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Short communication

Separation and identification of iron-chelating peptides from defatted walnut flake by nanoLC-ESI–MS/MS and *de novo* sequencing

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ABSTRACT

Walnuts are rich in unsaturated fatty acids and are processed to walnut oil. The by-product (defatted walnut flake) consists of approximately 50% protein and is usually treated as waste or low-value-use material. In this study, walnut protein was isolated and hydrolyzed to low-molecular-weight peptides. Iron-binding walnut peptides were prepared by iron-immobilized affinity chromatography (IMAC-Fe^{3+}). Some iron-chelated peptides adsorbed to the IMAC-Fe^{3+} column were easily broken by NaH_2PO_4, whereas part of the peptides isolated from IMAC-Fe^{3+} column remained as peptide-iron complexes and could only be disrupted by EDTA-2Na. Subsequently, the iron-binding walnut peptides were separated into approximately 10 fractions by RP-HPLC. The main peak, with retention time of 32 min, was further analyzed by nanoLC-ESI–MS/MS. Two main components in $F_{32\ min}$ were characterized as LAGNPDDEFRPQ and VEDELVAVV with *de novo* sequencing.

1. Introduction

Walnut is rich in unsaturated fatty acids, proteins, and polyphenols and possess many important physical and functional activities such as antioxidant activity, anti-inflammatory activity, prevention of cancer, lowering of cholesterol, reduction of cardiovascular risk, and promotion of cognition [1–6]. In addition to its use in food processing and for direct consumption, walnut is used to manufacture walnut oil. The by-product obtained from walnut oil processing consists of approximately 50% protein, containing albumin, globulin, prolamin, and glutelin. Food protein can be hydrolyzed to low-molecular-weight peptides that possess many functional activities that are different from the parent protein [7–9].

Iron is an important trace element for the human body. Iron deficiency results in anemia, poor cognitive development, increased maternal mortality, and decreased energy levels. Recently, peptides from food sources have been reported to possess iron-binding properties. The hydrolysates obtained from animal protein such as whey protein, porcine blood plasma protein, Alaska pollock skin protein, and anchovy muscle protein and plant protein (chickpea, rice bran, and palm kernel cake proteins) showed iron-chelating ability [10–17]. Thus, such peptides can be used for iron fortification. However, little is known about the walnut protein. This study aimed to identify the structure and properties of iron-binding walnut peptides (WPs) by RP-HPLC, nano LC electrospray ionization tandem mass spectrometry (nanoLC-ESI–MS/MS), and manual *de novo* sequencing.

2. Materials and methods

2.1. Materials

Defatted walnut flakes were provided by Jingwan Co. Ltd. (Shijiazhuang, China). Neutral protease was purchased from Novozymes Co. Ltd. (Beijing, China). Nickel-iminodiacetic acid (IDA)-Sepharose 6B was purchased from Seven Sea Biotechnology Co. Ltd (Shanghai, China). All other chemicals were of analytical grade.

2.2. Preparation of iron-chelating WPs with iron-immobilized affinity chromatography

Walnut protein isolates (WPIs) were obtained from defatted walnut flakes added to deionized water (water/flour = 10:l; w/v), pH 8.0 adjusted with 2 mol/L NaOH, for 1.5 h at room temperature. The mixture was then centrifuged (3000 × g, 20 min). The supernatant was adjusted to pH 4.5 with 2 mol/L HC1, kept for 0.5 h at room temperature, and then centrifuged at 3000 × g for 20 min. The method used to extract walnut protein was according to the properties of the protein [18]. The precipitate was washed twice with water to remove

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soluble residues. Following this, water was added, and the pH of the solution was adjusted to 6.5 to obtain WPIs. Subsequently, the solutions were denatured for 10 min in boiling water. Neutral protease was added (protein content = 20 g/L, E/S = 5:100; w/w) for enzymatic hydrolysis, and the solution was incubated at 50 °C for 60 min. Following the hydrolysis, the solutions were heated in boiling water for 5 min to deactivate the protease, cooled to room temperature, and then centrifuged at 3000 × g for 20 min. Finally, the supernatants were lyophilized and collected as WPs.

Nickel-IDA-Sepharose 6B (25 mL) was loaded onto a column (26 × 100 mm) and eluted with 0.05 mol/L EDTA disodium salt (EDTA-2Na) for 3–4 bed volumes to remove nickel, and then 8–9 bed volumes of distilled water was used to remove EDTA-2Na. Then, the IDA-Sepharose 6B was incubated with 40 mL of 0.2 mol/L $FeCl_3$ solution for 0.5 h. The gel was then washed with 8–9 bed volumes of distilled water to remove the unbound iron from the column. Subsequently, 0.05 mol/L sodium acetic acid buffer (pH 4.0) was used to remove any loosely bound iron. The immobilized iron column was then washed with 5–6 bed volumes of equilibrating buffer (0.05 mol/L sodium acetate-acetic acid, 0.1 mol/L NaCl, pH 5.5). Subsequently, 20 mg of WP was loaded onto the IMAC-Fe^{3+} column and washed with the equilibrating buffer to remove any unbound peptides. The bound peptides were then eluted with 0.01 and 0.02 mol/L Na_2HPO_4 separately. The peptide fraction was collected and lyophilized from the eluate obtained with 0.02 mol/L Na_2HPO_4 and called iron-chelating WPs (ICWPs).

2.3. Molecular weight of ICWP by size-exclusion HPLC

The molecular weights of the WPs were evaluated according to the procedure described by Lv [19].

2.4. Separation of ICWP with RP-HPLC

The ICWPs were applied to RP-HPLC on a peptide Zorbax SB-C18 column (9.4 × 150 mm, 5 μm) (Agilent Technologies). The column was equilibrated 0.1% trifluoroacetic acid (Sigma) in water at a flow rate of 1 mL/min. A linear gradient from 0% to 60% acetonitrile (ACN, Fisher) in 60 min was applied. The elution was monitored at 214 nm. The main peaks were collected and lyophilized.

2.5. The characteristics of ICWPs by MALDI TOF/TOF MS and nanoLC-ESI–MS/MS

The WPs (F_{32min}) obtained from RP-HPLC were dissolved in 30 μL of MilliQ deionized water or 50 mM EDTA-2Na solution. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF/TOF MS) was used to measure the molecular weight of the F_{32min} samples, and nanoLC–MS/MS was used to analyze the properties of the samples with or without EDTA-2Na treatment.

2.5.1. MALDI TOF/TOF MS

Peptide mass finger printings were collected by MALDI TOF/TOF tandem mass spectrometer ABI 4700 (Applied Biosystems, USA) equipped with a 337-nm nitrogen laser and operating in the reflectron mode for positive ions with an acceleration voltage of 20 kV, accumulating 2000 laser shots per spot at a 200-Hz laser frequency; the scanning range was set from *m/z* 450 to *m/z* 5000. Pulsed ion extraction was set to 330 ns. Calibration was performed using the Bruker peptide calibration standard. α-Cyano-4-hydroxycinnamic acid (Fluka) was used as the matrix. The *m/z*-intensity peaklist files with S/N > 10 and without smooth processing were created by the software DE 4.7 (ABSciex).

2.5.2. NanoLC-ESI–MS/MS

The WP (with or without EDTA-2Na) solutions were diluted with 0.2% formic acid (FA) at a 1:1 ratio and centrifuged at 20,000*g* for 30 min. Samples were analyzed by nanoLC-ESI–MS/MS using an Ultimate 3000 nano-HPLC (Dionex) coupled to an ESI-QTOF-MS (microTOF-Q II, Bruker). The separation was performed using a 75 μm × 15 cm nanoscale C18 column (Dionex) filled with beads of 3 μm particle size and 100 Å pore size; in addition, a 300 μm × 5 mm C18 trap column (Dionex) with beads of 5 μm particle size and 100 Å pore size was used. Samples were run at a flow rate of 350 nL/min with an injection volume of 10 μL. The mobile phases consisted of 0.1% FA (A) and ACN in 0.1% FA (B). A three-step linear gradient of 2% B from 0 to 5 min, 2–40% B from 5 to 45 min, and 40–80% B from 45 to 55 min was used throughout this study. The scan range was *m/z* 50–2500 for MS and MS/MS, and the scan speed was 0.5 s. The base peak chromatography (BPC) and extracted ion chromatography (EIC) of nanoLC–MS/MS were displayed by the software DA 4.0 (Bruker). The *m/z*-intensity peaklist files with S/N > 30 and without smooth processing were created for comparison between MALDI-TOF/TOF and ESI–MS peaks.

2.5.3. Database search and Manual de novo sequencing of WPs

The nanoLC-ESI tandem mass spectrum data were submitted using Mascot Daemon to our in-house Mascot server for searching in-house custom-designed databases. The Mascot generic format (mgf) files for database search were created from nanoLC–MS/MS raw data files with the intensity > 10^4 of parent ions and 10^3 of fragmentation ions. The database search was carried out using Mascot 2.1 with the following parameters: Swissprot protein sequence database, taxonomy is Green Plant (12179 sequences), mass error 0.3 Da for MS and 0.6 Da for MS/MS, variable modifications set to Oxidation (M) and Deamidation (NQ), enzyme is None. Peptide sequence analysis was carried out by BLAST on line (http://www.ncbi.nlm.nih.gov/BLAST) with the NCBInr protein sequence database. Peptide *De Novo* sequence analysis was carried out by Biotools v3.0 (Bruker). The MS-BLAST search results were considered significant if the resulting sequence coverage and identity were higher than 90%.

3. Results and discussion

3.1. Separation of WPs with RP-HPLC

WPs were first separated by iron-immobilized affinity column. The peptides that bound to the iron column were eluted with 0.02 mol/L of Na_2PO_4. Subsequently, these ICWP were analyzed by size-exclusion (SE)-HPLC and RP-HPLC. Fig. 1 (*inset*) shows the molecular weight (MW) distribution of the peptides. Most of the component are peptides with MW less than 3 kDa. Subsequently, the ICWPs were loaded onto RP-HPLC, and the profiles are presented in Fig. 1. Approximately 8–10 fractions were obtained; the main peak (F_{32min}) was collected, analyzed, and identified by MALDI TOF/TOF MS and nanoLC-ESI–MS/MS.

3.2. Characteristics of the iron-chelated peptides in F_{32min}

The F_{32min} sample was dissolved in deionized water and analyzed by MALDI TOF/TOF MS. The ion peak correction profile is presented in Fig. 2. Approximately 199 mass peaks were detected (signal to noise ratio, S/N ≥ 15). The main peptide components showed molecular weight between *m/z* 550 and *m/z* 2250, which is consistent with the results obtained with SE-HPLC (Fig. 1 *inset*).

Subsequently, the F_{32min} sample with or without EDTA-2Na was investigated by nanoLC–MS/MS. The base peak chromatogram (BPC) of the two samples is presented in Fig. 3. When the peak intensity and retention time of the two profiles were compared, obvious differences were observed in fraction T_1-T_4, which is illustrated in Table 1. The peak area ratios (before and after EDTA-2Na treatment) of T_1 and T_2 were 1/3.16 and 1/2.92, respectively. This indicates that both the peptides' amounts increased at the two retention times after EDTA-2Na