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NEW PEPTIDE FROM SEEDS OF *Cicer arietinum*

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The pure peptide YZDCM was isolated from seeds of Cicer arietinum *and assigned to the homologous class of defensins according to molecular weight and* N*-terminus amino-acid sequence.*

Keywords: chickpea, peptide, antioxidant activity, carcinolytic activity.

Seeds of plants have now yielded a large number of antimicrobial peptides that can be assigned according to their structural properties to various classes of antimicrobial peptides including defensins [1–3], lipid-transfer proteins [4], thionins, heveins, knottins, Ib-AMPs, cephedrins, snakins, and cyclotides. They play an important role in protecting seeds against microbial pathogens [5].

Chickpea (*Cicer arietinum* L.) is employed in Uigur medicine, has been cultivated in Xinjiang (PRC) for ~2500 years, and is used for treatment of bronchitis, two types of diabetes, and hyperlipemia and as nutritional food for the local population [6, 7]. Peptides with high antifungal activity were isolated previously from green chickpeas [8].

The goal of the present work was to isolate a pure peptide from chickpea seeds and to determine the partial *N*-terminus amino-acid sequence in order to identify the isolated peptide with known classes of antimicrobial peptides.

The pure peptide was isolated in several steps. Seeds of chickpea were first ground and defatted. Total proteins were extracted (4507.13 mg/100 g of seeds) with phosphate buffer. Then, fractional precipitation by ammonium sulfate from 30 to 80% saturation was performed.

The molecular weights of components remaining in the extracts after precipitation were determined by gel electrophoresis in PAAG (15%). Analysis of the resulting fractions showed that fraction I [precipitated by 30% $(NH_4)_2SO_4$; 391.0 mg protein per 100 g of seeds (8.68%)] contained primarily low-molecular-weight components. Fractions II and III [precipitated by 50% $(NH_4)_2SO_4$, 2124.8 mg/100 g of seeds (47.14%) and by 80% $(NH_4)_2SO_4$, 1991.33 mg/100 g of seeds (44.18%), respectively] contained high-molecular-weight components in the range 10–45 kDa (Fig. 1).

The resulting fractions were tested for antioxidant and carcinolytic activity [9] (Table 1).

It was found that fraction I had the highest activity in both tests. Then, it was separated by a series of chromatographic methods.

Fraction I was separated by ion-exchange chromatography over a Servacel DEAE-23SN anion-exchange column. Adsorbed peptides (261.97 mg/100 g of seeds, 5.81% yield) were separated by a linear gradient. Then, the peptide fraction (IV, 62.56 mg/100 g of seeds, 1.39% yield) that did not adsorb to the anion-exchange column was placed on a CM-TSK-650M column (30%). Peptides (V, 29.325 mg/100 g of seeds, 0.65% yield) that adsorbed to the CM-TSK-650M column were eluted by a linear NaCl gradient. This produced three fractions that were analyzed by gel electrophoresis in PAAG. It was found that fraction I-1 contained components with molecular weights in the range 6.5–65 kDa; fraction I-2, 5–30 kDa; fraction I-3, 5–65 kDa.

The resulting fractions were tested for antioxidant and carciolytic activity.

Fraction I-2, which contained the principal peptides, exhibited pronounced antioxidant and antitumor activities. Fraction I-2 was separated by gel filtration over an HW 40C column in order to isolate the pure peptides. Two fractions were obtained.

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TABLE 1. Antioxidant ($C_{1/2}$) and Antitumor (IC_{50}) Activity of Fractions from Seeds of *Cicer arietinum*

Fractions	$C_{1/2}$, μg/mL	IC_{50}, μg/mL	Fractions	$C_{1/2}$, μg/mL	IC_{50}, μg/mL
I	48	80	IV	35	62
II	41	248	V	62	248
III	70	250	I-2	23	38

TABLE 2. Comparison of *N*-Amino-acid Sequences of the Peptide from Seeds of *Cicer arietinum* with Related Peptides and Proteins

Peptide source	*N*-terminus sequence	Homology, %
YZDCM, peptide from chickpea seeds	ACCENLADTYRGPCF	100
Gymnin	KTCENLADDY	70
Defensin 1	KTCEHLADTY	70
Defensin 2	KTCENLSGTF	60
Precursor PR of protein 39	-TCEHLADTY	80
Precursor PR of protein 230	-TCENLAGSY	70
Precursor of antifungal protein of *Medicago sativa*	-TCENLADKY	80
Antimicrobial defensin DRR230-c	-TCEHLADTY	80
PDF1 *Vigna radiata*	KTCENLANTY	70

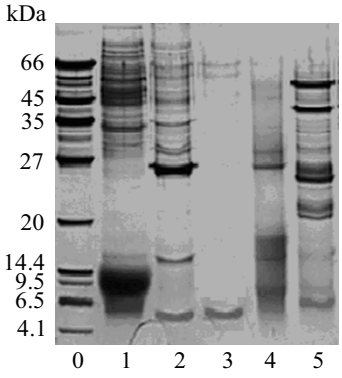

Fig. 1. Gel electrophoresis of protein–peptide fractions from seeds of *Cicer arietinum*: mixture of markers and standards (0); unadsorbed on CM-TSK-650M (30%) (1); adsorbed on CM-TSK-650M (30%) (2); pure peptide (3); precipitate, 30% $(NH_4)_2SO_4$ (4); adsorbed on DEAE-cellulose (30%) (5).

According to electrophoresis and mass spectrometric analysis, the second fraction contained a pure peptide that was called YZDCM and had molecular weight 5.4833 kDa. Stepwise Edman degradation determined the *N*-terminus amino-acid sequence as H_2N-Ala-Cys-Cys-Glu-Asn-Leu-Ala-Asp-Thr-Tyr-Agr-Gly-Pro-Cys-Phe.

A search for the obtained amino-acid sequence of the isolated peptide in the UniProt database using the BLAST program revealed a high degree of homology with defensins isolated earlier from other plant species.

Table 2 presents a comparison of the *N*-terminus sequence of the homogeneous peptide from chickpea seeds with related proteins and peptides.

Thus, our results indicated that a new peptide that was homologous to defensins from other plant species was isolated from chickpea seeds. It presumably had high inhibiting activity against various fungus species and tumor cells.

EXPERIMENTAL

Isolation of Total Peptides from Seeds. Peptides were extracted by buffer of the following composition: Na_2HPO_4 (15 mM), KCl (100 mM), EDTA (1.5 mM), thiourea (2 mM), α-toluenesulfonylfluoride (PMSF, 1 mM), and polyvinylpyrrolidone (1.5%) (pH 7.4). Chickpea seeds (300 g) were ground, defatted with hexane in a Soxhlet apparatus, dried, and extracted with cold buffer (1 L) for 2 h at 4°C with continuous stirring. The extract was clarified by centrifugation for 15 min at 12,000 rpm. The supernatant was treated with $(NH_4)_2SO_4$ until 30% saturated. The mixture was left overnight at 4°C in order to develop a precipitate. The precipitated proteins were separated by centrifugation. The supernatant was treated with $(NH_4)_2SO_4$ until 50% saturated. The precipitate that formed overnight was removed by centrifugation. The supernatant was treated with $(NH_4)_2SO_4$ until 80% saturated. The resulting precipitate was separated by centrifugation for 15 min at 12,000 rpm.

Ion-exchange Chromatography. Desalted extract from chickpea seeds [precipitated by 30% $(NH_4)_2SO_4$] was made basic (pH 8.7) with ammonia solution (50 mM) and placed on a Servacel DEAE-23SN column (3.0 × 15 cm, Reanal) equilibrated with ammonium acetate solution (50 mM, pH 8.7) at flow rate 0.8 mL/min. The fraction of peptides that did not adsorb to the sorbent was placed on a CM-TSK-650M column (2.0 × 10 cm, Tosoh Bioscience, Japan) equilibrated with ammonium acetate solution (50 mM, pH 5). Proteins that adsorbed to the sorbent were eluted by a linear NaCl gradient (300 mL, from 0 to 1 M) in ammonium acetate (50 mM, pH 6) at flow rate 0.5 mL/min.

Gel filtration of the principal peptides from chickpea was performed on an HW-40C column (2.0 × 85 cm) using distilled water at flow rate 30 mL/h.

The protein concentration was determined by the Bradford method [10] using trypsin and ovalbumin as standard proteins.

Electrophoretic analysis of proteins was carried out by the Laemmli method [11] using PAAG (15%) with Na-DDS (0.1%, pH 8.9).

Mass spectrometric analysis of antioxidant peptides was carried out in a Finnigan LCQ-MS instrument. Purified peptide was dissolved in H_2O:MeOH (1:1, v/v) to concentration 5 pM and analyzed by an electron-capture method [9].

***N*-Amino-acid sequence** was determined by the Edman method [12] using a Perkin–Elmer (USA) automated sequencer.

Antioxidant activity was determined using ABTS [2,2′-azino-*bis*(3-ethylbenzothiazoline-6-sulfonate] (Sigma, St. Louis, MO, USA) dissolved in H_2O to a final concentration of 7 mg/L. Radical cations of ABTS were produced by reaction with potassium persulfate (2.45 mL/L, Sigma, St. Louis, MO, USA) and by storing the stock solution in the dark at room temperature for 12 h. Peptides were extracted by H_2O containing phosphate buffer (0.1 M) in order of increasing solubility. The ABTS solution was diluted with phosphate buffer until the optical density was 0.70 ± 0.02 at wavelength 734 nm and 30°C before determining the antioxidant activity. Then, ABTS solution (184 μL) was treated with peptide solution (200 μL). The absorption was measured three times on a plate reader. The antioxidant activity was calculated as the equivalent to the antioxidant volume.

Analysis of Inhibiting Activity on Colon Cancer Cells. Human colon cancer cells Caco-2 were cultivated in DMEM medium with 10% fetal bovine serum at 37°C in a humid atmosphere with 5% CO_2. Passage of the cells was carried out every 3–4 d.

Inhibition of cell activity was analyzed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in 96-well plates. Each well was treated after 48 h of incubation with MTT (50 μL) and incubated for another 4 h. Plates were centrifuged (1,000 rotations per 5 min). The liquid medium was removed. Cells from each well were dissolved in DMSO (150 μL). Absorption at 550 nm was measured using a SpectraMax M5/M5e spectrophotometer (America). Inhibition of cell activity (ICA) was expressed in percent and calculated using the equation:

$$\text{degree of inhibition (\%)} = 100 - [\text{ICA}]\ \text{test}/[\text{ICA}] \times \text{control} \times 100.$$

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