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ISOLATION OF TWO ANTIOXIDANT PEPTIDES FROM SEEDS OF *Apium graveolens* INDIGENOUS TO CHINA[a]

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Recently, interest has emerged in the search for natural antioxidants for use in the food industry or in medical materials as replacements for synthetic antioxidants, the use of which is limited by their carcinogenicity [1, 2]. Peptides of various bioactivities have been isolated from seeds of several plants, such as antimicrobial peptides [3, 4] and antioxidative peptides [5]. Many antioxidative peptides are released from animals by enzymatic hydrolysis. However, antioxidative peptides from plants are little studied, in particular, natural antioxidative peptides. Moreover, peptides from plants of *Apium graveolens* L. are also little studied. The results of our search previously showed that an antioxidative peptide of molecular weight 5.0005 kDa was isolated and purified from the neutral fraction of *Apium graveolens* [6]. The goal of the present work was to further investigate antioxidant peptides from *Apium graveolens* seed as a potential new source of natural antioxidants, to develop chromatographic methods for isolating and quantifying them using ion-exchange and gel-permeation chromatography, and to determine the partial *N*-terminus sequence in order to identify the new peptides.

An acid peptide named QCZDE was obtained by gel filtration of the acid peptide fraction of *Apium graveolens* on Sephadex G-75, whose molecular weight was estimated as 6.5 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (15%) (PAGE) under dissociating conditions [7]. However, its molecular mass cannot be estimated by mass spectrometry, nor its *N*-terminus sequence. The acid peptide QCZDE, with many negative charges, binds electrons with difficulty, so it cannot be effectively estimated by mass spectrometry. This peptide, which is acidic with many carboxyl groups (-COOH), can form H^+. The amidogens (-NH_2) in the peptide react with H^+ to form cation NH_3^+, which cannot react with phenylisothiocyanate. Thus it was difficult to determine the *N*-terminus amino-acid sequence of the peptide by the Edman method [8].

Using the same methods as above, we obtained a basic peptide named QCZCM from the basic-peptide fraction of *Apium graveolens* purified by reversed-phase high-performance liquid chromatography (RP-HPLC). As shown by gel electrophoresis (15%) (PAGE) in sodium dodecyl sulfate–polyacrylamide, the molecular weight of this peptide was between the 9.5–14.4 kDa, which agreed with the 11.390 kDa determined by ESI-MS [9]. The partial *N*-terminus sequence was determined by the Edman method [8] as H_2N-Ala1-Gln-Glu-Glu-Thr-Gln-Glu-Arg-Gln-Pro. A search for this amino-acid sequence in the BLAST database did not reveal a homology with previously isolated known peptides.

A study of the antioxidative activity of the two isolated peptides showed that both peptides had a strong inhibiting effect on ABTS free radical, and the IC_{50} values of QCZDE and QCZCM for ABTS* radical scavenging activities were 158.7 and 193.2 μg/mL, respectively. The free radical scavenging activity of purified peptide (QCZDE) was weaker than that of its crude fraction (IC_{50} 8.228452 μg/mL) [6]. These results indicate that the purified peptide may possess synergistic antioxidation potentials with the other antioxidants in the crude acid fraction from celery. But the antioxidative activity of both purified peptides was stronger than that of peptides from other sources [9].

A study of the biological activity of the two peptides showed that they both had no effect on *Candida albicans*, *Escherichia coli*, and *Staphyloccus aureus*. It was reported that the activity of the peptides were related to the amino-acid composition, the amino-acid sequence, and the MW [5].

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Preparation of Samples. The seeds of *Apium graveolens* L. from Xinjiang of China were used; the plant was identified by Guan-mian Shen from Xinjiang Institute of Ecology and Geography, Chinese Academy of Sciences.

The preparation of the acidic and basic fractions was conducted according to the method of M. Q. Ling and A. Yili [6]. After ion-exchange chromatography on DEAE-SN-23 and on KM-TSK-650M, the fraction of proteins and peptides binding to DEAE-SN-23 was the acidic fraction, and that binding to KM-TSK-650M was the basic fraction.

Purification of Peptides. Gel filtration chromatography of the acidic fraction from celery seeds was performed on a G-75 Sephadex column (20 × 800 mm) using distilled water at a flow rate of 15 mL/h.

The basic fraction from celery seeds was purified by RP-HPLC, and the mobile phase was carried out with 10% B in 5 min and a linear gradient from 10 to 55% B in another 70 min at 1.1 mL/min flow rate: eluting solvent A: 0.1% TFA, B: acetonitrile; detection wavelength: 225 nm. Fractions were manually collected according to the chromatogram.

Mass spectrometry of the purified peptides was performed on a Finnigan LCQ-MS instrument, and their *N*-Terminus amino-acid sequences were determined by a PPSQ-33A protein sequencer from Shimadzu (Shanghai Applied Protein Technology Co. Ltd.). The purified peptides were dissolved in water–methanol (1:1 v/v) to a concentration of 5 pmol/mL and analyzed by an electron-impact method.

The purity of peptides was checked through SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Electrophoresis of proteins was carried out by the Laemmli method [10] using PAAG (15%) and Na-DDS (0.1%) at pH 8.9 [6]. After electrophoresis, the gel was stained with silver. The molecular mass of peptides was determined by comparison of its electrophoretic mobility with those of molecular mass marker proteins from Amersham Biosciences.

Protein concentration was determined by the Bradford method [11] using trypsin and ovalbumin as standards.

Mass spectrometry of the antioxidant peptide was performed in a Finnigan LCQ-MS instrument. The purified peptide was dissolved in H_2O–MeOH (1:1, v/v) to a concentration of 5 pmol/mL and analyzed using electron-capture ionization.

The *N*-terminus aminoacid sequence was determined by the Edman method using an automated sequencer (Perkin–Elmer Co.) [8]. Phenylthiohydantoin derivatives were analyzed by reversed-phase HPLC on a C18 column.

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 the reaction with potassium persulfate (2.45 mmol/L, Sigma, St. Louis, USA) (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 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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