Bull. Fac. Agr., Saga Univ. No. 90 : 55~61 (2005)

Fluorescent-Labeled *Ac*-AMPs with Antifungal and Chitin-Binding Properties: Usefulness of an anthraniloyl group as a fluorescent-labeling group

Saki HYAKUTAKE, Shoji ANDO*, Kiyohiko SEKI, Yasushi UCHIDA**,
and Toshihisa UEDA
(Laboratory of Biomolecular Chemistry)
Received September 21, 2004

Summary

Chitin-binding antifungal peptides *Ac*-AMPs (a mixture of *Ac*-AMP 1 and *Ac*-AMP 2), isolated from the seeds of *Amaranthus caudatus*, were easily fluorescent-labeled with an anthraniloyl (Ant) group using isatoic anhydride. The mass spectrum showed that monoAnt-*Ac*-AMP 1 and diAnt-*Ac*-AMP 1 have been synthesized. The fluorescent *Ac*-AMPs (a mixture of monoAnt- and diAnt-*Ac*-AMP 1) had an affinity to chitin and a fungus, *Valsa ceratosperma* Maire. The binding with the latter gave the clear image of the hyphal structure with a fluorescence microscope. Furthermore, they retained an antifungal activity against *Valsa ceratosperma* Maire. These results show that an Ant group is a good fluorophore without perturbing the properties which parent *Ac*-AMPs have. The fluorescent *Ac*-AMPs prepared in this study can be a good tool to know the relationship between a chitin-affinity and an antifungal activity.

Key words: *Ac*-AMP, antifungal activity, chitin-binding, fluorescent-labeling, fluorescence microscopy

Many antifungal peptides, obtained from various plants, have been shown to have an affinity to chitin (Fig. 1). Chitin, a β-1,4-linked polymer of *N*-acetyl-D-glucosamine, is a major component of exoskeletons of arthropods, and found in the cell walls of diatoms, fungi, and higher plants. So far, no clear relationship between an antifungal activity and a chitin-binding property of the peptides has been shown.

The seeds of *Amaranthus caudatus* contain antifungal chitin-binding peptides *Ac*-AMPs, a

hevein[1]	EQCGRQAGGKLCPNNLCCSQWGWCGSTDEYCSPDHNCQSNCKDS
WGA[2]	QRCGEQGSNNECPNNLCCSQYGYCGMGGDYCGKGCQDGACWTS
Ac-AMP 1[3]	VGECVRGRCPSGMCCSQFGYCGKGPKYCG
Ac-AMP 2[3]	VGECVRGRCPSGMCCSQFGYCGKGPKYCGR

Fig. 1 Amino acid sequences of antifungal chitin-binding peptides: hevein, the A domain of wheat germ agglutinin (WGA), *Ac*-AMP 1, and *Ac*-AMP 2.

Faculty of Agriculture, Saga University, Saga 840-8502, Japan
* Faculty of Medicine, Saga University, Saga 849-8501, Japan
** Department of Food Nutrition, Saga Junior College, Saga 840-0806, Japan

mixture of *Ac*-AMP 1 and *Ac*-AMP 2[3]. Both peptides have almost the same amino acid sequence and the same disulfide bridge pattern. Only the difference is that the latter has an extra Arg residue at C-terminus.

A fluorescent-labeled peptide is a very good tool to study the interaction between a peptide and a fungus because it is very sensitive. Fluorescein isothiocyanate (FITC, Fig. 2-a) has been widely used for fluorescent-labeling of proteins[4] or peptides[5], because this fluorescent dye has many advantages such as a strong fluorescence intensity and a high quantum yield. However, FITC is so bulky that it would perturb a biological property of a target peptide. A small biological peptide, vasopressin (n=9), was fluorescent-labeled with FITC via an alkyl chain to reduce the influence of a FITC molecule on a peptide[6]. Therefore, if a small peptides are to be labeled, it is not likely that FITC is the best fluorophore.

On the other hand, an anthraniloyl group (Ant, Fig. 2-b), an acyl type of anthranilic acid, has been used successfully for fluorescent-labeling of nucleotides[7] or proteins[8]. Although a molecular size of an Ant group is much smaller than that of FITC, an Ant group fluoresces a blue color enough for observation. And it is easily introduced into an amino group of a target molecule using isatoic anhydride (Fig. 2-c), which is an activated derivative of anthranilic acid.

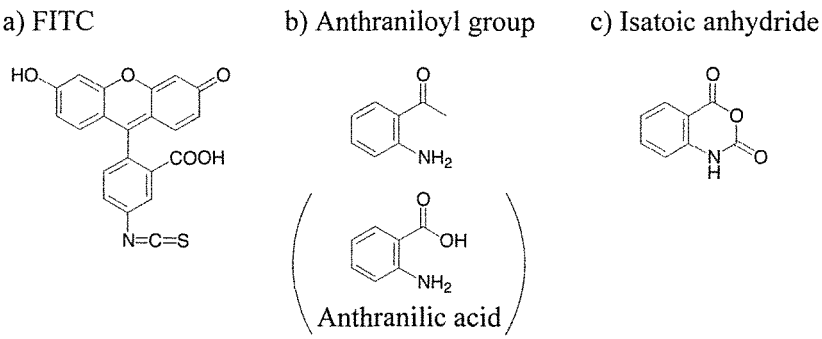

Fig. 2 Structures of fluorescent-labeling reagents.

In this paper, we report the preparation of *Ac*-AMPs labeled with a fluorescent Ant group, and show their binding property to chitin or a fungus and antifungal activity.

Material and Methods

Solvents and reagents were commercial products of high purity. Isatoic anhydride and *N*-methylmorpholine (NMM) were products of Wako Pure Chemical Industries, Ltd. (Japan) and ALDRICH Chemical Company Inc. (USA), respectively. Seeds of *Amaranthas caudatus* were commercially available. Chitin powder was a product of Seikagaku Corporation (Japan).

Isolation of peptides. The phosphate buffer solution (pH 6.0) of crude peptides, which had been extracted from the seeds of *Amaranthus caudatus* with 2% aqueous AcOH, was passed through a chitin-affinity column. Desired *Ac*-AMPs were adsorbed by the chitin, and subsequently eluted with 0.2 mol/l aqueous AcOH. The ion-exchange column chromatography to separate *Ac*-AMP 1 and *Ac*-AMP 2 was not performed. Identification was done by fast-atom bom-

bardment (FAB) mass spectrometry on a JMS-HX 110 A (JEOL Ltd., Japan).

Antifungal activity. A suspension of spore of *Valsa ceratosperma* Maire in 1.5% potato dextrose agar, was pored into a Petri dish. The aqueous peptide solution (100 µl) was dropped on a filter paper, which was placed on the agar. The sample was kept at 25℃ for 3 days. An appearance of a clear zone around the filter paper was checked.

Introduction of an anthraniloyl group (fluorescent-labeling). *Ac*-AMPs (2.9 mg, *ca*. 1 µmol) was dissolved in distilled water (1.5 ml), and pH was adjusted to 8.8 with NMM. Isatoic anhydride (1 µmol) was added to the solution. The solution was stirred at 38℃. After the reaction was completed, the solution was neutralized with 6.0 N aqueous HCl and the volume was reduced *in vacuo*. The sample was identified by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry on a Voyager RP (PerSeptive Biosystems Inc., USA).

Fluorescence microscopic observation. Chitin powder or *Valsa ceratosperma* Maire was incubated with Ant-labeled *Ac*-AMPs overnight and washed with distilled water. The sample and a drop of distilled water were placed on a slide glass and covered with a cover-glass. Excitation was done with an ultraviolet ray (330-380 nm), and the blue fluorescent image of the sample was observed with a fluorescence microscope BX 51 equipped with BX-RFA (Olympus Corp., Japan).

Result and Discussion

Both *Ac*-AMP 1 and *Ac*-AMP 2 were successfully isolated as a mixture. Only two peaks were seen in the HPLC profile (data not shown). FAB mass spectrum showed peaks at 3025 (*Ac*-AMP 1, $[M+H]^+$) and 3181 (*Ac*-AMP 2, $[M+H]^+$). The mixture was used for a fluorescent-labeling and an antifungal activity test without further separation. Because both peptides are expected to be similar in antifungal and chitin-binding abilities, judging from the reports that the antifungal activity of *Ac*-AMP 1 is almost identical to that of *Ac*-AMP 2[3] and that both peptides were eluted from a chitin column under the same pH condition.

To find out a reaction condition suitable for an introduction of an Ant group with isatoic anhydride, a few kinds of bases and solvents were tested. As a result, the reaction using NMM as a base in distilled water gave a good result.

The MALDI-TOF mass spectrum showed that prepared Ant-labeled *Ac*-AMPs contained two main peptides showing the peak at 3146 and 3265, respectively. The simulation about an isotopic pattern of the fluorescent *Ac*-AMPs indicated that the former peak corresponds to monoAnt-*Ac*-AMP 1 (3144, $[M+H]^+$) and the latter to diAnt-*Ac*-AMP 1 (3263, $[M+H]^+$). Small peaks corresponding to parent *Ac*-AMPs were detected. But no peaks corresponding to Ant-derivatives of *Ac*-AMP 2 were seen, the reason being unclear. The sample of Ant-labeled *Ac*-AMPs was used for the further study without separation.

Localization of the Ant groups was not done. *Ac*-AMP 1 has three amino groups: one α-amino group of Val^1 and two ε-amino groups of Lys^{23} and Lys^{26}. Values of pKa are generally 7.0 for an α-amino group and 9.5 for an ε-amino one. Therefore, at pH 8.8, an α-amino group at N-terminus is deprotonated and preferentially substituted with an Ant group to yield a monoAnt-labeled peptide. On the other hand, under this pH condition, the reaction at an ε-amino group of a Lys residue should be slow because the amino group is mainly protonated. However, the find-

ing of diAnt-*Ac*-AMP shows that the second Ant group was introduced to the ε-amino group of either Lys^{23} or Lys^{26}. Bromer *et al*. reported that, when insulin was treated with FITC at pH 9.1, the substitution of the ε-amino group of Lys^{29} of B-chain occurred[9].

The result of the binding test of Ant-*Ac*-AMPs to chitin is shown in Fig. 3. Chitin treated with Ant-*Ac*-AMPs did show the blue fluorescent image, while neither chitin untreated with the peplides (Fig. 3) nor chitin treated with anthranilic acid (data not shown) had fluorescence emission. This suggests that the Ant moiety of Ant-*Ac*-AMPs does not prevent *Ac*-AMPs from binding to chitin.

A fungus, *Valsa ceratosperma* Maire, was treated with Ant-*Ac*-AMPs, the result being shown in Fig. 4. A compartment structure of hyphae was clearly seen. Moreover, a strong fluorescence was observed at a tip, indicating that a chitin wall at a tip is exposed.

These results indicate that fluorescent *Ac*-AMPs can bind not only chitin but also a fungus and that they can work as a fluorescent probe.

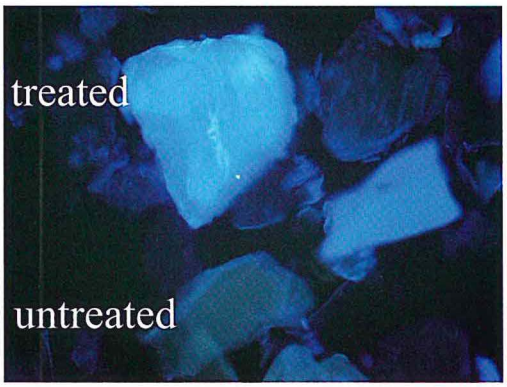

Fig. 3 Fluorescence micrograph of chitin treated and untreated with Ant-*Ac*-AMPs.

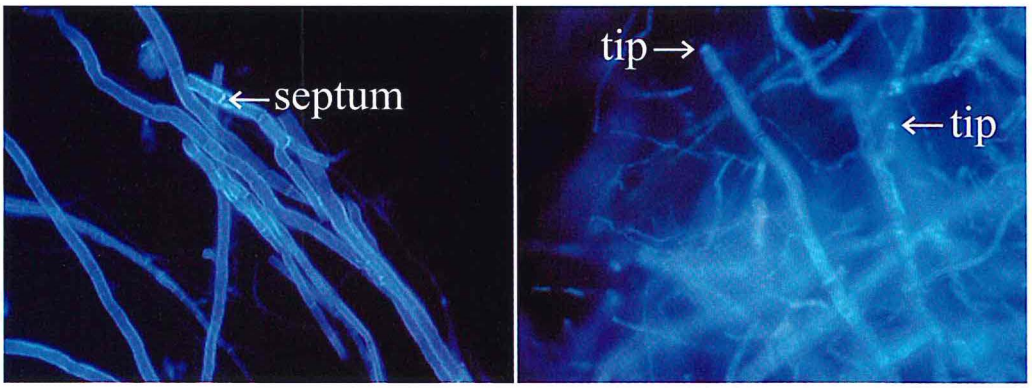

Fig. 4 Fluorescence micrographs of *Valsa ceratosperma* Maire treated with Ant-*Ac*-AMPs.

Ciopraga *et al.* reported that WGA causes the morphological changes of microorganisms such as a swollen tip or an appearance of vacuole[7]. In this study, however, no such changes of *Valsa ceratosperma* Maire were observed. The reason is not clear at this time.

Verheyden *et al.* reported that the residues, Val[1], Phe[18], Tyr[20], and Tyr[27], are involved in the chitin-binding site of *Ac*-AMP 2[10]. And, NMR study by Martin *et al.* about the structure of *Ac*-AMP 2 in water showed that three amino groups of the residues, Val[1], Lys[23] and Lys[26], are located at the edge of the chitin-binding pocket[11]. The MALDI-TOF mass spectrum showed Ant-labeled *Ac*-AMPs prepared in this study contained both monoAnt- and diAnt-*Ac*-AMP 1. And both peptides should have the Val[1] residue having the α-amino group modified with an Ant group for the reason described above. Nevertheless, Ant-labeled *Ac*-AMPs gave the fluorescent image of chitin. Clearly, the Ant group is small enough that it does not prevent *Ac*-AMP 1 from accepting chitin. There still remains a problem that both Ant-labeled peptides bind to chitin.

The result of an antifungal activity of the peptides is shown in Fig. 5. Ant-*Ac*-AMPs retains their antifungal activity *Valsa ceratosperma* Maire, at 2.9 mg/ml concentration. This suggests that the introduced Ant group did not perturb the antifungal action of *Ac*-AMP 1.

a) control b) Ant-Ac-AMP (2.9 mg/ml)

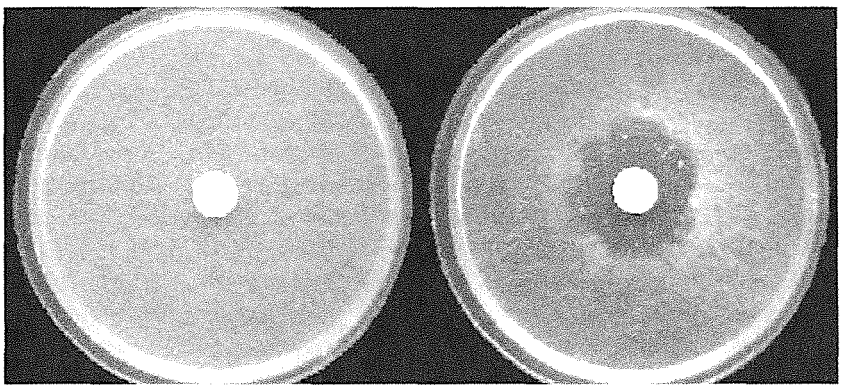

Fig.5 Antifungal activity of Ant-*Ac*-AMPs against *Valsa ceratosperma* Maire.

An anthraniloyl group was easily introduced to *Ac*-AMPs using isatoic anhydride as a labeling reagent. Importantly, Ant-*Ac*-AMPs have a chitin-affinity and an antifungal activity even after modification. Theses results show that an anthraniloyl group is very useful for fluorescent-labeling of a relatively small peptide. Furthermore, the Ant-labeled peptide with chitin-binding and antifungal properties can be a good tool to study the relationship between those two properties.

Acknowledgment

The authors thank Professor T. Shinmyozu and Ms. Eri Okazaki, IMCE of Kyushu University, for a measurement of mass spectrometry, and Associate Professor T. Anai and Mr. Y. Kodama, Faculty of Agriculture, Saga University, for an observation with a fluorescence microscope.

References

1. Walujono, L., R. A. Sholma, J. J. Beintema, A. Mariono, and A. M. Hahn, (1976). in *Atlas of Protein Sequences and Structure (Dayhoff, M. O., Ed.)* **Vol. 5.** Suppl. 3, National Biomedical Press, Washington, DC., p 308.
2. Wright, C. S., F. Gavilanes, and D. L. Peterson (1987). Primary Structure of Wheat Germ Agglutinin Isolectin 2. Peptide Order Deduced from X-ray Structure. *Biochemistry* **23**, 280-287.
3. Broekaert, W. F., W. Marien, F. R. G. Terras, M. F. C. De Bolle, P. Proost, J. Van Damme, L. Dillen, M. Claeys, S. B. Rees, J. Vanderleyden, and B. P. A. Cammue (1992). Antimicrobial Peptides from *Amaranthus caudatus* Seeds with Sequence Homology to the Cysteine/Glycine-Rich Domain of Chitin-Binding Proteins. *Biochemistry* **31**, 4308-4314.
4. Rao, U. R., R. C. Lowrie, Jr., A. C. Vickery, B. H. Kwa, and K. Nayar (1990). A DIRECT FLUORESCENCE TECHNIQUE FOR THE RAPID DETECTION OF SHEATHED MICROFILARIAE IN BLOOD SMEARS. *International J. Parasitology* **20**, 1099-1103.
5. Ciopraga J., O. Gozia, R. Tudor, L. Brezuica, and R.J. Doyle (1999). *Fusarium* sp. growth inhibition by wheat germ agglutinin. *Biochimica et Biophysica Acta* **1428**, 424-432.
6. Lutz, W. H., J. M. Londowski, and R. Kumar (1990). The Synthesis and Biological Activity of Four Novel Fluorescent Vasopressin Analogs. *J. Biol. Chem.* **265**, 4657-4663.
7. Hiratsuka, T (1982). New fluorescent Analogs of cAMP and cGMP Available as Substrates for Cyclic Nucleotide Phosphodiesterase. *J. Biol. Chem.* **257**, 13354-13358.
8. Taki, M., T. Hohsaka, H. Murakami, K. Taira, and M. Shishido (2001). A non-natural amino acid for efficient incorporation into proteins as a sensitive fluorescent probe. *FEBS Letters* **507**, 25-38.
9. Bromer, W.W., S.K. Sheehan, A.W. Berns, and E. R. Arquilla (1967). Preparation and Properties of Fluoresceinthiocarbamyl Insulins. *Biochemistry* **6**, 2378-2388.
10. Verheyden, P., J. Pletinckx, D. Maes, H. A. M. Pepermans, L. Wyns, R. Willem, and J. C. Martins (1995). ^{1}H NMR study of the interacting of *N,N',N"* -triacetyl chitotriose with *Ac*-AMP 2, a sugar binding antimicrobial protein isolated from *Amaranthus caudatus. FEBS Letters* **370**, 245-249.
11. Martins, J. C., D. Maes, R. Loris, H. A. M. Pepermans, L. Wyns, R. Willem, and P. Verheyden (1996). ^{1}H NMR Study of the solution Structure of Ac-AMP 2, a Sugar Binding Antimicrobial Protein Isolated from *Amaranthus caudatus. J. Mol. Biol.* **258**, 322-333.

抗真菌性とキチン結合性をもつ蛍光ラベル化された *Ac*-AMPs: アントラニロイル基の蛍光ラベル基としての有用性について

百武　早紀・安藤　祥司・関　清彦・内田　泰・上田　敏久
（機能高分子化学研究室）
平成16年9月21日　受理

摘　　要

Amaranthus caudatus の種子由来の抗真菌性・キチン結合性ペプチド *Ac*-AMPs（*Ac*-AMP 1と *Ac*-AMP 2の混合物）を、アントラニロイル基で蛍光ラベルした。質量スペクトルから、1個または2個のアントラニロイル基が結合した *Ac*-AMP 1が合成されていることがわかった。蛍光ラベル化された *Ac*-AMPs はキチンと結合した。また、このペプチドはキチン質を含むリンゴ腐らん病菌とも結合性を示し、蛍光顕微鏡でその菌糸構造をはっきりと観察できた。さらに、この蛍光性 *Ac*-AMPs はリンゴ腐らん病菌に対して抗真菌性を維持していた。これらの結果から、アントラニロイル基は *Ac*-AMPs のもつ特性を乱すことがなく、有用な蛍光ラベル基であることがわかった。今回の研究で合成した蛍光性 *Ac*-AMPs は、キチン結合性と抗真菌活性の関係を研究するためのよい道具となりうるであろう。