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FUNGICIDAL LIPID-TRANSFER PEPTIDE FROM *Daucus carota sativa* SEEDS

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A non-specific lipid-transfer peptide (nsLTP) with fungicidal activity was isolated from Daucus carota sativa *carrot seeds. Peptides were purified by a method including aqueous extraction, anion-exchange chromatography over CM-TSK-650M, and HPLC over a column of 250/8/4 Protein@Peptide C_{18} using an acetonitrile gradient. The molecular weight of the peptide was determined as 9624 Da by mass spectrometry. The peptide was found to have fungicidal activity against the pathogenic fungus* Verticillium dahliae. *The partial* N*-terminal sequence, which was highly homologous to the* N*-terminal sequences of lipid-transfer peptides from seeds of rice, tobacco, and maize, was determined using Edman automated sequencing.*

Key words: carrot seeds, fungicidal lipid-transfer peptides, *N*-terminal amino-acid sequence.

Many fungicidal peptides from plant seeds have already been described. These include lipid-transfer peptides, main proteins of molecular weight 9-10 kDa that can bind and transfer various lipids through membranes [1]. We isolated and characterized previously two peptides from *Daucus carota sativa* (Apiaceae) carrot seeds that were similar to lipid-transfer peptides in molecular weight according to PAAG electrophoresis [2]. The goal of the present work was to isolate a homogeneous non-specific lipid-transfer peptide (nsLTP) from carrot seeds, determine the *in vitro* fungicidal activity against the pathogen *Verticillium dahliae*, and to identify its partial *N*-terminal sequence.

The method for purifying cationic peptides from carrot seeds consisted of several steps. Proteins and peptides were extracted from seeds by phosphate buffer and precipitated from the extract by ammonium sulfate at 30-80% saturation. High-molecular-weight proteins were removed by precipitation at 80°C with subsequent centrifugation. Table 1 gives the protein yields for the purification steps.

The fraction of thermally stable peptides that was precipitated by ammonium sulfate at 80% saturation was then purified by anion-exchange chromatography over a column of Servacel DEAE 23SN. Then the peptide fraction that did not adsorb to the anion-exchange column was placed on a cation-exchange column with KM-TSK-650M. It was shown earlier that fungicidal proteins and peptides are adsorbed at low ionic strength onto strongly acidic SP-Toypearl cation-exchange resin or onto an affinity column with Affi-gel blue gel [3, 4]. Then the peptide fraction bound to a column of KM-TSK-650M was eluted by a NaCl gradient to produce two fractions of cationic peptides AFP-1 (anti-fungal peptide 1) and AFP-2, which were collected, desalted by gel-chromatography over Molselect G-15, and used for further analysis.

The molecular weights of the cationic peptides were determined by gel-electrophoresis in PAAG (15%) under dissociating conditions [5]. Figure 1 shows the results, from which it can be seen that the molecular weights of the peptides were 2.5-9 kDa. This agrees with the values for known antimicrobial peptides (AMP) [6-8].

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TABLE 1. Carrot-Seed Peptide Purification Steps

Protein purification step	Amount of protein, mg/100 g of seeds	Protein yield, %
Protein extract	380.5	100
Precipitation		
30% $(NH_4)_2SO_4$	110.2	28.9
80% $(NH_4)_2SO_4$	100.1	26.3
Chromatography over		
DEAE Servacel 23SN	39.0	10.2
CM TSK-650M	10.2	2.6

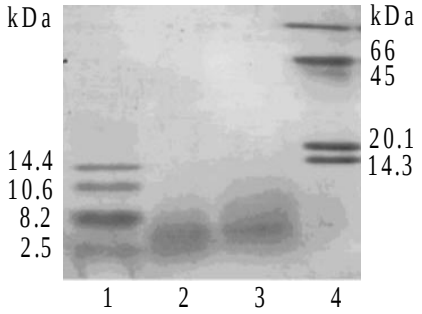

Fig. 1.

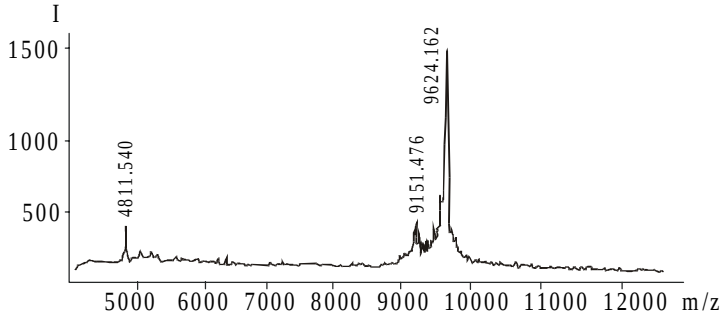

Fig. 2.

Fig. 1. Fractions of cationic peptides from carrot seeds analyzed by gel-electrophoresis in PAAG (15%): marker proteins (1, 4), AFP-1 (2), AFP-2 (3).

Fig. 2. Mass spectrometric analysis of peptide P22 from carrot seeds.

The sensitivity of pathogenic fungus *Verticillium dahliae* (*in vitro*) to the two isolated peptide fractions AFP-1 and AFP-2 was investigated. The effective concentrations required for 50% growth inhibition of the fungus were 67.9 and 56.6 μg/mL, respectively. The non-specific lipid-transfer peptide Ace-AMP1 isolated from onion seeds is known to exhibit fungicidal activity against *V. dahliae* at a concentration of 10 μg/mL [6]. Peptides from *Triticum aestivum* wheat (Ta-nsLTP) [9] and *Zea mays* maize seeds (Zm-nsLTP) [8] did not exhibit fungicidal activity at this same protein concentration in solution.

The cationic peptide fractions from carrot seeds were combined and fractionated over a reversed-phase HPLC column. This produced 33 fractions that were tested for fungicidal activity. Peptide fractions 1, 2, 9, 18, 22, and 28-30 had the greatest fungicidal activity. The most active homogeneous peptide P22 with a toxicity against *V. dahliae* of 38 μg/mL was selected for structural studies.

Mass spectrometry determined the molecular weight of P22 as 9624 Da (Fig. 2). This suggested that this peptide belongs to the known class of lipid-transfer proteins that were isolated from wheat and maize seeds [8, 9]. Determination of the *N*-terminal amino-acid sequence of homogeneous P22 by the Edman method [10] gave the structure H_2N-Ile-Ser-Cys-Gly-Gln-Val-Thr-Ser-Ser-Ile-.

This peptide was identified as a lipid-transfer protein by comparison of its *N*-terminal amino-acid sequence with the literature (Table 2). Carrot-seed P22 had a highly conserved structure and was 90% homologous to lipid-transfer peptide from maize seeds and 70%, to peptides from rice and tobacco seeds.

Thus, the *N*-terminal conserved parts of the lipid-transfer peptide from carrot seeds was highly homologous to analogous polypeptides isolated from seeds of other plants. The fungicidal peptide from carrot seeds with molecular weight 9624 Da had an *N*-terminal sequence Ile-Ser-Cys-Gly-Gln-Val-Thr-Ser-Ser-Ile-. According to the literature, this peptide had a toxicity, molecular weight, and homologous *N*-terminal sequence similar to fungicidal non-specific lipid-transfer peptides.

TABLE 2. Comparison of *N*-Terminal Sequences of Non-specific Lipid-Transfer Peptide from Carrot Seeds and Peptides from Other Plants

Source of peptide	*N*-terminal sequence	Hemologous, %	Literature data
Daucus carota sativa	ISCGQVTSSI	100	-
Zea mays	ISCGQVASAI	90	8
Oryza sativa	ISCGQVNSVS	70	14.8
Nicotiana tabacum	ITCGQVTSNL	70	14.8
Citrus sinensis	ITCGQVSGSL	60	14
Spinacia oleraceae	ITCGMVSSKL	50	15

EXPERIMENTAL

Isolation of Total Peptides from Seeds. Peptides were extracted by the following buffers: Na_2HPO_4 (10 mM), NaH_2PO_4 (15 mM), KCl (100 mM), EDTA (1.5 mM), thiourea (2 mM), α-toluenesulfonyl flouride (PMSF, 1 mM), and polyvinylpyrrolidone (1.5%, pH 7.4). Carrot seeds (250 g) were ground in a coffee grinder, defatted by hexane in a Soxhlet apparatus, dried, and extracted by cold buffer (1000 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 ammonium sulfate to produce 30% saturation and left overnight at 6°C to form a precipitate. The precipitated proteins were separated by centrifugation (30 min, 6000 rpm). The supernatant was treated with ammonium sulfate to produce 80% saturation. The precipitate that formed overnight was separated by centrifugation (30 min, 6000 rpm) and dissolved in a minimum amount of distilled water. The solution was stored at 80°C for 10 min to denature and precipitate high-molecular-weight proteins. The precipitate of thermally labile proteins was removed by centrifugation (30 min, 6000 rpm). The supernatant was exhaustively dialyzed against distilled water using Spectra/Por® 3 tubes with pore size allowing retention of proteins up to 3.5 kDa.

Ion-exchange Chromatography. The desalted extract of carrot seeds was made basic until the pH was 9.0 using ammonia solution (12 M) and passed over a column of Servacel DEAE-23SN (2.0 × 10 cm, Reanal) equilibrated with ammonium acetate solution (50 mM, pH 9) at flow rate 0.5 mL/min. Fractions of peptides not binding to the sorbent were basic proteins. The resulting effluent was treated with HCl until the pH was 6 and placed on a column of KM-TSK-650M (2.0 × 10 cm, Tosoh Bioscience, Japan) equilibrated with ammonium acetate (0.050 M, pH 6). Proteins bound by sorbent were eluted by a linear gradient of NaCl (300 mL, 0 to 1 M) in ammonium acetate (0.050 M, pH 6) at flow rate 0.5 mL/min. Proteins were detected at 280 nm.

Reversed-phase HPLC. Peptides were separated on a Du-Pont 8800 chromatograph using a 250/8/4 Protein@Peptide C_{18} column and solution A (TFAA, 0.1%) and B (CH_3CN) at flow rate 1 mL/min; absorption at 226 nm; and gradient 0-5%/0-5 min, 5-60%/5.1-35 min, 60-60%/35.1-40 min, and 60-5%/40.1-45 min [11].

Protein concentration was determined by the Bradford method [12] using trypsin and ovalbumin as standard proteins.

Fungicidal activity of biocidal peptides at all isolation and purification steps was analyzed by a turbidimetric method [13].

Electrophoretic analysis of proteins used the Laemmli method [5] and PAAG (15%) with Na-DDS (0.1%) at pH 8.9.

Mass spectrometric analysis of the lipid-transfer peptide used a Finnigan LCQ-MS instrument. Purified peptide was dissolved in water:methanol (1:1 v/v) to a concentration of 5 pmol/mL and analyzed by an electron-impact method [14].

***N*-Terminal amino-acid sequence** was determined by the Edman method [10] using a Perkin—Elmer (USA) Applied Biosystem 491A sequencer. PTH (phenylthiohydantoin) derivatives were analyzed by HPLC over a C_{18} capillary column [14].

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REFERENCES

1. J. C. Kader, *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **47**, 627 (1996).
2. A. Yili, H. Aisa, V. V. Maksimov, O. N. Veshkurova, I. A. Arzanova, and Sh. I. Salikhov, *Chem. Nat. Comp.*, **42**, 201 (2006).
3. X. Y. Ye and T. B. Ng, *J. Pept. Res.*, **57**, 330 (2000).
4. X. Y. Ye and T. B. Ng, *Biochem. Biophys. Res. Commun.*, **273**, 1111 (2000).
5. U. K. Laemmli, *Nature (London)*, **227**, 680 (1970).
6. B. P. Cammue, K. Thevissen, M. Hendriks, K. Eggermont, I. J. Goderis, P. Proost, J. Van Damme, R. W. Obsorn, F. Guerbette, and J. C. Kader, *Plant Physiol.*, **109**, 445 (1995).
7. P. T. Coutos-Thevenot Jounenne, O. Maes, F. Guerbette, M. Grosbois, J. P. Le Caer, M. Boulay, A. Deloire, J. C. Kader, and J. Guern, *Eur. J. Biochem.*, **217**, 885 (1993).
8. A. Molina, A. Segura, and F. Garcia-Olmedo, *FEBS Lett.*, **16**, 119 (1993).
9. A. Molina, J. Gorlach, S. Volrath, and J. Ryals, *Mol. Plant Microbe Interact.*, **12**, 53 (1999).
10. P. Edman and A. Henschen, *Protein Sequences Determination*, S. B. Needleman, 2nd Ed., Springer, Berlin, Heidelberg, New York (1975), p. 232.
11. A. Yili, H. A. Aisa, X. Imamu, V. V. Maksimov, Zh. F. Ziyavitdinov, O. N. Veshkurova, N. Zh. Sagdiev, and Sh. I. Salikhov, *Khim. Prir. Soedin.*, 588 (2006).
12. M. M. Bradford, *Anal. Biochem.*, **72**, 248 (1976).
13. M. S. Almeida, K. S. Cabral, R. B. Zingali, and E. Kurtenbach, *Arch. Biochem. Biophys.*, **378**, 278 (2000).
14. Sh. Y. Wang, J. H. Wu, T. B. Ng, X. Y. Ye, and P. F. Rao, *Peptides*, **25**, 1235 (2004).
15. W. R. Bernhard, S. Thoma, J. Botella, and C. R. Somerville, *Plant Physiol.*, **95**, 164 (1991).