to confirm this.

In this study, we evaluated collagen- and gelatin-degrading activities of a serine protease from the latex extract of *F. carica* cv. Masui Dauphine. The results showed that the serine protease degraded denatured collagen, but not undenatured collagen with the stiff triple-helical structure, indicating that this serine protease is not a collagenase. Furthermore, in contrast to a previous report (Raskovic *et al*. 2014), the activity of this serine protease was not specific to collagen. Amino acid sequence analysis identified the serine protease as a subtilisin-like protease. It was previously reported that many subtilisin-like proteases from plants have broad substrate specificity (Rautengarten *et al*. 2005); consistent with this, the serine protease from *F. carica* acts on several substrates.

## Materials and methods

*Materials*. Ficin from *F. carica* tree latex was purchased from Tokyo Chemical Industry (Tokyo, Japan), with titer 400–1,000 milk clotting units (MCU)/mg where one MCU is defined as the amount that coagulates 25 mL milk at pH 6.0 at 40 ℃ , according to information from the manufacturer. Collagenase from *Clostridium histolyticum* (Chcol) (Lot. M6M0414) was purchased from Nacalai Tesque (Kyoto, Japan). Collagenase from *Grimontia hollisae* (Ghcol) was prepared as described previously (Teramura et al. 2011; Takita et al. 2018; Hayashi et al. 2020). The cysteine protease inhibitor E-64 and the serine protease inhibitor Pefabloc SC (AEBSF, 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride) were purchased from Sigma-Aldrich (St. Louis, MO). Bovine skin acid-solubilized collagen (bovine ASC) was purchased from Nippi Inc. (Tokyo, Japan). Rat tail ASC (rat ASC) was purchased from Sigma-Aldrich. Polyvinylidene fluoride (PVDF) membranes were purchased from Sigma-Aldrich. Bovine serum albumin (BSA) was purchased from Fujifilm Wako Pure Chemical (Osaka, Japan). Protein concentration was determined using Protein Assay CBB Solution (Nacalai Tesque) with BSA as a standard.

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

*Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)*. SDS-PAGE was performed in gradient (5% to 20%) polyacrylamide gels under reducing conditions. Samples were mixed with four volumes of SDS-PAGE sample buffer [0.25 M Tris-HCl (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 to the gel. A constant current of 25 mA was applied for 60 min. After electrophoresis, proteins were stained with Coomassie Brilliant Blue R-250 or silver.

*Digestion of ASC*. 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 ℃ ) 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.

*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 ℃ 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.

*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% (v/v) Triton X-100, 1% (w/v) dithiothreitol (DTT)] was applied to agarGel. After IEF, the gels were expelled from the tubes and equilibrated in 2.5% (w/v) trichloroacetic acid for 3 min, followed by immersion in water. 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 water for 3 min, followed by immersion in water. 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, gels were soaked first in 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, then in reaction buffer [50 mM Tris-HCl buffer (pH 8.0), 0.1% Tween 20, 5 mM $CaCl_2$] at 37℃ for 1h for gelatin hydrolysis. After the reaction, gels were stained with 0.25% Coomassie Brilliant Blue R-250, 50% methanol, and 7% acetic acid.

*N-Terminal cleavage analysis*. Samples were analyzed by SDS-PAGE as described above. Proteins were transferred to PVDF membranes by wet blotting using a Safety Blotting Cell STB-8 (TEFCO, Tokyo, Japan) as described by the manufacturer. Bands were visualized by staining with Coomassie Brilliant Blue R-250, and degradation products were excised and sequenced by Edman degradation (Aguda et al. 2014).

*Liquid chromatography-MS/MS (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 (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) (Meier et al. 2015; Meier et al. 2018). 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.

*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 respectively frozen at −80℃, 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℃ overnight. In Method (ii), the fruit, leaves, and branches (each 45 g) were respectively frozen at −80℃ and broken 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℃ overnight. The suspensions obtained in Methods (i) and (ii) were filtered with gauze. The filtrates were centrifuged at 15,000 × *g* at 4℃ for 10 min, and the supernatants were collected. Proteins in the extracts thus prepared were precipitated with ammonium sulfate or acetone. For 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℃ overnight. For 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℃ for 2 h. These solutions were centrifuged at 17,300 × *g* at 4℃ 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℃.

*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), 0.098 mM 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℃. 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 (PerkinElmer, Waltham, MA) for 3 min.

## Results and discussion

*Collagen-degrading activities of the serine protease from* F. carica c*v. Masui Dauphine*

We previously examined the bovine ASC-degrading activities of the ficin and serine protease contained in the branch extract of *F. carica* cv. Masui Dauphine and

reported the following (Nishimura *et al*. 2020): (i) In the absence of cysteine protease inhibitor E-64 where both ficin and the serine protease were active, bovine ASC was completely degraded by incubation with the extract at 40–55℃ for 30 min; (ii) in the presence of E-64 where ficin was inactive and the serine protease was active, bovine ASC was not completely degraded, indicating that the bovine ASC-degrading activity of the serine protease was considerably lower than that of ficin.

In this study, we evaluated the collagen-degrading activity of the serine protease in the latex extract from *F. carica* cv. Masui Dauphine. Bovine and rat ASC were used as substrates to determine the effect of collagen denaturation on degradation. We also used Chcol, a representative collagenase, for comparison.

On SDS-PAGE under reducing conditions, the ficin preparation and the latex extract of Masui Dauphine showed 26- and 28-kDa bands, respectively (**Fig. 1A**), each corresponding to an isoform of ficin, as previously reported (Zare *et al*. 2013; Volunt *et al*. 2015). The latex extract showed a weak 41-kDa band, which corresponded to the serine protease from Brown Turkey, as previously reported (Raskovic *et al*. 2014). Chcol was visualized as a single 66-kDa band.

**Fig. 1B** shows the digestion of bovine ASC. Untreated bovine ASC yielded six protein bands corresponding to γ, variant β 11, β 11, β 12, α 1, and α 2 chains, in order from the largest to the smallest (Sato *et al*. 2009; Qian *et al*. 2016). The temperature was set between 32–42℃ for ASC digestion, which is close to the complete denaturation temperature ($T_d$) of type I collagen [40℃ for bovine type I collagen (Komsa *et al*. 1996; Komsa *et al*. 1999; Duan *et al*. 2009) and 36℃ for rat type I collagen (Brown *et al*. 2000; Leikina *et al*. 2002)]. 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 serine protease were active, bovine ASC incubated with the extract at 32℃ or 37℃ for 24h was partially degraded. However, bovine ASC incubated at 42 ℃ 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 serine protease was active, bovine ASC incubated with the extract at 32℃ or 37℃ was hardly degraded at all, while it was partially degraded at 42℃. 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℃ ) (Qian *et al*. 2016). In the presence of E-64

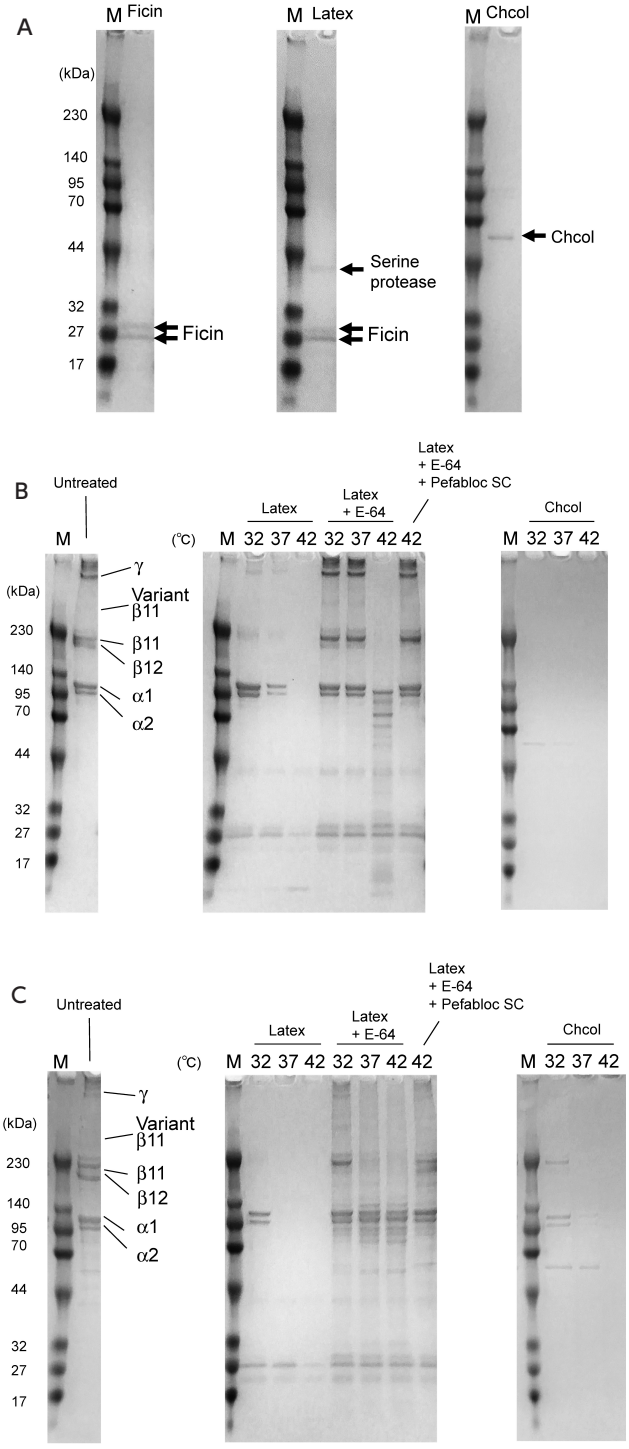


**Fig. 1.** Digestion of bovine and rat acid-solubilized collagen (ASC).

A Coomassie Brilliant Blue-stained gradient (5% to 20%) polyacrylamide gel is shown. (A) A commercial ficin preparation, the *Ficus carica* cv. Masui Dauphine latex extract we prepared, and a commercial *Clostridium histolyticum* collagenase (Chcol) preparation; 180 ng of each protein were applied to the gel. (B, C) The reaction was carried out with 0.33 mg/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 ℃ for 24 h. Lane M, molecular-mass markers.

and Pefabloc SC where both ficin and the serine protease were inactive, bovine ASC incubated with the extract at 42℃ was hardly degraded. Bovine ASC incubated with Chcol at 32–42℃ was completely degraded, indicating that Chcol cleaved the stiff triple-helical structure of collagen.

**Fig. 1C** 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℃ for 24h was degraded completely, differing from the case with bovine ASC (**Fig. 1B**). This might be because the $T_d$ of rat ASC (36℃ ) is lower than that of bovine ASC (40℃ ). In the presence of E-64 and in the absence of Pefabloc SC, rat ASC incubated at 32 ℃ was hardly degraded at all; on incubation at 37 or 42 ℃ , it was partially degraded. This result was consistent with a previous study reporting that the serine protease in the latex extract of *F. carica* cv. Brown Turkey degraded rat ASC at 37 ℃ (Raskovic *et al*. 2014). In the presence of E-64 and Pefabloc SC, rat ASC incubated at 42 ℃ was hardly degraded. Rat ASC incubated with Chcol at 32–42 ℃ for 24 h was almost completely degraded. In summary, the *F. carica* cv. Masui Dauphine serine protease degraded heat-denatured ASC, but not ASC with the stiff triple-helical structure.

*Cleavage sites of bovine ASC by the serine protease from* F. carica *cv. Masui Dauphine*

Bovine ASC was incubated with the latex extract of Masui Dauphine plus E-64, ficin, Chcol, or Ghcol at 42℃ for 24h. **Figure 2** 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 S1** and **S2** show the amino acid sequences of the α 1 and α 2 chains, respectively, of bovine type I collagen in which the identified sequences are marked in bold and underlined. These results indicate that the serine protease 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 our previous report that in the hydrolysis of MOCAc-KPLGL(DPA)-AR, MOCAc-KPLG was produced by ficin while MOCAc-KPL was produced by the serine protease (Nishimura *et al*. 2020).

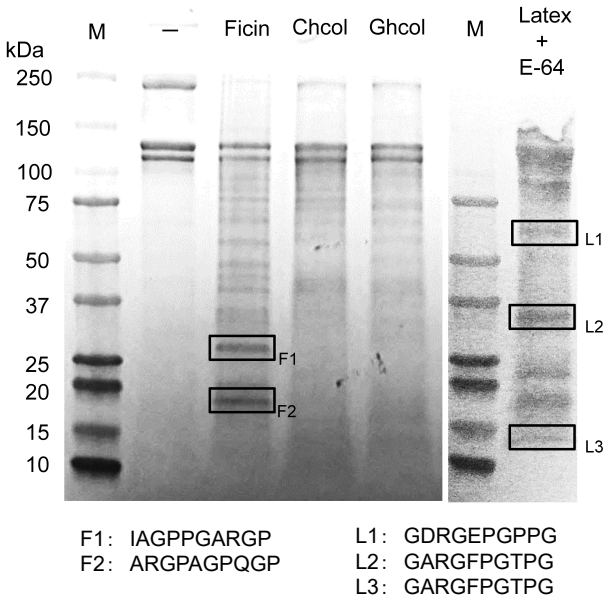


**Fig. 2.** Analysis of cleavage sites of bovine ASC.

A Coomassie Brilliant Blue-stained gradient (5% to 20%) polyacrylamide gel is shown. 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℃ for 24 h. Lane M, molecular-mass markers.

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   1 MLSFVDTRTLLLLAVTSCLATCQSLQEATARKGPSGDRGPRGERGPPGPPGRDGDDGIPG
  61 PPGPPGPPGPPGLGGNFAAQFDAKGGGPGPMGLMGPRGPPGASGAPGPQGFQGPPGEPGE
                                                      L2, L3
 121 PGQTGPAGARGPPGPPGKAGEDGHPGKPGRPGERGVVGPQ**GARGFPGTPG**LPGFKGIRGH
 181 NGLDGLKGQPGAPGVKGEPGAPGENGTPGQTGARGLPGERGRVGAPGPAGARGSDGSVGP
 241 VGPAGPIGSAGPPGFPGAPGPKGELGPVGNPGPAGPAGPRGEVGLPGLSGPVGPPGNPGA
 301 NGLPGAKGAAGLPGVAGAPGLPGPRGIPGPVGAAGATGARGLVGEPGPAGSKGESGNKGE
 361 PGAVGQPGPPGPSGEEGKRGSTGEIGPAGPPGPPGLRGNPGSRGLPGADGRAGVMGPAGS
 421 RGATGPAGVRGPNGDSGRPGEPGLMGPRGFPGSPGNIGPAGKEGPVGLPGIDGRPGPIGP
 481 AGARGEPGNIGFPGPKGPSGDPGKAGEKGHAGLAGARGAPGPDGNNGAQGPPGLQGVQGG
 541 KGEQGPAGPPGFQGLPGPAGTAGEAGKPGERGIPGEFGLPGPAGARGERGPPGESGAAGP
 601 TGPIGSRGPSGPPGPDGNKGEPGVVGAPGTAGPSGPSGLPGERGAAGIPGGKGEKGETGL
 661 RGDIGSPGRDGARGAPGAIGAPGPAGANGDRGEAGPAGPAGPAGPRGSPGERGEVGPAGP
 721 NGFAGPAGAAGQPGAKGERGTKGPKGENGPVGPTGPVGAAGPSGPNGPPGPAGSRGDGGP
 781 PGATGFPGAAGRTGPPGPSGISGPPGPPGPAGKEGLRGPRGDQGPVGRSGETGASGPPGF
                                                              F1
 841 VGEKGPSGEPGTAGPPGTPGPQGLLGAPGFLGLPGSRGERGLPGVAGSVGEPGPLG**IAGP**
 901 **PGARGP**PGNVGNPGVNGAPGEAGRDGNPGNDGPPGRDGQPGHKGERGYPGNAGPVGAAGA
 961 PGPQGPVGPVGKHGNRGEPGPAGAVGPAGAVGPRGPSGPQGIRGDKGEPGDKGPRGLPGL
1021 KGHNGLQGLPGLAGHHGDQGAPGAVGPAGPRGPAGPSGPAGKDGRIGQPGAVGPAGIRGS
1081 QGSQGPAGPPGPPGPPGPPGPSGGGYEFGFDGDFYRADQPRSPTSLRPKDYEVDATLKSL
1141 NNQIETLLTPEGSRKNPARTCRDLRLSHPEWSSGYYWIDPNQGCTMDAIKVYCDFSTGET
1201 CIRAQPEDIPVKNWYRNSKAKKHVWVGETINGGTQFEYNVEGVTTKEMATQLAFMRLLAN
1261 HASQNITYHCKNSIAYMDEETGNLKKAVILQGSNDVELVAEGNSRFTYTVLVDGCSKKTN
1321 EWQKTIIEYKTNKPSRLPILDIAPLDIGGADQEIRLNIGPVCFK
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**Fig.S1.** Amino acid sequence of α 1 chain of bovine type I collagen (Accession No.P02453).

Sequences identified by the N-terminal amino acid analysis are marked in bold.

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  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
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**Fig.S2.** Amino acid sequence of α 2 chain of bovine type I collagen (Accession No.P02465).

Sequences identified by the N-terminal amino acid analysis are marked in bold.

*Digestion of BSA by the serine protease from Masui Dauphine*

Results so far indicated that the serine protease in *F. carica* cv. Masui Dauphine latex extracts has different characteristics from Chcol. To examine whether the serine protease 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℃ 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. At pH 8.0, 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 serine protease degraded BSA more than Chcol did. The difference between the results at pHs 5.5 and 8.0 might be due to the pH-dependence of the activity of the serine protease. The results in **Figs. 1B** and **1C** also suggested that the activity of the serine protease was not specific to collagen.

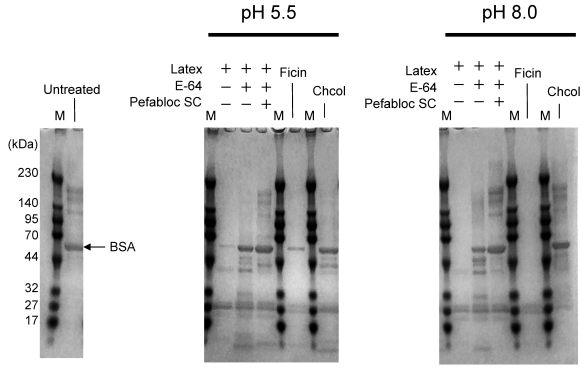


**Fig. 3.** Digestion of bovine serum albumin (BSA).

A Coomassie Brilliant Blue-stained gradient (5% to 20%) polyacrylamide gel is shown. The reaction was carried out with 0.33 mg/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℃ for 24 h. Lane M, molecular-mass markers.

*Amino acid sequence analysis of the serine protease from* F. carica *cv. Masui Dauphine*

We previously reported that in gelatin zymography in the presence of E-64, a branch extract of Masui Dauphine showed a band corresponding to 45–66 kDa (Nishimura *et al*. 2020). This result showed that the gelatin was degraded by the serine protease in the extract. To purify the serine protease, we performed two-dimensional gel electrophoresis of the latex extract of Masui Dauphine in the presence of E-64 (**Fig. 4**). 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. 4A**). However, no band appeared in the gelatin zymography (**Fig. 4B**), possibly because the serine protease was inactivated by trichloroacetic acid (Saitoh *et al*. 2007). When the gel was not treated with trichloroacetic acid after IEF, one band (named S-3) corresponding to 45–66 kDa was observed (**Fig. 4C**). The band appeared at the same position in gelatin zymography (**Fig. 4D**).

S-1, S-2, and S-3 were excised from the gel and subjected to sequence analysis by trypsin digestion and LC-MS/MS. Five sequences were obtained from S-1 (**Table 1**). The same four sequences were obtained from 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.

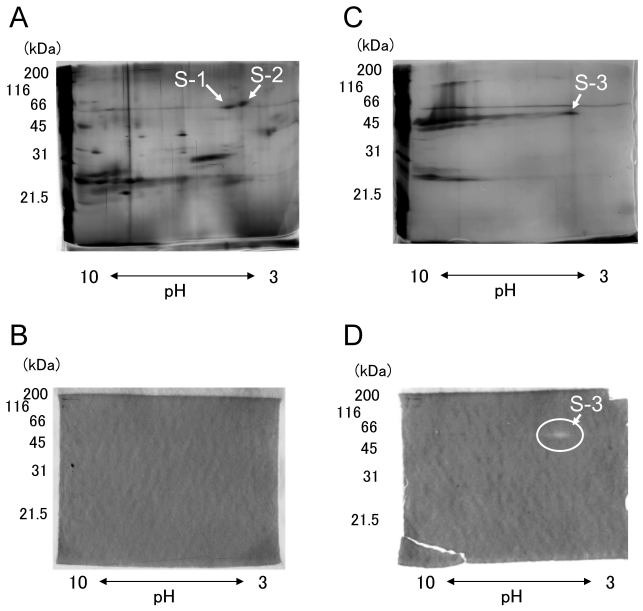


**Fig. 4.** Two-dimensional gel electrophoresis analysis of the latex extract of *F. carica* cv. Masui Dauphine.

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 gradient (5% to 20%) polyacrylamide gel. (A, C) Silver staining of the gel. (B, D) Gelatin zymography.

BBD74161), and S-2 and S-3 were derived from a subtilisin-like protease of *F. carica* (Accession No. BBD74156). This strongly suggested that the serine protease contained in the latex extract of Masui Dauphine was a subtilisin-like protease. **Figure 5** 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 (Gallagher *et al*. 1995).

It was reported that cucumisin from melon, a well-known plant-derived subtilisin-like protease, has broad substrate specificity (Uchikoba *et al*. 1995; Yonezawa *et al*. 2000). The subtilisin-like protease of *F. carica* showed high amino acid sequence homology (94%) to cucumisin (Yamagata *et al*. 1994). Another subtilisin-like protease, myroicolsin, from the deep-sea microbe *Myroides profundi*, cleaves the stiff triple-helical structure of collagen (Ran *et al*. 2014). The subtilisin-like protease of *F. carica* showed low amino acid sequence homology (23%) to myroicolsin. This evidence supports our finding that the subtilisin-like protease of *F. carica* cv. Masui Dauphine showed nonspecific protease activity and did not cleave the stiff triple-helical structure of collagen.

Our results indicated that following about the serine protease of Masui Dauphine: 1) It cleaved denatured, but not undenatured, ASC; 2) it cleaved at the N-terminal side of Gly residues in collagen; 3) its protease activity was not specific to collagen; and 4) it was a subtilisin-like protease. In addition, we previously reported that the branch extract of 23 *F. carica* cultivars including Masui Dauphine and Brown Turkey exhibited both ficin and serine protease activities (Nishimura *et al*. 2020). We presume that the activity of serine proteases in *F. carica* cultivars including Brown Turkey might not be specific to collagen. Further research is required to clarify this.

*Comparison of the serine protease activities of branch, leaf, and fruit extracts of Masui Dauphine*

The *F. carica* cv. Masui Dauphine latex contains the serine protease abundantly. However, the yield of the latex is limited compared with that of branches, leaves, and fruit. Therefore, we compared the serine protease activities of branch, leaf, and fruit extracts of Masui Dauphine. We also compared two preparation methods for 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 broken 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 2**). 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). This indicated that leaf and fruit were more suitable than branch as sources of the serine protease. Considering that the fruit has high value as food, leaf is the most appropriate material for industrial use. There was no difference in recovery of total enzyme between the two preparation methods, nor was there a difference between precipitation with ammonium sulfate and acetone.

**Table 1.** Identification of proteins purified from the latex extract of Masui Dauphine.

| Spot number | Protein name | Accession No. | Number of matched peptides | Protein score | Matched peptide sequence | Peptide score |
|---|---|---|---|---|---|---|
| S-1 | (R)-mandelonitrilelyase2-like [*Ficus carica*] | BBD74161 | 21 | 402 | EFPADVVLGNSR | 60 |
| | | | | | EFPADVVLGNSR | 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 |
| S-2 | subtilisin-like protease 2, partial [*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 |
| S-3 | subtilisin-like protease 2, partial [*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 |

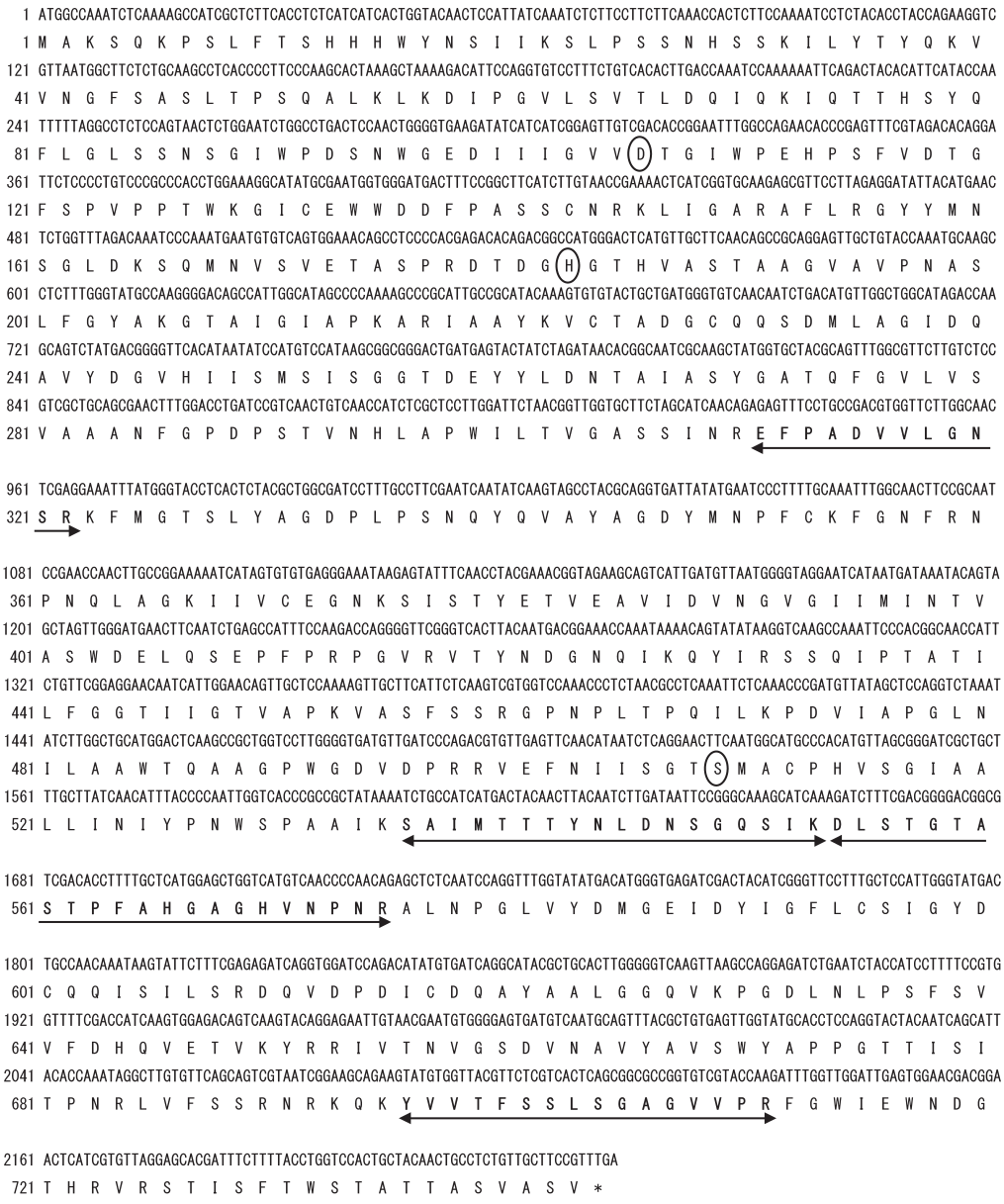


**Fig. 5.** Amino acid sequence of subtilisin-like serine protease from *F. carica* cv. Masui Dauphine (Accession No. BBD74156).

Amino acids marked in bold indicate the regions matching the sequences determined by liquid chromatography-MS/MS analysis. The putative catalytic triad amino acid residues (Asp, His, and Ser) based on sequence comparison with cucumisin (Accession No. Q39547) are indicated by circles.

**Table 2.** 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.
Hydrolysis of MOCAc-KPLGL(DPA)-AR by the Masui Dauphine extracts. 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 ℃ . One unit is defined as the amount which exhibits $1.0 \times 10^5$ fluorescence intensity in the reaction solution (200 μL) in 1 s. Relative activity is the total enzyme activity compared to that of leaf extract obtained from freeze-dried powders using ammonium sulfate for the precipitation.
Average and SD values of triplicate determination are shown.

## Author contributions

K. Nishimura, K. Kojima, T. Takita, T. Abe, T. Takahashi, and K. Yasukawa designed the research; K. Nishimura, K. Higashiya, and N. Ueshima performed the research; K. Nishimura, K. Kojima, T. Takita, T. Abe, T. Takahashi, and K. Yasukawa analyzed data; K. Nishimura, K. Higashiya, N. Ueshima, and K. Yasukawa wrote the manuscript.

## Disclosure statement

No potential conflict of interest was reported by the authors.

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## Data Availability Statement

The authors confirm that the data supporting the findings of this study are available within the article and/or its supplementary materials.

## References

Aguda AH, Panwar P, Du X, *et al*. Structural basis of

collagen fiber degradation by cathepsin K. *Proc. Natl. Acad. Sci. USA* 2014;**111**(49):17474–9.

Badgujar SB, Patel VV, Bandivdekar AH, *et al*. Traditional uses, phytochemistry and pharmacology of *Ficus carica*: a review. *Pharm. Biol.* 2014; **52**(11): 1487–503.

Brown EM, Farrell HMJ, Wildermuth RJ. Influence of neutral salts on the hydrothermal stability of acid-soluble collagen. *Protein J*. 2000; **19**(2): 85–92.

Duan R, Zhang J, Du X, *et al*. Properties of collagen from skin, scale and bone of carp (*Cyprinus carpio*). *Food Chem*. 2009; **112**(3): 702–6.

Gallagher T, Gilliland G, Wang L, *et al*. The prosegment-subtilisin BPN' complex: crystal structure of a specific 'foldase'. *Structure* 1995; **3**(9): 907–14

Hamed MB, El-Badry MO, Kandil EI, *et al*. A contradictory action of procoagulant ficin by a fibrinolytic serine protease from Egyptian *Ficus carica* latex. *Biotechnol. Rep. (Amst)* 2020; **27**: e00492.

Hayashi K, Ikeuchi T, Morishita R, *et al*. The roles of histidine and tyrosine residues in the active site of collagenase in *Grimontia hollisae*. *J Biochem* 2020; **168**(4): 385–92.

Kitajima S, Aoki W, Shibata D, *et al*. Comparative multi-omics analysis reveals diverse latex-based defense strategies against pests among latex-producing organs of the fig tree (*Ficus carica*). *Planta* 2018; **247**(6): 1423–38.

Komsa-PR, Koynova R, Kostov G, *et al*. Thermal stability of calf skin collagen type I in salt solutions. *Biochim Biophys. Acta* 1996; **1297**(2): 171–81.

Komsa-PR, Koynova R, Kostov G, *et al*. Discrete reduction of type I collagen thermal stability upon oxidation. *Biophys. Chem.* 1999; **83**(3): 185–95.

Leikina E, Mertts MV, Kuznetsova N *et al*. Type I collagen is thermally unstable at body temperature. *Proc. Natl. Acad. Sci. USA* 2002; **99**(3): 1314–8.

Li Z, Scott K, Hemar Y, *et al*. Purification and characterisation of a protease (tamarillin) from tamarillo fruit. *Food Chem*: 2018; **256**: 228–34.

Meier F, Beck S, Grassl N, *et al*. Parallel accumulation – serial fragmentation (PASEF): Multiplying sequencing speed and sensitivity by synchronized scans in a trapped ion mobility device. *J. Proteome Res.* 2015; **14**(12): 5378–87.

Meier F, Brunner AD, Koch S, *et al*. Online parallel accumulation–serial fragmentation (PASEF) with a novel trapped ion mobility mass spectrometer. *Mol. Cell Proteomics* 2018; **17**(12): 2534–45.

Nishimura K, Higashiya K, Ueshima N, *et al*. Characterization of proteases activities in *Ficus carica* cultivars. *J. Food Sci.* 2020; **85**(3): 535–44.

Qian J, Okada Y, Ogura T, *et al*. Kinetic analysis of the digestion of bovine type I collagen telopeptides with porcine pepsin. *J. Food Sci.* 2016; **81**(1): C27–34.

Ran LY, Su HN, Zhou MY, *et al*. Characterization of a novel subtilisin-like protease myroicolsin from deep sea bacterium *Myroides profundi* D25 and molecular insight into its collagenolytic mechanism. *J. Biol .Chem*. 2014; **289**(9): 6041–53.

Raskovic B, Bozovic O, Prodanovic R, *et al*. Identification, purification and characterization of a novel collagenolytic serine protease from fig (*Ficus carica* var. Brown Turkey) latex. J. *Biosci. Bioeng* 2014; **118**(6): 622–27.

Rautengarten C, Steinhauser D, Büssis D, *et al*. Inferring hypotheses on functional relationships of genes: Analysis of the *Arabidopsis thaliana* subtilase gene family. *PLoS Comput. Biol.* 2005; **1**(4): e40.

Saitoh E, Yamamoto S, Okamoto E, *et al*. Identification of cysteine proteases and screening of cysteine protease inhibitors in biological samples by a two-dimensional gel system of zymography and reverse zymography. *Anal. Chem. Insights* 2007; **2**: 51–9.

Sato K, Ebihara T, Adachi E, *at al*. Possible involvement of aminotelopeptide in self-assembly and thermal stability of collagen I as revealed by its removal with proteases. *J. Biol. Chem.* 2009; **275**(33): 25870–5.

Takita T, Qian J, Geng H, *et al*. Comparative studies on the activities of collagenases from *Grimontia hollisae* and *Clostridium hystoliticum* in the hydrolysis of synthetic substrates. *J. Biochem.* 2018; **163**(5): 425–31.

Teramura N, Tanaka K, Iijima K, *et al*. Cloning of a novel collagenase gene from the gram-negative bacterium *Grimotia (Vibrio) hollisae* 1706B and its efficient expression in *Brevibacillus choshinensis. J. Bacteriol.* 2011; **193**(12): 3049 – 56.

Uchikoba T, Yonezawa H, Kaneda M, *et al*. Cleavage specificity of cucumisin, a plant serine protease. *J. Biochem*. 1995; **117**(5): 1126–30.

Volant DB, Matagne A, Mahyaoui RE, *et al*. A novel form of ficin from *Ficus carica* latex: Purification and characterization. *Phytochemistry* 2015; **117**: 154–67.

Yamagata H, Masuzawa T, Nagaoka Y, *et al*. Cucumisin, a serine protease from melon fruits, shares structural homology with subtilisin and is generated from a large precursor. *J. Biol. Chem*. 1994; **269**(52):

32725–31.

Yonezawa H, Kaizuka H, Uchikoba T, *et al*. Substrate specificity of cucumisin on synthetic peptides. *Biosci*. *Biotechnol*. *Biochem*. 2000; **64**(10): 2104–8.

Zare H, Moosavi-Movahedi AA, Salami M, *et al*. Purification and autolysis of the ficin isoforms from fig (*Ficus carica* cv. Sabz) latex. *Phytochemistry* 2013; **87**: 16–22.

Zhang YZ, Ran LY, Li CY *et al*. Diversity, Structures, and Collagen-Degrading Mechanisms of Bacterial Collagenolytic Proteases, *Appl*. *Environ*. *Microbiol*. 2015; **81**(18): 6098–107.