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Structure and Antibiotic Activity of Fragment Peptides of Antifungal Protein Isolated From *Aspergillus giganteus*

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Abstract In order to determine the functional region of the antifungal protein (AFP) isolated from *Aspergillus giganteus* responsible for growth inhibitory activity and the promotion of phospholipid vesicle aggregation, overlapping peptides covering the complete sequence of AFP were synthesized. The antibiotic activity against bacterial, fungal, and tumor cells, and the vesicle-aggregation activity of the synthetic peptides were investigated. The AFP functional sequence responsible for antibiotic and vesicle-aggregation activity was determined to be located within the region between AFP residues 19 to 32. AFP (19-32) exhibited an α-helical conformation in a cell membrane-like environment. AFP (19-32) displayed potent antibiotic activity against bacterial, fungal, and tumor cells without peptide toxicity as indicated by hemolysis. Accordingly, AFP (19-32) could be used as a good model for the design of effective antibiotic agents with powerful antibiotic activity yet without any cytotoxic effects against the host organism.

Key words: *Aspergillus giganteus*, antifungal protein, fragment peptides, functional sequence

Aspergillus giganteus is known to produce mainly two novel extracellular proteins with antifungal activity [11, 16, 19, 20, 21]. One of them is α-sarcin, a cytotoxic ribonuclease toxin [21]. The other is a very basic small-sized protein, the so-called antifungal protein (AFP) [11, 19, 20]. This small protein can be isolated in a relatively high amount from an *A. giganteus* culture medium [11, 19, 20]. AFP consists of 51 amino acid residues with a high content of tyrosine and lysine residues (6 and 12 residues, respectively) containing eight cysteine residues which are all involved in four-intramolcular disulfide linkages [11, 19, 20]. Its apparent toxic character resembles other plant proteins known as thionins which occur in both monocotyledonous and various dicotyledonous plants [2, 16]. As assessed by two-dimensional nuclear magnetic resonance, AFP has a five-stranded antiparalled β-sheet structure that is stabilized by four intrachain disulfide bridges [3].

Until now, the AFP functional region responsible for the growth inhibitory activity against bacterial, fungal, and tumor cells is still undetermined. Therefore, in this study, a series of peptide fragments based on the AFP five antiparallel β-strand sequences were synthesized [3]. The cell membrane lytic activities of the synthetic peptide fragments against gram-positive and negative bacteria, filamentous fungi, and human small-cell lung cancer (SCLC) cell lines were measured and two peptide fragments with potent activity were determined. Also, in order to determine the minimum AFP functional sequence for antibiotic activity, we synthesized the peptides with various lengths including the short region overlapping the amino acid sequence of the two peptide fragments with potent antibiotic activity, and then their antibiotic activity in various organisms was tested. The secondary structure of the peptides was also investigated in various conditions using circular dichroism (CD). Finally, the lipid aggregation ability induced by the peptides was investigated.

Materials and Methods

Reagents

Fmoc-Wang-resins and Fmoc-amino acids were obtained from Nova Biochem. (San Diego, U.S.A.). Chemical reagents for the solid phase peptide synthesis, phosphatidylcholine (PC) and phosphatidylserine (PS) were purchased from Sigma Chemical Co. (St. Louis, U.S.A.).

Bacteria, Fungi, Human Erythrocytes, and Tumor Cells

Escherichia coli (ATCC 33694), *Bacillus subtilis* (ATCC 6633), *Aspergillus flavus* (KCTC 1375), *Aspergillus fumigatus* (KCTC 6145), *Fusarium oxysporum* (KCTC 6076), and

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Fusarium solani (KCTC 6326) were obtained from the Korean Collection for Type Cultures, Korea Research Institute of Bioscience and Biotechnology, Taejon, Korea. Human SCLC cell lines, NCI-H126 and NCI-H148, were received from the Cell Bank of Seoul National University, Korea. Human red blood cells were purchased from the Blood Center of the Korean Red Cross.

Peptide Synthesis

The synthesis of the peptides was carried out manually by the solid-phase strategy [10]. The α-amino group of amino acids were protected by 9-fluorenylmethyloxycarbonyl (Fmoc). Fmoc-Wang-resin was used as the starting support. The side-chain protecting group were as follows: Arg, 2,2,5,7-pentamethylchroman-*O*-sulfonyl (Pmc); Asn and Gln, trityl (Trt); Ser and Glu, t-butyl (tBu); Lys, t-butyloxycarbonyl (Boc). All coupling stages were performed with a 10-fold excess of protected amino acid derivatives with an equimolar mixture of DCC (N,N′-dicyclohexylcarbodiimide)/HOBt (1-hydroxybenzotriazole) as the coupling agent. The coupling cycle was performed according to the following steps: 1. NMP wash; 2. 20% (vol/vol) piperidine/NMP (3 min); 3. 20% piperidine/NMP (17 min); 4. NMP wash (4×); 5. Coupling (60 min); 6. NMP wash (4×). After coupling the last amino acid, the Fmoc-group was removed with 20% piperidine/NMP, and the protected peptide-resins were cleaved and protected with a mixture of Reagent K containing triisopropylsilane (85% TFA, 4% phenol, 2% 1, 2-ethandithiol, 4% H_2O, 4% thioanisole, and 1% triisopropylsilane, vol/vol) for 2 h at room temperature, precipitated with diethylether, and then dried in a vacuum. The crude peptides were purified using a reversed-phase preparative HPLC (Waters) on a Delta-Pak C_{18} column, 3.9×15 cm. Purity of the purified peptides was then checked by an analytical reversed-phase HPLC (Waters) on a Delta-Pak C_{18} column, 3.9×50 cm. The purified peptides were hydrolyzed with 6 N HCl containing 2% phenol at 110°C for 22 h, and then dried in a vacuum. The residues were finally dissolved in 0.02 N HCl and subjected to amino acid analysis (Hitachi Model, 8500 A, Japan).

Antibacterial Activity Assay

Escherichia coli (ATCC 33694) and *Bacillus subtilis* (ATCC 6633) were grown in a Luria-Bertani medium (LB, 10 g of bactotryptone, 5 g of yeast extract, and 10 g of NaCl per litre) at 37°C overnight and diluted in a basal medium of 1% bactopeptone (Difco) to 1:200 [i.e. final bacterial suspension containing 2~8×10^6 colony forming units (CFU)/ml]. Peptide 1:2 serial dilutions were prepared from 200 μg/ml stock solutions to provide a range of 100~0.78 μg/ml in a 1% bactopeptone media. 100 μl of peptide stock solution was added to the 100 μl of bacterial suspension and then incubated at 37°C overnight. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of peptide in which there was no change in optical density (OD) at 620 nm after 18 h.

Antifungal Activity Assay

The fungal strains were grown at 28°C in a YM medium (1% glucose, 0.3% malt extract, 0.5% peptone, and 0.3% yeast extract). The fungal conidia were seeded on 96-well plates (NUNC, U.S.A.) at a density of 1×10^2 spores per well in a volume of 100 μl of YM media. 10 μl of the serially diluted peptides was added to each well, and the cell suspension was incubated for 24 h at 28°C. After incubation, 10 μl of a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) solution [5 mg/ml MTT in phosphate-buffered saline (PBS), pH 7.4] was added to each well, and the plates were further incubated at 37°C for 4 h. 30 μl of a 20% (w/v) SDS solution containing 0.02 M HCl was added and the reaction incubated at 37°C for 16 h to dissolve the formed formazan crystals. The turbidity of each well was measured at 570 nm using a microtiter ELISA reader (Molecular Devices, Sunnyvale, California).

Antitumor Activity Assay

Human SCLC cell lines (NCI-H126 and NCI-H148) were grown in RPMI-1640 medium supplemented with heat-inactivated 10% Fetal bovine serum (FBS), 100 U/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate. The cells suspended in the RPMI-1640 medium with 10% FBS were plated in 96-well plates (Nunc, F96 microtiter plates) at a density of 2.0×10^4cells/well. After incubating the plates overnight at 37°C in 5% CO_2 atmosphere, 20 μl of the serially diluted-peptides was added to each well and incubated for 3 days. 20 μl of a MTT solution (5 mg/ml, MTT in PBS) was then added to each well and the plates were incubated for 4 h at 37°C. 30 μl of a 20% sodium dodecyl sulfate (SDS) solution containing 0.02 M HCl was added to each well, and then incubated for 3 h at 37°C to dissolve the dark blue crystals (MTT-formazan product). The absorbance was monitored at 570 nm using an ELISA plate reader.

Hemolytic Activity Assay

The hemolytic activity of the peptides was determined using human red blood cells (hRBCs). Fresh hRBCs were rinsed three times with PBS (35 mM phosphate buffer, 0.15 M NaCl, pH 7.0), centrifuged for 15 min at 900×g, and resuspended in PBS. 100 μl of a 0.4% (vol/vol) suspension in PBS was plated in 96-well plates (Nunc, F96 microtiter plates), and then 100 μl of the peptide solution dissolved in PBS was added (the final peptide concentration: 100 μg/ml). The plates were incubated for 1 h at 37°C, and then centrifuged at 1000×g for 5 min. One hundred μl aliquots of the supernatant were transferred to 96-well microtiter plates. The release of hemoglobin was monitored by measuring the absorbance at 414 nm with an ELISA

plate reader (Molecular Devices). 0 and 100% hemolysis were determined in PBS and 0.1% Triton-X 100, respectively. The hemolysis percentage was calculated as [($A_{414\ nm}$ in the peptide solution - $A_{414\ nm}$ in PBS)/($A_{414\ nm}$ in 0.1% Triton-X 100- $A_{414\ nm}$ in PBS)]×100.

Circular Dichroism Analysis

The CD spectra of the peptides were recorded using a Jasco J720 spectropolarimeter (Japan). All samples were maintained at 25°C during the analysis. Four scans per sample were performed over a wavelength range of 190~250 nm at 0.1 nm intervals. The spectra were measured in a 10 mM sodium phosphate buffer (pH 7.2) in the presence of TFE or SDS at 25°C using a 1 mm pathlength cell. The peptide concentrations were 50 μM. The mean residue ellipticity, [θ], is given in deg·cm^2·dmol^{-1}: $[\theta]=[\theta]_{obs}$ (MRW/10*lc*), where $[\theta]_{obs}$ is the ellipticity measured in millidegrees, MRW is the mean residue molecular weight of the peptide, *c* is the concentration of the sample in mg/ml, and *l* is the optical path length of the cell in cm. The peptide percent helicities were calculated using the following equation [1, 5, 9, 12].

$$\% \text{ helicity} = 100\ ([\theta]_{222} - [\theta]^{0}_{222}) / [\theta]^{100}_{222}$$

where $[\theta]_{222}$ is the experimentally observed mean residue ellipticity at 222 nm. Values for $[\theta]^{0}_{222}$ and $[\theta]^{100}_{222}$, corresponding to 0% and 100% helical content at 222 nm, were estimated to be -2,000 and -30,000 deg·cm^2/dmol, respectively [1, 4, 5, 9].

Preparation of Small Unilamellar Vesicles (SUVs) and Vesicle-Aggregation Test

SUVs were prepared by the sonication of a mixture of phosphatidylcholine (PC)/phosphatidylserine (PS) (4:1, w/w) or PC/PS(1:1, w/w). The phospholipid mixture was dried to a thin film in a 25 ml round bottomed flask using a rotary evaporator, and then dried overnight in a high vacuum. The resulting lipid film was suspended in a Hepes buffer (10 mM Hepes, pH 7.4/150 mM NaCl), and then sonicated until clear using a tip type sonicator. The phospholipid concentration used for the aggregation test was 35 M and the peptides were added from 0.2 nM to 0.4 nM. After 30 min at room temperature, the vesicle-aggregation activities induced by the peptides were determined at 400 nm using a spectrophotometer.

Results and Discussion

In order to identify the region of the AFP protein which is responsible for growth inhibitory activity against bacteria, fungi, and tumor cells, an overlapping series [AFP (1-17), AFP (13-29), AFP (22-38), AFP (28-44), and AFP (35-51)] of truncated peptides covering the complete sequence were synthesized (Table 1). The antibacterial activity of the peptides against *E. coli* and *B. subtilis* was determined as the minimum inhibitory concentration (MIC). Human small cell lung cancer (SCLC) cell lines and filamentous fungi, *Aspergillus flavus*, *Aspergillus fumigatus*, *Fusarium oxysporum*, and *Fusarium solani* were all used for determining the antitumor and antifungal activities of the peptides. The peptide antitumor and antifungal activities were represented as 50% growth inhibitory concentration (IC_{50}).

Among the five peptides, AFP (13-29) and AFP (22-38) showed a potent cytotoxic activity against both bacterial and tumor cells (Table 2). However, the other peptides, AFP (1-17), AFP (28-44), and AFP (35-51), did not display any antibacterial activity with a 50 μM peptide concentration.

Table 1. Amino acid sequences of the overlapping synthetic peptides of AFP Protein.

Peptides	Amino acid sequences
AFP (1-17)	ATYNGKCYKKDNICKYK
AFP (13-29)	ICKYKAQSGKTAICKCY
AFP (22-38)	KTAICKCYVKKCPRDGA
AFP (28-44)	CYVKKCPRDGAKCEFDS
AFP (35-51)	RDGAKCEFDSYKGKCYC

Table 2. Antibiotic activities of the AFP overlapping peptides against bacterial, fungal, human tumor, and human red blood cells.

Peptides	Bacterial cells (MIC: μM)		Tumor cells (SCLC) (IC_{50}: μM)		Fungal cells (IC_{50}: μM)				% Hemolysis (100 μM)
	E. coli	*B. subtilis*	NCI-H126	NCI-H148	*A. flavus*	*A. fumigatus*	*F. oxysporum*	*F. solani*	
AFP (1-17)	>50	>50	>100	>100	38.0	70	32.5	70.0	0
AFP (13-29)	12.5	6.125~12.5	58	38	10.1	11.0	10.8	15.0	0
AFP (22-38)	12.5~25.0	12.5	52	23	11.0	11.2	12.5	20.0	0
AFP (28-44)	>50	>50	>100	>100	30.0	55.0	25.0	50.0	0
AFP (35-51)	>50	>50	>100	>100	43.0	70.0	40.0	50.0	0

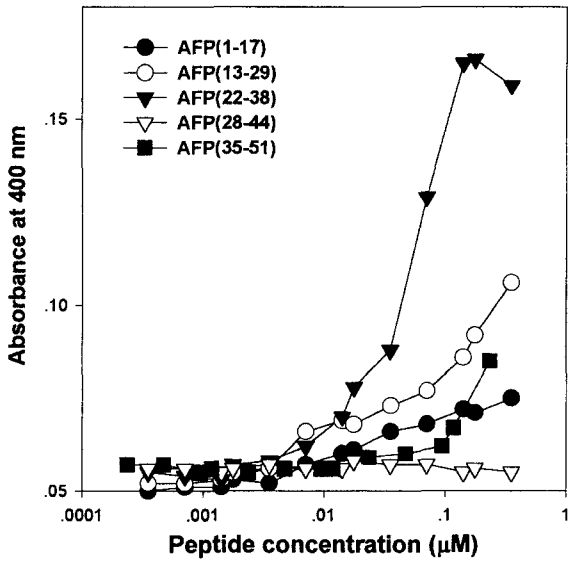

Fig. 1. Vesicle-aggregation activity at PC/PS (4:1) SUVs promoted by the peptides.

Furttermore, these peptides did not have any cytotoxic activity against tumor cells with a 100 μM. In several fungal cells, AFP (13-29) and AFP (22-38) exhibited higher antifungal activity than the other peptides. All five peptides showed no hemolytic activity against human red blood cells even at 100 μM.

The membrane fusion process is understood to consist of several stages: the close apposition of the vesicle, aggregation, vesicle destabilization, and finally the leakage of vesicle contents [12]. Several antibiotic peptides were reported to target cell membranes through the above process [13]. The vesicle-aggregation induced by the peptides was identified as the initial step of the vesicle-peptide interaction event. AFP (13-29) and AFP (22-38) were more active in the phospholipid vesicle-aggregation activity against negatively charged PC/PS(4:1) SUVs compared to the other peptides (Fig. 1). Therefore, these results suggest that the functional region of AFP is included within the region from the residues 13 to 38.

The secondary structures of the peptides, AFP(1-17), AFP (13-29), AFP (22-38), AFP (28-44), and AFP (35-51), were analyzed using CD spectra. In an aqueous solution (10 mM sodium phosphate buffer, pH 7.0) and a cell membranes-resembling environment of 50% TFE and 30 mM SDS, all of them had an almost random coil conformation (data not shown).

Table 3. Amino acid sequences of the peptides with various lengths derived from AFP (22-29).

Peptides	Amino acid sequences
AFP (22-29)	KTAICKCY
AFP (21-30)	GKTAICKCYV
AFP (20-31)	SGKTAICKCYVK
AFP (19-32)	QSGKTAICKCYVKK
AFP (18-33)	AQSGKTAICKCYVKKC

An analysis of the cell lytic activity of the peptides comprising the consecutive parts of the AFP primary sequence, as described in Table 2, indicated that the most active peptides encompassed the AFP region ranging between residues 13 to 38. Accordingly, the short region [AFP (22-29)] that overlapped the amino acid sequences of AFP (13-29) and AFP (22-38) was selected. In order to determine the AFP functional sequence responsible for antibiotic activity, five peptides which were extended by a one-by-one residue at both the N- and C-terminus of AFP (22-29) were synthesized as shown in Table 3. The cytotoxic activities of these peptide against various cells are summarized in Table 4. Although AFP (19-32) displayed more potent cytotoxic activity than the other four peptides against both bacterial and fungal cells, the other peptides exhibited similar antibacterial and antifungal activities to AFP (13-29) and AFP (22-38). Thus, the AFP functional site against bacterial and fungal cells would seem to be the region between residues 13 to 38. However, AFP (19-32) was more prominently active against tumor cells compared to the other peptides (Table 4). Several human tumor cells are commonly known to possess more exposed anionic phosphatidylserine (PS) on their surface [15, 16]. Therefore, AFP (19-32) and AFP (18-33) showed a similar activity at the vesicle-aggregation of PC/PS (4:1) SUVs, whereas AFP (19-32) exibited greater vesicle-aggregating activity at highly negatively charged PC/PS (1:1) SUVs than AFP (18-33) (Fig. 2). These results suggest that the AFP

Table 4. Antibiotic activities of the peptides with various lengths derived from AFP(22-29) against bacterial, fungal, tumor, and red blood cells.

Peptides	Bacterial cells (MIC: μM)		Tumor cells (SCLC) (IC_{50}: μM)		Fungal cells (IC_{50}: μM)				% Hemolysis (100 μM)
	E. coli	*B. subtilis*	NCI-H126	NCI-H148	*A. flavus*	*A. fumigatus*	*F. oxysporum*	*F. solani*	
AFP (22-29)	12.5	3.125-6.25	>100	>100	50.0	45.0	70.1	80.7	0
AFP (21-30)	25.0	6.25	>100	>100	21.0	25.0	40.2	30.2	0
AFP (20-31)	25.0	6.25	50.0	75.0	31.0	31.0	35.0	30.0	0
AFP (19-32)	12.5	0.78-1.56	22.0	43.0	12.0	16.0	10.0	10.1	0
AFP (18-33)	25.0	3.125	90.0	90.0	30.0	27.0	30.7	30.0	0

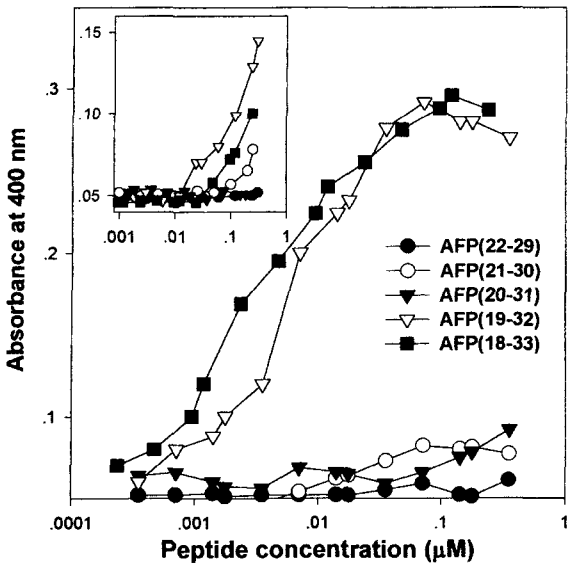

Fig. 2. Vesicle-aggregation activity at PC/PS (4:1) SUVs and PC/PS (1:1) SUVs (inset) promoted by the peptides.

functional region against tumor cells is focused on the region between residues 19 to 32.

In order to investigate the secondary structure of the AFP fragment peptides with antibiotic activity, AFP (22-29), AFP (21-30), AFP (20-31), AFP (19-32), and AFP (18-33) were analyzed using CD spectra. These peptides displayed a somewhat α-helical conformation in cell membrane mimicking environments such as TFE and SDS (Fig. 3 and Table 5). AFP (20-31), AFP (19-32), and AFP (18-33) exhibiting more potent antibiotic activity indicated higher α-helical contents in the presence of SDS and 50% TFE

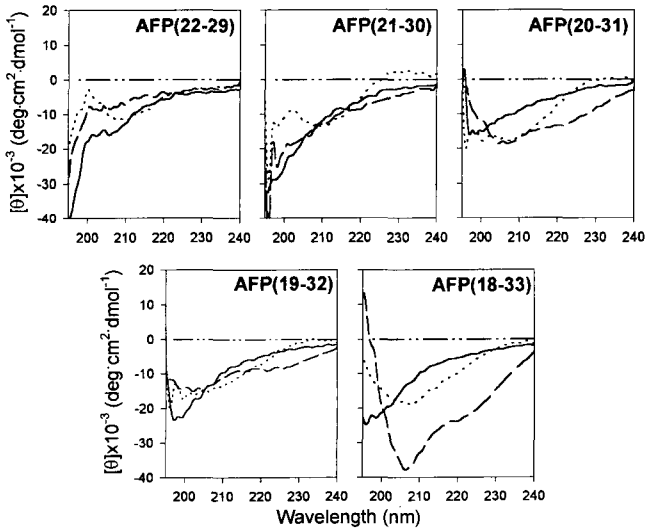

Fig. 3. CD spectra of AFP (22-29), AFP (21-30), AFP (20-31), AFP(19-32) and AFP (18-33) in a solution of 0% TFE (——), 50% TFE (- - - -) and 30 mM SDS (– – –) in 10 mM sodium phosphate, pH 7.2, respectively.

Table 5. Percentage α-helicity of the peptides in various media deduced from CD spectra.

Peptides	Buffer[a]	50 % TFE	5 mM SDS	15 mM SDS	30 mM SDS
AFP(22-29)	11.0	6.4	8.2	7.6	6.3
AFP(21-30)	10.1	3.0	19.1	17.9	16.8
AFP(20-31)	8.2	12.1	32.1	31.2	37.4
AFP(19-32)	8.5	12.1	25.6	29.1	23.5
AFP(18-33)	9.7	21.1	36.0	55.2	68.2

[a]10 mM sodium phosphate buffer (pH 7.2).

than AFP (22-29) and AFP (21-30) exhibiting less antibiotic activity. AFP (20-31), AFP (19-32), and AFP (18-33) showed somewhat higher α-helical contents in 30 mM SDS rather than 50% TFE. Although AFP (18-33) showed less antibiotic activity than AFP (19-32), AFP (18-33) indicated higher α-helix contents than AFP (19-32) in 30 mM SDS conditions. This result suggested that the α-helicity of an amphipathic antimicrobial peptide is not always correlated to its antibiotic activity. The similar results have been also found in our previous studies [6, 8, 14, 15].

Previous analysis of the three-dimensional structure of native AFP has revealed the presence of a putative cationic site formed by three Lys residues (Lys-9, Lys-10, and Lys-32) and the adjacent hydrophobic stretch formed by Tyr-29, Val-30, and Tyr-45 [3]. Accordingly, the cationic site and adjacent hydrophobic stretch present in the AFP protein are recognized to be responsible for antifungal activity [7]. The AFP functional region determined in this study, the AFP (19-32) peptide, contains four Lys residues and four hydrophobic residues (Ala-24, Ile-25, Tyr-29, and Val-30). Therefore, the positively charged and hydrophobic residues of the AFP (19-32) peptide would seem to be involved in the electrostatic interaction with negatively charged phospholipids on cell membranes and the pore and/or ion channel formation into cell membranes. AFP (19-32) is located in the region containing a β3 strand and the loop connecting β strands, the β3 and β4 in the three-dimensional structure of the native AFP protein [3]. AFP (19-32) exhibited a somewhat α-helical conformation in cell membrane-mimicking environments such as TFE or SDS micelles.

In conclusion, it was confirmed that the 19-32 portion of AFP was the shortest region displaying potent growth inhibitory activity against bacterial, fungal, and tumor cells, and promoting the aggregation of positively charged PC/PS vesicles. Native AFP protein exhibits potent antifungal activity against *Fusarium oxysporum*, however, it does not show growth inhibitory activity against bacteria [7]. Conversely, AFP (19-32) displayed powerful growth inhibitory activity against bacteria and tumor cells as well as fungi without peptide toxicity as indicated by hemolysis. Accordingly, AFP (19-32) could be used as a good model for the design of effective antibiotic agents with powerful activity without any cytotoxic effect against the host organism.

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