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Proteomic platform for the identification of proteins in olive (*Olea europaea*) pulp

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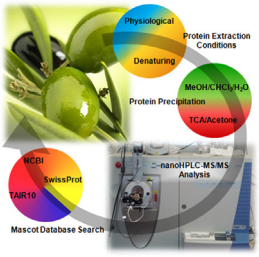
HIGHLIGHTS

- Gel-free proteomic study of pulp olive proteins.
- Two extraction protocols (denaturing and physiological conditions) based on different solubility properties.
- High resolution mass spectrometry and three different databases increase number of protein identification.

GRAPHICAL ABSTRACT



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ABSTRACT

The nutritional and cancer-protective properties of the oil extracted mechanically from the ripe fruits of *Olea europaea* trees are attracting constantly more attention worldwide. The preparation of high-quality protein samples from plant tissues for proteomic analysis poses many challenging problems. In this study we employed a proteomic platform based on two different extraction methods, SDS and CHAPS based protocols, followed by two precipitation protocols, TCA/acetone and MeOH precipitation, in order to increase the final number of identified proteins.

The use of advanced MS techniques in combination with the Swissprot and NCBI *Viridiplantae* databases and TAIR10 *Arabidopsis* database allowed us to identify 1265 proteins, of which 22 belong to *O. europaea*. The application of this proteomic platform for protein extraction and identification will be useful also for other proteomic studies on recalcitrant plant/fruit tissues.

1. Introduction

Olives (*Olea europaea*) are one of the most important crops in the Mediterranean area, both as the source of olive oil and as edible fruit. Several studies have focused on determining the main components in olives, such as fatty acids, polyphenols or sterols, and on this subject a great quantity of literature can be found [1–3]. On the other hand, although some authors have suggested that minor pulp components, such as proteins, play an important role in oil stability as well as having beneficial effects on human health [4,5], this line of research has been so far very limited. However, the characterization of olive proteins could lead to the discovery of new bioactive molecules, thus increasing the value of olive products. To date, only three studies have examined the olive proteome [6–8]. The most

Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; CMC, critical micelle concentration; DTT, dithiothreitol; FDR, false discovery rate; GO, gene ontology; PPO, polyphenol oxidase; RP, reversed-phase; RT, room temperature; RPK, receptor like protein kinase; SDS, sodium dodecyl sulphate; SPE, solid phase extraction; TCA, trichloroacetic acid.

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recent publication aimed at describing the olive pulp proteome exploited a new technology for protein extraction, the combinatorial (hexa)peptide ligand libraries. These affinity beads enable an increase in protein identifications, also for low abundance proteins, due to the capacity of equalizing protein concentration.

The proteomic analysis of olive pulp proteins poses two major difficulties: to begin with, because of the complexity of the matrices examined, it is difficult to obtain high quality protein extracts, and secondly, due to the lack of sequenced genomes, the database information available is limited.

The preparation of the samples is a critical step in proteomic research on plant tissues. The presence of interfering substances such as polysaccharides, lipids, phenolic compounds and secondary metabolites can impact on the protein separation and analysis. Moreover, the low concentration of soluble proteins and the abundance of proteases hinder proteomic analyses [9]. Finally, owing to the diversity of protein abundance, molecular weight, charge, isoelectric point, hydrophobicity, post-translational processing and modifications, and complexation with other molecules, no single extraction protocol is effective for all proteins.

For this reason a number of protein extraction protocols have been published [10,11]; those most often employed are the trichloroacetic acid (TCA) and acetone extraction and the phenol extraction. The first is based on protein denaturation under acidic and hydrophobic conditions, and has the advantage of assisting the concentration of proteins while removing contaminants; the second involves protein solubilization in the phenol phase, followed by precipitation with methanol and ammonium acetate and centrifugation. This method has been shown to generate high-quality protein extracts from a variety of plant species, but it is more time consuming, and it can be difficult to resolubilize the pellet.

Several strategies, based on biochemical, biophysical, or cellular properties, have been developed to improve the coverage and detection of specific groups of proteins, such as membrane proteins and low-abundant proteins. For example: phase partitioning by using organic solvents or Triton X-114 detergent [12,13], 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) [14,15], sodium dodecyl sulphate (SDS) [16], LC fractionation [17] and isolation of highly enriched organelles or subcellular compartments [18].

The elimination of interfering compounds after protein extraction involves the use of aqueous or phenol buffers for the extraction of proteins and their subsequent precipitation by acetone or methanol. Sometimes neither of these strategies is efficient enough, especially when the sample contains a great number of interfering compounds and a low percentage of proteins, as in olive pulp.

In studies on plant proteomics the use of model plant databases is an approach that is commonly employed. In traditional genetics Thale cress (*Arabidopsis thaliana*) has been used as a model because it is small and has a short life cycle. For this model organism the Arabidopsis Information Resource (TAIR) maintains a database of genetic and molecular biology data (www.arabidopsis.org, 2013). However, the main issue in proteomics for non-model plants is the limited availability of fully sequenced genomes. The number of plant species for which genomes are available has recently increased considerably, but in most cases they are incomplete and not fully annotated. Thus, in most cases protein identification for non-model species is performed by sequence homology to known plant genomes. Manually annotated databases, such as Swiss-Prot, are certainly more reliable, but the entries for plants other than Arabidopsis are very few and they are therefore of limited interest for proteomic searches [19]. On the other hand the NCBI database contains a very large number of protein sequences, but it is also highly redundant.

The main aim of this work was to establish an effective proteomic platform for the isolation of olive pulp proteins in order to provide an effective method for the identification of as many proteins present in olive pulp as possible. More specifically, we proposed two different extraction protocols for gel-free approaches, and two precipitation strategies were tested for each one. The extracted proteins were trypsin digested, and the resulting peptide mixture analyzed by reversed-phase (RP) nanoHPLC–tandem MS (MS/MS). Different protein databases, in particular *Viridiplantae* entries for SwissProt and NCBI, and TAIR10, were used for searching mass spectra and assigning peptides to proteins. Combining different approaches and different databases in each step of the analytical process might help to increase the final number of proteins identified.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals and reagents (β-mercaptoethanol, dithiothreitol (DTT), ammonium persulphate, urea, CHAPS, acetonitrile (ACN), TFA, SDS) were of analytical grade and were supplied by Sigma–Aldrich (Steinheim, Germany). Deionized water was prepared with an arium 611 VF system from Sartorius (Göttingen, Germany). Complete protease inhibitor cocktail tablets and sequencing grade trypsin were from Promega (Milano, Italy).

2.2. Samples

Olive fruits of the "Caninese" cultivar were collected in Northern Latium, Italy, during olive harvesting for oil production. An experimental design based on a complete sample pooling strategy was used for proteome analysis. Four trees growing in the same row were selected considering the similarity in size, number of branches, and high fruit yields. About 100 olives from the "Caninese" cultivar were cleaned from the leaves and stones, ground to a fine powder with liquid nitrogen and then transferred into falcon tubes and stored at −80 °C until analysis. Pooling reduces variability by minimizing individual variations and is an alternative approach to biological replicates in experiments concerning the characteristics of the population rather than of the individual. All samples from one developmental stage were pooled together and replicates were experimental replicates of this pooled sample.

2.3. Olive pulp protein extraction and quantification

Fig. 1 shows a diagram of protein extraction and precipitation.

Two extraction protocols were employed in two different conditions: denaturing (extraction A, SDS), or physiological (extraction B, CHAPS) conditions.

In extraction A the olive pulp was ground to a fine powder with liquid nitrogen. The protein extraction buffer was prepared using 0.125 mol L^{-1} Tris–HCl (pH 7.4), 5% (v/v) glycerol, 3% (m/v) SDS, 1% (v/v) protease inhibitor cocktail and 25 mmol L^{-1} DTT. For 1.5 g of ground pulp, then 4.5 mL of extracting buffer was added. The resulting suspension was vortexed for a few minutes and then was boiled for 10 min. The insoluble matter was removed by centrifugation at 10,000 rpm, at RT for 10 min. In extraction B, which was carried out in physiological conditions, 1.5 g of olive pulp were homogenized with 4.5 mL of a non-denaturing buffer containing 0.125 mol L^{-1} Tris–HCl (pH 7.4), 50 mmol L^{-1} NaCl, 3% (m/v) CHAPS and 1% (v/v) protease inhibitor cocktail. The mixture thus obtained was subjected to three cycles of ultrasounds, for 30 s at 30% amplitude, using an ultrasonic microprobe.

For both extractions, two different precipitation protocols were employed, MeOH/$CHCl_3$/H_2O (40:10:30) (v/v/v/) (precipitation 1) and TCA/acetone (precipitation 2).