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Purification and Characterization of Novel Proteinase Inhibitors from Dried Figs

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Two types of the natural organic matter P and B were isolated from dried figs by gel permeation and high-performance liquid chromatography. The characterizations of their molecular structures were also performed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and infrared absorption spectrometry. As a result, these samples were revealed to inhibit the serine and cysteine proteinases chymotrypsin and papain ($K_i = 10^{-6}-10^{-4}$ M). The optimal inhibitory pH values of the P and B samples were observed to be approximately 5.5 and 5.0, respectively. The analyses of their UV-vis absorption spectra and infrared absorption spectra indicated that they would be a kind of humic substance. The mass spectrometry analyses showed that they possessed relatively small heterogeneous molecules with molecular masses around 692, 845, and 1389 Da for the P sample and around 551, 704, and 909 Da for the B sample.

KEYWORDS: Dried figs; *Ficus carica*; humic substances; optimal inhibitory pH; proteinase inhibitor

INTRODUCTION

Humic substances are polyelectrolytes produced by the abiotic alteration of plant and animal tissue during the early decomposition of natural organic matter found in the soils, sediments, and surface waters on the earth (*1*). They are important components of soil refractory organic matter and play critical roles in sediment processes such as metal reduction and pollutant transport. Three fractions of humic substances are defined in terms of solubility aspects: humine which is insoluble in aqueous solutions at any pH value; humic acids which are insoluble in acidic medium; and fulvic acids which are soluble in aqueous solutions at any pH value. Humic substances were often considered, when studied in aqueous solutions, as macromolecules with molecular masses around 10000−20000 Da for humic acids and 2000−3000 Da for fulvic acids (*2*). However, recent studies showed that humic substances have a supermolecular nature in which relatively small heterogeneous molecules (masses around 500 Da) are self-assembled by hydrogen bonds, and also by weaker forces such as van der Waals, $\pi-\pi$, and CH$-\pi$ interactions, into large assemblies of only apparently high molecular mass (*3*, *4*).

On the other hand, the fig tree (*Ficus carica*) is cultivated for its fruit in temperate zones and has been investigated for its proteolytic enzyme ficin (*5*), organic acids (*6*), sugars (*7*), and natural rubber (*8*). The dried fruit has been a very familiar food for human beings since B.C. 3000, and California is especially famous for the production. The nutritional characteristic of dried figs is known for the abundance of calcium, potassium, and dietary fiber as compared with other fruits; on the other hand, dried figs have been taken as a laxative, an expectorant, or a hemorrhoidal drug since ancient times. Despite its high agricultural, economic, and medical values, little attention has been given to investigate the physiological and biochemical traits of the dry fruit.

Recently, I found that dried figs possess weak inhibitory activities against some proteinases. To obtain a better insight into the laxative effect, in this paper, the author purified the proteinase inhibitory components from dried figs, measured their inhibitory activities against some digestive enzymes, and characterized their molecular structures briefly. As a result, the inhibitory components were indicative of a kind of humic substance and were revealed to have moderate inhibitory activities toward a ficin-like proteinase papain as well as a digestive enzyme chymotrypsin. Furthermore, it was found that they consist of relatively small heterogeneous molecules with molecular masses around 500−1400 Da.

EXPERIMENTAL PROCEDURES

Materials. Dry fig fruit in California (Black Mission) was purchased from a local market, and purified chymotrypsin, papain, and trypsin were from Sigma-Aldrich (St. Louis, MO). All other reagents used were of the highest grade commercially available.

Isolation of Organic Matter from Dried Figs. One hundred and thirty-seven grams of the dry fruit were extensively washed with distilled water, added to 450 mL of distilled water, and then macerated to produce juice using a domestic juicer. The resulting juice was centrifuged using a Hitachi 55P-72 centrifuge (Hitachi, Tokyo, Japan) at 33570*g* for 30 min at 4 °C, and the clear supernate was thoroughly dialyzed against distilled water using a 1000 MWCO membrane (Spectrum Laboratories, Rancho Dominguez, CA) and freeze-dried. The sample solution was applied on a Sephadex G-50 column (2.2 cm × 100 cm) equilibrated with 50 mM Tris-HCl (pH 7.5) at room temperature. The resulting organic matter was finally purified on a RESOURCE RPC analytical column (3 mL) (Amersham Biosciences, Piscataway, NJ) equilibrated with 0.05% (v/v) trifluoroacetic acid (TFA)

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using a Shimadzu LC10-V_p high-performance liquid chromatography (HPLC) system (Kyoto, Japan). Elution was achieved at a flow rate of 1.0 mL/min with a linear gradient of 1−80% (v/v) acetonitrile in 0.065% TFA.

Confirmation of Nonprotein Nature. The presence of proteins was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a Tris−tricine buffer system (*9*). The gels were stained using 0.1% (w/v) Coomassie brilliant blue R250 and 40% (v/v) methanol and destained in acetic acid/methanol/H_2O (1:5:4). Furthermore, the nonprotein nature of the organic matter was confirmed using the biuret assay (*10*) with a minor modification. Biuret reagent was freshly prepared in 50 mL of H_2O by mixing 75 mg of $CuSO_4 \cdot 5H_2O$, 300 mg of Rochelle salt, and 1.5 g of NaOH. Working standards were prepared from crystalline bovine serum albumin (BSA) (0, 0.5, 1, 2, 4, 6, 8, and 10 mg/mL). The biuret reagent, 0.8 mL, was added to 0.2 mL of each sample solution (10 mg/mL) or each standard solution. The resulting solutions were incubated for 30 min or longer at room temperature. The absorbance at 550 nm was measured, and the protein concentration was calculated from a standard line, and the sample absorbance was made an appropriate correction for differences between the color value of the standard and the sample.

Characterization of Proteinase Inhibitory Activity. A volume of 50 μL of chymotrypsin (final concentration of ca. 300 nM), 50 μL of the inhibitor solution, and 2.350 mL of 20 mM sodium acetate buffer (pH 5.0) containing 25% (v/v) acetonitrile were mixed thoroughly in a 1 cm path length quartz cuvette which was placed in the thermostated cell compartment (25 °C) of a Beckman DU-640 spectrophotometer (Palo Alto, CA). After at least 2 min of preincubation, 50 μL of the substrate *p*-nitrophenyl benzyloxycarbonyl-L-phenylalanate (CPN) (20 μM) was added on the tip of a flattened glass rod, and then the solution was stirred for 10 s. The initial velocity (v_0) was calculated from an increase in absorbance at 317 nm of the released *p*-nitrophenol. The percent inhibition was calculated by the following equation:

$$\text{Inhibition (\%)} = [1 - (v_0 \text{ with inhibitor})/(v_0 \text{ without inhibitor})] \times 100$$

In the case of papain or trypsin, the buffers used were 20 mM sodium acetate buffer (pH 4.5) containing 1 mM β-mercaptoethanol or not, respectively; *p*-nitrophenyl benzyloxycarbonyl-L-lysinate (CLN) was used as a substrate for both enzymes. The concentrations of chymotrypsin, papain, and trypsin were calculated using the $E^{1\%}_{280}$ values 20.4, 25.0, and 14.4, respectively (*11*−*13*). The inhibition constant (K_i) value was calculated by a Dixon plot analysis (*14*). In the case of uncompetitive inhibition, apparent K_m values ($1/K_{m(app)}$) were estimated from Lineweaver−Burk plots, and the K_i values were calculated by plotting the $1/K_{m(app)}$ values versus inhibitor concentrations [I] in the following equation:

$$1/K_{m(app)} = [I]/K_iK_m + 1/K_m$$

The pH dependence of proteinase inhibitory activity was investigated by the same method as described above, except for the use of McIlvaine wide-range buffer (*15*).

Infrared Absorption (IR) Measurements. IR spectra were obtained using a Jasco A-702 infrared spectrophotometer (JASCO International, Tokyo, Japan) in a wavenumber range of 200−4000 cm^{-1}, after N_2 gas flow purging of the measurement chamber for 20 min to avoid CO_2 and moisture interference. Samples were freeze-dried prior to the preparation of pressed disks (2 cm in diameter) containing 3 mg of sample in 200 mg of KBr. Due to the hygroscopic nature, KBr powder of spectroscopic grade was kept in moisture-free atmosphere.

Mass Spectrometric Analysis. Mass spectrometry (MS) experiments were conducted using a Micromass TofSpec-2E matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer (JASCO International/Micromass, Manchester, U.K.). α-Cyano-4-hydrocinnamic acid (CHCA), dihydroxybenzoic acid (DHB), or dithranol was used as a matrix. Each matrix-saturated solution was prepared in a 1:1 mixture of acetonitrile and H_2O or in methanol. Eventually, six conditions (three matrixes × two solutions) were checked for each sample by MS.

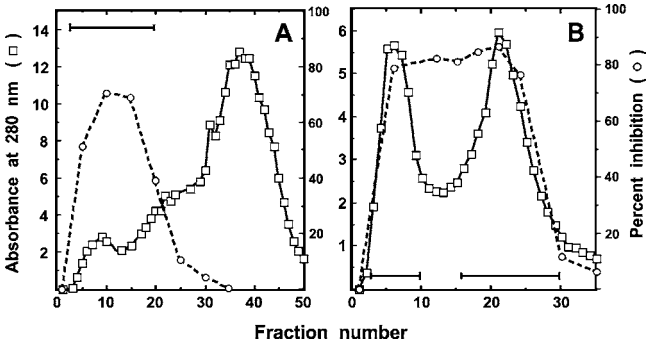

Figure 1. Fractionation of the chymotryptic inhibitory activity from dried figs. (**A**) Second gel permeation chromatography on a Sephadex G-50 column. The fraction size and flow rate were 7.5 mL and 0.4 mL/min, respectively. (**B**) Third gel permeation chromatography. Approximately 3 g of sample obtained by the previous step was applied to the column. The fractions with a bar were pooled.

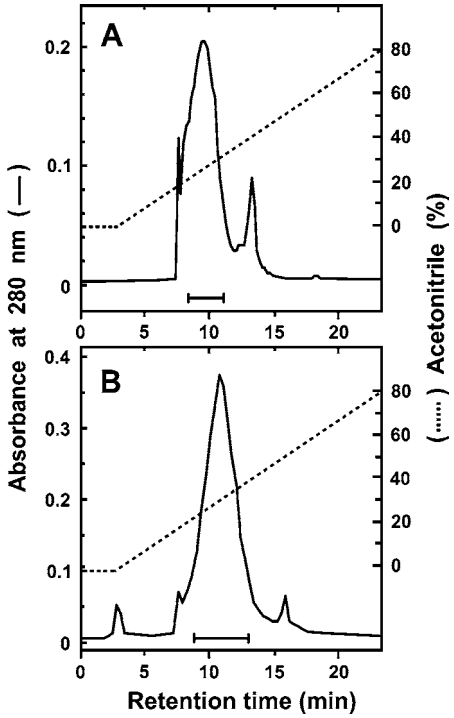

Figure 2. HPLC chromatogram of the fractions P (**A**) and B (**B**). Approximately 20 mg of the P fraction or 10 mg of the B fraction from the gel permeation chromatography was applied to a RESOURCE RPC column. Absorbance (solid line) was monitored at 280 nm, and elution was performed for 20 min with a linear gradient (dotted line) of 0–80% (v/v) acetonitrile at a flow rate of 1.0 mL/min.

RESULTS

Purification of the Organic Matter with Chymotryptic Inhibitory Activity. The supernatant of the fruit juice was thoroughly dialyzed for 3 days; however, the lysate still contained much sugar. Therefore, for the complete removal of the sugar, it was required to perform three chromatography applications by the Sephadex G-50 column. As shown in **Figure 1B**, the chymotryptic inhibitory activity was found both in the former (P) and later peaks (B), whose colors were pink and brown, respectively. On the other hand, the color of the main peak in **Figure 1A** was orange. It is notable that there was no stained band on the SDS−PAGE gel (data not shown). Moreover, the P and B samples (10 mg/mL) were revealed to contain 17 and 107 μg protein/mg by the biuret test, respectively (**Figure 3**). The overall yields of the fractions P and B were

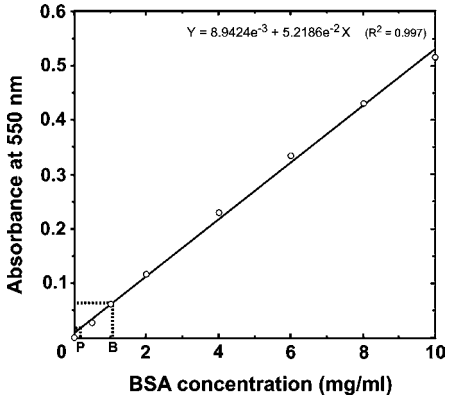

Figure 3. Biuret assay for the protein concentrations of the P and B samples. The numerical formula of the BSA standard line was calculated by linear least-squares minimization using the software Cricket Graph (Cricket Software, Malvern, PA) and is shown on the upper right. The dotted lines indicate absorbance at 550 nm and protein concentration for the sample solutions (10 mg/mL).

approximately 140 mg and 750 mg/137 g of dried figs, respectively. The elution profile of HPLC for the P fraction had two broad peaks at retention times of 9.7 and 13.4 min (**Figure 2A**), and that for the fraction B also had two broad peaks at 10.9 and 15.9 min (**Figure 2B**). The peaks at retention times of 3.0 and 7.8 min were revealed to be artifacts, since these peaks also appeared in the control experiment (data not shown). As judged from the peak height on each chromatogram, the peaks at retention times of 9.7 and 10.9 min are indicative of the P and B fractions, respectively. Hereafter, the pooled fractions (**Figure 2**, parts **A** and **B**) were used as the samples P and B for structural analyses.

Kinetic Properties and pH Dependence of the Proteinase Inhibitory Activities. As an example, the Lineweaver-Burk plots of the B sample are shown in **Figure 4A**−**C**. These plots indicated that the B sample noncompetitively inhibited the chymotrypsin and papain and uncompetitively inhibited trypsin. On the other hand, the P sample was revealed to inhibit the chymotryptic activity noncompetitively and to inhibit the papain activity uncompetitively (data not shown). The obtained K_i values of the P and B samples toward representative proteinases are listed in **Table 1**. In summary, the P and B samples were found to inhibit the serine and cysteine proteinases, but the P sample was unable to inhibit the tryptic activity. Furthermore, the P sample seems to possess more inhibitory activity than the B sample from comparison of their K_i values (**Table 1**). The optimal inhibitory pH of the P sample against chymotrypsin and papain was observed to be around 5.5, while that of the B sample was around 5.0 for chymotrypsin and papain and around 4.5 for trypsin, respectively (**Figure 4D**−**F**).

Table 1. Summary of the Inhibition Parameters of the P and B Samples against Several Proteinases

proteinase	K_i (M)[a]	inhibitory mode	optimal pH
	P Sample		
chymotrypsin	6.0×10^{-6}	uncompetitive	5.5
papain	9.1×10^{-6}	noncompetitive	5.5
trypsin	not detected		
	B Sample		
chymotrypsin	1.1×10^{-4}	noncompetitive	5.0
papain	2.4×10^{-5}	noncompetitive	5.0
trypsin	2.8×10^{-4}	uncompetitive	4.5

[a] The K_i values of the P and B samples were calculated from the molecular masses of 845 and 551 Da, respectively.

Spectroscopic Analyses. The UV-vis absorption spectra of the P and B samples are indicative of nonprotein (**Figure 5A**), which is consistent with their SDS-PAGE experiments. The IR spectra of samples P and B are shown in **Figure 5B**. The bands at 3300, 1500−1600, and 1050 cm^{-1} were assigned to the stretching vibration of hydrogen-bonding O−H, the stretching mode of aromatic C═C coupled with carbonyl or carboxylate, and the bending vibration of C−OH, respectively. It is noteworthy that the band at 3300 cm^{-1} of the B sample was not so strong as compared with that of the P sample. Moreover, the broad bands (400−600 cm^{-1}) of both samples are indicative of the presence of carboxylate dimers, amines, and/or amides. The mass spectra of the P and B samples in positive ion mode

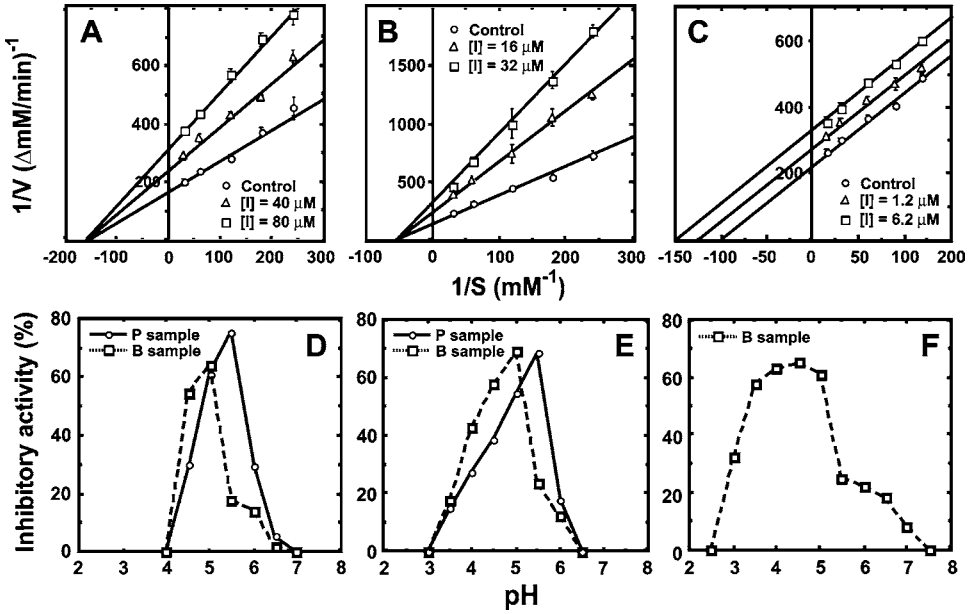

Figure 4. Lineweaver-Burk plots for CPN or CLN hydrolysis catalyzed by chymotrypsin (**A**), papain (**B**), and trypsin (**C**) in the presence and absence of the B sample. The data represent the mean values ± standard deviations of triplicate measurements. Panels **D**–**F** indicate the pH dependence of the inhibitory activities of the P and B samples toward chymotrypsin, papain, and trypsin, respectively.

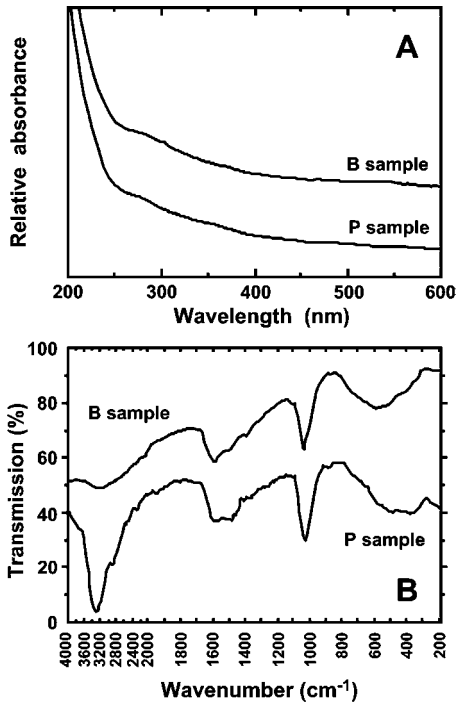

Figure 5. UV-vis absorption (**A**) and IR (**B**) spectra of the P and B samples.

are shown in **Figure 6**, parts **A** and **B**, respectively. As a result, the P sample possessed relatively small heterogeneous molecules with molecular masses around 692, 845, and 1389 Da; on the other hand, those of the B sample were around 551, 704, and 909 Da. The mass spectrum of the P sample in CHCA/methanol (data not shown) also showed almost the same ion distributions as that in DHB/methanol. On the other hand, in the case of the B sample, peaks with molecular masses around 698 and 909 Da were present on the spectra in CHCA/acetonitrile/H_2O, while the MS peak on the dithranol/methanol spectra was observed around 551 Da (data not shown).

Humic Substances. Generally, the absorption spectra of the humic substances, humic acid and fulvic acid, show no specific features but a strong steady increase with decreasing wavelength (*1*, *16*), as also found for the spectra of the samples P and B from the present study (**Figure 5A**). Furthermore, the IR spectra of humic substances in general indicate the following typical bands: 3400 (hydrogen-bonding O−H), 2920−2860 (aliphatic C−H), 1720 (carboxyl C═O), 1600−1650 (aromatic C═C coupled with C═O or COO^-), 1450 (aliphatic $-CH_3$), 1400 (carboxylate C═O or alcohol O−H), 1200 (carboxyl C−O), and 1050 (alcohol or ether C−OH) cm^{-1} (for instance, *16*). The above-mentioned IR bands were observed in the IR spectra of P and B samples (**Figure 5B**). Thus the P and B samples were revealed to have these characteristic features expected for humic and fulvic acids, so the author considered them to be a kind of humic or fulvic acid. Especially, the B sample was concluded to be fulvic acid on the basis of the high solubility in aqueous solutions at any pH value. On the other hand, it is likely that the P sample is a kind of humic acid, because the P sample showed poor solubility in a solution of 1% TFA.

DISCUSSION

As shown in **Figures 1** and **2**, the P and B fractions with chymotryptic inhibitory activity were isolated from dried figs by gel permeation and HPLC. However, it appears to be impossible to purify these fractions by ion-exchange chromatography. For instance, they did not adsorb on a column of CM-Sephadex under any condition, while they strongly adsorbed a DEAE-Sephadex column, and it was almost impossible to elute all the sample adsorbed even using a linear pH (4−2) or salt (0−5 M) gradient (data not shown). This indicates that the P and B fractions have many strong negative charges, probably carboxylate anions, which is consistent with the results of the

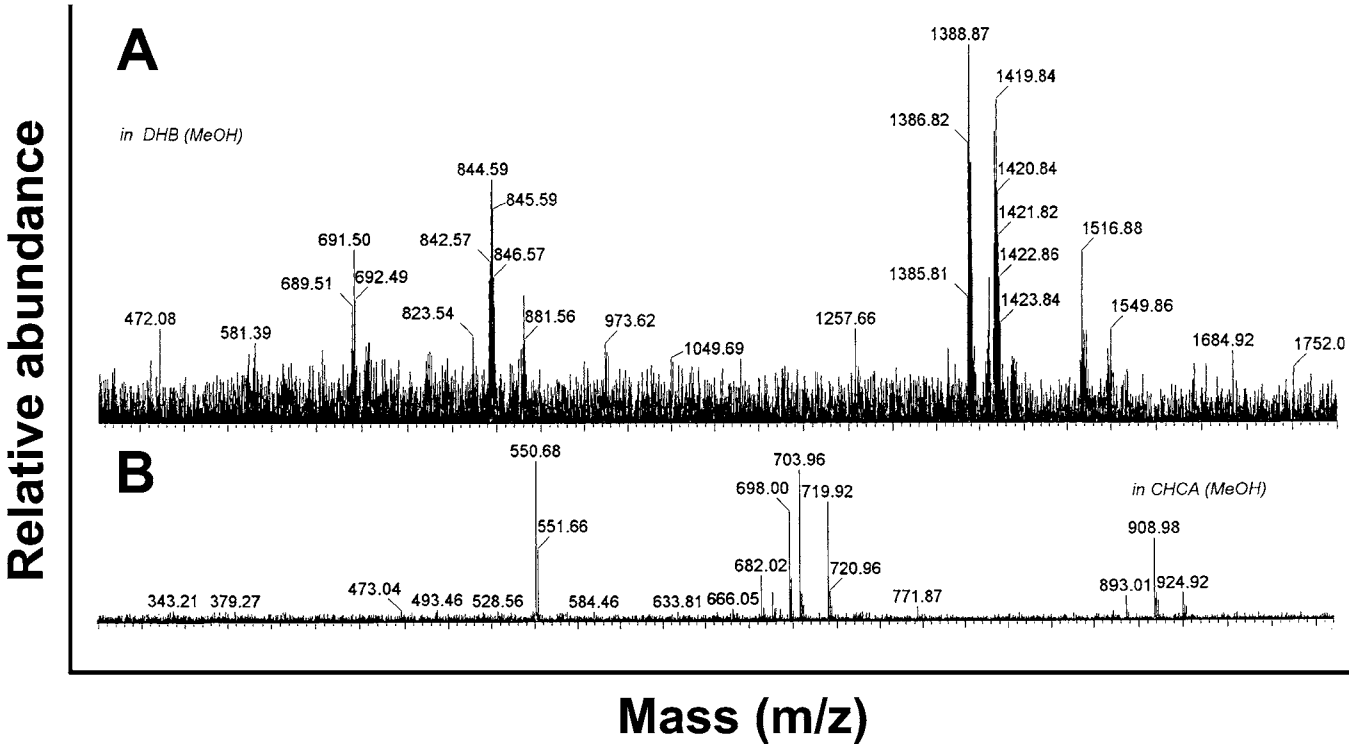

Figure 6. Positive ion MALDI-TOF mass spectra of the samples P (**A**) and B (**B**). DHB or CHCA in methanol was used as a matrix for the P and B samples, respectively.

UV-vis absorption and IR spectra (**Figure 5**). Therefore, the protein quantities of the samples did not show zero, since carboxylate anions in the samples might bind to copper ions in the biuret reagent and interfere with the color reaction. It seems reasonable to assume that the protonation of some carboxylates in the P and B samples is necessary for their inhibitory activities, since their optimal inhibitory pH values were observed over the range of pH 4.5−5.5 (**Table 1**). Furthermore, the pH-inhibition profile of the B sample exhibited an inhibitory peak at pH 5 and a shoulder around pH 6 (**Figure 4D−F**), indicating that the B sample is not pure or has two independent inhibitory sites in the same molecule. Originally, the author tried to find inhibitors such as bromein (*17*) in dried fig. The optimum inhibitory pH of bromein is about 4 (*18*). Therefore, the inhibitory activities toward several proteinases were checked under acidic pH conditions at first. Fortunately, this led to finding of such interesting inhibitors in the present work.

As shown in **Figure 6**, the signal-to-noise ratio of the B sample (ca. 18.3) was approximately 3 times as high as that of the P sample (ca. 6.5), so the ionization of the P sample would be more difficult than that of the B sample. As described in the Introduction, humic substances have been reported to consist of relatively small heterogeneous molecules ($m/z \sim 500$) and to be self-assembled by hydrogen bonds and weaker forces (*3*, *4*). The present research also revealed that both samples had ion distributions on the order of $m/z \sim 550-1400$ and that the reversed-phase HPLC profiles showed a broad elution peak, which is compatible with the plausible explanation for the structure of humic substances. As shown in **Figure 6**, the mass distribution of P sample ($m/z \sim 690-1420$) was a little higher than that of the B sample ($m/z \sim 550-910$). The IR experiment revealed that the band of hydrogen-bonding O−H (3300 cm^{-1}) in the P sample was much stronger than that in the B sample (**Figure 5B**). This evidence indicates that the intermolecular hydrogen bonds of the P sample would be stronger than those of the B sample. Therefore, the P sample might be able to exist as macromolecules in comparison with the B sample, which is consistent with the result of the gel permeation experiments (**Figure 1B**).

In the present paper, to clarify the relationship between dried figs and the effect as a laxative, the extract was screened for inhibitory activity against digestive enzymes. Moreover, the author investigated the inhibitory property toward papain as well as the digestive enzymes chymotrypsin and trypsin, since the latex contains a papain-like proteinase ficin (*5*). As a result, the dry fruit was found to possess the inhibitory components P and B against these proteinases, which leads to the laxative effect. The reason that the laxative effect of dried figs was weak would be that the optimum inhibitory pH 4.5−5.5 of the P and B samples was different from the optimum pH 6−7 of the target proteinases (*11*−*13*). It is notable that the P and B samples inhibited both serine and cysteine proteinases, since proteinase inhibitors usually block only one out of four proteinase categories (*19*). Generally, plants have a proteinase(s) and its inhibitor(s) at the same tissue for the regulation of the proteinase activity (cf., *17*). However, this study revealed that the inhibitors P and B were a sort of humic substance and were not produced by the fig tree itself; therefore, they would not be involved in the target proteinase regulation. Actually, there is not any proteinase activity in dried figs (data not shown). To the author's knowledge, this is the first report that organic matter with high molecular masses, such as humic substances, has inhibitory activities toward some proteinases. In the near future, it is planed to investigate the chemical and conformational structure of these humic substances in detail, which would contribute to further understanding of the inhibitory mechanism.

ABBREVIATIONS USED

CHCA, α-cyano-4-hydrocinnamic acid; CLN, *p*-nitrophenyl benzyloxycarbonyl-L-lysinate; CPN, *p*-nitrophenyl benzyloxycarbonyl-L-phenylalanate; DHB, dihydroxybenzoic acid; HPLC, high-performance liquid chromatography; IR, infrared absorption; K_i, inhibition constant; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid.

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