Chemistry of Natural Compounds, Vol. 45, No. 5, 2009

ISOLATION OF CYSTEINE-RICH PEPTIDES FROM *Nigella sativa* SPROUTS

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UDC 547.96:577.15

Peptides containing six and eight cysteines were isolated by acid extraction and subsequent three-step chromatography from Nigella sativa *sprouts. The molecular weights of the resulting fractions were determined by MALDI-TOF mass spectrometry.*

Key words: *Nigella sativa*, sprouts, peptides.

A whole arsenal of mechanisms, the most important of which is the synthesis of proteins and peptides with antimicrobial properties, is used to protect plants from pathogens [1]. Several families of antimicrobial peptides that differ in molecular structure and biological activity such as thionins, defensins, hevein- and knottin-like peptides, cyclotides, and lipid-transfer proteins have been observed in plants [2–4]. The study of antimicrobial peptides (AMP) has fundamental significance for explaining the molecular mechanisms of the acquired immunity of plants and is of great practical interest for developing a new strategy for battling pathogens and agricultural pests that is based on genetic engineering methods. The search for AMP from new plant sources is just as promising for developing a new generation of drugs and preservatives for the food industry.

Our goal was to develop methods for preparative isolation of AMP from two-week sprouts of *Nigella sativa* L. We have previously performed analytical separation of total extracts from seeds and sprouts [5] and isolated and characterized a lipid-transfer protein from seeds [6].

Acid extraction followed by three-step chromatography was used to isolate AMP from *N. sativa* sprouts. Two main fractions that eluted at different NaCl concentrations (100 and 500 mM) were obtained from Hi Trap Heparin HP affinity sorbent. The fractions were separated by exclusion chromatography (gel-filtration) over a column of Superdex^{TM} Peptide HR. Gel-filtration of the 100-mM fraction produced five fractions that were not protein or peptide; the 500-mM fraction, five fractions, two of which were protein or peptide.

Mass spectrometry of the 500-mM fraction showed that it contained fraction 3 with MW 9018 Da and fraction 4 with MW 4965 and 5071 Da that were characteristic of AMP. Therefore, these fractions were further separated into pure components.

The components were purified by RP-HPLC (Fig. 1). Chromatographic separation of fraction 3 produced two fractions with MW 9016 Da in the first and 8992 and 9153 Da in the second. Fractions 4–10 contained fractions with MW in the range 4862-5072 Da.

Fractions were rechromatographed by RP-HPLC under conditions analogous to those for purifying the components in order to establish the homogeneity of the isolated peptides. The peptide with MW 9016 Da and peptides with MW in the range 4862–5072 Da were homogeneous.

The isolated peptides were characterized by determining the number of cysteine units in the molecule. For this, peptides were reduced and alkylated. Then, the products were separated by RP chromatography over a column of Vydac C_{18} (4×250 mm). The resulting fractions were analyzed by mass spectrometry. The MW of the reduced and alkylated peptide of 9016 Da increased by 840 Da compared with the native peptide. This was consistent with eight cysteines in this peptide. The MW of peptides of fraction 4 (with MW in the range 4862–5072 Da) increased by 630 Da compared with the native peptides. This was consistent with six cysteines in them.

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 0009-3130/09/4505-0690

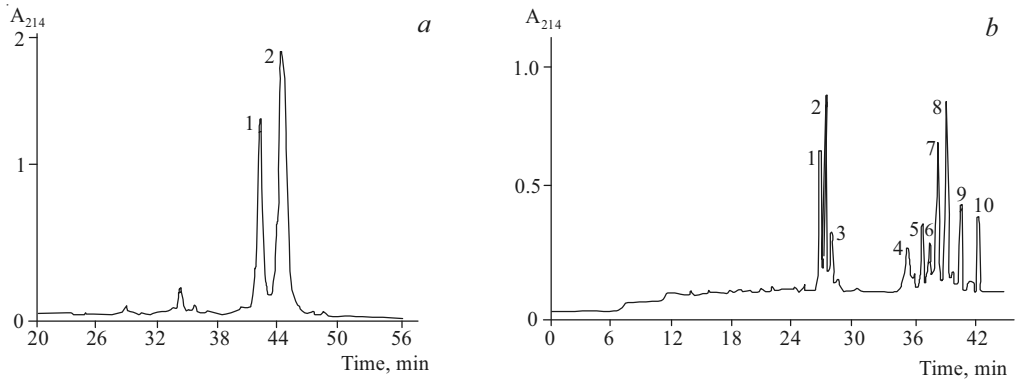

Fig. 1. Chromatographic separation by RP-HPLC over a Luna C_{18} column (4.6 × 150 mm) using a CH_3CN gradient (10–50%) over 40 min of fractions 3 (a) and 4 (b) obtained by separation of fraction (500 mM) over a Superdex^{TM} Peptide HR column at flow rate 0.8 mL/min.

Thus, 11 peptides, one of which with MW 9016 Da was preliminarily identified as a lipid-transfer peptide and the others, d- and γ-defensins, were isolated from two-week sprouts of *N. sativa*.

EXPERIMENTAL

Acid extraction of peptides and proteins, desalting of samples, and MALDI mass spectrometry were carried out as before [5].

Affinity Chromatography over a Hi Trap Heparin HP Column (2.5 × 5 cm) (Amersham Biosciences, England) Using a NaCl Gradient. The desalted extract was separated by affinity chromatography over a column equilibrated with Tris-HCl (10 mM, pH 7.2, buffer A). After elution of the unbound fraction, peptides and proteins were eluted by a stepwise concentration gradient of NaCl (0.1–0.5–1 M) in Tris-HCl (10 mM, pH 7.2) over 2 h at flow rate 1.2 mL/min and room temperature. Proteins and peptides were detected at 214 nm.

The collected fractions were evaporated to dryness in a Speedvac vacuum concentrator (Savant, USA) and redissolved in TFA (0.1%).

Gel Filtration over a Superdex^{TM} Peptide HR Column (10 × 300 mm). Desalted fractions were separated by gel filtration over a column equilibrated with CH_3CN (5%) in TFA (0.05%) at flow rate 15 mL/h. Proteins and peptides were detected at 214 nm.

RP HPLC over a Luna C_{18} Phenomenex Column (4.6 × 150 mm). Components from gel filtration were purified by RP HPLC over a column equilibrated with CH_3CN (10%) in TFA (0.1%). The elution took 40 min at flow rate 0.8 mL/min with a linear gradient from 10 to 50% CH_3CN in TFA (0.1%). Proteins and peptides were detected at 214 nm.

Reduction of Peptides Using Dithioerythritol and Alkylation Using 4-Vinylpyridine. Dried peptide was dissolved in guanidinium hydrochloride (35 μL, 6 M) and EDTA (2 mM) in Tris-HCl (0.5 M, pH 8.5) and treated with isopropanol (5 μL). The tube was shaken on a vortex stirrer, settled by centrifugation, treated with dithioerythritol (4 μL, 0.7 M) in *i*-PrOH (10 μL *i*-PrOH, 1 mg 1,4-dithioerythritol), left in a thermostat at 40°C for 4 h, treated with vinylpyridine (4 μL, 50%) in *i*-PrOH, shaken on the vortex stirrer, centrifuged, left in the dark at room temperature for 20 min, and treated with TFA (30 μL, 0.1%) to stop the reaction. The products were immediately separated by HPLC over a Vydac C_{18} column (4 × 250 mm).

RP HPLC over a Vydac C_{18} column (4.6 × 250 mm, Pharmacia Fine Chemicals, Sweden) was carried out as before [5].

ACKNOWLEDGMENT

The work was supported financially by The Coordinating Committee for Development of Science and Technology of the Republic of Uzbekistan Cabinet of Ministers (Grant FA-F3-T140) and the RFBR (Grant 08-04-90257).

REFERENCES

1. P. C. Selitrennikoff, *Appl. Environ. Microbiol.*, **67**, 2883 (2001).
2. W. F. Broekaert, B. P. A. Cammue, M. F. C. De Bolle, K. Thevissen, G. W. De Samblanx, and R. W. Osborn, *Crit. Rev. Plant Sci.*, **16**, 297 (1997).
3. F. Garcia-Olmedo, A. Molina, J. M. Alamillo, and P. Rodriguez-Palenzuela, *Biopolymers*, **47**, 479 (1998).
4. F. Garcia-Olmedo, P. Rodriguez-Palenzuela, A. Molina, J. M. Alamillo, E. Lopez-Salanilla, M. Berrocal-Lobo, and C. Poza-Carrion, *FEBS Lett.*, **498**, 219 (2001).
5. Yu. I. Oshchepkova, E. A. Rogozhin, O. N. Veshkurova, Ts. A. Egorov, and Sh. I. Salikhov, *Khim. Prir. Soedin.*, 247 (2009).
6. Yu. I. Oshchepkova, O. N. Veshkurova, E. A. Rogozhin, A. Kh. Musolyamov, A. N. Smirnov, T. I. Odintsova, Ts. A. Egorov, E. V. Grishin, and Sh. I. Salikhov, *Bioorg. Khim.*, **35**, 344 (2009).