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ISOLATION OF A NEW PEPTIDE FROM SEEDS OF *Apium graveolens* INDIGENOUS TO CHINA

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A new peptide that had anti-oxidant activity and molecular weight 5,000.5 Da according to PAAG data and mass spectrometry was isolated from celery seed using chromatographic methods.

Keywords: *Apium graveolens*, peptide, anti-oxidant activity, *N*-terminus amino-acid sequence.

Interest in the search for natural anti-oxidants for use in the food industry or medical materials as replacements for synthetic anti-oxidants and antibacterial peptides, the use of which is limited by their carcinogenicity, has recently risen [1, 2].

Peptides of various biological activity were isolated from seeds of several plants. However, peptides from plants of the family Apiaceae are little studied, in particular, from *Apium graveolens* L. (celery).

Our goal was to estimate the total content and anti-oxidant activity of peptides from celery seed as a potential new source of natural anti-oxidants, 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.

The method for purifying the peptides from celery seed consisted of several stages. Table 1 presents the protein yields in each purification stage. The molecular weights (MWs) of the peptides in the extracts were determined by PAAG gel-electrophoresis (15%) under dissociating conditions [3]. The isolated peptides had MWs 4.1–6.5 kDa upon precipitation by ammonium sulfate (50 and 80%). This agreed with the values for known biologically active peptides [4–6].

Determination of the anti-oxidant activity showed that the total peptides precipitated by 50% ammonium sulfate from the crude extract exhibited the greatest activity for cleavage of the free radical ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate). Next, pure bioactive peptides were isolated from this fraction by ion-exchange chromatography on Servacel DEAE-SN-23. The anti-oxidant activities of the protein and peptide fractions from celery seed are given below:

Sample	$C_{1/2}$, *mg/mL*	*Sample*	$C_{1/2}$, *mg/mL*
Extract	3.38654	DEAE-bound	8.228452
30% $(NH_4)_2SO_4$	34.2277	CM-bound	176.3234
50% $(NH_4)_2SO_4$	24.0791	CM-not bound	157.8625
80% $(NH_4)_2SO_4$	40.0125	Pure peptide	182.0615

Then the peptide fraction that was not adsorbed to the anion-exchange column was placed on a CM-TSK-650M cation-exchange column. Next, the peptide fractions bound to Servacel DEAE-SN-23 and CM-TSK-650M were eluted by a NaCl gradient (0–1 M) in order to isolate fractions of acidic and basic peptides. The fraction that did not adsorb to CM-TSK-650M was collected, desalted on Sephadex G-25, and used for further isolation of peptides with anti-oxidant activity.

The MWs of the proteins and peptides contained in the resulting fractions were analyzed by PAAG gel-electrophoresis. The resulting fractions contained a large amount of high- and low-MW proteins with MWs 4.1–45 kDa. The total peptides were separated after performing the two-stage ion-exchange chromatography into a fraction of acidic peptides that contained proteins with MW 20–65 kDa, the principal peptides with MW 2.5–14.5 kDa, and neutral peptides with MW of the order of 5 kDa.

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TABLE 1. Celery-Seed Peptide Purification Stages

Protein purification stages	Protein amount, mg	Protein yield, %	Protein purification stages	Protein amount, mg	Protein yield, %
Protein extract	4559.7	100	DEAE Servacel 23SN	62	2.09
Precipitation 30% $(NH_4)_2SO_4$	485.2	10.64	CM TSK-650M	54	1.82
Precipitation 50% $(NH_4)_2SO_4$	1112.0	24.39	Neutral peptides	12	0.37
Precipitation 80% $(NH_4)_2SO_4$	2962.5	64.97			

*Protein concentration determined by Bradford method.

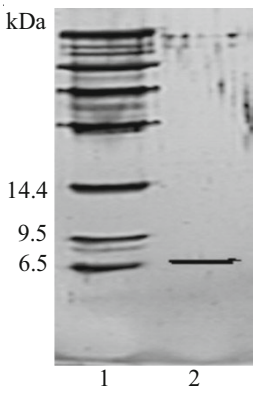

Fig. 1. Gel-electrophoresis of protein fraction from *Apium graveolens* seeds in PAAG (15%): standards (1), peptide after gel-filtration purification on HW-55F (2).

Then the fraction of neutral peptides was separated by gel-filtration on an HW-55F column into three fractions. The third fraction according to Na-DDS electrophoresis (Fig. 1) and mass spectrometry containd a pure peptide with MW 5005.5 Da. The partial *N*-terminus sequence was determined by the Edman method [7] as: Ala^1-Asp^2-Asn^3-Ala^4-Ala^5-Arg^6-Pro^7-Val^8-Arg^9-Glu^{10}-Thr^{11}-Asp^{12}-Ala^{13}-Val^{14}-Pro^{15}.

A search for this amino-acid sequence in the BLAST database did not reveal homology with previously isolated known peptides [8]. A study of the anti-oxidant activity of the acidic proteins and peptides showed that both the crude extract and the isolated peptide had the strongest inhibiting effect on ABTS free radical. A study of the biological activity of these peptides showed that they had no effect on *Candida albicans*, *Escherichia coli*, and *Staphylcoccus aureus* although the anti-oxidant activity of the peptides was stronger than that of peptides from other sources [9–11]. The activity of peptides is known to be related to the amino-acid composition, the amino-acid sequence, and the MW.

Thus, chromatographic methods including ion-exchange and gel-filtration column chromatographies were used to isolate a pure peptide with an amino-acid sequence not known among previously isolated analogs. The isolated peptide had high anti-oxidant activity.

EXPERIMENTAL

Isolation of Total Peptides from Seeds. Buffer for peptide extraction was prepared using Na_2HPO_4 (10 mM), KCl (100 mM), EDTA (1.5 mM), and polyvinylpyrrolidone (1.5%), pH 7.4 The solution was stirred for 2 h at 4°C and treated before use with thiourea (2 mM) and α-toluene sulphonyl flouride (PMSF) (1 mM) dissolved in isopropanol. Celery seeds (250 g) were ground, defatted with hexane in a Soxhlet apparatus, dried, and extracted with cold buffer (1000 mL) for 2 h at 4°C with constant stirring. The extract was clarified by centrifugation for 30 min at 6,000 rpm, treated with solid $(NH_4)_2SO_4$ until the supernatant was 30% saturated, and left overnight at 6°C to form a precipitate. The precipitated proteins were separated by centrifugation (30 min at 6,000 rpm). The supernatant was treated with $(NH_4)_2SO_4$ until 50% saturated. The precipitate that formed overnight was separated by centrifugation (30 min at 6,000 rpm). Precipitated proteins were also separated by centrifugation (under the same conditions). The supernatant was treated with $(NH_4)_2SO_4$ until 80% saturated. The precipitate that formed overnight was separated by centrifugation (30 min at 6,000 rpm). Precipitated peptides were

dissolved in the minimum amount of distilled water. The solution was stored at 80°C for 10 min for denaturation. High-MW proteins were precipitated. Centrifugation (30 min at 6,000 rpm) was repeated to remove the precipitate of thermally labile proteins. The supernatant was repeatedly dialyzed against distilled water using a Spectra/Por® 3 dialysis tube with a pore size retaining proteins up to 3.5 kDa.

Ion-exchange Chromatography. The dialyzed extract of proteins precipitated by 50% godium sulfate was adjusted to pH 9.0 with ammonium acetate (0.050M) and passed over a column of Servacel DEAE-23SN (2.0 × 10 cm, Reanal) equilibrated with NH_4Ac (12M, pH 9) at flow rate 0.5 mL/min. Fractions of proteins that did not bind to the sorbent contained the principal thermally stable proteins of celery seed. The resulting effluent was adjusted to pH 6 with HCl and placed on a KM-TSK-650M column (2.0 × 10 cm, Tosoh Bioscience, Japan) equilibrated with NH_4Ac (0.050M, pH 6). Proteins bound to the sorbent were eluted by a linear NaCl gradient (0→1 M) and NH_4Ac (0.050M, pH 6) at flow rate 0.5 mL/min.

Gel-filtration of neutral peptides from celery seed was performed on a column of HW-55F (2.5 × 85 cm) using distilled water at flow rate 60 mL/h.

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

Electrophoresis of proteins was carried out by the Laemmli method [3] using PAAG (15%) and Na-DDS (0.1%) at pH 8.9.

Mass spectrometry of the anti-oxidant 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.

***N*-Terminus amino-acid sequence** was determined by the Edman method using an automated sequencer (Perkin–Elmer Co.) [7]. Phenylthiohydantoin derivatives were analyzed by reversed-phase HPLC on a C_{18} column.

Determination of Anti-oxidant 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 0.1M. The ABTS solution was diluted with phosphate buffer before determining the anti-oxidant activity until the optical density was 0.70 ± 0.02 at wavelength 734 nm and 30°C. Then ABTS solution (184 μL) was added to the peptide solution (200 μL). The absorption was measured three times on a planchette reader. The anti-oxidant activity was calculated as the equivalent to the anti-oxidant volume.

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