LWT - Food Science and Technology 59 (2014) 376–382

Contents lists available at ScienceDirect

LWT - Food Science and Technology

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

Contribution of peptides and polyphenols from olive water to acrylamide formation in sterilized table olives

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ARTICLE INFO

Article history:
Received 13 May 2013
Received in revised form
28 February 2014
Accepted 25 April 2014
Available online 4 May 2014

Keywords:
Acrylamide
Olives
Peptides
Fractionation
Peptide–polyphenol complexes

ABSTRACT

To confirm the role of peptides as principal precursors of acrylamide formation in sterilized table olives, peptides from olive water were fractionated. After their partial fractionation by solid phase extraction (SPE) and ultrafiltration (<10,000 Da), respectively, small peptides from olive water were isolated by size-exclusion chromatography (SEC). In the fractions collected, peptides and polyphenolic compounds were determined colorimetrically, and acrylamide was quantitated by LC–MS/MS after heating of the samples. Subsequently, peptides were characterized by matrix-assisted laser desorption/ionization-tandem time of flight mass spectrometry (MALDI-TOF/TOF-MS), and polyphenols were analyzed by LC–MS in the respective fractions. Finally, peptides containing fractions were purified on a polymeric resin (Amberlite XAD 16HP) to remove unbound phenolic compounds by adsorption. The results of the different experiments performed in complete absence of free asparagine and reducing sugars strongly support small peptides bound to polyphenols to be the principal precursors of acrylamide in sterilized table olives.

1. Introduction

Acrylamide, a chemical compound classified as “probably carcinogenic to humans” by the International Agency for Research on Cancer (IARC, 1994), was first detected in heated carbohydrates-rich foods in 2002 (Tareke, Rydberg, Karlsson, Eriksson, & Tornqvist, 2002). The Maillard reaction between free asparagine (Asn) and reducing sugars and further carbonyl sources, respectively, has been confirmed as the major pathway of acrylamide formation in foodstuffs (Gökmen & Palakzaglu, 2008; Mottram, Wedzicha, & Dodson, 2002; Stadler et al., 2002). In general, the highest levels of acrylamide have been found in carbohydrate-rich foods from potato, wheat and other cereals, and coffee (Friedman, 2003).

High acrylamide levels (from 200 to 2000 μg/kg) have been detected in black ripe olives, one of the main types of table olive commercialized worldwide in which sterilization treatment is compulsory (Casado & Montaño, 2008; FDA, 2006). Nevertheless, it has been demonstrated that acrylamide formation in sterilized olives follows a different pathway, since acrylamide levels of olives did not correlate with the contents of free Asn and any of the reducing sugars determined prior to sterilization (Amrein, Andres, Escher & Amadò, 2007; Casado & Montaño, 2008). Compared with potato and cereal products, little information is available on acrylamide in sterilized black ripe and green ripe olives. Up to now, the mechanism of acrylamide formation in olives is still unknown.

A detailed study dealing with heated model peptides and olive water fractions has been reported previously (Casado, Montaño, Spitzner, & Carle, 2013). According to our findings, the role of free Asn and glucose, being the main reducing sugar in olives, as acrylamide precursors in these fruits has been ruled out, suggesting peptides smaller than 10,000 Da to be the principal precursors of acrylamide formation in heated table olives. In the absence of free Asn, only fractions containing peptides/proteins obtained by partial fractionation of olive water by solid phase extraction (SPE) and precipitation with cold acetone, respectively, generated significant amounts of acrylamide upon heating at usual sterilization conditions (121 °C for 30 min).

The aim of the present work was to fractionate and isolate peptides from olive water to confirm their putative role as precursors of acrylamide in sterilized olives. Due to the complex fruit

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http://dx.doi.org/10.1016/j.lwt.2014.04.050
0023-6438/

matrix, isolation of peptides was to be expected very challenging, considering the multiple interactions of peptides or proteins with phenolic compounds. Consequently, olive water from untreated green olives was subjected to size-exclusion chromatography (SEC) after partial fractionation by SPE and ultrafiltration. Peptides and polyphenolic compounds of the fractions collected were measured colorimetrically. For the quantitation of acrylamide in the heated samples, liquid chromatography–tandem mass spectrometry (LC–MS/MS) was applied. Subsequently, peptides were further characterized by matrix-assisted laser desorption/ionization-tandem time of flight mass spectrometry (MALDI-TOF/TOF-MS), and polyphenols were determined by liquid chromatography–mass spectrometry (LC–MS) in the respective fractions. Finally, phenolic compounds were removed from the peptides containing fractions using a polymeric resin to elucidate the mechanism of acrylamide formation in table olives.

2. Materials and methods

2.1. Chemical and materials

Acrylamide (99%) was purchased from ICN Biomedicals (Eschwege, Germany), 2,3,3-D_3-labeled acrylamide (98%) was from Cambridge Isotope Laboratories (Andover, MA, USA). Formic acid and acetonitrile (ACN), both gradient grade, ammonium acetate (99%), ethanol (98%), acetone, ethyl acetate, methanol, hydrochloric acid, potassium hexacyanoferrate (Carrez I) and zinc acetate (Carrez II) were provided by VWR (Darmstadt, Germany). Glacial acetic acid and trifluoroacetic acid (TFA) were from Merck (Darmstadt, Germany). 2,5-dihydroxybenzoic acid (DHB) and α-Cyano-4-hydroxycinnamic acid (HCCA), were obtained from Bruker Daltonics (Bremen, Germany). Deionized water (Sartorius Arium 611 Ultrapure water system) was used throughout. Solid phase extraction cartridges (Isolute Multimode, 1000 mg) were obtained from IST (Hengoed, Mid Glamorgan, UK). Silica-based bonded-phase cartridges (Sep Pak Vac 20 cc/5 g C18) were purchased from Waters (Milford, MA, USA). Chem Elut cartridges for solid phase supported liquid–liquid extraction and Hydromatrix diatomaceous earth were from Varian (Darmstadt, Germany).

2.2. Olive water extraction

Green olives (5 kg) of cv. 'Hojiblanca' (Seville, Spain) from a local processor were used. Olives were pitted and homogenized using a mixer. The olive water was obtained by pressing the mash using an hydraulic laboratory press (Hafico, HP-2, Düsseldorf, Germany) and twofold centrifugation at 20,000 *g* for 20 min at 20 °C to remove the olive oil. The olive water was stored at −20 °C until the different experiments were performed. After extraction, the pH of the olive water was 4.4.

2.3. Partial fractionation of olive water

Peptides from olive water were fractionated according to the scheme showed in Fig. 1. To the partial fractionation of olive water by SPE, Sep Pak Vac C18 cartridges were conditioned with methanol and water. After pH adjustment to 3.0 using HCl, aliquots (10 mL) of olive water were filtered through a 0.45 μm syringe filter, loaded, and slowly (1 mL/min) passed through the cartridges. Two different fractions were recovered: (1) fraction A, an aqueous fraction consisting of the eluate, and the retentate obtained after elution from the sorbent with 15 mL of water; and (2) fraction B, comprising the less polar fraction eluted from the sorbent with 15 mL of methanol. Whereas fraction A was discarded, fraction B was evaporated to dryness in a rotary evaporator, resuspended in 10 mL of water, and the pH was adjusted to the initial value (≈4.4). Fraction B was used for the fractionation of peptides by SEC after ultrafiltration.

2.4. Ultrafiltration

Fraction B (see Section 2.3) was re-circulated through a stirred ultrafiltration cell model 8003 containing a cellulose membrane with a molecular weight cut-off of M_r 10,000 (Millipore, Beverly, MA, USA). Aliquots of the retentate (fraction C) and permeate (fraction D), resuspended with water, were adjusted to pH 4.4. Fraction D was used for the fractionation of peptides by SEC.

2.5. Fractionation of peptides by size-exclusion chromatography

A 5 mL of fraction D (permeate) was concentrated to 1 mL under reduced pressure. The concentrate was applied to a glass column (Superformance, 600 × 26 mm, Merck, Darmstadt, Germany) filled with Toyopearl HW-55F (Tosoh, Stuttgart, Germany). Elution was performed at room temperature using 0.3 mol/L ammonium acetate buffer, pH 4.0. The flow-rate of 2 mL/min was produced using a HPLC compact pump (Bischoff, Leonberg, Germany). The absorbance at 280 nm was measured with a SPD-10AV UV/Vis detector (Shimadzu, Duisburg, Germany). The eluate was collected in 40 mL fractions. The fractionation was performed in sextuplicate. Appropriate fractions collected after six runs were pooled, freeze-dried, and resuspended in water. The pH of the fractions ranged from 5.7 to 6.0.

2.6. Adsorptive removal of phenolic compounds from olive water fractions using polymeric resin

Phenolic compounds from appropriate olive water fractions were removed by adsorption with a polymeric resin, Amberlite XAD 16 HP (Rohm and Haas, Philadelphia, PA, USA), following a method developed by Weisz, Schneider, Schweiggert, Kammerer, and Carle (2010) with several modifications.

The resin (15 g) was activated overnight by soaking in 96% EtOH (5 mL/g). The same volume of deionized water at 50 °C was used to remove the alcohol from the resin beads prior to the experiments. Subsequently, the adsorbent material was conditioned by purging with 3 mL/g of NaCl solution (1.3 mol/L, pH 6.0).

For each experiment, 15 mL olive water fraction adjusted to pH 6.0 was combined with 1 g of pre-treated resin in a 50 mL screw cap flasks. To prevent polyphenol oxidation, the flask was flushed with nitrogen. The suspension was stirred using a magnetic stirrer at 150 rpm in a thermostatted water bath at 20 °C during 120 min. After separation of the solution (fraction E), MeOH (20 mL) was added to the colored resin and the suspension was stirred at room temperature for 30 min to elute the retained compounds. Subsequently, the colored solution was evaporated to dryness in a rotary evaporator (30 °C, 40 mbar), and resuspended in 15 mL of water (fraction F). The pH of fraction F was adjusted to 6.0.

2.7. Heat treatment of model systems

Heat treatments were performed using 5 mL of the corresponding sample (aliquots of fraction B after ultrafiltration, SEC or adsorption treatment with resin) filled in a stainless tubular steel reactor (internal diameter 1 cm, length 8.5 cm). The reactor was sealed, and then heated in a thermostatted oil bath at 121 °C (±1 °C) for 30 min in all experiments to mimic sterilization conditions. After heating, the sample was immediately cooled in ice water for 3 min to stop the reaction. All heating experiments were performed in triplicate.