Sequence Analysis of Grape (*Vitis vinifera*) Berry Chitinases That Cause Haze Formation in Wines

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Chitinases account for ca. 50% of the soluble proteins in the berries of the grape vine (*Vitis vinifera* L. Muscat of Alexandria). The other major proteins present are thaumatin-like proteins. Both these groups of proteins persist through the vinification process and cause hazes and sediments in bottled wines. Four chitinases have been purified from Muscat of Alexandria fruit and characterized by both sequence and mass spectral analysis. Their protein sequences were highly similar, and all proteins were modified at their N-terminus. It was confirmed for three of the chitinases that the N-terminal group was a pyroglutamate residue. Comparative sequence analysis of two chitinases from berries and two from wine demonstrated that, despite their reputed resistance to proteolytic degradation, some limited proteolytic processing of these proteins occurs. The peptide fragments of the chitinases account for only 3% of the total soluble protein content of the fruit.

Keywords: *Pathogenesis related proteins; chitinases; protein sequence; pyroglutamate; proteolytic processing; Vitis vinifera; grapes; grape juice; wine; protein haze*

INTRODUCTION

A common cause of haze formation in white wines is protein. Grape-derived proteins are considered unstable in bottled wine because they can precipitate and cause unattractive and commercially unacceptable hazes and sediments (Bayly and Berg, 1967; Hsu and Heatherbell, 1987; Waters et al., 1992). In a recent paper, we identified the proteins that cause haze in wine as pathogenesis related (PR) proteins from the grape berry (Waters et al., 1996). Specifically, in Muscat of Alexandria wine, the major PR proteins were thaumatin-like proteins and chitinases.

PR proteins are produced by plants in defense against fungal pathogens (Boller, 1987; Linthorst, 1991). Thaumatin-like proteins have antifungal properties (Cheong et al., 1997; Liu et al., 1996; Malehorn et al., 1994; Stintzi et al., 1993), possibly by their ability to permeabilize cell membranes (Abad et al., 1996; Batalia et al., 1996; Yun et al., 1997). Their precise mechanism of action has not yet been elucidated. In contrast, there is strong evidence that chitinases have antifungal properties resulting from their activity toward chitin, a major structural component of many fungal cell walls (Boller, 1987; Graham and Sticklen, 1994; Punja and Zhang, 1993). Chitinases may also play a role in nonspecific stress responses and are sometimes developmentally regulated (Graham and Sticklen, 1994). The current classification scheme is based on primary structure, and although not entirely satisfactory (Beintema, 1994), it separates the chitinases into four classes. Many of the classes can occur simultaneously in the same plant (Flach et al., 1992; Graham and Sticklen, 1994; Punja and Zhang, 1993). Class I chitinases are basic and have a cysteine-rich putative chitin binding domain at the N-terminus; class II chitinases are acidic and lack the cysteine-rich domain, class III are dissimilar to classes I and II and share sequence homology to the bifunctional chitinase/lysozyme from *Hevea brasiliensis*; and class IV chitinases have structural similarities but sequence dissimilarities to class I chitinases. In general, all PR proteins, including chitinases and thaumatin-like proteins, are acid-soluble and resistant to proteases (Linthorst, 1991).

Given the known proteolytic resistance of PR proteins (Linthorst, 1991), strategies to remove these proteins from wine based on proteolysis under traditional wine-making conditions are probably futile. One potential approach may be to genetically engineer grapevines to lower the expression of the genes encoding for the specific PR proteins involved in wine haze problems. As a prerequisite to this, more information about the proteins implicated in wine haze formation is needed, in particular their amino acid sequence, to allow the cloning of the respective genes. We report here sequence analysis data on the soluble chitinases, and their derived fragments, that are present in Muscat of Alexandria grapes. These chitinases are a major part of the pool of berry-derived PR proteins that survive fermentation and cause protein haze formation in wine.

MATERIALS AND METHODS

Identification and Quantification of Soluble Protein from Muscat of Alexandria Grapes. *Vitis vinifera* cv. Muscat of Alexandria grapes from the 1996 season were harvested at commercial maturity (soluble solids of 21 °Brix) from irrigated vines at the Waite Campus of the University of Adelaide at Urrbrae, South Australia. 'Free run juice', the

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soluble protein fraction, was extracted by gently pressing fruit in a plastic bag by hand, followed by coarse filtration of the pressed berries through 2 mm mesh and then centrifugation (2000*g*, 20 min).

Free run juice samples were desalted on an Econo-Pac 10DG column (Bio-Rad Laboratories, Sydney, Australia) into water before HPLC analysis (Peng et al., 1997). Peaks were detected at 214 nm and identified by comparison to retention times of previously purified grape PR proteins (Waters et al., 1996) and from protein sequence analysis (see below). Proteins were quantified by comparison to the peak area of a protein standard: horse heart cytochrome *c* (Sigma Chemical Co., St Louis, MO).

Isolation of Total Protein and Purification of Chitinases from Muscat of Alexandria Grapes. The free run juice from mature Muscat of Alexandria grapes grown at the Waite Campus in 1997 was concentrated 10-fold by ultrafiltration through a 10 000-Da nominal molecular mass cutoff membrane (YM-10, Amicon Corporation, Danvers, MA) and then desalted on an Econo-Pac 10DG column (Bio-Rad Laboratories) into water.

Concentrated and desalted samples (1 mL) were loaded at 1 mL/min onto a semipreparative C18 column (10 × 250 mm, Vydac, Hesperia, CA) fitted with a C18 guard column (4.6 × 10 mm, Alltech, Sydney, Australia) equilibrated in a mixture of 39% (v/v) solvent A [0.05% (v/v) trifluoroacetic acid (TFA)] and 61% solvent B [70% (v/v) acetonitrile, 0.035% (v/v) TFA] and held at 40 °C. Proteins were eluted by a gradient of solvent B from 61% to 70% solvent B in the first 8 min, from 70% to 79% solvent B from 8 to 9 min, from 79% to 94% solvent B from 9 to 23 min, and then held at 94% for a further 5 min. Peaks were detected at 280 nm, and those eluting between 24.8 and 25.8 min (named ChitB), between 26.4 and 27.4 min (ChitC), between 27.4 and 28.4 min (ChitD), between 30.0 and 30.8 min (ChitE), and between 31.0 and 31.8 min (ChitF) were collected. Retention times vary from those observed during the separations on the analytical column (see above) because the gradient was optimized on the semipreparative column to purify the chitinases rather than separate all berry proteins. The injection and separation procedure was performed three times. The fractions containing ChitB were pooled, diluted with solvent A, reinjected under the same chromatographic conditions, and recollected. The fractions containing ChitC and the fractions containing ChitD were treated similarly. The resultant fractions containing ChitB, ChitC, and ChitD were lyophilized.

ChitA was purified from a Muscat of Alexandria juice supplied by a commercial wine producer (BRL Hardy Berri Estates, Waikerie, Australia). $(NH_4)_2SO_4$ precipitation and anion exchange chromatography was performed as described by Tattersall et al. (1997), except that the material that did not bind to the Q-Sepharose column (Pharmacia, Sydney, Australia) at pH 8.0 was pooled, concentrated by ultrafiltration through a 10 000-Da nominal molecular mass cutoff membrane (YM-10, Amicon Corp.), and buffer exchanged into 20 mM sodium acetate buffer, pH 5.0. The concentrate was then loaded onto a 2.6 × 10 cm SP-Sepharose column (Pharmacia) equilibrated with 20 mM sodium acetate buffer, pH 4.8. Proteins were eluted by a linear 0−400 mM NaCl gradient (in equilibration buffer) over 17.3 h at a flow rate of 32 mL/h. Fractions of 8 mL were collected, and a single fraction containing a large quantity of a protein with relative M_r of 30 K, as judged by SDS−PAGE according to Fling and Gregerson (1986) was selected for further study. This protein, named ChitA, was also present in the free run juice from the Muscat of Alexandria vines grown at Urrbrae. This was confirmed by comparing the chromatographic behavior of the proteins isolated from the two different juices using the HPLC system described above and by comparing the molecular masses of the two proteins by electrospray ionization mass spectrometry (see below).

Wine protein Ia and Fa were purified from a commercial Muscat of Alexandria wine supplied by Southcorp Wines, Nuriootpa, Australia, as described previously (Waters et al., 1996).

Reduction, Alkylation, and Digestion of the Chitinases. The purified proteins (∼50−500 μg) were dissolved in 400 mM Tris-HCl buffer (pH 8.5) containing 5 M guanidine-HCl and 2 mM EDTA (buffer C, 150 μL). Dithioerythritol (80 mM in buffer C, 20 μL) was added to give a final concentration of 9 mM dithioerythritol, and the solution was heated in the dark (40 °C, 3 h). On cooling, iodoacetic acid (500 mM in 1 M tris-HCl, pH 8.5, 17 μL) was added to give a final concentration of 45 mM iodoacetic acid, and the solution was incubated in the dark (25 °C, 1 h). The solution was then desalted by ultrafiltration (Centricon 10, Amicon Corp.) and diluted into either 25 mM Tris-HCl buffer (pH 7.5) containing 2 M urea and 2 mM EDTA for the juice chitinases or into 100 mM ammonium carbonate buffer (pH 8.7) containing 2 M urea and 1 mM $CaCl_2$ for the wine chitinases. The juice chitinases were then digested with endoproteinase Lys C and the wine chitinases with trypsin, as follows.

Endoproteinase Lys C (EC 3.4.99.30, Promega, Sydney, Australia, 100 mg/L in 25 mM NaH_2PO_4, pH 7.5, 5 μL, 30 × 10^{-3} U) was added to the reduced and alkylated juice chitinase solutions (∼10 μg/μL, 40 μL), and the mixture was incubated (23 °C, 22 h). Digestion was terminated by dilution in solvent A (100 μL), and an aliquot (135 μL) was loaded at 0.6 mL/min on a C18 column (4.6 × 250 mm, Vydac) fitted with a C18 guard column (4.6 × 10 mm, Alltech) equilibrated in solvent A. Peptides were eluted by a gradient of 1−70% solvent B in 90 min, detected at 214 nm, and collected.

Trypsin (EC 3.4.21.4, Sigma Chemical Co., 2 g/L in 0.1 mM HCl, 10 μL, 200 U) was added to the reduced and alkylated wine chitinase solutions (∼2 μg/μL, 80 μL), and the mixture was incubated (40 °C, 2h). Digestion was terminated by dilution in solvent A (200 μL), and the peptides in an aliquot (145 μL) were separated and collected as above.

Determination of the N-Terminal Amino Acid of the Juice Chitinases. The blocked N-terminal peptides from the Lys C digests of the reduced and alkylated ChitB, ChitC, and ChitD were tentatively identified by mass spectrometry (M_r of 1489, 1489, and 1547, respectively, as described below). The peptide fractions (approximately 1000 pmol) were lyophilized and then redissolved in 100 mM NaH_2PO_4, pH 8, containing 10 mM EDTA, 5 mM dithioerythritol, 5% (v/v) glycerol, and 1 M urea (40 μL). Pyroglutamate aminopeptidase (EC 3.4.19.3, Boehringer Mannheim, Sydney, Australia, 250 mg/L in water, 1 μL) was added, the reaction mixture overlaid with nitrogen gas, and then incubated (4 °C, 18 h; then 23 °C, 4 h). Digestion was terminated by dilution in solvent A (100 μL), and an aliquot (135 μL) was loaded at 0.6 mL/min onto a C18 column (4.6 × 250 mm, Vydac) fitted with a C18 guard column (4.6 × 10 mm, Alltech) equilibrated in solvent A. Reaction products were eluted by a gradient of 5−100% solvent B in 25 min, detected at 214 nm, and collected. Peptides that had lost their pyroglutamate residue were identified by mass spectrometry and then subjected to Edman degradation.

Amino Acid Sequencing. Automated Edman degradation was performed by the Nucleic Acid and Protein Chemistry Unit of the Department of Plant Science, University of Adelaide, on an Hewlett-Packard G1000A protein sequencer (Palo Alto, CA) according to the manufacturers' instructions.

Protein sequence databases were searched using OWL at http://www.biochem.ucl.ac.uk/bsm/dbbrowser/OWL/OWL.html. OWL is a nonredundant composite of four publicly available primary sources: SWISS-PROT, PIR (1-3), GenBank (translation), and NRL-3D (Bleasby et al., 1994).

Electrospray Mass Spectrometry. Purified proteins and peptides were subjected to mass spectrometric analysis using a PE Sciex API 300 with ion spray ionization (Perkin-Elmer, Thornhill, Ontario, Canada) at the Mass Spectrometry Facility of the Waite Campus, University of Adelaide, as described previously (Peng et al., 1997).

RESULTS AND DISCUSSION

Chitinases Are Major Components of Ripe Muscat of Alexandria Berries. The dominant protein

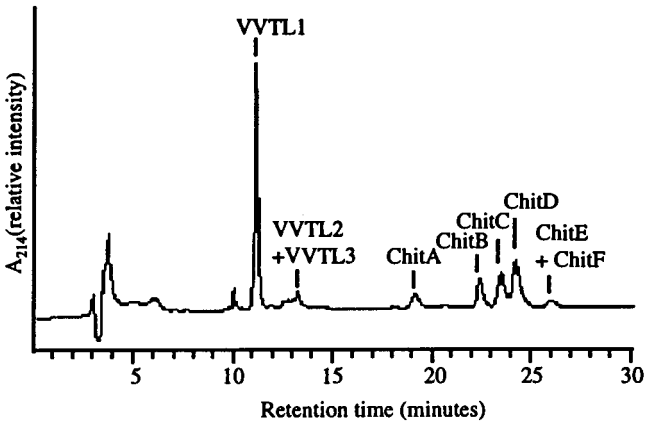

Figure 1. Protein composition of free run juice from Muscat of Alexandria berries by reverse-phase HPLC. Proteins were identified by comparison of their retention time to that of the purified and sequenced proteins under the same chromatographic conditions and from their amino acid sequence.

components of the free run juice from ripe Muscat of Alexandria grapes were chitinases and thaumatin-like proteins as shown by HPLC analysis (Figure 1).

At least three thaumatin-like proteins were identifiable by HPLC; the major one (named VVTL1, eluting at 11 min in Figure 1) is the thaumatin-like protein identified previously (Tattersall et al., 1997; Waters et al., 1996). The minor thaumatin-like proteins eluted later (12.5−13.5 min, named VVTL2 and VVTL3 see Figure 1) from the reversed-phase column and were also identified previously by amino acid sequencing as minor thaumatin-like proteins of Muscat of Alexandria wine (Waters et al., 1996).

Four major chitinases were separated by HPLC in the free run juice from the Muscat of Alexandria grapes: one eluting at 18.9 min (named ChitA) and the other three eluting as a group at 22.2, 23.3, and 24 min (named ChitB, ChitC, and ChitD, respectively). The chitinases were identified in this juice by amino acid sequence analysis of peptides generated from the proteins. In addition, ChitD eluted at the same retention time as a chitinase previously identified as a major component of Muscat of Alexandria wine (wine protein Ia; Waters et al., 1996). There was also an additional peak eluting at 25.8 min that appeared to contain two fragments of the chitinases (named ChitE and ChitF). The sequence analysis of all chitinases and their fragments is discussed in detail below. The total protein concentration of the juice was 237 mg/L with the chitinases accounting for 50% of the total protein (Table 1).

Chitinase activity, along with many other enzyme activities, has been previously reported in grapes (Derckel et al., 1996, Robinson et al., 1997) and other fruits such grapefruit (McCollum et al., 1997), kiwi fruit (McLeod and Poole, 1994; Wurms et al., 1997), orange (Nairn et al., 1997), and pumpkin (Esaka et al., 1993). It has also been recently observed that there is relatively high expression of chitinase genes in grape berries (Robinson et al., 1997). To our knowledge, however, this is the first report giving evidence that chitinases are indeed a major component of the soluble protein fraction of grapes.

The Four Major Chitinases in Muscat of Alexandria Grapes Have Similar Amino Acid Sequences. To confirm the identity of the proteins and to determine the differences among the major chitinases

Table 1. Protein Composition and Concentration of Free Run Juice from Muscat of Alexandria Grapes

protein	retention time (min)	concentration (mg/L)[a]	concentration (%)[b]
VVTL1	11.0	99.3	42
VVTL2 + VVTL3	12 to 13	19.8	8
ChitA	18.9	13.7	6
ChitB	22.2	21.1	9
ChitC	23.2	29.1	12
ChitD	24.0	46.3	20
ChitE + ChitF	25.8	7.4	3
total chitinases		117.6	50
total protein		236.7	100

[a] From peak areas and in cytochrome *c* equivalents. [b] As a percentage of the total protein of this fraction.

(ChitA, ChitB, ChitC, and ChitD), automated Edman degradation was performed to obtain their N-terminal amino acid sequences. The N-termini of all proteins were blocked to Edman degradation. Thus amino acid sequences of peptides derived from all of the proteins were determined and compared to each other and to the sequences deduced from cDNAs isolated from a *V. vinifera* cv. Shiraz cDNA library (VvChi4A and VvChi4B, Robinson et al., 1997). For ChitB, ChitC, and ChitD, the molecular weights of the peptides were also determined by electrospray ionization mass spectrometry.

The digest of ChitA, ChitB, ChitC, and ChitD with endoproteinase Lys C produced peptides (peptide 2, Figure 2) with extensive sequence homology to Shiraz berry chitinases (Robinson et al., 1997). These peptides thus identify the proteins as chitinases. The peptides are presumed to be close to the blocked N-termini of the Muscat of Alexandria proteins because the peptides have homology to the N-termini cysteine-rich chitin binding domain of the Shiraz berry chitinases. In this likely chitin binding domain, all four chitinases were highly similar, although the number and types of amino acids varied slightly. The mass spectral data for peptide 2 from ChitC and ChitD (Table 2) confirmed the data obtained from the sequencer.

The digest of ChitB, ChitC, and ChitD with endoproteinase Lys C also produced peptides from the C terminus of the proteins (peptide 6, Figure 2). The sequence of this peptide was identical in all three chitinases and identical to that of VvChi4B, a protein for which the sequence has been recently deduced from cDNA sequences from Shiraz berries. The measured molecular weights of the peptides (Table 2) confirmed the data obtained from the sequencer.

Internal sequence was obtained from the endoproteinase Lys C digests of ChitC and ChitD (peptide 3, Figure 2). The sequences of these peptides were identical except for two amino acid changes, and the sequence of peptide 3 from ChitC was the same as that recently deduced from cDNA sequences from Shiraz berries (Figure 2). The measured molecular weights of peptide 3 (Table 2) also confirmed the data obtained from the sequencer. Further sequence was obtained from the endoproteinase Lys C digests of ChitA (peptides 4 and 5, Figure 2), and again the sequences of these peptides were similar to those deduced recently from published cDNA sequences from Shiraz berries (Figure 2).

Thus, analysis of the protein sequence of the major chitinases (ChitA, ChitB, ChitC, and ChitD) in Muscat of Alexandria fruit revealed that they were closely related but not identical, suggesting that the chitinases

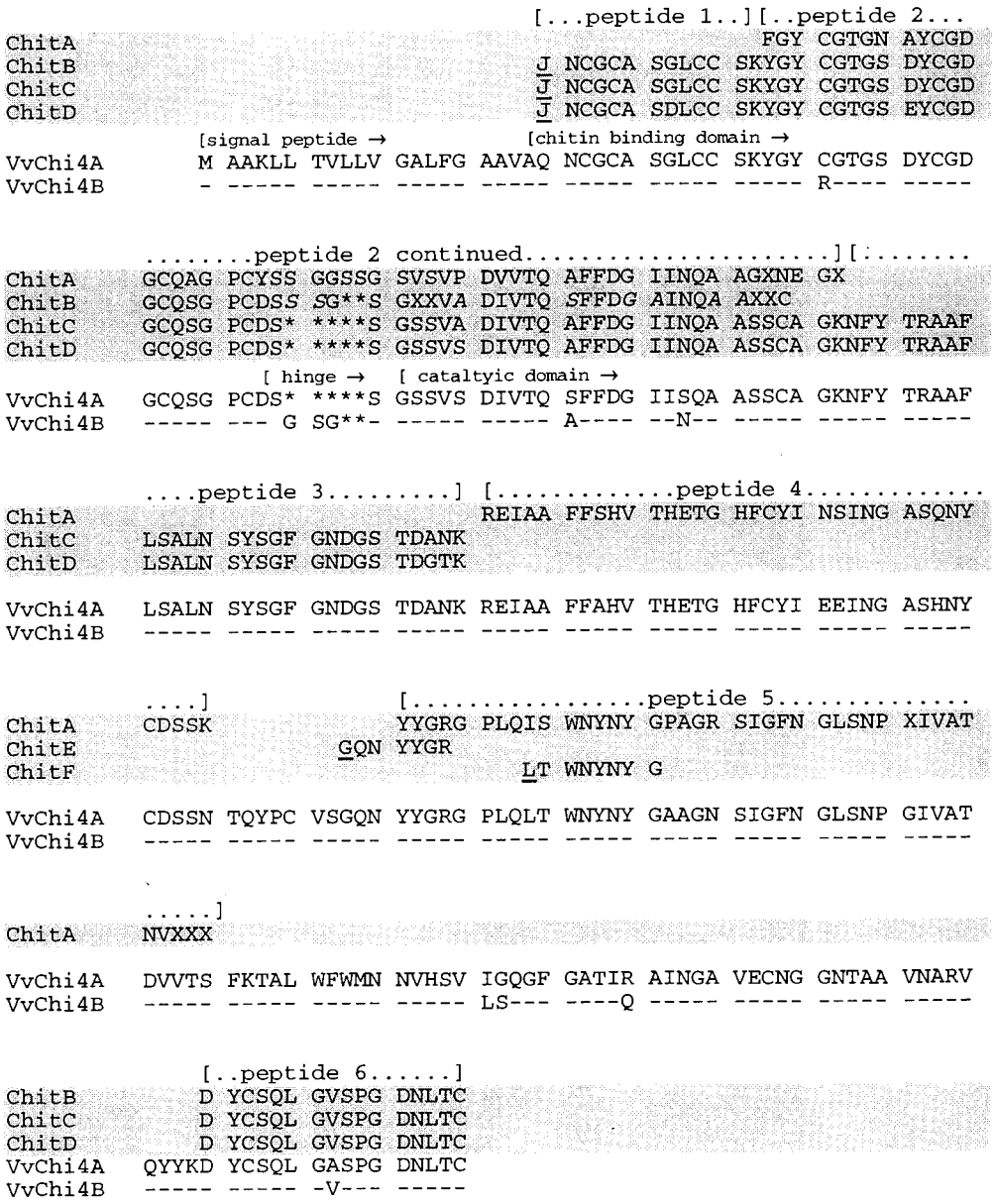

Figure 2. Protein sequences of peptides obtained from digests of ChitA, ChitB, ChitC, and ChitD and N-terminal protein sequences of ChitE and ChitF compared to that of the deduced protein sequences from two cDNA clones of chitinases from Shiraz berries (VvChi4A, residues 1−83, and VvChi4B, residues 1−86, Robinson et al., 1997) The protein sequences of ChitA, ChitB, ChitC, ChitD, ChitE, and ChitF are hatched, and the N-terminal amino acids of the proteins are underlined. The presumed domains of the Shiraz chitinases are indicated. Amino acids in italics are tentative assignments, X signifies an unknown residue, an asterisk (*) is a space added to facilitate alignments, and a minus sign (−) signifies that VvChi4B was identical to VvChi4A except where indicated.

Table 2. Molecular Masses of Peptides from ChitB, ChitC, and ChitD Determined Experimentally by Electrospray Mass Spectrometry and Predicted from Their Amino Acid Sequence

	measured M_r			predicted M_r[a]		
peptide	ChitB	ChitC	ChitD	ChitB	ChitC	ChitD
1	1489	1489	1547	(blocked to Edman degradation)		
1 after treatment[b]	1378	1378	1436	1378	1378	1436
2	nd[c]	5212	5242	nd	5212	5242
3	nd	2989	2962	nd	2989	2962
6	1788	1788	1788	1788	1788	1788

[a] M_r predicted from the experimental data obtained after sequencing the peptides. [b] N-terminal peptides were treated with pyroglutamate aminopeptidase, an enzyme that removes pyroglutamate residues (M_r of 111) from the N-terminus of peptides. [c] nd, not determined.

were different gene products. The existence of more than one chitinase gene is consistent with the data of Robinson et al. (1997) describing two alleles for VvChi4 in Shiraz fruit, that of Busam et al. (1997) describing two further chitinase genes in cultured grape cells, and the immunodetection of up to 13 isoforms of chitinases in various grape tissues by Derckel et al. (1996). One of the notable differences between the two Shiraz chitinases (VvChi4A and VvChi4B) described by Robinson et al. (1997) and among the Muscat of Alexandria

chitinases discussed here is sequence variation in the proposed hinge region (see Figure 2).Within this region, ChitC, ChitD, and VvChi4A were identical in sequence while ChitA, ChitB, and VvChi4B varied.

In addition to the differences in the protein sequences of the chitinases, their physical properties also varied. ChitB, ChitC, and ChitD all bind to an anion exchange column at pH 8.0, whereas ChitA, under the same conditions, does not bind (data not shown). Therefore ChitB, ChitC, and ChitD have lower isoelectric points and are thus more acidic than the basic ChitA. In addition, ChitA appears to be more hydrophilic in nature than the other chitinases since it elutes earlier from the HPLC system used in this study than do ChitB, ChitC, or ChitD, which elute in close proximity to one another (Figure 1).

Three of the Major Chitinases in Muscat of Alexandria Grapes Are N-Terminally Blocked with Pyroglutamate. The digest of three of the major chitinases (ChitB, ChitC, or ChitD) produced a peptide that eluted early from the reversed-phase HPLC column and was N-terminally blocked. The molecular mass of the peptides are given in Table 2. The mass of the peptide from ChitC and ChitB is correct for a peptide analogous to that present in VvChi4A residues 21−33 (i.e., peptide 1, Figure 2) with the following sequence modification: pyroglutamate-NCGCASGLCCSK and with the cysteines derivatized with carboxymethyl groups following reduction and alkylation of the protein prior to digestion with endoproteinase Lys C. It was confirmed that a pyroglutamate residue was blocking the N-termini of ChitB, ChitC, and ChitD as follows. The peptides were treated with pyroglutamate aminopeptidase, an enzyme that removes pyroglutamate from the N-terminus of peptides and proteins, and then subjected to mass spectral analysis (peptide 1, Table 2) and sequencing (peptide 1, Figure 2). The change in mass after this treatment (Table 2) demonstrated removal of the blocking group by the enzyme through the loss of 111 mass units and confirmed that the three chitinases were N-terminally blocked with a pyroglutamate residue. In addition, these data confirm the prediction of Robinson et al. (1997) that the mature chitinases in Shiraz grapes begin at residue 21 of the cDNA clones, a glutamine residue, since pyroglutamate can form at the N-terminus due to the cyclization of glutamine.

A yam chitinase is the only other plant chitinase that has been identified to date as being N-terminally blocked with a pyroglutamate (Araki et al., 1992a,b). N-terminal blockage to Edman degradation through acylation of the α-amino group is a widespread phenomenon, and the pyroglutamate residue is one of several fairly common acyl groups (Wold, 1981). Although pyrollation can be an enzymatic process, the spontaneous cyclization of N-terminal glutamine to form pyroglutamate and its apparent lack of biological significance (Chung and Webster, 1996) suggests that such N-terminal modification could be an artifact of protein isolation (Wold, 1981). Of all plant chitinases and deduced plant chitinase sequences from cDNAs sequenced to date (and searched through OWL), the N-terminal amino acid is glutamine in only 32% of the proteins. Thus pyroglutamate N-terminal groups, at their maximum potential occurrence, cannot be considered as a major feature of plant chitinases. Harris (1989) described the spontaneous formation of pyroglutamate from glutamine after cleavage of a basic amino acid doublet adjacent to the glutamine residue. None of the potential pyrollated plant chitinases, nor those confirmed as pyrollated proteins in this study, have this feature.

Chitinases from Muscat of Alexandria Grapes Are Processed. In the free run juice from the Muscat of Alexandria fruit examined here, it was possible to detect low levels of proteins with molecular masses approximately half that of the chitinases described above. These proteins (ChitE and ChitF) were identified as fragments of the chitinases from amino acid sequencing (Figure 2) and electrospray ionization mass spectrometry.

The N-terminal amino acid sequence of ChitE was identical to cDNA-deduced internal sequence of the chitinase from Shiraz berries (VvChi4A and VvChi4B) and the N-terminal amino acid of ChitE maps to residue 154 of VvChi4A. The predicted molecular mass of a fragment of VvChi4A from residue 154 to the C-terminus was 11 464. This value is close to the measured molecular mass of ChitE, which was 11 560 (difference of 96 mass units).

The N-terminal amino acid sequence of ChitF was also identical to cDNA-deduced internal sequence of the chitinase from Shiraz berries and the N-terminal amino acid of ChitF maps to residue 165 of VvChi4A. Similarly, the predicted molecular mass of a fragment of VvChi4A from residue 165 to the C-terminus was 10 229, again close to the measured molecular mass of ChitF, which was 10 325 (difference of 96 mass units). Since it is likely that there would be small differences in amino acid sequences of the chitinases from fruit of different cultivars, the small differences in molecular masses of the observed Muscat of Alexandria chitinase fragments and the predicted Shiraz chitinase fragments are not unexpected.

Previously, two chitinases (wine protein Ia and wine protein Fa) were detected in a commercial Muscat of Alexandria wine (Waters et al., 1996). Wine protein Ia was very similar to one of the major chitinases in the Muscat of Alexandria fruit examined here (ChitD), but wine protein Fa was not present nor was there a comparable protein in Muscat of Alexandria fruit. Since (i) Fa was of lower M_r than Ia (28K and 32K by SDS−PAGE, respectively; Waters et al., 1996) and (ii) Fa was not N-terminally blocked (Waters et al., 1996) whereas Ia was, it was considered possible that Fa could be a fragment of Ia. Thus the sequences of both Fa and Ia were further examined in order to determine whether they were separate gene products or if wine protein Fa was a degraded form of wine protein Ia.

The amino acid sequences of peptides derived from both wine protein Ia and Fa were determined and compared to each other and to the cDNA sequence from Shiraz berries (VvChi4A, Figure 3). Some minor differences between the current sequence and the previously published sequence of Ia (Waters et al., 1996) were found. The sequences presented here in Figure 3 have been confirmed by analyzing overlapping regions of sequence. The N-terminus of Fa mapped to the hinge region close to the start of the catalytic domain of the Shiraz berry chitinases. Apart from this difference, Fa was identical to Ia in all other portions of protein that were sequenced.

Since there were no differences between Fa and Ia other than size and an additional cysteine-rich domain

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                                                                  [.........
Ia                                                               YGYCG TGSEY

                        [signal peptide →            [chitin binding domain →
VvChi4A                  MAA KLLTV LLVGA LFGAA VAQNC GCASG LCCSK YGYCG TGSDY


                 .................peptide 7.....................] [.........
Ia               CGDGC QSGPC DSSGS SVSDI VTQAF FDGII NQAAS SCAGK NFYTR
Fa                         DSSGS SVSDI VTQAF FDGII NQAAS SCAGK NFYTR AAFLS
                         [ hinge →   [ cataltyic domain →
VvChi4A          CGDGC QSGPC DSSGS SVSDI VTQSF FDGII SQAAS SCAGK NFYTR AAFLS


                 .....peptide 8......][.............peptide 9.................]
Ia                                    E IAAFF AHVTH ETGHF CYIEE INGAS HNYCD
Fa               ALNSY SGFGN DGSTD GTHRE IAAFF AHVTH ETGHF CYIEE INGAS HNYCD
VvChi4A          ALNSY SGFGN DGSTD ANKRE IAAFF AHVTH ETGHF CYIEE INGAS HNYCD


                                    [..............peptide 10..............]
Ia                                  GPL QLTWN YNYGA AGNXI GFDGL SNPGI VATDV
Fa                                  GPL QLTWN YNYGA AGNSI GFDGL SNPGI VATDV
VvChi4A          SSNTQ YPCVS GQNYY GRGPL QLTWN YNYGA AGNSI GFNGL SNPGI VATDV


                       [........peptide 11.......][......peptide 12.....][..
Ia                     TALWF WMNNV HSVLD QGFGA TIRAI NGAVE CNGGN TAAVN ARVQY
Fa                     TALWF WMNNV HSVLD QGFGA TIRAI NGAVE CNGGN TAAVN ARVQY
VvChi4A          VTSFK TALWF WMNNV HSVIG QGFGA TIRAI NGAVE CNGGN TAAVN ARVQY


                 .....peptide 13.....]
Ia               YHDYC SQLGV SPGDN LTC
Fa               YHDYC SQLGV SPGDN LTC
VvChi4A          YKDYC SQLGA SPGDN LTC
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Figure 3. Protein sequences of peptides obtained from digests of Ia and Fa as compared to that of the complete deduced protein sequences from a cDNA clone of a chitinase from Shiraz berries (VvChi4A, Robinson et al., 1997) The protein sequences of Ia and Fa are hatched, and amino acid residues that differ between the proteins are underlined. The N-terminal amino acid of Fa is double underlined. The presumed domains of the Shiraz chitinases are indicated. Amino acids in italics are tentative assignments, and X signifies an unknown residue.

at the N-terminus of Ia, Fa is probably a processed form of Ia. Because we have not been able to detect Fa in grapes or juice, it is likely that this putative proteolytic processing event occurs during fermentation or aging of the wine. It cannot be excluded, however, that Fa is the product of a separate gene for a chitinase that lacks the N-terminal cysteine-rich domain of Ia but is otherwise identical to Ia.

It is noteworthy that, although wine protein Ia behaved identically to berry protein ChitD under the chromatographic conditions utilized in this study, there were two observable differences in the sequences of the two chitinases (Figures 2 and 3). The C-terminal fragment produced from ChitD by digestion with endoproteinase Lys C (peptide 6) was presumably cleaved from the protein at a lysine (K) residue. The C-terminal fragment produced from Fa (peptide 13) was longer than that from ChitD (peptide 6) and contained a histidine (H) residue in the position of the presumed lysine of peptide 6. The terminal amino acid of the internal peptide (peptide 8) produced from Ia, and presumably from Fa also, was H, whereas the terminal amino acid of the internal peptide (peptide 3) of ChitD was K.

One possible explanation for these sequence differences is that, although the proteins originated from the same grape cultivar, there may have been clonal differences between the Muscat of Alexandria vines that produced protein Ia and the Muscat of Alexandria vines that produced protein ChitD. Thus, in addition to the sequence variations of the berry chitinases, some limited proteolysis of the chitinases appears to occur. As described above, it was possible to detect fragments of the berry chitinases in free run juice, and there is some evidence for proteolytic degradation of the chitinases during fermentation or aging.

Proteolytic processing of bean root chitinases was recently demonstrated (Lange et al., 1996). In the bean chitinases, the processing occurred at the same site in the protein sequence that would produce Fa from Ia in grapes (i.e., between the chitin-binding domain and the catalytic domain).The bean root chitinases were induced by infection of the roots with *Fusarium solani*, the causal agent of dry root rot; therefore, either the plant or the fungus may have been the source of proteolytic activity. Since the source of the Muscat of Alexandria fruit studied here were vines grown in the field, pathogenic attack cannot be completely excluded. However, because the vines showed no signs of disease and were not stressed throughout the berry growth period, the grape berry chitinases appear to be constitutively expressed and in the absence of pathogenic attack. In addition, one of the putative chitinase fragments, Fa, was not present in juice but only detected in wine. Thus, it appears that the limited processing of the grape berry chitinases either is a function undertaken by the plant, occurs during crushing of the fruit, and/or occurs during fermentation.

On face value, this observation of degradation of the chitinases appears to be inconsistent with the known proteolytic resistance and acid stability of these PR proteins. The proportion of the total chitinase pool that was in a degraded form in the juice studied here was however minor. Since the total protein concentration of the juice was still relatively high (237 mg/L), degra-

dation of the chitinases had a very minor effect on the potential heat instability of this juice.

Although it must be borne in mind that the degradation of the chitinases observed here may have been acid catalyzed rather than enzymatic, these observations do indicate that plants might be the source of a proteolytic activity capable of degrading wine haze proteins. Should identification of such resident proteases be possible, their subsequent production through recombinant DNA technology may allow more efficient means of stabilizing wines against protein haze formation.

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