Taste Properties of Grape (*Vitis vinifera*) Pathogenesis-Related Proteins Isolated from Wine

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The major proteins in Sauvignon Blanc wine are grape (*Vitis vinifera*) thaumatin-like proteins. Although authentic thaumatin from miracle fruit (*Thaumattococcus danielli*) is intensely sweet, a panel of 26 participants did not identify a wine protein fraction containing 82% thaumatin-like proteins at either 22, 220, or 2200 mg/L in aqueous solution as sweet. Thus, it is highly unlikely that grape thaumatin-like proteins, which would be present in wine at a substantially lower concentration than the maximum concentration tested, contribute sweetness to wine.

Keywords: *Unstable protein; haze; turbidity; white wine; pathogenesis-related proteins; thaumatin-like proteins; taste; sweetness; Vitis vinifera*

INTRODUCTION

An unattractive and commercially unacceptable haze can form in white wine due to the precipitation of grape-derived proteins (Bayly and Berg, 1967). Recent work has shown that the haze-forming proteins in Muscat of Alexandria wine are pathogenesis-related (PR) proteins of grapevines. One of the major PR proteins in the wine was a thaumatin-like protein (Waters et al., 1996) and had sequence homology to the thaumatins, a group of intensely sweet tasting proteins from the fruit of an African shrub, *Thaumattococcus danielli*. On a molar basis, the thaumatins are 10 000 times sweeter than sucrose, and this sweetness lingers for several minutes after swallowing and rinsing the mouth with water (Van der Wel and Loeve, 1972). The thaumatins also enhance the perception of flavor compounds, mask metallic and bitter tastes, and act in concert with other flavor enhancers, such as monosodium glutamate, in a synergistic manner (Stephens, 1983). In contrast to these highly desirable properties of the thaumatins, wine proteins are a nuisance to commercial winemakers as a result of their potential to form a haze, necessitating their removal before bottling. The possibility that these wine proteins may share the taste properties of, as well as sequence homology to, the thaumatins is of potential significance to the sensory properties of wine. This paper describes (a) the identification of the major protein and two minor proteins from a Sauvignon Blanc wine as all being grape thaumatin-like proteins, (b) the isolation of the proteins from this wine, and (c) the investigation by difference testing of the possible contribution of these proteins to the perception of sweetness in wine.

MATERIALS AND METHODS

Materials. Water was purified by a Milli-Q reagent water system (Millipore Pty. Ltd., Australia). Thaumatin and horse heart cytochrome *c* were obtained from the Sigma Chemical Co. A Sauvignon Blanc wine from the 1996 vintage was collected from a commercial wine producer before bentonite fining had been performed. Technical grade ammonium sulfate was purchased from ACE Chemicals, Australia. Analytical grade sucrose was obtained from Merck Pty. Ltd., Australia.

Isolation of Grape PR Proteins from Wine. Five kilograms of $(NH_4)_2SO_4$ was gradually added over 30 min to 10 L of wine at 4 °C. The $(NH_4)_2SO_4$-saturated wine was then stirred for 1 h in a temperature-controlled room (18 °C), and the final temperature of the wine was 12 °C. The saturated wine was centrifuged (18000*g*, 30 min), and the precipitate was suspended in deionized water (200 mL, Milli RO6 plus water purification system, Millipore Pty. Ltd.) and dialyzed against deionized water (10 L, four changes). The dialyzed sample was centrifuged and the supernatant lyophilized. This procedure was repeated three times.

Analysis and Quantification of Protein. The protein composition of the wine and isolated protein fraction was determined by reversed phase HPLC. Wine samples before and after $(NH_4)_2SO_4$ precipitation were desalted on a PD6G column (Bio-Rad Laboratories, Australia) into water. Lyophilized samples were dissolved in water and filtered (0.45 μm) before loading at 1 mL/min onto a Vydac C_{18} column (4.6 × 250 mm) equilibrated in a mixture of 17% (v/v) solvent A [80% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid] and 83% solvent B [8% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid] and held at 35 °C. Proteins were eluted by a gradient of solvent A from 17% solvent A to 49% solvent A in the first 7 min, from 49 to 57% from 7 to 15 min, from 57 to 65% from 15 to 16 min, and then from 65 to 81% from 16 to 30 min. Peaks were detected at 220 nm and their identity assigned by comparison of their retention times to those of purified grape PR proteins (Waters et al., 1996) and quantified by comparison to the peak area of a protein standard: horse heart cytochrome *c*. Using this system, authentic thaumatin eluted at 10 min and the peak area of 10 μg of authentic thaumatin was equivalent to the peak area of 7.5 μg of cyctochrome *c*.

Protein content was separately determined by multiplying the nitrogen content of the sample by 6.25. Total nitrogen analysis was performed on the lyophilized sample on a NA1500 Series II nitrogen analyser (Carlo Erba Instruments) according to the manufacturer's instructions.

Purification of the Wine Proteins. The isolated protein fraction (2 g/L, 2 mL) was loaded at 2 mL/min on a preparative Vydac C_{18} column (10 × 250 mm) equilibrated in a mixture of 17% (v/v) solvent A and 83% solvent B and held at 35 °C. Proteins were eluted by the gradient of solvent A described above for the analytical column. The peaks of the major thaumatin-like protein and the chitinase were collected. The purity of the collected peaks was confirmed by SDS−PAGE analysis, performed as described by Laemmli (1970), and by analytical HPLC as described above.

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S0021-8561(97)00194-5 CCC: $14.00

Digestion of the Chitinase with Endoproteinase Lys C. The chitinase was reduced and alkylated prior to digestion. The lyophilized chitinase (50 μg) was dissolved in 500 mM Tris-HCl buffer (pH 8.5) containing 6 M guanidine−HCl and 5 mM EDTA (buffer C, 150 μL). Dithioerythritol (74 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 100 mM ammonium carbonate buffer (pH 8.5) containing 2 M urea and 1 mM $CaCl_2$. Endoproteinase Lys C (EC 3.4.99.30, Promega, 200 mg/L in 25 mM NaH_2PO_4, pH 7.5, 2 μL) was added to the reduced and alkylated protein solution (500 mg/L, 40 μL), and the mixture was incubated (23 °C, 22 h). Digestion was terminated by dilution in solvent D [0.05% (v/v) TFA, 100 μL], and an aliquot (135 μL) was loaded at 0.6 mL/min on a Vydac C_{18} column (4.6 × 250 mm) equilibrated in solvent D. Peptides were eluted by a gradient of solvent E [acetonitrile containing 0.045% (v/v) TFA; 1−70% solvent E in 90 min].

Amino Acid Sequencing and Mass Spectrometry. Sequencing of the N terminus of VVTL1 and peptides from the chitinase was performed on a Hewlett-Packard G1000A protein sequencer (Palo Alta, CA).

Grape thaumatin-like proteins from Sauvignon Blanc and Muscat of Alexandria wine were subjected to mass spectrometric analysis on a PE Sciex API 300 with ion spray ionization (Perkin-Elmer, Foster City, CA). The ion spray voltage was 5500 V, and the orifice potential was 30 V. The curtain (nitrogen) and nubulizer (air) gases were set at 8 and 10 units, respectively. Solutions of the proteins in water, or in mixtures of acetonitrile and water and containing 0.05% (v/v) TFA, were diluted 1- fold with 75% (v/v) acetic acid in acetonitrile and introduced into the mass spectrometer by a flow injector (8125, Rheodyne, Cotati, CA) with a 20 μL sample loop connected to the ion spray needle by PEEK tubing. The mobile phase [50% (v/v) acetonitrile containing 2% (v/v) acetic acid] was delivered using a dual-syringe pump (140B Solvent Delivery Systems, Applied Biosystems, Perkin-Elmer) at a flow rate of 5 μL/min.

Mass spectra were acquired under positive ion mode by repetitively scanning the mass analyzer over the range m/z 1600−3000 with a mass step of 0.1 Da and a dwell time of 0.5 ms. The mass spectrum of each scan was integrated over the entire protein peak by multichannel analysis mode and then processed to determine the molecular weight of the protein by deconvolution of the multiply charged ions using BioMultiview software (Perkin-Elmer)

Sensory Analysis. Paired comparison tests for the assessment of sweetness were performed using the method of Meilgaard et al. (1991), and the significance of the tests was determined from statistical tables. The judges were asked which sample of a pair was more sweet—one of the pair was deionized water, the other was the sample to be tested. All samples were aqueous solutions (20 mL, using deionized water) presented for assessment, in coded, covered cups at 18−22 °C, in isolated booths under red light. Judges (26) were recruited from staff and postgraduate students from The Australian Wine Research Institute. Samples assessed for sweetness were 20 g/L sucrose, 22 mg/L (1 μM) authentic thaumatin, and the isolated grape proteins at 22, 220, and 2200 mg/L. The samples were assessed in two sessions on consecutive days.

RESULTS AND DISCUSSION

Characterization of Proteins from Sauvignon Blanc Wine. HPLC analysis showed that the dominant protein components of the Sauvignon Blanc wine were thaumatin-like proteins (Figure 1a). There were three thaumatin-like proteins identifiable by HPLC, the major one, VVTL1, eluting at 10.9 min and the two minor

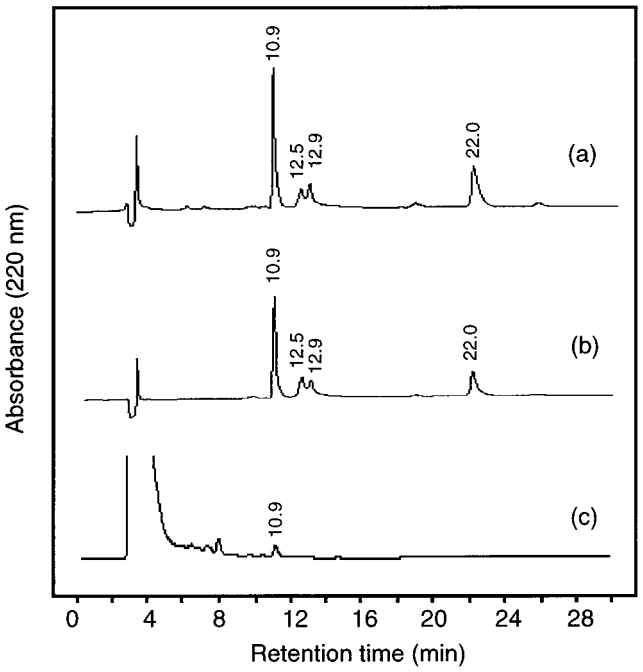

Figure 1. Separation of wine proteins by reversed phase HPLC. The wine before (a) and after (c) $(NH_4)_2SO_4$ precipitation was desalted before injection of a sample equivalent to 150 μL wine onto the reversed phase column. Protein precipitated by $(NH_4)_2SO_4$ (b) was dialyzed and lyophilized before injection of a sample containing protein from 150 μL of wine. The retention times of the protein peaks are given.

thaumatin-like proteins, VVTL2 and VVTL3, eluting at 12.5 and 12.9 min, respectively, from the reversed phase column (Figure 1). All three proteins were assigned by comparison of their retention times to those of purified grape PR proteins (Waters et al., 1996); thus, VVTL1 is the major thaumatin-like protein and VVTL2 and VVTL3 are the two minor thaumatin-like proteins of Muscat of Alexandria wine identified previously by amino acid sequencing (Waters et al., 1996). The assignment of VVTL1 in Sauvignon Blanc wine was then confirmed by N-terminal sequencing, SDS−PAGE, and electrospray mass spectrometry of the protein.

The N-terminal amino acid sequence of VVTL1 from Sauvignon Blanc wine, shown in Figure 2, was identical to that of the major thaumatin-like protein isolated from Muscat of Alexandria wine (Waters et al., 1996; Figure 2) and to the deduced amino acid sequence of a cDNA clone encoding VVTL1 isolated from Muscat of Alexandria grapes (Tattersall et al., 1997; Figure 2).

The relative molecular mass (M_r) estimated by SDS−PAGE of both VVTL1 from Sauvignon Blanc wine and the major thaumatin-like protein isolated from Muscat of Alexandria wine (Waters et al., 1996) was 24 000 (data not shown). By electrospray mass spectrometry, the M_r of VVTL1 from Sauvignon Blanc wine was 21 272, and that of the major thaumatin-like protein isolated from Muscat of Alexandria wine (Waters et al., 1996) and grapes (Tattersall et al., 1997) was also 21 272. Under all conditions of mass spectrometry, all samples of VVTL1 examined (i.e. both that from Sauvignon Blanc wine and that from Muscat of Alexandria wine) contained a minor component with M_r of 21 250, although the samples contained only one N-terminal sequence and appeared homogeneous under other chromatographic conditions.

In the analytical HPLC separation (Figure 1a), there was another protein peak eluting at 22.0 min. By SDS−

SB wine	ATFDI	LNKCT	YTVWA	AASP*	*****	GGGRR	LDSGQ	S			30
MA wine	ATFDI	LNKXT	YTVWA	AASP*	*****	GGGRR	LDSGQ	SWTIT	VNPGT	V	40
MA grape	ATFDI	LNKCT	YTVWA	AASP*	*****	GGGRR	LDSGQ	SWTIT	VNPGT	VNARI	44
Thaumatin	ATFEI	VNRCS	YTVWA	AASKG	**D**AALD	AGGRQ	LNSGE	SWTIN	VEPGT	KGGKI	50
MA grape	WGRTS	CTFDA	NGRGK	CETGD	CNGLL	ECQGY	GSPPN	TLAEF	ALNQP	NNLDY	94
Thaumatin	WARTD	CYFDD	SGRGI	CRTGD	CGGLL	QCKR**F**	GRPPT	TLAEF	SLNQY	GQ*DY	99

Figure 2. Protein sequence of the N terminus of VVTL1 isolated from Sauvignon Blanc wine (SB wine) compared to that of the N terminus of the major thaumatin-like protein from Muscat of Alexandria wine (MA wine; Waters et al., 1996), the first 94 residues of the deduced protein sequence from a cDNA clone encoding VVTL1 from Muscat of Alexandria grapes (MA grape; Tattersall et al., 1997), and the first 99 residues of the deduced protein sequence from a cDNA clone encoding for thaumatin II from *Thaumatococcus danielli* (thaumatin; Edens et al., 1982). Underlined residues and bold residues are those identified by Slootstra et al. (1995) as important to the taste properties of thaumatin and as the residues on the tips of two extruding loops, respectively; X is an unidentified residue, and * is a space added to facilitate alignment of sequences.

SB wine								YGY	CGTGS	DYCGD	
VvChi4A	M	AAKLL	TVLLV	GALFG	AAVAQ	NCGCA	SGLCC	SKYGY	CGTGS	DYCGD	46
VvChi4B	-	-----	-----	-----	-----	-----	-----	-----	R----	-----	
BN	MALTK	LSLVL	FVCFL	GLYSE	TVKSQ	NCGCA	PNLCC	SQFGY	CGSTD	AYCGT	50
SB wine	GCQSG	*****	PCDS*S*	*S*GSGX	XVADI	VTQ*S*F	FDG*A*I	NQ*AA*X	XC		
MA wine			DS*	**SGS	SVSDI	VTQAF	FDGII	NQAAS	SCAGK		30
VvChi4A	GCQSG	*****	PCDS*	**SGS	SVSDI	VTQSF	FDGII	SQAAS	SCAGK	NFYTR	88
VvChi4B	-----	*****	----G	SG---	-----	---A-	-----	N----	-----	-----	91
BN	GCRSG	PCRSP	GGTPS	PPGGG	SVGSI	VTQAF	FNGII	NQAGG	GCAGK	NFYTR	100

Figure 3. Protein sequence of peptide isolated from an endoproteinase Lys C digest of Sauvignon Blanc wine chitinase (SB wine) compared to that of the first 30 residues of a chitinase isolated from Muscat of Alexandria wine (MA wine; Waters et al., 1996), the first 88 and 91 residues of the deduced protein sequence from two closely related cDNA clones encoding chitinases from Shiraz grapes (VvChi4A and VvChi4B; Robinson et al., 1997), and the first 100 residues of the deduced protein sequence from a cDNA clone encoding a chitinase from *Brassica napus* (BN; Rasmussen et al., 1995). The sequence of VvChi4B is identical to that of VvChi4A except where indicated. Amino acids in italics are tentative assignments, X is an unidentified residue, and * is a space added to facilitate alignment of sequences.

Table 1. Protein Composition and Concentration of the Sauvignon Blanc Wine before and after Ammonium Sulfate Precipitation and of the Material Precipitated by Ammonium Sulfate

		concentration					
		wine before $(NH_4)_2SO_4$ precipitation		wine after $(NH_4)_2SO_4$ precipitation		$(NH_4)_2SO_4$ precipitated material	
protein	retention time (min)	mg/L of wine[a]	%[b]	mg/L of wine	%	mg/L of wine	%
VVTL1	10.9	110	47	12	100	77	52
VVTL2	12.5	22	9	0	0	20	14
VVTL3	12.9	33	14	0	0	26	18
chitinase	22.0	71	30	0	0	24	16
total thaumatin-like proteins (VVTL1 + VVTL2 + VVTL3)		165	70	12	100	123	84
total protein		236	100	12	100	147	100

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

PAGE, the estimated M_r of this protein was 32 000 (data not shown), but the protein was presumably derivatized at the N terminus and could not be sequenced. Digestion of this protein with endoproteinase Lys C and sequencing of one of the peptides showed that this protein had sequence homology to a chitinase isolated from Muscat of Alexandria wine (Waters et al., 1996), to the deduced amino acid sequence of two cDNA clones encoding chitinases isolated from Shiraz grapes (Robinson et al., 1997), and to a chitinase from *Brassica napus* leaves (Rasmussen et al., 1995). All sequences are shown in Figure 3.

The amino acid sequences of the proteins in the Sauvignon Blanc wine examined here were similar to, thus the protein identities were the same as, that of proteins in Muscat of Alexandria wine (Waters et al., 1996). By SDS−PAGE, the M_r values of the proteins in the Sauvignon Blanc wine examined here were also similar to that of proteins in wines vinified from different *Vitis vinifera* varieties (Correa et al., 1988; Dawes et al., 1994; Hsu and Heatherbell, 1987; Murphey et al., 1989; Paetzold et al., 1990; Pueyo et al., 1993; Waters et al., 1992). Thus, the proteins present in Sauvignon Blanc wine examined here are representative of white wine proteins in general.

The total protein content of the wine was 236 mg/L, and the thaumatin-like proteins accounted for 70% of the total protein (Table 1).

Isolation of Proteins from Sauvignon Blanc Wine. The majority of the Sauvignon Blanc wine proteins were precipitated by ammonium sulfate, since the supernatant wine after this procedure contained only 12 mg/L of total protein, which was 5% of that originally present (Table 1 and Figure 1c). The protein composition of the isolated precipitate (Figure 1b and Table 1) was similar to that of the wine before treatment, except that the isolated fraction was enriched in the thaumatin-like proteins (84% of total protein was thaumatin-like proteins) compared with that of the wine before treatment (70% of total protein was thaumatin-like proteins). Losses associated with the isolation procedure meant that 67% (147 mg/L) of the total material precipitated by ammonium sulfate (224 mg/L, difference between protein content of the wine before

Table 2. Results of Paired Taste Tests of Aqueous Sucrose, Thaumatin, and Wine Protein Solutions for Sweetness Compared to Water

sample	concentration (mg/L)	no. of judges who identified sample as more sweet than water[a]	significance[b]
sucrose	20000	26	$p < 0.001$
thaumatin	22	26	$p < 0.001$
wine proteins	22	11	not significant
	220	13	not significant
	2200	15	not significant

[a] Total of 26 judges. [b] From statistical table T8 of Meilgaard et al. (1991).

and after ammonium sulfate precipitation; Table 1) was recovered in the isolated fraction. A poor recovery of the chitinase contributed to the overall loss since the thaumatin-like proteins were recovered in high yields in the isolated fraction. The proportion of nitrogen (13.8% w/w) in the isolated fraction indicated that the total protein content of this material was 86% (w/w). From the HPLC analysis, the total protein content on a dry weight basis of this isolated material was estimated to be 81% (w/w).

Isolation of juice and wine proteins by ammonium sulfate precipitation is a common laboratory procedure. In previous work by some of us (Waters et al., 1991), two of the major wine proteins remained soluble after the addition of ammonium sulfate; precipitation of wine protein was not complete. In the current work, we have effected almost total precipitaion of all wine proteins. This was achieved by adding the solid ammonium sulfate slowly and at low temperature. It is also important to resolubilize the majority of the precipitated protein. Preliminary experiments indicated that dialysis of the resuspended precipitate, rather then dialysis of the soluble part only, was the method which gave the greatest recovery of precipitated protein. Our recovery of 67% of the total precipitated wine protein is higher than that reported by Yokotsuka et al. (1991) for the recovery of Koshu juice proteins after dialysis and then precipitation by ammonium sulfate. Other publications regarding the precipitation of juice or wine proteins by ammonium sulfate do not give values for the recovery of protein.

Sensory Analysis of Proteins from Sauvignon Blanc Wine. Informal assessment of the isolated protein fraction from the Sauvignon Blanc wine indicated that the proteins of this material in aqueous solution had no obvious taste or aroma. To formally establish whether the fraction had any sweet taste properties, paired comparison tests with a panel of 26 participants were carried out. In initial assessment, all participants correctly identified solutions containing 20 g/L sucrose or 22 mg/L authentic thaumatin as more sweet than water (Table 2). However, the isolated wine protein fraction at 22, 220, or 2200 mg/L was found to be not significantly sweeter than water (Table 2). Since the major proteins in the isolated fraction were the grape-derived thaumatin-like proteins, this result indicates that grape thaumatin-like proteins are not sweet at these levels.

The concentration of total protein in wine is reported to range from 1.5 mg/L (Yokotsuka et al., 1977) to 840 mg/L (Somers and Ziemelis, 1973) and was 236 mg/L in the wine used in this study (Table 1). Thus, wine proteins and, in particular, grape thaumatin-like proteins would not contribute any sweetness to wines even at levels far in excess of those at which they are naturally present.

It has been postulated that the presence and spatial position of lysine residues are important to the sweet properties of authentic thaumatin (Ogata et al., 1992; Van der Wel and Bel, 1976). The low levels of these basic residues in acidic proteins like wine proteins (Waters et al., 1995; Yokotsuka et al., 1994) may be a partial explanation for the lack of sweet taste properties of the acidic thaumatin-like proteins of wine. Using an immunological approach, Slootstra et al. (1995) identified two regions of amino acid sequence in authentic thaumatin as containing the taste determinants (shown underlined in Figure 2). These regions form two extruding loops. In these regions, VVTL1 shows only 37% homology to authentic thaumatin. In addition, the two amino acid residues on the tips of these extruding loops (in bold type in Figure 2), which allegedly form an aspartame-like site in authentic thaumatin, are missing in VVTL1. Thus, the lack of these putative sweet taste determinants in the amino acid sequence of VVTL1 is a likely explanation for its lack of sweet sensory properties. Thaumatin-like proteins from other plant tissues, such as tobacco leaves (Singh et al., 1987) and cherry fruit (Fils-Lycaon et al., 1996), also lack homology to authentic thaumatin in the regions of sequence containing the putative sweet taste determinants, and, like grape thaumatin-like proteins, these proteins do not taste sweet.

The sensory data accumulated in this work and reported for other plant thaumatin-like proteins, considered together with the amino acid sequences of the proteins, add further support to the identification by Slootstra et al. (1995) of the sweet taste determinants in authentic thaumatin. Thus, in spite of the general sequence and structural homology of thaumatin-like proteins to that of authentic thaumatin, it appears that the sweetness of thaumatin is a unique property of that protein which is not shared by the thaumatin-like proteins from other plants.

ACKNOWLEDGMENT

We thank Southcorp Wines (Nuriootpa) for the supply of wine and the participants of the sensory study for their cooperation. We also thank Nick Robinson and Jelle Lahnstein of the Department of Plant Science of The University of Adelaide and Yoji Hayasaka of the Australian Wine Research Institute for performing the nitrogen analysis, protein sequencing, and electrospray mass spectrometry, respectively. We also thank Simon Robinson of the Division of Horticulture, CSIRO, and Peter Høj, Robyn van Heeswijck and David Tattersall of the Department of Horticulture, Viticulture and Oenology, University of Adelaide, for valuable discussions about this work.

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Received for review March 12, 1997. Revised manuscript received July 14, 1997. Accepted September 4, 1997.[⊗] Financial support was provided by the Grape and Wine Research and Development Corp.

JF970194A

[⊗] Abstract published in *Advance ACS Abstracts*, October 15, 1997.