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ANTIOXIDANT PEPTIDES FROM *Cicer arietinum* OF XINJIANG, CHINA[a]

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Four peptides of molecular weights 1.148, 4.68, 5.41, and 9.086 kDa with antioxidant activity were isolated from chickpea sprout using two-step ion-exchange chromatographic and HPLC techniques for the first time. The partial N*-terminal amino acid sequences of the peptide named YZDBCM-1 (9.086 kDa) was determined as* $H_2N^{1\text{-}15}$*-*H_2*N-Ala-Ile-Thr-Cys-Gly-Arg-Val-Ser-Ala-Ala-Leu-Ala-Pro-Pro-Leu using the Edman automated sequencing apparatus, which was a new peptide rich in alanine. It was shown that the antioxidant activity of the peptide YZDBCM-1 was* IC_{50} *156.2 μg/mL (17.2 μmol/L) to the free radical of ABTS.*

Keywords: *Cicer arietinum* (chick pea sprout), antioxidant, peptides, *N*-terminal sequences.

Dietary intake of sprouts provides not only nutrients but also a highly interesting class of secondary metabolites beneficial to health. Peptides with biological activities have been isolated from plant sprouts [1, 2]. However, peptides from *Cicer arietinum* L. (chick pea sprout) have not been reported. Interest in the search for natural antioxidants for use in the food industry or medical materials as replacements for synthetic antioxidants and antibacterial peptides, the use of which is limited by their carcinogenicity, has recently risen [3, 4]. In continuation of our research on peptides from chick pea [5], herein we report the isolation and characterization of four low-molecular-weight peptides from two-week chickpea sprouts and investigate their antioxidant activity *in vitro* against the free radical of ABTS, as well as identify its partial *N*-terminal sequence. Proteins and peptides were extracted from seeds by phosphate buffer and precipitated by ammonium sulfate at 30–80% saturation. High molecular-weight proteins were removed by precipitation after 5 min boiling at 80°C with subsequent centrifugation. Table 1 gives the protein yields for the purification steps. The fraction of thermally stable peptides precipitated by ammonium sulfate at 30%, 50%, and 80% saturation was dissolved and dialyzed against distillated water overnight to form a white precipitate. The dialyzed solutions were centrifuged, and six fractions (YZDB-30% Y; YZDB-30% C; YZDB-50% Y; YZDB-50% C; YZDB-80% Y; YZDB-80% C) were prepared. The molecular weights of the peptide fractions were determined by gel-electrophoresis in PAGE (15%) under dissociating conditions [6]. It can be seen that the YZDB-30%Y and YZDB-50%Y fractions are rich in peptides and their molecular weights are in the range 4.1–14 kDa. This agrees with the values for known antimicrobial peptides (AMP) [7–9]. The two peptide fractions were combined and dialyzed against water and ammonium acetate buffer (0.05 M, pH 9.0). Then, the solution was separated successively by ion-exchange chromatography over columns of DEAE-23SN (2 × 15 cm, Toyopearl) and KM-TSK-650M (2 × 15 cm, Toyopearl).

The peptide fraction, bound to a KM-TSK-650 M (2 × 5 cm, Toyopearl) column, was eluted by NaCl in ammonium acetate solution (50 mM, pH 6) to produce the cationic peptide fraction, which was collected, desalted by gel-chromatography over Sephadex G-15, and used for further analysis.

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TABLE 1. Yield and Antioxidant Activity of Every Fraction from Chick Pea Sprout

Protein purification step	Amount of protein, mg/100 g of seeds	Protein yield, %	Mass/kDa	IC_{50}, μg/mL
Crude seed extract	11910.4	100	–	–
$(NH_4)_2SO_4$ precipitation (30%) Y	2273.3	19.09	4.1–66	> 250
$(NH_4)_2SO_4$ precipitation (30%) C	359.3	3.03	4.1–66	24.8
$(NH_4)_2SO_4$ precipitation (50%) Y	3232.7	27.14	4.1–66	53.6
$(NH_4)_2SO_4$ precipitation (50%) C	1810.5	15.20	4.1–66	3.8
$(NH_4)_2SO_4$ precipitation (80%) Y	2734.5	22.96	4.1–66	> 250
$(NH_4)_2SO_4$ precipitation (80%) C	1500.1	12.60	4.1–66	> 250
DEAE-23SN adsorbed (30%)	1061.97	8.92	–	> 250
KM-TSK-650M unabsorbed (30%)	429.325	3.61	4.1–14; 27	44.8
DEAE-23SN adsorbed (30%)	1051.82	8.83	–	> 250
KM-TSK-650M unabsorbed (50%)	345.21	2.90	4.1–14; 27	> 250
Total basic peptides	774.54	6.5	4.1–14	> 250
YZDBCM-4	3	0.025	1.148	> 250
YZDBCM-3	5	0.04	4.68	> 250
YZDBCM-2	5	0.04	5.41	> 250
YZDBCM-1	20	0.17	9.086	> 156.2

Bio-RAD Biologic LP Isothermal chromatography system at 4°C.

The cationic peptide fraction was further subjected to reversed-phase HPLC, and the results show that most of the peptides were appeared at 8 to 24 min. The peptides that appeared at 8.027, 19.033, 21.18, and 26.31 min were collected and their molecular mass determined by ESI-MS apparatus as 4.68, 1.148, 9.086, and 5.41 kDa, respectively.

The most active and largest amount of the homogeneous peptide that appeared at 21.18 min with antioxidant activity against free radical of IC_{50} 156.2 μg/mL (17.2 μmol/L) was selected for structural studies. Determination of the *N*-terminal aminoacid sequence of the homogeneous peptide by the Edman method [10] gave the structure as $H_2N^{1\text{-}15}$-Ala- Ile-Thr-Cys-Gly-Arg-Val-Ser-Ala-Ala-Leu-Ala-Pro-Pro-Leu.

A study of the biological activity of the crude and purified peptides showed that the peptides from the sprout had stronger biological activity than those from the seeds; the total peptide fraction of YZDB-50%C had strongest biological activity among all the crude peptides. The isolated peptide of YZDBCM-1 had no inhibiting effect on bacterium and on human colon cancer Caco-2 cells, but it had the strongest inhibiting effect on the ABTS free radical compared to the other purified peptides, and the IC_{50} value was 156.2 μg/mL (17.2 μmol/L).

EXPERIMENTAL

Isolation of Total Peptides from Chick Pea Sprouts. 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). Chickpea sprouts (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 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 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 chick pea sprout was made basic to pH 9.0 using ammonia solution (12 M) and passed over DEAE-23-SN column (2 × 10 cm, Toyopearl) and equilibrated with ammonium acetate solution (50 mM, pH 9) at flow rate 0.5 mL/min. The fractions of peptides not binding to the sorbent were basic proteins. The resulting effluent was treated with HCl to pH 6 and placed on a KM-TSK-650M column (2 × 15 cm, Toyopearl) equilibrated

with ammonium acetate (0.050 M, pH 6). Proteins bound by sorbent were eluted by a linear gradient of NaCl (300 mL, 0 – 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 Dupont 8800 chromatograph using a 250/8/4 Protein & Peptide C18 column and solution A (TFA, 0.1%) and B (CH_3CN) at flow rate 1 mL/min; absorption at 226 nm; 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.

The Laemmli method [6] and PAGE (15%) with Na-DDS (0.1%) at pH 8.9 were used for electrophoresis of proteins. A Finnigan LCQ-MS instrument was used for mass spectrometric analysis of the lipid-transfer peptide. 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 [13].

The *N*-terminal amino-acid sequence was determined by the Edman method [10] using a Perkin–Elmer Applied Biosystem 491A sequencer (USA). PTH (phenylthiohydantoin) derivatives were analyzed by HPLC over a C18 capillary column [13].

Determination of Antioxidant Activity. ABTS (Sigma, St. Louis, USA) was dissolved in water to a concentration of 7 mmol/L. ABTS radical cations were produced by reaction with potassium persulfate (2.45 mmol/L final concentration) with storage of the mother liquor in the dark at room temperature for 12 h. Peptides were extracted in the order of increasing solubility by water containing phosphate buffer at a concentration of 0.1 M. The ABTS solution was diluted with phosphate buffer before determination of the antioxidant activity until the optical density was 0.70 ± 0.02 at a wavelength 734 nm and 30°C. Then the ABTS solution (184 μL) was added to the peptide solution (200 μL). The absorption was measured three times on a planchette reader. The antioxidant activity was calculated as inhibition (%) of ABTS radical formation: inhibition (%) = [(Acontrol – Aextract)/Acontrol] × 100.

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