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

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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 IC₅₀ 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

the upper layer was evaporated, and then the hippuric acid was dissolved in 1.0 mL of distilled water, and the absorbance at 228 nm was measured. The inhibition was calculated from the equation $(1-(As-Asb)/(Ac-Acb)) \times 100$, where Ac is the absorbance from the reaction mixture of ACE without sample, Acb is the absorbance from Ac-blank (added denatured ACE), As is the absorbance from the reaction mixture of ACE with the sample, and Asb is the absorbance from As-blank (added denatured ACE with the sample). The data were expressed as mean $\pm$ SEM from triplicate determination.

Separation of WSP Hydrolysate

The WSP hydrolysates were dissolved in ultrapure water containing 0.1% trifluoroacetic acid (TFA) and applied to sequential filtration by Millex-GV (pore size: 0.22 μ m) and Millex-LG (pore size: 0.20 μ m). The hydrolysate in the filtrate were isolated by reversed-phase HPLC with a Mightysil RP-18GP column (4.6 $\times$ 150 mm) (Kanto Kagaku, Tokyo, Japan) using a linear gradient of acetonitrile (1-20%) containing 0.1% TFA at a flow rate of 1.0 mL/min. The peptides from the hydrolysate were detected by measuring at 228 nm.

Prediction of Peptides by MALDI-TOF/MS

The peptide sequences were predicted by Matrix Assisted Laser Desorption/Ionization Time Of Flight Tandem Mass Spectrometry (MALDI-TOF/MS) method using a 4700 Proteomics Analyzer with Denovo Explorer software (Applied Biosystems, Carlsbad, CA, USA). The predicted peptide sequences from WSP were calculated by PeptideCutter (https://web.expasy.org/peptide_cutter/).

Determination of Protein Concentration

The amount of phycobiliproteins was determined by the spectra using following equations: $PC = [OD_{615} - 0.474(OD_{652})]/5.34$; $APC = [OD_{652} - 0.208(OD_{615})]/5.09$; $PE = [(OD_{562}) - 2.41(PC) - 0.849(APC)]/9.62$ (Bennett and Bogorad 1973). The amount of Rubisco was determined by SDS-PAGE using ImageJ software (<https://imagej.nih.gov/ij/>) and BSA as a standard.

Collection of ACE Inhibitory Peptides from Database and *In Silico* Digestion of WSP

ACE inhibitory peptides were obtained from the biopep-uwm database (<http://www.uwm.edu.pl/biochemia/index.php/pl/biopep>) on 7 July 2019. From the database, 924 ACE

inhibitory peptides having IC₅₀ from 0.01 to 17,000 μM were extracted and peptides having IC₅₀ 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 IC₅₀ value (iSD₅₀) was obtained as follows: $1/iSD_{50} \text{ (L/g)} = [PE]/(\text{Sum of PE-IC}_{50}) + [PC]/(\text{Sum of PC-IC}_{50}) + [APC]/(\text{Sum of APC-IC}_{50}) + [Rbc]/(\text{Sum of Rbc-IC}_{50})$, 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-IC₅₀) was calculated from the sum of each protein 1/IC₅₀ 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 PE α (maximum) and 13 peptides in PC β (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 PE α , 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 PE α , PC α and RbcL, but not in APC α (Table 2). The indigestibility would be come from the amino acid sequence in the vicinity of target peptides. We then determined iSD₅₀ 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 (μ mol/g), respectively. Using the data from Table 2, iSD₅₀ value was determined as 0.971 (mg/mL). The value was considerably close to *in vitro* IC₅₀ value (1.02 mg/mL). This accuracy would come from using *in vitro* protein concentration and the abundance of IC₅₀ value of thermolysin. This data supported that proteins in main WSP were phycobiliproteins and Rbc. Among them, PE-iSD₅₀ 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 (IC₅₀) less than 100 μ M,

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

We attempted to understanding the correlation *in silico* and *in vitro* results using protein components in the sample. Several issues still remained. In this study, the unidentified peptides did not take into consideration. When *in silico* IC_{50} value was higher than that of *in vitro* values, the novel peptides exist in the sample. The *in silico* data was obtained by complete digestion using PeptideCutter. When the *in silico* IC_{50} value was lower than *in vitro* values, two reason was assumed in the understanding of *in vitro* assay: loss of bioactive peptides by incomplete digestion and overactivity. These were occurred by the lack of understanding of protease specificities. The accumulation of these data will increase reliability and accuracy in *in silico* analysis. On the other hand, we clarified that the iSD_{50} value of WSP-peptides was affected by several bioactive peptides. The prediction method will also apply to find the relationship between *in silico* analysis and the amounts of bioactive peptides in HPLC fractions.

Conclusions

In this study, we first determined the complete chloroplast genome sequence of *G. asiatica* and clarified the amino acid sequences of main WSP to perform *in silico* analysis. Then, we extracted WSP from red alga *G. asiatica*, which contained mainly PE, PC APC and Rbc, and prepared WSP hydrolysate by thermolysin, resulting that the hydrolysate showed ACE inhibitory activity. Consequently, 190 potential ACE inhibitory peptides existed in the main WSP sequences from *in silico* analysis, and 10 peptides were 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* IC₅₀ values differ from *in silico* data, the difference would lead to the finding of novel inhibitory peptides (in case of low *in vitro* IC₅₀) 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

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Conflict of interest

The authors declared that they have no conflict of interest.

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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	<i>rne</i>	<i>dnaB</i>								
RNA polymerase	5	<i>rpoA</i>	<i>rpoB</i>	<i>rpoC1</i>	<i>rpoC2</i>	<i>rpoZ</i>					
Transcription factor	4	<i>ntcA</i>	<i>ompR</i>	<i>rbcR</i>	<i>ycf29</i>						
Transcription	4	<i>infB</i>	<i>infC</i>	<i>tsf</i>	<i>tufA</i>						
Ribosomal protein											
		<i>rpl1</i>	<i>rpl2</i>	<i>rpl3</i>	<i>rpl4</i>	<i>rpl5</i>	<i>rpl6</i>	<i>rpl9</i>	<i>rpl11</i>	<i>rpl12</i>	<i>rpl13</i>
Large subunit	28	<i>rpl14</i>	<i>rpl16</i>	<i>rpl18</i>	<i>rpl19</i>	<i>rpl20</i>	<i>rpl21</i>	<i>rpl22</i>	<i>rpl23</i>	<i>rpl24</i>	<i>rpl27</i>
		<i>rpl28</i>	<i>rpl29</i>	<i>rpl31</i>	<i>rpl32</i>	<i>rpl33</i>	<i>rpl34</i>	<i>rpl35</i>	<i>rpl36</i>		
Small subunit	19	<i>rps1</i>	<i>rps2</i>	<i>rps3</i>	<i>rps4</i>	<i>rps5</i>	<i>rps6</i>	<i>rps7</i>	<i>rps8</i>	<i>rps9</i>	<i>rps10</i>
		<i>rps11</i>	<i>rps12</i>	<i>rps13</i>	<i>rps14</i>	<i>rps16</i>	<i>rps17</i>	<i>rps18</i>	<i>rps19</i>	<i>rps20</i>	
tRNA processing	1	<i>tisS</i>									
Protein quality control	4	<i>clpC</i>	<i>ftsH</i>	<i>dnaK</i>	<i>groEL</i>						
Photosystems											
Phycobilisomes	12	<i>apcA</i>	<i>apcB</i>	<i>apcD</i>	<i>apcE</i>	<i>apcF</i>	<i>cpcA</i>	<i>cpcB</i>	<i>cpcG</i>	<i>cpcS</i>	<i>cpeA</i>
		<i>cpeB</i>	<i>nblA</i>								
Photosystem I	13	<i>psaA</i>	<i>psaB</i>	<i>psaC</i>	<i>psaD</i>	<i>psaE</i>	<i>psaF</i>	<i>psaI</i>	<i>psaJ</i>	<i>psaK</i>	<i>psaL</i>
		<i>psaM</i>	<i>ycf3</i>	<i>ycf4</i>							
Photosystem II	19	<i>psbA</i>	<i>psbB</i>	<i>psbC</i>	<i>psbD</i>	<i>psbE</i>	<i>psbF</i>	<i>psbH</i>	<i>psbI</i>	<i>psbJ</i>	<i>psbK</i>
		<i>psbL</i>	<i>psbN</i>	<i>psbT</i>	<i>psbV</i>	<i>psb28</i>	<i>psbX</i>	<i>psbY</i>	<i>psbZ</i>	<i>ycf12</i>	
Cytochrome complex	11	<i>ccsA</i>	<i>ccsI</i>	<i>petA</i>	<i>petB</i>	<i>petD</i>	<i>petF</i>	<i>petG</i>	<i>petJ</i>	<i>petL</i>	<i>petM</i>
		<i>petN</i>									
Redox system	7	<i>grx</i>	<i>dsbD</i>	<i>firB</i>	<i>acsF</i>	<i>basI</i>	<i>pbsA</i>	<i>trxA</i>			
ATP synthesis											
ATP synthase	8	<i>atpA</i>	<i>atpB</i>	<i>atpD</i>	<i>atpE</i>	<i>atpF</i>	<i>atpG</i>	<i>atpH</i>	<i>atpI</i>		
Metabolism											
Carbohydrates	6	<i>rbcL</i>	<i>rbcS</i>	<i>cfxQ</i>	<i>pgmA</i>	<i>odpA</i>	<i>odpB</i>				
Lipids	5	<i>accA</i>	<i>accD</i>	<i>fabH</i>	<i>accB</i>	<i>acpP</i>					
Nucleotides	2	<i>carA</i>	<i>upp</i>								
Amino acids	8	<i>argB</i>	<i>gltB</i>	<i>ilvB</i>	<i>ilvH</i>	<i>syfB</i>	<i>syh</i>	<i>trpA</i>	<i>trpG</i>		
Cofactors	4	<i>chlI</i>	<i>moeB</i>	<i>preA</i>	<i>thiG</i>						
Transport											
Transport	9	<i>secA</i>	<i>secG</i>	<i>secY</i>	<i>cemA</i>	<i>ycf16</i>	<i>ycf24</i>	<i>ycf38</i>	<i>ycf43</i>	<i>ycf63</i>	
Unknown											
		<i>ORF58</i>	<i>ORF65</i>	<i>ORF83</i>	<i>ORF621</i>	<i>ycf17</i>	<i>ycf19</i>	<i>ycf20</i>	<i>ycf21</i>	<i>ycf22</i>	<i>ycf26</i>
Conserved ORFs	35	<i>ycf33</i>	<i>ycf34</i>	<i>ycf35</i>	<i>ycf36</i>	<i>ycf37</i>	<i>ycf39</i>	<i>ycf40</i>	<i>ycf45</i>	<i>ycf46</i>	<i>ycf52</i>
		<i>ycf53</i>	<i>ycf54</i>	<i>ycf55</i>	<i>ycf56</i>	<i>ycf60</i>	<i>ycf65</i>	<i>ycf80</i>	<i>ycf92</i>	<i>orf10</i>	<i>orf12</i>
		<i>orf13</i>	<i>orf14</i>	<i>orf18</i>	<i>orf24</i>	<i>orf25</i>					
Total	206										

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

Main WSP	PE α			PE β			PC α			PC β			APC α			APC β			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						
	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]
	<u>AR</u>	96	[36-37]	<u>AY</u>	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]	<u>AR</u>	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]	<u>LY</u>	5.1	[90-92]	IL	55	[42-43]	<u>VR</u>	53	[38-39]	ST	4.0	[66-67]	RF	93	[221-222]	ITF	49	[468-470]	NY	33	[46-47]
	<u>AKYSY</u>	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]	<u>LY</u>	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]	<u>AY</u>	19	[103-104]	<u>YW</u>	1.4	[285-286]				LVR	14	[111-113]
	NY	33	[94-95]	SY	66.3	[94-95]	YLR	5.8	[91-93]	<u>AY</u>	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]	<u>LY</u>	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]				<u>FY</u>	25	[151-152]	YN	51	[116-117]	<u>VK</u>	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]	<u>AY</u>	19	[147-148]	FYN	18	[348-350]						
	YVA	1.4	[127-129]				<u>LDY</u>	6.1	[154-156]							<u>LY</u>	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]	<u>LDY</u>	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]						
	<u>LDY</u>	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 [‡]	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-terminal 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	<i>Grateloupia asiatica</i>	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	
	<i>Grateloupia taiwanensis</i>	Identity ^{*4}	99	100	100	99	99	99	99	99			DePriest et al. 2013
		Peptides	25	17	22	12	17	21	61	17	114	192	NC_021618
		iSD	3	2	1	3	1	3	5	0	13	18	
	<i>Grateloupia filicina</i>	Identity	100	100	100	100	100	100	99	100			Zhang et al. 2018
		Peptides	25	17	22	13	17	21	59	16	115	190	NC_037841
		iSD	3	2	1	3	1	3	5	0	13	18	
	Gracilariaceae Gracilariales	Identity	91	93	92	93	98	96	95	88			Du et al. 2016
		Peptides	25	13	22	14	17	21	63	15	112	190	KP330491
		iSD	3	3	1	3	1	3	4	1	14	19	
Florideophyceae Gigartinales	<i>Gracilariopsis firma</i>	Identity	88	87	93	90	98	94	95	83			Ng et al. 2017
		Peptides	24	15	20	14	17	19	61	15	109	185	NC_033877
		iSD	3	3	0	3	1	3	4	0	13	17	
	<i>Mazzaella japonica</i>	Identity	91	91	96	95	98	96					Kitade et al. 2018
		Peptides	22	13	22	12	16	19	N.D.	N.D.	104	-	AB698819.2
		iSD	2	2	1	4	1	2			12	-	LC222737 LC222738
Florideophyceae Palmariales	<i>Palmaria palmata</i> in Japan	Identity	91	86	93	92	96	96	91	78			Kumagai et al. 2019
		Peptides	28	17	21	15	17	20	54	14	118	186	AB807662
		iSD	3	3	0	2	1	2	5	1	11	17	
Bangiophyceae Pyropia	<i>Pyropia yezoensis</i>	Identity	90	90	93	91	96	94	89	72			
		Peptides	25	19	27	11	17	21	57	16	120	193	NC_007932
		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'-AGAGCCTATAACCTTCATCAG-3'
Gap3 Fw	5'-CTCAAAAAGCGATAGTAGCCG -3'
Gap3 Re	5'-CCCAAATAACCTCACCATCAG-3'
Gap4 Fw	5'-CACCGAATGGCTTTTTGGA-3'
Gap4 Re	5'-ACCACATCCCATATAGTTGC-3'
Gap5 Fw	5'-CCCGATTCAATTGCCCACTA-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

Figure 2