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Profiling of Soluble Proteins in Wine by Nano-High-Performance Liquid Chromatography/Tandem Mass Spectrometry

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Wine proteins play an important role in a wine's quality as they affect taste, clarity, and stability. To enhance our understanding of the proteins in wine, nano-high-performance liquid chromatography (HPLC)/tandem mass spectrometry was used to profile soluble proteins in wine. Twenty proteins were identified from a Sauvignon Blanc wine including five proteins derived from the grape, 12 from yeast, two from bacteria, and one from fungi. The findings are somewhat peculiar at first glance, but reasonable explanations can account for the results. The grape proteins identified are less in number, which may be due to the availability of an incomplete database and possibly bentonite fining. The relatively large number of identified yeast proteins may be due to their complete protein database. The identified bacterial and fungal proteins could possibly be attributed to sources in the vineyard including natural infections and improper handling during harvest. The use of nano-HPLC/tandem mass spectrometry is an important tool for identifying wine proteins and understanding how they affect its characteristics.

KEYWORDS: Wine; nano-HPLC; tandem mass spectrometry

INTRODUCTION

Wine has many low molecular weight organic compounds such as catechin, *trans*-resveratrol, flavonoids, procyanidins, tartaric acid, and other polyphenolic compounds, which have been examined for their pharmacological impact via a variety of modern analytical methods (*1*−*8*). In contrast, nitrogen-containing high molecular weight compounds, such as proteins, are some of the least investigated compounds in wine. Despite the low amount of proteins in wine, they play important roles in various technological and enological issues, although they contribute minimally to its nutritive value (*9*−*16*).

A major characteristic that consumers look for in wine is its clarity. The clarity of wine is directly related to its quality and indicates the amount of fining it underwent during production. A poorly refined wine may contain proteins that precipitate out after production and contribute to the wine's haziness (*10*). This makes the wine less desirable, reduces its value, and returns no profit for the producers. Some yeast proteins were reported to reduce the haze formation in white wine while other grape proteins induce it (*14*, *15*). A number of other proteins contribute to the formation and stability of foam in champagne-based wines (*9*). In terms of biological properties, thaumatin-like proteins and chitinases are known to have antifungal activities (*10*, *16*). Understanding these factors and the role of proteins in wine and how they contribute to its quality can lead to better methods of production of higher quality wines.

In the past, several methods for the preparation and the detection of wine proteins have been employed to study their properties. These include dialysis, ultrafiltration, precipitation, exclusion chromatography, one- or two-dimensional electrophoresis, capillary electrophoresis, isoelectric focusing, affinity chromatography, immunodetection, high-performance liquid chromatography (HPLC), and fast protein liquid chromatography (*17*−*24*). These studies indicate that the majority of wine proteins are in the range of 20−30 kDa molecular mass, and proteins that are less abundant remained to be characterized.

The profiling of proteins in white wine using one dimensional sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) coupled with nano-HPLC/tandem mass spectrometry is reported for the first time in this study. Twenty proteins, identified from the Sauvignon Blanc wine, are found to originate from grape, yeast, bacteria, and fungi. The latter two suggest that nonwine components of this particular wine may trace back as far as the vineyard and harvest, being that the bacteria and fungi found are generally related to grape cultivation. Simultaneous identification of many proteins here has gone unreported in previous papers that employed other detection methods. Therefore, a nano-HPLC/tandem mass spectrometry would be a suitable analytical tool for the identification of proteins in wine.

EXPERIMENTAL PROCEDURES

Materials. The following reagents were purchased from commercial companies and used without further treatment. HPLC grade acetonitrile, water, and methanol were from EM Science (Gibbstown, NJ); trifluoroacetic acid (TFA) was from Fluka (Buchs, Switzerland); acetic acid was from Aldrich (St. Louis, MO); ammonium bicarbonate, ammonium sulfate, bromophenol blue, SDS, monobasic sodium phosphate, and dibasic sodium phosphate were from Fisher (Fair Lawn, NJ); am-

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monium persulfate, acrylamide, Tris-HCl, Tris base, glycine, glycerol, 2-mercaptoethanol, and bovine serum albumin were from Sigma (St. Louis, MO); TEMED and Colloidal Coomassie Blue staining kit were from Invitrogen (Carlsbad, CA); the protein standards solution was from Bio-Rad (Hercules, CA); and trypsin was from Promega (Madison, WI).

Extraction of Protein from Wine. A 2002 vintage Sauvignon Blanc wine (14.1% (v/v) alcohol and pH 3.1) purchased from a commercial wine market was used in this study. Twenty milliliters of wine was centrifuged at 10000*g* for 30 min and filtered with 0.22 μm pore size cellulose acetate membrane filter (Corning Inc., Corning, NY). The protein concentration was determined as 11.2 mg/L by simple colorimetric assay based on the Bradford dye-binding procedure (Bio-Rad Laboratories). The soluble wine proteins were generated by centrifugation at 4000*g* for 30 min using a 5 kDa regenerated cellulose membrane centrifugal filter tube (Millipore, Billerica, MA). Then salting-out precipitation was carried out by 80% (v/v) saturated ammonium sulfate aqueous solution, and white protein pellets were formed after being centrifuged at 14000*g* for 30 min.

Protein Separation and Digestion. The precipitated protein pellets were redissolved in 50 μL of SDS sample buffer [12.5% (v/v) 0.5 M Tris-HCl/10% (v/v) glycerol/2% (w/v) SDS/5% (v/v) 2-mercaptoethanol/0.001% (w/v) bromophenol blue] with a vortex and boiled for 5 min (*25*). Afterthe sample was cooled to room temperature and centrifuged at 10000*g* for 1 min, the supernatant was loaded onto a homemade 12% (w/v) polyacrylamide gel (80 mm × 70 mm × 1.5 mm) with protein standards. A constant voltage of 150 V was applied to the gel for 50 min at room temperature, and the gel was stained using Colloidal Coomassie Blue staining kit's (3 h staining and 7 h washing).

Protein bands were excised from the gel slab. Each protein band was cut into one cubic millimeter pieces and destained with 25 mM NH_4HCO_3 in 50% methanol/50% water (v/v) three times for 10 min. Then they were washed with 10% acetic acid/50% methanol/40% water (v/v/v) three times for an hour each time and swollen in water twice for 20 min each time. After that, the gel pieces were dehydrated with acetonitrile and dried in the SpeedVac (Thermo Savant, Holbrook, NY). The gel pieces were again rehydrated with modified porcine trypsin at the concentration of 10 ng/μL in 50 mM NH_4HCO_3 and then subjected to trypsin proteolytic digestion at 37 °C overnight. Tryptic peptides were sequentially extracted with 50% acetonitrile/45% water/5% TFA (v/v/v) and 75% acetonitrile/24.9% water/0.1% TFA (v/v/v) solutions. The peptide extracts were combined and dried in the SpeedVac. The peptide samples were cleaned with ZipTip C_{18} (Millipore, Bedford, MA) prior to nano-HPLC/tandem mass spectrometry analysis.

Nano-HPLC/Mass Spectrometry for Protein Identification. Nano-HPLC/tandem mass spectrometry analysis was performed using a LCQ DECA XP ion trap mass spectrometer (ThermoFinnigan, San Jose, CA) equipped with a nano ESI source (ThermoFinnigan). The electrospray source was coupled online with an Agilent 1100 series nano flow HPLC system (Agilent, Palo Alto, CA). Two microliters of the peptide solution in buffer A [2% acetonitrile/97.9% water/0.1% acetic acid (v/v/v)] was manually loaded into a capillary HPLC column (50 mm length × 75 μm ID, 5 μm particle size, 300 Å pore diameter) packed in-house with Luna C18 resin (Phenomenex, Torrance, CA). The peptides were eluted from the column with a gradient of 5−80% buffer B [90% acetonitrile/9.9% water/0.1% acetic acid (v/v/v)] in buffer A for 10 min. The flow rate of nano-HPLC was 0.3 μL/min. The eluted peptides were electrosprayed directly into the LCQ mass spectrometer.

The MS/MS spectra were acquired in a data-dependent mode that determined the masses of the parent ions and fragments of the three strongest ions. All MS/MS spectra were searched against the NCBI nonredundant protein sequence database using the Knexus program (Genomic Solutions, Ann Arbor, MI) for protein identification. All proteins with less than two peptides were sufficiently identified, and the redundant proteins were removed from the identification list to increase confidence in identification. All of the spectra were manually analyzed again to ascertain the accuracy of protein identification.

RESULTS AND DISCUSSION

Sample Preparation. The Colloidal Coomassie Blue staining method was chosen because of its relatively high sensitivity and compatibility with mass spectrometry. **Figure 1** shows wine proteins resolved in SDS−PAGE. Although a relatively high amount (56 μg) was loaded, only two distinct regions were apparent. This may explain why low abundant wine proteins have proven difficult to study in the past.

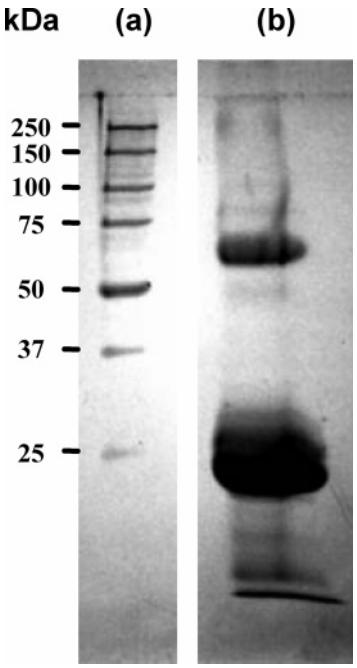

Figure 1. SDS–PAGE separation of wine proteins: The proteins were resolved in 12% (w/v) acrylamide and stained with Colloidal Coomassie Blue. (**a**) Molecular markers were run, and the molecular weight of each protein marker was shown in the left lane. (**b**) Wine proteins isolated by centrifugal filtration and precipitation were run.

Twenty-six gel slices were excised according to the density of proteins in the gel. The regions with dark or multiple bands were cut to 1 mm in width, and the regions with light bands were cut to 3 mm in width. Excised gel slices were subjected to in-gel digestion, and the tryptic peptides hydrolyzed at the carboxyl side of lysine and arginine residues by trypsin were analyzed by nano-HPLC/tandem mass spectrometry for protein identification.

Criteria for Protein Identification. Figure 2 shows a representative nano-HPLC/tandem mass spectrometry analysis of a peptide from a protein. The three strongest parent ions of one full MS spectrum were selected for fragmentation, and normally, 700−1300 spectra were generated during the analysis time. Each MS/MS spectrum was searched against the NCBI nonredundant protein sequence database with the program Knexus. The following search parameters were used in all Knexus searches: use of unmodified peptides, allowance for one missed trypsin cleavage, 4 Da error tolerance in the MS, and 0.6 Da error tolerance in the MS/MS.

Manual analysis was conducted to validate protein identification results. The following criteria were used for manual verification. All major isotope-resolved peaks should match fragment masses of the identified peptide. y, b, and a ions as well as their water loss or amine loss peaks are considered. The isotope-resolved peaks were emphasized because a single peak could come from an electronic spark and is less likely to be relevant to peptide fragments. Typically, >7 isotope-resolved peaks were matched to theoretical masses of the peptide fragments. All redundant proteins were removed by confirming the unique peptides. To confirm the unique peptides, all amino acid sequences of the identified proteins were listed and each

Table 1. List of Identified Proteins from Wine; Proteins Were Sorted by Species and Mass

identified protein	mass (kDa)	gi number	identified peptide	species
laccase 2	63.4	15022489	(K)SPANFNLVNPPR	*B. fuckeliana*
			(R)YDSSSTVDPTSVGVTPR	
succinyl-CoA synthetase	41.2	26990878	(K)ATIDPLVGAQPFQGR	*P. putida* KT2440
			(K)ELYLGAVVDR	
			(R)LEGNNAELGAK	
			(K)QLFAEYGLPVSK	
translation elongation factors	77.1	23470603	(K)IATDPFVGTLTFVR	*P. syringae pv. syringae* B728a
			(K)LAQEDPSFR	
YJU1	21.8	4814	(K)DGSSYIFSSK	*Saccharomyces cerevisiae*
			(K)EGSESDAATGFSIK	
			(K)FDDDKYAVVNEDGSFK	
			(K)LGSGSGSFEATITDDGK	
			(R)SGSDLQYLSVYSDNGTLK	
endo-β-1,3-glucanase	34.1	6321721	(K)AALQTYLPK	*S. cerevisiae*
			(K)ESTVAGFLVGSEALYR	
			(K)HWGVFTSSDNLK	
			(K)IKESTVAGFLVGSEALYR	
			(R)NDLTASQLSDK	
			(R)NDLTASQLSDKINDVR	
			(K)STSDYETELQALK	
			(R)SVVADISDSDGK	
GP38	37.3	297485	(R)GVLSVTSDK	*S. cerevisiae*
			(K)NAVGAGYLSPIK	
			(K)RGVLSVTSDK	
			(K)SALESIFP	
			(K)WFFDASKPTLISSDSIIR	
target of SBF	47.9	6319638	(K)AAVIFNSSDK	*S. cerevisiae*
			(R)EGIPAYHGFGGADK	
			(K)LISHIHDGQDGGTQDYFERPTDGTLK	
ECM33 protein precursor	48.3	1351738	(K)KVNVFNINNNR	*S. cerevisiae*
			(K)VGQSLSIVSNDELSK	
			(K)VNVFNINNNR	
putative glycosidase	49.9	6320795	(K)NSGGTVLSSTR	*S. cerevisiae*
			(K)YQYPQTPSK	
acid phosphatase	52.7	6319568	(K)QSETQDLK	*S. cerevisiae*
			(K)YDTTYLDDIAK	
			(R)YSYGQDLVSFYQDGPGYDMIR	
putative glycosidase	52.7	6321628	(R)GEFHGVDTPTDK	*S. cerevisiae*
			(K)TTWYLDGESVR	
			(K)VIVTDYSTGK	
β-1,3-glucanosyltransferase	59.5	6323967	(K)IPVGYSSNDDEDTR	*S. cerevisiae*
			(R)KIPVGYSSNDDEDTR	
			(K)KLNTNVIR	
			(K)LNTNVIR	
			(K)TLDDFNNYSSEINK	
			(K)YGLVSIDGNDVK	
invertase 4 precursor	60.5	124705	(K)FSLNTEYQANPETELINLK	*S. cerevisiae*
			(K)GLEDPEEYLR	
			(K)IEIYSSDDLK	
			(R)KFSLNTEYQANPETELINLK	
endo-β-1,3-glucanase	63.5	6320467	(R)QFIEAQLATYSSK	*S. cerevisiae*
			(K)SPVVGIQIVNEPLGGK	
			(K)TWITEDDFEQIK	
daughter cell specific secreted protein	121	6324395	(R)DVANPSEKDEYFAQSR	*S. cerevisiae*
			(K)DWVNSLVR	
			(K)IGSSVGFNTIVSESSSNLAQGILK	
			(K)NEESSSEDYNFAYAMK	
			(R)SETFVEEEWQTK	
basic extracellular β-1,3-glucanase precursor	14.6	4151201	(K)HWGLFLPNK	*V. vinifera*
			(K)TYNSNLIQHVK	
putative thaumatin-like protein	20.1	7406714	(R)CPDAYSYPK	*V. vinifera*
			(R)TNCNFDASGNGK	
			(K)TRCPDAYSYPK	
VVTL1	23.9	2213852	(K)CTYTVWAAASPGGGR	*V. vinifera*
			(R)LDSGQSWTITVNPGTTNAR	
			(R)RLDSGQSWTITVNPGTTNAR	
class IV endochitinase	27.5	2306813	(R)AAFLSALNSYSGFGNDGSTDANK	*V. vinifera*
			(R)AAFLSALNSYSGFGNDGSTDANKR	

Table 1. Continued

identified protein	mass (kDa)	gi number	identified peptide	species
vacuolar invertase 1	71.5	1839578	(R)DPTTMWVGADGNWR (K)GWASLQSIPR (R)ILYGWISEGDIESDDLK (K)KGWASLQSIPR (K)TFFCTDLSR (R)VLVDHSIVEGFSQGGR (R)ILYGWISEGDIESDDLKK (R)SSLAVDDVDQR (R)TAFHFQPEK (K)YENNPVMVPPAGIGSDDFR (R)VYPTEAIYGAAR (R)SCITTRVYPTEAIYGAAR	*V. vinifera*

Table 2. Biochemical Features of Yeast and Grape Proteins

yeast protein's name (systematic name, gene name)	molecular function	cellular component
β-1,3-glucanosyltransferase (YMR307W, GAS1)	1,3-β-glucanosyltransferase activity	plasma membrane
acid phosphatase (YBR092C, PHO3)	acid phosphatase activity	periplasmic space
invertase 4 precursor (N/A, SUC4)	β-fructofuranosidase activity	unknown
endo-β-1,3-glucanase (YGR282C, BGL2)	glucan 1,3-β-glucosidase activity	cell wall
endo-β-1,3-glucanase (YDR261C, EXG2)	glucan 1,3-β-glucosidase activity	cell wall
daughter cell specific secreted protein (YNR067C, DSE4)	glucan 1,3-β-glucosidase activity	cell wall, extracellular, septum
YJU1 (YKL096W, YJU1)	structural constituent of cell wall	cell wall
GP38 (YNL160W, YGP1)	unknown	cell wall
putative glycosidase (YGR189C, CRH1)	unknown	cell wall
putative glycosidase (YEL040W, UTR2)	unknown	cell wall
target of SBF (YBR162C, TOS1)	unknown	cell wall
ECM33 protein precursor (YBR078W, ECM33)	unknown	plasma membrane

grape protein's name	biological function
basic extracellular β-1,3-glucanase precursor	pathogenesis-related protein
putative thaumatin-like protein	cell wall and stress response protein
VVTL1	conjunction with the onset of sugar accumulation and berry softening
C class IV endochitinase	expression in grape berries during ripening
vacuolar invertase 1	sugar accumulation in grape berries

peptide was examined. The solid validation procedures and well-established confirmation criteria described above can allow wide dispersion of these methods in laboratories for the purpose of protein identification.

Eighty peptides corresponding to 20 proteins were unambiguously identified, and their theoretical molecular weights also corresponded well with their migration on SDS−PAGE. The proteins assigned by not less than two peptides were considered correctly identified. Individual proteins that contained only one properly identified matching peptide were excluded from the protein identification list even though their spectra matched perfectly and the score given by the program was high. On the basis of this strict criterion, the probability of false-positive protein identification was very low when compared to a list that includes the single peptide MS/MS. However, it is acknowledged that several pertinent proteins may be ignored using this method. Some proteins were identified from unseen bands as the detection limit of the present nano-HPLC/tandem mass spectrometry system is less than that of Colloidal Coomassie Blue staining.

Evaluation of Identified Proteins. In comparison to 12 yeast proteins, only five grape proteins were identified. The small number of identified grape proteins may result from the incomplete grape protein database or the bentonite fining used in the production of wine. The NCBI-nr grape protein database is small and incomplete when compared with the complete yeast protein database (ftp://ftp.ncbi.nih.gov/blast/db/FASTA/nr.gz). To overcome this problem, isolation of pure protein followed by N-terminal sequence would be needed to investigate the unknown proteins. If the proteins are not easily isolated, de novo sequencing method using ^{18}O water-containing trypsin could be used for the protein mixture samples (*26*). As for physical treatment, wine is treated with bentonite to remove grape proteins for the purpose of preventing haziness in white wines, which also helps to explain the smaller number of grape proteins found. Another possible reason for the low number of wine proteins is that suitable peptides may not be produced easily from the wine proteins pool. As for class IV endochitinase, an assigned protein of this study, trypsin could generate only a few pertinent peptides for mass spectrometry.

Table 1 shows the list of identified white wine proteins, and the biochemical features of proteins were displayed in **Table 2**. Interestingly, yeast invertase, characterized as having a haze preventative property against heat unstable grape proteins, was identified in this study. Grape thaumatin-like proteins and chitinases, which are known to be antifungal, acid soluble, and resistant to proteases, were also identified.

It is generally thought that there are no bacteria or fungi proteins in wine. However, three proteins from fungus and bacteria were identified as follows: laccase 2 from *Botryotinia fuckeliana*, succinyl-CoA synthetase from *Pseudomonas putida* KT2440, and translation elongation factors from *Pseudomonas*

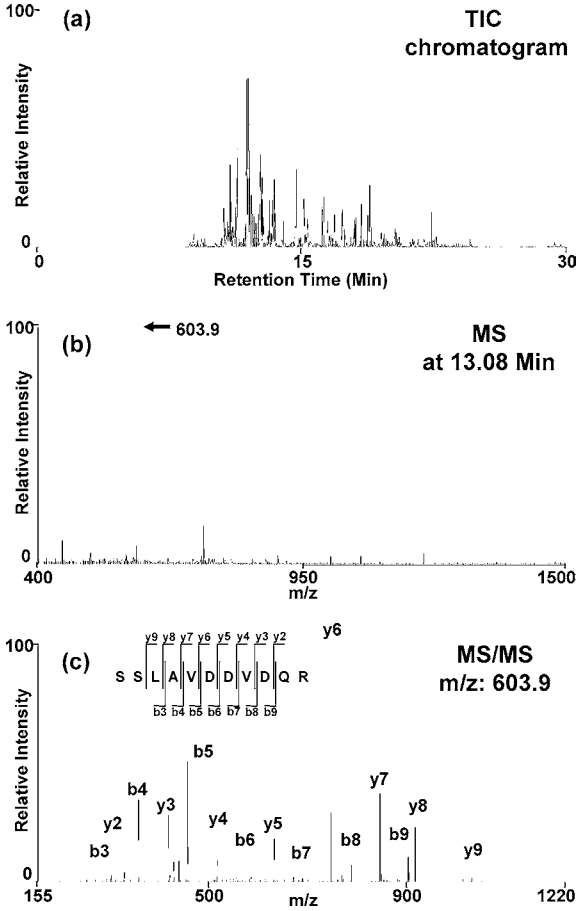

Figure 2. Example of nano-HPLC/tandem MS analysis results: (**a**) Total ion current (TIC) chromatogram of a nano-HPLC/tandem MS of the tryptic digest of gel slice 7 (MW range 60–75 kDa in SDS–PAGE). (**b**) Full MS spectrum at the retention time of 13.08 min (400–1500 *m*/*z*). (**c**) MS/MS spectrum of 603.9 *m*/*z*, which identified the peptide SSLAVDDVDQR, unique to vacuolar invertase 1 from *V. vinifera*.

syringae pv. syringae B728a (*27*−*29*). However, the fact that the above three species are generally related to plant cultivation makes these findings not surprising. *B. fuckeliana* is the causative agent of gray mold on fruits and vegetables and is an omnipresent plant pathogenic fungus. The *Pseudomonas* genus is the ubiquitous bacterium observed from many sources. *P. putida* KT2440 originates from restriction deficient derivatives of *P. putida* mt-2 and is a bacterium specific to soil. *P. syringae pv. Syringae* is an ordinary inhabitant of many plant species. It is necessary to mention that the principles of mass spectrometry and the data checking methods used in this experiment are sound, the protein database is incomplete, and there is a chance that proteins from other species are misidentified as proteins in the database. If wines are classified according to their identified proteins' species, then it may be possible to ascertain the quality of wine. Quite possibly, with mass spectrometry as a detection tool, wines have been found to have fewer bacteria and fungi proteins could be recognized as higher quality wines. Also, wine adulteration could be detected by mass spectrometry analysis since wine protein variations are caused primarily by grape species (*18*).

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