九州大学学術情報リポジトリ
Kyushu University Institutional Repository

Study on the intestinal absorption of small and oligopeptides in rats

ブ, ティ, ハン

https://doi.org/10.15017/1866364

出版情報：九州大学, 2017, 博士（農学）, 課程博士
バージョン：
権利関係：

KYUSHU UNIVERSITY

Study on the intestinal absorption of small and oligopeptides in rats

Vu Thi Hanh

Kyushu University

2017

List of contents

Chapter I

Introduction 1

Chapter II

Application of a standard addition method for quantitative mass spectrometric assay of dipeptides 17

1. Introduction 17

2. Materials and Methods 21

2.1. Materials and instrumentation 21

2.2. Preparation of peptide standard and soybean hydrolysate solutions.....22

2.3. Derivatization of dipeptides with TNBS 22

2.4. LC-TOF-MS analysis 23

3. Results and Discussion 24

3.1. ESI-MS detection of intact and TNBS-derivatized dipeptides 24

3.2. Application of a standard addition method for quantitative MS assay of dipeptides in soybean hydrolysate.....30

4. Summary 36

Chapter III

Intestinal absorption of oligopeptides in spontaneously hypertensive rats .37

1. Introduction 37

2. Materials and Methods 39

2.1. Materials 40

2.2. Animal experiments 40

2.3. Determination of absorbed oligopeptides in plasma 41

2.4. Statistical analyses 43

3. Results and Discussion 43

3.1. Absorption of a tripeptide model Gly-Sar-Sar in spontaneously hypertensive rats 43

3.2. Absorption of oligopeptide models Gly-Sar-Sar-Sar and Gly-Sar-Sar-Sar-Sar in spontaneously hypertensive rats 48

4. Summary 54

Chapter IV

Effect of aging on intestinal absorption of peptides in spontaneously hypertensive rats.....55

1. Introduction 55

2. Materials and Methods 57

2.1. Materials 57

2.2. Animal experiments 57

2.3. Determination of absorbed peptides in plasma 58

2.4. Western blotting analyses 61

2.5. Statistical analyses 63

3. Results and Discussion 64

3.1. Effect of aging on absorption of di-/tripeptides in spontaneously hypertensive rats 64

3.2. Effect of aging on PepT1 expression in spontaneously hypertensive rats ...72

3.3. Effect of aging on absorption of oligopeptides Gly-Sar-Sar-Sar and Gly-Sar-Sar-Sar-Sar in spontaneously hypertensive rats 74

4. Summary 79

Chapter V

Conclusion 81

References 86

Acknowledgements 101

Abbreviations

- ACE, angiotensin I-converting enzyme
- ACN, acetonitrile
- AUC, area under the curve
- C_{max}, maximum concentration
- EDTA, ethylenediamine tetraacetic acid
- ESI, electrospray ionization
- FA, formic acid
- IS, internal standard
- LC, liquid chromatography
- LOD, limit of detection
- LOQ, limit of quantitation
- MeOH, methanol
- MRM, multiple reaction monitoring
- MS/MS, tandem mass spectrometry
- P_{app}, apparent permeability
- PepT1, proton-coupled peptide transporter 1
- *m/z*, mass-to-charge ratio
- SBP, systolic blood pressure
- SD, Sprague-Dawley
- SHR, spontaneously hypertensive rat
- *S/N*, signal-to-noise ratio
- SEM, standard error of mean
- TJ, tight-junction
- t_{max}, time for maximum concentration
- $t_{1/2}$, elimination of half-life
- TNBS, 2,4,6-trinitrobenzene sulfonate
- TNP, trinitrophenyl
- TOF, time of flight

Chapter I

Introduction

In the modern society, lifestyle-related diseases concomitant with chronic diseases, such as atherosclerosis, heart disease, stroke, obesity, and type 2 diabetes, have been rapidly increased as a critical public health issue in the world [1]. It is estimated that there are approximately 60 million deaths worldwide each year, in which over half are related to lifestyle-related diseases. The classes of diseases can be improved by lifestyle changes and early treatments such as healthy diet, non-smoking, reducing excessive alcohol use, reducing stress level, and regular exercise [2].

It is well known that a healthy diet plays an important role in disease prevention or modulation. For this reason, food scientists have researched physiological activities of food compounds, in particular, bioactive peptides from food proteins, which can exert positive physiological responses in the body upon their basic nutritional compositions in provision of nitrogen and essential amino acids [4]. It has been demonstrated that bioactive peptides are essential in the prevention of lifestyle-related diseases such as hypertension [3–7], antioxidation [8], and inflammation [9]. Thus far, many peptides with

various bioactive functions have been discovered and identified [8,10–12]. It was known that peptides generally consisting 2 to 9 amino acids may elicit bioactivities [4,8]. Among them, small peptides showing antihypertensive activity by angiotensin-converting enzyme (ACE) inhibition, renin inhibition, and calcium channel blocking effects are in common [13].

The source of food-derived bioactive peptides is mainly from dietary proteins (milk, meat, egg, and soybean) [5,8,14–16]. So far reported, Sipola *et al.* [17] demonstrated that a long-term administration (12 weeks) of peptides (Ile-Pro-Pro and Val-Pro-Pro) or a sour milk containing both tripeptides to 12- and 20-wk spontaneously hypertensive rats (SHR) resulted in a significant decrease in systolic blood pressure (SBP) of 12 or 17 mmHg, respectively. A dipeptide, Val-Tyr, from sardine muscle hydrolysate, showed a significant clinical antihypertensive effect in mild hypertensive subjects [5]. Trp-His and His-Arg-Trp were reported to block L-type Ca^{2+} channel [18,19]. Vallabha *et al.* [11] identified peptides including Leu-Ile, Leu-Ile-Val, Leu-Ile-Val-Thr, and Leu-Ile-Val-Thr-Gln from soybean hydrolysate with ACE inhibitory activity. A series of oligopeptides Phe-Asp-Ser-Gly-Pro-Ala-Gly-Val-Leu and Asn–Gly-Pro-Leu-Gln-Ala-Gly-Gln-Pro-Gly-Glu-Arg from squid [20]; Asp-Ser-Gly-Val-Thr, Ile-Glu-Ala-Glu-Gly-Glu, Asp-Ala-Gln-Glu-Lys-Leu-Glu, Glu-Glu-Leu-Asp-Asn-Ala-Leu-Asn, and Val-Pro-Ser-Ile-Asp-Asp-Gln-Glu-Glu-Leu-Met in hydrolysates produced from porcine myofibrillar proteins [12] were found to have antioxidant activity. Other reported peptides were also

demonstrated to have physiological activities in preventing lifestyle-related diseases, as summarized in Table 1-1.

Although bioactive peptides from functional foods have been found to be less effective than therapeutic drugs by daily intake, peptides must play a crucial role as natural and safe diet in disease prevention. When any new functional food products are developed and released on market, industrial manufacturers must control the quality and quantity of functional products. Therefore, it is also essential to evaluate the amount of candidates in functional food products. Additionally, in Japan (2016), a serious social issue on the reliability of functional food products was reported [21]. From Japanese Government Report, an FOSHU (Food for Specified Health Use) product approved by the Government was decided to decline the approval due to the lack of the required amount of candidate ACE inhibitory peptide Leu-Lys-Pro-Asn-Met in the product.

Table 1-1. Reported physiological functions of peptides from food proteins

Source	Preparation	Peptides	Action	Reference
Sardine	Enzymatic hydrolysis	Val-Tyr, Met-Phe, Arg-Tyr, Met-Tyr, Leu-Tyr, Tyr-Leu, Ile-Tyr, Val-Phe, Gly-Arg-Pro, Arg-Phe-His, Ala-Lys-Lys, Arg-Val-Tyr	ACE inhibition	[5,22]
Soy bean	Enzymatic hydrolysis	Leu-Ile, Leu-Ile-Val, Leu-Ile-Val-Thr, Leu-Ile-Val-Thr-Gln	ACE inhibition	[11]
Milk	Fermentation	Ile-Pro-Pro, Val-Pro-Pro	Antihypertension	[14]
Buckwheat	Pepsin, chymotrypsin, trypsin hydrolysis	Val-Lys, Tyr-Gln, Tyr-Gln-Tyr, Pro-Ser-Tyr, Leu-Gly-Ile, Ile-Thr-Phe, Ile-Asn-Ser-Gln	ACE inhibitory	[23]
Squid	Trypsin hydrolysis	Phe-Asp-Ser-Gly-Pro-Ala-Gly-Val-Leu, Asn–Gly-Pro-Leu-Gln-Ala-Gly-Gln-Pro-Gly-Glu-Arg	Antioxidation	[20]
Porcine myofibrillar proteins	Enzymatic hydrolysis	Asp-Ser-Gly-Val-Thr, Ile-Glu-Ala-Glu-Gly-Glu, Asp-Ala-Gln-Glu-Lys-Leu-Glu, Glu-Glu-Leu-Asp-Asn-Ala-Leu-Asn, Val-Pro-Ser-Ile-Asp-Asp-Gln-Glu-Glu-Leu-Met	Antioxidation	[12]
Defatted soy protein	Thermolase hydrolysis	X-Met-Leu-Pro-Ser-Tyr-Ser-Pro-Tyr	Anticancer	[24]
Soybean glycinin	Enzymatic hydrolysis	Leu-Pro-Tyr-Pro-Arg	Hypocholesterolemia	[25]
α’ subunit of β-conglycinin	Enzymatic hydrolysis	Soymetide-13: Met-Ile-Thr-Leu-Ala-Ile-Pro-Val-Asn-Lys-Pro-Gly-Arg Soymetide-9: Met-Ile-Thr-Leu-Ala-Ile-Pro-Val-Asn Soymetide-4: Met-Ile-Thr-Leu	Immunostimulation; sometide-9 showed the most active in stimulating phagocytosis *in vitro*	[25]
Soybean conglycinin	Protease S hydrolysis	Val-Asn-Pro-His-Asp-His-Gln-Asn, Leu-Val-Asn-Pro-His-Asp-His-Gln-Asn, Leu-Leu-Pro-His-His, Leu-Leu-Pro-His-His	Antioxidation	[26]

Liquid chromatography-mass spectrometry (LC-MS) analysis is growing in any scientific fields such as biochemical, food, medicinal aspects owing to its highly selective and sensitive detection of analytes of a given mass/charge (*m/z*) at trace levels. In principle, analytes are eluted from a column attached to a liquid chromatograph (LC), and are then converted to a gas phase to produce ions by an ionization *e.g*., electrospray ionization (ESI). Analyte ions are fragmented in the mass spectrometer, and then fragments or molecular masses are used for MS detection. Furthermore, the potential of MS has been successfully applied for visualization of analytes [27,28]. Despite the advantages, interfering species may still cause the reduced MS ability due to low inherent sensitivity, matrix and/or poor solvent effects, leading to the poor ionization of analytes. In order to overcome the drawbacks, several techniques have been applied to solve the issues to improve ionization efficiency of analytes.

Sample clean-up such as column switching and solid phase extraction is commonly used to remove the matrix components from biological samples [29,30]. However, it is difficult to remove co-eluting substances from biological samples for the reduction of matrices completely. In addition, the time-consuming and multi-step preparation may cause the loss of analytes in samples.

Alternatively, chemical derivatization techniques are expected to improve the MS detectability of poor ionizable analytes [31–33]. Chemical

derivatization involves the chemical reaction of analyte with reagents to provide more ionizable characteristics [31–33]. It has been reported that several derivatization methods are available for determination of small amines such as amino acids [34,35], free advanced glycation end-products [33], small peptides [36]. So far reported, Fonteh *et al*. [34] revealed that a propyl chloroformate derivatization enhanced LC-MS/MS determination of amino acids and dipeptides in cerebrospinal fluids at pmol levels. Shimbo *et al*. [35] reported that 3-aminopyridyl-*N*-hydroxysuccinimidyl carbamate could be used to determine 23 amino acids at limit of detection (LOD) of 0.04 to 2.3 nmol/mL. An amine specific derivatization reagent, 2,4,6-trinitrobenzene sulfonate (TNBS), has excellent features for high sensitive LC-MS [33,36]. Small peptides such as Val-Tyr, Met-Tyr, and Gly-Tyr were easily derivatized with TNBS, and were detected at fmol/mL levels owing to enhanced ionization efficiency by induced hydrophobic trinitrophenyl (TNP) moiety (Figure 1-1) [36]. The TNBS-LC-MS technique was successfully applied for the living body to evaluate intact absorption and pharmacokinetics of basic dipeptide Trp-His [37]. Hence, a TNBS derivatization-aided high sensitive LC-MS method would be suitable for the evaluation of small peptide absorption to get insight on pharmacokinetics, distribution, and metabolism in tissues and/or blood circulation. However, the TNBS-LC-MS technique still suffers from interfering matrix contaminants, requiring the compensation of matrix effects for accurate peptide assay.

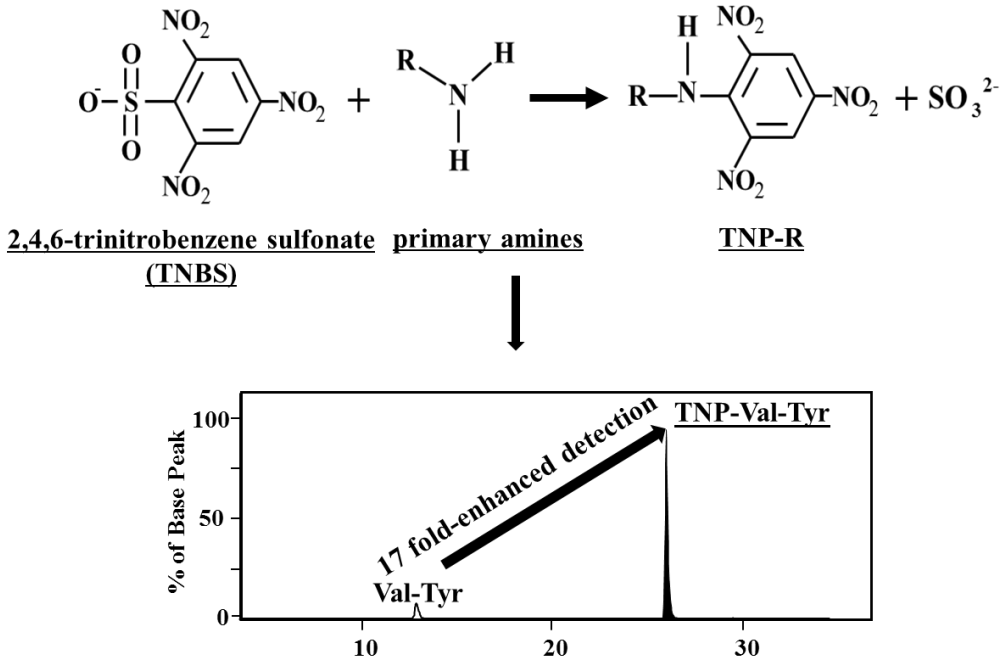


Figure 1-1. Enhanced MS detection of amines by TNBS derivatization [36]

Matrix contaminants cannot be completely eliminated or compensated from target analytes by any pretreatments. Appropriate calibration techniques are used to compensate (but do not eliminate) matrix contaminants. The following options are, thus, obtained:

i) A labeled internal standard (IS), which has the same chemical properties and retention time as non-labeled target, is useful for the correction of MS signal because they can compensate for matrix effects [33,38]. Although the best option to tackle matrix effects is the use of isotopically labeled targets, the isotope labeling IS technique would be limited by less available IS or high cost.

ii) A standard addition method may be sufficient for correcting matrix effects, in which a standard chemical is added to sample (Figure 1-2) [39,40]. So far reported, Ito *et al.* [40] showed that quantitative results of four diarrhetic shellfish poisoning toxins in scallops extracts by common external standards were lower 15-33% than those of a standard addition method because of matrix suppression effect. Fernández-Fígares *et al.* [41] evaluated the effect of different matrices (plasma, muscle, and liver) on physiological amino acid analysis, and revealed that the standard addition method was more useful for the correction of matrix effects compared to absolute calibration method; in turn, concentrations of amino acids such as Thr, Val, and Ala obtained from the absolute calibration method were much lower 46.8%, 37%, and 44.6% than those from the standard addition method, respectively, in all tested matrices

(plasma, muscle, and river). Cimetiere *et al.* [39] demonstrated the advantage of a standard addition method compared to conventional method (external calibration with internal standard correction) for the determination of 27 targeted pharmaceutical compounds at pg/mL levels in drinking water. For example, quantification of ofloxacin by the conventional method (external standard with internal standard correction) (8 ± 2 ng/L) showed a significant lower estimation compared to the standard addition method (22 ± 3 ng/L) in drinking water. Additionally, Ostroukhova *et al*. [42] recommended to use a standard addition method, since concentrations of pesticides in plant samples determined by an external standard method were 10–70% lower than those by the standard addition method. Taken together, the standard addition method may be suitable for the compensation of matrix effects.

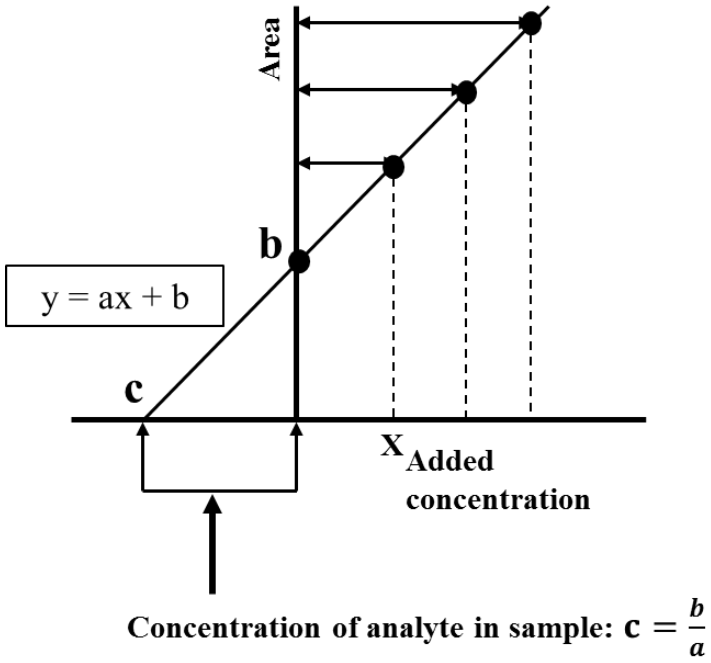


Figure 1-2. A standard addition method, where y: peak area or signal intensity of standard in sample; x: concentration of added standards in sample.

It was believed that dietary proteins were completely hydrolyzed into their constituent amino acids, and then absorbed into blood *via* specific amino acid transport systems until the report by Newey and Smyth, who provided the first convincing evidence that dipeptides could be absorbed in intact form [43]. After that, some researchers [44,45] have reported that a proton-coupled peptide transporter 1 (PepT1) was found to be expressed in the brush border membrane of small intestine, which plays a role in the intestinal absorption of di-/tripeptides. PepT1 is composed of 708 amino acids with 12 membrane-spanning domains. Although intestinal membrane expresses another type of proton-coupled peptide transporter, peptide/His transporter 1 (PHT1), by which His and di-/tripeptides can be transported, PepT1 was mainly responsible for the transport of an enormous range of substrate specificity for di-/tripeptides [44]. At intestinal epithelial cells, some small peptides (di-/tripeptides) can be transported across membrane in intact form with the help of PepT1 transporter, others are hydrolyzed to free amino acids by peptidases in the gut intestinal tract and/or plasma, and released into the portal circulation *via* the amino acid transporter located in the intestinal basolateral membrane (Figure 1-3). The early work by Boullin *et al*. [46] pointed out the absorption of six dipeptides (Gly-Gly, Gly-D-Phe, Gly-Phe, Gly-Pro, Pro-Gly, and carnosine (β-Ala-His)) in their intact forms into rat blood stream. There are some evidences on the bioavailability of bioactive peptides such as Val-Tyr [47] and Pro-Gly [10] in humans, and Trp-His in rats [37]. The detection of lactotripeptides, Ile-Pro-Pro and Val-Pro-Pro, in human after oral administration suggests the resistance of

the tripeptides to protease digestion [14]. However, there were few reports on the relationship between di-/tripeptide absorption and PepT1 expression, exceptional report by Jappar *et al.* [48], who demonstrated that fasting caused a significant upregulation of PepT1 in the small intestine, leading to a significant increase in *in vivo* pharmacokinetics of a model dipeptide glycyl-sarcosine (Gly-Sar) in wild-type and *Pept1* knockout mice. Additionally, the intestinal PepT1 was reported to alter the expression during the developmental stages in rats and chicks [49,52]. However, little information on relationship between small peptide absorption and PepT1 expression by aging is available.

Apart from the aforementioned di-/tripeptides, much work has been focused on the absorption of oligopeptides, since many oligopeptides have been demonstrated to play physiological preventive roles in events against lifestyle-related diseases [13, 53–55]. *In vitro* studies reported that oligopeptides could be transported across the brush border membrane (Figure 1-3). Recently, some reports demonstrated that an ACE inhibitory pentapeptide as Gln-Ile-Gly-Leu-Phe [54] and an octapeptide as Gly-Ala-Hyp-Gly-Leu-Hyp-Gly-Pro [55] derived from egg white and chicken collagen were passively transported across Caco-2 cell monolayers through tight-junction (TJ)-mediated passive route with P_{app} values of $9.11 \pm 0.19 \times 10^{-7}$ cm/s and $4.36 \pm 0.20 \times 10^{-7}$ cm/s, respectively. A series of oligopeptides such as Arg-Val-Pro-Ser-Leu [56], Lys-Val-Leu-Pro-Val-Pro [57], and Gly-Gly-Tyr-Arg [58] were demonstrated to be possibly transported *via* TJ route, along with the reduction in blood pressure in hypertensive rats after orally administered [56,58,59]. The aforementioned

results strongly implied that some oligopeptides may exert biological effect in body by their intact absorption into blood circulation. Thus, it is extremely important to clarify and get insight into the absorption and pharmacokinetic profiles of oligopeptides in living bodies. Recently, Hong *et al*. [60] successfully designed novel transport models of oligopeptides with high protease resistance on the basis of a Gly-Sar mother peptide skeleton, *i.e*., Gly-Sar-Sar as a tripeptide model, Gly-Sar-Sar-Sar as a tetrapeptide, and Gly-Sar-Sar-Sar-Sar as a pentapeptide. However, *in vivo* absorption of oligopeptides has remained unclear.

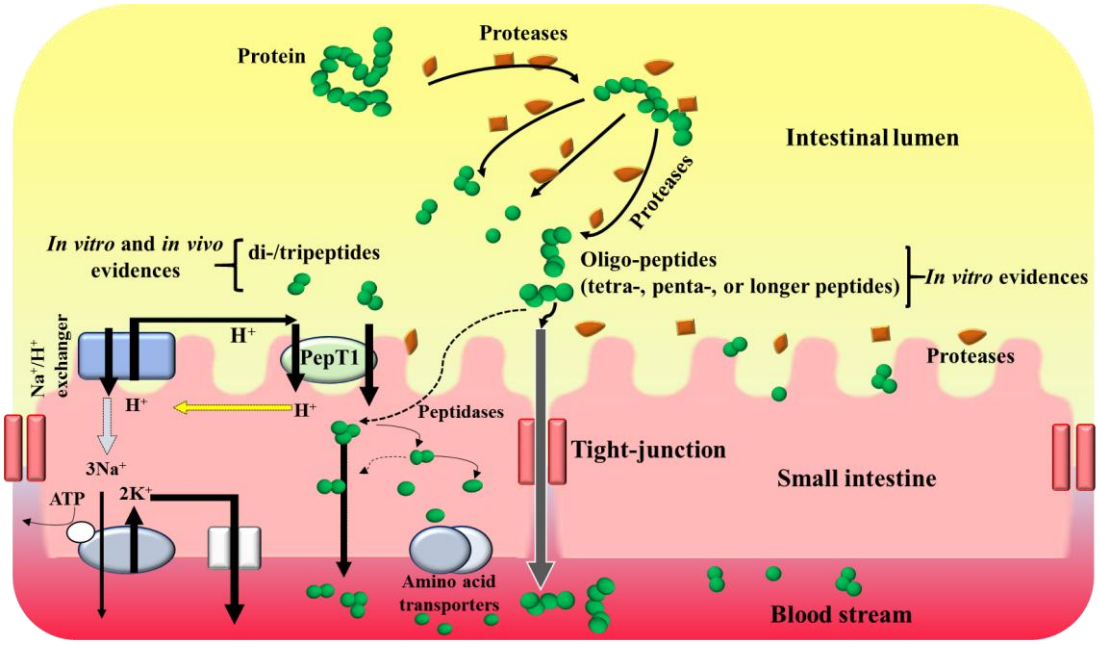


Figure 1-3. Schematic diagram for peptide absorption in intestinal tract

According to all of the above-mentioned points, the aim of the present work was to overcome issues on the development of a convenient and reliable quantification assay of peptides, and on their *in vivo* absorption behavior. The detailed objectives for each Chapter are detailed below:

1) **Chapter II** aimed to develop a convenient and reliable MS quantification assay for the analysis of small peptides in soybean hydrolysate, which contains a number of small bioactive peptides. To compensate matrix suppression, a standard addition method using target peptide standards was applied for this study, in combination with a TNBS derivatization-aided high sensitive LC-MS method. The proposed method provided excellent and convenient evaluation of peptide profiles in protein hydrolysate without the use of an isotope labeling technique.

2) A TNBS derivatization-aided high sensitive LC-MS method was applied to clarify the bioavailability of oligopeptides (tri- to pentapeptides) *in vivo*. Model oligopeptides including Gly-Sar-Sar as tripeptide, Gly-Sar-Sar-Sar as tetrapeptide, Gly-Sar-Sar-Sar-Sar as pentapeptide, were used in this study. **Chapter III** clearly demonstrated the first evidence that oligopeptides could be absorbed in their intact forms *in vivo* into the blood of 8-wk SHRs.

3) Based on the findings obtained above, the effect of aging on the absorption of peptide (di- to pentapeptides) in SHRs was investigated. In **Chapter IV,** it was demonstrated for the first time that aging may enhance the

absorption of di-/tripeptide through the enhanced PepT1 transport route, whereas the intestinal absorption of oligopeptides were not affected by aging of SHRs.

Chapter II

Application of a standard addition method for quantitative mass spectrometric assay of dipeptides

1. Introduction

To date, mass spectrometry (MS) is a powerful analytical tool in pharmaceutics, biochemistry, and food science fields for sensitive and quantitative detection of analytes of a given mass/charge (*m/z*). The potential of MS allows further analytical applications, *e.g.*, the visualization of analytes in tissues [27,28]. Despite these advantages, MS still has some restrictions such as poor detection of small molecules. This is because small analytes typically display low ionization efficiency caused by matrix and/or poor solvent effects [31]. Several chemical derivatization techniques have, therefore, been developed in order to overcome these disadvantages [31–33]. A preferred technique for small amines or peptides at fmol/mL levels has been established with TNBS derivatization [33,36] (Figure 1-1).

Although the successful derivatization of small analytes may be of benefit for improving MS detection by an enhanced solvent effect, the detection can still be complicated by interferences from matrix contaminants. In particular, food-related compounds with similar composition profiles, such

as peptides in enzymatic hydrolysate, may cause the difficulty of quantitative MS analysis. Internal standard (IS)-guided quantification methods using isotope labeled targets [33,38] is the best option for compensation for the effects of interfering matrix contaminants. However, isotope labeling technique may be limited due to cost-efficiency and the availability of isotope labeled compounds.

To date, many studies on bioactive compounds of food-derived compounds are addressing the physiological effects and potential health-benefits to develop functional products. A commercially available product, soybean hydrolysate (or protein hydrolysate), is known to contain a number of bioactive peptides [61], which could exhibit properties such as *in vitro* ACE inhibitory activity (IC_{50}: Gly-Tyr, 220 μM; Ile-Tyr, 3.7 μM) [62] and improved brain dysregulation effects (Ser-Tyr, Ile-Tyr) [63,68].

In Chapter II, we, thus, attempted to develop a convenient and reliable MS quantification assay for the analysis of bioactive dipeptides in soybean hydrolysate without the use of isotope labeling IS technique. In order to compensate for matrix signal suppression, a standard addition method using target peptide standard was applied to analyze peptides in soybean hydrolysate *via* combination with a TNBS derivatization-aided high sensitive LC-MS method [36]. Factors affecting simultaneous detection and quantification of target peptides (Gly-Tyr, Ile-Tyr, and Ser-Tyr) (*e.g.*, overlapped elution and/or suppressed ionization of targets on LC-MS) were examined. Dipeptides with

reversed sequences of the three targets, *i.e.*, Tyr-Gly, Tyr-Ile and Tyr-Ser, respectively, together with Leu-Tyr and Tyr-Leu (having the same molecular weight of 294.3468 Da as target Ile-Tyr), were also selected for the study (Figure 2-1).

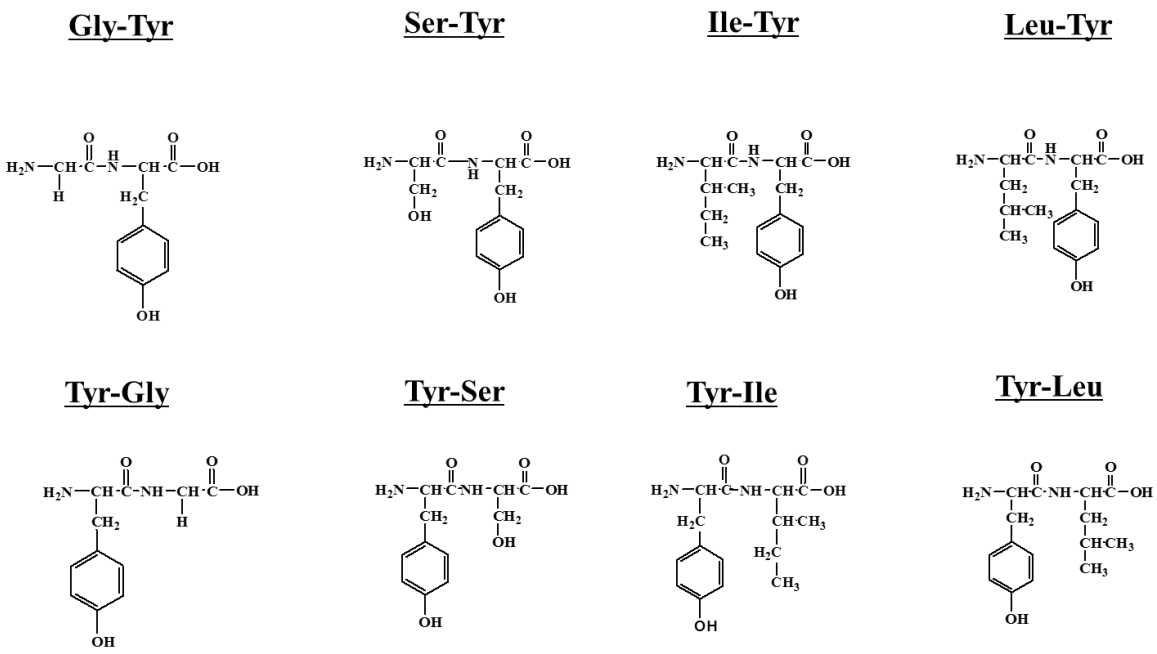


Figure 2-1. Target dipeptides in this study

2. Materials and Methods

2.1. Materials and instrumentation

Dipeptides (Gly-Tyr, Tyr-Gly, Ser-Tyr, Tyr-Ser, Ile-Tyr, Tyr-Ile, Tyr-Leu, and Leu-Tyr) were synthesized *via* the Fmoc solid phase synthesis according to the method provided by the manufacturer (Kokusan Chemicals, Osaka, Japan), and their sequences were confirmed on a PPSQ-21 amino acid sequencer (Shimadzu Co., Kyoto, Japan). Commercially available soybean hydrolysate (a peptide mixture having an average length of 3-6 peptides) was a product of FUJI OIL Co. (Hinute AM, Tokyo, Japan). Distilled water, methanol (MeOH), and formic acid (FA) were of LC-MS grade (Kanto Chemical, Tokyo, Japan). TNBS was purchased from Nacalai Tesque (Kyoto, Japan). All other chemicals were of analytical grade and were used without further purification.

High-performance liquid chromatography coupled with time-of-flight mass spectrometry (LC-TOF-MS) assays were performed on an Agilent 1200 HPLC (Agilent Technologies, Waldbronn, Germany) equipped with a degasser, binary pump, and column oven. The HPLC system was coupled to an ESI-micrOTOF II system (Bruker Daltonics, Bremen, Germany). Both instruments were controlled by a micrOTOF control 3.0 and a Bruker Compass HyStar 3.2.

2.2. Preparation of peptide standard and soybean hydrolysate solutions

Stock solutions of eight dipeptides (Gly-Tyr, Tyr-Gly, Ser-Tyr, Tyr-Ser, Ile-Tyr, Tyr-Ile, Tyr-Leu, and Leu-Tyr) were individually prepared by dissolving each peptide in distilled water to a concentration of 1.0 mg/mL and were stocked at -40 °C. Working standards were prepared daily prior to carrying out experiments by combination of the individual stock solutions and further dilution with distilled water. Standard solutions (0.5-4.0 μg/mL) were used to obtain absolute calibration curves. Soybean hydrolysate (Hinute AM) was dissolved in distilled water (50.0 mg/mL). The hydrolysate solution (final concentration, 10.0 mg/mL) was spiked with a series of standard peptide solutions with final concentration of 4.0, 8.0, and 16.0 μg/mL for the standard addition method. The calculation equation is as follows:

$$[\text{Analyte}] = \frac{\text{Measured signal in unadded sample (b)}}{\text{Slope of the standard addition calibration curve (a)}}$$

2.3. Derivatization of dipeptides with TNBS

TNBS derivatization of peptides was performed according to previously reported method [36]. To either a standard solution or a sample solution (40 μL), TNBS solution (10 μL of 150 mM) in 0.1 M borate buffer at pH 8.0 was added. After incubation at 30 °C for 30 min, 0.2% FA (50 μL) was added to the derivatized mixture. An aliquot (10 μL) of the resulting mixture was injected into LC-TOF-MS. The analyses of each solution of the four different concentrations were conducted in replicate in order to obtain calibration curves

and standard addition curves. No degradation of TNP-dipeptides was observed during storage of the solution at 4 °C for 24 h [33,36]. The results are expressed as the mean ± standard error of mean (SEM).

2.4. LC-TOF-MS analysis

Chromatographic separation was performed on a Waters Biosuite C_{18} column (2.1 mm x 150 mm, 3 µm particle size) (Waters, Milford, MA, USA). A linear gradient elution of MeOH (60-100% over 40 min) containing 0.1% FA at a flow rate of 0.25 mL/min was performed at 40 °C. For the separation of intact (or non-TNBS derivatized) dipeptides, an elution of 0-100% MeOH containing 0.1% FA was performed over 20 min. ESI-TOF-MS analysis was carried out in positive mode, and mass-detection range was set at *m/z* 100-1000. The conditions of ESI source were as follows: drying gas (N_2) flow rate = 8.0 L/min; drying gas temperature = 200 °C; nebulizing gas pressure = 1.6 bar; capillary voltage = 3800 V. All data acquisition and analyses were controlled by Bruker Data Analysis 3.2 Software. To ensure optimal conditions for the analyses, calibration of the detector was performed using sodium formate clusters (10 mM NaOH in water:acetonitrile (1:1, v/v)). The calibration solution was injected at the beginning of each run, and all spectra were calibrated prior to identification. The width was set at *m/z* 0.01 for mono-isotopic isolation of the target ions: Gly-Tyr and Tyr-Gly = *m/z* 239.1026; Ser-Tyr and Tyr-Ser = *m/z* 269.1026; Ile-Tyr, Tyr-Ile, Leu-Tyr and Tyr-Leu = *m/z* 295.1652; TNP- Gly-Tyr and TNP-Tyr-Gly = *m/z* 450.0892; TNP-Ser-Tyr and

TNP-Tyr-Ser = *m/z* 480.0097; TNP-Ile-Tyr, TNP-Tyr-Ile, TNP-Leu-Tyr and TNP-Tyr-Leu = *m/z* 506.1518.

3. Results and Discussion

3.1. ESI-MS detection of intact and TNBS-derivatized dipeptides

At the optimal LC-TOF-MS conditions (described in the Method section) with standard solution containing eight dipeptides (Gly-Tyr, Tyr-Gly, Ser-Tyr, Tyr-Ser, Ile-Tyr, Tyr-Ile, Tyr-Leu, and Leu-Tyr, at final concentration of each 0.4 μg/mL) with or without TNBS derivatization was injected into LC-ESI-TOF-MS system to gain insight into LC separation behavior of each target dipeptide in a single assay. As shown in Figure 2-2 and Table 2-1, intact (or non-derivatized) dipeptides were eluted within 30 min, but their detection/signal intensities or signal to noise (*S/N*) ratios were low. In contrast, highly enhanced detection (higher signal intensity or *S/N* ratio) for TNP-dipeptides was observed by TNBS derivatization (Figure 2-2 and Table 2-1). This suggested that the induced TNP moiety (+212 Da) to small and polar dipeptides may enhance their hydrophobicity, and therefore overcome the poor retention and sensitivity for intact dipeptides. Since dipeptides Tyr-Ile, Tyr-Leu, and Leu-Tyr have the same molecular weight as Ile-Tyr, they displayed the same retention time of 36 min on a Biosuite column, and the same *m/z* of 506.1815 for mono-isotopic TOF-MS detection. In this assay, we thus excluded dipeptides Tyr-Ile, Tyr-Leu, and Leu-Tyr due to their co-elution under the present LC conditions and the overlap of their signals during analysis by single

TOF-MS. A multi-reaction monitoring (MRM)-MS/MS technique could be, therefore, useful for the detection of such targets by their characteristic mono-isotopic fragmentations (TNP-Tyr-Ile, *m/z* 506.2 > 460.1; TNP-Leu-Tyr, *m/z* 506.2 > 232.0; TNP-Tyr-Leu, *m/z* 506.2 > 278.1) (see in Figure 2-3). Sora *et al.* [64] revealed the efficacy of the standard addition method-aided LC-MRM-MS/MS assay in generating reproducible ionization conditions with successful quantification of five terpene trilactones from *Ginkgo* extracts. Further modification of LC-MS (or LC-MRM-MS/MS) conditions will be required if the peptides of interest possess the same molecular properties such as in the case of Tyr-Ile and Leu-Tyr. However, for the purpose of the current objective, *i.e.*, establishment of a convenient and reliable quantitative LC-MS assay for bioactive dipeptides, a serial set of MRM segmentations for all eight targets would cause the influence on their robust ion source against operational MS parameters.

Figure 2-2 and Table 2-1 reveals the efficiency of TNBS derivatization for enhanced MS detection of the five dipeptides compared to non-derivatized peptides with 0.9-, 4.3-, 11.4-, 24.7-, and 30.5-fold higher signal intensities for TNP-Ile-Tyr, TNP-Ser-Tyr, TNP-Tyr-Ser, TNP-Tyr-Gly, and TNP-Gly-Tyr being observed, respectively, together with limits of detection of 0.05-0.22 μg/mL. The enhanced MS detection of the five TNP-dipeptides may be due to their improved ESI-ionization efficiency through solvent effects and/or hydrophobicity induced by the TNP moiety, as similar to other dipeptides (*e.g.*, Val-Tyr, Met-Tyr, Lys-Tyr, and His-Tyr) [36]. Further experiments were

performed for quantification of five dipeptides Ile-Tyr, Ser-Tyr, Tyr-Ser, Tyr-Gly, and Gly-Tyr in soybean hydrolysate.

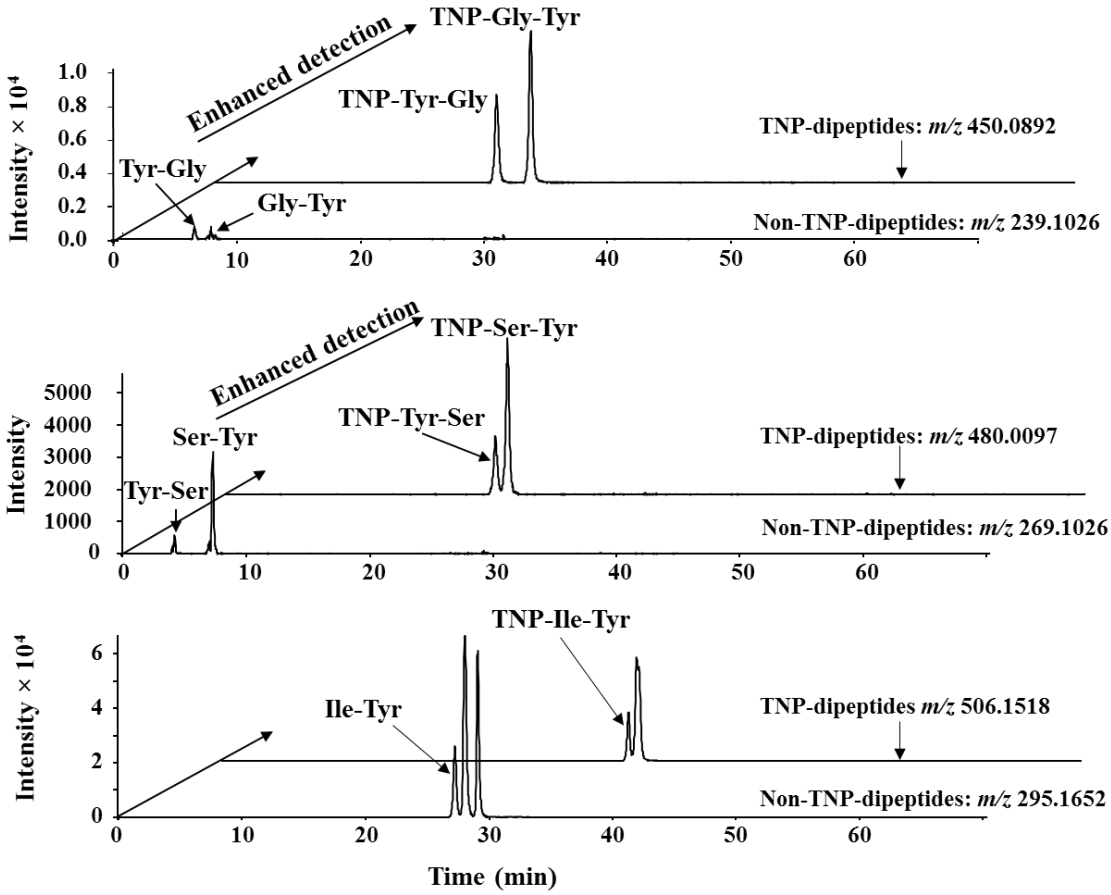


Figure 2-2. Typical MS chromatograms of non-TNBS and TNBS-derivatized dipeptides. Dipeptides (final concentration of each 0.4 µg/mL; Gly-Tyr, Tyr-Gly, Ser-Tyr, Tyr-Ser, Ile-Tyr) were subjected to a 150 mM TNBS derivatization at 30 ˚C for 30 min. Mono-isotopic TOF-MS detection of the corresponding molecular ions ($[M + H]^+$) was performed, as described in the Materials and Methods section. LC separations were performed on a Waters Biosuite C_{18} column (2.1 mm x 150 mm) with 0-100% MeOH in 0.1% FA for non-derivatized dipeptides, and 60-100% MeOH in 0.1% FA for TNBS-derivatized dipeptides at a flow rate of 0.25 mL/min at 40 ˚C.

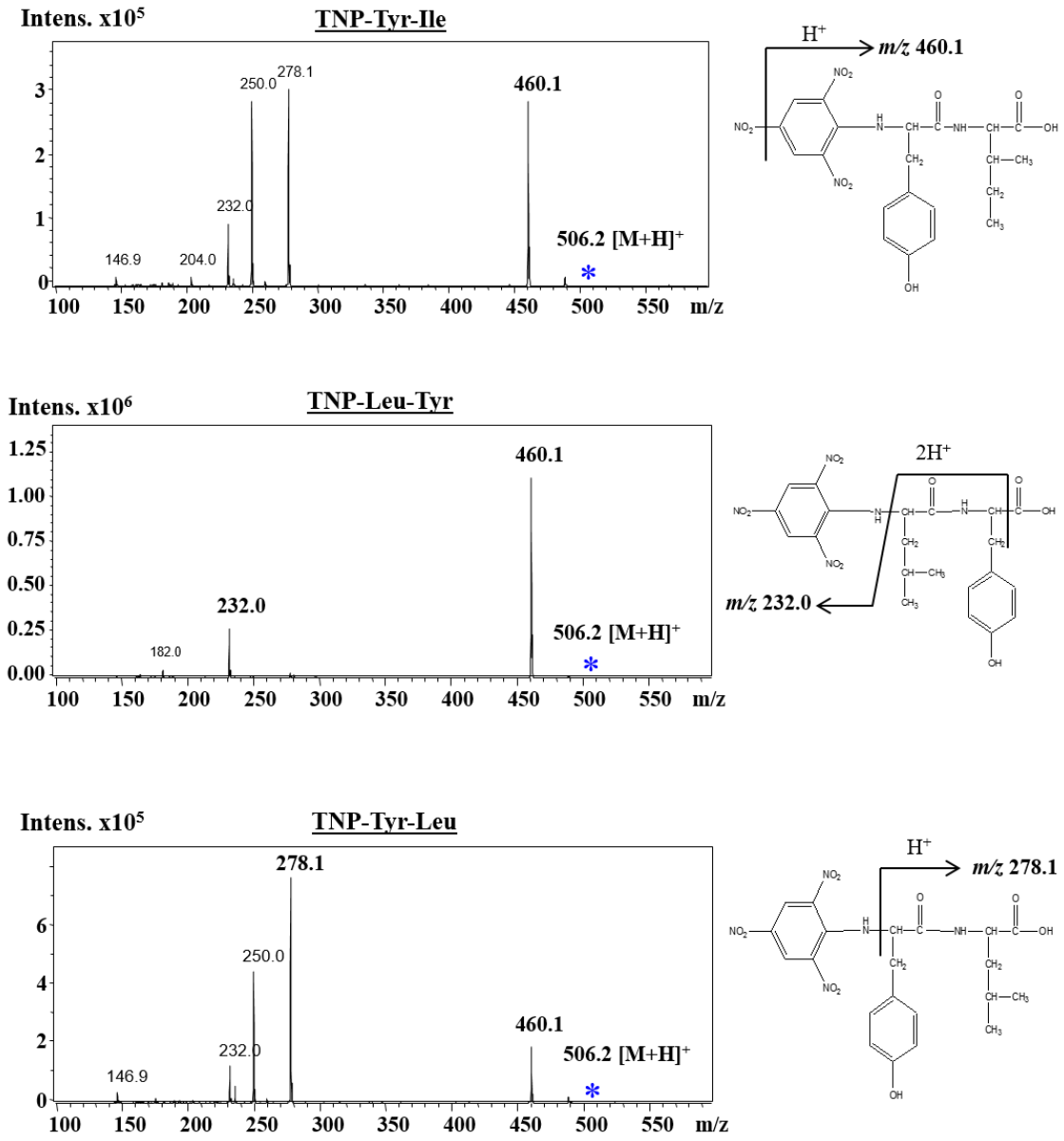


Figure 2-3. Product ion spectra of TNP-Tyr-Ile, TNP-Leu-Tyr, and TNP-Tyr-Leu. The infusion analyses of TNP-Tyr-Ile, TNP-Leu-Tyr, and TNP-Tyr-Leu were performed at a concentration of 20 μg/mL by MS/MS analysis. The transition of targeted precursor ion ($[M+H]^+$, *m/z*): TNP-Tyr-Ile, 506.2 > 460.1; TNP-Leu-Tyr, 506.2 > 232.0; TNP-Tyr-Leu, 506.2 > 278.1.

Table 2-1 Comparison of TOF-MS detection between non-derivatized and TNBS-derivatized dipeptides[a]

Parameter	Gly-Tyr		Tyr-Gly		Ser-Tyr		Tyr-Ser		Ile-Tyr	
	intact	derivatized	intact	derivatized	intact	derivatized	intact	derivatized	intact	derivatized
RT (min)[b]	8.0	26.9	6.6	23.9	7.7	23.9	4.6	22.9	27.2	34.2
Area (x10^6 AU)	5.3	161.4	5.0	123.4	21.7	94.2	2.9	33.1	229.9	200.6
Area ratio	1	30.5	1	24.7	1	4.3	1	11.4	1	0.9
LOD (μg/mL)[c]	ND[d]	0.12	ND	0.19	ND	0.13	ND	0.22	ND	0.05

[a] Concentration of each dipeptide was 0.4 μg/mL. TNBS derivatization and LC-TOF-MS conditions are described in the Materials and Methods section.

[b] RT: retention time on a Water Biosuite column

[c] LOD: limit of detection.

[d] ND: not determined.

3.2. Application of a standard addition method for quantitative MS assay of dipeptides in soybean hydrolysate

Figure 2-4 shows the typical MS chromatograms of the five target dipeptides in soybean hydrolysate (10.0 mg/mL), together with spiked target standards at final concentrations of 0, 4.0, 8.0, and 16.0 μg/mL. MS chromatograms of the non-spiked sample (Figure 2-4) revealed that the five target peptides, including bioactive Gly-Tyr, Ser-Tyr, and Ile-Tyr, were present in their TNP-dipeptide forms in soybean hydrolysate. These five peptides are found in glycinin and/or β-conglycinin protein sequences. MS signal intensity of the TNBS-derivatized targets increased with the concentration of spiked standards (Figure 2-4). The standard addition curves (Figure 2-5 and Table 2-2) revealed a good relationship between the concentration of spiked standards and MS signal intensity, with a correlation coefficient of $r^2 > 0.979$. This indicates that a TNBS derivatization reaction with the target dipeptides and spiked standards in soybean hydrolysate was successfully performed under the mild TNBS reaction conditions (within 30 min at 30 °C), compared with the reported naphthalene-2,3-dialdehyte (NDA) derivatization method within 60 min at 25 ^{o}C [65]. In addition, acceptable relative standard deviation values of 4.6 - 9.1% for all measurements indicated that the proposed standard addition method would be applicable for the quantitative MS assay of dipeptides without isotopic ISs. As summarized in Table 2-2, the five target dipeptides (Gly-Tyr, Tyr-Gly, Ser-Tyr, Tyr-Ser, Ile-Tyr) in soybean hydrolysate were successfully quantified (424 ± 12, 184 ± 5, 2188 ± 114, 327 ± 9, and

2211 ± 77 μg/g of hydrolysate, respectively).

An external standard method (or an absolute calibration method) was compared to the standard addition method to assess the matrix effects. Figure 2-5 and Table 2-2 demonstrated the advantage of the standard addition method for quantitative assays of dipeptides compared to the absolute calibration. It was observed a 7- to 24-fold decrease in the slope of the standard addition curves of hydrolysate compared to the absolute calibration curves of dipeptide standard solution (Figure 2-5 and Table 2-2). This indicated that the standard addition method can exclude the matrix suppression effect from contaminating peptides in soybean hydrolysate. In turn, the content of the target dipeptides in soybean hydrolysate may be over-estimated when the standard addition calibration curve was used. To avoid interference from matrix effects in the MS analysis of food products, a similar study was performed by Cai *et al*. [66]. They demonstrated the accurate quantitative analysis of histamine in beer by the standard addition method, coupled with an extractive nano-ESI-MS technique, without the requirement for matrix cleaning. In addition, these results were in consensus with previous reports on quantification of diarrhetic shellfish poisoning toxins in scallops [40], amino acids in different matrices (plasma, muscle, and liver) [41], and pesticides in plant samples [42]. They showed that the contents of analytes (diarrhetic shellfish poisoning toxins in scallops extracts, amino acids in all tested matrices, and pesticides in plant samples) obtained from the absolute calibration method were 15-33% [40], 37-46.8% [41], and 10–70% [42] lower than those from the standard addition method,

respectively. Taking these factors into account, the proposed TNBS derivatization-aided LC-MS quantification assay with the standard addition method could rule out the consideration of: 1) insufficient MS ionization due to matrix effects, and 2) insufficient TNBS derivatization efficiency.

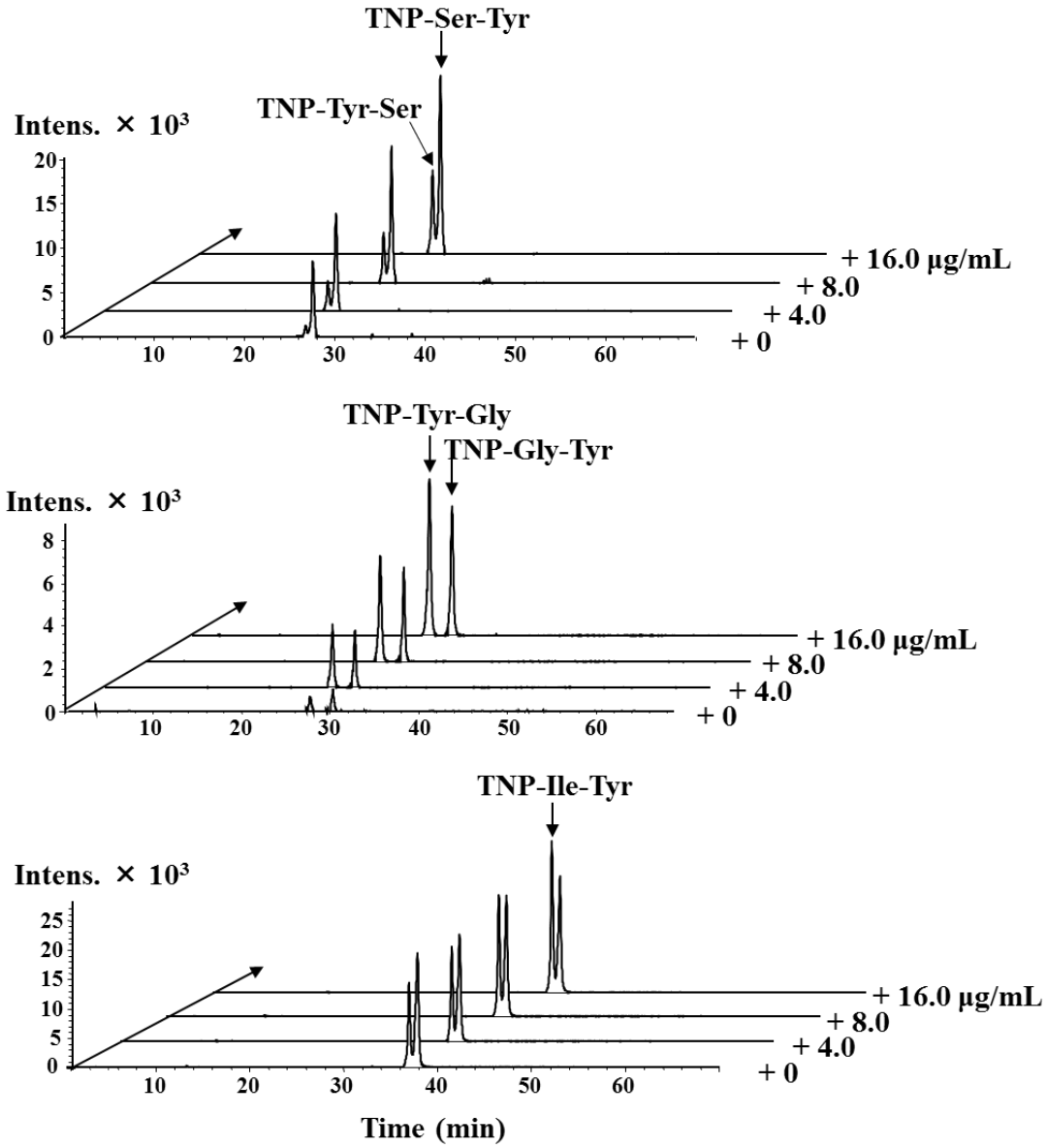


Figure 2-4. Typical MS chromatograms of soybean hydrolysate spiked with dipeptide standards. Standard solutions of dipeptides of Gly-Tyr, Tyr-Gly, Ser-Tyr, Tyr-Ser, and Ile-Tyr (0, 4.0, 8.0, and 16.0 µg/mL) were added to soybean hydrolysate (10.0 mg/mL) in order to obtain standard addition curves. TNBS derivatization and LC-MS conditions were the same as described in Figure 2-2.

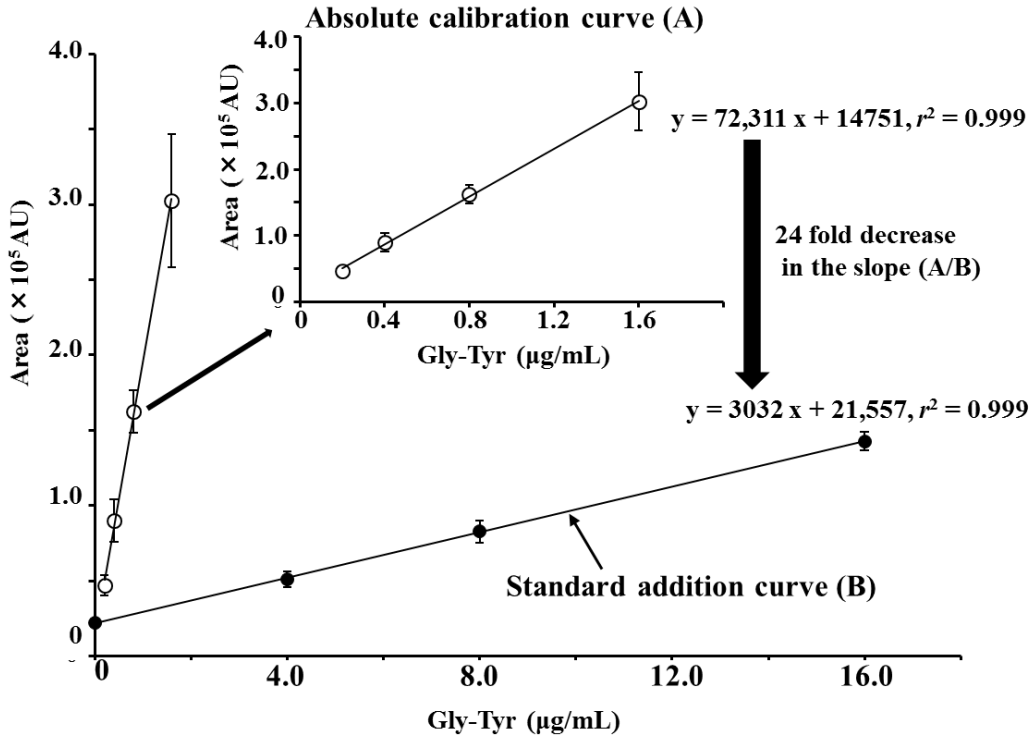


Figure 2-5. Absolute calibration curve (opened circle) and standard addition curve (closed circle) for TNP-Gly-Tyr. The absolute calibration curve was obtained from a standard water solution of Gly-Tyr (final concentration of 0.2-1.6 μg/mL). The standard addition curve was obtained from soybean hydrolysate (10.0 mg/mL) spiked with Gly-Tyr (final concentration of 4.0-16.0 μg/mL). TNBS derivatization and LC-MS conditions were the same as described in Figure 2-2. Results are expressed as the mean ± SEM (n = 3).

Table 2-2. Comparison between absolute calibration method and standard addition method[a]

TNP-peptide	Absolute calibration curve (A)		Standard addition curve (B)			
	Linear regression equation[b]	Correlation coefficient (r^2)	Linear regression equation[c]	Correlation coefficient (r^2)	Slope ratio (A/B)	Content in hydrolysate (μg/g)[d]
TNP-Gly-Tyr	y = 72,311 x + 14,751	0.999	y = 3032 x + 21,557	0.999	24	424 ± 12
TNP-Tyr-Gly	y = 51,374 x + 4626	0.997	y = 4522 x + 17,846	0.999	11	184 ± 5
TNP-Ser-Tyr	y = 53,312 x + 3678	0.998	y = 4552 x + 206,162	0.990	12	2188 ± 114
TNP-Tyr-Ser	y = 28,002 x + 2425	0.996	y = 3881 x + 33,415	0.997	7	327 ± 9
TNP-Ile-Tyr	y = 100,141 x + 24,921	0.984	y = 5686 x + 265,531	0.979	18	2211 ± 77

[a] TNBS derivatization and TOF-MS conditions are described in the Materials and Methods section. Results are expressed as the mean ± SEM (n=3).

[b] Linear regression equation of absolute calibration curve was obtained by dipeptide standard solutions (0.2 – 1.6 μg/mL).

[c] Linear regression equation of standard addition curve was obtained by soybean hydrolysate solution (10.0 mg/mL) spiked with dipeptide standard solutions (4.0 - 16.0 μg/mL).

[d] Content of dipeptide in soybean hydrolysate was calculated by standard addition curve.

4. Summary

In this Chapter II, a standard addition method with TNBS derivatization-aided LC-TOF-MS assay was established for reliable quantification of dipeptides in soybean hydrolysate. Under the optimal TNBS reaction conditions (pH 8.0, 30 min, 30 ^{o}C), five TNP-dipeptides, including Gly-Tyr, Tyr-Gly, Ser-Tyr, Tyr-Ser, and Ile-Tyr in soybean hydrolysate, were successfully detected within 40 min at a limit of detection of > 0.05 μg/mL. By spiking each target standard to hydrolysate, the five target dipeptides were individually quantified without interference from matrix effects. The advantage of the proposed TNBS-LC-TOF-MS aided-standard addition method would provide a convenient evaluation of peptide profiles in hydrolysate or food-derived bioactive peptides without the requirement of isotope labeled IS dipeptides.

Chapter III

Intestinal absorption of oligopeptides in spontaneously hypertensive rats

1. Introduction

Over the last century, it was thought that dietary proteins were completely hydrolyzed into constituent amino acids, and were absorbed *via* amino acid transport system. To date, the uptake of di-/tripeptides has been clearly demonstrated to be transported across the intestinal endothelium by PepT1 [44]. In previous studies, antihypertensive dipeptide Val-Tyr [67] and anti-atherosclerotic dipeptide Trp-His [37] were demonstrated to be absorbed in intact form into blood circulation system through PepT1 route with C_{max} of 3.7 pmol/mL-plasma and 28.7 pmol/mL-plasma, respectively. Dipeptides Ile-Tyr, Ser-Tyr, and Tyr-Pro derived from soybean hydrolysate (in Chapter II) had altered dopamine and noradrenaline metabolism in the mouse brain stem upon oral administration [68]. These observations imply that some dipeptides may display their functions in terms of intact intestinal absorption.

In accordance with studies on functional and absorbable dipeptides, many researchers have investigated that oligopeptides can play physiological preventive

roles in events against lifestyle-related diseases [13]. It has been reported that longer peptides from food-derived bioactive peptides, such as peptide casein α_{s1}-CN f(90-94) (Arg-Tyr-Leu-Gly-Tyr) and α_{s1}-CN f(143-149) (Ala-Tyr-Phe-Tyr-Pro-Glu-Leu), had an antihypertensive effect of 25 ± 3 mmHg and 20 ± 5 mmHg reduction, respectively, in SHRs when orally administered at each dose of 5 mg/kg [69]. Other researches also claimed the antihypertensive effect of oligopeptides *β*-casein Leu-His-Leu-Pro-Leu-Pro (ACE inhibitory activity: IC_{50} = 2.4 μM) [51], Leu-Lys-Pro-Asn-Met (IC_{50} = 2.4 μM) [52], α-lactorphin Tyr-Gly-Leu-Phe (IC_{50} = 1,260 μM) [70], and Ile-Val-Gly-Arg-Pro-Arg (ACE activity: IC_{50} = 300.0 μM) [71] in SHRs after oral administration. However, absorption behavior of oligopeptides still remain unclear. In *vitro* Caco-2 cell experiments, oligopeptides, such as Gly-Gly-Tyr-Arg [72], Gln-Ile-Gly-Leu-Phe [54], Arg-Val-Pro-Ser-Leu [56], and Gly-Ala-Hyp-Gly-Leu-Hyp-Gly-Pro [55], were possibly transported intact *via* paracellular pathway, which is regulated by TJ. In previous report, Hong *et al*. [60] successfully designed acceptable transport model oligopeptides based on the mother peptide skeleton of glycyl-sarcosine (Gly-Sar) with high resistance to protease digestion during intestinal absorption process (*i.e*., Gly-Sar-Sar as tripeptide model; Gly-Sar-Sar-Sar as tetrapeptide; Gly-Sar-Sar-Sar-Sar as peptapeptide). It was observed that these designed oligopeptides could be transported across Caco-2 cell monolayers *via* paracellular TJ route. However, there has been no *in vivo* evidence on intact absorption of oligopeptides so far.

Considering the results of recent *in vitro* studies on possible transport of oligopeptides across Caco-2 cells [54–56,60], the challenging aim of this Chapter III is to investigate whether oligopeptides (tri- to pentapeptides) can be absorbed into rat blood stream.

2. Materials and Methods

2.1. Materials

Gly-Sar, aprotinin, and chymostatin were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). [$^{13}C_2$, ^{15}N]-labeled Gly-Sar was obtained from Scrum (Tokyo, Japan). Gly-Sar-Sar was obtained from Bachem (Bubendorf, Switzerland). Gly-Sar-Sar-Sar and Gly-Sar-Sar-Sar-Sar were purchased from Biomatik Co. (Cambridge, ON, Canada). TNBS was purchased from Nacalai Tesque (Kyoto, Japan). Distilled water, methanol (MeOH), acetonitrile (ACN) and formic acid (FA) were of LC-MS grade (Merck, Darmstadt, Germany). All other reagents were of analytical reagent grade and were used without further purification.

2.2. Animal experiments

Seven-week-old male SHRs (7-wk SHR/Izm, Japan SLC, Shizuoka, Japan) were fed on a laboratory diet (MF, Oriental Yeast Co., Tokyo, Japan) and given water *ad libitum*. All rats were housed for 1 week at 21 ± 1 °C and 55 ± 5% humidity under controlled lighting from 8:00 to 20:00. Each rat was fasted for 16 h before a single oral administration of each peptide. Each peptide was dissolved in milli-Q water, and then was orally administered at a dose of 10 mg/kg to rats. At fixed time-schedules (0, 10, 20, 30, 60, and 90 min), about 100 μL of blood was collected from the tail vein into a tube containing ethylenediaminetetraacetic acid disodium salt (EDTA-2Na) and inhibitors (0.1 mg aprotinin and 0.1 mg chymostatin), then immediately centrifuged at

3,500 × *g* at 4 °C for 15 min to obtain plasma samples. All rat experiments in this study were handled in accordance with the Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science, and Technology in Japan. The Ethics Committee on Animal Experiment at Kyushu University approved all experimental protocols (Permit Number: A28-040).

2.3. Determination of absorbed oligopeptides in plasma

Measurements of plasma peptide levels were determined by using TNBS derivatization-aided LC-MS according to the previous report [37,60]. Namely, an aliquot (50 μL) of plasma solution was added to 150 μL of a solution containing 0.1% NaCl and 0.1% FA. An internal standard (IS; 10 μL) was then added to the plasma solution (0.2 nmol/mL [$^{13}C_2$, ^{15}N]-labeled Gly-Sar was used as IS for Gly-Sar-Sar assay; 0.1 nmol/mL Gly-Sar-Sar was used as IS for Gly-Sar-Sar-Sar and Gly-Sar-Sar-Sar-Sar assays). The resultant solution was loaded onto an Amicon Ultra 0.5-mL-3K Centrifugal filter (Millipore, Carrigtwohill, Ireland), followed by a centrifugation at 14,000 × *g* at 4 °C for 30 min. The obtained filtrate was evaporated to dryness, and then 50 μL of 150 mM TNBS solution (pH 10) was added for TNBS derivatization reaction at 30 °C for 30 min. The reaction was stopped by the addition of 50 μL of 0.2% FA solution, and an aliquot (20 μL) of the resultant trinitrophenyl (TNP)-derivative was injected into LC-MS system.

LC separation was performed using an Agilent 1200 series (Agilent, Waldbronn, Germany) on a Cosmosil 5C_{18}-AR-II column (2.0 mm × 150 mm, 3 μm particle size, Nacalai Tesque, Kyoto, Japan) at 40 ^{o}C with a linear gradient elution of ACN (0 - 100% over 20 min) containing 0.1% FA at a flow rate of 0.2 mL/min. ESI-TOF-MS analysis was carried out using a micrOTOF II (Bruker Daltonics, Bremen, Germany) with the target TNP-Gly-Sar, *m/z* 358.0630; TNP-[$^{13}C_2$,^{15}N]-Gly-Sar, *m/z* 361.0630; TNP-Gly-Sar-Sar, *m/z* 429.1001; TNP-Gly-Sar-Sar-Sar, *m/z* 500.1372; TNP-Gly-Sar-Sar-Sar-Sar, *m/z* 571.1743 in an ESI-positive mode. The conditions of the ESI source were performed as follows: drying gas (N_2) flow rate, 8.0 L/min, drying gas temperature, 200 ^{o}C; nebulizing gas pressure, 1.6 bar; capillary voltage, 3800 V; mass range *m/z* 100 - 1000. All of the acquisitions and analyses of data were controlled by a Bruker Data Analysis 3.2 Software. A calibration solution containing 10 mM sodium formate in 50% ACN was injected at the beginning of each run, and all of the spectra were calibrated prior to the identification.

Typical calibration graphs were used to determine concentrations of peptides in plasma, as follows: Gly-Sar-Sar, y = 2.6736x + 0.0374 (0.1 - 10 nmol/mL, r = 0.9992); Gly-Sar-Sar-Sar, y = 3.1849x + 0.1803 (0.01 - 2.5 nmol/mL, r = 0.9979); Gly-Sar-Sar-Sar-Sar y = 1.6207x + 0.0601 (0.05 - 1.0 nmol/mL, r = 0.9968), where y is the peak area ratio (observed peak area of target against that of each corresponding IS) and x is the peptide concentration (nmol/mL).

2.4. Statistical analyses

Pharmacokinetic parameters such as C_{max} and t_{max}, were analyzed from 0 to 90 min after oral administration. The elimination rate constant (k) was determined by linear regression analysis of data points plotted between the respective C_{max} and 90 min. The elimination of half-life ($t_{1/2}$) was calculated by plotting the plasma level against logarithmic time. The area under the plasmatic concentration-time curve ($AUC_{0 - 90\ min}$) was calculated using the trapezoidal rule. Results are expressed as the mean ± SEM. All pharmacokinetic analyses were performed using the GraphPad Prism5 (GraphPad Software, La Jolla, CA, USA).

3. Results and Discussion

3.1. Absorption of a tripeptide model Gly-Sar-Sar in spontaneously hypertensive rats

After oral administration of Gly-Sar-Sar to 8-wk SHRs at a dose of 10 mg/kg, blood from tail vein was taken at fixed time-schedules (0, 10, 20, 30, 90 min). As shown in Figure 3-1, Gly-Sar-Sar was not detected in 0-min blood sample by TNBS derivatization-added LC-TOF-MS. This indicated that no endogenous tripeptides corresponding to model peptides occurred in rat blood stream. By using IS-guided TNBS-LC-TOF-MS assay, absorbed Gly-Sar-Sar was successfully detected, and the concentration of Gly-Sar-Sar drastically increased as early as 10 min and reached the maximum concentration (C_{max}) of 4.6 ± 0.6 nmol/mL-plasma within 30 min after its single oral administration. The absorption of Gly-Sar-Sar ($AUC_{0 - 90\ min}$, 88.2 ± 17.6

nmol·min/mL plasma) was higher than the reported tripeptides Gly-Pro-Hyp, Gly-Pro-Ala, and Gly-Ala-Hyp ($AUC_{0\text{ - }240\text{ min}}$, 164.81 ± 41.94, 0.21 ± 0.02, and 0.07 ± 0.01 nmol·min/mL plasma, respectively), in which they were administered at each dose of 446 mg/kg to Wistar rats [73]. In order to confirm the intestinal protease resistance of the tripeptide model during absorption, MS analyses of the blood samples were conducted at 30 min after administration. As shown in Figure 3-2, no MS detection of an estimated metabolite, Gly-Sar, was observed in the circulation of SHRs, while it was reported that the mother peptides Gly-Pro-Hyp, Gly-Pro-Ala, and Gly-Ala-Hyp were degraded into their dipeptides Pro-Hyp, Gly-Pro, Pro-Ala, and Ala-Hyp after the absorption [73]. Val-Pro-Pro was also hydrolyzed by intestinal peptidases in the apical cell membrane, producing free Val and Pro [74]. This indicates that a tripeptide Gly-Sar-Sar remained stable during the *in vivo* absorption into rat blood systerm, being consistent with the reported high stability in *in vitro* Caco-2 cell and rat small intestinal transport studies [60]. Hence, Gly-Sar-Sar can be used as a possitive control to evaluate *in vitro* and *in vivo* absorption behavior of tripeptides.

Table 3-1. Pharmacokinetics of oligopeptides after oral administration at a dose of 10 mg/kg in 8 week-old male SHRs

Peptide	t_{max} (min)	C_{max} (nmol/mL-plasma)	$AUC_{0-90\ min}$ (nmol·min/mL-plasma)	$t_{1/2}$ (min)
Gly-Sar-Sar	30	4.6 ± 0.6	267.4 ± 34.3	72
Gly-Sar-Sar-Sar	30	1.5 ± 0.3	88.2 ± 17.6	87
Gly-Sar-Sar-Sar-Sar	60	1.1 ± 0.1	71.7 ± 2.8	*n.a.*

$t_{max,}$ time to reach maximum concentration; C_{max}, maximum concentration; AUC, area under the curve; $t_{1/2}$, half-life. Results are expressed as mean ± SEM (n=4). n.a, not applicable

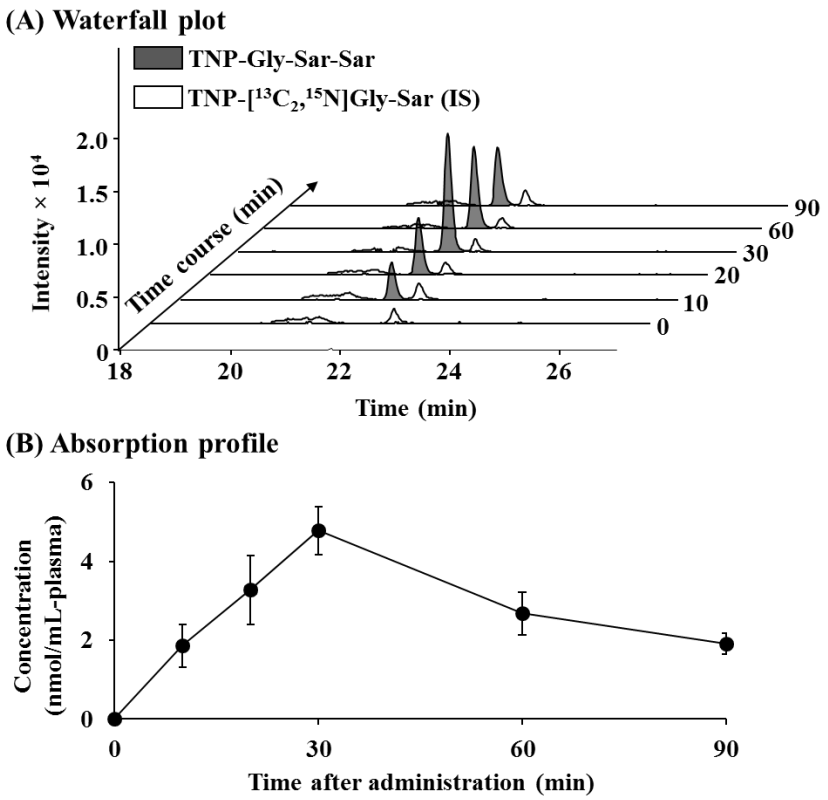


Figure 3-1. Stacked intensity-time chromatograms (waterfall plot) of Gly-Sar-Sar and IS ([$^{13}C_2$,^{15}N]-labeled Gly-Sar, 0.2 nmol/mL) arranged by sampling time of rat plasma (0 to 90 min) (A). Time course of concentration of Gly-Sar-Sar in plasma after single oral administration of 10 mg/kg in 8-wk SHRs (B). Blood (100 μL) was taken from the tail vein at fixed time intervals from 0 to 90 min. Plasma samples were subjected to TNBS derivatization. The extracted ions with *m/z* 361.0630 and *m/z* 429.1001 correspond to molecular ions of TNP-[$^{13}C_2$,^{15}N]-Gly-Sar (IS) and TNP-Gly-Sar-Sar, respectively. Data are expressed as the mean ± SEM (n = 4).

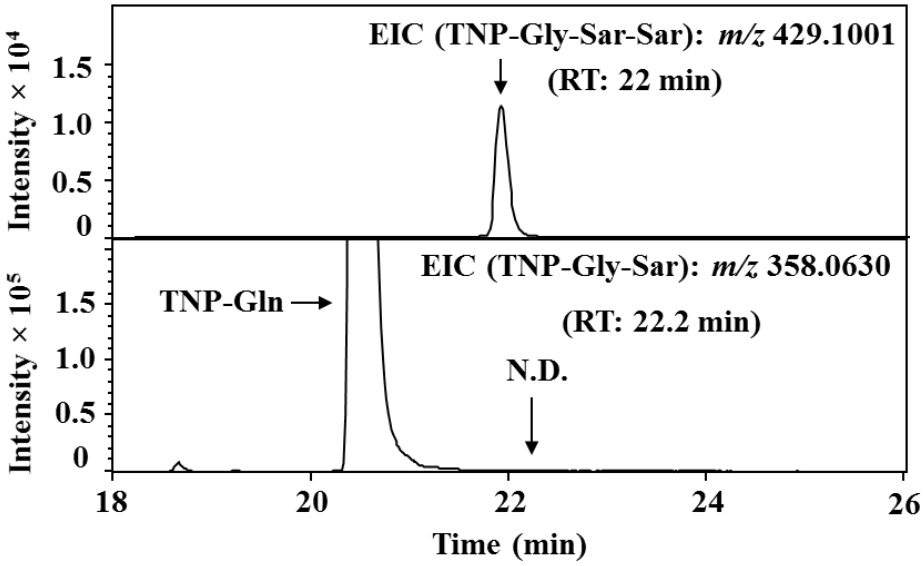


Figure 3-2. LC-TOF-MS detection of metabolites of Gly-Sar-Sar after single oral administration of 10 mg/kg to 8-wk SHRs. Plasma obtained from the tail vein at 30 min were assayed by LC-TOF-MS with TNBS derivatization technique. The extracted ions with *m/z* 358.0630 and *m/z* 429.1001 correspond to molecular ions of TNP-Gly-Sar and TNP-Gly-Sar-Sar, respectively. A huge peak at the monitoring of *m/z* 358.0630 at the retention time of 20.8 min was a TNP-Gln. N.D.: not detected.

3.2. Absorption of oligopeptide models Gly-Sar-Sar-Sar and Gly-Sar-Sar-Sar-Sar in spontaneously hypertensive rats

Gly-Sar-Sar-Sar and Gly-Sar-Sar-Sar-Sar, which can be transported through a paracellular or TJ transport route and have high resitance against protease digestion in *in vitro* Caco-2 cells [60], were orally administrated at a dose of 10 mg/kg to 8-wk SHRs. The blood samples were analyzed by the TNBS-LC-TOF-MS. As a result, intact absorption of both Gly-Sar-Sar-Sar and Gly-Sar-Sar-Sar-Sar was successfully detected as soon as 10 min after each oral administration to SHRs. As shown in Figures 3-3 and 3-5, no TNP-Gly-Sar-Sar-Sar and TNP-Gly-Sar-Sar-Sar-Sar were detected before oral administration, suggesting that no endogenous oligopeptides were present in rat blood. The C_{max} values were achieved at 30 and 60 min after administration of Gly-Sar-Sar-Sar (C_{max}, 1.5 ± 0.3 nmol/mL) (Table 3-1 and Figure 3-3) and Gly-Sar-Sar-Sar-Sar (C_{max}, 1.1 ± 0.1 nmol/mL) (Table 3-1 and Figure 3-5), respectively.

In order to clarify the production behavior of metabolites during *in vivo* absorption of either Gly-Sar-Sar-Sar or Gly-Sar-Sar-Sar-Sar, MS analyses of the blood samples obtained from the SHRs were conducted without spiking the internal standard Gly-Sar-Sar. Blood samples were used for this study at 30 min after the administration. As shown in Figures 3-4 and 3-6, no MS detections were observed for expected peptide fragments such as Gly-Sar and Gly-Sar-Sar from Gly-Sar-Sar-Sar; Gly-Sar, Gly-Sar-Sar, and Gly-Sar-Sar-Sar

from Gly-Sar-Sar-Sar-Sar, suggesting that both tetrapeptide Gly-Sar-Sar-Sar and pentapeptide Gly-Sar-Sar-Sar-Sar were resistant to digestive proteases, in agreement with the findings of *in vitro* and *ex vivo* transport studies using Caco-2 cell and rat intestinal membranes [60]. To the best of our knowledge, the present study was the first report on intact *in vivo* absorption of oligopeptides Gly-Sar-Sar-Sar and Gly-Sar-Sar-Sar-Sar without degradation. Sanchez-Rivera *et al.* [75] reported that a pentapeptide (His-Leu-Pro-Leu-Pro) was rapidly absorbed at a plasma level of > 10 pmol/mL-plasma, and then eliminated with $t_{1/2}$ of 3 min after a single oral administration to 9-wk Wistar rats (40 mg/kg), and hydrolyzed into smaller fragments Leu-Pro-Leu-Pro and His-Leu-Pro-Leu. Considering the unexpected rapid absorption (at > 3 min) to blood circulation by oral administration of the pentapeptide and its low stability against protease degradation [75], further studies must be needed for *in vivo* absorption of oligopeptides. The present study also demonstrated that *in vivo* absorption of oligopeptides might decrease with peptide length: Gly-Sar-Sar (267.4 ± 34.3 nmol·min/mL-plasma) > Gly-Sar-Sar-Sar (88.2 ± 17.6 nmol·min/mL-plasma) > Gly-Sar-Sar-Sar-Sar (71.7 ± 2.8 nmol·min/mL-plasma) (Table 3-1).

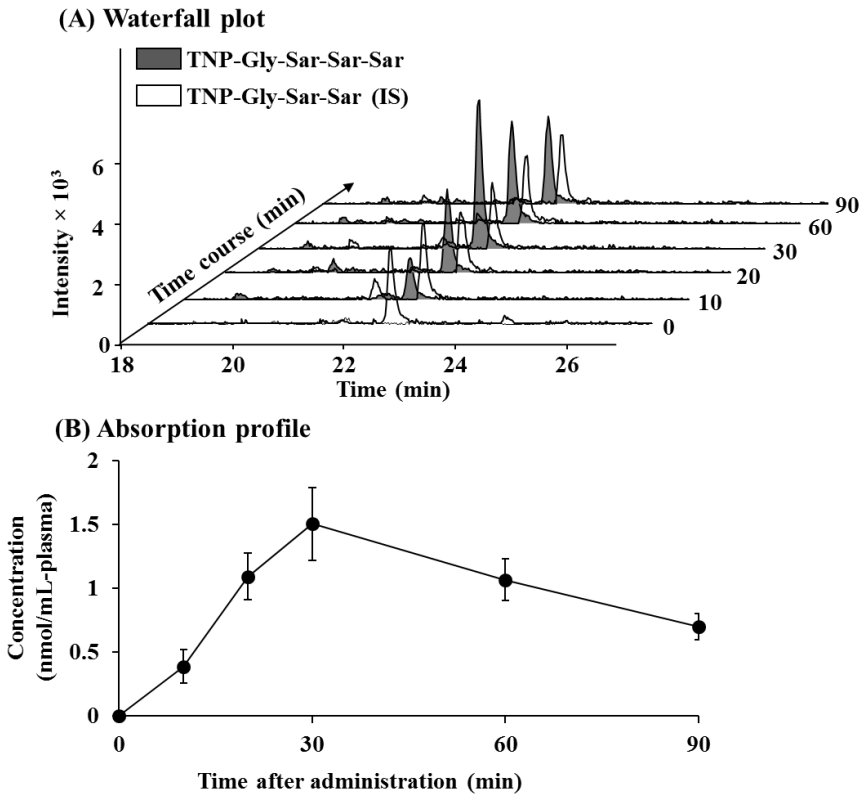


Figure 3-3. Stacked intensity-time chromatograms (waterfall plot) of Gly-Sar-Sar-Sar and IS (Gly-Sar-Sar, 0.1 nmol/mL) arranged by sampling time of rat plasma (0 to 90 min) (A). Time course of concentration of Gly-Sar-Sar-Sar in plasma after single oral administration of 10 mg/kg in 8-wk SHRs (B). Blood (100 μL) was taken from the tail vein at fixed time intervals from 0 to 90 min. Plasma samples were subjected to TNBS derivatization. The extracted ions with *m/z* 429.1001 and *m/z* 500.1372 correspond to molecular ions of TNP-Gly-Sar-Sar (IS) and TNP-Gly-Sar-Sar-Sar, respectively. Data are expressed as the mean ± SEM (n = 4).

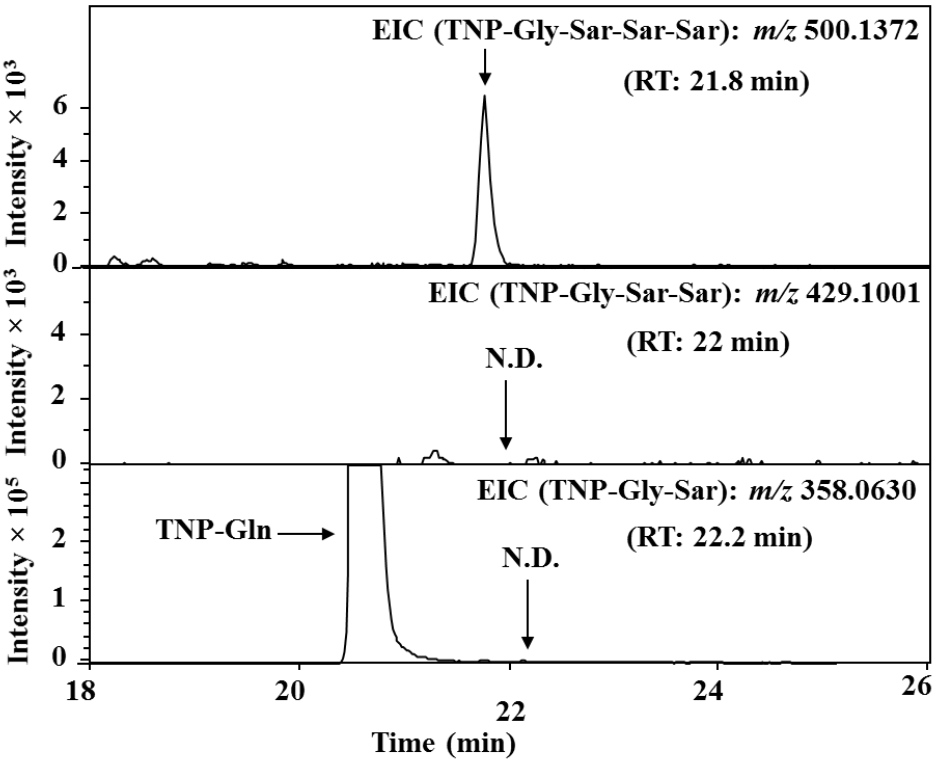


Figure 3-4. LC-TOF-MS detection of metabolites of Gly-Sar-Sar-Sar after single oral administration of 10 mg/kg to 8-wk SHRs. Plasma obtained from the tail vein at 30 min were assayed by LC-TOF-MS with TNBS derivatization technique. The extracted ions with *m/z* 358.0630, *m/z* 429.1001, and *m/z* 500.1372 correspond to molecular ions of TNP-Gly-Sar, TNP-Gly-Sar-Sar, and TNP-Gly-Sar-Sar-Sar, respectively. A huge peak at the monitoring of *m/z* 358.0630 at the retention time of 20.8 min was a TNP-Gln. N.D.: not detected.

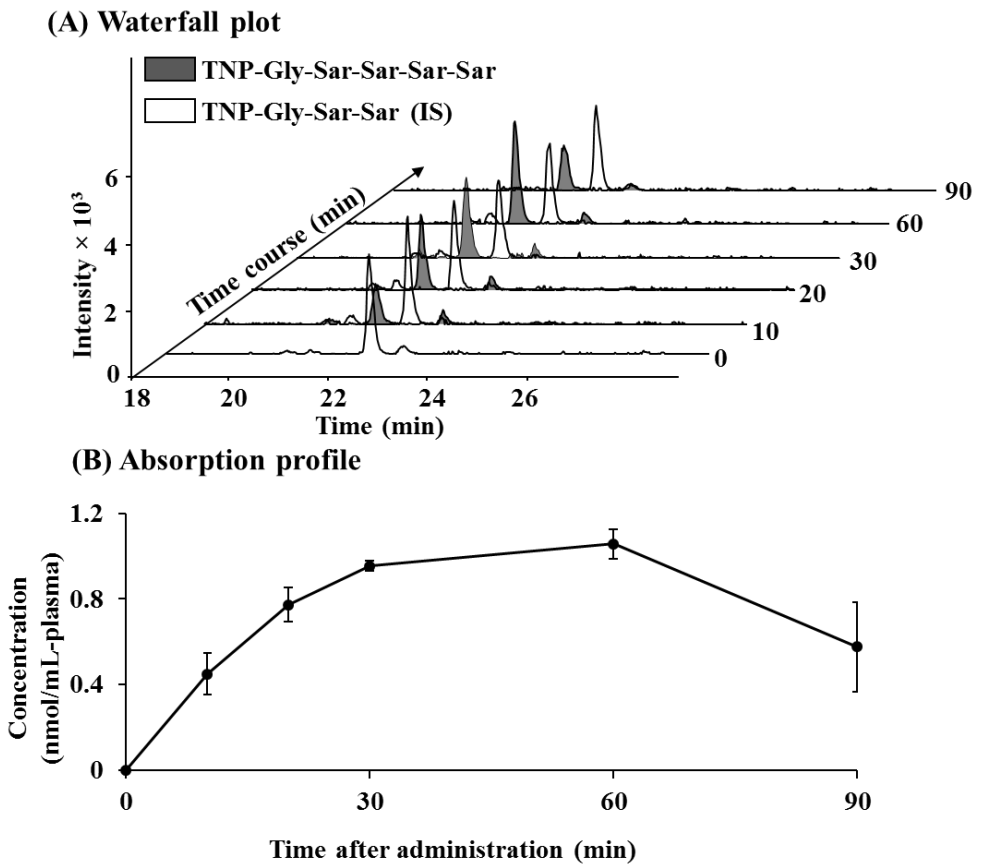


Figure 3-5. Stacked intensity-time chromatograms (waterfall plot) of Gly-Sar-Sar-Sar-Sar and IS (Gly-Sar-Sar, 0.1 nmol/mL) arranged by sampling time of rat plasma (0 to 90 min) (A). Time course of concentration of Gly-Sar-Sar-Sar-Sar in plasma after single oral administration of 10 mg/kg in 8-wk SHRs (B). Blood (100 μL) was taken from the tail vein at fixed time intervals from 0 to 90 min. Plasma sample was subjected to TNBS derivatization. The extracted ions with *m/z* 429.1001 and *m/z* 571.1743 correspond to molecular ions of TNP-Gly-Sar-Sar (IS) and TNP-Gly-Sar-Sar-Sar-Sar, respectively. Data are expressed as the mean ± SEM (n = 4).

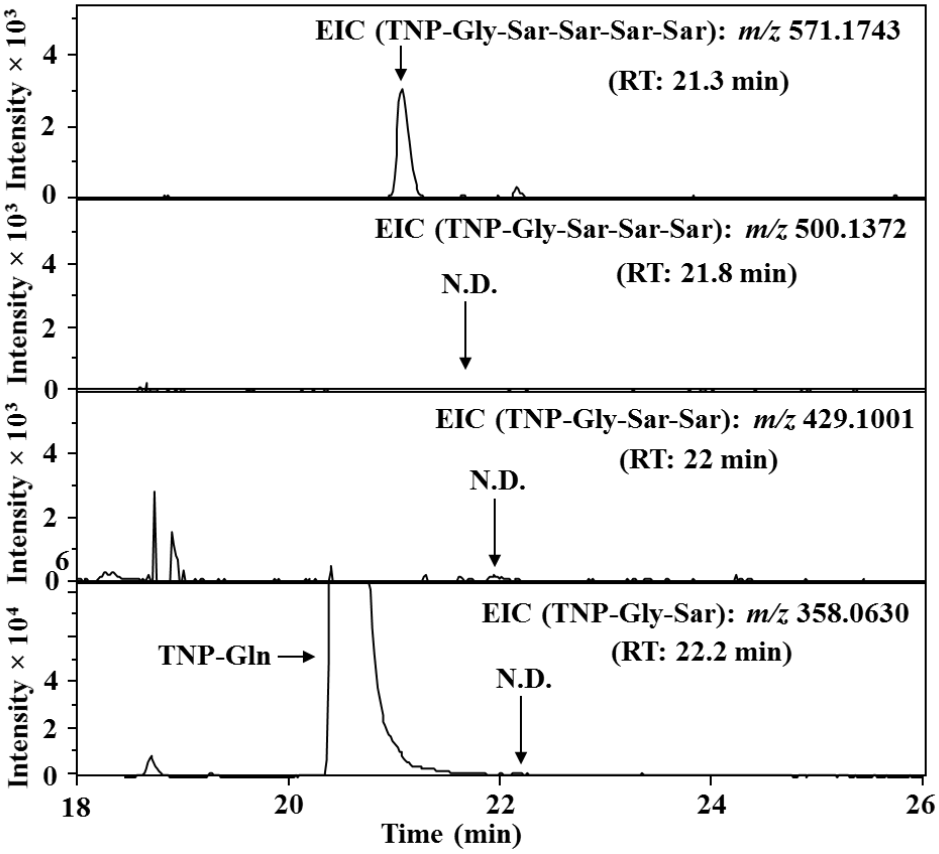


Figure 3-6. LC-TOF-MS detection of metabolites of Gly-Sar-Sar-Sar-Sar after single oral administration of 10 mg/kg to 8-wk SHRs. Plasma obtained from the tail vein at 30 min were assayed by LC-TOF-MS with TNBS derivatization technique. The extracted ions with *m/z* 358.0630, *m/z* 429.1001, *m/z* 500.1372, and *m/z* 571.1743 correspond to molecular ions of TNP-Gly-Sar, TNP-Gly-Sar-Sar, TNP-Gly-Sar-Sar-Sar, and TNP-Gly-Sar-Sar-Sar-Sar, respectively. A huge peak at the monitoring of *m/z* 358.0630 at the retention time of 20.8 min was a TNP-Gln. N.D.: not detected.

4. Summary

Bioactive peptides must be absorbed intact without protease hydrolysis in order to reach their target organs intact and exert their biological effects. Di-/tripeptides can be absorbed into the blood stream; however, it is not clear whether larger bioactive peptides (≥ 4 residues in length) are absorbed intact from the intestine and reached to target organs. In this Chapter III, we have demonstrated for the first time that tripeptide Gly-Sar-Sar, tetrapeptide Gly-Sar-Sar-Sar and pentapeptide Gly-Sar-Sar-Sar-Sar could be absorbed *in vivo,* and in a peptide length-dependent manner in the descending order of Gly-Sar-Sar > Gly-Sar-Sar-Sar > Gly-Sar-Sar-Sar-Sar. Furthermore, no metabolites of Gly-Sar-Sar, Gly-Sar-Sar-Sar, and Gly-Sar-Sar-Sar-Sar were observed in the blood samples, indicating that these peptides remained stable during the *in vivo* absorption into blood at > 0.4 nmol/mL-plasma levels. Therefore, these oligopeptides can be used as positive standard to evaluate *in vitro* and *in vivo* absorption of bioactive oligopeptides.

Chapter IV

Effect of aging on intestinal absorption of peptides in spontaneously hypertensive rats

1. Introduction

Aging may affect the absorption of various compounds in different manners caused by several factors such as pathophysiological state, genetic differences, life style choices, and dietary background [76–78]. So far reported, Yamamoto *et al.* [79] demonstrated that lipid absorption was decreased due to reduced pancreatic lipase activity in aged mice. Several researchers found out that a changed circulatory concentration of actively transported substances such as glucose and calcium was associated with aging [80,81].

Contrary to the aforementioned age-related reports, *in vivo* absorption behavior of peptides regarding aging still remains unclear. There have been clinical evidence that peptides, in particlar, small di-/tripeptides have a potency to improve lifestyle-related diseases such as hypertension [5,7]. In the small intestine, PepT1 has been demonstrated to be responsible for the predominant intestinal uptake of not only di-/tripeptides, but also peptidomimetics and peptide-like drugs [44,48]. Thus far, Metelsky has already reported that absorbable dipeptide Gly-Ala tended to increase with aging of male Wistar rats

due to enhanced PepT1 expression in small intestine [76]. Other reports also claimed that the promoting PepT1 expression in intestine could be induced by proinflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interferon-γ (INF-γ) [82], as well as when the rats were under fasting conditions [83]. Although the mechanism was not fully elucidated, the above observations indicated that PepT1 expression may change by any intestinal environment, which in turn there might be a positive link between intestinal PepT1 transporter expression and absorption of di-/tripeptides in terms of aging.

In Chapter III, it was observed that tripeptide Gly-Sar-Sar, tetrapeptide Gly-Sar-Sar-Sar, and pentapeptide Gly-Sar-Sar-Sar-Sar can be absorbed *in vivo,* and remained stable during *in vivo* absorption into blood. Therefore, Chapter IV comprises to investigate the effect of aging on the absorption of di- to pentapeptides in young (8-wk) and old (40-wk) SHRs. Target peptides used in this study are Gly-Sar as a PepT1 substrate [44], Trp-His as an absorbable anti-atherosclerotic dipeptide [3,37], captopril as a typical dipeptidomimetic antihypertensive drug [84], and absorbable Gly-Sar-Sar as a designed model tripeptide (Chapter III). Based on the findings obtained in Chapter III, the effect of aging on oligopeptide absorption was also performed in young and aged SHRs using model tetrapeptide (Gly-Sar-Sar-Sar) and pentapeptide (Gly-Sar-Sar-Sar-Sar) with high protease resistance.

2. Materials and Methods

2.1. Materials

Gly-Sar, captopril, aprotinin, and chymostatin were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). [$^{13}C_2$,^{15}N]-labeled Gly-Sar was obtained from Scrum (Tokyo, Japan). Gly-Sar-Sar was obtained from Bachem (Bubendorf, Switzerland). Gly-Sar-Sar-Sar and Gly-Sar-Sar-Sar-Sar were purchased from Biomatik Co. (Cambridge, ON, Canada). Trp-His was purchased from Medical & Biological Laboratories Co. (Nagoya, Japan) and His-Trp was purchased from Kokusan Chemical Co. (Osaka, Japan). 2,4,5-Trinitrobenzensulfonate (TNBS) was purchased from Nacalai Tesque (Kyoto, Japan). *p*-Bromophenacyl bromide (*p*-BPB) was obtained from Tokyo Chemical Ind. (Tokyo, Japan). Distilled water, methanol (MeOH), acetonitrile (ACN) and formic acid (FA) were of LC-MS grade (Merck, Darmstadt, Germany). All other reagents were of analytical reagent grade and were used without further purification.

2.2. Animal experiments

Seven- and 39-week-old (wk) male SHRs (SHR/Izm, Japan SLC, Shizuoka Japan) were fed on a laboratory diet (MF, Oriental Yeast Co., Tokyo, Japan) and given water *ad libitum*. All rats were housed for 1 week at 21 ± 1 °C and 55 ± 5% humidity under controlled lighting from 8:00 to 20:00 (systolic

blood pressure for 8-wk SHR: 186 mmHg; 40-wk SHR: 235 mmHg). Each rat was unfed for 16 h before a single oral administration of each peptide. Each peptide was dissolved in milli-Q water, and then was orally administered at a dose of 10 mg/kg to rats. At a fixed time-schedule (0, 10, 20, 30, 60, and 90 min), about 100 μL of blood was collected from the tail vein into a tube containing EDTA-2Na and inhibitors (aprotinin, 0.1 mg; chymostatin, 0.1 mg), then immediately centrifuged at 3,500 × *g* at 4 °C for 15 min to obtain plasma samples. All rat experiments in this study were handled in accordance with the Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science, and Technology in Japan. The Ethics Committee on Animal Experiment at Kyushu University approved all experimental protocols (Permit Number: A28-040).

2.3. Determination of absorbed peptides in plasma

Concentrations of Gly-Sar, Trp-His, Gly-Sar-Sar, Gly-Sar-Sar-Sar, and Gly-Sar-Sar-Sar-Sar in plasma after administration were determined by a TNBS derivatization-aided LC-MS method [36]. Namely, an aliquot (50 μL) of plasma solution was added into 150 μL of a solution containing 0.1% NaCl, and 0.1% FA. An internal standard (IS; 10 μL) used for each assay was as follows: (0.2 nmol/mL [$^{13}C_2$,^{15}N]-labeled Gly-Sar for Gly-Sar and Gly-Sar-Sar assays; 0.1 nmol/mL Gly-Sar-Sar for Gly-Sar-Sar-Sar and Gly-Sar-Sar-Sar-Sar assays; 0.02 nmol/mL His-Trp for Trp-His assay). The resultant solution was

applied to an Amicon Ultra 0.5-mL-3K centrifugal filter (Millipore, Carrigtwohill, Ireland), followed by the centrifugation at 14,000 × *g* at 4 °C for 30 min. The obtained filtrate was evaporated to dryness, followed by the addition of 50 µL of 150 mM TNBS solution (pH 10) for TNBS derivatization reaction for 30 min at 30 °C. The reaction was stopped by the addition of 50 µL of 0.2% FA solution, and an aliquot (20 µL) of the resultant trinitrophenyl (TNP)-derivative was injected into LC-MS system. The concentration of captopril was determined by a *p*-BPB derivatization method [85] with slight modifications. Briefly, an aliquot (20 µL) of 3.6 µM *p*-BPB in MeOH was added to 50 µL of the above-mentioned plasma solution, and incubated for 30 min at room temperature. The solution was then mixed with 80 µL of MeOH, followed by a centrifugation at 14,000 × *g* for 10 min at 4 °C. The resultant supernatant was applied onto a 0.45-µm Cosmonice filter (Nacalai Tesque, Kyoto, Japan), and an aliquot (20 µL) of the filtrate was injected into LC-MS system.

For the determinations of TNP-Gly-Sar, *p*-BPB-captopril, TNP-Gly-Sar-Sar, TNP-Gly-Sar-Sar-Sar, and TNP-Gly-Sar-Sar-Sar-Sar, an LC-time of flight (TOF)-MS was conducted. LC separation was performed using an Agilent 1200 series (Agilent, Waldbronn, Germany) on a Cosmosil 5C_{18}-AR-II column (2.0 × 150 mm, 3 µm, Nacalai Tesque) at 40 °C with a linear gradient elution of ACN (0 - 100% over 20 min) containing 0.1% FA at a flow rate of 0.2 mL/min. An electrospray ionization (ESI)-TOF-MS analysis was carried out using a micrOTOF II (Bruker Daltonics, Bremen, Germany) with monitoring ions of

m/z 358.0630, *m/z* 361.0630, *m/z* 429.1001, *m/z* 500.1372, *m/z* 571.1743, and *m/z* 416.0350 in ESI-positive mode for TNP-Gly-Sar, TNP-[$^{13}C_2$,^{15}N]-Gly-Sar, TNP-Gly-Sar-Sar, TNP-Gly-Sar-Sar-Sar, TNP-Gly-Sar-Sar-Sar-Sar, and *p*-BPB-captopril, respectively. ESI conditions were as follows: drying gas (N_2) flow rate, 8.0 L/min, drying gas temperature, 200 °C; nebulizing gas pressure, 1.6 bar; capillary voltage, 3,800 V; mass range, *m/z* 100 - 1,000. All of the acquisitions and analyses of data were controlled by a Bruker Data Analysis 3.2 Software. The calibration solution containing 10 mM sodium formate in 50% ACN was injected at the beginning of each run, and all of the spectra were calibrated prior to the identification.

For the determination of Trp-His (His-Trp as IS), an LC-multiple reaction monitoring (MRM)-MS/MS (Esquire 6000, Bruker Daltonics) by their characteristic TNBS-derivatized fragment ions was conducted, since an adequate separation of both peptides under the aforementioned LC-TOF-MS conditions was not achieved. LC separation was performed using an Agilent 1200 series on a Waters Atlantis T3 column (2.1 × 100 mm, 3 μm, Milford, MA, USA) with a linear elution gradient of 40 - 100% MeOH containing 0.1% FA over 20 min at 0.2 mL/min at 40 °C. MRM-MS/MS conditions *via* a time segment definition of 21 - 27 min in ESI-positive mode were as follows: nebulizer gas (N_2), 40 psi; dry gas (N_2), 8 L/min; dry temperature, 330 °C; HV capillary, 2,750 V; HV end plate offset, -500 V; resolution, *m/z* 0.25; target ion trap, 50,000; Oct1, 9.1 V; Oct2, 2.5 V; trap drive, 55.4 V; skimmer, 40.0 V.

Monoisotopic isolations (*m/z*) at the width of *m/z* 1.5 were 553.1>325.1 for TNP-Trp-His and 553.1>188.0 for TNP-His-Trp (IS).

For the determination of concentrations of peptides in plasma, each typical calibration graph for Gly-Sar, y = 3.031x + 0.3605 (0.1 - 10 nmol/mL, *r*=0.9986); Trp-His, y = 98.288x - 0.0528 (0.001 - 0.05 nmol/mL, *r* = 0.9917) ; captopril, y = 660052x - 19894 (0.01 - 2 nmol/mL, *r* = 0.9932); Gly-Sar-Sar, y = 2.6736x + 0.0374 (0.1 - 10 nmol/mL, *r* = 0.9992); Gly-Sar-Sar-Sar, y = 3.1849x + 0.1803 (0.01 - 2.5 nmol/mL, *r* = 0.9979); and Gly-Sar-Sar-Sar-Sar, y = 1.6207x + 0.0601 (0.05 - 1.0 nmol/mL, *r* = 0.9968), was used; where y is the peak area ratio (observed peak area of target against that of each corresponding IS) and x is the peptide concentration (nmol/mL).

2.4. Western blotting analyses

The expression of PepT1 and claudin-1 proteins in small intestine was evaluated by Western blotting analysis. Small intestines of both 8-wk and 40-wk SHRs were excised and divided equally into twelve segments. In this study, we selected the fourth (up-jejunum), eighth (mid-jejunum), and twelfth (ileum) segments below the duodenum. Each segment was washed with phosphate-buffered saline (PBS) buffer (137 mM NaCl, 2.7 KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 2 mM EDTA-2Na. After fat tissues were removed, each intestinal segment was homogenized in radio-immunoprecipitation assay (RIPA) buffer (150 mM NaCl, 0.5% sodium deoxycholate, 0.1% sodium sulfate, 1.0% Nonidet P-40,

and 50 mM Tris-HCl, pH 8.0) containing 1 mM PMSF and 2 mM EDTA-2Na on ice. The homogenate was sonicated 10 times for 10 s on ice, and then centrifuged at 14,000 × *g* for 15 min at 4 °C. The obtained supernatant was then mixed with an equal volume of sample buffer (20% glycerol, 4% SDS, 3% dithiothreitol, 0.002% bromophenol blue and 0.125 M Tris-HCl, pH 6.8), and boiled at 95 °C for 5 min. Protein concentration was determined using a Bio-Rad DC protein assay kit with bovine serum albumin as standard (Nacalai Tesque). An aliquot (60 μg-protein/lane) of the prepared sample was applied to 10% SDS-PAGE gel for 1 h at 40 mA, and transferred onto PVDF membrane (Hybond-P, GE Healthcare, Piscataway, NJ, USA) for 1.5 h at 80 mA. The membrane was blocked with 5% (w/v) enhanced chemiluminescence (ECL) blocking agent (GE Healthcare, Little Chalfont, UK) in TBS-Tween 20 (TBS-T) (20 mM Tris-HCl, 137 mM NaCl, and 0.05% Tween-20, pH 7.6) for 1.5 h at room temperature, and then probed with the primary antibodies to PepT1 (1:400 dilution), claudin-1 (1:350 dilution), and β-actin (1:6000 dilution) at 4 °C overnight. The membrane was washed with the TBS-T solution 3 times for each 10 min-interval, and then incubated with secondary antibody (anti-rabbit IgG antibody for PepT1 and for claudin-1; anti-mouse IgG antibody for β-actin, 1:1000 dilution) for 1 h at room temperature. The membrane was washed again with the TBS-T solution 3 times for each 10 min-interval, and analyzed by an ECL plus detection reagent using Quant LAS 4000 (GE Healthcare, Little Chalfont, UK). Quantification of the amount of target protein was determined by an Image Quant TL 7.0 software (GE Healthcare, Little

Chalfont, UK). The level of protein (PepT1 and claudin-1) was calculated by the ratio of the levels of each target protein to β-actin. The ratio of each target protein level in 8-wk SHR was denoted as 1.0.

2.5. Statistical analyses

Pharmacokinetic parameters such as C_{max} and t_{max} were analyzed from 0 to 90 min after oral administration. The elimination rate constant (k) was determined by linear regression analysis of data points plotted between the respective C_{max} and 90 min. The elimination of half-life ($t_{1/2}$) was calculated by plotting the plasma level against logarithmic time. The area under the plasmatic concentration-time curve ($AUC_{0\ -\ 90\ min}$) was calculated using the trapezoidal rule. Results are expressed as the mean ± (SEM). Statistical evaluation between two groups (8-wk and 40-wk SHR groups) was performed by unpaired Student's *t*-test. A P value of < 0.05 was considered as significant. All pharmacokinetic and statistical analyses were performed using the GraphPad Prism5 (GraphPad Software, La Jolla, CA, USA).

3. Results and Discussion

3.1. Effect of aging on absorption of di-/tripeptides in spontaneously hypertensive rats

Gly-Sar that has an *N*-methylated peptide bond with high protease resistance and plays a role as PepT1 substrate was primarily investigated to clarify the effect of aging on peptide absorption in SHRs. After oral administration of Gly-Sar to 8-wk and 40-wk SHRs at a dose of 10 mg/kg, blood from the tail vein was collected at fixed time-schedules (0, 10, 20, 30, 60, and 90 min). As shown in Figure 4-1A, no interfering contaminants corresponding to *m/z* 358.0630 of TNP-Gly-Sar were detected in 0-min blood by TNBS derivatization-aided LC-TOF-MS (0.2 nmol/mL [$^{13}C_2$,^{15}N]-labeled Gly-Sar as IS). After the administration, absorbed Gly-Sar was successfully detected by the assay, showing t_{max} of 10 min in both SHR groups (Figures 4-1A and B). As analyzed over 90 min after administration, the amount of absorption of Gly-Sar in 40-wk SHRs was significantly ($P < 0.05$) higher than that in 8-wk SHRs; 1.5-fold higher $AUC_{0\text{ - }90\text{ min}}$ was observed in 40-wk SHRs (Table 4-1). The effect of aging on the pharmacokinetics of anti-atherosclerotic dipeptide, Trp-His, which was absorbed intact into the blood stream of Sprague-Dawley (SD) rats *via* the intestinal PepT1 route [37], was then investigated in the present work. As shown in Figures 4-2A, B, and Table 4-1, significantly enhanced C_{max} and $AUC_{0\text{ - }90\text{ min}}$ with 2.1- and 1.8-fold changes, respectively, were observed in 40-wk SHRs, compared to 8-wk SHRs.

Enhanced absorption with aging was also observed for captopril that is a dipeptidomimetic antihypertensive drug and a good substrate for PepT1 transporter [44,86] (Figures 4-3A, B); C_{max} and $AUC_{0 - 90\ min}$ of captopril in 40-wk SHRs were 7.4- and 6.7-fold higher ($P < 0.01$), respectively, than those in 8-wk SHRs (Table 4-1).

As shown in Figures 4-4A, B, and Table 4-1, the enhanced absorption of a tripeptide, Gly-Sar-Sar, in 40-wk SHRs was obtained, as similar to the aforementioned dipeptides, indicating that aging of SHRs may greatly affect or promote the absorption of di-/tripeptides into rat blood stream. The results were consistent with the report by Metelsky [76], who revealed that the absorption of Gly-Ala through rat intestinal sections tended to increase with aging of male Wistar rats. Some *in vitro* studies also demonstrated a similar tendency in the absorption of dipeptides in guinea pig intestine segments [87] and rabbit intestinal mucosa [88]. It has been clarified that intestinal PepT1 transporter plays a major role in the transport of small peptides (di-/tripeptides) or peptidomimetics [44,48]. It was found that the intestinal PepT1 expression was changed from embryonic to adult age in SD rats [50]. Therefore, the enhanced di-/tripeptide transport in aged animals (Figures 4-1, 4-2, 4-3, 4-4, and Table 4-1) may be related to the expression of PepT1 transporter at the brush border membrane. However, there have been reports on the relationship between enhanced *in vivo* absorption and PepT1 expression in aged SHRs. Therefore, further interest on the mechanism of the promoting absorption of di-/tripeptides

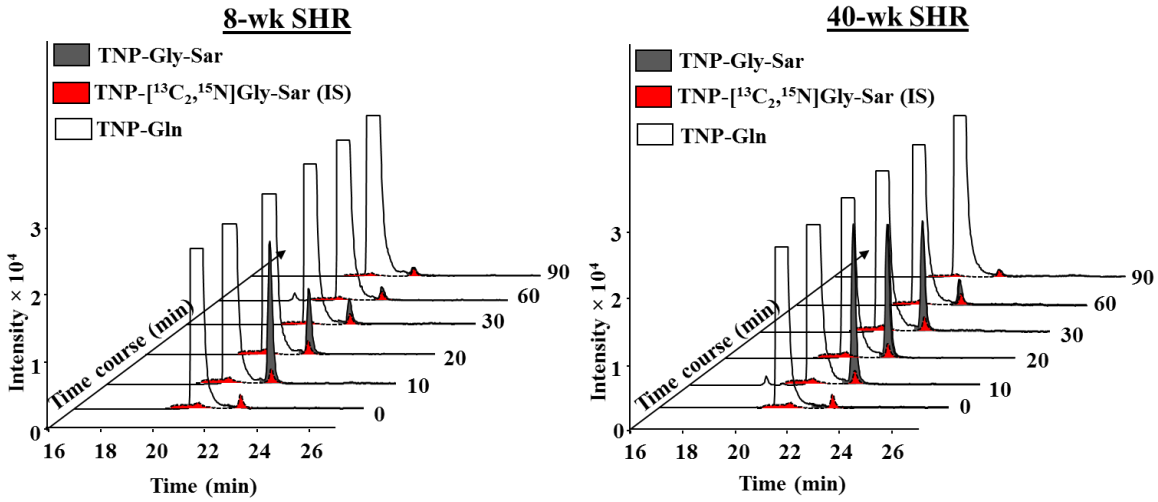
with aging was focused on the relationship between expression of PepT1 and aging of SHRs.

Table 4-1. Pharmacokinetics after a single oral administration of peptides at each dose of 10 mg/kg in 8-wk and 40-week-old male SHRs

Peptide	C_{max} (nmol/mL-plasma)		t_{max} (min)		$AUC_{0\text{-}90\ min}$ (nmol · min/mL-plasma)		$t_{1/2}$ (min)	
	8-wk	40-wk	8-wk	40-wk	8-wk	40-wk	8-wk	40-wk
Gly-Sar	12.2 ± 1.8	14.8 ± 1.1	10	10	341.9 ± 41.1	505.8 ± 51.3*	27	33
Trp-His	0.019 ± 0.001	0.042 ± 0.004**	10	20	0.76 ± 0.09	1.3 ± 0.1*	37	37
Captopril	1.3 ± 0.3	9.6 ± 3.2*	60	60	64.0 ± 17.6	430.6 ± 95.4**	*n.a.*	*n.a.*
Gly-Sar-Sar	4.6 ± 0.6	10.7 ± 1.5**	30	30	258.0 ± 32.1	621.0 ± 86.3**	75	83
Gly-Sar-Sar-Sar	1.5 ± 0.3	1.7 ± 0.7	30	20	82.6 ± 16.4	113.0 ± 37.3	87	139
Gly-Sar-Sar-Sar-Sar	1.1 ± 0.3	1.5 ± 0.2	60	60	71.7 ± 2.8	99.0 ± 14.8	*n.a.*	*n.a.*

C_{max} = maximum peak plasma concentration; t_{max} = time to reach peak plasma concentration; $t_{1/2}$ = elimination half-life; $AUC_{0\text{-}90\ min}$ = area under the plasma concentration time curve. *P < 0.05, **P < 0.01, significantly different between 8-wk and 40-week-old male SHRs by unpaired Student's t-test. Data are expressed as the mean ± SEM (n = 4 - 6). *n.a.*: not applicable.

A) Waterfall plot



B) Absorption profile

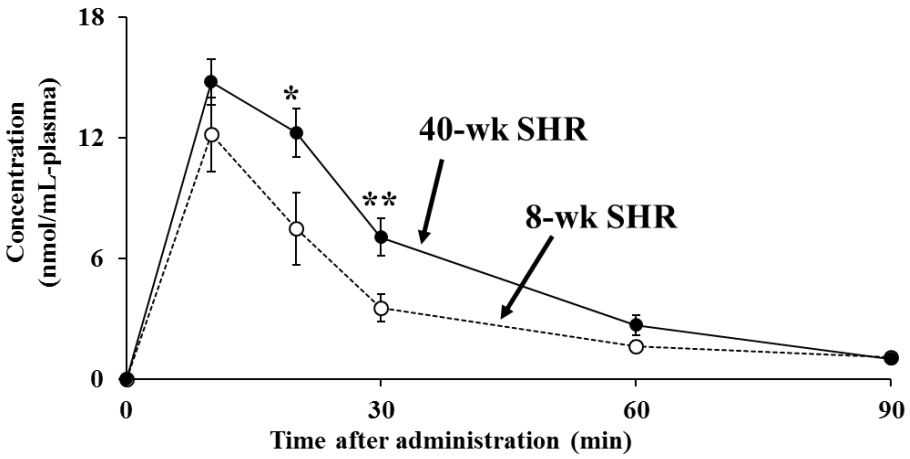
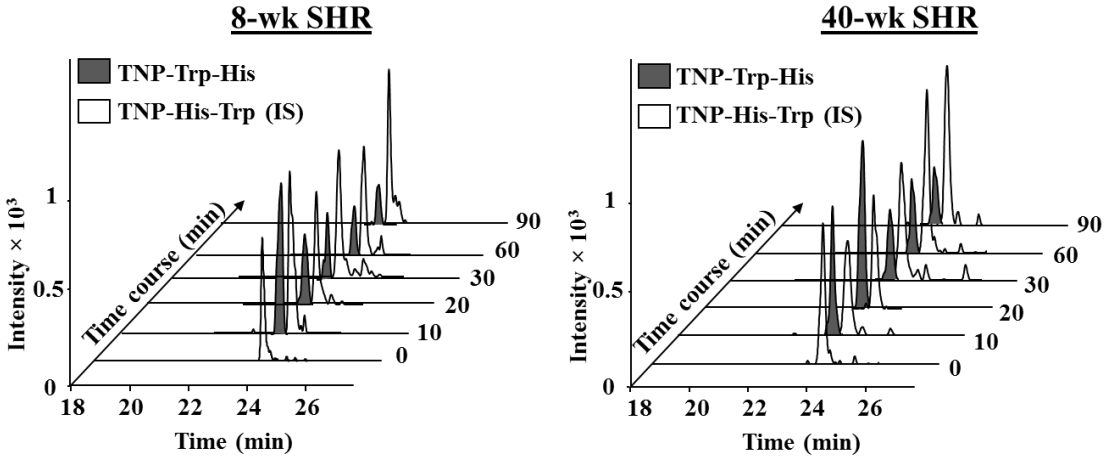


Figure 4-1. Stacked intensity-time chromatograms (waterfall plot) of Gly-Sar and IS ([$^{13}C_2$,^{15}N]-labeled Gly-Sar, 0.2 nmol/mL) arranged by sampling time of rat plasma (0 to 90 min) (A). Time-course of concentration of Gly-Sar in plasma after single oral administration at 10 mg/kg to 8-wk (n = 4) and 40-wk SHRs (n = 4) (B). Blood (100 µL) was taken from the tail vein at a fixed time of 0 - 90 min. Plasma sample was subjected to a TNBS derivatization-LC-TOF-MS analysis. Extracted ions with *m/z* 358.0630 and *m/z* 361.0630 correspond to molecular ions of TNP-Gly-Sar and TNP-[$^{13}C_2$,^{15}N]-Gly-Sar (IS), respectively. Data are expressed as the mean ± SEM (n = 4). *P < 0.05, **P < 0.01, significantly different between 8-wk and 40-wk SHRs by unpaired Student's *t*-test.

A) Waterfall plot



B) Absorption profile

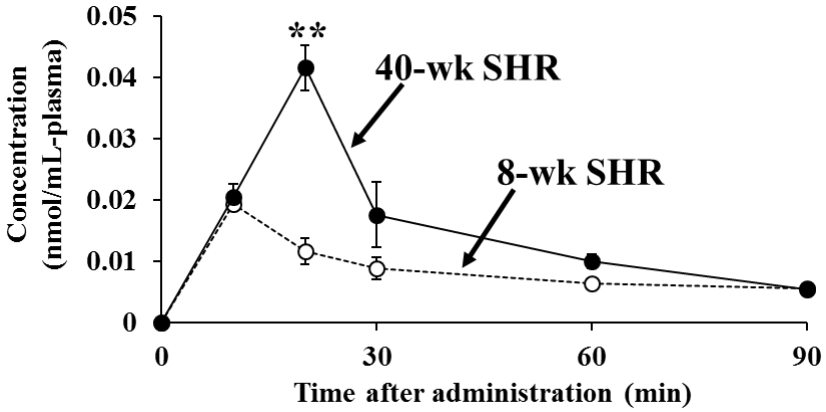
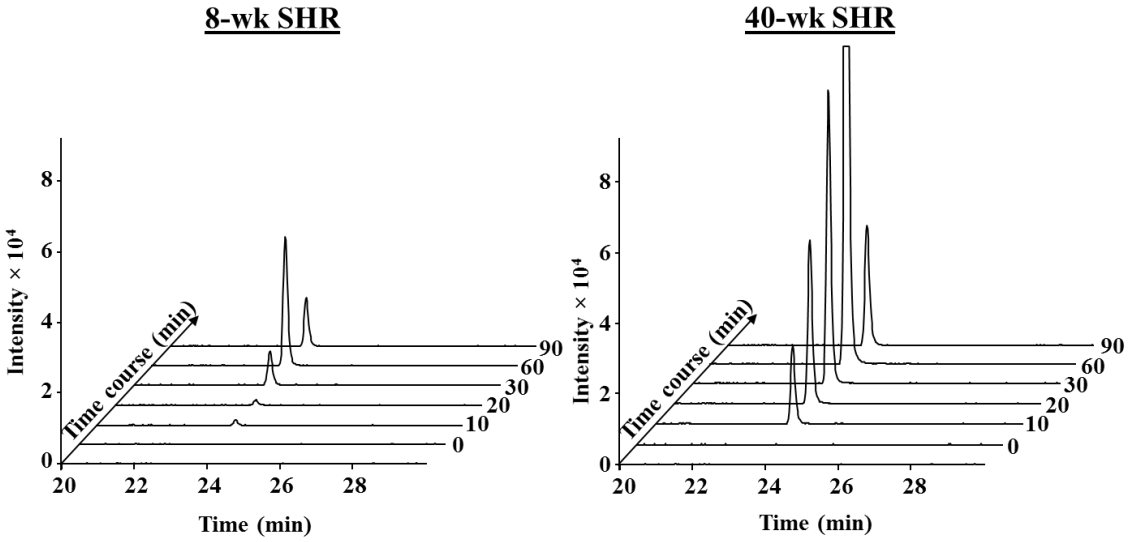


Figure 4-2. Stacked intensity-time chromatograms (waterfall plot) of Trp-His and IS (His-Trp, 0.02 nmol/mL) arranged by sampling time of rat plasma (0 to 90 min) (A). Time-course of concentration of Trp-His in plasma after single oral administration of 10 mg/kg to 8-wk (n = 4) and 40-wk SHRs (n = 4) (B). Blood (100 μL) was taken from the tail vein at fixed time intervals from 0 to 90 min. Plasma samples were subjected to TNBS derivatization-LC-MRM-MS/MS analysis *via* a time segment definition of 21 - 27 min for Trp-His and His-Trp (IS). Monoisotopic isolations (*m/z*) at the width of *m/z* 1.5 were 553.1>325.1 for TNP-Trp-His and 553.1>188.0 for TNP-His-Trp (IS). Data are expressed as the mean ± SEM (n = 4). ***P* < 0.01, significantly different between 8-wk and 40-wk SHRs by unpaired Student's *t*-test.

A) Waterfall plot



B) Absorption profile

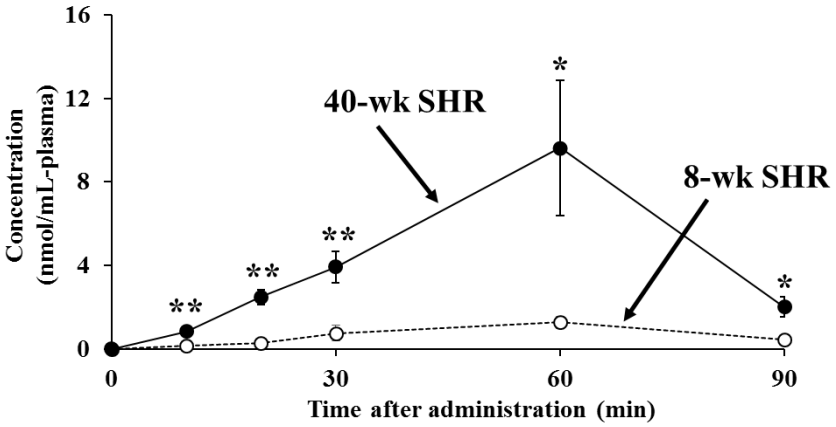
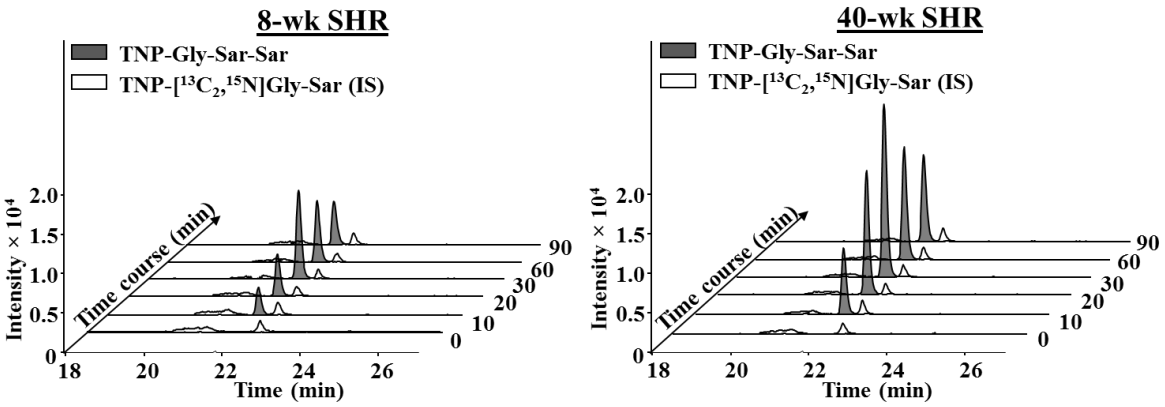


Figure 4-3. Stacked intensity-time chromatograms (waterfall plot) of captopril arranged by sampling time of rat plasma (0 to 90 min) (A). Time-course of concentration of captopril in plasma after single oral administration of 10 mg/kg to 8-wk (n = 4) and 40-wk SHRs (n = 4) (B). Blood (100 µL) was taken from the tail vein at fixed time intervals from 0 to 90 min. Plasma samples were subjected to *p*-BPB derivatization-LC-TOF-MS analysis. An extracted ion with *m/z* 416.0350 corresponds to a molecular ion of *p*-BPB-captopril. Data are expressed as the mean ± SEM (n = 4). **P* < 0.05, ***P* < 0.01, significantly different between 8-wk and 40-wk SHRs by unpaired Student's *t*-test.

A) Waterfall plot



B) Absorption profile

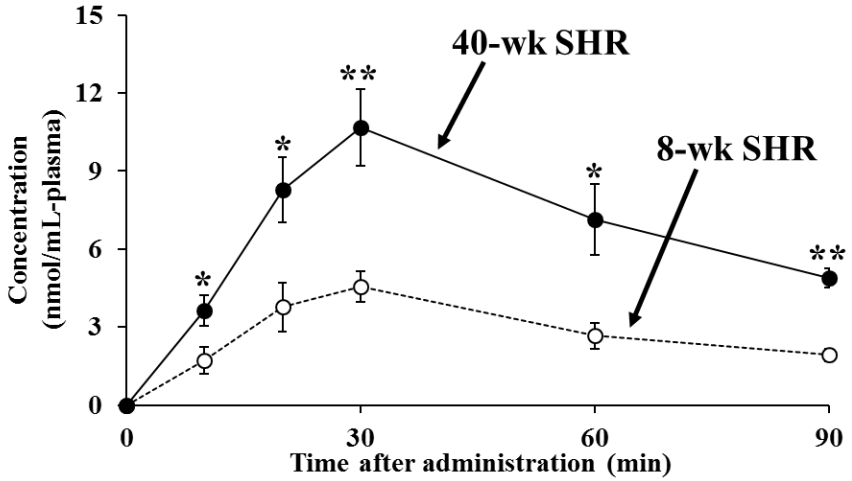


Figure 4-4. Stacked intensity-time chromatograms (waterfall plot) of Gly-Sar-Sar and IS ([$^{13}C_2$,^{15}N]-labeled Gly-Sar, 0.2 nmol/mL) arranged by sampling time of rat plasma (0 to 90 min) (A). Time-course of concentration of Gly-Sar-Sar in plasma after single oral administration of 10 mg/kg to 8-wk (n = 6) and 40-wk SHRs (n = 6) (B). Blood (100 μL) was taken from the tail vein at fixed time intervals from 0 to 90 min. Plasma samples were subjected to TNBS derivatization-LC-TOF-MS analysis. Extracted ions with *m/z* 361.0630 and *m/z* 429.1001 correspond to molecular ions of TNP-[$^{13}C_2$,^{15}N]-Gly-Sar (IS) and TNP-Gly-Sar-Sar, respectively. Data are expressed as the mean ± SEM (n = 6). **P* < 0.05, ***P* < 0.01, significantly different between 8-wk and 40-wk SHRs by unpaired Student's *t*-test.

3.2. Effect of aging on PepT1 expression in spontaneously hypertensive rats

Quantitative Western blotting analyses of intestinal PepT1 expression were performed using the up-jejunum, mid-jejunum, and ileum segments of 8-wk and 40-wk SHRs. As shown in Figure 4-5, PepT1 expression was the most abundant in the mid-jejunum among the three segments, suggesting that the mid-jejunum region may be mainly involved in di-/tripeptide absorption in SHRs. Surprisingly, Figure 4-5 also revealed that the expression of PepT1 in the mid-jejunum was significantly ($P < 0.05$) higher in 40-wk SHRs by approximately 1.5-fold, compared to 8-wk SHRs, while the protein expression of PepT1 in the upper jejunum and ileum did not show any significant difference between 8-wk and 40-wk SHR groups.

Thus far, some studies regarding intestinal PepT1 expression with age in rats and chicks have demonstrated that the protein expression decreased after birth, and then increased after weaning [49,50]. In this study, we pointed out that the intestinal PepT1 expression significantly increased in aged SHRs. It has been reported that pro-inflammatory cytokines such as TNF-α and IFN-γ stimulated the colonic PepT1 expression [89,90]. Thus, the enhanced expression of PepT1 in 40-wk SHRs (Figure 4-5) might be in part caused by increased pro-inflammatory cytokine levels associated with aging or promoting hypertension [91,92].

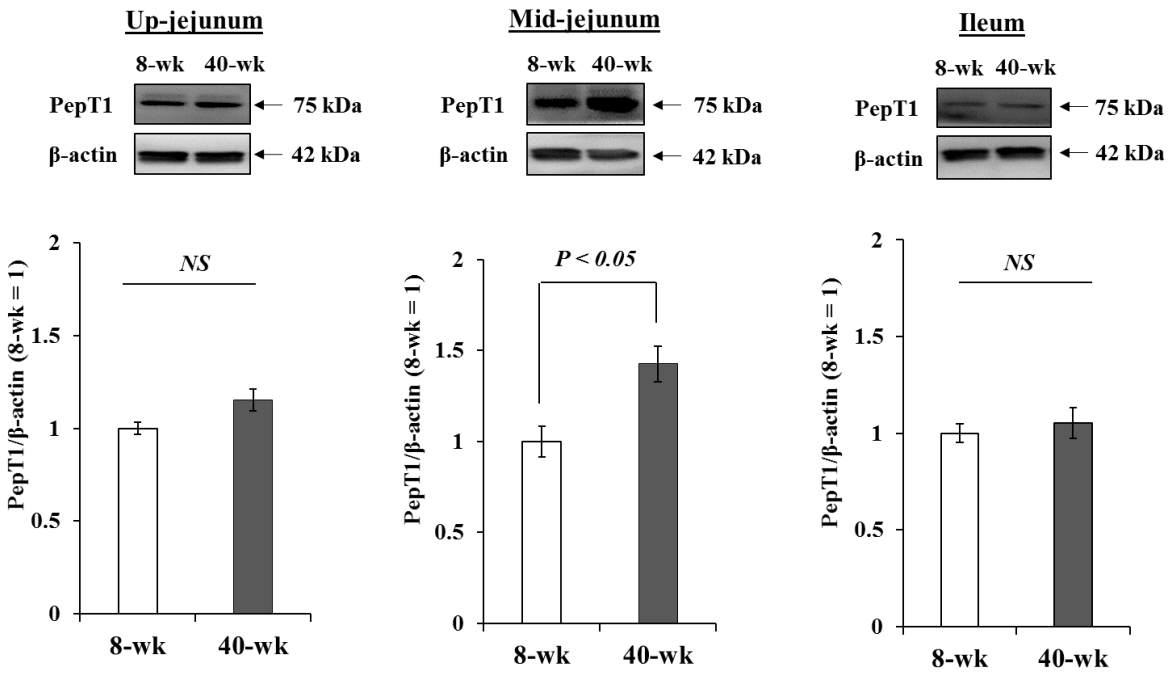


Figure 4-5. **Western blotting analyses of PepT1 expression in the upper-jejunum, mid-jejunum, and ileum regions from 8-wk (n = 4) and 40-wk SHRs (n = 4).** Values are expressed as the mean ± SEM (n = 4). Statistical analyses between two SHR groups were performed by unpaired Student's *t*-test. *NS* indicates no significant difference at $P > 0.05$.

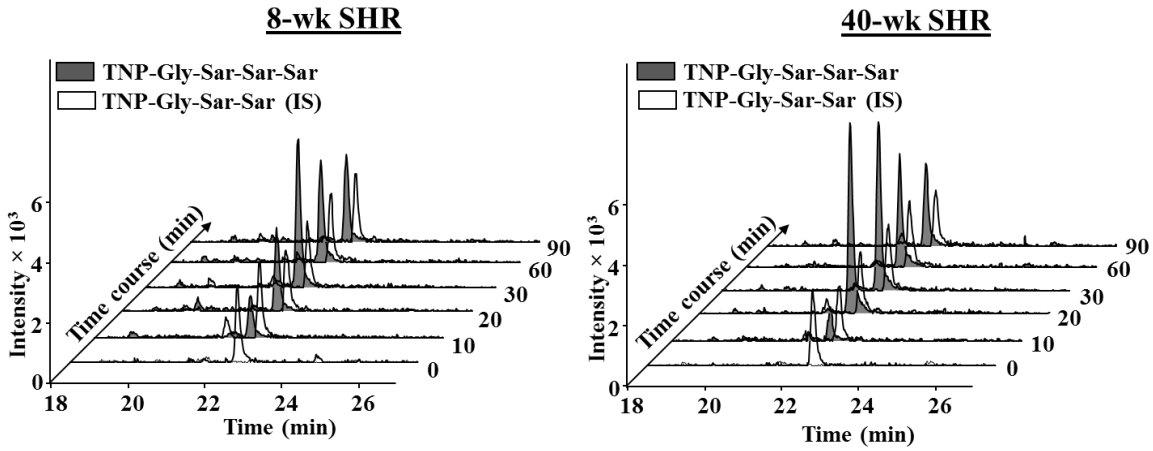
3.3. Effect of aging on absorption of oligopeptides Gly-Sar-Sar-Sar and Gly-Sar-Sar-Sar-Sar in spontaneously hypertensive rats

The effect of aging on the pharmacokinetics of oligopeptides Gly-Sar-Sar-Sar and Gly-Sar-Sar-Sar-Sar, which were absorbed intact into 8-wk SHRs (Chapter III), were examined. Gly-Sar-Sar-Sar and Gly-Sar-Sar-Sar-Sar were orally administered at each dose of 10 mg/kg to 8-wk and 40-wk SHRs. As shown in Figures 4-6A and 4-7A, the absorption of Gly-Sar-Sar-Sar and Gly-Sar-Sar-Sar-Sar were succesfully detected by TNBS-LC-TOF-MS after oral administration in both SHRs. Apart from the age-related absorption of di-/tripeptides (Figures 4-1 to 4-4 and Table 4-1), no significant difference in the absorption of the tetrapeptide Gly-Sar-Sar-Sar and pentapeptide Gly-Sar-Sar-Sar-Sar in terms of C_{max} and $AUC_{0\text{ - }90\text{ min}}$ between 8-wk and 40-wk SHRs was observed (Table 4-1, Figures 4-6A and B, and Figures 4-7A and B), suggesting that *in vivo* oligopeptide absorption might not be influenced by aging in SHRs. Some reports in SD rats revealed that aging or inflammation might lead to a reorganization of paracellular TJ proteins including zonua occludens (ZO-1), occludin, claudin-1, claudin-4, and junctional adhesion molecules (JAMs), thus weakened intestinal barrier [93–95]. Ren *et al*. [94] reported that the TJs of 48-wk and 96-wk SD rats were weakened with reduced mRNA and protein expressions of TJ proteins such as ZO-1 and occludin.

Taking these factors into account, claudin-1 protein, which is the initial promoter of TJ-close or open action [96], was determined by Western blotting

analysis to understand the mechanism of the absorption of oligopeptides between 8-wk and 40-wk SHRs. The results showed no significant difference in the expression of claudin-1 protein between 8-wk and 40-wk SHRs (Figure 4-8). Thus, it is possible to explain that no change in age-related absorption of oligopeptides Gly-Sar-Sar-Sar and Gly-Sar-Sar-Sar-Sar (Figures 4-6, 4-7, and Table 4-1) would be caused by the constant TJ protein expression in young and old SHRs.

A) Waterfall plot



B) Absorption profile

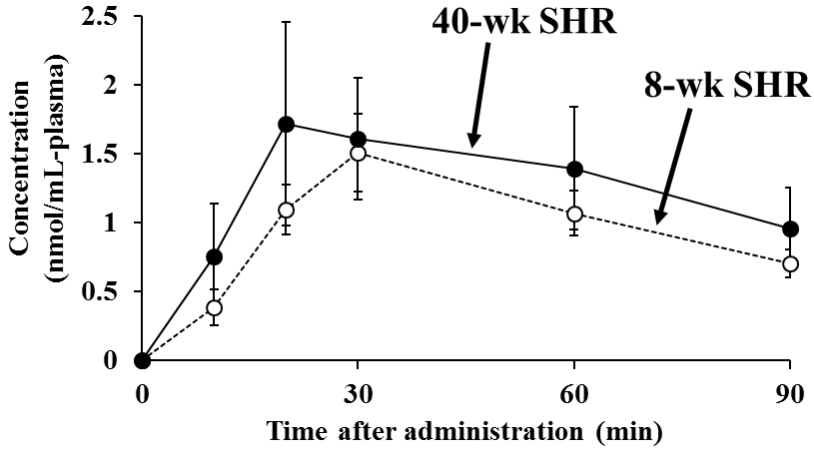
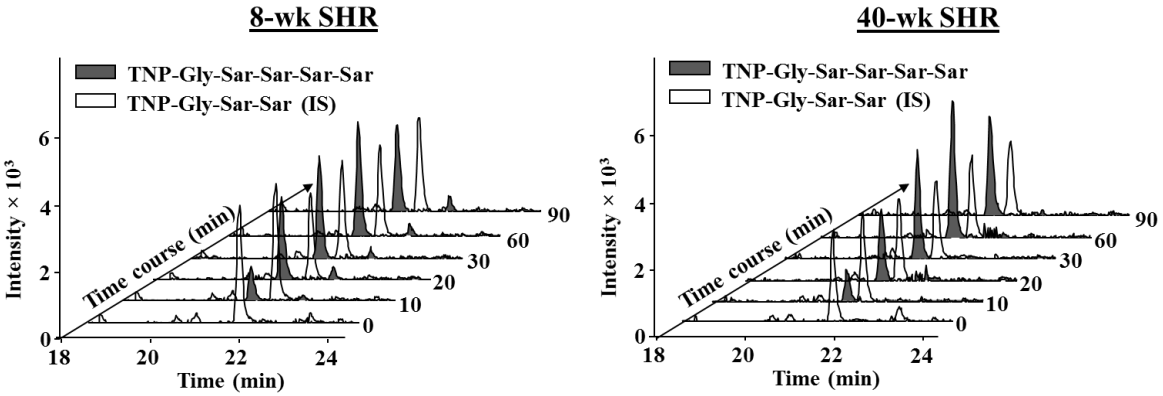


Figure 4-6. Stacked intensity-time chromatograms (waterfall plot) of Gly-Sar-Sar-Sar and IS (Gly-Sar-Sar, 0.1 nmol/mL) arranged by sampling time of rat plasma (0 to 90 min) (A). Time-course of concentration of Gly-Sar-Sar-Sar in plasma after single oral administration of 10 mg/kg to 8-wk (n = 4) and 40-wk SHRs (n = 4) (B). Blood (100 µL) was taken from the tail vein at fixed time intervals from of 0 to 90 min. Plasma samples were subjected to TNBS derivatization-LC-TOF-MS analysis. The extracted ions with *m/z* 429.1001 and *m/z* 500.1372 correspond to molecular ions of TNP-Gly-Sar-Sar and TNP-Gly-Sar-Sar-Sar, respectively. Data are expressed as the mean ± SEM (n = 6). Significant difference between 8-wk and 40-wk SHRs was analyzed by unpaired Student's *t*-test.

A) Waterfall plot



A) Absorption profile

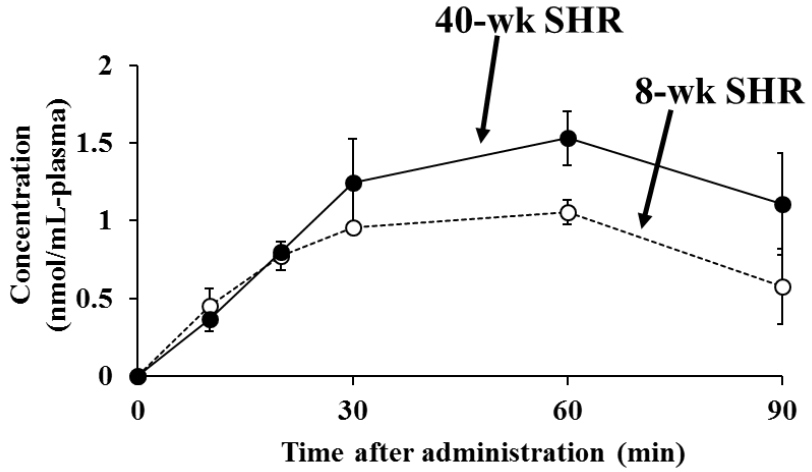


Figure 4-7. Stacked intensity-time chromatograms (waterfall plot) of Gly-Sar-Sar-Sar-Sar and IS (Gly-Sar-Sar, 0.1 nmol/mL) arranged by sampling time of rat plasma (0 to 90 min) (A). Time-course of concentration of Gly-Sar-Sar-Sar-Sar in plasma after single oral administration of 10 mg/kg to 8-wk (n = 4) and 40-wk SHRs (n = 4) (B). Blood (100 μL) was taken from the tail vein at fixed time intervals from 0 to 90 min. Plasma samples were subjected to TNBS derivatization-LC-TOF-MS analysis. The extracted ions with *m/z* 429.1001 and *m/z* 571.1743 correspond to molecular ions of TNP-Gly-Sar-Sar and TNP-Gly-Sar-Sar-Sar-Sar, respectively. Data are expressed as the mean ± SEM (n = 4). Significant difference between 8-wk and 40-wk SHRs was analyzed by unpaired Student's *t*-test.

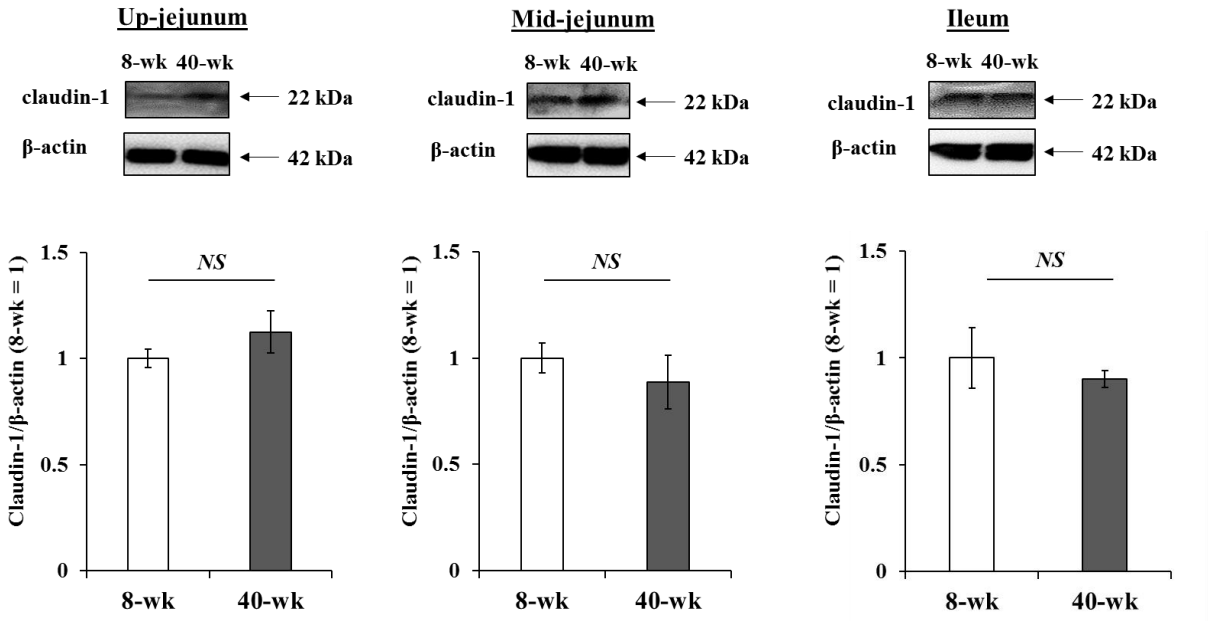


Figure 4-8. Western blotting analyses of claudin-1 expression in the upper-jejunum, mid-jejunum, and ileum regions from 8-wk (n = 4) and 40-wk (n = 4) SHRs. Statistical analyses between two SHR groups were performed by unpaired Student's *t*-test. *NS* indicates no significant difference at $P > 0.05$.

4. Summary

Chapter IV demonstrated that the absorption of di-/tripeptides may be enhanced with aging in SHRs, along with increased PepT1 expression in the intestinal mid-jejunum. The current work, together with the findings obtained in Chapter III, provided the first evidence on intact absorption of model oligopeptides Gly-Sar-Sar-Sar as tetrapeptide and Gly-Sar-Sar-Sar-Sar as pentapeptide in an age-independent manner. Chapter III and Chapter IV clearly demonstrated that peptide chain length affects *in vivo* peptide absorption in the following order: Gly-Sar > Gly-Sar-Sar > Gly-Sar-Sar-Sar > Gly-Sar-Sar-Sar-Sar. Greater attentions must be paid for *in vivo* absorption of oligopeptides as well as di-/tripeptides to gain more insights into the matter in future in respect to aging (Figure 4-9).

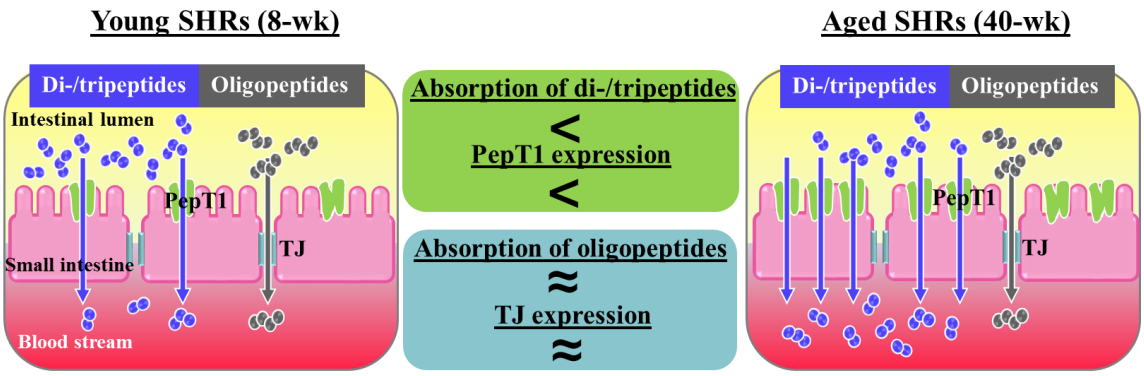


Figure 4-9. Proposed mechanism of *in vivo* absorption of peptides associated with aging.

Chapter V

Conclusion

Many researchers have focused on biologically active peptides present in the sequences of food proteins. Food-derived bioactive peptides as functional foods are expected to be effective in preventing lifestyle-related diseases, and maintaining physical and well-being of humans due to their physiological benefits such as antihypertensive [3–7], antioxidant [8], and anti-inflammatory effects [9]. Industrial manufacturers, thus, need to control the quality and quantity of peptides for the development of functional foods.

The potential physiological effect of a peptide depends on the ability to reach target organ in an active or intact form after oral administration [6]. Although the absorption of di-/tripeptides across intestinal membrane in humans and animals has been proven, no *in vivo* evidence on the absorption of larger peptides or oligopeptides (≥ tetrapeptides).

Thus, the present study was primarily aimed to develop a reliable and convenient method to quantify small bioactive peptides in food proteins. Further study was performed on *in vivo* absorption of oligopeptides. Finally, we aimed to elucidate the effect of aging on the absorption of peptides in SHRs.

Chapter II: Application of a standard method for quantitative mass spectrometric assay of dipeptides in soybean hydrolysate

In Chapter II, the proposed TNBS derivatization-aid LC-MS quantification assay using a standard addition method provided a reliable and convenient assay, without interference from matrix effects in protein hydrolysate, and without requirement for the use of isotope labeled IS. Substantially enhanced detection of TNP-dipeptides was obtained by TNBS derivatization when compared to the non-derivatized peptides with 0.9-, 4.3-, 11.4-, 24.7-, and 30.5-fold higher signal intensities for TNP-Ile-Tyr, TNP-Ser-Tyr, TNP-Tyr-Ser, TNP-Tyr-Gly, and TNP-Gly-Tyr, respectively. The matrix effects could be minimized by using the standard addition method for quantification of peptides in soybean hydrolysate. The result showed a 7- to 24-fold decrease in the slope of the standard addition curves of hydrolysate compared to the absolute calibration curves of the dipeptide standard solution. The advantage of the proposed TNBS-LC-MS-aided standard addition method laid the convenient and reliable peptide quantitative assays in food protein hydrolysate without the requirement for matrix cleaning, and without the use of isotope labeled IS.

Chapter III: Intestinal absorption of oligopeptides in spontaneously hypertensive rats

The aim of Chapter III was to investigate whether oligopeptides (tri- to pentapeptides) can be absorbed in intact form after single oral administration in rats by using TNBS-LC-MS technique. It was demonstrated for the first time that oligopeptides Gly-Sar-Sar (C_{max}, 4.6 ± 0.6 nmol/mL-plasma; AUC, 267.4 ± 34.3 nmol·min/mL-plasma), Gly-Sar-Sar-Sar (C_{max}, 1.5 ± 0.3 nmol/mL-plasma; AUC, 88.2 ± 17.6 nmol·min/mL-plasma), and Gly-Sar-Sar-Sar-Sar (C_{max}, 1.1 ± 0.1 nmol/mL-plasma; AUC, 71.7 ± 2.8 nmol·min/mL-plasma), were absorbed in their intact forms into SHR blood at each dose of 10 mg/kg. No expected metabolites from Gly-Sar-Sar, Gly-Sar-Sar-Sar, and Gly-Sar-Sar-Sar-Sar were observed in the blood samples, indicating that all oligopeptides were high resistant to protease hydrolysis. It was clear that the magnitute of *in vivo* absorption of peptides was dependent on peptide length in the descending order of Gly-Sar-Sar > Gly-Sar-Sar-Sar > Gly-Sar-Sar-Sar-Sar. Namely, molecular size (peptide length) is a critical determinant for *in vivo* absorption of bioactive peptides.

Chapter IV: Effect of aging on intestinal absorption of peptides in spontaneously hypertensive rats

Chapter IV aimed to elucidate the effect of aging on *in vivo* absorption of peptides in SHRs. Each peptide including Gly-Sar and Trp-His dipeptides, Gly-Sar-Sar tripeptide, Gly-Sar-Sar-Sar tetrapeptide, Gly-Sar-Sar-Sar-Sar pentapeptide, and captopril (a dipeptidomimetic antihypertensive drug) was administered at a dose of 10 mg/kg each to 8-wk and 40-wk SHRs. The peptides were all detected in their intact forms in the blood by TNBS-LC-MS. As a result, there was significantly enhanced absorption of di-/tripeptides in 40-wk SHRs, compared to 8-wk SHRs. In contrast, the absorption of oligopeptides Gly-Sar-Sar-Sar and Gly-Sar-Sar-Sar-Sar was not affected by aging. Quantitative Western blotting analyses of intestinal PepT1 and claudin-1 expression using the up-jejunum, mid-jejunum, and ileum segments from 8- and 40-wk SHRs showed that PepT1 expression in the mid-jejunum was significantly increased in 40-wk SHRs compared to 8-wk SHRs, whereas aging did not affect the expression of claudin-1, a TJ-related protein.

In conclusion, the present study provided the convenient and reliable peptide quantitative assays in food protein hydrolysate by using TNBS-LC-MS-aided standard addition method without the requirements of matrix cleaning and isotope labeled IS. Secondly, it was demonstrated for the first time that oligopeptides can be absorbed *in vivo* in a peptide length-dependent manner of di- > tri- > tetra- > pentapeptide length. Finally, we demonstrated

that SHR aging may enhance the absorption of di-/tripeptides by stimulated PepT1 expression, whereas oligopeptides may be absorbed in an age-independent manner.

References

[1] D.E.A. Yach, Global burden of chronic diseases: overcoming impediments to prevention and control, J. Am. Med. Assoc. 291 (2004) 2616–2622.

[2] W.C. Willett, J.P. Koplan, R. Nugent, P. Puska, T.A. Gaziano, Disease control priorities in developing contries: Prevention of chronic disease by means of diet and lifestyle changes, D.T. Jamison, J.G. Breman, A.R Measham, G. Alleyne, M. Claeson, D.B Evans, P. Jha, A. Mills, P. Musgrove (Eds.), Disease Control Priorities Project. (2006) 833–850 (Chapter 44).

[3] T. Matsui, M. Sato, M. Tanaka, Y. Yamada, S. Watanabe, Y. Fujimoto, K. Imaizumi, K. Matsumoto, Vasodilating dipeptide Trp-His can prevent atherosclerosis in apo E-deficient mice, Br. J. Nutr. 103 (2010) 309–313.

[4] R. Norris and R.J. FitzGerald, Bioactive food peptides in health and disease: Antihypertensive peptides derived from food proteins, B. Hernández-Ledesma and C.C. Hsieh (Eds.), Intech. (2013) 45-72 (Chapter 3).

[5] T. Kawasaki, E. Seki, K. Osajima, M. Yoshida, K. Asada, T. Matsui, Y. Osajima, Antihypertensive effect of valyl-tyrosine, a short chain peptide derived from sardine muscle hydrolyzate, on mild hypertensive subjects, J. Hum. Hypertens. 14 (2000) 519–523.

[6] B. Hernández-Ledesma, M. del Mar Contreras, I. Recio,

Antihypertensive peptides: production, bioavailability and incorporation into foods, Adv. Colloid Interface Sci. 165 (2011) 23–35.

[7] Y. Hata, M. Yamamoto, M. Ohni, K. Nakajima, Y. Nakamura, T. Takano, A placebo-controlled study of the effect of sour milk on blood pressure in hypertensive subjects, Am. J. Clin. Nutr. 64 (1996) 767–771.

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

[9] Y.F. Wang, X. Xu, X. Fan, C. Zhang, Q. Wei, X. Wang, W. Guo, W. Xing, J. Yu, J. L. Yan, H. P. Liang, A cell-penetrating peptide suppresses inflammation by inhibiting NF-κB signaling, Mol. Ther. 19 (2011) 1849–1857.

[10] Y. Shigemura, M. Nakaba, E. Shiratsuchi, M. Suyama, M. Yamada, T. Kiyono, K. Fukamizu, E.Y. Park, Y. Nakamura, K. Sato, Identification of food-derived elastin peptide, prolyl-glycine (Pro-Gly), in human blood after ingestion of elastin hydrolysate, J. Agric. Food Chem. 60 (2012) 5128–5133.

[11] V. Vallabha, P.K. Tiku, Antihypertensive peptides derived from soy protein by fermentation, Int. J. Pept. Res. Ther. 20 (2013) 161–168.

[12] A. Saiga, S. Tanabe, T. Nishimura, Antioxidant activity of peptides obtained from porcine myofibrillar proteins by protease treatment, J. Agric. Food Chem. 51 (2003) 3661–3667.

[13] N. Yamamoto, Antihypertensive peptides derived from food proteins,

Biopolymers 43 (1997) 129–134.

[14] Y. Nakamura, N. Yamamoto, K. Sakai, T. Takano, Antihypertensive effect of sour milk and peptides isolated from it that are inhibitors to angiotensin I-converting enzyme, J. Dairy Sci. 78 (1995) 1253–1257.

[15] Y. Yamada, K. Nishizawa, M. Yokoo, H. Zhao, K. Onishi, M. Teraishi, S. Utsumi, M. Ishimoto, M. Yoshikawa, Anti-hypertensive activity of genetically modified soybean seeds accumulating novokinin, Peptides 29 (2008) 331–337.

[16] M. Miguel, A. Aleixandre, Antihypertensive peptides derived from egg proteins, J. Nutr. 136 (2006) 1457–1460.

[17] M. Sipola, P. Finckenberg, J. Santisteban, R. Korpela, H. Vapaatalo, M.L. Nurminen, Long-term intake of milk peptides attenuates development of hypertension in spontaneously hypertensive rats, J. Physiol. Pharmacol. 52 (2001) 745–754.

[18] Z. Wang, S. Watanabe, Y. Kobayashi, M. Tanaka, T. Matsui, Trp-His, a vasorelaxant di-peptide, can inhibit extracellular Ca^{2+} entry to rat vascular smooth muscle cells through blockade of dihydropyridine-like L-type Ca^{2+} channels, Peptides 31 (2010) 2060–2066.

[19] M. Tanaka, S. Watanabe, Z. Wang, K. Matsumoto, T. Matsui, His-Arg-Trp potently attenuates contracted tension of thoracic aorta of Sprague-Dawley rats through the suppression of extracellular Ca^{2+} influx, Peptides 30 (2009) 1502–1507.

[20] E. Mendis, N. Rajapakse, H.G. Byun, S.K. Kim, Investigation of jumbo

squid (*Dosidicus gigas*) skin gelatin peptides for their *in vitro* antioxidant effects, Life Sci. 77 (2005) 2166–2178.

[21] http://www.caa.go.jp/foods/pdf/syokuhin1555.pdf.

[22] H. Matsufuji, T. Matsui, E. Seki, K. Osajima, M. Nakashima, Y. Osajima, Angiotensin I -converting enzyme inhibitory peptides in an alkaline protease hydrolyzate derived from sardine muscle, Biosci. Biotech. Biochem. 58 (1994) 2244–2245.

[23] C.H. Li, T. Matsui, K. Matsumoto, R. Yamasaki, T. Kawasaki, Latent production of angiotensin I-converting enzyme inhibitors from buckwheat protein, J. Pept. Sci. 8 (2002) 267–274.

[24] S.E. Kim, H.H. Kim, J.Y. Kim, Y.I. Kang, H.J. Woo, H.J. Lee, Anticancer activity of hydrophobic peptides from soy proteins, Biofactors 12 (2000) 151–155.

[25] M. Yoshikawa, H. Fujita, N. Matoba, Y. Takenaka, Bioactive peptides derived from food proteins preventing lifestyle-related diseases, BioFactors 12 (2000) 143–146.

[26] H.M. Chen, K. Muramoto, F. Yamauchi, Structural analysis of antioxidative peptides from soybean beta-conglycinin, J. Agric. Food Chem. 43 (1995) 574–578.

[27] S. Taira, K. Uematsu, D. Kaneko, H. Katano, Mass spectrometry imaging: applications to food science, Anal. Sci. 30 (2014) 197–203.

[28] S.M. Hong, M. Tanaka, S. Yoshii, Y. Mine, T. Matsui, Enhanced visualization of small peptides absorbed in rat small intestine by phytic-

acid-aided-matrix-assisted laser desorption/ionization-imaging mass spectrometry, Anal. Chem. 85 (2013) 10033−10039.

[29] T. Hyötyläinen, M.L. Riekkola, Solid-phase extraction or liquid chromatography coupled on-line with gas chromatography in the analysis of biological samples, J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci. 817 (2005) 13–21.

[30] M.C. Hennio, Solid-phase extraction: method development, sorbents, and coupling with liquid chromatography, J. Chromatogr. A. 856 (1999) 3–54.

[31] Y. Iwasaki, Y. Nakano, K. Mochizuki, M. Nomoto, Y. Takahashi, R. Ito, K. Saito, H. Nakazawa, A new strategy for ionization enhancement by derivatization for mass spectrometry, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 879 (2011) 1159–1165.

[32] T. Santa, Derivatization reagents in liquid chromatography/electrospray ionization tandem mass spectrometry, Biomed. Chromatogr. 25 (2011) 1–10.

[33] C. Hashimoto, Y. Iwaihara, S.J. Chen, M. Tanaka, T. Watanabe, T. Matsui, 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. 85 (2013) 4289–4295.

[34] A.N. Fonteh, R.J. Harrington, M.G. Harrington, Quantification of free amino acids and dipeptides using isotope dilution liquid chromatography

and electrospray ionization tandem mass spectrometry, Amino Acids 32 (2007) 203–212.

[35] K. Shimbo, S. Kubo, Y. Harada, T. Oonuki, T. Yokokura, H. Yoshida, M. Amao, M. Nakamura, N. Kageyama, J. Yamazaki, S. Ozawa, K. Hirayama, Automated precolumn derivatization system for analyzing physiological amino acids by liquid chromatography/mass spectrometry, Biomed Chromatogr. 24 (2010) 683–691.

[36] E.M.N. Nakashima, H.Q. Qing, M. Tanaka, T. Matsui, Improved detection of di-peptides by liquid chromatography-tandem mass spectrometry with 2,4,6-trinitrobenzene sulfonate conversion, Biosci. Biotech. Biochem. 77 (2013) 2094–2099.

[37] M. Tanaka, S.M. Hong, S. Akiyama, Q.Q. Hu, T. Matsui, Visualized absorption of anti-atherosclerotic dipeptide, Trp-His, in Sprague-Dawley rats by LC-MS and MALDI-MS imaging analyses, Mol. Nutr. Food Res. 59 (2015) 1541–1549.

[38] E.M.N. Nakashima, A. Kudo, Y. Iwaihara, M. Tanaka, K. Matsumoto, T. Matsui, 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. 414 (2011) 109–116.

[39] N. Cimetiere, I. Soutrel, M. Lemasle, A. Laplanche, Standard addition method for the determination of pharmaceutical residues in drinking water by SPE-LC-MS/MS, Environ. Technol. 34 (2013) 3031–3041.

[40] S. Ito, K. Tsukada, Matrix effect and correction by standard addition in quantitative liquid chromatographic-mass spectrometric analysis of diarrhetic shellfish poisoning toxins, J. Chromatogr. A. 943 (2002) 39–46.

[41] I. Fernández-Fígares, L.C. Rodríguez, A. González-Casado, ffect of different matrices on physiological amino acids analysis by liquid chromatography: Evaluation and correction of the matrix effect, J. Chromatogr. B. 799 (2004) 73–79.

[42] O.K. Ostroukhova, I.G. Zenkevich, A comparison of the external standard and standard addition methods for the quantitative chromatographic determination of pesticide concentrations in plant samples, J. Anal. Chem. 61 (2006) 442–451.

[43] H. Newey, D. Smyth, The intestinal absorption of some dipeptides, J. Physiol. (1959) 48–56.

[44] H. Daniel, G. Kottra, The proton oligopeptide cotransporter family SLC15 in physiology and pharmacology, Pflugers Arch. Eur. J. Physiol. 447 (2004) 610–618.

[45] S.A. Adibi, Regulation of expression of the intestinal oligopeptide transporter (PepT1) in health and disease, Am. J. Physiol. Gastrointest. Liver Physiol. 285 (2003) G779–G788.

[46] D.J. Boullin, R.F. Crampton, C.E. Heading, D. Pelling, Intestinal absorption of dipeptides containing glycine, phenyalanine, proline, β-alanine or histidine in the rat, Clin. Sci. Mol. Med. 45 (1973) 49–58.

[47] T. Matsui, K. Tamaya, E. Seki, K. Osajima, K. Matsumoto, T. Kawasaki,

Val-Tyr as a natural antihypertensive dipeptide can be absorbed into human circulatory blood system, Clin. Exp. Pharmacol. Physiol. 29 (2002) 204–208.

[48] D. Jappar, S.P. Wu, Y. Hu, D.E. Smith, Significance and regional dependency of peptide transporter (PepT) 1 in the intestinal permeability of glycylsarcosine: In situ single-pass perfusion studies in wild-type and *Pept1* knockout mice, Drug Metab. Dispos. 38 (2010) 1740–1746.

[49] B. Zwarycz, E.A. Wong, Expression of the peptide transporters PepT1, PepT2, and PHT1 in the embryonic and posthatch chick., Poult. Sci. 92 (2013) 1314–1321.

[50] H. Shen, D.E. Smith, F.C. Brosius, Developmental expression of PepT1 and PepT2 in rat small intestine, colon, and kidney, Pediatr. Res. 49 (2001) 789–795.

[51] A. Quirós, M. Ramos, B. Muguerza, M.A. Delgado, M. Miguel, A. Aleixandre, I. Recio, Identification of novel antihypertensive peptides in milk fermented with *Enterococcus faecalis*, Int. Dairy J. 17 (2007) 33–41.

[52] H. Fujita, M. Yoshikawa, Leu-Lys-Pro-Asn-Met: A prodrug-type ACE-inhibitory peptide derived from fish protein, Immunopharmacology 44 (1999) 123–127.

[53] W.C. Ko, M.L. Cheng, K.C. Hsu, J.S. Hwang, Absorption-enhancing treatments for antihypertensive activity of oligopeptides from tuna cooking juice: *In vivo* evaluation in spontaneously hypertensive rats, J. Food Sci. 71 (2006) S13–S17.

[54] L. Ding, Y. Zhang, Y. Jiang, L. Wang, B. Liu, J. Liu, Transport of egg white ACE-inhibitory peptide, Gln-Ile-Gly-Leu-Phe, in human intestinal Caco-2 cell monolayers with cytoprotective effect, J. Agric. Food Chem. 62 (2014) 3177–3182.

[55] K. Shimizu, M. Sato, Y. Zhang, T. Kouguchi, Y. Takahata, F. Morimatsu, M. Shimizu, The bioavailable octapeptide Gly-Ala-Hyp-Gly-Leu-Hyp-Gly-Pro stimulates nitric oxide synthesis in vascular endothelial cells, J. Agric. Food Chem. 58 (2010) 6960–6965.

[56] L. Ding, L. Wang, Y. Zhang, J. Liu, Transport of antihypertensive peptide Arg-Val-Pro-Ser-Leu, Ovotransferrin 328-332, in human intestinal Caco-2 cell monolayers, J. Agric. Food Chem. 63 (2015) 8143–8150.

[57] H. Sun, D. Liu, S. Li, Z. Qin, Transepithelial transport characteristics of the antihypertensive peptide, Lys-Val-Leu-Pro-Val-Pro, in human intestinal Caco-2 cell monolayers, Biosci. Biotechnol. Biochem. 73 (2009) 293–298.

[58] V. Vermeirssen, P. Augustijns, N. Morel, J. Van Camp, A. Opsomer, W. Verstraete, In vitro intestinal transport and antihypertensive activity of ACE inhibitory pea and whey digests, Int. J. Food Sci. Nutr. 56 (2005) 415–430.

[59] D. Liu, H. Sun, L. Zhang, S. Li, High-level expression of milk-derived antihypertensive peptide in Escherichia coli and its bioactivity, J. Agric. Food Chem. 55 (2007) 5109–5112.

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

[61] T. Nakamori, Bioactive protein and peptides as functional foods and nutraceuticals: Soy peptides as functional food materials, Y. Mine, E.C.Y. Li-Chan, and B. Jiang (Eds.), Wiley and Blackwell. (2010) 265–273 (Chapter 18).

[62] T. Matsui, K. Matsumoto, Lead molecules from natural Products: Discovery and new trends, M.T.H. Khan and A. Ather (Eds.), Elsevier, Netherlands. (2006) 259–276 (Chapter 15).

[63] K. Esaki, T. Ohmori, M. Maebuchi, T. Nakamori, T. Ohshima, S. Furuya, Increased tyrosine in the brain and serum of mice by orally administering dipeptide Ser-Tyr, Biosci. Biotechnol. Biochem. 77 (2013) 847–849.

[64] D.I. Sora, V. Stefanescu, V. David, A. Medvedovici, Validation of an LC-MS/MS assay of terpene trilactones in Ginkgo biloba extracts and pharmaceutical formulations through standard addition method, J. Pharm. Biomed. Anal. 50 (2009) 459–468.

[65] T. Ueno, M. Tanaka, T. Matsui, K. Matsumoto, Determination of antihypertensive small peptides, Val-Tyr and Ile-Val-Tyr, by fluorometric high-performance liquid chromatography combined with a double heart-cut column-switching technique, Anal. Sci. 21 (2005) 997–1000.

[66] J. Cai, M. Li, X. Xiong, X. Fang, R. Xu, Detection of histamine in beer

by nano extractive electrospray ionization mass spectrometry, J. Mass Spectrom. 49 (2014) 9–12.

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

[68] K. Moriyasu, T. Ichinose, A. Nakahata, M. Tanaka, T. Matsui, S. Furuya, The dipeptides Ile-Tyr and Ser-Tyr exert distinct effects on catecholamine metabolism in the mouse brainstem, Int. J. Pept. 2016 (2016) 1–5.

[69] M. del M. Contreras, R. Carrón, M.J. Montero, M. Ramos, I. Recio, Novel casein-derived peptides with antihypertensive activity, Int. Dairy J. 19 (2009) 566–573.

[70] M.L. Nurminen, M. Sipola, H. Kaarto, A Pihlanto-Leppälä, K. Piilola, R. Korpela, O. Tossavainen, H. Korhonen, H. Vapaatalo, Alpha-lactorphin lowers blood pressure measured by radiotelemetry in normotensive and spontaneously hypertensive rats, Life Sci. 66 (2000) 1535–1543.

[71] J.T. Ryan, R.P. Ross, D. Bolton, G.F. Fitzgerald, C. Stanton, Bioactive peptides from muscle sources: Meat and fish, Nutrients 3 (2011) 765–791.

[72] M. Shimizu, M. Tsunogai, S. Arai, Transepithelial transport of oligopeptides in the human intestinal cell, Caco-2, Peptides 18 (1997) 681–687.

[73] S. Yamamoto, F. Hayasaka, K. Deguchi, T. Okudera, T. Furusawa, Y. Sakai, Absorption and plasma kinetics of collagen tripeptide after peroral or intraperitoneal administration in rats, Biosci. Biotechnol. Biochem. 79 (2015) 2026–2033.

[74] M. Satake, M. Enjoh, Y. Nakamura, T. Takano, Y. Kawamura, S. Arai, M. Shimizu, Transepithelial transport of the bioactive tripeptide, Val-Pro-Pro, in human intestinal Caco-2 cell monolayers., Biosci. Biotechnol. Biochem. 66 (2002) 378–384.

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

[76] S.T. Metelsky, Rat small intestine absorption and membrane digestion in the process of aging, J. Biophys. Chem. 4 (2013) 66–71.

[77] F. Raul, F. Gosse, M. Doffoel, P. Darmenton, J.Y. Wessely, Age related increase of brush border enzyme activities along the small intestine, Gut 29 (1988) 1557–1563.

[78] L. Drozdowski, A.B.R. Thomson, Aging and the intestine, World J. Gastroenterol. 12 (2006) 7578–7584.

[79] K. Yamamoto, Y. Kitano, E. Shuang, Y. Hatakeyama, Y. Sakamoto, T. Honma, T. Tsuduki, Decreased lipid absorption due to reduced pancreatic lipase activity in aging male mice, Biogerontology 15 (2014) 463–473.

[80] A.A. Mangoni, S.H.D. Jackson, Age-related changes in pharmacokinetics and pharmacodynamics: Basic principles and practical applications, Br. J. Clin. Pharmacol. 57 (2004) 6–14.

[81] S. Natalucci, P. Ruggeri, C.E. Cogo, V. Picchio, A. Brunori, R. Burattini, Age-related analysis of glucose metabolism in spontaneously hypertensive and normotensive rats, Exp. Physiol. 88 (2003) 399–404.

[82] S.R. Vavricka, M.W. Musch, M. Fujiya, K. Kles, L. Chang, J.J. Eloranta, G.A.K. Ken, Tumor necrosis factor-α and interferon-γ increase PepT1 expression and activity in the human colon carcinoma cell line Caco-2/bbe and in mouse intestine, Pflügers Arch. 452 (2006) 71–80.

[83] K. Ma, Y. Hu, D.E. Smith, Influence of fed-fasted state on intestinal PepT1 expression and in vivo pharmacokinetics of glycylsarcosine in wild-type and Pept1 knockout mice, Pharm. Res. 29 (2012) 535–545.

[84] N.M. Hooper, Angiotensin converting enzyme: Implications from molecular biology for its physiological functions, Int. J. Biochem. 23 (1991) 641–647.

[85] S. Vancea, S. Imre, G. Donáth-Nagy, T. Béla, M. Nyulas, T. Muntean, R. Borka-Balás, Determination of free captopril in human plasma by liquid chromatography with mass spectrometry detection, Talanta 79 (2009) 436–441.

[86] T. Zhu, X. Chen, A. Steel, M.A. Hediger, D.E. Smith, Differential recognition of ACE inhibitors in xenopus laevis oocytes expressing rat PepT1 and PepT2. Pharm. Res. 17 (2000) 526–532.

[87] M. Himukai, T. Konno, T. Hoshi, Age-dependent change in intestinal absorption of dipeptides and their constituent amino acids in the guinea pig, Pediatr. Res. 14 (1980) 1272–1275.

[88] S. Guandalini, A. Rubino, Development of dipeptide transport in the intestinal mucosa of rabbits, Pediatr. Res. 16 (1982) 99–103.

[89] A. Ramezani, D.S. Raj, The gut microbiome, kidney disease, and targeted interventions, J. Am. Soc. Nephrol. 25 (2014) 657–670.

[90] S. A. Ingersoll, S. Ayyadurai, M. A. Charania, H. Laroui, Y. Yan, D. Merlin, The role and pathophysiological relevance of membrane transporter PepT1 in intestinal inflammation and inflammatory bowel disease, Am. J. Physiol. Gastrointest. Liver Physiol. 302 (2012) G484–G492.

[91] J.P. Granger, An emerging role for inflammatory cytokines in hypertension. Am. J. Physiol. Heart Circ. Physiol. 290 (2006) 923–924.

[92] M. Michaud, L. Balardy, G. Moulis, C. Gaudin, C. Peyrot, B. Vellas, M. Cesari, F. Nourhashemi, Proinflammatory cytokines, aging, and age related diseases. J. Am. Med. Dir. Assoc. 14 (2013) 877–882.

[93] D.R. Clayburgh, L. Shen, J.R. Turner, A porous defense: the leaky epithelial barrier in intestinal disease, Lab. Invest. 84 (2004) 282–291.

[94] W.R.K. Ren, X. Li, M. Luo, Age-related changes in small intestinal mucosa epithelium architecture and epithelial tight junction in rat models, Aging Clin. Exp. Res. (2014) 183–191.

[95] N.S. Harhaj, D.A. Antonetti, Regulation of tight junctions and loss of

barrier function in pathophysiology, Int. J. Biochem. Cell Biol. 36 (2004) 1206–1237.

[96] S. Angelow, R. Ahlstrom, A.S.L. Yu, Biology of claudins, Am. J. Physiol. Renal Physiol. 295 (2008) 867–876.

Acknowledgements

I would like to take this opportunity to express my gratitude to all those who helped me to finish my doctoral dissertation. First and foremost, I would like to thank from the bottom of my heart to my supervisor, Professor Toshiro Matsui, for his support, guidance, and encouragement and enthusiasm in scientific research. I have been extremely lucky to have a supervisor who has always helped me to figure out the right direction about my research with his erudition and profession; at the same time, his door was always open any question I had. I am forever indebted to Professor Toshiro Matsui for his positive role in my professional development.

I would like to express my profound gratitude to Professor Mitsuya Shimoda and Associate Professor Noriyuki Igura for their professional review and valuable advice on my doctoral dissertation.

I thank Associate Professor Yoshiyuki Miyazaki for his kindness and support. I am grateful to Assistant Professor Mitsuru Tanaka for his valuable suggestions, generous support during my academic activities, and for his care and help in my personal life in Japan. He has energized me to work more professionally and cooperatively with other members in a multi-cultural background. I thank Ms. Kaori Miyazaki for taking care of all official matters to keep studying without any official problems.

I also would like to acknowledge my laboratory members, Yutaro Kobayashi and Hu-Qiang Qing, for their indefatigable help and virtuous support during my research journey.

Many thanks to foreigner colleagues Kumrungsee Thanutchporn, Seong-Min Hong, Chen Sijing, Jian Guo, Nguyen Huu Nghi, Chung Hsuan, Dissanayake Mudiyasnselage Dilan Rasika, Jocelyn Risuko Sato Miyahira for their encouragement and friendship with a multicultural atmosphere. Specially, I would like to express my gratefulness to other members of Food Analysis Laboratory for kindness and good memories that make me happy. I wish all the best to them.

Thanks to financial support under the MEXT (The Ministry of Education, Culture, Sports, Science and Technology) Scholarship, I can focus on my study in comport without worrying about the living expenses.

Last, but not least, I sincerely thank my parents for their unconditional love, support, and encouragement. I truly thank my sisters and brother for their love and care.

I am most grateful to you all.