

**Study on small intestinal mucosa exopeptidases
in association with the bioavailability of food-derived peptides**

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General Introduction

Upon ingestion, proteins are first digested by endoproteinases such as pepsin, trypsin, and chymotrypsin, generating peptides. The peptides are further degraded into smaller peptides and amino acids by exopeptidases in the small intestine and blood circulation. Several exopeptidases have been identified in the gastrointestinal tract, such as carboxypeptidase A (CPA), leucine aminopeptidase (LAP), aminopeptidase N (APN), aminopeptidase A (APA), aminopeptidase B (APB), prolidase, dipeptidyl peptidase 3 (DPP3), and dipeptidyl peptidase 4 (DPP4) (Aganga et al., 2021; Endo et al., 1989; Gustafsson et al., 2011; Hiwada et al., 1981; Johansson et al., 2011; Lundquist & Artursson, 2016; Misiura & Mityk, 2020). LAP and APN are well-known exopeptidases that release amino-terminal amino acids of peptides. LAP preferentially releases Leu and other amino acids, including Pro, from the amino terminus but cannot release Arg and Lys (Gu and Walling, 2000). APN releases most amino acids, including Arg and Pro (slow), from the amino terminus (Niven et al., 1995). Conversely, APB preferentially releases Arg and Lys from the amino terminus (Nagata et al., 1991). Prolidase preferentially cleaves Pro residue at the carboxyl terminus (Wilk et al., 2017), while DPPs release dipeptides at the amino terminus (Ohara-Nemoto et al., 2002).

In general, dietary protein and peptides were considered to be completely degraded into amino acids. This concept was partially changed by the findings of food-derived peptides in the gastrointestinal tract and blood after ingestion of protein hydrolysates or peptides (Asai et al., 2019; Iwai et al., 2005; Ohara et al., 2007; Miyauchi et al., 2022; Shigemura et al., 2018; Taga et

al., 2014). After ingestion of collagen hydrolysate (CH), di- and tripeptides containing hydroxyproline (Hyp) increase in the human peripheral blood at μM levels (Asai et al., 2019; Iwai et al., 2005; Ohara et al., 2007; Shigemura et al., 2018; Taga et al., 2014). These facts clearly indicate that a part of food-derived peptides survives digestion by exopeptidases in the small intestine and blood.

The ingestion of collagen hydrolysate (CH) has been proven to exert many health benefits. Human clinical studies have indicated that ingestion of CH improved skin conditions, including moisture content, elasticity, less wrinkle, etc. (Inoue et al., 2016; Oba et al., 2013; Ohara et al., 2010), reduced joint pain (Nakatani et al., 2009; Zdzieblik et al., 2017), and improved wound healing of pressure ulcer (Sugihara et al., 2018; Yamanaka et al., 2017). Pro-Hyp is the major CH-derived peptide that significantly increases in human blood after CH ingestion (Asai et al., 2019; Iwai et al., 2005; Ohara et al., 2007; Shigemura et al., 2018; Taga et al., 2014). Animal experiments also revealed that Pro-Hyp is responsible for the acceleration of wound healing on the mice abdominal wall (Jimi et al., 2021). In vitro studies using cell culture also showed that Pro-Hyp enhances the proliferation of primary cultured fibroblasts attached on collagen fibrils, which can be associated with the improvement of wound healing (Asai et al., 2020).

The bioavailability of food-derived peptides is now believed to be crucial for exerting their bioactivity in vivo (Matsui, 2018; Sato, 2022). Numerous di- and tripeptides have been reported to enter enterocytes via peptide transporter 1 (PepT1) (Hanh et al., 2017; Rohm et al., 2019). Nonetheless, the bioavailability of food-derived peptides greatly differs by their structure (Sato, 2022). Thus, not only peptide transportation but another factor also plays a crucial role in

determining the bioavailability of food-derived peptides. In vitro and in silico digestion have been used to predict peptides with high bioavailability. Furthermore, a single or a combination of several endoproteinases have been intensively used for in vitro digestion (Guo et al., 2014; Aléman et al., 2013; Larder et al., 2021; Feng and Betti, 2017; Larder et al., 2021; Yang et al., 2019; Bai et al., 2023; Brodkorb et al., 2019). However, a previous study in the research group the author belongs to has shown that most short-chain food-derived peptides resist endoproteinases but are readily degraded by exopeptidases (Chen et al., 2019). Ejima et al. (2018) also reported that some dipeptides with proline (Pro) at the carboxyl terminus (X-Pro) remained after in vitro digestion of corn and wheat gluten using pancreatin and LAP. Yet, these peptides did not increase in human blood after ingestion of the corn and wheat gluten hydrolysates. These facts suggest that there is an exopeptidase responsible for X-Pro dipeptides degradation in the gastrointestinal tract, which was not used by the above in vitro digestion. The present author assumed that the susceptibility of peptides to certain exopeptidases in the gastrointestinal tract greatly contributes to the bioavailability of food-derived peptides. However, knowledge of the role of exopeptidases in the gastrointestinal tract in the digestion of food-derived peptides has been very limited. This study aimed to elucidate the role of exopeptidases in determining the bioavailability of food-derived peptides in the body.

In Chapter 1, the establishment of in vitro digestion using crude extract of rat's small intestinal mucosa to simulate in vivo digestion is described. Collagen hydrolysate (CH) was used in the study in this chapter since the structure of collagen-derived peptides in the blood plasma of humans and rats upon ingestion has been well-elucidated. Consequently, the author found

prolidase activity in the crude extract of rat's small intestinal mucosa played a crucial role in determining food-derived collagen peptides in the body upon ingestion of CH.

In Chapter 2, the identification of prolidase and aminopeptidase N in the crude extract of rat's small intestinal mucosa is described. Partially purified mucosa extract containing aminopeptidase N and prolidase rapidly and completely digested synthetic Gly-Pro, while Pro-Hyp remained in considerable amounts after the *in vitro* digestion. This finding can clarify why Pro-Hyp increased significantly in blood plasma after CH ingestion while Gly-Pro did not.

In Chapter 3, the impact of co-administration of CH with rice protein hydrolysate (RPH) on the bioavailability of Pro-Hyp is described. Pyroglutamyl (pGlu) peptides are the major food-derived peptides in the gastrointestinal tract after ingestion of RPH (Miyauchi et al., 2022). Interestingly, co-administration of CH with RPH increased the bioavailability of Pro-Hyp. The data suggest that several pGlu peptides showed inhibition on Pro-Hyp degradation by exopeptidases, including prolidase, in a crude extract of mucosa, where pGlu-Pro exerted a significant inhibition among the others.

Taken together, exopeptidases in the small intestinal mucosa play a crucial role in determining food-derived peptides. Therefore, the *in vitro* digestion using crude extract of rat's small intestinal mucosa established in this study can predict the bioavailability of food-derived peptides. Among the exopeptidases, prolidase is the key player for X-Pro and X-Hyp dipeptides degradation in the gastrointestinal tract, which greatly contributes to determining the bioavailability of CH-derived peptides. Furthermore, co-administration of RPH with CH enhanced the bioavailability of Pro-Hyp, a major collagen-derived bioactive peptide. The pGlu peptides,

derived from RPH, may interfere with prolidase, thus inhibiting the degradation of Pro-Hyp. Therefore, the bioavailability of bioactive is potentially enhanced by combining specific peptides which can hinder the peptide degradation by gastrointestinal exopeptidases.

Chapter 1

Substrate specificity of exopeptidases in small intestinal mucosa of rats determines the structure of food-derived collagen peptides in rat lumen and blood

1.1 Introduction

The bioavailability of food-derived peptides is crucial to exert their biological activity (Matsui, 2018; Sato, 2022). Conventionally, it was believed that ingested proteins or peptides were rapidly hydrolyzed into amino acids and smaller peptides during digestion. Certain di- and tripeptides have been demonstrated to enter enterocytes via peptide transporter 1 (PepT1) (Hanh et al., 2017; Rohm et al., 2019). Yet, it was also assumed that these peptides were rapidly degraded in enterocytes and blood circulation. However, it has been demonstrated that di- and tripeptides containing hydroxyproline (Hyp) significantly increase in the human peripheral blood at μM levels after ingestion of collagen hydrolysate (CH) (Asai et al., 2019; Iwai et al., 2005; Ohara et al., 2007; Shigemura et al., 2018; Taga et al., 2014). In human blood, collagen-derived peptides predominantly comprise Pro-Hyp, with the second most abundant being Hyp-Gly (Shigemura et al., 2011). In vitro studies using cell culture systems and animal studies have demonstrated that Pro-Hyp and Hyp-Gly exert some biological activities (Asai et al., 2020a; Asai et al., 2020b; Jimi et al., 2021; Nakatani et al., 2009; Ohara et al., 2010; Shigemura et al., 2009; Shigemura et al., 2011). Furthermore, human clinical studies have indicated that ingestion of collagen hydrolysates exerts health benefits such as improvement of skin (Inoue et al., 2016; Oba et al., 2013; Ohara et

al., 2010) and joint conditions (Nakatani et al., 2009; Zdzieblik et al., 2017), and improvement of wound healing of pressure ulcer (Sugihara et al., 2018; Yamanaka et al., 2017).

Collagen has a specific amino acid sequence in which every third residue is Gly, resulting in a repetition as Gly-Xa-Xb (Xa and Xb are any amino acids) in the triple helical domain. Pro residue at Xb is specifically changed to Hyp residue by prolyl hydroxylase during post-translational modification, producing Gly-Pro-Hyp-Gly as the most frequently observed motif (10.5% of total Hyp) in collagen (Ramshaw et al., 1998; Shoulders & Raines, 2009). Pro-Hyp, Hyp-Gly, Gly-Pro-Hyp, and Pro-Hyp-Gly, which notably increase in human peripheral blood upon ingestion of CH (Asai et al., 2019; Iwai et al., 2005; Shigemura et al., 2018), can be derived from this motif. Interestingly, ingestion of CH has shown either no significant increase or only a slight increase in Gly-Pro levels in human plasma (Asai et al., 2019; Iwai et al., 2005; Ohara et al., 2007; Shigemura et al., 2018; Taga et al., 2014; Taga et al., 2019; Yamamoto et al., 2016), despite its potential derivation from the same motif. Furthermore, in vitro studies using the Caco-2 monolayer system have reported that Gly-Pro can be passed through Caco-2 monolayer in intact form (Enjoh et al., 1996; Ganapathy & Leibachs, 1983; Rohm et al., 2019). However, inconsistencies in bioavailability have not been resolved. In this chapter, a crucial determining factor of the bioavailability of food-derived peptides using in vitro and in vivo digestion is described.

To predict peptides with high bioavailability, in vitro digestion has been carried out using a combination of some endoproteinases such as pepsin, trypsin, crude protease preparations such as gastric juice (containing pepsin and gastric lipase), and pancreatin (containing amylase, trypsin,

chymotrypsin, and lipase) (Guo et al., 2014; Aléman et al., 2013; Larder et al., 2021; Feng and Betti, 2017; Larder et al., 2021; Yang et al., 2019; Bai et al. 2023; Brodkorb et al., 2019). However, as mentioned in the General Introduction section, most short-chain food-derived peptides resist endoproteinases but are readily degraded by exopeptidases (Chen et al., 2019). The intestinal mucosa contains various exopeptidases secreted by enterocytes such as leucine aminopeptidase (LAP), aminopeptidases N (APN), aminopeptidase A (APA), aminopeptidase B (APB), prolidase, dipeptidyl peptidase 3 (DPP3), and dipeptidyl peptidase 4 (DPP4) (Aganga et al., 2021; Endo et al., 1989; Gustafsson et al., 2011; Hiwada et al., 1981; Johansson et al., 2011; Lundquist & Artursson, 2016; Misiura & Milyk, 2020). In mammals, aminopeptidases are produced by various tissues and organs, thus abundantly present in the liver, kidney, intestine, and placenta. Furthermore, APN is particularly abundant in the brush border membrane of the kidney, small intestine, and placenta (Turner, 2013). In addition, carboxypeptidase A (CPA) is produced by the pancreas and sent into the gastrointestinal tract. However, their role in determining the bioavailability of food-derived peptides has not been well examined.

Based on these facts, it was hypothesized that the substrate specificity of exopeptidases in the intestinal mucosa determines the structure of peptides with high bioavailability. A range of in vitro and rat in vivo digestions were used to elucidate the role of exopeptidases in small intestinal mucosa on the bioavailability of food-derived peptides. For in vitro digestions, CH was digested by several exopeptidase preparations, crude extracts from rat intestinal mucosa, and blood plasma. Consequently, the results showed that the exopeptidases in small intestinal mucosa generated Hyp-containing di- and tripeptides with low levels of peptides containing Pro at the carboxyl terminus

(X-Pro), while X-Pro peptides resisted LAP, CPA, and APN digestion. CH digestion by crude extract of mucosa generated a similar peptide profile to in vivo digestion, specifically having a high content of Pro-Hyp and a negligible content of Gly-Pro. These results indicate that prolidase activity in the small intestinal mucosa, in addition to LAP, CPA, and APN, plays a crucial role in determining collagen-derived peptides with high bioavailability.

1.2 Materials and Methods

Collagen hydrolysate

Collagen hydrolysate (CH, Marine Oligopeptide®) was a kind gift from the Japan Food Peptide Institute (Osaka, Japan). According to the supplier's information, CH was prepared from the scale of tilapia (*Oreochromis niloticus*) by two protease preparations and the average molecular weight of peptides in CH is approximately 500 Da.

Reagents

Acetonitrile (HPLC-grade), phosphate-buffered saline pH 7.4 (PBS, 10×), and porcine pancreatin were purchased from Nacalai Tesque (Kyoto, Japan). 6-Aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AccQ) was purchased from Toronto Research Chemical (Toronto, ON, Canada). A lysis buffer (CellLytic), aminopeptidase N (APN), leucine aminopeptidase (LAP) from porcine kidney, carboxypeptidase A (CPA) from bovine pancreas, L-leucine *p*-nitroanilide (Leu-pNA, substrate for EC 3.4.11.2) were purchased from Sigma-Aldrich (St. Louis, MO). 9-Fluorenyl methoxycarbonyl (Fmoc) amino acid derivatives, Fmoc amino acid-bound *p*-alkoxybenzyl alcohol

(Alko) resin, proline-bound 2-chlorotrityl chloride (Barlos) resin, and stable isotope labeled Fmoc-Gly (2,2-d₂)-OH were purchased from Watanabe Chemical Industries (Hiroshima, Japan). All other reagents were of analytical grade or higher.

Animal experiments

Five-week-old male Wistar/ST rats (120–140 g) were purchased from Japan SLC (Shizuoka, Japan). Every three rats were kept in a cage at 22–25°C and 40–70% relative humidity, with a 12-hour light/dark cycle. The rats were allowed free access to a normal rodent chow (solid type of certified diet MF; Oriental Yeast, Tokyo, Japan) and drinking water during an acclimatization period of one week. All the animals were treated and cared for according to the guidelines of the National Institutes of Health (NIH) for experimental animals. All experimental procedures were approved by the Animal Care Committee of the Louis Pasteur Center for Medical Research (Kyoto, Japan, approval number 20221). After the acclimatization period, rats were divided into the control group (n=3) and the collagen hydrolysate (CH) group (n=3). The CH groups were administered collagen hydrolysate water solution (300 µL) at a dose of 800 mg/kg body weight, while the control group was administered the same volume of distilled water. One hour after the oral administration, small intestine and blood from the abdominal vena cava were collected from rats under anesthesia using isoflurane inhalation.

The inner content of the small intestines was flushed using 10 mL of cold PBS. The effluent was added with three volumes of ethanol and centrifuged at 13,000 ×g at 4°C for 10 min. The supernatant was collected and used for the following experiments as the lumen sample. The washed small intestine was cut into small pieces. Aliquots of the intestine (100 mg) were

homogenized with PBS (100 μ L) using a Biomasher II (Nippi, Tokyo, Japan). The homogenate was mixed with three volumes of ethanol and centrifuged at 13,000 $\times g$ for 10 min at 4°C. The supernatant was collected and used as the small intestinal tissue extract for the following experiments. The blood was collected using a heparinized syringe and centrifuged at 1,500 $\times g$ at 4°C for 10 min. The resultant plasma was mixed with three volumes of ethanol and centrifuged as described above. The supernatant was used as a plasma sample for the following experiments.

Preparation of crude mucosa extract from rat small intestine

To prepare the crude mucosa extract, 6-week-old male Wistar/ST rats were sacrificed by drawing blood from the abdominal vena cava under anesthesia by isoflurane inhalation. The experiment procedures were approved by the Animal Care Committee of the Louis Pasteur Center for Medical Research (Kyoto, Japan, with approval number 20221). The whole small intestine was collected immediately. After flushing the inner content with cold PBS, the intestine was split open longitudinally with scissors. The mucosa was scraped using a spatula and then mixed with two volumes of a lysis buffer (CellLytic) before homogenization in Biomasher II. The suspension was centrifuged at 13,000 $\times g$ at 4°C for 15 min. The supernatant was used as a crude mucosa extract of small intestinal mucosa.

Leucine aminopeptidase (LAP) assay

LAP activity was measured using L-leucine-p-nitroanilide (Leu-pNA) as substrate according to the supplier test protocol (Sigma-Aldrich) with some modifications. Leu-pNA was dissolved in

methanol to give a concentration of 1.2 mM. PBS (90 μ L) was mixed with 5 μ L of the crude extract of small intestinal mucosa and 5 μ L of the substrate solution. The absorbances at 405 nm were immediately recorded before and after 5 min incubation at 37 °C. One unit was defined as the enzymes hydrolyzing 1.0 μ mole of Leu-pNA to Leu and pNA per minute under assay conditions. The enzyme activity was expressed as a unit/mL, which can be obtained by the following equation: Unit/mL= ((Δ absorbance at 405/min of sample- Δ absorbance at 405/min of blank) $\times$ total volume (0.1 mL) $\times$ dilution factor (20))/(9.9 $\times$ volume of enzyme (mL)). Where 9.9 was the millimolar extinction coefficient of pNA at 405 nm.

Prolidase activity assay

Prolidase activity of crude extract of small intestinal mucosa was measured using synthetic Gly-Pro as the substrate. Aliquots (180 μ L) of 0.5 mM Gly-Pro solution in PBS were reacted with 20 μ L of crude extract of mucosa (25 $\times$ dilution using PBS) and incubated at 37 °C for 10 min. The reaction was terminated with the addition of three volumes of ethanol. The content of free Pro in the supernatant (10 μ L) liberated from Gly-Pro was quantified by amino acid analysis as described in Bidlingmeyer, et al. (1984) with some modifications (Miyachi et al., 2022) without HCl hydrolysis. One unit was defined as the enzymes liberating 1.0 μ mole of Pro from Gly-Pro per minute under assay conditions. The enzyme activity was expressed as a unit/mL and was calculated using the following equation.

$$\text{Unit/mL} = (\text{free Pro at 10 min } (\mu\text{mol}) - \text{free Pro at 0 min } (\mu\text{mol})) \times \text{total volume (0.2 mL)} \times \text{dilution factor (250)} / (10 \text{ min} \times \text{volume of enzyme (0.02 mL)})$$

In vitro digestion using commercial enzymes

In vitro digestion was carried out using the method previously described (Ejima et al., 2018) with some modifications. Briefly, CH was dissolved in 1 mL of PBS to give a 2.5 mg/mL concentration. The resulting CH solution (1 mL) underwent digestion with a mixture containing pancreatin (100 µg), CPA (7.7 U), and LAP (2.45 U) at 37 °C for 4 h. The reaction was halted by freezing at -20°C. Similarly, another CH solution (1 mL) was digested with APN (0.8 U). The peptides in the digests were identified and quantified using LC-MS/MS, as described in the subsequent section.

In vitro digestion using crude extracts of small intestinal mucosa and blood plasma

CH (0.2 mg) was digested by crude extract of mucosa and blood plasma with 0.08 U leucine aminopeptidase (LAP) activity in 100 µL of PBS at 37°C for 4 h. Crude extract of mucosa was diluted with PBS to give suitable LAP activity. The reaction was terminated by adding 300 µL of ethanol. The supernatant was collected after centrifugation at 13,000 ×g at 4°C for 15 min and used as crude mucosa and blood plasma digests of CH. For the blank, crude extracts and blood plasma without CH were incubated and treated in the same manner as the samples. The peptides in the digests were identified and quantified using LC-MS/MS, as described in the subsequent section.

Identification of peptides in CH

Aliquot (10 µL) of the 2.5 mg/mL CH in PBS solution was directly injected into a liquid chromatography-tandem mass spectrometer (LC-MS/MS, LCMS 8040, Shimadzu, Kyoto, Japan)

equipped with an Inertsil ODS-3 column (2.1 mm i.d. × 250 mm, GL Science, Tokyo, Japan), which was maintained at 40°C. The elution was performed using a binary linear gradient using 0.1% formic acid (solvent A) and 0.1% formic acid containing 80% acetonitrile (solvent B) at 0.2 mL/min. The gradient program was as follows: 0–20 min, 0–30% B; 20–25 min, 30–100% B; 25–30 min, 100% B; 30–30.1 min, 100–0% B, and 30.1–40 min, 0% B. Peptides were detected by total ion scan at positive mode at scan ranges of mass to charge ratio (m/z), 100–200, 200–225, 225–250, 250–275, 275–300, 300–350, 350–400, 400–500, and 500–1000. Precursor ions in some major peaks were further analyzed by product ion scan mode with collision energy at -15, -25, and -35 eV.

Identification of peptides in the in vitro and in vivo digests of CH

CH was digested in vitro using the 3 different methods described above. Furthermore, the inner content of the small intestine, intestinal tissue extract, and blood plasma were collected from rats 1 h after administration of CH or vehicle. Ethanol-soluble fractions of these digests and extracts were prepared as described above. To improve the resolution of short-chain peptides, amino/imino groups of peptides and amino acids were derivatized with AccQ. Aliquots of the ethanol-soluble fraction (100 µL and 20 µL for digests and extracts, respectively) were dried in a microcentrifuge tube (1.5 mL) under vacuum and added with 20 µL of water, 60 µL of 50 mM sodium borate buffer, pH 8.8, and 20 µL of 0.3% AccQ-acetonitrile solution. The reaction was carried out at 50 °C for 10 min. The reactant was filtered using a Cosmonice filter. An aliquot (10 µL) of the filtrate was injected into the LC-MS/MS equipped with the Inertsil ODS-3 column. The same elution condition

as described above was used. The AccQ-derivatives were specifically detected by precursor ion scan at positive mode targeting AccQ-derived b1 ion with $m/z = 171.1$ in the scan ranges at $m/z = 325\text{--}350$, $350\text{--}375$, $375\text{--}400$, $400\text{--}450$, and $450\text{--}500$. To estimate peptide sequences, the precursor ions in major peaks were further subjected to product ion scan with collision energy at -15, -25, and -35 eV.

Peptide synthesis

The estimated peptides were synthesized using an Fmoc strategy with a solid-phase automatic peptide synthesizer (PSSM-8, Shimadzu), following the supplier's protocol. For the synthesis of peptides with Pro at the carboxyl terminus, proline-bound Barlos resin rather than Alko resin was used to suppress the formation of diketopiperazines. The synthesized peptides were purified by RP-HPLC using a Cosmosil MS-II (10 mm i.d. $\times$ 250 nm; Nacalai Tesque). The binary gradient was used to elute the peptides using 0.1% formic acid (solvent A) and 0.1% formic acid containing 80% acetonitrile (solvent B) at 2.0 mL/min flow rate. The gradient program was as follows: 0–20 min; B 0–50%, 20–30 min; B 50–100%, 30–35 min; B 100%, 35–35.1 min; B 100–0%, 35.1–45 min; B 0%. The column was maintained at 40°C. The concentration of each peptide was determined by amino acid analysis after HCl hydrolysis, as described by Bidlingmeyer et al. (1984) with some modifications (Miyauchi et al., 2022), and used as a standard for peptide quantification.

Peptide quantification

The content of each peptide in the in vitro digests of CH and samples from the animal experiments was determined using the LC-MS/MS in multi-reaction monitoring (MRM) in positive ion mode following the AccQ derivatization described above. The AccQ derivatives were resolved by the Inertsil ODS-3 column using the same eluents as described above. The elution program was as follows: 0-15 min, 0-50% B; 15-20 min, 50-100% B; 20-25 min, 100% B; and 25-35 min, 0% B. MRM conditions were optimized by LabSolutions LCMS Ver. 5.5 (Shimadzu) using the synthetic peptides.

Statistical analysis

The data were analyzed using SPSS version 28 (IBM, New York, USA). Student's *t*-test was used to determine the difference between the means of the two groups. One Way ANOVA with Tukey post hoc test was used to compare more than two groups. A *p*-value less than 0.05 was considered a significant difference.

1.3 Results

Identification of peptides in collagen hydrolysate (CH)

The total ion chromatograms of peptides in the CH are shown in Figure 1-1. Compounds in major peaks of CH before digestion were estimated based on precursor and product ions, as shown in Supplemental Table 1. Identified amino acids and peptides are indicated by one-letter abbreviation of amino acid on the peaks (Figure 1-1). Pro-Hyp (PO), of which elution position is indicated by

asterisks, was not detected in the CH before further in vitro digestion, while some amino acids and dipeptides were detected in the scan range of m/z 100-300. Most dipeptides consisted of hydrophobic amino acids such as Leu, Ile, Phe, and Met at the carboxyl terminus. In addition, many peaks were detected in the scan range of m/z 300-1000 (Figure 1-1), corresponding to tripeptides, tetrapeptides, pentapeptides, and larger ones. These results reveal that the CH before digestion mainly consists of peptides larger than tripeptide and does not contain Pro-Hyp.

Exopeptidases activity of crude extract of rats small intestinal mucosa

The crude extract of mucosa had both LAP and prolidase activities at 5.80 and 5.85 U/mL, respectively.

Peptides in the in vitro digests of CH

CH was further digested with four different protease preparations: a single enzyme aminopeptidase N (APN); a mixture of enzymes including pancreatin, carboxypeptidase A (CPA), and leucine aminopeptidase (LAP); rat small intestinal mucosal extract; and rat blood plasma. Amino compounds in the digests were derivatized with AccQ and analyzed by LC-MS/MS in the precursor ion mode targeting the b1 ion of AccQ moiety, as shown in Figure 1-2. The one-letter abbreviations indicate the amino acid peaks. Amino acid derivatives less than m/z 325 (amino acids less than 154 Da) were also observed but not shown in Figure 1-2. In addition to amino acids, several peptide peaks were observed in all digests marked with numbers. Conversely, minimal peptide peaks were detected with m/z values exceeding 500 in all digests (data not illustrated).

While some amino acid peaks were present in the mucosa and plasma blanks (Figures 1-2.D and F), no peptide peaks were detected in these samples. Sequences of peptides in the peaks marked with numbers were estimated based on the precursor and product ions, as shown in Table 1-1. Unexpectedly, a prominent peak of Gly-Pro (peak 1) was observed in the digest of CH with APN and the mixture of pancreatin, CPA, and LAP (Figure 2.A and B). In contrast, Gly-Pro could not be observed in the digests of CH with rat small intestinal mucosa extract and blood plasma (Figure 2.C and E). Pro-Hyp (peak 7) was observed in all digests at different levels.

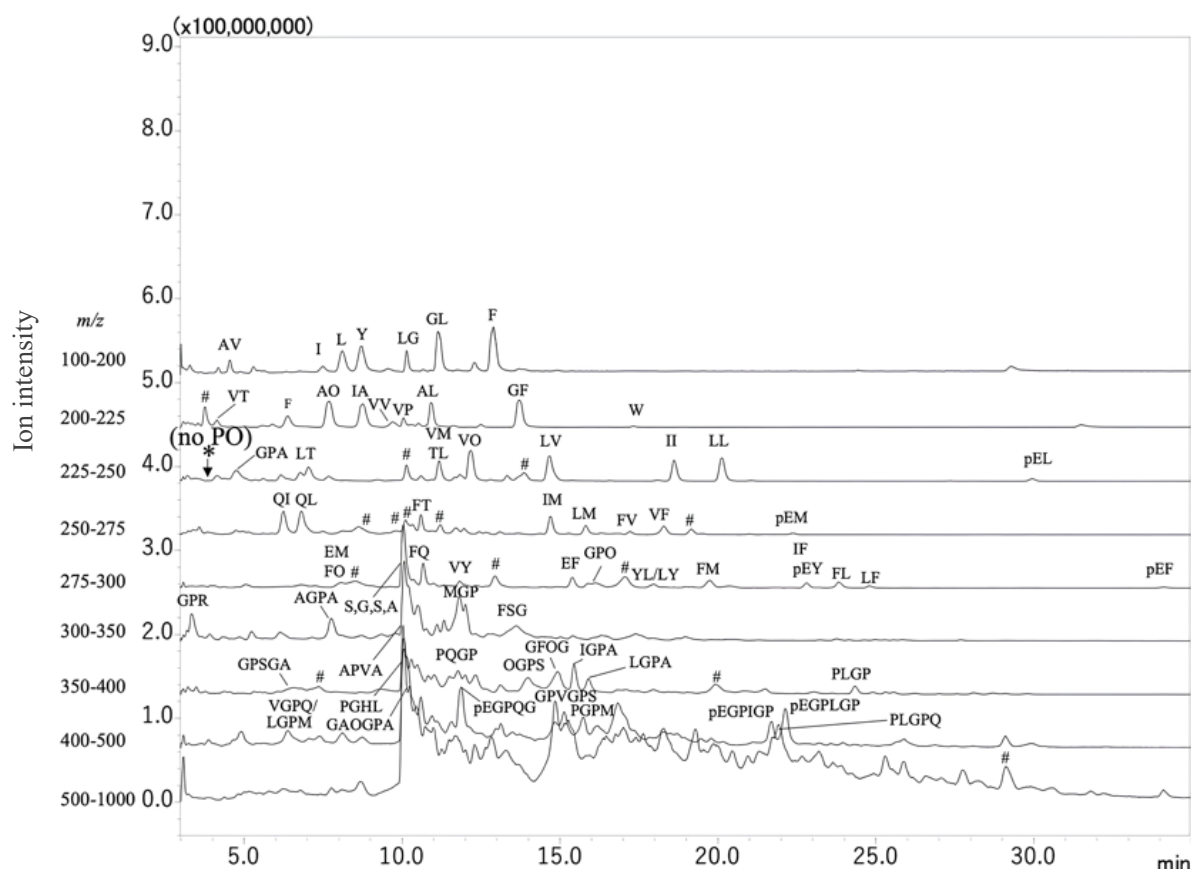


Figure 1-1. Mass spectrometry chromatograms of compounds in collagen hydrolysate (CH) before digestion

Non-derivatized compounds were resolved by reversed-phase high-performance liquid chromatography (RP-HPLC) and detected by a mass spectrometer (LC-MS/MS) in total ion scan at positive mode at different scan ranges across mass-to-charge ratio (m/z) 100–200, 200–225, 225–250, 250–275, 275–300, 300–350, 350–400, 400–500, and 500–1000.

Amino acids and peptides are indicated by a one-letter abbreviation for amino acids. O and pE represent hydroxyproline and pyroglutamic acid, respectively.

* Represents the position of the Pro-Hyp (PO) peak.

represents the peaks that could not be assigned to peptides with any combination of amino acids.

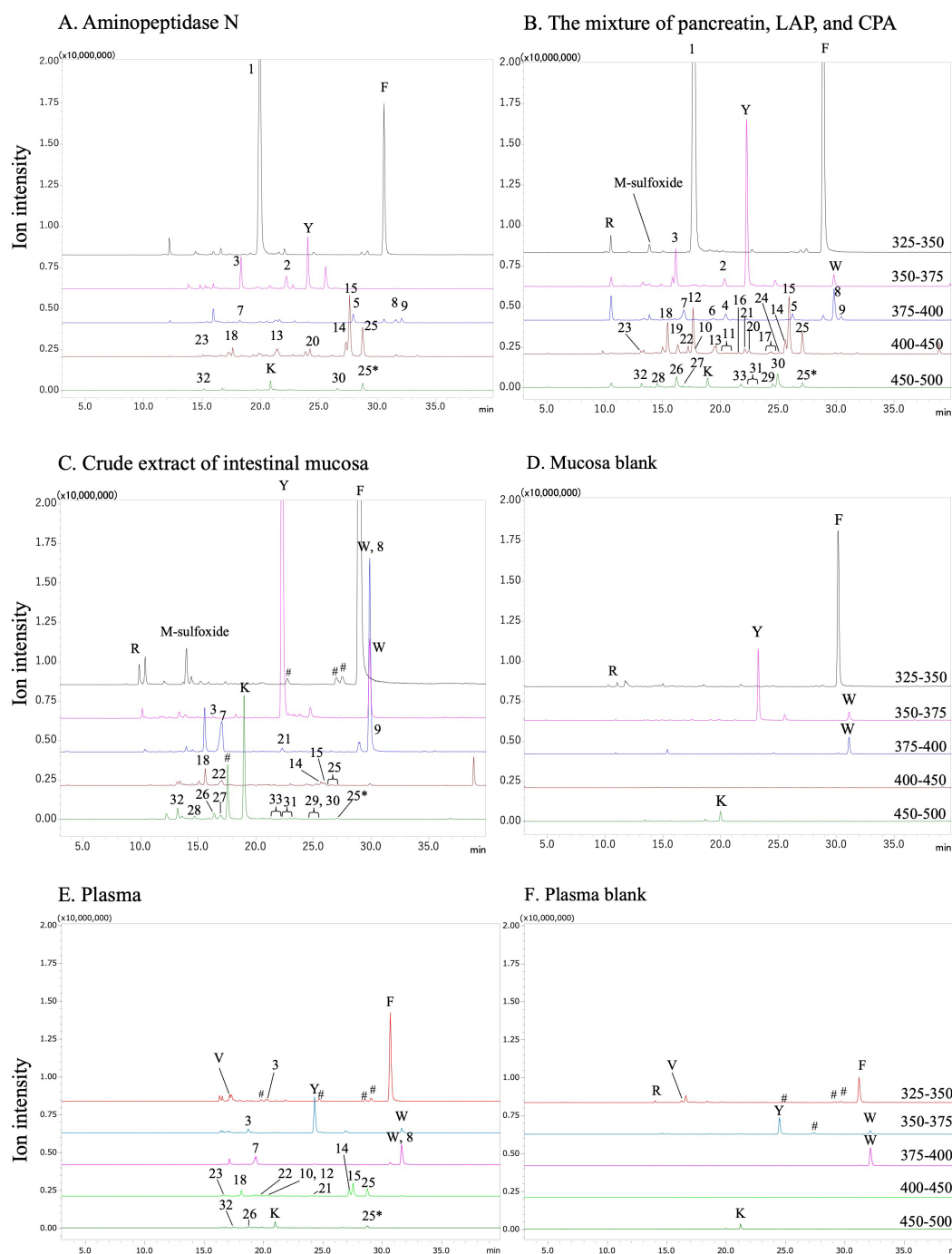


Figure 1-2. Mass spectrometry chromatograms of AccQ derivatives in digests of collagen hydrolysate (CH)

A: In vitro digest using aminopeptidase N, B: in vitro digest using a mixture of pancreatin, leucine aminopeptidases, and carboxypeptidases A, C: in vitro digest using crude extract of small intestinal mucosa of rats, D: crude extract of mucosa blank, E: in vitro digest using blood plasma of rats, and F: blood plasma blank. The AccQ-derivatives were specifically detected by LC-MS/MS in precursor ion scan targeting b1 ion of AccQ moiety ($m/z = 171$) in positive mode across m/z range of 325–250, 350–375, 375–400, 400–450, and 450–500. # represents the peaks that could not be assigned to peptides with any combination of amino acids.

Table 1-1. Summary of identified peptides in the digests of CH as shown in Figure 1-2

Peak	Precursor ion (<i>m/z</i>)	MW	Estimated sequence	Product ions (<i>m/z</i>)
1	343	172	Gly-Pro	116.1 (y1), 171.1 (AccQ, b1), 173.2 (y2), 228.1 (b2), 343.0 (y3)
2	357	186	Ala-Pro	44.2 (*Ala), 116.1 (y1), 171.1 (AccQ, b1), 187.1 (y2), 214.2, 242.0 (b2), 357.1 (y3)
3	373	202	Ala-Hyp	132.0 (y1), 171.1 (AccQ, b1), 203.1 (y2), 242.1 (b2), 373.1 (y3)
4	383	212	Pro-Pro	70.1 (*Pro), 116.2 (y1), 171.2 (AccQ, b1), 213.0 (y2), 268.1 (b2), 383.3 (y3)
5	385	214	Val-Pro	72.0 (*Val), 116.1 (y1), 171.0 (AccQ, b1), 215.0 (y2), 242.1, 270.1 (b2), 385.2 (y3)
6	387	216	Thr-Pro	74.3 (*Thr), 116.2 (y1), 171.2 (AccQ, b1), 217.1 (y2), 244.1, 272.3 (b2), 387.1 (y3)
7	399	228	Pro-Hyp	70.1 (*Pro), 86.1 (*Hyp), 132.2 (Hyp), 171.1 (AccQ, b1), 229.2 (y2), 268.1 (b2), 399.1 (y3)
8	399	228	Ile-Pro	70.1 (*Pro), 116.2 (y1), 145.2, 171.1 (AccQ, b1), 229.1, 256.1, 284.1 (b2), 399.2 (y3)
9	399	228	Leu-Pro	70.2 (*Pro), 116.1 (y1), 145.1, 171.3 (AccQ, b1), 229.2, 256.1, 284.1 (b2), 399.1 (y3)
10	401	230	Asp-Pro	70.1 (*Pro), 116.1 (y1), 171.0 (AccQ, b1), 213.1, 231.0 (y2), 286.1 (b2)
11	403	232	Asp-Val	72.0 (*Val), 88 (*Asp), 118.1 (y1), 145.2, 171.1 (AccQ, b1), 233.1 (y2), 259.1 (a2), 286.2 (b2), 403.1 (y3)
12	414	243	Gly-Pro-Ala	70.0 (*Pro), 127.1, 155.1, 171.2 (AccQ, b1), 187.3, 200.1, 228.2 (b2), 244.2, 414.4 (y4)
13	415	244	Glu-Pro	70.3 (*Pro), 84.1, 116.2 (y1), 145.0, 171.1, 227.1, 245.0 (y2), 272.1, 300.2 (b2), 415.1 (y3)
14	415	244	Ile-Hyp	86.1 (*Ile), 132.1 (y1), 171.0 (AccQ, b1), 245.0 (y2), 256.1, 283.9 (b2), 414.9 (y3)
15	415	244	Leu-Hyp	86.1 (*Leu), 132.1 (y1), 171.0 (AccQ, b1), 245.0 (y2), 256.1, 283.9 (b2), 414.9 (y3)
16	417	246	Leu-Asp	86.2 (*Ile/Leu), 88 (*Asp), 144.6, 170.9 (AccQ, b1), 256.1, 285.2, 416.9 (y3)
17	417	246	Met-Pro	56.3, 60.8, 104 (*Met), 115.8 (y1), 170.8 (AccQ, b1), 246.9 (y2), 273.9, 301.9 (b2), 416.7 (y3)
18	430	259	Ala-Hyp-Gly	44.4 (*Ala), 86.1 (*Hyp), 144.9, 171.0 (AccQ, b1), 189.0 (y2), 213.6 (a2), 242.1 (b2), 258.6 (y3), 430 (y4)
19	430	259	Gly-Pro-Ser	70.1 (*Pro), 88.3 (*Asp), 106 (y1), 127.0, 145, 171.1 (AccQ, b1), 200.1 (a2), 203.0 (y2), 227.8 (b2), 242.0, 260.0 (y3), 268.3 (b2), 429.7 (y4)
20	433	262	Me-Hyp	55.8, 86.5 (*Hyp), 104.3 (*Met), 132.0 (y1), 170.9 (AccQ, b1), 263.1, 302.3 (b2), 433.0 (y3)

21	442	271	Gly-Pro-Val	70.2 (*Pro), 72 (*Val), 116 (y1), 117.9 (y1), 145.0, 171.0 (AccQ, b1), 200 (a2), 215.3 (y2), 228 (b2), 272.1 (y3), 442.1 (y4)
22	444	273	Gly-Pro-Thr	70.0 (*Pro), 127.0, 171.0 (AccQ, b1), 186.9, 200 (a2), 217.0 (y2), 227.9 (b2), 256.1, 273.9 (y3), 444 (y4)
23	446	275	Ser-Hyp-Gly	60.1 (*Ser), 86.1 (*Hyp), 145.2, 171.0 (AccQ, b1), 189.8 (y2), 201.1, 229.6, 258.0 (b2), 275.9, 446.3 (y4)
24	449	278	Tyr-Pro	116.1 (y1), 136.2 (*Tyr), 171.0 (AccQ, b1), 278.8 (y2), 306.2 (a2), 334.1 (b2), 448.8 (y4)
25	449	278	Phe-Hyp	120.2 (*Phe), 132.0 (y1), 171.0 (AccQ, b1), 279.1 (y2), 289.9, 318.1 (b2), 448.9 (y4)
25*	450	279	Phe-Hyp*	120.1 (*Phe), 132.3 (y1), 144.6, 171.0 (AccQ, b1), 279.9 (y2), 290.5 (a2), 317.6 (b2), 449.7 (y4)
26	456	285	Pro-Hyp-Gly	70.3 (*Pro), 86.3 (*Hyp), 141.0, 171.1 (AccQ, b1), 189.5 (y2), 286.1 (y3), 455.9 (y4)
27	456	285	Gly-Pro-Hyp	30.1 (*Gly), 70.2 (*Pro), 132.1 (y1), 171.1 (accQ, b1), 229.1 (b2, y2), 286.2 (y3), 456.1 (y4)
28	460	289	Thr-Hyp-Gly	74 (*Thr), 86.1 (*Hyp), 145.1, 171.3 (AccQ, b1), 189.1 (y2), 244, 2 (a2), 71.7 (b2), 289.8 (y3), 459.3
29	472	301	Ile-Hyp-Gly	86.5 (*Ile), 171.0 (AccQ, b1), 188.9 (y2), 255.8 (a2), 283.7 (b2), 302 (y3), 472 (y4)
30	472	301	Leu-Hyp-Gly	86.2 (*Leu), 170.8 (AccQ, b1), 188.8 (y2), 226.4, 256.1 (a2), 283.9 (b2), 302 (y3), 471.8 (y4)
31	474	303	Gly-Pro-Met	70.0 (*Pro), 127.0, 155.2, 170.8 (AccQ, b1), 200.2 (a2), 227.7 (b2), 246.5 (y2), 285.3, 304.1 (y3), 473.1
32	488	317	Glu-Hyp-Gly	76.6, 85.2 (*Hyp), 102 (*Glu), 145.3, 170.6 (AccQ, b1), 189.3 (y2), 224.8, 272.1 (a2), 300.0 (b2), 487.8 (y4)
33	490	319	Met-Hyp-Gly	55.7, 85.5 (*Hyp), 104.3 (*Met), 131.9 (y1), 170.7 (AccQ, b1), 188 (y2), 273.7, 301.6 (b2), 319.4, 489.6 (y4)
34	506	335	Phe-Hyp-Gly	86.0 (*Hyp), 119.8 (*Phe), 145.2, 170.9 (AccQ, b1), 189 (y2), 289.7, 317.6 (b2), 335.8 (y3), 505.3
35	359	188	Hyp-Gly	70.3 (*Pro), 86 (*Hyp), 171.1 (AccQ, b1), 189.2 (y2), 359.1 (y3)
36	431	260	Glu-Hyp	76.6, 85.2 (*Hyp), 102 (*Glu), 132.1 (y1), 145.3, 170.6 (AccQ, b1), 261.2 (y2), 431.1 (y3)

All estimated peptides were quantified using LC-MS/MS in MRM mode. The peptide contents in CH digests are shown in Figure 1-3. After in vitro digestion of CH using four different protease preparations, di- and tripeptides containing Hyp (O) and Pro (P) were generated. Digestion with APN and the mixture of pancreatin, CPA, and LAP predominantly generated Gly-Pro with smaller contents of Hyp-containing peptides such as Pro-Hyp and some non-Hyp-containing dipeptides with a proline residue at the carboxyl terminus (Glu-Pro, Pro-Pro, Asp-Pro, etc.) (Figure 1-3.A and B). Conversely, digestion using crude mucosa extract yielded a negligible amount of Gly-Pro but predominantly Pro-Hyp (Figure 1-3.C), along with smaller quantities of Hyp-containing di- and tripeptides. Minimal amounts of non-Hyp-containing peptides with a proline residue at the carboxyl terminus (X-Pro) were observed. Digestion of CH with plasma preparation also predominantly resulted in Pro-Hyp and smaller amounts of other Hyp-containing peptides, resembling mucosa digestion. However, small but detectable quantities of non-Hyp peptides with Pro at the carboxyl terminus, such as Gly-Pro, Glu-Pro, and Asp-Pro, were generated (Figure 1-3.D). Additionally, the digestion with plasma preparation generated a larger content of Pro-Hyp than that with mucosa extract.

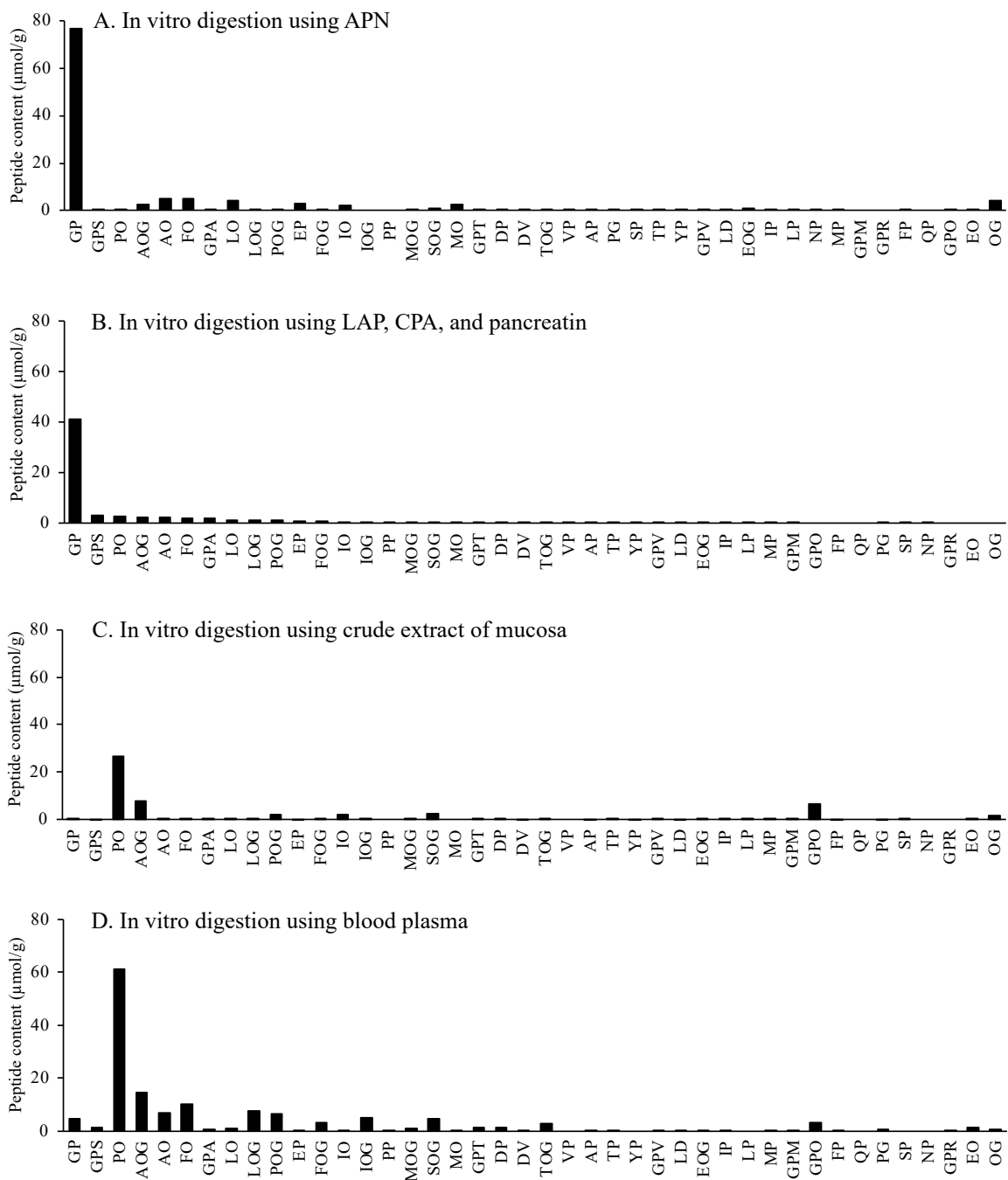


Figure 1-3. Peptide contents in different in vitro digests of CH

A: CH digest using APN, B: CH digest using a mixture of pancreatin, LAP, and CPA, C: CH digest using crude extract of small intestinal mucosa of rat, and D: CH digest with blood plasma of rat. All peptides were quantified by LC-MS/MS in multi-reaction monitoring mode (MRM). Estimated peptides were chemically synthesized and used for the optimization of MRM and external standards.

Food-derived peptides in rats after CH ingestion

CH was administered to rats by sonde. Peptides in the lumen, small intestinal tissue, and plasma were analyzed by LC-MS/MS in MRM mode. Figure 1-4 shows that Pro-Hyp was predominantly generated in the lumen and small intestinal tissue. In the lumen, Hyp-containing peptides significantly increased 1 h after ingestion. In addition, some non-Hyp-containing dipeptides, including Gly-Pro, also significantly increased at low content. However, in the intestinal tissue, Gly-Pro and other dipeptides with Pro at the carboxyl terminus (X-Pro) did not significantly increase.

Furthermore, in blood from the inferior vena cava, only Pro-Hyp, Ala-Hyp, Leu-Hyp-Gly, Glu-Hyp, and Hyp-Gly significantly increased after ingestion of CH. Pro-Hyp was not only the predominant peptide in the blood before the administration of CH but also increased most significantly after the administration compared to other Hyp-containing peptides, such as Pro-Hyp-Gly, Hyp-Gly, etc. Increased collagen peptide profiles in rats' small intestinal tissue and blood are similar to peptide profiles in CH digest with mucosal extract.

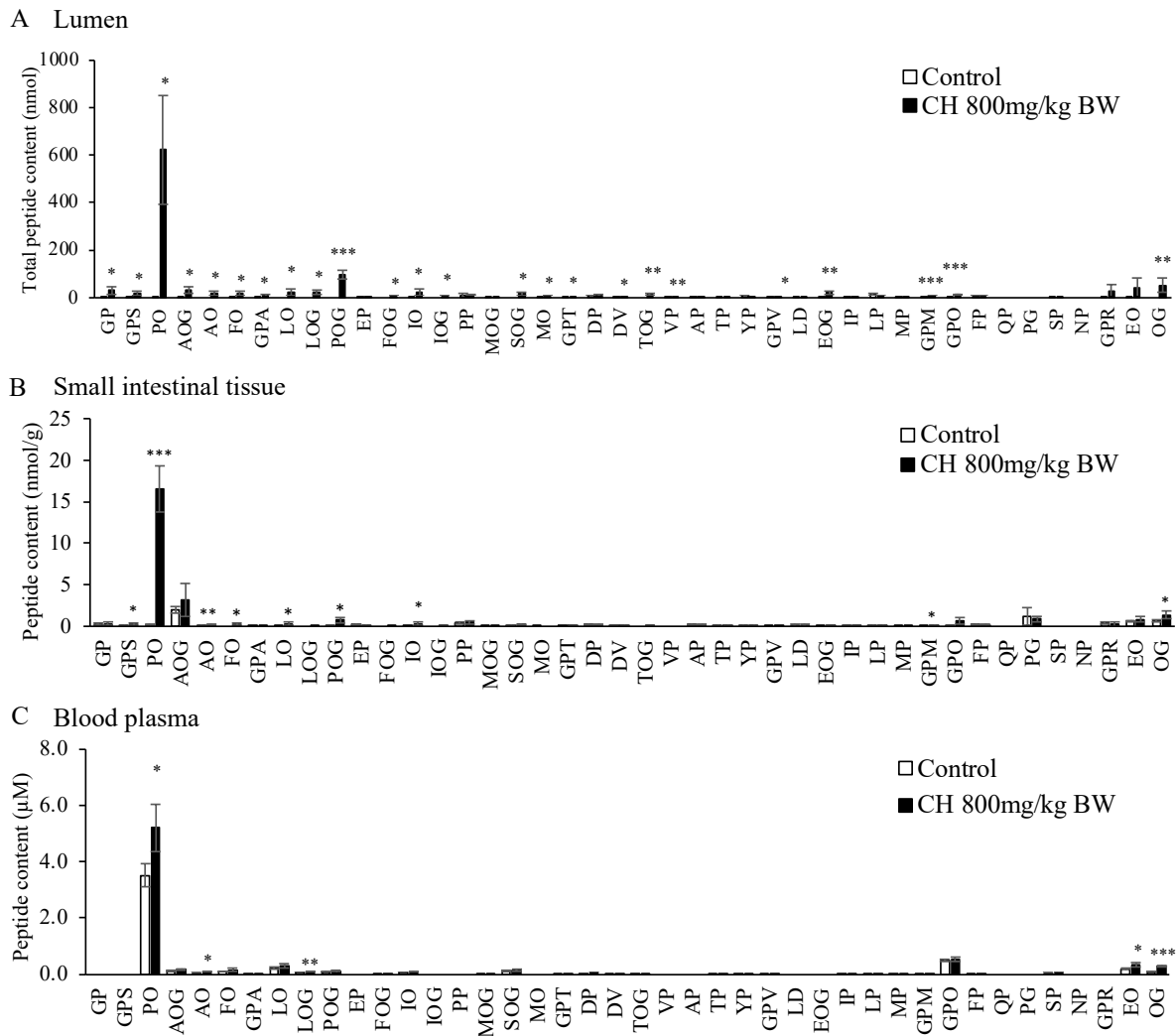


Figure 1-4. Peptide contents in the lumen (A), small intestinal tissue (B), and blood plasma (C) of rats after ingestion of CH

The collagen group was administered CH solution at 800 mg/kg BW, and the control group with 300 μ L water. After 1 h, the rats were sacrificed, and the peptides were extracted from the lumen, small intestinal tissue, and plasma. All peptides were derivatized using AccQ and quantified by LC-MS/MS in MRM mode. The results are presented as the mean $\pm$ SD. Asterisks (***), (**), and (*) represent significant differences, $p < 0.001$, $p < 0.01$, and $p < 0.05$, respectively, between the collagen group and control group by Student's t-test ($n = 3$).

1.4 Discussion

It has been demonstrated that food-derived collagen peptides such as Pro-Hyp, Hyp-Gly, etc. are circulating in human blood at relatively high concentrations ($> \mu\text{M}$ levels) for a few hours after ingestion of CH at 80-400 mg/kg body weight (Asai et al., 2019; Iwai et al., 2005; Ohara et al., 2007; Shigemura et al., 2018; Taga et al., 2014). Compared to humans, a lower concentration of collagen-derived peptides, including Pro-Hyp, was detected in rat blood 1h after ingestion of a higher dose of CH at 800 mg/kg (Figure 4), which is consistent with other animal experiments (Hanh et al., 2017; Kawaguchi et al., 2012; Wang et al., 2015). However, collagen peptide composition in the rat lumen and small intestinal tissue is similar to that observed in human blood. These facts indicate that peptides in CH are similarly digested by exopeptidases in the small intestinal lumen of humans and rats. In rats, the remaining peptides are, however, rapidly degraded when passing through the enterocyte to blood circulation and incorporated into cells from blood circulation, which may rapidly decrease blood levels of collagen-derived peptides in rats compared to humans.

Pro-Hyp and Hyp-Gly, the major food-derived collagen peptides in human blood, can be derived from the Gly-Pro-Hyp-Gly motif in larger collagen peptides by gastrointestinal digestion. After CH ingestion, Pro-Hyp reached $25 \mu\text{M}$ in human blood plasma, while Hyp-Gly at $3.5 \mu\text{M}$ and lower for other Hyp-peptides (Asai et al., 2019). As mentioned above, CH ingestion has little impact on Gly-Pro levels in human blood plasma, while Gly-Pro is potentially generated from the same motif. The present study also found that oral administration of CH did not impact Gly-Pro plasma levels in the rats (Figure 1-4). In addition, only a slight increase of Gly-Pro was observed

in the rat lumen. Furthermore, a single administration of Gly-Pro also did not impact the blood level of Gly-Pro (Wijanarti et al., 2024). Ejima et al. (2018) also reported that peptides with Pro residue at the carboxyl terminus (X-Pro) are degraded during the in vivo digestion and absorption process, while these X-Pro peptides resist digestion with LAP. The present in vitro digestion of CH by crude extract of rat's small intestinal mucosa generated only negligible amounts of X-Pro peptides, including Gly-Pro. In contrast, LAP, CPA, and APN predominantly generated Gly-Pro, which is consistent with the results by Ejima et al. (2018). These facts indicate that X-Pro peptides, including Gly-Pro, are degraded by exopeptidase in the small intestinal mucosa except for LAP, APN, or CPA. The digestion with blood plasma generated a peptide composition similar to that of mucosa digest with detectable amounts of Gly-Pro. The composition of the remaining peptides in the mucosa and plasma digests of CH is similar to that of collagen-derived peptides in human and rat blood (Asai et al., 2019; Iwai et al., 2005; Shigemura et al., 2011; Shigemura et al., 2018; Yamamoto et al., 2016).

The combination of LAP, CPA, and APN proved effective in degrading most peptides in CH (Figures 1-2. A and B) and other protein hydrolysates (Ejima et al., 2018; Chen et al., 2019), except for X-Pro dipeptides. However, X-Pro dipeptides can be degraded by prolidase. Thus, most peptides in the lumen are degraded into amino acids through sequential cleavage of exopeptidases. The combination of these exopeptidases can act as an intestinal blood barrier to food-derived peptides. Therefore, to get a reliable prediction of the structure of food-derived peptides, it is necessary to carefully design in vitro digestion using exopeptidases of small intestinal mucosa with different substrate specificities, such as LAP, CPA, APN, and prolidase.

In addition to Hyp-containing peptides, Pro-Gly can resist the digestion of these peptidases and increase in human blood more than μM levels (Shigemura et al., 2012), while Gly-Pro cannot. The reason why Pro-Gly resists the exopeptidases remains to be solved. Other modified peptides, such as pyroglutamyl and beta aspartyl-isopeptides, have been reported to significantly increase in the intestinal lumen and blood upon ingestion of protein hydrolysates (Ejima et al., 2019; Miyauchi et al., 2022) due to resistance to intestinal peptidases (Miyauchi et al., 2022).

Until now, the Caco-2 monolayer system has been used for in vitro simulation of intestinal absorption. However, Gly-Pro has been reported to pass through the Caco-2 cell monolayer as an intact form (Enjoh et al., 1996; Ganapathy & Leibachs, 1983; Rohm et al., 2019). Enterocytes secrete various exopeptidases, which accumulate in the brush border membrane and mucus layer for digestive purposes (Gustafsson et al., 2011; Hiwada et al., 1981; Johansson et al., 2011; Lundquist & Artursson, 2016; Rodríguez-Piñeiro et al., 2013). However, previous studies reported that compared to enterocytes in the human small intestine, the Caco-2 monolayer has a lower expression level of endoproteinases and exopeptidases in the brush border membrane (Lundquist & Artursson, 2016) and a lack of mucus layer (Frey et al., 1996). Taken together, some Caco-2 monolayers lack some exopeptidases, especially prolidase. Thus, some Caco-2 mono-cell layer cannot be used for the prediction of the bioavailability of peptides. Careful consideration regarding the transporter and the expression of exopeptidases is necessary for all in vitro absorption models. Similarly, for the in-silico digestion approach, the substrate specificity of exopeptidases, especially prolidase, should be considered to predict the bioavailability of peptides. Neglecting these factors may lead to erroneous results.

1.5 Conclusion

In the rat lumen, LAP, APN, and CPA degrade most food-derived peptides into amino acids but leave X-Pro dipeptides intact. Notably, X-Pro dipeptides, including Gly-Pro, are preferentially degraded by prolidase in the lumen. In contrast, Hyp-containing peptides resist both types of exopeptidases, which can explain the high bioavailability of Hyp-containing peptides such as Pro-Hyp and Hyp-Gly. To predict the bioavailability of food-derived peptides, both types of exopeptidases must be taken into consideration. The in vitro and in silico systems, without considering both types of exo peptidases, may lead to fallacious results.

Chapter 2

The identification of exopeptidases in the small intestinal mucosa of rats

2.1 Introduction

Endoproteinases and exopeptidases have been identified in the small intestines, such as trypsin, pepsin, leucine aminopeptidase (LAP), aminopeptidase A (APA), aminopeptidase B (APB), aminopeptidase N (APN), prolidase, dipeptidyl peptidase 3 (DPP3), dipeptidyl peptidase 4 (DPP4), etc. (Gustafsson et al., 2011; Hiwada et al., 1981; Johansson et al., 2011; Lundquist & Artursson, 2016; Rodríguez-Piñeiro et al., 2013). In vitro digestion and in silico systems have been developed by combining several digestive enzymes to simulate gastrointestinal digestion and predict the bioactive peptides from food proteins. However, until recently, only endoproteinases, such as pepsin, trypsin, chymotrypsin, or crude protease preparation (pancreatin or gastric juice), are considered in the in vitro digestion simulations (Aléman et al., 2013; Bai et al. 2023; Brodkorb et al., 2019; Feng and Betti, 2017; Guo et al., 2014; Larder et al., 2021; Yang et al., 2019). Therefore, some inconsistencies are often observed between in vitro and in vivo results (Amigo et al., 2020). Chen et al. (2019) reported that specific peptides resist endoproteinases but are readily cleaved by exopeptidases. Thus, the present author hypothesized that exopeptidases play crucial roles in determining the bioactive peptides upon the ingestion of food proteins. The role of exopeptidases in intestinal mucosa in determining the bioavailability of collagen peptides is reported in Chapter 1. Unexpectedly, the digestion of CH by a combination of well-known intestinal exopeptidases, such as LAP and CAP, with an endoprotease pancreatin, generated a

peptide profile that extensively differed from the collagen peptide profile in blood upon ingestion of CH. On the other hand, digestion of CH with crude extract of rat small intestinal mucosa generated a similar peptide profile to that in human and rat blood plasma after CH ingestion. These results suggest that CH was degraded similarly during *in vitro* digestion using crude extract of mucosa and in the gastrointestinal tract of humans and rats, indicating the similarity of enzyme composition. Furthermore, the results in Chapter 1 indicate the presence of prolidase in the crude extract of intestinal mucosa. This chapter aimed to identify the exopeptidases in the crude extract of small intestinal mucosa, especially confirming the presence and role of prolidase in the digestion of short-chain peptides.

Consequently, prolidase and APN were successfully identified at the protein level in this chapter. Furthermore, Gly-Pro and other dipeptides with Pro at the carboxyl terminus (X-Pro) resist some purified endoproteinases (trypsin and chymotrypsin) and some purified exopeptidases (LAP, CPA, and APN) but can be cleaved by purified prolidase. Interestingly, Pro-Hyp partially resisted prolidase degradation even after 4 h digestion, but Gly-Pro was degraded entirely in 0.5 h. These results confirmed that prolidase is present in the crude extract of small intestinal mucosa and plays an essential role in determining the bioavailability of CH-derived peptides. Prolidase is responsible for digesting Gly-Pro and other X-Pro dipeptides in the final stage of digestion, which LAP, CPA, and APN cannot digest.

2.2 Materials and Methods

Reagents

Tris(hydroxymethyl)aminomethane (Tris), acetonitrile (HPLC-grade), phosphate-buffered saline pH 7.4 (PBS, 10×), and porcine pancreatin were purchased from Nacalai Tesque (Kyoto, Japan). Lysis buffer (CellLytic), L-leucine p-nitroanilide (Leu-pNA, substrate for EC 3.4.11.2), and Coomassie Blue R-250 were purchased from Sigma-Aldrich (St. Louis, MO). Gly-Pro and Pro-Hyp were purchased from Bachem (Bubendorf, Switzerland). A dried acrylamide to concentrate the sample (Mizubutorikun) and 12.5% polyacrylamide gel were purchased from Atto (Tokyo, Japan). The prestained XL-Ladder broad range was purchased from Pharma Foods Apro Science Group (Tokushima, Japan). Pierce trypsin protease was purchased from Pierce (Waltham, MA, USA). All other reagents were of analytical grade or higher.

Preparation of crude extract of small intestinal mucosa

The crude extract of small intestinal mucosa was prepared from rats (Wistar ST, 6-week-old, n=3) using the method described in Chapter 1. The experiment procedures were approved by the Animal Care Committee of the Louis Pasteur Center for Medical Research (Kyoto, Japan, with approval number 20221).

Size exclusion chromatography (SEC)

Size exclusion chromatography (SEC) was used to partially purify proteins in crude mucosa. Two hundred microliters of crude extract of mucosa were filtered by passing through a centrifugal filter

(5.0 μm pore size, Ultrafree-MC UFC30SV00, Merck Millipore, Darmstadt, German) at 3,000 $\times g$ for 5 min before the injection. Two hundred microliters of the clarified sample were injected into a Superdex TM 75 10/300 GL (10 mm $\times$ 30 cm, GE Healthcare, Chicago, IL, USA) equilibrated with PBS at 0.5 mL/min flow rate. The elution of proteins was monitored by absorbance at 280 nm. Fractions were collected every 1 min for 60 min.

Anion exchange chromatography (AEC)

The crude extract of mucosa was also partially purified using anion exchange chromatography using a Cosmogel IEX Type Q (4.6 mm $\times$ 50 mm, Nacalai Tesque) equilibrated using 10 mM Tris HCl buffer, pH 7.5, at 0.5 mL/min flow rate. After injection of the sample (200 μL), proteins were eluted with a binary gradient of NaCl in the buffer: 0-15 min, NaCl 0-250 mM, 15-20 min, NaCl 250-500 mM, 20-25 min, NaCl 500 mM at 0.5 mL/min. The elution of proteins was monitored by absorbance at 280 nm. Fractions were collected every 1 min for 35 min.

Leucine aminopeptidase (LAP) activity assay

LAP activity was measured using L-leucine-p-nitroanilide (Leu-pNA) as substrate according to the supplier test protocol (Sigma-Aldrich) with some modifications. Leu-pNA was dissolved in methanol to give a concentration of 1.2 mM. PBS (90 μL) was mixed with 5 μL of the sample and 5 μL of the substrate solution. The absorbances at 405 nm were immediately recorded before and after 5 min incubation at 37 $^{\circ}\text{C}$. One unit was defined as the enzymes hydrolyzing 1.0 μmole of

Leu-pNA to Leu and pNA per minute under assay conditions. The enzyme activity (EA) was expressed as a unit/mL, which can be obtained by the following equation:

$$EA = \frac{(\Delta\text{absorbance at 405/min of sample} - \Delta\text{absorbance at 405/min of blank}) \times \text{total volume (0.1 mL)} \times \text{dilution factor (20)}}{9.9 \times \text{volume of enzyme (mL)}}$$

Where 9.9 was the millimolar extinction coefficient of p-nitroaniline at 405 nm.

Aliquots (100 μ L) of some SEC fractions were combined, as shown in Figure 2-1. Aliquots (5 μ L) of the combined fractions were used. For AEC fractions, an aliquot (5 μ L) of each fraction was used.

Prolidase activity assay

Prolidase activity was measured using the same method as described in Chapter 1. SEC and AEC fractions were assayed as mentioned above.

CH digestion using partially purified crude extract of mucosa

The amount of 80 μ L of 2.5 mg/mL CH in PBS was digested separately with aliquots (20 μ L) of SEC fractions A-G. The digestions were carried out at 37 °C for 4 h. Then, three volumes of ethanol were added to stop the reaction. The mixtures were centrifuged at 13,000 $\times g$ for 5 min, and the peptide contents in the supernatants were quantified as described in the subsequent section.

Digestion of synthetic Gly-Pro and Pro-Hyp by partially purified crude extract of the mucosa

To remove low molecular weight compounds, especially amino acids and peptides, exopeptidases in the crude extract of mucosa were partially purified using size exclusion chromatography (SEC),

as mentioned above. Synthetic Gly-Pro and Pro-Hyp (400 μ M) in 400 μ L of PBS were digested with the partially purified mucosa exopeptidase preparation with LAP and prolidase activity 0.01 U (Peak A in Figure 2-1). The mixture was incubated at 37 °C. Aliquots (50 μ L) were withdrawn at 0, 30, 60, 90, and 240 min of incubation, and released Pro was quantified as described by Bidlingmeyer et al. (1984) with some modifications (Miyauchi et al., 2022) without HCl hydrolysis. The degradation (%) was calculated using the following equation:

$$\text{Degradation rate (\%)} = \frac{\text{free Pro content in the supernatant (nmol)}}{\text{peptide concentration (20 nmol)}} \times 100$$

Peptide quantification

The content of each peptide in the in vitro digests of CH was determined using the LC-MS/MS in multi-reaction monitoring (MRM) in positive ion mode following the AccQ derivatization. For AccQ derivatization, 20 μ L of digest were dried in a microcentrifuge tube (1.5 mL) under vacuum and added with 20 μ L of water, 60 μ L of 50 mM sodium borate buffer, pH 8.8, and 20 μ L of 0.3% AccQ-acetonitrile solution. The reaction was carried out at 50 °C for 10 min. The reactant was clarified using a Cosmonice filter. The aliquots (10 μ L) were injected into LC-MS/MS. The AccQ derivatives were resolved by the Inertsil ODS-3 column using a binary linear gradient using 0.1% formic acid (solvent A) and 0.1% formic acid containing 80% acetonitrile (solvent B) at 0.2 mL/min. The elution program was as follows: 0-15 min, 0-50% B; 15-20 min, 50-100% B; 20-25 min, 100% B; and 25-35 min, 0% B. MRM conditions were optimized by LabSolutions LCMS

Ver. 5.5 (Shimadzu) using the synthetic peptides. Synthetic peptides, which have been prepared by the study in Chapter 1, were used for the optimization of MRM and external standards.

Protein identification

Aliquots of fractions (30 μ L) obtained by SEC and AEC were mixed 10 μ L of 4 $\times$ Laemmli's buffer consisting of 0.025 M Tris-HCl buffer, pH 6.8, 0.02% of bromophenol blue, 40% glycerol and 8% of SDS and heated at 98 $^{\circ}$ C for 5 min then electrophoresed using a 12.5% polyacrylamide gel in a Rapid Mini Slab Electrophoresis Tank AE-6500 (ATTO, Tokyo, Japan) and then stained with 0.25% Coomassie Blue R-250 (Sigma, Japan) in 10% acetic acid and 45% of ethanol. In some cases, the sample was concentrated by using dried acrylamide powder (Mizubutorikun, Atto) before mixing with Laemmli's buffer. Standard markers (molecular mass range 10-210 kDa; Pharma Foods Apro Science Group, Tokushima, Japan) were used. The protein bands and gel with no band (blank) were excised, and proteins in the gel were digested with trypsin by the method of Santoni et al. (2003) with minor modifications. Briefly, the selected protein bands were excised and cut into small pieces (approximately 1-2 mm) and then washed successively in 5% acetic acid containing 50% methanol overnight and then pure acetonitrile for 5 min. Samples were dried in a centrifugal concentrator under vacuum. For the reduction and alkylation of cystine residues, gel pieces were incubated with 10 mM dithiothreitol (DTT) in 100 mM NH_4HCO_3 for 30 min and then with 100 mM iodoacetamide (IAA) in 100 mM NH_4HCO_3 for 30 min at room temperature. The gel pieces were dehydrated in pure acetonitrile for 5 min, reswollen in 100 mM NH_4HCO_3 for 5 min, dehydrated in acetonitrile again for 5 min, and then completely dried under vacuum. Finally,

the alkylated protein in the gel pieces was digested with 50 μ L of 0.01 mg/mL trypsin (Pierce) in 100 mM NH_4HCO_3 for 24 h at 37 °C. The supernatant of the tryptic digest was collected, and the remaining tryptic peptides in gel pieces were extracted twice in 5% formic acid containing 50% acetonitrile for 10 min with occasional gentle agitation. The supernatants were pooled, and the volume was reduced to 5 μ L under vacuum. The final volume was adjusted to 10-20 μ L by adding 1% acetic acid. Tryptic peptides were analyzed by LC-MS in total ion scan mode using an LCMS-8040 (Shimadzu, Kyoto, Japan) equipped with an Inertsil ODS-3 column (2.1 mm $\times$ 250 mm). The mass-to-charge ratio (m/z) of tryptic peptides in the samples, which were not observed in the blank, were selected. Protein in gel was identified based on the m/z of its tryptic fragments using an open access software MS-Fit (<https://prospector.ucsf.edu/prospector/mshome.htm>, University of California, San Francisco, UCSF). For confirmation, the tryptic fragment peptides were sequenced by LC-MS/MS in product ion scan mode at positive mode. Different collision energies were used depending on the m/z of the precursor ions (-13 eV for $m/z < 320$, -15 eV for $320 < m/z < 400$, -18 eV for $400 < m/z < 500$, -22 and -24 eV for $500 < m/z < 600$, -24 and -26 eV for $600 < m/z < 700$). The product ion fragments of each tryptic peptide were matched with the database via MS-Fit.

2.3 Results

Fractionation of exopeptidases in crude extract of mucosa by chromatography

Exopeptidases in the crude extract of mucosa were fractionated based on their molecular size and charge using SEC and AEC, respectively. Figure 2-1 shows the chromatogram of SEC. LAP is a well-known mammalian exopeptidase, and prolidase activity was detected in a crude extract of rats' small intestinal mucosa (Chapter 1). Therefore, the activity of LAP and prolidase were measured to determine the active fractions. The highest LAP and prolidase activities were observed in a large peak after void volume (Peak A), while other peaks (B-G) show relatively low exopeptidase activities. Furthermore, CH was digested with peaks A-G, and the peptides were detected by LC-MS/MS in MRM mode, as used in Chapter 1. As shown in Figure 2-2, peak A generated Pro-Hyp and other Hyp-containing peptides. The other peaks (B-G) did not generate these Hyp-containing peptides, but Gly-Pro-Arg, Gly-Pro-Ala, and Gly-Pro-Ser were predominantly detected; these peptides were present in CH before the digestion (Figure 1-1), which also indicates that peak B-G has negligible exopeptidase activity. Figure 2-3 shows that Fr. 16 of peak A showed the highest LAP activity. Based on the elution volumes of standard proteins obtained from the supplier, the molecular weight of Fr. 16 is estimated to be approximately 110 kDa. The exopeptidase in fraction 16 was identified in the subsequent section.

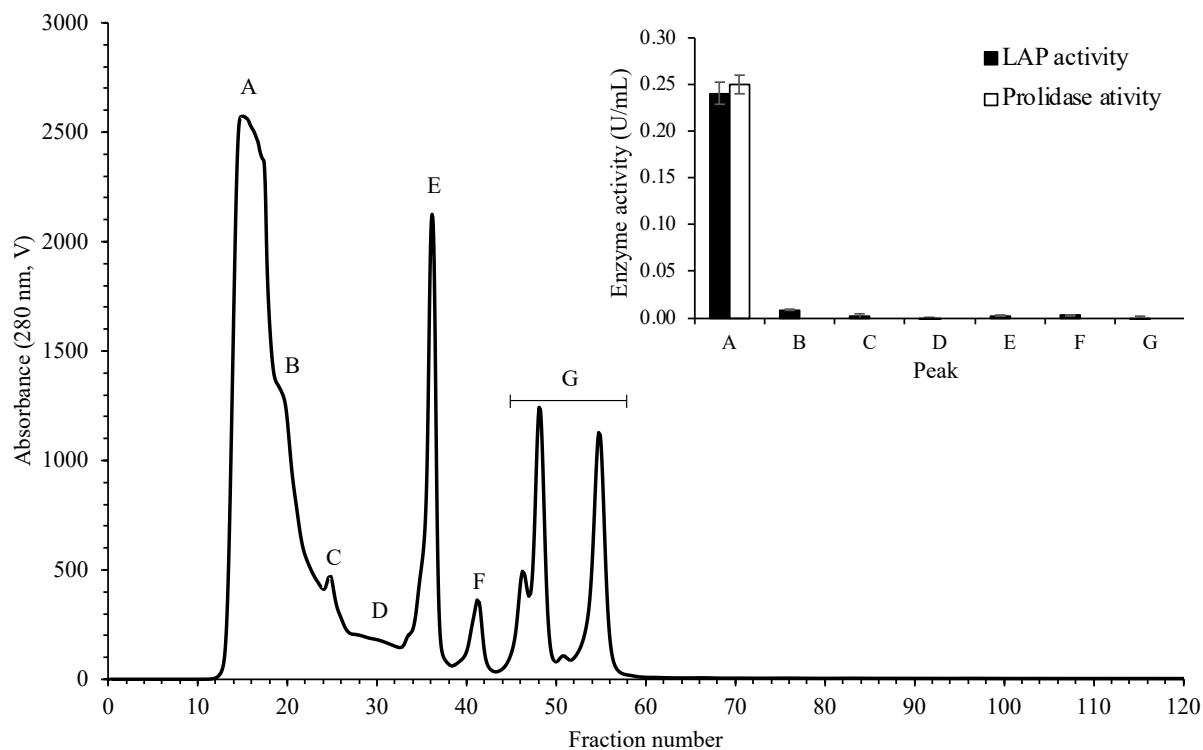


Figure 2-1. Protein separation in crude extract of mucosa using size exclusion chromatograph using Superdex 75 10/300 GL column

Peak A showed the highest exopeptidase activities and was selected for synthetic peptide digestion.

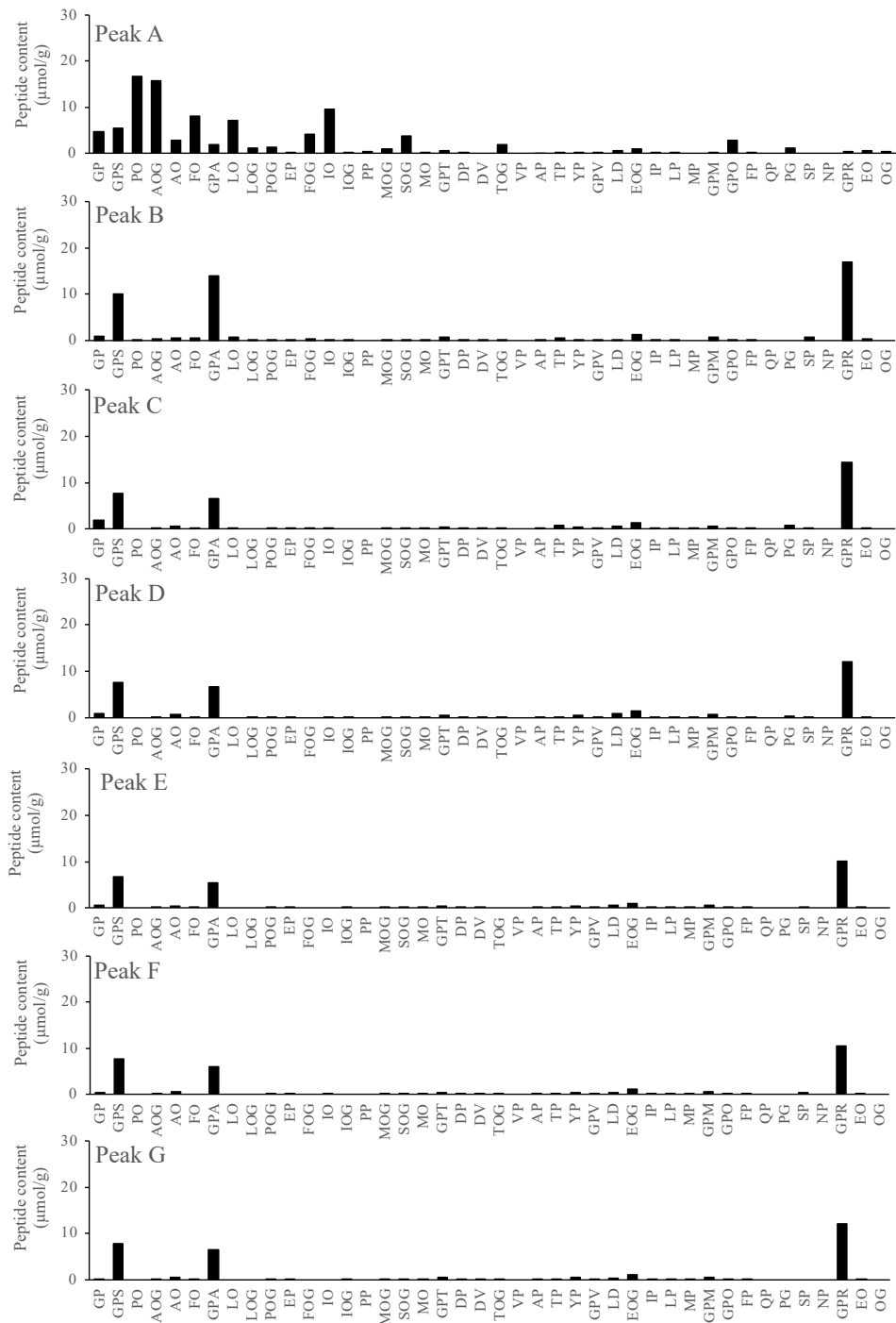


Figure 2-2. Peptide profiles in the CH digests after in vitro digestion using partially purified crude extract of mucosa (peak A-G)

Peak A generated a peptide profile similar to a crude extract of mucosa, while other peaks did different patterns.

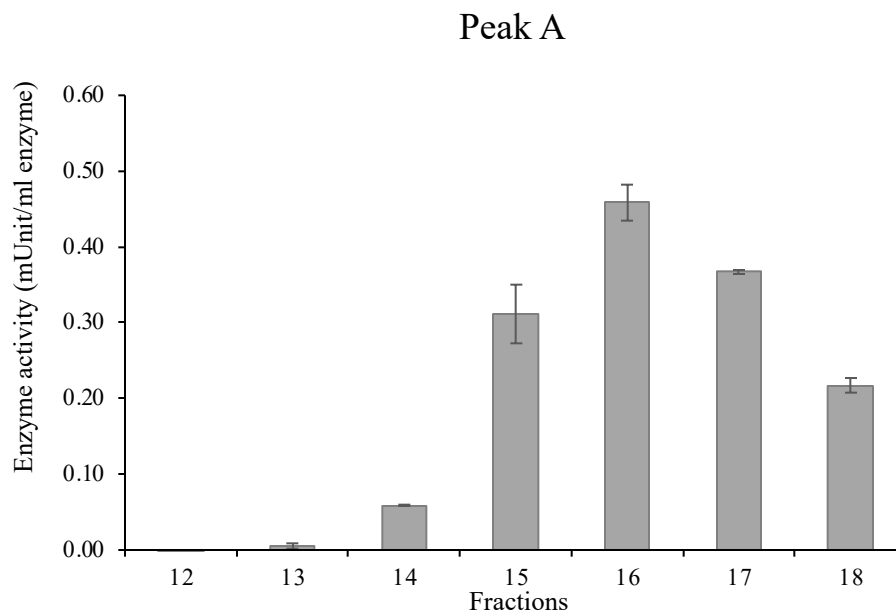


Figure 2-3. Leucine aminopeptidase (LAP) activity of peak A (Fr.12—Fr.18) after SEC
Fr. 16 showed the highest LAP activity. Thus, Fr. 16 was selected for protein identification.

As SEC could not resolve LAP and prolidase activities, proteins in the crude extract were fractionated by anion exchange chromatography (AEC). The chromatogram of AEC, prolidase activity, and LAP activity of each fraction are shown in Figure 2-4. Unlike the previous separation using SEC, this technique successfully separated LAP and prolidase activities in the crude extract of the mucosa. LAP activity was eluted in Fr. 11-13, while exopeptidase with prolidase activity was eluted in Fr. 15-18. Thus, Fr.12 and Fr. 16 were selected for identification protein as these fractions showed the highest enzyme activity for each type of exopeptidase.

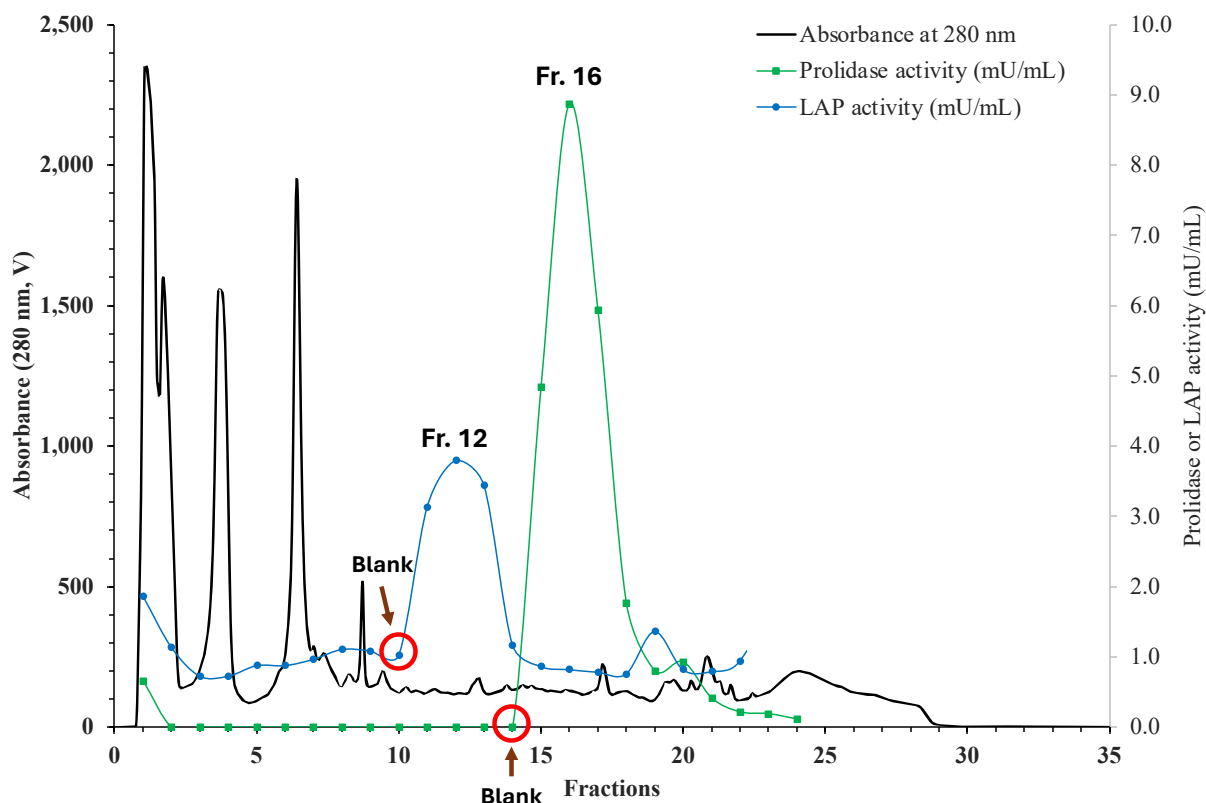


Figure 2-4. Separation of LAP and prolidase in crude mucosa extract using anion exchange chromatography

Identification of exopeptidases in chromatographic fractions

SEC Fr. 16, AEC Fr. 12 and 16 were selected for protein identification. The proteins in these fractions were further resolved by SDS-PAGE. SDS-PAGE patterns of SEC Fr. 15-18 are shown in Figure 2-5. The protein bands with a molecular mass of around 100-140 kDa in SEC Fr. 16 with the highest LAP activity were selected, which are indicated by the arrows and alphabets (a, b, and c). Fr. 15, 16, and 18 were used as comparison only as these fractions have lower LAP activity. The selected protein bands were excised and digested using trypsin. For the blank, the clear gel area was excised and digested in the same manner as the samples. Resultant tryptic

peptides were resolved and detected by LC-MS in total ion scan mode over the ranges of m/z 240-350, 350-450, 450-550, 550-650, 650-750, 750-900, and 900-1200. The total ion chromatograms of tryptic digests of band a and blank band in Figure 2-5 are shown in Figure 2-6, and other chromatograms of other bands are provided in Supplemental Figure S1. Peaks that were only observed in the digest of the band a were selected and considered as tryptic peptides (Figure 2-6). The protein in band a was estimated to be an aminopeptidase N (APN) (E.C. 3.4.11.2) based on the m/z of the tryptic peptides using MS fit. APN is also reported to be identified in human small intestine (Hiwada et al., 1981). Other bands were estimated to be structural proteins such as major vault protein (b) and α -actinin 4 (c). MS-fit search results are provided in Supplemental Data S1-S4. For confirmation, tryptic peptides in band a were subjected to LC-MS/MS in product ion scan mode to identify the peptide sequences. The product ions of each tryptic peptide were then assigned to the a, b, c, and x, y, z series of peptide fragments as defined by Papayannopoulos (1995) using MS-fit. As shown in Figures 2-7, five tryptic peptide sequences were obtained and matched with the sequence of aminopeptidase N, while negligible product ions were generated from the other 2 tryptic peptide peaks. These results confirmed that protein in the band a is aminopeptidase N, and the alignment of these tryptic peptides with the aminopeptidase N sequence is shown in Figure 2-8.

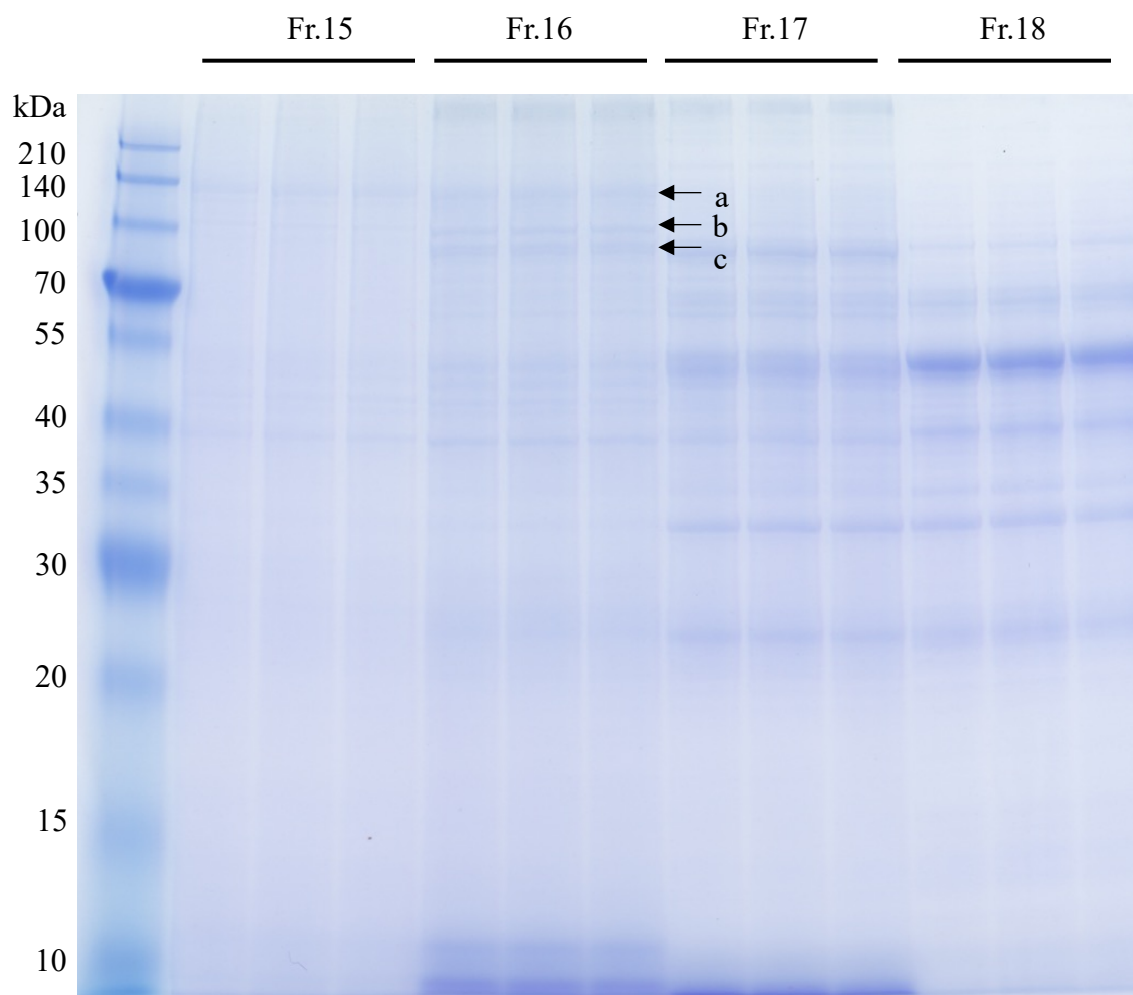


Figure 2-5. SDS-PAGE of SEC Fr.15-18

The SEC Fr. 16 was selected as it showed the highest LAP activity, while Fr. 15, 17, and 18 were selected as the comparison fractions with less LAP activity. The gel was loaded with 24 μ L of each sample, electrophoresed, and stained by Coomassie Brilliant Blue G-250. The arrows indicate the selected bands for further protein identification as they are only observed in the Fr. 16 but not in other Frs.

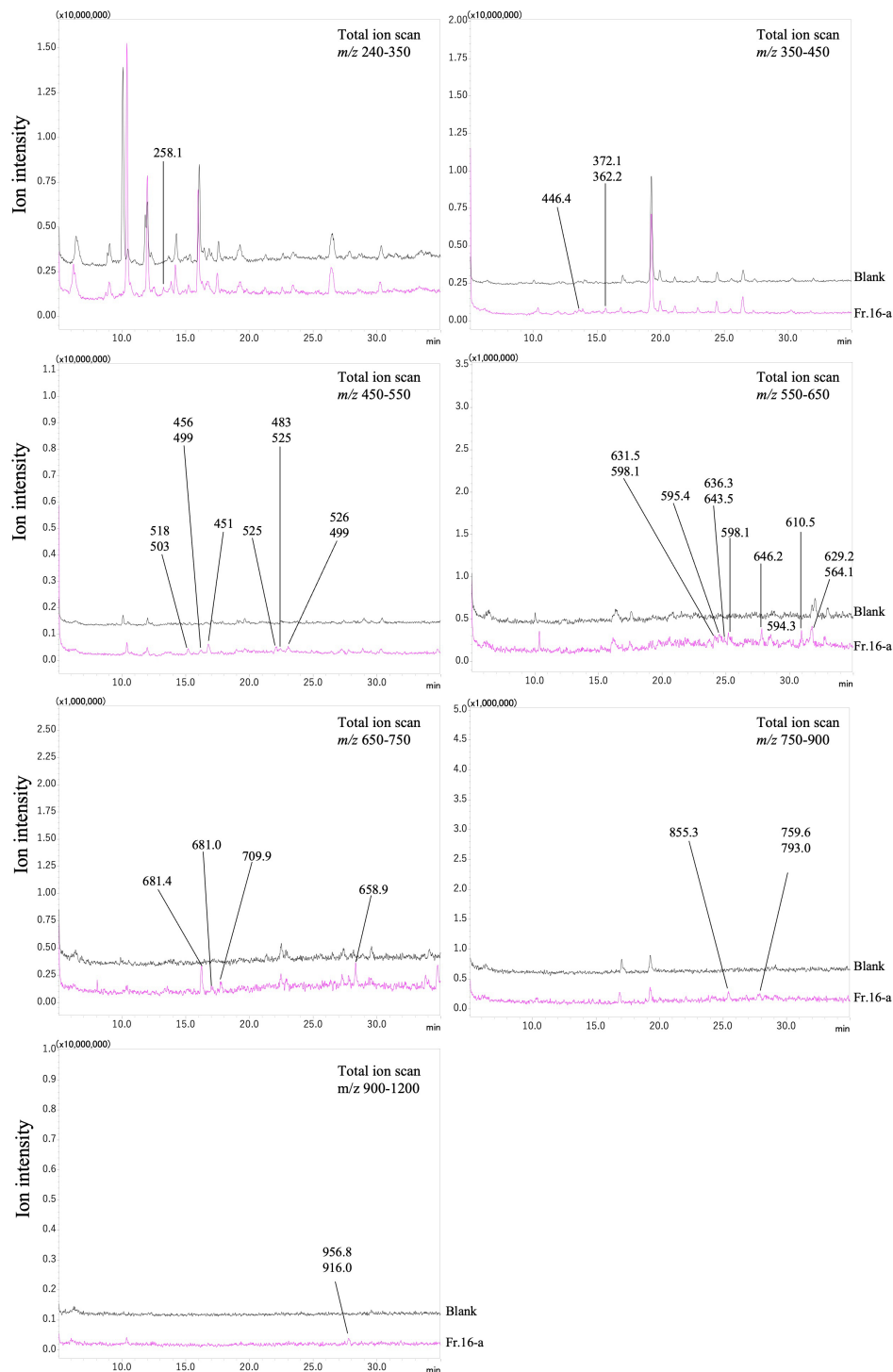


Figure 2-6. Total ion chromatograms of tryptic digest of the band a in SEC Fr. 16 and blank
 Elution was monitored by total ion mode in the scan ranges of m/z 240-350, 350-450, 450-550, 550-650, 650-750, 750-900, and 900-1200. Twenty-three peaks were detected only in the digest of band a, and the m/z of the peaks was used for protein prediction. The bands b and c are provided in Supplemental Figure S1.

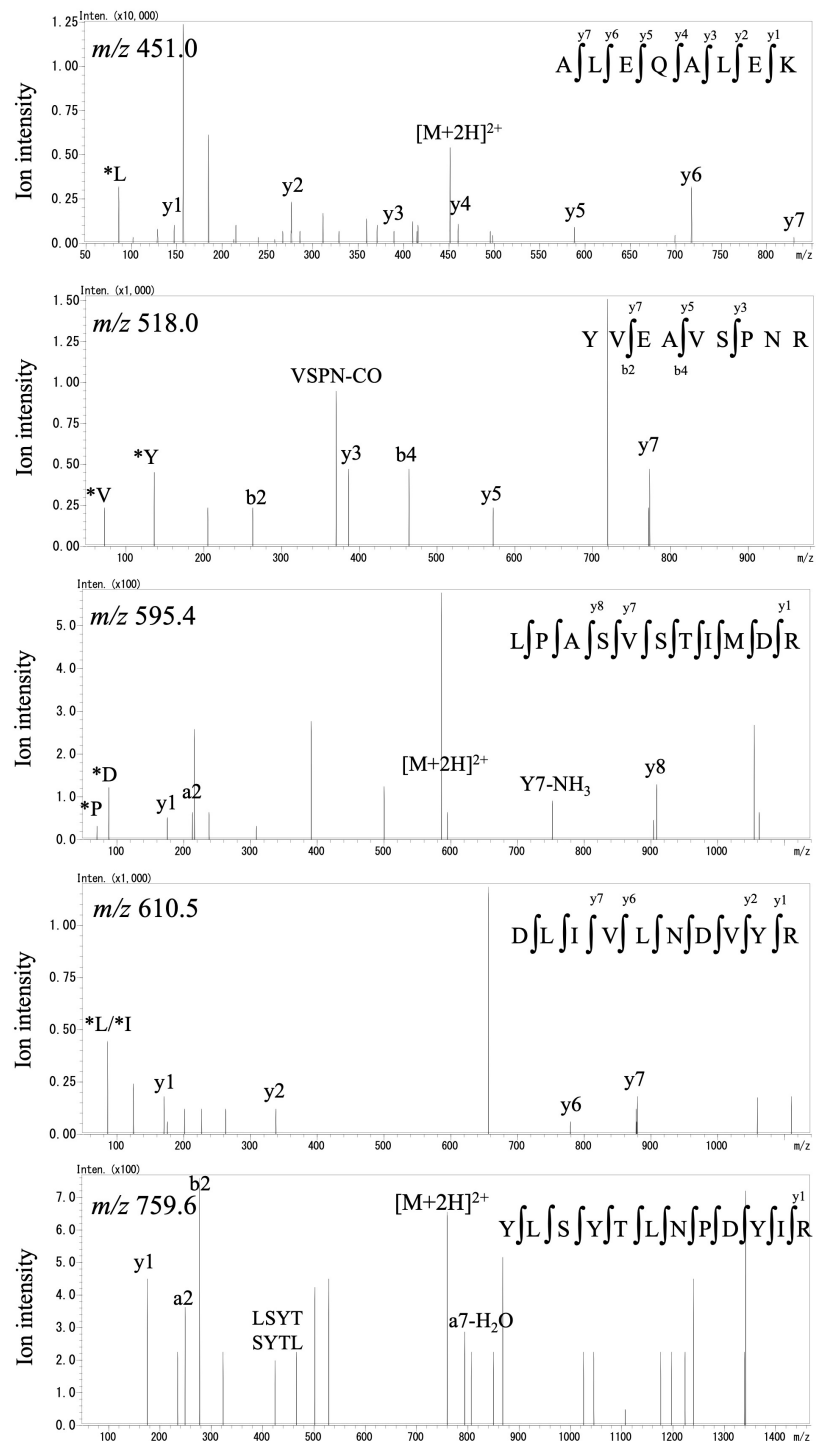


Figure 2-7. Peptide sequences of tryptic peptides from protein in the band a

Some precursor ions (m/z 451.0, 518.0, 595.4, 610.5, and 759.6), as shown in Figure 2-6, were subjected to product ion scan. Sequences were estimated by the N-terminal (a, b, c), carboxy-terminal ions (x, y, z), and internal ions. Internal ions are indicated by peptide sequence using a one-letter abbreviation. M represents the molecular weight of the peptide.

1	MA <u>K</u> GFYIS <u>K</u> T	LGILGILLGV	AAVCTIIALS	VVYAQE <u>K</u> <u>N</u> <u>R</u> <u>N</u>	AENSAIAPTL	PGSTSATTST	TNPAIDESKP	WNQY <u>R</u> LP <u>K</u> TL
81	IPDSYQVTLR	PYLTPNEQGL	YIF <u>K</u> GSSTV <u>R</u>	FTCNETTNI	IIHS <u>K</u> <u>K</u> LN <u>Y</u> T	N <u>K</u> GNHR <u>V</u> AL <u>R</u>	ALGDTAPNI	DTTELVER <u>T</u> E
161	YLVVHLQGS	V <u>K</u> GHQYEMDS	EFQGEIADDL	AGFY <u>R</u> SEYME	GGN <u>K</u> <u>K</u> VVATT	QMQAADAR <u>K</u> S	FPCFDEPAM <u>K</u>	ASFNITLIHP
241	NNLTALSNNL	PKDS <u>R</u> TLQED	PSWNVTEFHP	TP <u>K</u> MSTYLLA	YIVSEF <u>K</u> YVE	AVSPN <u>R</u> VQIR	IWARPSAIDE	GHGDYALQVT
321	GPILNFFAQH	YNTAYPLE <u>K</u> S	DQIALPDFNA	GAMENWGLVT	Y <u>R</u> ESALVFDP	QSSSISN <u>K</u> ER	VVTVIAHELA	HQWFGNLVTV
401	DWWNDLWLNE	GFASYVEFLG	ADYAEPTWNL	<u>K</u> DLIVLNDVY	<u>R</u> VMASDALAS	SHPLSSPANE	VNTPAQISEL	FDSITYS <u>K</u> GA
481	SVL <u>R</u> MLSSFL	TEDLF <u>K</u> <u>K</u> GLS	SYLHTFQYSN	TIYLDLWEHL	QQAVDSQTAI	<u>K</u> LPASVSTIM	DR WILQMGGP	VITVNTSTGE
561	IYQEHFLDLP	TSKPTRPSDF	NYLWIVPIPY	L <u>K</u> NG <u>K</u> EDHYW	LETE <u>K</u> NQSAE	FQTSSNEWLL	LNINVTGYYQ	VNYDENNW <u>R</u> <u>K</u>
641	IQNQLQTDLS	VIPVIN <u>R</u> AQI	IHDSFNLASA	G <u>K</u> LSITLPLS	NTLFLASETE	YMPWEAALSS	LNIFY <u>K</u> LMFDR	SEVYGPM <u>K</u> <u>R</u> <u>Y</u>
721	L <u>K</u> <u>K</u> QVTPFLA	YF <u>K</u> I <u>K</u> TNNWL	DRPPTLMEQY	NEINAISTAC	SSGLEECRDL	VVGLYSQWMN	NSDNNPIHPN	LRSTVYCNAI
801	AFGGEEEWNF	AWEQFR <u>K</u> ATL	VNEAD <u>K</u> L <u>R</u> SA	LACSNEVWIL	<u>N</u> <u>R</u> YLSYTLNP	DYIR <u>K</u> QDATS	TIVSIANNVV	GQTLVWDFV <u>R</u>
881	SNW <u>K</u> <u>K</u> LFEDY	GGGSFSFANL	IQGVTR <u>R</u> FSS	EFELQQLEQF	<u>K</u> EDNSATGFG	SGTR <u>A</u> LEQAL	E <u>K</u> T <u>K</u> ANIKWV	<u>K</u> EN <u>K</u> DVVL <u>K</u> <u>W</u>
961	FTENS							

Figure 2-8. Alignment of sequences of tryptic peptides to the sequence of aminopeptidase N (APN)

Lysyl and arginyl residues are highlighted by underline, of which the carboxyl side is potential cleavage site by trypsin. Sequences of the tryptic peptides are highlighted in red.

Proteins in the AEC Fr. 12 and Fr. 16, which had the highest LAP and prolidase activities, respectively, and Fr. 10 and Fr. 14, which had no activity, were resolved by SDS-PAGE as shown in Figure 2-9. Six protein bands specifically observed in AEC Fr. 12 and Fr. 16 were selected and marked with capital letters, as shown in Figure 2-9. The protein in each band was digested in the same manner as for SEC fractions and then subjected to LC-MS in total ion scan mode. The total ion chromatograms of tryptic peptides in band E are shown in Figure 2-10. The chromatograms of other bands are provided in Supplemental Figure S2. As mentioned above, tryptic peptide peaks were selected by comparing the sample bands with the blank band. Sixteen tryptic peptide peaks and twenty-eight precursor ions of tryptic peptide were detected. Based on the m/z of tryptic peptides, the protein in band E was estimated as rats Xaa-Pro dipeptidase (EC: 3.4.13.9), while other protein bands were identified as structural proteins. The protein predictions for all protein

bands in AEC fractions based on MS-Fit software are provided in Supplemental Data S5-S13. Xaa-Pro dipeptidase has many alternative names, such as proline dipeptidase, prolidase, X-Pro dipeptidase, peptidase D, or imidodipeptidase. Prolidase is selected to mention this enzyme in this report.

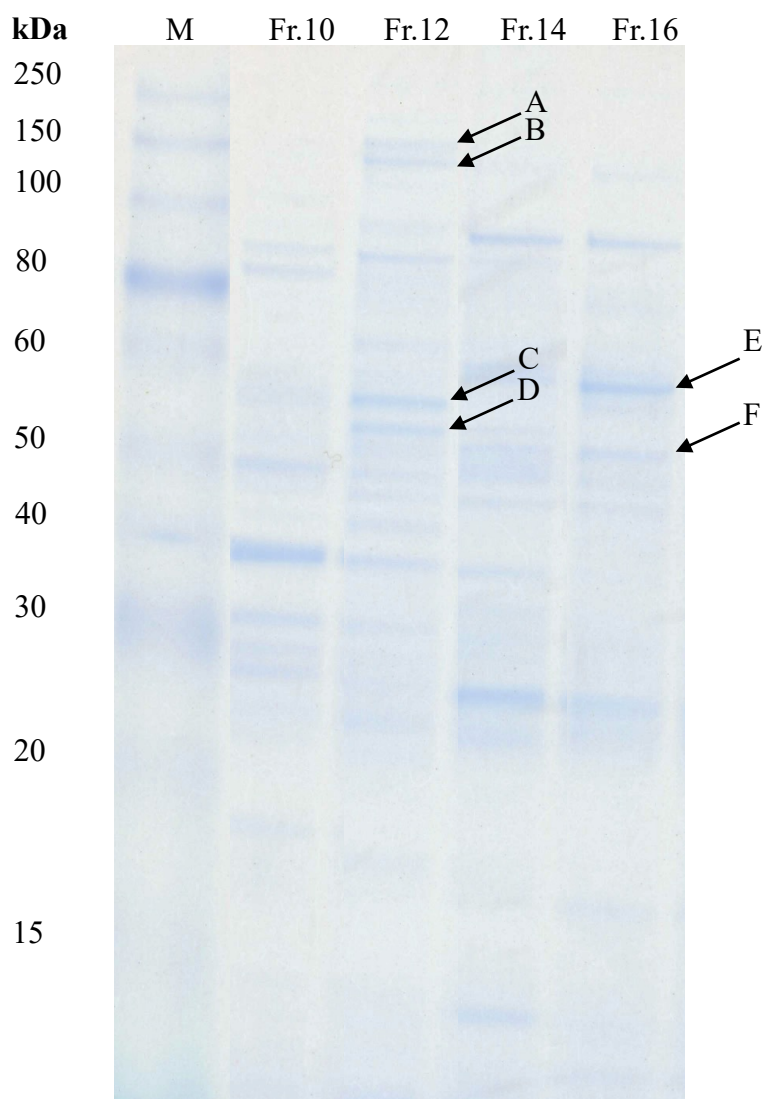


Figure 2-9. SDS-PAGE of AEC fractions

Aliquots of AEC Fr. 12 and 16 with the highest LAP and prolidase activities were concentrated and subjected to SDS-PAGE. Fr. 10 and 14 were selected as they eluted near Fr. 12 and 16 but showed no LAP and prolidase activity.

For confirmation, the tryptic peptides in band E were sequenced using LC-MS/MS in product ion scan mode. The product ions of each tryptic peptide were assigned to the a, b, c, and x, y, z series of peptide fragments, as previously mentioned. All the tryptic peptides in band E could be sequenced, as shown in Figure 2-11. The summary of the sequence analysis of tryptic peptides from protein in band E is shown in Table 2-1. Prolidase consists of 492 amino acid residues with a molecular weight of 54,751 Da (UniProtKB Q5I0D7). All sequences of tryptic peptides can be aligned in the prolidase sequence (Figure 2-12).

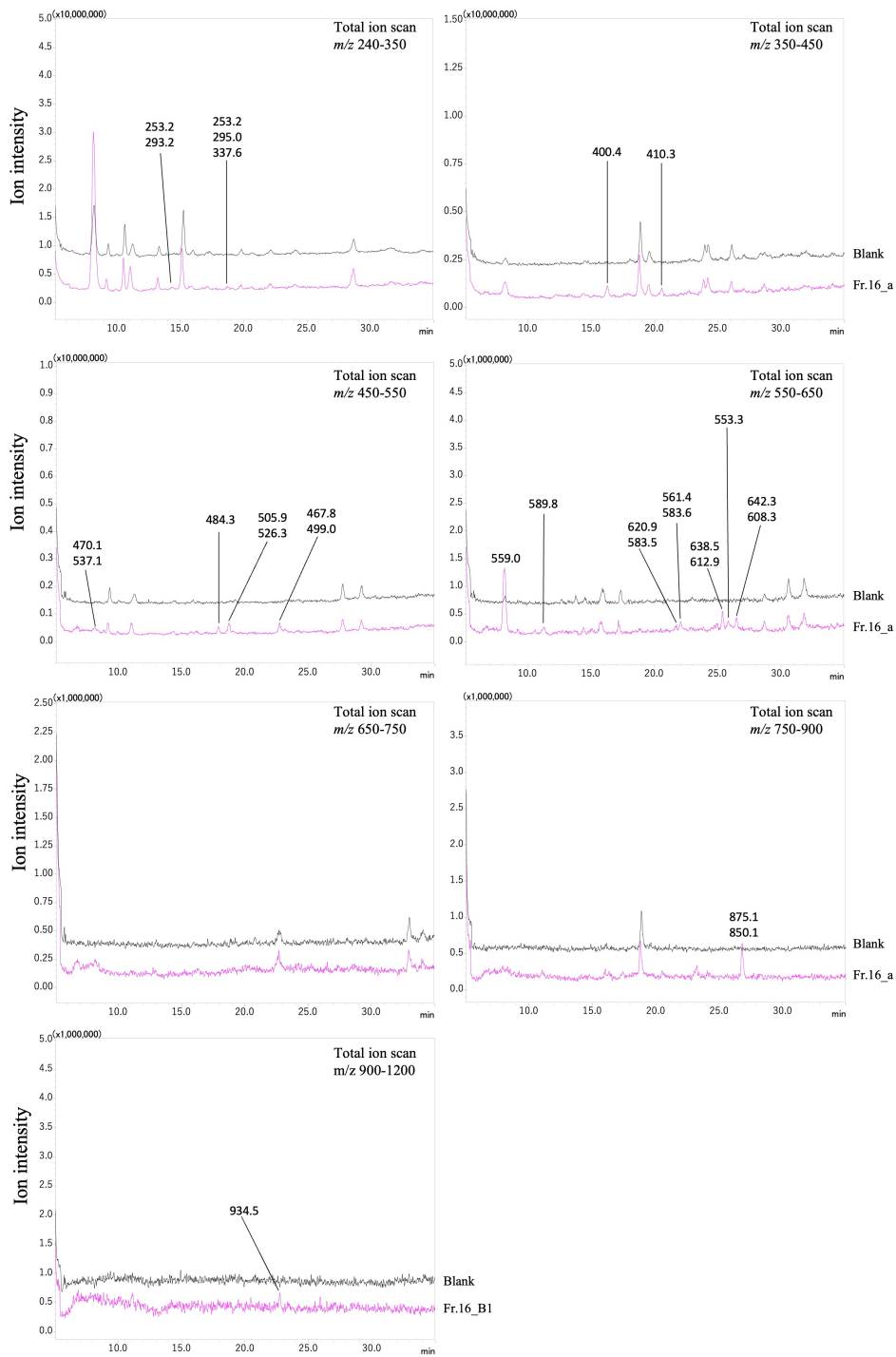
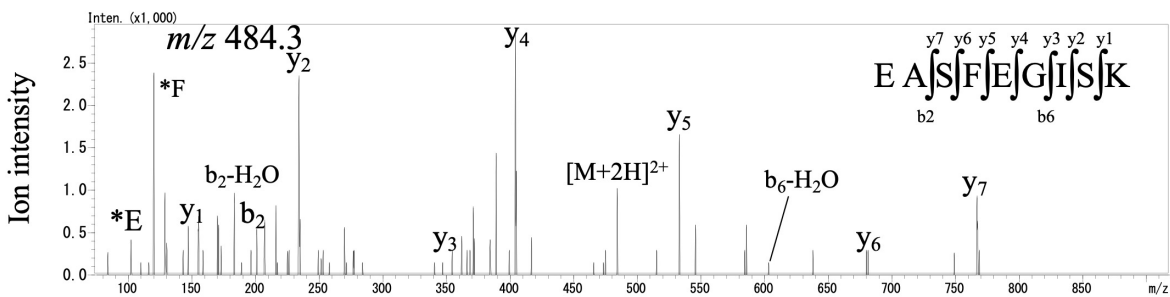
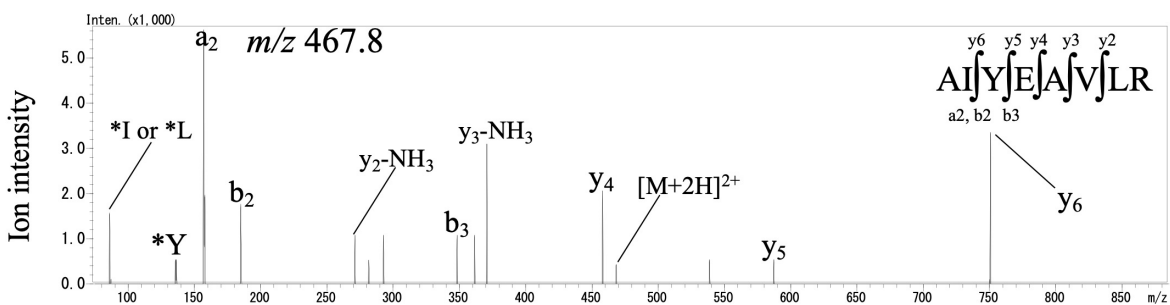
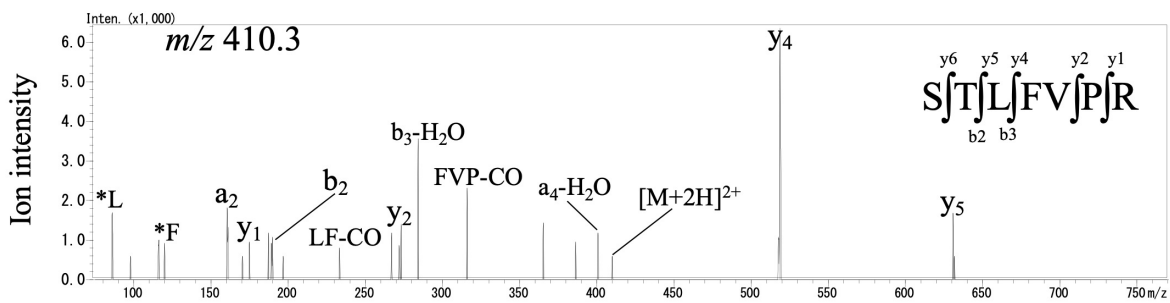
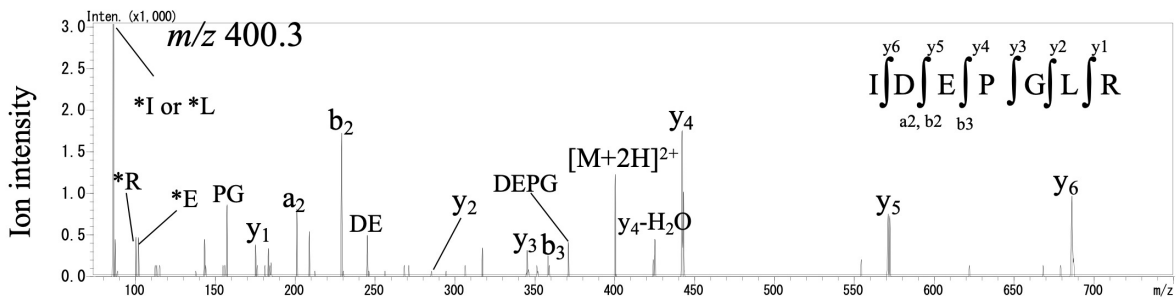


Figure 2-10. Total ion chromatograms of the tryptic digest of band E and blank band

Elution was detected in total ion mode in the scan ranges of m/z 240-350, 350-450, 450-550, 550-650, 650-750, 750-900, and 900-1200. Sixteen peaks were detected, and the m/z on peaks were used for protein prediction using MS-Fit.



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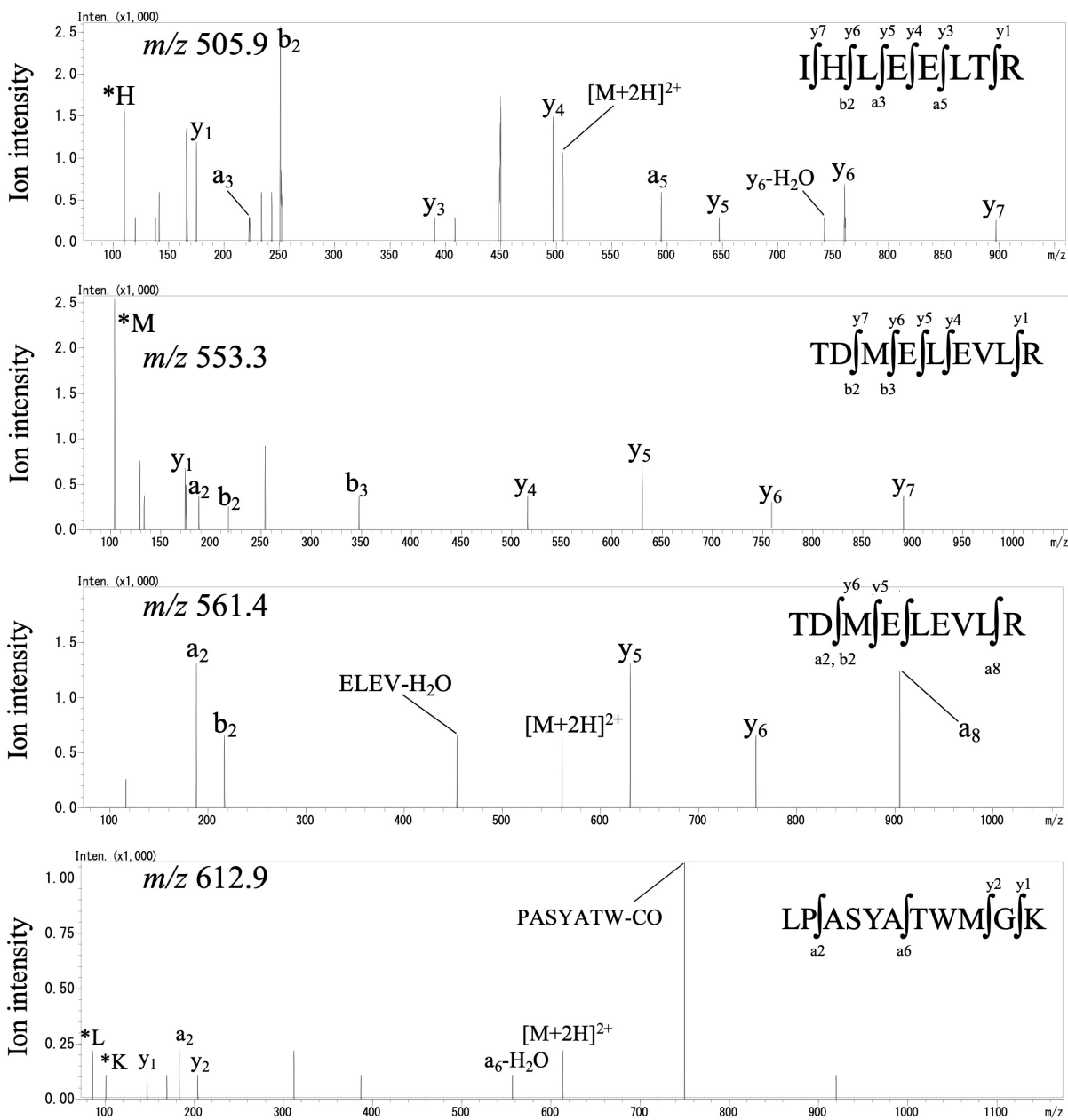


Figure 2-11. Peptide sequences of tryptic peptides from protein in the band E

Eight precursor ions (m/z 400.3, 410.3, 467.8, 484.3, 505.9, 553.3, 562.4, and 612.9), as shown in Figure 2-10, were subjected to product ion scan. Sequences were estimated by the N-terminal (a, b, c) and carboxy terminal ions (x, y, z) and internal ions. Internal ions are indicated by peptide sequence using one-letter abbreviation. M represents molecular weight of peptide.

1 MASTVRPSFS LGNETLKVPL ALFALNRRQL CERLRKNGAV QAGSAVVLQG GEEMQRYCTD TSIIFRQESF FHWAFGVIES
 81 GCGYVIDVDT GKSTLFVPRL **PASYATWMGK** IHSKEHFKEK YAVDDVQYA EIASVLTSRN PSVLLTLRGV NTDSGNVCR**E**
 161 **ASFEGISK**FT VNNTILHPEI VECRVFK**TDM** **ELEVL**RYTNR ISSEAHREVM KAVKVGMKEY EMESLFQHYC YSKGGMRTS
 241 YTCICCSGEN AAVLHYGHAG APNDRTIKDG DICLFDMGGE YYCFASDITC SFPANGKFTD DQK**AIYEAVL** **R**SCRTVMSTM
 321 KPGVWWPDMH RLADR**IHLEE** **LTR**IGLLSGS VDAMLQVHLG AVFMPHGLGH FLGLDVHDVG GYPEGVER**ID** **EPGL**SLRTA
 401 RHLEPGMVLV VEPGIYFIDH LLDQALADPA QACFFNQEV LQRFRNFGGVR IEEDVVVTDS GMELLTCVPR TVEEIEACMA
 481 GCDKALAPSG PK

Figure 2-12. Alignment of sequences of tryptic peptides to the sequence of prolidase (Xaa-Pro dipeptidase)

Lysyl and arginyl residues are highlighted by underline, of which the carboxyl side is potential cleavage site by trypsin. Sequences of the tryptic peptides identified in band E are highlighted in red.

Table 2-1. The sequence of tryptic fragments of protein in band E of AEC Fr.16 compare to the sequence from MS-Fit database

No.	m/z [M+2H ⁺]	m/z [M+H ⁺]	Start	End	Modifications	Sequence from MS-Fit database	Sequence from analysis
1	400.3	799.60	389	395		(R)IDEPGLR(S)	IDEPGLR
2	410.3	819.60	93	99		(K)STLFVPR(L)	STLFVPR
3	467.8	934.60	304	311		(K)AIYEAVLR(S)	AIYEAVLR
4	484.3	967.60	160	168		(R)EASFEGISK(F)	EASFEGISK
5	505.9	1010.80	336	343		(R)IHLEELTR(I)	IHLEELTR
6	553.3	1105.60	188	196		(K)TDMELEVLR(Y)	TDMELEVLR
7	561.4	1121.80	188	196	Oxidation	(K)TDMELEVLR(Y)	TDMELEVLR
8	612.9	1224.80	100	110		(R)LPASYATWMGK(I)	LPASYATWMGK
9	620.9	1240.80	100	110	Oxidation	(R)LPASYATWMGK(I)	Not analyzed
10	638.5	1276.00	57	66		(R)YC(Carbamidomethyl)TDTSIIFR(Q)	Not analyzed
11	875.1	1749.20	1	17	Met-loss+Acetyl	(-)MASTVRPSFSLGNETLK(V)	Not analyzed

Degradation of Gly-Pro and Pro-Hyp by partially purified mucosa exopeptidases

Synthetic Gly-Pro and Pro-Hyp were digested by partially purified mucosa exopeptidases using SEC. Peak A (in Figure 2-1) showed the highest enzyme activities for both LAP and prolidase, and thus, was used for Gly-Pro and Pro-Hyp digestion. In addition, Peak A is the only SEC fraction that maintains the degradation activity as in the crude extract of mucosa (Figure 2-2). Gly-Pro was completely degraded after 60 min, while only 15% of Pro-Hyp was degraded simultaneously, as shown in Figure 2-13. LC-MS/MS analysis also confirmed the absence of Gly-Pro after 60 min digestion (data not shown). On the other hand, Pro-Hyp remained even after 240 min digestion.

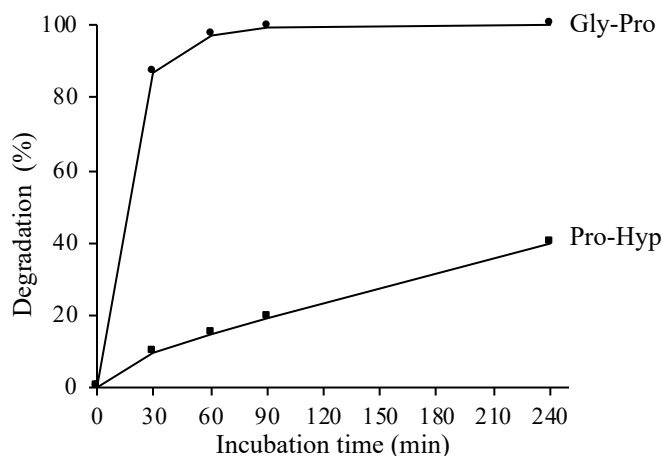


Figure 2-13. Degradation of synthetic Gly-Pro and Pro-Hyp by partially purified mucosa exopeptidases

The degradation rate (%) was calculated by quantifying the liberated Pro from substrates. The results are presented as the average of duplicate analysis.

2.4 Discussion

In Chapter 1, *in vitro* digestion of CH by a combination of pancreatin and well-known gastrointestinal exopeptidases, such as leucine aminopeptidase (LAP), carboxypeptidase A (CPA), and aminopeptidase N (APN), generated Gly-Pro as the major peptides. Interestingly, CH digestion using crude extract of small intestinal mucosa generated Pro-Hyp as the major peptide, while it generated only negligible amounts of Gly-Pro. These results suggest the presence of prolidase in the mucosa, as prolidase is reported to be able to cleave Xaa-Pro, where Xaa is any amino acid residue (Aganga et al., 2021). Thus, Gly-Pro can be cleaved by prolidase. Prolidase is generally considered a cytosolic metalloprotein enzyme essential for protein metabolism and collagen turnover within cells. Even though its activity has been reported in many organs (Liu et al., 2007), it remains unclear whether prolidase is secreted into the extracellular environment, even during collagen and ECM remodeling (Aganga et al., 2021). The present study showed that only a negligible amount of Gly-Pro was detected in the small intestinal lumen after oral administration of CH in rats (Chapter 1, Figure 1-4. A). Furthermore, no significant increase of Gly-Pro in the lumen was also observed after oral administration of synthetic Gly-Pro in rats (Wijanarti et al., 2024 in press). The results indicate that prolidase is not only present in the cytosol of cells but also secreted into the lumen and plays a crucial role in the digestion of small peptides.

At least 2 isoforms of prolidase (prolidase I and II) have been identified in rats and many animals (Liu et al., 2007). In addition, some microorganisms produce prolidases (Aganga et al., 2021). The origin of prolidase present in the small intestinal mucosa, however, remained unknown. The present study demonstrates the presence of host (rat) prolidase I (EC.3.4.13.9) with amino

peptidase N (EC. 3.4. 11.2) in small intestinal mucosa at the protein level. Prolidase I is considered a key player in collagen degradation to liberate free Pro for extracellular matrix remodeling and collagen resynthesis (Besio et al., 2013; Yu et al., 2012). The majority of research on prolidase focuses on intracellular collagen metabolism. A new finding reported that prolidase was shown as a ligand of epidermal growth factor receptor (EGFR) and stimulated downstream signaling protein in the EGFR pathway (Yang et al., 2013; Baszanowska et al., 2021). This activation occurred only when prolidase was present extracellularly. Since prolidase has no peptide signal, the presence of prolidase in the extracellular matrix is still intriguing. However, it is plausible prolidase may be released from damaged cells and tissue.

Furthermore, the roles of prolidase in cellular regulation, wound healing, and several diseases have also been well-studied (Aganga et al., 2021). However, the knowledge of prolidase function in food digestion is very limited. It has been reported that prolidase not only cleaves X-Pro but also X-Hyp (Aganga et al., 2021). This study confirms that prolidase I in the small intestinal mucosa cleaves both Gly-Pro and Pro-Hyp, but the degradation rate differs extensively. Gly-Pro was rapidly degraded by prolidase I, while Pro-Hyp was slowly degraded and remained after prolonged digestion. On the other hand, another main exopeptidase in the small intestinal mucosa, aminopeptidase N, leaves Gly-Pro intact after CH digestion (Chapter 1), indicating that it cannot cleave Gly-Pro. Taken together, prolidase I, rather than aminopeptidase N, plays a crucial role in determining the bioavailability of collagen-derived peptides at the final digestion process in the small intestinal mucosa.

The presence of Pro in peptides has been assumed to be associated with resistance against protease digestion (Markert et al., 2003). However, dipeptides with Pro at the amino terminus (Pro-X) can be degraded by LAP and APN (Gu and Walling, 2000; Niven et al., 1995). Carboxypeptidase A (CPA) cleaves amino acid residue at the carboxyl terminus of peptide except for Pro, Asp, Glu, Arg, Lys, and Hyp (Laethem et al., 1996). Therefore, X-Pro dipeptides are resistant to the above-mentioned exopeptidases but are preferentially cleaved by prolidase (Wilk et al., 2017). These facts indicate that most small peptides, including Pro at the amino and carboxyl terminus, are degraded by a combination of exopeptidases in the small intestinal mucosa. However, several types of small peptides have been shown to increase significantly in blood plasma following the ingestion of protein hydrolysates, such as Hyp-containing peptides, β -isopeptides, and pyroglutamyl peptides (Asai et al., 2019; Ejima et al., 2019; Miyauchi et al., 2022). Hyp-containing peptides consist of post-translational modified amino acid residue. Pyroglutamyl peptides, β -isopeptides, and D-aspartyl peptides are generated from normal peptides by chemical or enzymatical modifications. The unusual structure of the modified amino acids in the peptides may interrupt the enzyme-substrate recognition, hindering the degradation of these peptides.

2.5 Conclusion

Prolidase I and aminopeptidase N were successfully identified in the crude extract of small intestinal mucosa. Although prolidase is generally known as a cytosolic enzyme that acts within cells, the present study revealed the presence of prolidase I activity in the small intestinal lumen.

Prolidase I rapidly cleaves Gly-Pro but slowly Pro-Hyp, which can clarify why Hyp-containing peptides increase in rats and human blood plasma after CH ingestion while Gly-Pro and other X-Pro dipeptides do not. To the best of our knowledge, this study is the first report showing the role of prolidase I in the lumen of the small intestine in determining the bioavailability of collagen-derived peptides.

Chapter 3

Enhancement of bioavailability of Pro-Hyp in rat by co-administration of collagen hydrolysate (CH) with rice protein hydrolysate (RPH)

3.1 Introduction

The ingestion of CH has been clinically proven to exert many health benefits (Clark et al., 2008; Inoue, et al., 2016; Sugihara et al., 2018). Pro-Hyp, a major food-derived collagen peptide in blood, has been associated with the health benefits of CH. It has been reported that co-ingestion of other protein hydrolysates and non-peptide compounds can impact the bioavailability of CH-derived peptides, including Pro-Hyp. Iwasaki et al. (2020) reported that co-ingestion of collagen peptides with yogurt increased the absorption of collagen-derived peptides into human blood plasma. Co-administration of CH with other compounds also improved its health benefits. A double-blind, randomized, placebo-controlled clinical trial showed that the co-ingestion of collagen peptides with vitamins and other bioactive compounds such as chondroitin sulfate, glucosamine, and so on significantly improved skin and joint conditions compared to only collagen peptides (Czajka et al., 2018). There is an episode in China that consuming CH with RPH enhances its efficacies on skin conditions better than consuming only CH. However, the mechanism of improvement of the skin by coadministration of RPH with CH remains to be solved.

It has been reported that pyroglutamyl (pGlu) peptides significantly increased in the small intestinal lumen of rats after ingestion of another RPH preparation (Miyauchi et al., 2022). Furthermore, some studies also reported that pGlu-peptides exhibited some bioactivities such as

hepatoprotective effect, anti-obesity, and improved immune barrier system in the small intestine by increasing the host antimicrobial peptide, rattusin (Sato et al., 2013; Shirako et al., 2020; Shirako et al., 2019). Even though only a few pGlu-peptides were absorbed into the blood plasma, pGlu-peptides in the small intestinal lumen are stable and resist the degradation of digestive enzymes (Miyauchi et al., 2022). The presence of pGlu-peptides in the lumen may interact with exopeptidases in the small intestine and affect the peptide degradation. This chapter aimed to elucidate the effects of co-administration of RPH with CH on the bioavailability of Pro-Hyp, a major bioactive collagen peptide, in rats.

3.2 Materials and Methods

Protein hydrolysate

Collagen hydrolysate (CH, marine oligopeptide) and rice protein hydrolysate (RPH) were a kind gift from the Japan Food Peptide Institute (Osaka, Japan).

Reagents

Acetonitrile (HPLC-grade), methanol, formic acid, and phosphate-buffered saline pH 7.4 (PBS, 10×) were purchased from Nacalai Tesque (Kyoto, Japan). Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP), 1-hydroxybenzotriazole (HOBt), 9-fluorenylmethoxycarbonyl (Fmoc) amino acid derivatives, *N*-(*tert*-Butoxycarbonyl)-L-pyroglutamic acid (Boc-pyroGlu), L-proline *tert*-butyl ester hydrochloride (H-Pro-OtBu·HCl), L-leucine *tert*-butyl ester hydrochloride (H-Leu-OtBu·HCl), and L-glutamic acid *tert*-butyl ester

hydrochloride (H-Glu-OtBu·HCl) were purchased from Watanabe Chemical Industries (Hiroshima, Japan). 6-Aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AccQ) was purchased from Toronto Research Chemical (Toronto, ON, Canada). All other reagents were of analytical grade or higher. Prolidase I from porcine kidney was purchased from Sigma Aldrich (St. Louise, MO, USA).

Peptide synthesis

The pGlu-peptides were synthesized using an Fmoc strategy with a solid-phase automatic peptide synthesizer (PSSM-8, Shimadzu), following the supplier's protocol described in Chapter 1.

Animal experiment

All the animals were treated and cared for according to the guidelines of the National Institutes of Health (NIH) for experimental animals. All experimental procedures were approved by the Animal Care Committee of the Louis Pasteur Center for Medical Research (Kyoto, Japan, approval number 20211). Five-week-old male Wistar/ST rats (120–140 g) were purchased from Japan SLC (Shizuoka, Japan). The rats were acclimatized for one week with free access to a normal rodent chow (solid type of certified diet MF; Oriental Yeast, Tokyo, Japan) and drinking water. The room was kept at 22–24°C and 40–70% relative humidity, with a 12-hour light/dark cycle. After the acclimatization period, rats were divided into a control group, collagen hydrolysate (CH), rice protein hydrolysate (RPH), and co-administration of CH+RPH (CH+RPH) groups. The CH and RPH groups were administered 300 µL of CH and RPH water solution at a dose of 200 mg/kg

body weight, respectively. The CH+RPH group was administered the same volume of the mixture of CH and RPH at a dose of 200 mg/kg body weight for each protein hydrolysate, while the control group was administered the same volume of distilled water.

One hour after oral administration, small intestine and blood from the abdominal vena cava were collected from rats under anesthesia using isoflurane inhalation. The inner content of the small intestine was flushed with 10 mL of cold PBS and used as sample of the inner content of the small intestine or lumen. Pieces of tissue (approximately 100 mg) were collected from the center of the washed small intestine and cut into small pieces, then added with 100 μ L of PBS and homogenized in a Biomasher II (Nippi, Tokyo, Japan). The homogenate was mixed with 3 volumes of ethanol and centrifuged at 13,000 $\times g$ for 10 min at 4°C. The supernatant was collected and used as the small intestinal tissue extract for the following experiments. The blood was collected from the abdominal vena cava using a heparinized syringe and centrifuged at 1,500 $\times g$ at 4°C for 10 min. The resultant plasma was mixed with three volumes of ethanol and centrifuged at 13,000 $\times g$ for 10 min at 4°C. The supernatant was used as a plasma sample for the following experiments. The food-derived peptides in the inner content of intestines (lumen), intestinal tissue, and blood plasma were determined as described in the following subsection.

Crude extract of small intestinal mucosa was prepared from the same strain of rat by the method described in Chapter 1. All experimental procedures were approved by the Animal Care Committee of the Louis Pasteur Center for Medical Research (Kyoto, Japan, approval number 20231 and 20241).

Determination of food-derived peptides after oral administration of CH and RPH

Aliquots (100 μ L) of the ethanol-soluble fraction from the lumen were dried in a microcentrifuge tube (1.5 mL) under vacuum. The residue was added with 20 μ L of water, 60 μ L of 50 mM sodium borate buffer, pH 8.8, and 20 μ L of 0.3% AccQ-acetonitrile solution. The reaction was carried out at 50°C for 10 min. The reactant was filtered using a Cosmonice filter and used to identify the normal peptides with the amino group. The aliquots (10 μ L) of the filtered lumen sample of CH and RPH groups were injected into LC-MS/MS (LCMS 8040, Shimadzu). The AccQ derivatives were resolved by the Inertsil ODS-3 column using a binary linear gradient using 0.1% formic acid (solvent A) and 0.1% formic acid containing 80% acetonitrile (solvent B) at 0.2 mL/min. The gradient program was as follows: 0–20 min, 0–30% B; 20–25 min, 30–100% B; 25–30 min, 100% B; 30–30.1 min, 100–0% B, and 30.1–40 min, 0% B. The AccQ-derivatives (normal peptides) were specifically detected by precursor ion scan at positive mode targeting AccQ-derived b1 ion with $m/z = 171.1$ in the scan ranges at $m/z = 325–350$, $350–375$, $375–400$, $400–450$, and $450–500$. Pyroglutamyl peptides in the sample treated with AccQ were resolved by the same chromatographic condition and detected by precursor ion scan targeting the immonium ion of pyroglutamic acid with $m/z=84.1$ in the scan ranges at $100–200$, $200–225$, $225–250$, $250–275$, $275–300$, $300–350$, $350–400$, $400–500$, and $500–1000$. The precursor ions in major peaks were further subjected to product ion scan with collision energy at -15, -25, and -35 eV to estimate peptide sequences. The estimated peptides were synthesized as described in Chapter 1 and in a previous study (Shirako et al., 2020) and used as external standards for the quantification of food-derived peptides. The content of each peptide was determined in multi-reaction monitoring (MRM)

in positive ion mode. The elution program was as follows: 0-15 min, 0-50% B; 15-20 min, 50-100% B; 20-25 min, 100% B; and 25-35 min, 0% B. MRM conditions were optimized by a LabSolutions LCMS Ver. 5.5 (Shimadzu) using the synthetic peptides.

The effect of RPH and pGlu peptides on Pro-Hyp degradation

Aliquots (40 μ L) of 1% Pro-Hyp solution in PBS were mixed with 40 μ L of crude extract of mucosa and 320 μ L of RPH solution to yield a final concentration of 0.5%, 2.5%, and 5% of RPH. For the control, Pro-Hyp was digested without the addition of RPH. The digestion was carried out at 37 °C. Aliquots (50 μ L) were withdrawn at 0, 30, 60, 120, and 180 min of incubation and mixed with 3 volumes of ethanol. The content of free Hyp was quantified by the method of Bidlingmeyer et al. (1984) with modifications (Miyauchi et al., 2022). The degradation (%) was calculated using the following equation.

Pro-Hyp degradation rate (%) = (free Hyp content in the supernatant / Pro-Hyp content before digestion $\times$ 100%

The mixture of pGlu-peptides consisted of the same concentration of pGlu-Leu, pGlu-Glu, and pGlu-Pro. Aliquots (90 μ L) of 1 mM Pro-Hyp solution in PBS were mixed with 90 μ L of pGlu mixture and 20 μ L of the crude extract of mucosa. The final concentration of each pGlu peptide was set to 150 and 450 μ M. For the control, Pro-Hyp was digested without the addition of pGlu peptides by adding 90 μ L of PBS instead of the pGlu mixture solution. The final concentration of Pro-Hyp was 450 μ M. The reaction was carried out at 37 °C. Aliquots (50 μ L) were collected at 0,

30, 60, 120, and 240 min and mixed with 3 volumes of ethanol. The content of free Hyp was determined using the same method described above.

Aliquots (20 μ L) of 250 μ M Pro-Hyp were mixed with 25 μ L of each pGlu peptide (1 mM) and 5 μ L of crude extract of mucosa. The reaction was carried out at 37 °C and mixed with 3 volumes of ethanol to terminate the peptidase reaction. The content of the released Hyp was measured.

The type of enzyme inhibition was investigated using different concentrations of substrate and inhibitor, following a method in a recent review (Smirnoviene et al., 2021). The inhibition of prolidase by pGlu-Pro was performed in a total volume of 100 μ L with 3 concentrations of inhibitor, 0, 250, and 500 μ M pGlu-Pro. First, 10 μ L of prolidase (0.05 U) was mixed with 40 μ L of 625 or 1250 μ M pGlu-Pro as an inhibitor to obtain final concentrations of 250 and 500 μ M, respectively. For no inhibitor treatment, 40 μ L of PBS was added instead of pGlu-Pro solution. Each type of treatment was prepared in 7 microcentrifuge tubes. The mixtures were incubated at 37°C for 20 min. Then, 50 μ L of Pro-Hyp solutions as substrate were added to obtain the final concentration of 5, 25, 50, 100, 200, 400, and 500 μ M. The reaction was carried out at 37°C for 20 min and terminated by adding 3 volumes of ethanol. After centrifugation at 130,000 $\times$ g for 5 min, the supernatant was collected, and the content of released Hyp was determined using the same method described above.

Statistical Analysis

The data were analyzed using SPSS version 28 (IBM, New York, NY, USA). The student's t-test was used to determine the difference between the means of the two groups. One-way ANOVA with Tukey post hoc test was used to compare more than two groups. A *p*-value less than 0.05 was considered a significant difference.

3.2 Results

Determination of CH- and RPH-derived peptides in the small intestinal lumen

Food-derived peptides present in the lumen 60 min after oral administration of CH and RPH at a dose of 200 mg/kg body weight were detected and identified by LC-MS/MS in precursor ion and product ion scan modes. The mass chromatograms of precursor ion scans targeting pGlu residues (m/z 84.1) and AccQ-derived b1 ion (m/z 171.1) after oral administration of CH and RPH are shown in Figures 3-1. After CH administration, many peaks of AccQ-derivatives peptides (normal peptides with the amino group) were detected (numbers 13-37), while fewer peaks of pyroglutamyl peptides (numbers 1-12) were observed with prominent peaks of Lys, Glu, and Gln (Figure 3-1 A), as these amino acids also generated immonium ions of m/z 84. The major peaks were selected and sequenced to estimate the structure of peptides. Among the normal peptide peaks detected in the lumen, peak number 16 was the major one and identified as AccQ-Pro-Hyp based on m/z of product ions. The summary of estimated food-derived peptides after CH administration is shown in supplemental Tables S2 and S3.

As shown in Figure 3-1 B, many peaks of pyroglutamyl peptides (a-q) were detected in the lumen with high intensity 60 min after the administration of RPH. On the other hand, only small peaks of normal peptides (t-ab) were detected. These results indicate that pyroglutamyl peptides are predominantly generated in the lumen after RPH administration. The identified RPH-derived peptides in the lumen are listed in Supplemental Tables S4 and S5.

Contents of food-derived peptides in the lumen, intestinal tissue, and blood

All the estimated collagen peptides in the lumen and the peptides previously identified in Chapter 1 were quantified by LC-MS/MS in multi-reaction (MRM) mode. After administration of CH only, Pro-Hyp and many other Hyp-containing peptides significantly increased in the lumen of the small intestine (Figure 3-2. A). Pro-Hyp was the major collagen-derived peptide, while other Hyp-containing peptides were at lower levels. Some dipeptides with Pro at the carboxyl terminus (X-Pro), such as Gly-Pro, Pro-Pro, and Asp-Pro, were detected in the lumen after administration of CH. However, their contents were considerably lower than those of Hyp-containing peptides. In the small intestinal tissue, only some Hyp-containing peptides, including Pro-Hyp, Hyp-Gly, Gly-Pro-Hyp, Leu-Hyp, Phe-Hyp, Ala-Hyp, and Ile-Hyp, significantly increased after the administration (Figure 3-2. B). In blood plasma, Pro-Hyp content and other Hyp-peptides did not change substantially after the oral administration of CH at this dose (Figure 3-2. C). Pyroglutamyl peptides also did not change in the rat lumen (Figure 3-3. A), intestinal tissue (Figure 3-3. B), and blood plasma (Figure 3-3. C) after administration of CH.

Administration of RPH only did not impact Hyp-containing and X-Pro peptides in the lumen, as RPH does not contain Hyp-containing peptides (Figure 3-2. A). Similar results were observed in intestinal tissue (Figure 3-2. B) and blood plasma (Figure 3-2. C). On the other hand, pGlu-peptides including pGlu-Leu, pGlu-Val, pGlu-Gln, pGlu-Glu, pGlu-Lys, pGlu-Phe, pGlu-Ile, and pGlu-Ala significantly increased in the lumen (Figure 3-3. A) and intestinal tissue (Figure 3-3. B). However, only hydrophobic pyroglutamyl (pGlu) peptides such as pGlu-Leu and pGlu-Val significantly but slightly increased in blood plasma, while other pGlu-peptides remained unchanged (Figure 3-3. C).

Co-administration of RPH with CH did not change the profile of collagen-derived peptides in the lumen (Figure 3-2. A) but slightly increased the content of Pro-Hyp and other Hyp-peptides, including Hyp-Gly, Phe-Hyp, Leu-Hyp, Pro-Hyp-Gly, and Gly-Pro-Hyp in the intestinal tissue (Figure 3-2. B). Interestingly, Pro-Hyp content increased by 1 μ M in the blood plasma of the CH+RPH group after oral administration compared to other groups (Figure 3-2. C). However, no significant increase in Pro-Hyp and other Hyp-containing peptides was observed, except for Hyp-Gly. Co-administration of RPH with CH did not change the profile of pGlu-peptides in the lumen of the small intestine (Figure 3-3. A). However, the content of some pGlu-peptides such as pGlu-Leu, pGlu-Val, and pGlu-Gln significantly decreased in the intestinal tissue of the CH+RPH group compared to the RPH group (Figure 3-3. B), and only pGlu-Leu, pGlu-Val, and pGlu-Met significantly increased in blood plasma of CH+RPH group but pGlu-Leu levels were lower than those in RPH group.

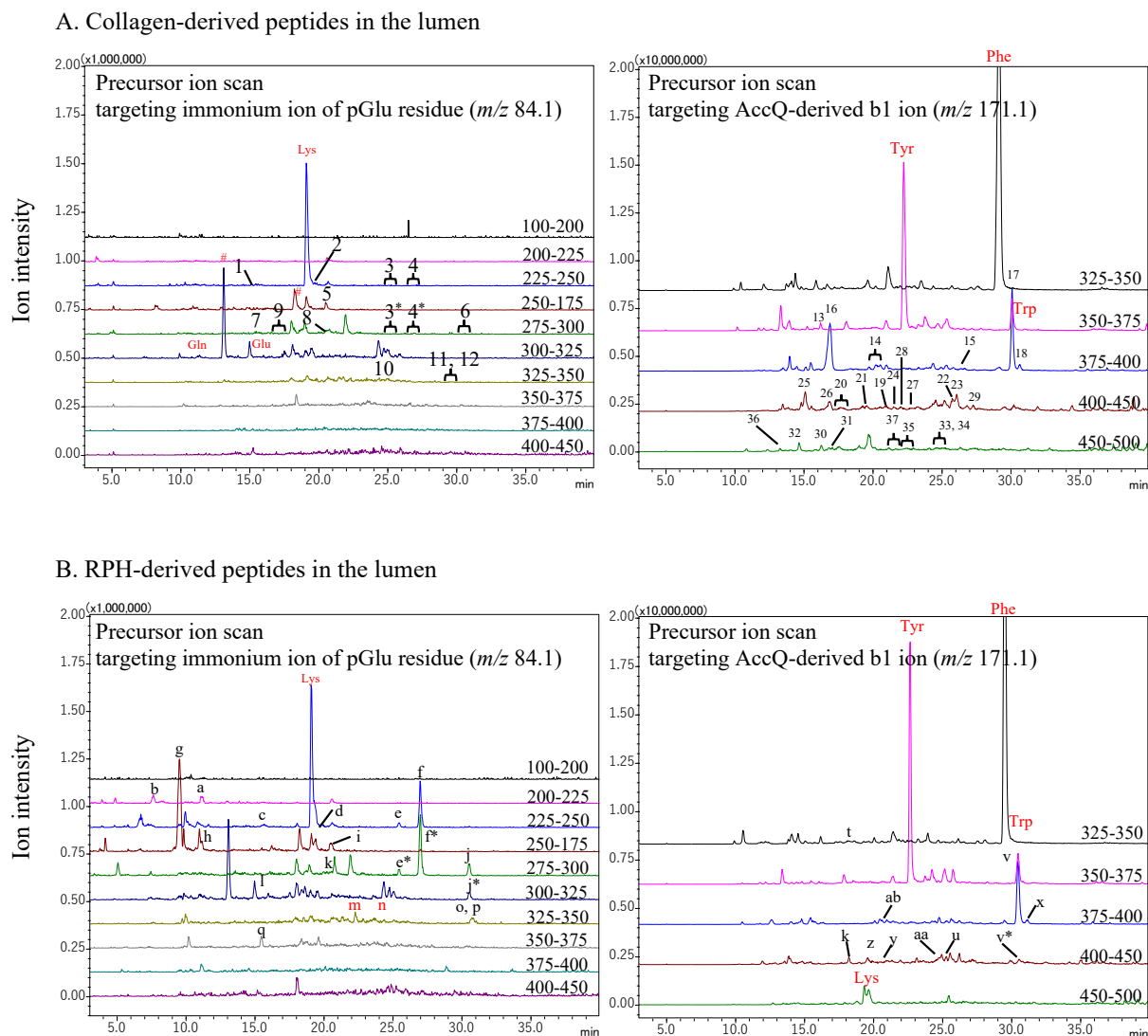


Figure 3-1. Mass spectrometry chromatograms in precursor ion scan mode targeting immonium ion of pGlu residue and AccQ-derived b1 ion of contents in the rat small intestinal lumen after oral administration of (A) CH and (B)RPH

The AccQ-derivatives (normal peptides) were specifically detected by precursor ion scan at positive mode targeting AccQ-derived b1 ion with $m/z = 171.1$. Pyroglutamyl peptides were detected by precursor ion scan targeting the immonium ion of pyroglutamic acid with $m/z=84.1$. The peptide peaks are indicated with alphabetic and the amino acids with three-letter abbreviations. The peptide peaks are indicated with numbers and the amino acids with three-letter abbreviations. In addition to pE, Lys, Glu, Gln residues also generated immonium ion of m/z 84.

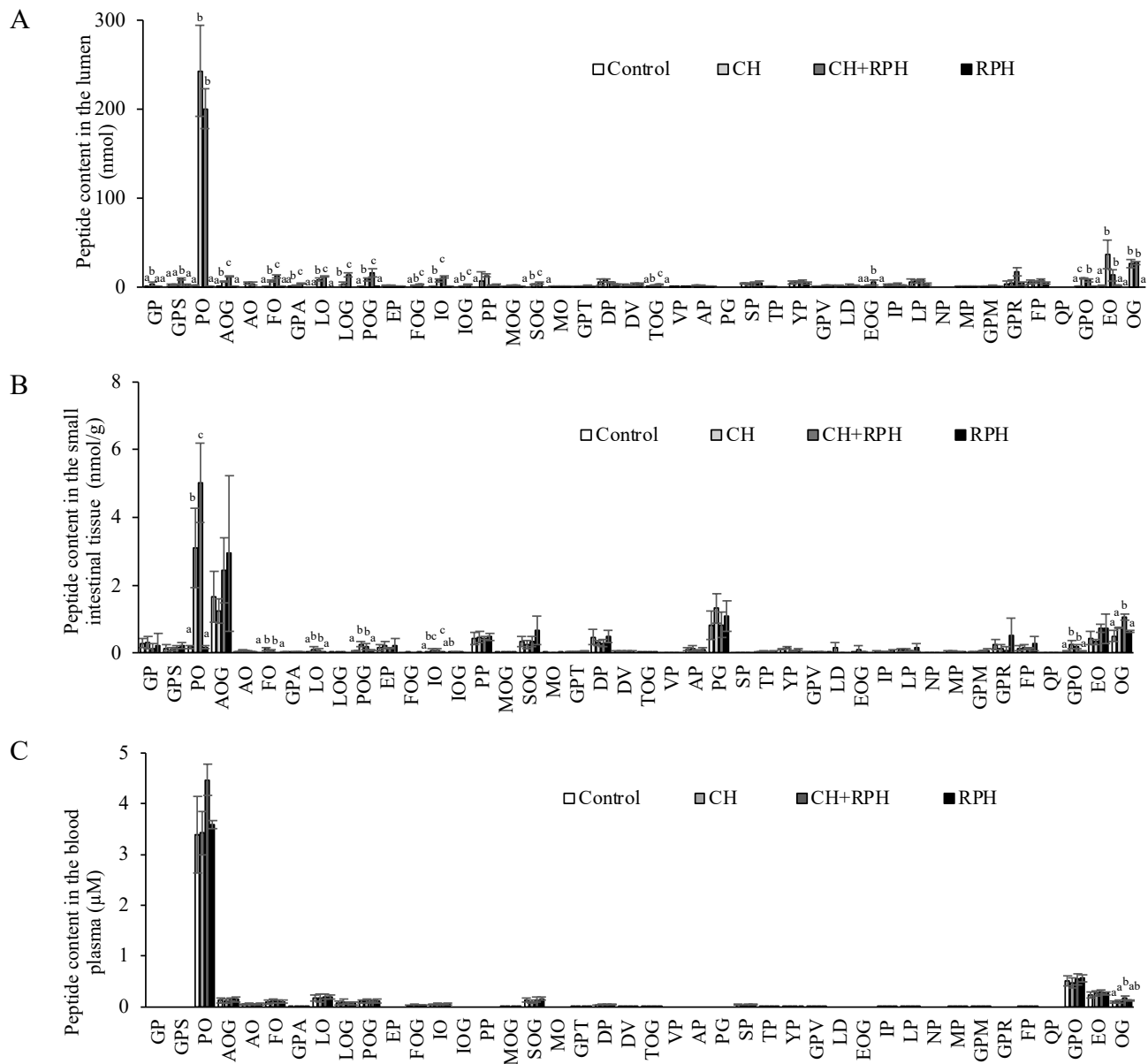


Figure 3-2. Food-derived peptides with amino group (A) in the lumen of the small intestine, (B) intestinal tissue, and (C) blood plasma of rats 1 h after oral administration of CH, RPH, and co-administration of CH+RPH

After ingestion of CH, many Hyp-peptides increased in the lumen of the small intestine, with the predominant constituent being Pro-Hyp (PO). Asterisks indicate a significant difference (One-way ANOVA, post hoc Tukey, $*p < 0.05$). Peptides are designated by one letter abbreviation of amino acid. O represents hydroxyproline (Hyp). The full-page graphs are provided in the Supplemental Figure S3-S5.

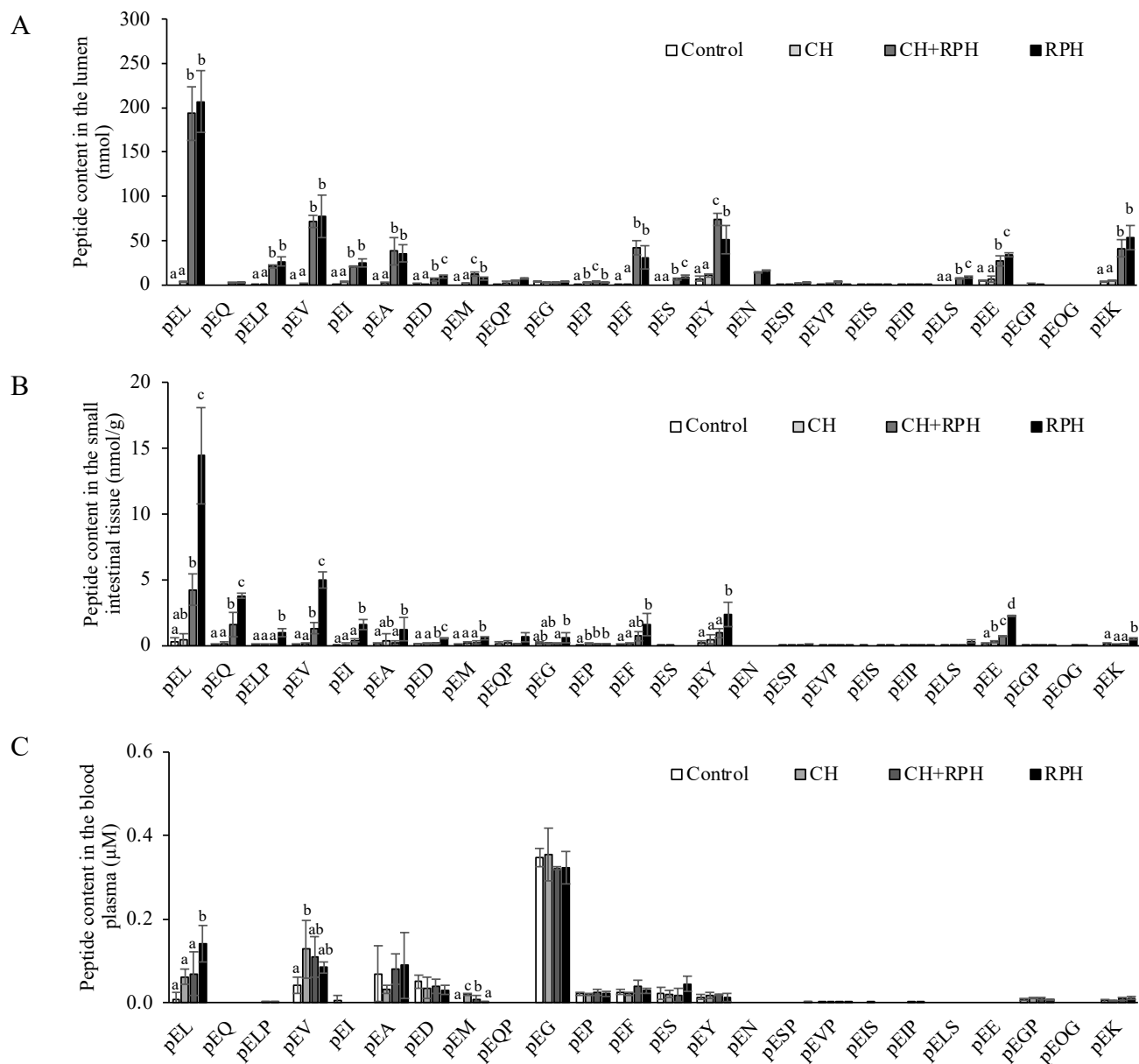


Figure 3-3. Pyroglutamyl peptides (A) in the lumen of the small intestine, (B) intestinal tissue, and (C) blood plasma of rats 1 h after ingestion of CH, RPH, and co-administration of CH+RPH

The ingestion of CH did not significantly increase pyroGlu-peptides in the lumen, while the ingestion of RPH significantly increased these peptides. Asterisks indicate a significant difference from the control (One-way ANOVA, post hoc Tukey, * $p < 0.05$). pE represents pyroglutamyl residue. Refer to the legend of Figure 3-2 for other abbreviations.

Inhibition of degradation of Pro-Hyp with intestinal mucosa exopeptidases by RPH and pGlu-peptides

The addition of RPH at a final concentration of 0.5%, 5 times higher concentration of Pro-Hyp, significantly inhibited degradation of Pro-Hyp by the crude extract of intestinal mucosa compared to the control. Increasing the concentration of RPH to 2.5% and 5% completely inhibited the degradation of Pro-Hyp even after 180 min (Figure 3-4). Furthermore, a mixture of the same concentration of pGlu-Pro, pGlu-Glu, and pGlu-Leu inhibited the degradation of Pro-Hyp by crude extract of small intestinal mucosa at two doses (Figure 3-5). Single-pGlu peptides, which increased in lumen after the administration of RPH, were checked for their inhibitory activity at the substrate-to-inhibitor ratio of 1:1. Among them, only pGlu-Pro significantly inhibited degradation of the Pro-Hyp (Figure 3-6).

Type of enzyme inhibition of prolidase by pGlu-Pro

The type of inhibition of prolidase on hydrolyzing Pro-Hyp by pGlu-Pro was evaluated. The Lineweaver-Burk plot of prolidase inhibition by pGlu-Pro is shown in Figure 3-7. No dose response was observed between 250 and 500 μM of pGlu-Pro. The addition of pGlu-Pro decreased V_{max} from 0.18 $\mu\text{M}/\text{min}$ without inhibitor to 0.12 and 0.13 $\mu\text{M}/\text{min}$ for 250 and 500 μM pGlu-Pro, respectively. Unexpectedly, K_m decreased from 117.60 μM without inhibitor to 80.75 and 81.89 μM for 250 and 500 μM pGlu-Pro, respectively.

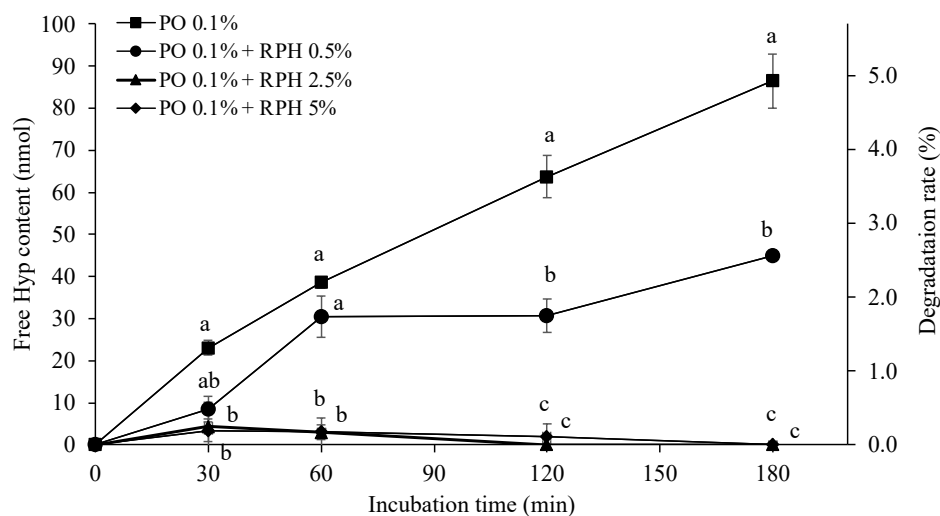


Figure 3-4. Suppression of the degradation of Pro-Hyp by crude extract of the small intestinal mucosa by different concentrations of RPH

Pro-Hyp was degraded by exopeptidase in crude extract of the mucosa into free Hyp and Pro during in vitro digestion. The degradation rate was calculated by dividing the free Hyp content in the supernatant after digestion by the total Pro-Hyp content before digestion.

One Way ANOVA, post hoc Tukey, $p < 0.05$.

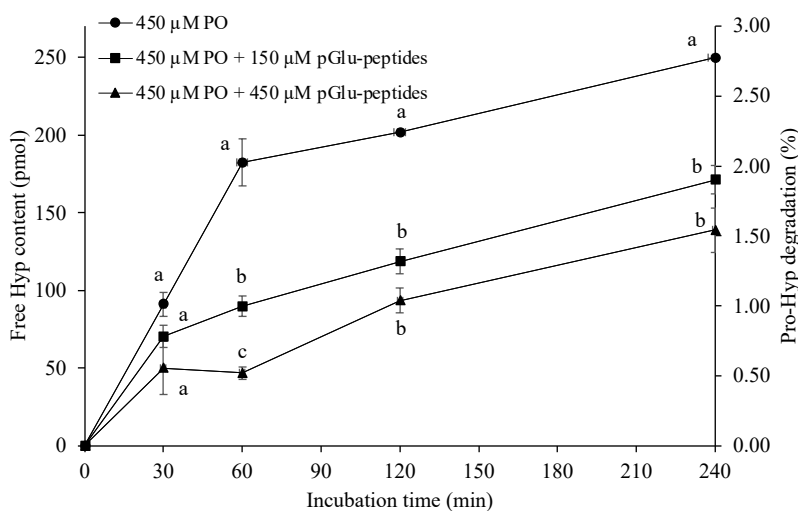


Figure 3-5. The mixture of pGlu-peptides solution decreased the degradation of Pro-Hyp

Pro-Hyp was mixed with the mixture of pGlu-peptide at 1:0.3 and 1:1 in molar ratio and incubated at 37°C. The contents of free Hyp were measured at 0, 30, 60, 120, and 240 min. One Way ANOVA, post hoc Tukey, $p < 0.05$.

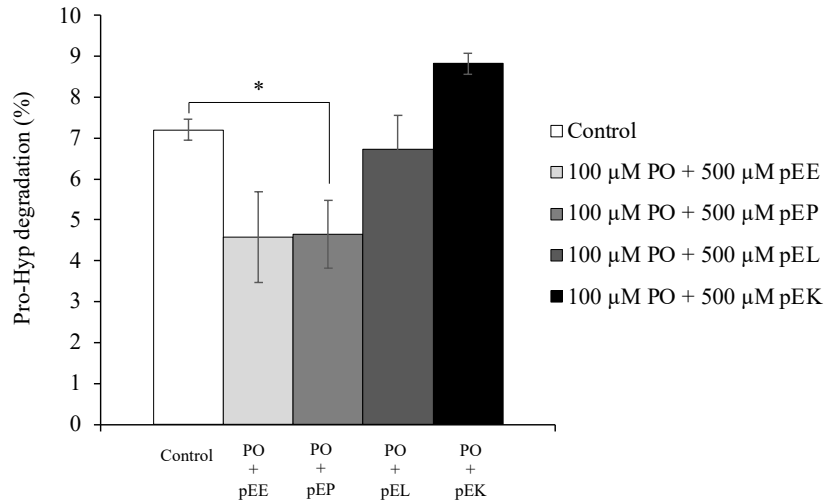


Figure 3-6. The effect of single pGlu peptide addition on the degradation of Pro-Hyp

Pro-Hyp was mixed with a single pGlu-peptide at a molar ratio of 1:5 and digested with the crude extract of mucosa. The used single pGlu-peptides are pGlu-Glu (pEE), pGlu-Pro (pEP), pGlu-Leu (pEL), and pGlu-Lys (pEK). The reaction was carried out at 37°C for 30 min. The asterisk indicated a significant difference compared to the control (Student *t*-test, **p*<0.05).

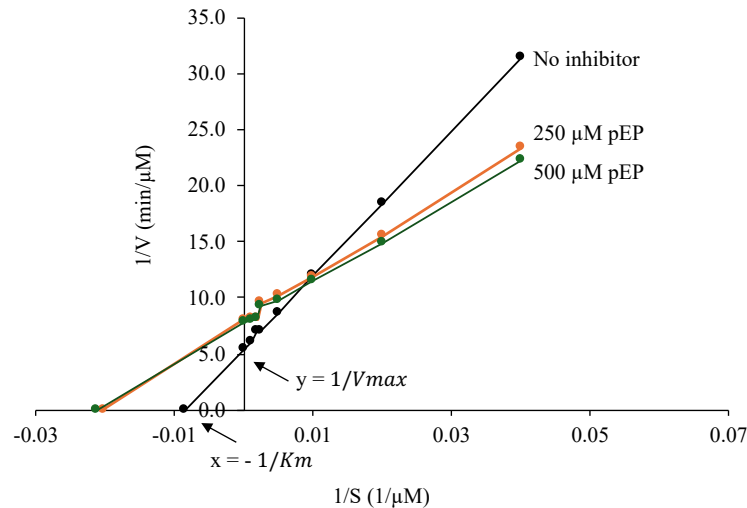


Figure 3-7 The Lineweaver-Burk plot of inhibition of prolidase by pGlu-Pro

Prolidase activity on hydrolyzing Pro-Hyp with and without adding pGlu-Pro as an inhibitor. Prolidase was incubated with 0, 250, and 500 μM pGlu-Pro at 37°C for 20 min. Then, Pro-Hyp was added at final concentrations of 5, 50, 100, 200, 400, and 500 μM and further incubated at the same condition. The addition of pGlu-Pro decreased both the maximum velocity (V_{max}) and the affinity for the substrate.

3.4 Discussion

Asai et al. (2019) demonstrated that Pro-Hyp significantly increased to approximately 25 μM in human blood plasma 60 min after ingestion of 13 g of CH, a different preparation from the CH used in the present study. However, in rats, Pro-Hyp and other CH-derived peptides did not significantly increase in blood plasma even after ingestion of CH at an equivalent dose (Figure 3-2. C). Interestingly, although no significant difference was observed, CH co-administered with RPH at the same dose increased Pro-Hyp by 1 μM in the rat blood plasma. When CH was administered alone, a higher dose (800 mg/kg body weight) was necessary to increase Pro-Hyp significantly in the rat blood plasma (Chapter 1, Figure 1-4). This finding shows that the co-administration of CH with RPH improves the bioavailability of Pro-Hyp, the major collagen-derived peptide that has been associated with the health benefits of CH (Inoue et al., 2016; Jimi et al., 2021; Sugihara et al., 2018). After the administration of RPH, pGlu peptides, such as pGlu-Leu, pGlu-Val, pGlu-Ala, pGlu-Phe, pGlu-Tyr, pGlu-Glu, and pGlu-Lys, significantly increased in the small intestinal lumen of rats.

pGlu can be derived from glutamine, which is rich in rice protein, with condensation between the amino group at the alpha position and a side chain in water. Peptides with a glutaminy residue at the amino-terminal position can be converted to pyroglutamyl peptide. The formation of pGlu-peptide is irreversible and proceeds even in low temperatures, and is favored by heating (Sato et al., 1998). Pyroglutamyl peptides are widely identified in various enzymatic hydrolysates of food proteins and fermented food (Ejima et al., 2018; Kiyono et al., 2013; Shirako et al., 2020; Kiyono et al., 2016; Paoletta et al., 2018), as well as exhibiting various health benefits (Shirako et

al., 2019; Kiyono et al., 2013; Kiyono et al., 2016). It has been reported that several pGlu-peptides derived from rice protein hydrolysate resist peptidases in the small intestine and can be absorbed as peptides into the rat blood plasma after oral administration (Miyauchi et al., 2022). Pyroglutamyl peptides were predominantly detected in the lumen, but only negligible amounts of normal peptides were detected, indicating that most of the normal peptides were hydrolyzed into amino acids by peptidases in the small intestine. In this chapter, the food-derived peptides after RPH oral administration showed similar results.

In the co-administration of RPH with CH, both Hyp-peptides and pGlu-peptides significantly increased in the lumen after oral administration. As mentioned above, the content of Pro-Hyp in small intestinal tissue increased considerably in the CH+RPH group. These results suggested that pGlu-peptides, the food-derived peptides in the lumen after RPH oral administration, can improve Pro-Hyp bioavailability. Iwasaki et al. (2020) reported that co-ingestion of collagen peptides with yogurt increased the short-chain Hyp-peptides in human blood plasma. In addition to the increasing short-chain Hyp-peptides via fermentation, yogurt matrices containing milk-derived peptides may influence the absorption of collagen peptides into the human blood plasma. Combining collagen peptides with vitamins and other bioactive compounds also enhanced collagen efficacy in improving skin elasticity and joint conditions (Czajka et al., 2018). However, the mechanism of improvement of the bioavailability of collagen peptides by non-collagen-derived peptides has not been discussed.

To address this problem, the effects of RPH-derived peptides on Pro-Hyp degradation were evaluated using a crude extract of mucosa prepared from the small intestine of rats. It was found

that the addition of RPH reduced the Pro-Hyp degradation in a dose-dependent manner. The degradation of Pro-Hyp was inhibited entirely with the addition of RPH at a final concentration of 2.5% (Figure 3-5). This result indicates that RPH can inhibit Pro-Hyp degradation by peptidases in the small intestine. To identify peptides responsible for the inhibition of the degradation of Pro-Hyp with peptidases in the small intestinal mucosa, a mixture of RPH-derived peptides, including pGlu-Pro, pGlu-Glu, and pGlu-Leu was checked for their inhibitory activities. The best inhibition was obtained with a substrate-inhibitor ratio of 1:1, which is the similar ratio of Pro-Hyp to pGlu-peptides in the lumen of CH+RPH group. Among the other peptides, pGlu-Pro showed significant inhibition in Pro-Hyp degradation by crude extract of mucosa peptidases.

It has been reported that prolidase is a crucial enzyme for cleaving X-Pro dipeptides, and later, it is also reported to cleave X-Hyp dipeptides (Aganga et al., 2021; present study Chapter 2). Therefore, inhibition of prolidase on hydrolyzing Pro-Hyp by pGlu-Pro was examined. The results showed that pGlu-Pro reduced K_m , indicating the increase of prolidase affinity for the substrate. Furthermore, pGlu-Pro slightly reduced V_{max} , resulting in a non-parallel Lineweaver-Burk plot between inhibited and non-inhibited lines (Figure 3-7). The decrease of the Michaelis constant (K_m) and the maximum reaction rate (V_{max}) are the characteristics of the uncompetitive inhibition type. The conventional uncompetitive inhibitor specifically binds to the enzyme-substrate complex (Herries, 1976) and stabilizes the complex. It effectively increases the enzyme affinity for the substrate but makes the enzyme less available to catalyze the reaction, which causes a lower V_{max} . The uncompetitive inhibition strategy is used in pharmacological and biological intervention (Ahmed et al., 2019). It is an effective and selective means of recognizing the enzyme-substrate

complex instead of free enzymes, as in competitive inhibition. pGlu-Pro has no positive charge as the amino group is blocked, making it possible to bind to enzyme-substrate basic sites, thus changing the conformation of the enzyme, increasing the affinity of prolidase to the substrate but decreasing the catalytic activity. The mechanism of inhibition of pyroglutamyl peptides on other small intestinal mucosa exopeptidases requires investigation in future works to support this finding.

3.5 Conclusion

The co-administration of CH with RPH enhanced the bioavailability of Pro-Hyp in the rat blood plasma at a low dose of CH administration (200 mg/kg body weight). Pyroglutamyl (pGlu) peptides, which are derived from RPH, can inhibit the degradation of Pro-Hyp during intestinal mucosa digestion. Further investigation of the inhibition mechanism of pGlu-peptides is required to support this finding.

Conclusion and Future Prospect

Food-derived peptides after the ingestion of CH and their health benefits have been well studied. The bioavailability of food-derived peptides is crucial for exerting health benefits in vivo. However, the crucial determining factors of bioavailability have not been fully understood. In this study, a novel in vitro digestion method using crude extract of small intestinal mucosa was established. Prolidase I and aminopeptidase N were identified in the crude extract of mucosa. This method generated a similar peptide profile to that in rat blood plasma and lumen upon ingestion of CH and RPH. Prolidase is responsible for the final degradation of X-Pro and X-Hyp dipeptides, which other exopeptidases could not degrade. The absence of prolidase led to a high portion of Gly-Pro or other X-Pro peptides after in vitro digestion, causing inconsistent results with in vivo digestion. Prolidase has been considered to be present in the cytosol. However, food-derived X-Pro peptides, including Gly-Pro, are extensively degraded in the intestinal lumen. Thus, it can be concluded that prolidase I is secreted into the lumen and plays a crucial role in the digestion of food-derived peptides in the lumen. Even though Pro-Hyp can also be degraded by prolidase in the crude extract of mucosa, the degradation rate is much lower compared to Gly-Pro. These findings can clarify the reason why Pro-Hyp significantly increases in human and rat blood plasma after ingestion of CH, while Gly-Pro does not, even though both can be derived from the same Gly-Pro-Hyp-Gly motif in collagen. Furthermore, the bioavailability of Pro-Hyp was enhanced by the co-administration of CH with RPH. Pyroglutamyl peptides, the food-derived peptides after oral administration of RPH, suppressed the degradation of Pro-Hyp by intestinal mucosa exopeptidases. Investigation of the type of inhibition of exopeptidases in the crude mucosa by pyroglutamyl

peptides is necessary for future work to support these findings. In addition, a comprehensive identification of the enzymes involved in peptide degradation, including endoproteinases and exopeptidases, can provide valuable knowledge to establish a robust in vitro digestion method that can be used to predict food-derived peptides, not only for CH.

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Supplemental Material

Supplemental Figure

Figure S1. Total ion chromatogram of B1-B4 compared to blank

Figure S2. Total ion chromatogram of B1-B7 compared to blank

Figure S3. Food-derived peptides with amino group in the small intestinal lumen of rats 1 h after oral administration of CH, RPH, and co-administration of CH+RPH

Figure S4. Food-derived peptides with amino group in the small intestinal tissue of rats 1 h after oral administration of CH, RPH, and co-administration of CH+RPH

Figure S5. Food-derived peptides with amino group in the blood plasma of rats 1 h after oral administration of CH, RPH, and co-administration of CH+RPH

Supplemental Table

Table S1. Estimated peptides in collagen hydrolysate (CH) before digestion

Table S2. Identified normal peptides in the small intestinal lumen after oral administration of CH in rats

Table S3. Identified pyroglutamyl peptides in the small intestinal lumen after oral administration of CH in rats

Table S4. Identified normal peptides with amino acid in the small intestinal lumen after oral administration of RPH in rats

Table S5. Identified pyroglutamyl peptides in the small intestinal lumen after oral administration of RPH in rats

Supplemental Data (MS-Fit results)

Supplemental data S1. MS-Fit prediction for B1

Supplemental data S2. MS-Fit prediction for B2

Supplemental data S3. MS-Fit prediction for B3
Supplemental data S4. MS-Fit prediction for B4
Supplemental data S5. MS-Fit prediction for B1
Supplemental data S6. MS-Fit prediction for B2
Supplemental data S7. MS-Fit prediction for B3
Supplemental data S8. MS-Fit prediction for B4
Supplemental data S9. MS-Fit prediction for B5
Supplemental data S10. MS-Fit prediction for B6

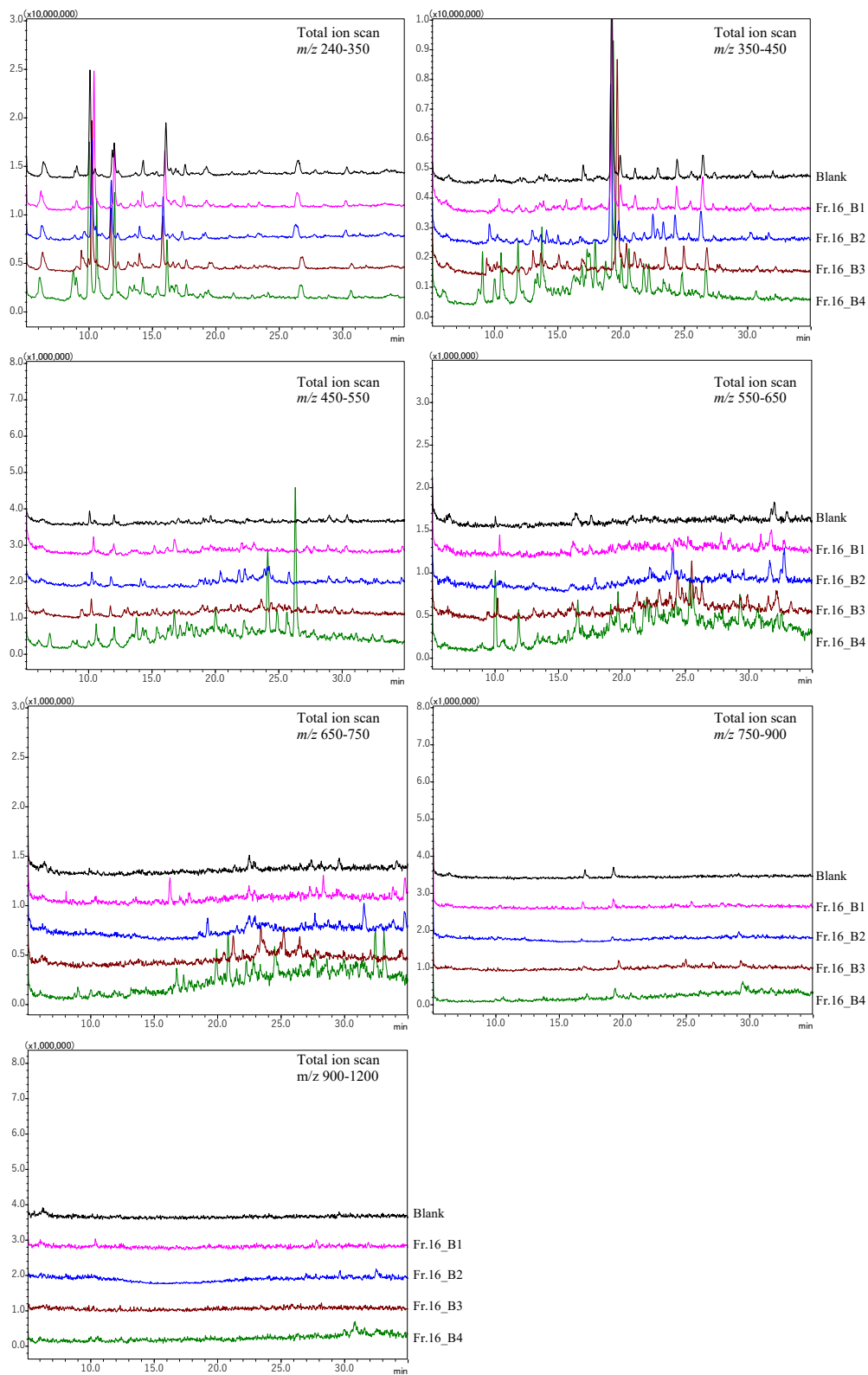


Figure S1. Total ion chromatograms of SEC Fr.16 band a-b compared to the blank band

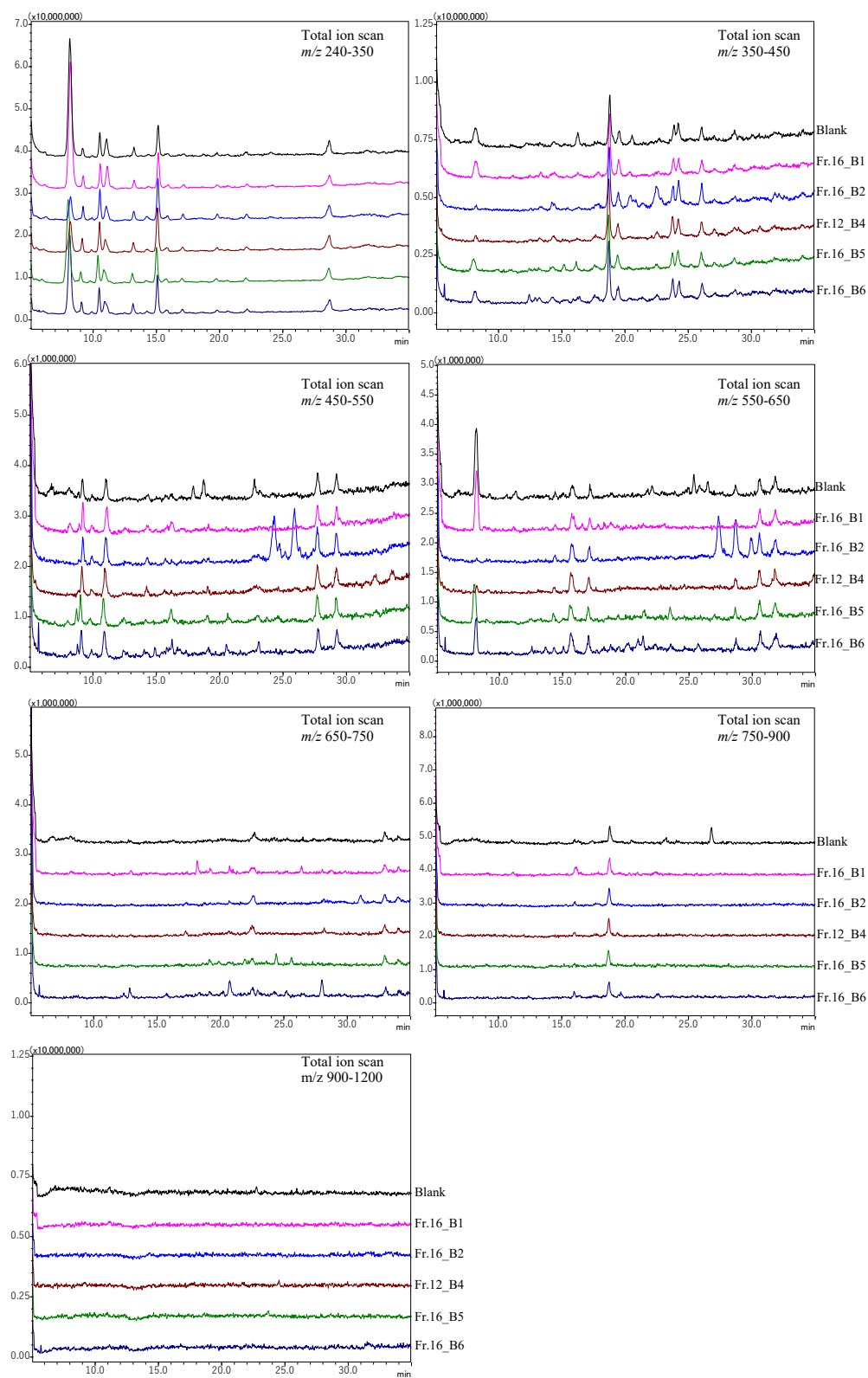


Figure S2. Total ion chromatograms of AEC Fr.16 band A-F compared to the blank band

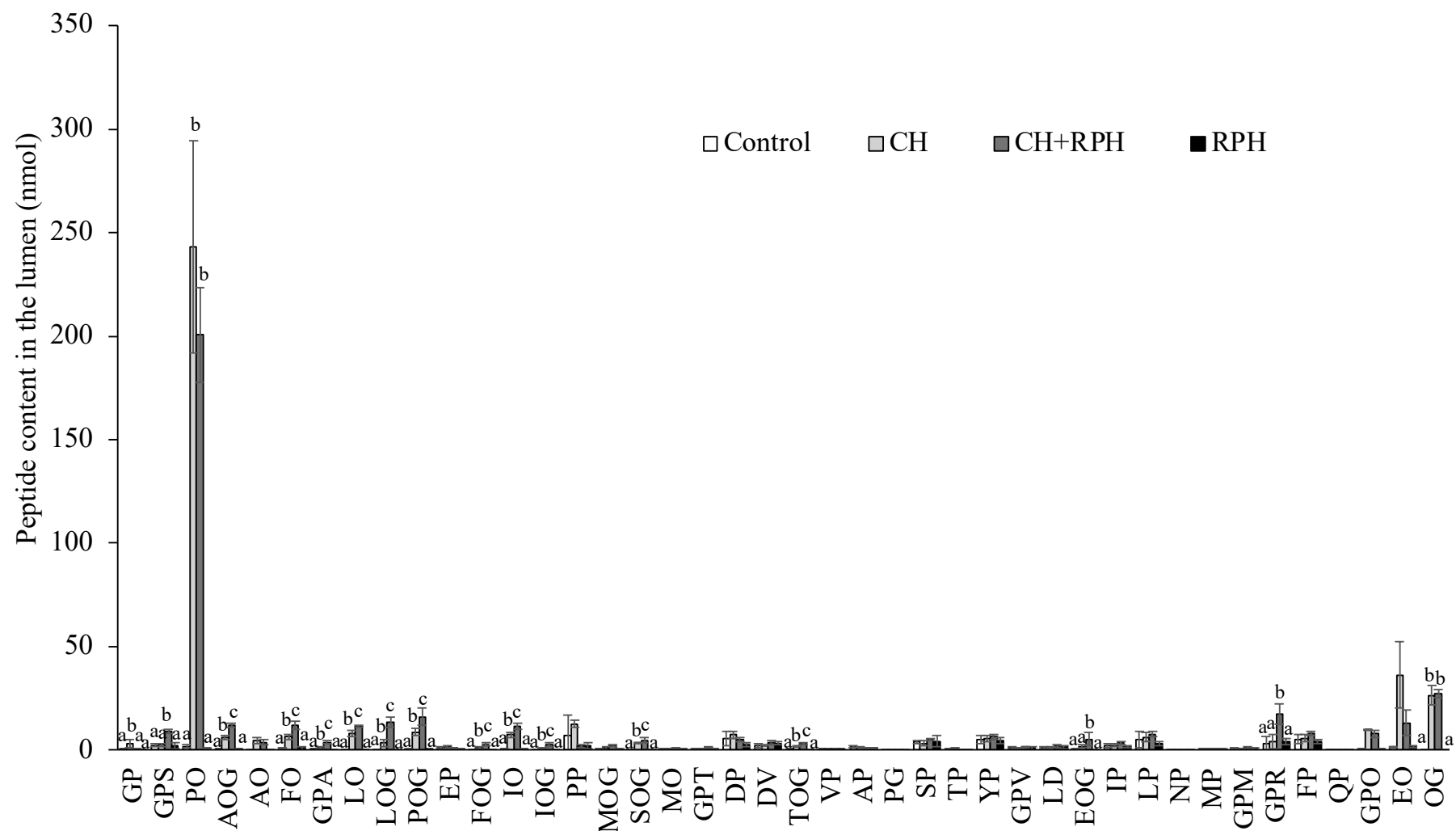


Figure S3. Food-derived peptides with amino group in the lumen of the small intestine of rats 1 h after oral administration of CH, RPH, and co-administration of CH+RPH

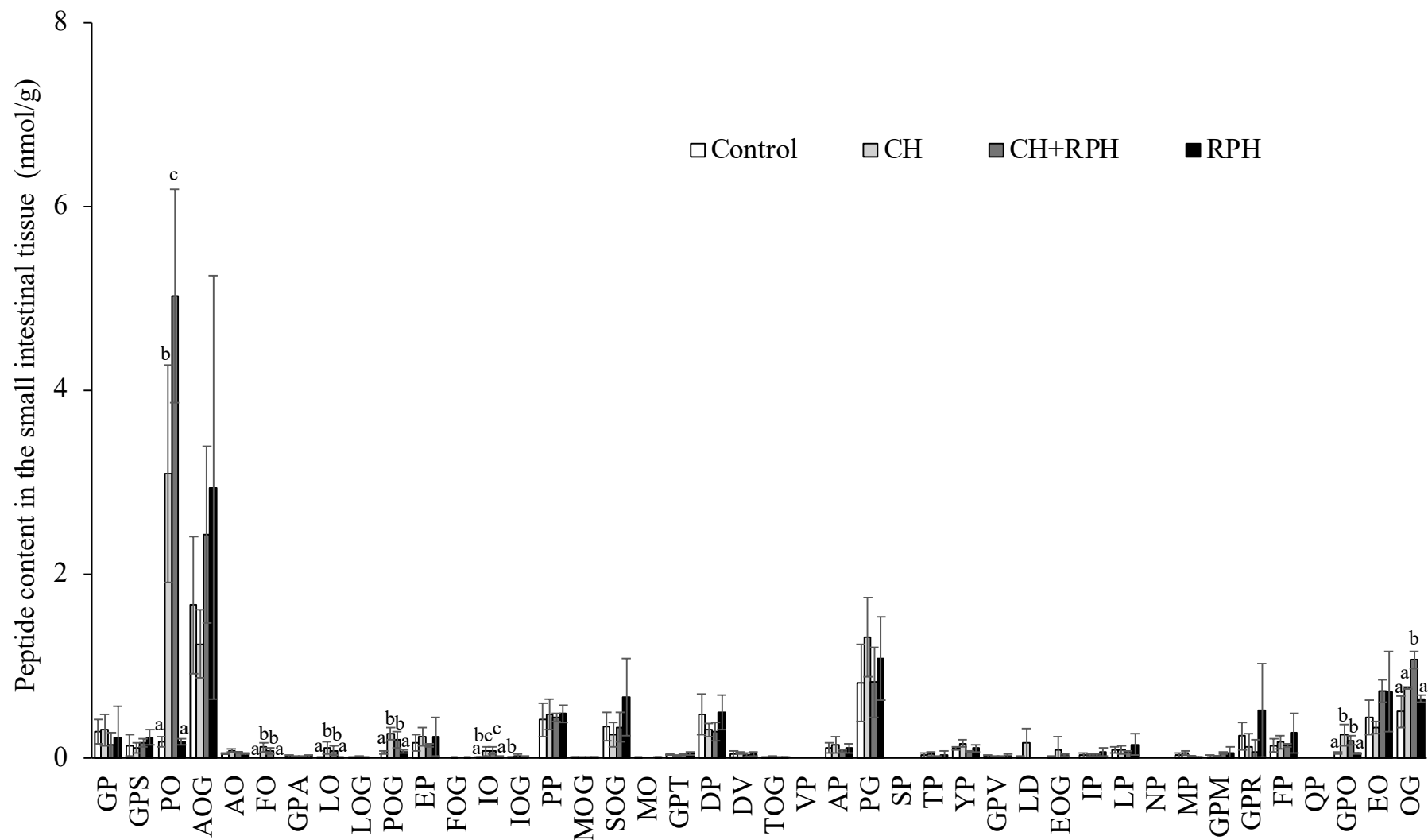


Figure S4. Food-derived peptides with amino group in the small intestinal tissue of rats 1 h after oral administration of CH, RPH, and co-administration of CH+RPH

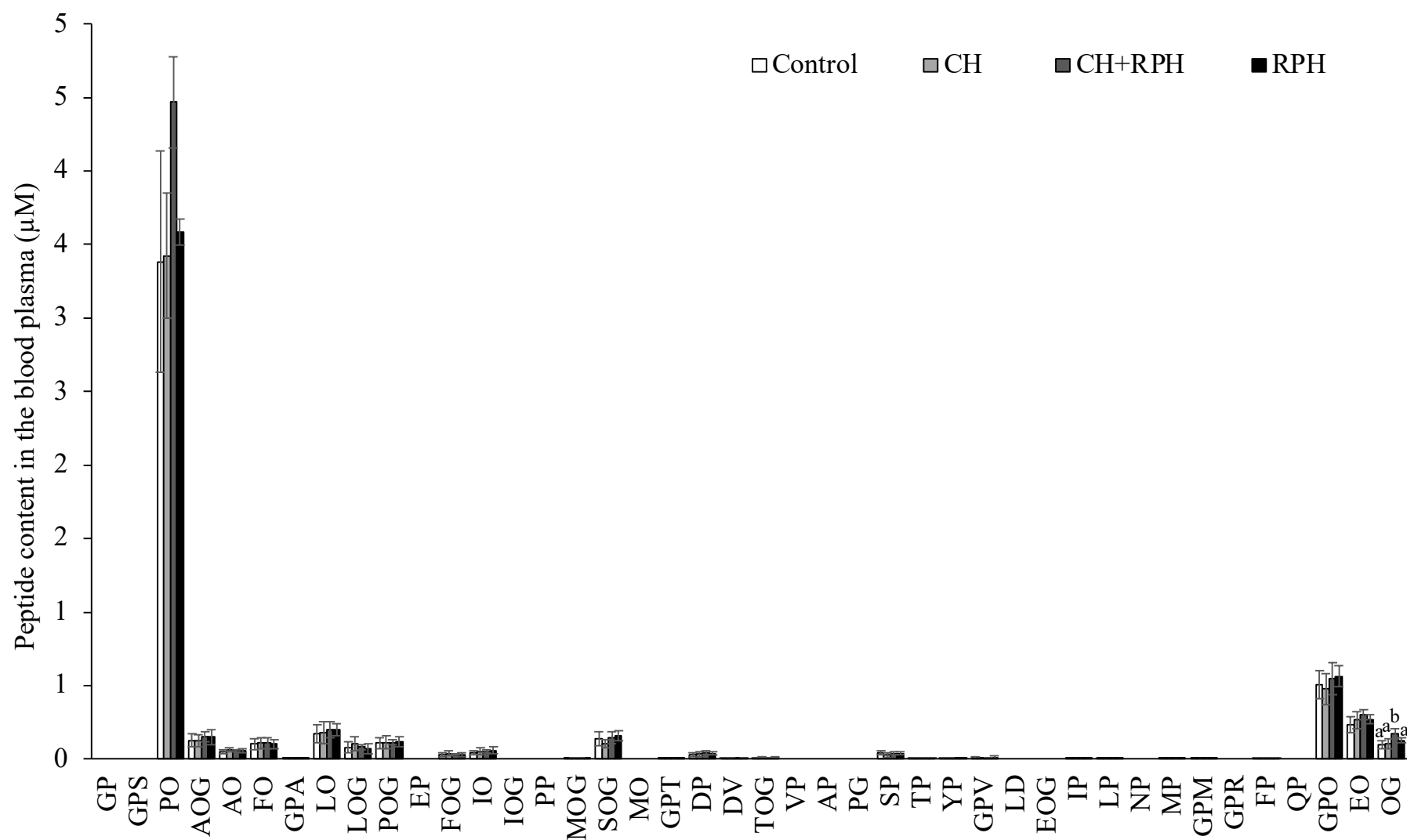


Figure S5. Food-derived peptides with amino group in the blood plasma of rats 1 h after oral administration of CH, RPH, and co-administration of CH+RPH

Supplemental Table S1. Estimated peptides in collagen hydrolysate (CH) before digestion.

Precursor ion (m/z)	MW	Estimated sequence (3 letter)	Estimated sequence (1 letter)	Product Ion (m/z)
132.1	131.1	Ile	I	30.3, 44.2, 69.1, 86.1 (*Ile), 132.2 (y1)
132.1	131.1	Leu	L	30.3, 44.2, 86.1 (*Leu), 132.3 (y1)
166.0	165.0	Phe	F	120.1 (*Phe), 166.1 (y1)
182.1	181.1	Tyr	Y	65.1, 77.1, 91.1, 107.0, 119.0, 136.1 (*Tyr), 165.0, 182.0 (y1)
189.1	188.1	Ala-Val	AV	22.2, 44.3 (*Ala), 72.2 (*Val), 118.0 (y1), 143.2, 189.0 (y2)
189.1	188.1	Leu-Gly	LG	30.3 (*Gly), 43.3, 44.2, 86.1 (*Leu), 189.0 (y2)
189.1	188.1	Gly-Leu	GL	30.3 (*Gly), 44.2, 86.1 (*Leu), 132.2 (y1), 189.0 (y2)
203.1	202.1	Ile-Ala	IA	30.2, 44.2, 69.2, 86.1 (*Ile)
203.1	202.1	Leu-Ala	LA	30.4, 44.3 (*Ala), 86.1 (*Leu)
203.1	202.1	Ala-Leu	AL	30.1, 44.3 (*Ala), 86.1 (*leu), 132.1 (y1)
205.1	204.1	Trp	W	159.1 (*Trp), 205.1 (y1)
207.1	206.1	Phe-ACN	F-acetonitrile	77.1, 79.2, 103.1, 120.0 (*Phe), 166.1 (y1)
214.6	213.6	Val-Pro	VP	41.9, 55.3, 70.2 (*Pro), 72.2 (*Val), 73.1, 86.0, 116.2 (y1), 129.2, 215.0 (y2)
217.1	216.1	Val-Val	VV	55.1, 72.2 (*Val), 118.0 (y1), 217.0 (y2)
219.1	218.1	Val-Thr	VT	29.5, 43.4, 55.2, 71.8 (*Val), 74.2 (Thr), 84.2, 101.9, 120.4 (y1)
219.1	218.1	Ser-Leu	SL	330.5, 42.3, 60.1, 86.1 (*Leu), 132.0 (y1), 219.0 (y2)
223.1	222.1	Gly-Phe	GF	30.1 (*Gly), 103.1, 120.1 (*Phe), 166.1 (y1)
231.1	230.1	Val-Ile	VI	30.4 (*Gly), 44.3 (*Ala), 69.2, 72.1 (*Val), 86.2 (*Ile)
231.1	230.1	Val-Leu	VL	30.4 (*Gly), 44.3 (*Ala), 69.2, 86.2 (*Leu)
233.1	232.1	Leu-Thr	LT	30.6, 44.3 (*Ala), 55.8, 86.2 (*Leu), 119.8 (y1), 152.1
233.1	232.1	Thr-Leu	TL	29.2, 56.3, 74.2 (*Thr), 86.1 (*Leu), 132.1 (y1), 233.1 (y2)
243.1	242.1	pGlu-Leu	pEL	30.7, 41.3, 56.3, 84.1 (*pE), 86.2 (*Leu), 142.0, 197.1
244.1	243.1	Gly-Pro-Ala	GPA	30.1 (*Gly), 70.1 (*Pro), 127.1, 155.0 (b2), 187.0 (y2), 226.9, 244.0 (y3)
245.1	244.1	Ile-Ile	II	30.3, 41.3, 44.3, 69.1, 86.2 (*Ile/Leu), 131.9 (y1)

Precursor ion (m/z)	MW	Estimated sequence (3 letter)	Estimated sequence (1 letter)	Product Ion (m/z)
245.1	244.1	Leu-Leu	LL	30.3, 41.3, 44.3, 86.2 (*Ile/Leu), 131.9 (y1)
249.1	248.1	Val-Met	VM	55.2, 72.1 (*Val), 104.1 (*Met), 133.1, 150.0 (y1)
260.1	259.1	Gln-Ile	QI	30.5, 44.2, 69.2, 84.0, 86.1 (*Ile), 101.1 (*Gln), 130.0, 147.1 (y1), 243.2, 260.0 (y2)
260.1	259.1	Gln-Leu	QL	30.5, 44.2, 84.0, 86.1 (*Leu), 101.1 (*Gln), 130.0, 147.1 (y1), 243.2, 260.0 (y2)
261.1	260.1	pGlu-Met	pEM	55.9, 61.2, 84.1 (pE), 87.0, 101.8, 104.2 (*Met), 130.3, 149.6 (y1), 167.2, 212.8, 261.1 (y2)
263.1	262.1	Ile-Met	IM	30.5, 44.2, 69.1, 86.2 (*Ile), 104.2 (*Met), 133.2, 150.0 (y1), 263.0 (y2)
263.1	262.1	Leu-Met	LM	30.4, 44.1, 70.1, 86.2 (*Leu), 103.9 (*Met), 133.2, 149.9 (y1), 262.9 (y2)
265.1	264.1	Phe-Val	FV	71.9 (*Val), 103.2, 118.1 (y1), 120.0 (*Phe), 137.1
265.1	264.1	Val-Phe	VF	55.1, 72.1 (*Val), 102.8, 120.1 (*Phe), 166.0 (y1), 264.9 (y2)
267.1	266.1	Phe-Thr	FT	56.3, 74.1 (*Met), 77.2, 103.1, 120.0 (*Phe or y1)
277.1	276.1	pGlu-Phe	pEF	41.2, 84.2 (*pGlu), 119.9 (*Phe), 165.1 (y1), 230.8, 277.2 (y2)
279.1	278.1	Glu-Met	EM	44.2, 56.1, 84.1, 86.1, 102.2, 104.1, 129.8, 132.9, 150.1, 167.1, 213.1, 214.9, 237.9, 242.8, 261.1, 279.2
279.1	278.1	Ile-Phe	IF	30.6, 41.4, 44.3, 69.1, 86.2 (*Ile), 103.2, 120.2 (*Phe), 166.0 (y1), 279.0 (y2)
279.1	278.1	Phe-Leu	FL	30.5, 86.1 (*Leu), 103.1, 120.1 (*Phe), 279.0 (y2)
279.1	278.1	Leu-Phe	LF	30.3, 44.0, 86.2 (*Leu), 103.2, 120.2 (*Phe), 166.0 (y1), 279.0 (y2)
281.1	280.1	Val-Tyr	VY	55.2, 72.1 (*Val), 91.2, 136.0 (*Tyr), 164.7, 182.0 (*Tyr)
286.1	285.1	Gly-Pro-Hyp	GPO	30.5, 70.2, 116.1 (y1), 127.1, 131.1 (y1), 155.1 (b2), 229.1 (y2), 286.0 (y3)
293.1	292.1	pGlu-Tyr	pEY	56.3, 83.9 (*pGlu), 90.8, 119.2, 122.7, 136.3 (*Tyr), 147.4, 181.9 (y1), 202.1, 247.0, 292.8 (y2)
294.1	293.1	Phe-Gln	FQ	84.1, 93.0, 100.9 (*Gln), 103.0, 120.1 (*Phe), 130.0, 147 (y1).0, 165.0, 242.2, 277.0, 294.0 (y2)
295.1	294.1	Glu-Phe	EF	41.2, 56.2, 84.0, 103.1, 120.1 (*Phe), 130.1, 166.0 (y1), 185.8, 231.2, 277.0
295.1	294.1	Tyr-Leu or Leu-Tyr	YL or LY	30.9, 73.8 (*Thr), 85.9 (*Leu), 91.0, 118.8, 136.1 (*Tyr), 220.8, 235.0, 248.9, 278.1, 294.9 (y2)
297.1	296.1	Phe-Met	FM	56.2, 84.1, 103.0, 120.1 (*Phe), 133.1, 150.0 (y1), 251.0, 297.0 (y2)
304.1	303.1	Gly-Pro-Met	GPM	70.2, 86.1, 104.2 (*Met), 127.1, 150.0 (y1), 154.9, 247.1, 286.8 (y2), 304.0 (y3)

Precursor ion (m/z)	MW	Estimated sequence (3 letter)	Estimated sequence (1 letter)	Product Ion (m/z)
309.7	308.7	Phe-Ser-Gly	FSG	60.1 (*Ser), 103.1, 120.1 (*Phe), 162.9, 207.0, 235.0, 291.9, 309.9 (y3)
315.1	314.1	Ala-Gly-Pro-Ala	AGPA	44.5, 70.2 (*Pro), 127.0, 155.2, 155.2, 187.1 (y2), 226.0 (b3), 244.1 (y3), 315.0 (y4)
329.1	328.1	Gly-Pro-Arg	GPR	30.1 (Gly), 60.1, 70.2 (*Pro), 116.2 (y1), 126.9, 175.1 (y1), 215.2, 255.0 (b2), 293.0, 329.0 (y3)
357.2	356.2	Ala-Pro-Val-Ala	APVA	44.6, 69.9, 72.4, 126.9, 158.9, 234.8, 249.4, 277.8, 357.4
357.2	356.2	Ile-Gly-Pro-Ala	IGPA	70.3, 86.3, 127.0, 155.0, 173.0, 186.8, 357.0
357.2	356.2	Leu-Gly-Pro-Ala	LGPA	70.2, 86.1, 127.2, 155.0, 173.0, 187.1, 357.0
373.2	372.2	Hyp-Gly-Pro-Ser	OGPS	70.3, 86.2, 127.0, 154.9, 202.7 (y2), 241.9, 241.9, 260.0, 372.9 (y4)
383.2	382.2	Pro-Leu-Gly-Pro	PLGP	70.2 (*Pro), 172.9 (y2)
388.1	387.1	Gly-Pro-Ser-Gly-Ala	GPSGA	30.1, 60.4, 70.2, 86.4, 126.8, 154.9, 170.8, 260.0 (b3), 387.8 (y5)
393.1	392.1	Gly-Phe-Hyp-Gly	GFOG	86.2, 120.1, 188.9 (y2)
398.2	397.2	Pro-Gln-Gly-Pro	PQGP	70.2 (*Pro), 116.2 (y1), 127.2, 155.0, 172.8, 226.0, 254.9 (y3), 285.0 (b3), 379.9, 397.6 (y4)
400.2	399.2	Val-Gly-Pro-Gln	VGPQ	70.3, 72.1, 84.1, 100.7, 127.0, 147.1, 154.9, 243.8 (y2), 301.1 (y3), 400.0 (y4)
401.0	400.0	Pro-Gly-Pro-Met	PGPM	70.2 (*Pro), 127.0, 154.9, 246.6, 303.8 (y3), 400.9
417.2	416.2	Leu-Gly-Pro-Met	LGPM	70.3 (*Pro), 86.1 (*Leu), 126.9, 150.0 (y1), 155.0, 247.0, 303.8 (y2), 417.0 (y4)
423.2	422.2	Pro-Gly-His-Leu	PGHL	44.0 (*Ala), 70.3 (*Pro), 186.9, 378.3, 422.7 (y4)
469.2	468.2	pGlu-Gly-Pro-Gln-Gly	pEGPQG	70.1 (*Pro), 127.1, 155.0, 187.0, 244.0, 282.1, 469.1
472.2	471.2	pGlu-Gly-Pro-Pro-Ile-Gly	pEGPPIG	70.0 (*Pro), 84.0 (*pGlu), 86.2 (*Ile), 116.0 (y1), 125.0, 143.0, 171.0, 189.0 (Pro-Ile), 284.0 (b3), 472.0 (y6)
472.2	471.2	pGlu-Gly-Pro-Pro-Leu-Gly	pEGPPLG	70.0 (*Pro), 84.0 (*pGlu), 86.2 (*Leu), 116.0 (y1), 125.0, 143.0, 171.0, 189.0 (Pro-Leu), 284.0 (b3), 472.0 (y6)
485.1	484.1	Gly-Ala-Hyp-Gly-Pro-Ala	GAOGPA	70.2 (*Pro), 86.0 (*Ile/Leu/Hyp), 127.0, 154.9, 171.0, 187.0, 244.0 (y3), 202.0, 339.0, 357.0, 484.9 (y6)
488.0	487.0	Hyp-Gly-Leu-Trp	OGLW	86.1 (*Hyp/Ile/Leu), 143.1, 147.8, 170.9, 205.0, 317.8 (y2), 488.1 (y4)
511.2	510.2	Pro-Leu-Gly-Pro-Gln	PLGPQ	70.2 (*Pro), 100.9 (*Gln), 106.0 (y1), 127.1, 154.9, 179.8, 229.1 (b2), 282.7 (y3), 285.8 (b3), 396.2, 510.8 (y5)
513.2	512.2	Gly-Pro-Val-Gly-Pro-Ser	GPVGPS	70.2 (*Pro), 72.4 (*Val), 106.0 (y1), 127.0, 155.0, 203.0 (Pro-Val), 260.0 (y3), 310.6, 408.0 (b5), 512.9 (y6)

Table S2. Identified normal peptides with amino acid in the small intestinal lumen after oral administration of CH in rats

Peak	precursor ion (m/z)	MW	Estimated sequence	Product Ion (m/z)
13	373	202	Ala-Hyp	132.2 (y1), 171.0 (AccQ, b1), 203.0 (y2), 242.0 (b2), 372.7
14	383	212	Pro-Pro	70.3 (*Pro), 116.3 (y1), 170.7 (AccQ, b1), 212.8 (y2), 268 (b2), 383.1
15	385	214	Val-Pro	72.3 (*Val), 116.1 (y1), 145.1, 170.8, 214.9 (y2), 241.8 (a=?), 270.0 (b2), 384.8
16	399	228	Pro-Hyp	70.1 (*Pro), 86 (*Hyp), 132.4 (Hyp), 171.0 (AccQ, b1), 228.9 (y2), 267.7 (b2), 399.5
17	399	228	Ile-Pro	70.2 (*Pro), 116.1 (y1), 145.4, 171.0 (AccQ, b1), 229.2, 255.8, 284 (b2), 399.1
18	399	228	Leu-Pro	70.2 (*Pro), 116.1 (y1), 145.4, 171.0 (AccQ, b1), 229.2, 255.8, 284 (b2), 399.1
19	403	232	Asp-Val	72.1 (*Val), 88 (*Asp), 118.3 (y1), 144.8, 171.0 (AccQ, b1), 232.9 (y2), 259 (a2), 285.9 (b2), 402.8
20	414	243	Gly-Pro-Ala	70.2 (*Pro), 127.1, 154.9, 171.0 (AccQ, b1), 187.0, 200, 227.8 (b2), 243.9, 414.0
21	415	244	Glu-Pro	70 (*Pro), 84.3, 116.1 (y1), 144.8, 170.8, 227.2, 244.7 (y2), 271.9 (a2=?), 300.0 (b2), 415.0
22	415	244	Ile-Hyp	86.1 (*Ile), 132.1 (y1), 171.0 (AccQ, b1), 245.0 (y2), 256.1, 283.9 (b2), 414.9
23	415	244	Leu-Hyp	86.1 (*Leu), 132.1 (y1), 171.0 (AccQ, b1), 245.0 (y2), 256.1, 283.9 (b2), 414.9
24	417	246	Leu-Asp	86.2 (*Ile/Leu), 88 (*Asp very low), 144.6, 170.9 (AccQ, b1), 256.1, 285.2 (b2 for Asn), 416.9
25	430	259	Ala-Hyp-Gly	44.4 (*Ala), 86.1 (*Hyp), 144.9, 171.0 (AccQ, b1), 189.0 (y2), 213.6 (a2), 242.1 (b2), 258.6 (y3), 430
26	430	259	Gly-Pro-Ser	70.1 (*Pro), 88.3 (*Asp), 106 (y1), 127.0, 145, 171.1 (AccQ, b1), 200.1 (a2), 203.0 (y2), 227.8 (b2), 242.0, 260.0 (y3), 268.3 (b2), 429.7
27	433	262	Met-Hyp	55.8, 86.5 (*Hyp), 104.3 (*Met), 132.0 (y1), 170.9 (AccQ, b1), 263.1, 302.3 (b2), 433.0
28	442	271	Gly-Pro-Val	70.2 (*Pro), 72 (*Val), 116 (y1), 117.9 (y1), 145.0, 171.0 (AccQ, b1), 200 (a2), 215.3 (y2), 228 (b2), 272.1 (y3), 442.1
29	449	278	Phe-Hyp	120.2 (*Phe), 132.0 (y1), 171.0 (AccQ, b1), 279.1 (y2), 289.9, 318.1 (b2), 448.9
30	456	285	Pro-Hyp-Gly	70.3 (*Pro), 86 (*Hyp), 141.0, 171.1 (AccQ, b1), 189.5 (y2), 286.1 (y3), 455.9
31	456	285	Gly-Pro-Hyp	30 (*Gly), 70 (*Pro), 132 (y1), 171 (accQ, b1), 22 (b2, y2), 286 (y3), 456.1 (y4)

32	460	289	Thr-Hyp-Gly	74 (*Thr), 86.1 (*Hyp), 145.1, 171.3 (AccQ, b1), 189.1 (y2), 244, 2 (a2), 71.7 (b2), 289.8 (y3), 459.3
33	472	301	Ile-Hyp-Gly	86.5 (*Ile), 171.0 (AccQ, b1), 188.9 (y2), 255.8 (a2), 283.7 (b2), 302 (y3), 472
34	472	301	Leu-Hyp-Gly	86.2 (*Leu), 170.8 (AccQ, b1), 188.8 (y2), 226.4 (?), 256.1 (a2), 283.9 (b2), 302 (y3), 471.8
35	474	303	Gly-Pro-Met	70.0 (*Pro), 127.0, 155.2, 170.8 (AccQ, b1), 200.2 (a2), 227.7 (b2), 246.5 (y2), 285.3, 304.1 (y3), 473.1
36	488	317	Glu-Hyp-Gly	76.6, 85.2 (*Hyp), 102 (*Glu), 145.3, 170.6 (AccQ, b1), 189.3 (y2), 224.8, 272.1 (a2), 300.0 (b2), 487.8
37	490	319	Met-Hyp-Gly	55.7, 85.5 (*Hyp), 104.3 (*Met), 131.9 (y1), 170.7 (AccQ, b1), 188 (y2), 273.7 (a2=?), 301.6 (b2), 319.4, 489.6
38	506	335	Phe-Hyp-Gly	86.0 (*Ile/Leu), 119.8 (*Phe), 145.2, 170.9 (AccQ, b1), 189 (y2), 289.7, 317.6 (b2), 335.8, 505.3

Table S3. Identified pyroglutamyl (pGlu) peptides in the small intestinal lumen after oral administration of CH in rats

Peak	Precursor ion (m/z)	MW	Estimated sequence	Product Ion (m/z)
1	227	226	pyroGlu-Pro	56, 70 (*Pro), 84 (*pGlu), 116 (y1 Pro), 181, 200
2	229	228	pyroGlu-Val	41, 72 (*Val), 84 (*pGlu), 118 (y1 Val), 183
3	243	242	pyroGlu-Ile	56, 84 (*pGlu), 85 (*Ile), 132 (y1 Ile), 197 (a2), 243
4	243	242	pyroGlu-Leu	56, 84 (*pGlu), 85 (*Leu), 132 (y1 Leu), 197 (a2)
5	261	260	pyroGlu-Met	56, 61, 84(*pGlu), 104 (*Met), 150
6	277	276	pyroGlu-Phe	84 (*pGlu), 103, 120 (*Phe), 166 (y1 Phe)
3*	284	283	pyroGlu-Ile+CH ₃ CN	56, 84 (*pGlu), 86 (*Ile), 132 (y1 Ile), 197, 225, 243 (y2), 284 (y2+CH ₃ CN)
4*	284	283	pyroGlu-Leu+CH ₃ CN	56, 84 (*pGlu), 86 (*Leu), 132 (y1 Leu), 197, 225, 243 (y2), 284 (y2+CH ₃ CN)
7	284	283	pyroGlu-Gly-Pro	70 (*Pro), 84 (*pGlu), 116 (y1 Pro), 169
8	293	292	pGlu-Tyr	23.5, 41.2, 56.2, 84.1 (*pGlu), 136, 165, 182 (y1 Tyr), 293
9	300	299	pyroGlu-Hyp-Gly	56, 84 (*pGlu), 86 (*Hyp), 197 (a2), 225
10	326	325	pyroGlu-Val-Pro	70 (*Pro), 72 (*Val), 84 (*pGlu), 116 (y1 Pro), 183
11	340	339	pyroGlu-Ile-Pro	70 (*Pro), 84 (*pGlu), 86 (*Leu), 116 (y1 Pro), 197, 225, 258, 339.8
12	340	339	pyroGlu-Leu-Pro	70 (*Pro), 84 (*pGlu), 86 (*Leu), 116 (y1 Pro), 197, 225, 258, 339.8

Table S4. Identified normal peptides with amino acid in the small intestinal lumen after oral administration of RPH in rats

Peak	Precursor ion (m/z)	MW	Estimated sequence	Product Ion (m/z)
t	343	172	Gly-Pro	116 (y1), 171.0 (AccQ, b1), 173.1 (y2), 227.9 (b2), 343
u	383	212	Pro-Pro	70.3 (*Pro), 116.3 (y1), 170.7 (AccQ, b1), 212.8 (y2), 268 (b2), 383.1
v	399	228	Ile-Pro	86.2 (*Ile), 116.0 (y1), 170.9 (AccQ, b1), 229.0 (y2), 256.0 (a=?), 283.9 (b2), 399.0
w	399	228	Leu-Pro	86.2 (*Leu), 116.0 (y1), 170.9 (AccQ, b1), 229.0 (y2), 256.0 (a=?), 283.9 (b2), 399.0
v*	400	229	Leu-Pro	86.2 (*Ile/Leu), 116.3 (y1), 171.3 (AccQ, b1), 229.1 (y2), 256.9 (a2), 284.9 (b2), 400
x	401	230	Asp-Pro	70.5 (*Pro), 115.7 (y1), 170.9 (AccQ, b1), 213.5, 231.3 (y2), 286.0 (b2)
y	403	232	Asp-Val	72.1 (*Val), 88 (*Asp), 118.3 (y1), 144.8, 171.0 (AccQ, b1), 232.9 (y2), 259 (a2), 285.9 (b2), 402.8
z	415	244	Glu-Pro	70 (*Pro), 84.3, 116.1 (y1), 144.8, 170.8, 227.2, 244.7 (y2), 271.9 (a2=?), 300.0 (b2), 415.0
aa	417	246	Leu-Asp	86.2 (*Ile/Leu), 88 (*Asp very low), 144.6, 170.9 (AccQ, b1), 256.1, 285.2 (b2 for Asn), 416.9
ab	449	278	Tyr-Pro	116.1 (y1), 136.2 (*Tyr), 171.0 (AccQ, b1), 278.8 (y2), 306.2 (a2), 334.1 (b2), 448.8

Table S5. Identified pyroglutamyl (pGlu) peptides in the small intestinal lumen after oral administration of RPH in rats

Peak	Precursor ion (m/z)	MW	Estimated sequence	Product Ion (m/z)
a	201	200	pyroGlu-Ala	44 (*Ala), 56, 84 (*pGlu), 90
b	217	216	pyroGlu-Ser	41, 60 (*Ser), 70 (*Pro), 84 (*pGlu), 106 (y1 Ser), 171 (AccQ)
c	227	226	pyroGlu-Pro	56, 70 (*Pro), 84 (*pGlu), 116 (y1 Pro), 181, 200
d	229	228	pyroGlu-Val	41, 72 (*Val), 84 (*pGlu), 118 (y1 Val), 183
e	243	242	pyroGlu-Ile	56, 84 (*pGlu), 85 (*Ile), 132 (y1 Ile), 197 (a2), 243
f	243	242	pyroGlu-Leu	56, 84 (*pGlu), 85 (*Leu), 132 (y1 Leu), 197 (a2)
g	258	257	pyroGlu-Gln	56, 84 (*pGlu), 130
h	259	258	pyroGlu-Glu	56, 84 (*pGlu), 101 (*Gln), 130/131
i	261	260	pyroGlu-Met	56, 61, 84(*pGlu), 104 (*Met), 150
j	277	276	pyroGlu-Phe	84 (*pGlu), 103, 120 (*Phe), 166 (y1 Phe)
e*	284	283	pyroGlu-Ile+CH ₃ CN	56, 84 (*pGlu), 86 (*Ile), 132 (y1 Ile), 197, 225, 243 (y2), 284 (y2+CH ₃ CN)
f*	284	283	pyroGlu-Leu+CH ₃ CN	56, 84 (*pGlu), 86 (*Leu), 132 (y1 Leu), 197, 225, 243 (y2), 284 (y2+CH ₃ CN)
k	293	292	pGlu-Tyr	23.5, 41.2, 56.2, 84.1 (*pGlu), 136, 165, 182 (y1 Tyr), 293
l	314	313	pyroGlu-Ser-Pro	60 (*Ser), 70 (*Pro), 84 (*pGlu), 116 (y1 Pro), 171 (AccQ), 203
j*	318	317	pyroGlu-Phe-ACN	84 (*pGlu), 103, 120 (*Phe), 166 (y1 Phe), 318 (y2+CH ₃ CN)
m	330	329	pyroGlu-Ile-Ser	60 (*Ser), 84 (*pGlu), 86 (*Ile), 88 (*Asp), 106 (y1 Ser), 197, 225
n	330	329	pyroGlu-Leu-Ser	60 (*Ser), 84 (*pGlu), 86 (*Leu), 88 (*Asp), 106 (y1 Ser), 197, 225
o	340	339	pyroGlu-Ile-Pro	70 (*Pro), 84 (*pGlu), 86 (*Leu), 116 (y1 Pro), 197, 225, 258, 339.8
p	340	339	pyroGlu-Leu-Pro	70 (*Pro), 84 (*pGlu), 86 (*Leu), 116 (y1 Pro), 197, 225, 258, 339.8
q	355	354	pyroGlu-Gln-Pro	70 (*Pro), 84 (*pGlu), 116 (y1 Pro), 129, 227, 240

Supplemental data S1. MS-Fit prediction for B1

MS-Fit Search Results

R 5/07/28 15:35

MS-Fit Search Results

[+] Parameters

[+] **Pre Search Results (SwissProt.2021.06.18.sub)**

Fraction-Spot-Run ID: 1-1-1

MS-Fit search selects **404** entries (results displayed for top **20** matches).

[–] Results Summary

Protein Hit Number	MOWSE Score	# pep # mat % mat 30 pks	% Cov	% TIC	Mean Err Da	Data Tot Da	# Hom Prot	MS- Digest Index #	Protein MW (Da)/pI	Accession #	Species	Protein Name
1	1371	8/8/27	7.8	26.7	0.159	0.773	No	16541	109449/5.3	P15684	RAT	Aminopeptidase N
2	570	11/11/37	10.0	36.7	-0.156	0.812	No	109002	108046/5.8	Q9EPA0	RAT	Dystrophin-related protein 2
3	559	8/8/27	4.7	26.7	-0.313	0.769	No	237990	165449/5.9	O88281	RAT	Multiple epidermal growth factor-like domains protein 6
4	397	8/8/27	5.4	30.0	-0.0505	0.830	No	17968	139208/5.9	P0C6S7	RAT	Ankyrin repeat and sterile alpha motif domain-containing protein 1B
5	258	12/14/47	8.3	46.7	0.163	0.722	No	562312	165540/9.2	Q499R0	RAT	Zinc finger protein 518A
6	232	14/12/40	12.3	40.0	-0.00991	0.872	No	346537	108819/6.2	Q811U3	RAT	ELKS/Rab6-interacting/CAST family member 1
7	220	11/9/30	7.7	30.0	0.128	0.836	No	497759	150288/8.9	Q6P3V7	RAT	Tetratricopeptide repeat protein 41
8	173	8/9/30	5.9	30.0	0.157	0.896	1	269602	128866/6.2	P55161	RAT	Nck-associated protein 1
9	172	7/7/23	6.6	23.3	0.0316	0.927	No	168058	123468/6.4	P23897	RAT	Heat-stable enterotoxin receptor
10	143	5/6/20	3.6	23.3	0.335	0.676	No	332951	106229/6.6	Q63259	RAT	Receptor-type tyrosine-protein phosphatase-like N
11	121	7/7/23	7.9	23.3	0.413	0.468	No	232735	100486/9.4	Q63560	RAT	Microtubule-associated protein 6
13	113	9/9/30	3.9	30.0	0.359	0.564	No	466554	172882/6.4	Q62910	RAT	Synaptojanin-1
14	107	6/5/17	6.7	16.7	0.0199	0.934	No	519565	100949/5.6	Q9R037	RAT	WD repeat-containing protein 44
15	107	6/6/20	8.1	20.0	0.149	0.806	No	31747	103021/5.8	Q8R4C1	RAT	Calcium-transporting ATPase type 2C member 2
16	89.9	11/10/33	9.3	33.3	-0.178	0.699	No	399259	127724/8.4	O54888	RAT	DNA-directed RNA polymerase I subunit RPA2
17	84.7	12/11/37	5.9	36.7	-0.166	0.844	No	110496	171559/7.7	Q9S45	RAT	Dual oxidase 2
18	84.2	7/8/27	5.8	26.7	-0.128	0.801	No	202597	130810/5.6	P18614	RAT	Integrin alpha-1
19	81.1	17/15/50	11.4	50.0	0.206	0.790	No	119040	163197/6.4	Q1JU68	RAT	Eukaryotic translation initiation factor 3 subunit A
20	80.8	9/8/27	4.3	26.7	0.0841	0.924	No	443332	157990/6.3	O7TP36	RAT	Protein Shroom2

16541	x	.	.	.	x	x	.	.	.	x	.	x	.	x	.	.	.	.	x	x	.	.
109002	.	x	.	.	.	x	x	x	.	.	x	x	x	.	x	.	.	.	.	.	.	x
237990	x	.	.	.	.	.	.	.	.	.	x	.	x	x	x	.	.	x	.	.	x	.
17968	.	x	.	x	.	.	.	.	.	.	x	.	x	x	.	x	.	.	x	x	.	.
562312	x	.	.	x	.	.	x	x	.	.	.	x	x	x	.	.	.	x	x	x	.	x
346537	.	.	x	.	.	.	x	x	x	x	.	.	.	x	x	x	.	.	x	.	.	.
497759	x	.	.	.	.	x	x	.	.	.	.	.	.	x	x	.	x	.	.	.	x	x
269602	x	.	.	.	x	x	x	x	.	x	x	.	.	.	.	.	.	x	.	.	.	.
168058	.	.	.	x	.	.	.	.	.	.	x	x	.	.	.	.	.	x	.	.	x	.
332951	.	.	.	x	.	.	x	x	.	.	.	x	x	x	.	.	.	.	.	.	.	.
232735	.	.	.	x	.	.	.	.	x	x	x	.	.	x	.	.	.	.	.	.	.	x
466554	.	.	.	.	.	.	x	x	.	x	x	.	x	x	.	.	.	x	.	.	.	.
519565	.	.	.	.	.	.	.	x	.	.	x	.	.	.	.	x	.	x	.	x	.	.
31747	.	.	.	.	.	x	.	.	.	.	.	.	.	.	.	.	.	x	.	.	x	x
399259	.	.	x	.	x	x	x	.	.	x	.	x	.	.	.	.	.	.	x	x	.	x
110496	.	x	x	x	x	.	.	.	x	.	.	.	.	.	.	.	.	x	x	x	x	.
202597	.	x	.	.	x	.	.	x	x	x	.	.	.	.	.	.	.	.	x	.	.	x
119040	x	.	x	.	x	x	x	x	x	.	.	.	x	x	x	.	.	.	x	.	.	x
443332	.	x	x	.	.	.	.	.	.	x	.	x	.	x	x	x	.	.	.	.	.	.

Similar Matches for Protein Hit 8

Protein Hit Number	MOWSE Score	# pep # mat % Cov	% TIC	Mean Err Da	Data Tol Da	# Hom Prot	MS-Digest Index #	Protein MW (Da)	Accession #	Species	Protein Name
8	173	8/9/30	5.9 30.0	0.157	0.896	1	269602	128866/6.2	P55161	RAT	Nck-associated protein 1
Protein Hit Number	MOWSE Score	# pep # mat % Cov	% TIC	Mean Err Da	Data Tol Da	# Hom Prot	MS-Digest Index #	Protein MW (Da)	Accession #	Species	Protein Name
12	114	15/14/47	6.1 46.7	-0.0315	0.908	1	44532	207892/5.5	D4A631	RAT	Brefeldin A-inhibited guanine nucleotide-exchange protein 1

[-] Detailed Results

1. 8/30 matches (26%).

Acc. #: P15684 **Uniprot ID:** AMPN_RAT **Species:** RAT **Name:** Aminopeptidase N**Organism:** Rattus norvegicus **OX=10116** **Gene:** Anpep **Existence:** Evidence at protein level **Version:** 2**Index:** 16541 **MW:** 109449 Da **pI:** 5.3

m/z Submitted	MH ⁺ Matched	Intensity	Delta Da	Modifications	Start End	Missed Cleavages	Sequence
515.0000	515.3300	100.0	-0.330		297 300	0	(R)VQIR(I)
901.0000	901.4989	100.0	-0.499		935 942	0	(R)ALEQALEK(T)
911.0000	910.4339	100.0	0.566		711 718	0	(R)SEVYGPMK(R)
1035.0000	1034.5265	100.0	0.473		288 296	0	(K)YVEAVSPNR(V)
1189.8000	1189.6245	100.0	0.175		532 542	0	(K)LPASVSTIMDR(W)
1220.0000	1219.6681	100.0	0.332		432 441	0	(K)DLIVLNDVYR(V)
1361.8000	1361.6842	100.0	0.116		206 218	0	(K)VVAITQMQAADAR(K)
1518.2000	1517.7635	100.0	0.437		843 854	0	(R)YLSYTLNPDYIR(K)

Num Unmatched Masses: 22

Search for disulfide linked peptides.

Do a non-specific cleavage search.

Search for another component.

The matched peptides cover 7.8% (75/965AA's) of the protein.

Coverage Map for This Hit (MS-Digest index #): 16541

2. 11/30 matches (36%).

Acc. #: Q9EPA0 **Uniprot ID:** DRP2_RAT **Species:** RAT **Name:** Dystrophin-related protein 2**Organism:** Rattus norvegicus **OX=10116** **Gene:** Drp2 **Existence:** Evidence at protein level **Version:** 2**Index:** 109002 **MW:** 108046 Da **pI:** 5.8

m/z Submitted	MH ⁺ Matched	Intensity	Delta Da	Modifications	Start End	Missed Cleavages	Sequence
723.0000	723.3276	100.0	-0.328	2Carbamidomethyl->Propionamide	610 614	0	(K)C(Carbamidomethyl)SIC(Carbamidomethyl)R(Q)
997.0000	997.5538	100.0	-0.554	1Gln->pyro-Glu	192 199	1	(R)QRIQNLSR(F)
997.0000	997.5538	100.0	-0.554	1Gln->pyro-Glu	192 199	1	(R)QRIQNLSR(F)
1005.0000	1005.5298	100.0	-0.530		615 622	1	(R)QC(Carbamidomethyl)PIKGFR(Y)
1189.8000	1189.5565	100.0	0.244		606 614	1	(K)HQTKC(Carbamidomethyl)SIC(Carbamidomethyl)R(Q)
1195.0000	1195.5062	100.0	-0.506	1Carbamidomethyl->Propionamide	16 24	0	(R)C(Carbamidomethyl)HEWHAADR(F)
1195.0000	1195.5062	100.0	-0.506	1Carbamidomethyl->Propionamide	16 24	0	(R)C(Carbamidomethyl)HEWHAADR(F)
1262.0000	1261.6423	100.0	0.358		204 214	0	(K)QATVASELWEK(L)
1271.0000	1270.7226	100.0	0.277		862 871	1	(K)QLESQQLRLR(E)
1271.0000	1270.7478	100.0	0.252		323 333	1	(K)QLQVSVAERLK(Q)
1291.0000	1290.7317	100.0	0.268		194 203	1	(R)IQNLSRFVWK(Q)
1291.0000	1291.5987	100.0	-0.599		143 153	0	(K)ETHAAFMEEVK(S)
1831.0000	1830.8513	100.0	0.149		628 642	0	(K)QFNVDIC(Carbamidomethyl)QTC(Carbamidomethyl)FLTGK(A)

Num Unmatched Masses: 19

Search for disulfide linked peptides.

Do a non-specific cleavage search.

Search for another component.

The matched peptides cover 10.0% (96/957AA's) of the protein.

Coverage Map for This Hit (MS-Digest index #): 109002

3. 8/30 matches (26%).

Acc. #: O88281 **Uniprot ID:** MEGF6 **RAT Species:** RAT **Name:** Multiple epidermal growth factor-like domains protein 6

Organism: Rattus norvegicus **OX=**10116 **Gene:** Megf6 **Existence:** Evidence at protein level **Version:** 1

Index: 237990 **MW:** 165449 Da **pI:** 5.9

m/z Submitted	MH ⁺ Matched	Intensity	Delta Da	Modifications	Start	End	Missed Cleavages	Sequence
515.0000	515.2725	100.0	-0.272		986	989	0	(R)WGPR(C)
1051.0000	1050.4646	100.0	0.535	1Carbamidomethyl->Propionamide	170	178	0	(R)AHNGGC(Carbamidomethyl)QHR(C)
1195.0000	1195.5313	100.0	-0.531	1Carbamidomethyl->Propionamide	1169	1178	0	(R)GWFGEAC(Carbamidomethyl)AQR(C)
1195.0000	1195.5313	100.0	-0.531	1Carbamidomethyl->Propionamide	1169	1178	0	(R)GWFGEAC(Carbamidomethyl)AQR(C)
1195.0000	1195.5380	100.0	-0.538	3Carbamidomethyl->Propionamide	1347	1355	1	(K)GC(Carbamidomethyl)ELKC(Carbamidomethyl)AC(Carbamidomethyl)R(N)
1195.0000	1195.5380	100.0	-0.538	3Carbamidomethyl->Propionamide	1347	1355	1	(K)GC(Carbamidomethyl)ELKC(Carbamidomethyl)AC(Carbamidomethyl)R(N)
1220.0000	1220.5187	100.0	-0.519		1258	1267	0	(R)YGP GC(Carbamidomethyl)EHIC(Carbamidomethyl)K(C)
1262.0000	1262.5187	100.0	-0.519	2Carbamidomethyl->Propionamide	1045	1053	0	(K)NC(Carbamidomethyl)QHSC(Carbamidomethyl)LC(Carbamidomethyl)R(N)
1316.8000	1316.5576	100.0	0.242		910	920	0	(R)QGYYPSC(Carbamidomethyl)EQK(C)
1518.2000	1518.6576	100.0	-0.458	2Carbamidomethyl->Propionamide	1335	1346	1	(R)C(Carbamidomethyl)EHGC(Carbamidomethyl)PQDRFGK(G)

Num Unmatched Masses: 22

Search for disulfide linked peptides.

Do a non-specific cleavage search.

Search for another component.

The matched peptides cover 4.7% (74/1574AA's) of the protein.

Coverage Map for This Hit (MS-Digest index #): 237990

4. 8/30 matches (26%).

Acc. #: P0C6S7 **Uniprot ID:** ANS1B **RAT Species:** RAT **Name:** Ankyrin repeat and sterile alpha motif domain-containing protein 1B

Organism: Rattus norvegicus **OX=**10116 **Gene:** Anks1b **Existence:** Evidence at protein level **Version:** 1

Index: 17968 **MW:** 139208 Da **pI:** 5.9

m/z Submitted	MH ⁺ Matched	Intensity	Delta Da	Modifications	Start	End	Missed Cleavages	Sequence
723.0000	723.3342	100.0	-0.334		1095	1100	0	(K)STEQMK(K)
773.0000	773.3716	100.0	-0.372		1255	1260	0	(K)YETTIF(-)
1051.0000	1050.5313	100.0	0.469		322	331	0	(K)SETVTGELSK(L)
1195.0000	1195.5841	100.0	-0.584		739	749	0	(K)SDSDLIAYPSK(D)
1195.0000	1195.5841	100.0	-0.584		739	749	0	(K)SDSDLIAYPSK(D)
1220.0000	1219.7297	100.0	0.270		1102	1112	0	(K)VPTILSVSYK(G)
1286.0000	1285.7362	100.0	0.264		244	255	0	(R)VLLETGIDANIK(D)
1361.0000	1360.6889	100.0	0.311		1	12	1	(-)MGKDQELLEAAR(T)
1418.8000	1418.6944	100.0	0.106	1Oxidation 1Acetyl	1	12	1	(-)MGKDQELLEAAR(T)

Num Unmatched Masses: 21

Search for disulfide linked peptides.

Do a non-specific cleavage search.

Search for another component.

The matched peptides cover 5.4% (68/1260AA's) of the protein.

Coverage Map for This Hit (MS-Digest index #): 17968

Supplemental data S2. MS-Fit prediction for B2

MS-Fit Search Results

R 5/07/28 16:45

MS-Fit Search Results

[÷] Parameters

[+] Pre Search Results (SwissProt.2021.06.18)

Fraction-Spot-Run ID: 1-1-1

MS-Fit search selects **601** entries (results displayed for top **20** matches).

[–] Results Summary

Protein Hit Number	MOWSE Score	# pep # mat % mat % Cov 37 pks	% TIC	Mean Err Da	Data Tol Da	# Hom Prot	MS- Digest Index #	Protein MW (Da)/pI	Accession #	Species	Protein Name
1	1.90e+8	25/23/62	24.6	62.2	0.191	0.430	No	263806	95799/5.4	RAT	Major vault protein
2	804	14/12/32	11.9	32.4	0.0431	0.684	No	56803	109555/6.6	RAT	Coiled-coil domain-containing protein 39
3	496	16/13/35	14.3	37.8	1.90e-4	0.763	No	211545	98668/8.8	RAT	Histone-lysine N-methyltransferase KMT5B
4	285	9/9/24	7.9	24.3	0.0623	0.724	No	231075	96308/5.8	RAT	Mitogen-activated protein kinase kinase kinase 12
5	271	14/12/32	9.2	32.4	0.122	0.595	No	313035	134498/6.4	RAT	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase beta-4
6	242	11/9/24	8.5	24.3	0.114	0.838	No	71631	105115/6.6	RAT	Complement component C6
7	240	13/10/27	13.7	27.0	0.132	0.696	No	273169	78394/5.3	RAT	Nexilin
8	229	10/8/22	8.2	24.3	0.134	0.528	No	476365	125311/6.6	RAT	Tudor domain-containing protein 7
9	221	10/8/22	9.5	21.6	0.0118	0.813	No	302049	104054/7.9	RAT	Proprotein convertase subtilisin/kexin type 6
10	187	8/8/22	6.2	21.6	-0.128	0.679	No	6710	115069/7.3	RAT	Activated CDC42 kinase 1
11	161	7/7/19	7.6	18.9	0.0855	0.800	No	152248	79010/6.1	RAT	Gametogenetin-binding protein 2
12	153	12/11/30	13.0	29.7	0.0479	0.689	No	24683	82338/5.8	RAT	LisH domain-containing protein ARMC9
13	140	9/9/24	7.6	24.3	0.0704	0.701	No	563345	116819/9.0	RAT	Zinc finger RNA-binding protein
14	131	7/7/19	6.4	18.9	-0.0859	0.650	No	123071	116210/6.4	RAT	Ephrin type-A receptor 6
15	130	11/11/30	8.3	29.7	0.0404	0.765	No	289775	126206/8.7	RAT	2'-5'-oligoadenylate synthase 3
16	129	7/7/19	9.7	18.9	0.162	0.618	No	160417	77076/7.8	RAT	Dihydroxyacetone phosphate acyltransferase
17	113	6/6/16	5.9	16.2	0.129	0.630	No	520290	98345/7.5	RAT	Whirlin
18	103	8/7/19	5.0	18.9	0.150	0.653	No	118606	124127/5.7	RAT	Pro-epidermal growth factor
19	98.7	10/9/24	7.2	24.3	0.00792	0.639	No	145150	94281/9.8	RAT	GLIS2-like protein 2
20	93.5	7/6/16	8.0	16.2	0.184	0.668	No	175846	82906/8.5	RAT	Hepatocyte growth factor
263806	. x x x x	. x x .	. x x x x	. . . x .	. . x . x x	. x . x x .	. x x x .	. x x x x x x x			
56803	. x . . .										
211545											
231075											
313035											
71631											
273169											
476365											
302049	x										
6710											
152248											
24683											
563345											
123071											
289775											
160417											
520290											
118606	x										
145150											

<https://prospector.ucsf.edu/prospector/cgi-bin/mssearch.cgi>

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[–] Detailed Results

Acc. #: Q62667 Uniprot ID: MVP RAT Species: RAT Name: Major vault protein

Organism: Rattus norvegicus OX=10116 **Gene:** Mvp **Existence:** Evidence at protein level **Version:** 4

Index: 263806 **MW:** 95799 Da **pI:** 5.4

m/z	MH ⁺	Intensity	Delta	Modifications	Start	End	Missed	Sequence
Submitted	Matched		Da				Cleavages	
515.0000	515.3412	100.0	-0.341		176	179	1	(R)LRAR(K)
517.0000	517.2729	100.0	-0.273		730	734	0	(R)AEAAR(I)
517.0000	517.3093	100.0	-0.309		574	578	0	(K)AIASR(V)
581.6000	581.3518	100.0	0.248		240	244	0	(R)GVLRH(T)
710.8000	710.4308	100.0	0.369		225	230	0	(K)TALHLR(A)
729.6000	729.4002	100.0	0.200		170	175	0	(K)QNQALR(L)
745.4000	745.3839	100.0	0.0161		680	685	0	(R)LEQEAR(G)
801.8000	801.4981	100.0	0.302		43	49	0	(R)VLFAFVR(M)
815.8000	815.3894	100.0	0.411		553	560	0	(R)NDPAEAAK(L)
815.8000	815.4444	100.0	0.356	1Oxidation	50	56	0	(R)MVTVPPI(H)
854.8000	854.3825	100.0	0.417		180	185	1	(R)KEC(Carbamidomethyl)FDR(E)
871.8000	871.5611	100.0	0.239		821	828	0	(K)LLQSLGLK(S)
950.6000	950.4829	100.0	0.117		125	132	0	(K)ALLDFEDK(N)
1016.8000	1016.5623	100.0	0.238		775	783	0	(R)AQLELVSK(A)
1024.6000	1024.5132	100.0	0.0868		767	774	0	(R)EMELIYAR(A)
1032.8000	1032.5208	100.0	0.279		693	701	0	(K)ILDQSEAK(A)
1060.8000	1060.5673	100.0	0.233		361	370	0	(R)GPLEYVPSAK(V)
1080.8000	1080.5473	100.0	0.253		307	315	0	(K)SFFLPQPER(L)
1088.8000	1088.5735	100.0	0.227		191	199	0	(R)VTGEEWLV(R)
1257.8000	1257.6507	100.0	0.149		809	820	0	(R)DLAVAGPEMQVK(L)
1275.0000	1274.6409	100.0	0.359		797	808	0	(K)EMTEALPGPIR(D)
1315.8000	1315.6576	100.0	0.142		57	67	0	(R)HYC(Carbamidomethyl)IVANPVSR(D)
1455.0000	1454.6984	100.0	0.302		561	573	0	(K)LFSPDFGVDC(Carbamidomethyl)K(A)
1459.0000	1458.6958	100.0	0.304		336	348	0	(K)ALQPLEEGSEEK(V)
1816.4000	1815.9527	100.0	0.447		92	107	0	(R)LAODPFPLYPGVELEK(D)

Search for disulfide linked peptides.

Do a non-specific cleavage search.

Search for another component.

The matched peptides cover **24.6%** (212/861 AA's) of the protein.

Coverage Map for This Hit (MS-Digest index #): 263806

Acc. #: D3Z8K2 Uniprot ID: CCD39 RAT Species: RAT Name: Coiled-coil domain-containing protein 39

Organism: Rattus norvegicus **OX=10116** **Gene:** Ccdc39 **Existence:** Inferred from homology **Version:** 1

Index: 56803 **MW:** 109555 Da **pI:** 6.6

m/z Submitted	MH ⁺ Matched	Intensity	Delta Da	Modifications	Start	End	Missed Cleavages	Sequence
487.4000	487.3239	100.0	0.0761		566	569	0	(K)ILQVK(R)
517.0000	517.2187	100.0	-0.219	1Gln->pyro-Glu 1Carbamidomethyl->Propionamide	802	805	0	(K)QC(Carbamidomethyl)GR(L)
517.0000	517.3344	100.0	-0.334		264	267	1	(R)EKIK(F)
929.8000	929.5302	100.0	0.270		26	33	0	(K)ILEDQLAK(L)
997.0000	996.4381	100.0	0.562		163	170	0	(K)YSQQDNNK(I)
1016.8000	1016.5193	100.0	0.281		52	60	0	(R)INSMSSHLK(N)
1024.6000	1024.4438	100.0	0.156		181	188	0	(K)LTMEC(Carbamidomethyl)NEK(R)
1032.8000	1032.5143	100.0	0.286	1Oxidation	52	60	0	(R)INSMSSHLK(N)

1032.8000	1032.5320	100.0	0.268				418	427	0	(K)ALGSEIEGTR(S)
1077.0000	1077.5171	100.0	-0.517				918	928	0	(R)SSSGSNSNIPK(G)
1202.4000	1202.5436	100.0	-0.144	1Gln->pyro-Glu			622	631	0	(R)QTVSSEFHER(L)
1248.8000	1248.5347	100.0	0.265	1Gln->pyro-Glu			590	599	0	(K)QQLC(Carbamidomethyl)TAMEER(V)
1455.0000	1454.7737	100.0	0.226				887	900	0	(R)SLEDSPISPTAK(V)
1816.4000	1816.9725	100.0	-0.572	1Oxidation			555	569	1	(K)EDLMIEDNLLKLQVK(R)

Num Unmatched Masses: 25

Search for disulfide linked peptides.

Do a non-specific cleavage search.

Search for another component.

The matched peptides cover 11.9% (111/934AA's) of the protein.

Coverage Map for This Hit (MS-Digest index #): 56803

3. 13/37 matches (35%).

Acc. #: P0C2N5 Uniprot ID: KMT5B RAT Species: RAT Name: Histone-lysine N-methyltransferase KMT5B

Organism: Rattus norvegicus OX=10116 Gene: Kmt5b Existence: Inferred from homology Version: 1

Index: 211545 MW: 98668 Da pI: 8.8

m/z Submitted	MH ⁺ Matched	Intensity	Delta Da	Modifications	Start	End	Missed Cleavages	Sequence
747.0000	747.3632	100.0	-0.363		875	880	0	(R)EDQSLR(L)
777.8000	777.3486	100.0	0.451		389	395	0	(K)DNATSNR(K)
777.8000	777.3923	100.0	0.408		171	176	1	(K)NKMQEK(L)
815.8000	816.3959	100.0	-0.596		721	727	1	(R)RHDSSSK(T)
950.6000	950.4360	100.0	0.164	1Carbamidomethyl->Propionamide	604	611	1	(K)SDSC(Carbamidomethyl)PSRK(K)
961.8000	961.4996	100.0	0.300	1Oxidation	9	16	1	(K)NMVVNGRR(N)
1010.2000	1010.4724	100.0	-0.272		100	108	0	(K)MNTSAFPSR(S)
1016.8000	1016.5809	100.0	0.219		470	478	0	(K)LEMGNLVLK(E)
1022.0000	1022.4975	100.0	-0.498	1Oxidation 1Acetyl	1	8	1	(-)MKWLGDSEK(N)
1032.8000	1032.5758	100.0	0.224	1Oxidation	470	478	0	(K)LEMGNLVLK(E)
1060.8000	1060.5673	100.0	0.233		556	565	0	(K)LAPSPLDGYK(N)
1077.0000	1076.5887	100.0	0.411		180	187	0	(K)EHVFTYLR(M)
1077.0000	1077.4517	100.0	-0.452		595	603	0	(R)EEASQEC(Carbamidomethyl)PK(S)
1080.8000	1080.6160	100.0	0.184		32	41	1	(K)IQHAGKDALK(T)
1088.8000	1088.6575	100.0	0.143	1Gln->pyro-Glu	615	624	1	(R)QGGKPVKHLAK(T)
1275.0000	1275.5535	100.0	-0.553		116	126	0	(K)ADSFSHNNPMR(F)

Num Unmatched Masses: 23

Search for disulfide linked peptides.

Do a non-specific cleavage search.

Search for another component.

The matched peptides cover 14.3% (126/883AA's) of the protein.

Coverage Map for This Hit (MS-Digest index #): 211545

4. 9/37 matches (24%).

Acc. #: Q63796 Uniprot ID: M3K12 RAT Species: RAT Name: Mitogen-activated protein kinase kinase 12

Organism: Rattus norvegicus OX=10116 Gene: Map3k12 Existence: Evidence at protein level Version: 1

Index: 231075 MW: 96308 Da pI: 5.8

m/z Submitted	MH ⁺ Matched	Intensity	Delta Da	Modifications	Start	End	Missed Cleavages	Sequence
517.0000	517.3093	100.0	-0.309		740	744	0	(R)AAVTR(S)
745.4000	745.3839	100.0	0.0161		494	499	0	(R)EQALER(R)
777.8000	777.3461	100.0	0.454	1Carbamidomethyl->Propionamide	378	382	0	(R)QC(Carbamidomethyl)WNR(K)
801.8000	802.3941	100.0	-0.594		317	323	0	(R)NEPVSEK(V)
815.8000	815.4734	100.0	0.327		487	492	1	(K)ERELLR(R)
854.8000	854.4254	100.0	0.375		285	292	0	(K)ISDFGTSK(E)
961.8000	961.5201	100.0	0.280		189	196	1	(R)DLKETDIK(H)

1202.4000	1202.6198	100.0	-0.220	41	50	0	(K)DLTPQC(Carbamidomethyl)VLR(D)
1455.0000	1454.7672	100.0	0.233	551	565	0	(K)LDAALSGVGLPGC(Carbamidomethyl)PK(A)

Num Unmatched Masses: **28**
 Search for disulfide linked peptides.
 Do a non-specific cleavage search.
 Search for another component.

The matched peptides cover **7.9%** (70/888AA's) of the protein.
 Coverage Map for This Hit (MS-Digest index #): 231075

5. 12/37 matches (32%).

Acc. #: Q9QW07 **Uniprot ID:** PLCB4 **RAT Species:** RAT **Name:** 1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase beta-4
Organism: Rattus norvegicus **OX=10116** **Gene:** Plcb4 **Existence:** Evidence at protein level **Version:** 2
Index: 313035 **MW:** 134498 Da **pI:** 6.4

m/z	MH ⁺	Intensity	Delta Da	Modifications	Start	End	Missed Cleavages	Sequence
Submitted	Matched							
729.6000	729.3865	100.0	0.214		149	153	1	(K)KHWMK(L)
745.4000	745.3814	100.0	0.0186	1Oxidation	149	153	1	(K)KHWMK(L)
747.0000	747.4247	100.0	-0.425		996	1001	1	(K)KETEK(I)
871.8000	871.3978	100.0	0.402		341	347	0	(K)SSVEMYR(Q)
997.0000	996.5724	100.0	0.428		447	455	0	(R)LLPSPNDLK(R)
1022.0000	1021.5135	100.0	0.486	1Gln->pyro-Glu 1Oxidation	618	625	1	(R)QMSRIYPK(G)
1022.0000	1022.5452	100.0	-0.545		618	625	1	(R)QMSRIYPK(G)
1024.6000	1024.6150	100.0	-0.0150		910	918	0	(K)GIELIPQVR(I)
1051.8000	1051.5055	100.0	0.295		772	780	0	(R)IAVYDDNNK(L)
1051.8000	1051.5782	100.0	0.222		154	163	0	(K)LAFLTNTSGK(I)
1248.8000	1248.6075	100.0	0.192	1Carbamidomethyl->Propionamide	138	148	0	(R)ANNVSPMTC(Carbamidomethyl)LK(K)
1257.8000	1257.6797	100.0	0.120		1002	1012	1	(K)IQLTSDHKSK(V)
1315.8000	1315.7369	100.0	0.0631		786	797	0	(R)ILPLDGLQAGYR(H)
1455.0000	1454.7460	100.0	0.254		209	219	1	(K)FYELTQKIC(Carbamidomethyl)PR(T)

Num Unmatched Masses: **25**
 Search for disulfide linked peptides.
 Do a non-specific cleavage search.
 Search for another component.

The matched peptides cover **9.2%** (108/1175AA's) of the protein.
 Coverage Map for This Hit (MS-Digest index #): 313035

6. 9/37 matches (24%).

Acc. #: Q811M5 **Uniprot ID:** CO6 **RAT Species:** RAT **Name:** Complement component C6
Organism: Rattus norvegicus **OX=10116** **Gene:** C6 **Existence:** Evidence at transcript level **Version:** 1
Index: 71631 **MW:** 105115 Da **pI:** 6.6

m/z	MH ⁺	Intensity	Delta Da	Modifications	Start	End	Missed Cleavages	Sequence
Submitted	Matched							
710.8000	710.3468	100.0	0.453		310	315	0	(K)DSSFVR(I)
710.8000	711.3533	100.0	-0.553		226	231	0	(R)TSNPIR(V)
854.8000	854.3825	100.0	0.417		144	150	0	(K)FLC(Carbamidomethyl)DSGR(C)
997.0000	996.4163	100.0	0.584		38	46	0	(K)SC(Carbamidomethyl)NSGTQSR(Q)
997.0000	997.5425	100.0	-0.543		301	309	1	(K)NAIEASHKK(D)
1010.2000	1010.4320	100.0	-0.232	1Carbamidomethyl->Propionamide	38	46	0	(K)SC(Carbamidomethyl)NSGTQSR(Q)
1022.0000	1021.5677	100.0	0.432		509	517	1	(R)KALQEYAAK(F)
1051.8000	1051.4585	100.0	0.341		417	424	1	(K)EDRC(Carbamidomethyl)TTNR(L)
1088.8000	1088.5847	100.0	0.215		310	318	1	(K)DSSFVRHKK(V)
1202.4000	1202.6164	100.0	-0.216		449	459	0	(R)SQQAALAWEK(G)
1275.0000	1274.6409	100.0	0.359		724	734	0	(K)IGESIELTC(Carbamidomethyl)PR(G)

Num Unmatched Masses: **28**

Supplemental data S3. MS-Fit prediction for B3

MS-Fit Search Results

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MS-Fit Search Results

[+] Parameters

[+] Pre Search Results (SwissProt.2021.06.18)

Fraction-Spot-Run ID: 1-1-1

MS-Fit search selects **136** entries (results displayed for top **20** matches).

[–] Results Summary

Protein Hit Number	MOWSE Score	# pep # mat % mat % mat 71 pks	% % % % TIC	Mean Err Da	Data Tot Da	# Hom Prot	MS- Digest Index #	Protein MW (Da)/pI	Accession #	Species	Protein Name	
1	5.66e+9	24/25/35	23.8	35.2	0.152	0.304	1	9313	104916/5.3	Q9QXQ0	RAT	Alpha-actinin-4
3	171910	31/23/32	18.0	32.4	0.0834	0.636	No	398809	159628/5.7	Q63644	RAT	Rho-associated protein kinase 1
4	117907	17/15/21	15.2	21.1	0.141	0.291	No	257668	99188/7.5	D4A1W8	RAT	Microsomal triglyceride transfer protein large subunit
5	62665	23/23/32	8.7	32.4	0.0890	0.633	No	44532	207892/5.5	D4A631	RAT	Brefeldin A-inhibited guanine nucleotide-exchange protein 1
6	42015	36/29/41	22.2	40.8	0.0799	0.580	No	445894	143206/7.5	Q9Z1M9	RAT	Structural maintenance of chromosomes protein 1A
7	40110	28/26/37	11.2	36.6	0.0953	0.604	No	449809	208161/5.7	Q9R095	RAT	Sperm flagellar protein 2
8	39557	19/20/28	9.4	28.2	0.0709	0.512	No	562312	165540/9.2	Q499R0	RAT	Zinc finger protein 518A
9	17037	22/18/25	8.7	25.4	-0.00907	0.658	No	497169	201280/6.9	P49816	RAT	Tuberin
10	10633	30/28/39	15.0	39.4	-0.0192	0.585	No	299767	149450/8.1	Q9Z340	RAT	Partitioning defective 3 homolog
11	10205	17/16/23	8.5	23.9	0.0468	0.576	No	107993	165308/5.5	O89042	RAT	DNA polymerase alpha catalytic subunit (Fragment)
12	9455	18/17/24	17.4	23.9	0.00630	0.717	No	13472	93495/6.4	O35774	RAT	A-kinase anchor protein 4
13	9078	19/16/23	8.6	22.5	-0.181	0.643	No	202674	200591/5.6	Q64632	RAT	Integrin beta-4
14	8273	16/15/21	8.1	21.1	0.0834	0.699	No	258175	166039/5.2	Q62635	RAT	Mucin-2 (Fragment)
15	8044	17/15/21	16.9	21.1	0.00746	0.671	No	313580	87962/9.2	D3ZRC4	RAT	Calcium-independent phospholipase A2-gamma
16	7428	18/18/25	13.4	25.4	0.114	0.635	No	515369	141585/6.5	Q9Z1A6	RAT	Vigilin
17	6949	18/17/24	9.2	23.9	0.0243	0.571	No	130215	163622/5.9	Q61V68	RAT	Fanconi anemia group D2 protein homolog
18	5805	17/16/23	8.5	22.5	0.0262	0.680	No	191151	197604/5.7	Q9JKU3	RAT	Intraflagellar transport protein 172 homolog
19	5119	18/16/23	12.8	22.5	0.118	0.549	No	313123	123815/9.0	P70496	RAT	Phospholipase D1
20	5105	14/16/23	25.8	22.5	-0.0498	0.724	No	482748	51061/8.3	O35094	RAT	Mitochondrial import inner membrane translocase subunit TIM44

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<https://prospector.ucsf.edu/prospector/cgi-bin/mssearch.cgi>

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Similar Matches for Protein Hit 1

Protein Hit Number	MOWSE Score	# pep # mat % mat 71 pks	% Cov	% TIC	Mean Err Da	Data Tot Da	# Hom Prot	MS-Digest Index #	Protein MW (Da)	Accession #	Species	Protein Name
1	5.66e+9	24/25/35	23.8	35.2	0.152	0.304	1	9313	104916/5.3	Q9QXQ0	RAT	Alpha-actinin-4
Protein Hit Number	MOWSE Score	# pep # mat % mat 71 pks	% Cov	% TIC	Mean Err Da	Data Tot Da	# Hom Pep	MS-Digest Index #	Protein MW (Da)	Accession #	Species	Protein Name
2	4.22e+6	19/20/28	16.7	28.2	0.0845	0.529	13	9300	102961/5.2	Q9ZIP2	RAT	Alpha-actinin-1

[–] Detailed Results

1. 25/71 matches (35%).

Acc. #: Q9QXQ0 Uniprot ID: ACTN4 RAT Species: RAT Name: Alpha-actinin-4

Organism: Rattus norvegicus OX=10116 **Gene:** Actn4 **Existence:** Evidence at protein level **Version:** 2

Index: 9313 **MW:** 104916 **Da** **pI:** 5.3

m/z Submitted	MH ⁺ Matched	Intensity	Delta Da	Modifications	Start End	Missed Cleavages	Sequence
734.4000	734.3502	100.0	0.0498		669 674	0	(K)MEEIGR(I)
739.6000	739.4461	100.0	0.154		96 101	0	(R)LPKPER(G)
748.6000	748.4240	100.0	0.176		234 239	0	(K)YLDIPK(M)
750.4000	750.3451	100.0	0.0549	1 Oxidation	669 674	0	(K)MEEIGR(I)
811.6000	811.5036	100.0	0.0964		626 632	0	(K)VQQLVPK(R)
825.6000	825.4465	100.0	0.154		411 417	0	(R)LDHLAEK(F)
864.6000	864.4825	100.0	0.117		115 122	0	(K)ALDFIASK(G)
884.4000	884.4771	100.0	-0.0771		433 439	1	(K)EAMLKHR(D)
911.6000	911.4945	100.0	0.105		597 604	0	(R)IAESNIHK(L)
936.4000	936.4170	100.0	-0.0170		806 813	0	(R)QGDAEFNR(I)
1005.8000	1005.5034	100.0	0.297		324 331	0	(K)TIQEMQQK(L)
1029.6000	1029.5364	100.0	0.0636		312 319	0	(R)TIPWLEDR(V)
1092.8000	1092.5684	100.0	0.232		860 868	0	(K)NFITAEELR(R)
1147.8000	1147.5888	100.0	0.211	1 Carbamidomethyl->Propionamide	274 283	1	(K)AETAANRIC(Carbamidomethyl)IK(V)
1174.8000	1174.6037	100.0	0.196		167 175	0	(K)EGLLWC(Carbamidomethyl)QR(K)
1199.8000	1199.6307	100.0	0.169		41 50	0	(R)DLLDPAWEK(Q)
1215.6000	1215.6732	100.0	-0.0732		301 310	0	(R)LASDLLLEWIR(R)
1216.0000	1215.6732	100.0	0.327		301 310	0	(R)LASDLLLEWIR(R)
1255.6000	1255.6052	100.0	-0.00524		440 450	0	(R)DYETATLSDIK(A)
1289.8000	1289.5896	100.0	0.210		900 911	0	(K)SFSTALYGESDL(-)
1353.0000	1352.6263	100.0	0.374		761 771	0	(K)GISQEQMQEFR(A)
1430.0000	1429.7645	100.0	0.235		746 757	0	(R)TINEVENQILTR(D)
1538.0000	1537.7744	100.0	0.226		153 166	0	(R)FAIQDISVEETSAK(E)
1683.8000	1683.8588	100.0	-0.0588	1 Gln->pyro-Glu	522 535	0	(K)QLETIDQLHLEYAK(R)
1742.4000	1741.8127	100.0	0.587		838 853	0	(R)ETDTDTADQVIAFFK(V)

Num Unmatched Masses: 46

Search for disulfide linked peptides.

Do a non-specific cleavage search.

Search for another component.

The matched peptides cover **23.8%** (217/911AA's) of the protein.

Coverage Map for This Hit (MS-Digest index #): 9313

2. 20/71 matches (28%).

Acc. #: Q9Z1P2 Uniprot ID: ACTN1 RAT Species: RAT Name: Alpha-actinin-1

Organism: Rattus norvegicus OX=10116 **Gene:** Actn1 **Existence:** Evidence at protein level **Version:** 1
Index: 9300 **MW:** 102961 **Da** **pI:** 5.2

m/z Submitted	MH ⁺ Matched	Intensity	Delta Da	Modifications	Start	End	Missed Cleavages	Sequence
734.4000	734.3502	100.0	0.0498		650	655	0	(K)MEEIGR(I)
748.6000	748.4240	100.0	0.176		215	220	0	(R)YLDIPK(M)
750.4000	750.3451	100.0	0.0549	1Oxidation	650	655	0	(K)MEEIGR(I)
768.6000	768.4839	100.0	0.116		609	614	1	(R)QLVPRR(D)
773.6000	773.4264	100.0	0.174		494	499	1	(R)REALER(T)
811.6000	811.3805	100.0	0.219		625	630	0	(R)QQHNER(L)
825.6000	825.4465	100.0	0.154		392	398	0	(R)LDHLAEK(F)
864.6000	864.4825	100.0	0.117		96	103	0	(K)ALDFIASK(G)
975.0000	975.5106	100.0	-0.511		495	502	1	(R)EALERTEK(L)
1028.0000	1028.5524	100.0	-0.552		293	300	0	(R)TIPWLENR(V)
1147.8000	1147.5888	100.0	0.211	1Carbamidomethyl->Propionamide	255	264	1	(K)AETAANRIC(Carbamidomethyl)K(V)
1174.8000	1174.6037	100.0	0.196		148	156	0	(K)EGLLLC(Carbamidomethyl)QR(K)
1199.8000	1199.6307	100.0	0.169		22	31	0	(R)DLLLPAWEK(Q)
1215.6000	1215.6732	100.0	-0.0732		282	291	0	(K)LASDLLEWIR(R)
1216.0000	1215.6732	100.0	0.327		282	291	0	(K)LASDLLEWIR(R)
1270.2000	1269.6209	100.0	0.579		421	431	0	(K)DYETATLSEIK(A)
1305.8000	1305.7175	100.0	0.0825		604	613	1	(K)WDHVRQLVPR(R)
1404.4000	1404.6610	100.0	-0.261		301	312	0	(R)VPENTMQAMQQK(L)
1430.0000	1429.7645	100.0	0.235		727	738	0	(R)TINEVENQLTR(D)
1538.0000	1537.7744	100.0	0.226		134	147	0	(R)FAIQDISVEETSAK(E)

Num Unmatched Masses: 51

Search for disulfide linked peptides.

Do a non-specific cleavage search.

Search for another component.

The matched peptides cover 16.7% (149/892AA's) of the protein.

Coverage Map for This Hit (MS-Digest index #): 9300

3, 23/71 matches (32%).

Acc. #: Q63644 **Uniprot ID:** ROCK1 **RAT Species:** RAT **Name:** Rho-associated protein kinase 1

Organism: Rattus norvegicus OX=10116 **Gene:** Rock1 **Existence:** Evidence at protein level **Version:** 1

Index: 398809 **MW:** 159628 **Da** **pI:** 5.7

m/z Submitted	MH ⁺ Matched	Intensity	Delta Da	Modifications	Start	End	Missed Cleavages	Sequence
531.4000	531.2885	100.0	0.111		873	876	0	(R)ENLR(K)
531.4000	531.3249	100.0	0.0751		1017	1020	1	(K)IDRK(K)
714.8000	714.3529	100.0	0.447		935	940	0	(K)DHTVSR(L)
739.6000	739.4171	100.0	0.183		100	105	1	(R)KVYAMK(L)
743.0000	743.4410	100.0	-0.441		14	19	0	(K)IDNLLR(D)
743.0000	743.4410	100.0	-0.441		14	19	0	(K)IDNLLR(D)
818.8000	819.3665	100.0	-0.567		567	573	0	(K)SHEMSK(S)
845.6000	845.5091	100.0	0.0909		858	864	1	(K)TQVKELK(E)
937.6000	937.4785	100.0	0.122	1Carbamidomethyl->Propionamide	1279	1285	1	(R)C(Carbamidomethyl)HVKSHR(D)
943.6000	943.5823	100.0	0.0177		784	791	0	(R)ILLQSELK(T)
975.0000	974.5153	100.0	0.485		878	885	0	(K)IQELQSEK(E)
975.0000	974.5452	100.0	0.455		1006	1013	1	(K)LAEMNRK(D)
985.8000	985.5023	100.0	0.298	1Carbamidomethyl->Propionamide	1292	1299	0	(K)EDLPPC(Carbamidomethyl)K(V)
988.4000	988.5534	100.0	-0.153		1342	1350	1	(R)ASPRTLSTR(S)
997.4000	997.4949	100.0	-0.0949		1300	1308	0	(K)VSVDVTSAR(D)
1075.6000	1075.5895	100.0	0.0105		85	94	0	(R)GAFGEVQLVR(H)
1102.8000	1102.5739	100.0	0.226		941	950	0	(R)LEEANNALTK(D)

1102.8000	1102.6103	100.0	0.190		877	885	1	(R)KIQLQSEK(E)
1147.8000	1147.6066	100.0	0.193		556	565	1	(R)TESDTAVRLR(K)
1147.8000	1147.6470	100.0	0.153		11	19	1	(R)FEKIDNLLR(D)
1161.8000	1161.5932	100.0	0.207		1074	1083	0	(R)NELQMQLASK(E)
1174.8000	1174.6062	100.0	0.194		730	739	1	(K)AENRVVETEK(Q)
1177.2000	1177.5881	100.0	-0.388	1Oxidation	1074	1083	0	(R)NELQMQLASK(E)
1212.0000	1211.5977	100.0	0.402		71	80	1	(R)MKAEDYEVVK(V)
1212.0000	1212.5421	100.0	-0.542		710	719	1	(K)SVAMC(Carbamidomethyl)EMKK(L)
1222.8000	1222.5806	100.0	0.219	1Carbamidomethyl->Propionamide	740	749	0	(K)QC(Carbamidomethyl)SMLDVLK(Q)
1222.8000	1222.6249	100.0	0.175		311	320	0	(K)NLIC(Carbamidomethyl)AFLTDR(E)
1270.2000	1269.6586	100.0	0.541		51	60	1	(K)NIDNLSRYK(D)
1270.2000	1270.7114	100.0	-0.511		1036	1045	1	(R)KLQLELNQER(E)
1430.0000	1429.6495	100.0	0.350		115	125	1	(K)RSDSAFFWEER(D)
1430.0000	1429.6636	100.0	0.336	1Carbamidomethyl->Propionamide	214	225	1	(K)LADFGTC(Carbamidomethyl)MKMNK(E)
1430.0000	1429.6740	100.0	0.326		653	664	1	(R)KEAQDMLNHSEK(E)

Num Unmatched Masses: 48

Search for disulfide linked peptides.

Do a non-specific cleavage search.

Search for another component.

The matched peptides cover 18.0% (246/1369AA's) of the protein.

Coverage Map for This Hit (MS-Digest index #): 398809

4. 15/71 matches (21%).

Acc. #: D4A1W8 Uniprot ID: MTP RAT Species: RAT Name: Microsomal triglyceride transfer protein large subunit

Organism: Rattus norvegicus OX=10116 Gene: Mtp Existence: Evidence at protein level Version: 1

Index: 257668 MW: 99188 Da pI: 7.5

m/z	MH ⁺	Intensity	Delta Da	Modifications	Start	End	Missed Cleavages	Sequence
Submitted	Matched							
515.2000	515.3664	100.0	-0.166		595	598	1	(R)RVLK(E)
531.4000	531.2959	100.0	0.104		267	271	0	(R)MVPKG(Q)
702.8000	702.4509	100.0	0.349		250	255	1	(K)IVSKQK(L)
714.8000	714.4509	100.0	0.349		272	278	0	(K)QVAGVIK(A)
937.6000	937.4738	100.0	0.126		51	58	0	(K)LQDSVGYR(I)
985.8000	985.5928	100.0	0.207		342	349	0	(R)EEILQILK(A)
1147.8000	1147.5953	100.0	0.205		383	392	0	(K)SDSSILQER(F)
1176.4000	1176.5466	100.0	-0.147		599	607	0	(K)EMIVHNYDR(F)
1192.6000	1192.5415	100.0	0.0585	1Oxidation	599	607	0	(K)EMIVHNYDR(F)
1196.8000	1196.7249	100.0	0.0751		559	569	0	(K)NILLSIGELPK(E)
1196.8000	1196.7249	100.0	0.0751		456	466	0	(K)LILGGLEKPEK(K)
1212.0000	1211.6994	100.0	0.301	1Gln->pyro-Glu	272	283	1	(K)QVAGVIKAIDSK(Y)
1289.8000	1289.6770	100.0	0.123		717	729	0	(K)MLSASGDPVSVVK(G)
1289.8000	1289.6848	100.0	0.115		833	843	1	(K)AEAPLRQFETK(Y)
1305.8000	1305.6719	100.0	0.128	1Oxidation	717	729	0	(K)MLSASGDPVSVVK(G)
1430.0000	1429.7209	100.0	0.279		36	48	0	(K)LTYSSTEVFLDGGK(G)
1510.0000	1509.7907	100.0	0.209		199	213	0	(R)SGFTTANQVLGVTSK(A)

Num Unmatched Masses: 56

Search for disulfide linked peptides.

Do a non-specific cleavage search.

Search for another component.

The matched peptides cover 15.2% (136/896AA's) of the protein.

Coverage Map for This Hit (MS-Digest index #): 257668

5. 23/71 matches (32%).

Acc. #: D4A631 Uniprot ID: BIG1 RAT Species: RAT Name: Brefeldin A-inhibited guanine nucleotide-exchange protein 1

Organism: Rattus norvegicus OX=10116 Gene: Arfgef1 Existence: Evidence at protein level Version: 1

Supplemental data S4. MS-Fit prediction for B4

MS-Fit Search Results

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MS-Fit Search Results

[+] Parameters

[+] Pre Search Results (SwissProt.2021.06.18)

Fraction-Spot-Run ID: 1-1-1

MS-Fit search selects 183 en

MS-Fit search selects **183** entries (results displayed for top 20 matches).

[–] Results Summary

Protein Hit Number	MOWSE Score	# pep # mat % mat 122 pps	% cov % TIC	Mean Err Da	Data Tot Da	# Hom Prot	MS-Digest Index #	Protein MW (Da)/pI	Accession #	Species	Protein Name
1	3.47E-6	10/97	56.8	7.4	0.105	0.605	1	169431	13990/10.4	Q06715	RAT Histone H2B type 1
3	2.5755	8/87	42.3	6.6	-0.0759	0.797	No	74930	11495/8.5	D3ZVN1	RAT Colipase-like protein 2
4	1.5986	8/76	40.0	6.6	0.169	0.358	1	87540	11743/9.3	P10715	RAT Cytoschrome c, testis-specific
5	7548	9/87	41.9	6.6	0.298	0.643	No	46076	9803/10.5	A1L1L1	RAT Centromere protein W
6	6850	8/97	44.0	7.4	-2.26E-4	0.541	No	418142	13742/10.1	P62853	RAT 40S ribosomal protein S25
7	5284	4/43	49.2	3.3	0.175	0.560	No	255305	6006/8.4	P52803	RAT Metallothionein-1
9	4035	6/76	35.2	6.6	0.139	0.374	No	503951	13711/11.6	P57690	RAT Urocoitin
10	3691	5/54	37.5	4.1	0.00252	0.395	No	446701	14094/9.3	P60192	RAT SNARE-associated protein Snapin
11	2485	3/32	10.8	2.5	0.378	0.405	No	82511	14981/6.2	Q8V188	RAT Cystatin-14
12	2464	6/43	38.3	5.7	-0.118	0.569	No	273927	14174/8.7	P55770	RAT NHP2-like protein
13	1954	3/32	24.8	2.5	0.355	0.576	No	210928	13511/8.9	P27158	RAT Thymidine kinase, cytosolic (Fragment)
14	1785	4/54	23.3	4.1	0.0744	0.421	No	99970	8308/9.3	Q3JZ87	RAT Beta-defensin 39
15	1678	5/65	56.5	4.9	0.0810	0.523	No	93742	9864/8.4	P56702	RAT Diacylglycerol-binding inhibitor-like 5
16	1629	5/54	34.2	4.1	0.122	0.614	No	99980	7901/9.0	Q30KJ2	RAT Beta-defensin 50
17	1622	4/43	24.6	3.3	-0.0281	0.381	No	58775	13790/8.9	P27274	RAT CD59 glycoprotein
18	1521	7/54	45.7	5.7	0.132	0.390	No	272251	12783/9.4	P52504	RAT NADH dehydrogenase [ubiquinone] iron-sulfur protein 6, mitochondrial
19	1455	7/76	55.7	5.7	0.0483	0.636	No	229911	10410/10.3	B2GV91	RAT LYR motif-containing protein 2
20	1382	4/43	40.5	3.3	0.0881	0.585	No	99946	9040/8.2	Q3JZ16	RAT Beta-defensin 15

[illegible]

Similar Matches for Protein Hit 1

Protein Hit Number	MOWSE Score	# pep # mat % mat 122 pts	% Cov	% TIC	Mean Err	Data Tot Da	# Hom Prot	MS-Digest Index #	Protein MW (Da)	Accession #	Species	Protein Name
1	3.47e6	10979	56.8	7.4	0.105	6.605	1	169431	13990/10.4	C00715	RAT	Histone H2B type 1

Protein Hit Number	MOWSE Score	# pep # mat % mat % cov TIC	Mean Err Data Tol	# Hom Pep	MS-Digest Index	Protein (Da)/pI	Accession	Species	Protein Name
2	57015	11/1008 67.7 9.8	0.0460 0.531	6	169392	14225/10.3	000729	RAT	Histone H2B type 1-A

Similar Matches for Protein Hit 4

Protein Hit	MOWSE Score	# pep # mat % % mat Cov TIC	Mean Data Err Tol Dn Dn	# Hom MS-Digest Protein MW Accession Prot Index # (Da)/pl # Species Protein Name
Number		122 aks		

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4	15896	8/7/6	40.0	6.6	0.169	0.358	1	87540	11743/9.3	P10715	RAT	Cytochrome c, testis-specific
Protein Hit Number	MOWSE Score	# mat	% mat	% Cov	Mean TIC	Data Err	# Hom	MS-Digest	Protein MW (Da)	Accession #	Species	Protein Name
		122 pks										
8	4950	8/5/4	45.7	6.6	0.195	0.382	4	87884	11606/9.6	P62898	RAT	Cytochrome c, somatic

[-] Detailed Results

1. 9/122 matches (7%).

Acc. #: Q00715 Uniprot ID: H2B1 RAT Species: RAT Name: Histone H2B type 1

Organism: Rattus norvegicus OX=10116 Existence: Evidence at protein level Version: 2

Index: 169431 MW: 13990 Da pI: 10.4

m/z	MH ⁺	Intensity	Delta Da	Modifications	Start	End	Missed Cleavages	Sequence
Submitted	Matched							
816.6000	816.4574	100.0	0.143		93	99	0	(R)EQTAVR(L)
901.4000	901.5003	100.0	-0.100		80	86	1	(R)LAHYNKR(S)
901.4000	901.5003	100.0	-0.100		79	85	1	(R)RLAHYNNK(R)
953.8000	953.6030	100.0	0.197		100	108	0	(R)LLLPGELAK(H)
1137.8000	1137.5463	100.0	0.254		36	44	0	(K)ESYSVYVYK(V)
1168.6000	1168.5957	100.0	0.00432		48	58	0	(K)QVHPDTGISSK(A)
1308.2000	1308.7093	100.0	-0.509		1	12	1	(-)MPEPAKSRPAK(K)
1744.2000	1743.8193	100.0	0.381		59	73	0	(K)AMGIMNSFVNDIFER(I)
1760.0000	1759.8142	100.0	0.186	1Oxidation	59	73	0	(K)AMGIMNSFVNDIFER(I)
1776.4000	1775.8091	100.0	0.591	2Oxidation	59	73	0	(K)AMGIMNSFVNDIFER(I)

Num Unmatched Masses: 113

Search for disulfide linked peptides.

Do a non-specific cleavage search.

Search for another component.

The matched peptides cover 56.8% (71/125AA's) of the protein.

Coverage Map for This Hit (MS-Digest index #): 169431

2. 10/122 matches (8%).

Acc. #: Q00729 Uniprot ID: H2B1A RAT Species: RAT Name: Histone H2B type 1-A

Organism: Rattus norvegicus OX=10116 Gene: H2bc1 Existence: Evidence at protein level Version: 2

Index: 169392 MW: 14225 Da pI: 10.3

m/z	MH ⁺	Intensity	Delta Da	Modifications	Start	End	Missed Cleavages	Sequence
Submitted	Matched							
703.0000	703.3733	100.0	-0.373		75	81	0	(R)IAGEASR(L)
703.6000	703.3733	100.0	0.227		75	81	0	(R)IAGEASR(L)
775.6000	775.4672	100.0	0.133		19	25	1	(K)AVTKTKQ(K)
816.6000	816.4574	100.0	0.143		95	101	0	(R)EQTAVR(L)
901.4000	901.5003	100.0	-0.100		82	88	1	(R)LAHYNKR(S)
953.8000	953.6030	100.0	0.197		102	110	0	(R)LLLPGELAK(H)
1165.8000	1165.5776	100.0	0.222		37	45	0	(K)ESYSIYIK(V)
1168.6000	1168.5957	100.0	0.00432		49	59	0	(K)QVHPDTGISSK(A)
1217.6000	1217.6736	100.0	-0.0736	1Met-loss	1	13	1	(-)MPEVSAKGTITISK(K)
1294.2000	1293.6725	100.0	0.527		36	45	1	(R)KESYSIYIK(V)
1390.8000	1390.7246	100.0	0.0754	1Acetyl	1	13	1	(-)MPEVSAKGTITISK(K)
1776.4000	1776.8295	100.0	-0.430	1Oxidation	60	74	0	(K)AMSIMNSFVTDIFER(I)

Num Unmatched Masses: 110

Search for disulfide linked peptides.

Do a non-specific cleavage search.

Search for another component.

The matched peptides cover 67.7% (86/127AA's) of the protein.

Coverage Map for This Hit (MS-Digest index #): 169392

3. 8/122 matches (6%).

Acc. #: D3ZVN1 Uniprot ID: COLL2 RAT Species: RAT Name: Colipase-like protein 2

Organism: Rattus norvegicus OX=10116 Gene: Clpsl2 Existence: Inferred from homology Version: 1

Index: 74930 MW: 11495 Da pI: 8.5

m/z	MH ⁺	Intensity	Delta Da	Modifications	Start	End	Missed Cleavages	Sequence
Submitted	Matched							
775.6000	775.3226	100.0	0.277		94	99	0	(K)DPMC(Carbamidomethyl)IPR(R)
788.8000	789.3382	100.0	-0.538	1Carbamidomethyl->Propionamide	94	99	0	(K)DPMC(Carbamidomethyl)IPR(R)
870.8000	871.3978	100.0	-0.598	1Carbamidomethyl->Propionamide	56	63	0	(R)SGAFC(Carbamidomethyl)ITSK(S)
901.4000	901.4560	100.0	-0.0560		86	93	0	(R)IGLSC(Carbamidomethyl)HSK(D)
945.6000	945.4393	100.0	0.161	1Carbamidomethyl->Propionamide	94	100	1	(K)DPMC(Carbamidomethyl)IPR(C)
1132.8000	1132.5602	100.0	0.240		77	85	0	(R)SLNILC(Carbamidomethyl)PC(Carbamidomethyl)R(I)
1288.2000	1288.6613	100.0	-0.461		76	85	1	(K)RSLNILC(Carbamidomethyl)PC(Carbamidomethyl)R(I)

1330.0000 1329.6321 100.0 0.368 22 32 1 (K)FDPSNSDLKK(A)

Num Unmatched Masses: 114
Search for disulfide linked peptides.
Do a non-specific cleavage search.
Search for another component.

The matched peptides cover 42.3% (44/104AA's) of the protein.
Coverage Map for This Hit (MS-Digest index #): 74930

4, 7/122 matches (5%).

Acc. #: P10715 Uniprot ID: CYC2_RAT Species: RAT Name: Cytochrome c, testis-specific
Organism: Rattus norvegicus OX=10116 Gene: Cyet Existence: Evidence at transcript level Version: 2
Index: 87540 MW: 11743 Da pI: 9.3

m/z Submitted	MH ⁺ Matched	Intensity	Delta Da	Modifications	Start End	Missed Cleavages	Sequence
648.4000	648.4079	100.0	-0.00792		10 14	0	(K)EFQK(C)
775.6000	775.3945	100.0	0.206	1Met-loss	1 9	1	(-)MGDAEAGKK(I)
779.6000	779.4884	100.0	0.152		81 87	0	(K)MIFAGIK(K)
906.6000	906.4349	100.0	0.165		1 9	1	(-)MGDAEAGKK(I)
1132.8000	1132.4874	100.0	0.313		15 23	0	(K)C(Carbamidomethyl)IAQC(Carbamidomethyl)HTVEK(G)
1217.6000	1217.6426	100.0	-0.0426		29 39	0	(K)TGPNLWGLFGR(K)
1345.8000	1345.7375	100.0	0.0625		29 40	1	(K)TGPNLWGLFGRK(T)
1776.4000	1775.8931	100.0	0.507	1Carbamidomethyl->Propionamide	10 23	1	(K)EFQK(Carbamidomethyl)IAQC(Carbamidomethyl)HTVEK(G)

Num Unmatched Masses: 114
Search for disulfide linked peptides.
Do a non-specific cleavage search.
Search for another component.

The matched peptides cover 40.0% (42/105AA's) of the protein.
Coverage Map for This Hit (MS-Digest index #): 87540

5, 8/122 matches (6%).

Acc. #: A1L1L1 Uniprot ID: CENPW_RAT Species: RAT Name: Centromere protein W
Organism: Rattus norvegicus OX=10116 Gene: Cenpw Existence: Inferred from homology Version: 1
Index: 60776 MW: 9803 Da pI: 10.5

m/z Submitted	MH ⁺ Matched	Intensity	Delta Da	Modifications	Start End	Missed Cleavages	Sequence
809.6000	809.3458	100.0	0.254		58 64	0	(R)TNAC(Carbamidomethyl)ESK(S)
823.8000	823.3614	100.0	0.439	1Carbamidomethyl->Propionamide	58 64	0	(R)TNAC(Carbamidomethyl)ESK(S)
971.2000	970.6044	100.0	0.596		28 35	1	(K)KPRLSLEK(R)
977.8000	977.5084	100.0	0.292	1Met-loss+Acetyl	1 9	0	(-)MVLSTTV(Carbamidomethyl)R(R)
1222.8000	1222.6395	100.0	0.161		1 10	1	(-)MVLSTTV(Carbamidomethyl)RR(I)
1294.2000	1293.6467	100.0	0.553		58 69	1	(R)TNAC(Carbamidomethyl)ESKSGVIK(K)
1294.2000	1294.6696	100.0	-0.461	1Oxidation 1Acetyl 1Carbamidomethyl->Propionamide	1 10	1	(-)MVLSTTV(Carbamidomethyl)RR(I)
1308.2000	1307.6624	100.0	0.538	1Carbamidomethyl->Propionamide	58 69	1	(R)TNAC(Carbamidomethyl)ESKSGVIK(K)
1495.0000	1494.6853	100.0	0.315		52 64	1	(K)LAESRTNAC(Carbamidomethyl)ESK(S)

Num Unmatched Masses: 114
Search for disulfide linked peptides.
Do a non-specific cleavage search.
Search for another component.

The matched peptides cover 41.9% (36/86AA's) of the protein.
Coverage Map for This Hit (MS-Digest index #): 60776

6, 9/122 matches (7%).

Acc. #: P62853 Uniprot ID: RS25_RAT Species: RAT Name: 40S ribosomal protein S25
Organism: Rattus norvegicus OX=10116 Gene: Rps25 Existence: Evidence at transcript level Version: 1
Index: 418142 MW: 13742 Da pI: 10.1

m/z Submitted	MH ⁺ Matched	Intensity	Delta Da	Modifications	Start End	Missed Cleavages	Sequence
815.8000	815.4258	100.0	0.374		19 25	1	(K)DKDPVYK(S)
850.2000	850.4781	100.0	-0.278		105 111	0	(R)AQVIYTR(N)
850.6000	850.4781	100.0	0.122		105 111	0	(R)AQVIYTR(N)
901.4000	901.4738	100.0	-0.0738		21 29	1	(K)DPVNSGGK(A)
972.6000	972.5724	100.0	0.0276		86 94	0	(R)AALQELLSK(G)
1012.0000	1012.5132	100.0	-0.513	1Carbamidomethyl->Propionamide	53 60	1	(K)ATYDKLC(Carbamidomethyl)IK(E)
1143.8000	1143.6382	100.0	0.162		103 111	1	(K)HRAQVIYTR(N)
1273.8000	1273.5655	100.0	0.235		112 125	1	(R)NTKGGDAPAAAGDA(-)
1383.8000	1383.8570	100.0	-0.0570		86 98	1	(R)AALQELLSKGLIK(L)

Num Unmatched Masses: 113
Search for disulfide linked peptides.

Supplemental data S5. MS-Fit prediction for B1

MS-Fit Search Results

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MS-Fit Search Results

[–] Parameters

Database searched: **SwissProt.2021.06.18**
 Digest Used: **Trypsin**
 Max. # Missed Cleavages: **1**
 Constant Modification: **Carbamidomethyl (C)**
 Minimum Matches: **4**
 Sort Type: **Score Sort**
 Considered modifications: **Peptide N-terminal Gln to pyroGlu | Oxidation of M | Protein N-terminus Acetylated | Acrylamide Modified Cys**
 Min Parent Ion Matches: **1**
 MOWSE On: **1**
 MOWSE P Factor: **0.4**
 Report Homologous Proteins: **Interesting**

[–] Pre Search Results (SwissProt.2021.06.18)

Number of entries in the database: **565254**
 Molecular weight search (**40000 - 60000 Da**) selects **121313** entries.
 Full pI range: **565254** entries.
 Taxonomy search **RATTUS NORVEGICUS** selects **8135** entries.
 Combined molecular weight, pI and taxonomy searches select **2168** entries.
 Pre searches select **2168** entries.

Fraction-Spot-Run ID: **1-1-1**

MS-Fit search selects **108** entries (results displayed for top **20** matches).

[–] Results Summary

Protein Hit Number	MOWSE Score	# pep # mat % mat 24 pks	% Cov	% TIC	Mean Err Da	Data Tol Da	# Hom Prot	MS-Digest Index #	Protein MW (Da)	Accession #	Species	Protein Name
1	34706	12/11/46	18.9	45.8	0.190	0.193	No	305238	54751/5.6	Q510D7	RAT	Xaa-Pro dipeptidase
2	207	5/6/25	14.8	25.0	-0.174	0.746	No	204081	50214/5.1	Q6IFU7	RAT	Keratin, type I cytoskeletal 42
3	193	5/5/21	14.1	20.8	-0.0440	0.723	No	447174	46794/6.2	B1H267	RAT	Sorting nexin-5
4	143	4/4/17	5.0	16.7	0.214	0.203	No	273124	42382/6.8	Q64627	RAT	Sialidase-2
5	134	5/5/21	11.3	20.8	-0.243	0.762	No	448905	56020/8.7	Q99MU5	RAT	Spermatogenesis-associated protein 6
6	112	6/6/25	12.2	25.0	0.197	0.658	No	181858	52712/6.9	P22449	RAT	Hepatocyte nuclear factor 4-alpha
7	95.8	5/5/21	11.4	20.8	0.325	0.223	No	64673	45101/5.2	O54783	RAT	Choline/ethanolamine kinase
8	95.5	4/4/17	6.3	16.7	0.268	0.407	No	204187	50709/5.7	Q6IG12	RAT	Keratin, type II cytoskeletal 7
9	82.3	5/5/21	10.2	20.8	-0.0736	0.601	No	3729	46429/6.7	P13221	RAT	Aspartate aminotransferase, cytoplasmic
10	80.7	4/4/17	8.4	16.7	0.191	0.342	No	81572	46290/5.6	Q68FS2	RAT	COP9 signalosome complex subunit 4
11	54.3	5/5/21	11.5	20.8	0.0548	0.698	No	18973	54908/7.5	O35827	RAT	Amyloid-beta A4 precursor protein-binding family B member 3
12	50.9	5/5/21	9.6	20.8	0.0302	0.777	No	128279	54764/6.3	P07953	RAT	6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase 1
13	48.6	3/4/17	6.8	16.7	-0.191	0.560	No	302709	49913/8.8	Q9Z1Z9	RAT	PDZ and LIM domain protein 7
14	41.9	5/5/21	8.1	20.8	-0.0538	0.627	No	135877	51451/5.5	Q9QVC8	RAT	Peptidyl-prolyl cis-trans isomerase FKBP4
15	40.5	3/4/17	4.9	16.7	-0.184	0.675	No	355455	45551/6.0	A1A5R1	RAT	RNA binding protein fox-1 homolog 2
16	40.1	3/4/17	5.8	16.7	0.359	0.386	No	124996	59153/8.9	Q62986	RAT	Estrogen receptor beta
17	36.3	4/4/17	7.7	16.7	0.0544	0.761	No	202716	53905/5.3	Q5PQQ8	RAT	Integrin beta-like protein 1
18	31.0	3/4/17	3.5	16.7	-0.187	0.570	No	132578	55356/4.9	D4ABP9	RAT	F-box only protein 3

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19	25.5	6/6/25	15.3	29.2	0.0961	0.612	No	477022	44799/5.3	Q5XIV0	RAT	Terminal nucleotidyltransferase 5C
20	24.6	3/4/17	5.7	16.7	-0.266	0.573	No	90433	48120/9.0	Q5VJ55	RAT	Dynein axonemal assembly factor 4
305238	x	x	x	x	x	x	x	x	x	x	x	x
204081	.	.	.	.	.	.	.	.	.	.	.	.
447174	.	.	.	.	.	.	.	.	.	.	.	.
273124	.	.	.	.	.	.	.	.	.	.	.	.
448905	.	.	.	.	.	.	.	.	.	.	.	.
181858	.	.	.	.	.	.	.	.	.	.	.	.
64673	.	.	.	.	.	.	.	.	.	.	.	.
204187	.	.	.	.	.	.	.	.	.	.	.	.
3729	.	.	.	.	.	.	.	.	.	.	.	.
81572	.	.	.	.	.	.	.	.	.	.	.	.
18973	.	.	.	.	.	.	.	.	.	.	.	.
128279	.	.	.	.	.	.	.	.	.	.	.	.
302709	.	.	.	.	.	.	.	.	.	.	.	.
135877	.	.	.	.	.	.	.	.	.	.	.	.
355455	.	.	.	.	.	.	.	.	.	.	.	.
124996	.	.	.	.	.	.	.	.	.	.	.	.
202716	.	.	.	.	.	.	.	.	.	.	.	.
132578	.	.	.	.	.	.	.	.	.	.	.	.
477022	.	.	.	.	.	.	.	.	.	.	.	.
90433	.	.	.	.	.	.	.	.	.	.	.	.

[-] Detailed Results

1. 11/24 matches (45%).

Acc. #: Q5I0D7 Uniprot ID: PEPD RAT Species: RAT Name: Xaa-Pro dipeptidase

Organism: Rattus norvegicus OX=10116 Gene: Pepd Existence: Evidence at transcript level Version: 1

Index: 305238 MW: 54751 Da pI: 5.6

m/z	MH ⁺	Intensity	Delta	Modifications	Start	End	Missed	Sequence
Submitted	Matched		Da				Cleavages	
799.6000	799.4057	100.0	0.194		201	207	0	(R)ISSEAHK(E)
799.6000	799.4308	100.0	0.169		389	395	0	(R)IDEPGLR(S)
819.6000	819.4723	100.0	0.128		93	99	0	(K)STLFVPR(L)
934.6000	934.5356	100.0	0.0644		304	311	0	(K)AIYEAVLR(S)
967.6000	967.4731	100.0	0.127		160	168	0	(R)EASFEGISK(F)
1010.8000	1010.5629	100.0	0.237		336	343	0	(R)IHLEELTR(I)
1105.6000	1105.5558	100.0	0.0442		188	196	0	(K)TDMELEVLK(Y)
1121.8000	1121.5507	100.0	0.249	1Oxidation	188	196	0	(K)TDMELEVLK(Y)
1224.8000	1224.6082	100.0	0.192		100	110	0	(R)LPASYATWMGK(I)
1240.8000	1240.6031	100.0	0.197	1Oxidation	100	110	0	(R)LPASYATWMGK(I)
1276.0000	1275.6038	100.0	0.396		57	66	0	(R)YC(Carbamidomethyl)TDTSIIFR(Q)
1749.2000	1748.9177	100.0	0.282	1Met-loss+Acetyl	1	17	0	(-)MASTVRPFSGLNETLK(V)

Num Unmatched Masses: 13

Search for disulfide linked peptides.

Do a non-specific cleavage search.

Search for another component.

The matched peptides cover 18.9% (93/492AA's) of the protein.

Coverage Map for This Hit (MS-Digest index #): 305238

2. 6/24 matches (25%).

Acc. #: Q6IFU7 Uniprot ID: K1C42 RAT Species: RAT Name: Keratin, type I cytoskeletal 42

Organism: Rattus norvegicus OX=10116 Gene: Krt42 Existence: Inferred from homology Version: 1

Index: 204081 MW: 50214 Da pI: 5.1

m/z Submitted	MH ⁺ Matched	Intensity	Delta Da	Modifications	Start	End	Missed Cleavages	Sequence
1117.0000	1117.5735	100.0	-0.574		430	439	0	(R)TIVEEVQDGK(V)
1166.0000	1166.5445	100.0	-0.545	1 Carbamidomethyl- >Propionamide	18	28	0	(K)GLC(Carbamidomethyl)APGMGFSR(M)
1166.2000	1166.5445	100.0	-0.345	1 Carbamidomethyl- >Propionamide	18	28	0	(K)GLC(Carbamidomethyl)APGMGFSR(M)
1196.6000	1196.6494	100.0	-0.0494		440	449	1	(K)VVSSREQVHR(S)
1699.2000	1698.8657	100.0	0.334	1 Met-loss+Acetyl	1	17	1	(-)MASTTSIRQFSTSGSVK(G)
1868.0000	1867.8637	100.0	0.136		40	58	0	(R)APSLGGGSC(Carbamidomethyl)SNMSVTSSR(F)

Num Unmatched Masses: 18

Search for disulfide linked peptides.

Do a non-specific cleavage search.

Search for another component.

The matched peptides cover **14.8%** (67/452AA's) of the protein.

Coverage Map for This Hit (MS-Digest index #): 204081

3, 5/24 matches (20%).

Acc. #: B1H267 **Uniprot ID:** SNX5 **RAT Species:** RAT **Name:** Sorting nexin-5

Organism: Rattus norvegicus **OX=10116** **Gene:** Snx5 **Existence:** Evidence at protein level **Version:** 1

Index: 447174 **MW:** 46794 Da **pI:** 6.2

m/z Submitted	MH ⁺ Matched	Intensity	Delta Da	Modifications	Start	End	Missed Cleavages	Sequence
967.6000	967.5571	100.0	0.0429		150	158	0	(R)LSSHPVLSK(D)
1166.0000	1166.5688	100.0	-0.569		315	324	0	(K)ALIDYENSNA(A)
1166.2000	1166.5688	100.0	-0.369		315	324	0	(K)ALIDYENSNA(A)
1215.6000	1215.6078	100.0	-0.00782		297	306	0	(R)YYMLNIEAAK(D)
1699.2000	1698.8771	100.0	0.323		124	137	1	(K)MKQLEAEYLAVFK(K)
1699.2000	1698.8843	100.0	0.316	1 Oxidation	375	388	1	(K)NLIEMSELEIKHAR(N)

Num Unmatched Masses: 19

Search for disulfide linked peptides.

Do a non-specific cleavage search.

Search for another component.

The matched peptides cover **14.1%** (57/404AA's) of the protein.

Coverage Map for This Hit (MS-Digest index #): 447174

4, 4/24 matches (16%).

Acc. #: Q64627 **Uniprot ID:** NEUR2 **RAT Species:** RAT **Name:** Sialidase-2

Organism: Rattus norvegicus **OX=10116** **Gene:** Neu2 **Existence:** Evidence at protein level **Version:** 1

Index: 273124 **MW:** 42382 Da **pI:** 6.8

m/z Submitted	MH ⁺ Matched	Intensity	Delta Da	Modifications	Start	End	Missed Cleavages	Sequence
1105.6000	1105.5380	100.0	0.0620		1	9	0	(-)METC(Carbamidomethyl)PVLQK(E)
1121.8000	1121.5329	100.0	0.267	1 Oxidation	1	9	0	(-)METC(Carbamidomethyl)PVLQK(E)
1224.8000	1224.5388	100.0	0.261		84	93	0	(R)SMNPC(Carbamidomethyl)PLYDK(Q)
1240.8000	1240.5337	100.0	0.266	1 Oxidation	84	93	0	(R)SMNPC(Carbamidomethyl)PLYDK(Q)

Num Unmatched Masses: 20

Search for disulfide linked peptides.

Do a non-specific cleavage search.

Search for another component.

The matched peptides cover **5.0%** (19/379AA's) of the protein.
Coverage Map for This Hit (MS-Digest index #): 273124

5. 5/24 matches (20%).

Acc. #: Q99MU5 **Uniprot ID:** SPAT6 **RAT Species:** RAT **Name:** Spermatogenesis-associated protein 6
Organism: Rattus norvegicus **OX=**10116 **Gene:** Spata6 **Existence:** Evidence at protein level **Version:** 2
Index: 448905 **MW:** 56020 Da **pI:** 8.7

m/z Submitted	MH ⁺ Matched	Intensity	Delta Da	Modifications	Start	End	Missed Cleavages	Sequence
934.6000	934.4887	100.0	0.111	1Carbamidomethyl- >Propionamide	179	185	1	(R)LQC(Carbamidomethyl)RTSR(S)
1073.2000	1073.6023	100.0	-0.402		17	26	0	(R)SVTC(Carbamidomethyl)PGVLLK(D)
1166.0000	1166.5688	100.0	-0.569		205	214	0	(K)NYEQPTISSK(S)
1166.2000	1166.5688	100.0	-0.369		205	214	0	(K)NYEQPTISSK(S)
1749.2000	1748.8564	100.0	0.344	1Carbamidomethyl- >Propionamide	29	42	0	(K)EDIYLSIC(Carbamidomethyl)VFGQYK(K)
1749.2000	1749.7730	100.0	-0.573	1Oxidation 2Carbamidomethyl- >Propionamide	287	300	1	(R)MHS DHPHLC(Carbamidomethyl)C(Carbamidomethyl)RAK(D)

Num Unmatched Masses: **19**

Search for disulfide linked peptides.
Do a non-specific cleavage search.
Search for another component.

The matched peptides cover **11.3%** (55/488AA's) of the protein.
Coverage Map for This Hit (MS-Digest index #): 448905

6. 6/24 matches (25%).

Acc. #: P22449 **Uniprot ID:** HNF4A **RAT Species:** RAT **Name:** Hepatocyte nuclear factor 4-alpha
Organism: Rattus norvegicus **OX=**10116 **Gene:** Hnf4a **Existence:** Evidence at protein level **Version:** 3
Index: 181858 **MW:** 52712 Da **pI:** 6.9

m/z Submitted	MH ⁺ Matched	Intensity	Delta Da	Modifications	Start	End	Missed Cleavages	Sequence
967.6000	967.3873	100.0	0.213		91	97	0	(K)NHMYSC(Carbamidomethyl)R(F)
997.0000	997.3978	100.0	-0.398	1Oxidation 1Carbamidomethyl- >Propionamide	91	97	0	(K)NHMYSC(Carbamidomethyl)R(F)
1117.0000	1116.5392	100.0	0.461		128	136	1	(K)EAVQNERDR(I)
1317.8000	1317.7274	100.0	0.0726		222	234	0	(R)AHAGEHLLLGATK(R)
1699.2000	1698.8414	100.0	0.359	1Carbamidomethyl- >Propionamide	254	267	1	(R)HC(Carbamidomethyl)PELAEMSRVSIR(I)
1749.2000	1748.7268	100.0	0.473		71	85	1	(K)HYGASSC(Carbamidomethyl)DGC(Carbamidomethyl)KGFFR(R)

Num Unmatched Masses: **18**

Search for disulfide linked peptides.
Do a non-specific cleavage search.
Search for another component.

The matched peptides cover **12.2%** (58/474AA's) of the protein.
Coverage Map for This Hit (MS-Digest index #): 181858

7. 5/24 matches (20%).

Acc. #: O54783 **Uniprot ID:** CHKB **RAT Species:** RAT **Name:** Choline/ethanolamine kinase
Organism: Rattus norvegicus **OX=**10116 **Gene:** Chkb **Existence:** Evidence at protein level **Version:** 3

Supplemental data S6. MS-Fit prediction for B2

R 6/03/22, 16:17

MS-Fit Search Results

[–] Parameters

Database searched: SwissProt.2021.06.18

Digest Used: **Trypsin**

Max. # Missed Cleavages: 1

Constant Modification: Carbamidomethyl (C)

Minimum Matches: 4

Sort Type: Score Sort

Considered modifications: | Peptide N-terminal Gln to pyroGlu | Oxidation of M | Protein N-terminus Acetylated | Acrylamide

Modified Cys

Min Parent Ion Matches: 1

MOWSE On: 1

MOWSE P Factor: 0.4

Report Homologous Proteins: Interesting

[–] Pre Search Results (SwissProt.2021.06.18)

Number of entries in the database: **565254**

Molecular weight search (40000 - 60000 Da) selects 121313 entries.

Full pl range: **565254** entries.

Taxonomy search **RATTUS NORVEGICUS** selects **8135** entries.

Combined molecular weight, pI and taxonomy searches select **2168** entries.

Pre searches select **2168** entries.

Fraction-Spot-Run ID: 1-1-1

MS-Fit search selects **64** entries (results displayed for top **20** matches).

[–] Results Summary

Protein Hit Number	MOWSE Score	# pep # mat % mat % Cov % TIC 20 pks	Mean Err Da	Data Tot Da	# Hom Prot	MS-Digest Index	Protein MW (Da)/pI	Accession #	Species	Protein Name
1	205	7/7/35 11.8 35.0	-0.200	0.535	No	513950	46398/8.0	Q35757	RAT	Vascular endothelial growth factor C
2	168	4/4/20 4.7 20.0	0.0257	0.935	No	486581	46509/6.4	Q5PQV5	RAT	Trophoblast glycoprotein
3	137	5/5/25 11.5 25.0	0.198	0.331	No	302612	56624/5.9	P11598	RAT	Protein disulfide-isomerase A3
4	132	5/5/25 10.2 25.0	-0.144	0.648	No	137880	59826/8.6	P36365	RAT	Dimethylalanine monooxygenase [N-oxide-forming]
5	97.6	7/7/35 12.5 35.0	0.0672	0.783	No	203995	56505/5.1	Q6IFW6	RAT	Keratin, type I cytoskeletal 10
6	81.2	4/4/20 7.3 20.0	0.0375	1.03	No	199244	46338/8.4	Q6AYC2	RAT	Immunity-related GTPase family M protein
7	42.3	5/4/20 14.0 20.0	-0.0548	0.919	No	67036	47035/8.9	Q6AYU0	RAT	Uncharacterized protein C12orf50 homolog
8	32.7	4/4/20 8.3 20.0	-0.0415	0.829	No	48426	58459/9.2	Q6AXU1	RAT	BTB/POZ domain-containing protein 16
9	30.7	5/4/20 9.0 20.0	0.126	0.425	No	70951	47269/9.0	P13233	RAT	2',3'-cyclic-nucleotide 3'-phosphodiesterase
10	27.0	4/4/20 9.8 20.0	-0.131	0.661	No	77849	55996/9.1	P17711	RAT	Cytochrome P450 2A1
11	25.2	4/4/20 9.0 20.0	-0.0919	0.518	No	489266	54955/6.9	Q9WV59	RAT	E3 ubiquitin-protein ligase TRIM17
12	23.7	4/4/20 11.7 20.0	-0.169	0.419	No	446910	47042/6.5	Q6P6S0	RAT	Synaptosomal-associated protein 47
13	23.1	4/4/20 7.9 20.0	-0.193	0.410	No	489358	59980/4.6	Q5PQN5	RAT	Tripartite motif-containing protein 55
14	22.6	4/4/20 8.7 20.0	-0.271	0.527	No	81520	53429/6.4	P97834	RAT	COF9 signalosome complex subunit 1
15	20.3	4/4/20 11.1 20.0	0.0524	0.999	No	319543	40766/6.7	Q6DGG0	RAT	Peptidyl-prolyl cis-trans isomerase D
16	19.6	4/4/20 11.5 20.0	0.220	0.376	No	9628	45804/6.0	Q6AYS7	RAT	Aminoacylase-1A
17	19.4	6/30/11 1.1 30.0	-0.139	0.767	No	212280	57818/6.6	P11980	RAT	Pyruvate kinase PKM
18	16.4	4/4/20 12.4 20.0	-0.0304	0.690	No	56922	43454/5.7	B2RZ86	RAT	Coiled-coil domain-containing protein 89
19	15.2	6/6/30 14.0 30.0	-0.0596	0.454	No	166295	46743/9.0	P18266	RAT	Glycogen synthase kinase-3 beta
20	14.5	4/4/20 6.3 20.0	0.0190	0.562	No	181858	52712/6.9	P22449	RAT	Hepatocyte nuclear factor 4-alpha

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203995	.	.	.	x	x	.	.	x	x	.	.	.	.	.	.	.	.	.	.	.
199244	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
67036	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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319543	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
9628	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
212280	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
56922	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
166295	x	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
181858	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

[-] Detailed Results

1. 7/20 matches (35%).

Acc. #: Q35757 Uniprot ID: VEGFC RAT Species: RAT Name: Vascular endothelial growth factor C

Organism: Rattus norvegicus OX=10116 Gene: Vegfc Existence: Evidence at protein level Version: 2

Index: 513950 MW: 46398 Da pI: 8.0

m/z Submitted	MH ⁺ Matched	Intensity	Delta Da	Modifications	Start	End	Missed Cleavages	Sequence
835.2000	835.4785	100.0	-0.278	1Gln->pyro-Glu	216	222	0	(R)QVHSIIR(R)
1165.4000	1165.5783	100.0	-0.178	1Carbamidomethyl->Propionamide	319	328	0	(K)LFNLSC(Carbamidomethyl)GANR(E)
1340.2000	1340.5756	100.0	-0.376	3Carbamidomethyl->Propionamide	309	318	1	(R)DSC(Carbamidomethyl)QC(Carbamidomethyl)VC(Carbamidomethyl)KNK(L)
1382.0000	1381.7005	100.0	0.300		342	353	1	(R)TC(Carbamidomethyl)PRNQPLNPGK(C)
1395.0000	1395.5681	100.0	-0.568		372	381	0	(K)FHHQTC(Carbamidomethyl)SC(Carbamidomethyl)YR(R)
1407.6000	1407.7161	100.0	-0.116	1Carbamidomethyl->Propionamide	317	328	1	(K)NKLFLNSC(Carbamidomethyl)GANR(E)
1409.4000	1409.5837	100.0	-0.184	1Carbamidomethyl->Propionamide	372	381	0	(K)FHHQTC(Carbamidomethyl)SC(Carbamidomethyl)YR(R)

Num Unmatched Masses: 13

Search for disulfide linked peptides.

Do a non-specific cleavage search.

Search for another component.

The matched peptides cover 11.8% (49/415AA's) of the protein.

Coverage Map for This Hit (MS-Digest index #): 513950

2. 4/20 matches (20%).

Acc. #: Q5PQV5 Uniprot ID: TPBG RAT Species: RAT Name: Trophoblast glycoprotein

Organism: Rattus norvegicus OX=10116 Gene: Tpbpg Existence: Evidence at protein level Version: 1

Index: 486581 MW: 46509 Da pI: 6.4

m/z Submitted	MH ⁺ Matched	Intensity	Delta Da	Modifications	Start	End	Missed Cleavages	Sequence
916.8000	916.4622	100.0	0.338		317	324	0	(K)ETEVVPDK(A)
1382.0000	1381.5293	100.0	0.471		62	73	0	(R)C(Carbamidomethyl)PAAC(Carbamidomethyl)EC(Carbamidomethyl)SEEAR(T)
1395.0000	1395.5450	100.0	-0.545	1Carbamidomethyl->Propionamide	62	73	0	(R)C(Carbamidomethyl)PAAC(Carbamidomethyl)EC(Carbamidomethyl)SEEAR(T)
1409.4000	1409.5606	100.0	-0.161	2Carbamidomethyl->Propionamide	62	73	0	(R)C(Carbamidomethyl)PAAC(Carbamidomethyl)EC(Carbamidomethyl)SEEAR(T)

Num Unmatched Masses: 16

Search for disulfide linked peptides.

Do a non-specific cleavage search.

Search for another component.

The matched peptides cover **4.7%** (20/426AA's) of the protein.
Coverage Map for This Hit (MS-Digest index #): 486581

3. 5/20 matches (25%).

Acc. #: P11598 **Uniprot ID:** PDIA3 **RAT Species:** RAT **Name:** Protein disulfide-isomerase A3

Organism: Rattus norvegicus **OX=**10116 **Gene:** Pdia3 **Existence:** Evidence at protein level **Version:** 2

Index: 302612 **MW:** 56624 Da **pI:** 5.9

m/z Submitted	MH ⁺ Matched	Intensity	Delta Da	Modifications	Start	End	Missed Cleavages	Sequence
877.8000	877.4890	100.0	0.311		297	304	0	(K)LNFAVASR(K)
1198.0000	1197.7201	100.0	0.280		423	433	1	(K)LSKDPNIVIAK(M)
1395.0000	1394.6587	100.0	0.341		162	173	0	(R)DLFSDGHSEFLK(A)
1396.8000	1396.6955	100.0	0.105		367	379	0	(K)SEPIPETNEGPK(V)
1593.8000	1593.8483	100.0	-0.0483		483	496	0	(R)EATNPPIQEEKPK(K)

Num Unmatched Masses: **15**

Search for disulfide linked peptides.

Do a non-specific cleavage search.

Search for another component.

The matched peptides cover **11.5%** (58/505AA's) of the protein.
Coverage Map for This Hit (MS-Digest index #): 302612

4. 5/20 matches (25%).

Acc. #: P36365 **Uniprot ID:** FMO1 **RAT Species:** RAT **Name:** Dimethylaniline monooxygenase [N-oxide-forming] 1

Organism: Rattus norvegicus **OX=**10116 **Gene:** Fmo1 **Existence:** Evidence at protein level **Version:** 2

Index: 137880 **MW:** 59826 Da **pI:** 8.6

m/z Submitted	MH ⁺ Matched	Intensity	Delta Da	Modifications	Start	End	Missed Cleavages	Sequence
916.8000	916.4921	100.0	0.308		451	458	0	(R)AMLLTDPR(L)
1063.4000	1063.4989	100.0	-0.0989		34	42	0	(R)SC(Carbamidomethyl)DLGGLWR(F)
1109.2000	1109.5473	100.0	-0.347		345	354	0	(K)VEDGQASLYK(Y)
1409.4000	1409.9454	100.0	-0.545		292	304	1	(R)IITGKVLKPSIK(E)
1593.8000	1593.8370	100.0	-0.0370		503	516	0	(R)TVQETPSTFETLLK(L)

Num Unmatched Masses: **15**

Search for disulfide linked peptides.

Do a non-specific cleavage search.

Search for another component.

The matched peptides cover **10.2%** (54/532AA's) of the protein.
Coverage Map for This Hit (MS-Digest index #): 137880

5. 7/20 matches (35%).

Acc. #: Q6IFW6 **Uniprot ID:** K1C10 **RAT Species:** RAT **Name:** Keratin, type I cytoskeletal 10

Organism: Rattus norvegicus **OX=**10116 **Gene:** Krt10 **Existence:** Inferred from homology **Version:** 1

Index: 203995 **MW:** 56505 Da **pI:** 5.1

m/z Submitted	MH ⁺ Matched	Intensity	Delta Da	Modifications	Start	End	Missed Cleavages	Sequence
877.8000	877.4486	100.0	0.351		490	497	1	(K)GSNTNKTR(V)
916.8000	916.3829	100.0	0.417		285	291	0	(K)NHEEEMK(D)
1109.2000	1109.4898	100.0	-0.290		334	342	0	(K)DAEAWFNEK(S)
1165.4000	1165.5848	100.0	-0.185		441	449	0	(R)LENEIQTYR(S)
1235.2000	1234.6790	100.0	0.521		235	244	1	(R)LYENEVALR(Q)
1243.8000	1243.6529	100.0	0.147		501	511	0	(K)THIEVTPEGR(V)
1340.2000	1340.6917	100.0	-0.492	1Gln->pyro-Glu	245	256	1	(R)QSVEADINGLRR(V)

Num Unmatched Masses: **13**

Supplemental data S7. MS-Fit prediction for B3

MS-Fit Search Results

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MS-Fit Search Results

[−] Parameters

Database searched: **SwissProt.2021.06.18**
 Digest Used: **Trypsin**
 Max. # Missed Cleavages: **1**
 Constant Modification: **Carbamidomethyl (C)**
 Minimum Matches: **4**
 Sort Type: **Score Sort**
 Considered modifications: | **Peptide N-terminal Gln to pyroGlu** | **Oxidation of M** | **Protein N-terminus Acetylated** | **Acrylamide Modified Cys** |
 Min Parent Ion Matches: **1**
 MOWSE On: **1**
 MOWSE P Factor: **0.4**
 Report Homologous Proteins: **Interesting**

[−] Pre Search Results (SwissProt.2021.06.18)

Number of entries in the database: **565254**
 Molecular weight search (**100000 - 200000 Da**) selects **22559** entries.
 Full pI range: **565254** entries.
 Taxonomy search **RATTUS NORVEGICUS** selects **8135** entries.
 Combined molecular weight, pI and taxonomy searches select **755** entries.
 Pre searches select **755** entries.

Fraction-Spot-Run ID: **1-1-1**
 MS-Fit search selects **47** entries (results displayed for top **20** matches).

[−] Results Summary

Protein Hit Number	MOWSE Score	# pep # mat % mat % Cov % TIC 11 pks	Mean Err Da	Data Tol Da	# Hom Prot	MS- Digest Index #	Protein MW (Da)/pI	Accession #	Species	Protein Name	
1	10.9	5/5/45	4.4	45.5	0.0452	0.238	No	505477	103242/5.9	Q32PZ3 RAT	Protein unc-45 homolog A
2	8.72	4/4/36	2.7	36.4	0.00823	0.0681	No	237671	110082/6.3	Q4V8B3 RAT	Mediator of RNA polymerase II transcription subunit 24
3	8.16	6/6/55	3.3	54.5	-0.0313	0.556	No	515003	150251/8.6	P53767 RAT	Vascular endothelial growth factor receptor 1
4	4.71	5/4/36	3.1	36.4	0.0446	0.0692	No	203367	129706/5.5	P97607 RAT	Protein jagged-2 (Fragment)
5	4.64	4/4/36	2.4	36.4	-0.0193	0.0782	No	356762	141944/6.6	Q5PQJ5 RAT	Rho GTPase-activating protein 29
6	4.24	6/5/45	2.3	45.5	0.0317	0.243	No	210459	182565/7.0	F1MSN7 RAT	Kinesin-like protein KIF21B
7	4.13	4/4/36	2.6	36.4	0.0555	0.330	No	264860	115284/5.4	Q10728 RAT	Protein phosphatase 1 regulatory subunit 12A
8	3.73	5/4/36	2.5	36.4	-0.0413	0.204	No	459036	172595/8.3	O70608 RAT	Synaptonemal complex protein 2
9	3.01	4/4/36	2.4	36.4	-0.199	0.520	No	494430	127630/6.6	Q8R455 RAT	Transient receptor potential cation channel subfamily M member 8
10	2.79	4/4/36	3.3	36.4	0.0404	0.153	No	109002	108046/5.8	Q9EPA0 RAT	Dystrophin-related protein 2
11	2.29	4/4/36	3.4	36.4	-0.0309	0.0792	No	47692	106720/9.2	D4A7T3 RAT	Bromodomain testis-specific protein
12	2.23	5/4/36	3.2	36.4	0.0114	0.275	No	282362	107210/5.3	P52590 RAT	Nuclear pore complex protein Nup107
13	2.15	4/4/36	2.3	36.4	0.0826	0.300	No	503693	176157/7.6	Q6IE24 RAT	Inactive ubiquitin carboxyl-terminal hydrolase 54
14	2.11	4/4/36	2.7	36.4	0.0477	0.104	No	276152	143103/8.7	Q62645 RAT	Glutamate receptor ionotropic, NMDA 2D
15	2.10	5/4/36	2.0	36.4	-0.236	0.626	No	106512	182775/8.5	Q9Z330 RAT	DNA (cytosine-5)-methyltransferase 1
16	2.07	4/4/36	2.5	36.4	0.0227	0.0769	No	562804	135301/8.7	Q5EXX3 RAT	Zinc finger and BTB domain-containing protein 38
17	1.90	6/4/36	2.9	36.4	0.0176	0.105	No	12432	172885/8.0	C0HL12 RAT	Adhesion G protein-coupled receptor B1
18	1.78	5/5/45	2.3	45.5	0.0361	0.172	No	107964	136857/6.4	Q9QYV8 RAT	DNA polymerase subunit gamma-1
19	1.66	4/4/36	2.1	36.4	0.0917	0.197	No	51844	165614/6.6	D4AEC2 RAT	Calmodulin-regulated spectrin-associated protein 2
20	1.64	5/4/36	3.1	36.4	-0.00345	0.0433	No	225089	156654/6.2	Q5SGE0 RAT	Leucine-rich PPR motif-containing protein, mitochondrial

505477	x	x	.	.	x	.	x	x	.
237671	.	x	x	.	.	x	x	.	.

<https://prospector.ucsf.edu/prospector/cgi-bin/mssearch.cgi>

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515003	x	.	x	x	.	.	x	.	x	.	x	.	x
203367	.	x	.	.	.	.	x	.	x	.	x	.	x
356762	.	.	.	.	.	.	x	x	x	.	x	.	.
210459	x	x	x	.	x	x	.	.	.	.	.	.	.
264860	x	.	.	.	.	.	x	.	x	x	.	.	.
459036	.	x	.	.	.	.	x	.	x	.	.	.	x
494430	.	x	.	.	.	.	x	.	x	.	.	.	.
109002	.	x	.	.	.	.	x	.	x	.	.	.	x
47692	.	x	.	.	.	.	x	.	x	.	.	.	.
282362	x	x	.	.	.	.	x	.	.	.	.	.	x
503693	x	.	.	.	.	.	.	.	.	.	x	x	x
276152	.	.	x	.	x	.	.	.	.	.	x	.	x
106512	.	x	.	.	.	.	x	.	x	.	.	.	.
562804	.	x	.	.	.	.	x	.	x	.	.	.	.
12432	.	x	.	.	.	.	.	.	.	.	x	.	.
107964	x	.	.	.	.	.	x	.	x	.	.	.	.
51844	x	.	.	.	.	.	.	.	.	.	x	.	x
225089	.	.	x	.	.	.	x	.	.	.	.	.	x

[-] Detailed Results

1. 5/11 matches (45%).

Acc. #: Q32P23 Uniprot ID: UN45A RAT Species: RAT Name: Protein unc-45 homolog A

Organism: Rattus norvegicus OX=10116 Gene: Unc45a Existence: Evidence at transcript level Version: 1

Index: 505477 MW: 103242 Da pI: 5.9

m/z	MH ⁺	Intensity	Delta Da	Modifications	Start	End	Missed Cleavages	Sequence
Submitted	Matched							
713.6000	713.3763	100.0	0.224	1Carbamidomethyl->Propionamide	65	70	0	(R)AAC(Carbamidomethyl)HLK(L)
719.4000	719.3206	100.0	0.0794		184	190	0	(R)EDAGA(EK)(I)
833.4000	833.4186	100.0	-0.0186		923	930	0	(R)AAAAC(Carbamidomethyl)LEK(A)
1099.6000	1099.5643	100.0	0.0357		618	626	0	(K)QHVPEQH(PK)(D)
1275.6000	1275.6943	100.0	-0.0943	1Gln->pyro-Glu	517	528	1	(K)QFAEGSTLKLAK(Q)

Num Unmatched Masses: 6

Search for disulfide linked peptides.

Do a non-specific cleavage search.

Search for another component.

The matched peptides cover 4.4% (42/944AA's) of the protein.

Coverage Map for This Hit (MS-Digest index #): 505477

2. 4/11 matches (36%).

Acc. #: Q4V8B3 Uniprot ID: MED24 RAT Species: RAT Name: Mediator of RNA polymerase II transcription subunit 24

Organism: Rattus norvegicus OX=10116 Gene: Med24 Existence: Evidence at protein level Version: 1

Index: 237671 MW: 110082 Da pI: 6.3

m/z	MH ⁺	Intensity	Delta Da	Modifications	Start	End	Missed Cleavages	Sequence
Submitted	Matched							
719.4000	719.3471	100.0	0.0529		720	725	0	(K)GWVDSR(S)
747.4000	747.4182	100.0	-0.0182		637	642	0	(K)SLQMIR(Q)
833.4000	833.4186	100.0	-0.0186		628	634	0	(R)MLGLDER(E)
865.4000	865.3832	100.0	0.0168		134	141	0	(R)C(Carbamidomethyl)TAASAER(L)

Num Unmatched Masses: 7

Search for disulfide linked peptides.

Do a non-specific cleavage search.

Search for another component.

The matched peptides cover 2.7% (27/987AA's) of the protein.

Coverage Map for This Hit (MS-Digest index #): 237671

3. 6/11 matches (54%).

Acc. #: P53767 **Uniprot ID:** VGFR1 **RAT Species:** RAT **Name:** Vascular endothelial growth factor receptor 1
Organism: Rattus norvegicus **OX=**10116 **Gene:** Flt1 **Existence:** Evidence at transcript level **Version:** 1
Index: 515003 **MW:** 150251 Da **pI:** 8.6

m/z Submitted	MH ⁺ Matched	Intensity	Delta Da	Modifications	Start	End	Missed Cleavages	Sequence
713.6000	713.3875	100.0	0.212		1017	1021	1	(R)KC(Carbamidomethyl)IHR(D)
747.4000	747.3784	100.0	0.0216		184	189	0	(R)IAWDSR(R)
768.8000	769.3661	100.0	-0.566		307	312	0	(K)GLYTC(Carbamidomethyl)R(V)
835.4000	835.4560	100.0	-0.0560		1147	1153	0	(R)FAELVEK(L)
1099.6000	1099.5353	100.0	0.0647		925	932	1	(K)RDFFC(Carbamidomethyl)LNK(D)
1363.8000	1363.6641	100.0	0.136		172	183	0	(K)FPFDALTPDQGR(I)

Num Unmatched Masses: 5

Search for disulfide linked peptides.

Do a non-specific cleavage search.

Search for another component.

The matched peptides cover 3.3% (44/1336AA's) of the protein.

Coverage Map for This Hit (MS-Digest index #): 515003

4. 4/11 matches (36%).

Acc. #: P97607 **Uniprot ID:** JAG2 **RAT Species:** RAT **Name:** Protein jagged-2 (Fragment)
Organism: Rattus norvegicus **OX=**10116 **Gene:** Jag2 **Existence:** Evidence at transcript level **Version:** 1
Index: 203367 **MW:** 129706 Da **pI:** 5.5

m/z Submitted	MH ⁺ Matched	Intensity	Delta Da	Modifications	Start	End	Missed Cleavages	Sequence
719.4000	719.3107	100.0	0.0893		734	739	0	(R)DGWEGR(T)
833.4000	833.3393	100.0	0.0607		806	812	0	(R)C(Carbamidomethyl)SC(Carbamidomethyl)PPGR(S)
833.4000	833.4046	100.0	-0.00465		1195	1201	1	(K)DVRC(Carbamidomethyl)AGR(E)
865.4000	865.3655	100.0	0.0345	2Carbamidomethyl->Propionamide	456	462	0	(K)C(Carbamidomethyl)ASSPC(Carbamidomethyl)R(R)
1275.6000	1275.5569	100.0	0.0431	2Carbamidomethyl->Propionamide	849	858	1	(R)C(Carbamidomethyl)LDGHRDC(Carbamidomethyl)SK(V)

Num Unmatched Masses: 7

Search for disulfide linked peptides.

Do a non-specific cleavage search.

Search for another component.

The matched peptides cover 3.1% (37/1202AA's) of the protein.

Coverage Map for This Hit (MS-Digest index #): 203367

5. 4/11 matches (36%).

Acc. #: Q5PQJ5 **Uniprot ID:** RHG29 **RAT Species:** RAT **Name:** Rho GTPase-activating protein 29
Organism: Rattus norvegicus **OX=**10116 **Gene:** Arhgap29 **Existence:** Evidence at protein level **Version:** 2
Index: 356762 **MW:** 141944 Da **pI:** 6.6

m/z Submitted	MH ⁺ Matched	Intensity	Delta Da	Modifications	Start	End	Missed Cleavages	Sequence
801.4000	801.3737	100.0	0.0263		920	926	0	(R)DSDHSLK(Q)
833.4000	833.3999	100.0	6.24e-5		396	402	0	(R)NDLENTK(R)
835.4000	835.4560	100.0	-0.0560		759	765	0	(K)EFIDLAK(E)
1275.6000	1275.6474	100.0	-0.0474	1Carbamidomethyl->Propionamide	386	395	1	(K)VC(Carbamidomethyl)VTNVEERR(N)

Num Unmatched Masses: 7

Search for disulfide linked peptides.

Do a non-specific cleavage search.

Search for another component.

Supplemental data S8. MS-Fit prediction for B4

MS-Fit Search Results

R 6/03/22, 16:20

MS-Fit Search Results

[−] Parameters

Database searched: **SwissProt.2021.06.18**
 Digest Used: **Trypsin**
 Max. # Missed Cleavages: **1**
 Constant Modification: **Carbamidomethyl (C)**
 Minimum Matches: **4**
 Sort Type: **Score Sort**
 Considered modifications: | **Peptide N-terminal Gln to pyroGlu** | **Oxidation of M** | **Protein N-terminus Acetylated** | **Acrylamide Modified Cys** |
 Min Parent Ion Matches: **1**
 MOWSE On: **1**
 MOWSE P Factor: **0.4**
 Report Homologous Proteins: **Interesting**

[−] Pre Search Results (SwissProt.2021.06.18)

Number of entries in the database: **565254**
 Molecular weight search (**100000 - 200000 Da**) selects **22559** entries.
 Full pI range: **565254** entries.
 Taxonomy search **RATTUS NORVEGICUS** selects **8135** entries.
 Combined molecular weight, pI and taxonomy searches select **755** entries.
 Pre searches select **755** entries.

Fraction-Spot-Run ID: **1-1-1**
 MS-Fit search selects **10** entries.

[−] Results Summary

Protein Hit Number	MOWSE Score	# pep # mat % mat % Cov	% TIC	Mean Err Da	Data Tol Da	# Hom Prot	MS-Digest Index #	Protein MW (Da)/pI	Accession #	Species	Protein Name	
1	5.01	4/4/50	3.2	50.0	0.0368	0.147	No	10188	128937/5.8	P21932	RAT	Adenylate cyclase type 3
2	4.29	5/4/50	5.1	50.0	0.0319	0.241	No	264860	115284/5.4	Q10728	RAT	Protein phosphatase 1 regulatory subunit 12A
3	2.52	5/4/50	3.0	50.0	-0.0353	0.246	No	562312	165540/9.2	Q499R0	RAT	Zinc finger protein 518A
4	2.17	4/4/50	3.4	50.0	-0.0286	0.175	No	56803	109555/6.6	D3Z8K2	RAT	Coiled-coil domain-containing protein 39
5	1.85	4/4/50	3.8	50.0	-4.34e-4	0.269	No	324238	106765/8.3	A1A5S1	RAT	Pre-mRNA-processing factor 6
6	1.71	5/4/50	3.2	50.0	-0.0171	0.186	No	110496	171559/7.7	Q9ES45	RAT	Dual oxidase 2
7	1.44	5/4/50	3.7	50.0	-0.0265	0.246	No	345437	153785/6.6	Q9JIL8	RAT	DNA repair protein RAD50
8	1.15	6/5/62	3.6	62.5	-0.0278	0.164	No	83921	192662/5.7	D3ZEF4	RAT	Cullin-7
9	0.929	5/4/50	2.8	50.0	-0.0396	0.252	No	360075	179656/9.6	Q9JIR4	RAT	Regulating synaptic membrane exocytosis protein 1
10	0.641	7/4/50	4.0	50.0	0.0346	0.173	No	313083	147736/6.4	P24135	RAT	1-phosphatidylinositol 4,5-bisphosphate phosphodiesterase gamma-2

10188	x	.	.	x	x	.	x	.
264860	x	.	x	.	x	.	x	.
562312	x	x	.	x	x	.	.	.

<https://prospector.ucsf.edu/prospector/cgi-bin/mssearch.cgi>

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56803	.	x	x	.	x	.	x	.
324238	x	.	x	.	.	x	x	.
110496	.	x	x	.	x	x	.	.
345437	.	x	.	x	x	x	.	.
83921	.	.	x	x	x	x	.	x
360075	.	x	x	x	x	.	.	.
313083	x	x	x	x	.	.	.	.

[-] Detailed Results

1. 4/8 matches (50%).

Acc. #: P21932 **Uniprot ID:** ADCY3 **RAT Species:** RAT **Name:** Adenylate cyclase type 3**Organism:** Rattus norvegicus **OX=**10116 **Gene:** Adcy3 **Existence:** Evidence at protein level **Version:** 1**Index:** 10188 **MW:** 128937 Da **pI:** 5.8

m/z	MH ⁺	Intensity	Delta	Modifications	Start	End	Missed	Sequence
Submitted	Matched		Da				Cleavages	
713.4000	713.3287	100.0	0.0713	1Oxidation	881	885	0	(R)VYEMR(R)
1067.6000	1067.6136	100.0	-0.0136		1112	1120	0	(K)GELLTFFLK(G)
1316.6000	1316.6337	100.0	-0.0337		286	296	1	(K)HVADEMLKDMK(K)
1418.8000	1418.6766	100.0	0.123		454	465	0	(R)VHISQSTMDK(Carbamidomethyl)LK(G)

Num Unmatched Masses: 4

Search for disulfide linked peptides.

Do a non-specific cleavage search.

Search for another component.

The matched peptides cover 3.2% (37/1144AA's) of the protein.

Coverage Map for This Hit (MS-Digest index #): 10188

2. 4/8 matches (50%).

Acc. #: Q10728 **Uniprot ID:** MYPT1 **RAT Species:** RAT **Name:** Protein phosphatase 1 regulatory subunit 12A**Organism:** Rattus norvegicus **OX=**10116 **Gene:** Ppp1r12a **Existence:** Evidence at protein level **Version:** 2**Index:** 264860 **MW:** 115284 Da **pI:** 5.4

m/z	MH ⁺	Intensity	Delta	Modifications	Start	End	Missed	Sequence
Submitted	Matched		Da				Cleavages	
713.4000	713.3035	100.0	0.0965		552	557	0	(R)SC(Carbamidomethyl)SFGR(R)
979.6000	979.3963	100.0	0.204		676	683	0	(R)DEESESQR(K)
1316.6000	1316.6627	100.0	-0.0627	1Oxidation	1005	1015	1	(K)MLPDLKADNQR(L)
1316.6000	1316.6917	100.0	-0.0917		463	475	1	(K)DTAGVIRSASSPR(L)
1418.8000	1418.7863	100.0	0.0137		195	209	1	(R)HAKSGGTALHVAAK(G)

Num Unmatched Masses: 4

Search for disulfide linked peptides.

Do a non-specific cleavage search.

Search for another component.

The matched peptides cover 5.1% (53/1032AA's) of the protein.

Coverage Map for This Hit (MS-Digest index #): 264860

3. 4/8 matches (50%).

Acc. #: Q499R0 **Uniprot ID:** Z518A **RAT Species:** RAT **Name:** Zinc finger protein 518A**Organism:** Rattus norvegicus **OX=**10116 **Gene:** Znf518a **Existence:** Evidence at transcript level **Version:** 1

Index: 562312 **MW:** 165540 Da **pI:** 9.2

m/z Submitted	MH ⁺ Matched	Intensity	Delta Da	Modifications	Start	End	Missed Cleavages	Sequence
713.4000	713.4556	100.0	-0.0556	1Gln->pyro-Glu	14	19	1	(K)QTILKK(I)
919.4000	919.5095	100.0	-0.109		816	823	1	(K)EEVIKSSK(D)
1067.6000	1067.4245	100.0	0.176	1Carbamidomethyl->Propionamide	181	188	0	(K)C(Carbamidomethyl)DIC(Carbamidomethyl)NSER(S)
1316.6000	1316.6515	100.0	-0.0515		802	812	0	(K)NLPLDC(Carbamidomethyl)DQTLK(K)
1316.6000	1316.7355	100.0	-0.135	1Oxidation	1404	1414	1	(K)MLKPVNSVKER(F)

Num Unmatched Masses: 4
 Search for disulfide linked peptides.
 Do a non-specific cleavage search.
 Search for another component.

The matched peptides cover **3.0%** (44/1478AA's) of the protein.
 Coverage Map for This Hit (MS-Digest index #): 562312

4. 4/8 matches (50%).

Acc. #: D3Z8K2 **Uniprot ID:** CCD39 **RAT Species:** RAT **Name:** Coiled-coil domain-containing protein 39
Organism: Rattus norvegicus **OX=**10116 **Gene:** Ccdc39 **Existence:** Inferred from homology **Version:** 1
Index: 56803 **MW:** 109555 Da **pI:** 6.6

m/z Submitted	MH ⁺ Matched	Intensity	Delta Da	Modifications	Start	End	Missed Cleavages	Sequence
919.4000	919.5030	100.0	-0.103		608	615	0	(K)AMLTQSIR(Y)
979.6000	979.5571	100.0	0.0429		571	578	1	(R)ARELLYSK(A)
1316.6000	1316.7056	100.0	-0.106		305	315	1	(R)NQLKDELDTLK(T)
1418.8000	1418.7485	100.0	0.0515		309	320	1	(K)DELDTLKTTLNR(T)

Num Unmatched Masses: 4
 Search for disulfide linked peptides.
 Do a non-specific cleavage search.
 Search for another component.

The matched peptides cover **3.4%** (32/934AA's) of the protein.
 Coverage Map for This Hit (MS-Digest index #): 56803

5. 4/8 matches (50%).

Acc. #: A1A5S1 **Uniprot ID:** PRP6 **RAT Species:** RAT **Name:** Pre-mRNA-processing factor 6
Organism: Rattus norvegicus **OX=**10116 **Gene:** Prpf6 **Existence:** Evidence at protein level **Version:** 1
Index: 324238 **MW:** 106765 Da **pI:** 8.3

m/z Submitted	MH ⁺ Matched	Intensity	Delta Da	Modifications	Start	End	Missed Cleavages	Sequence
713.4000	713.5032	100.0	-0.103		294	299	1	(K)ARLLLK(S)
979.6000	979.5142	100.0	0.0858		131	137	1	(K)YRMERPK(I)
1369.6000	1369.7263	100.0	-0.126		575	585	1	(K)SVWLRAAYFEK(N)
1418.8000	1418.6580	100.0	0.142		716	727	0	(K)GQIEEQGELMER(A)

Num Unmatched Masses: 4
 Search for disulfide linked peptides.
 Do a non-specific cleavage search.

MS-Fit Search Results R 6/03/22, 16:24

Similar Matches for Protein Hit 4

[–] Detailed Results

Acc. #: P25409 **Uniprot ID:** ALAT1 **RAT Species:** RAT **Name:** Alanine aminotransferase 1

Organism: Rattus norvegicus OX=10116 **Gene:** Gpt **Existence:** Evidence at protein level **Version:** 2

Index: 13887 **MW:** 55110 Da **pI:** 6.1

<https://prospector.ucsf.edu/prospector/cgi-bin/mssearch.cgi>

1419.0000	1418.7130	100.0	0.287		20	31	0	(K)VLTLDTMNP(Carbamidomethyl)VR(R)
1434.8000	1434.7079	100.0	0.0921	1Oxidation	20	31	0	(K)VLTLDTMNP(Carbamidomethyl)VR(R)
1474.0000	1473.7696	100.0	0.230		392	404	0	(K)LTEQVFNEAPGIR(C)

Num Unmatched Masses: **16**
 Search for disulfide linked peptides.
 Do a non-specific cleavage search.
 Search for another component.

The matched peptides cover **16.5%** (82/496AA's) of the protein.
 Coverage Map for This Hit (MS-Digest index #): 13887

2. 5/25 matches (20%).

Acc. #: P04694 **Uniprot ID:** ATTY_RAT **Species:** RAT **Name:** Tyrosine aminotransferase
Organism: Rattus norvegicus OX=10116 **Gene:** Tat **Existence:** Evidence at protein level **Version:** 1
Index: 40350 **MW:** 50636 Da **pI:** 5.3

m/z Submitted	MH ⁺ Matched	Intensity	Delta Da	Modifications	Start	End	Missed Cleavages	Sequence
989.6000	989.5666	100.0	0.0334		185	192	0	(K)LYNLLPEK(S)
989.6000	989.5666	100.0	0.0334		185	192	0	(K)LYNLLPEK(S)
1334.8000	1334.6158	100.0	0.184		41	51	0	(R)WDVRPSDMSNK(T)
1335.0000	1334.6158	100.0	0.384		41	51	0	(R)WDVRPSDMSNK(T)
1854.2000	1854.0630	100.0	0.137		312	328	1	(K)LSQRILGPC(Carbamidomethyl)TIVQGALK(S)

Num Unmatched Masses: **20**
 Search for disulfide linked peptides.
 Do a non-specific cleavage search.
 Search for another component.

The matched peptides cover **7.9%** (36/454AA's) of the protein.
 Coverage Map for This Hit (MS-Digest index #): 40350

3. 6/25 matches (24%).

Acc. #: Q66HC3 **Uniprot ID:** CI072_RAT **Species:** RAT **Name:** Guanine nucleotide exchange C9orf72 homolog
Organism: Rattus norvegicus OX=10116 **Existence:** Evidence at transcript level **Version:** 1
Index: 65675 **MW:** 54306 Da **pI:** 5.7

m/z Submitted	MH ⁺ Matched	Intensity	Delta Da	Modifications	Start	End	Missed Cleavages	Sequence
916.8000	916.5098	100.0	0.290		157	164	0	(K)IVLEGTER(M)
1334.8000	1334.7137	100.0	0.0863	1Carbamidomethyl->Propionamide	250	260	0	(R)TLC(Carbamidomethyl)LFLTPAER(K)
1335.0000	1334.7137	100.0	0.286	1Carbamidomethyl->Propionamide	250	260	0	(R)TLC(Carbamidomethyl)LFLTPAER(K)
1381.6000	1381.7733	100.0	-0.173		130	140	1	(R)VC(Carbamidomethyl)VDRLTHIIR(K)
1474.0000	1473.6461	100.0	0.354		262	273	1	(K)C(Carbamidomethyl)SRLC(Carbamidomethyl)EAESSFK(Y)
1854.2000	1853.9644	100.0	0.236		74	90	1	(R)NAESGAIDVKFFVLSEK(G)

Num Unmatched Masses: **19**
 Search for disulfide linked peptides.
 Do a non-specific cleavage search.
 Search for another component.

The matched peptides cover **12.3%** (59/481AA's) of the protein.
 Coverage Map for This Hit (MS-Digest index #): 65675

4. 5/25 matches (20%).

Acc. #: Q6IFU7 **Uniprot ID:** K1C42 **RAT Species:** RAT **Name:** Keratin, type I cytoskeletal 42

Organism: Rattus norvegicus **OX=10116** **Gene:** Krt42 **Existence:** Inferred from homology **Version:** 1

Index: 204081 **MW:** 50214 Da **pI:** 5.1

m/z Submitted	MH ⁺ Matched	Intensity	Delta Da	Modifications	Start	End	Missed Cleavages	Sequence
807.4000	807.3995	100.0	4.56e-4		174	180	0	(R)LAADDFR(T)
1153.0000	1152.5289	100.0	0.471		18	28	0	(K)GLC(Carbamidomethyl)APGMGFSR(M)
1166.2000	1166.5445	100.0	-0.345	1Carbamidomethyl->Propionamide	18	28	0	(K)GLC(Carbamidomethyl)APGMGFSR(M)
1193.0000	1193.5878	100.0	-0.588		29	39	1	(R)MSSVRIGGAC(Carbamidomethyl)R(A)
1391.2000	1390.6809	100.0	0.519		332	344	0	(K)ASLENSLAETEAR(Y)

Num Unmatched Masses: 20

Search for disulfide linked peptides.

Do a non-specific cleavage search.

Search for another component.

The matched peptides cover 9.3% (42/452AA's) of the protein.

Coverage Map for This Hit (MS-Digest index #): 204081

5. 4/25 matches (16%).

Acc. #: P11715 **Uniprot ID:** CP17A **RAT Species:** RAT **Name:** Steroid 17-alpha-hydroxylase/17,20 lyase

Organism: Rattus norvegicus **OX=10116** **Gene:** Cyp17a1 **Existence:** Evidence at protein level **Version:** 2

Index: 77706 **MW:** 57251 Da **pI:** 6.9

m/z Submitted	MH ⁺ Matched	Intensity	Delta Da	Modifications	Start	End	Missed Cleavages	Sequence
1153.0000	1152.6371	100.0	0.363		471	480	1	(K)QLPRLEGDPK(V)
1334.8000	1334.5901	100.0	0.210	1Carbamidomethyl->Propionamide	151	161	0	(K)SLC(Carbamidomethyl)DMMLAHDK(E)
1335.0000	1334.5901	100.0	0.410	1Carbamidomethyl->Propionamide	151	161	0	(K)SLC(Carbamidomethyl)DMMLAHDK(E)
1335.0000	1335.5522	100.0	-0.552	1Gln->pyro-Glu 1Oxidation	496	507	0	(R)QAWMDAQAEVST(-)
1381.6000	1381.6594	100.0	-0.0594		199	211	0	(K)TFTEGIVDATGDR(N)

Num Unmatched Masses: 21

Search for disulfide linked peptides.

Do a non-specific cleavage search.

Search for another component.

The matched peptides cover 9.1% (46/507AA's) of the protein.

Coverage Map for This Hit (MS-Digest index #): 77706

6. 4/25 matches (16%).

Acc. #: Q5PQN2 **Uniprot ID:** BFAR **RAT Species:** RAT **Name:** Bifunctional apoptosis regulator

Organism: Rattus norvegicus **OX=10116** **Gene:** Bfar **Existence:** Evidence at transcript level **Version:** 1

Index: 43724 **MW:** 53025 Da **pI:** 6.4

m/z Submitted	MH ⁺ Matched	Intensity	Delta Da	Modifications	Start	End	Missed Cleavages	Sequence
803.2000	803.3604	100.0	-0.160	1Acetyl	1	6	0	(-)MEEPQK(N)
803.2000	803.4662	100.0	-0.266		94	100	0	(K)LFDAIK(M)
1391.2000	1391.7390	100.0	-0.539		166	177	1	(R)SRESEHGLLVHK(A)
1419.0000	1418.6708	100.0	0.329		56	66	0	(R)HC(Carbamidomethyl)LALWWSSK(K)
1434.8000	1434.6657	100.0	0.134	1Oxidation	56	66	0	(R)HC(Carbamidomethyl)LALWWSSK(K)

Num Unmatched Masses: 21

MS-Fit Search Results

[−] Parameters

Database searched: **SwissProt.2021.06.18**

Digest Used: **Trypsin**

Max. # Missed Cleavages: **1**

Constant Modification: **Carbamidomethyl (C)**

Minimum Matches: **4**

Sort Type: **Score Sort**

Considered modifications: | **Peptide N-terminal Gln to pyroGlu** | **Oxidation of M** | **Protein N-terminus Acetylated** |

Acrylamide Modified Cys |

Min Parent Ion Matches: **1**

MOWSE On: **1**

MOWSE P Factor: **0.4**

Report Homologous Proteins: **Interesting**

[−] Pre Search Results (SwissProt.2021.06.18)

Number of entries in the database: **565254**

Molecular weight search (**50000 - 60000 Da**) selects **48751** entries.

Full pI range: **565254** entries.

Taxonomy search **RATTUS NORVEGICUS** selects **8135** entries.

Combined molecular weight, pI and taxonomy searches select **990** entries.

Pre searches select **990** entries.

Fraction-Spot-Run ID: **1-1-1**

MS-Fit search selects **100** entries (results displayed for top **20** matches).

[−] Results Summary

Protein Hit Number	MOWSE Score	# pep # mat % mat	% Cov	% TIC	Mean Err	Data Da	# Tol	# Hom	MS-Digest Index #	Protein MW (Da)	Accession #	Species	Protein Name
1	1980	10/9/36	16.5	36.0	0.141	0.237	No		13887	55110/6.1	P25409	RAT	Alanine aminotransferase 1
2	165	3/5/20	7.9	20.0	0.154	0.289	No		40350	50636/5.3	P04694	RAT	Tyrosine aminotransferase
3	129	5/6/24	12.3	24.0	0.180	0.390	No		65675	54306/5.7	Q66HC3	RAT	Guanine nucleotide exchange C9orf72 homolog
4	83.8	5/5/20	9.3	20.0	0.0117	0.977	1		204081	50214/5.1	Q6IFU7	RAT	Keratin, type I cytoskeletal 42
5	43.5	4/4/16	9.1	16.0	0.0742	0.790	No		77706	57251/6.9	P11715	RAT	Steroid 17-alpha-hydroxylase/17,20 lyase
6	42.9	5/4/16	8.0	16.0	-0.100	0.681	No		43724	53025/6.4	Q5PQN2	RAT	Bifunctional apoptosis regulator
7	42.7	4/4/16	10.1	16.0	0.00839	0.949	No		204187	50709/5.7	Q6IG12	RAT	Keratin, type II cytoskeletal 7
9	38.5	4/5/20	7.3	20.0	0.153	0.516	No		64670	51825/6.1	Q01134	RAT	Choline kinase alpha
10	35.6	8/8/32	12.3	36.0	0.210	0.372	No		161184	51012/8.8	Q4V8J4	RAT	Glycerol-3-phosphate acyltransferase 3
11	32.6	5/5/20	8.7	20.0	-0.245	0.751	No		397517	58038/8.4	Q4G055	RAT	RNA-binding region-containing protein 3
12	27.1	5/6/24	8.8	24.0	0.120	0.312	No		245852	53428/5.4	P03957	RAT	Stromelysin-1
13	24.7	6/7/28	10.9	28.0	0.153	0.349	No		466898	53333/8.1	Q5M7W7	RAT	Probable proline-tRNA ligase, mitochondrial
14	24.1	5/6/24	8.8	24.0	-0.0809	0.730	No		56190	59291/5.4	Q6AYN9	RAT	Coiled-coil domain-containing protein 181
15	24.0	4/4/16	8.0	16.0	-0.262	0.714	No		84690	54948/5.9	Q3KR64	RAT	Uncharacterized protein CXorf49 homolog
16	23.9	3/4/16	6.3	16.0	0.129	0.476	No		77849	55996/9.1	P11711	RAT	Cytochrome P450 2A1
17	23.0	4/5/20	6.9	20.0	0.177	0.352	No		132555	57400/6.4	B2RYN2	RAT	F-box only protein 31
18	19.3	4/4/16	7.3	16.0	-0.0334	0.609	No		316984	53512/7.6	D3ZW91	RAT	POC1 centriolar protein homolog B

Supplemental data S10. MS-Fit prediction for B6

MS-Fit Search Results

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MS-Fit Search Results

[–] Parameters

Database searched: SwissProt.2021.06.18

Digest Used: **Trypsin**

Max. # Missed Cleavages: 1

Constant Modification: Carbamidomethyl (C)

Minimum Matches: 4

Sort Type: Score Sort

Considered modifications: | **Peptide N-terminal Gln to pyroGlu** | **Oxidation of M** | **Protein N-terminus Acetylated** | **Acrylamide**

Modified Cys |

Min Parent Ion Matches: 1

MOWSE On: 1

MOWSE P Factor: 0.4

Report Homologous Proteins: Interesting

[–] Pre Search Results (SwissProt.2021.06.18)

Number of entries in the database: 565254

Molecular weight search (50000 - 60000 Da) selects 48751 entries.

Full pl range: **565254** entries.

Taxonomy search **RATTUS NORVEGICUS** selects **8135** entries.

Combined molecular weight, pI and taxonomy searches select **990** entries.

Pre searches select **990** entries.

Fraction-Spot-Run ID: 1-1-1

MS-Fit search selects **231** entries (results displayed for top **20** matches).

[–] Results Summary

Protein Hit Number	MOWSE Score	# pep # mat % cov % TIC 39 pks	Mean Err Da	Data Tot Da	# Hom Prot	MS-Digest Index	Protein MW (Da)/pI	Accession #	Species	Protein Name
1	1400	10/9/23 20.2 23.1	0.0949	0.579	No	515408	53733/5.1	P31000	RAT	Vimentin
2	563	6/8/21 11.0 20.5	0.0634	0.591	No	244942	58586/5.9	O88368	RAT	Microphthalmia-associated transcription factor
3	411	4/5/13 7.7 12.8	0.186	0.542	No	448396	53205/9.7	Q3KRF1	RAT	Sp110 nuclear body protein
4	196	5/5/13 10.1 12.8	0.198	0.512	No	174370	56573/5.3	Q6AZ54	RAT	Hemogen
5	191	3/4/10 8.1 10.3	0.180	0.195	No	295639	51480/8.3	P51578	RAT	P2X purinoceptor 5
6	169	5/7/18 10.9 17.9	0.211	0.444	No	211102	55655/6.2	Q68G30	RAT	Kinesin light chain 3
7	165	5/5/13 11.4 12.8	0.0858	0.695	No	77942	55946/7.7	Q35293	RAT	Cytochrome P450 2F2
8	163	4/4/10 10.0 10.3	-0.00762	0.467	No	141899	57972/5.0	Q2A121	RAT	Alpha-ketoglutarate-dependent dioxygenase FTO
9	162	5/6/15 11.8 15.4	0.115	0.475	No	108596	53257/9.2	Q5XIR6	RAT	Dynein regulatory complex protein 10
10	126	5/6/15 11.7 15.4	-0.279	0.643	1	204081	50214/5.1	Q6IFU7	RAT	Keratin, type I cytoskeletal 42
11	113	4/5/13 8.6 12.8	-0.281	0.806	No	448905	56020/8.7	Q99MU5	RAT	Spermatogenesis-associated protein 6
12	105	4/5/13 7.4 12.8	0.190	0.151	No	132315	50161/4.5	Q9WVH8	RAT	Fibulin-5
13	102	5/5/13 9.1 12.8	0.0882	0.333	No	158021	53673/8.7	P24524	RAT	Glycine receptor subunit alpha-3
14	95.3	4/5/13 4.1 12.8	0.136	0.432	No	56059	59680/9.5	Q4V8B5	RAT	Coiled-coil domain-containing protein 116
15	90.7	4/5/13 10.0 12.8	-0.0695	0.300	No	483717	52359/9.6	Q5U4F4	RAT	Transmembrane protein 135
16	88.9	4/4/10 7.0 12.8	0.0926	0.855	No	52081	51589/7.2	Q08163	RAT	Adenyl cyclase-associated protein 1
17	85.5	4/5/13 9.7 12.8	0.120	0.121	No	564280	55532/5.5	Q5PPG4	RAT	Zinc finger protein 639
18	75.9	4/5/13 6.6 15.4	0.0230	0.654	No	519331	54326/8.3	O08816	RAT	Neural Wiskott-Aldrich syndrome protein
19	75.5	4/5/13 7.3 12.8	-0.109	0.684	No	355920	55692/8.3	P49803	RAT	Regulator of G-protein signaling 7

515408	.	.	.	X	.	.	.	X	X	X	.	.	.	.	.	.	.	.	X	.	.	X	X	.	.	X	.	.	.
244942	.	.	.	.	.	.	.	.	X	X	.	.	.	.	.	.	.	.	X	.	.	X	.	.	X	X	.	.	X
448396	.	.	.	.	.	.	.	.	.	.	X	X	X	.	.	.	.	.	.	.	.	.	.	.	.	X	X	.	.
174370	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	X	.	.	.	X	X	.	.	.	X	X	.

[illegible]

Protein Hit Number	MOWSE Score	# pep # mat % mat 39 pks	% Cov	% TIC	Mean Err Da	Data Tol Da	# Hom Prot	MS-Digest Index #	Protein MW (Da)/pI	Accession #	Species	Protein Name
10	126	56/15	11.7	15.4	-0.279	0.643	1	204081	50214/5.1	Q6IFU7	RAT	Keratin, type I cytoskeletal 42
Protein Hit Number	MOWSE Score	# pep # mat % mat 39 pks	% Cov	% TIC	Mean Err Da	Data Tol Da	# Hom Prot	MS-Digest Index #	Protein MW (Da)/pI	Accession #	Species	Protein Name
12	112	78/21	11.6	20.5	0.221	0.649	1	203995	56505/5.1	O6IFW6	RAT	Keratin, type I cytoskeletal 10

Acc. #: P31000 **Uniprot ID:** VIME_RAT **Species:** RAT **Name:** Vimentin
Organism: Rattus norvegicus **OX:** 10116 **Gene:** Vim **Existence:** Evidence at protein level **Version:** 2
Index: 515408 **MW:** 53733 Da **pI:** 5.1

m/z	MIH ⁺	Intensity	Delta Da	Modifications	Start	End	Missed Cleavages	Sequence
Submitted	Matched							
873.0000	872.4108	100.0	0.589		5	12	0	(R)SVSSSSYR(R)
1060.8000	1060.5269	100.0	0.273		160	168	0	(R)QVDQLTNDK(A)
1060.8000	1060.5633	100.0	0.237		402	410	1	(R)KLLGEESR(I)
1121.6000	1121.5837	100.0	0.0163		382	390	0	(R)EYQDLLNVK(M)
1122.0000	1121.5837	100.0	0.416		382	390	0	(R)EYQDLLNVK(M)
1125.2000	1125.6051	100.0	-0.405		114	122	1	(R)FANFYDKVRF(F)
1309.4000	1309.6059	100.0	-0.206		283	292	0	(K)NLQEAEEWYK(S)
1361.8000	1361.6842	100.0	0.116	1Oxidation	176	186	1	(R)DNLAEDIMRLR(E)
1405.8000	1405.6376	100.0	0.162	1Oxidation 1Acetyl	1	12	1	(-)MSTRSVSSSSYR(R)
1405.8000	1405.7573	100.0	0.0427		224	235	0	(K)IVLEQEEIAFLK(K)
1587.6000	1587.7973	100.0	-0.197		101	113	1	(R)TNEKVELQELNDR(F)

Num Unmatched Masses: 30
Search for disulfide linked peptides.
Do a non-specific cleavage search.
Search for another component.

The matched peptides cover **20.2%** (94/466AA's) of the protein.
Coverage Map for This Hit (MS-Digest index #): 515408

2. 8/39 matches (20%).
Acc. #: O88368 **Uniprot ID:** MITF **RAT Species:** RAT **Name:** Microphthalmia-associated transcription factor
Organism: Rattus norvegicus **OX=**10116 **Gene:** Mitf **Existence:** Evidence at transcript level **Version:** 2
Index: 244942 **MW:** 58586 **Da** **pI:** 5.9

m/z Submitted	MIH ⁺ Matched	Intensity	Delta Da	Modifications	Start End	Missed Cleavages	Sequence
1121.6000	1121.5837	100.0	0.0163	1Gln->pyro-Glu	141 150	0	(K)QYLSTTLANK(H)
1122.0000	1121.5837	100.0	0.416	1Gln->pyro-Glu	141 150	0	(K)QYLSTTLANK(H)
1220.4000	1220.6205	100.0	-0.220		80 89	0	(K)LQAAQFMQQR(V)
1352.0000	1352.5093	100.0	-0.509	1Carbamidomethyl->Propionamide	515 526	0	(R)SSMSAEETEHAC(Carbamidomethyl)(-)
1475.8000	1475.6002	100.0	0.200		205 217	0	(R)AESEC(Carbamidomethyl)PGMNTHSR(A)
1475.8000	1475.6002	100.0	0.200		205 217	0	(R)AESEC(Carbamidomethyl)PGMNTHSR(A)
1489.8000	1489.6158	100.0	0.184	1Carbamidomethyl->Propionamide	205 217	0	(R)AESEC(Carbamidomethyl)PGMNTHSR(A)
1717.0000	1716.7792	100.0	0.221	1Gln->pyro-Glu	61 73	1	(R)QQLMREQMQEQR(R)

Num Unmatched Masses: 31

Search for disulfide linked peptides.

Do a non-specific cleavage search.

Search for another component.

The matched peptides cover 11.0% (58/526AA's) of the protein.

Coverage Map for This Hit (MS-Digest index #): 244942

3. 5/39 matches (12%).

Acc. #: Q3KRF1 Uniprot ID: SP110 RAT Species: RAT Name: Sp110 nuclear body protein

Organism: Rattus norvegicus OX=10116 Gene: Sp110 Existence: Evidence at protein level Version: 1

Index: 448396 MW: 53205 Da pI: 9.7

m/z Submitted	MIH ⁺ Matched	Intensity	Delta Da	Modifications	Start End	Missed Cleavages	Sequence
1166.0000	1165.5670	100.0	0.433		92 101	0	(R)EYPSLGAIC(Carbamidomethyl)R(S)
1166.0000	1165.5670	100.0	0.433		92 101	0	(R)EYPSLGAIC(Carbamidomethyl)R(S)
1179.8000	1179.5827	100.0	0.217	1Carbamidomethyl->Propionamide	92 101	0	(R)EYPSLGAIC(Carbamidomethyl)R(S)
1489.8000	1489.7468	100.0	0.0532		419 431	0	(K)C(Carbamidomethyl)IQNEAGAWLTVK(E)
1587.6000	1587.8047	100.0	-0.205		50 63	0	(K)ESLEAC(Carbamidomethyl)QNLVPLSK(V)

Num Unmatched Masses: 34

Search for disulfide linked peptides.

Do a non-specific cleavage search.

Search for another component.

The matched peptides cover 7.7% (37/478AA's) of the protein.

Coverage Map for This Hit (MS-Digest index #): 448396

4. 5/39 matches (12%).

Acc. #: Q6AZ54 Uniprot ID: HEMGN RAT Species: RAT Name: Hemogen

Organism: Rattus norvegicus OX=10116 Gene: Hemgn Existence: Evidence at protein level Version: 1

Index: 174370 MW: 56573 Da pI: 5.3

m/z Submitted	MIH ⁺ Matched	Intensity	Delta Da	Modifications	Start End	Missed Cleavages	Sequence
1244.0000	1243.6681	100.0	0.332		400 410	0	(K)EFTVPIVSSHK(T)
1352.0000	1351.5439	100.0	0.456	1Carbamidomethyl->Propionamide	182 192	0	(K)MC(Carbamidomethyl)QDMAEPEAR(S)
1383.8000	1383.5337	100.0	0.266	2Oxidation 1Carbamidomethyl->Propionamide	182 192	0	(K)MC(Carbamidomethyl)QDMAEPEAR(S)
1587.6000	1587.8159	100.0	-0.216		294 307	0	(K)IC(Carbamidomethyl)QDVARPDVLSK(T)
1658.0000	1657.8465	100.0	0.153		336 351	0	(K)TLPSQSDVTPMSVTPGK(N)

Num Unmatched Masses: 34

Search for disulfide linked peptides.

Do a non-specific cleavage search.

Search for another component.

The matched peptides cover 10.1% (52/514AA's) of the protein.

Coverage Map for This Hit (MS-Digest index #): 174370

5. 4/39 matches (10%).

Acc. #: P51578 Uniprot ID: P2RX5 RAT Species: RAT Name: P2X purinoceptor 5

Organism: Rattus norvegicus OX=10116 **Gene:** P2rx5 **Existence:** Evidence at transcript level **Version:** 1
Index: 295639 **MW:** 51480 Da **pI:** 8.3

m/z Submitted	MH ⁺ Matched	Intensity	Delta Da	Modifications	Start	End	Missed Cleavages	Sequence
1335.0000	1334.6739	100.0	0.326		240	251	0	(R)WAGADFQDIALK(G)
1405.8000	1405.6607	100.0	0.139		285	296	0	(K)HTHISISSGYNFR(F)
1475.8000	1475.6721	100.0	0.128		407	419	0	(R)QDEQSQELAQSQR(K)
1475.8000	1475.6721	100.0	0.128		407	419	0	(R)QDEQSQELAQSQR(K)

Num Unmatched Masses: 35

Search for disulfide linked peptides.

Do a non-specific cleavage search.

Search for another component.

The matched peptides cover 8.1% (37/455AA's) of the protein.

Coverage Map for This Hit (MS-Digest index #): 295639

6. 7/39 matches (17%).

Acc. #: Q68G30 **Uniprot ID:** KLC3 **RAT Species:** RAT **Name:** Kinesin light chain 3

Organism: Rattus norvegicus OX=10116 **Gene:** Klc3 **Existence:** Evidence at protein level **Version:** 1

Index: 211102 **MW:** 55655 Da **pI:** 6.2

m/z Submitted	MH ⁺ Matched	Intensity	Delta Da	Modifications	Start	End	Missed Cleavages	Sequence
973.6000	973.4949	100.0	0.105		232	239	0	(R)QALEDLER(S)
1033.6000	1033.4796	100.0	0.120		121	128	0	(R)EELEETQR(R)
1121.6000	1121.5949	100.0	0.00505		324	334	0	(K)VLGADHPDVAK(Q)
1122.0000	1121.5949	100.0	0.405		324	334	0	(K)VLGADHPDVAK(Q)
1166.0000	1165.5452	100.0	0.455		453	464	1	(R)GESMAGAAAGMKR(A)
1166.0000	1165.5452	100.0	0.455		453	464	1	(R)GESMAGAAAGMKR(A)
1715.8000	1715.8680	100.0	-0.0680		465	480	1	(R)AMSLNMLNVDPRAAR(M)

Num Unmatched Masses: 32

Search for disulfide linked peptides.

Do a non-specific cleavage search.

Search for another component.

The matched peptides cover 10.9% (55/505AA's) of the protein.

Coverage Map for This Hit (MS-Digest index #): 211102

7. 5/39 matches (12%).

Acc. #: O35293 **Uniprot ID:** CP2F2 **RAT Species:** RAT **Name:** Cytochrome P450 2F2

Organism: Rattus norvegicus OX=10116 **Gene:** Cyp2f2 **Existence:** Evidence at transcript level **Version:** 1

Index: 77942 **MW:** 55946 Da **pI:** 7.7

m/z Submitted	MH ⁺ Matched	Intensity	Delta Da	Modifications	Start	End	Missed Cleavages	Sequence
1122.0000	1122.5174	100.0	-0.517		110	120	0	(K)GNGLAFSDGER(W)
1158.8000	1158.5500	100.0	0.250		263	271	0	(R)DFIDC(Carbamidomethyl)FLTK(M)
1185.0000	1184.6568	100.0	0.343		434	443	1	(R)RLC(Carbamidomethyl)LGEPLAR(M)
1206.6000	1206.7093	100.0	-0.109		75	85	0	(R)VIVLSGYQTVK(E)
1717.0000	1716.8374	100.0	0.163		344	358	0	(R)ASMPYTDVIEVQR(F)

Num Unmatched Masses: 34

Search for disulfide linked peptides.

Do a non-specific cleavage search.

Search for another component.

The matched peptides cover 11.4% (56/491AA's) of the protein.

Coverage Map for This Hit (MS-Digest index #): 77942