Journal of Proteomics 142 (2016) 62–69

Contents lists available at ScienceDirect

Journal of Proteomics

journal homepage: www.elsevier.com/locate/jprot

# Mass spectrometric analysis of allergens in roasted walnuts

Melanie L. Downs [a,b,*], Joseph L. Baumert [a], Steve L. Taylor [a], E.N.C. Mills [b]

[a] Food Allergy Research and Resource Program, Department of Food Science and Technology, University of Nebraska-Lincoln, Lincoln, NE, USA
[b] Centre for Respiratory Medicine and Allergy, Respiratory Institute of Inflammation and Repair, Manchester Academic Health Sciences Centre and Manchester Institute of Biotechnology, University of Manchester, Manchester, UK

ARTICLE INFO

*Article history:*
Received 8 December 2015
Received in revised form 30 March 2016
Accepted 29 April 2016
Available online 3 May 2016

*Keywords:*
Food allergy
Label-free quantification
Protein mass spectrometry
Thermal processing
Tree nut
Walnut

ABSTRACT

Thermal processing of allergenic foods can induce changes in the foods' constituent allergens, but the effects of heat treatment are poorly defined. Like other commonly allergenic tree nuts, walnuts often undergo heat treatment (e.g. roasting or baking) prior to consumption. This study evaluated the changes in solubility and detectability of allergens from roasted walnuts using tandem mass spectrometry methods. Walnuts were roasted (132 °C or 180 °C for 5, 10, or 20 min) and prepared for LC-MS/MS using sequential or simultaneous extraction and tryptic digestion protocols. The LC-MS/MS data analysis incorporated label-free quantification of relevant allergens and Maillard adduct screening. In some proteins (2S albumin, LTP, and the 7S globulin N-terminal region) minor changes in relative abundance were observed following roasting. The mature 7S and 11S globulins, however, showed significantly increased detection following roasting at 180 °C for 20 min when using the simultaneous extraction/digestion protocol, possibly due to increased digestibility of the proteins. The results of this study indicate that individual walnut allergens respond differently to thermal processing, and the detection of these proteins by LC-MS/MS is dependent on the protein in question, its susceptibility to proteolytic digestion, the degree of thermal processing, and the sample preparation methodology.

*Significance:* Understanding the behavior of food allergens in the context of relevant food matrices is critical for both food allergen management and for elucidating matrix and processing-associated factors influencing protein allergenicity. The use of mass spectrometry to identify food allergens and detect allergenic food residues has been increasingly developed due to the advantages associated with the direct, sequence-level analysis possible with MS. To date, however, few studies have implemented MS technology to analyze the effects of thermal processing on allergenic food proteins. The MS analysis results presented in this study revealed not only information about the molecular-level effects of roasting on walnut allergens but also data pertinent to the development of MS-based detection methods for walnut residues in food products.



## 1. Introduction

Thermal processing operations used to achieve safe and palatable food products can have a multitude of physical and chemical effects on proteins in foods, including allergens. Physical changes due to heating (protein denaturation and aggregation) can affect structural characteristics of food proteins, potentially altering their allergenic activity [1,2]. These physical changes can also affect the detection of food allergens by impacting protein solubility [3,4].

In addition to physical changes, chemical reactions occurring during thermal processing can also affect food allergens. One of the dominant chemical reactions occurring during heat treatment of foods is the Maillard reaction, a series of reactions involving food proteins and reducing sugars. The initial phase of the Maillard reaction is a condensation reaction between a reducing sugar and an amine group, typically lysine or arginine residue side chains. Following this condensation, a cascade of reactions produces a multitude of intermediates and final products known as advanced glycation end products (AGEs) [5–8]. Many of these AGEs have the desirable brown color and roasted flavor compounds commonly associated with thermal processing. It has been suggested that Maillard reactions involving some food allergens impact their allergenic activity [9–11]. While it is assumed that food allergens participate in Maillard-type reactions during thermal processing, as they often represent the most abundant proteins in a food, very little is known about the specific reactions that take place in a complete food system.

Unlike many traditional protein chemistry techniques, mass spectrometry, specifically LC-MS/MS, has the desirable ability to function at the protein sequence level. Depending on the approach, LC-MS/MS has the ability to assess changes in individual proteins or individual amino acid residues in a protein sequence. LC-MS/MS methods are also able to provide relative or absolute quantitative data, which is particularly valuable in food protein analysis, given the issue of non-

*Abbreviations:* DWNF, defatted walnut flour.

* Corresponding author at: 262 Food Innovation Center, 1901 North 21st Street, University of Nebraska, Lincoln, NE 68588-6205, USA.
*E-mail address:* mdowns2@unl.edu (M.L. Downs).

http://dx.doi.org/10.1016/j.jprot.2016.04.045
1874-3919/

uniform heat transfer in most food processing systems. In initial investigations such as this one, the primary interest is in relative changes to key allergenic proteins as a result of thermal processing. The types of specific effects on food allergens and which sites they impact are largely unknown, making a non-targeted method for relative quantitative analysis ideal. As such, a label-free quantification method was used in conjunction with LC-MS/MS to give the desired flexibility for such a dynamic system.

Walnuts, specifically English walnuts (*Juglans regia*), are among the most commonly allergenic tree nuts and frequently undergo some form of thermal processing prior to consumption [12,13]. To date, four allergens have been identified from *J. regia*: Jug r 1, a 2S albumin; Jug r 2, a 7S vicilin-like globulin; Jug r 3, a non-specific lipid transfer protein (nsLTP) and Jug r 4, an 11S legumin-like globulin [14–17]. Very little is known, however, about how individual walnut proteins are affected by heating in the context of a food system. The objective of this study was to use label-free LC-MS/MS analysis to evaluate changes in the solubility and detectability of allergens from roasted walnuts. Mass spectrometry was also used to screen for the presence of Maillard-type adducts on the walnut allergens.

## 2. Materials and methods

### 2.1. Walnut sourcing and preparation

Walnut sourcing, roasting, and defatted walnut flour (DWNF) production were performed as described previously [18]. Briefly, raw English walnuts (*J. regia* cv. Chandler; Gold River Orchards, Oakdale, CA) were dry roasted at 132 °C or 180 °C for 5, 10, or 20 min. In addition, an unheated sample of walnuts was also retained. After allowing the walnuts to cool to room temperature, the nuts were frozen until used for the production of hexane-defatted walnut flour, as described previously [18]. Briefly, the frozen walnuts were ground and subsequently defatted with hexane at a 1:5 (v:v) ratio at room temperature for 1 h, with constant stirring. Following removal of the hexane phase, the partially defatted flour was allowed to dry overnight, and the grinding and defatting processes were repeated. The sugar content (mono and disaccharides) of the unheated walnuts was determined by HPLC (UKAS C-TM-004; Premier Analytical Services, High Wycombe, UK).

### 2.2. Extraction and in-solution trypsin digestion

Two different techniques were used for the in-solution digestion of walnut proteins: a traditional, sequential workflow of protein extraction from DWNF into aqueous buffer, followed by digestion of the soluble proteins with trypsin, and a simultaneous in-solution tryptic digestion performed directly on the DWNF samples. Our hypothesis was that the simultaneous protocol could serve as a complete extraction/digestion, and the sequential protocol would deliver information on soluble proteins only. The sequential method used a high salt buffer developed in previous experiments (25 mM Tris-HCl, pH 7.5, containing 1.5 M NaCl and 3.5% (w/v) PVPP) to conduct the protein extraction of the unheated and roasted DWNF samples at a 1:50 (w:v) ratio (20 mg DWNF in 1.0 mL buffer) for 1 h at room temperature followed by centrifugation (4000 × *g*, 30 min). A subsample (25 μL) of the supernatant was combined with an equal volume (25 μL) of 0.2% (w/v) acid labile surfactant (*Rapi*Gest™ SF; Waters Corporation, Manchester, UK) in 50 mM ammonium bicarbonate to achieve a final concentration of 0.1% (w/v) surfactant. For the simultaneous protocol, DWNF (1.0 mg) was directly combined with 50 μL of 0.1% (w/v) acid-labile surfactant in 50 mM ammonium bicarbonate.

Following addition of the surfactant solutions, both types of extraction samples were reduced in 5 mM DL-dithiothreitol (DTT) at 60 °C for 30 min, by adding 50 mM DTT (40.5 and 81 μL, to sequential and simultaneous samples, respectively) and 50 mM ammonium bicarbonate (314.5 and 679 μL to sequential and simultaneous samples, respectively). Samples were subsequently alkylated in 15 mM iodoacetamide at room temperature for 30 min in the dark, by adding 45 and 90 μL of 150 mM iodoacetamide to the sequential and simultaneous samples, respectively. The sample proteins were digested with trypsin (proteomics grade, dimethylated trypsin from porcine pancreas, Sigma-Aldrich, St. Louis, MO) at a 1:100 (w:w) trypsin to protein ratio at 37 °C for 3 h (addition of 25 or 50 μL of 0.1 μg/μL trypsin solution to sequential or simultaneous samples, respectively), followed by digestion at a 1:50 (w:w) ratio at 31 °C overnight (by addition of the same volumes of trypsin working solution as before).

Digestion was stopped and the acid-labile surfactant was hydrolyzed by combining equal volumes of digest and 1.0% (v/v) trifluoroacetic acid. The digests were heated at 37 °C for 45 min and centrifuged at 14,000 × *g* for 10 min, as directed by the surfactant manufacturer. The supernatant was reserved and cleaned-up with commercial C18 spin columns (Pierce™, Rockford, IL). Following the elution of peptides from the spin columns, the peptide solutions were dried in a vacuum centrifuge and resuspended in 0.1% (v/v) formic acid (FA) prior to LC-MS/MS.

### 2.3. Mass spectrometry

LC-MS/MS was conducted with an UltiMate® 3000 Rapid Separation LC (RSLC, Dionex Corporation, Sunnyvale, CA), connected in-line to an Orbitrap Elite (Thermo Fisher Scientific, Waltham, MA) mass spectrometer. The liquid chromatography consisted of UPLC with a 75 mm × 250 μm i.d. 1.7 μM BEH C18 analytical column (Waters Corporation; Elstree, UK) and peptide separation over a gradient of 92% solution A (0.1% (v/v) FA in water) and 8% solution B (0.1% (v/v) FA in acetonitrile) to 33% B, in 44 min at 300 nL $min^{-1}$. Data-dependent acquisition mode and dynamic exclusion were enabled on the Orbitrap Elite. Precursor ions were analyzed in the Orbitrap with normal mass range (*m/z* 350–1500) and 120,000 resolution. CID was performed on the top six precursor ions, and the product ions were analyzed in the ion trap under normal mass range and rapid scan rate conditions.

### 2.4. Label-free quantification and data analysis

Label-free quantification analysis of the Orbitrap data was conducted with the Progenesis LC-MS software package (Nonlinear Dynamics, Newcastle upon Tyne, UK). Automatic alignment of all sample runs was performed, with no data refinement. Default peak picking was performed on all runs, and all features were retained for quantification. Peptide and protein identification was performed with the compiled mgf file (generated by Progenesis) and a local Mascot server (version 2.2.06). Due to the very small number of walnut protein sequences available (74 total *J. regia* sequences in UniProt at the time of analysis), the search was performed against a custom, non-redundant protein database, which consisted of the following: all of the protein entries from the AllergenOnline database, version 12 (Food Allergy Research & Resource Program, www.allergenonline.org), all of the proteins from the Juglandaceae family-level taxonomy in UniProt, all of the proteins from the peanut (*Arachis hypogaea*) species-level taxonomy in UniProt, and a standard list of contaminants (e.g. keratin). The Mascot search parameters allowed for one missed tryptic cleavage and included carbamidomethyl (C) and oxidation (M) as fixed and variable modifications, respectively. The peptide tolerance was set to 5 ppm and the MS/MS tolerance to 0.6 Da. Only peptides with charges of +2 or +3 were included in the search.

Following feature identification, protein quantification was conducted in Progenesis based on all peptides assigned to each protein of interest. The software can also perform quantification based on unique peptides only, but the database used included a substantial number of proteins with very similar (up to 97% identical) sequences originating from other related species (e.g. pecan and black walnut). For the purposes of quantification in this experiment, these conflicts perceived by