Identification of ACE inhibitory peptides from red alga *Mazzaella japonica*

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Abstract Angiotensin I converting enzyme (ACE) inhibition is one of the key factors for cardiovascular disease, and inhibition of ACE activity is related to the prevention of high blood pressure. Until now, some ACE inhibitory peptides have been found in the protein hydrolysates of red algae, and most of them were derived from phycobiliproteins and rubisco. In this study, we modified the preparation method to evaluate the potential of ACE inhibitory activity from protein hydrolysate of red alga *Mazzaella japonica* except for phycobiliproteins and rubisco. As a result, we identified 11 peptides (YRD, VSEGLD, TIMPHPR, GGPAT, SSNDYPI, SRIYNVKSNG, VDAHY, CPYDWV, YGDPDHY, NLGN and DFGVPGHEP) in the hydrolysate by the reversed phase-HPLC and MALDI-TOF/MS/MS. Among them, YRD would be derived from phycoerythrin α-subunit. However, the others would be from other proteins encoded in chloroplast or nuclear genomes. This study revealed that not only phycobiliproteins and rubisco but also the other proteins in red algae have the potential for the source of ACE inhibitory peptide.

Keywords ACE inhibitory activity · Antihypertension · Red alga · *Mazzaella japonica* · Protein hydrolysate

Introduction

High blood pressure (a systolic blood pressure equal to or higher than 140 mmHg and a diastolic blood pressure equal to or higher than 90 mmHg) is a worldwide problem, because patient is estimated to be more than one billion people, in other word, one in three adults aged 25 and older [1]. High blood pressure has been thought one of the risk factors for cardiovascular diseases, and angiotensin I converting enzyme (ACE; EC 3.4.15.1) plays an important role in the regulation of blood pressure [2]. There are some anti-hypertensive drugs including captopril, enalapril, lisinopril and alaceril, but these chemically synthesized inhibitors usually associated with unacceptable side effects such as dry cough, angioedema, taste disturbance and skin rash [3, 4]. Therefore, ACE inhibitory peptides derived from nature sources are desirable for prevention of hypertension.

Proteins in foods have been interested in the attractive attention for bioactive peptide sources, since foods have been guaranteed the safety, low toxicity and metabolism in human body. The promotion of human health by bioactive peptides were reported as decrease in blood pressure, blood glucose level, cholesterol and lipid and increase in immune responses [5]. Above things, importance of food nutrition has been spread worldwide. In Japan, Ministry of Agriculture, Forestry and Fisheries promotes Shokuiku “Food and Nutrition Education” (https://www.maff.go.jp/e/policies/tech_res/shokuiku.html). The guideline promotes eating well-balanced meals combined with vegetables, fruits, milk products, beans and fish. These are followed by the evidence-based guideline, requiring information on nutritional and bioactive function of foods. In recent years, ACE inhibitory peptides have been found in enzymatic hydrolysates of various marine food proteins [6-10]. Macroalgal proteins are getting regarded as the relatively unused bioactive nitrogenous resources and thought as the protein sources for bioactive peptides [11]. However, the studies on the ACE inhibitory peptides derived from marine algae were limited [12-21] especially the relationship between their structures and the original proteins.

In the previous study, we revealed the ACE inhibitory activity of protein hydrolysate from red algae dulse *Palmaria palmata* in Japan and *Grateloupia asiatica* [22, 23]. The protein hydrolysates showed the high ACE inhibitory activity, and contained several novel ACE inhibitory peptides (e.g., LRY, IC_{50}: 0.044 μM and VYRT, IC_{50}: 0.14 μM) [22]. Although most of peptides were from phycobiliproteins and rubisco in the preparation method (cold water extraction), bioinformatics analysis suggested the potential of rest of proteins will be the source of ACE inhibition by determination of complete chloroplast and mitochondrial genome of dulse [24, 25].

Moreover, we recently investigated ACE inhibitory peptide from red alga *Mazzaella japonica* in Hokkaido because of the high protein content (about 25%) in dry weight [26]. *M. japonica* showed the high

viscosity in solution, resulting in the difficulty of extraction of protein. Therefore, we modified the protein preparation method by dulse and *G. asiatica*. Namely, the peptides from two red algal proteins were prepared from cold water extraction proteins and then hydrolyzed by proteolysis. On the other hand, the peptide from *M. japonica* was prepared by proteolysis without water extraction, and the hydrolysate was obtained by dialysis. ACE inhibitory activity of the protein hydrolysate from *M. japonica* showed the highest among them. In this study, we attempted to identify ACE inhibitory peptides in the protein hydrolysate from *M. japonica* to clarify the potential of protein hydrolysate except for phycobiliproteins and rubisco.

Materials and methods

Materials

M. japonica was collected on the coast of Kushiro, Hokkaido, Japan in July 2015, and preserved at -30 °C until use. ACE from rabbit lung was purchased from Sigma Chemical Co. (Mo, USA). Millex-GV (pore size: 0.22 μm) and Millex-LG (pore size: 0.20 μm) were purchased from Merck Millipore Ltd. (Darmstadt, Germany). Hyppuryl-L-histidyl-L-leucine (Hip-His-Leu), thermolysin (EC 3.4.24.27) from *Bacillus thermoproteolyticus*, trifluoroacetic acid (TFA), and all other regents were purchased from FUJIFILM Wako Pure Chemical (Osaka, Japan).

Preparation of protein hydrolysate from *M. japonica*

Preparation of the protein hydrolysate from *M. japonica* was carried out by the same method in our previous paper [26]. Frozen samples were lyophilized and ground into a fine powder by cyclone sample mill, CSM-S1 (Shizuoka Seiki Co., Ltd., Shizuoka, Japan). The powder was suspended in 25 v/w of distilled water and dialyzed against distilled water using dialysis membrane (molecular weight cut off: approximately 10 kDa, EIDIA Co., Ltd., Tokyo, Japan) at 4 °C for 4 h. Then, proteins in the dialysate were hydrolyzed by 1.0 wt% of thermolysin at 37 °C for 5 h, and the reaction was ended by heating at 100 °C for 10 min. The solution was dialyzed against distilled water at 4 °C for 12 h. The dialysis outer solution via a dialysis membrane was lyophilized into *M. japonica* protein hydrolysate.

Isolation of peptides from *M. japonica* protein hydrolysate

The protein hydrolysate of *M. japonica* were separated by RP-HPLC. The hydrolysate was dissolved (20 mg/mL) in ultra-pure water containing 0.1% TFA, and applied to the sequential filtration by Millex-GV and Millex-LG. An aliquot of the sample (100 μL) was applied to a Mightysil RP-18GP column (4.6 × 150 mm) (Kanto Kagaku, Tokyo, Japan), and peptides was eluted with a linear gradient of acetonitrile (1-20%) containing 0.1% TFA at a flow rate of 1.0 mL/min. Absorbance of the eluent was monitored at 228 nm. The eluted peptide fractions, named 1-27, were collected and subjected to ACE inhibitory assay [26].

Analysis of amino acid sequences of ACE inhibitory peptides

The peptide sequence was analyzed by the same method in our previous paper [22, 23]. The alignment of phycobiliproteins and rubisco from *M. japonica* was performed using ClustalW (https://www.genome.jp/tools-bin/clustalw) using the data sets of AB698819.2, LC222737 and LC222738. BlastP analysis was performed in the obtained peptides by using mitochondria (NC_001677.2), chloroplast (HF562234) and nuclear (GCF_000 350225) genome from *Chondrus crispus*.

ACE inhibitory peptide from database

ACE inhibitory peptides were obtained from the biopep-uwm database (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep) on 7 March 2020. From the database, ACE inhibitory peptide structures in the obtained peptides were analyzed.

Results and discussion

Separation of ACE inhibitory peptides from the protein hydrolysate

We previously showed the ACE inhibitory activity in the protein hydrolysate from *M. japonica* [26]. The IC_{50} value of the protein hydrolysate was determined as 0.262 mg/mL. The IC_{50} value of ACE inhibitor captopril was reported as 17-21 nM, which was 1,000 times higher than that of *M. japonica* [27, 28]. On the other hand, the IC_{50} values of marine protein hydrolysates were reported ranging from 0.17-501.7 mg/mL [15], and the protein

hydrolysates from red alga and Blue–green alga (*Spirulina platensis*) had the high ACE inhibitory activity in the report. The tryptic hydrolysate of red alga *Gracilariopsis lemaneiformis* showed 57% ACE inhibitory activity in the concentration of 2 mg/mL [19], and many researchers concluded that protein sources are the key factor for the bioactive peptides [5, 15, 19], indicating that *M. japonica* has the potential to be an ingredient of functional food.

We here attempted to identify the ACE inhibitory peptides from protein hydrolysate. To detect the ACE inhibitory peptides, the protein hydrolysate was separated by RP-HPLC (Fig. 1A). The eluted peaks, named fraction number (FN1–27), were pooled and subjected to ACE inhibitory assay (Fig. 1B). FN 11 shows the highest inhibitory activity followed by FN 6 and 20. FN 26 is undetectable and lowest one was FN 14. The ratio of ACE inhibitory activity in fractions were from 1.3 to 6.7% of the total ACE inhibition, indicating that each fraction was important for the total ACE inhibitory activity.

We previously evaluated the thermolysin-digested-protein hydrolysate from water soluble protein of red algae [22, 23]. The HPLC patterns and ACE inhibitory activity in fractions differed. Namely, the highest ACE inhibitory fractions of protein hydrolysates from *P. palmata* in Japan and *G. asiatica* were found in the 7-8% acetonitrile concentrations; whereas, that of *M. japonica* was found in 10% acetonitrile concentration (FN11). In addition, peptides from the protein hydrolysate were eluted higher than 16% acetonitrile concentration (FN23-27), which were not found in previous study. The difference in the HPLC pattern would be come from the sample preparation. Former protein hydrolysate samples were prepared from water soluble proteins by thermolysin hydrolysis. We also employed thermolysin for the preparation of protein hydrolysate in this study. *M. japonica* contains highly viscous polysaccharide, carrageenan, which was differed in dulse [29, 30]. Of the character of polysaccharide, we cannot separate soluble and insoluble fractions, resulting in the different peptide patterns. We then analyzed peptides in the fractions by MALDI-TOF/MS/MS.

Prediction of peptide structures from protein hydrolysate

MALDI-TOF/MS/MS analysis was performed in the fractions. Among them, peptides in fractions 7, 8, 11, 12, 14, 16, 17 and 22 were determined (Table 1). The obtained peptide sequences were as follows: peptides YRD and VSEGLD from FN7, peptides TIMPHPR and GGPAT from FN8, SSNDYPI from FN11, SRIYNVKSNG from FN12, VDAHY and CPYDWV from FN14, YGDPDHY from FN16, NLGN from FN17, and DFGVPGHEP from FN22. We previously showed that the main component of ACE inhibitory peptides in red algae was

phycobiliproteins and rubisco [23]. Therefore, we performed ClustalW search in the peptides using the protein sequences from *M. japonica*, resulting that YRD was conserved in PEα. The other peptide sequences were not found in the proteins. Since the complete chloroplast genome of *M. japonica* was not determined, we employed chloroplast genome from the same family Gigartinaceae, *Chondrus crispus* (Accession No. HF562234) for the finding of peptide sources. Although the identical peptide sequences were not found, the highly conserved sequences were listed in Table 1 (E-value less than 1.0), i.e., IMPAPR from dnaK and PHPR from rbcS for the sequence of TIMPHPR (FN8), SNDY from rpoB for the sequence of SSNDYPI (FN11), and RIFKSNG from ycf45 and RIFNV from atpB for the sequence of SRIYNVKSNG (FN12). We then attempted to BlastP search using nuclear genome from *Chondrus crispus* (Accession No. GCF_000350225). The same peptides of VSEGLD and GGPAT were found in unnamed protein product (upp) (XP_005712105 and XP_005715754, respectively). The peptide of NLGN was also found in 34 upp because of the short peptide sequence. Most of peptides identified from nuclear genome were upp. We also performed in the BlastP search using mitochondrial proteins *Chondrus crispus* (Accession No. NC_001677.2). Peptide VSIGL from 150-154 amino acid residues of nad1 was found as the homolog of VSEGLD with the 83% cover, 80% identity and 3.8 E-value. However, the upp (XP_005712105) from nuclear showed the high E-value (0.84), indicating that the peptide would come from the nuclear protein. Evolution of nuclear genome was faster than mitochondria and chloroplast genome [31-36]. It was not sure that peptide sequences from nuclear genome were conserved between *M. japonica* and *C. crispus*. However, our data showed that red alga proteins except for phycobiliproteins and rubisco were also the potential for ACE inhibitory peptides.

ACE inhibitory structure in peptides

The IC_{50} value of the obtained peptides were not reported. Therefore, we investigated the ACE inhibitory activity from the partial structure of the peptides using BIO-PEP database. ACE inhibitory peptides were found in all peptides except for CPYDWV (Table 2). Each peptide possessed several ACE inhibitory peptide-structures. Among them, peptides having strong IC_{50} value were found, i.e., IY (2.1 μM) in SRIYNVKSNG and PR (4.1 μM) in TIMPHPR. ACE inhibitor captopril is a small compound and inhibits ACE activity by binding of zinc ion, S1' and S2' pockets [6]. The peptides from *M. japonica* were relatively long, and inhibitory mechanisms were unclear, i.e., a part of peptide structures work as a ACE inhibitor, the peptides were precursors and work as ACE inhibitory peptides by proteolysis, etc. Recently, the molecular docking and kinetic analysis revealed the possible binding of

relatively long peptide and ACE [37], showing that mix inhibitory mechanism of competitive and noncompetitive inhibition were reported by binding of active and substrate binding sites. We found N- or C-terminal of peptides from *M. japonica* possessed the ACE inhibitory structure, meaning the potential for mix inhibitory mechanism. ACE inhibitory activity of the obtained peptides needs to investigate. Prior to the analysis, we need to clarify the peptide sources by determining genome sequencing. In this study, we found the potential ACE inhibitory peptides from red alga *M. japonica* proteins except for phycobiliproteins and rubisco. This finding would help for the comprehensive understanding of whole utilization of red algae.

Conclusions

In this study, we prepared the protein hydrolysate of red alga *M. japonica* by the modified preparation method, resulting that 11 peptides were identified. Peptide YRD would be derived from phycoerythrin α-subunit. However, the others would be from other proteins encoded in chloroplast or nuclear genomes. This study revealed that not only phycobiliproteins and rubisco but also the other proteins in red algae have the potentail for the source of ACE inhibitory peptide. In this study, we could not determine the peptide sources. Our next plan is to clarify the whole primary sequence by next generation sequencer and develop red algal proteins as bioactive peptide sources for human health.

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Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflicts of interest resulting from the contents of this article.

Compliance with ethics requirements

This article does not contain any studies with human or animal subjects.

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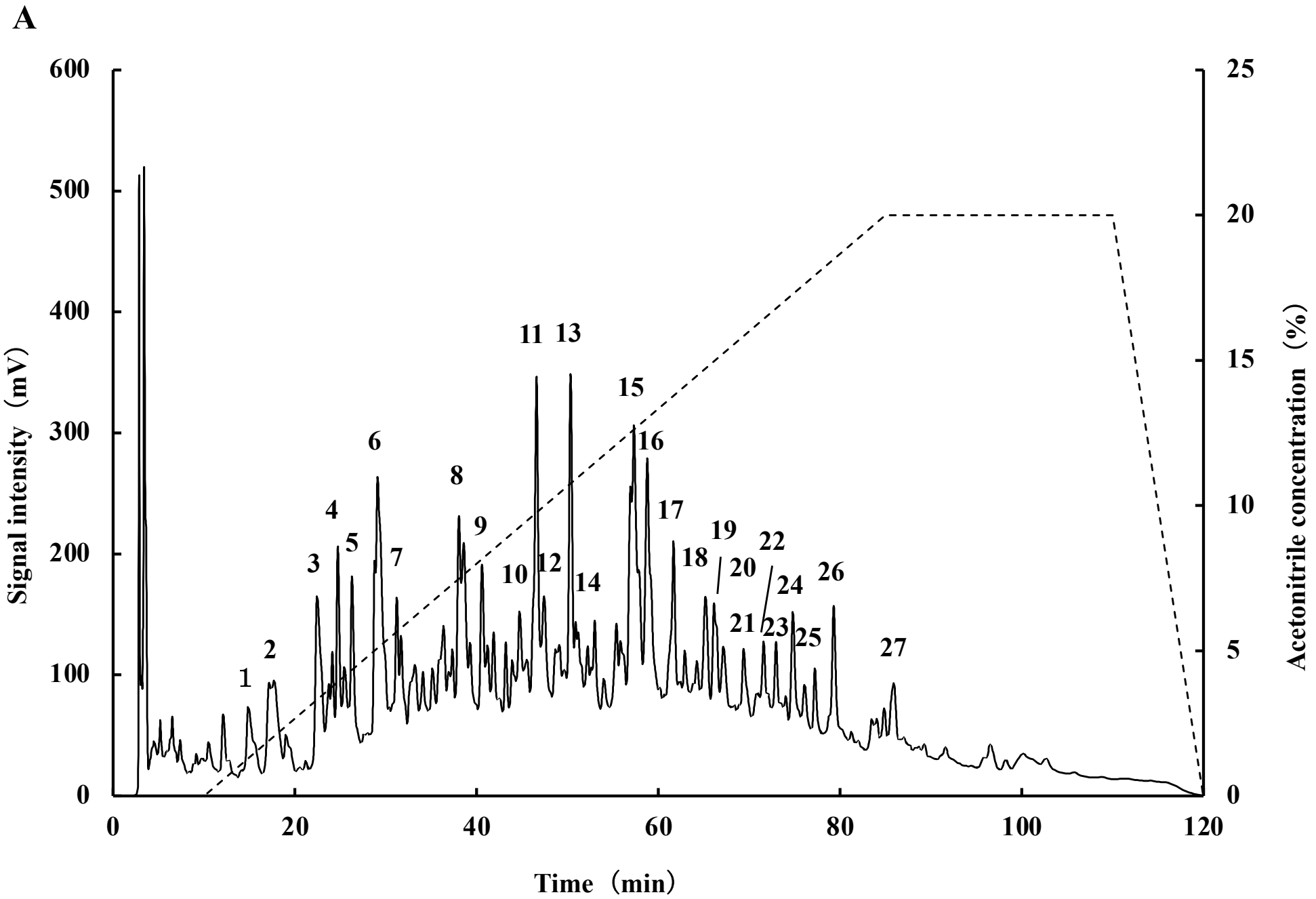
Captions to figure

Fig. 1 Isolation of ACE inhibitory peptides from *M. japonica* protein hydrolysates. (A) Separation of peptides on Mightysil RP-18GP column. The peaks marked 1-27 indicate pooled fractions. (B) ACE inhibitory activity of each fraction.

Fig. 1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Signal intensity (mV)
Acetonitrile concentration (%)
Time (min)
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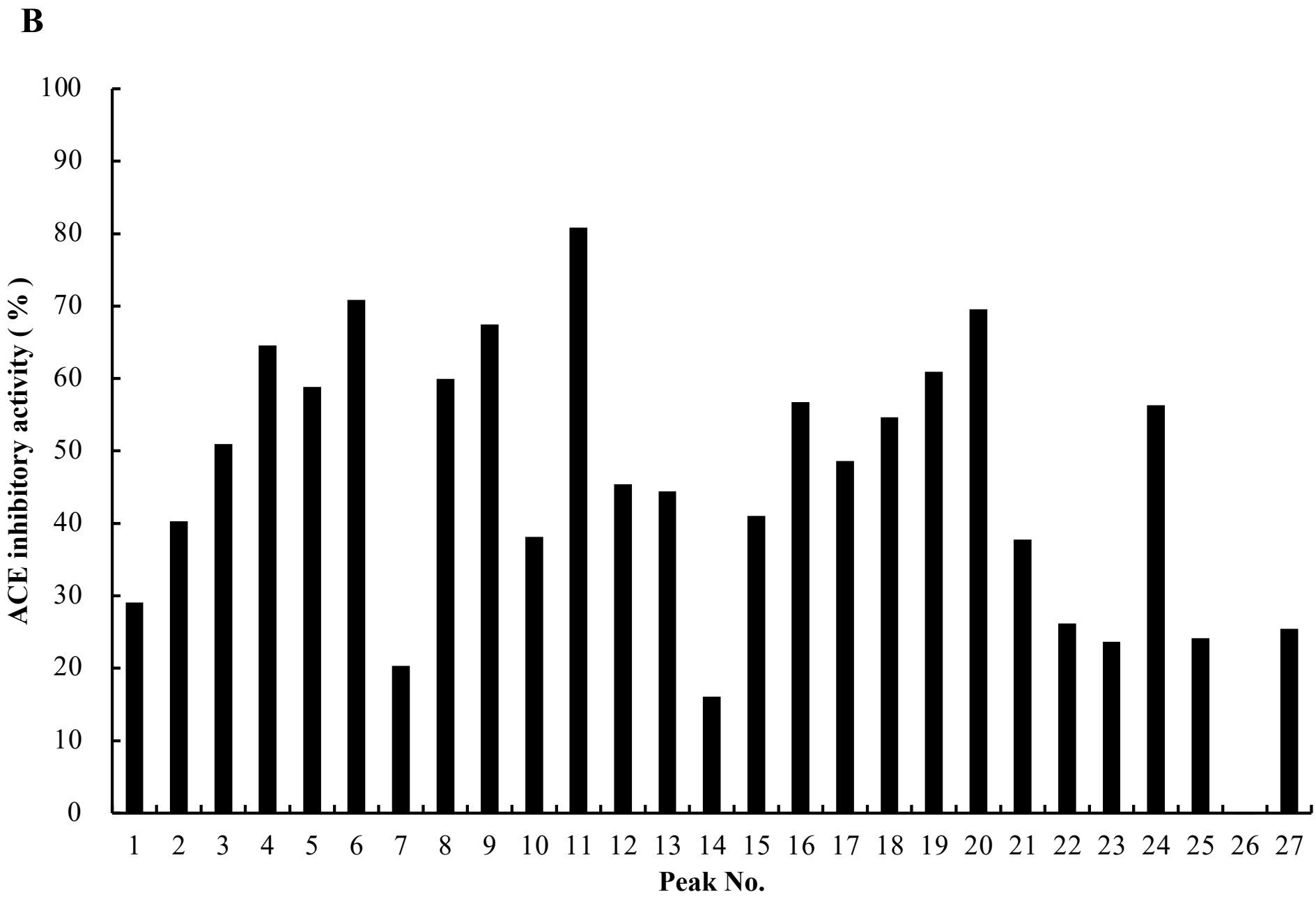
Fig. 1


B
ACE inhibitory activity (%)
100
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50
40
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0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27
Peak No.

Table 1 Peptide sequences and their homolog from *Chondrus crispus*

FN	MALDI-TOF/MS/MS (score)	Chloroplast (HF562234)							Nuclear (GCF_000350225)							
		Protein		Sequence [a]		Cover (%)	Identity (%)	E-value [b]	Protein		Sequence		Cover (%)	Identity (%)	E-value	Accession No.
7	YRD (35.86) VSEGLD (68.16)	PEα [c]	83	YRD	85	100	100	N.D.	upp [d]	194	VSEGLD	199	100	100	0.84	XP_005712105
8	TIMPHPR (80.18)	dnaK	457	IMPAPR	462	85	83	0.13	upp	122	MPHPR	126	71	100	0.53	XP_005715162
		rbcS	41	PHPR	44	57	100	0.37								
	GGPAT (79.81)								upp	1096	GGPAT	1100	100	100	15	XP_005715754
11	SSNDYPI (74.66)	rpoB	519	SNDY	522	57	100	0.53	clathrin heavy chain	265	SNDFPI	270	85	83	2.2	XP_005717586
12	SRIYNVKSNG (64.85)	ycf45	318	RIFKSNG	324	90	66	0.53	upp	97	RIYDVKS	103	70	86	0.26	XP_005715342
		atpB	90	RIFNV	94	50	80	0.75								
14	VDAHY (70.62)								upp	138	VDSHY	142	100	80	11	XP_005717520
	CPYDWV (70.01)								upp	482	PYDW	485	66	100	2.4	XP_005717633
16	YGDPDHY (78.33)								upp	240	YGDPD	244	71	100	2.2	XP_005714534
17	NLGN (65.31)								43 proteins [e]				100	100	43	
22	DFGVPGHEP (80.84)								upp	242	FGDAPGHEP	250	100	78	1.7	XP_005712565

[a] Numbers represent the position of amino acid residue from N-terminus
[b] Peptides having E-value less than 1 was employed for chloroplast protein
[c] PEα from *M. japonica* (AB698819)
[d] upp: unnamed protein product
[e] 43 proteins had the identical sequence (XP_005715212, XP_005716483, XP_005716537, XP_005710358, XP_005714002, XP_005713469, XP_005712869, XP_005711244, XP_005715776, XP_005712961, XP_005717512, XP_005714548, XP_005716465, XP_005715944, XP_005714952, XP_005718488, XP_005714129, XP_005711252, XP_005710407, XP_005716993, XP_005713251, XP_005718259, XP_005716224, XP_005718647, XP_005713466, XP_005710262, XP_005718183, XP_005711033, XP_005717430, XP_005711063, XP_005715493, XP_005710135, XP_005712875, XP_005716148)

1

2

3

Table 2 Structure of ACE inhibitory peptides

Peptide	Sequence	IC_{50} (μM)	ID [a]
YRD	**YR**PY	320	7679
VSEGLD	**EG**	10,000	7622
	GL	2,500	7599
	D**GL**	2.1	9056
TIMPHPR	**PH**	N.D. [b]	7843
	HP	N.D.	7842
	PR	4.1	3537
GGPAT	**GPA**	405	3342
	GG	7,200	7616
	GP	253	7512
SSNDYPI	LK**YPI**	27.1	9004, 9175, 9280
	DY	100	9072
	YP	720	3666
SRIYNVKSNG	**RIY**	28	7821
	IY	2.1	3383
	YN	51	9185
	VK	13	7558
	NG	12,000	7624
VDAHY	**VD**SDVVKG	13.3	9140
	DA	3,800	7606
	AH	N.D.	7835
	HY	26.1	3494
YGDPDHY	**YG**	1,523	3553
	GD	9,200	7620
	HY	26.1	3494
NLGN	RM**LGN**TPTK	N.D.	7573
DFGVPGHEP	**DFG**	44.7	9193

[a] ID from BIO-PEP

[b] Not determined

4