Purification, Characterization, and Sequencing of a Novel Type of Antimicrobial Peptides, Fa-AMP1 and Fa-AMP2, from Seeds of Buckwheat (Fagopyrum esculentum Moench.)

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Novel antimicrobial peptides (AMP), designated Fa-AMP1 and Fa-AMP2, were purified from the seeds of buckwheat (Fagopyrum esculentum Moench.) by gel filtration on Sephadex G75, ion-exchange HPLC on SP COSMOGEL, and reverse-phase HPLC. They were basic peptides having isoelectric points of over 10. Fa-AMP1 and Fa-AMP2 had molecular masses of 3,879 Da and 3,906 Da on MALDI-TOF MS analysis, and their extinction coefficients in 1% aqueous solutions at 280 nm were 42.8 and 38.9, respectively. Half of all amino acid residues of Fa-AMP1 and Fa-AMP2 were cysteine and glycine, and they had continuous sequences of cysteine and glycine. The concentrations of peptides required for 50% inhibition (IC50) of the growth of plant pathogenic fungi, and Gram-positive and -negative bacteria were 11 to 36 μg/ml. The structural and antimicrobial characteristics of Fa-AMPs indicated that they are a novel type of antimicrobial peptides belonging to a plant defensin family.

Key words: antimicrobial peptide (AMP); Fa-AMP; pathogenic related protein; buckwheat; Fagopyrum esculentum

Antimicrobial peptides are important substances functioning as self-defense against infection by various harmful pathogens. They are isolated from various sources among animals, plants, and bacteria, and have been characterized.1-3) Plants don’t have typical immune systems such as seen in mammals, but have different mechanisms for protecting themselves against infection by various pathogens. For example, there are some enzymes: β1,3-glucanase and chitinase, which degrade fungal cell walls; and ribosome-inactivating proteins having antiviral and antifungal properties.4-8) In addition to these substances, various peptides 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. Antimicrobial peptides of animal origin are classified on the basis of the primary structures. Antimicrobial peptides belonging to the defensin family9,10) from animals contain a large amount of cysteine and several disulfide bonds in the molecules. The cecropin family from insects have simple α-helical structure in the molecules. The glycine-rich peptide family is a kind of certain amino acid rich peptide family.11)

Some plants also have antimicrobial peptides similar in the amino acid sequence to those from animals. Since their primary structures are similar to those of the defensin family, antimicrobial peptides discovered from plants are called the plant defensin family. The relationship between the structure and the antimicrobial activity and mechanism of antimicrobial action in plants has not been clarified. In this paper, we describe the purification and some properties of novel antimicrobial peptides from Fagopyrum esculentum, which have a potent and wide antimicrobial activity against various pathogens.

Materials and Methods

Materials. Buckwheat seeds were harvested at Kagoshima prefecture in Japan. The following bacteria and fungi were used: Erwinia carotovora subsp. carotovora MAFF 106567, Agrobacterium rhizogenes MAFF 210265, Agrobacterium radiobacter MAFF 520028, Curtobacterium michiganensis subsp. michiganensis MAFF 301044, Curtobacterium flac-

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Abbreviations: AMP, antimicrobial peptide; AFP, antifungal peptide; MeCN, acetonitrile; 2-PrOH, 2-propanol; TFA, trifluoroacetic acid; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid; PITC, phenylisothiocyanate
**cum faciens** pv. oortii MAFF 301203, *Fusarium oxysporum* IFO 6384, *Geotrichum candidum*. They were donated by the Laboratory of Phytopathology, Department of Agriculture, Kagoshima University. All strains were cultured at 30°C in a constant-temperature incubator. All other chemicals were of the highest purity commercially available.

**Purification of antimicrobial peptides (Fa-AMP1 and Fa-AMP2).** Purification at all steps was done at 4°C. Seeds of buckwheat (300 g) were homogenized with 10 volumes of 50 mM sodium acetate buffer (pH 4.8). After this was stirred overnight, the homogenate was filtered through gauze and the filtrate was centrifuged at 10,000 rpm for 20 min. The clear supernatant was concentrated under reduced pressure, and saturated with ammonium sulfate. The precipitate was dissolved in a small amount of 50 mM sodium acetate buffer (pH 4.8), and then put through a Sephadex G75 column (Bio-Rad, USA) previously equilibrated with 50 mM sodium acetate buffer (pH 5.0). The protein fraction with antimicrobial activity was collected and saturated with ammonium sulfate. The precipitate was dialyzed against 10 mM sodium phosphate buffer (pH 7.5) containing 2.5 mM CaCl₂, and then 18 nmol of thermolysin was added to the solution. After incubation at room temperature for 4 h, pyridylethylated 4-vinylpyridine was added. After incubation at room temperature for 4 h, 20 μl of 4-vinylpyridine was added. After incubation at room temperature for 4 h, 20 μl of 4-vinylpyridine was added. After incubation at room temperature for 4 h, pyridylethylated Fa-AMPs were purified by reverse-phase HPLC using a Mightysil RP-4 column with a gradient of 0–30% MeCN:2-ProOH (3:7, v/v) in 0.1% TFA.

**Tricine SDS-PAGE.** Tricine SDS-PAGE was done at pH 8.9 under reducing conditions by 2-mercaptoethanol with a 6.0% polyacrylamide gel as described by Schagger and Jagow.12

**Isoelectric point.** Isoelectric focusing was done by the method of Wrigley13 on 5% carrier Bio-Lyte of the pH range of 3–10 (Bio-Rad, USA).

**Carbohydrate content.** Neutral carbohydrates were measured by the phenol-sulfuric acid method,14,15 and expressed as the amount of glucose.

**MALDI-TOF MS.** For measuring the molecular mass, Fa-AMP1 and Fa-AMP2 were analyzed on a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF MS) (Voyager DE-RP, Perseptive 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: Fa-AMPs = 9:1, v/v) was spotted 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%; guidewire voltage, 0.050%; low mass gate 500.0; and laser intensity, 2,400. Signals from 26–256 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 Corp., Salem, NH).

**Assay of antimicrobial activity.** Antimicrobial activity was measured by a method similar to that of Broekaert et al.10 using PD medium (Sigma, USA) containing 2.5 × 10⁴ fungal spores/ml for antifungal activity, and 1 × 10⁸ colony-forming units of bacteria/ml for antibacterial activity. A peptide solution (20 μl) sterilized by ultrafiltration with a nitrocelulose membrane, and the sterilized culture solution (80 μl), were successively put into a microplate leader’s well, and incubated for 24 h at 30°C. The turbidity of the solution was measured at 490 nm.

**Pyridylethylation of Fa-AMPs.** Lyophilyzed Fa-AMPs (100 nmol) were dissolved in 75 μl of 8 M guanidine hydrochloride, and 25 μl of 2 M Tris-HCl buffer containing 10 mM EDTA and 20 μl of 2-mercaptoethanol were successively added to the solutions. After incubation at room temperature for 4 h, 20 μl of 4-vinylpyridine was added. After incubation at room temperature for 4 h, pyridylethylated Fa-AMPs were purified by reverse-phase HPLC using a Mightysil RP-4 column with a gradient of 0–30% MeCN:2-ProOH (3:7, v/v) in 0.1% TFA.

**Amino acid composition and sequence analyses.** Pyridylethylated Fa-AMP1 and Fa-AMP2 (10 nmol) were hydrolyzed with 6.0 N HCl containing 0.05% 2-mercaptoethanol (200 μl) in sealed evacuated tubes at 130°C for 3 h. After hydrolysis, each amino acid was modified with PITC and analyzed with a Waters Pico-Tag amino acid analyzer (Waters 2690 Separations Module, USA). The content of tryptophan was measured by the method of Edelhoch.17 The amino acid sequences of the Fa-AMP1 and Fa-AMP2 were analyzed with an Applied Biosystems Procise 492 protein sequencer.18

**Thermolytic digestion of Fa-AMPs and separation of peptide fragments.** Pyridylethylated Fa-AMP1 and Fa-AMP2 (360 nmol) were dissolved in 50 mM Tris-HCl buffer (100 μl), pH 8.0, containing 6 M urea and 2.5 mM CaCl₂, and then 18 nmol of thermolysin was added to the solution. After incubation at 30°C for 3 h, the mixture was diluted with 0.1% TFA and then concentrated under reduced pressure. The clear supernatant was dialyzed against a solution containing 2.5 mM CaCl₂, and then 18 nmol of thermolysin was added to the solution. After incubation at room temperature for 4 h, pyridylethylated Fa-AMPs were purified by reverse-phase HPLC using a Mightysil RP-4 column with a gradient of 0–30% MeCN:2-ProOH (3:7, v/v) in 0.1% TFA.
filtration on Sephadex G75 (Fig. 1A), and was further purified by cation-exchange HPLC on a SP COSMOGEL column with a linear gradient from 0 to 0.5 M NaCl (Fig. 1B). The active fraction was put through reverse-phase HPLC on Mightysil RP-4 (Fig. 1C) to give two active fractions, followed by rechromatography. Two antimicrobial peptides were obtained in purified forms, Fa-AMP1 being obtained from the first peak, and Fa-AMP2 from the second one. Yields of purified Fa-AMP1 and Fa-AMP2 were approximately 1.8 mg and 1.4 mg, respectively. As shown in Fig. 2, reduced Fa-AMP1 and Fa-AMP2 migrated as single bands with apparent molecular weight of about 4 kDa.

The isoelectric points of purified Fa-AMP1 and Fa-AMP2 were 10.2 and 10.8, respectively. On MALDI-TOF MS, singly protonated molecular ions (M+H)+ of Fa-AMP1 and Fa-AMP2 were at m/z 3880.1 and 3907.1, respectively (Fig. 3), indicating that the molecular masses of Fa-AMP1 and Fa-AMP2 were 3879.1 and 3906.1, respectively. No carbohydrate was detected by the method using phenol-H2SO4. The extinction coefficients of Fa-AMP1 and Fa-AMP2 of 1% aqueous solutions at 280 nm (E1%280) were 42.8 and 38.9, respectively.

Antimicrobial activities of Fa-AMPs
The antimicrobial activities of Fa-AMPs against plant pathogenic fungi and Gram-negative and -positive bacteria were evaluated as the concentrations required for 50% growth inhibition, IC50, (Table 1). The values of IC50 varied from 11 to 36 μg/ml, depending on the tested microorganisms. The antifungal potency of Fa-AMP1 was almost identical to that

Fig. 1. Purification of Fa-AMPs by Column Chromatography. Active fraction is shown by the bold line. (A) Gel filtration chromatogram. Crude antimicrobial peptide preparation was passed through a Sephadex G75 column (φ 4.4 × 120 cm) previously equilibrated with 50 mM sodium acetate buffer (pH 5.0). (B) Cation-exchange chromatogram. The active fraction dissolved in 10 mM sodium phosphate buffer (pH 6.8) was put on a SP COSMOGEL HPLC column (φ 0.5 × 5 cm) previously equilibrated with 10 mM sodium phosphate buffer (pH 6.8). The column eluted with a linear gradient from 0 to 0.5 M NaCl in 10 mM sodium phosphate buffer (pH 6.8). (C) Reverse-phase HPLC column chromatogram. The active fraction was purified by reverse-phase HPLC using a Mightysil RP-4 column (φ 4.6 × 250 mm) with a linear gradient of 0–30% MeCN:2-PrOH (3:7, v/v) in 0.1% TFA.

Results

Purification and characterization of Fa-AMPs
The antimicrobial peptides of buckwheat seeds (300 g) were purified by three kinds of chromatography. The active fraction was obtained by gel added. After incubation at 25°C for 1.5 h, fragment peptides were separated by reverse-phase HPLC with a Mightysil RP-4 column, using a linear gradient of 0–30% MeCN:2-PrOH (3:7, v/v) in 0.1% TFA.
MALDI-TOF MS indicated that Fa-AMP1 and Fa-AMP2 have monoisotopic molecular masses of 3880.1 and 3907.1, respectively. The corresponding doubly charged protonated molecules at \( m/z \) 1939.7 and 1953.0, respectively.

Table 1. Antimicrobial Activities of Fa-AMP1 and Fa-AMP2

<table>
<thead>
<tr>
<th>Microorganisms</th>
<th>IC(_{50}) (μg/ml) Fa-AMP1</th>
<th>IC(_{50}) (μg/ml) Fa-AMP2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram-negative bacteria</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Erwinia carotovora</td>
<td>11</td>
<td>15</td>
</tr>
<tr>
<td>Agrobacterium radiobacter</td>
<td>24</td>
<td>17</td>
</tr>
<tr>
<td>Agrobacterium rhizogenes</td>
<td>20</td>
<td>24</td>
</tr>
<tr>
<td>Gram-positive bacteria</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clavibacter michiganensis</td>
<td>14</td>
<td>17</td>
</tr>
<tr>
<td>Curtobacterium flaccumfaciens</td>
<td>13</td>
<td>15</td>
</tr>
<tr>
<td>Fungi</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fusarium oxysporum</td>
<td>19</td>
<td>29</td>
</tr>
<tr>
<td>Geotrichum candidum</td>
<td>36</td>
<td>25</td>
</tr>
</tbody>
</table>

Table 2. Amino Acid Composition of Fa-AMPs

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Fa-AMP1 (residues/molecule)</th>
<th>Fa-AMP2 (residues/molecule)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asx</td>
<td>0.7 (1)</td>
<td>0.7 (1)</td>
</tr>
<tr>
<td>Glx</td>
<td>4.3 (4)</td>
<td>4.1 (4)</td>
</tr>
<tr>
<td>Ser</td>
<td>3.0 (3)</td>
<td>2.9 (3)</td>
</tr>
<tr>
<td>Gly</td>
<td>10.7 (10)</td>
<td>10.5 (10)</td>
</tr>
<tr>
<td>His</td>
<td>0.1 (0)</td>
<td>0.1 (0)</td>
</tr>
<tr>
<td>Arg</td>
<td>0.1 (0)</td>
<td>1.0 (1)</td>
</tr>
<tr>
<td>Thr</td>
<td>2.4 (2)</td>
<td>2.1 (2)</td>
</tr>
<tr>
<td>Ala</td>
<td>4.3 (4)</td>
<td>4.3 (4)</td>
</tr>
<tr>
<td>Pro</td>
<td>2.3 (2)</td>
<td>2.2 (2)</td>
</tr>
<tr>
<td>Tyr</td>
<td>1.2 (1)</td>
<td>1.3 (1)</td>
</tr>
<tr>
<td>Val</td>
<td>0.1 (0)</td>
<td>0.1 (0)</td>
</tr>
<tr>
<td>Met</td>
<td>0.0 (0)</td>
<td>0.1 (0)</td>
</tr>
<tr>
<td>Cys</td>
<td>8.3 (8)</td>
<td>8.6 (8)</td>
</tr>
<tr>
<td>Ile</td>
<td>0.4 (0)</td>
<td>0.2 (0)</td>
</tr>
<tr>
<td>Leu</td>
<td>1.0 (1)</td>
<td>1.0 (1)</td>
</tr>
<tr>
<td>Phe</td>
<td>0.1 (0)</td>
<td>0.0 (0)</td>
</tr>
<tr>
<td>Lys</td>
<td>2.1 (2)</td>
<td>1.4 (1)</td>
</tr>
<tr>
<td>Trp</td>
<td>— (2)</td>
<td>— (2)</td>
</tr>
</tbody>
</table>

The number in parenthesis are from the sequence determined.

The sequence of Fa-AMP1 was compared with those of various antimicrobial peptides in the plant defensin family (Figs. 5A and B).\(^1\)\(^,\)\(^19\) These plant defensins can be divided into two types (type 1 and type 2) according to the number and the position of cysteine residues in the molecules; type 1 (knotting type) and type 2 plant defensins contain 6 and 8 cysteines, respectively. Although Fa-AMPs have 8 cysteine residues, their cysteine arrangements were clearly different from other type 2 plant defensins (Fig. 5B), but strikingly similar to those of type 1 in the first 6 cysteine residues from N-terminus (Fig. 5A). Fa-AMPs, as well as type 1 plant defensins, have a continuous sequence of cysteine (–CC–). In addition to the –CC– sequence, Fa-AMPs contained continuous sequences of glycine (–GGG– and –GG–), as seen in the glycine-rich peptides family.

Fig. 3. MALDI-TOF MS Spectra of Fa-AMP1 and Fa-AMP2. MALDI-TOF MS indicated that Fa-AMP1 and Fa-AMP2 had wide antimicrobial spectra against various plant-pathogenic fungi and bacteria.

Amino acid compositions and sequences of Fa-AMPs

Amino acid compositions of Fa-AMP1 and Fa-AMP2 are shown in Table 2. Fa-AMP1 and Fa-AMP2 were particularly rich in cysteine and glycine. The complete amino acid sequences of Fa-AMP1 and Fa-AMP2 are shown in Fig. 4. The amino acid sequence of the first 25 residues from the amino-terminus was not different between Fa-AMP1 or Fa-AMP2. The sequence from the 25th residue to the carboxy-terminus was found by analyzing thermolytic peptides designated pep2-1 and pep2-2 (25 amino acid residues) of Fa-AMP1 and Fa-AMP2, respectively. In pep2-1 and pep2-2, the first 10 residues from the amino-terminus corresponded to the sequence from the 16th to 25th residue of Fa-AMP1 and Fa-AMP2. Fa-AMP1 and Fa-AMP2 consisted of 40 amino acid residues. The difference of values between MALDI-TOF MS analysis and theoretical masses calculated from the amino acid sequence data were within 0.6 Da, assuming that all eight cysteines form four disulfide bonds and that all other amino acids are unmodified. The amino acid sequences of Fa-AMP1 and Fa-AMP2 were the same except for the carboxy-terminus, the 40lysine of the carboxy-terminus of Fa-AMP1 being replaced by 40arginine in Fa-AMP2.
Amino Acid Sequences of Fa-AMP1 and Fa-AMP2.

The N-terminal amino acid sequence of Fa-AMP was analyzed after treatment with pyridylethylation. The amino acid sequence of the first 25 residues from amino-terminus was not different between Fa-AMP1 and Fa-AMP2 (N-terminal). Further sequence data were obtained from thermolytic peptides (pep 1, pep 2-1, and pep 2-2, respectively). Vertical arrow indicate replacement of an amino acid.

Fig. 4. Amino Acid Sequences of Fa-AMP1 and Fa-AMP2.

The amino acid sequences of Fa-AMP1 and Fa-AMP2 were highly basic and simple peptides. Both Fa-AMP1 and Fa-AMP2 consisted of 40 amino acid residues, and 45% of the total amino acid residues were glycine and cysteine.

Discussion

A novel type of antimicrobial peptides, designated Fa-AMP1 and Fa-AMP2, were purified. They were highly basic and simple peptides. Both Fa-AMP1 and Fa-AMP2 consisted of 40 amino acid residues, and 45% of the total amino acid residues were glycine and cysteine.

Antimicrobial peptides which contain many cysteine residues in the molecules are classified into the defensin family. The primary structures of Fa-AMP1 and Fa-AMP2 were similar to the defensin family from animals (Fig. 5C).20 In recent years, many antimicrobial peptides that contain cysteine residues abundantly have been isolated from plants, and these are classified into the plant defensin family.21 It is clear from the sequences that Fa-AMP1 and Fa-AMP2 also belong to this plant defensin family. Many cysteine residues seem to contribute to forming the stable tertiary structure which is necessary for...
antimicrobial activity. In animal defensins, the number of cysteine residues does not exceed 7. Antimicrobial peptides that contain 8 cysteine residues have been isolated from plants only. Antimicrobial peptides with a continuous sequence of cysteine (–CC–) are found in type 1 of the plant defensin family that contains 6 cysteine residues, but are not in type 2 that contains 8 cysteine residues. Consequently, Fa-AMPs are unique peptides with respect to their sequences having 8 cysteine residues and the continuous sequence of cysteines (–CC–) and thus should be classified into a subtype of type 1 and type 2 plant defensin family (Fig. 5A). Peptide chains of Fa-AMPs were comparatively short, compared with those of the plant defensin family with 8 cysteine residues (Fig. 5B).

Antimicrobial peptides with a large quantity of a specific amino acid, e.g. the glycine-rich peptide family and the proline-rich peptide family, have been found only in animals. Both Fa-AMP1 and Fa-AMP2 contained 10 glycine residues, and continuous sequences of glycine (–GGG– and –GG–). It can be concluded from these results that Fa-AMP1 and Fa-AMP2 are novel and peculiar antimicrobial peptides which have characteristics of both the defensin family and the glycine-rich peptide family.

The relationship between structures of antimicrobial peptides and antimicrobial activity are fairly made clear; the defensin family is specific to Gram-positive bacteria, and the glycine-rich peptides family predominantly to Gram-negative bacteria. Fa-AMPs were sensitive to both Gram-negative and -positive bacteria, as well as to fungi, in contrast to Ac-AMP and Mj-AMP; these peptides belong to the defensin family, and were not active against Gram-negative bacteria. The reason why Fa-AMPs had a wide antimicrobial spectrum seems to be that Fa-AMPs have the primary structural features of both the defensin family and the glycine-rich peptides family.

Cell walls of bacteria consist of peptidoglycan, and those of fungi contain chitin. Although the cell-wall compositions of bacteria and fungi are different, Fa-AMPs had antimicrobial activity on both. In case of bacteria, it seems that a highly basic peptide is bound to a cell wall peptidoglycan charged negatively. Antimicrobial peptides may show activity on fungi by a different action mechanism. Indeed, it is made clear that Fa-AMPs are combined with a microchitin column (data not shown). However, the relationship between antimicrobial activity and chitin binding avidity have not been clarified.

Mammals have gained a skillfull self-defense mechanism which is called “immunity“, but plants don’t have “immunity“. Plants are considered to have developed another very skillful and original self-defense system in the process of evolution. Defensin was found for the first time in animal. After that, antimicrobial peptides, to which the structures are very similar, came to be found in plants, indicating a possibility that the self-defense mechanism through these peptides can be widely generalized not only for the invertebrate animals but for plants. However, in a self-defense action, it is hard to think that these antimicrobial peptides react at the same stage and by the same mechanism.

In this paper, we describes the existence of new antimicrobial peptides, of which the structures and an antimicrobial spectra have not been reported until now. Fa-AMP1 and Fa-AMP2 had potent antimicrobial activity against both bacteria and fungi. It is necessary to add to the knowledge about the structure-function relationships of these antimicrobial peptides.

In the results of the sequence and antimicrobial activity of Fa-AMP1 and Fa-AMP2, they had characteristic features in both defensin and glycine-rich peptides families, and had broad antimicrobial activity. The unique properties of Fa-AMPs as potent antimicrobial compounds suggest that they may have evolved as a skillful defense mechanism for protecting the plant body.

References


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