Food Control 72 (2017) 14–19

Contents lists available at ScienceDirect

Food Control

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

Enzyme-assisted extraction of proteins from *Citrus* fruits and prediction of their cultivar using protein profiles obtained by capillary gel electrophoresis

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ARTICLE INFO

Article history:
Received 28 April 2016
Received in revised form
18 July 2016
Accepted 20 July 2016
Available online 25 July 2016

Keywords:
Citrus
Cultivar
Protein profile
Capillary gel electrophoresis
Enzyme-assisted extraction

ABSTRACT

The suitability of protein profiles established by capillary gel electrophoresis (CGE) as a tool to discriminate between 11 cultivars of *Citrus* (orange and tangerine) peel and pulp was evaluated in this work. Before CGE analysis, different extraction buffers (which included enzyme-assisted treatments) were compared. The best results were achieved using 5% (*v/v*) Celluclast® 1.5 L and 5% (*v/v*) Palatase® 20,000 L buffers for *Citrus* peel and pulp protein extracts, respectively. The resulting protein profiles obtained were used to construct linear discriminant analysis (LDA) models able to distinguish *Citrus* peel and pulp samples according to their cultivar. In both cases, all samples were correctly classified with an excellent resolution among all categories, which demonstrated that protein patterns are a powerful tool to discriminate *Citrus* samples coming from different cultivars.

1. Introduction

Orange (*Citrus sinensis*) and tangerine (*Citrus tangerine*) fruits and juices are frequently consumed in Europe and North America. The total annual *Citrus* production was estimated at over 145 million tons in 2013 (FAO, 2010). Within *Citrus* fruit production, orange juice processing constitutes the largest volume percentage (75%) of the world marker (FAO, 2010). In this sense, it is of prime importance to the *Citrus* industry to maintain and improve product quality, to remain competitive and to fulfill consumer demands (Klavons, Bennett, & Vannier, 1991).

Among the different compounds present in *Citrus* fruits, flavonoids (Kawaii, Tomono, Katase, Ogawua, & Yano, 1999), carotenoids (Goodner, Rouseff, & Hofsommer, 2001;; Moulya, Gaydoub, & Corsettia, 1999) and phenolic compounds (Cieslik, Greda, & Adamus, 2006; Khan, Abert-Vian, Fabiano-Tixier, Dangles, & Chemat, 2010) have been largely described in comparison to other components, such as proteins, that have been scarcely studied. This could be due to the fact that the extraction of fruit proteins is difficult due to the low solubility of these biomacromolecules. This fact is due to the complex formed between protein and pectin compounds (Klavons et al., 1991). In this regard, different extraction protocols have been described for the extraction of proteins from *Citrus* tissues (Saravanan & Rose, 2004; Zukas & Breksa, 2005). Saravanan and Rose (2004) compared two protein extraction methods (trichloroacetic acid and phenol extraction protocols) to obtain a high number of protein spots from 2D electrophoresis gel of several tissues (tomato, avocado, banana and orange peel). The best results were obtained using the phenol extraction protocol, which provided the greatest protein yield (2.08 mg/g fresh weight). On the other hand, Zukas and Breksa (2005) compared three extraction protocols (Tris–HCl, KCl and phenol) followed by precipitation to purify and concentrate proteins from *Citrus* leaves coming from 6 different varieties (Sour and Navel orange, mandarin, citron and lemon). In this case, the best results were obtained using the Tris-HCl extraction protocol. However, these latter extraction protocols employed several cleanup steps, which are laborious, time-consuming and in some cases non-environmentally friendly protocols.

Enzyme-assisted protein extraction constitutes an alternative method due to its mild extraction conditions and lower environmental impact (Sari, Bruins, & Sanders, 2013; Shen, Wang, Wang,

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http://dx.doi.org/10.1016/j.foodcont.2016.07.025
0956-7135/

Wua, & Chen, 2008; Vergara-Barberán, Lerma-García, Herrero-Martínez, & Simó-Alfonso, 2014a; Vergara-Barberán, Lerma-García, Herrero-Martínez, & Simó-Alfonso, 2015). Thus, several specific enzymes have been employed in protein extraction in tea leaves (Shen et al., 2008) in Leguminosae gums (Sebastián-Francisco, Simó-Alfonso, Mongay-Fernández, & Ramis-Ramos, 2004), in different oilseed meals (Sari et al., 2013) and in olive fruit and leaves (Vergara-Barberán et al., 2014a, 2015), providing improved protein extraction yields compared to alkaline or acidic treatments. However, to our knowledge, this methodology has not been applied to *Citrus* fruit protein extraction.

In previous works, it has been demonstrated that protein profile is a good marker of sample origin (Lliso, Tadeo, Phinney, Wilkerson, & Talón, 2007; Montealegre, Marina, & Ruiz, 2010; Vergara-Barberán, Lerma-García, Herrero-Martínez, & Simó-Alfonso, 2014b; Vergara-Barberán, Lerma-García, Herrero-Martínez, & Simó-Alfonso, 2014c; Wang et al., 2014).

As an example, the protein profiles established by both capillary gel electrophoresis (GCE) (Vergara-Barberán et al., 2014b) and by capillary zone electrophoresis (Vergara-Barberán et al., 2014c), followed by linear discriminant analysis (LDA), have been used to classify olive leaves and pulps according to their cultivar. On the other hand, Montealegre et al. (2010) have used sodium dodecyl sulfate (SDS)-CGE to differentiate proteins from raw and table olive samples from some cultivars. Moreover, Wang et al. (2014) studied different protein profiles of peanut cultivars using SDS- polyacrylamide gel electrophoresis (PAGE) followed by LDA, finding that the different varieties could be grouped according to their storage proteins.

In this work, different enzyme-assisted extraction protocols have been studied in order to obtain a satisfactory protein yield. To monitor the extraction, the total protein content was measured using the standard Bradford assay. Next, protein extracts were analyzed by CGE, and the resulting protein profiles were used to develop LDA models able to distinguish *Citrus* peel and pulp samples according to their cultivar.

2. Materials and methods

2.1. Chemicals

Tris (hydroxymethyl)-aminomethane (Tris), SDS, sodium chloride (NaCl), 3-[3-cholamidopropyl dimethylammonio]-1-propanesulfonate (CHAPS) and bovine serum albumin (BSA) were from Sigma-Aldrich (St. Louis, MO, USA). 2-Mercaptoethanol, sodium hydroxide (NaOH) pellets and hydrochloric acid (HCl) were purchased from Merck (Darmstadt, Germany). Molecular mass standard mixture solution (eight proteins comprised between 6.5 and 66 kDa) provided by Sigma-Aldrich was used. In order to perform Bradford protein assay, a Protein Quantification Kit-Rapid from Fluka (Steinheim, Germany) was employed. Different enzymes, which were kindly provided by Novozymes (Bagsvaerd, Denmark) were used: lipase (Palatase® 20,000 L; activity 2000 LU-MM/g (LU = lipase unit); working conditions, pH 7 and 25–45 °C) and cellulase (Celluclast® 1.5 L; activity 1500 NCU/g (NCU = novo cellulose unit); working conditions, pH 4.5–6.0 and 50–60 °C). SDS-MW gel buffer (pH 8, 0.2% SDS) (Beckman Coulter, Inc., Fullerton, CA) was utilized for CGE analysis. Deionized water (Barnstead deionizer, Sybron, Boston, MA) was also used.

2.2. Instrumentation

In order to quantify proteins using Bradford assay, UV–vis data were recorded at 595 nm using a diode array UV–visible spectrophotometer from Agilent Technologies (Waldbronn, Germany) equipped with a 1-cm optical path quartz cell from Hellma (Müllheim, Germany).

Protein separations by CGE were performed using an HP^{3D} CE system from Agilent, which is equipped with a diode-array spectrophotometric detector. Bare fused-silica capillaries of 33.5 cm (25 cm effective length) and 375 μm od × 50 μm id (Polymicro Technologies, Phoenix, AZ, USA) were used. These capillaries were initially activated with 1 and 0.1 M NaOH and water at 60 °C for 10 min each before its first use.

Every day, the capillary was sequentially conditioned with 0.1 M NaOH for 5 min, 0.1 M HCl and water for 2 min each, and SDS-MW gel buffer for 10 min before use. The protein samples were injected at −30 kV × 7 s. Separations were performed at −15 kV at 25 °C. UV detection was done at 214 nm.

2.3. Citrus fruits and protein extraction

Citrus fruit samples (both oranges and tangerines) coming from 11 different cultivars were employed in this study (see Table 1). In all cases, the cultivar origin of samples was guaranteed by the supplier (Fontestad S.A., Museros, Valencia, Spain).

Both, *Citrus* peel and pulp were subjected to protein extraction. For this purpose, the surface of *Citrus* fruits was washed with lukewarm 1% SDS solution, in order to eliminate bacterial and surface contamination from human hands. Next, the thin flavedo peel was carefully excised and reduced to very small fragments via a treatment in a fruit blender for 10 min at maximum power. Regarding pulp, 200 g were first lyophilized and the resulting powder next homogenized before their use.

Three different extraction buffers were tested with both, *Citrus* peel and pulp. The native buffer contained 50 mM Tris–HCl (pH 7.2), 50 mM NaCl and 2% (*w/v*) CHAPS (buffer I) (Lerma-García, D'Amato, Simó-Alfonso, Righetti, & Fasoli, 2016), and buffer II and III containing 5% (*v/v*) Celluclast® 1.5 L and 5% (*v/v*) Palatase® 20,000 L, respectively. The extraction procedure was carried out as follows. 15 g of minced peel or 5 g of lyophilized pulp were mixed with 40 and 30 mL, respectively, of each extraction buffer, and mildly shaken for 8 h at different temperatures depending on the optimal working conditions of each buffer (Vergara-Barberán et al., 2014b) (buffer I, 25 °C; buffer II, 55 °C and buffer III, 30 °C). Next, the homogenates were centrifuged at 10,000 rpm for 10 min and the supernatants of protein extracts from *Citrus* peel and pulp were used for further analysis.

A calibration curve up to 1 mg mL^{-1} of BSA was prepared for Bradford assay (Bradford, 1976). The procedure indicated in the Bradford assay Protein Quantitation Kit-Rapid (Fluka) was followed in order to measure sample protein content. A sample blank containing the corresponding buffer was also made in order to remove its contribution to the final sample absorbance.

For CGE, a proper volume of protein extract was taken to provide a final concentration comprised between 0.2 and 2 mg mL^{-1}. Then, this aliquot was mixed with 100 μL of sample buffer (0.1 M Tris–HCl pH 9.0, 1% (w/v) SDS, and 1% (v/v) 2-mercaptoethanol). Next, this mixture was heated at 95 °C for 5 min and allowed to cool in a water bath for 5 min. Next, samples were analyzed per triplicate into the CE system. Method reproducibility was also evaluated by measuring the migration times of the three replicates of each sample.

2.4. Molecular masses determination and construction of LDA models

The molecular mass standard mixture solution was injected under the selected CGE conditions, and employed to determine the molecular masses of sample protein peaks. For this purpose, a