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Effect of gallic acid on peptides released by trypsin digestion of bovine α-casein

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In this study, the effects of gallic acid (GA) on trypsin digestion of commercial α-casein (α-CN), which contains α_{s1}-CN and α_{s2}-CN, and the peptides released during digestion were investigated. Gallic acid showed no effect on the initial rate of digestion. However, the apparent degree of hydrolysis achieved its maximum value after 1 h, then decreased in the presence of GA, suggesting the cross-linking between peptides once released from α-CN during digestion. In the presence of GA, three peaks derived from α_{s1}-CN disappeared and three new peaks appeared in high-performance liquid chromatography (HPLC) analysis. In these peptides, two Met residues corresponding to the Met^{135} and Met^{196} in α_{s1}-CN were oxidized to Met sulfoxide residues. The oxidation of Met^{196} was quicker than that of Met^{135}. The inhibitory activity of TTMPLW (α_{s1}-CN 193–199) against angiotensin I-converting enzyme was reduced slightly by the oxidation of its Met residue.

[**Key words:** Angiotensin I-converting enzyme inhibitory activity; α-Casein; Gallic acid; Liquid chromatography/ion-trap time-of-flight mass spectrometry (LC/IT-TOF-MS); Methionine sulfoxide; Trypsin digestion]

Phenolic compounds (PCs) are a diverse group of plant secondary metabolites. More than 8000 PCs are known, and all share the common structural feature of the presence of at least one hydroxyl-substituted aromatic ring moiety (1). Gallic acid (3,4,5-trihydroxybenzoic acid; GA), a PC with well known antioxidant properties, is widely distributed in many medicinal plants (2,3). Gallic acid is known to bind to proteins and key minerals such as iron, zinc and calcium, and affect their bioavailability by forming insoluble complexes (4–6).

Since PCs are frequently present in foods, the interaction of individual food proteins with PCs may influence the functional and nutritional properties of foods (7). In our previous study, the effect of GA on trypsin digestion of bovine β-lactoglobulin (β-LG) was investigated as a model system to evaluate the effect of PCs on the enzymatic hydrolysis of food proteins and their products (8). It was shown that GA oxidized some methionine (Met) residues in the peptides released from β-LG by trypsin digestion. Accordingly, further studies are needed to clarify whether GA induces Met oxidation in peptides from other food proteins during trypsin digestion. In this study, α-casein (α-CN) was selected as a model of food protein with a disordered structure (9,10) which is different from the compact globular structure of β-LG. α-Casein is the major protein of bovine milk, accounting for more than 40% of total milk proteins, and can be subdivided into α_{s1}-CN and α_{s2}-CN. Because of the disordered structure, α-CN can be easily digested by trypsin (11), degraded into numerous peptide fragments by enzymatic proteolysis and serving as a source of bioactive peptides (12). In this study, the process and products of trypsin digestion of α-CN in the absence and presence of GA were characterized using liquid chromatography/ion-trap time-of-flight mass spectrometry (LC/IT-TOF-MS). The effect of Met oxidation on the physiological activity of an α-CN peptide released by trypsin digestion was also investigated.

MATERIALS AND METHODS

Materials α-CN from bovine milk, trypsin from porcine pancreas (T4799), L-leucine (reagent grade), GA, hippuryl-L-histidyl-L-leucine (HHL), hippuric acid (HA) and angiotensin I-converting enzyme (ACE) from rabbit lung were purchased from Sigma–Aldrich (St. Louis, MO, USA). 2,4,6-Trinitrobenzene sulfonic acid sodium salt dehydrate (TNBS), trichloroacetic acid (TCA), sodium dodecyl sulfate (SDS), trifluoroacetic acid (TFA, HPLC grade) and formic acid (HPLC grade) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Acetonitrile (ACN, HPLC grade) and distilled water (HPLC grade) were purchased from Kishida Chemical (Osaka, Japan). Milli-Q water was prepared by treatment with a Milli-Q water purification unit (Elix 3, Millipore, Billerica, MA). TTMPLW peptide (H-TTMPLW-OH, purity 90%) was synthesized by BEX (Tokyo, Japan). All other common reagents and solvents of analytical grade were obtained from Wako Pure Chemical Industries.

Trypsin digestion α-CN was digested by trypsin in the presence or absence of GA as described previously (8). Briefly, α-CN, GA and trypsin were separately

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dissolved in 20 mM potassium phosphate buffer (pH 7.8). α-Casein was mixed with GA and pre-incubated for 10 min at 37°C in a water bath with continuous shaking. Trypsin was then added to the medium. The final concentrations of α-CN, GA and trypsin were 0.5 g/L, 0.5 mmol/L and 0.05 g/L, respectively. Samples (1 mL) were taken after 0 min, 10 min, 20 min, 0.5 h, 1 h, 1.5 h, 2 h, 4 h, and 8 h of trypsin digestion. One milliliter of 10% (w/v) TCA was added to each sample immediately after collection to precipitate protein. After centrifugation at 21,000 ×*g* for 30 min, the supernatants (1 mL) were collected, their pH neutralized by the addition of 236 μL of 1 M Na_2CO_3, and the samples were then freeze-dried for further analysis.

Determination of the degree of α-CN hydrolysis The degree of α-CN hydrolysis (DH) in the presence or absence of GA after trypsin digestion was determined using the TNBS method as described previously (8,13). L-Leucine (0–2.0 mM) was used to generate a standard curve. DH values were calculated using Eq. 1:

$$\mathrm{DH}\% = 100 \times \frac{AN_2 - AN_1}{N_{\mathrm{pb}}} \quad (1)$$

where AN_1 is the amino nitrogen content of the protein substrate before hydrolysis (mg/g protein), AN_2 is the amino nitrogen content of the protein substrate after hydrolysis (mg/g protein), and N_{pb} is the nitrogen content of the peptide bonds in the protein substrate (mg/g protein). The total amino nitrogen content was obtained by hydrolysis of 50 μL of 1 mg native protein in 6 M HCl for 24 h at 110°C in sealed tubes. The values of AN_1, AN_2 and N_{pb} were obtained by referring to the standard curves of absorbance at 340 nm versus mg/L amino nitrogen. These values were then divided by the protein content of the test samples to give milligrams of amino nitrogen per gram of protein.

Characterization and fractionation of peptides by high-performance liquid chromatography The freeze-dried hydrolysates obtained as described in the trypsin digestion section were dissolved in 400 μL of eluent A (0.1% formic acid), and were then analyzed using reversed-phase (RP)-high-performance liquid chromatography (HPLC) with a C18 column (Inertsil ODS-3, 3 μm, 4.6 × 150 mm, GL Sciences, Tokyo, Japan) as described previously (8). Separated peptides were detected by a diode array detector (SPD-M10AVP) at a wavelength of 210 nm. The injection volume was 20 μL and the flow rate was 0.6 mL/min. HPLC was performed under the following conditions: 100% eluent A for 3 min, followed by a linear increase in eluent B (0.1% formic acid in a mixture of ACN and Milli-Q water 60:40), using a 0–45% gradient of B over 90 min, then 100% B for 10 min and finally 100% A for 17 min. Main peaks in the samples after 4 h digestion were isolated by manual fractionation and analyzed by mass spectrometry. The kinetics of the release of some peaks following digestion was analyzed by plotting their relative areas measured by absorbance at 210 nm.

Liquid chromatography–mass spectrometric analysis of peptides Hydrolysates of α-CN following trypsin digestion in the presence or absence of GA for 20 min, 4 h and 8 h as well as fractionated peptides were analyzed by mass spectrometry (MS). HPLC-MS analysis for peak identification was carried out using a Shimadzu LC/IT-TOF-MS equipped with a Prominence HPLC system as described previously (8). An Inertsil ODS-3 column (3 μm, 4.6 × 150 mm, GL Sciences) was operated at 40°C. The column was first equilibrated with eluent A (0.1% formic acid in distilled water). An aliquot (10 μL) of each α-CN hydrolysate and peptide was injected and its components were eluted at a flow rate of 0.6 mL/min using a 0–50% gradient of eluent B (0.1% formic acid in ACN) over 130 min. The column was then rinsed with 100% B for 10 min and re-equilibrated with eluent A for 10 min before the next injection. Electrospray ionization (ESI)-MS was performed in the positive mode under the operating parameters previously described (8). Manual *de novo* sequencing of each peptide was performed with the aid of the ProteinProspector V5.2.2 (http://prospector.ucsf.edu/prospector/mshome.htm).

Oxidation of TTMPLW Peptide TTMPLW (1 mmol) was mixed with GA (1 mmol) in 1 ml of 20 mM potassium phosphate buffer (pH 7.8) and incubated for 4 h at 37°C in a water-bath with continuous shaking. Reverse-phase (RP)-HPLC was then performed to separate TTMPLW and its Met-oxidized form TTM(O)PLW (Figs. S1 and S2). The HPLC system and methods are same as described above with a modified gradient program of eluent: 100% A for 3 min, followed by a linear increase of B from 0 to 45% over 37 min, 100% B for 5 min and finally 100% A for 15 min. The TTMPLW and TTM(O)PLW peptides were isolated by manual fractionation.

Assay for ACE inhibitory activity of TTMPLW and TTM(O)PLW The determination of ACE inhibitory (ACEI) activity was performed by the method of Cushman and Cheung (14) with minor modifications. Hippuryl-L-histidyl-L-leucine (HHL) was dissolved (2.5 mM) in 0.1 M sodium borate buffer (pH 8.3) containing 0.4 M NaCl. ACE was dissolved in the same buffer at a concentration of 8 mU/mL. A mixture containing 50 μL of ACE solution and 100 μL of sample [1×10^{-4}, 1×10^{-3}, 1×10^{-2}, 1×10^{-1}, 1, 10 and 100 μM of TTMPLW and TTM(O)PLW] was incubated at 37°C for 15 min, then 50 μL of HHL solution was added and incubated for 30 min. The reaction was stopped on an ice bath. HA liberated by ACE was determined by RP-HPLC. The flow rate was 1 mL/min with a linear gradient (0–70% in 24 min) of eluent B (100% ACN) in eluent A (0.1% TFA in distilled water). The effluent was monitored with a diode array detector at 228 nm. ACEI activity was calculated according to Eq. 2:

$$\text{Inhibitory activity (\%)} = \frac{A_{\mathrm{control}} - A_{\mathrm{sample}}}{A_{\mathrm{control}}} \times 100 \quad (2)$$

where A_{control} is area of HA peak liberated by ACE using buffer without peptides and A_{sample} is area of HA peak liberated by ACE with peptides. The IC_{50} value was defined as the concentration of peptide in mg/mL required for 50% inhibition of ACE determined by regression analysis of ACE inhibition (%) versus peptide concentration.

RESULTS

Effect of GA on trypsin digestion of bovine α-CN α-Casein was digested for 8 h by trypsin in the presence or absence of GA and the DH of digestion at various time points was determined using the TNBS method (Fig. 1). At the initial phase (0–1 h), GA had no effect on the trypsin digestion of α-CN. However, after 1 h of digestion, the apparent DH of α-CN achieved a maximum value in the presence of GA then decreased, while that of α-CN without GA increased gradually up to 8 h, suggesting the cross-linking occurred between peptides once released from α-CN. This time course is quite different from that of trypsin digestion of β-LG in the presence of GA, in which no maximum value of DH was observed (8).

As in our previous study on β-LG, in the present study, GA solution turned green after 0.5 h, then gradually changed to blue-green irrespective of the presence or absence of α-CN during the incubation. Additionally, black TCA-insoluble precipitates were formed after 1.5 h of digestion in the presence of GA.

Identification of the peptides released from α-CN after trypsin digestion The hydrolysates of α-CN obtained in the presence or absence of GA were analyzed by HPLC and the profiles are shown in Fig. 2. The amino acid sequences of the peptides corresponding to the peaks shown in Fig. 2 were identified using LC/IT-TOF-MS (Table 1). In total, 39 different peptides including three phosphorylated peptides were identified. Among them, 31 and 8 peptides were from α_{s1}-CN and α_{s2}-CN, respectively. This may be due to the fact that α_{s2}-CN accounts for only 10–20% of an α-CN preparation (15). Of these peptides, 19 peptides (corresponding to peaks 1–6, 10, 11, 15–18, 21, 27, 30, 32–34 and 36) may have been released by the action of trypsin, which splits peptide bonds next to Arg or Lys (16). In other peptides, the

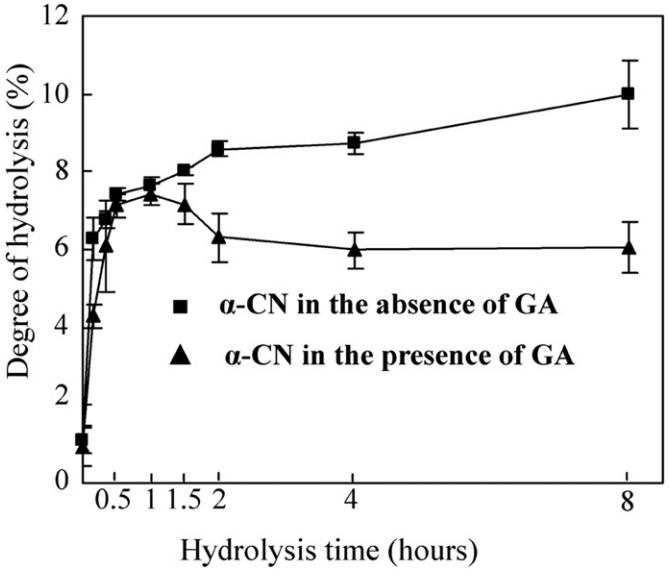

FIG. 1. Effect of GA on the DH of α-CN during trypsin digestion. The DH of α-CN in the presence (filled triangles) or absence (filled squares) of GA during digestion with trypsin (0–8 h) was assayed. Data are expressed as means ± SD (shown as vertical bars) ($n = 3$).