<table>
<thead>
<tr>
<th>Title</th>
<th>Structural characteristics of phycobiliproteins from red alga Mazzaella japonica</th>
</tr>
</thead>
<tbody>
<tr>
<td>Author(s)</td>
<td>Kitade, Yumi; Miyabe, Yoshikatsu; Yamamoto, Yohei; Takeda, Hirohumi; Shimizu, Takeshi; Yasui, Hajime; Kishimura, Hideki</td>
</tr>
<tr>
<td>Citation</td>
<td>Journal of Food Biochemistry, 42(1), e12436 [<a href="https://doi.org/10.1111/jfbc.12436">https://doi.org/10.1111/jfbc.12436</a>]</td>
</tr>
<tr>
<td>Issue Date</td>
<td>2017-09-27</td>
</tr>
<tr>
<td>Doc URL</td>
<td><a href="http://hdl.handle.net/2115/71537">http://hdl.handle.net/2115/71537</a></td>
</tr>
<tr>
<td>Rights</td>
<td>This is the peer reviewed version of the following article: Kitade Y, Miyabe Y, Yamamoto Y, et al. Structural characteristics of phycobiliproteins from red alga Mazzaella japonica. JFoodBiochem. 2018;42:e12436, which has been published in final form at <a href="https://doi.org/10.1111/jfbc.12436">https://doi.org/10.1111/jfbc.12436</a>. This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Use of Self-Archived Versions.</td>
</tr>
<tr>
<td>Type</td>
<td>article (author version)</td>
</tr>
<tr>
<td>File Information</td>
<td>Kishimura_JFB.pdf</td>
</tr>
</tbody>
</table>

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP
STRUCTURAL CHARACTERISTICS OF PHYCObILIPROTEINS FROM RED ALGA MAZZAELLA JAPONICA

YUMI KITADE 1, YOSHIKATSU MIYABE 1, YOHEI YAMAMOTO 1, HIROHUMI TAKEDA 2, TAKESHI SHIMIZU 3, HAJIME YASUI 4, HIDEKI KISHIMURA 5,6

1 Chair of Marine Chemical Resource Development, Graduate School of Fisheries Sciences, Hokkaido University, Hakodate, Hokkaido 041-8611, Japan
2 Hokkaido Research Organization, Fisheries Research Department, Abashiri Fishries Research Institute, 7-8-5 Minato-cho Monbetsu, Hokkaido 094-0011, Japan
3 Department of research and Development, Hokkaido Industrial Technology Center, Hakodate, Hokkaido 041-0801, Japan
4 Laboratory of Humans and the Ocean, Faculty of Fisheries Sciences, Hokkaido University, Hakodate, Hokkaido 041-8611, Japan
5 Laboratory of Marine Chemical Resource Development, Faculty of Fisheries Sciences, Hokkaido University, Hakodate, Hokkaido 041-8611, Japan
6 Corresponding author. TEL/FAX: 81-138-40-5519 EMAIL: kishi@fish.hokudai.ac.jp

Short title: Structural properties of phycobiliproteins from M. japonica
ABSTRACT

We determined the primary structures of phycoerythrin, phycocyanin and allophycocyanin from red alga *Mazzaella japonica*. The phycobiliproteins consist of α- and β-subunits like other red algae. *M. japonica* phycobiliproteins all conserved Cys residues for chromophore attachment site. The amino acid sequences of *M. japonica* phycobiliproteins showed considerably high identities with those of other red algae (81 ~ 100%). In addition, the sequences (YRD, LDY, LRY, VY, LF and FY), which were angiotensin I converting enzyme (ACE) inhibitory peptides from other algae, were detected in the primary structures of *M. japonica* phycobiliproteins. Then, we prepared the protein hydrolysates from *M. japonica* and measured its ACE inhibitory activity. Consequently, *M. japonica* protein hydrolysates indicated considerably high ACE inhibitory activity.

PRACTICAL APPLICATIONS

*M. japonica* is an abundant resource in Japan, which contains a lot of phycobiliproteins. Then, *M. japonica* protein hydrolysates strongly inhibited the activity of ACE. Therefore, it has the potential to be an ingredient of functional food.

KEYWORDS: ACE inhibitory activity; *Mazzaella japonica*; Phycobiliprotein; Primary-structure; Red algae
INTRODUCTION

Seaweeds have been used extensively as food in Japan since ancient times, and several edible seaweeds are well known worldwide such as Kombu (Saccharina sp.), Wakame (Undaria pinnatifida) and Nori (Pyropia sp.). Kombu and Wakame, which belong to brown algae, are rich in polysaccharides such as alginate, laminarin and fucans (Mabeau & Fleurence 1993). In contrast, red algae have high protein levels (Mabeau & Fleurence 1993; Fleurence et al., 2012). For example, it was reported that the crude protein content of Nori (Pyropia tenera or Pyropia yezoensis) is up to 47% of dry weight which is comparable with that of soy (Fleurence et al. 2012). Although the amino acid composition corresponding to several species of algae is known, the structure and biological properties of algal proteins are still relatively poorly documented (Harnedy & FitzGerald 2011). The main proteins of red algae are phycobiliproteins (up to 50% of the total protein content), and they constitute phycobilisomes attached on the cytoplasmic surface of thylakoid membranes (Cian et al. 2015). Major phycobiliproteins include phycoerythrin (PE), phycocyanin (PC), allophycocyanin (APC), and phycoerythrocyanin (PEC) (Denis et al. 2009).

Recently, we have begun exploring the health benefits of dulse (Palmaria palmata) to advance its use as a functional food material. Dulse is a red alga distributed mainly in high-latitude coastal areas, and is popular in Ireland, Iceland, Scandinavia and Atlantic Canada as a food and a source of minerals (Mouritsen et al. 2013). In Japan, dulse is rarely eaten and is even removed from Kombu farming areas in Hokkaido Prefecture, because it slows the growth of Kombu. Thus, we were going to focus on the protein components from dulse, because dulse was rich in proteins (approximately 40%), which were mainly composed of phycobiliproteins. The principal component of dulse phycobiliproteins was PE followed by PC and APC, so we determined the primary structures of dulse phycobiliproteins and the
3-D structure of dulse PE (Miyabe et al. 2017). Moreover, we examined the angiotensin I converting enzyme (ACE) inhibitory activity of dulse protein hydrolysates (Furuta et al. 2016). The dulse protein hydrolysates showed high inhibitory activity, and nine ACE inhibitory peptides (YRD, AGGEY, VYRT, VDHY, IKGHY, LKNPG, LDY, LRY, FEQDWAS) were isolated. Then, the sequences of YRD, AGGEY, VYRT, VDHY, LKNPG LDY and LRY were detected in the primary structures of dulse phycobiliproteins. From these results, it was suggested that the dulse ACE inhibitory peptides were mainly derived from phycobiliproteins.

On the other hand, there are many commercially important red algae in Japan, such as Pyropia sp., Gelidium sp., Gracilaria sp., Gloiopeltis sp., Chondrus sp., Mazzaella sp., Meristotheca sp., and so on. Among these, Mazzaella japonica distributes in northern part of Japan, especially Hokkaido Prefecture, and it has been used for food since long ago. M. japonica also shows considerably high content of protein (about 25%, unpublished data). However, there is no information about gene structures and primary structures of phycobiliproteins from M. japonica, which are very important to reveal the mechanisms of these functions. In addition, there are few studies on gene structures of red algal PC and APC comparing with that of PE. Therefore, in this study, we elucidated the gene structures of phycobiliproteins from M. japonica and characterized their primary sequences.
MATERIALS AND METHODS

Materials

*M. japonica* and dulse were collected on the coast of Hokkaido Prefecture, Japan.

ACE (EC 3.4.15.1) from rabbit lung was purchased from Sigma Chemical Co. (Mo, USA). Cetyltrimethylammonium bromide (CTAB), Hyppuryl-L-histidyl-L-leucine (Hip-His-Leu), thermolysin (EC 3.4.24.27) from *Bacillus thermoproteolyticus*, and all other regents were purchased from Wako Pure Chemical (Osaka, Japan).

Isolation and sequencing of chloroplast DNA

Chloroplast DNA was extracted from the thalli of *M. japonica* according to the CTAB DNA extraction method with some modifications (Wilson 2003). The nucleotide sequences of chloroplast DNA were analyzed by using a next generation sequencer, Ion PGM System (Thermo Fisher SCIENTIFIC, MA, USA). The data were assembled with CLC Genomics Workbench 9.5.4 (QIAGEN, Hilden, Germany). Nucleotide and deduced amino acid sequences of *M. japonica* phycobiliprotein genes were aligned using CLUSTAL W program (Thompson *et al.* 1994). Molecular weight and isoelectric point of *M. japonica* phycobiliproteins were calculated from deduced amino acid sequences by using Compute pI/Mw tool (Bjellqvist *et al.* 1993; Bjellqvist *et al.* 1994; Hoogland *et al.* 2000).

Prediction of 3-D structures of M. japonica phycobiliproteins

We predicted the 3-D structures of *M. japonica* phycobiliproteins on the basis of the primary

Preparation of protein hydrolysates from M. japonica and dulse

Phycobiliproteins comprise about 60% of the soluble protein in these cells (Apt et al., 1995). So, we tried to prepare the protein extract from *M. japonica*. Frozen samples were lyophilized and ground into a powder by Wonder Blender WB-1 (OSAKA CHEMICAL Co., Osaka, Japan). Proteins in the powder was extracted by 20 v/w of distilled water at 4 °C for 12 h, and the solution was centrifuged at 4 °C, 10,000 x g for 10 min. However, we hardly obtained a supernatant, since *M. japonica* has a lot of viscous polysaccharides. Therefore, we prepared the protein hydrolysates by the following method.

*M. japonica* frozen samples were lyophilized and ground into a fine powder by cyclone sample mill, CSM-S1 (Shizuoka Seiki Co., Ltd., Shizuoka, Japan). The powder (1.5 g) was suspended in 25 v/w of distilled water using dialysis membrane (molecular weight cut off: approximately 10 kDa, EIDIA Co., Ltd., Tokyo, Japan), and the suspended solution was dialyzed against distilled water at 4 °C for 4 h to remove low molecular weight contaminants. Then, proteins in the dialysate were hydrolyzed by adding 1.0 wt% of thermolysin at 37 °C for 5 h, and the reaction was ended by heat treatment at 100 °C for 10 min. The solution was dialyzed against distilled water using dialysis membrane (MWCO: approximately 10 kDa) at 4 °C for 12 h. The dialysis outer solution via a dialysis membrane was lyophilized into *M. japonica* protein hydrolysates (56.1 mg).

Preparation of dulse protein hydrolysates was carried out by the same method in our previous paper (Furuta et al. 2016).
ACE inhibitory assay

ACE inhibitory assay was carried out according to the method of Cheng and Cushman (1973) with some modifications. Fifteen microliters of sample solution (1.0 mg/mL) were added to 30 µL of ACE (0.2 units/mL), and the mixture was pre-incubated at 37 °C for 5 min. Thirty 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 hour, the reaction was stopped by adding 75 µL of 1.0 M HCl. The released hippuric acid was extracted with 450 µL of ethyl acetate. Four hundred microliters of the upper layer were evaporated, and then the hippuric acid was dissolved in 1.5 mL of distilled water. The absorbance at 228 nm of the solution was measured by a spectrophotometer. The inhibition was calculated from the equation (1- (As-Asb) / (Ac-Acb)) x 100, where Ac is the absorbance of the buffer, Acb is the absorbance when the stop solution was added to the buffer before the reaction, As is the absorbance of the sample, and Asb is the absorbance when the stop solution was added to the sample before the reaction. The data were expressed as mean ± SEM from triplicate determination, and statistical analysis was performed using Student’s t test.
RESULTS AND DISCUSSION

Nucleotide sequences of phycobiliprotein genes from M. japonica

In this study, we obtained 175,332 bp of nucleotide sequences (19 contigs) for *M. japonica* chloroplast DNA, and PE, PC and APC genes were detected in the sequences: PE (*rpeB* and *rpeA*, GenBank accession number AB698819.2) (Fig. 1a), PC (*rpcB* and *rpcA*, GenBank accession number LC222737) (Fig. 1b) and APC (*rapcA* and *rapcB*, GenBank accession number LC222738) (Fig. 1c). The structures of *rpeB* and *rpeA* of *M. japonica* were identical to those of our previous results analyzed by cDNA cloning method (unpublished data). The *M. japonica* PE, PC and APC genes were constituted of α- and β-subunit genes and A/T-rich spacer (Fig. 1). AT contents of the spacers in *M. japonica* PE, PC and APC genes were 79% (49 bp/62 bp), 86% (48 bp/56 bp) and 91% (48 bp/53 bp), respectively. Bernard et al. (1992) reported that *rpeB* gene of *Rhodella violacea* is split by intervening sequence and the sequence has a feature of group II intron which is typical in eukaryotic organisms, however the *M. japonica* phycobiliprotein genes have no introns. The *rpeB* and *rpcB* of *M. japonica* were present in prior to the *rpeA* and *rpcA* (Fig. 1a, b), but the order was opposite in dulse APC gene (Fig. 1c). The position of *rapcA* and *rapcB* was the same as those of other Florideophycidae algae, for example *Gracilaria tenuistipitata* (AY673996) (Hagopian et al. 2004) and *Chondrus crispus* (HF562234), but was different from the case of Bangiales (*P. yezoensis*, *Pyropia haitanensis* and *Pyropia purpurea*) (Tajima et al. 2012; Wang et al. 2013). The nucleotide sequences of *M. japonica* phycobiliprotein genes showed considerably high identities (78 ~ 98%) with those of other red algae, especially *C. crispus* (96 ~ 98%) which belongs to Gigartinaceae similar to *M. japonica* (Table 1). The GC contents in *M. japonica* phycobiliproteins were from 34.6% to 38.8% (*rpeB*: 38.8%, *rpeA*: 37.2%, *rpcB*: 36.8%, *rpcA*:
36.4%, \textit{rapcA}: 35.4%, \textit{rapcB}: 34.6%). The GC content of \textit{M. japonica} phycobiliprotein genes (Av=36.5%) showed very high similarity to those of \textit{G. tenuistipitata} (36.8%) and \textit{C. crispus} (36.1%), whereas it was a little lower than those of \textit{P. palmata} (39.3%), \textit{P. haitanensis} (39.2%), \textit{P. yezoensis} (39.2%) and \textit{P. purpurea} (39.4%) (Table 1). The Shine-Dalgarno sequence (5’-AGGAGGT-3’) acting as a binding site with 16S rRNA was searched in the \textit{M. japonica} phycobiliprotein genes. As a result, putative motifs were found at upstream regions of the phycobiliprotein genes (\textit{rpeB}: AAGGAGA, \textit{rpeA}: AGGATA, \textit{rpcB}: AAGGAGA, \textit{rpcA}: AAGGAAA, \textit{rapcA}: AAGGAGT, and \textit{rapcB}: AAGGAAA) (dotted underlines in Fig. 1a, b, c).

Primary structures of \textit{M. japonica} phycobiliproteins

The deduced amino acid sequences of \(\alpha\)- and \(\beta\)-subunits of \textit{M. japonica} PE, PC and APC are shown in Fig. 1. The \(\alpha\)-subunits of PE, PC and APC were composed by 164 (MW: 17,772; pI: 5.19), 162 (MW: 17,566; pI: 6.57) and 161 (MW: 17,465; pI: 5.06) amino acids, respectively, and the \(\beta\)-subunits of them were 177 (MW: 18,333; pI: 5.38), 172 (MW: 18,100; pI: 4.94) and 161 (MW: 17,589; pI: 5.47) amino acids, respectively. It is well known that three kinds of chromophores (phycoerythrobilin, phycocyanobilin and phycourobilin) covalently bind to some Cys residues in red algal phycobiliproteins (Apt \textit{et al}. 1995). The residues for chromophore attachment sites were all conserved in \textit{M. japonica} phycobiliproteins (PE: \(\beta\)Cys50, \(\beta\)Cys61, \(\beta\)Cys82, \(\beta\)Cys158, \(\alpha\)Cys82, \(\alpha\)Cys139; PC: \(\beta\)Cys82, \(\beta\)Cys153, \(\alpha\)Cys84; APC: \(\alpha\)Cys81, \(\beta\)Cys81) (Fig. 1). For amino acid sequence, \textit{M. japonica} phycobiliproteins showed remarkably high identities (95 ~ 100%) with those of \textit{C. crispus} (Table 1).

In 1992, Ficner \textit{et al}. reported the three-dimensional structure of \((\alpha\beta)_6\) hexamer of PE
isolated from red alga *Porphyridium sordidum*. They revealed that the intersubunit pairs of αSer3-βAsp3, αAsp13-βArg93, αAsp13-βTyr94, αAsp13-βArg110 and αArg17-βTyr97 are formed in (αβ) heterodimer. The specific hydrogen bonds of neighboring (αβ) in (αβ)₃ trimeric disc are also formed: 1αArg93-2βTyr76, 1α111 main chain carbonyl oxygen-2βAsn78, 1βAsp13-2βTyr76. In addition, (αβ)₃ trimer aggregates each other by electrostatic interactions (1αLys2-6αAsp23, 1αArg17-6αAsp108). Moreover, αAsn47, αAla74, αLys80, αArg86, αAsp87, αArg139, αArg140d, βAsn35, βAsp39, βAsp54, βThr77, βArg79, βArg80, βArg86, βAsp87, βArg131, βThr148a, βAla151, βGly153 are important in polar and ionic chromophore-protein interactions. In the α- and β-subunits of *M. japonica* PE, these amino acid residues were highly conserved (αLys2, αSer3, αAsp13, αArg17, αAsp23, αAsn47, αAla72, αLys78, αLys81, αArg84, αAsp85, αArg91, αAsp106, αArg137, αArg142, βAsp3, βAsp13, βAsn35, βAsp39, βTyr74, βThr75, βAsn76, βArg77, βArg78, βAla81, βArg84, βAsp85, βArg91, βTyr92, βTyr95, βArg108, βArg129, βGly156) (Fig. 2a).

Similar to PE, it has been clarified that several amino acids in PC molecule are related to the specific intersubunit co-relation (Apt et al., 1995; Schirmer et al. 1986). That is to say, the residues for interaction between α- and β-subunits were conserved in *M. japonica* PC (αAsp13, αArg93, αTyr97, βAsp13, βArg91, βTyr92, βTyr95) (Fig. 2b). In addition, αArg17 involving (αβ)₃ trimer-trimer interaction was exist in PC α-subunit of *M. japonica*. Moreover, the residues related to chromophore interaction (αArg86, αAsp87, βAsn72, βArg77, βArg78, βAla81) were found in *M. japonica* PC (Fig. 2b).

The crystal structure of APC from red alga *P. yeoensis* was determined by molecular replacement method (Liu et al. 1999). In the crystal, two (αβ)₃ trimers associate face to face into a (αβ)₆ hexamer. In the (αβ) heterodimer, the ionic- and polar-interacting pairs between the two subunits are αSer2-βAsp3, αAsp13-βTyr94, αAsp13-βArg110, αArg17-βTyr97,
\[\alpha\text{Tyr}_{18}\beta\text{Arg}_{93}, \alpha\text{Asp}_{89}\beta\text{Tyr}_{18}, \alpha\text{Arg}_{93}\beta\text{Asp}_{13}\text{ and }\alpha\text{Tyr}_{97}\beta\text{Asp}_{13}.\] In addition, the polar and charged interactions between the two \((\alpha\beta)_3\) trimers are \(1\alpha\text{Asp}_{25}\beta\text{Arg}_{37}, \ 1\alpha\text{Gly}_{22}\beta\text{Arg}_{26}, \ 1\alpha\text{Asp}_{25}\beta\text{Glu}_{151}, \ 1\alpha\text{Asp}_{25}\beta\text{Tyr}_{155}\text{ and }1\alpha\text{Lys}_{28}\beta\text{Asp}_{147}.\]

The chromophores at \(\alpha\text{Cys}_{84}\) and \(\beta\text{Cys}_{84}\) have similar hydrophobic environment; i.e., there are three aromatic residues close to \(\alpha\text{Tyr}_{90}, \alpha\text{Tyr}_{91}, \alpha\text{Tyr}_{119}, \beta\text{Tyr}_{90}, \beta\text{Tyr}_{91},\) and \(\beta\text{Tyr}_{119}.\)

In the \(\alpha\)- and \(\beta\)-subunits of \(M.\ japonica\) APC, these amino acid residues were highly conserved \((\alpha\text{Ser}_{2}, \alpha\text{Asp}_{12}, \alpha\text{Arg}_{16}, \alpha\text{Tyr}_{17}, \alpha\text{Gly}_{21}, \alpha\text{Asp}_{24}, \alpha\text{Arg}_{25}, \alpha\text{Lys}_{27}, \alpha\text{Arg}_{36}, \alpha\text{Asp}_{86}, \alpha\text{Tyr}_{87}, \alpha\text{Tyr}_{88}, \alpha\text{Arg}_{90}, \alpha\text{Tyr}_{94}, \alpha\text{Tyr}_{116}, \alpha\text{Asp}_{145}, \alpha\text{Glu}_{148}, \alpha\text{Tyr}_{152}, \beta\text{Asp}_{3}, \beta\text{Asp}_{13}, \beta\text{Tyr}_{18}, \beta\text{Tyr}_{87}, \beta\text{Tyr}_{88}, \beta\text{Arg}_{90}, \beta\text{Tyr}_{91}, \beta\text{Tyr}_{94}, \beta\text{Arg}_{107}, \beta\text{Tyr}_{116})\) (Fig. 2c).

The 3-D structures of \(\alpha\)- and \(\beta\)-subunits of \(M.\ japonica\) phycobiliproteins were predicted on the basis of their primary structures. As shown in Fig. 3, the predicted backbone conformations of all subunits are highly similar, and they have nine \(\alpha\)-helixes (X, Y, A, B, E, F, F’, G, and H) as a dominant secondary structure element. These properties are similar to the known 3-D structures (Miyabe et al. 2017; Ficner et al. 1992; Schirmer et al. 1986; Jiang et al. 2001). From the results, it is suggested that the \(\alpha\)- and \(\beta\)-subunits of \(M.\ japonica\) phycobiliproteins would form an \((\alpha\beta)_3\) trimer in the phycobilisome.

ACE inhibitory activity of M. japonica protein hydrolysates

ACE is a key enzyme in the regulation of peripheral blood pressure catalyzing the production of angiotensin II in the renin-angiotensin-aldosterone system and the destruction of bradykinin in the kinin-kalikrein system (Cheung et al. 1980). The specific inhibitors of the enzyme therefore have been considered with effective antihypertensive drugs. In addition to the drugs, ACE inhibitory peptides from daily food also useful for maintaining blood pressure at a healthy level. Although the potency of peptide is lower than drug, it does not have side
effect (Balti et al. 2015). It is well known that the peptides containing hydrophobic amino acid residues with aromatic (Y, F, W) or branched (L, I, V) side chains possess a strong ACE inhibitory effect (Saiga et al., 2008). For instance, Suetsuna (1998) obtained four ACE inhibitory peptides (IY, MKY, AKYSY and LRY) from red alga P. yezoensis. In 2004, Suetsuna et al. also isolated ten ACE inhibitory peptides (YH, KW, KY, LF, FY, VW, VF, IY, IW and VY) from brown alga U. pinnatifida (Wakame), and they clarified both single and repeated oral administrations of synthetic peptide (YH, KY, FY or IY) significantly decreased the blood pressure in spontaneously hypertensive rats. Recently, we found that the dulse phycobiliprotein hydrolysates possess strong inhibitory effect to ACE (Furuta et al., 2016). The dulse protein hydrolysates showed high inhibitory activity, and nine ACE inhibitory peptides (YRD, AGGEY, VYRT, VDHY, IKGHY, LKNPG, LDY, LRY, FEQDWAS) were isolated. Then, the sequences of YRD, AGGEY, VYRT, VDHY, LKNPG LDY and LRY were detected in the primary structures of dulse phycobiliproteins. From these results, it was suggested that the dulse ACE inhibitory peptides were mainly derived from phycobiliproteins.

In this study, the primary structures of M. japonica phycobiliproteins were determined, and they showed high amino acid sequence identities (81 ~ 100%) with those of other red algae (Table 1). It was clarified that M. japonica phycobiliproteins are rich in hydrophobic amino acid residues (46.0 ~ 52.3%), especially aromatic (Y and F) or branched (V, L and I) side chain residues. In addition, similar sequences of above ACE inhibitory peptides (YRD: PE α-subunit; LDY: PE α-subunit, PC α-subunit, APC α-subunit and APC β-subunit; LRY: PE β-subunit, PC β-subunit and APC α-subunit; VY: PE α-subunit, PC α-subunit and PC β-subunit; LF: PC β-subunit and APC α-subunit; FY: APC α-subunit; TKYSY: PE α-subunit; VYRA: PE α-subunit) were detected in the primary structures of M. japonica phycobiliproteins (Fig. 2). So, it was suggested that ACE inhibitory peptides are also derived from M. japonica phycobiliproteins. Then, we prepared the protein hydrolysates
from *M. japonica* and measured its ACE inhibitory activity. As a result, *M. japonica* protein hydrolysates showed significantly higher inhibitory activity than that of dulse (Fig. 4). Therefore, we concluded that *M. japonica* also has the potential to be an ingredient of functional food.

**CONCLUSION**

We determined the primary structures of phycoerythrin, phycocyanin and allophycocyanin from red alga *Mazzaella japonica*. The amino acid sequences of *M. japonica* phycobiliproteins showed considerably high identities with those of other red algae. In addition, the sequences (YRD, LDY, LRY, VY, LF and FY), which were angiotensin I converting enzyme (ACE) inhibitory peptides from other algae, were detected in the primary structures of *M. japonica* phycobiliproteins. Then, we prepared the protein hydrolysates from *M. japonica* and measured its ACE inhibitory activity. Consequently, *M. japonica* protein hydrolysates indicated considerably high ACE inhibitory activity. Therefore, we concluded that *M. japonica* has the potential to be an ingredient of functional food.

**Acknowledgment**

This study was supported in part by the “Science and technology research promotion program for agriculture, forestry, fisheries and food industry”.
REFERENCES


Food Chem. 170, 519-525.


Electrophoresis 15, 529-539.

CHEUNG, H.S. and CUSHMAN, D.W. 1973. Inhibition of homogeneous


Molecular Biology. John Wiley & Sons Inc, Chapter 2: Unit 2.4
FIG. 1  NUCLEOTIDE AND DEDUCED AMINO ACID SEQUENCES OF M. JAPONICA PHYCOBILIPROTEIN GENES.

a: M. japonica phycoerythrin gene.
b: M. japonica phycocyanin gene.
c: M. japonica allophycocyanin gene.

PE, PC, and APC represent phycoerythrin, phycocyanin and allophycocyanin, respectively. Asterisks show stop codon. Dotted underline is putative RNA polymerase-binding motif (Shine-Dalgano Sequence).

FIG. 2  ALIGNMENT OF AMINO ACID SEQUENCES OF PHYCOBILIPROTEIN SUBUNITS FROM SOME RED ALGAE.

a: Phycoerythrin.
b: Phycocyanin.
c: Allophycocyanin.

PEβ, PEα, PCβ, PCα, APCα and APCβ represent phycoerythrin β-subunit, phycoerythrin α-subunit, phycocyanin β-subunit, phycocyanin α-subunit, allophycocyanin α-subunit and allophycocyanin β-subunit, respectively. Asterisks show characteristic amino acid residues in the molecule.

M. japonica: Mazzaella japonica (in this study).
P. palmata: Palmaria palmata (AB625450.2, AB679831.1, AB742300.1).
G. tenuistipitata: Gracilaria tenuistipitata (NC_006137.1).
C. crispus: Chondrus crispus (NC_020795.1).
P. yezoensis: Pyropia yezoensis (KC517072.1).
FIG. 3  PREDICTED 3-D STRUCTURES OF M. JAPONICA PHYCOBILIPROTEIN SUBUNITS.

PEβ: Phycoerythrin β-subunit.
PEα: Phycoerythrin α-subunit.
PCβ: Phycocyanin β-subunit.
PCα: Phycocyaninα-subunit.
APCα: Allophycocyanin α-subunit.
APCβ: Allophycocyanin β-subunit.

FIG. 4  ACE INHIBITORY ACTIVITY OF PROTEIN HYDROLYSATES PREPARED FROM M. JAPONICA.

P. palmata: protein hydrolysates prepared from Palmaria palmata.
M. japonica: protein hydrolysates prepared from Mazzaella japonica.
Data are the mean ± SEM from triplicate determination.
*: P < 0.05 (the data of M. japonica compared with that of P. palmata by Student’s t test).
<table>
<thead>
<tr>
<th>Organism</th>
<th>Gene name</th>
<th>GC content (%)</th>
<th>Nucleotide identity to <em>M. japonica</em> (%)</th>
<th>Amino acid identity to <em>M. japonica</em> (%)</th>
<th>Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Mazzaella japonica</em></td>
<td>PE β-subunit</td>
<td>38.8</td>
<td>96</td>
<td>95</td>
<td>AB698819.2</td>
</tr>
<tr>
<td></td>
<td>α-subunit</td>
<td>37.2</td>
<td>-</td>
<td>-</td>
<td>AB698819.2</td>
</tr>
<tr>
<td></td>
<td>PC β-subunit</td>
<td>36.8</td>
<td>-</td>
<td>-</td>
<td>LC222737</td>
</tr>
<tr>
<td></td>
<td>α-subunit</td>
<td>36.4</td>
<td>-</td>
<td>-</td>
<td>LC222737</td>
</tr>
<tr>
<td></td>
<td>APC α-subunit</td>
<td>35.4</td>
<td>-</td>
<td>-</td>
<td>LC222738</td>
</tr>
<tr>
<td></td>
<td>β-subunit</td>
<td>34.6</td>
<td>-</td>
<td>-</td>
<td>LC222738</td>
</tr>
<tr>
<td><em>Chondrus crispus</em></td>
<td>PE β-subunit</td>
<td>37.2</td>
<td>96</td>
<td>95</td>
<td>NC_020795.1</td>
</tr>
<tr>
<td></td>
<td>α-subunit</td>
<td>39.1</td>
<td>97</td>
<td>96</td>
<td>NC_020795.1</td>
</tr>
<tr>
<td></td>
<td>PC β-subunit</td>
<td>35.6</td>
<td>98</td>
<td>100</td>
<td>NC_020795.1</td>
</tr>
<tr>
<td></td>
<td>α-subunit</td>
<td>35.6</td>
<td>97</td>
<td>100</td>
<td>NC_020795.1</td>
</tr>
<tr>
<td></td>
<td>APC α-subunit</td>
<td>34.6</td>
<td>98</td>
<td>100</td>
<td>NC_020795.1</td>
</tr>
<tr>
<td></td>
<td>β-subunit</td>
<td>34.2</td>
<td>98</td>
<td>100</td>
<td>NC_020795.1</td>
</tr>
<tr>
<td><em>Gracilaria tenuistipitata</em></td>
<td>PE β-subunit</td>
<td>38.8</td>
<td>80</td>
<td>81</td>
<td>NC_006137.1</td>
</tr>
<tr>
<td></td>
<td>α-subunit</td>
<td>37.0</td>
<td>84</td>
<td>86</td>
<td>NC_006137.1</td>
</tr>
<tr>
<td></td>
<td>PC β-subunit</td>
<td>37.6</td>
<td>83</td>
<td>82</td>
<td>NC_006137.1</td>
</tr>
<tr>
<td></td>
<td>α-subunit</td>
<td>36.8</td>
<td>81</td>
<td>93</td>
<td>NC_006137.1</td>
</tr>
<tr>
<td></td>
<td>APC α-subunit</td>
<td>36.2</td>
<td>82</td>
<td>96</td>
<td>NC_006137.1</td>
</tr>
<tr>
<td></td>
<td>β-subunit</td>
<td>34.6</td>
<td>84</td>
<td>92</td>
<td>NC_006137.1</td>
</tr>
<tr>
<td><em>Palmaria palmata</em></td>
<td>PE β-subunit</td>
<td>40.5</td>
<td>79</td>
<td>83</td>
<td>AB625450.2</td>
</tr>
<tr>
<td></td>
<td>α-subunit</td>
<td>40.2</td>
<td>82</td>
<td>87</td>
<td>AB625450.2</td>
</tr>
<tr>
<td></td>
<td>PC β-subunit</td>
<td>38.9</td>
<td>79</td>
<td>91</td>
<td>AB679831.1</td>
</tr>
<tr>
<td></td>
<td>α-subunit</td>
<td>39.3</td>
<td>79</td>
<td>91</td>
<td>AB679831.1</td>
</tr>
<tr>
<td></td>
<td>APC α-subunit</td>
<td>40.7</td>
<td>79</td>
<td>95</td>
<td>AB742300.1</td>
</tr>
<tr>
<td></td>
<td>β-subunit</td>
<td>36.0</td>
<td>81</td>
<td>94</td>
<td>AB742300.1</td>
</tr>
<tr>
<td><em>Pyropia haitanensis</em></td>
<td>PE β-subunit</td>
<td>41.4</td>
<td>81</td>
<td>88</td>
<td>HM008260.1</td>
</tr>
<tr>
<td></td>
<td>α-subunit</td>
<td>41.2</td>
<td>81</td>
<td>85</td>
<td>HM008260.1</td>
</tr>
<tr>
<td></td>
<td>PC β-subunit</td>
<td>39.9</td>
<td>80</td>
<td>91</td>
<td>HM008261.1</td>
</tr>
<tr>
<td></td>
<td>α-subunit</td>
<td>38.2</td>
<td>80</td>
<td>91</td>
<td>HM008261.1</td>
</tr>
<tr>
<td></td>
<td>APC α-subunit</td>
<td>38.7</td>
<td>81</td>
<td>94</td>
<td>AY372218.1</td>
</tr>
<tr>
<td></td>
<td>β-subunit</td>
<td>35.6</td>
<td>81</td>
<td>93</td>
<td>AY372218.1</td>
</tr>
<tr>
<td><em>Porphyra yezoensis</em></td>
<td>PE β-subunit</td>
<td>41.2</td>
<td>80</td>
<td>88</td>
<td>KC517072.1</td>
</tr>
<tr>
<td></td>
<td>α-subunit</td>
<td>42.2</td>
<td>81</td>
<td>85</td>
<td>KC517072.1</td>
</tr>
<tr>
<td></td>
<td>PC β-subunit</td>
<td>38.9</td>
<td>81</td>
<td>91</td>
<td>KC517072.1</td>
</tr>
<tr>
<td></td>
<td>α-subunit</td>
<td>39.3</td>
<td>79</td>
<td>91</td>
<td>KC517072.1</td>
</tr>
<tr>
<td></td>
<td>APC α-subunit</td>
<td>37.2</td>
<td>83</td>
<td>95</td>
<td>KC517072.1</td>
</tr>
<tr>
<td></td>
<td>β-subunit</td>
<td>36.4</td>
<td>80</td>
<td>93</td>
<td>KC517072.1</td>
</tr>
<tr>
<td><em>Porphyra purpurea</em></td>
<td>PE β-subunit</td>
<td>42.0</td>
<td>81</td>
<td>87</td>
<td>NC_000925.1</td>
</tr>
<tr>
<td></td>
<td>α-subunit</td>
<td>41.8</td>
<td>80</td>
<td>85</td>
<td>NC_000925.1</td>
</tr>
<tr>
<td></td>
<td>PC β-subunit</td>
<td>40.7</td>
<td>80</td>
<td>91</td>
<td>NC_000925.1</td>
</tr>
<tr>
<td></td>
<td>α-subunit</td>
<td>38.7</td>
<td>78</td>
<td>90</td>
<td>NC_000925.1</td>
</tr>
<tr>
<td></td>
<td>APC α-subunit</td>
<td>36.8</td>
<td>83</td>
<td>95</td>
<td>NC_000925.1</td>
</tr>
<tr>
<td></td>
<td>β-subunit</td>
<td>36.2</td>
<td>81</td>
<td>93</td>
<td>NC_000925.1</td>
</tr>
</tbody>
</table>
FIG. 1

5' –

GATACAAATATATTATTTGTGACACTAAATACATCGGCTTGTCAAAATATGACAGCTGAACAGCTAAGTCCCTTTTACAAAAATACAAAGGAGAGTCC
100
ATGCTGTATCGATTTCTAGATTTGTAGTTAATCTCATCAGACCTGAAGCTTTAGTGAGTGTCAGTCTACAGTCTCTTTACAAAATCTGAGCAAT
200
MLDAFSRVRVVSNDAKAAVVGGGSDDLQALTFTIAD
300
GTACAAACGCTTTAGTCTGAATTCTTTCTCTAGCTGAGCCTATGCAGACCTTTAGTGATTTGTTTTTCGCAAATCTGGCTATAT
400
GNKRLDVNVSNASCVSDAVSGMICENSGGLI
TGCTCCAGGAGCCAATTGCTATACATATCTAGCTCAGCTGCTTTCCAGTGATGGAAGAATTATATCGTAAAAATATCAAT
500
APGGNCYTNRMAACLDRGEDIIARRYISYALLAG
GATCGTCTGTATTAGAAGATCTGCTGCAATTATTTGATTTAAAAGAAAATATATATTGGCTTGGCGTACCATATTATTTCTGCTGATGCTGAAAT
600
DASVLEDRCNLNGKETYIALGVPNSSSARSVAI
TGAATCTGACGCTTGGATTATGTTAGATATCCAGCACCTCAACATGCGCAACATGACTATGGCTTTCTCGCTATTCTCGAGAGTGGCAAG
700
MKSAAVAFVSNSASQRTMPTTSGDCSALSAEVA
CTACTGTGATAGATTACCCGCTGCACTTTACAGCAATAAGCATAATAATTAAAAATAGTACTCAATACATATTAGGATATAAAAATGA
800
YCDRTVAALG*
AATCGATATCTATCTATATCATCAAGCTGTAGCTGCTGCTGTGTCCTTCTGACTAGCTCAGTCTTGAAATCAGTCAAAAGAATATTACAGTGAAG
900
KSIVITTISSAADAGGGFLTSSDLSESQVQGNIQRAG
TGCAAGATATAGACCAGTGCAAAAATCTGAGAATACTGAAAGCTTGAAATAGAGAAAAGGACAGGAGATCCTGTGTTTTACTAAATATTACATCTAAAT
TCAGGTGAAGCTGGACAGCTCAAGAAAAATTTAATATGTTAGATATAGATATCTATTATATGCTGCTAAATATGGTACGGAA
1,000
SGEAGDSQEKVNKCYRDIDHMYMRILNIALIVGG
CAGAGACTCTTGATAGGGGTATTGCTGGACGAGTTTTACAGACATTAATCTACCATCTGCTCTTATTTGGCTGGATGCTTTAATCG
1,100
TGPLDEWGIAGAREVYRALNLPASASYIIAIAAA
TGATAGATCTTGTTGCTGCTAGATATAGGCGGATACGGTGCTGCTATTGGTACATTATATTATATATTAACATCTCTAATTATATATATATATAT
1,200
DRLCVPQDMASAOAGLEYGAADLYVINSES*
CTTCGAAAACAATATATATAGTTAAAAATAAAAATAAAAAATATAATAATAGTGGTTTATTTATTTATTTATTTATTTATTTATTTATTTATTTATTTAAT
1,300

−3'
b

5' –

AATTCAATATTTTATCGATTTTGAATGATAAAATCTAATATCTTAGTATATAAACCTTTGGTATATAATTCCATGTGAGAGAAATCAAT
ATGTTAGATGGTTGCAAAGGTTAATCTGATCTCAAGTGAGATCTAGTAAATGAATTTACACATAATTTAGTCATTTTGAATGTTGCAGAAG
M L D A F A K I V A Q A D A R G E F L S N T Q L D A L V A M V A E
GATATAAAGATGAGATGTGGAATATAAATATATCCTAAGCTCAGCTATTTGCTAACTACACTTGCGTCTTTTGGCAGAACAACACACATATG
G N K R L D V N S N A S A I V T N S A R A L F A E Q P C O L V
TCAACCTGGAGGAAATGCTTATACGATAGAAAGATGAGCTCATCTTGTAGAGACAGATGAAATAGTTTAAAGATATGTAAGCTATGCTAGTATGTCGG
Q P G G N A Y T S R R M A A C L R D M E I V L R Y V S Y A M I A G
GATCTAGTGGTTTAAAGTGACAGTGTATTTGTTAAGAGAAACCTTATCAACGATATTAGCACACCAGGAATCTCTGATAGAGTCTGTCACAAAA
500
D S S V L D D R C L N G L R E T Y Q A L G T P G T S V A A V A V Q K
TGAAGAGGCTTTCAATTGCACTAGCTAATGACTTAAATGGAGACCTTTTGGAGACTGTGTTTCATGACTGCTGAACTAGTTGATATTTTGGACTGTC
M K E A S I A L A N D L N G V P L G D C S S L T A E L V V Y F D R A
TGCAGTTGCTAGTCTATAATTACACTAATAATACCAAGTTAAGAAATAAATATATATTATATAAGAAAAATTTATTATGAAACACCTTACACAGAAGCTA
A V A V V * M K T P I T E A
TTCATCGTCTGACAGTCAAGGAAATTTTGGAGAATGATATTCAATAATGAGATATCAAAGAGCTACGCTAGTACATAGGAGGACAAAA
800
I A S A D S Q O R F L S N G E L Q S I N G R Y Q R A N A S I E A A K
ATCTTTAATATTAAAGGACAAAGATAATTACTGAGACTGCTCGTCAAGCCTATATACAAAAATATCCATTGCTAACAAATTCGACGCCACACCTTATGCA
900
S L T N N A Q R L I T G A A A Q A Q A V Y T K Y P F V T Q M P G T Y A
TCAAGTGACTATGGAAAAGCCAAAGTGACAGTGTATTTGATATTATTTAAAGATAGACTAGTATTGCTTAGTTTGAGGACAGAGGACATGACG
1,000
S S A I G K A K C A R D I G Y Y L R M T T Y C L V G A T G P M D
AGATTTTGGTGCCCTGTTTAAAGGAATATTACCCAGGCTTTTGAACCTTTACCAAGTGAGCTATTGACATTTAAAGAATAGCTGAGTT
1,100
E Y L V A G L E E I N R S F E L S P S W Y I E A L Q Y I K V S H G L
AGCTGGAACAGACGAAATGAGAAACAAATACTATTTAGATTGACATATAACACATTATGTTAACGTATTTCTAGTTAAGACTTATATATTAAAGATGA
1,200
A G Q A A N E A N T Y L D Y A I N T L S *
CTAGAAAAAGCATATTATATATATATTGACCTTACCTTTCTGTTTTTATTTTATATTGCAATAAAATTATATAATTTTTTCTATATATTTCT
1,300
–3'
5' –

TAGCATAATTATGTATTTGTATAATAGTATTTATATATAATAATTGATTTCTGTATTTAAAATATACAAAATAGCTAAGGAGTTATAAA 100
ATGAGTATAGTAACTAAATCAATTTGAAATGTGAGATGAGATGATTATATTATCGCGGAGTTTTATATAGCTGATTAATAGTTTTTTTTATTCTGA 200
MSIVTKSIVNADEARYLSPGELDRIKSFVLSG 300
AAGCGAGATTAAGATTATGCTAGTAGTTAACAAGAATACAGAGCTAATTTGATTTAAAACAGTGTCGAGATTTATTTAAGGATTTATTTATCTGGAT 400
KRRRLRIAQVLDNDRELIVKQGGQLFQKRDPDVS
TCCAGGAGGAAAATGCTATGTTAAGAAATGACAGCTACTTTCTGTTACGGTTTTAGACTATTATTTAATGCTTAGTAAATATCTTACCCAGGGAAT 500
PGNAYGEEMTATCTLRDLRDLDDYLRLVTYGIVAGD
GTACACCATTGAAGAAATTGAGTTAGACATGCTGTTAAGGATTAATATATTAGTAACTCCATATTCTCTGTGATTAGAGATCTCGTCTATGA 600
VTPIEEIGLGVKEMYNSSLGTPISGVAGESRM
AAAGTGCACTTTGTTCAATGTCGAGAGACTGTCGACAGACAGGTTTTTTATTTGTTATGATTATTTAAGGAGAATGCAGTAATAAATAACTAAAGA 700
KSAACSLLSGEDSAEAGFYFDYTLGAMO*
TATATTTTATATATATATGTAAAGGAGGTTAATTTAGGAAGAATAGCAGTTAATCTCTGTAATTTATACACGCGAGTGCAAAGAAAATATTAGACG 800

MODAITSVINTADVQGKYLD
ATAACTGATTGAAATATACGTGTATTATTTCAAACGCGTGACATAAGTAGTACGAGTGCAGCTGAGCTACGAGCGGAATCGAGCGACTATCAAAGA 900
DNSLEKLRGYYFQTGELRVRAAATIKAATAATIKE
ATCAGTGCTAAGGTTGATTCCAATATTACTAGACCTGTTGATATTGATACACAAACAGCCTAGTACGACATATTAGAGATATTGATTAT 1,000
SVAKALLLSDITRPGGNMYTTRRYYAACIRDLDDY
TATTTAGATATGCTACTATTTGTTAGGAAAGGAGTTCCATTATCTCTTATAGAAGACGGTGCTTTGAATTTAAAAGAACACATAATATCATTAGGTT 1,100
LRYATYGMMLAGDPSSILDERVVLNLKETYNSLGV
TACCAATCGGACGAGTTCCACGATCATACAAAGAAGATTACGGCTACTAATGCTGCTGATGCTGAAAGAATAGGGCTATATTTTGAGGAG 1,200
PIGATIQTIMAKKEVTAKKLGVSDAGKEMSFLYFD
TTATAATTTGCTTGTAGTAAATTGTTATATATTATATATAAAATCAATAGGAAGCTGAAATAACTTCTTATGATAATTTTTAAGGTTTAAATATTAT 1,300
YICSGLS*
TAATATTTTATAGCCTGTATTTCTAGCAGCTGTTCTTTCCGAAATTTTTGTTGTTCTCTATCTCTTATATTCTTTTCTGTTCTGATTAAAGCAATAGT 1,400

−3’
**FIG. 2**

**a**

**PEβ**

<table>
<thead>
<tr>
<th>1</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>60</th>
<th>70</th>
<th>80</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>M. japonica</strong></td>
<td>MLDAFSRVVNSDKAYVGGSDLQALKTFIADGNKRLAVNISVNASCVSADAVSGMICENSGLIKPGNCYTRMRMA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>C. crispus</strong></td>
<td>MLDAFSRVVNSDKAYVGGSDLQALKTFIADGNKRLAVNISVNASCVSADAVSGMICENPLGIAPGNCYTTRRMMA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>G. tenuistipitata</strong></td>
<td>MLDAFSRVVDSDTKAYVGGSLQLRKFEGNQLAVNISVNASCVSADAVSGMICENPLGALTSPGNCYTTRRMMA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>P. palmata</strong></td>
<td>MLDAFSRVVNSDAKAYVGGSDLQALKFIDGNKRLAVNISVNASCVSADAVSGMICENPLGIAPGNCYTTRRMMA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>P. yeozaensis</strong></td>
<td>MLDAFSRVVNSDKAYVGGSDLQALKFIDGNKRLDVNASCVSADAVSGMICENPLGIAPGNCYTTRRMMA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**ACLRDGEIILRYISYALLAGDASVLEDRCNGLKETYIALGVNNSSSARSVA**MKSAAVAFVSNASQRMTPTTSGDCCASSISAEVASYCDRTVAALG

**ACLRDGEIILRYISYALLAGDASVLEDRCNGLKETYIALGVNNSSRSVMKAAAVAFVNNTASQRKMATTSGDCCASSISAEVASYCDRGAALS

**ACLRDGEIILRYISYALLAGDPVLREDRCNGLKETYIALGPITSSAVIMKASVAAFLNTAPGRMDTASGDTCLASEVGYSYDRTCAIIS

**ACLRDGEIILRYASYALLAGDPVLREDRCNGLKETYIALGVPTNSSVRAVIMKASATAFVSGTASDRKMACPDGDCALLASELGYCDRTAIS

**ACLRDGEIILRYVSYALLAGDPVSLEDRCNGLKETYIALGVPTNSSVRAVIMKAAAVAFIITASQRKMATADGGDCALLASEVSYCDRTAIS

**PEα**

<table>
<thead>
<tr>
<th>1</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>60</th>
<th>70</th>
<th>80</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>M. japonica</strong></td>
<td>MKSVITIIISAADADGRFLTSSDLESVQNIORAGARLEAEKLANHEAVKFEAGDACFTKSYLNSGAECDSEQEKVN</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>C. crispus</strong></td>
<td>MKSVITIIISAADADGRFLTSSDLESVQNIORAGARLEAEKLANHEAVKFEAGDACFRKSYLNSGAECDSEQEKVN</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>G. tenuistipitata</strong></td>
<td>MKSVITIIISAADAFRGPSDLESVQNIORASARLEAEKLANHDAVKFEAGDACFGKSYLNSGAECDSEQEKVN</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>P. palmata</strong></td>
<td>MKSVMTTIISAADGRPSDLESVQNIORARLEAEKLANHEAVKFEAGDACFTKSYLNSGAECDSEQEKVN</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>P. yeozaensis</strong></td>
<td>MKSVTITIIISAADAFRGPSDLESVQNIORAAELEKLANHEAVKFEAGDACFTKSYLNSGAECDSEQEKVN</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**KCYRDHVMRLINYALVGGTGPLEWDGIAAGARYRLNLPSAYIAAFAFTDRDLCVPSDMSAQAGLEYAGLARYINSLS

**KCYRDHVMRLINYALVGGTGPDEWDGIAAGARYRLNLPSAYIYAFTRDLCVPSDMSAQAGLEYAGLARYINSLS

**KCYRDHVMRLVNYSLVGGTGPDEWAIAGAREYRTLNLPSAYVAFTRDLCVPSDMSAQAGVEYTTYALIDYINSLS

**KCYRDHVMRLVNYSLVGGTGPDEWAIAGAREYRTLNLPSAYVAAFTDRDLCVPSDMSAQAGGYVAALDLYINLALT

**KCYRDHVMRLVNYCLVGGTGPDEWDGIAAGAREYRTLNLPSAYVAFARDRLCVPSDMSAQAGVEYAGNLIDYINLSC

**** ** ** ** ** ** ** ** **
b

PC β

| M. japonica | MLDFAKIVAQADARFEGLSNTOLDLAMVAEGNKRLDENVNKSNASAVTSNARALFAEQPOLVQPNNAYTSRMA |
| C. crispus | MLDFAKIVAQADARFEGLSNTOLDLAMVAEGNKRLDENVNKSNASAVTSNARALFAEQPOLVQPNNAYTSRMA |
| G. tenuistipitata | MLDFAKIVAQADARFEGLSNTOLDLANMAEGNKRLDENVNKSNASAVTNARSFLFAEQPOLVQPNNAYTNRMA |
| P. palmata | MLDFAKIVAQADARFEGLSNTOLDLSTMVNEGGKRLDENVNKSNASAVTSNARALFAEQPOLVQPNNAYTSRMA |
| P. yezoensis | MLDFAKIVAQADARFEGLSNTOLDLSMMAEKNKRLDENVNKSNASAVTSNARALFAEQPOLVQPLQPNNAYTSRMA |

PC α

| M. japonica | MKTPITEIASDQGRFSLNGELQNSINGRYORANASIAEAKSLTNQAQLTGAAAVYTKPFTQMPGPYASSAIG |
| C. crispus | MKTPITEIASDQGRFSLNGELQNSINGRYORANASIAEAKSLTNQAQLTGAAAVYTKPFTQMPGPYASSAIG |
| G. tenuistipitata | MKTPITEIASDQGRFSLNGELQNSINGRYORASASLAEARSLTNSAQRLTGAAQSVYTKPFTQMPGPYASSAIG |
| P. palmata | MKTPITEIASDQGRFSLNSAEQLNGYERASSSAEEAAASLTNSAQRLTGAAOAVYTKPFTQMPGPYASSAIG |
| P. yezoensis | MKTPITEIASDQGRFSLNGELQAINGROASASSSLGAARSLTNQAQLTGAAQSVYTKPFTQMPGPYASSAIG |

KAKCARDGYYLRTMTYCLVGVATPMEYLVAGLEEINRSFELSPWSYIEALQYI1KVSHGLAGAANEANTYLDYAINLS
KAKCARDGYYLRTMTYCLVGVATPMEYLVAGLEEINRSFELSPWSYIEALQYI1KVSHGLAGAANEANTYLDYAINLS
KAKCARDGYYLRTMTYCLVGVATPMEYLVAGLEEINRSFELSPWSYIEALQYI1KNSHLSGAGAANEANTYLDYAINLS
KAKCARDGYYLRTMTYCLVGVATPMEYLVAGLEEINRSFELSPWSYIEALQYI1KNSHLSGAGAANEANTYLDYAINLS
KAKCARDGYYLRTMTYCLVGVATPMEYLVAGLEEINRSFELSPWSYVEALQY1KSHGLSG01GNEANVYLDYAINLS

*  **  *  **
<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>60</th>
<th>70</th>
<th>80</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>APC α</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. japonica</em></td>
<td>MSIVTSIVNADAERYLSPGELDRIKSFVLGSKRRLRIQVLTNDRELIVKGOGQQLFKRPPDVSPGNNAYGEEMTAT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. crispus</em></td>
<td>MSIVTSIVNADAERYLSPGELDRIKSFVLGSKRRLRIQVLTNDRELIVKGOGQQLFKRPPDVSPGNNAYGEEMTAT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>G. tenuistipitata</em></td>
<td>MSIVTSIVNADAERYLSPGELDRIKSFVLGSKRRLRIQVLTNDRELIVKGOGQQLFKRPPDVSPGNNAYGEEMTAT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. palmata</em></td>
<td>MSIVTSIVNADAERYLSPGELDRIKSFVLGSKRRLRIQVLTNDRELIVKGOGQQLFKRPPDVSPGNNAYGEEMTAT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. yezoensis</em></td>
<td>MSIVTSIVNADAERYLSPGELDRIKSFVLGSKRRLRIQVLTNDRELIVKGOGQQLFKRPPDVSPGNNAYGEEMTAT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>APC β</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>M. japonica</em></td>
<td>MODAITSVINTADVQKYLDDNSLEKLRLGYFOGLGRRQAIANAITAIKEVSIAKALLSYDIIPGNNMYTTRYAA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. crispus</em></td>
<td>MODAITSVINTADVQKYLDDNSLEKLRLGYFOGLGRRQAIANAITAIKEVSIAKALLSYDIIPGNNMYTTRYAA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>G. tenuistipitata</em></td>
<td>MODAITSVINAADVQGRYLDNLSLEKLRLGYFOGLGRRQAIANAITAIKEVSIAKALLSYDIIPGNNMYTTRYAA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. palmata</em></td>
<td>MODAITSVINAADVQKYLDDSSVEKLRLGYFOGLGRRQAIANAITAIKEVSIAKALLSYDIIPGNNMYTTRYAA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. yezoensis</em></td>
<td>MODAITSVINAADVQKYLDDSSVEKLRLGYFOGLGRRQAIANAITAIKEVSIAKALLSYDIIPGNNMYTTRYAA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- **APC α**:

<table>
<thead>
<tr>
<th>90</th>
<th>100</th>
<th>110</th>
<th>120</th>
<th>130</th>
<th>140</th>
<th>150</th>
<th>160</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- **APC β**:

<table>
<thead>
<tr>
<th>90</th>
<th>100</th>
<th>110</th>
<th>120</th>
<th>130</th>
<th>140</th>
<th>150</th>
<th>160</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
FIG. 3

PE β

PE α

PC β

PC α

APC α

APC β