Nuisance Proteins of Wine Are Grape Pathogenesis-Related Proteins

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INTRODUCTION

An unsightly cloudiness or haze irretrievably mars consumer perception of even the finest white wine. A common cause of haze in wine is the precipitation of grape-derived proteins (Bayly and Berg, 1967; Hsu and Heatherbell, 1987a; Waters et al., 1992). Haze formation is traditionally prevented by protein removal through adsorption onto bentonite but, because this procedure is wasteful and flavor denuding (Miller et al., 1985), there is much interest in alternative removal practices. An understanding of the protein substrates is a prerequisite to the development of any alternative industrially viable winemaking practice. These proteins, which are apparently similar in wines vinified from different *Vitis vinifera* varieties (Correa et al., 1988; Dawes et al., 1994; Hsu and Heatherbell, 1987b; Murphey et al., 1989; Paetzold et al., 1990; Pueyo et al., 1993; Waters et al., 1992), are stable at the acid pH of wine and have proteolytic resistance (Feuillat and Ferrari, 1982; Waters et al., 1992, 1995a). A hitherto unrelated aspect of *V. vinifera* protein biochemistry concerns pathogenesis-related (PR) proteins. These are produced by plants as a defense against fungal pathogens and are sought after because of their potential as biocontrol agents (Boller, 1987; Linthorst, 1991). Proteins belonging to the acidic class of plant PR proteins are stable at acid pH and are highly proteolytically resistant (Linthorst, 1991). We report that the ubiquitous, acid-stable, proteolytically resistant, and troublesome proteins of wine are PR proteins of grapevines that are present in grapes.

MATERIALS AND METHODS

White wine vinified from *V. vinifera* cv. Muscat of Alexandria grapes was obtained from an Australian wine producer. The residual wine proteins were collected by ammonium sulfate precipitation and then separated by anion exchange chromatography as described previously (Waters et al., 1995a). The three major wine proteins were collected in pooled fractions A, F, and I (Waters et al., 1995a).

Wine protein Ib was purified from fraction I by reversed phase HPLC: Fraction I (200 μg) in 0.05% (v/v) trifluoroacetic acid (TFA, 100 μL) was loaded at 0.6 mL/min onto a Vydac C_{18} column (4.6 × 250 mm) equilibrated in 0.05% (v/v) TFA. Proteins were eluted by a gradient of acetonitrile containing 0.045% (v/v) TFA (solvent B; 1−40% solvent B in 5 min, then to 70% solvent B between 5 and 25 min); wine protein Ib eluted at 12 min.

Wine protein Ia was purified from fraction I by reversed phase HPLC, as described above, eluting at 22 min. After lyophilization, the protein was dissolved in 100 mM ammonium carbonate buffer (pH 8.5) containing 8 M urea and heated (90 °C, 1 h). On cooling, the protein solution was diluted 4-fold with 100 mM ammonium carbonate buffer (pH 8.5) and either trypsin (EC 3.4.21.4, Sigma) or staphylococcal protease (strain V8, EC 3.4.21.19, Boehringer Mannheim) added at an enzyme to substrate ratio of 1:10. After incubation (37 °C, 2 h for trypsin, 6 h for staphylococcal protease), digestion was terminated by dilution in TFA (2% v/v) and the mixture loaded at 0.6 mL/min on a Vydac C_{18} column (4.6 × 250 mm) equilibrated in 0.05% (v/v) TFA. Peptides were eluted by a gradient of acetonitrile containing 0.045% (v/v) TFA (solvent B; 1−70% solvent B in 90 min).

Sequencing was performed on a Hewlett-Packard G1000A protein sequencer, and SDS−PAGE was performed as described by Laemmli (1970).

RESULTS AND DISCUSSION

The N-terminal sequence of the protein present in fraction A, a single band at M_r 24 000 by SDS−PAGE, showed homology to thaumatin and to a number of plant thaumatin-like proteins (Figure 1).

Fraction F contained a major protein band at M_r 28 000 and a minor protein band at M_r 24 000. Two N-terminal sequences were observable in the mixture: a minor one identical to that of fraction A and a major one with homology to plant chitinases (Fa, Figure 2).

Fraction I contained a major band at M_r 32 000 (Ia) which was presumably derivatized at the N terminus and could not be sequenced. Digestion of the major protein (Ia) with trypsin provided two peptides (T1 and

protein	1																																				
A	ala	thr	phe	asp	ile	leu	asn	lys	X	thr	tyr	thr	val	trp	ala	ala	ala	ser	pro	gly	gly	gly	arg	arg	leu	asp	ser	gly	gln	ser	trp	thr	ile	thr	val	asn	pro	gly	thr	thr
Ib	ala	thr	phe	asn	ile	gln	asn	his	X	ser	tyr	thr	val	trp	ala	ala	ala	val	pro	gly	gly	gly	met	gln	leu	gly	ser	gly	gln	ser	-	-	-	-	-	-	-	-	-	-
TD	ala	thr	phe	glu	ile	val	asn	arg	cys	ser	tyr	thr	val	trp	ala	ala	ala	ser	lys	gly	asp	ala	ala	leu	asp	ala	gly	gly	arg	gln	leu	asn	ser	gly	glu	ser	trp	thr	ile	asn
NT	ala	thr	phe	asp	ile	val	asn	gln	cys	thr	tyr	thr	val	trp	ala	ala	ala	ser	pro	gly	gly	gly	arg	gln	leu	asn	ser	gly	gln	ser	trp	ser	ile	asn	val	asn	pro	gly	thr	val
LE	ala	thr	phe	glu	val	arg	asn	asn	cys	pro	tyr	thr	val	trp	ala	ala	ser	thr	pro	ile	gly	gly	gly	arg	arg	leu	asp	arg	gly	gln	thr	trp	val	ile	asn	ala	pro	arg	gly	thr
OS	ala	thr	phe	thr	ile	thr	asn	arg	cys	ser	phe	thr	val	trp	pro	ala	ala	thr	pro	val	gly	gly	gly	val	gln	leu	ser	pro	gly	gln	thr	trp	thr	ile	asn	val	pro	ala	gly	thr
TA	ala	thr	phe	asn	ile	lys	asn	asn	cys	gly	phe	thr	ile	trp	pro	ala	gly	ile	pro	val	gly	gly	gly	phe	ala	leu	gly	ser	gly	gln	thr	ser	ser	ile	asn	val	pro	ala	gly	thr

Figure 1. Amino acid sequences of wine protein fraction A and wine protein Ib, compared to those of *T. danielli* thaumatin (TD, M_r 23 000) (Edens et al., 1982) and other thaumatin-like proteins from *Nicotiana tabacum* (NT, tobacco, M_r 25 000) (van Kan et al., 1989), *Lycopersicon esculentum* (LE, tomato, M_r 25 000) (Rodrigo et al., 1993), *Oryza sativa* (OS, rice, M_r 17 000) (Reimmann and Dudler, 1993), and *Triticum aestivum* (TA, wheat, M_r 17 000) (Rebmann et al., 1991). Residue numbers refer to the mature protein. X indicates that no residue was detected; − indicates that sequencing was not performed. Identical residues in the sequences are shaded.

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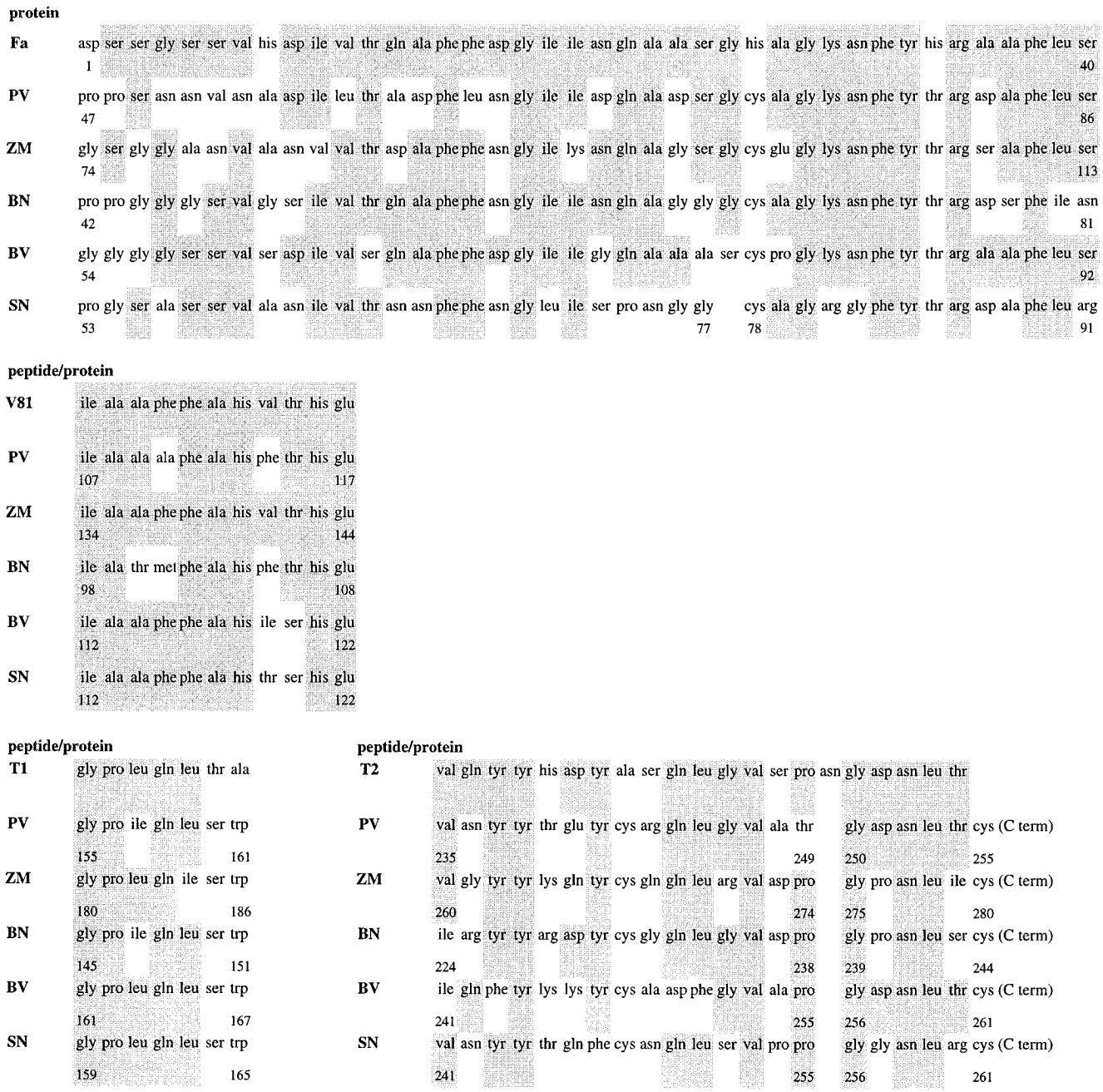

Figure 2. Amino acid sequences of wine protein Fa and peptides obtained from digests of wine protein Ia with staphylococcal protease (strain V8, V81) and trypsin (T1 and T2), compared to those of plant chitinases from *Phaseolus vulgaris* (PV, kidney bean) (Margis-Pinheiro et al., 1991), *Zea mays* (ZM, maize) (Huynh et al., 1992), *Brassica napus* (BN, rape) (Rasmussen et al., 1992), *Beta vulgaris* (BV, sugar beet) (Nielsen et al., 1994), and *Sambucus nigra* (SM, elder, EMBL accession Z46948). Residue numbers refer to the mature protein. Identical residues in the sequences are shaded.

T2), and digestion with staphylococcal protease (strain V8) provided another peptide (V81). All three peptides had sequence homology to plant chitinases (Figure 2). The N-terminal sequences of the minor protein species in fraction I had homology to thaumatin and other plant thaumatin-like proteins. One was identical to and another (Ib) was similar to fraction A (Figure 1). Both possessed M_r 24 000 by SDS−PAGE.

Thaumatin-like proteins and chitinases belong to a group of plant PR proteins that are believed to play a role in plant resistance (Boller, 1987; Linthorst, 1991). Thaumatin-like proteins have sequence homology to the thaumatins, a group of intensely sweet tasting proteins from the fruit of an African shrub, *Thaumatococcus danielli* (Edens et al., 1982). Although taste tests have shown that the thaumatin-like proteins from tobacco are not sweet tasting (Singh et al., 1987), the possibility that a protein with sweet taste properties occurs naturally in wine is tantalizing and of potential significance to the wine industry. The physiological role of thaumatin-like proteins in plants, especially in resistance to pathogens, has not yet been elucidated.

In contrast to thaumatin, there is strong correlative 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). Our analyses have shown that there are at least two forms of grape chitinases in wine (Fa and Ia) differentiated by electrophoretic mobility, with one being evidently N-terminally blocked (Ia). The apparent M_r difference between the two chitinases is not due to glycosylation or complexation with polyphenolic components (Waters et al., 1995a,b). It also seems unlikely to be due to proteolytic degradation because these wine proteins (Waters et al.,

1995a), and PR proteins in general (Linthorst, 1991), are highly resistant to proteolytic attack.

This resistance to proteolysis and stability at acid pH means that winemaking is a selective extraction procedure for grape berry PR proteins. Crushing of the fruit destroys the compartmentalization of the berry, releasing vacuolar acids and hydrolytic enzymes. Subsequent fermentation of the must by yeast further augments the proteolytic enzyme pool (Lagace and Bisson, 1990). This combination of low pH (3.0−3.8) and proteolytic activity ensures that only proteins resistant to these conditions, such as PR proteins, survive the winemaking process. Indeed, winemaking or similar processes based on acid conditions and proteolysis may prove to be an appropriate and easy initial purification procedure for researchers working on grapevine PR proteins. Strategies based on proteolysis for the subsequent removal of protein from wine, however, have proved unsuccessful in practice (Feuillat and Ferrari, 1982; Waters et al., 1992, 1995a), and, given the nature of PR proteins, are probably futile.

Recognition of the nature and origin of residual wine proteins suggests that appropriate viticultural practices, rather than postharvest processing, may hold the key to controlling the level of protein in wine. Identification of factors that cause accumulation of PR proteins in berries could allow the development of tactics to minimize expression of PR proteins in fruit without compromising the disease resistance of grapevines.

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