In Silico Analysis of ACE Inhibitory Peptides from Chloroplast Proteins of Red Alga *Grateloupia asiatica*

Kana Sumikawa[1] • Kentaro Takei[1] • Yuya Kumagai[2] • Takeshi Shimizu[3] • Hajime Yasui[4] • Hideki Kishimura[2, *]

[1] Chair of Marine Chemical Resource Development, Graduate School of Fisheries Sciences, Hokkaido University, Hakodate, Hokkaido 041-8611, Japan

[2] Laboratory of Marine Chemical Resource Development, Faculty of Fisheries Sciences, Hokkaido University, Hakodate, Hokkaido 041-8611, Japan

[3] Department of Research and Development, Hokkaido Industrial Technology Center, Hakodate, Japan

[4] Laboratory of Humans and the Ocean, Faculty of Fisheries Sciences, Hokkaido University, Hakodate, Hokkaido 041-8611, Japan

* Hideki Kishimura: i-dulse@fish.hokudai.ac.jp, +81-138-40-5519

Abstract

Inhibition of angiotensin I converting enzyme (ACE) is one of the key factors to repress high blood pressure. Although many studies have been reported that seaweed protein hydrolysates showed the ACE inhibitory activity, the comprehensive understanding of the relationship was still unclear. In this study, we employed chloroplast genome for *in silico* analysis and compared it with *in vitro* experiments. We first extracted water-soluble proteins (WSP) from red alga *Grateloupia asiatica*, which contained mainly PE, PC, APC and Rbc, and prepared WSP hydrolysate by thermolysin, resulting that the hydrolysate showed ACE inhibitory activity. Then, we determined the complete chloroplast genome of *G. asiatica* (187,518 bp: 206 protein coding genes, 29 tRNA and 3 rRNA) and clarified the amino acid sequences of main WSP, i.e., phycobiliproteins and Rubisco, to perform *in silico* analysis. Consequently, 190 potential ACE inhibitory peptides existed in the main WSP sequences, and 21 peptides were obtained by *in silico* thermolysin digestion. By comparing *in vitro* and *in silico* analyses, *in vitro* ACE inhibitory activity was correlated to the IC_{50} value from *in silico* digestion. Therefore, *in silico* approach provides insight into the comprehensive understanding of the potential bioactive peptides from seaweed proteins.

Keywords Red alga • *Grateloupia asiatica* • ACE inhibitory peptide • Chloroplast genome • *In silico* analysis

Abbreviations

ACE, angiotensin I converting enzyme; APC, allophycocyanin; CBB, coomassie brilliant blue; Rbc, ribulose-1, 5-bisphosphate carboxylase/oxygenase; PC, phycocyanin; PCG, protein coding genes; PE, phycoerythrin; WSP, water-soluble proteins

Introduction

High blood pressure or hypertension is the major risk for cardiovascular diseases (Daskaya-Dikmen et al. 2017) and serious public health problem regarding the loss of workforce and the increase in social security. Angiotensin I converting enzyme (ACE: EC 3.4.15.1) is a dipeptidyl carboxypeptidase, which regulates the blood pressure by the renin-angiotensin system. ACE converts the inactive decapeptide angiotensin I to an active octapeptide angiotensin II, resulting in the increase in the blood pressure (Schmieder et al. 2007). Therefore, regulation of ACE activity is a major therapeutic way to modulate hypertension. Synthetic inhibitors such as alcacepril, captopril, enalapril, lisinopril or ramipril are used for hypertensive patients, however, side effects of these inhibitors such as cough, skin rashes, angioedema have been reported (Daskaya-Dikmen et al. 2017; Schmieder et al. 2007). Therefore, the demand of peptides from natural food has been required. In addition, protein sources, which have a potential of ACE inhibitory peptides, are also demanded, since the active compound would be produced during digestion.

The nutrient components of seaweeds have been elucidated and spread as a healthy food worldwide (Holdt and Kraan 2011). Seaweeds are often used as a source of thickener in food processing, and the main components were polysaccharides. Some of the hydrolysates such as oligosaccharides showed biological activity on human health (Chen et al. 2006; Lee and Mooney 2012; Ruiter and Rudolph 1997; Yamamoto et al. 2019). Although the amount of proteins differed in species, season and environmental conditions, the content of red algae tend to higher than those of brown and green algae (Mai et al. 1994). The main proteins in red algae are phycobiliproteins, which play a role of light-harvesting for photosynthesis (Glazer 1985). Phycobilisomes are primarily consisted of phycoerythrin (PE), phycocyanin (PC) and allophycocyanin (APC), and the proteins are commonly constructed by α- and β-subunits. The phycobiliproteins form two domains in phycobilisomes: the core (APC) and the rods (PE and PC). Among the rods, PE is located at the tip of the rods; therefore, PE is easily obtained in water extraction accompanying with large- and small-subunits of Rubisco (RbcL and RbcS) (Sfriso et al. 2018). There are many reports for health benefits of hydrolyzed peptides from them, i.e., ACE inhibitory activity (Suetsuna and Nakano 2000; He et al. 2007; Cian et al. 2013; Fitzgerald et al. 2014; Beaulieu et al. 2016; Furuta et al. 2016; Cao et al. 2017; Kitade et al. 2018; Miyabe et al. 2017), DPP-IV inhibitory activity (Harnedy et al. 2015) and antioxidant activity (Cian et al. 2015; Fitzgerald et al. 2011; Sato et al. 2018; Sonani et al. 2014). However, the amino acid sequences of the proteins differ in the red algae.

Genome sequencing was more easily accessible, and many seaweed genomes were determined (Costa et al. 2016; Kumagai et al. 2019a; Yang et al. 2015). Phycobiliproteins and Rbc genes were coded on chloroplast genome, meaning that protein information would be employed in *in silico* analysis for the evaluation of the possible bioactive peptides in sequences and the speculation of the available peptides. Red algae are classified into more than 7,000 species in the world (Yang et al. 2016) and 800 species in Japan. However, limited red alga species are used for foodstuff, such as Nori (*Pyropia* sp.) and Agar (*Gelidium* sp. and *Gracilaria* sp.). Therefore, finding the potential bioactive components would lead to an effective utilization for food material.

In this study, we attempted to a comprehensive understanding of the relationship between *in vitro* and *in silico* analysis. First, we extracted water-soluble proteins (WSP) from *Grateloupia asiatica* and prepared WSP hydrolysate by thermolysin and then measured ACE inhibitory activity of the hydrolysate. In addition, we determined the complete nucleotide sequence of *G. asiatica* chloroplast genome and revealed the primary structures of main WSP (PE, PC, APC and Rbc) for the *in silico* enzyme digestion. As a result, we obtained *in vitro* and *in silico* data of *G. asiatica*. These analyses would show that the analysis of chloroplast genome lead to the understanding of the potential bioactive peptides from seaweeds.

Materials and Methods

Materials

Grateloupia asiatica was collected on the coast of Hakodate, Hokkaido Prefecture, Japan in February 2015 and preserved at -30 °C until use. ACE from rabbit lung was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Cetyltrimethylammonium bromide (CTAB), hyppuryl-L-histidyl-L-leucine, thermolysin (EC 3.4.24.27) and all other reagents were purchased from FUJIFILM Wako Pure Chemical (Osaka, Japan).

Chloroplast Genome Construction

Genomic DNA was extracted using the CTAB method (Cota-Sánchez et al. 2006) with some modification as previously (Watanabe et al. 2019; Kumagai et al. 2019b). The genome sequence data were generated using the Ion PGM System (Thermo Fisher SCIENTIFIC, Waltham, MA, USA). The data were assembled with CLC Genomics Workbench 9.5.4 (QIAGEN, Hilden, Germany). The contigs coding

chloroplasts genome were selected using BLASTn referring to *Grateloupia taiwanensis* chloroplast genome (NC_021618.1). After the reassembly, a circular chloroplast genome was obtained. The genes coding proteins were manually annotated using RNAmmer v1.2 server (http://www.cbs.dtu.dk/services/RNAmmer/), tRNAscan-SE 2.0 (http://lowelab.ucsc.edu/tRNAscan-SE/), ORF finder (https://www.ncbi.nlm.nih.gov/orffinder/). Gaps in genes were confirmed by using PCR amplification and Sanger sequencing (Online Resource Table S1). The annotated chloroplast genomes were visualized using GenomeVX (http://wolfe.ucd.ie/GenomeVx/).

Preparation of Protein Hydrolysate from *G. asiatica*

WSP of *G. asiatica* were extracted as previously (Furuta et al. 2016; Kitade et al. 2018). Namely, frozen samples were lyophilized and ground into a powder by Wonder Blender WB-1 (OSAKA CHEMICAL Co., Osaka, Japan). The powder was dissolved in 20 w/v of distilled water at 4 °C for 12 h, and WSP were recovered by centrifugation at 4 °C, 10,000×g for 10 min. WSP was dialyzed against distilled water using dialysis membrane (molecular weight cut off: approximately 10 kDa, EIDIA Co., Ltd., Tokyo, Japan) at 4 °C for 24 h to remove low molecular weight contaminants. After the dialysis, WSP was lyophilized. The obtained WSP was hydrolyzed by adding 1.0 wt% of thermolysin at 70 °C for 3 h, and the reaction was stopped by heating at 100 °C for 10 min. The solution was dialyzed against distilled water using the dialysis membrane at 4 °C for 12 h. The dialysis outer solution was lyophilized and obtained as WSP hydrolysate. Components of WSP and WSP hydrolysate were confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970). The band was visualized by CBB, and fluorescence images of phycobiliproteins were detected by gel documentation LED illuminator (VISIRAYS AE-6935GN: ATTO, Tokyo, Japan).

ACE Inhibitory Assay

ACE inhibitory assay was carried out according to the method of Cheung and Cushman (1973) with some modifications. Namely, ten microliters of sample (0.8-5.0 mg/mL) or buffer were added to 20 μL of ACE (0.2 units/mL), and the mixture was preincubated at 37 °C for 5 min. Twenty microliters of Hip-His-Leu solution (12.5 mM in 0.1 M sodium borate buffer containing 400 mM NaCl at pH 8.3) were added to the mixture. After incubation at 37 °C for 1 h, the reaction was stopped by adding 50 μL of 1.0 M HCl. The released hippuric acid was extracted with 300 μL of ethyl acetate. Two hundred sixty-five microliters of

130 the upper layer was evaporated, and then the hippuric acid was dissolved in 1.0 mL of distilled water, and
131 the absorbance at 228 nm was measured. The inhibition was calculated from the equation
132 (1-(As-Asb)/(Ac-Acb))×100, where Ac is the absorbance from the reaction mixture of ACE without
133 sample, Acb is the absorbance from Ac-blank (added denatured ACE), As is the absorbance from the
134 reaction mixture of ACE with the sample, and Asb is the absorbance from As-blank (added denatured
135 ACE with the sample). The data were expressed as mean±SEM from triplicate determination.
136
137 **Separation of WSP Hydrolysate**
138 The WSP hydrolysates were dissolved in ultrapure water containing 0.1% trifluoroacetic acid (TFA) and
139 applied to sequential filtration by Millex-GV (pore size: 0.22 μm) and Millex-LG (pore size: 0.20 μm).
140 The hydrolysate in the filtrate were isolated by reversed-phase HPLC with a Mightysil RP-18GP column
141 (4.6 × 150 mm) (Kanto Kagaku, Tokyo, Japan) using a linear gradient of acetonitrile (1-20%) containing
142 0.1% TFA at a flow rate of 1.0 mL/min. The peptides from the hydrolysate were detected by measuring at
143 228 nm.
144
145 **Prediction of Peptides by MALDI-TOF/MS**
146 The peptide sequences were predicted by Matrix Assisted Laser Desorption/Ionization Time Of Flight
147 Tandem Mass Spectrometry (MALDI-TOF/MS) method using a 4700 Proteomics Analyzer with Denovo
148 Explorer software (Applied Biosystems, Carlsbad, CA, USA). The predicted peptide sequences from
149 WSP were calculated by PeptideCutter (https://web.expasy.org/peptide_cutter/).
150
151 **Determination of Protein Concentration**
152 The amount of phycobiliproteins was determined by the spectra using following equations: PC =
153 $[OD_{615}-0.474(OD_{652})]/5.34$; APC = $[OD_{652}-0.208(OD_{615})]/5.09$; PE =
154 $[(OD_{562})-2.41(PC)-0.849(APC)]/9.62$ (Bennett and Bogorad 1973). The amount of Rubisco was
155 determined by SDS-PAGE using ImageJ software (https://imagej.nih.gov/ij/) and BSA as a standard.
156
157 **Collection of ACE Inhibitory Peptides from Database and *In Silico* Digestion of WSP**
158 ACE inhibitory peptides were obtained from the biopep-uwm database
159 (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep) on 7 July 2019. From the database, 924 ACE

inhibitory peptides having IC_{50} from 0.01 to 17,000 μM were extracted and peptides having IC_{50} less than 100 μM were collected (total 462 peptides). The peptide sequences in chloroplast proteins were manually annotated. The thermolysin-digestion products of WSP were predicted using PeptideCutter (https://web.expasy.org/peptide_cutter/). The ACE inhibitory activities in the predicted peptides were manually evaluated by the extraction data from biopep-uwm. *In Silico* thermolysin IC_{50} value (iSD_{50}) was obtained as follows: $1/iSD_{50}$ (L/g) = [PE]/(Sum of PE-IC_{50}) + [PC]/(Sum of PC-IC_{50}) + [APC]/(Sum of APC-IC_{50}) + [Rbc]/(Sum of Rbc-IC_{50}), where [*in vitro* Protein concentration (μmol/g)] was calculated from the section of protein concentration (μg/g) divided by each molecular weight (PE, 36,028; PC, 35,519; APC, 34,706 and Rbc, 70,035 μg/μmol), and (Sum of Protein-IC_{50}) was calculated from the sum of each protein $1/IC_{50}$ value (L/μmol) produced by thermolysin digestion from Table 2.

Results and Discussion

General Features of *G. asiatica* Chloroplast Genome

For a comprehensive understanding of ACE inhibitory peptides from WSP, we determined the complete sequence of *G. asiatica* chloroplast genome using a next-generation sequencing method. The contigs coding chloroplast genome were collected using BLASTn and aligned to construct a draft sequence of circular chloroplast genome. The genes in the chloroplast genome were annotated manually, and the gap or deletion in protein coding genes (PCG) was confirmed by Sanger sequencing using specific primers (Table S1). As a result, a total of 187,518 bp of the chloroplast genome was sequenced (Fig. 1). The genome contained 206 PCG (Table 1), 29 tRNA, 3 rRNA with the GC content of 30.8%. The chloroplast genome sequence was deposited in DNA Data Bank of Japan (DDBJ) as AP018129. The genes of main WSP (*cpeA* (PEα), *cpeB* (PEβ), *cpcA* (PCα), *cpcB* (PCβ), *apcA* (APCα), *apcB* (APCβ) in photosystems and *rbcL* (RbcL), *rbcS* (RbcS) in metabolism) were found in chloroplast genome (Table 1).

ACE Inhibitory Activities of WSP and WSP Hydrolysate

It has been studied that many seaweed-protein hydrolysates possess ACE inhibitory activity (Suetsuna and Nakano 2000; He et al. 2007; Cian et al. 2013; Fitzgerald et al. 2014; Beaulieu et al. 2016; Furuta et al. 2016; Cao et al. 2017; Kitade et al. 2018; Miyabe et al. 2017). To evaluate the ACE inhibitory activity from *G. asiatica* protein hydrolysate, we prepared WSP and WSP hydrolysate. As a result, 0.47 g of WSP were extracted from 15.0 g of *G. asiatica* powder with the yield of 3.1%, and WSP hydrolysate were

prepared by thermolysin digestion. The components of WSP and the hydrolysate were examined by SDS-PAGE (Fig. 2a). Two major bands (approximately 20 kDa: phycobiliproteins; approximately 55 kDa: RbcL) were detected in WSP (lane 1), and these protein bands were completely degraded by thermolysin (lane 2). Phycobiliproteins are fluorescent proteins that were detected under blue light (LED illuminator VISIRAYS AE-6935GN: ATTO, Tokyo, Japan), but not RbcL (lane 3). The fluorescence could not detect in WSP hydrolysate (lane 4).

ACE inhibitory activities of WSP and the hydrolysate were measured. The ACE inhibitory activity of WSP hydrolysate (77%) was higher than that of WSP 5.2% at a concentration of 5.0 mg/mL (Fig. 2b). Therefore, IC_{50} value of WSP hydrolysate was determined as 1.02 mg/mL. This result was similar to our previous studies (Furuta et al. 2016; Kitade et al. 2018), indicating WSP hydrolysate from *G. asiatica* possessed ACE inhibitory peptides.

ACE Inhibitory Peptides Prediction

To determine the ACE inhibitory peptides, the WSP hydrolysate was separated by reversed-phase high-performance liquid chromatography (HPLC) (Fig. 2c). The eluted peaks, named 1–18, were pooled and subjected to ACE inhibitory assay (Fig. 2d). Relatively high inhibitory activities were detected in fraction number 6, 7, 10 and 15. The peptides in fractions were analyzed by MALDI-TOF/MS, and then the masses were compared with the masses obtained by the thermolysin digestion of phycobiliproteins and Rubisco using PeptideCutter. Among them, peptide VYRT was found in fraction 7 and peptides LRY and LDY were found in fraction 15. These peptides were also determined as ACE inhibitory peptides by the water-soluble protein hydrolysate from dulse (Furuta et al. 2016), indicating that most red algae may possess ACE inhibitory peptides in WSP, and the peptides from WSP would be come from phycobiliproteins and Rubisco. The prediction of the ACE inhibitory peptides by MALDI-TOF/MS, which were deposited in database, was possible; however, several peptides were detected in each fraction. The comprehensive understanding of the relationship between activity and the peptides in the sample was difficult. We therefore attempted to *in silico* analysis using chloroplast genome.

***In Silico* Analysis of ACE Inhibitory Peptides from the Main WSP**

ACE inhibition is one of the key factors to repress a high blood pressure. Therefore, many ACE inhibitory peptides were reported from various materials such as seaweeds, fishes, plants, animals and human (Lee

and Hur 2017) and deposited in database. Utilization of the database information accelerates a development of the potential bioactive peptides. So, *in silico* analysis was performed in the main WSP (Table 2), resulting in a detection of 190 potential ACE inhibitory peptides from *G. asiatica*. Although the number of amino acid sequences of phycobiliproteins were similar in PE, PC and APC, the number of potential peptides varied, i.e., 25 peptides in PEα (maximum) and 13 peptides in PCβ (minimum). RbcL sequence contained 59 potential peptides, which would be due to the molecule size (488 amino acids). The main peptides were dipeptide. The potential ACE inhibitory peptides were distributed in the whole protein sequences of main WSP, and the overlapped peptide sequences were found, e.g., AKYSY (amino acid positions at 61-65) and KY (amino acid positions at 62-63) in PEα, which were found in *Pyropia yezoensis* (Suetsuna 1998; Suetsuna and Nakano 2000).

Then, we performed *in silico* thermolysin digestion in the main WSP. Five to one-*in silico* thermolysin-digested peptides were found in RbcL and phycobiliproteins, while no peptides were detected in RbcS. Although amino acid sequences coding the same ACE inhibitory peptides were conserved in WSP, not all the peptides were obtained by thermolysin digestion, e.g., LDY peptide was obtained in PEα, PCα and RbcL, but not in APCα (Table 2). The indigestibility would be come from the amino acid sequence in the vicinity of target peptides. We then determined iSD_{50} value as follows. First, the protein concentrations of main WSP components (PE, PC, APC and Rbc) were determined according to the section of determination of protein concentration in materials and methods, resulting that protein concentration of PE, PC, APC and Rbc were 0.770, 0.098, 0.070 and 0.114 (μmol/g), respectively. Using the data from Table 2, iSD_{50} value was determined as 0.971 (mg/mL). The value was considerably close to *in vitro* IC_{50} value (1.02 mg/mL). This accuracy would come from using *in vitro* protein concentration and the abundance of IC_{50} value of thermolysin. This data supported that proteins in main WSP were phycobiliproteins and Rbc. Among them, PE-iSD_{50} value was 1.183 mg/mL, indicating that PE is the key ACE inhibitory peptide source.

Correlation between *In Vitro* and *In Silico* Thermolysin digestion

Then, we compared the potential ACE inhibitory peptides with other red algae (Table 3). Amino-acid sequence-identities of the main WSP from *G. asiatica* were high (72-100%), and the number of potential ACE inhibitory peptides in phycobiliproteins (104-120) and in phycobiliproteins+Rbc (185-193) were mostly the same in these red algae. Although we selected ACE inhibitory activity (IC_{50}) less than 100 μM,

250 the value obtained by the thermolysin digestion was 69-fold difference in maximum (IC_{50} of VW = 1.4)
251 and minimum (IC_{50} of AR = 96) with an average IC_{50} of 25.4. The peptides having IC_{50} less than 10 μM
252 considerably affected *in silico* value. In addition, the amount of protein components highly reflected on *in*
253 *silico* value. PE was the main component of WSP in *G. asiatica*. Therefore, peptides AKYSY and LDY in
254 PEα and LRY in PEβ were the key peptides in ACE inhibitory in *G. asiatica*. We then compared the
255 number of potential ACE inhibitory peptides from *in silico* thermolysin digestion among red algae. The
256 number of the peptides in Florideophyceae was almost the same (17-19). Although Bangiophyceae
257 possessed slightly more peptides (23), ACE inhibitory peptides possessing strong IC_{50} value were
258 conserved. Although data of Table 3 means that the results of *in silico* digestion among red algae were
259 quite similar, protein concentrations in the samples differed by species and growth conditions. Therefore,
260 the prediction of ACE inhibitory activity in the samples would increase the precision using protein
261 components in WSP.

262 We attempted to understanding the correlation *in silico* and *in vitro* results using protein
263 components in the sample. Several issues still remained. In this study, the unidentified peptides did not
264 take into consideration. When *in silico* IC_{50} value was higher than that of *in vitro* values, the novel
265 peptides exist in the sample. The *in silico* data was obtained by complete digestion using PeptideCutter.
266 When the *in silico* IC_{50} value was lower than *in vitro* values, two reason was assumed in the
267 understanding of *in vitro* assay: loss of bioactive peptides by incomplete digestion and overactivity. These
268 were occurred by the lack of understanding of protease specificities. The accumulation of these data will
269 increase reliability and accuracy in *in silico* analysis. On the other hand, we clarified that the iSD_{50} value
270 of WSP-peptides was affected by several bioactive peptides. The prediction method will also apply to find
271 the relationship between *in silico* analysis and the amounts of bioactive peptides in HPLC fractions.
272
273 **Conclusions**
274 In this study, we first determined the complete chloroplast genome sequence of *G. asiatica* and clarified
275 the amino acid sequences of main WSP to perform *in silico* analysis. Then, we extracted WSP from red
276 alga *G. asiatica*, which contained mainly PE, PC APC and Rbc, and prepared WSP hydrolysate by
277 thermolysin, resulting that the hydrolysate showed ACE inhibitory activity. Consequently, 190 potential
278 ACE inhibitory peptides existed in the main WSP sequences from *in silico* analysis, and 10 peptides were
279 obtained by *in silico* thermolysin digestion. The IC_{50} values of *in vitro* and *in silico* were considerably

close. This accurately is due to the using of *in vitro*-protein concentration and the availability of abundant thermolysin digested ACE inhibitory peptides in database. On the other hand, when *in vitro* IC_{50} values differ from *in silico* data, the difference would lead to the finding of novel inhibitory peptides (in case of low *in vitro* IC_{50}) or the understanding of protease specificities (the loss of bioactive peptides by incomplete digestion and overactivity). Using four chloroplast-genes for the *in silico* analysis, we think this approach provides insight into the comprehensive understanding of the potential bioactive peptides from seaweed proteins.

Compliance with Ethical Standards

Funding

This study was partially supported by the "Regional Innovation Cluster Program (Global Type), Ministry of Education, Culture, Sports, Science and Technology, Japan" and the "Science and technology research promotion program for agriculture, forestry, fisheries and food industry (27004B)".

Conflict of interest

The authors declared that they have no conflict of interest.

References

Beaulieu L, Sirois M, Tamigneaux É (2016) Evaluation of the *in vitro* biological activity of protein hydrolysates of the edible red alga, *Palmaria palmata* (dulse) harvested from the Gaspe coast and cultivated in tanks. J Appl Phycol 28:3101-3115

Bennett A, Bogorad L (1973) Complementary chromatic adaptation in a filamentous blue-green alga. J Cell Biol 58:419-435

Cao D, Lv X, Xu X, Yu H, Sun X, Xu N (2017) Purification and identification of a novel ACE inhibitory peptide from marine alga *Gracilariopsis lemaneiformis* protein hydrolysate. Eur Food Res Technol 243:1829-1837

Chen H, Yan X, Zhu P, Lin J (2006) Antioxidant activity and hepatoprotective potential of agaro-oligosaccharides *in vitro* and *in vivo*. Nutr J 5:31

Cheung HS, Cushman DW (1973) Inhibition of homogeneous angiotensin-converting enzyme of rabbit lung by synthetic venom peptides of *Bothrops jararaca*. Biochim Biophys Acta - Enzymol 293:451-463

Cian RE, Alaiz M, Vioque J, Drago SR (2013) Enzyme proteolysis enhanced extraction of ACE inhibitory and antioxidant compounds (peptides and polyphenols) from *Porphyra columbina* residual cake. J Appl Phycol 25:1197-1206

Cian RE, Garzón AG, Ancona DB, Guerrero LC, Drago SR (2015) Hydrolyzates from *Pyropia columbina* seaweed have antiplatelet aggregation, antioxidant and ACE I inhibitory peptides which maintain bioactivity after simulated gastrointestinal digestion. LWT - Food Sci Technol 64:881-888

Costa JF, Lin SM, Macaya EC, Fernandez-Garcia C, Verbruggen H (2016) Chloroplast genomes as a tool to resolve red algal phylogenies: A case study in the Nemaliales. BMC Evol Biol 16:1-13

Cota-Sánchez JH, Remarchuk K, Ubayasena K (2006) Ready-to-use DNA extracted with a CTAB method adapted for herbarium specimens and mucilaginous plant tissue. Plant Mol Biol Report 24:161-167

Daskaya-Dikmen C, Yucetepe A, Karbancioglu-Guler F, Daskaya H, Ozcelik B (2017) Angiotensin-I-converting enzyme (ACE)-inhibitory peptides from plants. Nutrients 9:316

DePriest MS, Bhattacharya D, López-Bautista JM (2013) The Plastid Genome of the Red Macroalga *Grateloupia taiwanensis* (Halymeniaceae). Plos One 8:e68246

Du Q, Bi G, Mao Y, Sui Z (2016) The complete chloroplast genome of *Gracilariopsis lemaneiformis* (Rhodophyta) gives new insight into the evolution of family Gracilariaceae. J Phycol 52:441-450

Fitzgerald C, Gallagher E, Tasdemir D, Hayes M (2011) Heart health peptides from macroalgae and their potential use in functional foods. J Agric Food Chem 59:6829-6836

Fitzgerald C, Aluko RE, Hossain M, Rai DK, Hayes M (2014) Potential of a renin inhibitory peptide from the red seaweed *Palmaria palmata* as a functional food ingredient following confirmation and characterization of a hypotensive effect in spontaneously hypertensive rats. J Agric Food Chem 62:8352-8356

Furuta T, Miyabe Y, Yasui H, Kinoshita Y, Kishimura H (2016) Angiotensin I converting enzyme inhibitory peptides derived from phycobiliproteins of dulse *Palmaria palmata*. Mar Drugs 14:32

Glazer AN (1985) Light harvesting by phycobilisomes. Ann Rev Biophys Chem 14:47–77

Harnedy PA, O'Keeffe MB, FitzGerald RJ (2015) Purification and identification of dipeptidyl peptidase (DPP) IV inhibitory peptides from the macroalga *Palmaria palmata*. Food Chem 172:400-406

He HL, Chen XL, Wu H, Sun CY, Zhang YZ, Zhou BC (2007) High throughput and rapid screening of marine protein hydrolysates enriched in peptides with angiotensin-I-converting enzyme inhibitory activity by capillary electrophoresis. Bioresour Technol 98:3499-3505

Holdt SL, Kraan S (2011) Bioactive compounds in seaweed: functional food applications and legislation. J Appl Phycol 23:543-597

Kitade Y, Miyabe Y, Yamamoto Y, Takeda H, Shimizu T, Yasui H, Kishimura H (2018) Structural characteristics of phycobiliproteins from red alga *Mazzaella japonica*. J Food Biochem 42:e12436

Kumagai Y, Miyabe Y, Takeda T, Adachi K, Yasui H, Kishimura H (2019a) *In silico* analysis of relationship between proteins from plastid genome of red alga *Palmaria* sp. (Japan) and angiotensin I converting enzyme inhibitory peptides. Mar Drugs 17:190

Kumagai Y, Tsubouchi R, Miyabe Y, Takeda T, Adachi K, Yasui H, Kishimura H (2019b) Complete sequence of mitochondrial DNA of red alga dulse *Palmaria palmata* (Linnaeus) Weber & Mohr in Japan. Mitochondrial DNA Part B 4:3177-3178

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685

Lee KY, Mooney DJ (2012) Alginate: Properties and biomedical applications. Prog Polym Sci 37:106-126

Lee SY, Hur SJ (2017) Antihypertensive peptides from animal products, marine organisms, and plants. Food Chem 228:506-517

Mai K, Mercer JP, Donlon J (1994) Comparative studies on the nutrition of two species of abalone, *Haliotis tuberculata* L. and *Haliotis discus hannai* Ino: II. Amino acid composition of abalone and six species of macroalgae with an assessment of their nutritional value. Aquaculture 128:115-130

Miyabe Y, Furuta T, Takeda T, Kanno G, Shimizu T, Tanaka Y, Gai Z, Yasui H, Kishimura H (2017) Structural properties of phycoerythrin from dulse *Palmaria palmata*. J Food Biochem 41:e12301

Sato N, Furuta T, Takeda T, Miyabe Y, Ura K, Takagi Y, Yasui H, Kumagai Y, Kishimura H (2018) Antioxidant activity of proteins extracted from red alga dulse harvested in Japan. J Food Biochem 43:e12709

Ng PK, Lin SM, Lim PE, Liu LC, Chen CM, Pai TW (2017) Complete chloroplast genome of *Gracilaria firma* (Gracilariaceae, Rhodophyta), with discussion on the use of chloroplast phylogenomics in the subclass Rhodymeniophycidae. BCM Genomics 18:40

Schmieder RE, Hilgers KF, Schlaich MP, Schmidt BMW (2007) Renin-angiotensin system and cardiovascular risk. Lancet 369:1208-1219

Ruiter GA De, Rudolph B (1997) Carrageenan biotechnology. Trends Food Sci Technol 8:389–395

Sfriso AA, Gallo M, Baldi F (2018) Phycoerythrin productivity and diversity from five red macroalgae. J

Appl Phycol 30:2523-2531

Sonani RR, Singh NK, Kumar J, Thakar D, Madamwar D (2014) Concurrent purification and antioxidant activity of phycobiliproteins from *Lyngbya* sp. A09DM: An antioxidant and anti-aging potential of phycoerythrin in *Caenorhabditis elegans*. Process Biochem 49:1757-1766

Suetsuna K (1998) Purification and identification of angiotensin I-converting enzyme inhibitors from the red alga *Porphyra yezoensis*. J Mar Biotechnol 6:163-167

Suetsuna K, Nakano T (2000) Identification of an antihypertensive peptide from peptic digest of wakame (*Undaria pinnatifida*). J Nutr Biochem 11:450-454

Watanabe K, Kishimoto T, Kumagai Y, Shimizu T, Uji T, Yasui H, Kishimura H (2019) Complete sequence of mitochondrial DNA of *Gloiopeltis furcata* (Postels and Ruprecht) J. Agardh. Mitochondrial DNA Part B 4:2543-2544

Yamamoto Y, Kishimura H, Kinoshita Y, Saburi W, Kumagai Y, Yasui H, Ojima T (2019) Enzymatic production of xylooligosaccharides from red alga dulse (*Palmaria* sp.) wasted in Japan. Process Biochem 82:117-122

Yang EC, Kim KM, Kim SY, Lee JM, Boo GH, Lee JH, Nelson WA, Yi G, Schmidt WE, Fredericq S, Boo SM, Bhattacharya D, Yoon HS (2015) Highly conserved mitochondrial genomes among multicellular red algae of the Florideophyceae. Genome Biol Evol 7:2394-2406

Yang EC, Boo SM, Bhattacharya D, Saunders GW, Knoll AH, Fredericq S, Yoon HS (2016) Divergence time estimates and the evolution of major lineages in the florideophyte red algae. Sci Rep 6:21361

Zhang J, Tang X, Chen WZ, Liu T, Li Y (2018) The complete plastid genome of *Grateloupia filicina* (Rhodophyta) and phylogenetic analysis. Mitochondrial DNA B 3:1172-1173

Figure Captions

Fig. 1 The chloroplast genome map of *G. asiatica*.

Fig. 2 Components and ACE inhibitory activities of WSP and its hydrolysate. (a) SDS-PAGE. SDS-PAGE was performed in 13.75% acrylamide gel. CBB detection in lane 1 (WSP) and lane 2 (WSP hydrolysate). Fluorescent detection in lane 3 (WSP) and lane 4 (WSP hydrolysate). (b) ACE inhibitory activity. Symbols; closed circle, WSP hydrolysate; open circle, WSP. Data are the mean±SEM from triplicate determination and error bars are within the symbols. (c) Separation of WSP hydrolysate. Peptides were

400 separated by Mightysil RP-18GP column. The numbers on the peaks (1-18) were pooled. (d) ACE
401 inhibitory activity of each fraction.
402

Table 1 PCG in chloroplast genome of *G. asiatica*

Function	No.	PCG									
Genetic systems											
maintenance	2	*rne*	*dnaB*								
RNA polymerase	5	*rpoA*	*rpoB*	*rpoC1*	*rpoC2*	*rpoZ*					
Transcription factor	4	*ntcA*	*ompR*	*rbcR*	*ycf29*						
Transcription	4	*infB*	*infC*	*tsf*	*tufA*						
Ribosomal protein											
Large subunit	28	*rpl1*	*rpl2*	*rpl3*	*rpl4*	*rpl5*	*rpl6*	*rpl9*	*rpl11*	*rpl12*	*rpl13*
		rpl14	*rpl16*	*rpl18*	*rpl19*	*rpl20*	*rpl21*	*rpl22*	*rpl23*	*rpl24*	*rpl27*
		rpl28	*rpl29*	*rpl31*	*rpl32*	*rpl33*	*rpl34*	*rpl35*	*rpl36*		
Small subunit	19	*rps1*	*rps2*	*rps3*	*rps4*	*rps5*	*rps6*	*rps7*	*rps8*	*rps9*	*rps10*
		rps11	*rps12*	*rps13*	*rps14*	*rps16*	*rps17*	*rps18*	*rps19*	*rps20*	
tRNA processing	1	*tilS*									
Protein quality control	4	*clpC*	*ftsH*	*dnaK*	*groEL*						
Photosystems											
Phycobilisomes	12	*apcA*	*apcB*	*apcD*	*apcE*	*apcF*	*cpcA*	*cpcB*	*cpcG*	*cpcS*	*cpeA*
		cpeB	*nblA*								
Photosystem I	13	*psaA*	*psaB*	*psaC*	*psaD*	*psaE*	*psaF*	*psaI*	*psaJ*	*psaK*	*psaL*
		psaM	*ycf3*	*ycf4*							
Photosystem II	19	*psbA*	*psbB*	*psbC*	*psbD*	*psbE*	*psbF*	*psbH*	*psbI*	*psbJ*	*psbK*
		psbL	*psbN*	*psbT*	*psbV*	*psb28*	*psbX*	*psbY*	*psbZ*	*ycf12*	
Cytochrome complex	11	*ccsA*	*ccs1*	*petA*	*petB*	*petD*	*petF*	*petG*	*petJ*	*petL*	*petM*
		petN									
Redox system	7	*grx*	*dsbD*	*ftrB*	*acsF*	*bas1*	*pbsA*	*trxA*			
ATP synthesis											
ATP synthase	8	*atpA*	*atpB*	*atpD*	*atpE*	*atpF*	*atpG*	*atpH*	*atpI*		
Metabolism											
Carbohydrates	6	*rbcL*	*rbcS*	*cfxQ*	*pgmA*	*odpA*	*odpB*				
Lipids	5	*accA*	*accD*	*fabH*	*accB*	*acpP*					
Nucleotides	2	*carA*	*upp*								
Amino acids	8	*argB*	*gltB*	*ilvB*	*ilvH*	*syfB*	*syh*	*trpA*	*trpG*		
Cofactors	4	*chlI*	*moeB*	*preA*	*thiG*						
Transport											
Transport	9	*secA*	*secG*	*secY*	*cemA*	*ycf16*	*ycf24*	*ycf38*	*ycf43*	*ycf63*	
Unknown											
Conserved ORFs	35	*ORF58*	*ORF65*	*ORF83*	*ORF621*	*ycf17*	*ycf19*	*ycf20*	*ycf21*	*ycf22*	*ycf26*
		ycf33	*ycf34*	*ycf35*	*ycf36*	*ycf37*	*ycf39*	*ycf40*	*ycf45*	*ycf46*	*ycf52*
		ycf53	*ycf54*	*ycf55*	*ycf56*	*ycf60*	*ycf65*	*ycf80*	*ycf92*	*orf10*	*orf12*
		orf13	*orf14*	*orf18*	*orf24*	*orf25*					
Total	206										

Table 2 ACE inhibitory peptide sequences in the main WSP from *G. asiatica*

Main WSP	PEα			PEβ			PCα			PCβ			APCα			APCβ			RbcL						RbcS			
	Peptide	IC_{50}	Position*	Peptide	IC_{50}	Position	Peptide	IC_{50}	Position	Peptide	IC_{50}	Position	Peptide	IC_{50}	Position	Peptide	IC_{50}	Position	Peptide	IC_{50}	Position	Peptide	IC_{50}	Position	Peptide	IC_{50}	Position	Peptide	IC_{50}	Position
	RF	93	[17-18]	KA	31.5	[15-16]	TE	18	[6-7]	AR	96	[14-15]	AR	96	[15-16]	AI	3.4	[4-5]	RY	11	[15-16]	KP	22	[179-180]	DY	100	[439-440]	TF	18	[7-8]
	AR	96	[36-37]	**AY**	19	[17-18]	AI	3.4	[8-9]	AI	3.4	[50-51]	ARY	1.3	[15-17]	GKY	3.92	[16-18]	YW	11	[28-29]	KL	50	[181-182]	YVA	1.4	[440-442]	NY	33	[24-25]
	KL	50	[43-44]	TF	18	[29-30]	RF	93	[17-18]	**AR**	96	[56-57]	RY	11	[16-17]	KY	13	[17-18]	DY	100	[32-33]	NY	46	[188-189]	IL	55	[447-448]	AI	3.4	[26-27]
	AVV	67	[50-52]	DG	12.3	[33-34]	RY	11	[30-31]	LVQ	14	[66-68]	LSP	1.7	[18-20]	KL	50	[26-27]	LFR	32	[43-45]	VY	7.1	[193-194]	GPL	2.7	[456-458]	AI	3.4	[32-33]
	VK	13	[52-53]	PGL	13.9	[64-66]	VY	7.1	[59-60]	AY	19	[73-74]	IAQ	35	[39-41]	FQ	51	[31-32]	PG	33	[50-51]	RW	16	[217-218]	LW	50	[464-465]	PR	4.1	[43-44]
	CF	2	[59-60]	IAP	2.7	[67-69]	KF	28.3	[62-63]	**LRY**	5.1	[90-92]	IL	55	[42-43]	**VR**	53	[38-39]	ST	4.0	[66-67]	RF	93	[221-222]	ITF	49	[468-470]	NY	33	[46-47]
	AKYSY	1.5	[61-65]	IAPG	11.4	[67-70]	AI	3.4	[78-79]	RY	11	[91-92]	VK	13	[51-52]	KA	32	[58-59]	VW	1.4	[73-74]	LY	18	[223-224]	TF	18	[469-470]	YW	11	[47-48]
	KY	13	[62-63]	DG	12.3	[85-86]	IG	32	[79-80]	SY	66	[94-95]	FQ	51	[59-60]	LY	18	[61-62]	LY	18	[83-84]	HY	26	[242-243]	NY	46	[471-472]	LW	50	[50-51]
	SY	66	[64-65]	IL	55	[89-90]	KA	28	[81-82]	AI	3.4	[130-131]	VSP	10	[66-68]	RY	11	[77-78]	KA	32	[87-88]	MY	0.6	[254-255]	ST	4.0	[474-475]	GLP	1.6	[52-54]
	YRD	5.8	[83-85]	**LRY**	5.1	[90-92]	AR	96	[85-86]	PLG	4.7	[149-151]	AY	19	[72-73]	LDY	6.1	[85-87]	AY	19	[88-89]	AI	3.4	[279-280]				AY	19	[104-105]
	HY	26	[88-89]	RY	10.5	[91-92]	IG	32	[88-89]	AEL	57	[158-160]	LDY	6.1	[85-87]	DY	100	[86-87]	**AY**	19	[103-104]	**VW**	1.4	[285-286]				LVR	14	[111-113]
	NY	33	[94-95]	SY	66.3	[94-95]	YLR	5.8	[91-93]	**AY**	19	[162-163]	DY	100	[86-87]	YLR	5.8	[88-90]	IAY	13	[105-107]	AR	96	[287-288]				VR	53	[112-113]
	GTG	5.5	[101-103]	DG	12.3	[101-102]	LRM	0.15	[92-94]	AVV	67	[170-172]	YLR	5.8	[88-90]	**LRY**	5.1	[89-91]	AY	19	[106-107]	IL	55	[293-294]				KY	13	[122-123]
	TGP	71	[102-104]	VR	52.8	[128-129]	TGP	79	[104-106]				VK	13	[112-113]	RY	11	[90-91]	VF	9.2	[128-129]	ST	4.0	[302-303]				SY	66	[127-128]
	GPL	2.7	[103-105]	KA	31.5	[135-136]	GPM	17	[105-107]				YN	51	[116-117]	IL	55	[103-104]	KA	32	[132-133]	KW	1.6	[319-320]				KP	22	[132-133]
	AR	96	[113-114]	VAF	35.8	[139-141]	EY	2.7	[109-110]				**FY**	25	[151-152]	YN	51	[116-117]	**VK**	13	[134-135]	WM	96	[320-321]				RY	11	[137-138]
	VY	7.1	[116-117]	AI	3.41	[175-176]	LEE	100	[115-117]				DY	100	[154-155]	AI	3.4	[129-130]	KA	32	[135-136]	KL	50	[337-338]						
	VYRT	0.14	[116-119]				LSP	1.7	[124-126]							VGP	26	[141-143]	VAY	16	[146-148]	FY	25	[348-349]						
	SY	66	[126-127]				GSH	32	[138-140]							GLY	8.9	[150-152]	**AY**	19	[147-148]	FYN	18	[348-350]						
	YVA	1.4	[127-129]				**LDY**	6.1	[154-156]							**LY**	18	[151-152]	TF	18	[151-152]	YN	51	[349-350]						
	VAA	13	[128-130]				DY	100	[155-156]							DY	100	[154-155]	FQ	51	[152-153]	**LDY**	6.1	[394-396]						
	PR	4.1	[141-142]				AI	3.4	[157-158]										KF	28	[168-169]	DY	100	[395-396]						
	EY	2.7	[151-152]																GRP	20	[170-172]	FGG	83	[405-407]						
	LDY	6.1	[156-158]																VK	13	[178-179]	DG	12	[414-415]						
	DY	100	[157-158]																VKP	1.3	[178-180]	AR	96	[433-434]						
Total peptides	25			17			22			13			17			21								59			16		
Di-peptides	15			12			14			8			11			15								31			14		
Tri-peptides	8			4			8			5			6			6								11			2		
Tetra- and penta-peptide	2			1			0			0			0			0								0			0		
Overlap*2	9			3			4			1			4			5								15			2		
Thermolysin-peptides	3			2			1			3			1			3								5			0		

Bold with underline letters mean peptide sequences produced by *in silico* thermolysin digestion.

* The number from N-turminal amino acid residue of each protein.

*2 Overlap means ACE inhibitory peptides were coded in the same amino acid.

Table 3 Comparison of potential ACE inhibitory peptides in the main WSP from red algae

Class	Species		PE-α	PE-β	PC-α	PC-β	APC-α	APC-β	Rbc-L	Rbc-S	Sum*	Sum*2	Reference Accession No.
Florideophyceae Halymeniales	*Grateloupia asiatica*	No. of amino acid	164	177	162	172	161	161	488	138			
		Peptides	25	17	22	13	17	21	59	16	115	190	AP018129
		iSD*3	3	2	1	3	1	3	5	0	13	18	
	Grateloupia taiwanensis	Identity*4	99	100	100	99	99	99	99	99			DePriest et al. 2013 NC_021618
		Peptides	25	17	22	12	17	21	61	17	114	192	
		iSD	3	2	1	3	1	3	5	0	13	18	
	Grateloupia filicina	Identity	100	100	100	100	100	100	99	100			Zhang et al. 2018 NC_037841
		Peptides	25	17	22	13	17	21	59	16	115	190	
		iSD	3	2	1	3	1	3	5	0	13	18	
Florideophyceae Gracilariales	*Gracilariopsis lemaneiformis*	Identity	91	93	92	93	98	96	95	88			Du et al. 2016 KP330491
		Peptides	25	13	22	14	17	21	63	15	112	190	
		iSD	3	3	1	3	1	3	4	1	14	19	
	Gracilariopsis firma	Identity	88	87	93	90	98	94	95	83			Ng et al. 2017 NC_033877
		Peptides	24	15	20	14	17	19	61	15	109	185	
		iSD	3	3	0	3	1	3	4	0	13	17	
Florideophyceae Gigartinales	*Mazzaella japonica*	Identity	91	91	96	95	98	96					Kitade et al. 2018 AB698819.2 LC222737 LC222738
		Peptides	22	13	22	12	16	19	N.D.	N.D.	104	-	
		iSD	2	2	1	4	1	2			12	-	
Florideophyceae Palmariales	*Palmaria palmata* in Japan	Identity	91	86	93	92	96	96	91	78			Kumagai et al. 2019 AB807662
		Peptides	28	17	21	15	17	20	54	14	118	186	
		iSD	3	3	0	2	1	2	5	1	11	17	
Bangiophyceae Pyropia	*Pyropia yezoensis*	Identity	90	90	93	91	96	94	89	72			NC_007932
		Peptides	25	19	27	11	17	21	57	16	120	193	
		iSD	4	3	2	2	1	3	5	3	15	23	

N.D. means not determined.

* Sum without Rbc-L and Rbc-S.

*2 Sum with Rbc-L and Rbc-S.

*3 The number of peptides obtained from *in silico* digestion by thermolysin.

*4 Amino acid identity to that of *G. asiatica* (%).

Table S1 Primers used in this study

Primer	Sequence
Gap1 Fw	5'-CCTGAAAAGAATGGAGTATGCC-3’
Gap1 Re	5'-AGATGAAGGTATGGCTGGTGA-3’
Gap2 Fw	5'-GCAGGAGCATTAGCATCA-3’
Gap2 Re	5'-AGAGCCTATACCTTCATCAG-3’
Gap3 Fw	5'-CTCAAAAAGCGATAGTAGCCG -3’
Gap3 Re	5'-CCCAAATAACCTCACCATCAG-3’
Gap4 Fw	5'-CACCGAATGGCTTTTTGGA-3’
Gap4 Re	5'-ACCACATCCCATATAGTTGC-3’
Gap5 Fw	5'-CCCGATTCATTGCCCACTA-3’
Gap5 Re	5'-CTTTGACCGTGGTAGGAAACA-3’
Gap6 Fw	5'-CTAGATTACGCCATACGGTT-3’
Gap6 Re	5'-TGACAGCACCTCTATCATCA-3’
Gap7 Fw	5'-CCGCGCCCGCTAAATAGAAA-3’
Gap7 Re	5'-GGAAGTGTATCACCAACGGG-3’
Gap8 Fw	5'-GTAAGGATAAGGAGGCATAGCA-3’
Gap8 Re	5'-TTGGGGTATTGGTCACAGCA-3’
Gap9 Fw	5'-TCGACAACAAATCCATCAGGC-3’
Gap9 Re	5'-TTGAAGCTAGGGCAGACAAA-3’
Gap10 Fw	5'-AAATCGTTGTAGCCCACCAA-3’
Gap10 Re	5'-AGACCTCTAAGCAAAGGAGA-3’

Figure 1

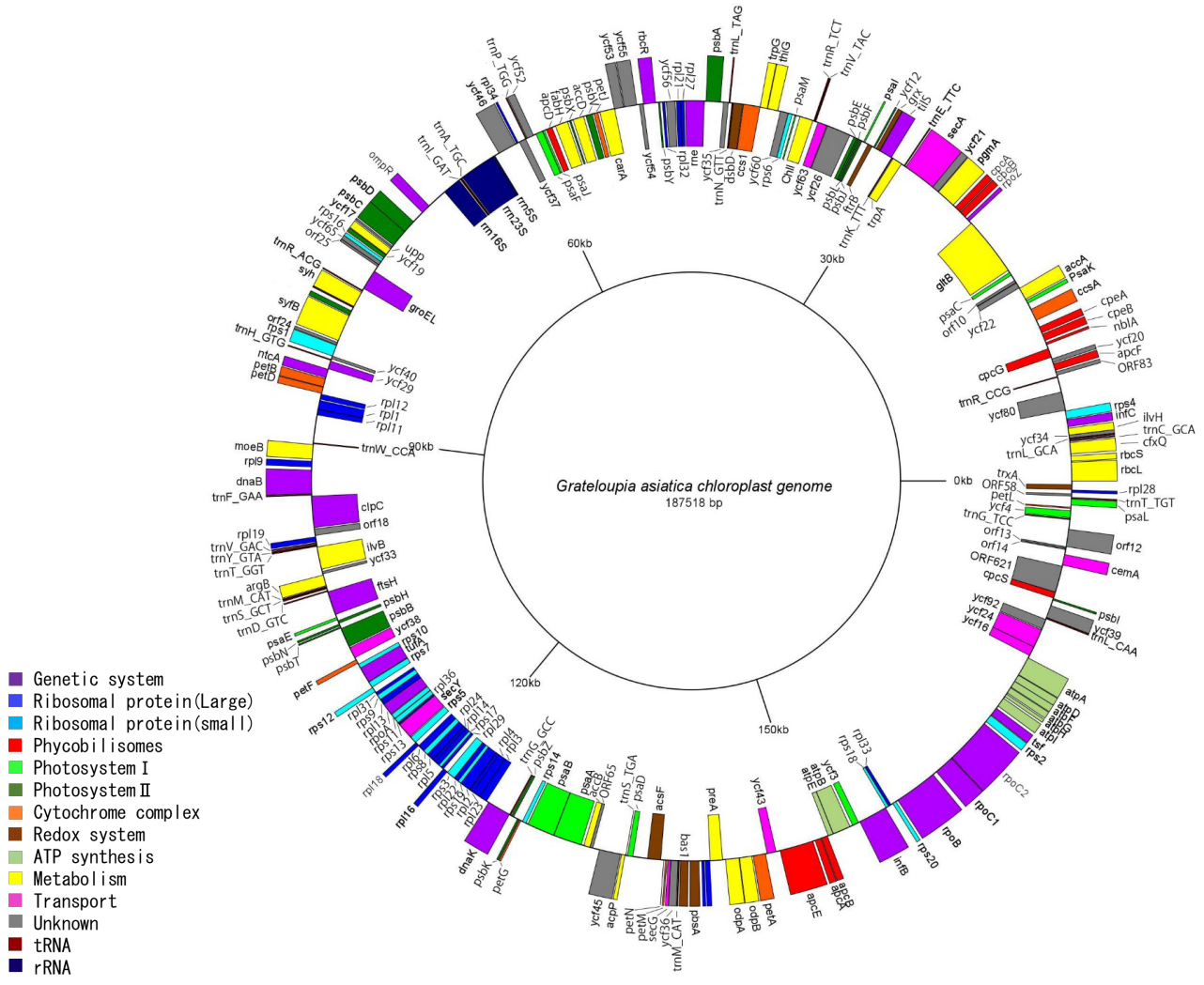
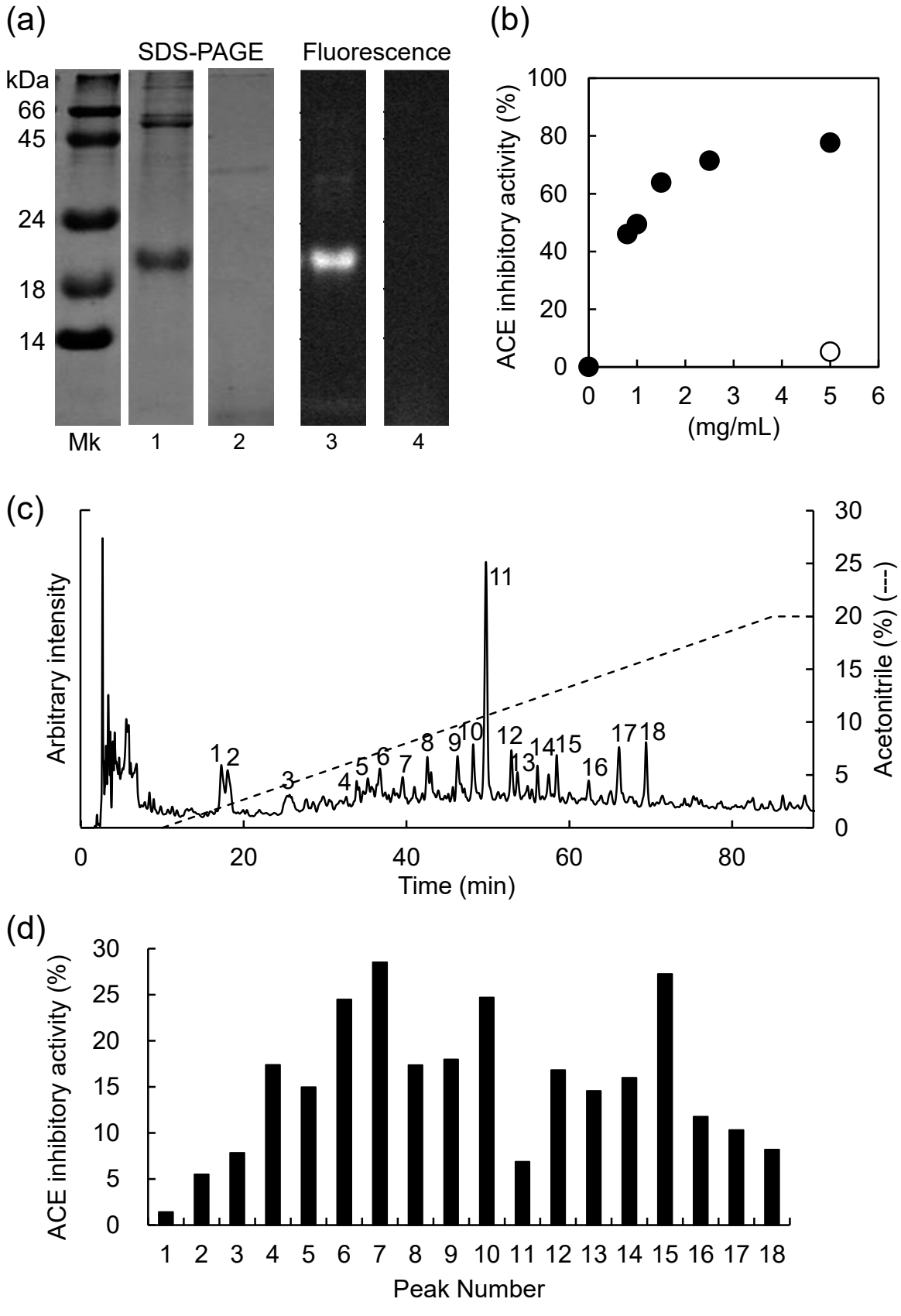

Grateloupia asiatica chloroplast genome
187518 bp
0kb
30kb
60kb
90kb
120kb
150kb
Genetic system
Ribosomal protein(Large)
Ribosomal protein(small)
Phycobilisomes
Photosystem Ⅰ
Photosystem Ⅱ
Cytochrome complex
Redox system
ATP synthesis
Metabolism
Transport
Unknown
tRNA
rRNA

Figure 2


(a)
SDS-PAGE
Fluorescence
kDa
66
45
24
18
14
Mk
1
2
3
4
(b)
ACE inhibitory activity (%)
100
80
60
40
20
0
0
1
2
3
4
5
6
(mg/mL)
(c)
Arbitrary intensity
Acetonitrile (%) (---)
30
25
20
15
10
5
0
1 2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
0
20
40
60
80
Time (min)
(d)
ACE inhibitory activity (%)
30
25
20
15
10
5
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Peak Number