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Identification of Olive (*Olea europaea*) Pulp Proteins by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry and Nano-Liquid Chromatography Tandem Mass Spectrometry

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ABSTRACT: Proteins in the pulp of olive (*Olea europaea*) constitute a minor fraction. They have been sparsely studied despite their suggested role in oil stability and olive allergenicity. The analysis of a pulp protein extract by sodium dodecyl sulfate—polyacrylamide gel electrophoresis (SDS-PAGE) showed a major band at 24 kDa that was subjected to tryptic in-gel digestion. Peptide extracts were analyzed by MALDI-TOF MS and nanoLC-MS/MS. The use of different search engines enabled the assignment of a number of fragmentation spectra to peptide sequences, identifying a major band as a thaumatin-like protein and other low-abundant proteins such a drought-induced protein SDi-6-like, an acyl carrier protein, Cu/Zn and Mn superoxide dismutases, a small heat shock protein, and an ATP-dependent protease subunit. Many of the produced spectra did not give good matches in the database searches, due to the scarce presence of *O. europaea* entries in protein databases. Nevertheless, a huge number of spectra corresponded to peptides, which showed a high degree of homology with others from sequenced organisms. These results proved that database searching with MS/MS spectra constitutes a promising approach for the characterization of olive pulp proteins.

KEYWORDS: LC-MS/MS, MALDI-TOF, olive, proteomics, thaumatin

INTRODUCTION

Olive is one of the main crops in Mediterranean countries. Olive fruit may be used for direct consumption as table olives, as well as for the production of oil. Olive oil is one of the most representative foods of the Mediterranean diet, and its consumption has been related with a decrease of incidence of cardiovascular diseases, cancer, and Alzheimer's disease.[1] Numerous studies have been done for the determination of main components in both olives and olive oil, producing a huge amount of information and a wide knowledge. Nevertheless, minor pulp components, such as proteins, have been poorly studied, despite different authors suggesting they play an important role in oil stability.[2−4] Characterization of olive proteins could increase the value of olive products by the discovery of new bioactive molecules.

Furthermore, there are studies showing that proteins present in olive fruit and oil may elicit allergic reactions in sensitive individuals. Although allergy to olive fruit or oil is uncommon, some cases of allergenic contact dermatitis[5−11] or allergy associated with olive or olive oil ingestion[7,12−15] have been reported. Moreover, some patients presented cross-reactivity of olive pollen allergen and olive fruit allergen.[14,16] Reliable detection methods for olive allergens are necessary to improve consumer protection.

In contrast to olive seed proteins, pulp proteins have been insufficiently studied.[17] For a long time, an oleosin-like polypeptide of 4.6 kDa was described as the main protein in olive pulp and oil.[18,19] Moreover, in recent work by our research group, the analysis by sodium dodecyl sulfate—polyacrylamide gel electrophoresis (SDS-PAGE) of a protein extract from the olive pulp allowed the detection of a main band at a molecular mass of 20−25 kDa.[20] An olive pulp protein having a M_r in the same range (23 kDa) was described and identified, by Edman degradation, as an allergenic thaumatin-like protein.[21]

Extraction of proteins from the olive pulp is problematic because of its high content in interfering compounds (lipids and phenolic compounds) and due to the low amount of proteins (approximately 2%).[19] This probably explains why scarce research has been devoted to the identification and characterization of pulp proteins. Only our research group has developed a methodology enabling the extraction of pulp proteins without interferences.[20] The procedure consisted of a precleaning step followed by Tris/SDS/dithiothreitol extraction, protein precipitation with acetone, and final protein solubilization in a Tris-HCl buffer.

The main difficulty associated with the proteomic analysis of olive pulp proteins is the limited information available in databases. Although gel electrophoresis and chromatographic techniques have been employed to analyze olive pulp protein extracts, mass spectrometry methodologies have not been applied so far to the study of these proteins. Thus, the main aim of this work was

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to identify as many of the proteins present in the olive pulp as possible, making use of the different proteomic mass spectrometry methodologies. Different protein database search engines, such as SEQUEST and MASCOT, were used to assign fragmentation spectra to characterize olive pulp proteins present in the only band appearing by SDS-PAGE using proteomic strategies.

MATERIALS AND METHODS

Chemicals and Samples. Acetonitrile and water for LC-MS (Scharlau, Barcelona, Spain) and formic acid (Fluka, Buchs, Switzerland) were used in the preparation of mobile phases. Tris(hydroxymethyl)-aminomethane (Tris), hydrochloric acid, sodium dodecyl sulfate (SDS) (Merck, Darmstadt, Germany), dithiothreitol (Sigma, St. Louis, MO), trichloroacetic acid (Panreac, Barcelona, Spain), and reagent grade acetone (Scharlau) were used for the extraction and precipitation of olive pulp proteins. Laemmli buffer, 40% acrylamide/Bis solution, *N,N,N′N′*-tetramethylethylenediamine (TEMED), ammonium persulfate, commercial Mini-PROTEAN Precast Gels, Bio-Safe Coomassie stain, Precision Plus Protein Standards (all from Bio-Rad, Hercules, CA), 2-mercaptoethanol (Sigma), methanol, ethanol, and glacial acetic acid (Scharlau) were employed for the SDS-PAGE separation of proteins. Ammonium bicarbonate and iodoacetamide (Sigma) and Trypsin Gold (Promega, Madison, WI) were used for protein digestion. Bradykinin fragment 1−7, angiotensin II (human), $P_{14}R$ synthetic peptide, and ACTH fragment 18−39 (human) mass spectrometry standards (Sigma) were used for calibrating MALDI-MS. α-Cyano-4-hydroxycinnamic acid matrix for MALDI was purchased from Fluka. [Glu^1]-fibrinopeptide used to tune ESI-LTQ was obtained from Sigma. Olives of 'Picual' variety from Spain, 'Frantoio' variety from Italy, 'Bent al Kadi' from Syria, and 'Misión de San Vicente' from Mexico were supplied by the World Olive Germplasm Bank of IFAPA at Córdoba (Junta de Andalucía, Spain). All fruit samples were collected at the same maturity index (violet). Olive fruits were manually depulped, and pulp was stored at −20 °C until use.

Protein Extraction. Protein extraction was based on a method previously developed by our research group.[20] Olive pulp was ground with a domestic miller without thawing. For 1 g of pulp, 10 mL of 10% (m/v) trichloroacetic acid in acetone was added. After vortexing thoroughly for 1 min, the sample was kept at −20 °C for 30 min to allow complete protein precipitation. The sample was centrifuged for 5 min, and the resulting pellet was washed twice with 10% (m/v) trichloroacetic acid in acetone, twice with aqueous 10% (m/v) trichloroacetic acid, and finally once with 80% (v/v) acetone in water. All of the trichloroacetic acid/acetone, trichloroacetic acid/water, and acetone/water solutions were kept at 4 °C. The resulting pellet was mixed with 10 mL of an extracting solution containing 125 mM Tris-HCl (pH 7.5), 1% (m/v) SDS, and 0.5% (m/v) dithiothreitol. After centrifugation for 10 min, proteins in the supernatant were precipitated by adding 50 mL of acetone and kept at −20 °C for 1 h. The proteins were recovered by centrifugation during 10 min and dried at room temperature. The final pellet was resuspended in 30 μL of Laemmli buffer before SDS-PAGE separation.

SDS-PAGE. Proteins were separated by SDS-PAGE using a Bio-Rad Mini-Protean system (Hercules, CA). 2-Mercaptoethanol (0.5%, v/v) was added to samples diluted in Laemmli buffer. Afterward, samples were heated for 5 min at 95 °C and centrifuged for 10 s. Proteins were separated in 3% T stacking gel and 12.5% T acrylamide separating gels containing 10% SDS (m/v) or in commercial Mini-PROTEAN Precast Gels. Electrophoresis was carried out at 12 mA in stacking gel and at 20 mA in separating gel with the homemade gels and at 100 V during 100 min with the commercial gels, using in both cases Tris/glycine/SDS as running buffer. Protein standards (Precision Plus Protein Standards) consisting in recombinant proteins expressed by *Escherichia coli* were used for the estimation of molecular masses. For staining, proteins were fixed with 100 mL of 10% (v/v) glacial acetic acid and 40% (v/v) MeOH for 30 min, stained with 50 mL of Bio-Safe Coomassie G-250 with gentle shaking for 1 h, and washed with water for at least 2 h.

In-Gel Tryptic Digestion. The most intensely stained region at the center of the protein band was cut into 1 mm^3 segments and destained with 25 mM ammonium bicarbonate in acetonitrile/water (1:1, v/v). The gel pieces were dehydrated with acetonitrile and dried in a SpeedVac (Eppendorf, Hamburg, Germany). The proteins were then reduced by adding to the gel pieces a solution containing 10 mM dithiothreitol in 25 mM ammonium bicarbonate and incubating for 30 min at 56 °C. The dithiothreitol solution was removed, and the gel pieces were dehydrated by adding acetonitrile and dried in a SpeedVac. As a following step, the proteins were carbamidomethylated by incubation of the gel pieces in 55 mM iodoacetamide and 25 mM ammonium bicarbonate in the dark for 20 min at room temperature. After removal of the iodoacetamide solution, gel pieces were dehydrated and dried as described above. The gel pieces were rehydrated with 13 ng/μL porcine trypsin in 25 mM ammonium bicarbonate and subjected to digestion overnight at 37 °C by shaking at 300 rpm. Prior to LC-MS/MS analysis, peptides were extracted with 5% (v/v) formic acid/acetonitrile in a 1:2 (v/v) ratio. Then, the supernatant was dried in a SpeedVac, and peptides were suspended in 15 μL of 0.1% (v/v) TFA. Peptides were desalted using reverse-phase ZipTip C_{18} minicolumns (Millipore, Bedford, MA) according to the manufacturer's recommendations and eluted with 10−15 μL of 70% (v/v) acetonitrile containing 0.1% (v/v) TFA.

MALDI-TOF MS Analysis. In-gel tryptic digests resulting from SDS-PAGE separation were analyzed by a MALDI-TOF mass spectrometer with a Voyager DE-PRO (Applied Biosystems, Foster City, CA). The instrument parameters were set as follows: detector, reflector mode; accelerating voltage, 20 kV; grid, 75%; guide wire, 0.002; delay time, 150 ns; laser intensity, 2300. Acquisition was made in the range m/z 700−4000. A total of 100 shots were performed per spectrum, and 5−10 spectra were accumulated per sample to increase the S/N ratio. Spectra were acquired in the positive ion mode.

Samples were prepared using a saturated solution of hydroxycinnamic acid in 50% (v/v) acetonitrile and 0.1% (v/v) TFA as matrix. A volume of 0.5 μL of digested sample was deposited onto a stainless steel MALDI sample target and air-dried. Next, 0.5 μL of hydroxycinnamic acid solution was deposited and dried in the same way. External calibration was performed using the monoisotopic masses of the singly charged ions produced by a solution containing 2.0 μM bradykinin fragment 1−7 (757.3997 Da), angiotensin II (1046.5423 Da), $P_{14}R$ synthetic peptide (1533.8582 Da), and ACTH fragment 18−39 (2465.1989 Da). Internal calibration was performed using trypsin autolysis or keratin peaks. When no peak from trypsin autolysis was found, 0.2 μM of the same standards used for external calibration were added to the sample. Calibrated monoisotopic peak mass lists were applied to peptide mass fingerprint searches using Mascot (Matrix Science, London, U.K.). Searches were performed against the NCBI protein sequence database allowing for up to 100 ppm error tolerance and up to one missed trypsin cleavage site. The carbamidomethylation of cysteines and methionine oxidation were selected as variable modifications during the search.

NanoLC-MS/MS. Tryptic digests were analyzed online by nanoLC-ESI-LIT-MS/MS using a CapLC (Waters) HPLC system coupled to a lineal ion trap LTQ XL (Thermo Scientific, San Jose, CA) equipped with a nanoelectrospray (nESI) source (Proxeon, Odense, Denmark). The instrumental parameters were tuned with a 2 μM Glu-fibrinipeptide solution, prepared in 70% (v/v) acetonitrile containing 0.1% (v/v) formic acid. The peptides were loaded onto a 1 × 0.3 mm i.d. C_{18} precolumn (LC Packings) at a flow rate of 3 μL/min. After washing, flow was reversed through the precolumn. Peptide separation was performed on a 15 cm × 75 μm i.d. fused silica capillary C_{18} HPLC column (LC Packings PepMap) connected to a fused silica distal end-coated tip nanoelectrospray needle (New Objective, Woburn, MA).) Formic acid

(0.1%, v/v) plus 2% (v/v) acetonitrile in water was used as mobile phase A and 0.1% (v/v) formic acid in acetonitrile as mobile phase B. The flow rate was set to 200 nL/min with the following gradient: 5−15% B in 5 min, 15−40% B in 90 min, 40−81% B in 10 min, 81% B during 10 min, 81−5% B in 0.1 min, and 5% B during 4.9 min. During the analysis, the parameters were typically set to spray voltage, 1.70 kV; capillary temperature, 200 °C; capillary voltage, 30 V; and tube lens, 105 V. Collision-induced dissociation (CID) at 35% energy was used for MS^2 experiments. Spectra were acquired in the positive ion mode, over the range m/z 400−1600.

MS/MS fragmentation spectra were searched using SEQUEST (Bioworks 3.1 package, Thermo Fisher) against the complete and general database UniProtKB (release 15.0). Identifications were preliminarily considered as correct when presenting a parametric value of P >30. Afterward, results corresponding to peptides assigned to Viridiplantae species were validated by a search with the MASCOT engine against the protein database NCBI. The following constraints were used for the searches: tryptic cleavage, up to two missed cleavage sites, and tolerances of 2 Da for precursor ions and 0.5 or 1 Da for MS/MS fragment ions. In both cases the variable modifications allowed were methionine oxidation (Mox) and carbamidomethylation of cysteines (C*).

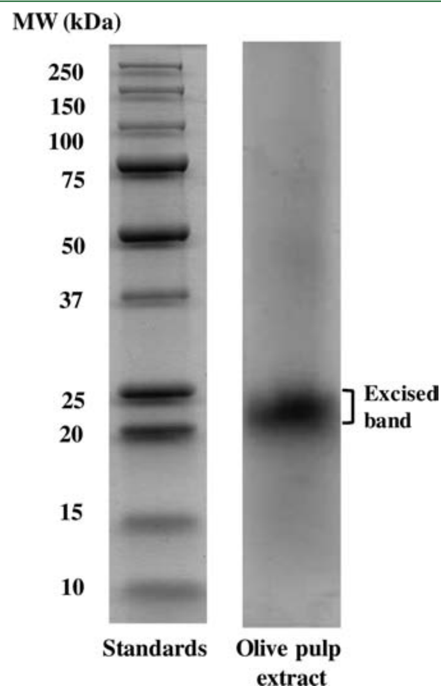

Figure 1. Coomassie-stained SDS-PAGE gel corresponding to the extract obtained from olive pulp. Separation was performed in a Mini-PROTEAN Precast Gel.

■ RESULTS AND DISCUSSION

SDS-PAGE Separation. Olive pulp proteins were separated using SDS-PAGE, and the gel was stained with Coomassie Blue. Figure 1 shows the gel separation of proteins. The Coomassie Blue staining enabled the visualization of a clear band appearing at a molecular mass of 20−25 kDa, as in the previous work.[20] This band was cut, in-gel trypsin-digested, and analyzed by mass spectrometry.

Peptide Mapping by MALDI-TOF MS. The peptides resulting from the excised band digestion were analyzed by MALDI-TOF. The resulting peptide map, shown in Figure 2, was searched against the NCBI database for matching proteins using the MASCOT search engine. Only six peptides, marked with an asterisk in Figure 2, matched a thaumatin-like protein from *Olea europaea* with a score of 59, out of the confidence zone, when all entries were selected for the taxonomy field. Relatively low sequence coverage, around 38%, was obtained, mainly due to the scarcity of arginine and lysine cleavage points in the thaumatin sequence. Trypsin cleavage in this region produces large peptides, which are not easily recoverable. When the observed peptide map was compared with the predicted map containing only peptides below 4000 Da, the coverage reached 69%. The other high-intensity peaks in the spectrum did not match any expected peptide of this protein.

LC-MS/MS Analysis. To detect the presence of the other peptides of this thaumatin-like protein and to find possible low-abundance proteins, further analysis of the tryptic peptides was carried out using LC-MS/MS. A huge amount of peptides appeared in the digested bands from the four selected olive varieties. Unfortunately, little information is available in the databases on the

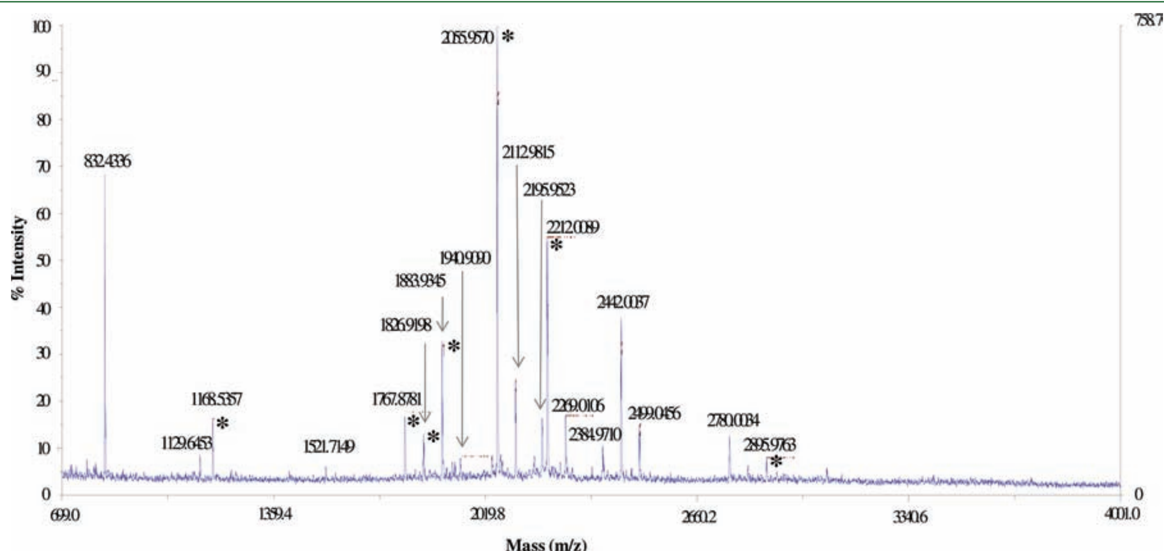

Figure 2. MALDI-TOF-MS peptide map from the 22−24 kDa band after trypsin in-gel digestion.

Table 1. Peptides Identified in the Digested Gel Band, Belonging to *Olea europaea* Proteins, Already Sequenced and Present in Databases

protein identity	$[MH]^+$	amino acid sequence	sequence fragment	NCBI accession
thaumatin-like protein	1292.63	YTVWAAASPGGGR	35−47	gi|34334177 *Gossypium hirsutum*
	2056.02	LDQGQSWNINVAPGTTQAR	49−67	gi|269996497 *Olea europaea*
	2212.12	RLDQGQSWNINVAPGTTQAR	48−67	gi|269996497 *Olea europaea*
	1111.45/1168.48	TNCNFDANGR	72−81	gi|269996497 *Olea europaea*
	1883.95	LVCNAPIVQQCPSELR	143−158	gi|269996497 *Olea europaea*
	2040.04	RLVCNAPIVQQCPSELR	142−158	gi|269996497 *Olea europaea*
	1486.59	CPDAYSYPQDD**R**	197−208	gi|220683825 *Zingiber zerumbet*
	2896.21	CPDAYSYPQDDPTSLFTCPAGTNYR	197−221	gi|269996497 *Olea europaea*
	2898.25	CPDAYSYP**K**DDPTSLFTCP**T**GTNY**K**	197−121	gi|255537367 *Ricinus communis*
	2919.24	CPDAYSYPQDD**K**TSLFTC**TS**GTNY**K**	197−121	gi|7406716 *Vitis vinifera*
	3181.36	ERCPDAYSYPQDDPTSLFTCPAGTNYR	195−221	gi|269996497 *Olea europaea*
drought-induced protein SDi-6-like	1606.80	VSGAAADVLGAAEQYGK	37−53	gi|154259313 *Olea europaea*
	1626.81	GIGQYVDKAEDYLR	59−72	gi|154259313 *Olea europaea*
	1028.52	STPTATEPPK	90−99	gi|154259313 *Olea europaea*
acyl carrier protein	1486.77	QLALPADSAVTGESK	68−82	gi|16518983 *Olea europaea*
	1614.86	KQLALPADSAVTGESK	67−82	gi|16518983 *Olea europaea*
Cu/Zn superoxide dismutase	1335.69	AVVVH**A**DPDDLG**K**	115−127	gi|58616005 *Nelumbo nucifera*
	1351.68	AVVVH**S**DPDDLG**K**	115−127	gi|162135925 *Dimocarpus longan*
	1363.69	AVVVH**A**DPDDLGR	115−127	gi|262089837 *Musa formosana*
	1379.69	AVVVH**S**DPDDLGR	115−127	gi|160962613 *Olea europaea*
	1471.74	A**F**VVH**ELE**DDLG**K**	115−127	gi|195618190 *Zea mays*
	1412.68	GGHELSLSTGNAGGR	128−142	gi|195618190 *Zea mays*
Mn superoxide dismutase	1627.89	LVVETTANQDPLVTK	111−125	gi|16507114 *Olea europaea*
small heat shock protein	1526.70	SDISNETSQFAATR	41−54	gi|307837689 *Olea europaea*
ATP-dependent protease subunit	1723.75	SPGEEDASWVDVYNR	12−26	gi|283794992 *Olea europaea*

O. europaea proteins. Table 1 summarizes peptide identification obtained from the SDS-PAGE band. From these peptides it was possible to identify proteins sequenced for *O. europaea* or peptides of homologous proteins from the clade Viridiplantae. Ten different peptides corresponding to sequenced fragments of thaumatin-like protein were observed, demonstrating that this is a major protein present in this band and, consequently, in olive pulp. As an example, Figure 3 shows the MS/MS spectrum of the peak at m/z 942.47 ($[\text{LVC\#NAPIVQQC\#PSELR}]^{2+}$) in which b- and y-ion series could be found. It was possible to observe a certain degree of microheterogeneity in thaumatin. In all four variants a discrepancy was observed in the peptide covering the sequence between amino acids 197 and 221, with a M_r of 2898.25. Up to three amino acid substitutions were found (marked in bold in Table 1). Changes in the amino acid sequence were also observed in a peptide with a M_r of 2919.21, in the 'Picual' olive variety. In the 'Bent al Kadi' olive variety a shorter peptide, due to a proline to arginine substitution, was observed with a M_r of 1486.59.

Peptides corresponding to another six previously sequenced proteins from *O. europaea* were also observed: three from a drought-induced protein SDi-6-like protein, two from an acyl carrier protein, two from a Cu/Zn superoxide dismutase, and one peptide from a Mn superoxide dismutase, a putative small heat shock protein, and an ATP-dependent protein subunit. In the particular case of Cu/Zn SOD, up to five different variations were found in the same position of the sequence, although they were not specific for any particular olive variety.

The information currently accessible in databases for olive is far from complete. As a result, searches with MASCOT and/or SEQUEST with good spectra produced a high percentage of matches that did not result in a valid protein identification. An amount of peptides that did not correspond to any protein sequence described for *O. europaea* showed homology to sequences from other plant organisms present in databases, which, like *Oryza sativa*, *Vitis vinifera*, and *Arabidopsis thaliana*, have a completely sequenced genome. The complete list of peptide sequences obtained after digestion with trypsin and identified in other Viridiplantae species different to *O. europaea* by SEQUEST and MASCOT is summarized in Table 2. In this table, the total of 56 sequenced proteins are classified depending on their location in the cell: membrane, cytosol, nucleus, or organelles (chloroplasts or ribosomes). Proteins with enzymatic activity are pooled in a separated section. The presence of a large amount of ribosomal proteins should be highlighted, which, in conjunction with rRNA, make up the ribosomal subunits involved in the cellular process of translation. Some additional found proteins were a germin-like protein, which is thought to play a significant role during zygotic and somatic embryogenesis, salt and heavy metal stress, and

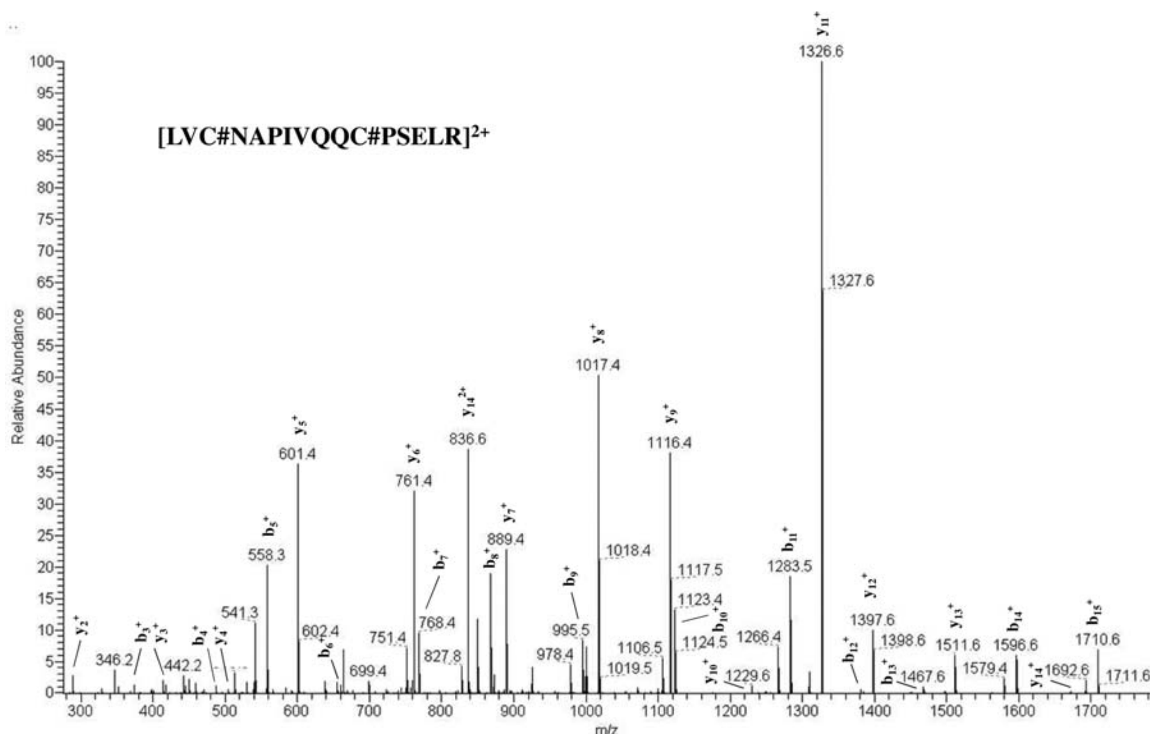

Figure 3. LC-MS/MS spectrum of the 2+ ion at m/z 942.47.

pathogen elicitation; and some cyclophilins, which have peptidyl prolyl isomerase activity or proteins from proteasome subunits where the unneeded or damaged proteins are degraded.

Identified Proteins. The identification of seven olive proteins was possible, as follows.

Thaumatin-like Protein. Thaumatin-like proteins (TLPs) are the products of a large, highly complex gene family involved in host defense and a wide range of developmental processes in fungi, plants, and animals.[22] Most TLPs have molecular masses ranging from 21 to 26 kDa with a relatively high amount of conserved cysteine residues. In pollen and fruits TLPs have been described as a new family of allergens.[23] Allergenic TLPs have been described in apple, sweet cherry, tomatoes, kiwi, grape, and cedar pollen.[22] In the case of olive, just one work has described the presence of a thaumatin-like protein.[21] In this case, a part of the sequence of the purified protein was obtained by Edman degradation. Besides this, this protein has been sequenced and has been described in the databases as an allergenic thaumatin, a new olive allergen named *Ole e 13*. Despite widespread consumption, just some cases of contact dermatitis[5−8] and food allergies[12−15] to olive fruit and derivative products have been reported. Nonetheless, in highly sensitized individuals, even the intake of minute amounts of allergens can provoke an allergenic response with dramatic consequences, making the study of food allergens a first necessity. The study of this protein is also important due to their host defense character, making it a possible target in the creation of new genetic modified organisms. Several transgenic plants have been developed to express variety of TLP genes for enhanced resistance against pathogens. Transgenic plants constitutively overexpressing TLPs often show an enhanced fungal resistance.[24] This is an important point taking into account that fungal infections such as the ones suffered from infection by *Verticillium dahliae* or *Spilocaea oleagina* are important diseases in the olive crop. These fungi have shown resistance to chemical compounds and are responsible for severe yield losses in many olive-growing regions in the world.

Drought-Induced Protein SDi-6-like. This protein seems to be synthesized at stress plant conditions due to water deficit. This is a 14.2 kDa protein never detected before with DNA sequenced by Guerrero et al. (unpublished results). No biological function has been demonstrated so far for this protein.

Acyl Carrier Protein. The gene of this olive protein has been already isolated and identified by Guerrero et al. (unpublished results). In olive, this acyl carrier protein has a molecular mass of 14 kDa. Acyl carrier protein is a universal and highly conserved carrier of acyl intermediates during fatty acid biosynthesis.[25]

Cu/Zn and Mn Superoxide Dismutases. Superoxide dismutases (SODs) are a family of metalloenzymes with a molecular mass of 16 kDa present in prokaryotic and eukaryotic cells being an important part of the cellular defense system against oxidative stress. Cu/Zn SOD has been widely described in olive pollen, being classified as allergen Ole e 5,[26−29] and, on just one occasion, in olive leaves.[30] Mn SOD has also been described in leaves.[30] The activity of total SOD has been spectrometrically measured, being determined the influence of salinity-induced changes, both in olive leaves[31] and in different varieties of olive pulp.[32] This could be a case of nonspecific gene transcription due to the presence of the same protein in different tissues from the same tree, and its presence in the olive pulp could explain some cases of allergy to the olive fruit after olive pollen immunotherapy.[14,16]

Small Heat Shock Protein. This is a protein of 18.3 kDa; its gene has been sequenced by Assab et al. (unpublished results). Although its biological function has not been indicated so far, it is known that it is implicated in the organism response to stress.

ATP-Dependent Protease Subunit. This is a chloroplast protein of 22.0 kDa involved in the hydrolysis of proteins to small peptides in the presence of ATP and magnesium. The entire plastome, the chloroplast genome, of olive was sequenced by Mariotti et al.[33]

Table 2. Peptides Found in the Digest of the Gel Band at 22 kDa Which Are Homologous to Other Proteins Described in Different Vegetable Organisms

protein identity	$[MH]^+$	amino acid sequence	NCBI accession and species
nonplast proteins			
cytosol and membrane proteins			
expansins	945.55	AGIVPVAYR	gi|285265634 *Coffea arabica*
	1101.67	GGIVPVLYKR	gi|11191999 *Solanum lycopersicum*
	2047.03	QHFDLAEPAFLQIAQYR	gi|285265634 *Coffea arabica*
lipid-associated protein	978.50	GNLDIFSGR	gi|15236014 *Arabidopsis thaliana*
germin-like protein	1326.77	IPGLNTLGVSLSR	gi|219522380 *Glycine max*
	2272.20	AAVTPAFDAQFPGVNGLGISIAR	gi|196122024 *Glycine max*
calmodulin	1809.91/1810.89	VFDKDQDGFISAAELR	gi|166655 *Arabidopsis thaliana*
	1927.85	EADVDGDGQINYEEFVK	gi|166655 *Arabidopsis thaliana*
Kunitz-type proteinase inhibitor	1348.64	YNSDVGPSGTPVR	gi|73920951 *Solanum tuberosum*
GTP-binding protein	1491.73	IDIPYAASEDELR	gi|225437537 *Vitis vinifera*
	2155.10	LVQHQPTQYPTSEELSIGK	gi|225437537 *Vitis vinifera*
nucleus proteins			
histone 4	1190.62	ISGLXYEETR	gi|195617808 *Zea mays*
	1325.75	DNIQGITKPAIR	gi|195617808 *Zea mays*
proteasome subunit $\alpha 2$	1556.88	KLPSILVDETSVQK	gi|195640986 *Zea mays*
proteasome subunit $\beta 5$	1225.69	LLANILYSYR	gi|14594931 *Nicotiana tobacum*
	1981.92	FSVGSGSPYAYGVLDSGYR	gi|14594931 *Nicotiana tabacum*
proteasome subunit $\beta 7$	1543.83	FNPLWNSLVLGGVK	gi|14594935 *Nicotiana tabacum*
proteasome subunit $\beta 6{,}9$	1554.73	SGSAADSQIVSDYVR	gi|255574159 *Ricinus communis*
enzymatic proteins			
cyclophilin	1131.58	SLSRSASPNGR	gi|22329097 *Arabidopsis thaliana*
	2007.01	IVIGLYGDDVPQTAENFR	gi|255547634 *Ricinus communis*
	1495.73/1511.73	VFFDMTIGGQPAGR	gi|145049729 *Ipomoea batatas*
	1624.90	HVVFGQVVEGLDVVK	gi|145049729 *Ipomoea batatas*
	1376.74/1392.74	IVMELYADVVPK	gi|52788398 *Pinus halepensis*
dimethylmenaquinone methyltransferase	1071.61	VLVVDGGGSLR	gi|15232963 *Arabidopsis thaliana*
	1090.58	VFEDNVLVR	gi|15241161 *Arabidopsis thaliana*
	1291.71	ALQPVFQIYGR	gi|15232963 *Arabidopsis thaliana*
β-hydroxyacyl-ACP dehydratase	1285.72/1301.72	KPVVAGDTLVMR	gi|75706919 *Picea mariana*
	1445.79	VVEYNPGVSAVAIK	gi|75706919 *Picea mariana*
	1903.90	NVTINDNFFPGHFPER	gi|75706919 *Picea mariana*
ribose-5-phosphate isomerase	2120.11	RGSLAGITAVTSSVLSASEADK	gi|226504086 *Zea mays*
chitinase	1741.85	GPIQLSYNYNYGPAGK	gi|38371990 *Bambusa oldhamii*
	1109.55/1125.55	YGGIMLWNR	gi|189095136 *Ananas comosus*
maturase K	2275.30	FLNKLILSNLFDQINLLEK	gi|198034201 *Equisetum scirpoides*
PII protein	1092.54	GFGAQGGLTER	gi|38231570 *Solanum lycopersicum*

Table 2. Continued

protein identity	$[MH]^+$	amino acid sequence	NCBI accession and species
ascorbate peroxidase	884.44	TEPPVEGR	gi|1389654 *Nicotiana tabacum*
	1555.88	ALLSDPAFRPLVEK	gi|1389654 *Nicotiana tabacum*
cysteine protease	1749.79	YNGGLETESAYPYTGK	gi|53748483 *Plantago major*
	2239.80	GGSNDDCDMAPGDEEEDDRR	gi|76574404 *Zea diploperennis*
β-fructofuranosidase	1100.58	GWASVQSIPR	gi|19849288 *Cucumis melo*
protein kinase	1493.72	SEVGGGGPSAGKFTDK	gi|15230753 *Arabidopsis thaliana*
serine/threonine protein kinase	1562.79	WDLGATGDLPARYK	gi|325511375 *Arabidopsis thaliana*
	1587.69	YILTTKGMAAMMEK	gi|15224934 *Arabidopsis thaliana*
nucleoside diphosphate kinase	1354.67	NVIHGSDAVESAR	gi|297809115 *Arabidopsis* lyrata
	1553.82	IIGATNPADSAPGTIR	gi|19387233 *Musa acuminata*
	1567.84	IIGATNPAESAPGTIR	gi|284433792 *Jatropha curcas*
	1695.93	KIIGATNPAESAPGTIR	gi|284433792 *Jatropha curcas*
chloroplast proteins			
chlorophyll *a*/*b*-binding protein	1312.69	FFDPLGFAGTLK	gi|8954298 *Vigna radiata*
	1315.66	NPGSVNQDPIFK	gi|195617028 *Zea mays*
	1811.81	TAENFANATGEQGYPGGK	gi|8954298 *Vigna radiata*
photosystem I precursor	1270.66	GVGSVVAVDQDPK	gi|121489737 *Phillyrea latifolia*
photosystem II precursor	1734.94	VAMLGFAASLLGEAVTGK	gi|1304215 *Oryza sativa*
glyceraldehyde 3-phosphate dehydrogenase	1268.63	LEKSASYEDVK	gi|255764584 *Jasminum multiflorum*
ribosomal proteins			
40S ribosomal protein S7	1055.55	LETFAGVYR	gi|20139964 *Avicennia marina*
40S ribosomal protein S9	1166.56	YGLLDESQNK	gi|15242241 *Arabidopsis thaliana*
40S ribosomal protein S10	1249.59	GGAPADYQPAFR	gi|81076822 *Solanum toberosum*
40S ribosomal protein S11	1151.55	CPFTGDVSIR	gi|89242341 *Chlamydomonas* sp.
40S ribosomal protein S14	1054.56	TPGPGAQSAXR	gi|146454918 *Sonneratia caseolaris*
40S ribosomal protein S16	1128.64	AMEPIILLGR	gi|255074629 *Micromonas* sp.
	1689.98	INGVPIELVQPEILR	gi|159138763 *Helianthus annuus*
40S ribosomal protein S19	833.46	GGIGVGAFR	gi|225457277 *Vitis vinifera*
	1057.53	DVSPHEFVK	gi|225423503 *Vitis vinifera*
40S ribosomal protein S20	1318.72	VIDLFSSPDVVK	gi|15232789 *Arabidopsis thaliana*
40S ribosomal protein S23	1133.69	VSGVSLLALFK	gi|195634715 *Zea mays*
40S ribosomal protein S24	1600.78	STGFGLIYDSVENAK	gi|307136061 *Cucumis melo*
40S ribosomal protein S26	999.45	NIVEQAAVR	gi|255558550 *Ricinus communis*
40S ribosomal protein S28	1360.69	EGDVLTLLESER	gi|255559537 *Ricinus communis*
60S ribosomal protein L7	1542.78	VEPYVTYGYPNLK	gi|30689617 *Arabidopsis thaliana*
60S ribosomal protein L10	1154.72	VAIGQVLLSVR	gi|18408550 *Arabidopsis thaliana*

Table 2. Continued

protein identity	[MH]+	amino acid sequence	NCBI accession and species
60S ribosomal protein L11	1573.85	AGGAYTLNTASAVTVR	gi|226530763 *Zea mays*
60S ribosomal protein L12	1267.79	VTVVPSAAALVIK	gi|255557008 *Ricinus communis*
	1343.71	VTGGEVGAASSLAPK	gi|297827223 *Arabidopsis* lyrata
60S ribosomal protein L15	1085.59	PTNQGVTQLK	*Arabidopsis thaliana*
	1702.94	GIVYGKPTNQGVTQLK	gi|255579641 *Ricinus communis*
	1731.82	VLNSYWINEDSTYK	gi|255579641 *Ricinus communis*
60S ribosomal protein L17	961.48	NAESNAEVK	gi|15223501 *Arabidopsis thaliana*
60S ribosomal protein L18	1181.70	APLGQNTVLLR	gi|15241061 *Arabidopsis thaliana*
	1182.68	APLGENTVLLR	gi|87280934 *Saccharum* hybrid cultivar
	1550.76	AGGECLTFDQLALR	gi|15241061 *Arabidopsis thaliana*
60S ribosomal protein L21	815.46	AVGVEVNK	gi|18406015 *Arabidopsis thaliana*
60S ribosomal protein L23	1213.68	VNTLIRPDGTK	gi|255580570 *Ricinus communis*
	1434.71	LTPDYDALDVANK	gi|255580570 *Ricinus communis*
60S ribosomal protein L26	1072.55	AHFTAPSSVR	gi|3747050 *Zea mays*
	1087.57	KDDEVQVVR	gi|3747050 *Zea mays*
	1408.75	VNGSTVNVGIHPSK	gi|3747050 *Zea mays*
60S ribosomal protein L27	1513.76	APQIDVTQFGYFK	gi|195617904 *Zea mays*
60S ribosomal protein L30	1613.91	IALTDNSIIEQVLGK	gi|87162669 *Medicago truncatula*
60S ribosomal protein L32	1157.65	AAQLDVVVTNK	gi|255557443 *Ricinus communis*
	1493.70	TYCAEIAHNVSTK	gi|195658369 *Zea mays*
60S ribosomal protein L35	751.43	GTILGYK	gi|255573911 *Ricinus communis*
	1399.85	LSIAQVLTVISQK	gi|255580548 *Ricinus communis*
60S ribosomal protein L34	1106.59	AYGGVLSGGAVR	gi|255562848 *Ricinus communis*
60S ribosomal protein L37	1551.81	AGGAYTLNTASAVTVR	gi|255552928 *Ricinus communis*

In conclusion, there is a lack of knowledge on olive proteins. A proteomic approach based on the use of nanoLC-MS/MS is proposed for the characterization of pulp olive tryptic digests. The detection of peptides from a thaumatin-like protein has been possible, with reasonable sequence coverage obtained taking into account the limited amount of basic residues in this protein. Results obtained with four different olive varieties showed slight differences in the same peptides corresponding to the thaumatin-like protein. Some peptides from other *O. europaea* proteins have been sequenced: those from a drought-induced protein SDi-6-like, an acyl carrier protein, Cu/Zn and Mn superoxide dismutases, a small heat shock protein, and an ATP-dependent protease subunit, were identified. Despite the obtained results, the identification of olive pulp proteins has been limited by the lack of available olive protein sequences in databases. Nevertheless, a large number of the fragmented peptides were assigned, by either Mascot or Sequest, to proteins from other completely sequenced vegetable organisms, such as *A. thaliana*, *V. vinifera*, or *O. sativa*, due to probable high degree of homology between certain proteins of these organisms and the corresponding proteins from *O. europaea*.

This work constitutes the first proteomic study focused on the identification of olive proteins, and it is the first time that most of them have been isolated, detected, and identified. In this regard, the study of proteins present in olive pulp opens new possibilities in the new foodomics field, and it can also be the base of new nutrigenomic studies.

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