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Isolation and Characterization of a Novel Antifungal Peptide from *Aspergillus niger*

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A novel antifungal peptide (termed as Anafp) was isolated from the culture supernatant of the filamentous fungi, *Aspergillus niger*. The whole amino acid sequence of Anafp was determined and the peptide was found to be composed of a single polypeptide chain with 58 amino acids including six cysteine residues. The peptide shows some degree of sequence homology to a cysteine-rich antifungal peptides reported from the seeds of *Sinapis alba* and *Arabidopsis thaliana* or the extracellular media of *Aspergillus giganteus* and *Penicillium chrysogenumsome*. Cysteine-spacing pattern of Anafp was similar to that of the antifungal peptide from *Penicillium chrysogenum*. The Anafp exhibited potent growth inhibitory activities against yeast strains as well as filamentous fungi at a range from 4 to 15 μM. In contrast, Anafp did not show antibacterial activity against *Echerichia coli* and *Bacillus subtilis* even at 50 μM.

The rapid emergence of fungal pathogens that resistant to currently available antibiotics has triggered considerable interest in the isolation of new antifungal peptides with no cytotoxicity against mammalian cells in recent years. Numerous antifungal peptides with inter-disulfide bonds such as α-, β-defensins, protegrins, IB-AMPs, thanatin, Hs-AFP1 and Rs-AFP2 have been identified from a variety of sources, including mammals, insects, and plants (1–10). However, only two antifungal peptides with disulfide bonds were characterized from the fungi until now (11–15). The antifungal peptide purified from *Aspergillus giganteus* consists of 51 amino acid residues with four disulfide bridges (11–14), whereas the antifungal peptide from *Penicillium chrisogenum* possesses 55 amino acid residues with three-disulfide linkages (15). These antifungal peptides are thought to play an important role in the host defense against invasive infection. However, a cysteine-rich antifungal peptides had not been discovered from the filamentous fungi, *Aspergillus niger*. Here, we report the isolation of a novel endogeneous antifungal peptide with 58 amino acids containing six cysteines from the culture supernatant of *A. niger*. This peptide exhibits potent antifungal activity against filamentous fungi and yeast and shows sequence homology to a cationic cysteine-rich antifungal peptides isolated from the seeds of *Sinapis alba* and *Arabidopsis thaliana* and the culture supernatant of *Aspergillus giganteus* and *Penicillium chrysogenum*.

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MATERIALS AND METHODS

Materials. Trypsin and endopeptidase Glu-C were purchased from Sigma Chemical Co. (St. Louis, MO). Acetonitrile (analytical grade) was from Burdick & Jackson Inc. (Muskegon, MI). Yeast extracts, Bacto peptone, and dextrose were from Difco Inc. (Detroit, MI). Other chemicals and reagents were analytical grade.

Bacterial and fungal strains. *Echerichia coli* (KCTC 1682), *Bacillus subtilis* (KCTC 1918), *Aspergillus flavus* (KCTC 1375), *Aspergillus fumigatus* (KCTC 6145), *Aspergillus niger* (KCTC 2025), *Fusarium oxysporum* (KCTC 6076), *Fusarium solani* (KCTC 6326), *Saccharomyces cerevisiae* (KCTC 2805), *Candida albicans* (KCTC 1940) *and Trichosporon beigelii* (KCTC 7251) were obtained from the Korean Collection for Type Cultures (KCTC), Korea Research Institute of Bioscience & Biotechnology (KRIBB), Taejon, Korea.

Purification of antifungal peptide. *A. niger* conidia was innoculated in to a 2 liters YPD medium (2% glucose, 1% peptone, 0.5% yeast extract, pH 5.5), and incubated at 30°C for 72–96 h in a shaking incubator (140 rpm). After incubation, the culture supernatants were collected by filtering through 3-layers of cheesecloth and adjusted to pH 7.0 by adding 1 M Tris solution. After filtering the solution by ultrafiltration using Amicon YM-30 membrane (MW 30,000 cutoff). Basic proteins from the culture supernatant were isolated by loading the filter onto a CM-Sepharose column (Pharmacia Biotech, Uppsala, Sweden) (2.3 × 20 cm) preequilibrated with 100 mM Tris-HCl buffer (pH 7.0). The basic proteins were then eluted by a 500 ml linear gradient from 0 to 1 M NaCl in 100 mM Tris-HCl buffer (pH 7.0) and 6 ml fractions were collected. The peak fractions were pooled and assayed for antifungal activity. The pooled peak fractions were concentrated and desalted by ultrafiltration using YM3 membrane (MW 3,000 cutoff) with several changes of 100 mM Tris-HCl buffer (pH 7.0). The solution was then applied to an

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Ultrasphere C_{18} RP-HPLC (4.6 × 250 mm), equilibrated with aqueous 0.05% trifluoroacetic acid (TFA) and eluted with a linear gradient of 0 to 80% acetonitrile in aqueous 0.05% TFA. Each peak fractions was collected and assayed for antifungal activity. The peak fraction showing the highest antifungal activity was lyophilized and stored at −20°C until use.

Reduction, S-carboxyamidomethylation and enzymatic digestion. The native peptide (20 μg) was dissolved in 50 μl of 0.2 M ammonium bicarbonate, pH 8.0 containing 4 M guanidine hydrochloride mixed with 5 μl of 45 mM dithiothreitol. The mixture was flushed with nitrogen gas and then incubated at 50°C for 30 min. The reduced protein was treated with 5 μl of 100 mM iodoacetamide at 37°C for 12 h. Sixty μl of H_2O was added to the solution of S-carboxyamidomethylated peptide and then digested with trypsin (enzyme: protein = 1:25, w:w) at 37°C for 24 h. Also, S-carboxyamidomethylated peptide was digested with endopeptidase Glu-C as described above. The reaction was stopped by freezing on dry ice. Peptide fragments resulting from enzymatic digestions were purified by a Microbore HPLC system ABI 172 (Applied Biosystem, USA) equipped with Perkin Elmer Brownlee C_{18} reversed phase column (Spheri-5 RP-18, 5 μm, 0.2 × 220 mm) equilibrated with buffer A (0.1% trifluoroacetic acid). Peptides were eluted from the column with a linear gradient (15%–80%, 1.083%/min, 60 min) of buffer B (80% acetonitrile containing 0.1% trifluoroacetic acid) at a flow rate of 210 μl/min at 25°C. Absorbance at 214 nm was measured, and peptide fragments were collected manually for amino acid sequence analysis. Peptides were numbered according to the order of the elution from the HPLC column. Peptides derived from trypsin and endopeptidase Glu-C were designated as T and G, respectively. The peptide fragments were sequnced by Edman degradation.

Amino acid microsequencing. Automated Edman degradation of the S-carboxyamidomethylated peptide and detection of phenylthiohydantion derivatives were performed on an automatic protein sequencer (Applied Biosystems, Model 476A).

Molecular weight determination of antifungal peptide by matrix-assisted laser desorption ionization mass spectroscopy (MALDI-MS). To determine the molecular weight of the purified native peptide, 1 ml of the peptide (6.5 mg/ml) in aqueous 0.1% TFA was mixed with 2 μl sinapinic acid (70 mg/ml). The peptide mixture was loaded onto a flat inert metal (e.g., silver, platinum) probe and the solvent was removed by warm airflows. The sample was then transferred to a vacuum chamber. The molecular weight of the peptide was determined by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) (16).

Antibacterial activity. The bacteria were grown to the midlog phase in a medium (g/l) [10 bactotryptone/5 yeast extract/10 NaCl (pH 7.0)]. Peptide solution diluted with 1% bactopeptone was added to the well of a 96-microtiter plate (100 μl per well), and the well were serially diluted twofold. The final concentrations of the peptide mixtrure ranged from from 1.56 μM to 50 μM. To this, 100 μl of the bacterial strains suspended in the growth medium (final bacterial suspension: 2×10^6 CFU/ml). The plates were incubated for 18 h at 37°C. Assays were performed in triplate for the peptide. The minimal inhibitory concentration (MIC) was defined as the lowest concentration of peptide which gave no visible growth on the plate.

Antifungal activity. The fungal strains were grown at 28°C in YM medium (1% glucose, 0.3% malt extract, 0.5% peptone, and 0.3% yeast extract). The fungal conidia were seeded on the well of a 96-microtiter plate of YM media at a density of 1×10^2 spores (100 μl per well). Ten μl of the serially diluted-peptide solution were added to each well, and the cell suspension was incubated for 24 h at 28°C. Ten 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 wells, and then the plates were incubated further at 37°C for 4 h. Thirty μl of 20% (w/v) SDS solution containing 0.02 M HCl was added, and then the plates were incubated at 37°C for 16 h to dissolve the formazan crystals that had formed. The turbidity of each well was measured at 570 nm using a microtiter ELISA reader (Molecular Devices, Sunnyvale, CA).

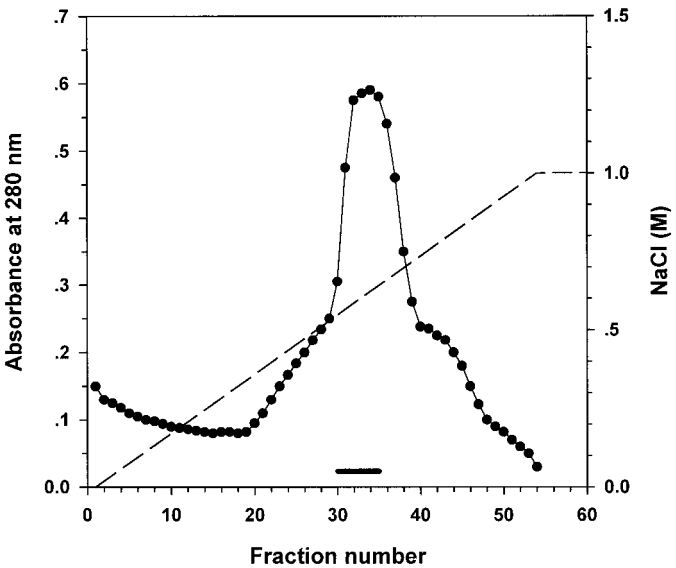

FIG. 1. Elution profile of CM-Sepharose the concentrated culture supernatants. The column (2.3 × 20 cm) was eluted with a linear gradient of 0–1.0 M NaCl containing 100 mM Tris-HCl buffer (pH 7.0). The solid bar indicates the fractions with antifungal activity. The dotted line indicates a linear salt gradient from 0 to 1.0 M NaCl.

RESULTS

Purification of antifungal peptide. The small molecular weight peptides secreted to the *A. niger* culture media were filtered through Amicon YM-30 membrane (MW cutoff; 30,000) and applied to a CM-Sepharose ion exchange column (2.3 × 20 cm) pre-equilibrated with 100 mM Tris-HCl, pH 7.0. Peptides were eluted by 500 ml linear gradient from 0 to 1.0 M NaCl and peak fractions were pooled (Fig. 1). The pooled fractions were concentrated and desalted using an Amicon YM-3 membrane (MW cutoff; 3,000), and then the antifungal activity of each fraction was measured. The fractions with antifungal activity were purified by analytical C_{18} reverse phase (RP)-HPLC. As shown in Fig. 2A, the concentrated peptide solution was resolved to two major peaks of which the first major peak, designated as peak 1, showed antifungal activity (data not shown). The peak 1 was then purified by an analytical C_{18} RP-HPLC (Fig. 2B). The purity of peak 1 was also confirmed by Tricine-SDS-polyacrylamide gel electrophoresis (Fig. 3). The purified peptide, designated as Anafp showed a molecular weight of 6582.63 by MALDI mass spectroscopy (Fig. 4).

Amino acid sequence analysis. Following reduction and S-carboxyamidomethylation of Anafp direct sequencing gave 46 amino acid residues (Fig. 5). Only one

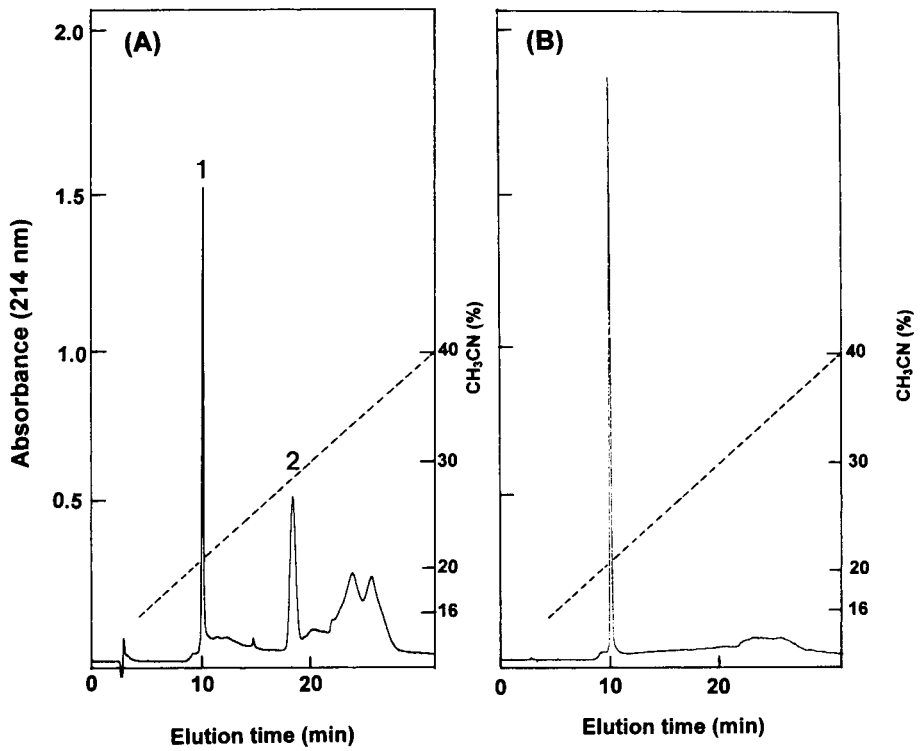

FIG. 2. (A) RP-HPLC profile by Ultrasphere C_{18} column (4.6 × 250 mm) of the desalted fractions 30–35 separated from CM-Sepharose column. Peak 1 with antifungal activity was pooled and subjected to RP-HPLC. (B) RP-HPLC profile by Ultrasphere C_{18} column (4.6 × 250 mm) of the final purified Anafp. After an initial 4 min wash in 0.05% tirfluoroacetic acid/water, the proteins were eluted with a linear gradient (1% increase/min) of 20–60% (by vol.) acetonitrile containing 0.05% trifluoroacetic acid, at a flow rate of 1 ml/min. The effluent was monitored at 214 nm.

amino acid sequence was found in amino acid sequencing of the carboxyamidomethylated peptide indicating that this peptide was indeed composed of a single polypeptide chain. The total sequence was confirmed by overlapping the peptide fragments obtained by either trypsin or endoproteinase Glu-C digestion. When S-carboxyamidomethylated Anafp was digested with endoproteinase Glu-C followed by separation of the peptides with Perkin Elmer Brownlee C_{18} reversed phase HPLC column, the four peptide fragments (G1, G2, G3, and G4) were obtained. Tryptic digestion produced nine peaks (T1 T9). All fragments were sequenced by Edman degradation. The whole amino acid sequence of Anafp with 58 amino acid residues is shown in Fig. 5.

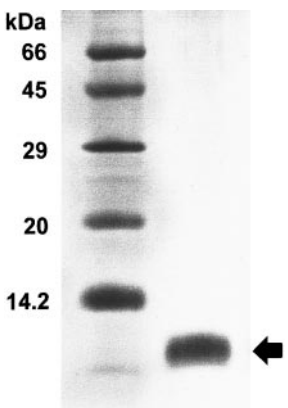

FIG. 3. Tricine gel electrophoresis of the purified Anafp. Lane 1: molecular size marker (66 kDa, Bovine serum albumin; 45 kDa, Ovalbumin; 29 kDa, Carbonic anhydrase; 20 kDa, Trypsin inhibitor; 14.2 kDa, α-Lactalbumin). Lane 2: 1 μg of purified Anafp.

Antifungal and antibacterial activities. The antifungal and antibacterial activities of the native peptide against two bacterial, five filamenteous fungal and two yeast strains were measured using growth inhibition assay (Table 1). The peptide displayed potent antifungal activity against *A. flavus* (MIC = 8 μM), *A. fumigatus* (MIC = 4–8 μM), *F. oxysporum* (MIC = 8–15 μM), *F. solani* (MIC = 8 μM), and *T. beigelii* (MIC = 8–15 μM). Furthermore, it was found to be active against two yeast strains, *C. albican* (MIC = 8–15 μM) and *S. cerevisiae* (MIC = 8 μM). In contrast, this peptide did not display antibacterial activity against *E. coli* and *B. subtilis* even at 50 μM.

DISCUSSION

A new antifungal peptide with six cysteines designated as Anafp was successfully isolated from the cul-

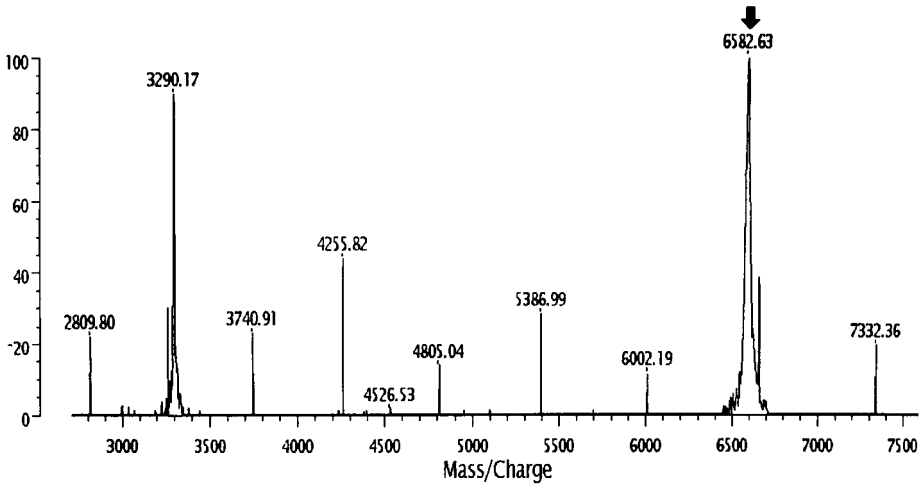

FIG. 4. MALDI mass spectra of the purified Anafp. The arrow shows the molecular weight (6582.6) of purified Anafp. This value was confirmed to be correct. The result was calculated from amino acid sequence analysis.

ture medium of *A. niger* and its complete amino acid sequence of 58 amino acid residues was determined by direct sequencing and enzyme-mapping using trypsin and endopeptidase Glu-C. The Anafp was rich in positively charged residues including five Lys, three Arg and six His, and contained six cysteine residues. The mass (6582.63) observed by MALDI-MS analysis was consistent with the calculated mass (6583.0) of Anafp

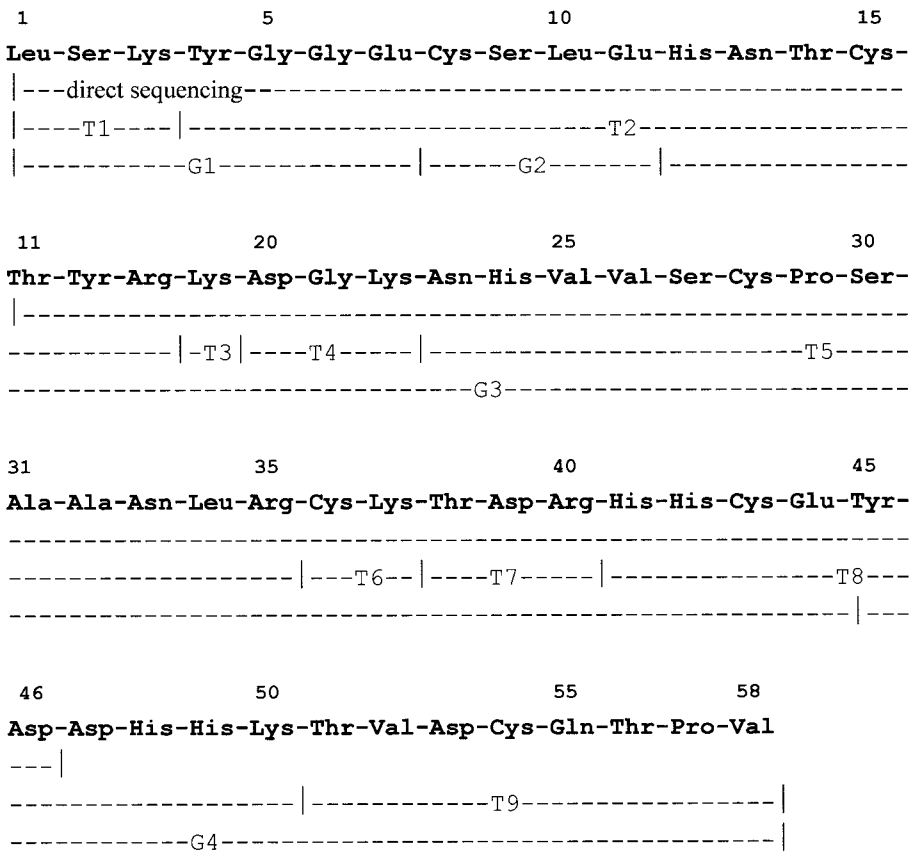

FIG. 5. Complete amino acid sequence of S-carboxyamidomethylated Anafp. The direct sequence was determined by automated Edman degradation without fragmentation. T(T1 – T9) and G(G1 – G4) denote the peptides derived from trypsin and endoproteinase Glu-C digestions of S-carboxyamidomethylated Anafp, respectively.

(Fig. 4). A protein sequence homology search using the Swiss-Prot data base indicates that Anafp isolated from *A. niger* is a novel sequence and possesses some homologies with several antifungal peptides from plants, *Sinapis alba* (17) and *Arabidopsis thaliana* (18) and filamentous fungi, *A. giganteus* (14) and *P. chrysogenum* (15) (Fig. 6). Anafp also possesses six cysteine residues. In particular, its six cysteinyl spacing pattern (-C-X_6-C-X_{12-13}-C-X_7-C-X_6-C-X_{10}-C-) was most similar to that of the antifungal peptide derived from *P. chrysogenum* (15). The topology of three disufude bridges of Anafp will be determined in further studies. The antifungal peptide isolated from *A. giganteus* showed no effect on the yeast growth even at 200 μM (14), whereas, Anafp displayed the potent antifungal activity against the yeast strains tested as well as the filamentous fungi (Table 1).

α-, β-defensins from mammals neutrophil and Agafp from *A. giganteus* are predominantly β-sheet conformation stabilized by three or four disulfide bonds and form highly amphipathic structure (9, 10, 14). They electrostatically bind to target cell membranes, causing the formation of multimeric pores and the leakage of essential minerals and metabolites (9). In contrast, the other antifungal peptide, IB-AMPs with two disulfide bonds derived from seeds of *Impatiens balsamina*, do not cause ion channel/pore formation on cell membranes and have a different antibiotic mechanism of DNA synthesis inhibition by the interaction with the target protein (19). Whether Anafp, the novel antifungal protein isolated from *A. niger* in this study interacts directly with cell membranes or whether it has a target protein will be evaluated in the further studies.

In conclusion, a novel antifungal peptide with 58 amino acids including six cysteines, Anafp, was newly isolated from *A. niger*. Cysteine-spacing pattern of Anafp was similar to that of the antifungal peptide derived from *P. chrysogenum*. This novel antifungal peptide might be useful for the understanding of the biological function and mechanism of endogeneous antifungal peptides secreted from fungi.

TABLE 1

The MIC Values of Anafp against Different Microorganisms

Microorganisms	MIC (μM)
Bacteria	
E. coli	>50
B. subtilis	>50
Filamentous fungi	
A. flavus	8
A. fumigatus	4–8
F. oxysporum	8–15
F. solani	8
T. beigelii	8–15
Yeast strains	
C. albicans	8–15
S. cerevisiae	8

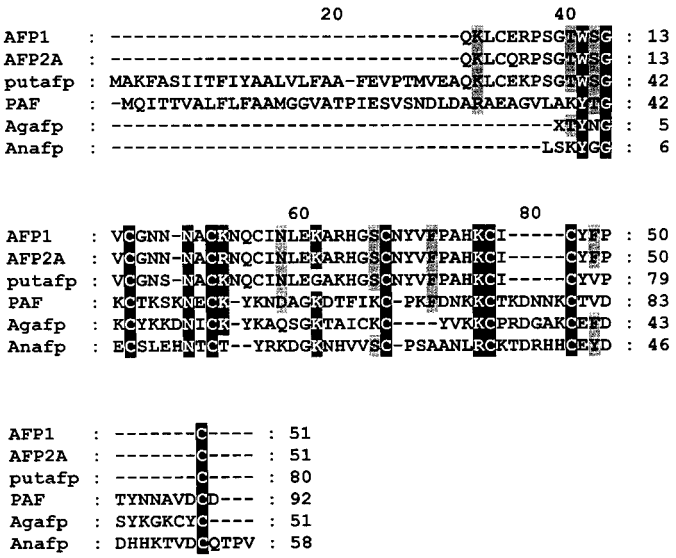

FIG. 6. Sequence comparison of Anafp and various antifungal proteins. *Dark boxes* show the conserved sequences, and the *shaded boxes* indicate similar residues. AFP1 and AFP2A: *Sinapis alba* (17), putafp: *Arabidopsis thaliana* (18), PAF: *Penicillium chrysogenum* (15), Agafp: *Aspergillus giganteus* (14).

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