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The chitin-binding capability of *Cy*-AMP1 from cycad is essential to antifungal activity

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Antimicrobial peptides are important components of the host innate immune responses by exerting broad-spectrum microbicidal activity against pathogenic microbes. *Cy*-AMP1 found in the cycad (*Cycas revoluta*) seeds has chitin-binding ability, and the chitin-binding domain was conserved in knottin-type and hevein-type antimicrobial peptides. The recombinant *Cy*-AMP1 was expressed in *Escherichia coli* and purified to study the role of chitin-binding domain. The mutants of *Cy*-AMP1 lost chitin-binding ability completely, and its antifungal activity was markedly decreased in comparison with native *Cy*-AMP1. However, the antimicrobial activities of the mutant peptides are nearly identical to that of native one. It was suggested that the chitin-binding domain plays an essential role in antifungal, but not antimicrobial, activity of *Cy*-AMP1. Copyright © 2009 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: cycad; antimicrobial peptide; chitin-binding capability; pGEX-4T1 vector; CD spectrum; antimicrobial activity; antifungal activity

Introduction

Antimicrobial peptides are important substances functioning of self-defense against infection by various pathogens. They are isolated from various sources of animals, plants and bacteria and have been characterized [1–3]. Some plants have antimicrobial peptides that have similar amino acid sequence to those from animals. Plant peptides having primary structures similar to those of the defensin family, most of which are found in animals, are called the plant defensin family [4,5]. Most of the plant defensins can be classified into three types: hevein type [6,7], knottin type [8,9] and thionin type [10,11], according to the number and position of cysteine residues in the molecules.

One of the actions of antimicrobial peptides is thought to exert their activity through destruction of the cell membrane of infections pathogens, but the molecular mechanism still remains unclear. For antimicrobial peptides, three models of membrane destruction mechanisms have been proposed: barrel-stave, carpet and toroidal models [12–16]. In these models, the interaction with the surface of pathogens serves as a key step of the antimicrobial activity. The characteristic, positive charge and hydrophobicity of antimicrobial peptides are considered to participate in antimicrobial activity [17,18]. Furthermore, the chitin-binding domain is conserved in knottin-type and hevein-type antimicrobial peptides (Figure 1). However, the relationship between the chitin-binding capability and the antimicrobial activity has not been studied.

Antimicrobial peptide (AMP) derived from cycad (*Cycas revoluta*) seeds, *Cy*-AMP1, has been isolated and characterized [19]. It has been shown that *Cy*-AMP1 is unique in having two knottin motifs and possesses antimicrobial activity against a wide range of microorganisms, including Gram-positive and Gram-negative bacteria and fungi. In addition, *Cy*-AMP1 has the chitin-binding capability.

To investigate the relationship between chitin-binding capability and antimicrobial activity, the recombinant *Cy*-AMP1 was produced in which highly conserved Ser residue is altered to Gly residue and the conserved aromatic amino acid to Asn or Val. The effect of these point mutations on the chitin-binding capability and the antimicrobial activity is discussed. This is the first report on the relationship between chitin-binding capability and antifungal activity.

Materials and Methods

Materials

The following bacteria and fungi were used for testing antimicrobial activity: *Erwinia carotovora* subsp. carotovora MAFF 106 567. They were kindly provided by Dr Iwai of the Laboratory of Phytopathology, Faculty of Agriculture, Kagoshima University. *Lactococcus lactis* subsp. *lactis* NBRC 12 007, *Geotrichum candidum* NBRC 4597, *Streptococcus mutans* NBRC 13 955, *Enterobacter cloacae* NCBR 12 935, *Clavibacter michiganensis* NBRC 13 763 and *Fusarium oxysporum* cucumerium NBRC 6384 were obtained from National Institute of Technology and Evaluation, Biological Resource Center (NBRC). The *Escherichia coli* strain JM109 was used for all routine cloning purpose and the strain BL21(DE3) was used for overproduction of recombinant proteins. All strains

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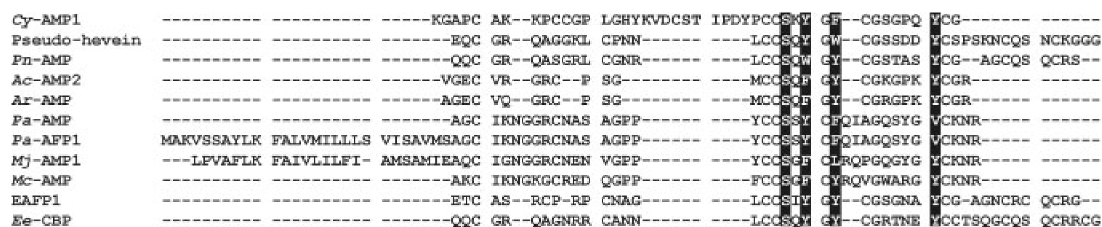

Figure 1. Sequences comparison of *Cy*-AMP1 with plant defensins. The amino acid sequence of *Cy*-AMP1 was compared with those of typical plant defensins. The well-conserved amino acids, indicated in black, are involved in chitin-binding activity. Pseudo-hevein, antimicrobial peptide from *Hevea brasiliensis* [20]; *Pn*-AMP1, antimicrobial peptide from *Pharbitis nil* [7]; *Pa*-AMP1, antimicrobial peptide from *Phytolacca americana* [9]; *Mj*-AMP1, antimicrobial peptide from *Mirabilis jalapa* [8]; *Ar*-AMP1, antimicrobial peptide from *Amaranthus retroflexus* [21]; *Ac*-AMP1, antimicrobial peptide from *Amaranthus caudatus* [22]; EAFP1, hevein-type antimicrobial peptide from the *Euonymus europaeus* [23]; Ee-CBP, antimicrobial peptide from *Euonymus europaeus* [24]; *Mc*-AMP, antimicrobial peptide from *Mesembryanthemum crystallinum*.

Table 1. Theoretical characteristic and amino acid sequence of *Cy*-AMP1 and its derivate

	Amino acid sequence	MW	GRAVY	Net charge
Cy-AMP1	KGAPCAKKPCCGPLGHYKVDCSTIPDYP<u>CCSKYGFCGSGPQY</u>CG	4591.3	−0.341	+3
r*Cy*-AMP1	GSKGAPCAKKPCCGPLGHYKVDCSTIPDYP<u>CCSKYGFCGSGPQY</u>CG	4735.4	−0.352	+3
S31G	GSKGAPCAKKPCCGPLGHYKVDCSTIPDYP<u>CC**G**KYGFCGSGPQY</u>CG	4705.4	−0.343	+3
Y33V	GSKGAPCAKKPCCGPLGHYKVDCSTIPDYP<u>CCSK**V**GFCGSGPQY</u>CG	4671.4	−0.233	+3
Y33N	GSKGAPCAKKPCCGPLGHYKVDCSTIPDYP<u>CCSK**N**GFCGSGPQY</u>CG	4686.4	−0.400	+3
F35V	GSKGAPCAKKPCCGPLGHYKVDCSTIPDYP<u>CCSKYG**V**CGSGPQY</u>CG	4687.4	−0.322	+3
F35N	GSKGAPCAKKPCCGPLGHYKVDCSTIPDYP<u>CCSKYG**N**CGSGPQY</u>CG	4702.4	−0.489	+3
Y42V	GSKGAPCAKKPCCGPLGHYKVDCSTIPDYP<u>CCSKYGFCGSGPQ**V**</u>CG	4671.4	−0.233	+3

were cultured at 30 °C in a constant-temperature incubator. All other chemicals were of the highest purity commercially available.

Plasmids and Site-specific Mutagenesis

The gene encoding 44 amino acid sequence of *Cy*-AMP1 was synthesized using the favored codons for *E. coli* (Figure 1A). The *Cy*-AMP1 gene was amplified by PCR with the following primers (forward primer 1: AAGGATCCAAGGGTGCACCGTGT and reverse primer 2: AAGAATTCTTAACCACAATATTGCGG) using *Cy*-AMP1 complementary DNA cDNA as template. The PCR-amplified DNA fragments were digested by *Bam*HI and *Eco*RI, and then ligated via multi-cloning site into pGEX-4T1 vector (GE Healthcare, UK), using DNA ligation kit Ver2.1 (TAKARA BIO, Japan) (Figure 1C). Construction of plasmid including site-specific mutant *Cy*-AMP1 cDNA was amplified from the above-mentioned vector by PCR using the following primer sets (S31G: primer1 CGTGTTGTGGTAAATACGGCTTCTGCGGTAGCG, primer2 CGCTACCGCAGAAGCCGTATTTACCACAACACG, Y33V: primer1 GTTGTAGTAAAGGTGGCTTCTGCGGTAGCGGTCC, primer2 GGACCGCTACCGCAGGAGCCAACTTTACTACAAC, Y33N: primer1 GTTGTAGTAAAAATGGCTTCTGCGGTAGCGGTCC, primer2 GGACCGCTACCGCAGAAGCCATTTTTACTACAAC, F35V: primer1 GTTGTAGTAAATACGGCGTTTGCGGTAGCGGTCC, primer2 GGACCGCTACCGCAAACGCCGTATTTACTACAAC, F35N: primer1 GTTGTAGTAAATACGGCAATTGCGGTAGCGGTCC, primer2 GGACCGCTACCGCAATTGCCGTATTTACTACAAC, Y42V: primer1 GTAGCGGTCCGCAAGTTTGTGGTTGAGAAT, primer2 ATTCTCAACCACAACTTGCGGACCGCTAC). And then, the PCR products were digested by *Dpn*I.

The resulting vectors were transformed into the *E. coli* JM109 cell (Promega, USA), and after isolation and purification, subjected to the DNA sequence analysis. The theoretical characteristic and amino acid sequence of *Cy*-AMP1 and its derivate were shown in Table 1.

Expression and Purification of Recombinant Peptides

A single colony was inoculated to 100 ml of Luria–Bertani LB medium supplemented with 100 µg/ml ampicillin and 1% (w/v) glucose, and cultured overnight at 37 °C. The culture was then used to inoculate the fresh LB medium at the volume ratio of 1 : 100. Cells were incubated to the mid-log phase (absorbance at 600 nm = 0.6), and then induced by the addition of isopropyl β-D-1-thiogalactopyranoside to a final concentration of 0.5 mM. Expression was carried out at 27 °C for 36 h. The cells were harvested from 1 l of culture by centrifugation at 1700× g for 20 min and were resuspended in 50 ml phosphate buffered saline. After sonicating for 30 min on ice, the suspension was centrifuged at 24 000×g for 20 min. The clear supernatant was put through the Glutathione Sepharose™ 4B column (GE Healthcare, UK) according to the manufacturer's instructions. To release r*Cy*-AMP1, the fusion protein was digested by thrombin at 16 °C for 36 h, and then passed through SP Sepharose column. The r*Cy*-AMP1 was further purified by reversed-phase HPLC on a Mightysil RP-4 column (particle diameter, 5 µm; Φ 4.6 × 250 mm; Kanto Chemical, Japan), using a linear gradient of 0–40% MeCN in 0.1% TFA.

Maldi-Tof Ms

For measuring the molecular mass, *Cy*-AMP1s were analyzed on a MALDI-TOF MS (Voyager DE-RP, Applied Biosystems, USA), which used a pulsed nitrogen laser emitting at 337 nm. Pressure in the ion chamber was kept between 1×10^{-7} and 4×10^{-7} Torr. The matrix solution contained saturated α-cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.1% TFA. Matrix mixture solution (matrix:

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Cy-AMP1s = 9 : 1, v/v) was put on the sample plate and then air-dried completely. Mass spectra were collected by using the linear delayed extraction mode (DE mode) and positive ion mode with the following parameters: accelerating voltage, 20 000 V; grid voltage, 95.500%; guide wire voltage, 0.050%; low mass gate 500.0 and laser intensity, 2,400. Signals from 26 to 256 excitation pulses were accumulated and averaged to yield each recorded mass spectrum. The spectra were externally calibrated with the α chain $[(M + H)^+ = 3497.0$ Da] and β chain of insulin $[(M + H)^+ = 5731.6$ Da] and analyzed using GRAMS/386 software (Galactic Industries Coro., Salem, New Hampshire, USA).

Measurement of CD Spectrum

CD spectra were measured on a Jasco J-820 spectropolarimeter (JASCO corporation, Japan). Measurement carried out at 27 °C using a 0.2-mm pathlength quartz cell, with five scans per spectrum at a scan speed of 50 nm/min. The peptide samples (500 µg/ml) were dissolved in Milli-Q water cont. 20% TFE. The molar ellipticity per residue $[\theta]_R$ (deg·cm^2/dmol) was calculated using the following equation:

$$[\theta]_R = \theta \cdot 100 \cdot (l \cdot C \cdot A)^{-1},$$

where θ is the experimental ellipticity (mdeg), l is cuvette path length (cm), C is protein concentration (M) and A is the number of residues in the protein.

Measurements of Chitin-binding Capability of Peptides

Chitin-binding capability was measured by a method similar to that of Fujimura *et al*. [25]. The peptides were put through the chitin column (φ1.5 × 15 cm) equilibrated with the 20 mM sodium bicarbonate buffer (pH 8.5). A chitin-flow through fraction was collected by loading the same buffer. Then, a chitin-binding fraction was eluted at a flow rate of 0.5 ml/min with 20 mM acetic acid (pH 3.0). A chitin-binding fraction and flow through fraction were applied to Tricin SDS-PAGE analysis as described by Schgger and Jagow [26].

Assay of Antimicrobial Activity

Antimicrobial activity was measured by a method similar to that of Broekaert *et al*. [27] using PD medium (Siguma Aldrich, USA) containing 2.5 × 10^4 fungal spores per milliliter for antifungal activity and 1 × 10^5 colony-forming units of bacteria per milliliter for antibacterial activity. A peptide solution (20 µl) sterilized by ultrafiltration with a nitrocellulose membrane, and the sterilized culture solution (80 µl), were successively put into a microplate and incubated at 30 °C for 24 h. The turbidity of the solution was measured at 490 nm [25].

Result

Expression and Purification of Recombinant Peptides

The nucleotide sequence of the constructed pGEX-*Cy*AMP1 and mutants were confirmed by DNA sequencing and were transformed into the expression host *E. coli* BL21(DE3). As shown in Figure 2, the resulting expression level of fusion protein reached >10% of the total protein, and >60% of the target proteins were in soluble form. The existence of the GST-tag on the carrier protein enables an effective purification of the fusion protein by

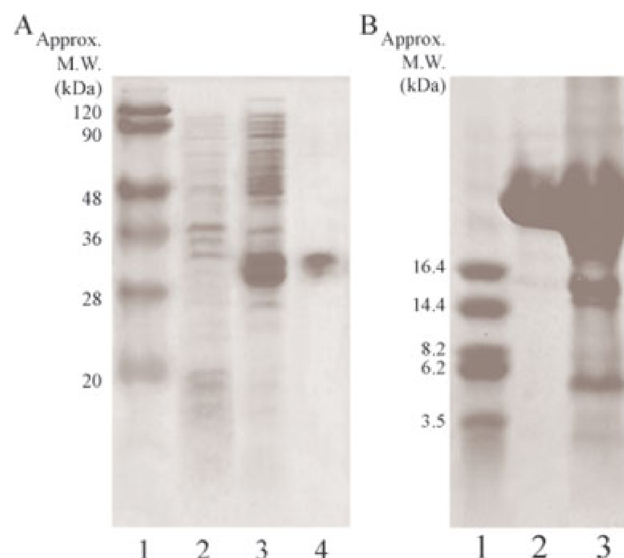

Figure 2. a, SDS-PAGE analysis of fusion proteins expressed in *E. coli,* and purified fusion protein. Lane 1, protein molecular weight marker; Lane 2, precipitation of bacterial lysate; Lane 3, supernatant of bacterial lysate; Lane 4, purified GST-*Cy*AMP1. b, Tricine SDS-PAGE analysis of digestion of fusion protein by thrombin. Lane 1, protein molecular weight marker; Lane 2, purified GST-*Cy*AMP1; Lane 3, purified GST-*Cy*AMP1 digestion product with Thrombin. This figure is available in colour online at www.interscience.wiley.com/journal/jpepsci.

Glutathione Sepharose™ 4B column. SDS-PAGE analysis revealed the purity of target peptides.

The desalted fusion protein was cleaved by thrombin at 16 °C for 36 h. The cleaved fusion partner and some containing proteins could be removed rapidly by SP Sepharose column, and then further purified by reverse-phase HPLC on a Mightysil RP-4 column using a linear gradient of 0–40% MeCN;2-PrOH (3 : 7, v/v) in 0.1% TFA. The major peak was confirmed to be the target peptide by analyses with a Waters Pico-Tag amino acid analyzer, MALDI-TOF/MS and amino acid sequencer. About 1 mg of target peptides was obtained from 1 l culture.

Estimation of the Secondary Structure of Native and Recombinant *Cy*-AMP1 (r*Cy*-AMP1)

To compare the structure of r*Cy*-AMP1 and mutant *Cy*-AMP1s with its native counterpart, *Cy*-AMP1, these peptides were analyzed by CD (Figure 3). Each of *Cy*-AMP1, r*Cy*-AMP1 and S31G presented comparable spectra that were characteristic of structured peptide with a negative maximum at 198 and 215 nm, which are indicative of a predominantly random coil and β-strand structure, respectively. In contrast, other mutant *Cy*-AMP1s, Y33V, Y33N, F35V, F35N and Y42V, showed reduction in a negative maximum at 215 nm.

Chitin-binding Capability of the Peptides

To confirm that *Cy*-AMP1 and its analogs have chitin-binding capability, these peptides were applied to chitin column, then collected flow through fraction and absorbed fraction. These fractions were applied to Tricin SDS-PAGE. The *Cy*-AMP1 and r*Cy*-AMP1 were present in absorbed fraction (Figure 4A). However, S31G, Y33V, Y33N, F35V, F35N, Y42V and Y42N were present in flow through fraction (Figure 4B).

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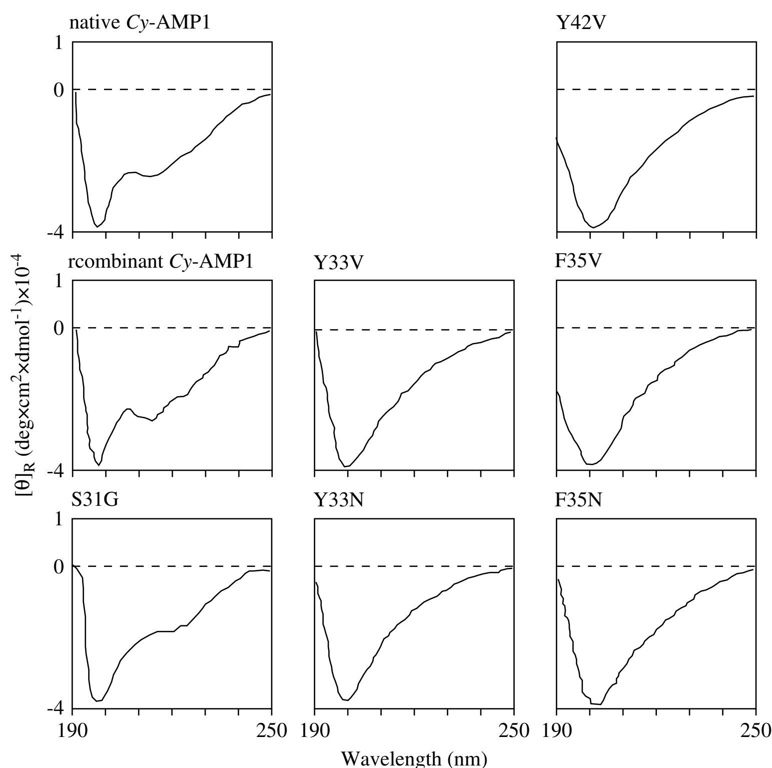

Figure 3. The feature comparison of native *Cy*-AMP1 and derived *Cy*-AMP1s. The comparison of the secondary structure by CD. The spectra were measured at 27 °C and the peptides were dissolved in 20% TFE.

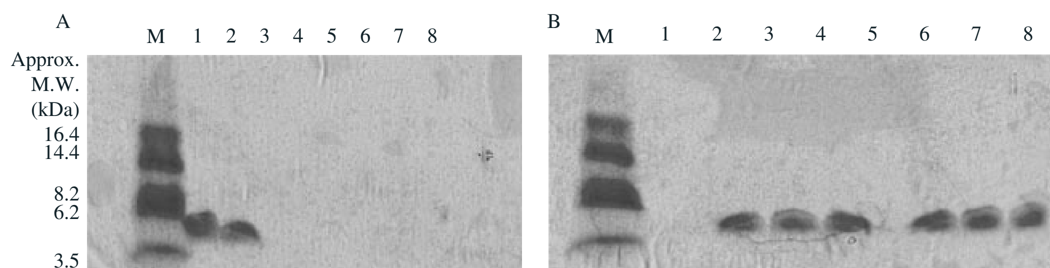

Figure 4. SDS-PAGE analysis of chitin-binding capability. a, Flow through fractions; b, Absorbed fractions; Lane M, protein molecular weight marker (16.9 kDa, 14.4 kDa, 8.2 kDa, 6.2 kDa, 3.5 kDa); Lane 1, native *Cy*-AMP1; Lane 2, r*Cy*-AMP1; Lane 3, S31G; Lane 4, Y33N; Lane 5, Y33N; Lane 6, F35W; Lane 7, F35V; Lane 8, F35N; Lane 9, Y42V.

Antimicrobial Activity of Recombinant Peptides

The antimicrobial activity of recombinant peptides against plant pathogenic fungi and Gram-negative and -positive bacteria were evaluated as equivalent concentration for growth inhibition. The growth inhibition ratios of the peptides are shown in Table 2. The r*Cy*-AMP1 and mutant *Cy*-AMP1s showed similar antimicrobial activity in comparison with native *Cy*-AMP1. In addition, the r*Cy*-AMP1 retained antifungal activity equivalent to native one. In contrast, antifungal activity of the mutant *Cy*-AMP1s, S31G, Y33V, Y33N, F35V, F35N and Y42V, was markedly decreased in comparison with *Cy*-AMP1 and r*Cy*-AMP1. These *Cy*-AMP1 mutants showed only ∼10% of antifungal activity of native one.

Discussion

Plant defensins that contribute to defense against pathogens have been recently reported. Although these peptides commonly have antimicrobial activity against pathogens, there are many variations in their primary structures. For relationship between structure and activity, linearization of cyclic antimicrobial peptides generally alters their activities as well as their ability to interact with cell membranes [28–33]. Furthermore, Peng *et al*. reported that the positive patch and the hydrophobic surface were both important for the antifungal function of *Pa*-AMP from poke weed (*P. americana*) seeds [34]. Most of antimicrobial peptides with chitin-binding capability were classified into knottin type or hevein type. These peptides have well-conserved amino acid sequence [C-C-S-X-aromatic amino acid residue-X-aromatic amino acid residue-(6 ∼ 8X)-aromatic amino acid residue)] (Figure 1), and these conserved amino acid are considered to participate in chitin-binding capability [35–37]. *Cy*-AMP1 has a potent antimicrobial activity and a model peptide for studying the relationships between structure, charge, affinity and antimicrobial activity. In this study, to investigate the effect of elimination of chitin-binding capability on the activity of *Cy*-AMP1, the 31 serine residue

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Table 2. Antimicrobial activity of *Cy*-AMP1, r*Cy*-AMP1 and mutant *Cy*-AMP1s

Microorganisms		IC50 (µg/ml)							
		Cy-AMP1	r*Cy*-AMP1	S31G	Y33V	Y33N	F35V	F35N	Y42V
Fungi	*F. oxysporum*	6.0	6.0	45	67	37	21	29	33
	G. candidum	7.4	8.2	56	55	41	31	68	72
Gram-positive	*L. lactis*	10	8.0	12	5.6	11	9.5	19	12
	S. mutans	8.0	9.0	7.0	9.0	11	5.1	4.2	10
	C. michiganensis	5.0	4.0	10	4.6	5.9	5.1	4.6	2.8
Gram-negative	*E. carotovora*	6.0	4.0	5.0	4.9	9.0	2.4	14	3.7
	E. cloacae	8.5	7.4	8.1	9.9	2.0	3.2	3.5	9.6

considered important in a conserved region was substituted with glycine residue, the aromatic amino acid residue, Tyr 33 and Phe 35 which exists in a chitin-binding domain were substituted with Val or Asp residue, in addition, the aromatic amino acid residue Tyr 42 which exists downstream of a chitin-binding domain was also substituted with Val residue.

In the CD spectra, r*Cy*-AMP1 and S31G were similar to native one. In contrast, Y33V, Y33N, F35V, F35N and Y42V showed a slight alteration in CD spectra. This result has suggested the influence on the secondary structure or CD spectra by the substitution of amino acid residue. The three aromatic amino acid residues might be important for keeping secondary structure.

Harata *et al*. suggested that CH-π interaction involving three aromatic residues is a major determinant of the affinity of hevein domain for chitin, and this interaction is further strengthened by a hydrogen bonding with Ser of hevein domain [38]. The substitution of Tyr 42 of *Cy*-AMP1 to Val caused loss of a chitin-binding capability. The substitution by Asp residue or Val residue of aromatic amino acid residue, the Tyr 33 and Phe 35 in a chitin-binding domain, resulted in disappearance of chitin-binding capability. In addition, the chitin-binding capability of *Cy*-AMP1 was lost by substitution of Ser 31 to Gly without change of secondary structure as compared with native one. This result suggested that the interaction of *Cy*-AMP1 and chitin, the π-stacking interaction of chitin and 33rd, 35th and 42nd aromatic rings and a hydrogen bond between the hydroxyl group of Ser 31 and the carbonyl group of chitin, is important for chitin-binding capability.

Mutants of *Cy*-AMP1 that were incapable of binding to chitin were shown to have reduced antifungal activity (Table 2). They had, however, same level of antibacterial activity against Gram-positive and Gram-negative bacteria in comparison with native *Cy*-AMP1 and r*Cy*-AMP1. This result suggested that chitin-binding capability of *Cy*-AMP1 plays a crucial role in its antifungal activity. The antifungal mechanism might be differing from the antibacterial mechanism. In the antifungal mechanism of knottin-type or hevein-type antimicrobial peptide, it is considered that binding to chitin at a fungus surface serves as first interaction of antifungal activity.

In conclusion, we created the mutants *Cy*-AMP1s that were substituted of serine residue or aromatic amino acid in chitin-binding domain without severe alteration in secondary structure and demonstrated the relationship between chitin-binding capability and antifungal activity. The biological role of *Cy*-AMP1 has not been clarified. The finding of this relationship may help contribute to the understanding of the antimicrobial mechanism.

References

1 Osborn RW, Genoveva W, Samblanx D, Thevissen K, Goderis I, Torrekens S, Leuven FV, Attenborough S, Rees SB, Broekaert WF. Isolation and characterisation of plant defensins from seeds of Asteraceae, Fabaceae, Hippocastanaceae and Saxifragaceae. *FEBS Lett.* 1995; **368**: 257–262.
2 Lehrer RI, Lichtenstein AK, Ganz T. Defensins: antimicrobial and cytotoxic peptides of mammalian cells. *Annu. Rev. Immunol.* 1993; **11**: 105–128.
3 Lowenberger C, Charlet M, Vizioli J, Kamal S, Richman A, Christensen BM, Bulet P. Antimicrobial activity spectrum, cDNA cloning, and mRNA expression of a newly isolated member of the cecropin family from the mosquito vector *Aedes aegypti*. *J. Biol. Chem.* 1999; **274**: 20092–20097.
4 Sitaram N, Nagaraj R. Host-defense antimicrobial peptides: importance of structure for activity. *Curr. Pharm. Des.* 2002; **8**: 727–742.
5 Zhang G, Hiraiwa H, Yasue H, Wu H, Ross CR, Troyer D, Blecha F. Cloning and characterization of the gene for a new epithelial β-defensin. *J. Biol. Chem.* 1999; **274**: 24031–24037.
6 Chrispeels MJ, Raikhel NV. Lectins, lectin genes, and their role in plant defense. *Plant Cell* 1991; **3**: 1–9.
7 Koo JC, Lee SY, Chun HJ, Cheong YH, Choi JS, Kawabata SI, Miyagi M, Tsunasawa S, Ha KS, Bae DW, Han CD, Lee BL, Cho MJ. Two hevein homologs isolated from the seed of *Pharbitis nil* L. exhibit potent antifungal activity. *Biochem. Biophys. Acta* 1998; **1382**: 80–90.
8 Cammue BPA, De Bolle MFC, Terras FRG, Proost P, Damme JV, Rees SB, Vanderleyden J, Broekaert WF. Isolation and characterization of a novel class of plant antimicrobial peptides from *Mirabilis jalapa* L. seeds. *J. Biol. Chem.* 1992; **267**: 2228–2233.
9 Minami Y, Higuchi S, Yagi F, Tadera K. Isolation and some properties of the antimicrobial peptide (*Pa*-AMP) from the seeds of pokeweed (*Phytolacca americana*). *Biosci. Biotechnol. Biochem.* 1998; **62**: 2076–2078.
10 Ozaki Y, Wada K, Hase T, Matsubara H, Nakanishi T, Yoshizumi H. Amino acid sequence of a purothionin homolog from barley flour. *J. Biochem.* 1980; **87**: 549–555.
11 Colilla FJ, Rocher A, Mendez E. γ-Purothionins: amino acid sequence of two polypeptides of a new family of thionins from wheat endosperm. *FEBS Lett.* 1990; **270**: 191–194.
12 Yeaman MR, Yount NY. Mechanisms of antimicrobial peptide action and resistance. *Pharmacol. Rev.* 2003; **55**: 27–55.
13 Ojcius DM, Young JD. Cytolytic pore-forming proteins and peptides: is there a common structural motif. *Trends Biochem. Sci.* 1991; **16**: 225–229.
14 Brogden KA. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria. *Nat. Rev. Microbiol.* 2005; **3**: 238–250.
15 Shai Y, Oren Z. From "carpet" mechanism to de-novo designed diastereomeric cell-selective antimicrobial peptides. *Peptides* 2001; **22**: 1629–1641.
16 Uematsu N, Matsuzaki K. Polar angle as a determinant of amphipathic alpha-helix-lipid interactions: a model peptide study. *Biophys. J.* 2000; **79**: 2075–2083.
17 Dathe M, Nikolenko H, Meyer J, Beyermann M, Bienert M. Optimization of the antimicrobial activity of magainin peptides by modification of charge. *FEBS Lett.* 2001; **501**: 146–150.

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18 Wieprecht T, Dathe M, Krause E, Beyermann M, Maloy WL, MacDonald DL, Bienert M. Modulation of membrane activity of amphipathic, antibacterial peptides by slight modifications of the hydrophobic moment. *FEBS Lett.* 1997; **417**: 135–140.
19 Yokoyama S, Kato K, Koba A, Minami Y, Watanabe K, Yagi F. Purification, characterization, and sequencing of antimicrobial peptides, *Cy*-AMP1, *Cy*-AMP2, and *Cy*-AMP3, from the Cycad (*Cycas revoluta*) seeds. *Peptides* 2008; **29**: 2110–2117.
20 Soedjanaatmadja UMS, Hofsteenge J, Jeronimus-Stratingh CM, Bruins AP, Beintema JJ. Demonstration by mass spectrometry that pseudo-hevein and hevein have ragged C-terminal sequences. *Biochim. Biophys. Acta* 1994; **1209**: 144–148.
21 Lipkin A, Anisimova V, Nikonorova A, Babakov A, Krause E, Bienert M, Grishin E, Egorov T. An antimicrobial peptide *Ar*-AMP from amaranth (*Amaranthus retroflexus* L.) seeds. *Phytochemistry* 2005; **66**: 2426–2431.
22 Martins JC, Maes D, Loris R, Pepermans HAM, Wyns L, Willem R, Verheyden P. NMR study of the solution structure of *Ac*-AMP2, a sugar binding antimicrobial protein isolated from *Amaranthus caudatus*. *J. Mol. Biol.* 1996; **258**: 322.
23 Huang RH, Xiang Y, Liu XZ, Zhang Y, Hu Z, Wang DC. Two novel antifungal peptides distinct with a five-disulfide motif from the bark of *Eucommia ulmoides Oliv*. *FEBS Lett.* 2002; **521**: 87–90.
24 Van den Bergh KP, Proost P, Van Damme J, Coosemans J, Van Damme EJ, Peumans WJ. Five disulfide bridges stabilize a hevein-type antimicrobial peptide from the bark of spindle tree *(Euonymus europaeus L.)*. *FEBS Lett.* 2002; **530**: 181–185.
25 Fujimura M, Minami Y, Watanabe K, Tadera K. Purification, characterization, and sequencing of a novel type of antimicrobial peptides, *Fa*-AMP1 and *Fa*-AMP2, from seeds of buckwheat (*Fagopyrum esculentum* Moench.). *Biosci. Biotechnol. Biochem.* 2003; **67**: 1636–1642.
26 Schagger H, Jagow GV. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the range from 1 to 100 kDa. *Anal. Biochem.* 1987; **166**: 368–379.
27 Broekaert WF, Terras FRG, Cammue BP, Vanderleyden J. An automated quantitative assay for fungal growth inhibition. *FEMS Microbiol. Lett.* 1990; **69**: 61–66.
28 Tamamura H, Ikoma R, Niwa M, Funakoshi S, Murakami T, Fujii N. Antimicrobial activity and conformation of tachyplesin I and its analogues. *Chem. Pharm. Bull.* 1993; **41**: 978–980.
29 Rao AG. Conformation and antimicrobial activity of linear derivatives of tachyplesin lacking disulfide bonds. *Arch. Biochem. Biophys.* 1999; **361**: 127–134.
30 Matsuzaki K, Yoneyama S, Fujii N, Miyajima K, Yamada KI, Kirino Y, Anzai K. Membrane permeabilization mechanisms of a cyclic antimicrobial peptide, tachyplesin I, and its linear analogue. *Biochemistry* 1997; **36**: 9799–9809.
31 Matsuzaki K, Nakayama M, Fukui M, Otaka A, Funakoshi S, Fujii N, Bessho K, Miyajima K. Role of disulfide linkages in tachyplesin-lipid interactions. *Biochemistry* 1993; **32**: 11704–11710.
32 Hirakura Y, Kobayashi S, Matsuzaki K. Specific interactions of the antimicrobial peptide cyclic β-sheet tachyplesin I with lipopolysaccharides. *Biochim. Biophys. Acta* 2002; **1562**: 32–36.
33 Satoh K, Okuda H, Horimoto H, Kodama H, Kondo M. Synthesis and properties of a heterodetic cyclic peptide: gramicidin S analog containing disulfide bond. *Bull. Chem. Soc. Jpn.* 1990; **63**: 3467–3472.
34 Penga C, Donga C, Houa Q, Xub C, Zhao J. The hydrophobic surface of PaAMP from pokeweed seeds is essential to its interaction with fungal membrane lipids and the antifungal activity. *FEBS Lett.* 2005; **579**: 2445–2450.
35 Aboitiz N, Vila-Perelló M, Groves P, Asensio JL, Andreu D, Cañada FJ, Jiménez-Barbero J. NMR and modeling studies of protein-carbohydrate interactions: synthesis, three-dimensional structure, and recognition properties of a minimum hevein domain with binding affinity for chitooligosaccharides. *Chembiochem.* 2004; **9**: 1245–1255.
36 Suetake T, Tsuda S, Kawabata S, Miura K, Iwanaga S, Hikichi K, Nitta K, Kawano K. Chitin-binding proteins in invertebrates and plants comprise a common chitin-binding structural motif. *J. Biol. Chem.* 2000; **275**: 17929–17933.
37 Muraki M, Morii H, Harata K. Chemically prepared hevein domains: effect of C-terminal truncation and the mutagenesis of aromatic residues on the affinity for chitin. *Protein Eng.* 2000; **6**: 385–399.
38 Harata K, Muraki M. Crystal structures of *Urtica dioica* agglutinin and its complex with tri-N-acetylchitotriose. *J. Mol. Biol.* 2000; **297**: 673–681.

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