Doctoral Dissertation

Effect of gallic acid on trypsin digestion of bovine milk proteins

(没食子酸が牛乳タンパク質のトリプシン消化に与える影響)

Ping Lai

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Abbreviations

ACEI	Angiotensin I-converting enzyme inhibitory
ACN	Acetonitrile
CID	Collision-induced dissociation
α-CN	α-Casein
DH	Degree of hydrolysis
ESI	Electrospray ionization
GA	Gallic acid
HA	Hippuric acid
HHL	Hippuryl-L-histidyl-L-leucine
LC/IT-TOF-MS	Liquid chromatography/ion trap time-of-flight mass spectrometry
β-LG	β-Lactoglobulin
Met(O)	Met sulfoxide residue
PCs	Phenolic compounds
RP-HPLC	Reversed-phase high-performance liquid chromatography
TCA	Trichloroacetic acid
TNBS	2,4,6-Trinitrobenzene sulfonic acid sodium salt dehydrate

Symbols for amino acids

Three letter symbol	One letter symbol	Amino acid
Ala	A	Alanine
Arg	R	Arginine
Asn	N	Asparagine
Asp	D	Aspartic acid
Cys	C	Cysteine
Glu	E	Glutamic acid
Gln	Q	Glutamine
Gly	G	Glycine
His	H	Histidine
Ile	I	Isoleucine
Leu	L	Leucine
Lys	K	Lysine
Met	M	Methionine
Phe	F	Phenylalanine
Pro	P	Proline
Ser	S	Serine
Thr	T	Threonine
Trp	W	Tryptophan
Tyr	Y	Tyrosine
Val	V	Valine

Abstract of the dissertation

Phenolic compounds (PCs) are frequently present in foods. However, little is known about the effect of PCs on enzymatic digestion process of food proteins and their products. In this thesis, the effect of gallic acid (GA) on trypsin digestion of two bovine milk proteins, β-lactoglobulin (β-LG) and α-casein (α-CN) was evaluated. β-Lactoglobulin has a compact globular structure, whereas α-CN (contains α_{s1}-CN and α_{s2}-CN) possesses an amphipathic primary structure with a disordered conformation. During experiments, milk proteins were mixed with GA, then trypsin was added to the medium and incubated at 37°C. The GA solutions turned green after 0.5 h, then this green color progressively changed to blue-green as the reaction time increased, irrespective of the presence or absence of β-LG and α-CN. Additionally, trichloroacetic acid (TCA)-insoluble precipitates were formed during digestion in the presence of GA. The color change of protein solutions and the black precipitate formed during digestion in the presence of GA, indicated that GA auto-oxidized to produce colored quinones, which might precipitate peptides.

2,4,6-Trinitrobenzene sulfonic acid sodium salt dehydrate (TNBS) method was used to determine the degree of hydrolysis (DH) of these two proteins in the presence and absence of GA during trypsin digestion. The results showed that GA had almost no effect on the initial rate of their digestion. In the presence of GA, the apparent DH of α-CN achieved a maximum value after 1 h digestion then decreased, while that of α-CN without GA increased gradually up to 8 h. 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, although it was lower than that without GA after 1.5 h digestion. The peptides released from β-LG and α-CN were characterized by reversed-phase high-performance liquid chromatography (RP-HPLC) and liquid chromatography/ion trap time-of-flight mass spectrometry (LC/IT-TOF-MS). The time courses of the release of individual peptides from these two proteins following digestion were analyzed by plotting the relative area of the RP-HPLC peak corresponding to each peptide versus time. The results showed that the areas of relatively hydrophilic peptides showed similar profiles irrespective of the presence or absence of GA. In contrast, the areas of the majority of hydrophobic peptides with retention time more than 43 min on the RP-HPLC were lower in the presence of GA in accordance with the difference of DHs. These results strongly suggested that the quinones which were generated as auto-oxidized products of GA might have induced cross-linking of hydrophobic peptides, leading to smaller DH values than those obtained in the absence of GA.

In the study of β-LG, three of the four Met residues in β-LG were identified. In the RP-HPLC chromatogram, five peaks disappeared, while four new peaks appeared following trypsin digestion of β-LG in the presence of GA. Met^{7}, Met^{24} and Met^{145} in the peptides corresponding to these four new peaks were all oxidized to their Met-sulfoxide form after 20 min digestion in the presence of GA. In the case of α-CN, three of the five Met residues in α_{s1}-CN were identified. In the RP-HPLC chromatogram, four peaks disappeared, while three new peaks derived from α_{s1}-CN appeared in the presence of GA. Two Met residues, Met^{135} and Met^{196} in the peptides corresponding to these three new peaks were oxidized to Met sulfoxide residues. The

oxidation of Met^{196} was quicker than that of Met^{135}. Another identified Met^{54} in α_{s1}-CN remained intact even in the presence of GA.

Finally, to clarify whether Met oxidation influences physiological properties of peptides, the effect of GA-induced Met oxidation on angiotensin I-converting enzyme inhibitory (ACEI) activity of a peptide TTMPLW (α_{s1}-CN 93–199), was also investigated. The result showed that the ACEI activity of TTMPLW was only slightly reduced by the oxidation of its Met residue.

As far as the author knows, this is the first report that describes how GA affect the trypsin digestion processes and products of food proteins, and elucidates the Met-oxidation occurrence pattern of produced peptides during digestion.

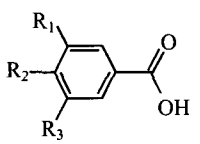
Chapter 1

General introduction

1.1 Phenolic compounds

Phenolic compounds (PCs) are a diverse group of chemicals. They are widely distributed in the plant kingdom and, therefore, commonly found in plant-based foods and beverages. More than 8,000 PCs are known, and all share the common structural feature of the presence of at least one hydroxyl-substituted aromatic ring moiety (Balasundram, Sundram & Samman, 2006). PCs range from simple molecules such as phenolic acid, to large highly polymerized compounds, such as tannins. PCs can be divided into several classes based on several principles, such as according to the number of constitutive carbon atoms in conjunction with the structure of the basic phenolic skeleton, or according to a function of the numbers of phenol ring and of the structural elements that bind these rings to one another, in which phenolic acids, flavonoids and tannins are regarded as the main dietary PCs (Figs. 1 and 2) (Bravo, 1998; King & Young, 1999; Manach, *et al.*, 2004; O'Connell & Fox, 2001).

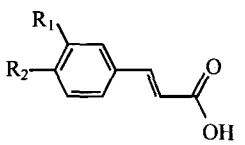
Hydroxybenzoic acids



R_1=R_2=OH, R_3=H: Protocatechuic acid

R_1=R_2=R_3=OH: Gallic acid

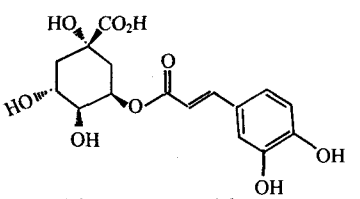
Hydroxycinnamic acids



R_1=OH: Coumaric acid

R_1=R_2=OH: Caffeic acid

R_1=OCH_3, R_2=OH: Ferulic acid



Chlorogenic acid

Fig. 1 Chemical structures of phenolic acids.

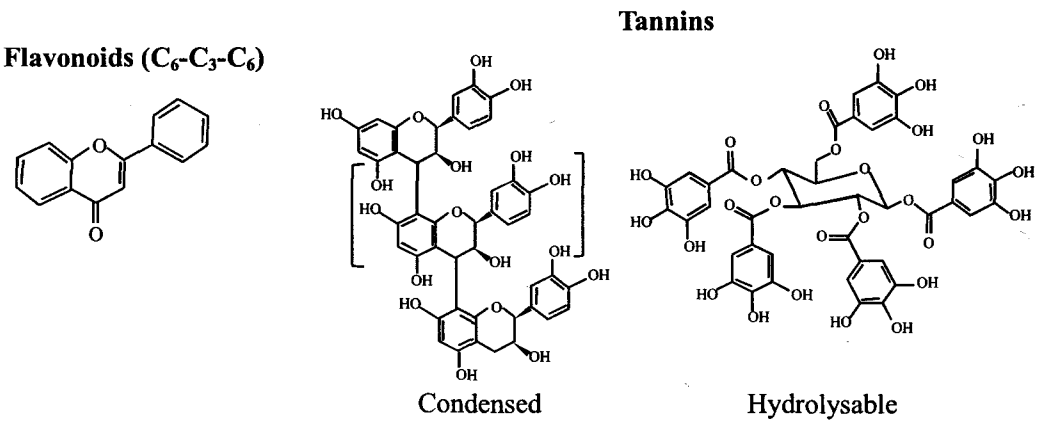
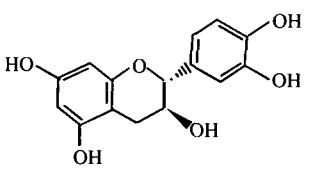


Fig. 2 Basic skeletons of flavonoids and tannins.

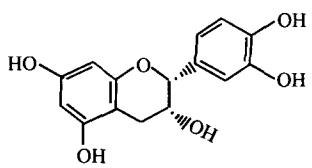
In general, phenolic acids are described as phenols with a phenolic ring and a carboxylic acid group (C_6-C_1 skeleton). They can be divided into three categories, monohydroxybenzoic acids, dihydroxybenzoic acids, and trihydroxybenzoic acids. When describing plant metabolites, phenolic acids also can be referred to two distinct groups of organic acids, hydroxybenzoic acid and hydroxycinnamic acid (Fig. 1) (Robbins, 2003). It widely spreads throughout the plant kingdom, and is responsible for the quality and organoleptic properties of plant-derived foods (Maga & Katz, 1978). There is currently an increasing interest in the potential health benefits associated with phenolic acids and their metabolism. Many investigations have reported that phenolic acids might possess antioxidant and antitumor activities (Rao, *et al.*, 1993; Rice-Evans, Miller & Paganga, 1997).

1.1.1 Gallic acid and its derivatives

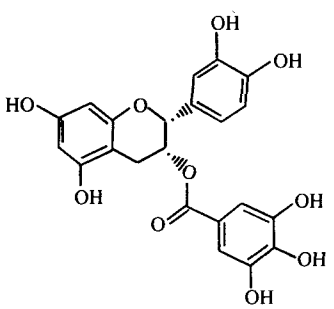
Gallic acid (3,4,5-trihydroxybenzoic acid; GA) (Fig. 1), a PC with well-known antioxidant properties, and its derivatives are widely distributed in plant kingdom (Aruoma, Murcia, Butler & Halliwell, 1993). For example, GA is present in grapes, hops, and various medicinal plants, such as some ayurvedic herbs, *Terminalia chebula*, *Phyllanthus emblica* and Triphala (Borde, Pangrikar & Tekale, 2011; Lu, Nie, Belton, Tang & Zhao, 2006). GA is rich in tea leaves, which contain about 4.5 g/kg of fresh weight (Tomás-Barberán & Clifford, 2000). In plants, it is present in the free form, galloyl conjugates of catechin derivatives, i.e., flanvan-3-ols, or as a part of tannin molecule. Its derivatives, such as propyl gallate, octyl gallate, lauryl gallate and dodecyl gallate, are widely used both as food additives to reduce rancidity and as preservatives (Serrano, *et al.*, 1998; Van der Heijden, Janssen & Strik, 1986).



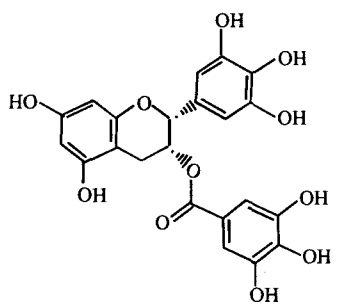
Catechin



Epicatechin



Epicatechin gallate



Epigallocatechin gallate

Fig. 3 Chemical structures of representative flavan-3-ols.

The predominant forms of flavan-3-ols are (+)-catechin, (–)-epicatechin, (+)-gallocatechin, (–)-epigallocatechin and their gallic acid esters: (–)-epicatechin gallate and (–)-epigallocatechin gallate (Fig. 3) (Hollman & Arts, 2000). (+)-Catechin and (–)-epicatechin are epimers and reported to have various physiological properties such as antioxidant activity (Jovanovic, Steenken, Tosic, Marjanovic & Simic, 1994). (–)-Epigallocatechin gallate is the most abundant catechin found in green tea. Several reports have suggested that (–)-epigallocatechin gallate possess many health benefits, including anticarcinogenic, antioxidant, antiangiogenic, and antiviral activities (Nagle, Ferreira & Zhou, 2006).

1.1.2 Phenolic compounds autoxidation

It is reported that PCs display both antioxidant and pro-oxidant effects. The pro-oxidant activity may be due to their auto-oxidation accompanied by the formation of active oxygen forms. Akagawa, Shigemitsu and Suyama (2003) reported that under *quasi*-physiological conditions (pH 7.4, 37°C), GA, (+)-catechin, (-)-epicatechin and other PCs were auto-oxidized, generating intensive hydrogen peroxide (H_2O_2). The proposed mechanism of auto-oxidation of GA and (+)-catechin is shown in Fig. 4. Semiquinones and quinones are highly reactive species that may spontaneously react with phenols, amino acids or proteins, yielding a complex mixture of brown products (Long, Lan, Hsuan & Halliwell, 1999; Mochizuki, Yamazaki, Kano & Ikeda, 2002; Nicolas, Cheynier, Fleuriet & Rouet-Mayer, 1993).

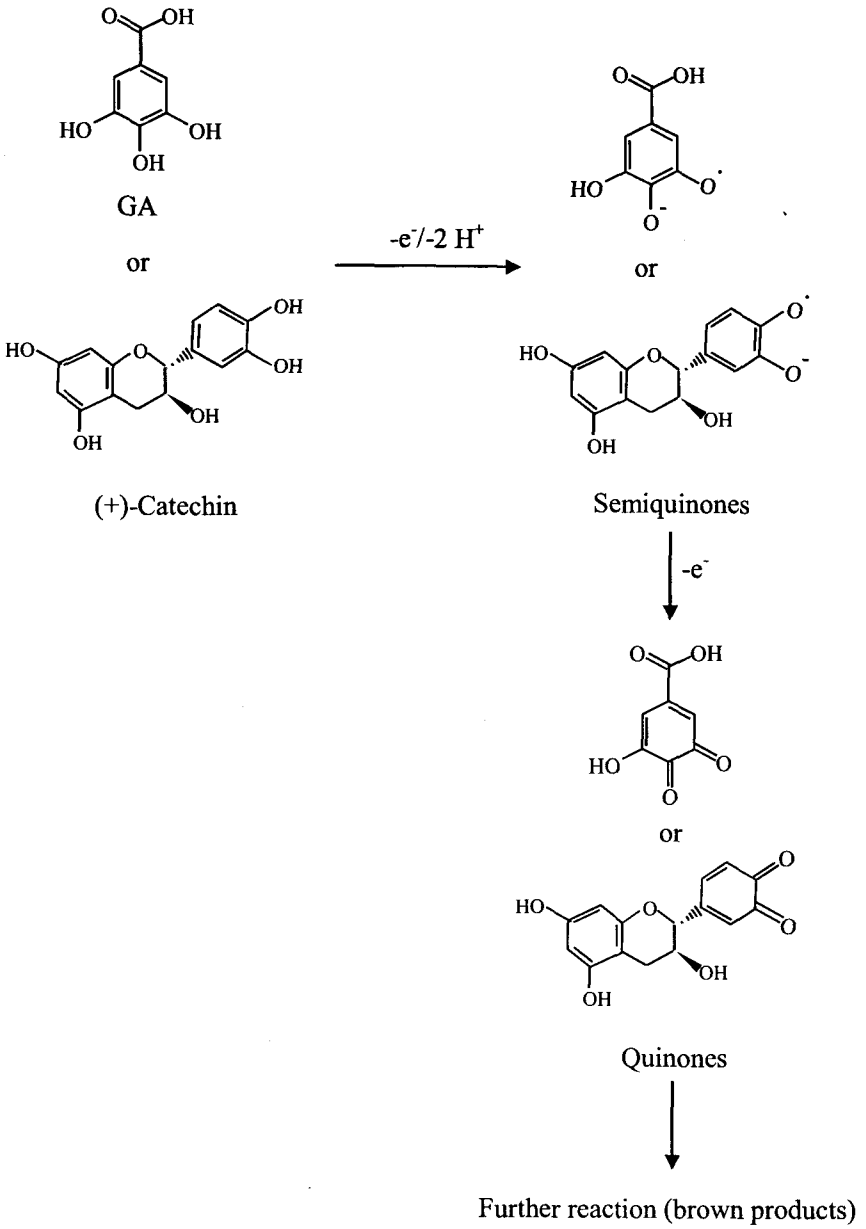


Fig. 4 Proposed mechanism of autoxidation of gallic acid and (+)-catechin.

1.2 Milk proteins

Milk proteins are the only proteins synthesized by mammals in order to feed their offspring. Milk proteins are comprised of around 78.3% caseins, 19% whey proteins and 2.7% miscellaneous proteins (Cheison, *et al.*, 2010). Milk proteins have received much attention as potential ingredients of health-promoting foods for their specific biological properties. At present, milk proteins are considered one of the most important sources of bioactive peptides. These bioactive peptides are inactive within the sequence of parent protein, but they can be liberated through gastrointestinal digestion, fermentation of milk with proteolytic starter cultures, or hydrolysis by proteolytic enzymes. Kitts and Weiler (2003) defined bioactive peptides as specific protein fragments that positively impact on body functions or conditions, and ultimately may influence health. These peptides have been found to have opioid, angiotensin-I-converting enzyme-inhibitory (ACEI), immunomodulating, antimicrobial and antithrombotic activities and so on (Meisel, 2004). Examples of bioactive peptides derived from milk proteins are shown in Table 1 (Meisel, 1998). Functionality of milk protein-derived bioactive peptides and their potential health targets are shown in Fig. 5 (Korhonen, 2009).

Table 1 Examples of bioactive peptides derived from milk proteins

Bioactive peptides	Precursor protein	Bioactivity
Casomorphins	α-Casein, β-casein	Opioid agonist
α-Lactorphin	α-Lactalbumin	Opioid agonist
β-Lactorphin	β-Lactoglobulin	Opioid agonist
Lactoferrosins	Lactoferrin	Opioid antagonist
Casoxins	κ-Casein	Opioid antagonist
Casokinins	α-Casein, β-casein	ACE-inhibitor
Immunopeptides	α-Casein, β-casein	Immunomodulator
Lactoferricin	Lactoferrin	Antimicrobial
Casoplatelins	κ-Casein, transferrin	Antithrombotic
Phosphopeptides	α-Casein, β-casein	Mineral binder

(Cited from Meisel, 1998)

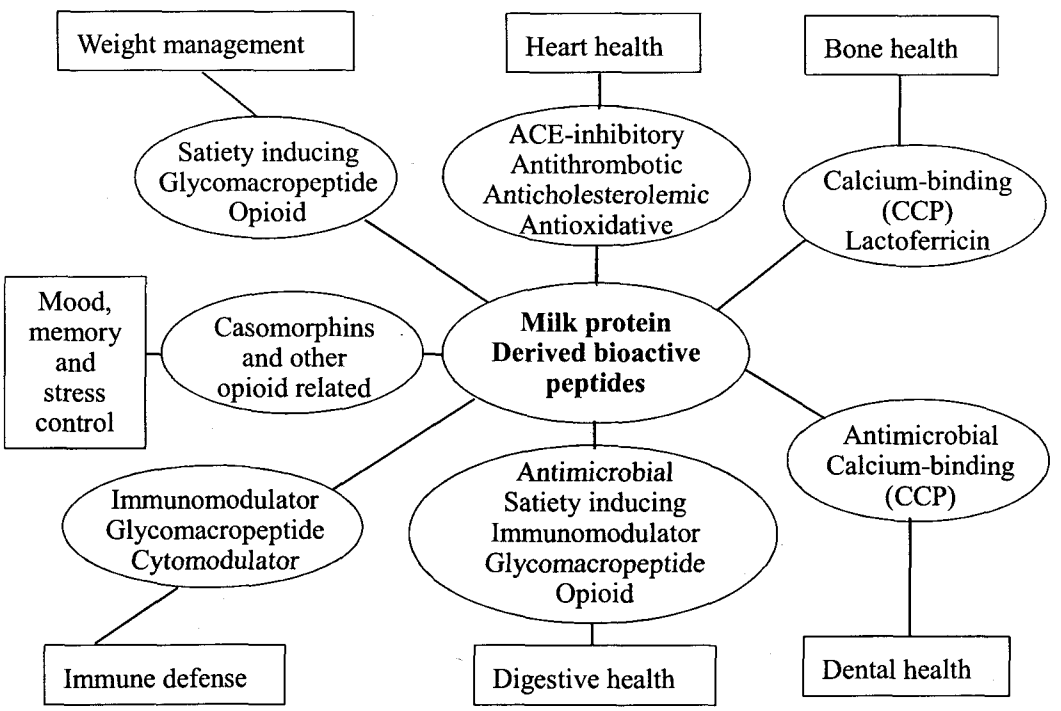


Fig. 5 Functionality of milk protein-derived bioactive peptides and their potential health targets. (cited from Korhonen, 2009)

1.3 Protein–phenolic compounds interactions

PCs frequently occur in milk and dairy products. The occurrence may be a consequence of several factors, such as dietary intake, consumption of particular fodder crops by cattle, and addition as specific flavoring or functional ingredients. For example, addition of polyphenol-rich wine extract to milk and yoghurt as a nutritional additive, has been patented (Howard, Nigdikar, Rajput-Williams & Williams, 2000). PCs can interact with food proteins, which can be either detrimental or beneficial. For example, GA is known to bind with proteins and affect their bioavailability by forming insoluble complexes (Brune, Rossander & Hallberg, 1989; Reddy & Love, 1999; Zhu, Phillipson, Greengrass, Bowery & Cai, 1997). However, knowledge about dietary protein–PC interactions is scarce and mainly limited to their nutritional significance. It is reported that PCs can interact with (or precipitate) proteins, particularly proline-rich proteins, such as the salivary proteins and caseins (Luck, *et al.*, 1994; Spencer, *et al.*, 1988). PCs provide the aromatic nuclei and the hydroxyl groups of the aromatic ring as the principle binding sites for protein-PC complexation. Under mild conditions, the principal cohesive forces between protein molecules and phenolic groups are hydrogen bonding and hydrophobic interaction (Haslam, 1998; Haslam, Lilley & Butler, 1988; Luck *et al.*, 1994; Spencer, *et al.*, 1988). In addition, it is reported that the binding of chlorogenic acid to sunflower proteins involved hydrogen bonding and covalent linkages between oxidized PCs and nucleophilic amino-acid side chains, such as lysine or cysteine (Sastry & Rao, 1990). It is now well known that PCs are very susceptible to oxidation when exposed to air, through a series of biochemical reactions to form their corresponding quinones (Cilliers & Singleton, 1991; Friedman, 1997).

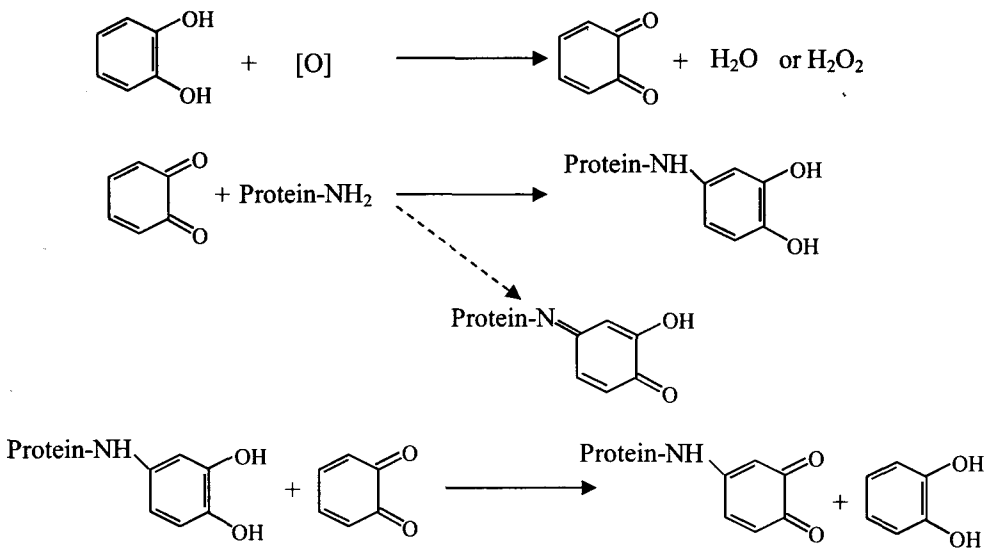


Fig. 6 Proposed mechanisms of phenolic acids auto-oxidation and quinone-protein

The proposed mechanisms of PCs auto-oxidation and quinone-protein interaction are illustrated in Fig. 6. When 1,2-dihydroxy or 1,2,3-trihydroxy phenolic groups are oxidized, quinones are formed (Loomis, 1974). This reaction can occur spontaneously or may be enzymatically catalyzed (Mason, 1955A; 1955B). Quinones are highly reactive, and normally can react further with other quinones (Kroll, Rawel & Seidelmann, 2002). Being reactive electrophilic intermediates, the quinones can readily undergo attack by nucleophiles such as lysine, methionine, cysteine and tryptophan residues in a protein chain, resulting in the cross-linking of protein molecules (Gil-Longo & González-Vázquez, 2010; Hurrell & Finot, 1984; Rawel, Kroll & Rohn, 2001).

1.4 Objective of this study

A broad range of PCs occur in food products. However knowledge about the effect of PCs on functional properties of foods is scarce. In preliminary experiments, GA, (+)-catechin, (–)-epicatechin, ferulic acid and chlorogenic acid were chosen as representative PCs to investigate the effect of their interaction with bovine α-CN, the main protein of bovine milk, on its digestion. As results, no new peptide peak appeared in the high-performance liquid chromatography (HPLC) chromatograms of the products obtained by the trypsin digestion of α-CN in the presence of (+)-catechin, (–)-epicatechin, ferulic acid or chlorogenic acid. On the other hand, some peaks newly appeared in the presence of GA.

Since GA is frequently present in foods, the functional properties of foods may be influenced by the interaction of individual food proteins with GA. However, there is little research about the effect of GA on enzymatic digestion process of food proteins and their products. The objective of this thesis is to determine such effects, using the model system in which the effect of the interaction between GA and two bovine milk proteins, β-lactoglobulin (β-LG) and α-casein (α-CN), on their trypsin digestion were evaluated.

Chapter 2

Effect of gallic acid on trypsin digestion of bovine β-lactoglobulin

2.1 Introduction

The β-LG was selected as a model protein because it is the major whey protein in cows' milk (3 g/L) (Bell & McKenzie, 1964), which is a widely used food. β-LG has a highly ordered secondary structure and a compact globular tertiary structure at physiological pH and temperature. This protein consists of 162 amino acids, and contains two disulfide bridges (Cys^{66}–Cys^{160} and Cys^{106}–Cys^{119}), which stabilize its compact globular conformation, as well as a free thiol group at Cys^{121} (Creamer, Parry & Malcolm, 1983). Five genetic variants of bovine β-LG have been described, of which the variants A and B are predominant. The bovine β-LG variant A differs from the variant B by only two amino acids, Asp^{64} and Val^{118} in variant A are substituted by Gly and Ala, respectively, in variant B (Eigel, *et al.*, 1984). Because of its stable tertiary structure, native β-LG is fairly resistant to hydrolysis by pepsin and chymotrypsin (Reddy, Kella & Kinsella, 1988), although, it is susceptible to porcine trypsin (Madsen, *et al.*, 1997). In the present study, liquid chromatography/ion trap time-of-flight mass spectrometry (LC/IT-TOF-MS) was used to characterize the effect of the presence of GA on the peptide fragments generated by trypsin digestion of bovine β-LG. For excluding the influencing factor of enzyme-PC interaction during digestion, a relatively high enzyme concentration was used in this work, because as a kind of proteins, the enzyme also can be precipitated by PCs which may influence the digestion of proteins (He, Lv & Yao, 2006; Zhu, Phillipson, Greengrass, Bowery & Cai, 1997).

2.2 Materials and methods

2.2.1 Materials

β-LG from bovine milk, trypsin from porcine pancreas, L-leucine (reagent grade), and GA were purchased from Sigma–Aldrich (St. Louis, MO). 2,4,6-Trinitrobenzene sulfonic acid sodium salt dehydrate (TNBS), trichloroacetic acid (TCA), sodium dodecyl sulphate (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 a Milli-Q water purification unit (Elix 3, Millipore, Billerica, MA). All other common reagents and solvents of analytical grade were obtained from Wako Pure Chemical Industries.

2.2.2 Trypsin digestion of β-lactoglobulin in the presence or absence of gallic acid

Trypsin was used to study the trypsin digestion of β-LG protein. For this study, β-LG, GA and trypsin were separately dissolved in 20 mM potassium phosphate buffer (pH 7.8). β-LG 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 β-LG, 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.

2.2.3 Determination of the degree of β-lactoglobulin hydrolysis

The degree of hydrolysis (DH) was determined using the TNBS method that was described previously by Adler-Nissen (1979). Samples and standard solutions were prepared in 1% (w/v) SDS. Duplicate aliquots (0.25 mL) of test or standard solutions were added to test tubes containing 2 mL of sodium phosphate buffer (0.21 M, pH 8.2). The TNBS reagent (0.1% w/v, 2 mL) was then added to each tube, which was then mixed and incubated at 50°C for 1 h in the dark. After this incubation, the reaction was stopped by the addition of 0.1 M HCl (4 mL) to each tube. The samples were cooled to room temperature for 30 min before the absorbance of the solution was measured at 340 nm using an UV-1600 spectrophotometer (Shimadzu, Kyoto, Japan).

L-Leucine (0-2 mM) was used to generate a standard curve. DH values were calculated using the equation [1]:

$$\mathrm{DH}\% = 100 \times \frac{AN_2 - AN_1}{N_{pb}} \qquad [1]$$

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.

2.2.4 Characterization and fractionation of β-lactoglobulin peptides by high-performance liquid chromatography

The freeze-dried trypsin hydrolyzates obtained described in the section 2.2.2 were dissolved in 400 μL of eluent A (0.1% formic acid), and were then analyzed using reversed-phase HPLC (RP-HPLC) with a C18 column (Inertsil ODS-3, 3 μm, 4.6 × 150 mm, GL Sciences, Tokyo, Japan). 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% to 45% gradient of B over 90 min, then 100% B for 10 min and finally 100% A for 17 min. HPLC was performed using an LC workstation (Shimadzu) with CLASS-VP Ver. 6.1 software, consisting of a system controller (SCL-10Avp), a column oven (CTO-10A), an autosampler (SIL-10Axl), and two pumps (LC-10AT). Separated peptides were detected by a diode array detector (SPD-M10AVP) at a wavelength of 210 nm. Main peaks in the samples after 4 h digestion were isolated by manual fractionation and analyzed by mass spectrometry (MS). The kinetics of the release of some peaks following digestion was analyzed by plotting their relative areas measured by absorbance at 210 nm.

2.2.5 Liquid chromatography-mass spectrometric analysis of β-lactoglobulin peptides

Hydrolyzates of β-LG following trypsin digestion in the presence or absence of GA for 20 min, 4 h and 8 h as well as fractionated peptides from HPLC 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 consisting of an SIL-20AC autosampler, LC-20AD pumps, a DGU-20A_5 degasser, a CBM-20A communications bus module, and an electrospray ionization (ESI) interface (Shimadzu). 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 β-LG hydrolyzate 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. ESI-MS was performed in the positive mode under the following operating parameters: mass range, *m/z* 100–2000 in MS and MS^n mode; CDL temperature, 200°C; heat block temperature, 200°C; detector voltage, 1.65 kV; ESI nebulization gas flow, 1.5 L/min; ion accumulation time, 30 ms in MS mode and 100 ms in MS^n mode. Automated data-dependent functions were set to acquire five scans for each precursor detected using the most intense ion signal as a trigger. 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). In addition, the PROWL tool of Rockefeller University (http://prowl.rockefeller.edu/prowl/peptidemap.html) was also used to detect disulfide linkages in peptides (Cheison, *et al.*, 2010).

2.3 Results

2.3.1 Effect of gallic acid on trypsin digestion of β-lactoglobulin

β-LG was digested for 8 h by trypsin in the presence or absence of GA and digestion at various time points was determined by measurement of DH (Fig. 7). GA alone was similarly treated as a negative control.

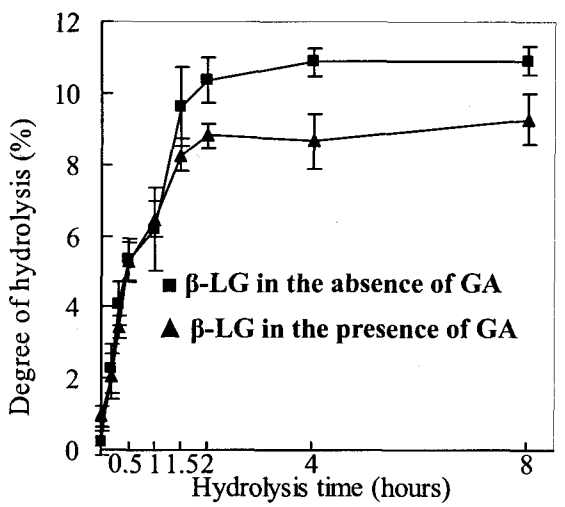


Fig. 7 Effect of gallic acid on the degree of β-lactoglobulin hydrolysis during trypsin digestion. The DH of β-LG in the presence (filled triangles: ▲) or absence (filled squares: ■) of GA during digestion with trypsin (0–8 h) was assayed. Data are expressed as means ± SD (shown as vertical bars) (n = 3).

In Fig. 7, similar curves were observed for β-LG digestion in the presence or absence of GA from 0 to 1.5 h of digestion, indicating that GA did not affect the initial rate of the trypsin digestion of β-LG. However, after 1.5 h of digestion, the DH of β-LG in the presence of GA was slightly lower than that in the absence of GA. During

digestion, the GA solution turned green after 0.5 h. This green color progressively changed to blue-green as the reaction time increased, irrespective of the presence or absence of β-LG. Additionally, a TCA-insoluble precipitate was formed after 1.5 h of digestion in the presence of GA.

2.3.2 Identification of the peptides released from β-lactoglobulin after trypsin digestion

The hydrolyzates of β-LG obtained by trypsin digestion in the presence or absence of GA were analyzed by RP-HPLC. These HPLC profiles are shown in Fig. 8. The amino acid sequences of the peptides corresponding to the peaks shown in Fig. 8 were identified using LC/IT-TOF-MS (Table 2). In total, 30 different peptides, which spanned 86% of the total β-LG sequence, were identified (Fig. 9).

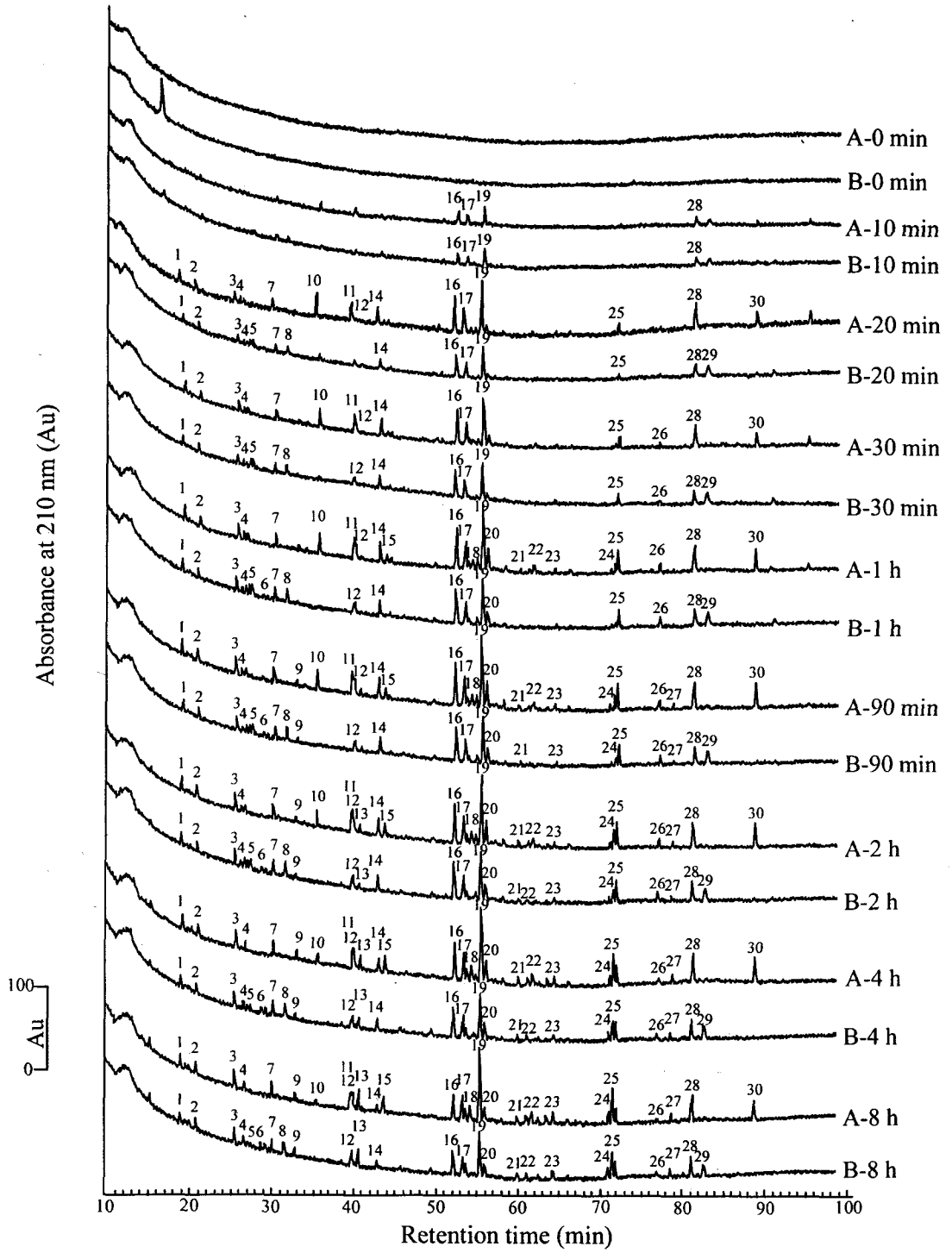


Fig. 8 Reversed-phase high-performance liquid chromatography analysis of the effect of gallic acid on trypsin digestion of β-lactoglobulin. RP-HPLC profiles (λ = 210 nm) of the trypsin digestsion of (A) β-LG or (B) β-LG in the presence of GA, after 0 min, 10 min, 20 min, 0.5 h, 1 h, 1.5 h, 2 h, 4 h and 8 h.

Table 2

Identity of peptides released from β-lactoglobulin by trypsin digestion in the presence or absence of gallic acid

Peak number[a]	Sequence[b]	β-LG residue	*m/z*	
			Observed	Theoretical
1	[L]RVY	40 – 42	437.2446	437.2507
2	[K]IIAEK	71 – 75	573.3604	573.3606
3	[F]KIDALNENK	83 – 91	1044.5670	1044.5684
4	[L]DAQSAPLR	33 – 40	857.4466	857.4476
5	[L]P**M(O)**HIR	144 – 148	669.3550	669.3501
6	[K]ALP**M(O)**	142 – 145	447.1989	447.2272
7	[K]GLDIQK	9 – 14	673.3904	673.3879
8	[-]LIVTQT**M(O)**K	1 – 8	949.5374	949.5387
9	[L]VLDTDYK	94 – 100	853.4300	853.4302
10	[L]PMHIR	144 – 148	653.3612	653.3552
11	[-]LIVTQTMK	1 – 8	933.5406	933.5438
12	[L]DAQSAPL	33 – 39	701.3426	701.3464
13	[R]TPEVDDEALEK	125 – 135	1245.5778	1245.5845
14	[K]VLVLDTDYKK	92 – 101	1193.6806	1193.6776
15	[K]ALPM	142 – 145	431.2258	431.2323
16	[R]TPEVDDEALEKFDK	125 – 138	1635.7687	1635.7748
17	[K]VLVLDTDYK	92 – 100	1065.5796	1065.5827
18	[K]PTPEGDLEILLQKW	48 – 61	1637.6114	1638.8737
19	[K]WENGEC⁺AQKK [R]LSFNPTQLEEQC⁺HI	[61-70] +[c] [149-162]B[d]	2849.3568	2849.10
	[K]WENDEC⁺AQKK [R]LSFNPTQLEEQC⁺HI	[61-70] +[c] [149-162]A[e]	2907.3396	2907.10
20	[K]VAGTWY	15 – 20	696.3258	696.3352
21	[K]WENGEC⁺AQK [R]LSFNPTQLEEQC⁺HI	[61-69] +[c] [149-162]B[d]	2721.2228	2719.20
22	[K]WENDEC⁺AQK [R]LSFNPTQLEEQC⁺HI	[61-69] +[c] [149-162]A[e]	2779.2052	2777.21
23	[K]IPAVF	78 – 82	546.3208	546.3286
24	[Y]VEELKPTPEGDLEIL	43 – 57	1681.8912	1681.8895
25	[Y]VEELKPTPEGDLEILLQK	43 – 60	2052.1336	2051.1271
26	[R]VYVEELKPTPEGDLEILLQK	41 – 60	2313.2662	2313.2588
27	[R]VYVEELKPTPEGDLEIL	41 – 57	1945.0130	1944.0212
28	[K]YLLF	102 – 105	555.3102	555.3177
29	[Y]SLA**M(O)**AASDISLL	21 – 32	1207.6186	1207.6239
30	[Y]SLAMAASDISLL	21 – 32	1191.6268	1191.6209

[a]Peak numbers are consistent with the peak numbers shown in Fig. 9.

[b]The single letter code is used for amino acid residues. The amino acid residues in square brackets are prior to the N-terminal residues of individual peptide. **M(O)** indicates a Met sulfoxide residue.

[c]A "+" indicates a disulfide-link between Cys residues.

[d]B indicates β-LG B variant.

[e]A indicates β-LG A variant.

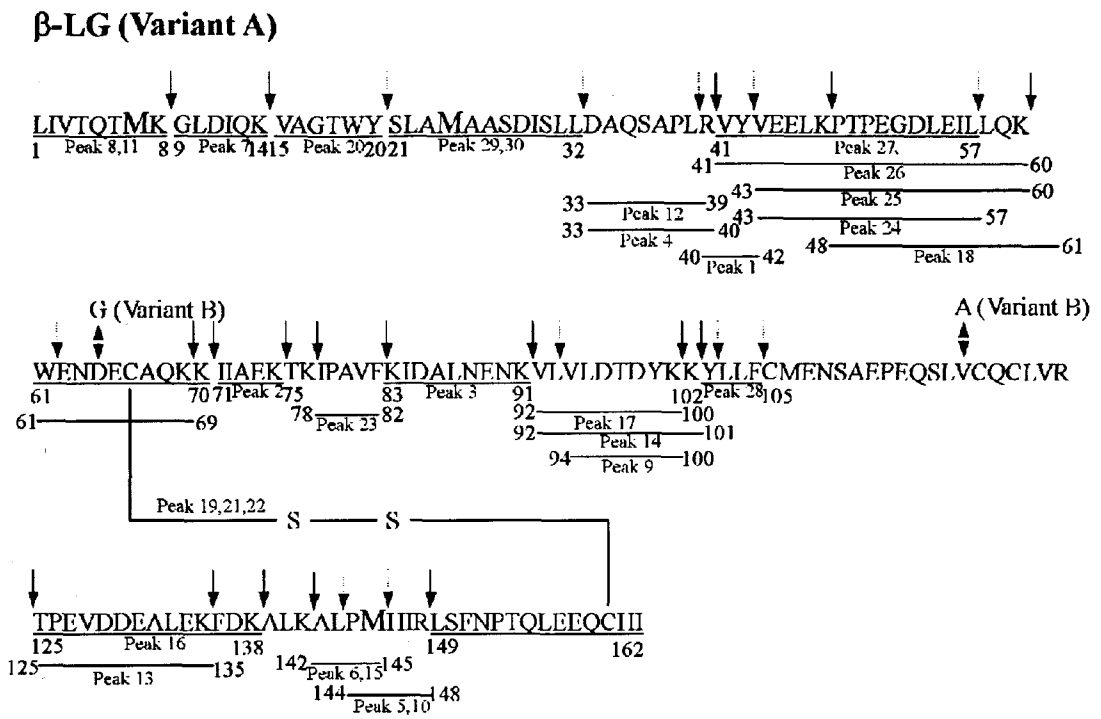


Fig. 9 Assignment of β-lactoglobulin (variants A and B) peptides released by trypsin digestion. Compared to β-LG A, β-LG B has two substituted amino acids: glycine (G) for aspartic acid (D) at position 64 and alanine (A) for valine (V) at position 118. All β-LG peptides that were identified are underlined. Trypsin and chymotrypsin cleavage sites are indicated by solid and dashed arrows, respectively. S–S indicates a disulfide link. A larger font M indicates a Met residue that was oxidized in the presence of GA.

Of these identified 30 peptides, 12 peptides (corresponding to peaks 2, 7, 8, 11, 13, 14, 16, 17, 19, 21, 22 and 26) could have been released by the action of trypsin, which splits peptide bonds next to Arg or Lys (Olsen, Ong & Mann, 2004). However, the release of the other peptides could not be explained by only the action of trypsin. The presence of aromatic amino acid residues prior to the N-terminus, or at the C-terminus, of the peptides in peaks 1, 3, 18, 20, 23–25 and 28–30, suggested the involvement of chymotrypsin in their release (Appel, 1986). Moreover, one of the

hydrophobic amino acid residues, Leu or Met, was situated prior to the N-terminus, or at the C-terminus, of the peptides corresponding to peaks 1, 4–6, 9, 10, 12, 15, 24, 27, 29 and 30. Chymotrypsin might also be involved in the release of these peptides as evidenced in the case of β-lactotensin (β-LG 146–149) released, which was shown to be released with cleavage of peptide bonds next to Met^{145} and Leu^{149} by chymotrypsin (Yamauchi, *et al.*, 2003). Chymotrypsin-dependent release of these peptides during trypsin digestion is possible because commercial trypsin preparations sometimes contain a small amount of chymotrypsin. Therefore, the peptides corresponding to peaks 1, 12, 24, 29 and 30 might be released only by the action of chymotrypsin and the peptides in peaks 3–6, 9, 10, 15, 18, 20, 23, 25, 27 and 28 might be released by the cooperation of trypsin and chymotrypsin. Thus, 18 trypsin cleavage sites and nine chymotrypsin cleavage sites were confirmed in this study (Fig. 9).

It is notable that peaks 10, 11, 15, 18 and 30 were not observed following digestion of β-LG in the presence of GA. On the other hand, the four peaks: 5, 6, 8 and 29, were only observed when β-LG was digested with GA (Fig. 8). These four peaks were collected for MS analysis. The MS/MS spectrum of peak 5 is shown in Fig. 10A. Based on the typical *a*- and *y*-type fragments generated by collision-induced dissociation (CID), this fragment pattern was matched to PM(O)HIR, in which Met^{145} was oxidized to the Met sulfoxide residue [Met(O)], which corresponded to the Met-oxidized form of the peptide of peak 10 (β-LG 144–148). Similarly, the MS/MS and MS/MS/MS spectra of the peptides in peaks 6, 8 and 29 that are shown in Figs. 10B–D matched the Met-oxidized forms of peaks 15, 11 and 30, respectively. Thus, three of the four Met residues in β-LG, Met^{7}, Met^{24} and Met^{145}, were detected as an oxidized form when β-LG was digested in the presence of GA. However, it was

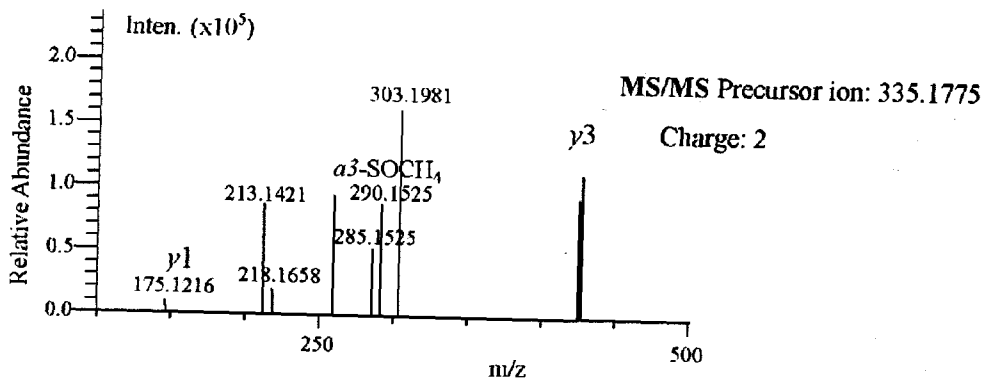
unclear whether Met^{107} was oxidized or not in the presence of GA, since peptides containing Met^{107} could not be detected following digestion, even in the absence of GA.

Several MS-based methods have recently been used to characterize Met(O) in proteins and peptides. MS^n analysis is a particularly attractive method for this purpose, because the peptide sequence and the position of Met(O) can be directly determined using the CID technique. A distinct fragmentation pathway that has been reported in the CID of Met(O)-containing peptides is the neutral loss of 64 Da from the precursor and/or product ions, which is consistent with the loss of methanesulfenic acid (CH_3SOH, calculated mass 63.998) (Lagerwerf, van de Weert, Heerma & Haverkamp, 1996; Schey & Finley, 2000). The fragmentation maps shown in Figs. 10A–C show that the obtained CID-spectra contained almost all of the information required for the detection and localization of the Met(O) in these three small peptides (corresponding to peaks 5, 6 and 8). The product ion *a*3 of peak 5, ion *y*2 of peak 6, and ion *y*2–*y*6 of peak 8 were accompanied by the loss of CH_3SOH (64 Da). The CID of peak 29 only yielded limited backbone fragmentation (Fig. 10D), with two out of a total of 11 inter-residue bonds being cleaved. However, the product ion *a*6 was accompanied by the loss of CH_3SOH, which indicated the occurrence of Met oxidation.

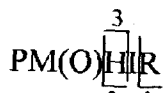
As seen in Fig. 8, peak 19 was the highest peak over the entire period of digestion. Analysis of the obtained MS spectra using PROWL revealed that peak 19, which displayed an *m/z* of 2849.4 and of 2907.3, corresponded to two peptides, Trp^{61}–Lys^{70} and Leu^{149}–Ile^{162}, which were linked by a disulfide bond (Fig. 9). The reason why two different disulfide-linked peptides were obtained is due to the existence of two genetic variants of β-LG, the A and B variants, which have different amino acids at position 64

and 118 (Eigel, *et al.*, 1984). Peak 19 was clearly observed starting from 10 min of hydrolysis and persisted for up to 8 h. No monomeric form of Trp^{61}–Lys^{70} or Leu^{149}–Ile^{162} was detected.

A

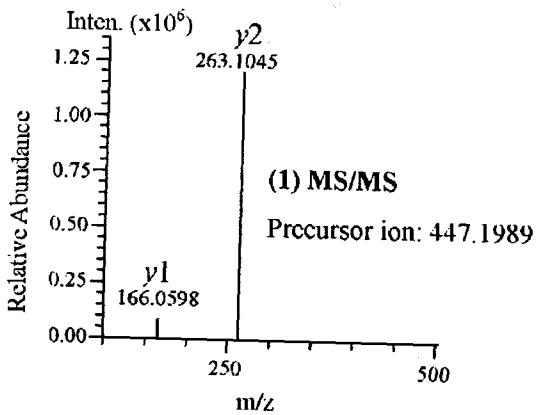


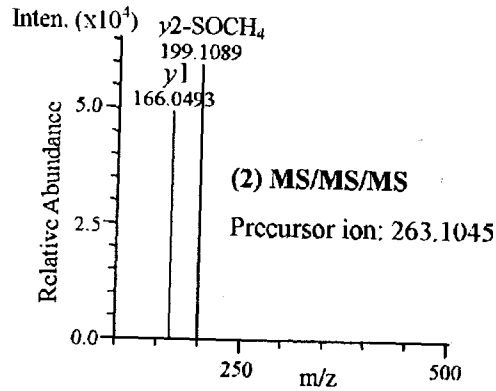
β-LG 144-148 (peak 5) *a* ion



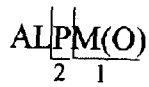
y ion

B



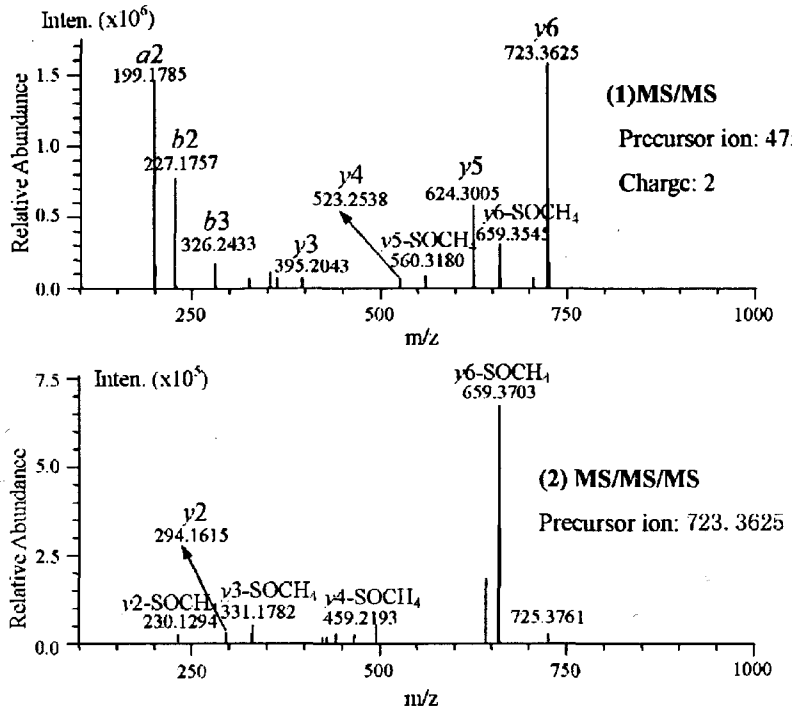


β-LG 142-145 (peak 6)



y ion

C



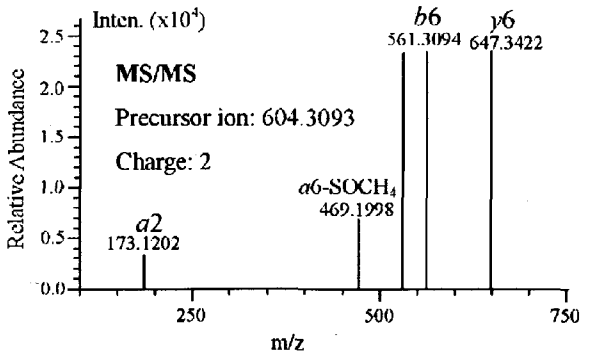
β-LG 1-8 (peak 8) *a*, *b* ion 2 3

LIVTQTM(O)K

6 5 4 3 2

y ion

D



β-LG 21-32 (peak 29) *a*, *b* ion 2 6

SLAM(O)AASDISLL

6

y ion

Fig. 10 Mass spectra of Met-oxidized peptide peaks from β-lactoglobulin digestion in the presence of gallic acid. The mass spectra of (A) peak 5 (*m/z* 335.1775^{2+}, calculated. mass 669.3501); (B) peak 6 (*m/z* 447.1989, calculated. mass 447.2272); (C) peak 8 (*m/z* 475.2687^{2+}, calculated. mass 949.5387); and (D) peak 29 (*m/z* 604.3093^{2+}, calculated. mass 1207.6239) are shown.

2.3.3 Kinetics of the release of individual peptides during trypsin digestion of β-lactoglobulin in the presence or absence of gallic acid

The time course of the release of individual peptides following digestion was analyzed by plotting the relative area of the HPLC peak corresponding to each peptide versus time (Fig. 11). Since peaks 4, 9, 21–23, 26 and 27 were too small to be quantified, and peaks 11 and 24 overlapped peaks 12 and 25, respectively, the areas of these peaks were not plotted. Most peaks appeared at the early phase of digestion. However, peak 13 only appeared after 2 h of digestion. The probable reason for the late appearance of peak 13 is that the peptide corresponding to peak 13 might be released from peak 16 as shown in Fig. 9. Generation of peak 13 from peak 16 may also be the reason why the peak area of peak 16 achieved its maximum at 1.5 h of digestion, after which it then decreased.

It should be noted that the areas of peaks with numbers larger than 14 (15–17, 19, 20, 28 and 30) were reduced during digestion in the presence of GA, while the areas of peaks with smaller numbers (1–3, 7, 10, 13 and 14) were not. This result might be related to the fact that peptides that have a longer retention time in HPLC analysis on an ODS column are more hydrophobic than those with a shorter retention time. Such hydrophobic peptides might associate with each other through hydrophobic interaction, which may facilitate their cross-linking by secondary products derived from GA. As a result, the areas of peaks that were eluted at a later time were selectively decreased. This selective decrease in the area of peaks with higher numbers may also be related to the observed decrease in the DH of β-LG after 1.5 h of trypsin digestion in the presence of GA, as was observed in Fig. 7.

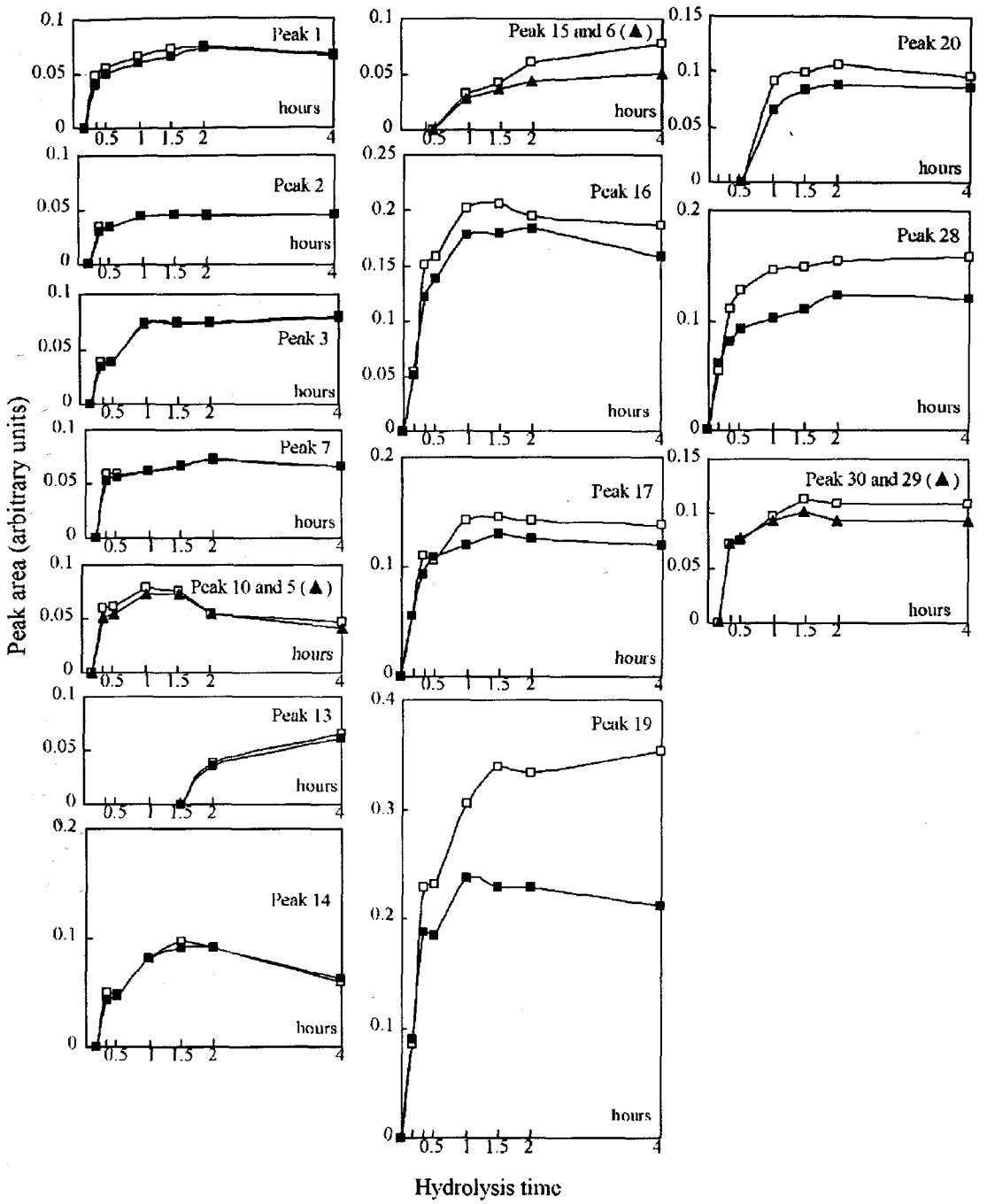


Fig. 11 Quantification of the effect of galic acid on the release of the main β-lactoglobulin peptides by trypsin digestion. The time-dependent release of the main β-LG peptides by trypsin digestion in the presence (filled squares: ■) or absence (open squares: □) of GA was quantified by measurement of the area of the corresponding peaks in the HPLC analysis. Filled triangles (▲) indicate Met-oxidized peptides. Peak numbers are consistent with the peak labels in Fig. 9 and Table 2.

Based on the data shown in Figs. 7 and 11, it is speculated that GA might induce cross-linking of hydrophobic peptides (corresponding to peaks 15–17, 19, 20, 28 and 30), thereby leading to DH values that were lower than those observed in the absence of GA (Fig. 7).

The results showed that GA had almost no effect on the initial rate of trypsin digestion of β-LG. However, after 1.5 h of digestion, GA might have induced cross-linking of hydrophobic peptides (corresponding to peaks 15–17, 19, 20, 28 and 30), thereby leading to DH values that were lower than those observed in the absence of GA. The most striking effect of GA on the peptides that were released from β-LG by trypsin digestion was the oxidation of Met residues to Met(O).

Chapter 3

Effect of gallic acid on trypsin digestion of bovine α-casein

3.1 Introduction

In this study, α-CN was selected as a model of food protein with a disordered structure (Gaspar, Appavou, Busch, Unruh & Doster, 2008; Kumosinski, Brown & Farrell, 1991) 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 (Qi, He & Shi, 2003), degraded into numerous peptide fragments by enzymatic proteolysis and serving as a source of bioactive peptides (Silva & Malcata, 2005). In this study, the process and products of trypsin digestion of α-CN in the absence and presence of GA were characterized using LC/IT-TOF-MS. The effect of Met oxidation on the physiological activity of an α_{s1}-CN peptide released by trypsin digestion was also investigated.

3.2 Materials and methods

3.2.1 Materials

α-CN from bovine milk, trypsin from porcine pancreas, 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. TNBS, TCA, SDS, TFA (HPLC grade) and formic acid (HPLC grade) were purchased from Wako Pure Chemical Industries. ACN (HPLC grade) and distilled water (HPLC grade) were purchased from Kishida Chemical. Milli-Q water was prepared by a Milli-Q water purification unit (Elix 3, Millipore). 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.

3.2.2 Trypsin digestion of α-casein in the presence or absence of gallic acid

α-CN was digested by trypsin in the presence or absence of GA as described in section 2.2.2.

3.2.3 Determination of the degree of α-casein hydrolysis

The DH of α-CN in the presence or absence of GA after trypsin digestion was determined by TNBS method as described in section 2.2.3.

3.2.4 Characterization and fractionation of α-casein peptides by high-performance liquid chromatography

The freeze-dried hydrolyzates obtained as described in section 3.2.2 were dissolved in 400 μL of eluent A (0.1% formic acid), and were then analyzed using RP-HPLC with a C18 column (Inertsil ODS-3, 3 μm, 4.6 × 150 mm) as described in section 2.2.4. Main peaks in the samples after 4 h digestion were isolated by manual fractionation and analyzed by MS. The kinetics of the release of some peaks following digestion was analyzed by plotting their relative areas measured by absorbance at 210 nm.

3.2.5 Liquid chromatography-mass spectrometric analysis of α-casein peptides

Hydrolyzates of α-CN following trypsin digestion in the presence or absence of GA for 20 min, 4 h and 8 h (section 3.2.3) as well as fractionated peptides (section 3.2.4), were analyzed by MS. HPLC-MS analysis for peak identification was carried out using a Shimadzu LC/IT-TOF-MS as described in section 2.2.5. Manual *de novo* sequencing of each peptide was performed with the aid of ProteinProspector V5.2.2 (http://prospector.ucsf.edu/prospector/mshome.htm).

3.2.6 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. RP-HPLC was then performed to separate TTMPLW and its Met-oxidized form TTM(O)PLW (Figs. 18 and 19). The HPLC system and method are same as described in section 2.2.4. 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.

3.2.7 Assay for angiotensin I-converting enzyme inhibitory activity of TTMPLW and TTM(O)PLW

The determination of angiotensin I-converting enzyme inhibitory (ACEI) activity was performed by the method of Cushman and Cheung (1971) with minor modifications. 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 samples [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 the equation [2]:

$$\text{Inhibitory activity}(\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100 \qquad [2]$$

$A_{control}$: area of HA peak liberated by ACE using buffer without peptides; A_{sample}: 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.

3.3 Results

3.3.1 Effect of gallic acid on trypsin digestion of α-casein

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. 12).

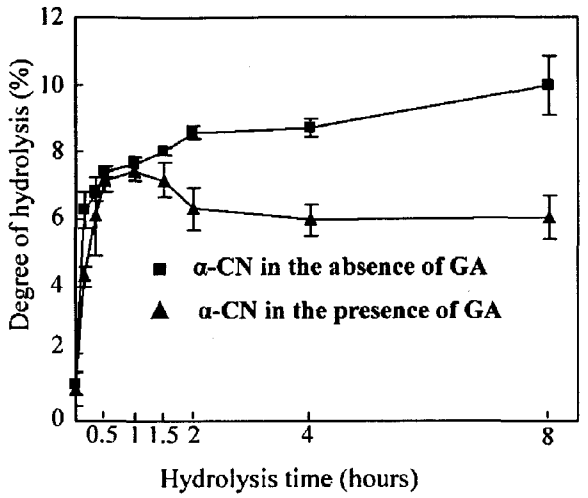


Fig. 12 Effect of gallic acid on the degree of α-casein hydrolysis during trypsin digestion. The DH of α-CN in the presence (filled triangles: ▲) or absence (filled squares: ■) of GA during digestion with trypsin (0–8 h) was assayed. Data are expressed as means ± SD (shown as vertical bars) (n = 3).

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.

As in the study on β-LG, 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.

3.3.2 Identification of the peptides released from α-casein after trypsin digestion

The hydrolyzates of α-CN obtained in the presence or absence of GA were analyzed by HPLC and the profiles are shown in Fig. 13. The amino acid sequences of the peptides corresponding to the peaks shown in Fig. 13 were identified using LC/IT-TOF-MS (Table 3).

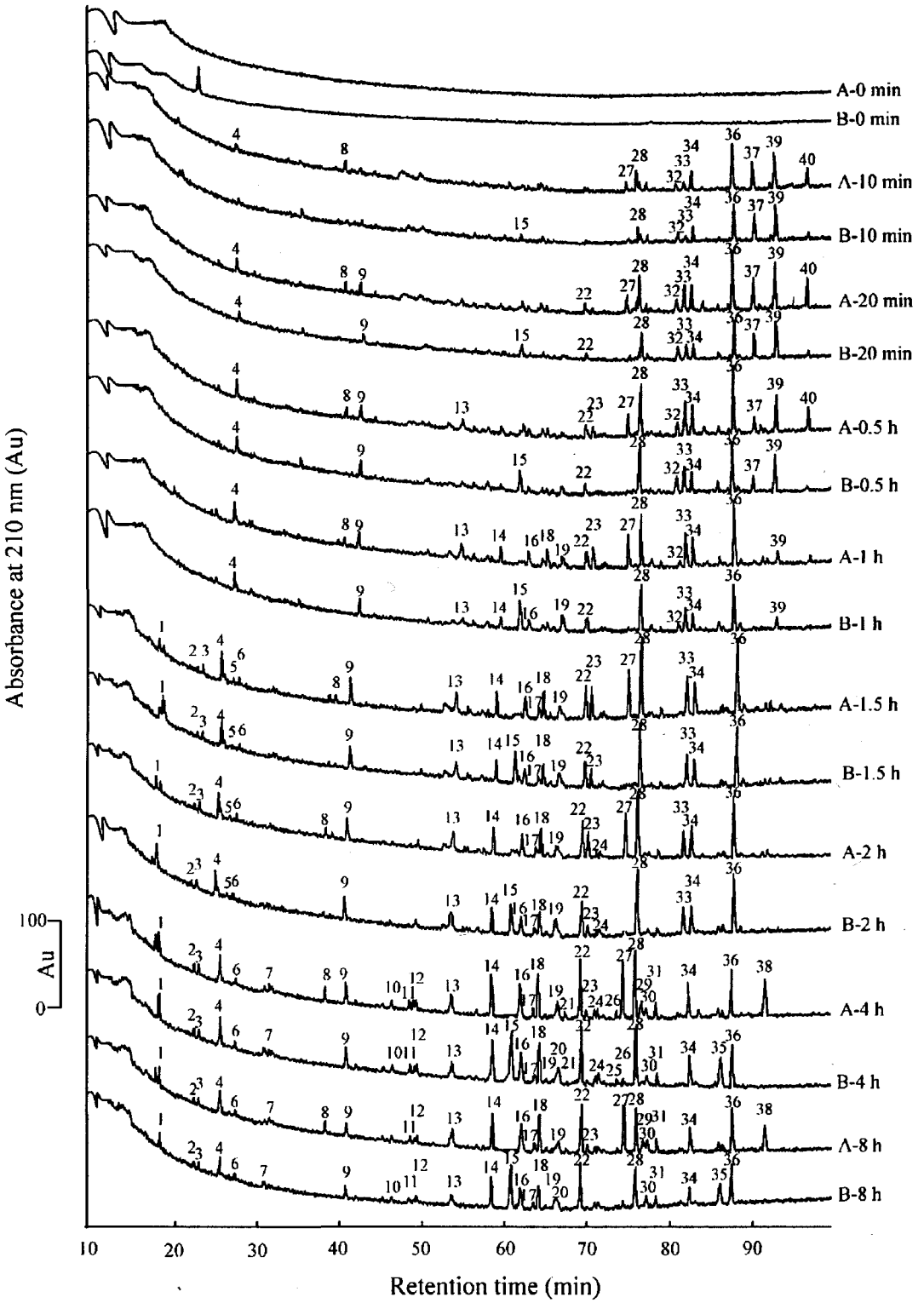


Fig. 13 Reversed-phase high-performance liquid chromatography analysis of the effect of gallic acid on trypsin digestion of α-casein. RP-HPLC profiles (λ = 210 nm) of the trypsin digestion of (A) α-CN or (B) α-CN in the presence of GA after 0 min, 10 min, 20 min, 0.5 h, 1 h, 1.5 h, 2 h, 4 h and 8 h.

Table 3

Identity of peptides that were released from α-casein by trypsin digestion in the presence or absence of gallic acid

Peak Number[a]	Sequence[b]	α-CN Residue	*m/z*	
			Observed	Theoretical
1	[K]EGIHAQQK	α_{s1}125 – 132	910.4608	910.4741
2	[K]EKVNELSK	α_{s1}35 – 42	946.5208	946.5204
3	[K]VNELSK	α_{s1}37 – 42	689.3898	689.3828
4	[K]HIQKEDVPSER	α_{s1}80 – 90	1337.6974	1337.6808
5	[K]ITVDDK	α_{s2}71 – 76	690.3768	690.3668
6	[K]EDVPSER	α_{s1}84 – 90	831.3870	831.3843
7	[Y]LEQLLR	α_{s1}95 – 100	771.4788	771.4723
8	[K]AMKPW	α_{s2}189 – 193	632.3318	632.3225
9	[R]QFY	α_{s1}152 – 154	457.2022	457.2082
10	[R]LNFLK	α_{s2}161 – 165	634.3970	634.3923
11	[R]NAVPITPTLNR	α_{s2}115 – 125	1195.6806	1195.6739
12	[R]YLGY	α_{s1}91 – 94	515.2709	515.2500
13	[F]YPELFR	α_{s1}146 – 151	824.4328	824.4301
14	[Y]YVPLGTQY	α_{s1}166 – 173	940.4763	940.4775
15	[K]TT**M(O)**PLW	α_{s1}194 – 199	764.3636	764.3647
16	[K]HQGLPQEVLNENLLR	α_{s1}8 – 22	1759.9495	1759.9450
17	[K]FALPQYLK	α_{s2}174 – 181	979.5604	979.5611
18	[K]YKVPQLEIVPNS^{P}AEER[c]	α_{s1}104 – 119	1952.9641	1951.9862
19	[Y]FYPELFR	α_{s1}145 – 151	971.4918	971.4985
20	[K]EGIHAQQKEP**M(O)**IGVNQELAYF	α_{s1}125 – 145	2419.162	2418.1758
21	[K]VPQLEIVPNS^{P}AEER[c]	α_{s1}106 – 119	1660.7794	1660.8279
22	[Y]TDAPSFSDIPNPIGSENSEK	α_{s1}174 – 193	2105.9592	2104.9669
23	[Y]QGPIVLNPWDQVK	α_{s2}101 – 113	1493.8124	1493.8111
24	[K]ALNEINQFY	α_{s2}81 – 89	1111.5472	1111.5419
25	[Y]LGYLEQLLR	α_{s1}92 – 100	1104.6618	1104.6412
26	[F]YPELF	α_{s1}146 – 150	668.3359	668.3290
27	[K]TTMPLW	α_{s1}194 – 199	748.3663	748.3698
28	[F]YQLDAYPSGAW	α_{s1}154 – 164	1270.5736	1270.5739
29	[K]EGIHAQQKEPMIGVNQELAYF	α_{s1}125 – 145	2403.1939	2402.1809
30	[K]DIGS^{P}ES^{P}TEDQAMEDIK[c]	α_{s1}43 – 58	1927.6850	1927.6916
31	[Y]LYQGPIVLNPWDQVK	α_{s2}99 – 113	1770.9642	1769.9585
32	[R]FFVAPFPEVFGKEK	α_{s1}23 – 36	1642.8838	1641.8675
33	[K]EGIHAQQKEPMIGVNQELAYFYPELFR	α_{s1}125 – 151	3208.5808	3207.5932
34	[R]YLGYLEQLLR	α_{s1}91 – 100	1267.7094	1267.7045
35	[K]EP**M(O)**IGVNQELAYF	α_{s1}133 – 145	1526.7196	1526.7196
36	[R]FFVAPFPEVFGK	α_{s1}23 – 34	1384.7318	1384.7300
37	[R]QFYQLDAYPSGAWY	α_{s1}152 – 165	1709.7736	1708.7642
38	[K]EPMIGVNQELAYF	α_{s1}133 – 145	1510.7242	1510.7246
39	[Y]YVPLGTQYTDAPSFSDIPNPIGSENSEKTTMPLW	α_{s1}166 – 199	3757.8010	3755.7785
40	n.d.[d]	n.d.[d]	n.d.[d]	n.d.[d]

[a]Peak numbers are consistent with the peak numbers shown in Fig. 2.
[b]The single letter code is used for amino acid residues. The amino acid residues in square brackets are prior to the N-terminal residues of individual peptides. **M(O)** indicates Met sulfoxide residue.
[c]S^{P} indicates phosphorylated Ser residue.
[d]Not determined.

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 (Bernard, Créminon, Yvon & Wal, 1998). 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 (Olsen, Ong & Mann, 2004). In other peptides, the presence of aromatic amino acid residues prior to the N-terminus, or at the C-terminus, suggests the involvement of chymotrypsin in their release (Appel, 1986; Yamauchi, *et al.*, 2003). After all, 80% of amino acid residues in α_{s1}-CN were recovered in peptides identified, while only 27% of amino acid residues in α_{s2}-CN were recovered as shown in Fig. 14.

In the presence of GA, peaks 8, 27, 29, 38 and 40 were not formed. Among them, peak 8 was derived from α_{s2}-CN while peaks 27, 29, 38 were derived from α_{s1}-CN. On the other hand, three new peaks (15, 20 and 35) appeared (Fig. 13). These three peaks were collected for MS analysis. The MS/MS and MS/MS/MS spectra of peak 15 are shown in Fig. 15A. Based on the typical *y* and *b*-type fragments generated by CID, this fragment pattern was matched to TTM(O)PLW, the Met-oxidized form of the peptide of peak 27 (α_{s1}-CN 194–199), in which Met^{196} was oxidized to the Met(O). Similarly, the MS/MS and MS/MS/MS spectra of the peptides in peaks 20 and 35 matched the Met-oxidized forms of peaks 29 and 38, both of which contain Met^{135} residue, respectively (Figs. 15B and C). Thus, two of the five Met residues in α_{s1}-CN, Met^{135} and Met^{196}, were detected in the oxidized form when α-CN was digested in the presence of GA. Met^{54} was identified as the un-oxidized form in peak 30 (α_{s1}-CN 43–58) even in the presence of GA. However, it was unclear whether or not Met^{60} and

Met^{123} were oxidized in the presence of GA, since peptides containing Met^{60} and Met^{123} could not be detected following digestion, even in the absence of GA.

Peak 8 (AMKPW) corresponding to α_{s2}-CN (189–193) also disappeared in the presence of GA. However, new peak corresponding to the oxidized form of peak 8 could not be detected in the presence of GA. Peak 40 also disappeared in the presence of GA. We could not determine the sequence of peak 40 from the MS/MS data. Ionization efficiency of this peak might be too low to be detected by the method.

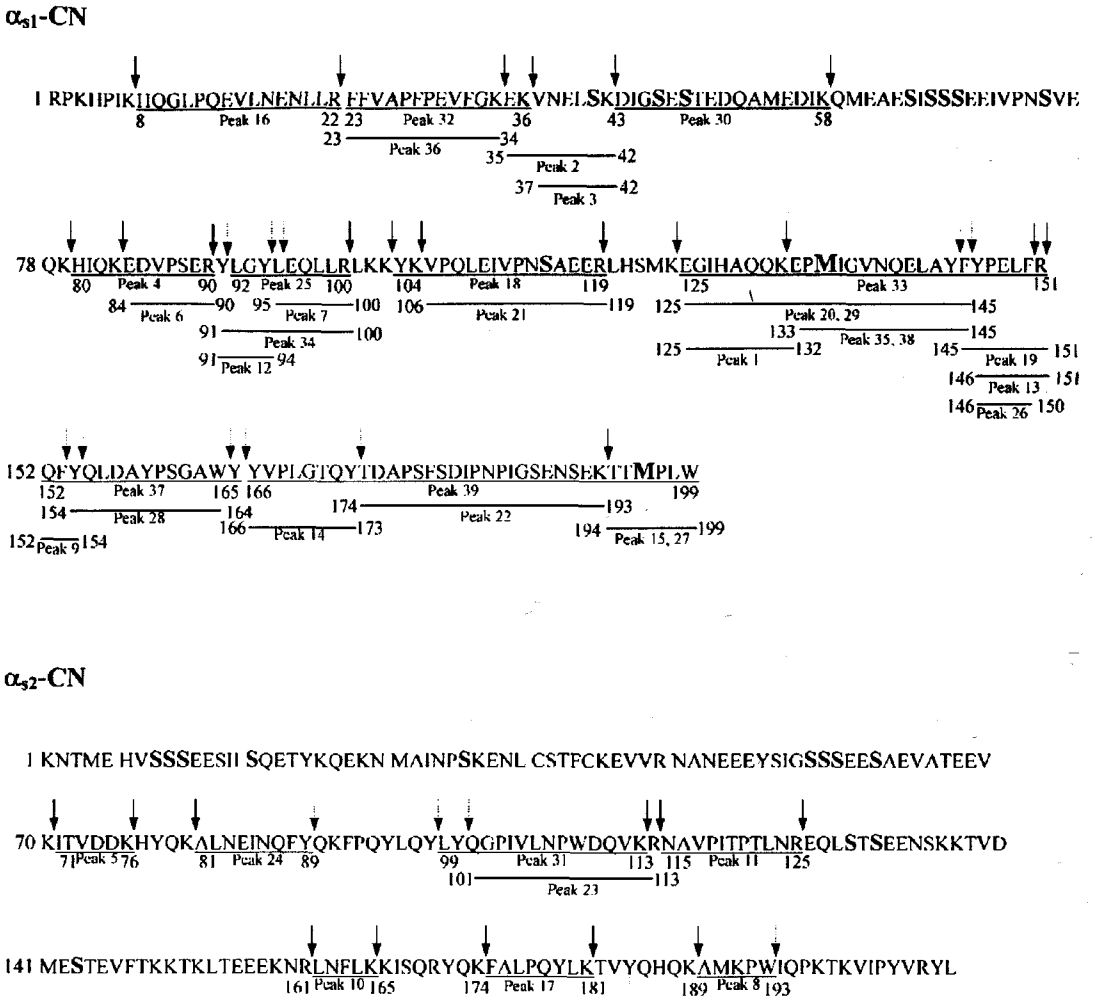
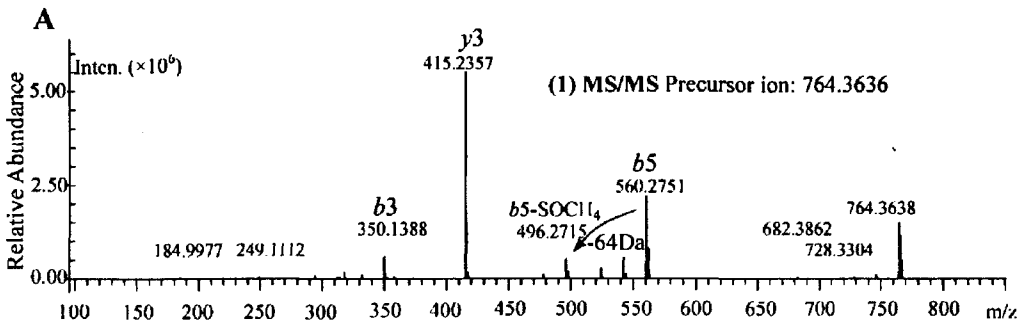
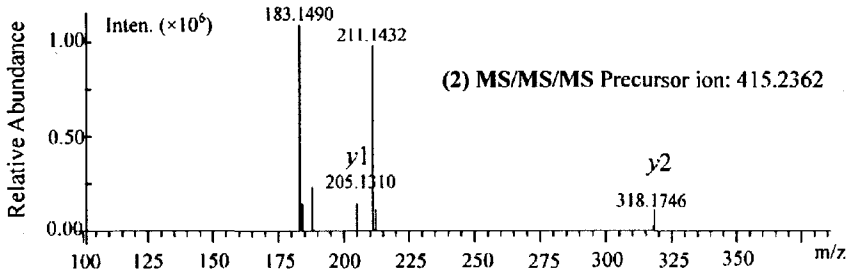
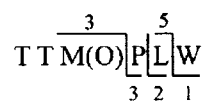


Fig. 14 Assignment of α_{s1}-casein and α_{s2}-casein peptides released by trypsin digestion. All α_{s1}-CN and α_{s2}-CN peptides that were identified are underlined. Trypsin and chymotrypsin cleavage sites are indicated by solid and dashed arrows, respectively. A bold font **M** indicates a Met residue that was oxidized in the presence of GA. A bold font **S** indicates a phosphorylated Ser residue.

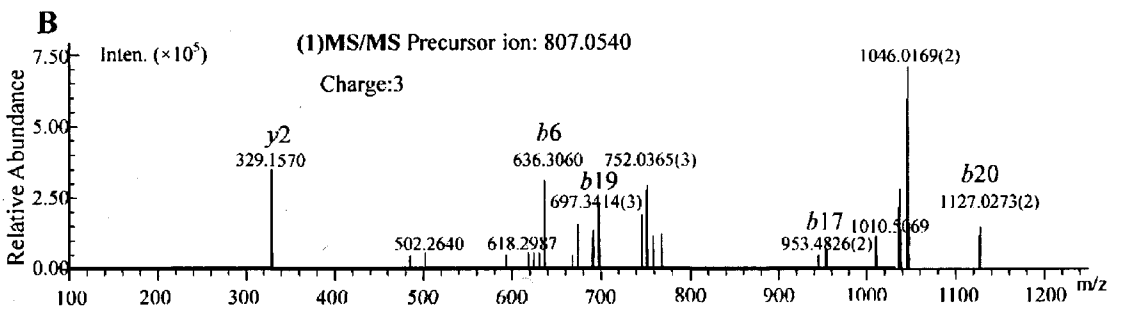


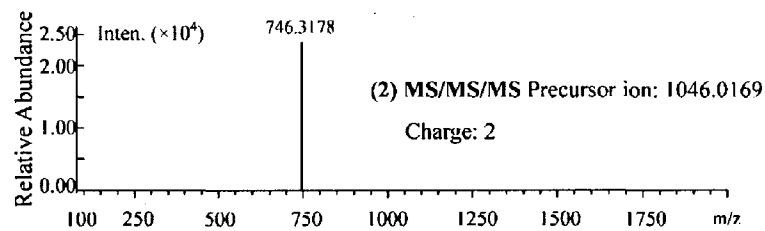


α_{s1}-CN 194-199 *b* ions

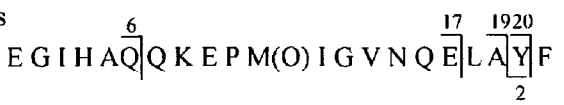


y ions





α_{s1}-CN 125-145 *b* ions



y ions

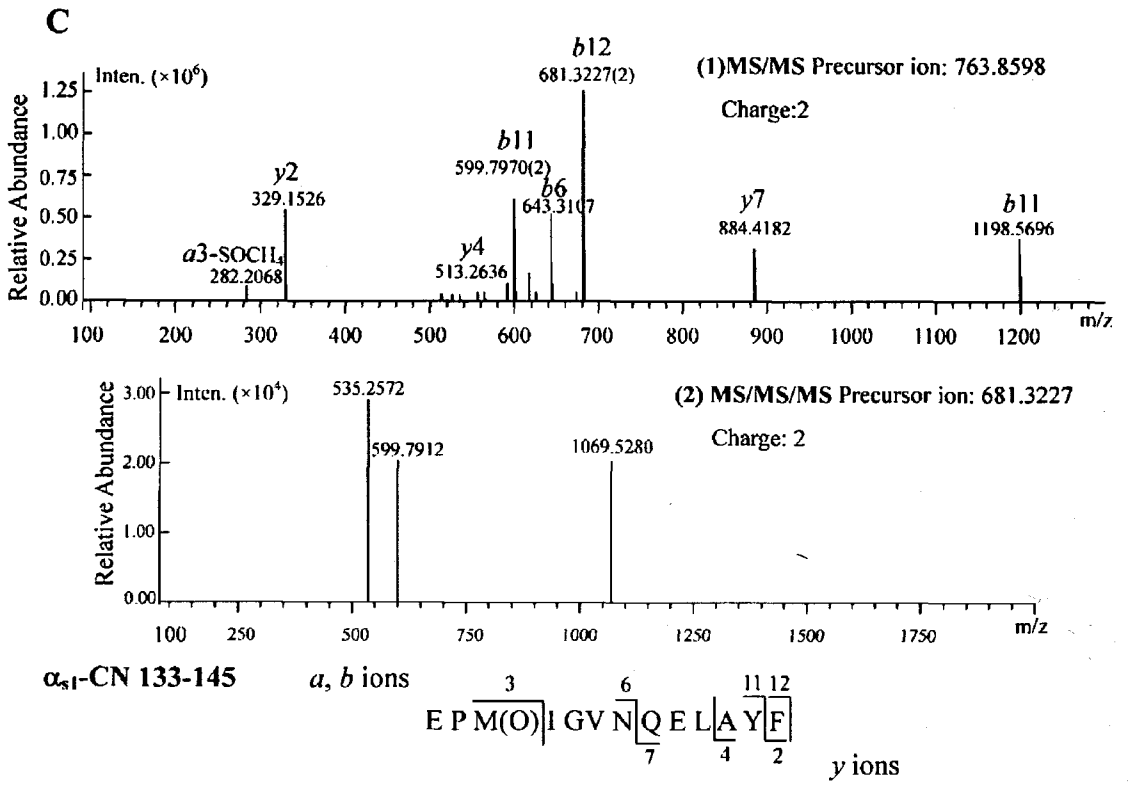


Fig. 15 Mass spectra of Met-oxidized peptide peaks from α_{s1}-casein digestion in the presence of gallic acid. The mass spectra of (A) peak 15 (*m/z* 764.3636, calculated. mass 764.3647); (B) peak 20 (*m/z* 807.0540^{3+}, calculated. mass 2418.1758); and (C) peak 35 (*m/z* 763.8598^{2+}, calculated. mass 1526.7196) are shown.

3.3.3 Kinetics of the release of individual peptides during trypsin digestion of α-casein in the presence or absence of gallic acid

The time course of the release of individual peptides following digestion was analyzed by plotting the relative area of the HPLC peak corresponding to each peptide versus time (Fig. 16). Since peak 1 overlapped the peak of GA, peaks 2, 3, 5–8, 10–12, 17, 21, 24–26, 29 and 30 were too small to be quantified, and peak 19 overlapped peak 20, the areas of these peaks were not plotted.

As can be seen in Fig. 16, the amount of relatively hydrophilic peptides (peaks 4 and 9) showed similar time course irrespective of the presence or absence of GA. In contrast, the amount of majority of hydrophobic peptides (peaks 13, 14, 16, 18, 22, 23, 27, 28, 31, 33, 34, 36 and 38) tended to become lower in the presence of GA, which is a similar pattern to that observed in β-LG. However, peaks 32, 37 and 39 behaved differently compared with other hydrophobic peptides, showing almost the same time course (maximum values after 20 min, disappearing within 1.5 h) regardless of the presence of GA. These peptides may be further degraded during digestion. As shown in Figs. 13 and 16, area of peak 32 (α_{s1}-CN 23–36) was reduced after 1 h of digestion giving rise to peak 36 (α_{s1}-CN 23–34). Similarly, peak 37 (α_{s1}-CN 152–165) was converted to peaks 9 (α_{s1}-CN 152–154) and 28 (α_{s1}-CN 154–164), and peak 39 (α_{s1}-CN 166–199) was converted to peaks 14 (α_{s1}-CN 166–173), 22 (α_{s1}-CN 174–193), and 27 (α_{s1}-CN 194–199) or 15 (Met-oxidized α_{s1}-CN 194–199), respectively. GA-induced cross-linking may not occur on these intermediary peptides since their areas were not affected by GA.

Although peak area was reduced in the presence of GA from early phase of digestion, peak 33 (α_{s1}-CN 125–151) behaved differently compared with the other hydrophobic peptides, decreasing after 1.5 h. Almost parallel to this decrease, peak 38 (α_{s1}-CN 133–145) and its Met-oxidized form peak 35 appeared after 4 h of digestion. These observations suggest that these two peptides were not directly released from α_{s1}-CN but from the intermediary peptide peak 33.

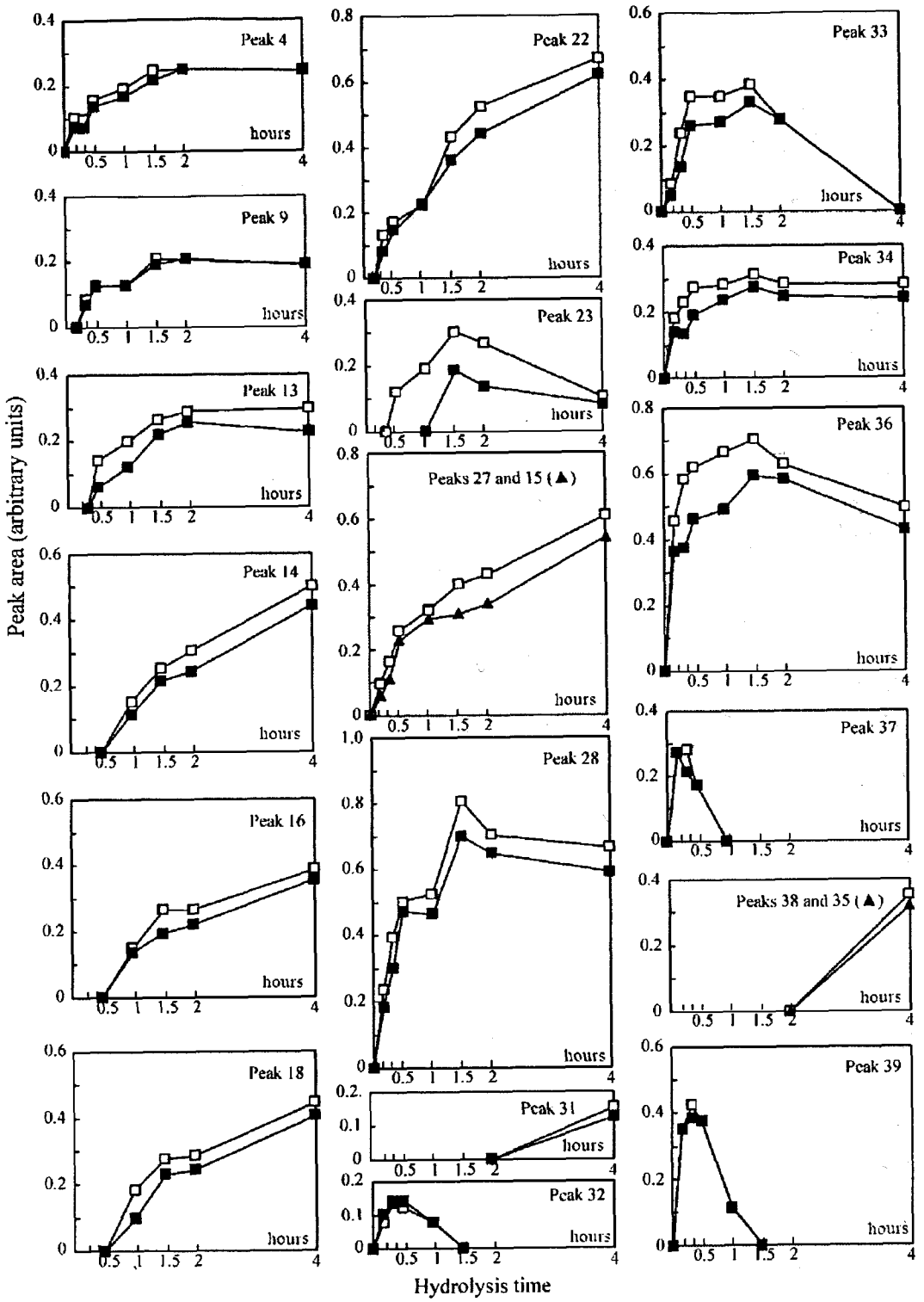

Peak area (arbitrary units)
Peak 4
Peak 9
Peak 13
Peak 14
Peak 16
Peak 18
Peak 22
Peak 23
Peaks 27 and 15 (▲)
Peak 28
Peak 31
Peak 32
Peak 33
Peak 34
Peak 36
Peak 37
Peaks 38 and 35 (▲)
Peak 39
hours
Hydrolysis time

Fig. 16 Quantification of the effect of gallic acid on the release of the main α-casein peptides by trypsin digestion. The time-dependent release of the main α-CN peptides by trypsin digestion in the presence (filled squares: ■) or absence (open squares: □) of GA was quantified by measurement of the area of the corresponding peaks in the HPLC analysis. Filled triangles (▲) indicate Met-oxidized peptides. Peak numbers are consistent with the peak labels in Fig. 14 and Table 3.

Peak 29 (α_{s1}-CN 125–145) and its Met-oxidized form peak 20 are the intermediary forms between peak 33 and peaks 35 and 38, respectively. These two peaks were not plotted in Fig. 16 because their areas were too small to be quantified. However, it is evident from Fig. 13 that peaks 29 and 20 also appeared after 4 h. These suggest that peaks 29 and 20 were also released not directly from intact α_{s1}-CN but from the intermediary peak 33. There is a possibility that peaks 38 and 35 were released not only directly from peak 33 but also via peaks 29 and 20 as a minor route.

Areas of peaks 23, 28 and 36 were partially reduced after 1.5 h of digestion. This might be due to these peptides having sites cleavable by chymotrypsin, although resultant peptides could not be detected.

As well as peak 39 (α_{s1}-CN 166–199) derived from the carboxyl terminus of the protein, TTMPLW, peak 27 (α_{s1}-CN 194–199), and its Met-oxidized form peak 15 could be detected from the early phase of digestion. This suggests that these peptides were released not only via peak 39 but also directly from α_{s1}-CN.

3.3.4 Effect of gallic acid-induced Met-oxidation on angiotensin I-convertion enzyme inhibitory activity of TTMPLW

TTM(O)PLW purified from GA-oxidized TTMPLW was used to study the effect

of Met oxidation on its ACEI activity. The IC_{50} values of TTMPLW and TTM(O)PLW were calculated to be 22.9 and 30.3 μM, respectively. Thus, ACEI activity of TTMPLW was only slightly reduced by the oxidation of its Met residue (Fig. 17).

ACE is one of the major regulators of peripheral blood pressure and acts mainly via the renin-angiotensin system with angiotensin I and bradykinin as natural substrates. Angiotensin I is cleaved by ACE into angiotensin II, a potent vasoconstrictor, whereas bradykinin, which is a vasodilator, is inactivated by ACE. Inhibition of ACE thus leads to decreased blood pressure (Skeggs, Kahn & Shumway, 1956). The peptide TTMPLW from α_{s1}-CN has been reported to exhibit ACEI activity and possess immunomodulatory properties (Maruyama & Suzuki, 1982; Migliore-Samour, Floc'h & Jolles, 1989). In this study, Met oxidation resulted in only slight suppression of the ACEI activity of TTMPLW.

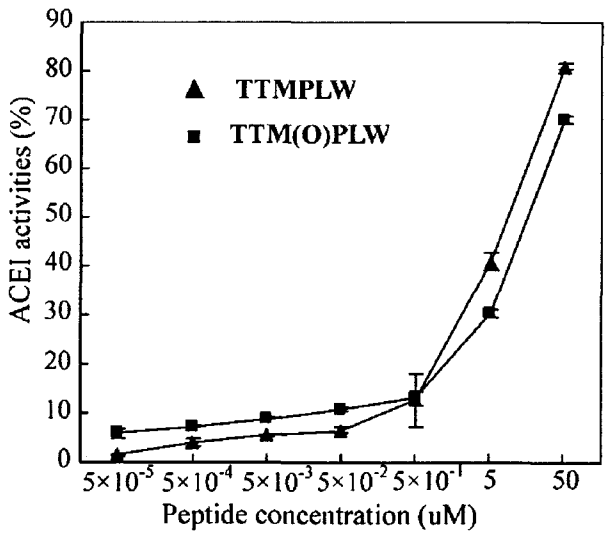


Fig. 17 Angiotensin I-converting enzyme inhibitory activity of isolated TTMPLW (filled triangles: ▲) and TTM(O)PLW (filled squares: ■).

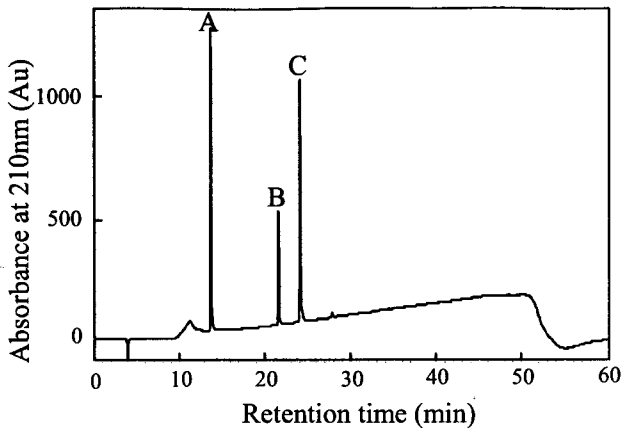


Fig. 18 Reversed-phase high-performance liquid chromatography profile of peptide TTMPLW in the presence of gallic acid. Peptide TTMPLW (1 mmol) (peak C) was mixed with GA (1 mmol) (peak A) in 1mL of 20 mM potassium phosphate buffer (pH 7.8) and incubated for 4 h at 37°C. A portion of TTMPLW were oxidized to TTM(O)PLW (peak B).

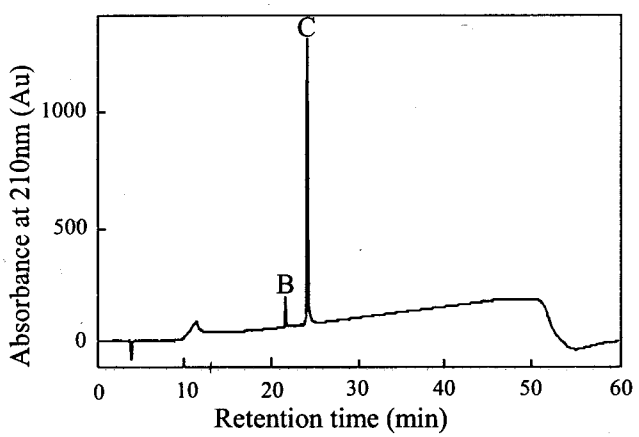


Fig. 19 Reversed-phase high-performance liquid chromatography profile of peptide TTMPLW in the absence of gallic acid. Peptide TTMPLW (1 mmol) (peak C) was incubated for 4 h at 37°C in 1 mL of 20 mM potassium phosphate buffer (pH 7.8). In the absence of GA, a small amount of TTM(O)PLW (peak B) was also formed.

The results show that GA had almost no effect on the initial rate of trypsin digestion of α-CN. However, the apparent degree of hydrolysis achieved its maximum value after 1 h, then decreased in the presence of GA, strongly suggesting that the cross-linking is occurring between hydrophobic peptides once they released from α-CN by trypsin digestion. In the presence of GA, three peaks derived from α_{s1}-CN disappeared and three new peaks appeared in 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.

Chapter 4

Discussion

4.1 Cross-linking between hydrophobic peptides

In the two studies, it was found that GA lowered DH value of β-LG and α-CN after 1.5 h trypsin digestion. Gil-Longo and González-Vázquez (2010) reported that GA spontaneously auto-oxidizes to form quinones in oxygenated aqueous solutions, which is accompanied by the production of hydrogen peroxide (H_2O_2). These quinones further react with other quinones to produce colored compounds of high molecular weight. During experiment, the color of the GA solution changed as the reaction time increased, indicating that GA might have auto-oxidized to produce colored quinones under these conditions. Rawel, Kroll and Rohn (2001) reported that quinones can react with free amino groups of proteins, resulting in the cross-linking of protein molecules. The DH of β-LG and α-CN were lower in the presence of GA than that in the absence of GA from 2 to 8 h of trypsin digestion (Figs. 7 and 12). This effect of GA is probably due to the cross-linking of peptides that was induced by quinones derived from GA. Based on the data shown in Figs. 11 and 16, it is speculated that the quinones derived from GA might induce cross-linking of hydrophobic peptides, thereby leading to DH values lower than those observed in the absence of GA. Such cross-linking of peptides might also be related to the fact that precipitates were formed after 1.5 h of β-LG and α-CN by trypsin digestion in the presence of GA.

As shown in Fig. 11, the areas of hydrophobic peptides with retention time greater than 43 min on the HPLC were lower in the presence of GA in accordance with the difference of DHs. Based on these results, we speculated that cross-linking was occurring between at least some of the hydrophobic peptides following their releases from β-LG during digestion. In Fig. 16, the areas of α-CN hydrophobic peptides eluted

after 43 min on the HPLC also tended to be lower in the presence of GA. These results strongly support the previous speculation that GA quinones may react with free amino groups of peptides, induce cross-linking between hydrophobic peptides once these have been released from proteins during digestion. The reason why the time course of α-CN in the presence of GA gave a maximum value during digestion while that of β-LG did not, might be related to the difference in the ratio of hydrophobic peptides derived from these two proteins. The number of peptides from α-CN with retention time greater than 43 min on the HPLC was more than those from β-LG. α_{s1}-Casein possesses an amphipathic primary structure with polar and hydrophobic domains, and self-associates each other through hydrophobic domains (Swaisgood, 1993). Hydrophobic peptides derived from the hydrophobic domain might associate with each other through hydrophobic interaction, which facilitate the cross-linking between them induced by GA quinones. On the other hand, the number of peptides which areas were not decreased in the presence of GA was only two for α-CN, while it was seven for β-LG (Table 4). These peptides were regarded as hydrophilic from their retention times on HPLC and amino acid compositions.

Table 4
Peptides released from β-lactoglobulin and α-casein after trypsin digestion in the presence of gallic acid

	β-LG	α-CN
Numbers of hydrophilic peptides which peak areas were not reduced by GA	**7**	**2**
Numbers of hydrophobic peptides which peak areas were reduced by GA	**7**	**13**

4.2 Oxidation of Met residues in peptides

It is well-known that methionine can be oxidized to methionine sulfoxide and sulfone during the conservation and storage of feedstuffs. In biological systems, the reaction that yields methionine sulfone is less common than that which yields methionine sulfoxide (Vogt, 1995). It is known that methionine can be extensively oxidized by quinones to its sulfoxide form (Hurrell & Finot, 1984). The ability of GA to function as an antioxidant has recently been extensively studied, and GA has been shown to have potentially positive effects on health (Lu, Nie, Belton, Tang & Zhao, 2006). Moreover, it has been reported that GA may act as a pro-oxidant, thereby stimulating the oxidation of methionine into its sulfoxide form in cell culture media and inducing apoptotic cell death in human myelogenous leukemia cell lines (Sakagami, *et al.*, 1997). In these two studies, GA appeared to act as an oxidizing reagent for the Met residues present in the peptides that were released from β-LG and α-CN by trypsin digestion. Akagawa, Shigemitsu and Suyama (2003) reported that GA is easily oxidized to the corresponding quinones with dioxygen under a *quasi*-physiological pH and temperature (pH 7.4, 37°C), and produces a significant amount of H_2O_2. The quinones and/or H_2O_2 produced by the autoxidation of GA might oxidize Met residues in β-LG and α-CN. It was reported that Met, Cys, Trp, Tyr and His residues on the surface of proteins are susceptible to oxidization by many different forms of reactive oxygen species, including H_2O_2, ozone, hypochlorous acid, alkyl peroxides and peroxynitrite (Kim, Berry, Spencer & Stites, 2001). In these experiments, it is probable that the quinones, rather than the H_2O_2 derived from GA, might act as a mild pro-oxidant to promote the oxidation of Met residues in β-LG and α-CN peptides, since oxidized forms

of peptides containing Cys, Trp, Tyr and His residues were not detected.

In the first study, three of the four Met residues of β-LG were identified in peptides by LC/MS. These three Met residues (Met^{7}, Met^{24} and Met^{145}) were all oxidized to their Met-sulfoxides form after 20 min digestion in the presence of GA. In the second study, three of the five Met residues in α_{s1}-CN were identified. Of these, only two, Met^{135} and Met^{196}, were oxidized to sulfoxides, while Met^{54} remained intact even in the presence of GA. The reason why Met^{54} was resistant to oxidation might be related to the negative charges around Met^{54} which prevent access of GA. During the experiment, peak 15, containing oxidized Met^{196}, appeared after 10 min of digestion, while peak 35, containing oxidized Met^{135}, appeared after 4 h of digestion. This difference might be interpreted as follows. The oxidation of Met^{135} occurs only on peaks 29 and 38 after their slow releases from peak 33, since the Met-oxidized form of peak 33 could not be detected at any time during the digestion process and the peak remained intact in the presence of GA. It seems that Met^{135} may be protected from GA-induced oxidation in the hydrophobic area of intact α_{s1}-CN, or in the hydrophobic core segment of the peptide peak 33 (Kumosinski, Brown & Farrell, 1991). In contrast, the oxidation of Met^{196} may occur quickly in intact α_{s1}-CN, or concurrently with the release of peak 37 from the protein, since the Met-oxidized form of peak 39 could not be detected at any time and the peak remained intact in the presence of GA. At first it was thought that Met residues in α-CN might be oxidized quickly in the presence of GA, because of the disordered structure of α-CN. However, Met^{135} in α_{s1}-CN was oxidized slower than Met^{196}. This means that the susceptibilities of individual Met residues to GA induced oxidation might be related to the hydrophilic environment of the residue in the peptides (Table 5). Thus, it is suggested that the complex time courses as for both apparent DH

and Met-oxidation during the digestion of α-CN in the presence of GA mainly depend on the amphiphatic nature rather than the disordered structure of the protein.

Table 5

Effect of gallic acid on Met-residues in peptides released from β-lactoglobulin and α_{s1}-casein after trypsin digestion

Met residues	β-LG (compact globular protein)	α_{s1}-CN (disordered protein with amphiphatic structure)
Quickly oxidized	3	1 (Met^{196})
Slowly oxidized	0	1 (Met^{135})
Unoxidized	0	1 (Met^{54})
Unidentified	1	2
Total	4	5

Besides GA, the effect of (+)-catechin, (–)-epicatechin, chlorogenic acid and ferulic acid on the trypsin hydrolysis of α-CN have been investigated by HPLC analysis as the preliminary experiment. There was no new peak appeared in the chromatogram with those PCs (data not shown). It is speculated that trihydroxy PCs such as GA and its derivatives, (–)-epicatechin gallate and (–)-epigallocatechin gallate, may induce the Met-oxidation in peptides during proteolytic hydrolysis.

The ACEI assay of TTM(O)PLW and TTMPLW indicated that Met oxidation might not affect biological properties of this peptide. It is reported that both amino acid residues in the C-terminal position and the amino acid sequence might be important for ACEI activity (López-Fandiño, Otte & van Camp, 2006). In this study, the Met-oxidation just resulted in little suppression of ACEI activity of TTMPLW, suggesting that the Met residue is not very important for the ACEI activity of this

hexapeptide. However, the Met-oxidation changes the peptide towards more hydrophilic. With some enzymes, the oxidation of Met residues can lead to loss of biological activity, or sometimes even an increase in activity (Vogt, 1995). So it is necessary to study whether GA affects the bioactivity of other Met-containing peptides released during digestion of food proteins.

Methionine is an essential amino acid that plays several vital physiological roles in the body. Oxidation might influence the nutrient value of methionine. It has been reported that methionine sulfoxide, but not methionine sulfone, can be reduced to methionine in biological systems (Vogt, 1995). If this is the case, then GA-mediated oxidation of Met residues in β-LG and α-CN peptides might not reduce their nutrient value.

The results in this study add new information to current understanding of the interaction of PCs and proteins, indicating some PCs, or at least GA, may affect the digestion process and product of proteins during digestion. This study accentuates the importance of investigation of the effect of different class of PCs on food protein digestion with diverse proteinases to evaluate their accurate products and nutrient values. Since PCs and proteins are frequently-encountered, there is a high possibility that unexpected peptides are released during digestion because of their interaction.

Chapter 5

General conclusion

In conclusion, using two model bovine milk proteins, β-LG (a protein with a compact globular structure) and α-CN (a protein possesses an amphipathic primary structure with disordered structure), the effects of GA on the trypsin digestion of proteins were clarified. Similar results were observed in β-LG and α-CN studies, which are the color change of protein solutions and the black precipitate formed during digestion in the presence of GA, indicating that GA auto-oxidized to colored quinones. The produced quinones might lower the apparent DH value after 1.5 h by inducing the cross-linking between hydrophobic peptides once they released from proteins during digestion. Furthermore, the GA quinones similarly induced the oxidation of some Met residues in their released peptides. Meanwhile, differences were observed between these two studies. In the study on β-LG, it was found that GA quinones lowered the apparent increasing rate of DH after 1.5 h digestion, but not decreased the DH value itself. In the study on α-CN, the apparent DH of α-CN in the presence of GA achieved a maximum value after 1 h of digestion then decreased as the reaction time increased. Additionally, GA quinones had induced Met oxidation in all Met-containing β-LG peptides which could be identified by LC/IT-TOF-MS after 20 min digestion in the presence of GA. However in the study on α-CN, three of the five Met residues in α_{s1}-CN were identified. Of these, only two Met residues were oxidized to sulfoxides at different digestion time.

The ACEI assay showed that ACEI activity of TTMPLW derived from α_{s1}-CN was only slightly reduced by the oxidation of its Met residue, indicating that Met oxidation might not affect biological properties of this peptide.

In interpreting the findings of these studies, two points are worth highlighting. First, GA showed no effect on the initial rate of β-LG and α-CN trypsin digestion.

Second, GA induced two types of reactions on β-LG and α-CN digestion products, oxidation of Met residues and cross-linking between hydrophobic peptides once these have been released from proteins during digestion. Due to the difference between the structures of β-LG and α-CN, the occurrence patterns of GA induced Met oxidation during trypsin digestion were elucidated. It was speculated that susceptibility of individual Met residues to GA induced oxidation in proteolytic digestion might depend on their positions in the peptides.

This is the first study showing that GA influenced the profile of peptide-fragmentation and induced Met-residues oxidation during trypsin digestion of proteins, and elucidating that the positions of individual Met residues in the peptides may influence their susceptibility to GA induced Met-oxidation.

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List of publications

This thesis is based on the work contained in the following papers:

1. Lai, P., Okazawa, A., Izumi, Y., Bamba, T., Fukusaki, E., Yoshikawa, M., and Kobayashi, A.: Gallic acid oxidizes Met-residues in peptides released from bovine β-lactoglobulin by *in vitro* digestion. *Journal of Bioscience and Bioengineering.* 114, 297–305, 2012.

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