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The diversity of pathogenesis-related proteins decreases during grape maturation

Sara Monteiro [a,b,*], Maria A. Piçarra-Pereira [b,c], Virgílio B. Loureiro [b], Artur R. Teixeira [b], Ricardo B. Ferreira [a,b]

[a] *Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Apartado 127, 2781-901 Oeiras, Portugal*
[b] *Departamento de Botânica e Engenharia Biológica, Instituto Superior de Agronomia, Universidade Técnica de Lisboa, 1349-017 Lisboa, Portugal*
[c] *Escola Superior Agrária, Instituto Politécnico de Castelo Branco, 6001-909 Castelo Branco, Portugal*

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Abstract

It was recently shown that wines contain typically a huge diversity of structurally similar polypeptides that exhibit a high degree of homology to pathogenesis-related (PR) proteins. This observation suggested the existence of one or a few precursors in mature grapes, common to most or all the wine PR proteins. Limited proteolysis and chemical modification of the precursor(s) during fruit ripening and winemaking could then generate the large number of distinct wine polypeptides. However, the patterns of PR proteins extracted from grape berries regularly harvested from the onset of development until maturity did not confirm the previous hypothesis. Two different methodologies, involving 2-D immunoblotting and a combination of FPLC cation/anion exchange chromatographies with 1-D immunoblotting, indicate that the total concentration of PR proteins is increased but its diversity is reduced from the early stages of berry development until maturity. These results indicate that PR proteins are synthesized in a wide variety of forms from the early stages of grape development, eliminating the hypothesis previously formulated on the existence of one or few precursors common to the wine proteins.

Keywords: *Vitis vinifera*; Vitaceae; Grapevine; Wine; Grape berries; PR proteins

1. Introduction

Pathogenesis-related (PR) proteins are generally considered as plant defence proteins, functioning in preventing or limiting pathogen multiplication and/or spread. Fourteen different classes of structurally and functionally unrelated proteins have been considered, numbered PR-1 to PR-14 (van Loon, 1999), some of which have been detected in grapevine (*Vitis vinifera*) (Ferreira et al., 2004). These include PR-5 (thaumatin-like proteins and osmotins), which are thought to create transmembrane pores and have therefore been termed permatins; PR-2 (β-1,3-glucanases) and PR-3, -4, -8 and -11 (chitinases), which attack β-1,3-glucans and chitin, respectively, components of the cell walls in most higher fungi (Ferreira et al., 2004).

PR proteins accumulate in grapes during the growing season (Tattersall et al., 2001). They are synthesized in healthy grape berries in a developmentally dependent manner as a normal part of the ripening process, with véraison (the French term used by viticulturalists to denote the inception of ripening) apparently being the trigger for PR gene expression (Van de Rhee et al., 1994; Ferreira et al., 2002). There is a significant increase in total grape protein content after véraison, with only a small number of proteins being synthesized in significant amounts during ripening (Tattersall et al., 1997). The two most prominent soluble proteins accumulated in grapes during ripening have been identified as chitinase and thaumatin-like proteins (Robinson and Davies, 2000), with chitinase alone

* Corresponding author. Fax: +351 213653238.
E-mail address: smonteiro@isa.utl.pt (S. Monteiro).

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being reported to account for half of the soluble protein in ripe grapes (Waters et al., 1998).

In addition, PR proteins are also induced in pre-véraison berries in response to environmental and stress-related stimuli, such as wounding, chemical elicitors, pathogen attack or abiotic stress, by the expression of specific PR genes (Jacobs et al., 1999; Robinson and Davies, 2000; Monteiro et al., 2003a,b). Taken together, these processes modulate the levels and proportions of the PR proteins in grapes, so that the environmental conditions prevailing during vegetative growth determine the precise pattern of major polypeptides that accumulate in mature berries (Monteiro et al., 2003a,b).

Given the physiological role attributed to PR proteins, genetically modifying vines in order to overexpress these proteins has been reported to obtain plants with enhanced resistance to pathogens (Ferreira et al., 2004). However, due to their inherent resistance to proteolytic attack and to the low pH values characteristics of musts and wines, vinification may be considered as a purification strategy for the grape PR proteins (Ferreira et al., 2002). The consequent accumulation of the grape PR proteins in wines is a technological nuisance because they greatly affect the clarity and stability of wines (Ferreira et al., 2002).

A simple electrophoretic analysis of the wine proteins detects the presence of only a few major polypeptides, ranging in molecular mass from 15 to 30 kDa. However, a more detailed examination of the whole protein fraction, by a combination of techniques including cation/anion exchange chromatographies or two-dimensional electrophoresis, reveals that wines contain, typically, a very large number (many tens and, possibly, many more) of distinct polypeptides, exhibiting similar molecular masses but different electrical charges. Most of these polypeptides are structurally similar and exhibit a high degree of homology to PR proteins (Monteiro et al., 2001).

All these observations suggest the existence of one or a few precursors in the grape, common to most or all the wine proteins, which could generate during grape maturation and/or winemaking all the detected polypeptides by limited proteolysis and/or chemical modification. For this reason, the initial purpose of this work was to locate the presence of such putative precursors at the maturing stage of the grape berry where they first appear, to isolate them and to study the proteolytic process that leads to the structural diversity observed in the wine proteins. To this end, using the total wine protein as a control, the total soluble protein was extracted from grapes at different stages of development, from post-flowering to maturity. This was a surprisingly difficult task due to the high content of secondary metabolites in the berries during most of their developmental stages. Antibodies highly specific for the grape PR proteins were subsequently employed to detect the pattern of synthesis and accumulation of these proteins during berry development.

2. Results

2.1. Specificity of the polyclonal antibodies for the grape PR proteins

Polyclonal antibodies were developed in rabbits against a highly purified 20 kDa polypeptide extracted from Assario wine as previously described (Monteiro et al., 1999). These antibodies were shown to be highly specific for the major 20 kDa Assario wine polypeptide (Monteiro et al., 2001). Two abundant polypeptides from Assario wine were selected and subsequently purified by a combination of FPLC cation/anion exchange chromatographies. These polypeptides (Fig. 1a) are recognized by the anti-20 kDa Assario polypeptide antibodies (Fig. 1b) and were identified by N-terminal sequencing as grape osmotin (Figs. 1a and b, lane 1) and grape thaumatin-like protein (Figs. 1a and b, lane 2; Table 1). In fact, the anti-20 kDa Assario polypeptide antibodies were found to specifically recognize a very wide diversity of structurally similar proteins that are present in grapes and wines, prepared from white or red varieties, regardless of the variety, year or region (Ferreira et al., 2000). This very wide diversity of structurally similar proteins, or protein isoforms, explains the multiple bands visible on the western blot shown in Fig. 1b (Monteiro et al., 2001). N-Terminal sequencing analyses revealed that these are PR proteins with high sequence homologies with osmotin, thaumatin-like protein and chitinase (Monteiro et al., 2001).

2.2. Two-dimensional analysis of the pattern of total grape PR proteins from post-flowering to maturity

After establishing that the anti-wine protein antibodies were highly specific towards the grape PR proteins, our attention moved towards the pattern of synthesis and accumulation of PR proteins during grape development. To this end, *Vitis vinifera* L. (cv Moscatel) grapes were regularly

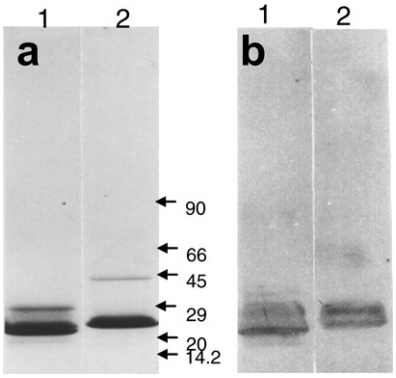

Fig. 1. Identification of the polypeptides recognized by the anti-pathogenesis-related protein antibodies. Selected wine polypeptides (lanes 1 and 2: 100 μg protein in (a) or 50 μg in (b)) were purified by a combination of cation/anion exchange fast protein liquid chromatography, subjected to SDS–PAGE and either stained for total protein (a) or probed with the antibodies (b). Molecular masses of markers are indicated in kDa.

Table 1
N-terminal sequencing of the wine polypeptides presented in Figs. 1a and b and comparison with the sequences of proteins from other sources[a]

Protein	Source	Alignment	Reference	Percent identity
Figs. 1a and b, lane 3	This work	^{1}ATFNIQNHHSYTVVAAAVPGGGMQL^{25}	–	–
Osmotin-like protein precursor	*Vitis vinifera* (grape)	^{25}ATFNIQNHCSYTVWAAAVPGGGMQL^{49}	AN P93621 (TrEMBL)	92.00
Figs. 1a and b, lane 2	This work	^{1}ATFDILNKKTYTVVAAAA^{18}	–	–
Thaumatin-like protein	*Vitis vinifera* (grape)	^{25}ATFDILNKCTYTVWAAAS^{42}	AN Q04708 (TrEMBL)	83.33

[a] The database consulted was Swiss-Prot/TrEMBL (available online).

(weekly) harvested from the onset of development until maturity, the respective proteins were extracted, quantified and studied.

In 1999, grape berries were regularly harvested from post-flowering week until maturity, for 11 consecutive weeks. The results presented in Table 2 show the changes in grape total soluble protein and PR protein content. As reported in the literature, there is a gradual increase in total soluble protein during grape development. However, the increment in grape PR proteins is particularly evident after the 7th week. The polypeptide pattern of the protein extracted from these berries is shown in Fig. 2a. Comparison of the 11 lanes of Fig. 2a reveals a relatively complex polypeptide pattern that gradually simplifies towards the final stages of grape development. The most distinct feature is the accumulation of major proteins with molecular masses ranging from 20 to 45 kDa, which is clearly distinguishable at and after the 7th harvesting week. This result is in good agreement with the data reported in the literature. For example, about 300 spots were detected by two-dimensional electrophoresis of the soluble proteins from grapevine ripe berries extracted from six different cultivars (Sarry et al., 2004). Sixty seven of these spots were subsequently identified using matrix-assisted laser desorption/ionization-mass spectrometry analysis. About 34%, 19% and 13% of identified proteins play, respectively, a role in energy metabolism, defence and stress response, and primary metabolism. As previously documented, this study identified PR proteins such as chitinase and thaumatin-like proteins as prevalent proteins in mature grapes (Sarry et al., 2004).

Table 2
Protein content of the Moscatel grapes harvested in 1999

Post flowering week	Total soluble protein[a] (μg/g fresh weight)	Total PR proteins[a] (μg/g fresh weight)
Berry set		
1st (03/07/1999)	12.37 ± 1.41	3.70 ± 0.45
3rd (17/07/1999)	16.87 ± 1.15	5.97 ± 1.30
5th (08/08/1999)	41.25 ± 0.70	11.00 ± 0.65
Véraison		
7th (17/08/1999)	60.00 ± 2.18	27.00 ± 2.10
9th (31/08/1999)	102.00 ± 0.74	78.10 ± 1.90
11th (14/08/1999)	245.00 ± 2.37	180.00 ± 3.84

[a] Ten to fifteen replicates were made for each grape harvest. The grape pulp was the source of biological material utilized for the total soluble protein and total PR protein assays.

The Moscatel polypeptides present in each of the 11 lanes of Fig. 2a were transferred onto a membrane and probed with the anti-20 kDa Assario wine polypeptide antibodies (Fig. 2b). These highly specific antibodies only recognized proteins present in grapes at and after the 7th harvesting week, showing a strong accumulation of PR-proteins in the 10th and 11th week.

However, the resolving power of one-dimensional electrophoresis does not allow the identification of the major changes that occur to the pulp polypeptide pattern during berry development. In other words, the data illustrated in

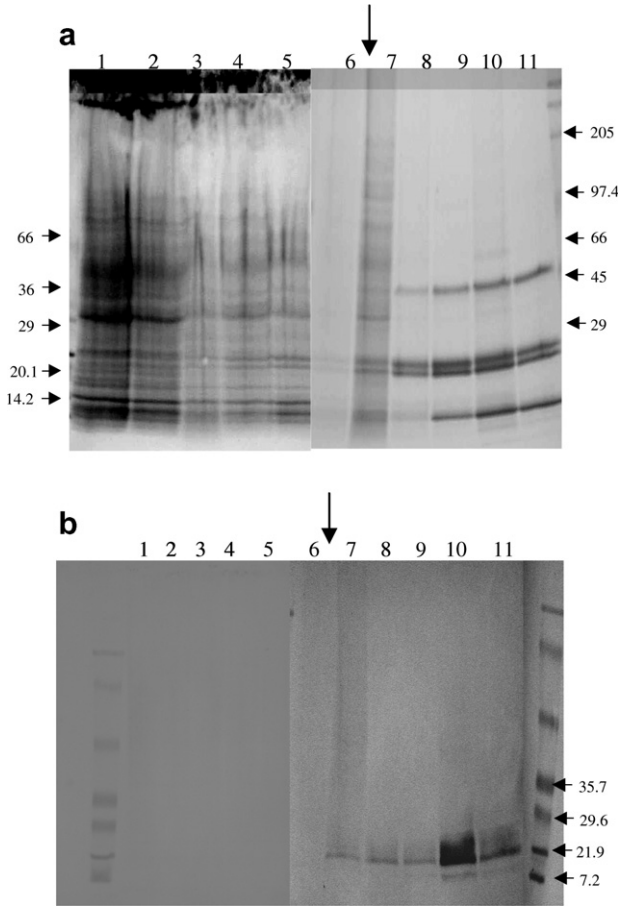

Fig. 2. SDS–PAGE of the total protein fraction extracted from 1999 Moscatel grape berries harvested along 11 consecutive weeks (marked on top of the gel) from post-flowering week to maturity (a) and probed with anti-20 kDa Assario wine polypeptide antibodies (b). Arrow indicates véraison. Each lane contained 100 μg (a) or 20 μg (b) of protein. Molecular mass standards (kDa) are indicated for the gel and the immunoblot.

Fig. 2 indicate that PR proteins are first detected in the grape berry at the 7th harvesting week, but accumulate at very high levels only during the final stages of development. No information is provided concerning the diversity with which PR proteins are synthesized.

To better understand these changes, the protein extracted from the berries was also analysed by two-dimensional electrophoresis and subjected to immunoblotting. *V. vinifera* (cv Moscatel) grapes were harvested at weekly intervals, in 2000, from post-flowering week until ripeness. The protein extracted from the berries was resolved by two-dimensional electrophoresis (pH 3–10) and stained for total protein (data not shown). Although the pulp protein content increased sharply along the ripening process, the number of distinct polypeptides undergoes a dramatic reduction from the onset of grape development to maturity. Furthermore, in the mature stage, the pulp polypeptides show a narrower range of p*I*'s (3–4) and molecular masses (from 22 to 30 kDa).

The Moscatel polypeptides present in the two-dimensional gels were transferred onto a membrane and probed with the anti-20 kDa Assario wine polypeptide antibodies (Fig. 3). In the 1st post-flowering week no protein could yet be detected by immunoblotting. The results presented in Fig. 3 do not support at all the previous hypothesis on the existence of one or a few precursors in the grape, common to most or all wine proteins. Rather, they indicate that the grape berry PR proteins, which comprise the wine proteins, are already synthesized in a variety of forms (Fig. 3b) during the early stages of berry development. In other words, the wide diversity of PR proteins that seems so typical of the wine polypeptides is not only already present in the mature grape, but also appears to be higher in earlier stages of berry development (Figs. 3f–b). There is, nevertheless, a large increment in total PR protein concentration during grape maturation, confirming the results shown in Table 2 and Fig. 2b.

The lower level of PR proteins complexity as grapes approach maturity is in part compensated by the appearance of some basic pH spots in the latest stages of maturity. Accumulation of a basic chitinase during berry ripening has been reported (Salzman et al., 1998).

2.3. Further evidence that the wide diversity of wine or mature grape proteins does not originate from a common precursor, but rather decreases during grape maturation

The profiles of the polypeptides extracted and separated by FPLC cation exchange chromatography from grape berries harvested at different stages of development in 1999 (Figs. 4a–c) show a few differences. Again, and as previously observed, along the ripening process the polypeptide pattern becomes less diverse but the protein concentration of the remaining peaks increases. This effect is clearly shown in Fig. 4d for the thaumatin-like protein and osmotin.

Each of the nine main peaks shown in Fig. 4b was collected, desalted, lyophilised and further fractionated by SDS–PAGE (Fig. 5a). It can be observed that each of the peaks eluted from the Mono S column contains several different polypeptides. When these proteins were transferred from the gel to a membrane and probed with the anti-20 kDa Assario wine polypeptide antibodies (Fig. 5b) only the bands corresponding to proteins with molecular masses from 22 kDa to around 30 kDa were recognized by the antibodies. The same set of experiments was

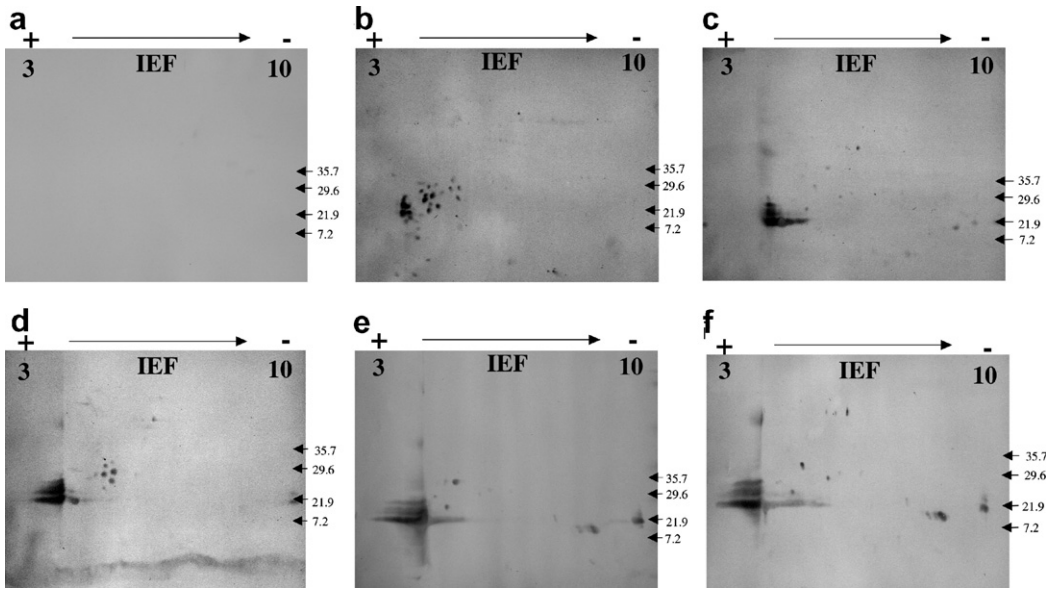

Fig. 3. PR protein pattern of Moscatel grape berries at different stages of development, harvested every other week, in 2000, from post-flowering week to maturity. The protein extracted from the berries and resolved by two-dimensional electrophoresis (pH 3–10) was transferred to a membrane and probed with the anti-20 kDa Assario wine polypeptide antibodies. Each gel contained 60 μg of protein. Molecular mass standards (kDa) are indicated for each harvest. (a), (b), (c), (d), (e) and (f): 1st, 3rd, 5th, 7th, 9th and 11th week, respectively.

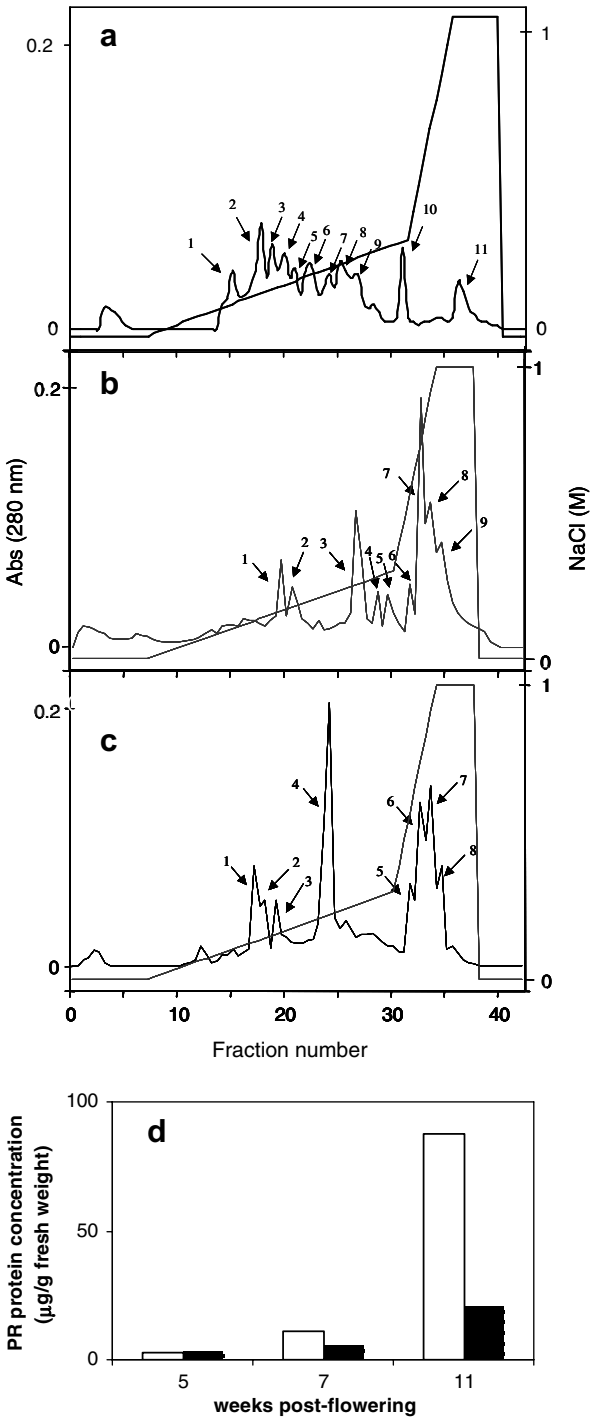

Fig. 4. Fractionation of the 1999 Moscatel grape pulp proteins by FPLC cation exchange chromatography. Protein dissolved in 20 mM citrate–NaOH buffer, pH 2.5, was loaded into the Mono S column of the FPLC, equilibrated in the same buffer. The bound proteins were eluted with a continuous gradient of NaCl (0–1 M). Profiles corresponding to the proteins extracted from grapes harvested in the 5th week (a), 7th week (b) and the 11th week (c) are shown. The polypeptide peaks corresponding to thaumatin-like protein (□) and to osmotin (■) were quantified (d).

performed with each of the eight main peaks shown in Fig. 4c (Figs. 5c and d).

The results obtained in Fig. 5 agree with those of Fig. 4. The two gels (Fig. 5a – proteins extracted from grape berries harvested in the 7th week and Fig. 5c – proteins extracted from berries harvested in the 11th week) show that, although the diversity of proteins present in the berries decreases along the ripening process, there is a sharp accumulation of the remaining proteins at maturity. These are the PR proteins shown on both blots (Figs. 5b and d) and recognized by the antibodies.

To further assess the high level of diversity of the PR proteins present in berries harvested in the 7th week, the nine main peaks separated by FPLC cation exchange chromatography (Fig. 4b) were desalted, lyophilised and subsequently subjected to FPLC anion exchange chromatography (Mono Q column, pH 9.8). Some of the peaks eluted from the Mono Q column were further separated by SDS–PAGE and subjected to immunoblotting using the anti-20 kDa Assario wine polypeptide antibodies (Fig. 6).

As a whole, Figs. 4–6 confirm and support the conclusions drawn from the analysis of Fig. 3 and contradict the hypothesis on the existence of one or a few precursors in the grape berry common to the wine proteins. Indeed, the wide diversity of distinct polypeptide structures characteristic of wines is not only already present in the mature grape but appears to decrease from early stages of development to maturity. That is, the grape PR proteins are apparently synthesized during the initial stages of berry development in a wide diversity of forms, accumulating in high concentrations as maturity approaches.

These experiments show that Moscatel grapes contain a large number (several tens and, possibly, many more) of polypeptides with different p*I*'s but similar molecular masses. These polypeptides are structurally similar, differing only by a few amino acid residues, as previously observed in the wines (Monteiro et al., 2001). The methodology employed does not allow the identification of different polypeptides with similar molecular mass and identical p*I*.

The 2D-immunoblots presented in Fig. 3 show that grape PR proteins are heterogeneous, comprising a considerable number of spots that vary both in electric charge (i.e. p*I*) and mass. Using different and independent approaches (different extraction and analytical procedures), evidence is provided indicating that these spots correspond to real and distinct PR protein molecules and are not the result of some artefact in 2D-electrophoresis. (i) Extraction – two distinct extraction procedures were employed in this work: one methodology suitable for protein extraction under native conditions, and utilized for the preparation of the data presented in Fig. 6, where strong evidence is presented for the variation in size and charge of grape PR proteins; the other methodology was performed under denaturing conditions and used to extract the polypeptides for the 2D-immunoblots shown in Fig. 3, where, once again, a considerable variation in size and charge is observed among the berry PR proteins. (ii) Analyses – the variation in the size of PR proteins detected in the 2D-immunoblots (Fig. 3) corresponds approximately to the range of molecular masses observed for mature grape PR proteins (24–

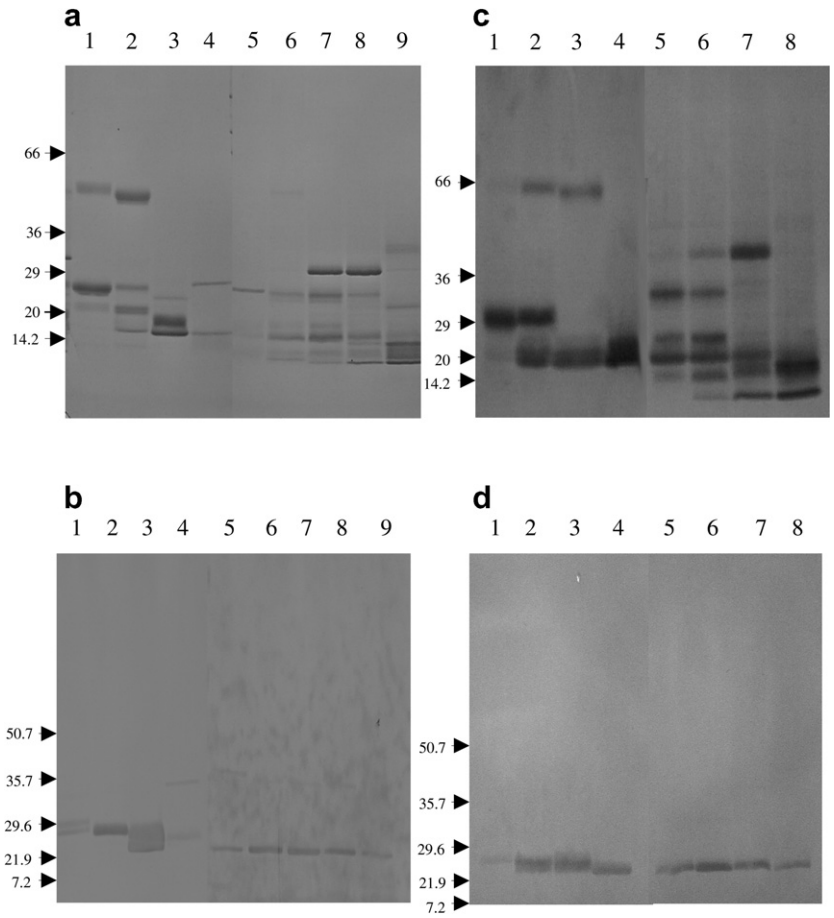

Fig. 5. The protein peaks previously separated by FPLC cation exchange chromatography (see Figs. 4b and c) were desalted, lyophilised and subjected to SDS–PAGE, and the polypeptides either stained with Coomassie Brilliant blue ((a) – 7th harvest and (c) – 11th harvest) or probed with the anti-20 kDa Assario wine polypeptide antibodies ((b) – 7th harvest and (d) – 11th harvest). Each lane contained 100 μg ((a) and (c)) or 20 μg ((b) and (d)) of protein. The molecular mass standards, in kDa, are shown on the gels and immunoblots.

28 kDa) obtained by Sarry and co-workers (Sarry et al., 2004) using MS after 2D-gel electrophoresis. This is in very good agreement with the results presented in Figs. 5b and d and 6 (insets, a–i). Variation in the charge of PR proteins is also clearly demonstrated in Fig. 6, where the individual PR proteins were first fractionated by cation exchange chromatography at pH 2.5 (Fig. 4), followed by anion exchange chromatography at pH 9.8 of each of the peaks obtained. The multitude of PR forms thus obtained were subsequently detected by immunoblotting (insets in Figs. 6a–i).

3. Discussion

The concentration of total soluble protein during the development of Moscatel grapes increases exponentially from berry set to maturity (Table 2). However, the diversity of polypeptides is greatly reduced during berry development, so that only a few, extremely abundant polypeptides are present in the berries in their later stages of development (Fig. 2a). Moscatel grape PR proteins follow a different pattern of synthesis and accumulation. Their concentration also increases along berry development but they represent an increasing fraction of the total grape proteins, comprising approximately 75% of the total protein at grape maturity (Table 2 and Fig. 4d). As reported before véraison acts as the trigger for the development-dependent accumulation of PR proteins in healthy grapes (Van de Rhee et al., 1994; Ferreira et al., 2002; and Table 2 and Fig. 4d). It is the case, for example, of VVTL1 (for *Vitis vinifera* thaumatin-like protein 1), which is encoded by a single gene and expressed in the berry only, in conjunction with the onset of sugar accumulation and softening (Tattersall et al., 2001). Also, the expression of two closely related chitinase genes (Vv*Chi*4A and Vv*Chi*4B) is fruit-specific and is induced at high rates throughout the sugar accumulation phase of ripening in grapes (Robinson et al., 1997). Pocock et al. (2000) reported the post-véraison accumulation of thaumatin-like proteins and chitinases as major soluble protein components in five cultivars of *V. vinifera*. These authors detected similar levels of PR proteins in both irrigated and water stressed, disease-free vines. The simultaneous accumulation of a basic chitinase and grape osmotin and sugars during berry ripening was correlated with the characteristic development of pathogen resistance that occurs in fruits during ripening (Salzman

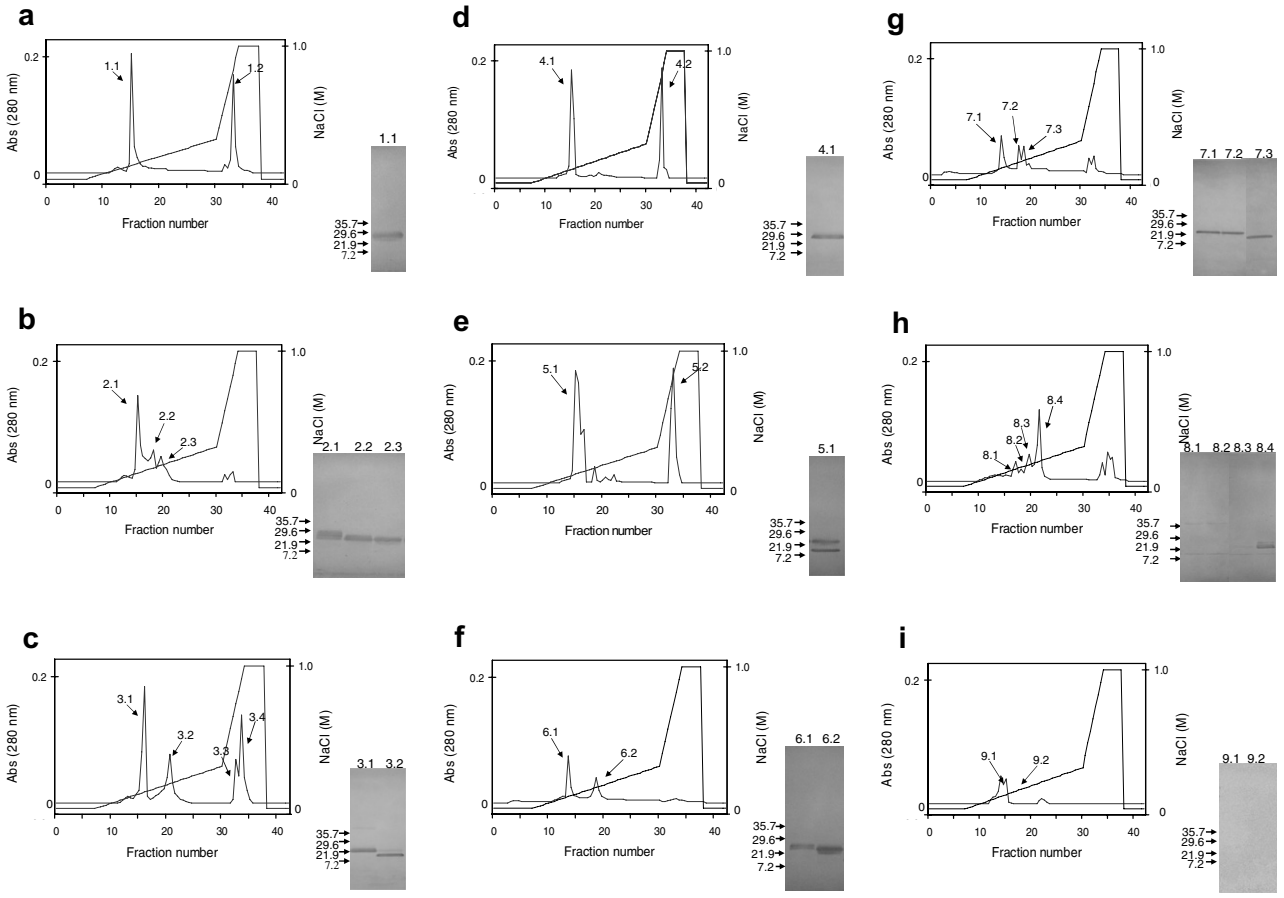

Fig. 6. Fractionation of the proteins separated by FPLC cation exchange chromatography (see Fig. 4) by FPLC anion exchange chromatography. Each of the nine main peaks obtained previously (Fig. 4b) was desalted, lyophilised and further separated by anion exchange chromatography on the Mono Q column of the FPLC at pH 9.8 ((a), (b), (c), (d), (e), (f), (g), (h) and (i), respectively). The bound proteins were eluted with a continuous gradient of NaCl (0–1 M). The insets show the immunoblot analysis (using the anti-20 kDa Assario wine polypeptide antibodies) of selected protein peaks eluted from the Mono Q column. Each lane contained 20 μg of protein. The molecular mass standards, in kDa, are shown for each blot.

et al., 1998). In this respect, it has been observed that the high levels of free hexoses induce the synthesis of a number of defence-related genes (Johnson and Ryan, 1990; Tsukaya et al., 1991; Herbers et al., 1995). Perhaps the expression of the majority of ripening-related genes is directly or indirectly caused by the onset of sugar accumulation, possibly due to the presence of regulatory "sugar boxes" within the promoters of these genes (Tsukaya et al., 1991).

As evidenced by 2D-immunoblots and by a combination of FPLC cation/anion exchange chromatographies followed by 1D-immunoblotting, grape PR proteins are synthesized in a wide variety of forms (Figs. 3–6). However, as maturity approaches, a decrease in diversity is observed among the PR proteins accumulated in the grapes, concomitantly with a large increase in their total concentration. These results do not support the previous hypothesis (Monteiro et al., 2001) on the existence of one or a few precursors, common to most of the proteins present in mature grapes or in wine, which could generate all the detected polypeptides by limited proteolysis and/or chemical modification. Rather, with the onset of grape ripening and sugar accumulation, there is a dramatic increase in concentration of PR proteins and the disappearance of other polypeptides present at the earlier stages of maturity. In fact the PR proteins are already synthesized with a great structural diversity.

The synthesis of the grape PR proteins in a wide diversity of structures is not surprising if we consider the information available in the literature. The vast array of PR proteins, the fact that many of these proteins are encoded by gene families (Jayaraj et al., 2004) and the pattern of expression of these proteins (discussed below for the grapevine PR proteins) may explain the results obtained in this work. Higher plants synthesize seven different classes of chitinases, which differ in protein structure, substrate specificity, mechanism of catalysis and sensitivity to inhibitions. The wide diversity of plant chitinases as well as the observation that many of these enzymes do not exhibit anti-fungal activity in *in vitro* assays has led to the suggestion that plant chitinases are involved not only in defense-related processes or general stress response, but also in numerous physiological events, including growth and development (Meins et al., 1994; Kasprzewska, 2003). A single study detected the presence of 13 chitinase isoforms

in grapevine tissues (Derckel et al., 1996). Since thaumatin-like proteins and chitinase resist proteolysis and precipitation by tannins during winemaking (Ferreira et al., 2002), their mass spectrometric determination was recently proposed as a new tool for spotting fraud on cultivars (Weiss et al., 1998; Hayasaka et al., 2001). Cluster analysis was also suggested to ascertain the origin of grape variety by considering the presence or absence of 11 spots detected on native PAGE gels (Moreno-Arribas et al., 1999). However, the application of these methodologies may be invalidated due to the partial inducible nature of the grapevine PR proteins (Ferreira et al., 2004). In this respect, three genomic sequences encoding chitinases were isolated by PCR walking on *V. vinifera* DNA. Two of these belong to class I chitinases with a putative vacuolar (*Vvchit 1a*) and extracellular (*Vvchit 1b*) localization while the third sequence belongs to class III (*Vvchit III*) (Robert et al., 2002). For the very limited number of environmentally conditions tested, this study indicated that chitinases are differentially expressed depending either on the developmental stage of berries or on the type of infecting pathogens.

In addition, to the development-dependent synthesis and accumulation of PR proteins, these proteins can also be induced in leaves and pre-véraison berries as part of an induced defence against the classical PR protein gene inducers (wounding, chemical elicitors, pathogen attack or abiotic stress), by the expression of specific PR genes (Ferreira et al., 2002). Taken together, these processes modulate the levels and proportions of the PR proteins in grapes, in a way that seems to depend on the cultivar, region, climate and agricultural practices. Thus, the actual pattern of proteins present in mature grapes appears to depend on the precise environmental and pathological conditions that occurred during vegetative growth. Indeed, a careful inspection of the published data reveals inconsistencies as to which proteins accumulate in mature grapes and a recent work showed that the environmental conditions prevailing during vegetative growth determine the exact pattern of major polypeptides that accumulate in mature grapes (Monteiro et al., 2003a,b).

4. Experimental

4.1. Biological material

Grapes (*Vitis vinifera* L. cv. Moscatel) were harvested in 1999 and 2000, at José Maria da Fonseca, Palmela, Portugal. The white wine used in the preparation of the antibodies was prepared from the single grape variety Assario. Ripened Assario grapes were harvested in 1994 in the Dão region, Portugal, and processed into wine by a conventional microvinification procedure according to the classical white wine technology. Wine and grapes were stored frozen at −80 °C until required.

Antibodies were previously and specifically produced against a major 20 kDa Assario wine polypeptide (Monteiro et al., 1999, 2001).

4.2. Extraction of total wine proteins

Wine aliquots (75 ml) were thawed and centrifuged at 15,800*g* for 5 min and the supernatant desalted at 4 °C on a PD-10 (Amersham Biosciences) column previously equilibrated with water. The protein samples (105 ml) were subsequently lyophilized and the dried residue resuspended and solubilized in 9 ml of 20 mM citrate–NaOH buffer, pH 2.5.

4.3. Extraction of the total protein from grapes

Two different extraction procedures were followed to isolate the proteins from mature grapes. In the experiments involving one-dimensional electrophoresis and FPLC ion exchange chromatography, the grape proteins were extracted following a methodology suitable to extract proteins from plant tissues rich in phenolic compounds (Pierpoint, 1996): The pulps from mature grapes (100 g) were ground to a fine powder under liquid nitrogen and homogenized in 250 ml of ice cold 250 mM Hepes/200 mM Tris buffer, pH 7.7, containing 10 mM $NaHCO_3$, 10 mM $MgCl_2$, 0.1 mM EDTA, 10 mM 2-mercaptoethanol, 10 mM sodium diethyldithiocarbamate, 0.1% (v/v) Tween 80 and 6 g PVPP. The solution was mixed in a blender and centrifuged at 10,000*g* for 40 min at 4 °C. The supernatant was passed through filter paper, desalted on a PD-10 column pre-equilibrated with water (pH adjusted to 7.5), frozen and lyophilized.

In the experiments involving two-dimensional electrophoresis, the protein from the grapes was extracted following the procedure described by (Tattersall et al., 1997). Grape pulps (4 g) were ground to a fine powder under liquid nitrogen and homogenized in 8 ml of 500 mM Tris–HCl buffer, pH 8.0, containing 5% (w/v) SDS, 10 mM dithiothreitol and 10 mM sodium diethyldithiocarbamate. The homogenate was incubated at 95 °C for 5 min and centrifuged at 12,000*g* for 5 min. The total protein was precipitated with trichloroacetic acid (10% w/v final), incubated for 15 min at 0 °C and centrifuged at 12,000*g* for 15 min. The resulting pellet was washed twice with an ice cold solution of ethanol:ethyl acetate (2:1, v/v). The pellet was dried under nitrogen, resuspended in a solution containing 7 M urea, 2 M thiourea, 2% (v/v) NP-40 and 1% (w/v) dithiothreitol, solubilized in a sonicator and desalted in NAP-10 columns (Amersham Biosciences) previously equilibrated in water. After lyophilization, the dried residue was solubilized in the same solution added with 0.5% (v/v) IPG-buffer, pH 3–10 (Amersham Biosciences).

4.4. Electrophoresis and immunoblotting (one dimension)

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed in standard sized gels,

by a modification (Christy et al., 1989) of the methods described by Weber and Osborn (1969) and Laemmli (1979). The molecular mass polypeptide standards used ranged from the 205 kDa subunit of rabbit muscle myosin to the 14.2 kDa of bovine milk α-lactalbumin. Immunoblotting was performed as described before (Ferreira et al., 2000).

4.5. Electrophoresis and immunoblotting (two dimensions)

Isoelectric focusing (first dimension) was performed using the IPGphor System (Amersham Biosciences). Immobiline Drystrip gel strips (IPG strips) (13 cm, pH 3–10) were obtained from Amersham Biosciences. IPG strips were rehydrated with 250 μl of a solution containing 0.5% (v/v) IPG-buffer, pH 3–10, 7 M urea, 2 M thiourea, 2% (v/v) NP-40, 1% (v/v) dithiothreitol and protein samples in the IPGphor strip holders. The program utilised for isoelectric focusing included the following steps: rehydration – 30 V, 12 h; step 1 – 200 V, 1 h; step 2 – 500 V, 2 h; step 3 – 1000 V, 2 h; step 4 – 8000 V, 3.5 h. After focusing, the gel strips were immediately frozen at −80 °C.

SDS–PAGE (second dimension) was performed as described above except that the gel contained only the separating gel. The gel strips were thawed and equilibrated for 15 min, with agitation, in 50 mM Tris–HCl buffer, pH 8.8, containing 6 M urea, 26% (v/v) glycerol, 2% (w/v) SDS and 1% (w/v) dithiothreitol. The strips were subsequently equilibrated for another 15 min, with agitation, in a similar solution that contained 2.5% (w/v) iodoacetamide (instead of the dithiothreitol), placed on top of the SDS–PAGE gel, sealed with 0.5% (w/v) agarose and electrophoresed (220 V, 15 mA for 15 min, followed by 220 V, 30 mA).

The preparation of the immunoblots from the 2 D-gels was performed as described above (Ferreira et al., 2000). A minimum of three replicates of each 2D-immunoblot were performed for each grape harvest.

4.6. Fractionation of the grape proteins by FPLC ion exchange chromatography

The solution containing the grape proteins, dissolved in 20 mM citrate–NaOH buffer, pH 2.5, was cleared by passage through a carboxymethyl-cellulose column equilibrated in the same buffer. The bound proteins were eluted with buffer containing 1 M NaCl, desalted in 20 mM citrate–NaOH buffer, pH 2.5, and loaded in the cation exchange Mono S HR5/5 column (Amersham Biosciences) previously equilibrated in the same buffer. The flow rate was 1.5 ml min^{-1} and the bound proteins were eluted with a continuous gradient (0–1 M) of NaCl.

Each of the peaks obtained from the continuous gradient was, subsequently, desalted in PD-10 Sephadex G-25M columns previously equilibrated with water, lyophilised, dissolved in 20 mM piperazine buffer, pH 9.8, and fractionated by FPLC anion exchange chromatography on a Mono Q HR5/5 column (Amersham Biosciences) previously equilibrated in the same buffer. The bound proteins were eluted with a continuous gradient (0–1 M) of NaCl.

4.7. Protein determination

Protein concentrations were measured according to the method described by Bensadoun and Weinstein (1976).

4.8. Polypeptide sequencing

All the solutions used in the gels and the electrophoresis buffers were freshly prepared and filtered (Whatman 3MM). The SDS used was either ultra-pure or twice recrystallised from ethanol and water (Hunkapiller et al., 1983). To prevent blockage of the amino terminals of the polypeptides the gels were subjected to a pre-electrophoresis (30 min at 200 V) in 350 μl glutathione 10 mM per 70 ml of the upper buffer (to avoid degradation of tryptophan and methionine residues). Electrophoresis was, then, performed by adding 70 μl of thioglycolic acid 100 mM to 70 ml of the upper buffer (Applied Biosystems, Foster City, CA) according to manufacturer's instructions. These precautions are meant to remove charged impurities, non-charged reactive species like acrylamide monomers and other reactive substances, and to reduce peroxides and residual radicals (Choli and Wittmann-Liebold, 1990). The membrane used for electroblotting (Harlow and Lane, 1988) was a ProBlot poly-vinylidene difluoride polymer (PVDF) from Applied Biosystems. The buffer used for electroblotting was a 10 mM solution of 3-(cyclohexamide)-1-propanesulfonic acid (CAPS) in 10% (v/v) methanol (LeGendre and Matsudaire, 1989). Electroblotting was performed for 1 h and 15 min at 15 V and 4 °C in a Trans-Blot Semi-Dry Transfer Cell from BioRad. The membranes were stained with Ponceau S (Applied Biosystems) according to manufacturer's instructions. The polypeptides immobilised on the membranes were, then, sequenced (Edman degradation) on a protein sequencer (Perkin–Elmer – Applied Biosystems, model 477A) online with an HPLC analyser (model 120A).

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