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The proteins of the grape (*Vitis vinifera L.*) seed endosperm: Fractionation and identification of the major components

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ABSTRACT

In the present study, grape (*Vitis vinifera L.*) seed endosperm proteins were characterized after sequential fractionation, according to a modified Osborne procedure. The salt-soluble fraction (albumins and globulins) comprised the majority (58.4%) of the total extracted protein. The protein fractions analysed by SDS–PAGE showed similar bands, indicating different solubility of the same protein components. SDS–PAGE in non-reducing and reducing conditions revealed the polypeptide composition of the protein bands. The main polypeptides, which were similar in all the grape varieties analysed, were identified by LC–MS/MS as homologous to the 11S globulin-like seed storage proteins of other plant species, while a monomeric 43 kDa protein presented high homology with the 7S globulins of legume seeds.

The results provide new insights about the identity, structure and polypeptide composition of the grape seed storage proteins.

1. Introduction

Grape is one of the world's most widely grown fruit crops, with a reported annual production higher than 69 million tons (FAOSTAT, 2011), which is almost completely transformed in wine. The main winemaking by-product is grape pomace, constituting 13% of the total grape weight (≈10 million tons) and containing 38–52% (on a dry matter basis) of grape seeds (Maier, Schieber, Kammerer, & Reinhold, 2009), which are then produced in several tons per year. A small amount of this is used for the extraction of the seed oil and natural antioxidants (Arvanitoyannis, Ladas, & Mavromatis, 2006), and recently a grape seed protein extract was proposed as a valuable fining agent for wine (Vincenzi et al., 2013). However, most of the grape seeds are treated as a waste material. The nutritional quality of the grape seed proteins have been considered for a long time, with these proteins having nutritional characteristics similar to those of other oilseeds and cereals (Igartuburu et al., 1991). Moreover, grape seed proteins show some functional properties such as good solubility and emulsifying activity (Zhou, Zhang, Liu, & Zhao, 2011).

Although several papers have been published on the identification and characterisation of the grape pulp and skin proteins (for a review see Giribaldi & Giuffrida, 2010, and the citations therein), grape seed proteins have received little attention. The protein content of the grape seed was the object of early studies, which reported varying values ranging from 8.44% (Igartuburu et al., 1991) to 25.9% (Fazio, Gattuso, Cilluffo, & Arcoleo, 1983). In contrast, only a few papers have been published on the characterisation of the different protein components present in the grape seed (Gianazza et al., 1989; Zhou, Li, Zhang, Bai, & Zhao, 2010).

In general, plant seeds contain different types of proteins, most of which can be classified as storage proteins, a nitrogen reserve for early seedling growth (Shewry, Napier, & Tatham, 1995). Early studies by Osborne divided the seed proteins according to their solubility in different solvents into albumins (water-soluble), globulins (salt-soluble), prolamins (aqueous alcohol-soluble) and glutelin (acidic or alkaline solution-soluble) (Osborne, 1924). This approach is still considered a valid method to fractionate and classify seed proteins. In several plant species, the main storage proteins belong to the globulin fraction, commonly divided into 11S and 7S according to their sedimentation behaviour (Shewry et al., 1995).

The major protein of the grape endosperm was initially shown to be a 60 kDa globulin containing disulfide-linked 19–21 kDa and 38–44 kDa peptides (Gianazza et al., 1989). More recently, a protein made of two polypeptides with molecular masses of 25.5 and 40.0 kDa was isolated and purified from grape seeds and identified by mass spectrometry as an 11S globulin-like protein (Zhou et al., 2010). However, no other information was available on the

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identity of the other protein components of the grape seed, nor on their structural organisation. Moreover, during a study on the effects of water-deficit stress on grape berry tissues, several grape seed proteins were identified by the proteomic approach, most of them belonging to the 11S globulins with storage function, although in this case their structural organisation in the seed was not investigated (Grimplet et al., 2009). Other studies focused on grape varieties differentiation by their seed protein compositions (Pesavento et al., 2008) although the proteins were not characterised.

In this paper, the proteins of the grape seed endosperm were fractionated according to their solubility and characterised by electrophoretic analyses in terms of polypeptide compositions. Moreover, the main electrophoretic bands, which were present in several grape varieties as major components, were identified by mass spectrometry.

2. Materials and methods

2.1. Plant material

Seeds were manually extracted from the berries of different white (Glera, Trebbiano, Durella, Cortese, Moscato Colli Euganei, Manzoni Bianco, Moscato Fiori d'Arancio, Garganega) and red (Raboso Piave and Corvina) grape (*Vitis vinifera L.*) varieties, harvested in the Conegliano area. For each variety, one hundred randomly selected, fully ripe seeds were used. Seeds were washed with distilled water and left to dry at room temperature. By means of a razor blade, seeds were dissected and the integuments were removed, while the endosperm was recovered and immediately frozen for storage.

2.2. Lipid extraction

Approximately 2 g (*cv*. Glera) or 400 mg (other varieties) of endosperm was ground to a fine flour in a mortar in the presence of an excess of *n*-hexane. The suspension was filtered at 0.45 μm with filters for organic solvents (polyethersulfone, PESU, Sartorius, Goettingen, Germany). The delipidated endosperm was recovered, weighed and stored at 4 °C.

2.3. Seed protein sequential fractionation based on solubility

Protein fractions were sequentially extracted at room temperature according to a modified Osborne procedure (Sogi, Arora, Garg, & Bawa, 2002) as follows. The defatted flour (1.4 g for *cv*. Glera and 100 mg for the other varieties) was extracted with 0.5 M sodium chloride (1:10, w/v) with constant stirring for 30 min. The slurry was centrifuged at 14,000*g* for 5 min. The supernatant was recovered and two further extractions were performed on the pellet, each stirring for 15 min. The three supernatants were pooled, filtered at 0.45 μm and extensively dialyzed (3 kDa MW cut-off) against distilled water. After dialysis, the suspension was centrifuged (14000*g*, 5 min). The precipitate (globulins) and the supernatant (albumins) were separated and freeze-dried.

The insoluble pellet resulting from the salt extraction was then treated with 70% v/v aqueous ethanol (1:10 w/v) and centrifuged (14,000*g*, 5 min). The procedure was repeated three times and the supernatants were pooled, filtered at 0.45 μm and dialyzed against distilled water. Finally, the content of the dialysis tube (prolamins) was freeze-dried.

The insoluble pellet from the previous extraction was treated with 0.05 M acetic acid (1:10 w/v). The suspension was stirred for 30 min and centrifuged (14,000*g*, 5 min.). After dialysis against distilled water, the fraction (glutelin) was freeze-dried.

The remaining insoluble material was finally extracted by stirring (20 min.) at 100 °C, using a solution containing 2% Sodium Dodecyl Sulphate (SDS) (Bio-Rad Laboratories, Milan, Italy). The sample was centrifuged and the residue extracted again with the same buffer. Supernatants were combined, dialyzed against water and freeze-dried (un-extractable proteins, UP). A scheme of the whole extraction procedure is reported in the Supplementary data.

2.4. Protein content determination

Total nitrogen of the defatted grape seed endosperm (*cv*. Glera) and of the extracted fractions was determined after sample mineralisation by a HACH Digesdahl apparatus (HACH Company, Loveland, CO, USA). Ammonia was quantified with the Nessler reagent (Vogel & Svehla, 1979), and protein content was computed as ammonia × 6.25.

2.5. Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE)

SDS–PAGE analyses were performed according to Laemmli (1970) in a Mini-Protean III apparatus (Bio-Rad). Aliquots of freeze-dried albumin, globulin, prolamin and glutelin fractions were solubilised in 10 μl of 0.5 M Tris–HCl buffer, pH 6.8, containing 15% (w/v) glycerol, 1.5% (w/v) SDS (Bio-Rad) and 4% (v/v) 2-mercaptoethanol (2-ME) (Sigma–Aldrich, Milan, Italy) (loading buffer). Samples were heated at 100 °C for 5 min. before loading. For SDS–PAGE analyses under non-reducing conditions, the reducing agent 2-ME was omitted from the loading buffer. Electrophoresis was carried out at 25 mA constant current until the tracking dye Bromophenol Blue ran off the gel. The molecular weight standard proteins were the Broad Range Molecular Weight Markers (Bio-Rad). 1.5 mm thick gels were prepared with $T = 16\%$ (acrylamide-N, N′ methylenebisacrylamide 29:1; Sigma–Aldrich), and stained with Colloidal Coomassie Brilliant Blue G-250 (Sigma–Aldrich).

2.6. Two-dimensional (transversal) SDS–PAGE (non-reducing × reducing)

After a standard SDS–PAGE performed in non-reducing conditions (first dimension), the gel lane of interest was cut using a razor blade, placed in a tube with 5 ml of loading buffer containing 4% (v/v) 2-ME and heated for 5 min at 100 °C. After cooling, the gel slice was placed horizontally on the top of a second gel (second dimension) and fixed seeping a 0.5% (w/v) agarose solution. Runs were performed under the same conditions previously mentioned for SDS–PAGE.

2.7. Liquid chromatography–tandem mass spectrometry (LC–MS/MS) analyses

After electrophoretic separation, the selected bands were excised from the gel and subjected to in-gel protein digestion. Briefly, cysteines were reduced with 10 mM dithiotreitol (DTT) (1 h, 56 °C, in the dark) and alkylated with 55 mM iodoacetamide (1 h, room temperature, in the dark). After washes with 50 mM NH_4HCO_3 and acetonitrile, digestion was performed at 37 °C overnight using sequencing grade modified trypsin (Promega, Madison, WI) (12.5 ng/μL). Peptides were extracted with 50% acetonitrile/1% formic acid, dried under vacuum and dissolved in 10 μl of 0.1% formic acid. Liquid chromatography–tandem mass spectrometry (LC–MS/MS) analyses were performed with a 6520 Q-TOF mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) coupled to a chip-based chromatographic interface. 4 μl of samples were loaded into the enrichment column (C18, 4 mm, 40 nl volume) at a flow rate of 4 μl/min. Peptides were separated in the C18