In Silico Analysis of Relationship between Proteins from Plastid Genome of Red Alga Palmaria sp. (Japan) and Angiotensin I Converting Enzyme Inhibitory Peptides

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Abstract: Plastid proteins are one of the main components in red algae. In order to clarify the angiotensin I converting enzyme (ACE) inhibitory peptides from red alga Palmaria sp. (Japan), we determined the plastid genome sequence. The genome possesses 205 protein coding genes, which were classified as genetic systems, ribosomal proteins, photosystems, adenosine triphosphate (ATP) synthesis, metabolism, transport, or unknown. After comparing ACE inhibitory peptides between protein sequences and a database, photosystems (177 ACE inhibitory peptides) were found to be the major source of ACE inhibitory peptides (total of 751). Photosystems consist of phycobilisomes, photosystem I, photosystem II, cytochrome complex, and a redox system. Among them, photosystem I (53) and II (51) were the major source of ACE inhibitory peptides. We found that the amino acid sequence of apcE (14) in phycobilisomes, psaA (18) and psaB (13) in photosystem I, and psbB (11) and psbC (10) in photosystem II covered a majority of bioactive peptide sequences. These results are useful for evaluating the bioactive peptides from red algae.

Keywords: dulse; Palmaria sp. (Japan); ACE inhibitory peptide; plastid genome

1. Introduction

Marine algae contain proteins, lipids, carbohydrates, vitamins, and minerals as nutrition. The amount of these elements vary depending on season and the area of production [1,2]. Seaweed can be used as a source of polysaccharides, such as alginate, carrageenan, and agar [3,4]. Asia has a long tradition of consuming seaweed and seaweed has recently become considered a health food worldwide [5].

Among seaweeds, red algae contain a high amount of protein compared to green and brown algae [1,6]. The amount of protein varies according to environmental conditions and ranges from 7% to 30% [1,7]. The main components of protein in red algae are phycobiliproteins and ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). Phycobiliproteins form the complex structure of phycobilisomes, with phycobiliproteins and chromophores that capture light energy for
photosynthesis [8]. The chromophores are used as the antioxidant materials in this process [9,10]. The proteinase hydrolysate of the rod-shaped protein of phycobiliproteins and Rubisco has different bioactivities, such as inhibition of both angiotensin I converting enzyme (ACE) and dipeptidyl peptidase IV (DPP IV) [11–22]. Bioactive peptides have been reported in various protein sources [18,23]. The typical strategy for the identification of peptides includes a series of steps: peptide production using proteinases, preparation, inhibitory activity measurement, identification of peptide sequences, and confirmation of the activity using a synthesized peptide [12–14,24]. Some studies have confirmed this peptide inhibitory activity in animal experiments [24]. This method is useful for the identification of novel and major peptide sequences in samples. However, it is difficult to identify a small amount of peptide that has strong activity in a sample, as the peptide contributes its activity to the whole hydrolysate sample. The data for peptide sequences and inhibitory concentration (IC_{50}) can be found in a database (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep). These data were obtained from various protein sources. It has been speculated that the same value of biological activity would be expressed by peptides obtained from different sources. Therefore, it was hypothesized that finding the peptide sequences in the protein sequences from genomes would unveil functional peptides from natural sources.

In this study, we determined the complete plastid genome sequence of *Palmaria* sp. (Japan) and annotated protein coding genes (PCGs), which are the main source of proteins in red algae. To discover functional peptides, the relationship between protein sequences in the plastid and the database was evaluated.

2. Results and Discussion

2.1. General Features of *Palmaria* sp. (Japan) Plastid Genomes

The complete plastid genomes of *Palmaria* sp. (Japan) were determined using next-generation sequencing (NGS) methods. The contigs coding plastid were assembled using BLASTn before we obtained the draft circular plastid genome. The genes in the plastid were annotated manually and the gap or deletion in PCGs were confirmed using PCR amplification followed by Sanger sequencing using specific primers (Table S1). As a result, a total of 192,409 nt of the plastid genome was sequenced (Figure 1). The average coverage for the plastid genomes was 630×. The genome contained 205 PCGs (Table 1). The plastid sequence was deposited in DNA Data Bank of Japan (DDBJ) as AB807662.

When comparing the architecture of plastid genomes between *Palmaria* sp. (Japan) and the related species, the plastid genome was most similar to that of *Palmaria palmata*. This similarity was namely in terms of two introns, 205 PCGs, 33 tRNAs, and two copies of the ribosomal RNA operon (Table 2). Although the genes were completely conserved, *Palmaria* sp. (Japan) had a small total number of nt (192,409) and high GC content (34.6%) compared to *P. palmata*, which had 192,960 nt and 33.9% GC content.
Figure 1. The plastid genome map of Palmaria sp. (Japan).
### Table 1. Protein coding genes (PCGs) in *Palmaria* sp. (Japan).

| Classification | No. | Gene |
|----------------|-----|------|
| **Genetic System** |     |      |
| Maintenance     | 2   | *dnaB* | *rne* |
| RNA polymerase  | 5   | *rpoA* | *rpoB* |
| Transcription factors | 4 | *ntcA* | *ompK* |
| Translation     | 4   | *infB* | *infC* |
| **Ribosomal Proteins** |     |      |
| Large subunit   | 28  | *rpl1* | *rpl12* |
|                 |     | *rpl13* | *rpl22* |
|                 |     | *rpl14* | *rpl33* |
|                 |     | *rpl23* | *rpl34* |
|                 |     | *rpl24* | *rpl35* |
|                 |     | *rpl27* | *rpl36* |
| Small subunit   | 19  | *rps1* | *rps9* |
|                 |     | *rps10* | *rps18* |
|                 |     | *rps19* | *rps20* |
| **tRNA processing** | 1 | *tilS* | *tilS* |
| Protein quality control | 4 | *clpC* | *dnaK* |
| **Photosystems** |     |      |
| Phycobilisomes   | 12  | *apcA* | *apcB* |
|                 |     | *apcC* | *apcD* |
|                 |     | *apcE* | *apcF* |
|                 |     | *psaA* | *psaA* |
|                 |     | *psaB* | *psaB* |
|                 |     | *psaL* | *psaL* |
| Photosystem I    | 13  | *psbA* | *psbA* |
|                 |     | *psbB* | *psbB* |
|                 |     | *psbK* | *psbK* |
|                 |     | *psbX* | *psbX* |
| Photosystem II   | 19  | *psaA* | *psaA* |
|                 |     | *psaB* | *psaB* |
|                 |     | *psaC* | *psaC* |
|                 |     | *psaD* | *psaD* |
|                 |     | *psaE* | *psaE* |
|                 |     | *psaF* | *psaF* |
|                 |     | *psaG* | *psaG* |
|                 |     | *psaH* | *psaH* |
|                 |     | *psaI* | *psaI* |
| Cytochrome complex | 11 | *ccsI* | *ccsA* |
|                 |     | *petA* | *petN* |
|                 |     | *petB* | *petD* |
|                 |     | *petF* | *petG* |
|                 |     | *petH* | *petG* |
| Redox system    | 7   | *acsF* | *bas1* |
| ATP Synthesis   | 8   | *atpA* | *atpB* |
|                 |     | *atpC* | *atpD* |
| Metabolism      |     |      |
| Carbohydrates   | 6   | *cfsQ* | *pdbA* |
|                 |     | *pdbB* | *pdbB* |
|                 |     | *pdbC* | *pdbC* |
|                 |     | *pdbD* | *pdbD* |
|                 |     | *pdbE* | *pdbE* |
| Lipoicosides    | 5   | *accA* | *accB* |
|                 |     | *accD* | *accD* |
|                 |     | *apcP* | *apcP* |
| Nucleotides     | 2   | *carA* | *carA* |
| Amino acids (AAs) | 8 | *argB* | *glhB* |
|                 |     | *ilvB* | *ilvB* |
|                 |     | *ileH* | *ileH* |
|                 |     | *hisS* | *hisS* |
| Cofactors       | 8   | *chlB* | *chlB* |
|                 |     | *chlI* | *chlI* |
|                 |     | *chlN* | *chlN* |
| Secondary metabolites | 1 | *dfr* | *dfr* |
| Transport       | 9   | *cerA* | *secA* |
|                 |     | *secG* | *secY* |
| Unknown         | 23  | *orfA* | *orfB* |
|                 |     | *orfC* | *orfD* |
|                 |     | *orfE* | *orfF* |
|                 |     | *orfG* | *orfH* |
|                 |     | *orfI* | *orfJ* |
| Unique ORFs     | 6   | *orf55* | *orf56* |
|                 |     | *orf57* | *orf58* |
| Total genes     | 205 |       |      |

### Table 2. Comparison of general plastid structure in red algae similar to *Palmaria* sp. (Japan).

| Subclass              | Species                                    | General Characteristics | RNAs | GenBank Accession | Reference |
|-----------------------|--------------------------------------------|-------------------------|------|------------------|-----------|
|                       |                                            | Total nt | GC% a | Introns | PCG *2* | tRNA | rRNA |                     |           |
| **Nemaliophycidae**   | *Palmaria* sp. (Japan)                     | 192,410 | 34.6  | 2       | 205     | 33   | 6    | AB807662             | This study|
|                       | *Palmaria palma*                           | 192,960 | 33.9  | 2       | 205     | 33   | 6    | NC_031114            | [25]      |
|                       | *Kumanoa americana hys120*                 | 184,025 | 29.3  | 2       | 202     | 33   | 6    | NC_031178            | [25]      |
|                       | *Thorea hispida hsy077*                    | 175,193 | 28.3  | 2       | 194     | 31   | 6    | NC_031171            | [25]      |
| **Corallinophycidae** | *Calliarthus tuberculatus*                 | 178,981 | 29.2  | 2       | 202     | 33   | 6    | NC_021075            | [26]      |
|                       | *Sporolithon durum*                        | 191,464 | 29.3  | 2       | 207     | 30   | 3    | NC_029857            | [27]      |
| **Ahnfeltiophycidae** | *Ahnfeltia plicata*                       | 190,451 | 32.5  | 1       | 207     | 31   | 6    | NC_031145            | [28]      |
| **Rhodymeniophycidae**| *Asparagopsis taxiformis*                  | 177,091 | 29.4  | 2       | 205     | 33   | 6    | NC_031148            | [28]      |
|                       | *Ceramium japonicum*                      | 171,634 | 27.8  | 1       | 202     | 32   | 3    | NC_031174            | [28]      |
|                       | *Rhodymenia pseudopalmata*                | 194,153 | 32.0  | 1       | 202     | 32   | 6    | NC_031144            | [28]      |
|                       | *Vertebrata lanosa*                       | 167,158 | 30.0  | 0       | 193     | 28   | 3    | KP368997             | [29]      |

* A percentage of guanine and cytosine in a plastid genome DNA; *2 protein coding genes.
2.2. Comparison of Amino Acid (AA) Composition between Palmaria sp. (Japan) Plastid Proteins and Proximate AA in *P. palmata*

The AA compositions of marine algae have been studied for a long period of time [30]. The AA compositions, which are an important source of protein, differ between algae species. This suggests that the differences may reflect the composition of the final product. Therefore, the AA composition of plastid proteins and the real composition in *P. palmata* were compared (Table 3). The AA composition was quite similar, except for aspartic acid and glycine in real protein, and isoleucine and leucine in real AA and protein. Mai et al. reported on the AA composition in various types of seaweed, and showed that the amount of aspartic acid and glycine was mostly stable in seaweed [6, 31]. Therefore, we focused on the amounts of isoleucine and leucine. The amounts of isoleucine (9.0%) and leucine (10.1%) in plastid proteins was higher than the true AA and protein (isoleucine 5.3% and 3.7%; leucine 7.8% and 7.1%). The proportions found in plastid proteins showed that the proteins were equally expressed. Focusing on the classification of protein function, the amount of isoleucine and leucine in ribosomal protein (8.7% and 8.7%) and isoleucine in phycobilisomes (7.5%) was low. Therefore, considering the fact that ribosomal protein and phycobilisomes proteins are the main red algae proteins, the percentage of AA in the real seaweed would be close to the composition of plastid proteins. Although there is currently no information on nuclear and mitochondrial genomes, it would be expected that the proteins from these genomes would contain low amounts of isoleucine and leucine.

Table 3. Composition of AAs in *Palmaria* sp. (Japan) plastid protein and AAs in *P. palmata*.

| AA          | Plastid | GS | RP | PS | ATP | Meta | TP | UK | % of Total AA a or Protein b |
|-------------|---------|----|----|----|-----|------|----|----|----------------------------|
| Alanine     | 6.4     | 5.6 | 6.8 | 7.6 | 8.7 | 6.4  | 5.8 | 4.5 | 7.5 a 6.7 b               |
| Arginine    | 4.6     | 5.1 | 6.8 | 4.1 | 3.8 | 4.0  | 3.6 | 4.1 | 6.2 a 5.1 b               |
| Aspartic acid | 4.5   | 5.6 | 4.1 | 4.0 | 4.6 | 5.4  | 3.9 | 3.8 | 9.3 a 18.5 b             |
| Asparagine  | 5.5     | 5.9 | 5.2 | 4.8 | 4.2 | 5.6  | 5.6 | 6.4 | 13 a 9.9 b               |
| Cystine     | 1.1     | 0.8 | 0.7 | 1.0 | 0.2 | 1.4  | 1.2 | 1.5 | 13 a 0 b                 |
| Glutamic acid | 5.7  | 6.4 | 6.2 | 4.6 | 7.2 | 6.0  | 6.2 | 4.7 | 20 a 9.9 b               |
| Glutamine   | 4.2     | 4.5 | 4.1 | 3.8 | 5.5 | 4.3  | 3.9 | 4.2 | 13 a 9.9 b               |
| Glycine     | 6.3     | 5.7 | 7.0 | 7.6 | 7.1 | 6.6  | 5.4 | 3.7 | 7.2 a 13.3 b             |
| Histidine   | 1.9     | 1.9 | 1.9 | 1.9 | 0.7 | 2.3  | 1.4 | 2.1 | 21 a 0.5 b               |
| Isoleucine  | 9.0     | 10.0| 8.7 | 7.5 | 9.3 | 9.0  | 11.1| 10.0| 53 a 3.7 b               |
| Leucine     | 10.6    | 10.1| 8.7 | 10.4| 12.0| 10.4 | 12.2| 12.9| 78 a 7.1 b               |
| Lysine      | 6.5     | 7.3 | 9.3 | 4.4 | 5.5 | 5.8  | 5.3 | 7.0 | 8.2 a 3.3 b              |
| Methionine  | 2.2     | 1.8 | 2.1 | 2.6 | 2.0 | 2.4  | 1.9 | 1.8 | 19 a 2.7 b               |
| Phenylalanine | 4.1  | 3.2 | 2.7 | 5.8 | 3.4 | 3.5  | 4.8 | 5.1 | 52 a 5.1 b               |
| Proline     | 3.7     | 3.6 | 3.6 | 4.1 | 3.7 | 3.9  | 3.1 | 3.4 | 44 a                     |
| Serine      | 7.4     | 7.0 | 6.3 | 7.8 | 6.8 | 7.2  | 7.8 | 8.6 | 46 a 6.3 b               |
| Threonine   | 5.6     | 5.2 | 5.8 | 5.6 | 5.9 | 5.6  | 6.0 | 5.3 | 45 a 3.6 b               |
| Tryptophan  | 1.0     | 0.5 | 0.5 | 1.9 | 0.8 | 0.4  | 0.8 | 0.9 | 1.3                     |
| Tyrosine    | 3.6     | 3.5 | 2.6 | 3.9 | 2.4 | 3.4  | 4.2 | 4.7 | 45 a 3.4 b               |
| Valine      | 6.3     | 6.6 | 7.0 | 6.6 | 6.9 | 6.2  | 5.9 | 4.9 | 7.3 a 6.9 b              |

*GS*: genetic system; *RB*: ribosomal proteins; *PS*: photosystems; *ATP*: ATP synthesis; *Meta*: metabolism; *TP*: transport; *UK*: unknown.

2.3. ACE Inhibitory Peptides in Plastid

ACE inhibitory peptides have been found in red algae proteins, which are namely the rod-like proteins of phycobilisomes and Rubisco, because these are the major components of soluble red algae proteins [32]. The increase in accessibility to the protein was previously studied [5, 33]. However, 205 PCGs exist in the *Palmaria* sp. (Japan) plastid genome, which indicates that the insoluble or
membrane proteins have potential as a source of bioactive peptides. Therefore, we screened the plastid proteins to confirm the possibility of using them as bioactive peptides. ACE inhibitory tripeptides with IC$_{50}$ less than 20 µM were extracted from the biopep-uwm database, and a total of 89 peptides were selected. Although di-, tetra-, or longer peptides with ACE inhibitory activity were deposited in the database, we employed the tripeptide database to reduce overestimation. A large proportion of these peptides consisted of proline (34 peptides) or tyrosine (20 peptides) at the C-terminus. After comparing the plastid proteins and the peptide sequences, a total of 751 ACE inhibitory peptides were found (Table 4). When the peptide sources were classified according to protein function, photosystems contained the highest number with 177 peptides, followed by metabolism (176) and ribosomal proteins (128). The smallest number of peptides were involved in ATP synthesis (28), according to functional classification. This was due to a small proportion of total AAs involved in ATP synthesis.

**Table 4. Angiotensin I converting enzyme (ACE) inhibitory peptides from *Palmaria* sp. (Japan) plastid.**

| Peptide * | Database | Plastid | GS | RP | PS | ATP | Meta | TP | UK |
|-----------|----------|---------|----|----|----|-----|------|----|----|
| XXP       | 34       | 260     | 48 | 38 | 62 | 10  | 61   | 20 | 21 |
| XXY       | 20       | 140     | 21 | 13 | 30 | 7   | 31   | 13 | 25 |
| XXX       | 6        | 66      | 9  | 11 | 21 | 5   | 13   | 3  | 4  |
| XXL       | 5        | 78      | 5  | 11 | 27 | 2   | 19   | 5  | 9  |
| XWX       | 5        | 4       | 0  | 0  | 3  | 0   | 1    | 0  | 0  |
| XXG       | 3        | 51      | 5  | 16 | 8  | 0   | 16   | 2  | 4  |
| XXR       | 3        | 31      | 4  | 10 | 3  | 0   | 7    | 0  | 7  |
| XXV       | 3        | 33      | 5  | 8  | 10 | 1   | 6    | 2  | 1  |
| XXF       | 2        | 7       | 0  | 0  | 3  | 0   | 2    | 0  | 2  |
| XXX       | 2        | 8       | 12 | 3  | 3  | 8   | 1    | 4  |    |
| XXX       | 2        | 5       | 0  | 1  | 1  | 0   | 2    | 0  | 1  |
| XXX       | 4        | 39      | 8  | 8  | 6  | 0   | 10   | 5  | 2  |

Total 89 751 113 128 177 28 176 51 80

Total AA 50,333 7010 8981 11,017 1975 11,213 3184 6953

Peptide/AA (%) 1.49 1.61 1.43 1.61 1.42 1.57 1.60 1.15

* The peptide structures and related proteins are listed in Table S2; *² No. of peptides having IC$_{50}$ (>20 µM) are obtained from BIO-PEP-UWM database; *³ Four tripeptide sequences: LVQ, LVE, IWH, GPM; GS: genetic system; RB: ribosomal proteins; PS: photosystems; ATP: ATP synthesis; Meta: metabolism; TP: transport; UK: unknown.

2.4. ACE Inhibitory Peptides in Photosystems

It has been reported that the proteins from photosystems are the major components of soluble proteins, with these proteins containing various types of bioactive peptides [23]. Photosystems contain a large number of ACE inhibitory peptides (Table 4). Therefore, we investigated the peptides in photosystems. The function of photosystem proteins was classified into phycobilisomes, photosystem I, photosystem II, cytochrome complex, and redox system. Among them, photosystem I had the highest number with 53 peptides, followed by photosystem II (51), and phycobilisomes (42) (Table 5). The ratio of the number of peptides to the total AA (peptide/AA (%)) was high in photosystem I (2.00%) and photosystem II (1.98%) compared with photosystems (1.59%) and plastid (1.49%). After this, we focused on the number of ACE inhibitory peptides in proteins. We found that the proteins of apcE, psaA, psaB, psbA, psbB, and psbC possessed a large number of the peptides (Table 6). The photosystem proteins psaA, psbA, psbB, and psbC are the components of the integral membrane proteins in photosystem I and II, which are not easily obtained through water extraction as soluble proteins. Most ACE inhibitory peptides from red algae were from soluble proteins, that is, from the rod-like proteins of phycobilisomes and Rubisco. These data are useful for finding novel bioactive peptides from red algae proteins.
Table 5. ACE inhibitory peptides from photosystems.

| Peptide * | PBS | PSI | PSII | Cc | Red |
|-----------|-----|-----|------|----|-----|
| XXP       | 11  | 21  | 17   | 7  | 6   |
| XXY       | 9   | 8   | 7    | 3  | 3   |
| XXA       | 6   | 4   | 8    | 1  | 2   |
| XXL       | 6   | 12  | 4    | 5  | 0   |
| XXW       | 0   | 3   | 0    | 0  | 0   |
| XXG       | 3   | 1   | 4    | 0  | 0   |
| XXR       | 1   | 1   | 1    | 0  | 0   |
| XXV       | 3   | 2   | 3    | 2  | 0   |
| XXF       | 0   | 0   | 2    | 1  | 0   |
| XXK       | 0   | 1   | 2    | 0  | 0   |
| XNX       | 0   | 0   | 0    | 0  | 1   |
| XXX **    | 3   | 0   | 3    | 0  | 0   |
| **Total** | 42  | 53  | 51   | 19 | 12  |

| Total AA  | 2644 | 2654 | 2582 | 1784 | 1353 |
| Peptide/AA (%) | 1.59 | 2.00 | 1.98 | 1.07 | 0.89 |

* The peptide structures and related proteins are listed in Table S3; ** LVQ, LVE, IWH, GPM; PS: photosystems; PBS: phycobilisomes; PSI: photosystem I; PSII: photosystem II; Cc: cytochrome complex; Red: redox system.

Table 6. ACE inhibitory peptide in photosystem proteins.

| PBS No. * | PSI No. * | PSII No. * | Cc No. * | Red No. * |
|-----------|-----------|------------|----------|-----------|
| apcA 2    | psaA 18   | psbA 9     | psbV 2   | ccs1 5    | acsF 2   |
| apcB 2    | psaB 13   | psbB 11    | psbW 1   | ccsA 0    | bas1 1   |
| apcD 3    | psaC 1    | psbC 10    | psbX 0   | petA 1    | dsbD 4   |
| apcE 14   | psaD 1    | psbD 6     | psbY 1   | petB 3    | ftrB 1   |
| apcF 3    | psaE 2    | psbE 1     | psbZ 2   | petD 1    | grx 0    |
| cpcG 0    | psaF 5    | psbF 0     | psb30 0  | petF 3    | pbsA 2   |
| cpcS 2    | psal 1    | psbH 3     |           | petG 1    | trxA 2   |
| rpcA 2    | psaj 0    | psbl 0     |           | petj 0    |         |
| rpcB 3    | psal 3    | psbj 1     |           | petL 0    |         |
| rpeA 6    | psam 1    | psbK 1     |           | petM 1    |         |
| rpeB 4    | psbH 1    | psbL 1     |           | petN 0    |         |
| nblA 1    | ycf3 3    | psbN 0     |           |         |         |
| ycf4 4    |           | psbT 2     |           |         |         |
| **Total** | 42        | 53         | 51        | 19        | 12        |

* No. of ACE inhibitory peptides; PBS: phycobilisomes; PSI: photosystem I; PSII: photosystem II; Cc: cytochrome complex; Red: redox system.

2.5. Comparison of ACE Inhibitory Peptides in Palmaria sp. (Japan) and P. palmata

The plastid genomes of *Palmaria* sp. (Japan) and *P. palmata* were similar, and the number of PCGs was the same (205). To clarify the differences in ACE inhibitory peptides between *Palmaria* sp. (Japan) and *P. palmata*, the ACE inhibitory peptides were compared (Table 7). A total of 742 peptides were found in *P. palmata*, which was less than that found in *Palmaria* sp. (751). The difference was due to an unknown protein that had 80 peptides in *Palmaria* sp. and 72 peptides in *P. palmata*. Although the number of peptides among the other protein functional groups was almost the same, the peptide sequences differed between these groups (Table 4; Table 7). These data are useful for selecting peptide producing proteinases.
Table 7. ACE inhibitory peptides from *P. palmata* plastid.

| Peptide * | Database *2 | Plastid | GS | RP | PS | ATP | Meta | TP | UK |
|-----------|-------------|---------|----|----|----|-----|------|----|----|
| XXP       | 34          | 263     | 47 | 39 | 63 | 10  | 63   | 20 | 21 |
| XXY       | 20          | 133     | 23 | 13 | 29 | 7   | 28   | 12 | 21 |
| XXA       | 6           | 68      | 8  | 13 | 20 | 6   | 13   | 3  | 5  |
| XXL       | 5           | 79      | 6  | 10 | 25 | 2   | 20   | 5  | 11 |
| XXW       | 5           | 4       | 0  | 0  | 3  | 0   | 1    | 0  | 0  |
| XXG       | 3           | 51      | 6  | 16 | 8  | 0   | 16   | 2  | 3  |
| XXR       | 3           | 27      | 2  | 10 | 3  | 0   | 7    | 0  | 5  |
| XXV       | 3           | 33      | 5  | 8  | 9  | 1   | 7    | 2  | 1  |
| XXF       | 2           | 6       | 0  | 0  | 3  | 0   | 2    | 0  | 1  |
| XXK       | 2           | 37      | 9  | 11 | 3  | 4   | 7    | 1  | 2  |
| XXX       | 2           | 5       | 0  | 1  | 1  | 0   | 2    | 0  | 1  |
| XXXX      | 4           | 36      | 7  | 8  | 6  | 0   | 9    | 5  | 1  |

| Total AA | 50,229 | 7009 | 8981 | 11,013 | 1970 | 11,237 | 3188 | 6831 |

Peptide/AA (%) 1.48 1.61 1.44 1.57 1.52 1.56 1.57 1.05

* The peptide structures and related proteins are listed in Table S4; *2 No. of peptides having IC<sub>50</sub> (>20 µM) were obtained from the BIO-PEP-UWM database; *3 LVQ, LVE, IWH, GPM; GS: genetic system; RB: ribosomal proteins; PS: photosystems; ATP: ATP synthesis; Meta: metabolism; TP: transport; UK: unknown.

3. Materials and Methods

3.1. Plastid Genome Construction

*Palmaria* sp. was collected from Usujiri, Japan in February 2012. Genomic DNA was extracted using the hexadecyltrimethylammonium bromide (CTAB) method [34]. The genome sequence data were generated using the GS Junior Titanium Series system (Roche). After this, the DNA library was subjected to emulsion PCR (emPCR) using the emPCR Reagents kit (Lib-A) (Roche) according to the manufacturer’s protocol. After emPCR, DNA beads were enriched and placed on a picotiter plate (Roche) before we ran generation sequencing on this DNA using the GS Junior equipment (Roche). The contigs coding plastids were assembled with BLASTn using the red algal *P. palmata* plastid genes as a reference (NC_031147.1). After the reassembly, a circular plastid 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/). A gap in genes was confirmed by PCR amplification and Sanger sequencing (Table S1). The annotated plastid genomes were visualized using OrganellarGenomeDraw v1.2 [35].

3.2. Collection of ACE Inhibitory Peptides and Comparison with Plastid Proteins

ACE inhibitory peptides were obtained from the biopep-uwm database (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep) on 28 January 2019. From the database, tripeptides with IC<sub>50</sub> less than 20 µM were selected. The peptide sequences in plastid proteins were manually annotated.

4. Conclusions

We determined the complete plastid genome sequence of the red alga *Palmaria* sp. (Japan) and annotated 205 PCGs. Comparing the plastid protein sequences and ACE inhibitory peptide sequences to a database, a large part of the peptide sequences was classified into photosystems (177) and metabolism (176). Among the photosystems, the proteins from apcE, psaA, psaB, psbA, psbB, and psbC possessed a large number of the peptides. Comparing protein sequences between *Palmaria* sp. (Japan) and *P. palmata*, the number of ACE inhibitory peptides was similar, although they had a different composition of peptides. We previously prepared ACE inhibitory peptides from water-extracted dulse protein as thermolysin hydrolysate [15]. The peptide sequences identified were
mainly from phycobiliproteins. We therefore could not identify peptides from membrane proteins such as photosystem I and II. *In silico* analysis showed both the potential of membrane proteins for ACE inhibitory peptides and the characteristic C-terminal structure of ACE inhibitory peptides. Digestive enzymes such as pepsin (Aps, Glu, Leu, Phe, Trp, and Tyr), chymotrypsin (Phe, Trp, and Tyr), elastase (Ala, Gly, Ile, Leu, Ser, and Val), and prolyl endopeptidase (Pro) hydrolyzed the C-terminus of proteins, and would produce ACE inhibitory peptides. We expected that peptides from membrane proteins, which were not identified in in vitro experiments, would play a role in the inhibition of high blood pressure. In addition to ACE inhibitory activity, DPP IV inhibitory peptides were also identified in red algae protein hydrolysates, and *in silico* analysis would apply for finding novel bioactive peptides from red algae proteins.

**Supplementary Materials:** The following are available online at http://www.mdpi.com/1660-3397/17/3/190/s1, Table S1: Primers for *Palmaria* sp. (Japan) plastid sequence, Table S2: ACE inhibitory peptide from *Palmaria* sp. (Japan) plastid, Table S3: ACE inhibitory peptide from *Palmaria* sp. (Japan) photosystems, Table S4: ACE inhibitory peptide from *Palmaria palmata* plastid.

**Author Contributions:** H.K. and H.Y. conceived and designed the research; H.K., Y.M., and H.Y. contributed to sample collection; Y.M., T.T., K.A., and Y.K. performed the experiments and analyzed the data. Y.K. and H.K. contributed to writing and editing the manuscript.

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