The Wide Diversity of Structurally Similar Wine Proteins

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In the present work, single grape variety wines, Moscatel and Arinto, were used. Analysis by denaturing polyacrylamide gel electrophoresis of the wine proteins revealed the presence of only a few 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, showed that these wines contain a very large number (many tens and, possibly, many more) of distinct polypeptides, exhibiting similar molecular masses but different electrical charges. The results obtained using highly specific antibodies and N-terminal sequencing indicate that there is structural similarity among most of the wine polypeptides. These observations can be explained by the existence of a common precursor to most or all of the wine proteins, which could generate all of the detected polypeptides by limited proteolysis. Comparison of the N-terminal sequences of the polypeptides isolated from Moscatel wine with proteins from other sources revealed a high degree of homology to pathogenesis-related proteins.

Keywords: *Wine proteins; protein structure; haze; PR proteins; antibodies; FPLC; immunoblotting; electrophoresis; sequencing*

INTRODUCTION

Wines, like many other natural food products, contain varying amounts of different nitrogenous substances (*1*). Of these, proteins are of most concern to winemakers, not due to their insignificant contribution to the nutritional value of wines (protein concentration in wines varies typically from 15 to 230 mg/L) but because they may become insoluble and precipitate. The appearance of a haze or deposit in the bottled wine would indicate that it is unstable and therefore unacceptable for sale.

Work pertaining to wine proteins and their influence on stability was initiated in California by Colby (*2*). Several techniques have been used by different groups of researchers in these studies: ion exchange FPLC (*3*−*5*), chromatofocusing on FPLC (*6*), HPLC (*7*, *8*), size exclusion chromatography (*9*), affinity chromatography (*4*, *9*), capillary electrophoresis (*10*), isoelectric focusing (*6*, *8*, *11*−*14*), and protein blotting (*11*−*13*).

More recently (*15*), polyclonal antibodies were raised against specific proteins as well as against the total protein from Assario wine. FPLC cation exchange chromatography was used to isolate the total protein fraction and, when in combination with denaturing electrophoresis, to purify individual wine polypeptides. The antibodies produced were shown to be highly specific for the corresponding antigens. When the total protein composition of grape skin, pulp, seed, stem, leaf, and yeast was analyzed, the Assario wine proteins were found to derive entirely from the pulp (*16*). Important changes were noted to occur to the pulp proteins during vinification. On the other hand, grapes from different, white or red, varieties were found to contain a set of polypeptides identical to those present in the Assario grapes.

In the present work, the proteins from two single grape variety wines, Moscatel and Arinto, were purified and studied. To easily probe the presence of the wine proteins, antibodies were raised against a 20 kDa polypeptide from the single grape variety wine Assario. The N-terminal sequences of 13, randomly selected, polypeptides isolated from the Moscatel wine were compared with those of proteins from other sources.

MATERIALS AND METHODS

Preparation of Wine. The white wines used were prepared from single grape varieties as previously described (*16*). Ripened Moscatel and Arinto grapes were harvested in 1997 and 1998, respectively, in Estremadura, Portugal. Ripened Assario grapes were harvested in 1994 in the Dão region of Portugal.

Protein Purification by FPLC Ion Exchange Chromatography. Wine aliquots were thawed and centrifuged at 15000*g* for 5 min, and the supernatant was desalted at 4 °C on prepacked PD-10 Sephadex G-25M columns (Pharmacia/LKB, Uppsala, Sweden), previously equilibrated with water (Milli-Q plus, Millipore, Bedford, MA). The protein samples were subsequently lyophilized (Edwards Micro Modulyo freeze-drier, Crawley, Sussex, U.K.), and the dried residue was solubilized in 20 mM citrate−NaOH buffer, pH 2.5.

A sample containing the wine (Moscatel and Arinto) total proteins was purified or fractionated by FPLC cation exchange chromatography on a Mono S HR5/5 column (Pharmacia/LKB) previously equilibrated in 20 mM citrate−NaOH buffer, pH 2.5. The bound proteins were eluted with a step gradient (0/1

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M, for the isolation of total proteins) or a continuous gradient (0−1 M, for the fractionation of the individual wine proteins) of NaCl. Each of the peaks obtained with the continuous gradient was subsequently desalted in PD-10 Sephadex G-25M columns previously equilibrated with water, lyophilized, dissolved in 20 mM piperazine buffer, pH 9.8, and fractionated by FPLC anion exchange chromatography on a Mono Q HR5/5 column (Pharmacia/LKB) previously equilibrated in the same buffer. The bound proteins were eluted with a continuous gradient (0−1 M) of NaCl.

For the purification of the 20 kDa Assario polypeptide a wine sample was subjected to FPLC cation exchange chromatography on the Mono S HR5/5 column (Pharmacia/LKB) previously equilibrated in 20 mM citrate−NaOH buffer, pH 2.5. The bound proteins were eluted with a continuous gradient (0−1 M) of NaCl. The 20 kDa Assario polypeptide was further purified by preparative SDS-PAGE, as previously described (*15*).

Electrophoresis, Western Blotting, and Immunoblotting. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to a modification (*17*) of the methods described by Weber and Osborn (*18*) and Laemmli (*19*). The modification included the use of *m*-cresol purple as the tracking dye and the inclusion of 0.1 M sodium acetate in the anode buffer to allow the resolution of polypeptides with molecular masses ranging from 2.5 to >200 kDa. 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.

Western blotting was performed using a semidry electrophoretic transfer cell (Trans-Blot SD, Bio-Rad, Richmond, CA) as described before (*15*). After protein transfer, the proteins were immobilized on nitrocellulose membranes (*20*), visualized by incubation in an amido black 10B/methanol/acetic acid solution, and destained in 2-propanol/acetic acid. The blots destined for immunoblotting were processed (*21*) including Tween 20 (0.05% v/v) in the antibody-containing solutions to reduce unspecific binding, and nonfat dried milk was used as a blocking agent to saturate the remaining protein binding sites.

Production of Antibodies and Preparation of Antisera. New Zealand female rabbits were immunized with purified antigen (400 μg of the 20 kDa Assario polypeptide) in complete Freund's adjuvant. To obtain a high titer, three boosters injections of 400 μg of antigen each were given every 2 weeks in complete Freund's diluted 1:10 with incomplete adjuvant. Total blood was taken from the heart 12 days after the third booster injection. Blood samples were allowed to clot, and the serum was collected, centrifuged, and stored at −70 °C.

Isolation of the Total Soluble Proteins from *Lemna minor*. The aquatic, higher plant, *L. minor* L., was grown as described in ref *22*. *Lemna* fronds were frozen in liquid nitrogen, ground to a fine powder, and the total soluble protein extracted in 100 mM Tris-HCl buffer, pH 7.5, containing 1 mM phenylmethylsulfonylfluoride (PMSF). The homogenate was filtered through two layers of cheesecloth and centrifuged for 10 min at 40000*g* and 2 °C, and the supernatant was desalted at 2 °C on a PD-10 column previously equilibrated with 20 mM Tris-HCl buffer, pH 7.5.

General Assays. Protein content was measured using a modification of the Lowry method (*23*). Total polysaccharides were also determined (*24*, *25*).

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 ultrapure or twice recrystallized from ethanol and water (*26*). 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 of 10 mM glutathione per 70 mL of the upper buffer (to avoid degradation of tryptophan and methionine residues). Electrophoresis was, then, performed, by adding 70 μL of 100 mM thioglycolic acid to 70 mL of the upper buffer (*27*). These precautions are meant to remove charged impurities and noncharged reactive species such as acrylamide monomers and other reactive substancess and to reduce peroxides and residual radicals (*28*). The membrane used for electroblotting (*29*) was a ProBlot polyvinylidene difluoride polymer (PVDF) from Applied Biosystems (*30*). The buffer used for electroblotting was a 10 mM solution of 3-[cyclohexamide]-1-propanesulfonic acid (CAPS) in 10% (v/v) methanol (*30*, *31*). Electroblotting was performed for 1 h and 15 min at 15 V and 4 °C in a TransBlot semidry transfer cell from Bio-Rad. The membranes were stained with Ponceau S (*30*). The polypeptides immobilized in the membranes were, then, sequenced—Edman degradation—in a protein sequencer from Perkin-Elmer-Applied Biosystems (model 477A) on-line with an HPLC analyzer (model 120A).

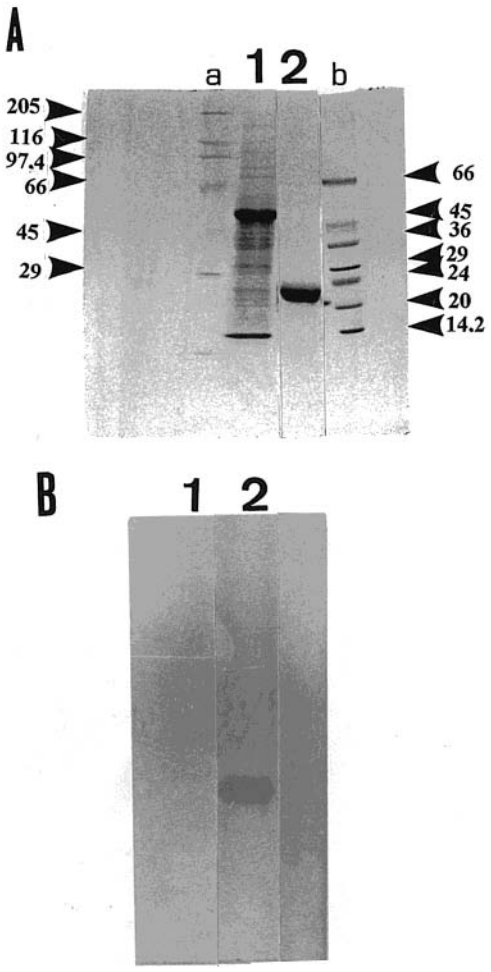

Figure 1. SDS-PAGE of the purified total protein fraction (Pt) of Moscatel wine (A) and Arinto wine (B). The protein loaded in each well was 100 μg. Lanes a and b are molecular mass standards (kDa).

RESULTS AND DISCUSSION

Each of the white wines was prepared from the single grape varieties Moscatel and Arinto. The use of a modification of the Lowry method (*23*) showed that the protein contents of the Moscatel and Arinto wines were 160 and 230 mg/L, respectively.

A simple SDS-PAGE analysis of the purified Moscatel and Arinto wine proteins revealed the presence of polypeptides ranging in molecular mass from 15 to 30 kDa (Figure 1).

Purification of the individual Moscatel proteins by FPLC cation exchange chromatography (Mono S column; pH 2.5) showed the presence of 12 major components (Figure 2A). The wine components with no positive charge at pH 2.5 did not bind to the column

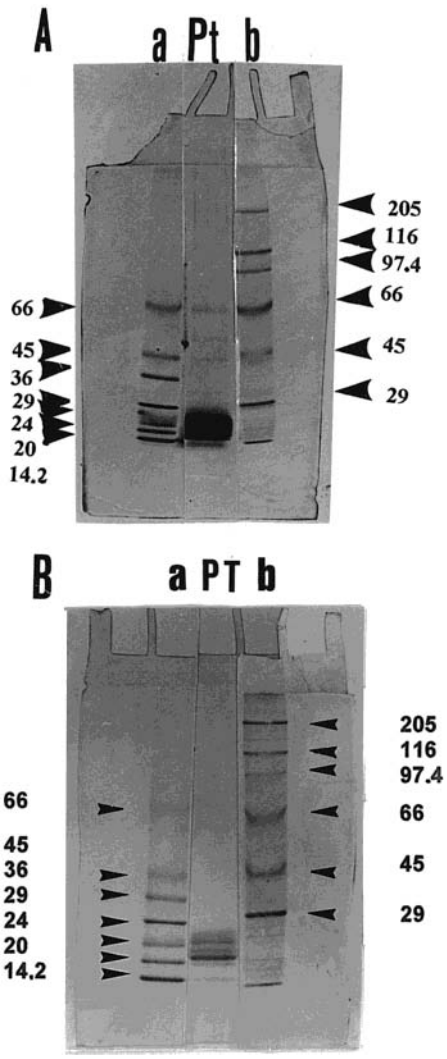

Figure 2. Fractionation of the total Moscatel wine proteins by FPLC and subsequent analysis by SDS-PAGE. (A) A wine sample was centrifuged, desalted, lyophilized, and fractionated by cation exchange chromatography on the Mono S column of the FPLC, at pH 2.5. The bound proteins were eluted with a continuous gradient of NaCl (0−1 M). Each of the 10 major protein peaks obtained previously was desalted, lyophilized, and further fractionated by anion exchange chromatography on the Mono Q column of the FPLC, at pH 9.8. The bound proteins were eluted with a continuous gradient of NaCl (0−1 M). (B) SDS-PAGE of the protein peaks eluted from the Mono Q column. The protein loaded was 100 μg/lane. Lanes a and b are molecular mass standards (kDa). Lanes L and Pt are *L. minor* total soluble protein and Moscatel wine total protein, respectively.

and appeared as a large A_{280} peak (peak 1), whereas the components with positive charge were eluted sequentially with a continuous gradient (0−1 M) of NaCl (peaks 2−12). Peak 1 contains polysaccharides, and peaks 3−12 revealed the presence of protein (*15*). Each of the 10 major protein peaks was subsequently desalted, lyophilized, and further fractionated by FPLC anion exchange chromatography on the Mono Q column of the FPLC, at pH 9.8, into several major distinct peaks with a continuous gradient of NaCl (0−1 M). SDS-PAGE of each of the peaks eluted from the Mono Q column (Figure 2A) shows that each can still be resolved into several different polypeptides (Figure 2B). Therefore, these experiments indicate that the Moscatel wine contains a large number (many tens) of polypeptides with different p*I* values but similar molecular masses.

Probably, these polypeptides are structurally similar, differing only by a few amino acid residues. In fact, it was observed that two Moscatel polypeptides resolved by native electrophoresis at pH 8.8 showed the same molecular mass by SDS-PAGE and the same electrical charge at both pH 2.5 (Mono S column) and pH 9.8 (Mono Q column) (data not shown). This observation raised the possibility that many more distinct polypeptides may be present in wines. However, only those differing in a significant way with regard to size or charge can be resolved by the techniques employed in this work.

In an attempt to determine if there is structural similarity among the large number of wine polypeptides, immunological methods were employed using highly specific polyclonal antibodies produced against a major 20 kDa polypeptide from the single grape variety, white wine, Assario.

To test the specificity of the antibodies produced against the major 20 kDa Assario wine polypeptide, *L. minor* was used as a control, not only because *Lemna* cells contain a very wide range of different polypeptides but also because two of them are extremely abundant—the large (52 kDa) and small (14.5 kDa) subunits of the photosynthetic enzyme ribulose bisphosphate carboxylase (*21*). These antibodies proved to be highly specific for the Assario polypeptide (Figure 3). The SDS gel presented (Figure 3A) shows the protein patterns of *L. minor* cells and of the 20 kDa Assario polypeptide. When the proteins present in the gel were transferred onto a nitrocellulose membrane and the resulting blot stained with amido black (results not shown), it was observed that the transfer process had been highly efficient. When a similar blot was processed and probed with the anti-20 kDa Assario polypeptide antibodies (Figure 3B), no signal was produced with the *Lemna* proteins, despite the extremely large number of different structures (proteins) present and the high abundance of some of them. This result clearly indicates that these antibodies possess an extremely high specificity and can be used in various aspects of wine protein science and technology. The specificity of the antibodies was also assessed using positive controls such as protein extracts prepared from Assario and other grapes or from Assario and other wines (*15*, *16*).

The Moscatel polypeptides present in each of the peaks eluted from the Mono Q column (Figure 2A) were fractionated by SDS-PAGE (Figures 2B and 4A), transferred to a membrane, and probed with the anti-20 kDa Assario polypeptide antibodies. Most polypeptides produced strong signals (Figure 4). These results strongly suggest not only that there is structural similarity between the 20 kDa Assario polypeptide and the Moscatel polypeptides but also that the majority of the Moscatel polypeptides are structurally related. The data obtained support the hypothesis of the existence of a common precursor to most or all of the Moscatel wine proteins, which subsequently generates a large number of polypeptides by limited proteolysis.

To confirm the structural similarity of the Moscatel polypeptides, 13 of these polypeptides were randomly selected and, subsequently, subjected to N-terminal sequencing. The sequences and percent homologies compared with those of proteins from other sources are presented in Table 1. The polypeptide corresponding to peak 3.1 (Figure 2 and Table 1), a peptide from which 21 amino acid residues were identified, exhibits a very

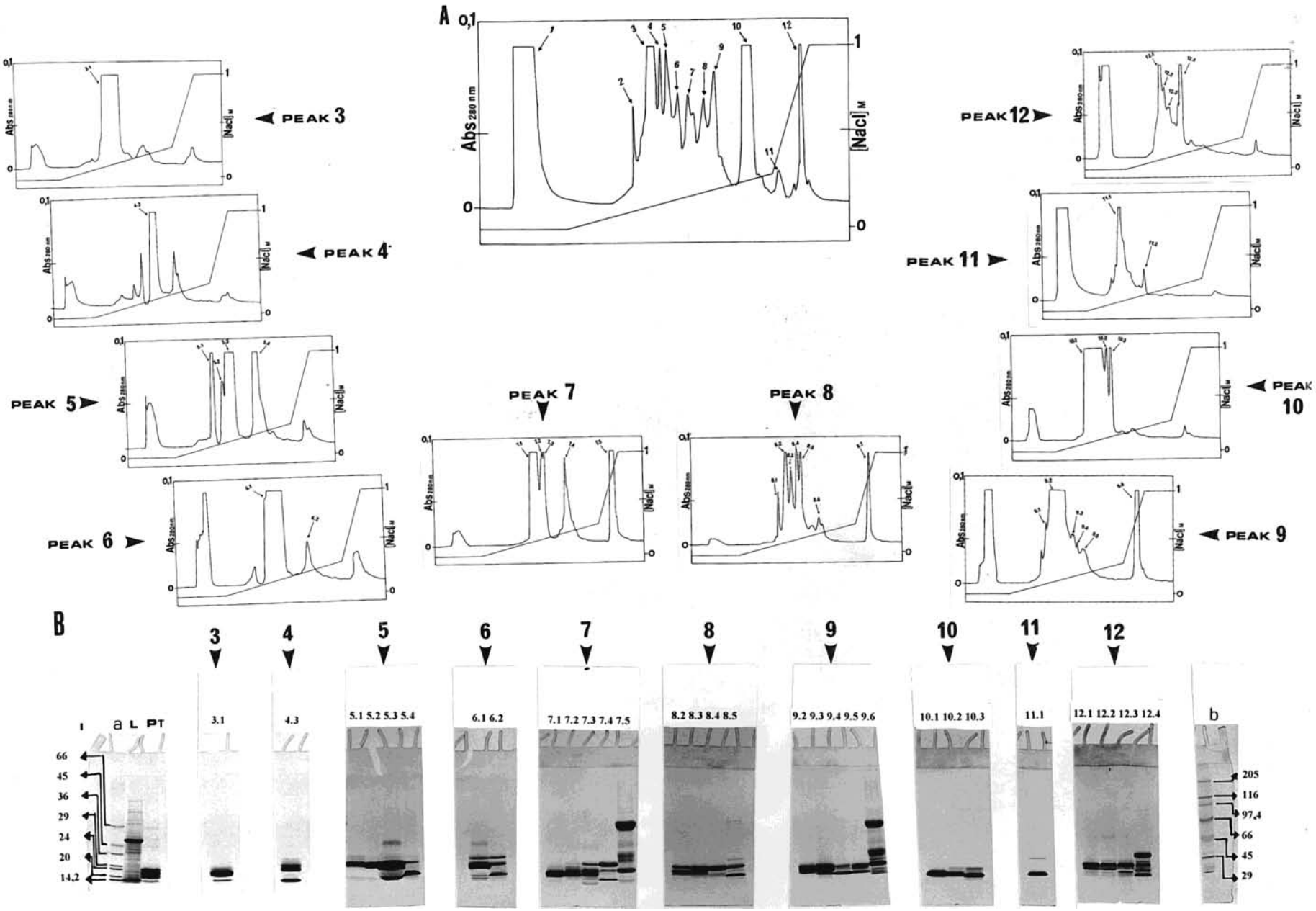

Figure 3. Specificity of the antibodies. Total protein from *L. minor* (lane 1) and the 20 kDa Assario polypeptide (lane 2) were subjected to SDS-PAGE and the polypeptides stained with Coomassie Brilliant Blue (A) or subjected to immunoblotting and probed with the anti-20 kDa Assario polypeptide antibodies (B). Lanes a and b are molecular mass standards (kDa). Lane 1 is *L. minor* total soluble protein [40 μL of extract in (A) and 20 μL in (B)]; lane 2 is 20 kDa Assario polypeptide [80 μg of protein in (A) and 9 μg in (B)].

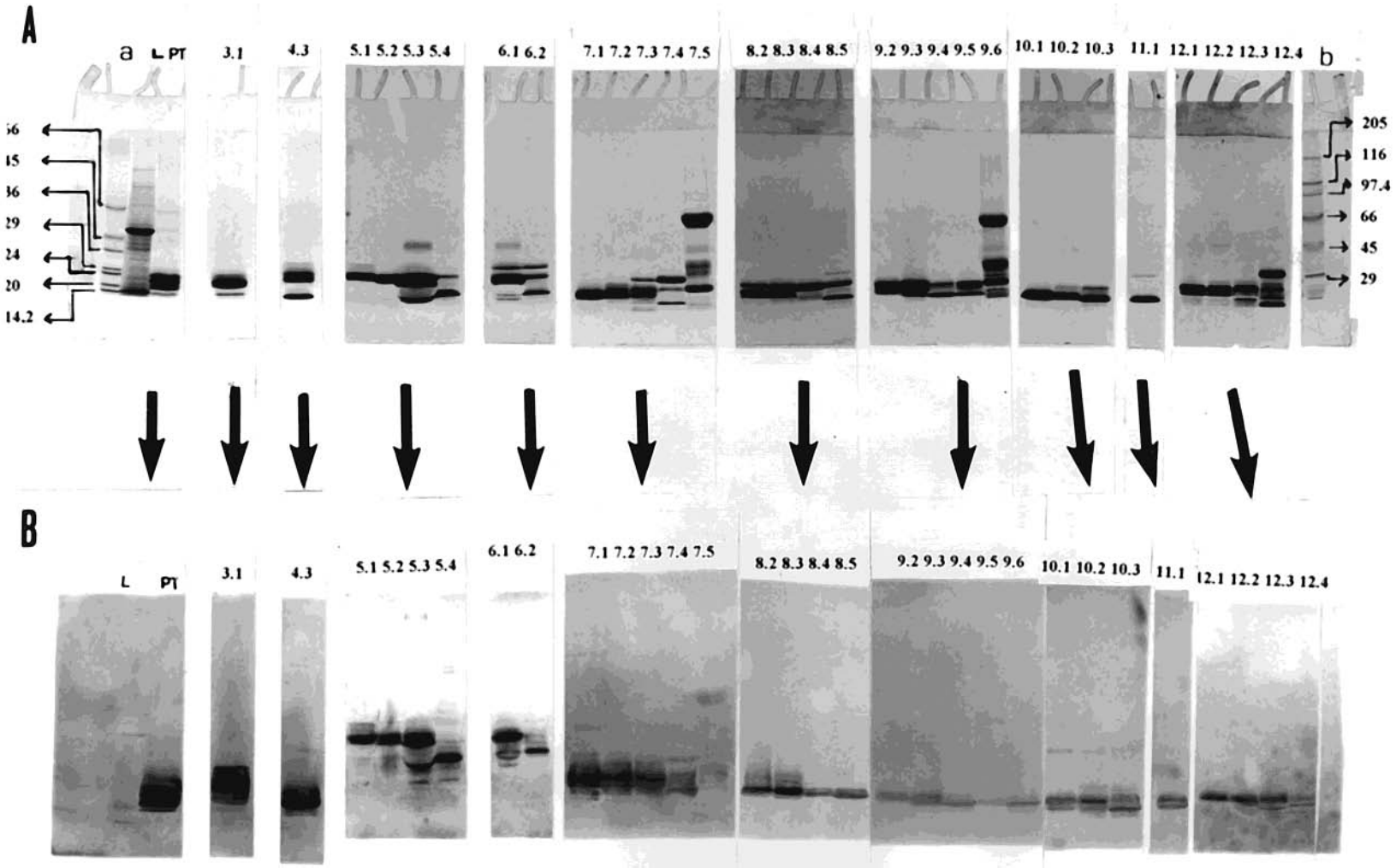

Figure 4. Search for structurally similar proteins in different Moscatel protein fractions. The Moscatel polypeptides were fractionated by FPLC ion exchange chromatography, as explained in the legend to Figure 3, subjected to SDS-PAGE (A) or probed with anti-20 kDa Assario polypeptide antibodies (B). The protein loaded on each lane was 100 μg (A) or 20 μg (B). Lanes a and b are molecular mass standards (kDa). Lanes L and Pt are *L. minor* total soluble protein and Moscatel wine total protein, respectively.

Table 1. Sequences of the Moscatel Wine Polypeptides and Comparison with the Sequences of Proteins from Other Sources[a]

PROTEIN	SOURCE	ALIGNMENT			PERCENT IDENTITY
Peak 3.1	Moscatel wine	1	LSQSDGRVNLVELGGYVVDIR	21	
PAU5 YEAST	*Sac.cerevisiae* (a)	26	LSQSDERVNLVELGVYVSDIR	46	85.71
PAU6 YEAST	*Sac.cerevisiae* (b)	26	LSQSDERVNLVELGVYVSDIR	46	85.71
YAJO YEAST	*Sac.cerevisiae* (c)	26	LSQSDERVNLVELGVYVSDIR	46	85.71
PAU1 YEAST	*Sac.cerevisiae* (d1)	24	LTQSDERVNLVELGVYVSDIR	44	80.95
PAU2 YEAST	*Sac.cerevisiae* (d2)	24	LAQSDERVNLVELGVYVSDIR	44	80.95
Peak 9.5	Moscatel wine	1	YDDTTAMFAAQ	11	
GLUTAMINE SYNTHETASE	*Vitis vinifera* (grape) (e)	53	YDDTTVVF	60	75.00
PUTATIVE PHOSPHO-2-D	*Schizosac. pombe* (f)	348	WDDTTAVF	355	75.00
GAS VESICLE PROTEIN	*Halobac.salinarium* (g)	184	FDDTAASFAA	193	70.00
RIBOSOME RECYCLING F	*Strept. coelicolor* (h)	161	DDTTAKYVAQ	170	70.00
C-GVPC PROTEIN	*Halobac.salinarium* (i)	184	FDDTAASFAA	193	70.00
Peak 10.1	Moscatel wine	1	ATFDILNKKTYTVVAAAA	18	
Peak 11.1	Moscatel wine	1	ATFDILNKKTYTVVAAAA	18	
THAUMATIN-LIKE PROTEIN	*Vitis vinifera* (grape)(j)	25	ATFDILNKCTYTVWAAAS	42	83.33
PRR1 TOBAC	*Nicotiana tabacum* (k1)	26	ATFDIVNKCTYTVWAAAS	43	77.77
PRR2 TOBAC	*Nicotiana tabacum* (k2)	26	ATFDIVNQCTYTVWAAAS	43	72.22
OSL3 ARATH	*Arabidopsis thaliana* (l)	23	ATFEILNQCSYTVWAAAS	40	66.67
OSMOTIN PRECURSOR	*Arabidopsis thaliana* (m)	23	ATFEILNQCSYTVWAAAS	40	66.67
Peak 12.4	Moscatel wine	1	LSNAGGSPSQLNNDEGQG	18	
R13H4.6 PROTEIN	*Caenorhabditis elegans* (n1)	3	ARAGGNPSQLN	13	72.73
YJH1 YEAST	*Sac.cerevisiae* (o)	190	LNNAGGIPSGERND	203	64.29
Y53C12A.3 PROTEIN	*Caenorhabditis elegans* (n2)	50	SNAGGSPDEQNPQFG	64	60.00
Peak 4.3	Moscatel wine	1	ATFNIQNHHSYTVVAAAVPGGGMQL	25	
Peak 5.1	Moscatel wine	1	ATFNIQNHHSYTVVAAAVPGGGMQL	25	
Peak 5.4	Moscatel wine	1	ATFNIQNHHSYTVVAAAVPGGGMQL	25	
Peak 6.2	Moscatel wine	1	ATFNIQNHHSYTVVAAAVPGGGMQL	25	
Peak 7.1	Moscatel wine	1	ATFNIQNHHSYTVVAAAVPGGGMQL	25	
Peak 8.5	Moscatel wine	1	ATFNIQNHHSYTVVAAAVPGGGMQL	25	
Peak 9.2	Moscatel wine	1	ATFNIQNHHSYTVVAAAVPGGGMQL	25	
Peak 10.3	Moscatel wine	1	ATFNIQNHHSYTVVAAAVPGGGMQL	25	
OSMOTIN-LIKE PROTEIN PREC.	*Vitis vinifera* (grape) (p)	25	ATFNIQNHCSYTVWAAAVPGGGMQL	49	92.00
RIPENING-ASSOCIATED PROTEIN	*Musa acuminata (q)*	27	ATFXIVNRCSYTVWAAAVPGGGRQL	51	76.00
BASIC CHITINASE	*Citrus sinensis* (r)	1	ATFDIRNNXPYTVWAAAVPGGGRRL	25	68.00
THAUMATIN-LIKE PROTEIN	*Actinidia deliciosa* (s)	1	ATFNIINNCPFTVWAAAVPGGGKRL	25	68.00
PRR2 TOBAC	*Nicotiana tabacum* (k2)	26	ATFDIVNQCTYTVWAAASPGGGRQL	50	68.00

[a] The databases consulted were Swiss-Prot/TrEMBL (www.expasy.ch/cgi-bin/) and EMBL Outsation, European Bioinformatics Institute (www2.ebi.ac.uk). (a) Reference *32*; AN P43575 (TrEMBL). (b) Reference *33*; AN P52921 (TrEMBL). (c) Reference *34*; AN P39545 (TrEMBL). (d) Reference *35*. (d1) AN P338924. (d2) AN P322612 (TrEMBL). (e) Refernce *36*; AN P51118 (TrEMBL). (f) Reference *38*; AN PQ09755 (TrEMBL). (g) Reference *39*; AN P08958 (TrEMBL). (h) Reference *44*; AN O86770 (TrEMBL). (i) Reference *40*; AN CAA45993 (EMBL). (j) Reference *45*; AN Q04708 (TrEMBL). (k) Reference *46*. (k1) AN P13046. (k2) AN P07052 (TrEMBL). (l) Reference *47*; AN P50700 (TrEMBL). (m) Reference *48*; AN CAB39936 (EMBL). (n) Reference *60*. (n1) AN P90952. (n2) AN O18212 (TrEMBL). (o) Reference *59*; AN P40360 (TrEMBL). (p) Reference *61*; AN P93621 (TrEMBL). (q) Reference *62*; AN O22322 (TrEMBL). (r) Reference *63*; AN G1881844 (TrEMBL). (s) Reference *64*; AN P81370 (TrEMBL).

high degree of homology (80.95− 85.71%) to several *Saccharomyces cerevisiae* protein segments (*32*−*35*). The yeast *S. cerevisiae* has been the focus of intensive studies as a model eukaryote. Some of the genes found are newly sequenced and yet of unknown function. Proteins with homologous sequences, highly conserved, may play identical roles in different organisms. It seems likely that the polypeptide corresponding to peak 3.1 derives from the yeast, although it has been shown that the bulk of the wine proteins typically originate in the pulp (*16*).

The polypeptide corresponding to peak 9.5 (Figure 2 and Table 1), from which 11 amino acid residues were identified, shows high homology with segments of five other proteins, apparently unrelated. Homology of 75.00% with glutamine synthetase from *Vitis vinifera* (*36*), a key enzyme in the nitrogen metabolism of higher plants responsible for the initial step in ammonia assimilation (*37*), is seen. Peak 9.5 also presents a homology of 75.00% (*38*) with putative phospho-2-dehydro-3-deoxyheptonate aldolase, a yeast enzyme important in the first step in the biosynthesis from chorismate of the aromatic amino acids (the shikimate pathway). The same peak is 70.00% homologous (*39*, *40*) to a gas vesicle protein present in aquatic bacteria, which provides buoyancy and, thus, increases the availability of light and oxygen to cells (*41*−*43*). Peak 9.5 also shows 70.00% homology with bacterial ribosome recycling factor (*44*), a cytoplasmic (by similarity) protein responsible for the release of ribosomes from messenger RNA at the termination of protein biosynthesis. It may increase the efficiency of translation by recycling ribosomes from one round of translation to another (by similarity). This homology of the polypeptide present in peak 9.5 with proteins present in microorganisms may indicate that it is of microbial origin. Alternatively, it may represent a protein derived from the grape pulp that has not yet been purified and sequenced in this tissue.

The polypeptides present in peaks 10.1 and 11.1 (Figure 2 and Table 1), with 18 amino acid residues sequenced each, are identical. They exhibit a high degree of homology with segments of pathogenesis-related (PR) proteins and with the polypeptides from peaks 4.3, 5.1, 5.4, 6.2, 7.1, 8.5, 9.2, and 10.3. In fact, they have an 83.33% homology with one of the most abundant proteins in extracts of mature grape berries (*Vitis vinifera* cv. Muscat of Alexandria). This protein was purified and identified by amino acid sequence to be a thaumatin-like protein, VVTL1 (*45*). VVTL1 is found in the berry only and is encoded by a single gene that is expressed in combination with the onset of sugar accumulation and softening. The exact role of VVTL1 is unknown, but the timing of its accumulation correlates with the inability of fungal pathogen powdery mildew (*Uncinula necator*) to initiate new infections of the berry. The presence of thaumatin-like proteins in ripening fruit might be a widespread phenomenon. Tattersall et al. (*45*) reported that thaumatin-like proteins are the major protein species in a range of fruits. The same peaks show 77.77 and 72.22% homology, respectively, with a segment of PR proteins R1 and R2 from *Nicotiana tabacum*, two isoforms in tobacco that accumulate in the intercellular spaces of many plants as a result of hypersensitive reaction to a pathogen (*46*). These proteins are homologous to the sweet-tasting protein thaumatin. The same peaks have a 66.67% homology with an osmotin-like protein from *Arabidopsis thaliana* (*47*, *48*). PR proteins have been distributed in 11 recognized classes (*49*). The members of class 5, the PR-5 proteins, are known as thaumatin-like (thau-1) because they show a certain degree of similarity to the sweet-tasting protein thaumatin isolated from the fruits of the West African monocotyledon plant *Thaumatococcus daniellii* Benth (*50*). PR-5 proteins are evolutionarily conserved in the plant kingdom and usually consist of acidic forms (*51*). In addition, a basic isoform has been originally isolated from salt-adapted tobacco cells and called osmotin (*52*). Osmotin and closely related proteins are usually referred to as osmotin-like (osm-1) proteins. These proteins can be considered as forming a subclass of thau-1 proteins. Osmotin was shown to accumulate in the vacuole of the cell. Although the physiological function of PR-5 proteins is not fully understood, several experiments have demonstrated that PR-5 proteins have an antifungal activity in vitro (*53*−*56*). In this respect, they could have an important role in plant defense. It was also reported that transgenic potato plants which expressed constitutively high levels of tobacco osmotin or a potato osm-1 protein exhibited delayed development of fungal disease symptoms (*57*, *58*).

The polypeptide presented in peak 12.4 (Figure 2 and Table 1), from which an 18 amino acid peptide was sequenced, has 64.29% homology to a segment of a protein from a 17.1 kb DNA fragment from chromosome X of *S. cerevisiae* (*59*) and 72.73 and 60.00% homologies to segments of proteins from the free-living nematode *Caenorhabditis elegans* (*60*).

The remaining polypeptides sequenced (peaks 4.3, 5.1, 5.4, 6.2, 7.1, 8.5, 9.2, and 10.3, Figure 2 and Table 1), each with 25 amino acid residues sequenced, show 92.00% homology to an osmotin-like protein precursor from *V. vinifera* (grape) (*61*), 76.00% homology to a banana ripening-associated protein fragment (*62*), and 68.00% homology with fragments of basic chitinase from *Citrus sinensis* L. Osbeck cv. Valencia (*63*), thaumatin-like protein from *Actinidia deliciosa* (*64*), and PRR2 Tobac from *N. tabacum* (*46*).

During banana (*Musa acuminata* L.) fruit ripening, ethylene production triggers a developmental cascade that is accompanied by a massive conversion of starch to sugars, an associated burst of respiratory activity, and an increase in protein synthesis. Identification of the mRNAs expressed reveals that many encode proteins thought to be associated with pathogenesis, senescence, or stress responses in plants (*62*) and that increased early in ripening.

Chitinases (*65*) degrade chitin, a β-1,4-linked polymer of *N*-acetyl-D-glucosamine that often comprises the cell walls of fungal pathogens and the exoskeletons of arthropods. These enzymes are interesting because they are widely found in nature and can often be multifunctional: they can possess chitosanase and/or lysozyme activities. Chitosanases hydrolyze chitosan (deacetylated chitin), which is a component of fungal cell walls. Lysozyme hydrolyzes murein (a peptidoglycan), which is a bacterial cell wall component. Purified lysozyme and lysozyme/chitinases have been shown to inhibit the growth of some fungal and bacterial pathogens in vitro (*66*−68). The expression of cloned bacterial phage T4 lysozyme genes in vivo can increase pathogen resistance in plants (*69*, *70*). Due to their apparent antipathogen activities and their inducibility in plants upon infection

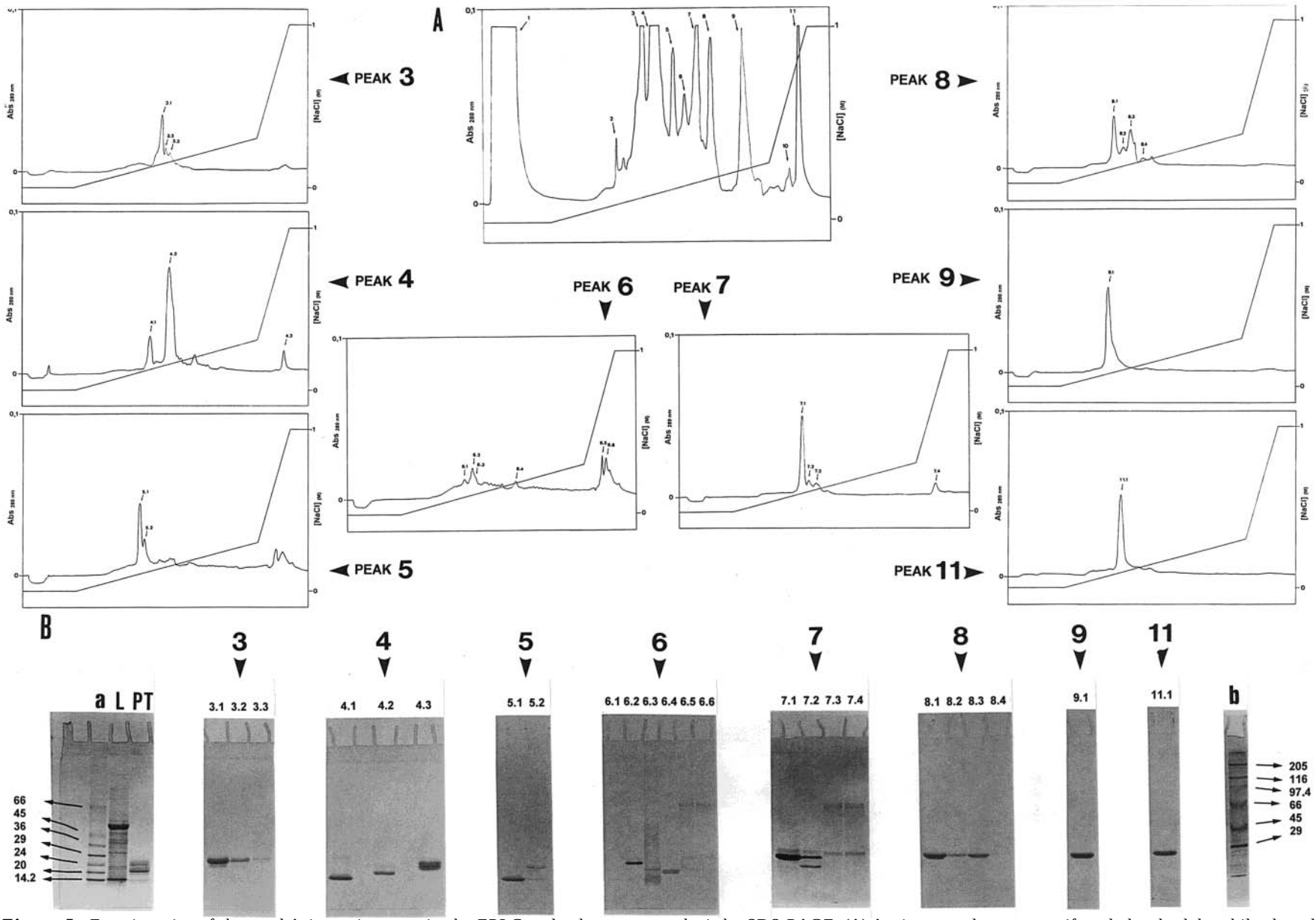

Figure 5. Fractionation of the total Arinto wine proteins by FPLC and subsequent analysis by SDS-PAGE. (A) A wine sample was centrifuged, desalted, lyophilzed, and fractionated by cation exchange chromatography on the Mono S column of the FPLC, at pH 2.5. The bound proteins were eluted with a continuous gradient of NaCl (0−1 M). Each of the eight major protein peaks obtained previously was desalted, lyophilized, and further fractionated by anion exchange chromatography on the Mono Q column of the FPLC, at pH 9.8. The bound proteins were eluted with a continuous gradient of NaCl (0−1 M). (B) SDS-PAGE of the protein peaks eluted from the Mono Q column. The protein loaded was 100 μg/lane. Lanes a and b are molecular mass standards (kDa). Lanes L and Pt are *L. minor* total soluble protein and Arinto wine total protein, respectively.

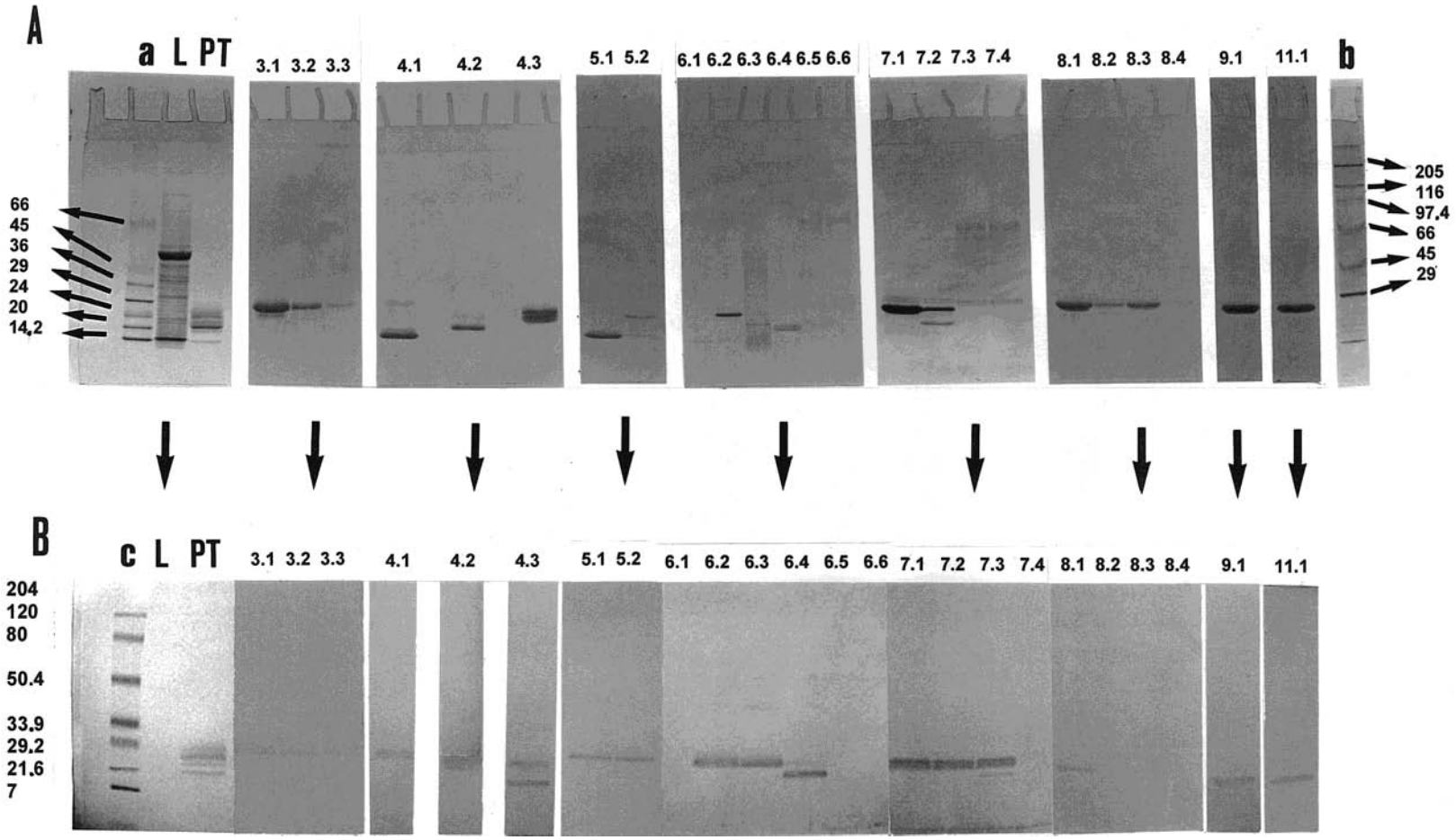

Figure 6. Search for structurally similar proteins in different Arinto wine protein fractions. The Arinto polypeptides were fractionated by FPLC ion exchange chromatography, as explained in the legend to Figure 5, and subjected to SDS-PAGE (A) or probed with anti-20 kDa Assario polypeptide antibodies (B). The protein loaded on each lane was 100 μg (A) or 10 μg (B). Lanes a−c are molecular mass standards (kDa). Lanes L and Pt are *L. minor* total soluble protein and Arinto wine total protein, respectively.

by pathogens (*66*, *71*, *72*), the lysozymes/chitinases have been classified as PR proteins.

Various types of environmental stress induce the systemic synthesis of several families of homologous proteins with protective functions in vegetative tissues of plants (*54*). Comparison of the N-terminal sequences of the polypeptide peaks isolated from Moscatel wine with proteins from other sources revealed a high degree of homology to PR proteins. Other authors (*73*−*77*) have, also, isolated PR proteins from wine and grape berries. These proteins seem to be responsible for haze formation in wines and resistant to proteolysis (*3*).

In a recent work, Ferreira et al. (*16*) employed immunological techniques to demonstrate that at least most of the wine proteins derive from the grape pulp. Important changes are known to occur to the pulp proteins during vinification, because most of these are lost during the fermentation process (*16*). In fact, the proteins that end up in wines are those that are highly resistant to proteolysis and to the low pH values characteristic of these beverages (*3*). As mentioned before, the data reported in Table 1 suggest that the polypeptide present in peak 9.5 may be of microbial origin. Alternatively, it may represent a protein derived from the grape pulp that has not yet been studied and sequenced in this tissue. Supporting this hypothesis is the observation that the polypeptide subjected to N-terminal sequencing in peak 9.5 (Table 1) was recognized by the anti-20 kDa Assario polypeptide antibody (Figure 4).

To confirm that the diversity of structurally similar proteins is not a characteristic specific of Moscatel wine, some of the experiments previously described for this wine were repeated with another single grape variety white wine, Arinto. Fractionation of the Arinto proteins by FPLC cation exchange chromatography (Mono S column; pH 2.5) resolved several major components (Figure 5A). As observed with the Moscatel wine proteins (Figure 2), the polysaccharides (peak 1) did not bind to the column, and the eight Arinto peaks with a higher protein content (peaks 3−9 and 11) were treated as described in the legend to Figure 5. Again, it can be observed that the poor diversity of the Arinto wine proteins shown in Figure 1 is only apparent. These experiments indicate that the Arinto wine contains a large number (several tens and, possibly, many more) of polypeptides with different p*I* values but similar molecular masses. Probably, these polypeptides are structurally similar, differing only by a few amino acid residues. It was observed that when the Arinto polypeptides were probed with the anti-20 kDa Assario polypeptide antibodies, strong signals were achieved (Figure 6) in most cases.

These results suggest not only that there is structural similarity between the Assario polypeptide and the Arinto polypeptides but also that most of the selected Arinto polypeptides are structurally related and, consequently, structurally related to the Moscatel polypeptides. Consequently, it was not considered to be necessary to sequence the several Arinto wine polypeptides.

The results, obtained in this laboratory, strongly suggest the existence, in the grape berries, of a common precursor to most of the proteins present in wine, which could generate all of the detected polypeptides by limited proteolysis. Experiments are underway to locate the presence of the precursor at the maturing stage of the grape berry where it first appears, to isolate it, and to study the proteolytic process that leads to the structural diversity observed in the wine proteins.

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