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Size-Exclusion Chromatography of Tea Tannins and Intercepting Potentials of Peptides for the Inhibition of Trypsin−Caseinolytic Activity by Tea Tannins

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Molecular-weight distribution and characterization of tea tannin were investigated by high-performance liquid chromatography and the equivalent preparative exclusion gel chromatography using Sephadex G-25. The characteristics of the fractions were studied regarding the amounts of terminal catechin, sugar, and gallic acid, the color reaction of the Folin−Chiocalteu reagent, the UV absorbance, and the inhibition activity for the trypsin−caseinolytic activity per weight. Furthermore, we investigated the intercepting activities of the inhibition by the amino acids, peptides, their analogues, poly(ethylene glycol)s (PEGs), and histatin 5 using the inhibition of trypsin−caseinolytic activity by tea. Arg, Lys, and their peptides had strong intercepting activities for the inhibition, but only a weak activity was detected in the Pro peptides or gelatin-like peptides of (Pro-Pro-Gly)$_n$ (n = 5 or 10). The guanidyl group of Arg and the amino methylene group of Lys were important for the intercepting activity, but the activity was weakly dependent upon the peptide bond formation. The intercepting activity of the peptides or PEG exponentially increased with the number of polymerizations. Histatin 5 did not have a remarkably strong intercepting activity considering the peptide length. The activity of the synthetic histatin 5 in which all of the Lys and Arg were substituted by Ala was at the same level as histatin 5.

KEYWORDS: Tea tannin; trypsin; arginine; lysine; histatin

INTRODUCTION

Tannin is a generic name for the polymeric polyphenol compounds of plants, which exist in fruit, leaves, and so on, and is divided into two groups: the hydrolyzable tannin, which consists of the galleates of glucose, and the condensed tannin, which is made of condensed flavonoids such as epicatechin and catechin (*1*, *2*). As for the tannins containing the polyphenols, their antioxidation activity has recently been reported (*3*−*7*). However, the tannin or polyphenolic compounds form an insoluble substance with the proteins and have an inhibitory activity for various enzymes (*8*−*12*). Several papers of cell necrosis by tannin were reported (*13*−*17*). These activities are like a double sword and have not yet been studied in detail. Concerning the food production field, the relations between tannin and protein are important (*18*−*24*). Generally, the tannin combines with the protein. This insoluble precipitation is well-known, and the interaction is quite characteristic and important. As examples, the fruit aging degree in wine and juice affects the astringency and insoluble substances and also affects the digestive influence of animal feeds (*18*−*20*). Many researchers have been trying to clarify the nature and characteristics or their application (*25*−*27*). There are very difficult problems because the tannin and polyphenolic compounds have complicated structures, various molecular weights, and a strong absorptability. Their characteristics and classification are not yet clear (*27*).

As for the interaction with tannin and protein, a powerful interaction has been well-known with the histatins and the proline-rich proteins in saliva (*28*−*33*). Both subjects have been well-studied by various means, but their characteristics are not clear yet (*27*). It is not clear whether Pro strongly binds to tannin and what is important for the binding element of the histatin (*27*). The tannin of tea is generally in our foods. The catechin in the tea was changed to the condensed tannin or polyphenol by polyphenol oxidase in the tea, which leaves during the fermentation process. The food function of the low molecular polyphenolic compounds was recently studied (*7*). The tea tannin has also been studied. The trypsin or amylase was strongly inhibited by tea tannin, and the enzymes and the tannin form a precipitate (*8*, *10*). Fickel et al. already reported this result using a hydrolyzable tannin from tea and a synthetic substrate for trypsin (*8*). We found that the tea tannin strongly inhibited the trypsin−caseinolytic activity compared to the hydrolysis activity for the synthetic substrate and thought that the inhibition in the trypsin−caseinolytic assay system provided a better detection.

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The interaction of various amino acids, peptides, and compounds with the tea tannin could be estimated using the trypsin−caseinolytic assay system.

In this paper, we show the high-performance liquid chromatography (HPLC) analysis, the equivalent fractionation of the tea tannin, and several characteristics per weight containing the inhibition activity of the fractions. Furthermore, we show the intercepting activity for the inhibition against the caseinolytic activity of trypsin by various amino acids, peptides, analogue compounds, poly(ethylene glycol)s (PEGs), histatin 5, and synthetic histatin 5, in which Ala is substituted for Arg and Lys of the histatin 5.

MATERIALS AND METHODS

Tea. The tea (red tea bags made by Lipton Co., Ltd.) was purchased from a local market. A tea bag was dipped in 200 mL of hot water (90 °C) for 2 min. The tea was eluted through a Sephadex G-25 column (2.5 × 4 cm) and then washing with 100 mL of water. The absorbed tea was extracted with 200 mL of 50% ethanol, and the extraction was concentrated and freeze-dried. The tea or the diluted tea solution was used for the trypsin−caseinolytic inhibition.

HPLC Analysis. The extracted tea solution (0.1 mL) and tested sample solution of various proteins, peptides, and chemicals (0.1 mL buffer solution) were placed in an eppen tube. The mixture was hand-shaken several times and centrifuged (6200 rpm for 10 min). The supernatant (0.1 mL) and ethanol (0.4 mL) were mixed in another tube, hand-shaken, and centrifuged again. The supernatant (0.4 mL) was diluted with water (0.28 mL), and the solution (10 μL) was injected into the HPLC system.

The HPLC system (DP8020, Toso, Tokyo, Japan) consisted of a column (Sephadex G-25, 6 × 100 mm, handmade column). The flow rate was 0.1 mL/min (50% ethanol), and the detection was monitored at 280 nm (optical photometer UV8020, Toso).

Preparative Exclusion Column of Sephadex G-25. Sephadex G-25 (fine grade) was purchased from Amersham Biosciences ABm Uppsala, Sweden. The column (5 × 52 cm) was equilibrated with 50% ethanol, and the extracted tea (40 mg) was applied to the column.

Estimation of Sugar, Catechin, Gallic Acid, and Polyphenolic Compounds. The total sugar was estimated by the phenol−sulfuric acid method (*34*). The catechin was estimated by the modified vanillin assay (*35*); polyphenolic compounds were estimated by the Folin−Chiocalteu reagent (*36*); and gallic acid was estimated by rhodamine (*37*). Each amount was calculated using a colorimetric standard curve with D-glucose, catechin, and gallic acid as the standards.

Enzyme and Peptides. Trypsin (code T-0303) was purchased from the Sigma Chemical Co., St. Louis, MO. Casein was a reagent from Wako Pure Chemistry Co., Ltd., Osaka, Japan. The natural amino acids were reagents of Wako Pure Chemistry Co., Ltd. and Kanto Chemicals Co., Ltd., Tokyo, Japan. The peptides of Lys-Lys-Lys acetate (H-41750.0050), Arg-Arg acetate (G-1465.0250), Lys-Arg acetate (G-2635.0250), Arg-Lys acetate (G-1505.0050), Arg-Arg-Arg acetate (H-1790.0050), and histatin 5 (G3144.1000) were purchased from Bachem AG, Budendorf, Switzerland. Peptides of Gly-Gly (code 3028), Gly-Gly-Gly (code 3061), $(Pro\text{-}Pro\text{-}Gly)_5 \cdot 4H_2O$ (code 4005), Pro-Pro HCl (code G-3060.0250), Pro-Pro-Pro HCl (code H4795.0250), $(Pro\text{-}Pro\text{-}Gly)_{10} \cdot 9H_2O$ (code 4006), and the synthetic substrate of Bz-L-Arg-*p*-NA HCl (code 3057) were from the Peptide Institute Co., Ltd., Osaka, Japan. The peptide of Lys-Lys 2HCl (code 2546663) was from Kokusan Kagaku Co., Ltd., Tokyo, Japan. Histatin 5 (code H-3144) was purchased from Bachem AG, Budendorf, Switzerland, and the synthetic histatin 5, which substituted Ala for Arg and Lys of histatin 5, was from AnaSpec, Inc., San Jose, CA.

Chemicals. Bovine serum albumin (BSA, albumin fraction V) was purchased from Merk KGaA, Darmstadt, Germany. 1,6-Diaminohexane, 1,3-diaminopropane, 6-amino-1-hexanol, 6-amino-hexanoic acid, PEG with the average molecular weights of 200, 1000, 4000, and 6000, guanidine hydrochloride, urea, ammonium chloride, and the Folin−Chiocalteu reagent were purchased from Kanto Chemicals Co., Ltd. (+)-Catechin, cyanidine chloride, and procyanidin B2 were from Funakoshi Co., Ltd., Tokyo, Japan. Theaflavin was from Wako Pure Chemistry, Co., Ltd. All other reagents were of reagent grade.

Trypsin Assay System. The trypsin−caseinolytic activity and its inhibition by tea were measured by the Casein−Folin method (*38*). The reaction buffer system used 0.1 M Tris-HCl buffer at pH 7.0. Trypsin (25 μg/100 μL) and various amounts of intercepting compounds (100 μL) or water were mixed and preincubated at 37 °C for 10 min, and then diluted tea (100 μL) was mixed and preincubated at 37 °C for another 10 min. After the preincubation, 0.8 mL of a 1.33% casein solution (0.1 M Tris-HCl buffer at pH 7.0) was added, and then the enzyme reaction was done for 10 min. The reaction was stopped by the addition of 1 mL of 0.44 M trichloroacetic acid, and then the mixture was filtered through filter paper (number 2, Toyo-Roshi, Tokyo, Japan). A 0.5 mL aliquot of the filtrate was mixed with 2.5 mL of 0.44 M Na_2CO_3; 0.5 mL of 2-fold diluted Folin−Chiocalteu reagent was added; and then the mixture was kept at 37 °C for 20 min. The colored mixture was measured at 660 nm using an optical photometer.

Intercepting Activity for the Inhibition of Trypsin−Caseinolytic Activity by Tea Tannin. The inhibitory activity of tea for trypsin was assayed by the method of caseinolytic activity described above. The inhibition rate of the caseinolytic activity against the various concentrations of tea was calculated by the following equation:

$$\text{inhibition (\%)} = [(E - E_b) - (I - I_b)]/(E - E_b) \times 100$$

where E, E_b, I, and I_b represent the optical density of the enzyme control, enzyme blank, sample, and sample blank, respectively. IA50 (50% intercepting activity) was defined as the concentration of a compound that produced the 50% intercepting ratio for the 50% inhibition of the trypsin−caseinolytic activity by tea, that is, a concentration in which the inhibition of trypsin (50% inhibition) is reduced to 25% by a compound. The IA50 values were obtained from the plotting and curve fitting of the inhibitions at four different concentrations. The intercepting activity of the various peptides and histatin 5 were assayed on a small scale in eppen tubes ($^1/_5$ or $^1/_{10}$) of the described method. The supernatant was obtained by centrifugation (3000 rpm for 5 min). The optical density of the colored solution was measured at 630 nm using a microplate reader (Microplate reader EZS-ABS, IWAKI Co., Ltd., Tokyo, Japan).

Trypsin Hydrolysis Activity for Synthetic Substrate. The reaction buffer system used the 0.1 M Tris-HCl buffer at pH 7.0. A solution of trypsin (100 μL) and tea (100 μL) was mixed and preincubated at 37 °C for 10 min (*39*), and then Bz-L-Arg-*p*-NA (0.6 mM, 2.3 mL) was mixed and incubated at 37 °C for 10 min. The reaction was stopped by the addition of 0.5 mL of 30% acetic acid. The yellow-colored mixture was measured at 410 nm using an optical photometer. The inhibition was calculated using the inhibition equation described above.

RESULTS

Tea-Inhibition Activity for Trypsin−Caseinolytic Digestion and Synthetic Substrate Hydrolysis. Figure 1 shows the tea-inhibition activity for the trypsin−caseinolytic digestion (TITC) and hydrolysis activity for the synthetic substrate of Bz-L-Arg-*p*-NA. The tea strongly inhibited the caseinolytic activity, and the inhibition was almost linear up to a 95% inhibition. The hydrolysis activity of the synthetic substrate was not strongly inhibited when compared with the caseinolytic activity. The tea was mixed with trypsin and produced a precipitate at pH 7 in the reaction mixture. The precipitated trypsin−tea tannin was estimated as 1:0.95 in weight. Catechin, gallic acid, protocatecuic acid, theaflavin, procyanidine HCl, and procyanidine C2 did not show any inhibition activity for trypsin. TITC was stable during boiling, and under acid conditions, the TITC was completely absorbed on Sephadex G-25 and about 70% of the activity was eluted with 50% ethanol or 1% PEG 6000.

HPLC Analysis of the Tea Tannin and Change in the Elution Profile with Trypsin. The HPLC analyses using Sephadex G-25 for the tea tannin were investigated. **Figure 2**

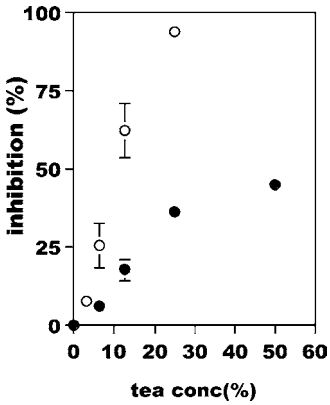

Figure 1. Inhibition of the trypsin–caseinolytic activity and hydrolysis activity of the synthetic substrate by tea. Plot of inhibition (%) of the trypsin–caseinolytic activity and hydrolysis activity of the synthetic substrate of Bz-L-Arg-*p*-NA versus various concentrations of tea. (○) Inhibition of the caseinolytic activity of trypsin. (●) Inhibition of the hydrolysis activity of trypsin. Each plot represents the average ± standard deviation (SD) ($n = 4$).

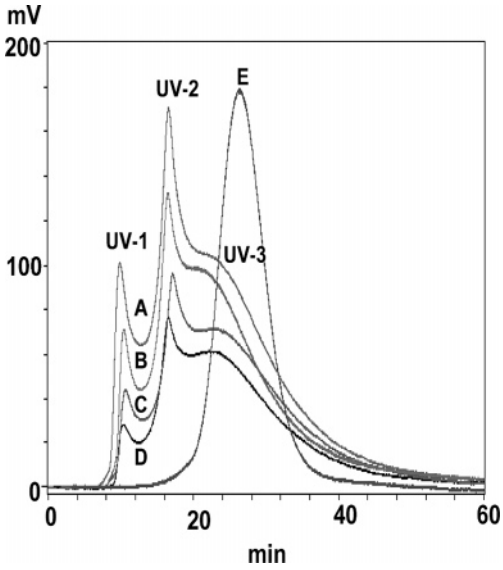

Figure 2. Change of the HPLC elution profile of the tea tannin by the addition of trypsin. The tea tannins and trypsin were mixed, and the supernatant was analyzed by HPLC using a column of Sephadex G-25 (fine grade). The HPLC analysis gave three UV peaks. The equivalent preparative chromatography also gave a similar profile (**Figure 3**), and the molecular weights were considered to be UV-1 > UV-2 > UV-3 from the results of **Table 1**. Catechin gave a single UV peak after UV-3. All of the UV peaks (UV-1, UV-2, and UV-3) decreased according to the addition of trypsin. (A) 0 mg/mL, (B) 0.88 mg/mL, (C) 1.76 mg/mL, (D) 2.64 mg/mL, and (E) catechin, 2 mg/mL. Sample size, 10 μL; column size, 6 × 100 mm; flow rate, 0.1 mL/min; detector, 280 nm.

shows the elution profile of the HPLC analysis. The chromatography conditions were optimized in detail, and three peaks (UV-1, UV-2, and UV-3 in **Figure 2**) were detected. The decreasing of all of the peaks depended upon the addition of the trypsin. Catechin was detected as the broad peak after UV-3. Catechin, cyanidine chloride, and procyanidin B2 did not provide any inhibition. The elution profile was broad, and no signal decrease was found with the addition of trypsin.

Fractionation by Gel Chromatography with Sephadex G-25. The large-scale open column of Sephadex G-25, which was equivalent to the HPLC analysis, also gave a very similar

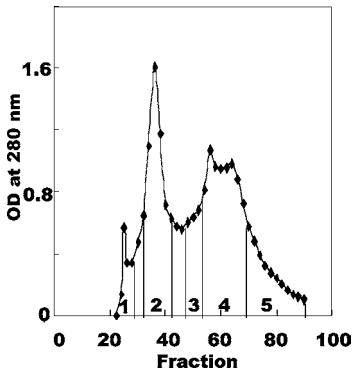

Figure 3. Elution profile of tea tannins on the preparative Sephadex G-25 column. The elution profile agreed well with the HPLC analysis. Numbers 1–5 represent fractionated parts. Each fraction group (parts 1–5) was collected, condensed, and freeze-dried. Sample size, 40 mg; column size, 50 × 5000 mm; flow rate, 0.3 mL/min; detection, 280 nm.

elution profile of the HPLC analysis. **Figure 3** shows a typical elution profile. The chromatography was repeated 5 times, and five parts were collected, concentrated, and freeze-dried. Part 1 (0.6 mg), part 2 (45.0 mg), part 3 (12.5 mg), part 4 (51.8 mg), and part 5 (30.8 mg) were obtained in the results. **Table 1** summarizes the characteristics of the color reaction using the Folin−Chiocalteu reagent, an optical density at 280 nm, amounts of terminal cathechin, sugar, and gallic acid, and the inhibition activity for trypsin per weight. All fractions were similar in principle; however, the terminal chatechin per weight was part 5 > part 4 > part 3 > part 2 > part 1. The trypsin inhibition activity per weight was strong as part 1 > part 2 > part 3 > part 4 > part 5. The elution profile was similar to the result of the apple tannin reported by Yanagida et al. (*40*). The molecular weight would be part 1 > part 2 > part 3 > part 4 > part 5.

Intercepting Activity for TITC by Amino Acids. Table 2 summarizes the intercepting activity of natural amino acids for the TITC. Natural amino acids, except for Arg and Lys, showed a weak intercepting activity. The intercepting activity of Pro and His was also not strong compared to the activities of Lys and Arg. Tyr has a low solubility at the stated pH, and the saturated concentration was not effective for the intercepting activity. The predicted intercepting activity using curve fitting was stronger than that of aliphatic amino acids; however, the intercepting activity was weaker than that of Lys and Arg. Both Lys and Arg were specifically effective for the intercepting activity for TITC.

Intercepting Activity for TITC by Lysine and Arginine Peptides. Table 3 summarizes the intercepting activity (IA50 values) of various compounds. The IA50 values of Lys, Arg, Lys-Lys, Lys-Arg, Arg-Lys, Arg-Arg, Lys-Lys-Lys, and Arg-Arg-Arg were described in groups a and b of **Table 3**. The di- and tripeptides of Lys and Arg showed a strong intercepting activity. The di- and tripeptides showed that the intercepting activity exponentially increased. The intercepting activities of Lys and Arg were almost equal, while Arg-Lys and Lys-Arg had the same level of activity. Gly, Gly-Gly, and Gly-Gly-Gly (group c) have weak intercepting activities for the TITC, although the activity exponentially increased. This result shows that the bond moiety in the peptides could have a weak intercepting activity and that the activity was due to the side chain of the amino acids.

Investigation of the Activity of the Side Chain of Lys and Arg for TITC. Group e of **Table 3** shows the intercepting activity for the TITC by some analogues having the partial side chain of Lys and Arg: ammonium chloride, guanidine HCl,

Table 1. Characteristics of Fractions Obtained from Preparative Exclusion Chromatography of Sephadex G-25[a]

part number	terminal catechin[b] (mg/g)	sugar[c] (mg/g)	gallic acid[d] (mg/g)	Folin–Chiocalteu[e] (mg/g)	OD_{280}[f]	inhibition activity[g] (IU/mg)
part 1	30.1 ± 2.6	90.1 ± 5.3	4.7 ± 0.4	2.0 ± 0.05	17.7 ± 1.1	350.6 ± 19.9
part 2	72.8 ± 3.3	93.3 ± 10.6	36.4 ± 0.7	2.1 ± 0.03	18.9 ± 0.6	158.5 ± 6.2
part 3	103.2 ± 8.8	111.6 ± 10.6	43.2 ± 4.2	2.2 ± 0.05	19.0 ± 0.9	78.9 ± 4.8
part 4	185.0 ± 4.3	139.4 ± 5.3	90.2 ± 3.4	2.5 ± 0.14	19.2 ± 0.7	50.5 ± 3.2
part 5	195.8 ± 10.4	127.3 ± 5.3	29.8 ± 1.8	2.0 ± 0.04	14.5 ± 1.3	17.0 ± 5.8

[a] The freeze-dried samples of the fraction group of parts 1–5 by repeating preparative Sephadex G-25 column chromatography (**Figure 2**) were estimated on the basis of the color reaction of the Folin–Chiocalteu reagent, absorbance at 280 nm, terminal catechin, sugar, gallic acid, and inhibition of trypsin–caseinolytic activity per weight. Each value was expressed as the average ± SD ($n = 4$). [b] Vanillin assay for proanthocyanidins. The standard was catechin (ref *35*). [c] Phenol–sulfuric acid. Tannic acid was 45.7 ± 3.5 mg/g. The standard was glucose (ref *34*). [d] Determination of gallotannin with rhodanine. Tannic acid of reagent grade was 436.6 ± 8.2 mg/g. The standard was gallic acid (ref *37*). [e] Standard was catechin (4.5 ± 0.11 mg/g) (ref *36*). [e] Optical density at 280 nm of 1 mg/mL aqueous. [f] Inhibition unit (1 IU = amount to inhibit 50% for the trypsin–caseinolytic activity).

Table 2. Intercepting Activity of Natural Amino Acids for the Inhibition of Trypsin–Caseinolytic Activity by Tea Tannins

amino acids	inhibition (%)[a]	amino acids	inhibition (%)[a]
water	100.0	cysteine	105.7
glycine	82.5	serine	93.2
alanine	98.3	threonine	72.9
valine	101.9	aspartic acid	90.4
leucine	112.4	glutamic acid	80.2
isoleucine	98.9	histidine	88.9
proline	82.6	lysine	36.3
phenylalanine	95.8	arginine	27.5
tyrosine	60[b]	asparagine	96.9
tryptophan	99.2[c]	glutamine	83.1
methionine	71.3		

[a] Intercepting activity is expressed as (inhibition with tea and the examined amino acid/inhibition by tea) × 100 (%). The examined concentration was 33.3 mM in the preincubation, except for tyrosine and tryptophan. Each value was an average ($n = 3$), and the error was within ±5%. [b] Tyrosine has a low solubility, and the activity was calculated by extrapolation. [c] Tryptophan also has a low solubility, and the activity was evaluated at 16.7 mM.

and urea. The intercepting activity of the guanidine HCl was similar to the activity of Lys. The guanidyl group of Arg was important for the activity. Urea had a very weak activity. This result meant that the ionized guanidyl or amino group was essential for the intercepting activity. Ammonium chloride had a weak activity; thus, the ϵ-amino group of Lys would be essential. The intercepting activities for the TITC of Lys and Arg were nearly equal; thus, the long methylene moiety could also be important.

Intercepting Activity for TITC by Analogues of Lys. Group f of **Table 3** shows the intercepting activity for the TITC by the side chain analogues of Lys. Both the 6-aminohexanoic acid and 6-amino-1-hexanol showed similar levels of activity; the IA50 values were 29.4 and 20.5 mM (IA50 of Lys = 23.4 mM), respectively. The side chain of the analogues was an essential moiety for the activity. In the formula $H_2N-CH_2-CH_2-CH_2-CH_2-CH_2-R$, the examined R group was the alcohol and carboxylic acid. The R was not essential; therefore, it did not need an amino acid moiety such as a carboxylic acid or amino group.

Effect of the Side-Chain Length of the Aminohexyl Group of Lys. The IA50 values of 1,6-diaminohexane and 1,3-diaminopropane (group f of **Table 3**) were 3.4 and 23.6 mM, respectively. The methylene length of the molecule was important for the intercepting activity of the TITC. 6-Aminohexane and 1,6-dimino-hexane were also examined to investigate the effect of the number of amino groups. 1,6-Diaminohexane was about 6-fold stronger than 6-amino-1-hexanol. The IA50 values of Lys-Lys (5.5 mM) and 1,6-diaminohexane (3.4 mM) were nearly equal. This result also suggested that the peptide bond was not essential for the intercepting activity for the TITC, but the amino group and the methylene moiety of the side chain of Lys and/or Arg were important.

Effects of the Intercepting Activity for TITC by Glycols. Group g of **Table 3** summarizes the PEGs and glycols. The high-molecular PEG showed a very strong intercepting activity for the TITC. Ethylene glycol, propylene glycol, and glycerin were not effective for the intercepting of the TITC. **Figure 4** shows the relationship of the IA50 values versus the polymerization number of the PEGs; an exponential increasing activity for the number of polymers, such as the peptides, was shown. The specific activity per weight was estimated as the strongest in this study, although the polymerization number was higher.

Comparison of the Intercepting Activity for TITC by Histatin 5, Synthetic Histatin 5, in which Alanine is Substituted for Arg and Lys of Histatin 5, the Dimer and Trimer of Arginine, Lysine, Glycine, and Proline, and Gelatin-like Peptides. Figure 5 shows plots of the intercepting activity for the TITC of histatin 5 (peptide of 23 amino acids), peptides of Arg, Lys, Gly, and Pro, and the gelatin-like peptides (Pro-Pro-Gly)$_n$, $n = 5$ or 10. All of the activities exponentially increased with the peptide length. The gelatin-like peptides of (Pro-Pro-Gly)$_n$ were considered as kinds of Pro-rich peptides or proteins. The intercepting activity was not remarkably strong. Histatin 5 showed a strong activity; however, the IA50 value was 24.7 μM, and the activity was at the same level as the gelatin-like peptide (IA50 value of (Pro-Pro-Gly)$_{10}$ = 13.8 μM). The value of histatin 5 was about 1/18-fold of the Lys-Lys-Lys value and was about 2-fold of the (Pro-Pro-Gly)$_{10}$ value. This result suggested that histatin 5 was not special when considering the peptide length. Histatin 5 contained Lys, which is a hydrolyzable amino acid by trypsin, but histatin 5 was not hydrolyzed by trypsin during the preincubation with tea because a new amino group was not detected in the reaction mixture by a fluorescence measurement using the *O*-phthalaldehyde method. The intercepting activity of the synthetic histatin 5, in which Ala is substituted for Arg and Lys of histatin 5 (IA50 = 25.0 μM), was equal to that of histatin 5. The Ala substitution to Lys and Arg of histatin 5 did not show a directive effect. The peptide length could be a factor.

Change in the Elution Profile of the HPLC Analysis of Sephadex G-25 by the Addition of Lys-Lys and 1,6-Diaminohexane. Lys-Lys or 1,6-diaminohexane was a strong interceptor of the tea tannin in the results described above. These compounds quickly produced a precipitate with the tea tannin. The change was investigated using the HPLC analysis of Sephadex G-25. **Figure 6** shows the results. Both compounds produced the decrease in the initial peak and shift change of

Table 3. Intercepting Potentials of Various Compounds for Inhibition of Trypsin–Caseinolytic Activity by Tea Tannins

group	examined compounds	IA50 (mM)[a]	group	examined compounds	IA50 (mM)
	amino acids and peptides			analogues of Arg and Lys	
a	Lys	23.4	e	NH_4Cl	80.2
	Lys-Lys	5.5		guanidine HCl	28.2
	Lys-Arg	4.9		urea	>400
	Lys-Lys-Lys	0.45		6-amino-hexanoic acid	29.4
b	Arg	4.3	f	6-amino-hexanol	20.5
	Arg-Agr	3.7		1,6-diaminohexane	3.4
	Arg-Lys	2.7		1,3-diaminopropane	23.6
	Arg-Arg-Arg	0.48		glycols[b]	
c	Gly	148.7		PEG 200	79.7
	Gly-Gly	66.7		PEG 1000	3.3
	Gly-Gly-Gly	29.3	g	PEG 4000	0.05
d	Pro	140.7		PEG 6000	0.001
	Pro-Pro	37.0		ethylene glycol	>500
	Pro-Pro-Pro	15.5		propylene glycol	>500
	$(Pro\text{-}Pro\text{-}Gly)_5$	1.25		glycerol	>500
	$(Pro\text{-}Pro\text{-}Gly)_{10}$	0.0138			

[a] IA50 (concentration of 50% intercepting activity) was defined as the concentration of a compound that produced the 50% intercepting ratio for the 50% inhibition of the trypsin–caseinolytic activity by tea, that is, a concentration (in millimolars) in which the inhibition of trypsin (50% inhibition) is reduced to 25% by a compound. The values were obtained from the plotting and curve fitting of the inhibitions at four different concentrations ($n = 4$). The detailed methods were described in "Trypsin Assay System" and "Intercepting Activity for the Inhibition of Trypsin–Caseinolytic Activity by Tea Tannin" of the Materials and Methods. [b] The number of PEGs presents the average molecular weight.

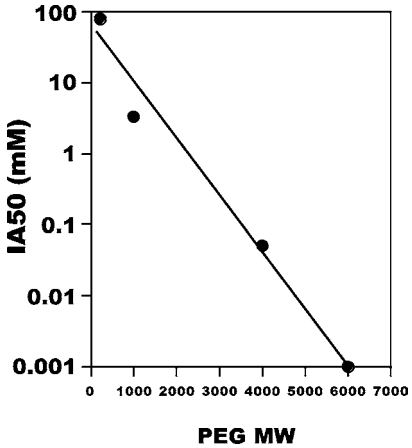

Figure 4. Intercepting activity of PEGs for the inhibition of trypsin–caseinolytic activity by tea. Plots of IA50 (in millimolars) of PEGs (PEG 200, 1000, 4000, and 6000) versus the logarithmic molecular weight. Each plot represents the average of the IA50 values ($n = 4$). The values are same as in **Table 2**.

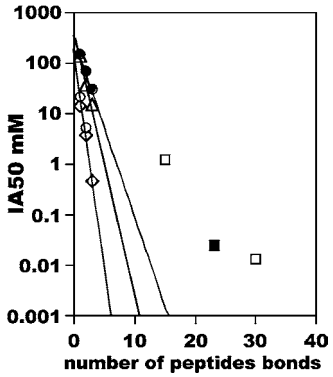

Figure 5. Intercepting activity (IA50 values) of gelatin-like peptides, Gly, Lys, Pro peptides, histatin 5, and synthetic histatin 5 for the inhibition of trypsin–caseinolytic activity by tea tannins. Plots of IA50 values (in millimolars) of monomer, dimer, and trimer peptides of Lys, Arg, Pro, and Gly versus the number of peptide bonds. IA50 values of the gelatin-like peptides of $(Pro\text{-}Pro\text{-}Gly)_5$, $(Pro\text{-}Pro\text{-}Gly)_{10}$, histatin 5, and synthetic histatin 5 in which Ala is substituted for Arg and Lys of histatin 5 were plotted and overlapped ($n = 4$). (○) Lys, Lys-Lys, and Lys-Lys-Lys; (◇) Arg, Arg-Arg, and Arg-Arg-Arg; (△) Pro, Pro-Pro, and Pro-Pro-Pro; (●) Gly, Gly-Gly, and Gly-Gly-Gly; (□) $(Pro\text{-}Pro\text{-}Gly)_5$ and $(Pro\text{-}Pro\text{-}Gly)_{10}$; (■) histatin 5. Hisitatin 5 (IA50 = 24.7 μM) and synthetic histatin 5 (IA50 = 25.0 μM) in which Ala is substituted for Arg and Lys of histatin 5 overlapped. The plots were average values, which were obtained by plotting the inhibitions at the four different concentrations ($n = 4$). The values are same as in **Table 2**.

the latter peaks. The results showed that the compounds would have a stronger interaction to the precipitate with the initial peak of the HPLC and would be a high polymeric group of the tea tannin.

DISCUSSION

Tea tannin was investigated by HPLC and equivalent preparative gel chromatography using Sephadex G-25. The characteristics of the fractions were studied on the basis of the amounts of terminal catechin, sugar, and gallic acid, the color reaction of the Folin−Chiocalteu reagent, the UV absorbance, and the inhibition activity for trypsin per weight, and the interaction with trypsin was investigated by the change in the HPLC elution profile. All of the fractions were principally similar in composition; thus, the size would be the only difference. The equivalent profile of the HPLC and the preparative column chromatography would be useful for various analyses and applications. In the HPLC analysis, trypsin would be combined with the whole tea tannin. The tea tannin and trypsin were coagulated, and the caseinolytic activity of the trypsin was quantitatively inhibited by the tea tannin. The inhibition of trypsin for the caseinolytic activity was the highest in part 1 (**Figure 3** and **Table 1**), but the actual inhibition would be due to the entire tea tannin (**Figure 2**). It was difficult to distinguish the absorbent low-molecular-weight tea tannin and compounds such as catechin; thus, an improvement will be needed (**Figure 2**). Furthermore, the evaluation of the intercepting activity of the amino acids, peptides, PEGs, and their analogues for tea was carried out using the trypsin−caseinolytic system, and the results are summarized as follows: (1) Arg and Lys significantly intercept the inhibition of trypsin by tea, but

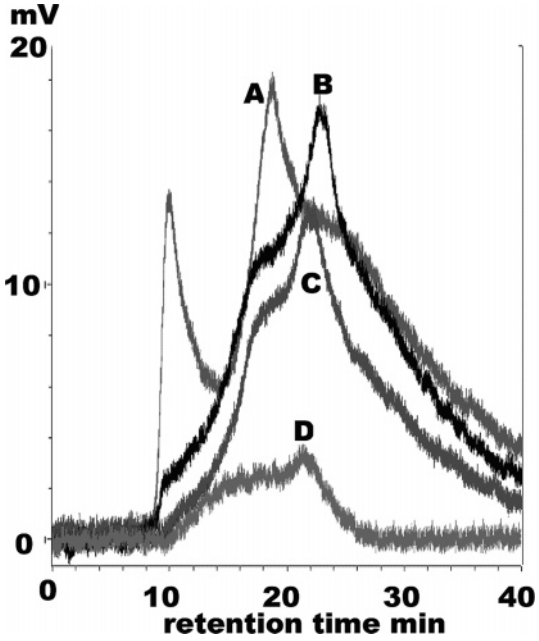

Figure 6. Change of the HPLC elution profile of the tea tannin by the addition of Lys-Lys. The tea tannin and Lys-Lys were mixed, and the supernatant was analyzed by a HPLC column of Sephadex G-25 (fine grade). The change in the elution profile was different from those of trypsin, BSA, or PEGs. Only the initial two UV peaks decreased according to the addition of Lys-Lys. (A) 0 mg/mL, (B) 11.5 mg/mL, (C) 23 mg/mL, and (D) Lys-Lys (11.5 mg/mL). Sample size, 10 μL; column size, 6 × 100 mm; flow rate, 0.1 mL/min (50% ethanol); detector, 280 nm.

the activities of Pro and His were weak. (2) The intercepting activity of the peptides or the polymer of PEG exponentially increased with the length of the peptides or the number of polymerizations. (3) The guanidyl group of Arg and the amino group of Lys were important for producing the strong intercepting activity. (4) The intercepting activity of Lys also depends upon the length of the methylene chain in the side chain. (5) The intercepting activity was weakly dependent upon the peptide bond formation. (6) Histatin 5 strongly intercepted the inhibition of trypsin by tea, but the activity was not remarkable and was within a significant range. (7) High molecular PEGs very strongly intercepted the inhibition of trypsin by tea. The PEG 6000 had the strongest inhibition intercepting activity by weight of the examined compounds.

Many studies of the interaction of the proteins and peptides with tannins have been carried out. Recently, Wróblewski et al. reported the interaction of epigallocatechin gallate (EGCG) or pentagalloyl glucose (PGG) and histatins by NMR, and they showed a strong interaction of Arg, Lys, His, Phe, and Tyr of histatin (*27*). Pro is also expected to be an amino acid of the Pro-rich protein, which has a strong interaction with tannin (*28*−*30*). However, Pro, His, Phe, and Tyr did not have a strong intercepting activity for the TITC in our assay system, but Arg and Lys had strong activities. Arg and Lys showed a stronger effect as the dimer or trimer. It was the same result that Arg and Lys would be important amino acids to bind with tannin or polyphenolic compounds. Wróblewski et al. reported the interactions of histatins and the low molecular phenolic compounds of EGCG and PGG; however, the high molecular group of tannin would be principally the same. Peptide bonding was also expected as the moiety to have the interaction with tannins (*41*). It was considered that the peptide linkage did not have a strong but a weak activity based on the results of the Gly peptides. The intercepting activity of the 1,6-diamino-hexane was the same as that of Lys-Lys, and this result also supported the fact that the peptide bond was not considered to be essential for the activity. This result suggested that the carboxyl and α-amino groups in the amino acid were not needed for the intercepting activity. Urea and ammonium chloride did not have a strong intercepting activity; the activity was not due to only the amino group. The length of the methylene is necessary to have a strong activity because 1,6-diaminohexane was stronger than 1,3-diaminopropane. The inhibitory effect of the methylene chain was considered to affect the activity of the terminal amino group. It would also have a partial hydrophobic-like effect. From the modeling of the peptides of Lys and Arg, it is predicted that the 3−4 methylene chains of the side chains of Lys and Arg could produce a preferential long side chain, which has enough of an end amino group to combine with the tannin (*27*). Lys and Arg are considered to be favorable when compared with other amino acids. Some studies support the fact that Pro was an amino acid having an interaction with tannin (*21*, *29*). It was reported that Pro-Pro was a favorable moiety for binding to the tannin (*29*). However, our results showed that Pro-Pro, Pro-Pro-Pro, and the gelatin-like peptides of $(Pro\text{-}Pro\text{-}Gly)_n$ (n = 5 or 10) were not strong ones.

As for the histatin 5, the arrangement of Arg and Lys in the molecule may be important for the strong binding to the tannin. Seven Arg or Lys peptides are contained in the histatin 5 molecule (*25*−*27*). The Arg-Lys, Arg-Lys-Arg, Arg, and Lys sequences were placed at intervals of 3 or 4 amino acid residues in the histatin. Peptides generally form a helical turn of six peptides; therefore, each location of Arg and Lys would be suitable for binding with the tannin. The IA50 value of the histatin 5 was approximately $^1/_{18}$−$^1/_{19}$ for the Arg or Lys trimers. Peptide formation of the sequential Arg and Lys had a ∼7−10-fold activity per peptide forming. The tetramer or pentamer of Lys or Arg would have an intercepting activity equal to or greater than that of histatin 5. The sequential Arg and Lys peptides are not simply contained in the histatin 5; the peptides were separately placed. Therefore, now, it is a problem of how to evaluate the activity of the molecule (*27*). Direct proof would provide a clear resolution by the histatin 5, which substituted Arg and Lys for Ala (*27*). The synthetic histatin 5 in which Ala is substituted for all of the Arg and Lys had the same intercepting activity as a result (the IA50 values were 24.7 and 25.0 μM). Wróblewski et al. also showed the precipitation activity of the Ala-substituted histatin variants, and PGG was similar to the one of native hishtatin (*27*). Arg and Lys are important amino acids to bind with tannin or polyphenol, but the chain length of the peptides would also be an important factor. Histatins could not provide a remarkably strong binding to the tannin or polyphenols in our study. The tannin-binding activity would be due to the total weak effects of the peptide length or bonding and strong effects of continuous sequences of Arg or Lys; the evaluation method should be studied.

The PEGs showed the strongest intercepting activity for the TITC. The number of polymerizations increased, and as for that effect, it was found that the intercepting activity also exponentially increased. A unit of a single ethylene glycol or propylene glycol did not have any activity. Many regular methylene and ether combinations are made possible by the easy combining of the long molecule of the tannin as well. Some research showed the effects of PEG on the animal feed, which detoxified the tannin with good digestibility (*42*−*46*). The sequential Arg and/or Lys continuing in the peptides would have a greater interaction than the PEG 6000 when it becomes more like octamers. Considering the toxicity of polyamines, PEG is more

economical and may be safer than the polyamine of Arg and/or Lys (*48*−*50*).

As for the fruit and brewing, the tannin and polyphenols are very important compounds in food processing (*18*−*22*). Our HPLC analysis and the results would be useful for understanding the characteristics of the tannin and would allow for a simple and easy analysis. We would like to proceed with the research that uses the tannin assay system in the agricultural and food fields.

ABBREVIATIONS USED

TITC, tea-inhibition activity for the trypsin−caseinolytic digestion; PEG, poly(ethylene glycol).

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