ISOLATION OF BIOCIDAL PEPTIDES FROM *Anethum graveolens* SEEDS

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Three peptides with fungicidal activity with molecular weights 4-8 kDa were isolated from Anethum graveolens *L. seeds. The cationic peptides were tested in vitro and inhibited the pathogenic strain* Verticillium dahliae. *The effective concentrations required for 50% inhibition (IC_{50}) growth of fungi were in the range 1.3-31 μg/mL. Peptide D-AFP-3 was the most active biocidal peptide.*

Key words: *Anethum graveolens* L. (dill), peptides, fungicidal activity, *Verticillium dahliae*.

Fungitoxic peptides make up a broad class of proteins of various molecular weight that are an important component of the natural system of protection against both animal and plant pathogens and directly inhibit the growth, multiplication, and distribution of microbes [1, 2]. The isolation and purification of effective antimicrobial peptides (EAP) from seeds of various plants have been described [3, 4].

Herein we report the isolation from dill seeds (*Anethum graveolens* L., Apiaceae, Umbelliferae) of three low-molecular-weight peptides and the investigation of their fungicidal activity in vitro against the pathogenic strain *Verticillium dahliae*.

The purification procedure of low-molecular-weight cationic peptides from dill seeds consisted of several steps. Total proteins and peptides were extracted from seeds by phosphate buffer and precipitated from the extract by $(NH_4)_2SO_4$ (from 30 to 70% saturated). High-molecular-weight proteins were denatured at 80°C for 10 min and removed by centrifugation. Table 1 gives the purification steps and the peptide yields of the fractions.

The total fraction of thermally stable peptides was placed on an anion-exchange column of Servacel DEAE 23SN (2.0 × 10 cm). The main peptide fraction that was not bound to the anion-exchange column was placed on a CM-TSK-650M cation-exchange column (2.0 × 5.0 cm). The peptide fraction bound to the CM-TSK-650M column was eluted by an NaCl gradient (0.050-1.5 M). Fractions of cationic peptides AFP-D1 and AFP-D2 were collected, desalted by dialysis, and analyzed for fungitoxic activity against the pathogenic strain *Verticillium dahliae* (Fig. 1).

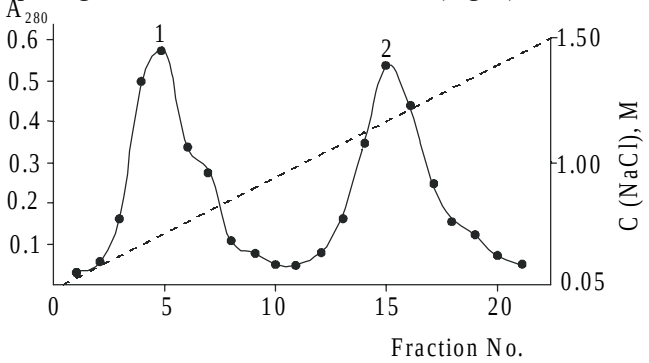

Fig. 1. Ion-exchange chromatography of cationic-peptide fractions over a column of CM-TSK-650M: peptide fraction AFP-D1 (1) and peptide fraction AFP-D2 (2).

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TABLE 1. Steps in Purification Procedure for Dill Seed Peptides

Protein isolation step	Total protein, mg*	Protein yield, %
Raw extract from seeds (100 g)	623	100
$(NH_4)_2SO_4$ precipitation		
30%	190	30.5
70%	422	67
Chromatography over Servacel DEAE 23SN	107	17
Chromatography over CM TSK-650M		
peptide fraction AFP-D1	16	2.4
peptide fraction AFP-D2	12	2.0

*Protein determined by Bradford method.

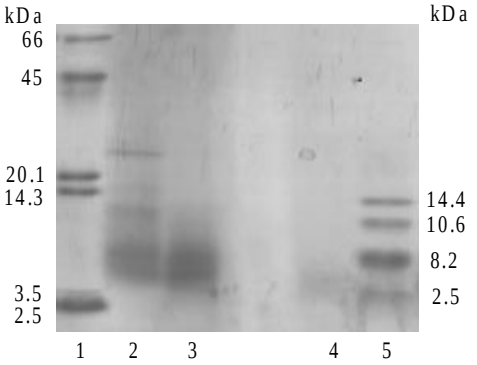

Fig. 2

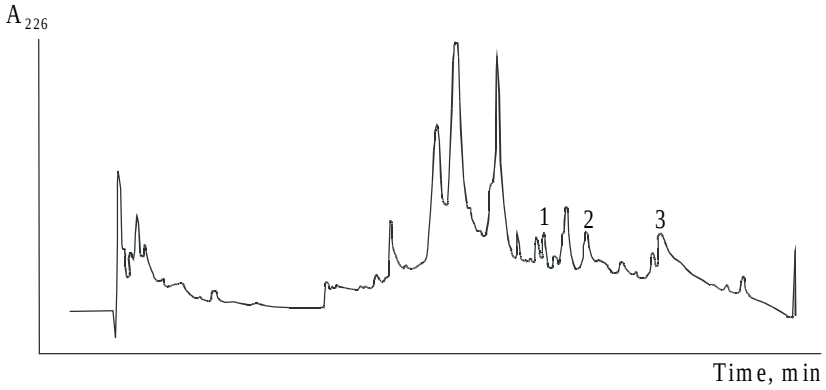

Fig. 3

Fig. 2. Gel electrophoresis of cationic peptides and standard proteins in PAAG (15%): peptide-marker mixture insulin chain A and B (2.4 and 3.5 kDa), lysozyme (14.3 kDa), soy trypsin inhibitor (20.1 kDa), ovalbumin (45 kDa), bovine serum albumin (66 kDa) (1); peptide mixture precipitated by $(NH_4)_2SO_4$ (70%) (2); AFP-D1 (3), AFP-D2 (4), protein-marker mixture: myoglobin (1-131) (14.4 kDa), myoglobin (56-153) (10.6 kDa), myoglobin (56-131) (8.2 kDa), myoglobin (132-153) (2.5 kDa). Fig. 3. Isolation of fungicidal cationic peptides of dill seeds by reversed-phase preparative high-performance liquid chromatography over a protein-peptide column. Numbers 1, 2, and 3 denote peptides D-AFP-1, D-AFP-2, and D-AFP-3, respectively.

The total peptide fraction that was obtained by $(NH_4)_2SO_4$ (70%) precipitation from the extract showed weak fungicidal activity toward *V. dahliae*. The 50% inhibition of fungal growth (IC_{50}) was observed at a protein concentration of 475 µg/mL. Cationic peptide fraction AFP-D1 from CM-TSK-650M showed significant fungicidal activity. At a concentration of 17 µg/mL it caused 50% inhibition of fungal growth (IC_{50}) whereas peptide fraction AFP-D2 exhibited low fungicidal activity. A concentration of 119 µg/mL caused 50% inhibition of fungal growth after 48 h incubation.

Molecular weights of cationic peptides AFP-D1 and AFP-D2 were determined by electrophoresis in PAAG (15%) with SDS-PAAG (0.1%) under denaturing conditions (Fig. 2). After treatment with reagents to reduce the protein disulfide bonds, all peptides gave protein bands of about 4-8 kDa, which is close to the molecular weight of other known EAP [3-5].

The electrophoresis results indicated that the precipitate obtained by precipitation with $(NH_4)_2SO_4$ (70%) (line 2 in Fig. 2) was rich in peptides of molecular weight between 2.5-10.6 kDa whereas cationic peptides of fractions AFP-D1 and AFP-D2 that were purified by two-stage ion-exchange chromatography gave concentrated protein bands and were rich in peptides with molecular weight between 4-8 kDa. However, clear peptide bands were not observed on the electrophoregram (line 4 in Fig. 2) because of the relatively low peptide concentration in fraction AFP-D2. The fungicidal activity of peptide fraction AFP-D1 was much higher than that of the initial fraction. Fraction AFP-D1 that was relatively richer in cationic peptides was purified by preparative reversed-phase HPLC (Fig. 3). This isolated three high-purity biocidal peptides D-AFP-1, D-AFP-2,

and D-AFP-3. The effective concentrations required for 50% inhibition of fungal growth were 13.2, 31, and 1.3 μg/mL, respectively.

The low molecular weight of isolated peptide D-AFP-3 in combination with its high fungitoxic activity makes it a likely candidate for a detailed investigation of its biological activity. In vitro screening of compounds with fungitoxic activity can identify compounds of plant origin with useful properties for use in further biochemical and pharmacological investigations.

Different types of antimicrobial plant and animal peptides are highly protective against different groups of infectious agents [6]. The peptides isolated by us from dill seeds had higher fungicidal activity than peptides from seeds of other plants [7, 8]. The results confirm that dill seeds contain a powerful fungitoxic peptide that strongly inhibits the growth of pathogenic fungus in vitro with IC_{50} 1.3 μg/mL (peak 3 in Fig. 3).

Our results on the isolation of biocidal peptides and screeing of fungicidal activity, especially the electrophoresis results, indicated that the precipitation and isolation methods are very promising for finding peptides in seeds of other plants (Fig. 2). Detailed further analysis of the peptides by amino-acid analysis and comparison with the composition and amino-acid sequence of known antimicrobial cationic peptides in appropriate databases will enable the isolated peptides to be assigned to certain known classes (thionins, defensins, lipid-transfer proteins, etc. [9]). Therefore, work on the determination of the antimicrobial activity of the peptides toward other pathogens should continue.

EXPERIMENTAL

Isolation of Total Peptides from Seeds. Buffer for peptide extraction was prepared using Na_2HPO_4 (10 mM), NaH_2PO_4 (15 mM), KCl (100 mM), EDTA (1.5 mM), and polyvinylpyrrolidone (1.5%) at pH 7. The suspension was stirred for 2 h at 4°C and was treated with thiourea and α-toluenesulfonyl fluoride (PMSF) dissolved in isopropanol to final concentrations of 2 and 1 mM, respectively [10].

Dill seeds (100 g) were ground, defatted with hexane in a Soxhlet apparatus, dried, and extracted with cold buffer (400 mL) for 2 h at 4°C with constant stirring. The extract was clarified by centrifugation for 30 min at 6000 rpm. The supernatant was treated with solid $(NH_4)_2SO_4$ to attain 30% saturation. The mixture was left for 18 h at 6°C with cautious stirring to form a precipitate. The precipitated proteins were separated by centrifugation (30 min at 6000 rpm). The supernatant was treated with $(NH_4)_2SO_4$ to attain 70% saturation. The precipitate that formed over 18 h at 6°C was separated by centrifugation (30 min at 6000 rpm). The precipitated peptides were dissolved in a minimum of distilled water. The solution was held at 80°C for 10 min for denaturation and precipitation of high-molecular-weight proteins. The centrifugation (30 min at 6000 rpm) was repeated to remove the precipitate of thermally labile proteins. The supernatant was dialyzed repeatedly against distilled water using a dialysis tube with a pore diameter that retained proteins up to 1 kDa.

Ion-exchange Chromatography. The dialyzed extract was adjusted to pH 9 with ammonium acetate (0.050 M) and passed over a column of Servacel DEAE-23SN (2.0 × 10 cm, Reanal) equilibrated with ammonium acetate (0.050 M, pH 9) at flow rate 0.5 mL/min. Fractions of non-bonded proteins were mainly thermally stable proteins. The resulting eluate was adjusted to pH 6 with acetic acid and placed on a column of CM-TSK-650M (2.0 × 5 cm, Tosoh Bioscience) equilibrated with ammonium acetate (0.50 M, pH 6). Proteins bound to the sorbent were eluted with a linear gradient of NaCl (from 0.050 M to 1.5 M, 200 mL) in ammonium acetate (0.050 M, pH 6) at flow rate 0.5 mL/min. Proteins were detected at 280 nm. Two peptide fractions were obtained, lyophilized after dialysis, and used for further purification.

Reversed-phase HPLC. Peptides that were eluted from the cation-exchange column were dialyzed against distilled water and separated in a chromatograph (DuPont 8800) using a 250/8/4 Protein@Peptide C_{18} column. Solution A: TFA (0.1%); B: CH_3CN; flow rate 1 mL/min; absorption at 226 nm; gradient (%/min): 0-5%/0-5 min, 5-60%/5.1-35 min; 60%/35.1-40 min; 60-5%/40.1-45 min.

Protein concentration was determined by the Bradford method [11] using trypsin as the standard protein.

Analysis of Fungicidal Activity. Fungicidal activity at all stages of isolation of biocidal peptides was determined by turbidimetry in microplanchettes [12]. The microplanchettes were incubated at 27°C for 48 h. Fungus growth was estimated from the change of optical absorption at 490 nm using an automated planchette reader (Bio-Rad, Model 3550). The analyses were performed in triplicate.

Electrophoresis of Proteins. Electrophoresis of proteins used the Laemmli method [13] with PAAG (15%) and SDS-Na (0.1%) at pH 8.9.

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