Study on the mass spectrometric identification of peptide metabolites from dietary proteins in rat bloodstream

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2022

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Chapter III   Identification of peptide metabolites from orally administered glycinin in rat bloodstream by a Coumarin-tagged MALDI-MS

Abbreviations

- ACE, angiotensin I-converting enzyme
- ACN, acetonitrile
- APDS, 3-aminopyridyl-*N*-hydroxy-succinimidyl carbamate
- AS, ammonium sulfate
- CHCA, α-cyano-4-hydroxycinnamic acid
- Cou, *N*-succinimidyl-7-methoxy-coumarin-3-carboxylate
- CVD, cardiovascular disease
- DBU, 1,8-diazabicyclo[5.4.0]-7-undecene
- DCM, dichloromethane
- DHB, 2,5-dihydroxybenzoic acid
- DMF, *N*, *N*-dimethylformamide
- DRI, Dietary Reference Intake
- EDTA, ethylenediaminetetraacetic acid
- EIC, extracted ion chromatogram
- ESI, electrospray ionization
- FA, formic acid
- FAO, Food and Agriculture Organization
- Gly-Sar, Gly-*N*-methyl-Gly
- GSS, Gly-Sar-Sar
- GSSS, Gly-Sar-Sar-Sar
- GSSSS, Gly-Sar-Sar-Sar-Sar
- HDL, high density lipoprotein
- HPLC, high-performance liquid chromatography
- IS, internal standard
- ITO, indium tin oxide
- LC, liquid chromatography
- MALDI, matrix-assisted laser desorption/ionization
- MeOH, methanol
- MS, mass spectrometry
- *m/z*, mass-to-charge ratio
- NMM, *N*-methylmorpholine
- OLETF, Otsuka Long-Evans Tokushima Fatty
- PepT1, peptide transporter 1
- RT, retention time
- SA, sinapic acid
- SD rat, Sprague-Dawley rat
- SHRs, spontaneously hypertensive rats
- SPI, soy protein isolate
- *S/N*, signal-to-noise ratio
- T2DM, type 2 diabetes
- TEAB, triethylammonium bicarbonate
- TG, triacylglycerol
- THAP, 2′,4′,6′-trihydroxyacetophenone
- TIS, triisopropylsilane
- TJ, tight-junction
- TNBS, 2,4,6-trinitrobenzene sulfonate
- TNP, trinitrophenyl
- TOF, time-of-flight

Chapter I

Introduction

Proteins play a crucial role in maintaining our homeostasis. The nutritional value of proteins has been widely recognized and the Dietary Reference Intake (DRI, United States National Academies) recommends a protein intake of more than 0.75 g/kg-body weight/day. In recent years, with the improvement in living standards, protein intake increased by ~30% worldwide according to the United Nations Food and Agriculture Organization (FAO). As shown in Fig. 1-1, in China, protein consumption doubled from 40 g/day in 1961 to 100 g/day in 2017. Even for several developed countries such as the United States and the United Kingdom, protein consumption was also in increasing trends. On average, the daily protein intake was increased by 20 g/day in the whole world from 1961 to 2017, and animal foods and plant foods, such as seafood, lean meats and poultry, eggs, legumes, nuts, seeds, and soy products, are excellent protein sources.
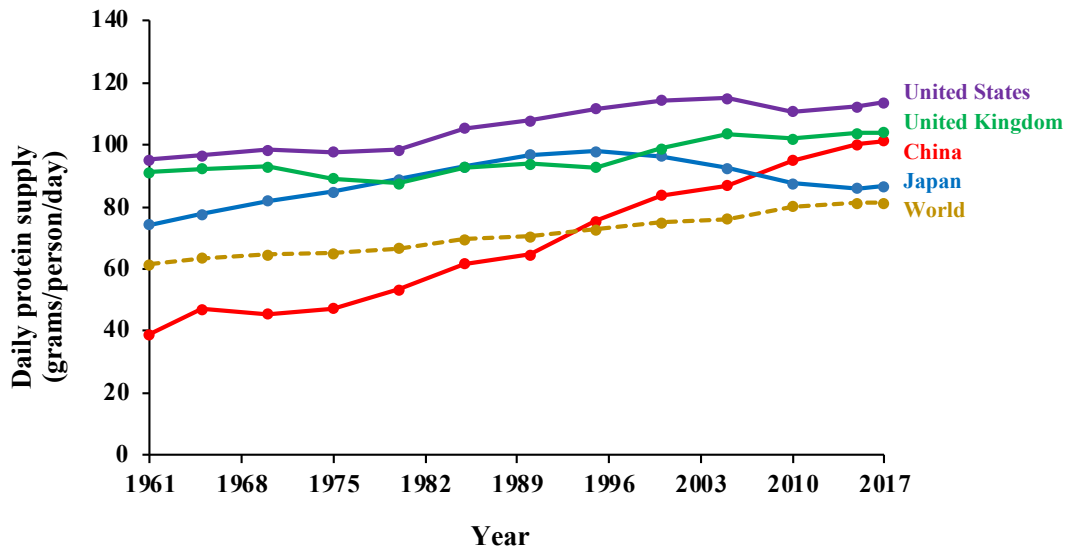
Fig. 1-1. Daily per capita protein supply from 1961 to 2017 in China, Japan, United States, United Kingdom, and the World. The data are retrieved from United Nations Food and Agriculture organization (FAO). https://ourworld indata.org/food-supply.

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In addition to the nutritional value, the tertiary function of proteins has been attracting attentions. The tertiary function of food, that is, modulation of physiological systems, was advocated in the Grant-in-Aid for Special Research Project "Systematic Analysis and Development of Food Functions (1984–1986)" by Ministry of Education, Science and Culture [currently Ministry of Education, Culture, Sports, Science and Technology (MEXT)] of Japan [1]. This notion encouraged innumerable studies on the physiological roles of food components. For instance, it was demonstrated that a milk intake reduced the risk of type 2 diabetes (T2DM), cardiovascular disease (CVD), and cancer [2]. The daily intake of dairy products (cheese [3, 4] and yogurt [5, 6]) also caused an improved insulin secretion in T2DM patients. It is believed that the insulinogenic proteins in dairy products are responsible for such modulatory functions [7]. Similarly, dietary protein has been demonstrated to exert health promoting effects against lifestyle-related diseases, such as hyperlipidemia [8], diabetes [9, 10], and obesity [11]. Daily intake of a milk protein lactoferrin caused a decrease in visceral fat accumulation in Sprague-Dawley (SD) rats [12]. Intake of whey protein and casein was reported to improve insulin response and blood glucose disposal in T2DM patients [13]. Choi *et al.* [14] demonstrated that the intake of casein, milk whey protein, and soybean protein improved lipid metabolism in SD rats. Some reported health effects of proteins are summarized in Table 1-1.

Table 1-1 Health benefits of proteins or protein foods

Food source	Protein	Health effect	Reference
Dairy food (milk, cheese, yogurt)	Casein	Anti-diabetic	[15]
		Anti-cancer	[16, 17]
		Anti-obesity	[11]
	Whey protein	Anti-diabetic	[10, 18–20]
		Anti-cancer	[16, 17, 19]
		Anti-obesity	[21]
Egg	Egg protein	Anti-cancer	[16]
		Anti-inflammatory	[22]
		Anti-hypertensive	[23]
Soybean	Soy protein	Anti-cancer	[16]
		Anti-CVD	[24]
		Anti-diabetic	[25]
Seafood	Cod proteins	Anti-cancer	[16]
		Anti-diabetic	[26]
Wheat	Gluten	Anti-cancer	[16]

Soybean-derived foods, for example, tofu, natto, miso, soy sauce, simmered soybean, are traditionally consumed in Japan. According to the Report of Utilization of Food-Grade Soybeans in Japan, soybean protein has steadily represented approximately 10 % (8.7 g/day/capita) of the total daily protein intake during the last 40 years [27]. Soybean is a rich source of high-quality proteins containing all the essential amino acids found in animal proteins with less saturated fats and no cholesterol [28]. Soybean protein has been reported to possess a variety of physiological functions including anti-diabetic, anti-hypertensive, and anti-obesity effects, etc [28]. Long-term intake of soybean proteins (20 g/kg/day) in human for 7 weeks was reported to reduce plasma lipid levels and improve kidney function in T2DM [25]. Typical soybean proteins, glycinin and β-conglycinin, are regarded as the essential functional components eliciting such physiological functions [29]. Especially, β-conglycinin was reported to improve lipid metabolism in humans, as 12-week β-conglycinin intake significantly lowered serum triacylglycerol (TG) levels [30]. Inoue *et al.* [31] also disclosed that the daily intake of β-conglycinin (20%/diet) in Wistar rats over a period of 4 weeks lowered serum lipid levels through an acceleration in carbohydrate consumption in terms of increased adiponectin. Physiological functions of dietary soybean proteins are summarized in Table 1-2.

Table 1-2 Dietary soybean proteins and their physiological functions

Protein	Human/Animal	Physiological effect	Reference
Soybean	Human	Renal metabolism	[25]
	Sprague-Dawley (SD) rats	Lipid metabolism	[32]
	SD rats	Cholesterol metabolism	[14]
	SD rats	Hepatic catabolism	[33]
	SD rats	Lipid metabolism	[34]
	SD rats and wild-type and *ob/ob* mice	Lipid metabolism	[35]
	SPRD-*cy* rats	Renal metabolism	[36]
	SPRD-*cy* rats	Renal metabolism	[37]
	SPRD-*cy* rats	Renal metabolism	[38]
	Wistar rats	Hepatic metabolism	[39]
Soybean protein isolate (SPI)	SD rats	Cholesterol lowering	[40]
	Wistar rats	Skeletal muscle regulation	[41]
SPI	Wistar rats	Serum TG lowering	[42]
Glycinin	Obese mice	Serum TG lowering	[29]
	Wistar rats	HDL-C improvement	[43]
β-Conglycinin	Obese mice	Serum TG lowering	[29]
	Wistar rats	Serum TG lowering	[42]
	Spontaneously diabetic Goto-Kakizaki (GK) rats	Hepatic metabolism	[44]
	Otsuka Long-Evans Tokushima Fatty (OLETF) rats	Hepatic metabolism	[45]
	C57BL6 mice	Hepatic and systemic metabolism	[46]
	OLETF rats	Lipid metabolism	[47]

	Human	Serum TG lowering	[30]
	Human	Lipid metabolism	[48]
α′-Subunit of β-conglycinin	SD rats	Cholesterol lowering	[49]
α′-Subunit of β-conglycinin in rice	SD rats	Cholesterol lowering	[50]

It has been demonstrated that soybean protein-derived small peptides also have an ability to elicit hypolipidemic [51], anti-hypertensive [52, 53], and anti-diabetic [54] effects *in vivo*. Nagaoka *et al.* [51] identified a cholesterol absorption-inhibitory peptide, VAWWMY (Val-Ala-Trp-Trp-Met-Tyr, designated as soystatin), derived from soybean protein in Wistar rats. VAWWMY intake significantly reduced cholesterol absorption *in vivo* via the inhibition of micelles formation in the gut [51]. Soymorphin-5 (YPFVV) derived from β-conglycinin β-subunit was reported to improve glucose and lipid metabolism in KK-A^y mice via the activation of adiponectin and PPARα system after YPFVV (10 mg/kg/day) intake for 5 weeks [55]. Soymorphin-6 (YPFVVN) and Soymorphin-7 (YPFVVNA) were also reported to show anxiolytic activities after oral administration at doses of 10–30 mg/kg in mice [56]. Physiological functions of soybean protein-derived peptides are summarized in Table 1-3.

Table 1-3 Physiological functions of soybean protein-derived peptides

| Peptide	Soybean protein	Physiological function	Reference
Soybean peptide	Soybean protein	Suppresses cognitive decline in SAMP8 mice	[57]
Soybean peptide	Soybean protein	Hypotensive effect in SHRs	[58]
WGAPSL	Soybean protein	Hypocholesterolemic effect in rats	[59]
FVVNATSN	Soybean protein	Hypocholesterolemic effect in rats	[59]
KA	Glycinin, β-conglycinin	Triglyceride-lowering effect in OLETF rats	[60]
VK	Glycinin, β-conglycinin	Triglyceride-lowering effect in OLETF rats	[60]
SY	Glycinin, β-conglycinin	Triglyceride-lowering effect in OLETF rats	[60]
VAWWMY	Glycinin	Improved lipid metabolism in rats	[51]
LPYPR	Glycinin	Hypocholesterolemic effect in mice	[61–63]
NWGPLV	Glycinin	ACE inhibition in SHRs	[62]
YPFVV	β-Conglycinin	Anti-diabetic effect in KK-A^y mice, immunostimulating effect in mice	[55, 56]
YPFVVN	β-Conglycinin	Anti-diabetic effect in KK-A^y mice, immunostimulating effect in mice	[55, 56]
YPFVVNA	β-Conglycinin	Anti-diabetic effect in KK-A^y mice, immunostimulating effect in mice	[55, 56]
VRIRLLQRFNKRS	β-Conglycinin	Appetite suppressant in SD rats	[64]
MITL	β-Conglycinin	Immunostimulating effect in neonatal rat model	[61, 65]

MITLAIPVN	β-Conglycinin	Immunostimulating effect in neonatal rat model	[61, 65]
MITLAIPVNKPGR	β-Conglycinin	Immunostimulating effect in neonatal rat model	[61, 65]

Upon oral intake, proteins are digested in the gut system by proteases and peptidases eventually to generate absorbable small peptides and amino acids [66]. Apparently, large protein molecules are not absorbable in intact forms into blood circulation [67]. In contrast, as described above and summarized in Table 1-3, it is likely that peptide fragments by protein digestion may be in line with the physiological functions of proteins. Small di-/tripeptides such as Val-Tyr (VY) and Trp-His (WH) have been reported to be absorbed into blood circulation by intestinal peptide transporter 1 (PepT1) upon oral intake [68]. After a single oral administration of VY (PepT1-transportable dipeptide, 10 mg/kg) to 18-week-old SHR, absorbed VY was subsequently accumulated in the kidney, lung, heart, mesenteric artery, and abdominal aorta [69]. Recent studies demonstrated that even longer oligopeptides with more than four amino acid residues could be absorbed into blood circulation, probably by passive diffusion via the paracellular tight-junction (TJ) [70–72]. For instance, pentapeptides, Gly-Sar-Sar-Sar-Sar (GSSSS) [70], Asp-Gly-Tyr-Met-Pro (DGYMP) [71], and His-Ile-Pro-Ile-Pro (HLPLP) [72] were detected in their intact forms in rat bloodstream after single oral administration. Schematic diagram for soybean protein digestion and peptide absorption in intestine tract is depicted in Fig. 1-2, speculating that peptides from dietary proteins upon oral intake may be candidates for health promotion. Thus, to understand the physiological functions of soybean proteins, identification of absorbable peptides in blood circulation is of vital importance.
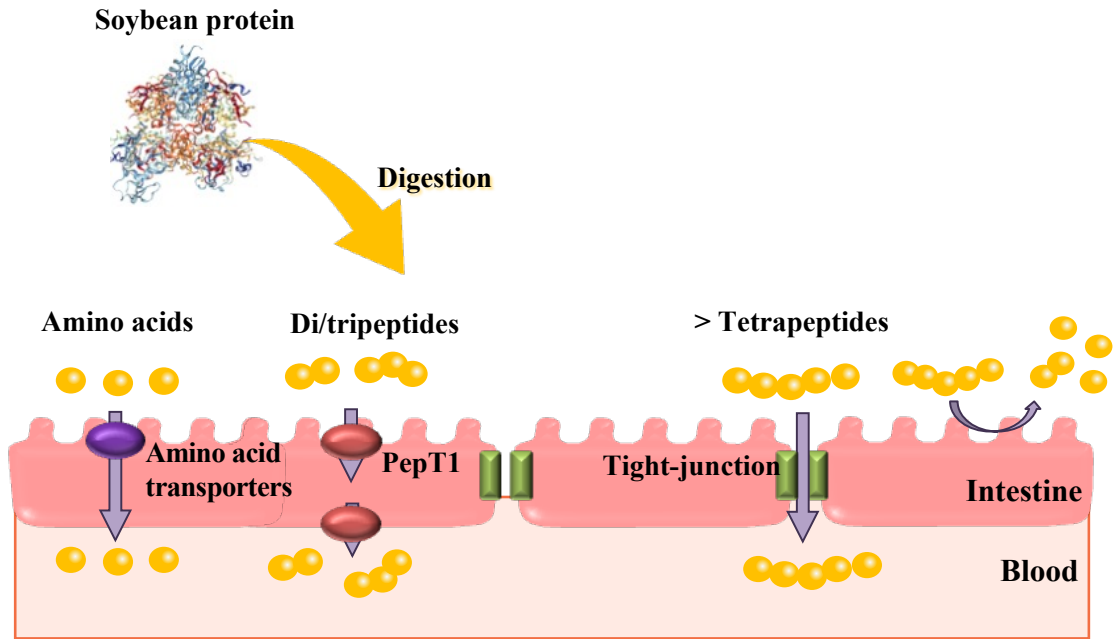
Fig. 1-2 Schematic diagram for soybean protein digestion and peptide absorption in intestinal tract. PepT1: peptide transporter 1.

An efficient, robust, and reliable analytical method is a key for successful detection and identification of peptides in complicated biological matrices. Liquid chromatography-mass spectrometry (LC-MS) has been applied to investigate the intestinal absorption of small peptides due to its high selectivity and sensitivity [70]. However, it remains challenging to systematically evaluate small peptides generated from oral protein intake in blood. Meanwhile, matrix-assisted laser desorption/ionization (MALDI)-MS is capable of fast and high-throughput detection of the entire molecular species in a small amount of sample [73], and thus has been used for biomarker discovery by these features [74, 75]. Therefore, in the present study, we attempted to use MALDI-MS as a tool for initial non-target screening of peptides in blood after dietary protein intake.

In combination with MALDI-MS, LC-time-of-flight (TOF)/MS was used as a complementary technique for identification of peptides in blood. LC-TOF/MS could detect not only target peptides but also peptide metabolites simultaneously. For signals detected by MADLI-MS, LC separation of peptides could furtherly provide a characteristic retention time for peptide sequence and a high resolution TOF/MS analyzer could measure the precise m/z values. Therefore, in this study, MALDI-MS in combination with LC-TOF/MS are applied for identification of absorbable peptides in blood after oral administration of dietary protein in animal experiments (Fig. 1-3).

(A) Non-target analysis by MALDI-MS
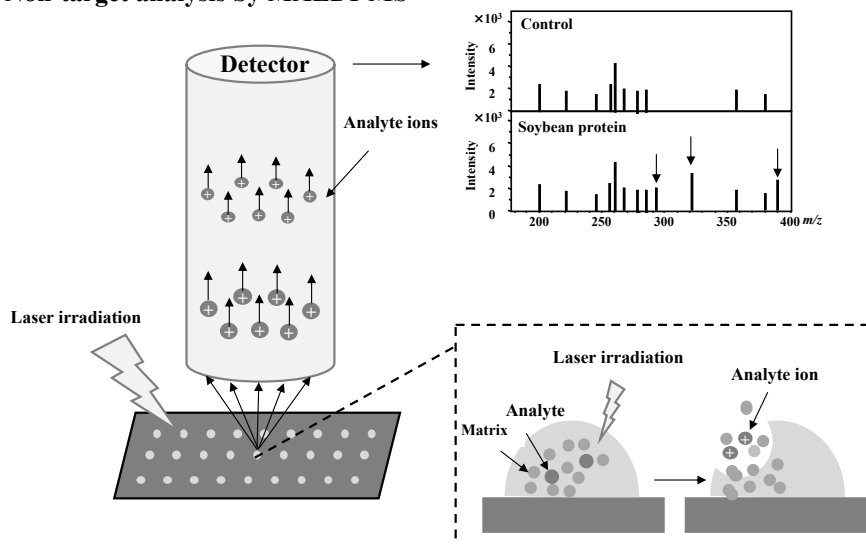
(B) Acquisition of retention time (RT) on LC column and exact mass data by TOF/MS
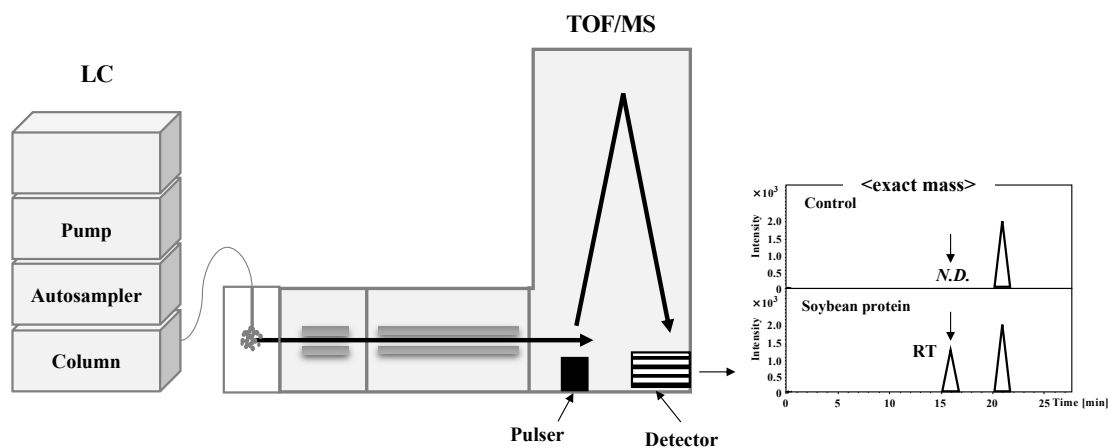
Fig. 1-3 Schematic workflow of MALDI-MS and LC-TOF/MS. (A) Non-target screening by MALDI-MS. (B) Acquisition of retention time on LC column and precise mass data by TOF/MS. Arrows indicate characteristic peaks *N.D.*: not detected. RT: retention time.

According to the aforementioned research background, knowledge on the absorption of proteins in blood circulation is crucial to understand their claimed preventive effects against lifestyle-related diseases. Thus, the aim of this study is to provide striking evidence on the appearance of peptides in bloodstream after protein oral administration. Establishment of an appropriate assay for peptide identification in bloodstream is also investigated in this study. The objectives for each Chapter in this study are described as follows:

1) In **Chapter II**, β-conglycinin, a reported bioactive protein was used to provide evidence for the production of peptide metabolites in rat bloodstream. In this Chapter, an oral administration of β-conglycinin to rats (100 mg/kg) was performed to clarify β-conglycinin-derived peptides in blood. MALDI-MS was primarily used for non-target screening of characteristic MS signals in the bloodstream of β-conglycinin-administered rats. LC-TOF/MS in combination with a solid-phase peptide synthesis was used to identify peptides sequences.

2) In **Chapter III**, to achieve high sensitivity and efficiency, we attempted to establish a novel MALDI-MS assay for peptide metabolites in blood by exploiting amine derivatization techniques. Three amine derivatization techniques, using 2,4,6-trinitrobenzene sulfonate (TNBS), 3-aminopyridyl-*N*-hydroxy-succinimidyl carbamate (APDS), and *N*-succinimidyl-7-methoxy-coumarin-3-carboxylate (Cou), were investigated in this study. Amine derivatization aided-MALDI-MS technique was applied to evaluate peptides in blood after glycinin administration to rats (100 mg/kg).

Taken together, the present study demonstrates the production of peptide metabolites in rat bloodstream after protein oral administration. Moreover, analytical assay of peptide metabolites in blood by amine derivatization-MALDI-MS technique may be useful for clarifying the systemic absorption and metabolism behavior of bioactive proteins in the body.

Chapter II

Identification of peptide metabolites from orally administered β-conglycinin in rat bloodstream by MS analyses

1. Introduction

It has been demonstrated that various dietary proteins potentially play a role in physiological regulation in the body system. For example, β-conglycinin (7S globulin), a major constituent of soybean protein [76, 77] have been extensively studied regarding their bioactivities. In obese KK-A^y mice, it was revealed that daily intake of β-conglycinin (2 g diet/day) for 2 weeks improved fatty acid metabolism, including promotion of β-oxidation, suppression of fatty acid synthesis, and elevation of fecal excretion of fatty acids, resulting in a significant reduction in serum triacylglycerol level, as well as decreased serum

glucose and insulin levels [29]. The effect was also reported in rats, in which dietary intake of β-conglycinin caused a lowering of serum triacylglycerol [30] and enhanced lipid metabolism [45]. However, macromolecules like proteins may undergo dynamic digestion in the gastrointestinal tract upon oral intake. While extensive studies on bioactivity of β-conglycinin have been reported, bioavailability of proteins has not been fully understood.

Even a larger peptide (> tetrapeptide), such as HVSSSEE, PVRGPFPIIV, and DIGSESTEDQ, were detected in human blood after a consecutive consumption of 100 g/day of Parmigiano Reggiano cheese for 1 week [78]. A consumption of chicken breast has been reported to cause an increase of plasma dipeptides (GV and KE) in healthy human volunteers [79]. These studies strongly suggested that in addition to amino acids, various peptides were generated during the gastrointestinal digestion and absorbed into blood circulation. Although little is known regarding the absorption of β-conglycinin, systematic evaluation of peptide production in the bloodstream upon oral intake could provide new insights of bioactive peptides from dietary proteins in the living body.

Taken together, the aim of **Chapter II** was to provide evidence for the presence of peptides in the bloodstream after the oral administration of β-conglycinin. A non-targeting approach using MALDI-MS was applied to screen peptide metabolites by characteristic MS signals in β-conglycinin-administered blood of Wistar rats. According to the MALDI-MS analysis, precise mass of

characteristic MS signals was acquired by LC-TOF/MS, and eventually

identified with the help of peptide synthesis.

2. Materials and methods

2.1. Materials

β-Conglycinin was prepared according to the method reported by Saito *et al* [76]. Briefly, defatted soymilk was extracted with 10-fold volume of water and centrifuged to obtain the supernatant. The supernatant was treated by phytase (pH 6.0, 1 h, 40 °C) and centrifuged (3,000 × *g*, 10 min, 25 °C). The supernatant was then adjusted to pH 5.0 and centrifuged to obtain β-conglycinin precipitate.

Phytic acid and ammonium sulfate (AS), *N*-methylmorpholine (NMM), dichloromethane (DCM), 1,8-diazabicyclo[5.4.0]-7-undecene (DBU) were purchased from Nacalai Tesque Inc. (Kyoto, Japan). 2,5-Dihydroxybenzoic acid (DHB), and $[^{13}C_5,^{15}N]Val^3$-angiotensin II were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Distilled water, acetonitrile (ACN), and formic acid (FA) were LC-MS grade, purchased from Merck Co. (Darmstadt, Germany). Methanol (MeOH) and *N, N*-dimethylformamide (DMF) were purchased from Kanto Chemical Co. (Tokyo, Japan). Sevoflurane was purchased from Maruishi Pharmaceutical Co. Ltd. (Osaka, Japan). Peptides used in this study were synthesized by an Fmoc-solid phase synthesis method, according to manufacturer's instructions (Kokusan Chemicals, Osaka, Japan) as described in the latter Section 2.6. Fmoc-AA-OH ・ H_2O, Fmoc-AA-Wang resin, 1-hydroxybenzotriazole monohydrate (HOBT ・ H_2O), benzotriazol-1-yloxytripyrrolidinophospho-nium hexafluorophosphate (PyBOP) were

purchased from Kokusan Chemicals Co. (Osaka, Japan). All other reagents were of analytical reagent grade and were used without further purification.

2.2. Animal experiments

Ten-week-old male Wistar rats were used in this study (SPF/VAF Crj; Charles River Japan; Kanagawa, Japan, 340–390 g). All rats (n = 9) were individually housed under a temperature of 21 ± 1 °C and humidity of 55 ± 5%. Rats were fed an AIN 93G (Oriental Yeast Co.; Tokyo, Japan) and water *ad libitum* for 1 week. The rats were fasted for 16 h before protein administration to avoid the influence of feeding on the expression of intestinal oligopeptide transporter [80, 81]. Milli-Q water was administered to Wistar rats 1 week before the β-conglycinin administration experiments, by taking into consideration the administration stress by gavage to rats. Approximately 300 μL of blood (Control) were collected at 1, 2, 4, and 8 h after Milli-Q oral administration from the rat tail vein. One week later, β-conglycinin dissolved in Milli-Q water was orally administered to the same Wistar rats at a dose of 100 mg/kg-body weight (B.W.). Blood from the tail vein was collected at the same blood sampling schedule as mentioned above. Blood was collected into a 1.5-mL Eppendorf tube (Hamburg, Germany) containing EDTA-2Na and centrifuged at 3,500 × g for 15 min at 4 °C to obtain plasma. The outline of blood sampling schedule in this study is shown in Fig. 2-1. All the animal experiments were performed according to the Guidelines for Animal Experiments in the Faculty of Agriculture in the Graduate

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Course of Kyushu University and according to the Law (No. 105, 1973) and

Notification (No. 6, 1980 of the Prime Minister's Office and No. 71, 2006, of the

Ministry of Health, Labor and Welfare) of the Japanese Government. All the

experiments were reviewed and approved by the Animal Care and Use

Committee of Kyushu University (permit number: A30-015).
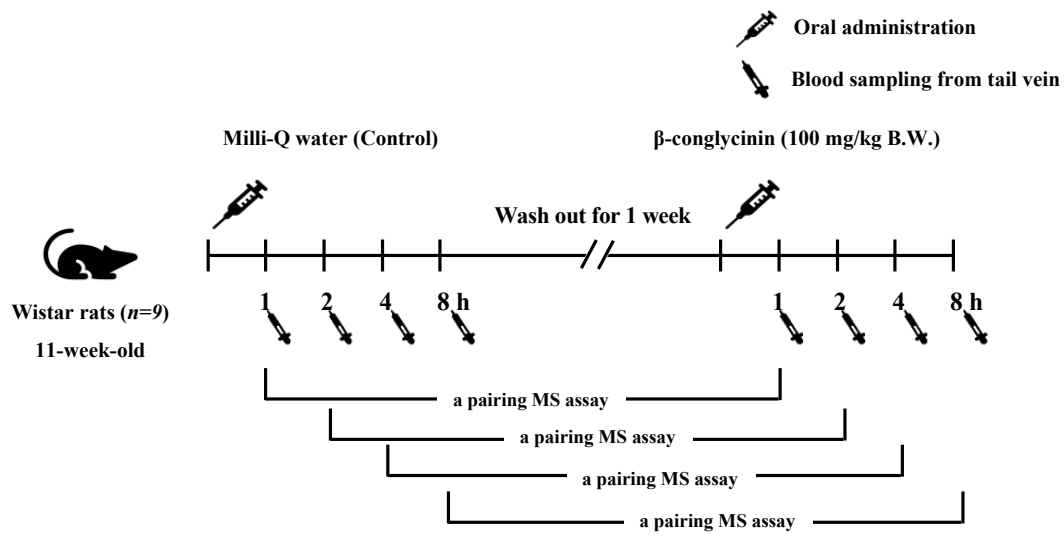
**Fig. 2-1 Blood sampling schedule in this study.**

2.3. Preparation of rat plasma for MS analysis

An aliquot (100 μL) of plasma was mixed with 400 μL of 20% ACN containing 0.1% FA and 0.1% NaCl. $[^{13}C_5,^{15}N]Val^3$-angiotensin II (final concentration, 1 nmol/mL) was spiked into plasma as internal standard (IS). Sample was filtrated by centrifugation with an Amicon Ultra 0.5-mL-3K centrifugal filter (Millipore; Carrigtwohill, Ireland) at 14,000 × g for 30 min at 4 °C. The Amicon filter was rinsed with 0.1 mol/L NaOH followed by a second spin of Milli-Q water at 14,000 × g for 5 min at 4 °C to remove any interfering compounds for MS analysis prior to sample filtration. The filtrate was then mixed with 100 μL of 0.1% FA and loaded onto a Waters Sep-Pak C18 column (Waters; MA, USA). Peptide fraction was obtained by the elution with 3 mL of 100% ACN/0.1% FA. The obtained fraction was evaporated to dryness and stored at -20 °C prior to MS analysis.

2.4. MALDI-MS analysis

MALDI-MS analysis of plasma sample was conducted with a Bruker Autoflex III mass spectrometer equipped up with a SmartBeam III (Bruker Daltonics; Bermen, Germany). Matrix used for MALDI-MS analysis was DHB (10 mg/mL) containing 10 mmol/L phytic acid [82] and 50 mmol/L ammonium sulfate [83] (Fig. 2-2). The dried plasma samples were dissolved in 10 μL of DHB solution, and an aliquot (1 μL) was spotted onto an indium tin oxide (ITO)-coated glass slide. MALDI-MS data were acquired in a positive ion-linear mode

of 100–2,000 *m/z*. MS parameters were as follows: ion source 1, 20.00 kV; ion source 2, 18.80 kV; lens voltage, 7.50 kV; gain, 10.0; laser offset, 60%; laser value, 6.5%; laser power, 34%; laser frequency, 100 Hz; shot, 5,000. MS spectra were analyzed by Bruker Data Analysis 3.3 software. The remaining solution was used for LC-TOF/MS analysis.
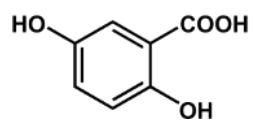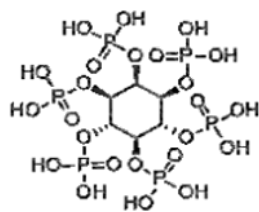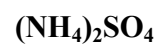
| DHB | Phytic acid | Ammonium sulfate |

Fig. 2-2 Structure of matrices for MALDI-MS analysis used in this study.

2.5. LC-TOF/MS analysis

LC separation of plasma samples was performed using an Agilent 1200 series HPLC (Agilent Technologies; Waldbronn, Germany) on a Cosmosil 5C$_{18}$-MS-II column (2.0 × 150 mm, Nacalai Tesque). TOF-MS analysis was performed on a Bruker micrOTOF-II mass spectrometer in the electrospray ionization (ESI)-positive mode. Plasma samples were eluted with a linear gradient elution of 0.1% FA to MeOH containing 0.1% FA over 20 min at a flow rate of 0.2 mL/min at 40 °C. MS parameters were as follows: drying gas (N$_2$), 8.0 L/min; drying temperature, 350 °C; nebulizer pressure, 1.6 bar; capillary voltage, 3,800 V; mass range, 100–2,000 *m/z*. Data were analyzed and acquired by a Bruker Data Analysis 4.0 software. A calibration solution contained 10 mmol/L sodium formate in 50% ACN was injected at the start of each run. Sequence Editor analysis (Bruker Daltonics biotools, version 3.2) was used for predicting sequence of peptide metabolites from β-conglycinin-administered plasma samples.

2.6. Fmoc-solid phase peptide synthesis

Peptides were synthesized by an Fmoc-solid phase synthesis. *C*-terminal amino acid Fmoc-AA-Wang resin (50 μmol) was weighed into a reactor column and swollen with 2 mL of DCM for 15 min by stirring. Then, 2 mL of DMF was added into the reactor column and stirred for 1 min to wash the resin. The washing process was repeated for 5 times. Fmoc-AA-OH (125 μmol), HOBT·

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H$_2$O (38 mg) and PyBOP (167 mg) were weighed and placed in the peptide synthesizer. After the synthesis reaction, 2 mL of ACN was added to remove DMF for 5 times, and a 1 h-vacuum drying was performed.

3. Results and discussion

3.1. MALDI-MS analysis of peptides derived from β-conglycinin in rat blood

MALDI-MS measurement, capable of non-targeting detection of ionizable compounds [82, 84, 85], was performed to obtain information on peptide metabolites from orally administered β-conglycinin (100 mg/kg) in the circulating blood of Wistar rats. Nine rats (named as **A, B, C, D, E, F, G, H,** and **I**) fed with casein-based diet (AIN-93G) were used in this study. Blood was taken from the tail vein at a time-schedule of 1, 2, 4, and 8 h after β-conglycinin oral administration. Considering that individual difference of rats may cause different production behavior of peptides and to avoid administration stress by gavage to rats [86], blood samples were taken at the above time-schedules from the corresponding rats one week prior to oral administration of β-conglycinin and used as control to distinguish β-conglycinin-derived MS peaks from endogenous peaks.

MALDI-MS spectra of control and β-conglycinin administered blood samples (rat **B,** 1 h) are depicted in Fig. 2-3. Many MS signals were nominated in β-conglycinin-administered plasma in a mass range of 100–1,000 *m/z,* compared to control plasma. Most MS signals were detected in a mass range of 200–800 *m/z,* corresponding to di- to hexa-oligopeptide chain lengths. No characteristic signals were obtained between 800–2,000 *m/z,* suggesting that

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larger oligopeptides might not be present in β-conglycinin-administered blood

or could not be detected under the current MALDI-MS detection capacity.
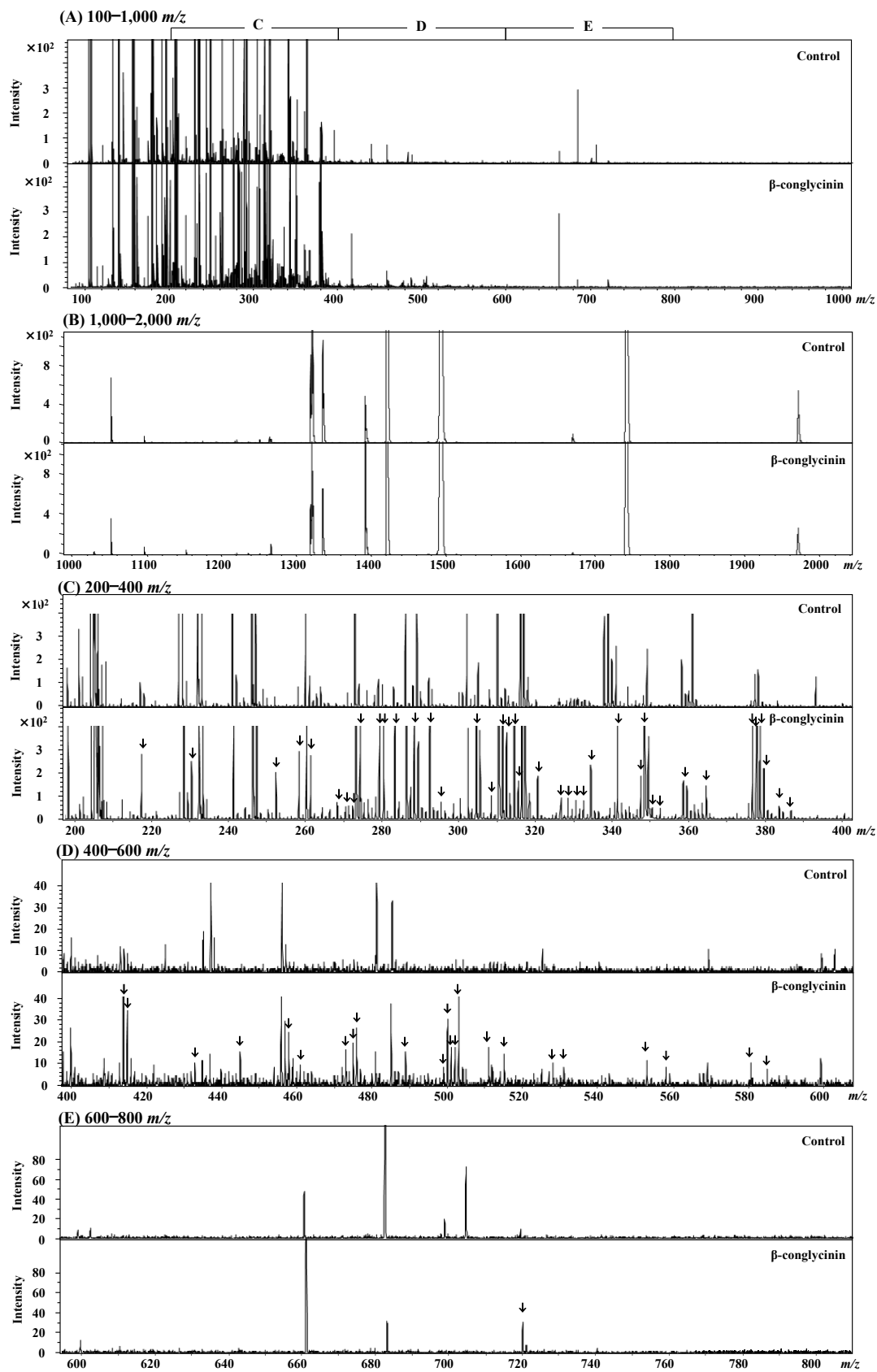
Fig. 2-3 MALDI-MS spectra of plasma sample (rat B, 1 h) after β-conglycinin oral

administration (β-conglycinin) or Milli-Q water (Control). Arrows indicate

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characteristic peaks in β-conglycinin-administered blood. Each MALDI-MS spectrum

in a positive ion-linear mode corresponds to *m/z* ranges of (A) 100–1,000, (B) 1,000–

2,000, (C) 200–400, (D) 400–600, and (E) 600–800.

Similarly, plasma samples of nine rats were subjected to MALDI-MS analysis. As summarized in Table 2-1, a total of 126 characteristic signals for β-conglycinin-administered plasma were obtained. Three signals, namely, 286.2 *m/z* (rats **F, G, I**), 288.2 *m/z* (rats **F, H, I**), and 599.2 *m/z* (rats **A, B, C**), were commonly observed. These results suggested that the production patterns of peptide metabolites after β-conglycinin administration may greatly differ from individual. Digestion of proteins in the gut is affected by individual villi and microbiota, cytosolic hydrolysis in intestinal epithelial cells, and protease activity. It has been reported that the gut microflora proportions in SD rats (*Lactobacillus reuteri, Akkermansia municiphila*, etc) were largely different in SD individuals [87]. Egger *et al.* [88] demonstrated that different peptides were produced in individual pig intestinal tracts. Moreover, Caira *et al.* [78] also reported the impact of individual differences on peptide production in plasma after casein intake in humans. Taken together, a variety of *m/z* signals in individual Wistar rats would be an acceptable peptide production feature.

According to β-conglycinin primary sequence (subunits: α, UniProtKB-P0DO16; β, UniProtKB-P25974; α', UniProtKB-P11827), peptide sequences were predicted using a Sequence Editor software (Bruker Daltonics biotools, version 3.2). As summarized in Table 2-1, the predicted peptides were composed of di- to octapeptide lengths. However, for the identification of peptide metabolites detected by the MALDI-MS (Table 2-1), precise *m/z* value by TOF/MS analysis may be required when the Sequence Editor is applied, since

different peptides are predicted as the same ones by rough *m/z* value with the

first chemical digit (e.g., KG and QG; K, 147.11; Q, 147.07, in Table 2-1).

Table 2-1 Observed *m/z* values ([M+H⁺]) in β-conglycinin-administered plasma by MALDI-MS analysis

m/z	Predicted sequence	*m/z*	Predicted sequence
204.1 (4 h; **A**)*	KG, QG, GQ, GK, AN, NA	286.2 (4 h; **F**, **G**, **I**)	GPL, VPA
205.1 (4 h; **A**)	SV, VS, W, EG, GE, AD, DA	288.2 (4 h; **F**, **H**, **I**)	GLV, LR, RI, IR, RL, LVG, VGL, LGV, VGI, PGD, GVI
207.2 (4 h; **A**)	ST, TS	290.3 (2 h; **F**, **H**)	LAS, ALS, LGT
217.2 (1 h; **B**)	VV, TP	295.1 (1 h; **B**)	FE, EF, YL, LY, IY
218.2 (1 h; **D**)	QA, AQ, AK, KA	300.2 (4 h; **A**)	AIP, IPA
219.0 (4 h; **F**)	LS, SL, EA, IS, SI, AE, TV	301.2 (1 h; **A**)	QPG, KPG, KGP
221.1 (4 h; **D**)	DS, TT, AM, SD, VC, CV	302.2 (4 h; **F**, **H**)	LLG, LGL, PGE, WP, VPS, AIV, IGI, ILG
229.2 (1 h; **D**)	PL, IP, PI, LP	303.1 (2 h; **B**)	RQ, QR, RK, KR, LNG, HF, VNA, GIN, FH, GRA, NLG
230.1 (1 h; **B**, **C**)	NP, PN	304.3 (1 h; **B**)	VSV, ER, RE, TAI, TLA, VDA, SVV, EGV
231.1 (1 h; **C**)	LV, VL, IV, VI, PD, DP	305.3 (1 h; **B**)	AKS, SKA, SAK
243.1 (4 h; **D**)	SH, HS	306.3 (1 h; **B**)	MR, LSS, ISS, RM
247.2 (4 h; **A**)	VE, NN, DL, LD, DI, EV	308.2 (2 h; **B**)	SVC, CVS
260.2 (2 h; **F**)	GIA, LQ, LK, QL, PSG, QI KL, GAL, AIG, LAG, PGS LGA, IAG, IK	310.2 (1 h, 2 h; **B**; 2 h; **H**, **I**)	SFG, FGS, QY, GFS, KY, AGY, HPG
263.1 (4 h; **A**)	FP, PF, MI, ED, DE	312.1 (1 h; **B**, **D**)	HR, RH, GHV
264.1 (4 h; **A**)	SGT, MN	316.2 (2 h; **H**, **I**)	AIL, LAI, ALL, VVV, IPS, PLS, PSI
265.1 (4 h; **A**)	VF, DM, FV	321.1 (4 h; **D**)	QAC, KSS, TSN, SQS
269.1 (1 h; **D**)	SY, YS, LH	324.2 (1 h; **A**)	YAA, AAY
272.2 (1 h; **B**)	PR, RP	328.2 (2 h; **H**)	VIP, IPV
274.1 (1 h; **D**)	RV, VR, GVV	329.1 (1 h; **D**)	VNP, PVN, PGR, YF, FY
275.1 (1 h; **D**)	QQ, QK, KQ, KK	334.2 (1 h; **B**)	ESV, AFP, SDL, MIA, WE, EEG, EGE, EW, GEE, SNN, MIA
277.1 (4 h; **A**, **B**, **D**)	EE, GSN	336.2 (1 h; **B**)	STE, ETS, GYP, FGI, LFG
278.1 (4 h; **A**)	RC, GTT, ATS, SGD	342.1 (4 h; **A**)	HAD, HGE, WH
279.1 (1 h; **B**)	FL, LF, IF, YP	343.2 (1 h; **B**)	LPN, VPAG, NPI
284.1 (4 h; **D**)	KH, QH, HQ, HK	347.2 (1 h; **B**)	QLS, LQS, ISQ, SQI, NTL, NTI, ISK, LSK, IKS, SLK, LAGS, DNI, EAQ

Table 2-1 *Continued*

m/z	Predicted sequence	m/z	Predicted sequence
348.2 (1 h; **A**, **B**, **F**; 2 h, 3 h, 4 h; **F, G, H, I**)	SEL, NSK, EIS, SNK, SKN, ELS, LSE, GKGS, GSKG, VMV, DDV	378.1 (1 h; **B**;4 h; **A, D**)	DED, EDD, LVF, VFL, VLF, IFV, FVI, YPV, LFV, FVL
350.1 (1 h; **B**)	SED, EDS, DSE, PPH, AYP, FPS, ALF, FAI, FLA	379.1 (1 h; **B**;4 h; **A, D**)	DMN, SEGS, GSES, GRF, FGR, ASVC
351.2 (1 h; **B, D**)	QGF, FGK, GKF	381.2 (1 h; **B**)	PKH, PQH, PHQ, QHP, QPH, HQP, FSK, SFQ, FQS
352.2 (1 h; **B**)	VSF, ADF, FGE	383.2 (1 h; **B**)	HAR, NLH, HNI, YSN, SYN
355.2 (8 h; **I**)	KHA, HAK	384.2 (1 h; **B**)	DSY, TTY, SYD, FFA, FAF
357.1 (4 h; **D**)	PQL, QPL, IPAG, QIP, SHN	391.3 (1 h; **A**)	SVSV, VSVS, SER, QFP, RES, SRE, KPF, AFPG, CLR, EKD, DKE, EDK, CLR, MIAG
358.0 (2 h; **B**)	NPK, EIP, KNP, KPN, NKP, NPQ, PLE, QNP, EPL	402.3 (4 h; **D**)	RAR, RNI, NLR, VRK, VVNA, KVR, INR, RRA, VQR, VRQ, RQV
359.0 (1 h; **B**)	NLL, LKV, VLQ, RSP, ILN, NLI, ALR, VPSG, NIL, KVL, IRA, RAL, RAI, DKP, IVQ, PRS, PGDA, LRA	409.2 (1 h; **F**)	FSR, YRA, VSFG, HPR, RPH, EFN, YNL, FEN, FRS
361.1 (1 h; **A**)	RGE, PSGT, GRE, DLN, DIN, QDV, KTI, GTAI, IAGT, ITQ, GTL	414.2 (1 h; **B, H**; 2 h; **F, H, I**; 4 h; **F, G, H, I**; 8 h; **H, I**)	EEH, EHE, GQIP, KGPL
363.1 (1 h; **B**)	ASVS, KCL, CLQ, NDD, DTK, QES, SKE, SEQ, SEK, KES, ESQ, SQE, DND, QSE, MVN	415.2 (1 h; **B**)	LLLG, LLGL, GEIP, NKPG, AIVI
364.2 (1 h; **B**)	NKC, SEE, ESE, EES, VDM, HHA, MIT, VFV, FVV	433.2 (2 h; **I**)	ERE, REE, EER, GSNR, AQQS, SIVD, GSAQA, SVDI
372.2 (1 h; **F**)	PQQ, QQP, QPK, PKK, QPQ, HSE	435.1 (1 h; **A**)	NQAC, ISSE, GYPV, TSDL
375.4 (2 h; **E**)	KVE, VEK, NKN, GDAL, ENL, EIN, LRS, INE, NIE, RAE, QDI, VQE, EVQ, GNKG, KDI, IEN, LEN, VLSG, LSR	446.2 (1 h; **F**)	RALS, ANIE, GLKE, INAE, VISQ, QAVE, AVEK, EGQI, TLVN, PAGTT, EANI, GIKE, AKDI, VSLK, SLKV, DNVV, TYY
376.2 (1 h; **A, B**)	FPL, IPF, PFL, SNR, NNE, NEN, SRN, GDAN, ENN, QDN, SAQA, VEE, YGH, FLP, KDN, DIE	447.3 (8 h; **H**)	EGEI, ESVI, VEIS, SNLN, NAEN, AENN, LAFP, EGNK, TQAQ, GFSH, VELS, SKGR, PFPS
377.3 (1 h; **B**; 2 h, 4 h, 8 h; **H**)	MRA, RDS, NPF, PFN, SRD	456.3 (2 h; **F**)	NHAD, LLPN, VIPAG, RIPA

Table 2-1 *Continued*

m/z	Predicted sequence	m/z	Predicted sequence
460.2 (2 h; **B**, **D**; 4 h; **D**; 8 h; **I**)	VPSGT, SILGA, GQQQ, QQQG, DNVI, QELA, GPLSS, PDND, VDIN, RER, NTIL, NTLL, ISQI, LIKS	504.2 (1 h; **B**)	EEIN, HFNS, EQDI, PAGYP, ENNQ, EGNKG, QYGH, ENQN, FSHN, KDIE, GPFPS, VEKE, SELR, NQRS, TAILS, LAFPG, KVEE, NSKR, NKRS, RELS, GSKGR
463.2 (1 h; **B**)	ARCN, EEGE, DSEL, SQDN, VVDM, SKDN, RSSN, GEEE, LSED, FPSI, ALFL	505.2 (4 h; **C**)	NSER, RCNL, KNPF, KPFN, NEGDA, EGDAN, ATSNL, VEEE, PHDH, EKDN
475.2 (1 h; **B**)	LNGTA, NGTAI, EAQQ, DLDI, FVIP, LAGSQ, QDNV, SAQAV, YGHV, SQQL, ISKK, LAGSK, IKSQ, ILET, KDNV	515.3 (4 h; **A**; 8 h; **G**)	SYFV, LKVR, NLRL, RIVQ, RNIL, VILNG, VLQR, VRQI, RVLQ, RVPQG
476.2 (2 h; **D**)	LQES, LSEQ, FYF, ISKE, LASVS, PGRF, EISK, ELSK, LSKE	517.1 (2 h; **I**)	NLRD, ILNGT, GQQQG, KEQI, LKEQ, AQAVE, IKEQ, LAGEK, ITQR, RKTI, SKAIV
477.2 (2 h; **I**)	NKCL, GEKGS, NNDD, QSGDA, IVDM, ELSE, AGSQD, VFVI, NSEK, GKGSE, EDDV, AGSKD, GSAKD, EGSKG, SFHS, GSAQD, SSRK, VVFL, MITL, YPVV, EDDV	519.3 (2 h; **E**)	QRES, KCLR, ESQR, SQRE, AGEKD, EGEAN, EKEN, EQDK, FHSE, HSEF, QDKE, NEGEA, NDDR, LQSGD, SAQPV, SFHE
485.2 (4 h; **D**)	PQLQ, SYDT, SHNK, SESY, YFR	521.2 (2 h; **I**)	EEED, EEDE, EDEE, LFKN, ILEF, LFEI, MNEGA, AFGIN, FGINA, NFLAG, VQFQ
486.2 (4 h; **D**)	CHAR, HARC, KNPQ, GALLL, KQNP, VRVL, EQPL, QPLE, PQLE, PSILG, ILGAL	525.3 (2 h; **I**)	RPRP, PRPR, QQSY, YPFV, QPRQ, SGTTY, HADAD, HEQK, HQKE, HKQE, QEKH, EKHQ, KLAIP, DFFL
487.2 (4 h; **B**)	PGDAQ, EEPL, EPLE, AIGIN, AQRI, ENPK, IGINA, KENP, KNIL, KRSP, LQNL, NLIK, NLKI, NLLK, NVVR, LLKN, QIRA, QRSP, RSPQ, EKNP, PEKN	528.2 (1 h; **B**)	QPRQ, SGTTY, HADAD
488.3 (8 h; **H**)	RNQA, LKVE, DEQP, NILE, EDKP, NIEL, RAEL, VQEL, VEKL, KGRK, SILR, IENL, ENLI, LENL, LEVQ	541.4 (2 h; **F**)	HEQK, HQKE, HKQE, QEKH, EKHQ, KLAIP, DFFL
489.2 (1 h; **B**; 8 h; **F**)	EKRG, KRGE, RGEK, NENL, SRNI, GEQR, NLRS, GDANI, DAQR, SRRA, EDED, EPLL, LFLP	542.1 (8 h; **F**)	HQEE, EHEQ
501.2 (1 h; **B**)	HNKC, EPQQ, GROR, INRV, KGPLS, NVRI, VVRQ, VVVNA, NRVL	553.2 (1 h; **B**)	CLQSC, PNHAD, EFEE

Table 2-1 *Continued*

m/z	Predicted sequence	m/z	Predicted sequence
565.3 (2 h; **I**)	NRFE, VRKY, PRRH, ASVCVS, VQRY, RYRA, SSEDK, SEKDS, CEEGQ, DMNEG	708.2 (8 h; **F**)	QGFSRN, DPIYSN, ESQESE, FENQNG
568.2 (4 h; **D**)	AYWE, NPSHN, RQQH, RYDD, AAYPF	713.3 (4 h; **H**)	HARCNL, PQLENL, GRIRVL, LRVPAGT, PEPRR
569.3 (2 h; **I**)	HEQR, LLLPN, HRKE	717.5 (2 h; **F**)	SGDALRV, RVPSGTT, ISKEQI, ERERQ, SQQLQN, SGRAILT, LSKEQI, AQDVER, NLRMIA
599.2 (4 h; **A**, **B**, **C**)	PQLQN, HGEKE, EEKHG, KHEW, HEWQ, EWQH	719.4 (1 h; **B**)	DNDENL, KVLFGR, LFEITP, VNATSDL, GSAKDIE, FYFRS
600.2 (4 h; **B**, **C**)	HARCN, KNKNP, RSPQL, SFFLS, FFLSS, EHEW, RVPAGT, PQLEN	743.6 (2 h; **F**)	SKAIVIL, KLLKNQ, AILTLVN, GRAILTL
605.2 (8 h; **F**)	NKCLQ, DNNEN, PGRFE, EISKE, ELSEQ, AGYPVV, VNATSN, YLVNP, ELSKE	768.3 (4 h; **B**)	DTKFEE, PHQEEE, SYNLQSG, YYLVNP
611.5 (2 h; **F**)	EIPRP, PVNKPG, LGKFF	792.6 (2 h; **F**)	VRKYRA, KRFQTL
622.2 (8 h; **F**)	DEDED, DYLIV, SSTEAQ, DLDIF FLAGSQ, ESEEE, SEEEE, LFEIT QRYR, MVNATS	806.3 (8 h; **F**)	KGSEEEQ, HKQEKH, QGKESEE, ASVCVSLK, ELSEDDV, RNFLAGE, FYFRSS
627.5 (2 h; **F**)	LLLGLV, ITLAIP, NPQLR, AIVILV, GRKGPL, LLVLLG, RNPQL	815.3 (8 h; **F**, **G**)	QQPGEKE, PDNNENL, QQQRQQ, QQRQQQ, LNFFAFG, NFFAFGI, FFAFGIN, PQQKEEG, YNLHPGD, FHEINR
660.8 (1 h; **B**)	SELRR	830.6 (1 h; **D**)	LRVPSGTT, FFLSSTE, EITPEKN, LVINEGDA, GDANIELV, AQDVERL, DANIELVG, GLKEQQQ, GEQPRPF, SQQLQNL, GIKEQQQ, ENQNGRI
669.5 (2 h; **F**)	IVILVI, LRAFY, HKQEK, KQEKH	849.4 (8 h; **F**)	EGALLLPH, VLFGREE, IVQFQSK, NKPGRYD, RVLFGEE
685.5 (2 h; **F**)	QREPR, TPEKNP, FYLRS, RLLQR	857.3 (8 h; **F**)	SHNKCLR, HNKCLRS, KEEHEW, EEKHEW, YLVNPHD, AGTTFYVV, EITQRNP
693.2 (4 h; **H**)	DADFLL, VLFGEE	874.4 (8 h; **F**)	LKVEKEE, DNNENLR, LKNQRES, REEGQQQ, QQQGEER, SVIVEISK, EGEANIEL, EQRQQEG

*Parenthesis indicates a sampling time of 1, 2, 4, or 8 h in individual nine rats (**A** to **I**).

3.2. LC-TOF/MS analysis of peptides derived from β-conglycinin in rat blood

LC-TOF/MS analysis was performed to furtherly obtain the exact mass and LC retention time of acquired MALDI-MS signals (Table 2-1). Extracted ion chromatograms (EICs) at the width of ± 0.005 *m/z* were conducted from the *m/z* values by MALDI-MS analysis. Fig. 2-4 shows an example of EIC analyses (348.1813 *m/z* and 460.2765 *m/z*) of β-conglycinin-administered plasma from rat **B**. Both signals were detected only in β-conglycinin-administered plasma, but not in control plasma. As a result of EIC analyses, out of the 126 specific signals detected by MALDI-MS (Table 2-1), 37 signals were detected by LC-TOF/MS analysis (Table 2-2). The decreased detection of signals in LC-TOF/MS analysis may be due to low detection capacity of TOF/MS compared to MALDI-MS. By a Sequence Editor analysis, 37 signals were found in the peptide sequence of β-conglycinin. However, the application of exact *m/z* fails to identify the peptide sequence, e.g., 263.1391 *m/z* for FP and PF, except for 515.2302 *m/z* as SYFV in β-conglycinin sequence (Table 2-2).
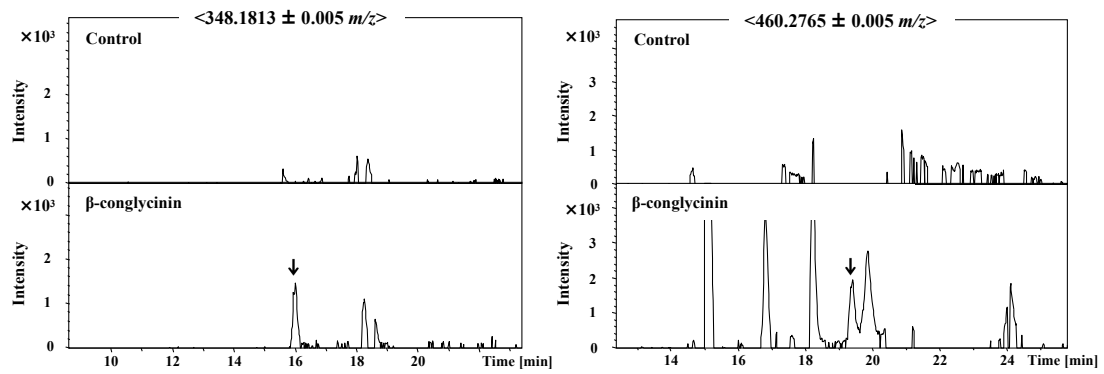
Fig. 2-4 EIC-LC-TOF/MS chromatograms of β-conglycinin-administered plasma from rat B monitoring at 348.1813 *m/z* and 460.2765 *m/z*. Arrows indicate peaks specific for β-conglycinin-administered plasma.

Table 2-2 Observed *m/z* values ([M+H$^+$]) specific for β-conglycinin-administered plasma by MALDI-MS and LC-TOF/MS analyses

| MALDI-MS	LC-TOF/MS	Predicted sequence	MALDI-MS	LC-TOF/MS	Predicted sequence
263.1	263.1391	FP, PF, MI	383.2	383.1334	YSN, SYN
278.1	278.1745	RC, GTT, ATS	383.2	383.2010	HAR, NLH, HNI
286.2	286.1634	GPL, VPA	414.2	414.2766	EEH, EHE, GQIP, KGPL
295.1	295.1209	FE, EF, YL, LY, IY	446.2	446.2630	RALS, ANIE, GLKE, INAE, VISQ, etc.
305.3	305.2112	AKS, SKA, SAK	460.2	460.2765	VPSGT, SILGA, DNVI, QELA, GPLSS, etc.
324.2	324.1856	YAA, AAY	463.2	463.2060	ARCN, EEGE, DSEL, SQDN, ARCN, etc.
334.2	334.1369	ESV, AFP, SDL, MIA, WE, etc.	487.2	487.2353	PGDAQ, EEPL, EPLE
336.2	336.1107	STE, ETS, GYP	489.2	489.2758	EKRG, KRGE, RGEK, NENL, SRNI, etc.
347.2	347.2128	NTL, NTI, ISK, LSK, IKS, etc.	501.2	501.1964	HNKC, EPQQ
348.2	348.1813	SEL, NSK, EIS, SNK, SKN, etc.	504.2	504.1654	EEIN, HFNS, EQDI, PAGYP, ENNQ, etc.
350.1	350.1403	SED, EDS, DSE, PPH, AYP, etc.	515.3	515.2302	SYFV
352.2	352.1846	VSF, ADF, FGE	517.1	517.2722	NLRD, ILNGT, GQQQG, KEQI, LKEQ, etc.
358.0	358.1666	NPK, EIP, KNP, KPN, NKP, etc.	519.3	519.2738	QRES, KCLR, ESQR, SQRE
359.0	359.2265	NLL, LKV, VLQ, RSP, ILN, etc.	553.2	553.2532	CLQSC, PNHAD, EFEE
361.1	361.1446	RGE, PSGT, GRE, DLN, DIN, etc.	565.3	565.2777	NRFE, VRKY, PRRH, ASVCVS, VQRY, etc.
364.2	364.1423	NKC, SEE, ESE, EES, VDM, etc.	599.2	599.2802	PQLQN, HGEKE, EEKHG, KHEW, HEWQ, etc.
375.4	375.2311	KVE, VEK, NKN, GDAL, ENL, etc.	713.3	713.3401	HARCNL, PQLENL, GRIRVL
378.1	378.1306	DED, EDD, LVF, VFL, VLF, etc.	830.6	830.3573 830.4335	LRVPSGTT, FFLSSTE, EITPEKN, LVINEGDA, GDANIELV, etc.
379.1	379.0008	DMN, SEGS, GSES, GRF, FGR, etc.			

3.3. Identification of peptides derived from β-conglycinin in rat blood

A complete identification of the predicted peptides (Table 2-2) was performed by matching the LC retention time with that of synthetic peptide. As shown in Fig. 2-5, although at 348.1813 *m/z,* EIS, ELS, LSE, SEL, and VMV, were a candidate peptide, the retention time of synthetic SEL (15.5 min) was in agreement with that of β-conglycinin plasma. According to the identification procedures, nine peptides, namely, SEL (α185–187; RT, 15.5 min), KGPL (α594–597, α'610-613; RT, 15.5 min), SILGA (β433–437; RT, 19.3 min), DSEL (α184–187; RT, 16.8 min), GDANI (α468–472, β302–306; RT, 16.5 min), SYFV (α576–579, α'592-595, β410–413; RT, 20.4 min), CLQSC (α38–42; RT, 16.3 min), GEQPRPF (α'97–103; RT, 17.0 min), and LVINEGDA (α463–470, β297–304; RT, 19.2 min) were successfully identified as β-conglycinin-derived peptide in the circulating blood of Wistar rats after oral administration of β-conglycinin (100 mg/kg). The respective EICs are shown in Fig. 2-6.
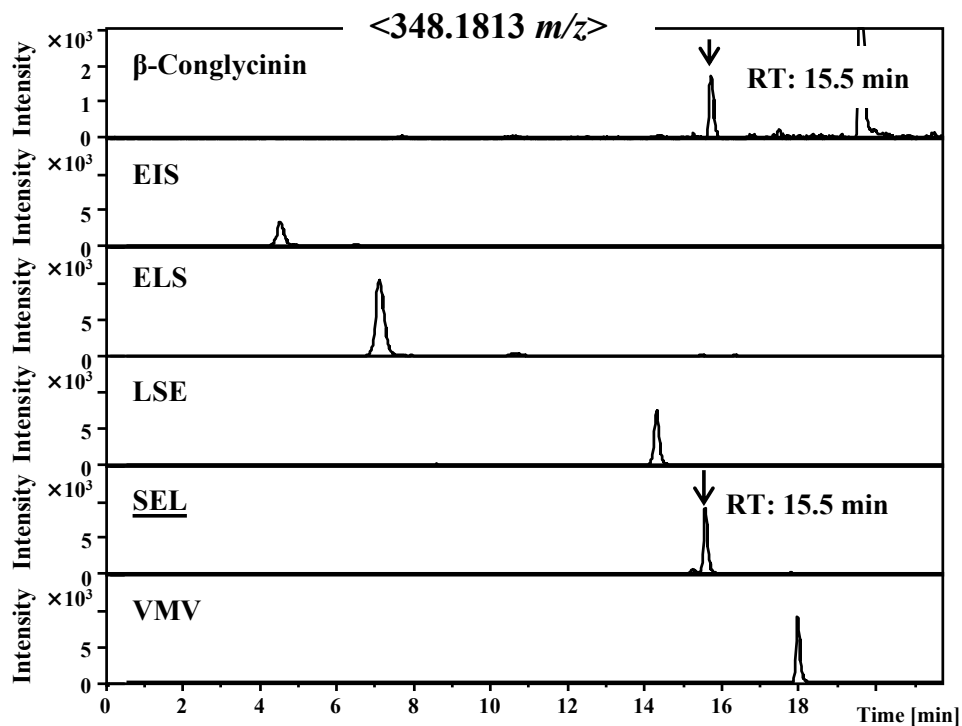
Fig. 2-5 EIC-LC-TOF/MS chromatograms of β-conglycinin-administered plasma and synthetic peptides of EIS, ELS, LSE, SEL, and VMV with 348.1813 *m/z*. LC-TOF/MS analysis was performed in positive ESI mode. RT: retention time.
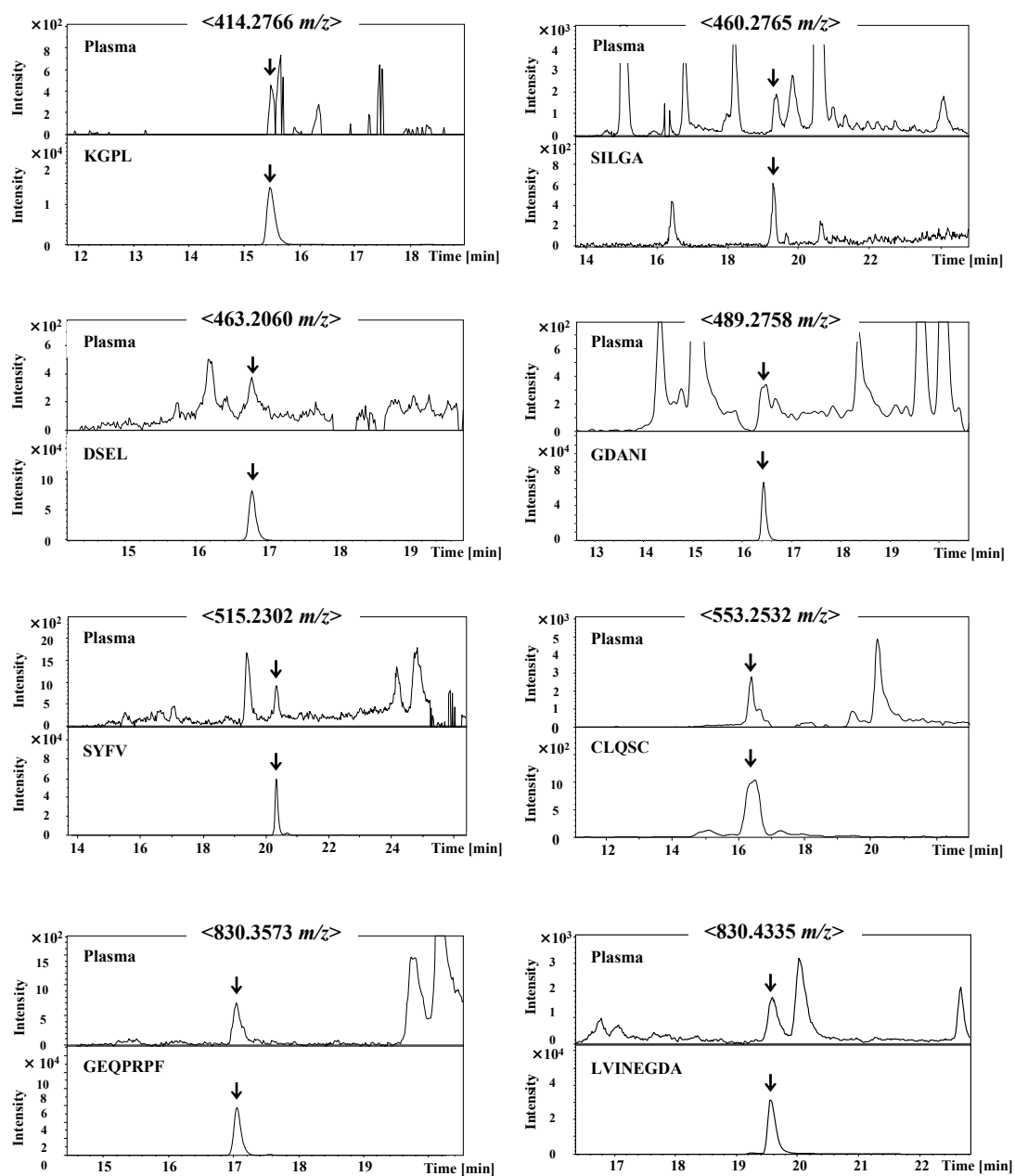
Fig. 2-6 EIC-LC-TOF/MS chromatograms of β-conglycinin-administered plasma and synthetic peptides (SEL, KGPL, SILGA, DSEL, GDANI, SYFV, CLQSC, GEQPRPF, and LVINEGDA) monitoring at 348.1813, 414.2766, 460.2765, 463.2060, 489.2758, 515.2302, 553.2532, 830.3573, and 830.4335 *m/z*, respectively. Arrows indicate target LC-MS peaks on each EIC.

Eight of the nine identified peptides were oligopeptides with > tetrapeptide length except for SEL. Thus far, we have revealed that not only di- and tripeptides but also longer oligopeptides could be absorbed, while peptide absorption across the intestinal membrane may be different from peptide chain length; a transportability of tetrapeptide across Caco-2 cell monolayers was 1/1,000-fold lower than that of dipeptide [89, 90]. It has been revealed that oral administration of protein hydrolysate to SHRs resulted in a preferable absorption of small peptides in circulating bloodstream [91]. It has been also reported that longer peptides such as octa- and decapeptides were liable to digestive degradation before entering bloodstream [92]. Taken together, the presence of > tetrapeptide length in the blood following β-conglycinin administration (Table 2-3) suggested that long oligopeptides digested from β-conglycinin in the gut might be absorbed while receiving protease degradation to smaller oligopeptides or amino acids.

With the aid of $[^{13}C_5,^{15}N]Val^3$-angiotensin II as an internal standard (IS) and synthetic peptide standards, plasma concentration of the nine identified peptides was roughly estimated. A factor f is introduced to correct the difference in ESI efficiency between peptide and IS in water solution. Plasma concentration of each identified peptide was estimated by the following equation:

$$\text{Conc. of peptide} = \text{Conc. of IS} \times \frac{\text{Int. of peptide in plasma}}{\text{Int. of IS in plasma}} \times f$$

$$f = \frac{\text{Int. of IS in water}}{\text{Int. of peptide in plasma}}$$

Hence, plasma concentration of SEL was calculated to be 20.4 pmol/mL. As a result, plasma concentrations of peptides were calculated to be in the range of 0.75 pmol/mL for DSEL to 756 pmol/mL for CLQSC (Table 2-3). The pmol/mL concentrations of these peptides were the same order as reported peptides such as VY (4.11 pmol/mL-plasma), LPP (11 pmol/mL-plasma), and IPP 12 pmol/mL-plasma [68, 93, 94].

Plasma (after β-conglycinin administration)

SEL (348.1816 *m/z*)

[$^{13}C_5$,^{15}N]Val^3-angiotensin II (526.7980 *m/2z*, 1 nmol/mL)

Intensity 1500

Intensity 2.5 × 10^6
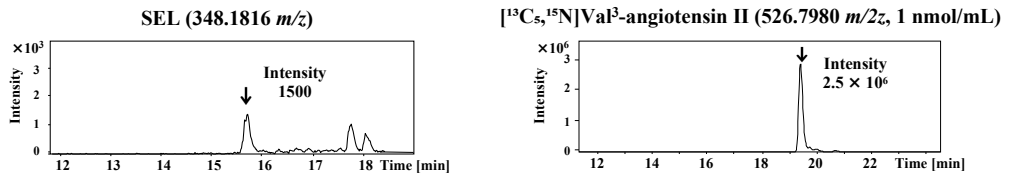
Standard (1 nmol/mL SEL and 1 nmol/mL [$^{13}C_5$,^{15}N]Val^3-angiotensin II)

SEL (348.1816 *m/z*)

[$^{13}C_5$,^{15}N]Val^3-angiotensin II (526.7980 *m/2z*)

Intensity 1.0 × 10^4

Intensity 3.4 × 10^5
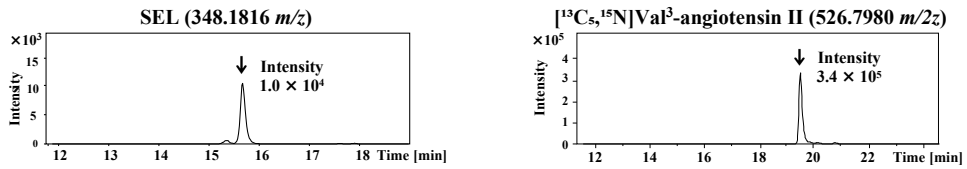
Fig. 2-7 LC-TOF/MS chromatograms of SEL and [$^{13}C_5$,^{15}N]Val^3-angiotensin II as IS. Plasma SEL concentration was estimated to be 20.4 pmol/mL.

Table 2-3 Identified peptides in β-conglycinin-administered plasma

m/z	Sequence	Position in β-conglycinin	Conc. in plasma (pmol/mL)
348.1813	SEL	α subunit 185-187	20.4 (rat **F**, 1 h)
414.2766	KGPL	α subunit 594-597, α' subunit 610-613	4.85 (rat **H**, 2 h)
460.2765	SILGA	β subunit 433-437	378 (rat **D**, 4 h)
463.2060	DSEL	α subunit 184-187	0.75 (rat **B**, 1 h)
489.2758	GDANI	α subunit 468-472, β subunit 302-306	2.27 (rat **B**, 1 h)
515.2302	SYFV	α subunit 576-579, α' subunit 592-595, β subunit 410-413	1.89 (rat **A**, 4 h)
553.2532	CLQSC	α subunit 38-42	756 (rat **B**, 1 h)
830.3573	GEQPRPF	α' subunit 97-103	0.94 (rat **D**, 1 h)
830.4335	LVINEGDA	α subunit 463-470, β subunit 297-304	52.9 (rat **D**, 1 h)

As depicted in Table 2-4, the appearance of the nine identified peptides was apparently different from individual rat and blood sampling time. The variability of peptides between rat groups (Table 2-4) can be explained by color-coded amino acid count analysis [88, 95]. The characteristic peptide signals in Table 2-1 were mapped onto the protein sequence of β-conglycinin. As depicted in Fig. 2-8, the red bands which contain the sequence of the nine identified peptides and their related sequences are frequently observed in all the groups. Even though the nine identified peptides in Table 2-4 were not observed in all rat groups, peptides related to the nine identified peptide sequences (e.g., SE, SL, DSEL, etc. from SEL) are co-produced as relating peptide precursor and/or degraded forms in all the groups with high frequency.

Table 2-4 Occurrence of identified peptides in β-conglycinin-administered plasma

Sequence	[M + H]⁺	Time (h)	Rat								
			A	B	C	D	E	F	G	H	I
SEL	348.1765	1	○	○				○			
		2						○	○	○	○
		4						○	○	○	○
		8						○	○	○	○
KGPL	414.2766	1		○						○	
		2						○		○	○
		4						○	○	○	○
		8								○	○
SILGA	460.2765	1									
		2		○		○					
		4				○					
		8									○
DSEL	463.2060	1		○							
		2									
		4									
		8									
GDANI	489.2758	1		○							
		2									
		4									
		8						○			
SYFV	515.2302	1									
		2									
		4	○								
		8							○		
CLQSC	553.2532	1		○							
		2									
		4									
		8									
GEQPRPF	830.3573	1							○		
		2									
		4									
		8									
LVINEGDA	830.4335	1							○		
		2									
		4									
		8									
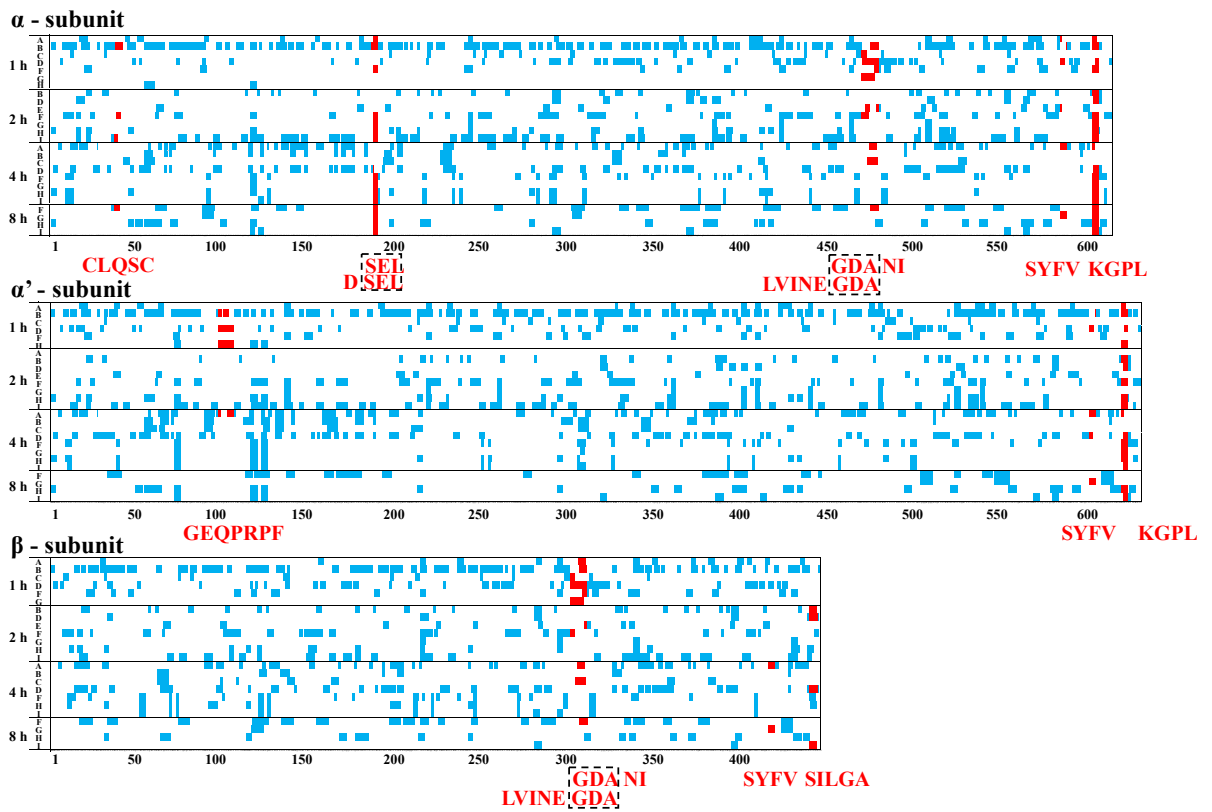
Fig. 2-8 Production mapping of peptides predicted by MALDI-MS in the sequence of β-conglycinin (α, α', and β subunits). Peptide sequences are mapped from individual rats (**A** to **I**) at 1–8 h. Red bands indicate the sequences of the nine identified peptides and their related peptides. Blue bands indicate the sequences of peptides predicted by MALDI-MS (from Table 2-1).

Health benefits of dietary β-conglycinin diet *in vivo* have been reported, including serum triglyceride-reducing [30], anti-diabetes [42], and anti-obesity [45], but the candidates responsible the effects remained unclear. In this study, nine peptides were identified in the rat bloodstream (Table 2-3), while there were no evidential reports on their physiological effect. The finding that LSEL [96] and PGPL [97] showed anti-diabetic effects in animals led to the expectation for the physiological potential of SEL, DSEL, and KGPL among the nine peptides (Table 2-3). SY, a fragment of SYFV, was reported to inhibit the secretion of apolipoprotein B in HepG2 cells [60]. Therefore, not only the nine identified peptides, but also their related metabolites must be considered to explain the overall health effects of orally administered β-conglycinin.

4. Summary

In this **Chapter II,** we aimed to clarify the production of peptide metabolites in bloodstream after protein oral administration. As a result of the study, the intake of β-conglycinin (100 mg/kg) to Wistar rats caused the production of nine oligopeptides (SEL, KGPL, SILGA, DSEL, GDANI, SYFV, CLQSC, GEQPRPF, and LVINEGDA) in the circulating bloodstream by non-targeting MALDI-MS and targeting LC-TOF/MS analyses. Although 117 MS signals remained unidentified, a variety of peptide metabolites produced by β-conglycinin digestion would contribute to the health benefits of β-conglycinin reported so far.

Chapter III

Identification of peptide metabolites from orally administered glycinin in rat bloodstream by a Coumarin-tagged MALDI-MS

1. Introduction

As mentioned earlier in **Chapter I**, although dietary proteins have been demonstrated to possess potential health promoting effects, such as anti-hyperlipidemic [40], anti-diabetic [9], and anti-adipogenic effects [12], the fact that protein macromolecules could not be absorbed led to the hypothesis that any metabolites derived from dietary proteins may be involved in the health benefits. As demonstrated in **Chapter II,** nine peptides were successfully identified in the circulatory blood of Wistar rats after oral administration of a soy protein β-conglycinin (100 mg/kg) by combinatory MALDI-MS and LC-TOF/MS analyses. These results suggested that peptides could be a key factor to

understand the diverse biological functions of dietary proteins. Although MALDI-MS and LC-MS are promising analytical tools for the identification of peptides in blood [98, 99], there were still limitations due to huge interfering peaks from matrix and blood in the region of < 400 *m/z* corresponding to di- to pentapeptides [100, 101]. The MS limitations at low *m/z* regions may avoid the extensive study on small candidates responsible for health benefits of dietary proteins. Thus, appropriate analytical advances are required for comprehensive evaluation of metabolites in a small amount of blood sample.

Thus far, in order to achieve highly sensitive and selective detection of amines, a variety of amine-derivatization techniques have been proposed for ESI-MS detection. Dansyl derivatization enhanced a MS detection of amino acids at the limit of detection (LOD) of > 2.8 fmol/mL [102]. 6-Aminoquinolyl-*N*-hydroxysuccinimidyl carbamate derivatization also improved MS detection of amino acids at the LOD of > 10.2 fmol/mL [103]. However, the reported derivatization conditions were optimized for amino acids [102, 103], so that the conditions might cause any unexpected degradation of target peptides. For the derivatization of peptides, TNBS [104, 105], APDS [106], and Cou derivatizations [107] have been conducted for selective and enhanced LC-MS detection of peptides at rapid and mild (slight alkaline pHs) derivatization conditions.

Therefore, in **Chapter III,** we aimed to develop a novel MALDI-MS method in combination to amine derivatization technique for comprehensive

characterization of peptide metabolites in blood. The strategy of **Chapter III** was thus oriented to establish a convenient peptide identification method by a tag-incremented *m/z* shift of peptides to silent or less matrix-derived *m/z* regions. Glycinin, a soybean protein, was used in this study, since HDL-C improvement [43] and serum TG lowering [29] potentials were reported by the intake.

2. Materials and methods

2.1. Materials

Glycinin was prepared from defatted soymilk, according to previously reported method [76]. Briefly, defatted soymilk was extracted with 10-fold volume of water and centrifuged to obtain the supernatant. The supernatant was treated by phytase (pH 6, 1 h, 40 °C) and centrifuged to obtain glycinin precipitate. Gly-Sar-Sar (GSS), Gly-Sar-Sar-Sar (GSSS) and Gly-Sar-Sar-Sar-Sar (GSSSS) were obtained from Biomatik Co. (Cambridge, ON, Canada). *N*-Succinimidyl-7-methoxycoumarin-3-carboxylate (Cou) and 2′,4′,6′-trihydroxyacetophenone (THAP) were purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). Triethylammonium bicarbonate (TEAB) buffer and 3-aminopyridyl-*N*-hydroxy-succinimidyl carbamate (APDS) were purchased from FUJIFILM Wako Pure Chemical Co. (Osaka, Japan). 2,4,6-Trinitrobenzene sulfonate (TNBS), phytic acid, and ammonium sulfate were purchased from Nacalai Tesque Inc. (Kyoto, Japan). 2,5-Dihydroxybenzoic acid (DHB), *α*-cyano-4-hydroxycinnamic acid (CHCA), and sinapic acid (SA) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Distilled water, ACN, and FA of LC-MS grade were purchased from Merck Co. (Darmstadt, Germany). MeOH was purchased from Kanto Chemical Co. (Tokyo, Japan). All other reagents were of analytical reagent grade and were used without further purification.

2.2. Animal experiments

Eleven-week-old male SD rats (SPF/VAF Crj; Charles River Japan; Kanagawa, Japan, 395–440 g, $n = 3$) were used in this study. Rats **A**, **B**, and **C** were individually housed for 1 week under a temperature of 21 ± 1 °C and humidity of 55 ± 5%. Rats were given access to an AIN-93G diet (Oriental Yeast Co.; Tokyo, Japan) and water *ad libitum*. Each rat was fasted for 16 h prior to glycinin administration. Glycinin was orally administered to 12-week-old SD rats at a dose of 100 mg/kg-B.W. Before administration (noted as 0 h), approximately 300 µL of blood was collected from the tail vein and used as control. Glycinin-administered blood samples were collected at 0.5, 1, 2, 4, and 8 h after administration. Blood samples were collected into 1.5-mL Eppendorf tubes (Hamburg, Germany) containing EDTA-2Na, and immediately centrifuged at 3,500 × g for 15 min at 4 °C to obtain plasma. All the animal experiments were performed according to the Guidelines for Animal Experiments in the Faculty of Agriculture in the Graduate Course of Kyushu University and according to the Law (No. 105, 1973) and Notification (No. 6, 1980 of the Prime Minister's Office and No. 71, 2006, of the Ministry of Health, Labor and Welfare) of the Japanese Government. All the experiments were reviewed and approved by the Animal Care and Use Committee of Kyushu University (Permit Number: A20-095).

2.3. Chemical derivatization of peptides

GSS, GSSS, and GSSSS were used to optimize MALDI-MS detection of amine derivatized peptides. For APDS derivatization [106], the peptide mixture (10 µL) was added to 185 µL of borate buffer (100 mmol/L, pH 8.5), followed by the addition of 5 µL of 80 mmol/L APDS solution. After the reaction at 60 °C for 15 min, 200 µL of 2% FA was added to stop the reaction. For TNBS derivatization [105], the peptide mixture (10 µL) was added to 100 µL of 150 mmol/L TNBS in borate buffer (pH 8.5). The solution was incubated at 30 °C for 30 min, followed by the addition of 200 µL of 2% FA to stop the reaction. Each APDS or TNBS derivatization solution was then desalted by a Waters Sep-Pak C18 column (Waters Co.; Milford, MA, USA). Chemically derivatized peptides were eluted with 3 mL of ACN/0.1% FA. The eluate was evaporated to dryness and used for MALDI-MS analysis. For Cou derivatization [107], the peptide mixture (10 µL) was added to 5 µL of TEAB buffer (1 mol/L, pH 8.5), followed by the addition of 5 µL of 0.3 mmol/L Cou solution in ACN. The reaction was carried out at room temperature for 30 min. The solution was evaporated to dryness and used for MALDI-MS analysis. The reaction scheme of each derivatization is shown in Fig. 3-1.
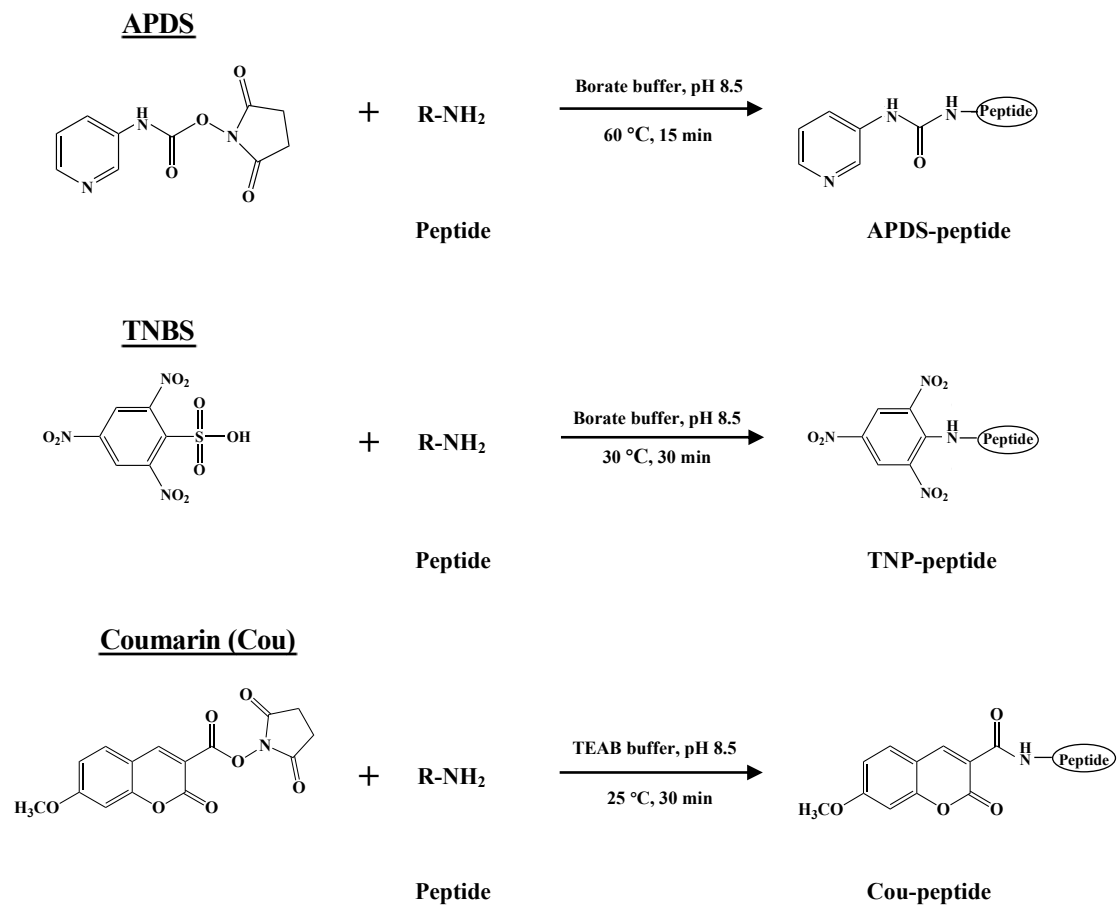
Fig. 3-1 Reaction scheme of APDS, TNBS, and Coumarin derivatization of peptides.

2.4. Coumarin derivatization of plasma sample

An aliquot (50 µL) of plasma was mixed with 250 µL of 10% ACN containing 0.1% FA and 0.1% NaCl, and filtrated by centrifugation with an Amicon Ultra 0.5-mL-3K centrifugal filter (Millipore Co.; Carrigtwohill, Ireland) at 14,000 × g for 30 min at 4 °C. For Cou derivatization, 100 µL of TEAB buffer (1 mol/L, pH 9.0) was added to the filtrate, followed by the addition of 50 µL of 30 mmol/L Cou solution in ACN. After the incubation for 30 min at 25 °C, 200 µL of 2% FA was added to stop the reaction. The solution was loaded to Waters Sep-Pak C18 column and eluted with 3 mL of ACN/0.1% FA. For comparative MALDI-MS study of Cou-derivatized plasma sample with non-derivatized (intact) one, intact plasma sample was prepared according to the aforementioned steps without the addition of Cou solution. Intact and Cou-derivatized plasma samples were evaporated to dryness and used for MALDI-MS analysis.

2.5. MALDI-MS analysis

The Cou-derivatized plasma samples were dissolved in 10 µL of matrix solution. Matrix reagents used in this study were DHB, CHCA, SA, and THAP at a concentration of 10 mg/mL. DHB was dissolved in 50% MeOH. CHCA, SA, and THAP were dissolved in 60% ACN. All matrix solutions contain 10 mmol/L phytic acid [82], and 50 mmol/L ammonium sulfate [83]. An aliquot (1 µL) was spotted onto an ITO-coated glass slide. The remaining solution (9 µL) was evaporated to dryness and used for further LC-TOF/MS analysis.

Bruker Autoflex III mass spectrometer equipped with a SmartBeam III (Bruker Daltonics; Bermen, Germany) was used for MALDI-MS analysis in a positive ion-linear mode. MALDI-MS data were acquired in the range of 100–2,000 m/z. MS parameters were as follows: ion source 1, 20.00 kV; ion source 2, 18.80 kV; lens voltage, 7.50 kV; gain, 10.0; laser offset, 60%; laser value, 6.5%; laser power, 35%; laser frequency, 100 Hz; shot, 1,000. MS spectra were analyzed by Bruker Data Analysis 3.3 software.

2.6. LC-TOF/MS analysis

To obtain exact masses of m/z signals characterized by MALDI-MS, LC-TOF/MS analysis was performed for Cou-derivatized plasma samples. LC separation was performed on an Agilent 1200 series HPLC (Agilent Technologies; Waldbronn, Germany) on a Cosmosil 5C18-MS-II column (2.0 × 150 mm, Nacalai Tesque). The dried plasma sample was dissolved in 25 µL of 0.2% FA, and an aliquot of 20 µL was injected to the LC-TOF/MS system. LC separation was performed in a linear gradient of 0.1% FA to MeOH containing 0.1% FA (20 min) at a flow rate of 0.2 mL/min at 40 °C. TOF/MS analysis was conducted on a Bruker micrOTOF-II mass spectrometer in an ESI-positive mode. MS parameters were as follows: drying gas (N_2), 8.0 L/min; drying temperature, 350 °C; nebulizer pressure, 1.6 bar; capillary voltage, 3,800 V; mass range, 100–2,000 m/z. A calibration solution (10 mmol/L sodium formate in 50% ACN) was injected at the start of each run. Data were analyzed and acquired by a Bruker

Data Analysis 4.0 software.

2.7. Identification of peptide sequence by LC-TOF/MS

Peptide sequence of Cou-tagged MALDI-MS signals specific for glycinin-administered plasma was identified by the following m/z correction calculation, using a Sequence Editor analysis (Bruker Daltonics biotools, version 3.2). Namely, exact m/z value of peptides in glycinin-administered plasma was obtained by subtracting exact m/z value corresponding to [tag-peptide + H]$^+$ with exact m/z value of Cou-tag moiety ($\Delta m/z$); e.g., for Cou-GSS, 420.1390 m/z ([tag-peptide+H]$^+$) – $\Delta m/z$ [203.0336 m/z (Cou moiety) – 1.0073 m/z (H)] = 218.1127 m/z ([GSS+H]$^+$) (Table 3-1). The mean $\Delta m/z$ value obtained by the measurements of Cou-GSS, Cou-GSSS, and Cou-GSSSS was 202.0263 ± 0.0003 m/z, indicating the high reproducibility within the tolerance of ± 0.005 under the present LC-TOF/MS conditions. The calculated exact m/z values of target peptides were then applied to a Sequence Editor software to predict the sequence in the primary sequences of glycinin composed of G1, UniProtKB-P04776; G2, UniProtKB-P04405; G3, UniProtKB-P11828; G4, UniProtKB-P02858; and G5, UniProtKB-P04347.

Table 3-1 Observed *m/z* values of intact and Cou-derivatized model peptides by LC-TOF/MS

	$[M + H]^+$	$[M\text{-}Cou + H]^+$	$\Delta m/z$*	mean	SD
GSS	218.1128	420.1390	202.0262		
GSSS	289.1523	491.1790	202.0267	202.0263	0.0003
GSSSS	360.1888	562.2148	202.0260		

*$\Delta m/z$ indicates the neutral loss of *m/z* by subtracting $[M\text{-}Cou + H]^+$ with $[M + H]^+$. Thus, the

*m/z* of Cou moiety is calculated to be $\Delta m/z$ + 1.0073 of H = 203.0336.

3. Results and discussion

3.1. Amine derivatization for a tag-aided peptide detection in blood by MALDI-MS

Three amine derivatizations, namely, APDS [106], TNBS [105], and Cou [107], were applied for peptide derivatization under mild derivatization conditions of pH < 9 and < 30 min to avoid unexpected degradation of target peptides during reaction. Using synthetic oligopeptides GSS, GSSS, and GSSSS, which are stable against enzymatic and chemical degradations under mild reaction conditions [90], the three derivatizations were conducted accordingly, and the resultants were analyzed by MALDI-MS to selectively detect derivatized peptides by *m/z* increment of derivatization tag moiety. The *m/z* increments of APDS, TNBS, and Cou reagents with GSS ([M + H]$^+$, 218.1 *m/z*), GSSS ([M + H]$^+$, 289.1 *m/z*), and GSSSS ([M + H]$^+$, 360.2 *m/z*) were expected to be +120, +211, and +202 *m/z*, respectively. As shown in Fig. 3-2, no MALDI-MS signals of APDS- and TNBS-derivatized synthetic peptides were detected, whereas successful MS detection was obtained for Cou-derivatized peptides when DHB was used as a matrix reagent. It has been reported that APDS, TNBS, and Cou derivatizations for amines resulted in an enhanced ionization efficiency in ESI ionization [105–107]. In this study, the detection intensity of derivatized peptides was enhanced only by Cou derivatization in MALDI-MS. One possible reason is that absorption efficiency of MALDI-laser energy (Nd:YAG = 355 nm) was markedly increased by the Cou-tag (λ_{max} of Cou = 358 nm, [108]) to allow for

the enhanced MS detection of the peptides, since ultraviolet（UV）absorption has been considered as a prerequisite for the efficient ionization of small molecules in MALDI ion source [109–111]. For GSSS and GSSSS, Cou derivatization caused 3- and 12-fold higher MS intensities, respectively compared to intact ones (Fig. 3-2). Moreover, it was clear that a +202 *m/z* increment by Cou moiety was sufficient to shift the mass of target peptides to a "silent" (free noise signals) regions (> 400 *m/z*), as shown in Fig. 3-2.
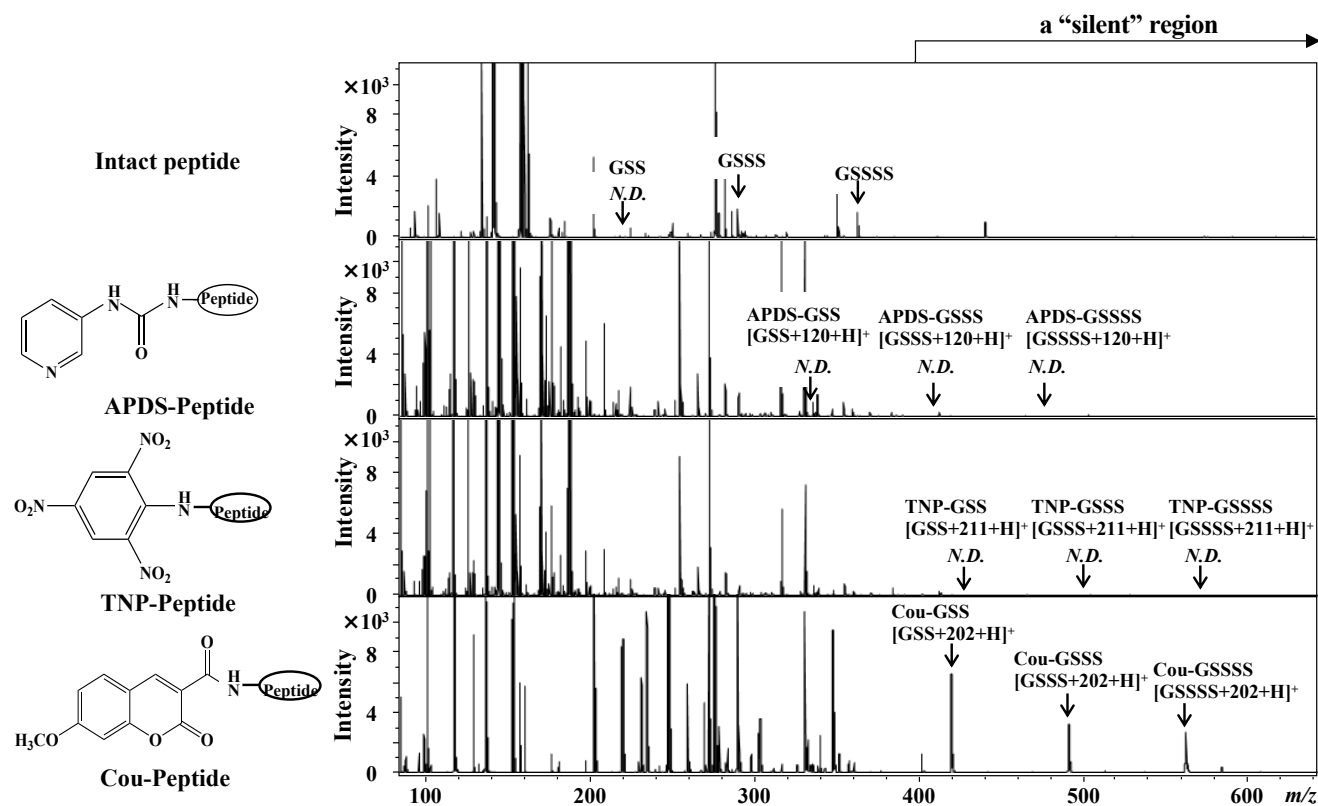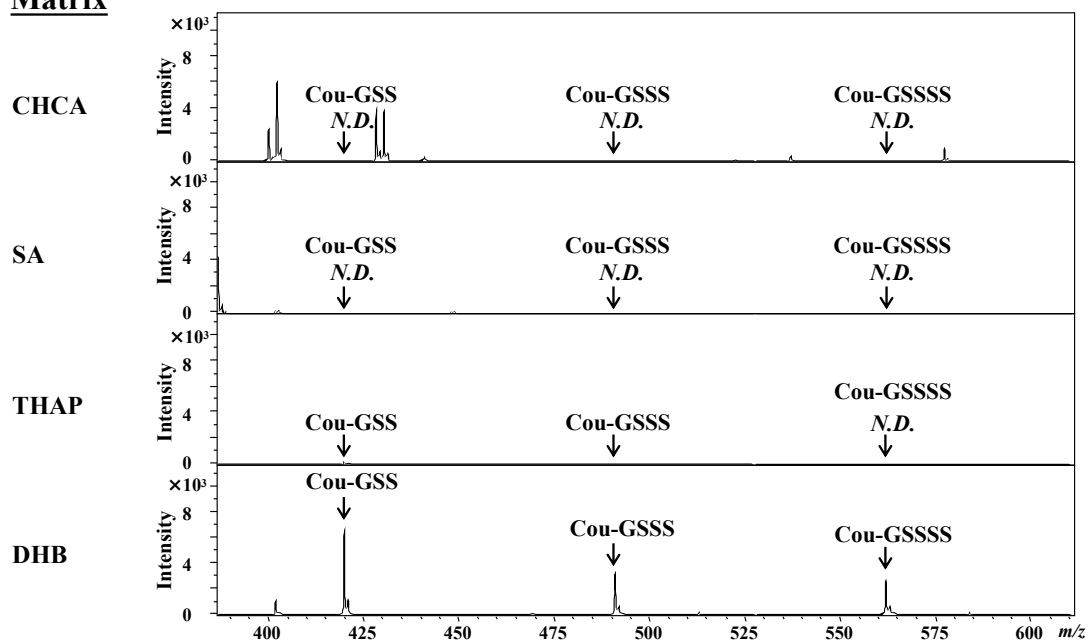
Fig. 3-2 MALDI-MS spectra of model peptides (GSS, GSSS, GSSSS) derivatized with or without APDS, Cou, and TNBS. DHB

was used as MALDI matrix. The mass shift by each derivatization is +120 *m/z* for APDS, +211 *m/z* for TNBS, and +202 *m/z* for Cou.

MALDI-MS spectra in a positive ion-linear mode were shown in the mass range of 100–600 *m/z*. *N.D.*, not detected.

In order to optimize the apparent MALDI-MS detection of Cou-derivatized peptides, effect of matrix reagent on the detection was investigated. CHCA, SA, THAP, and DHB were used for the detection of Cou-derivatized GSS, GSSS, and GSSSS at a concentration of 100 fmol/spot. As shown in Fig. 3-3, CHCA, SA, and THAP, which are commonly used for positively ionized compounds [112, 113], had no ability to ionize Cou-derivatized peptides, while only DHB did provide an efficient MS detection for Cou-GSS, -GSSS, and -GSSSS (Fig. 3-3). Therefore, DHB was used as MALDI matrix suitable for Cou-derivatized peptides for further study.
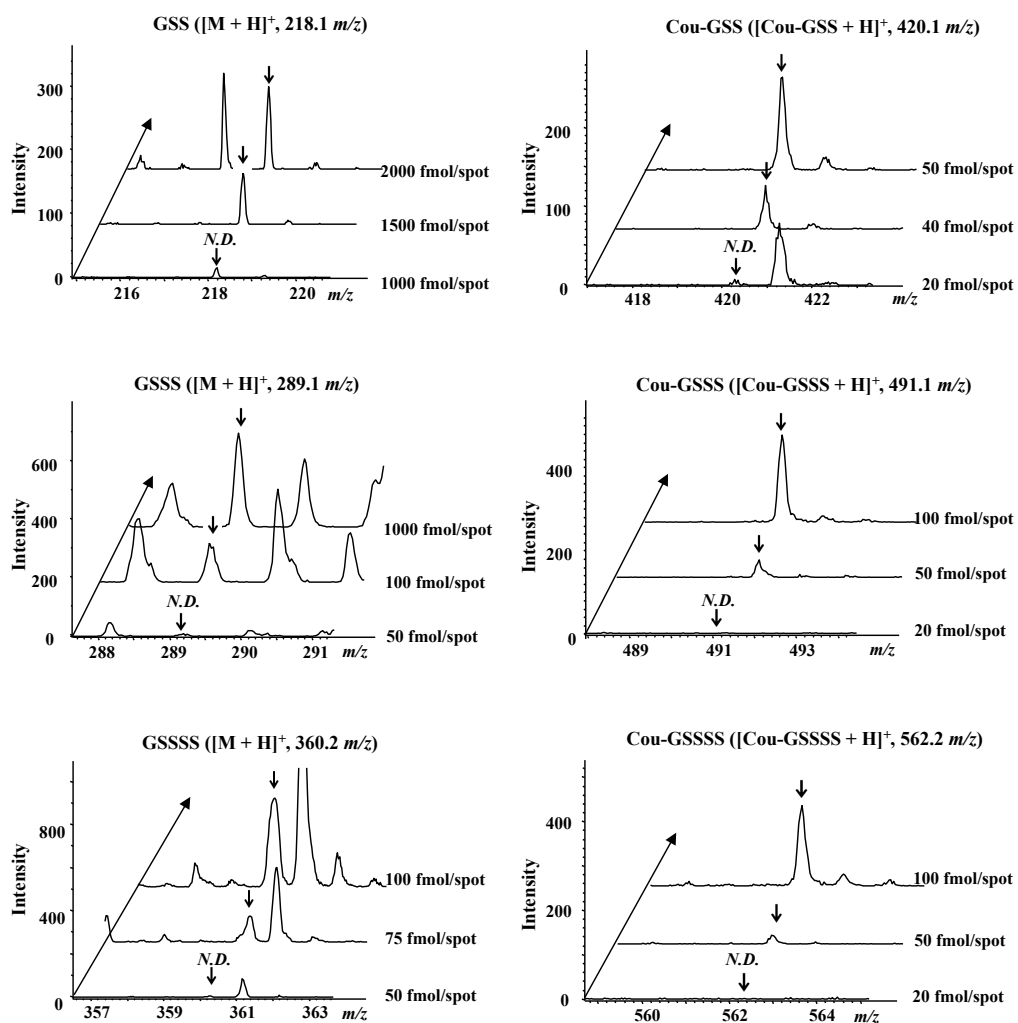
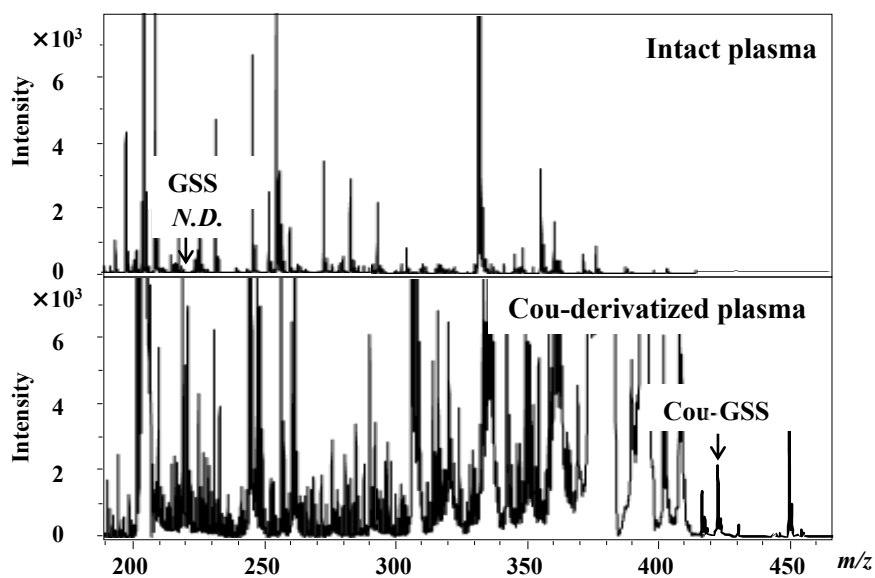**Fig. 3-3 MALDI-MS spectra of Cou-derivatized model peptides (Cou-GSS, Cou-GSSS, Cou-GSSSS) in CHCA, SA, THAP, and DHB as matrix reagents.** MALDI-MS spectra in a positive ion-linear mode are shown in the mass range of 400–600 *m/z*. *N.D.*, not detected.

MALDI-MS detection capacity for Cou-derivatized peptides (20–50 fmol/spot) was examined in DHB matrix. As depicted in Fig. 3-4, higher amounts of 1,500 fmol/spot for GSS, 100 fmol/spot for GSSS, and 75 fmol/spot for GSSSS were required for significant MALDI-MS detection in their intact forms. In contrast, it was apparent that their derivatization with Cou caused an enhanced detection as low as 40–50 fmol/spot. This indicates that Cou-derivatization of peptides has great advantage on not only the *m/z* shift to silent regions (selectivity), but also the enhancement of MS detection (sensitivity). Moreover, no MS signals for remaining underivatized GSSS and GSSSS in Cou-derivatized solution were observed (Fig. 3-4), which suggested a high reaction efficiency of Cou with peptides under the present reaction conditions (30 mmol/L, 30 min, 25 °C). Using di- to pentapeptide standards, Cou derivatization of peptides with different lengths was detected by LC-ESI-MS [114]. Pashkova *et al.* [107] also reported that Cou could react with longer oligopeptide (>hexapeptides). Together with our results, it is suggested that Cou could react with *N*-terminal amino groups of peptides without consideration of chain lengths and form stable Cou-tagged peptide derivatives [107, 114].

Fig. 3-4 MALDI-MS spectra of intact (left) and Cou-derivatized peptides (right) at different concentrations (20–2000 fmol/spot). The $[M+H]^+$ of intact peptides (GSS, m/z 218.1; GSSS, m/z 289.1; GSSSS, m/z 360.2) and Cou-derivatized peptides (Cou-GSS, m/z 420.1; Cou-GSSS, m/z 491.1; Cou-GSSSS, m/z 562.2) were analyzed by MALDI-MS. DHB was used as a matrix reagent.

Spike experiments of GSS in rat plasma were conducted to validate the Cou derivatization technique for MALDI-MS analysis. As shown in Fig. 3-5, although intact GSS was not detected in plasma, under the current Cou-tagged MALDI-MS conditions, Cou-GSS was successfully detected in plasma spiked at 5 nmol/mL. These results indicated that Cou derivatization under the current reaction conditions (30 mmol/L Cou, 30 min, 25 °C) enabled enhanced detection of peptides in plasma at > 5 nmol/mL-plasma concentrations.

Fig. 3-5 MALDI-MS spectra of GSS spiked in rat plasma without (intact, upper) or with (lower) Cou derivatization. GSS was spiked to SD rat plasma at a concentration of 5 nmol/mL. DHB was used as a matrix reagent for MALDI-MS analysis. *N.D.*, not detected.

3.2. Characterization of peptides in glycinin-administered blood of Sprague-Dawley rats by Coumarin-tagged MALDI-MS

Plasma samples of three SD rats (rats **A**, **B**, and **C**) at time-intervals from 0.5 h to 8 h after glycinin administration (100 mg/kg) were collected from the tail vein and were subjected to the optimized Cou-tagged MALDI-MS analysis. Rat plasma collected after glycinin administration were analyzed with or without Cou derivatization and typical MS spectra at 0 h (before) and 4 h are shown in Fig. 3-6 and Fig. 3-7. For plasma samples without Cou derivatization, a few characteristic peaks were detected, while many noise signals interfering peptide detection by MALDI-MS were also observed in the range of 100–400 *m/z* (Fig. 3-6). In contrast, for Cou-derivatized plasma samples, many characteristic peaks were detected in the range of > 400 *m/z* in 4 h-administered plasma, as compared to 0 h-plasma (Fig. 3-7). These results strongly suggested that any peptides in plasma were successfully derivatized with Cou and glycinin-derived peptides were shifted to higher mass ranges by the increment of +202 *m/z* of Cou tag. Therefore, the established Cou-tagged MALDI-MS analysis was capable of a rapid (< 1 min per one-run) and specific selection of glycinin-derived peptides in a small volume (< 10 μL) of blood sample.
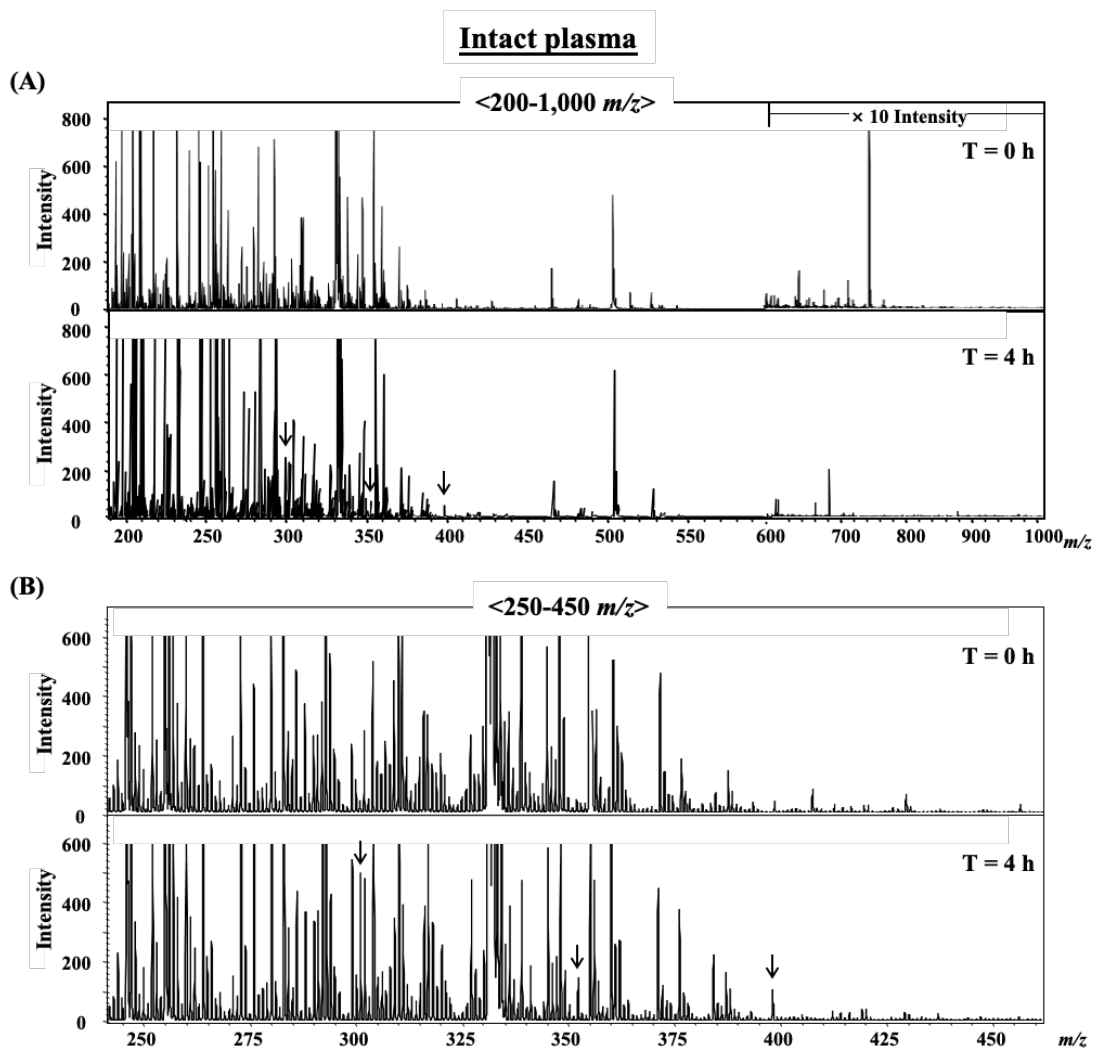
Fig. 3-6 Typical MALDI-MS spectra of intact plasma of rat A collected before (T = 0 h) and after (T = 4 h) the oral administration of glycinin (100 mg/kg). Arrows indicate peaks specific for glycinin-administered plasma compared to 0 h (Control). MALDI-MS spectra in a positive ion-linear mode were shown in full ranges of 200–1,000 m/z (A) and enlarged ranges of 250–450 m/z (B). DHB was used as a matrix reagent for MALDI-MS analysis.
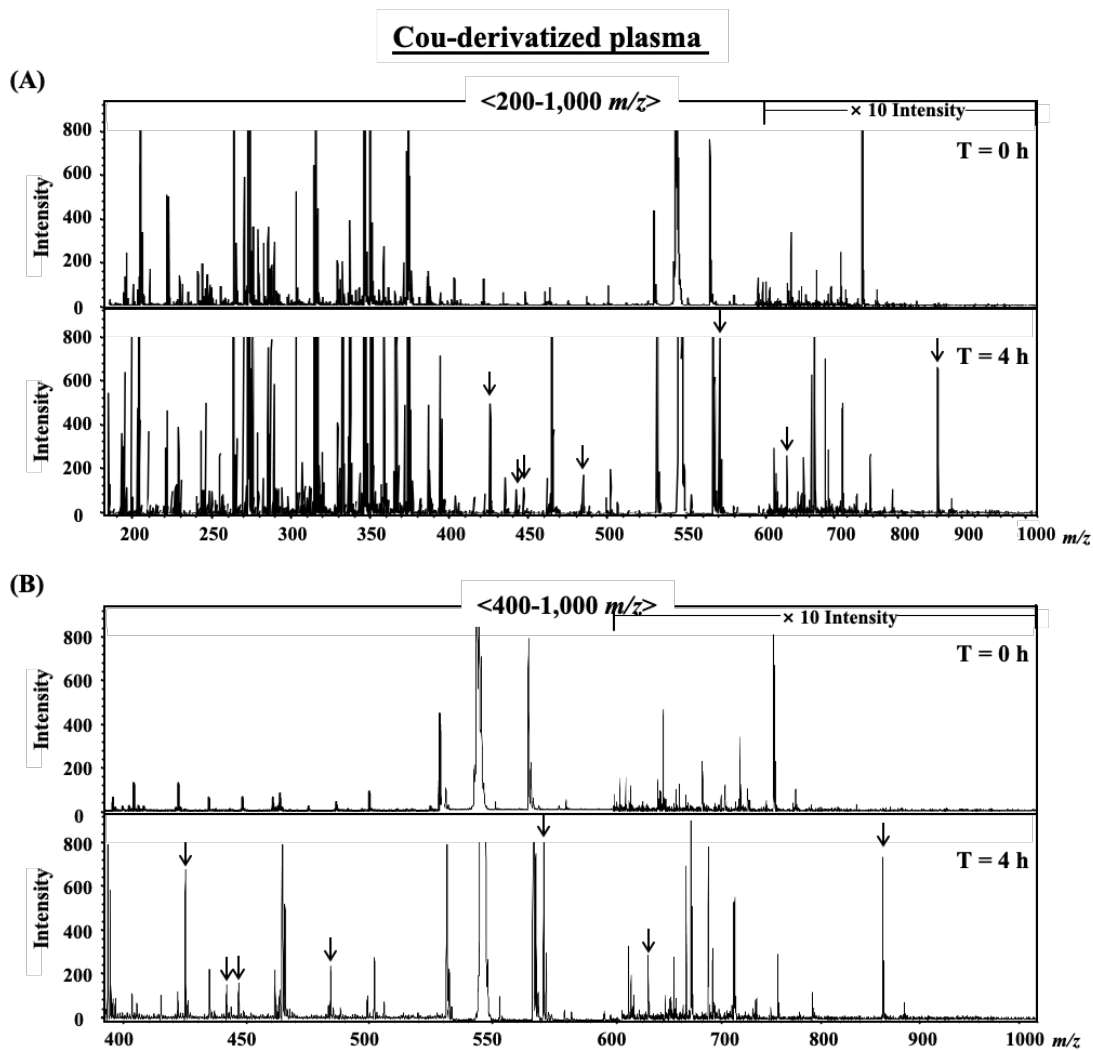
Fig. 3-7 Typical MALDI-MS spectra of Cou-derivatized plasma of rat A collected before (T = 0 h) and after (T = 4 h) the oral administration of glycinin (100 mg/kg). Arrows indicate peaks specific for glycinin-administered plasma compared to 0 h (Control). MALDI-MS spectra in a positive ion-linear mode were shown in full ranges of 200–1,000 *m/z* (A) and enlarged ranges of 400–1,000 *m/z* (B). DHB was used as a matrix reagent for MALDI-MS analysis.

Applying the Cou-tagged MALDI-MS approach to glycinin-administered plasma samples, we successfully detected a total of 36 characteristic *m/z* signals at 400–900 *m/z* in plasma samples of rats **A**, **B**, and **C** taken at 0.5 to 8 h after glycinin administration (Fig. 3-8); rat **A**: 17 signals (424.2, 440.2, 446.2, 482.2, 518.3, 543.2, 547.1, 565.3, 569.2, 578.5, 597.3, 603.1, 623.3, 625.1, 705.4, 731.3, 861.2 *m/z*); rat **B**: 14 signals (405.2, 474.3, 494.3, 498.2, 530.2, 531.2, 546.3, 576.4, 587.3, 618.3, 624.2, 630.3, 715.4, 734.4 *m/z*); rat **C**: 6 signals (446.2, 461.2, 510.1, 564.3, 683.3, 715.2 *m/z*). Although 446.2 *m/z* signal was commonly observed in plasma of rats **A** and **C**, other signals were different among rat individuals. The unique production dynamics of peptide metabolites depending on rat individual after the intake of dietary proteins in blood were not scarce, since the production of a variety of oligopeptides was reported after the intake of casein [78] and β-conglycinin as described in **Chapter II**. Taken together, exploiting the Cou-tagged MALDI-MS analysis established in the current study, it was evident that the intake of glycinin caused the production of a variety of peptide metabolites in rat bloodstream (Fig. 3-8).

Rat A
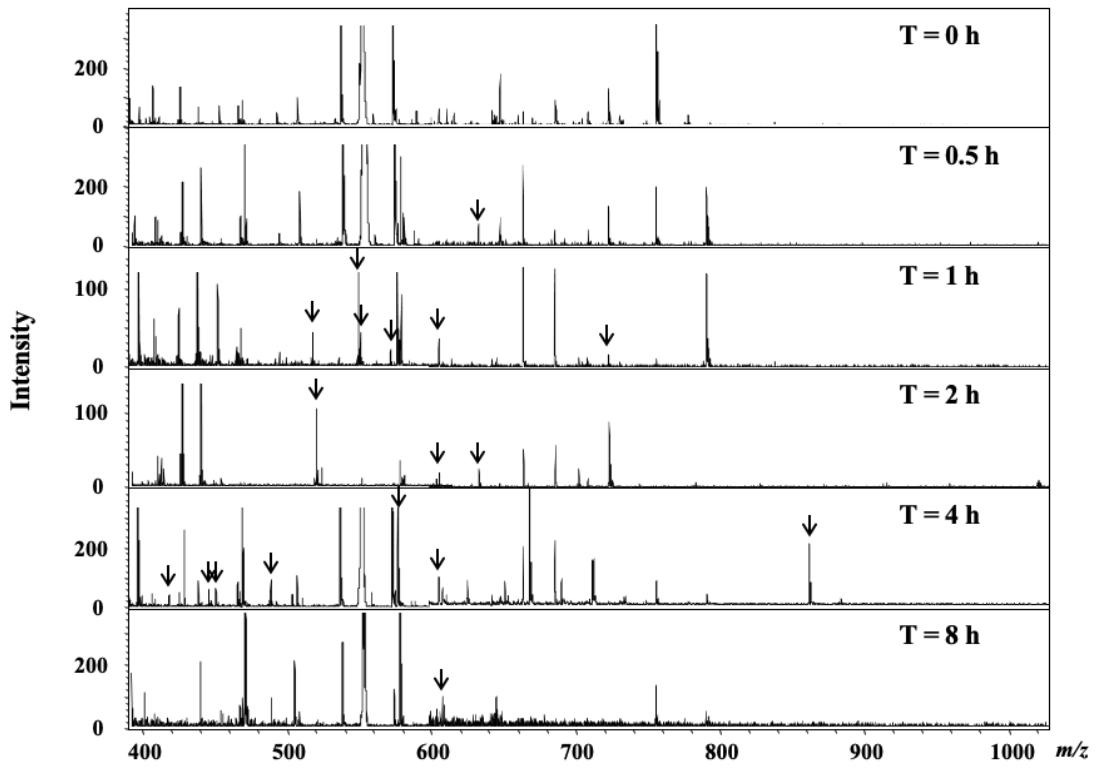
Rat B
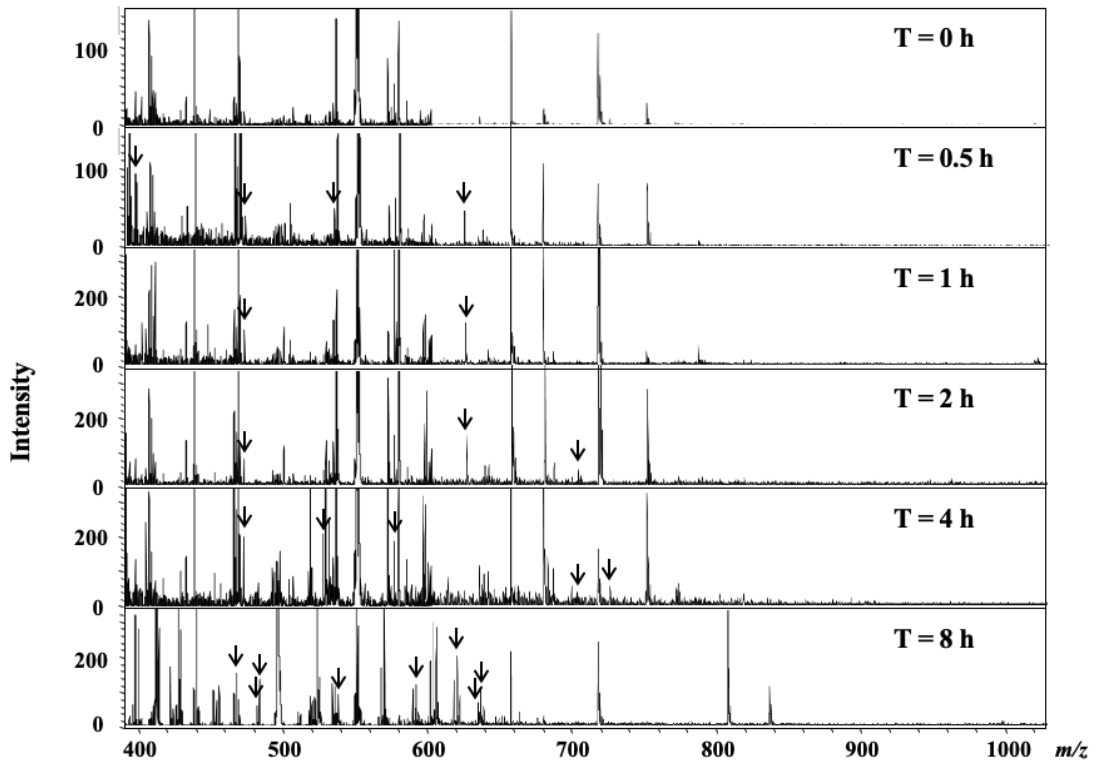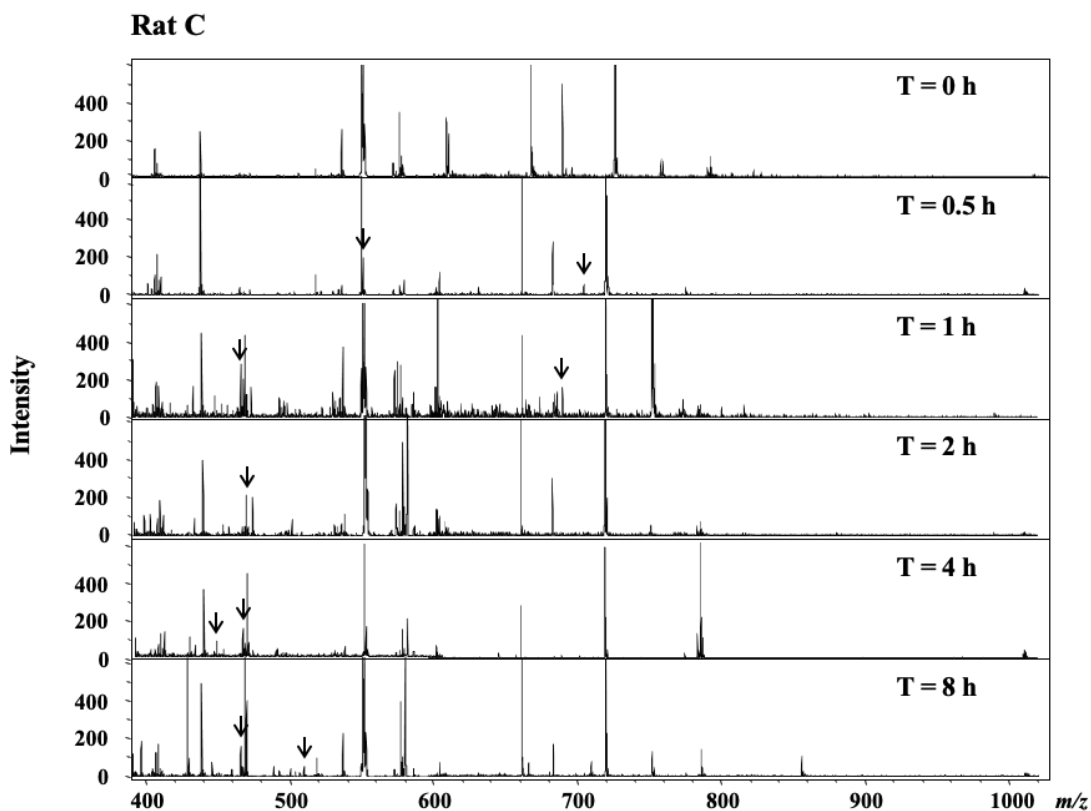
Fig. 3-8 Cou-tagged MALDI-MS analysis of plasma samples taken at 0, 0.5, 1, 2, 4, and 8 h after the oral administration of glycinin (100 mg/kg) to rats A, B, and C. Arrows indicate peaks specific for glycinin-administered plasma compared to 0 h (control). MALDI-MS spectra were obtained in a positive ion linear mode in 400–1,000 *m/z*. DHB was used as a matrix reagent for MALDI-MS analysis.

3.3 Identification of peptides from orally administered glycinin to Sprague-Dawley rats by LC-TOF/MS

The 36 characteristic *m/z* signals in glycinin-administered plasma obtained from Cou-tagged MALDI-MS analysis (Fig.3-8) were further analyzed by LC-TOF/MS to acquire information on the precise mass of each signal and to allow their sequence identification by a Sequence Editor software. As shown in Fig. 3-9, LC-TOF/MS analysis of glycinin-administered plasma successfully achieved the detection of 15 signals out of the 36 signals detected by Cou-tagged MALDI-MS at randomly in 0.5 to 8 h schedule. The diverse *m/z* values in glycinin-administered blood were in good agreement with the results in **Chapter II** (Fig. 2-8) and other reports [78, 88].

Measured exact masses of the 15 detected signals as $[M\text{-Cou} + H]^+$ (Fig. 3-9) were then converted to $[M + H]^+$ by subtracting the increment mass of $\Delta m/z$ (202.0263 *m/z*) from $[M\text{-Cou} + H]^+$. Table 3-2 summarizes the exact $[M + H]^+$ value for 15 signals, and their predicted sequences in glycinin by a Sequence Editor analysis.
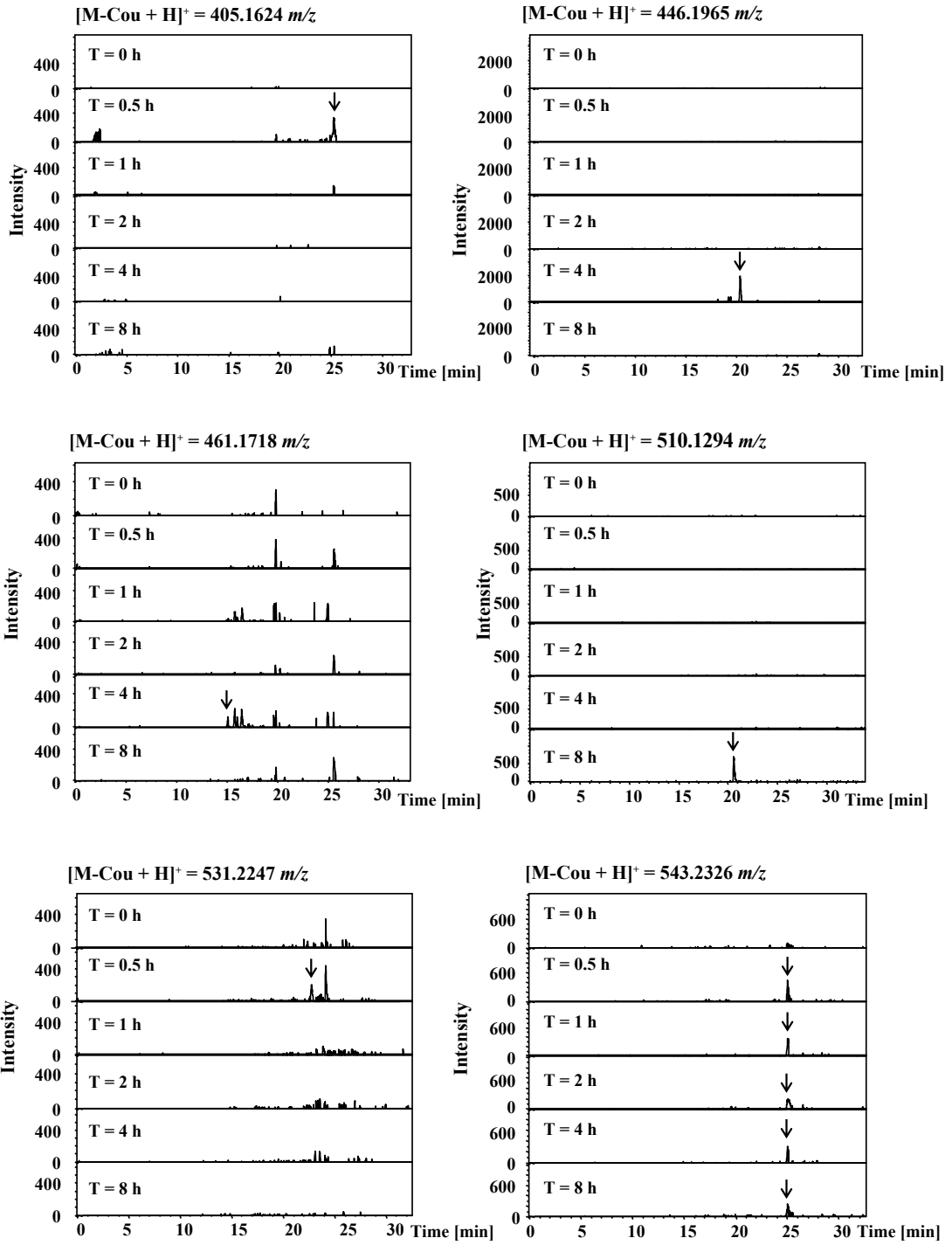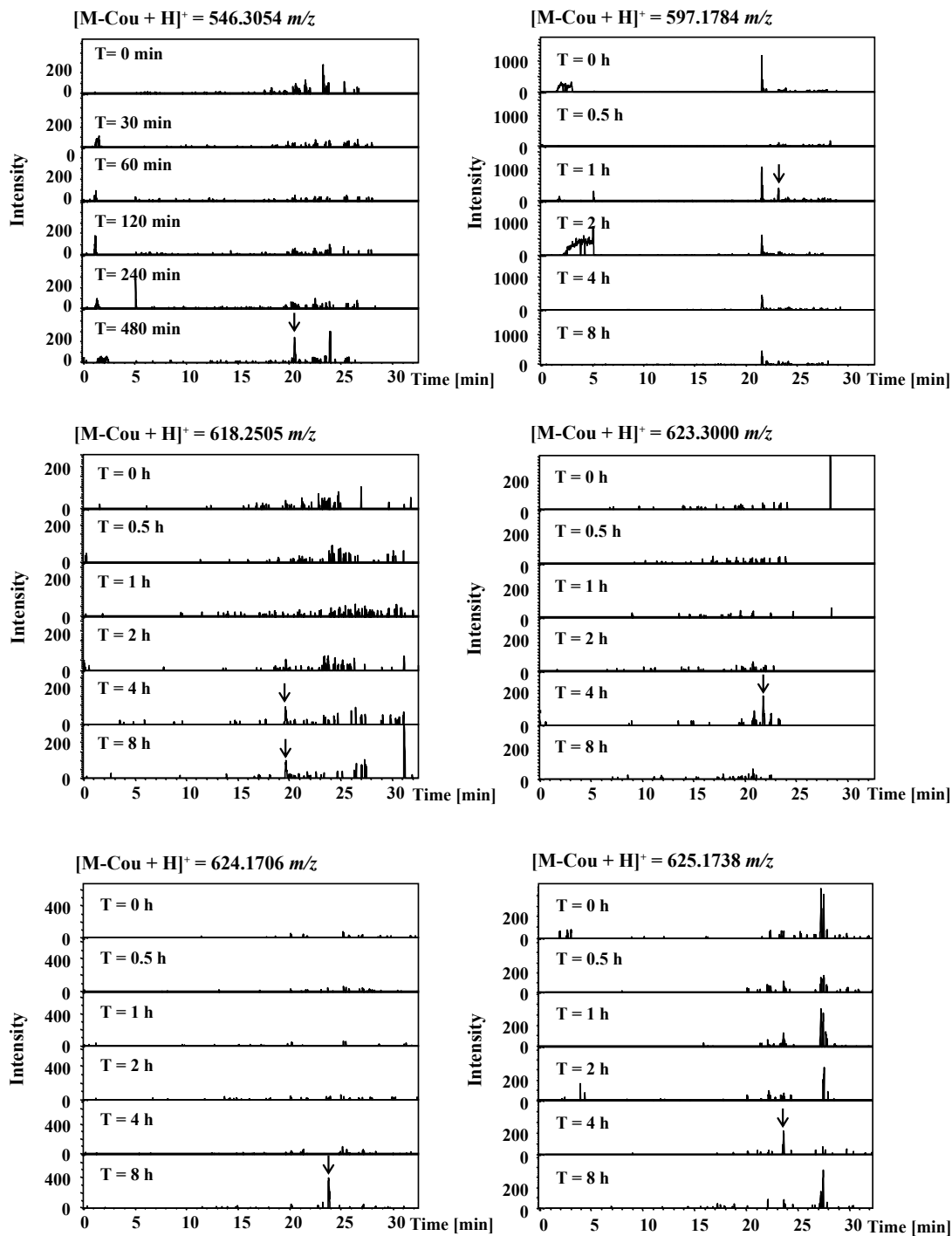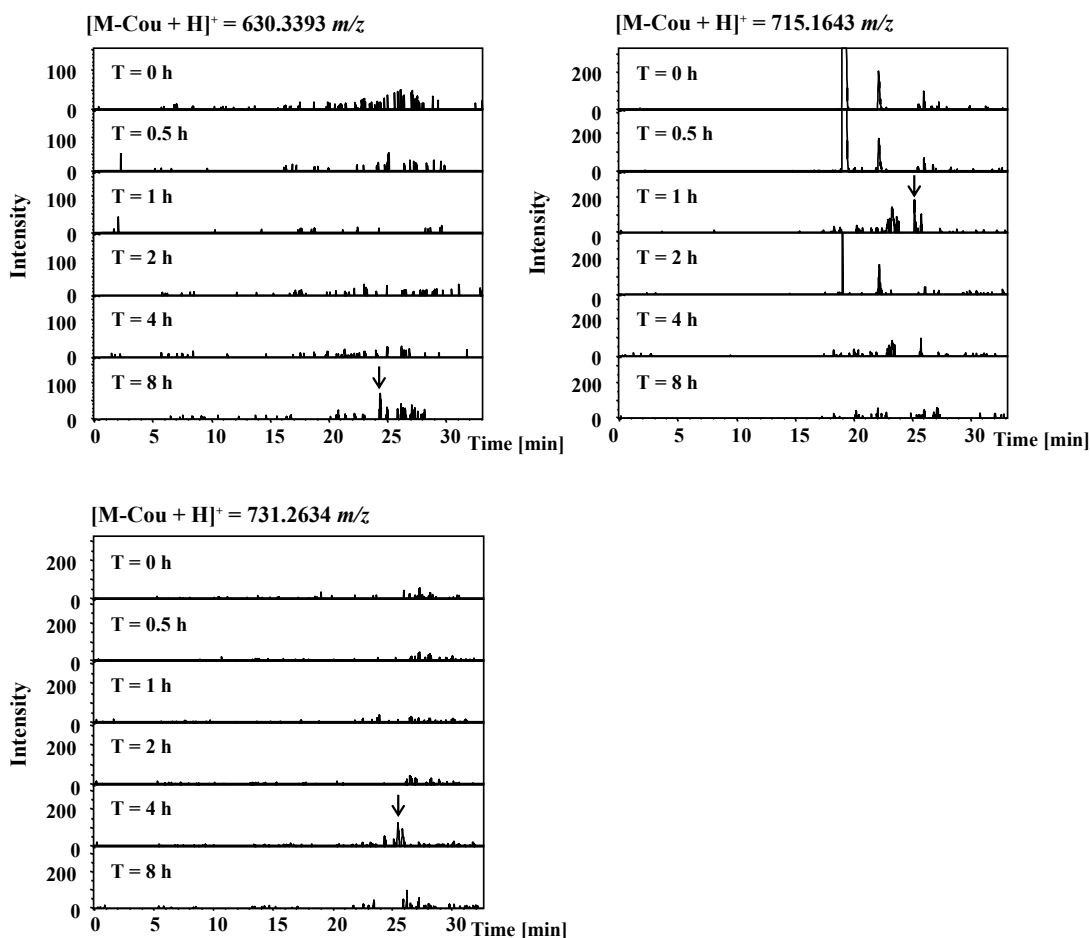
Fig. 3-9 EIC chromatograms of LC-TOF/MS analysis of Cou-derivatized plasma taken at 0, 0.5, 1, 2, 4, and 8 h after the oral administration of glycinin (100 mg/kg). The EIC targeted *m/z* values [M-Cou + H]$^+$ with a tolerance of $\pm$ 0.005 *m/z* were obtained from the MALDI-MS results of Cou-derivatized plasma samples (Fig. 3-8). LC-TOF/MS analysis was performed in a positive ESI mode. Arrows indicate peaks specific for glycinin-administered plasma.

Table 3-2 Peptides from glycinin in blood after the oral administration to SD rats (100 mg/kg).

MALDI-MS [M-Cou + H]$^{+}$	LC-TOF/MS [M-Cou + H]$^{+}$	[M + H]$^{+ b}$	Predicted sequence	Position in glycinin
405.2 (0.5 h; **B**) [a]	405.1624	203.1361	AI	G1 subunit 254-255, G2 subunit 251-252, G3 subunit 253-254 etc.
			AL	G1 subunit 38-39, 68-69, 78-79, 351-352 etc.
			IA	G1 subunit 141-142, 239-240, G2 subunit 139-140 etc.
			LA	G1 subunit 184-185, 445-446, G2 subunit 181-182 etc.
424.2 (4 h; **A**)	–	–	–	
440.2 (4 h; **A**)	–	–	–	–
446.2 (4 h; **A, C**)	446.1965	244.1702	KP	G1 subunit 40-41, 60-61, 266-267, 287-288 etc.
			PK	G3 subunit 472-473, G4 subunit 274-275, 404-405 etc.
461.2 (1, 2, 4, 8 h; **C**)	461.1718	259.1455	HC	G1 subunit 296-297, G3 subunit 289-290
474.3 (1, 2, 4, 8 h; **B**)	–	–	–	–
482.2 (4 h; **A**)	–	–	–	–
494.3 (8 h; **B**)	–	–	–	–
498.2 (8 h; **B**)	–	–	–	–
510.1 (8 h; **C**)	510.1294	308.1031	GCE	G4 subunit 373-375
518.3 (1, 2 h; **A**)	–	–	–	–
530.2 (4 h; **B**)	–	–	–	–
531.2 (0.5 h; **B**)	531.2247	329.1984	PRG	G5 subunit 335-337
543.2 (1 h; **A**)	543.2326	341.2063	KPP	G1 subunit 266-268
			PPK	G3 subunit 471-473
546.3 (8 h; **B**)	546.3054	344.2791	IIV	G5 subunit 93-95
			LIV	G1 subunit 412-414, G2 subunit 402-404, G3 subunit 398-400
			LLV	G4 subunit 479-481, G5 subunit 444-446
			LVI	G4 subunit 147-149, G5 subunit 147-149
			LVL	G2 subunit 4-6, G3 subunit 4-6
			VLI	G1 subunit 411-413, G2 subunit 401-403, G3 subunit 397-399
547.1 (1, 2 h; **A**)	–	–	–	–
564.3 (0.5 h; **C**)	–	–	–	–

565.3 (1 h; **A**)	–	–	–	–
569.2 (4 h; **A**)	–	–	–	–
576.4 (4 h; **B**)	–	–	–	–
578.5 (2 h; **A**)	–	–	–	–
587.3 (8 h; **B**)	–	–	–	–
597.3 (1, 2 h; **A**)	597.1784	395.1523	DNF	G1 subunit 427-429, G2 subunit 417-419, G3 subunit 413-415
603.1 (8 h; **A**)	–	–	–	–
618.3 (8 h; **B**)	618.2505	416.2242	PRGS	G1 subunit 300-303
			GSPR	G4 subunit 556-559
623.3 (4 h; **A**)	623.3000	421.2737	RVF	G3 subunit 386-388, G2 subunit 390-392, G4 subunit 185-187 etc.
			VFR	G4 subunit 516-518, G5 subunit 481-483
			FRV	G5 subunit 482-484
624.2 (8 h; **B**)	624.1706	422.1443	CNGE	G1 subunit 396-399, G2 subunit 386-389, G3 subunit 382-395
625.1 (4 h; **A**)	625.1738	423.1475	FPGC	G2 subunit 101-104, G3 subunit 104-107, G5 subunit 106-109
630.3 (8 h; **B**)	630.3393	428.3130	ALKP	G1 subunit 38-41, G2 subunit 35-38, G3 subunit 38-41
683.3 (1 h; **C**)	–	–	–	–
705.4 (1 h; **A**;	–	–	–	–
715.2 (0.5 h; **C**)	715.1643	513.1380	DIEH	G5 subunit 194-197
715.4 (2, 4 h; **B**)	–	–	–	–
731.3 (4 h; **A**)	731.2634	529.2371	YSPY	G4 subunit 85-88
734.4 (4 h; **B**)	–	–	–	–
861.2 (4 h; **A**)	–	–	–	–

[a] Parenthesis indicates a sampling time of 0.5, 1, 2, 4, or 8 h in SD rats **A**, **B**, and **C**.

[b] $[M+H]^+ = [M\text{-Cou}+H]^+ - 202.0263$ *m/z* (from Table 3-1).

By matching the precise masses with primary sequence of glycinin, some signals were identified as HC for 259.1455 *m/z*, GCE for 308.1031 *m/z*, RPG for 329.1984 *m/z*, DNF for 395.1523 *m/z*, CNGE for 422.1443 *m/z*, FPGC for 423.1475 *m/z*, ALKP for 428.3130 *m/z*, DIEH for 513.1380 *m/z*, and YSPY for 529.2371 *m/z* (Table 3-2). Unfortunately, other signals were not identified because of possible peptide isomers. However, by comparing the LC retention time of detected signal with that of synthetic peptide, a complete identification of peptide can be done in the Cou derivatization analysis (an example was AI in Fig. 3-10).
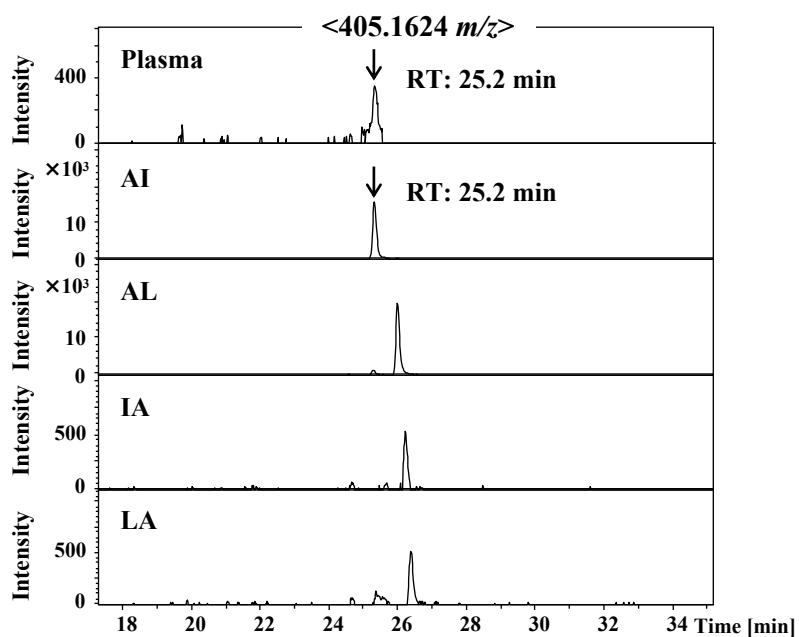
Fig. 3-10 EIC chromatograms of LC-TOF/MS analysis of Cou-derivatized plasma and synthetic peptides (AI, AL, IA, and LA). The EIC monitoring of 405.1624 *m/z* within a tolerance of ± 0.005 *m/z* was conducted for glycinin-administered plasma. Arrows indicate targeting peptide peak identified as AI. LC-TOF/MS analysis was performed in positive ESI mode. RT: retention time.

In this study, we tentatively identified 15 di- to tetrapeptides in glycinin-administered blood of SD rats by Cou-tagged MALDI-MS in combination to LC-TOF/MS analysis. MS signals corresponding to doubly tagged peptides (446.1965 m/z for KP or PK), containing Lys (to form $(Cou)_2$-Lys) were not detected in glycinin-administered plasma by the current Cou-tagged MALDI-MS analysis. In this study, Cou derivatization was applied to characterize specific peptides in glycinin-administered blood of SD rats because its high sensitivity and high selectivity in MALDI-MS analysis. Wu *et. al.*, [115] reported the use of functionalized fullerene (C60) to facilitate the detection of amino acids in MALDI-MS. A mass shift of 880 Da was observed and high sensitivity detection of amino acids at fmol level was achieved by C60 derivatization. Pashkova *et.al.*, [107, 108] reported the use of Coumarin tags to improve signal intensity of large peptide fragments > 1,000 Da. However, the performance of Cou tags on small peptides and the tolerance of the method with biomatrices such as urine, plasma, etc., was not clear. In this study, it concluded that Cou derivatization could be an effective method for the detection of small plasma peptides from protein oral administration by MALDI-MS. In combination with LC-TOF/MS analysis, the Cou-tagged MALDI-MS analysis established in the present study may conveniently and rapidly provide useful information on the metabolic profile of peptides in blood system.

4. Summary

In **Chapter III,** we aimed to establish a novel analytical method using MALDI-MS in combination with derivatization techniques to effectively detect peptide metabolites in bloodstream. Among amine derivatization techniques, Cou was an appropriate derivatization reagent, because of its mild derivatization conditions for peptides (30 mmol/L, 30 min, 25 °C). Under the condition, it showed a higher MS detection of peptides, compared to intact peptides. Moreover, a significant *m/z* shift of targets (+202.0 *m/z*) to silent MS regions (> 400 *m/z*) by Cou-derivatization can help an easy discrimination of peptide metabolites in glycinin-administered blood. After glycinin (100 mg/kg) oral administration to SD rats, 15 di- to tetrapeptides were successfully characterized in plasma as glycinin-derived metabolites. It demonstrated that the Cou-tagged MALDI-MS is an appropriate characterization technique for overall identification of peptide metabolites in the body system.
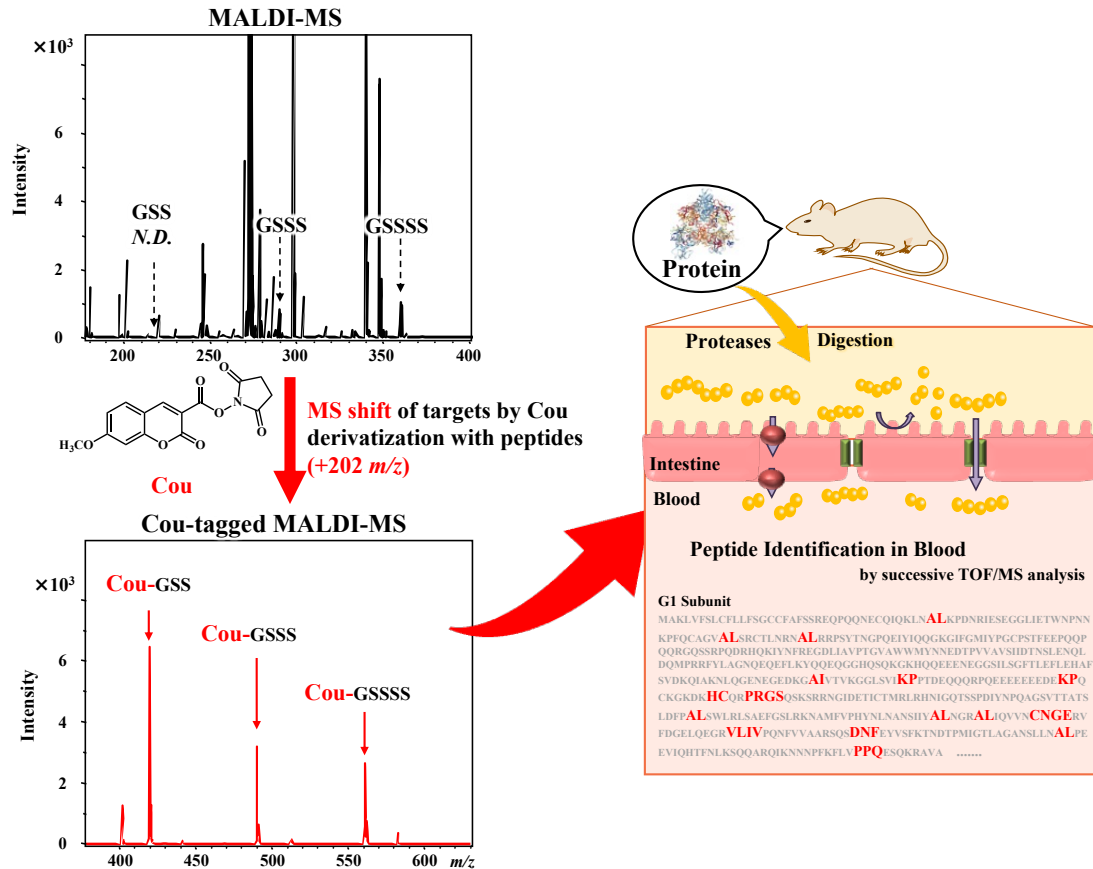
Fig. 3-11 Strategy for the identification of peptide metabolites by Cou-tagged MALDI-MS after protein oral administration.

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Chapter IV

Conclusion

Dietary protein as an essential macronutrient in our daily life provide high impact on nutritional value and health promoting effects against lifestyle-related diseases, such as hyperlipidemia [8], diabetes [9, 10], and obesity [11]. Moreover, protein-derived fragments, i.e., peptides, are also demonstrated to elicit *in vivo* hypolipidemic [51], anti-hypertensive [52, 53] and anti-diabetic effects [54]. Meanwhile, in the gastrointestinal tract, proteins are degraded by digestive proteases and peptidases eventually to generate absorbable small peptides and amino acids [66]. Apparently, large protein molecules are not absorbable in intact forms into blood circulation, so production and absorption behavior of digestion peptide metabolites from proteins in blood circulation is a key factor to understand their physiological potentials.

Thus, the present study aimed to provide evidence on the appearance of peptide metabolites in bloodstream after protein oral administration and to establish a comprehensive identification method of peptide metabolites by advanced MS approaches.

Chapter II Identification of peptide metabolites from orally administered β-conglycinin in rat bloodstream by MS analyses

Dietary β-conglycinin can suppress serum triglyceride level and improve lipid metabolism. In the **Chapter II**, β-conglycinin (100 mg/kg B.W.) was orally administered to Wistar rats (male, 11-week-old) and blood was collected from the tail vein at a time-schedule of 1, 2, 4, and 8 h following administration. MALDI-MS was primarily used for a rough non-target screening of MS signals specific for the blood of β-conglycinin-administered Wistar rats. LC-TOF/MS in combination with peptide synthesis was a good analytical tool to identify peptides, according to marker MALDI-MS signals. Among the 37 signals in β-conglycinin-administered blood by MALDI-MS and LC-TOF/MS analyses, 9 signals were successfully identified as β-conglycinin-derived peptides (i.e., SEL, KGPL, SILGA, DSEL, GDANI, SYFV, CLQSC, GEQPRPF, and LVINEGDA), at plasma concentrations of 0.75–756 pmol/mL. It provided the first evidence to

produce oligopeptides in blood circulation from the oral administration of β-conglycinin.

In order to elucidate the health-benefits of dietary β-conglycinin in animals [29, 42] and humans [30, 48], the physiological functions of these identified peptides must be elucidated in future study. However, it is worth that diverse oligopeptides are produced in blood circulation when we consider the potentials of dietary protein, β-conglycinin.

Chapter III Identification of peptide metabolites from orally administered glycinin in rat bloodstream by a Coumarin-tagged MALDI-MS

Although in the **Chapter II**, the production of peptide metabolites in rat bloodstream was confirmed after β-conglycinin intake, comprehensive identification approach for peptide metabolites is required. In the **Chapter III**, Cou derivatization method in combination with MALDI-MS was proposed for initial screening of peptide metabolites in rat blood after glycinin administration. Cou derivatization can be done at rapid (30 min) and mild (25 °C, pH 8.5) conditions; for synthetic oligopeptides, their MS intensities were enhanced by Cou-derivatization. In addition, MS shifts of target by Cou moiety (+202.0 m/z) allowed an easy detection of peptide metabolites from administered glycinin. In plasma obtained after oral administration of glycinin (100 mg/kg B.W.) to SD rats, 15 di- to tetrapeptides were successfully identified as glycinin-derived

metabolites. Therefore, the proposed Cou-tagged MALDI-MS and subsequent LC-TOF/MS analysis would be extensively used for overall identification of amine metabolites in the body by their high selective (+ Cou increment) and high sensitive (> 5 pmol/spot) properties.

In the perspective, the proposed Cou-tagged MS assays will lead us to elucidate the candidates and mechanism on health-benefits of dietary protein from the aspect of bioactive peptide production behavior in the body system.

References

[1] Arai, S. Studies on functional foods in Japan-State of the art. *Biosci. Biotechnol. Biochem.* **1996**, *60* (1), 9–15.

[2] McGregor, R. A.; Poppitt, S. D. Milk protein for improved metabolic health: a review of the evidence. *Nutr. Metab.* **2013**, *10* (1), 1–13.

[3] Pasin, G.; Comerford, K. B. Dairy foods and dairy proteins in the management of type 2 diabetes: a systematic review of the clinical evidence. *Adv. Nutr.* **2015**, *6* (3), 245–259.

[4] Gannon, M. C.; Nuttall, F. Q.; Lane, J. T.; Burmeister, L. A. Metabolic response to cottage cheese or egg white protein, with or without glucose, in type II diabetic subjects. *Metabolism* **1992**, *41* (10), 1137–1145.

[5] Ejtahed, H. S.; Mohtadi-Nia, J.; Homayouni-Rad, A.; Niafar, M.; Asghari-Jafarabadi, M.; Mofid, V.; Akbarian-Moghari, A. Effect of probiotic yogurt containing lactobacillus acidophilus and bifidobacterium lactis on lipid profile in individuals with type 2 diabetes mellitus. *J. Dairy Sci.* **2011**, *94* (7), 3288–3294.

[6] Ejtahed, H. S.; Mohtadi-Nia, J.; Homayouni-Rad, A.; Niafar, M.; Asghari-Jafarabadi, M.; Mofid, V. Probiotic yogurt improves antioxidant status in type 2 diabetic patients. *Nutrition* **2012**, *28* (5), 539–543.

[7] Gannon, M. C.; Nuttall, F. Q.; Krezowski, P. A.; Billington, C. J.; Parker, S. The serum insulin and plasma glucose responses to milk and fruit

products in type 2 (non-insulin-dependent) diabetic patients. *Diabetologia* **1986**, *29* (11), 784–791.

[8]   Nagaoka, S.; Futamura, Y.; Miwa, K.; Awano, T.; Yamauchi, K.; Kanamaru, Y.; Tadashi, K.; Kuwata, T. Identification of novel hypocholesterolemic peptides derived from bovine milk β-lactoglobulin. *Biochem. Biophys. Res. Commun.* **2001**, *281* (1), 11–17.

[9]   Beasley, J. M.; Wylie-Rosett, J. The role of dietary proteins among persons with diabetes. *Curr. Atheroscler. Rep.* **2013**, *15* (9), 348.

[10]   Bumrungpert, A.; Pavadhgul, P.; Nunthanawanich, P.; Sirikanchanarod, A.; Adulbhan, A. Whey protein supplementation improves nutritional status, glutathione levels, and immune function in cancer patients: a randomized, double-blind controlled trial. *J. Med. Food* **2018**, *21* (6), 612–616.

[11]   Zhao, F.; Song, S.; Ma, Y.; Xu, X.; Zhou, G.; Li, C. A short-term feeding of dietary casein increases abundance of lactococcus lactis and upregulates gene expression involving obesity prevention in cecum of young rats compared with dietary chicken protein. *Front. Microbiol.* **2019**, *10*, 1–12.

[12]   Ono, T.; Morishita, S.; Fujisaki, C.; Ohdera, M.; Murakoshi, M.; Iida, N.; Kato, H.; Miyashita, K.; Iigo, M.; Yoshida, T.; Sugiyama, K.; Nishino, H. Effects of pepsin and trypsin on the anti-adipogenic action of lactoferrin against pre-adipocytes derived from rat mesenteric fat. *Br. J. Nutr.* **2011**, *105* (2), 200–211.

[13]   Tessari, P.; Kiwanuka, E.; Cristini, M.; Zaramella, M.; Enslen, M.; Zurlo, C.; Garcia-Rodenas, C. Slowversus fast proteins in the stimulation of beta-cell response and the activation of the entero-insular axis in type 2 diabetes. *Diabetes. Metab. Res. Rev.* **2007**, *23* (5), 378–385.

[14]   Choi, Y. S.; Ikeda, I.; Sugano, M. Dietary fats modulate age-dependent effects of dietary proteins on cholesterol metabolism in rats. *J. Nutr. Sci. Vitaminol. (Tokyo).* **1990**, *36* (Supplement II), S181–S184.

[15]   Manders, R. J. F.; Wagenmakers, A. J. M.; Koopman, R.; Zorenc, A. H. G.; Menheere, P. P. C. A.; Schaper, N. C.; Saris, W. H. M.; Van Loon, L. J. C. Co-ingestion of a protein hydrolysate and amino acid mixture with carbohydrate improves plasma glucose disposal in patients with type 2 diabetes. *Am. J. Clin. Nutr.* **2005**, *82* (1), 76–83.

[16]   Azuma, N.; Suda, H.; Iwasaki, H.; Yamagata, N.; Saeki, T.; Kanamoto, R.; Iwami, K. Antitumorigenic effects of several food proteins in a rat model with colon cancer and their reverse correlation with plasma bile acid concentration. *J. Nutr. Sci. Vitaminol.* **2000**, *46* (2), 91–96.

[17]   McIntosh, G. H.; Regester, G. O.; Le Leu, R. K.; Royle, P. J.; Smithers, G. W. Dairy proteins protect against dimethylhydrazine-induced intestinal cancers in rats. *J. Nutr.* **1995**, *125* (4), 809–816.

[18]   Vasconcelos, Q. D. J. S.; Bachur, T. P. R.; Aragão, G. F. Whey protein supplementation and its potentially adverse effects on health: a systematic review. *Appl. Physiol. Nutr. Metab.* **2021**, *46* (1), 27–33.

[19]   Teixeira, F. J.; Santos, H. O.; Howell, S. L.; Pimentel, G. D. Whey protein in cancer therapy: a narrative review. *Pharmacol. Res.* **2019**, *144*, 245–256.

[20]   Kennedy, R. S.; Konok, G. P.; Bounous, G.; Baruchel, S.; Lee, T. D. G. The use of a whey protein concentrate in the treatment of patients with metastatic carcinoma: a phase I-II clinical study. *Anticancer Res.* **1995**, *15*(6), 2643–2649.

[21]   Haidari, F.; Aghamohammadi, V.; Mohammadshahi, M.; Ahmadi-Angali, K. Effect of whey protein supplementation on levels of endocannabinoids and some of metabolic risk factors in obese women on a weight-loss diet: a study protocol for a randomized controlled trial. *Nutr. J.* **2017**, *16* (1), 1–5.

[22]   Ratliff, J. C.; Mutungi, G.; Puglisi, M. J.; Volek, J. S.; Fernandez, M. L. Eggs modulate the inflammatory response to carbohydrate restricted diets in overweight men. *Nutr. Metab. (Lond).* **2008**, *5* (1), 1-9.

[23]   Rouhani, M. H.; Rashidi-Pourfard, N.; Salehi-Abargouei, A.; Karimi, M.; Haghighatdoost, F. Effects of egg consumption on blood lipids: a systematic review and meta-analysis of randomized clinical trials. *J. Am. Coll. Nutr.* **2018**, *37* (2), 99–110.

[24]   Ramdath, D.; Padhi, E.; Sarfaraz, S.; Renwick, S.; Duncan, A. Beyond the cholesterol-lowering effect of soy protein: a review of the effects of dietary

soy and its constituents on risk factors for cardiovascular disease. *Nutrients* **2017**, *9* (4), 324.

[25] Azadbakht, L.; Shakerhosseini, R.; Atabak, S.; Jamshidian, M.; Mehrabi, Y.; Esmaill-Zadeh, A. Beneficiary effect of dietary soy protein on lowering plasma levels of lipid and improving kidney function in type II diabetes with nephropathy. *Eur. J. Clin. Nutr.* **2003**, *57* (10), 1292–1294.

[26] Ouellet, V.; Marois, J.; Weisnagel, S. J.; Jacques, H. Dietary cod protein improves insulin sensitivity in insulin-resistant men and women: a randomized controlled trial. *Diabetes Care* **2007**, *30* (11), 2816–2821.

[27] Sasatani D. & Rakhovskaya M. Report Name : utilization of food-grade soybeans in Japan **2021**. https://www.fas.usda.gov/data/japan-utilization-food-grade-soybeans-japan.

[28] Chatterjee, C.; Gleddie, S.; Xiao, C. W. Soybean bioactive peptides and their functional properties. *Nutrients* **2018**, *10* (9), 8–11.

[29] Moriyama, T.; Kishimoto, K.; Nagai, K.; Urade, R.; Ogawa, T.; Utsumi, S.; Maruyama, N.; Maebuchi, M. Soybean β-conglycinin diet suppresses serum triglyceride levels in normal and genetically obese mice by induction of β-oxidation, downregulation of fatty acid synthase, and inhibition of triglyceride absorption. *Biosci. Biotechnol. Biochem.* **2004**, *68* (2), 352–359.

[30] Kohno, M.; Hirotsuka, M.; Kito, M.; Matsuzawa, Y. Decreases in serum triacylglycerol and visceral fat mediated by dietary soybean β-conglycinin. *J. Atheroscler. Thromb.* **2006**, *13* (5), 247–255.

[31] Inoue, N.; Fujiwara, Y.; Kato, M.; Funayama, A.; Ogawa, N.; Tachibana, N.; Kohno, M.; Ikeda, I. Soybean β-conglycinin improves carbohydrate and lipid metabolism in wistar rats. *Biosci. Biotechnol. Biochem.* **2015**, *79* (9), 1528–1534.

[32] Osada, K.; Inoue, T.; Nakamura, S.; Sugano, M. Dietary soybean protein moderates the deleterious disturbance of lipid metabolism caused by exogenous oxidized cholesterol in rats. *Biochim. Biophys. Acta - Gen. Subj.* **1999**, *1427* (3), 337–350.

[33] Bosisio, E.; Ghiselli, G. C.; Kienle, M. G.; Galli, G.; Sirtori, C. R. Effects of dietary soy protein on liver catabolism and plasma transport of cholesterol in hypercholesterolemic rats. *J. Steroid Biochem.* **1981**, *14* (11), 1201–1207.

[34] Frigolet, M. E.; Torres, N.; Uribe-Figueroa, L.; Rangel, C.; Jimenez-Sanchez, G.; Tovar, A. R. White adipose tissue genome wide-expression profiling and adipocyte metabolic functions after soy protein consumption in rats. *J. Nutr. Biochem.* **2011**, *22* (2), 118–129.

[35] Torre-Villalvazo, I.; Gonzalez, F.; Aguilar-Salinas, C. A.; Tovar, A. R.; Torres, N. Dietary soy protein reduces cardiac lipid accumulation and the

ceramide concentration in high-fat diet-fed rats and ob/ob mice. *J. Nutr.* **2009**, *139* (12), 2237–2243.

[36] Fair, D. E.; Ogborn, M. R.; Weiler, H. A.; Bankovic-Calic, N.; Nitschmann, E. P.; Fitzpatrick-Wong, S. C.; Aukema, H. M. Dietary soy protein attenuates renal disease progression after 1 and 3 weeks in han: SPRD-*cy* weanling rats. *J. Nutr.* **2004**, *134* (6), 1504–1507.

[37] Aukema, H. M.; Housini, I. Dietary soy protein effects on disease and IGF-I in male and female han:SPRD-*cy* rats. *Kidney Int.* **2001**, *59* (1), 52–61.

[38] Peng, C. Y.-C.; Sankaran, D.; Ogborn, M. R.; Aukema, H. M. Dietary soy protein selectively reduces renal prostanoids and cyclooxygenases in polycystic kidney disease. *Exp. Biol. Med.* **2009**, *234* (7), 737–743.

[39] Lavigne, C.; Marette, A.; Jacques, H. Cod and soy proteins compared with casein improve glucose tolerance and insulin sensitivity in rats. *Am. J. Physiol. - Endocrinol. Metab.* **2000**, *278* (3), 491–500.

[40] Sugano, M.; Goto, S.; Yamada, Y.; Yoshida, K.; Hashimoto, Y.; Matsuo, T.; Kimoto, M. Cholesterol-lowering activity of various undigested fractions of soybean protein in rats. *J. Nutr.* **1990**, *120* (9), 977–985.

[41] Tada, O.; Yokogoshi, H. Effect of different dietary protein composition on skeletal muscle atrophy by suspension hypokinesia/hypodynamia in rats. *J. Nutr. Sci. Vitaminol. (Tokyo).* **2002**, *48* (2), 115–119.

[42] Tachibana, N.; Iwaoka, Y.; Hirotsuka, M.; Horio, F.; Kohno, M. β-Conglycinin lowers very-low-density lipoprotein-triglyceride levels by

increasing adiponectin and insulin sensitivity in rats. *Biosci. Biotechnol. Biochem.* **2010**, *74* (6), 1250–1255.

[43] Fassini, P. G.; Noda, R. W.; Ferreira, E. S.; Silva, M. A.; Neves, V. A.; Demonte, A. Soybean glycinin improves HDL-C and suppresses the effects of rosuvastatin on hypercholesterolemic rats. *Lipids Health Dis.* **2011**, *10*, 1–7.

[44] Tachibana, N.; Yamashita, Y.; Nagata, M.; Wanezaki, S.; Ashida, H.; Horio, F.; Kohno, M. Soy β-conglycinin improves glucose uptake in skeletal muscle and ameliorates hepatic insulin resistance in Goto-Kakizaki rats. *Nutr. Res.* **2014**, *34* (2), 160–167.

[45] Kawabeta, K.; Hase-Tamaru, S.; Yuasa, M.; Suruga, K.; Sugano, M.; Koba, K. Dietary β-conglycinin modulates insulin sensitivity, body fat mass, and lipid metabolism in obese otsuka long-evans tokushima fatty (OLETF) rats. *J. Oleo Sci.* **2019**, *68* (4), 339–350.

[46] Hashidume, T.; Kato, A.; Tanaka, T.; Miyoshi, S.; Itoh, N.; Nakata, R.; Inoue, H.; Oikawa, A.; Nakai, Y.; Shimizu, M.; Inoue, J.; Sato, R. Single ingestion of soy β-conglycinin induces increased postprandial circulating FGF21 levels exerting beneficial health effects. *Sci. Rep.* **2016**, *6* (1), 28183.

[47] Wanezaki, S.; Tachibana, N.; Nagata, M.; Saito, S.; Nagao, K.; Yanagita, T.; Kohno, M. Soy β-conglycinin improves obesity-induced metabolic

abnormalities in a rat model of nonalcoholic fatty liver disease. *Obes. Res. Clin. Pract.* **2015**, *9* (2), 168–174.

[48] Ma, D.; Taku, K.; Zhang, Y.; Jia, M.; Wang, Y.; Wang, P. Serum lipid-improving effect of soyabean β-conglycinin in hyperlipidaemic menopausal women. *Br. J. Nutr.* **2013**, *110* (9), 1680–1684.

[49] Duranti, M.; Lovati, M. R.; Dani, V.; Barbiroli, A.; Scarafoni, A.; Castiglioni, S.; Ponzone, C.; Morazzoni, P. The α′ subunit from soybean 7S globulin lowers plasma lipids and upregulates liver β-VLDL receptors in rats fed a hypercholesterolemic diet. *J. Nutr.* **2004**, *134* (6), 1334–1339.

[50] Cabanos, C.; Kato, N.; Amari, Y.; Fujiwara, K.; Ohno, T.; Shimizu, K.; Goto, T.; Shimada, M.; Kuroda, M.; Masuda, T.; Takaiwa, F.; Utsumi, S.; Nagaoka, S.; Maruyama, N. Development of a novel transgenic rice with hypocholesterolemic activity via high-level accumulation of the α′ subunit of soybean β-conglycinin. *Transgenic Res.* **2014**, *23* (4), 609–620.

[51] Nagaoka, S.; Nakamura, A.; Shibata, H.; Kanamaru, Y. Soystatin (VAWWMY), a novel bile acid-binding peptide, decreased micellar solubility and inhibited cholesterol absorption in rats. *Biosci. Biotechnol. Biochem.* **2010**, *74* (8), 1738–1741.

[52] Matsui, T.; Matsumoto, K. Antihypertensive peptides from natural resources. In *Advances in Phytomedicine* **2006**, 2, 255–271.

[53] Miralles, B.; Amigo, L.; Recio, I. Critical review and perspectives on food-derived antihypertensive peptides. *J. Agric. Food Chem.* **2018**, *66* (36), 9384–9390.

[54] Yu, Z.; Yin, Y.; Zhao, W.; Liu, J.; Chen, F. Anti-diabetic activity peptides from albumin against α-glucosidase and α-amylase. *Food Chem.* **2012**, *135* (3), 2078–2085.

[55] Yamada, Y.; Muraki, A.; Oie, M.; Kanegawa, N.; Oda, A.; Sawashi, Y.; Kaneko, K.; Yoshikawa, M.; Goto, T.; Takahashi, N.; Kawada, T.; Ohinata, K. Soymorphin-5, a soy-derived μ-opioid peptide, decreases glucose and triglyceride levels through activating adiponectin and pparα systems in diabetic KK-A[y] Mice. *Am. J. Physiol. Metab.* **2012**, *302* (4), E433–E440.

[56] Ohinata, K.; Agui, S.; Yoshikawa, M. Soymorphins, novel μ opioid peptides derived from soy β-conglycinin β-subunit, have anxiolytic activities. *Biosci. Biotechnol. Biochem.* **2007**, *71* (10), 2618–2621.

[57] Katayama, S.; Imai, R.; Sugiyama, H.; Nakamura, S. Oral administration of soy peptides suppresses cognitive decline by induction of neurotrophic factors in SAMP8 mice. *J. Agric. Food Chem.* **2014**, *62* (16), 3563–3569.

[58] Wu, J.; Ding, X. Hypotensive and physiological effect of angiotensin converting enzyme inhibitory peptides derived from soy protein on spontaneously hypertensive rats. *J. Agric. Food Chem.* **2001**, *49* (1), 501–506.

[59] Yoshikawa, M. Bioactive peptides derived from natural proteins with respect to diversity of their receptors and physiological effects. *Peptides* **2015**, *72*, 208–225.

[60] Inoue, N.; Nagao, K.; Sakata, K.; Yamano, N.; Gunawardena, P. E. R.; Han, S.-Y.; Matsui, T.; Nakamori, T.; Furuta, H.; Takamatsu, K.; Yanagita, T. Screening of soy protein-derived hypotriglyceridemic di-peptides i*n vitro* and *in vivo. Lipids Health Dis.* **2011**, *10* (1), 1-10.

[61] Yoshikawa, M.; Fujita, H.; Matoba, N.; Takenaka, Y.; Yamamoto, T.; Yamauchi, R.; Tsuruki, H.; Takahata, K. Bioactive peptides derived from food proteins preventing lifestyle-related diseases. *BioFactors* **2000**, *12* (1–4), 143–146.

[62] Erdmann, K.; Cheung, B. W. Y.; Schröder, H. The possible roles of food-derived bioactive peptides in reducing the risk of cardiovascular disease. *J. Nutr. Biochem.* **2008**, *19* (10), 643–654.

[63] Wang, W.; de Mejia, E. G. A new frontier in soy bioactive peptides that may prevent age-related chronic diseases. *Compr. Rev. Food Sci. Food Saf.* **2005**, *4* (4), 63–78.

[64] Nishi, T.; Hara, H.; Asano, K.; Tomita, F. The soybean β-conglycinin β 51-63 fragment suppresses appetite by stimulating cholecystokinin release in rats. *J. Nutr.* **2003**, *133* (8), 2537–2542.

[65] Tsuruki, T.; Kishi, K.; Takahashi, M.; Tanaka, M.; Matsukawa, T.; Yoshikawa, M. Soymetide, an immunostimulating peptide derived from

soybean β-conglycinin, is an FMLP agonist. *FEBS Lett.* **2003**, *540* (1–3), 206–210.

[66] Onuh, J. O.; Aluko, R. E. Metabolomics as a tool to study the mechanism of action of bioactive protein hydrolysates and peptides: a review of current literature. *Trends Food Sci. Technol.* **2019**, *91*, 625–633.

[67] Foltz, M.; Meynen, E. E.; Bianco, V.; Van Platerink, C.; Koning, T. M. M. G.; Kloek, J. Angiotensin converting enzyme inhibitory peptides from a lactotripeptide-enriched milk beverage are absorbed intact into the circulation. *J. Nutr.* **2007**, *137* (4), 953–958.

[68] Shen, W.; Matsui, T. Intestinal absorption of small peptides: a review. *Int. J. Food Sci. Technol.* **2019**, *54* (6), 1942–1948.

[69] Matsui, T.; Imamura, M.; Oka, H.; Osajima, K.; Kimoto, K. I.; Kawasaki, T.; Matsumoto, K. Tissue distribution of antihypertensive dipeptide, Val-Tyr, after its single oral administration to spontaneously hypertensive rats. *J. Pept. Sci.* **2004**, *10* (9), 535–545.

[70] Hanh, V. T.; Shen, W.; Tanaka, M.; Siltari, A.; Korpela, R.; Matsui, T. Effect of aging on the absorption of small peptides in spontaneously hypertensive rats. *J. Agric. Food Chem.* **2017**, *65* (29), 5935–5943.

[71] Nakao, R.; Shen, W.; Shimajiri, Y.; Kainou, K.; Sato, Y.; Ulla, A.; Ohnishi, K.; Ninomiya, M.; Ohno, A.; Uchida, T.; Tanaka, M.; Akama, K.; Matsui, T.; Nikawa, T. Oral intake of rice overexpressing ubiquitin ligase

inhibitory pentapeptide prevents atrophy in denervated skeletal muscle. *npj Sci. Food* **2021**, *5* (1), 1–10.

[72] Sánchez-Rivera, L.; Ares, I.; Miralles, B.; Gómez-Ruiz, J. Á.; Recio, I.; Martínez-Larrañaga, M. R.; Anadón, A.; Martínez, M. A. Bioavailability and kinetics of the antihypertensive casein-derived peptide HLPLP in rats. *J. Agric. Food Chem.* **2014**, *62* (49), 11869–11875.

[73] Dale, M. J.; Knochenmuss, R.; Zenobi, R. Graphite/liquid mixed matrices for laser desorption/ionization mass spectrometry. *Anal. Chem.* **1996**, *68* (19), 3321–3329.

[74] Tiss, A.; Smith, C.; Menon, U.; Jacobs, I.; Timms, J. F.; Cramer, R. A well-characterised peak identification list of MALDI MS profile peaks for human blood serum. *Proteomics* **2010**, *10* (18), 3388–3392.

[75] Zimmerman, L. J.; Wernke, G. R.; Caprioli, R. M.; Liebler, D. C. Identification of protein fragments as pattern features in MALDI-MS analyses of serum. *J. Proteome Res.* **2005**, *4* (5), 1672–1680.

[76] Saito, T.; Kohno, M.; Tsumura, K.; Kugimiya, W.; Kito, M. Novel method using phytase for separating soybean β-conglycinin and glycinin. *Biosci. Biotechnol. Biochem.* **2001**, *65* (4), 884–887.

[77] Wu, S.; Murphy, P. A.; Johnson, L. A.; Reuber, M. A.; Fratzke, A. R. Simplified process for soybean glycinin and β-conglycinin fractionation. *J. Agric. Food Chem.* **2000**, *48* (7), 2702–2708.

[78] Caira, S.; Pinto, G.; Vitaglione, P.; Dal Piaz, F.; Ferranti, P.; Addeo, F. Identification of casein peptides in plasma of subjects after a cheese-enriched diet. *Food Res. Int.* **2016**, *84*, 108–112.

[79] Rohm, F.; Skurk, T.; Daniel, H.; Spanier, B. Appearance of di- and tripeptides in human plasma after a protein meal does not correlate with PepT1 substrate selectivity. *Mol. Nutr. Food Res.* **2019**, *63* (5), 1801094.

[80] Dranse, H. J.; Waise, T. M. Z.; Hamr, S. C.; Bauer, P. V.; Abraham, M. A.; Rasmussen, B. A.; Lam, T. K. T. Physiological and therapeutic regulation of glucose homeostasis by upper small intestinal PepT1-mediated protein sensing. *Nat. Commun.* **2018**, *9* (1), 1118.

[81] Ma, K.; Hu, Y.; Smith, D. E. Influence of fed-fasted state on intestinal PepT1 expression and *in vivo* pharmacokinetics of glycylsarcosine in wild-type and PepT1 knockout mice. *Pharm. Res.* **2012**, *29* (2), 535–545.

[82] Hong, S. M.; Tanaka, M.; Yoshii, S.; Mine, Y.; Matsui, T. Enhanced visualization of small peptides absorbed in rat small intestine by phytic-acid-aided matrix-assisted laser desorption/ionization-imaging mass spectrometry. *Anal. Chem.* **2013**, *85* (21), 10033–10039.

[83] Sugiyama, E.; Masaki, N.; Matsushita, S.; Setou, M. Ammonium sulfate improves detection of hydrophilic quaternary ammonium compounds through decreased ion suppression in matrix-assisted laser desorption/ionization imaging mass spectrometry. *Anal. Chem.* **2015**, *87* (22), 11176–11181.

[84]   Cucu, T.; De Meulenaer, B.; Devreese, B. MALDI based identification of soybean protein markers - possible analytical targets for allergen detection in processed foods. *Peptides* **2012**, *33* (2), 187–196.

[85]   Terracciano, R.; Pasqua, L.; Casadonte, F.; Frascà, S.; Preianò, M.; Falcone, D.; Savino, R. Derivatized mesoporous silica beads for MALDI-TOF MS profiling of human plasma and urine. *Bioconjug. Chem.* **2009**, *20* (5), 913–923.

[86]   Kent, P.; Awadia, A.; Zhao, L.; Ensan, D.; Silva, D.; Cayer, C.; James, J. S.; Anisman, H.; Merali, Z. Effects of intranasal and peripheral oxytocin or gastrin-releasing peptide administration on social interaction and corticosterone levels in rats. *Psychoneuroendocrinology* **2016**, *64*, 123–130.

[87]   Beaumont, M.; Jaoui, D.; Douard, V.; Mat, D.; Koeth, F.; Goustard, B.; Mayeur, C.; Mondot, S.; Hovaghimian, A.; Le Feunteun, S.; Chaumontet, C.; Davila, A. M.; Tomé, D.; Souchon, I.; Michon, C.; Fromentin, G.; Blachier, F.; Leclerc, M. Structure of protein emulsion in food impacts intestinal microbiota, caecal luminal content composition and distal intestine characteristics in rats. *Mol. Nutr. Food Res.* **2017**, *61* (10), 1–12.

[88]   Egger, L.; Schlegel, P.; Baumann, C.; Stoffers, H.; Guggisberg, D.; Brügger, C.; Dürr, D.; Stoll, P.; Vergères, G.; Portmann, R. Physiological comparability of the harmonized INFOGEST *in vitro* digestion method to *in vivo* pig digestion. *Food Res. Int.* **2017**, *102*, 567–574.

[89] Matsui, T. Are peptides absorbable compounds? *J. Agric. Food Chem.* **2018**, *66* (2), 393–394.

[90] Hong, S. M.; Tanaka, M.; Koyanagi, R.; Shen, W.; Matsui, T. Structural design of oligopeptides for intestinal transport model. *J. Agric. Food Chem.* **2016**, *64* (10), 2072–2079.

[91] Miguel, M.; Manso, M.; Aleixandre, A.; Alonso, M. J.; Salaices, M.; López-Fandiño, R. Vascular effects, angiotensin I-converting enzyme (ACE)-Inhibitory activity, and antihypertensive properties of peptides derived from egg white. *J. Agric. Food Chem.* **2007**, *55* (26), 10615–10621.

[92] Xu, F.; Zhang, J.; Wang, Z.; Yao, Y.; Atungulu, G. G.; Ju, X.; Wang, L. Absorption and metabolism of peptide WDHHAPQLR derived from rapeseed protein and inhibition of HUVEC apoptosis under oxidative stress. *J. Agric. Food Chem.* **2018**, *66* (20), 5178–5189.

[93] Nakashima, E. M. N.; Kudo, A.; Iwaihara, Y.; Tanaka, M.; Matsumoto, K.; Matsui, T. Application of ^{13}C stable isotope labeling liquid chromatography-multiple reaction monitoring-tandem mass spectrometry method for determining intact absorption of bioactive dipeptides in rats. *Anal. Biochem.* **2011**, *414* (1), 109–116.

[94] Ten Have, G. A. M.; Van Der Pijl, P. C.; Kies, A. K.; Deutz, N. E. P. Enhanced lacto-tri-peptide bio-availability by co-ingestion of macronutrients. *PLoS One* **2015,** *10* (6), e0130638.

[95] Egger, L.; Ménard, O.; Delgado-Andrade, C.; Alvito, P.; Assunção, R.; Balance, S.; Barberá, R.; Brodkorb, A.; Cattenoz, T.; Clemente, A.; Comi, I.; Dupont, D.; Garcia-Llatas, G.; Lagarda, M. J.; Le Feunteun, S.; JanssenDuijghuijsen, L.; Karakaya, S.; Lesmes, U.; Mackie, A. R.; Martins, C.; Meynier, A.; Miralles, B.; Murray, B. S.; Pihlanto, A.; Picariello, G.; Santos, C. N.; Simsek, S.; Recio, I.; Rigby, N.; Rioux, L.-E.; Stoffers, H.; Tavares, A.; Tavares, L.; Turgeon, S.; Ulleberg, E. K.; Vegarud, G. E.; Vergères, G.; Portmann, R. The harmonized INFOGEST *in vitro* digestion method: from knowledge to action. *Food Res. Int.* **2016**, *88*, 217–225.

[96] Nakaoka, F.; Sasakawa, Y.; Yamamoto, K.; Nakao, M.; Nakamura, M.; Tong, C.; Fukuhama, C.; Kagawa, K. Anti-diabetic effects of globin digest and its active ingredient Leu-Ser-Glu-Leu in ICR mice, streptozotocin-induced diabetic mice and KK-A^y mice. *Life Sci.* **2010**, *86* (11–12), 424–434.

[97] Shubina, T. A.; Grigor'eva, M. E.; Lyapina, L. A.; Obergan, T. Y.; Myasoedov, N. F.; Andreeva, L. A. Hypoglycemic and anticoagulant effects of tetrapeptide Pro-Gly-Pro-Leu in hypercholesterolemia. *Bull. Exp. Biol. Med.* **2014**, *158* (1), 30–33.

[98] Hortin, G. L. The MALDI-TOF mass spectrometric view of the plasma proteome and peptidome. *Clin. Chem.* **2006**, *52* (7), 1223–1237.

[99] Raimondo, F.; Corbetta, S.; Chinello, C.; Pitto, M.; Magni, F. The urinary proteome and peptidome of renal cell carcinoma patients: a comparison of different techniques. *Expert Rev. Proteomics* **2014**, *11* (4), 503–514.

[100] Qiao, Z.; Lissel, F. MALDI matrices for the analysis of low molecular weight compounds: rational design, challenges and perspectives. *Chem.-An Asian J.* **2021**, *16* (8), 868–878.

[101] Piovesana, S.; Cerrato, A.; Antonelli, M.; Benedetti, B.; Capriotti, A. L.; Cavaliere, C.; Montone, C. M.; Laganà, A. A clean-up strategy for identification of circulating endogenous short peptides in human plasma by zwitterionic hydrophilic liquid chromatography and untargeted peptidomics identification. *J. Chromatogr. A* **2020**, *1613*, 460699.

[102] Kurihara, T.; Jun, Z. M.; Toyo'oka, T.; Fukushima, T.; Inagaki, S. Determination of fluorescence-labeled asparaginyl-oligosaccharide in glycoprotein by reversed-phase ultraperformance liquid chromatography with electrospray ionization time-of-flight mass spectrometry. *Anal. Chem.* **2007**, *79* (22), 8694–8698.

[103] Salazar, C.; Armenta, J. M.; Shulaev, V. An UPLC-ESI-MS/MS assay using 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate derivatization for targeted amino acid analysis: application to screening of arabidopsis thaliana mutants. *Metabolites* **2012**, *2* (3), 398–428.

[104] Hashimoto, C.; Iwaihara, Y.; Chen, S. J.; Tanaka, M.; Watanabe, T.; Matsui, T. Highly-sensitive detection of free advanced glycation end-

products by liquid chromatography-electrospray ionization-tandem mass spectrometry with 2,4,6-trinitrobenzene sulfonate derivatization. *Anal. Chem.* **2013**, *85* (9), 4289–4295.

[105] Hanh, V. T.; Kobayashi, Y.; Maebuchi, M.; Nakamori, T.; Tanaka, M.; Matsui, T. Quantitative mass spectrometric analysis of dipeptides in protein hydrolysate by a TNBS derivatization-aided standard addition method. *Food Chem.* **2016**, *190*, 345–350.

[106] Shimbo, K.; Oonuki, T.; Yahashi, A.; Hirayama, K.; Miyano, H. Precolumn derivatization reagents for high-speed analysis of amines and amino acids in biological fluid using liquid chromatography/electrospray ionization tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* **2009**, *23* (10), 1483–1492.

[107] Pashkova, A.; Moskovets, E.; Karger, B. L. Coumarin tags for improved analysis of peptides by MALDI-TOF MS and MS/MS. 1. Enhancement in MALDI MS signal intensities. *Anal. Chem.* **2004**, *76* (15), 4550–4557.

[108] Pashkova, A.; Chen, H.-S.; Rejtar, T.; Zang, X.; Giese, R.; Andreev, V.; Moskovets, E.; Karger, B. L. Coumarin tags for analysis of peptides by MALDI-TOF MS and MS/MS. 2. Alexa Fluor 350 tag for increased peptide and protein identification by LC-MALDI-TOF/TOF MS. *Anal. Chem.* **2005**, *77* (7), 2085–2096.

[109] Nasser Abdelhamid, H.; Wu, H.-F. Furoic and mefenamic acids as new matrices for matrix assisted laser desorption/ionization-(MALDI)-mass spectrometry. *Talanta* **2013**, *115*, 442–450.

[110] He, H.; Qin, L.; Zhang, Y.; Han, M.; Li, J.; Liu, Y.; Qiu, K.; Dai, X.; Li, Y.; Zeng, M.; Guo, H.; Zhou, Y.; Wang, X. 3,4-Dimethoxycinnamic acid as a novel matrix for enhanced in situ detection and imaging of low-molecular-weight compounds in biological tissues by MALDI-MSI. *Anal. Chem.* **2019**, *91* (4), 2634–2643.

[111] Wang, X.; Han, J.; Chou, A.; Yang, J.; Pan, J.; Borchers, C. H. Hydroxyflavones as a new family of matrices for MALDI tissue imaging. *Anal. Chem.* **2013**, *85* (15), 7566–7573.

[112] Zhu, Z.; Shen, J.; Wang, D.; Chen, C.; Xu, Y.; Guo, H.; Kang, D.; Hamada, N.; Dong, J.; Wang, G.; Liang, Y. An auxiliary matrix for routine analysis of small molecules and biological macromolecules using matrix-assisted laser desorption ionization mass spectrometry. *Anal. Bioanal. Chem.* **2019**, *411* (5), 1041–1052.

[113] Dong, X.; Cheng, J.; Li, J.; Wang, Y. Graphene as a novel matrix for the analysis of small molecules by MALDI-TOF MS. *Anal. Chem.* **2010**, *82* (14), 6208–6214.

[114] Shen, W.; Ono, K.; Tanaka, M.; Matsui, T. Characteristics of electrospray-ionization detection of synthetic di- to penta-oligopeptides by amine derivatizations. *Anal. Sci.* **2021**, 1–16.

[115] Wu, P.; Xiao, H. M.; Ding, J.; Deng, Q. Y.; Zheng, F.; Feng, Y. Q. Development of C60-based labeling reagents for the determination of low-molecular-weight compounds by matrix assisted laser desorption ionization mass (i): determination of amino acids in microliter biofluids. *Anal. Chim. Acta* **2017**, *960*, 90–100.

Acknowledgements

Undertaking this doctoral dissertation has been a enriching experience for me and it would not have been possible to finish without the guidance and support that I received from many people.

First and foremost, I would like to extend my sincere gratitude to my supervisor, Prof. Toshiro Matsui for his intellectual guidance, inspiration, encouragement and continuous support throughout my Ph.D. study and research. During the three and half years, his enthusiasm, integral view on research and his strict requirement for high-quality work has made a deep impression on me. I have learned extensively from him, including how to raise new possibilities, how to approach a problem by systematic thinking. I could not have imaged having a better supervisor for my Ph.D. study. Besides my supervisor, I would like to thank the rest of my thesis committee Prof. Masao Sato and Assoc. Prof. Mitsuru Tanaka for their insightful comments and encouragement.

In addition, I would like to express my truthful gratitude to Prof. Satoshi Nagaoka in Gifu University for his insightful suggestion during my academic activities. I would also like to thank Mrs. Kaori Miyazaki for taking care of all official matters that I can focus on my research.

I must express my gratitude to all members of Food Analysis Laboratory for their kind help and expect nothing. They make me feel the warmth like at home in Japan. A special gratitude I would like to give to my partners including Yusuke Amiya, Marika Hashimoto, Risa Katagihara for their scientific support and always giving me words of encouragement.

I would like to thank Chinse embassy for recommendation and the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) for the financial support during my Ph.D. study. I also appreciate all Kyushu University staffs for their kind support.

Most of all, I would like to express my gratitude to my family for their understanding and encouragement.