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Systematic Identification of Yeast Proteins Extracted into Model Wine during Aging on the Yeast Lees

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Total protein and protein-associated mannan concentrations were measured, and individual proteins were identified during extraction into model wines over 9 months of aging on the yeast lees following completion of fermentations by seven wine strains of *Saccharomyces cerevisiae*. In aged wines, protein-associated mannan increased about 6-fold (±66%), while total protein only increased 2-fold (±20%), which resulted in a significantly greater protein-associated mannan/total protein ratio for three strains. A total of 219 proteins were identified among all wine samples taken over the entire time course. Of the 17 "long-lived" proteins detected in all 9 month samples, 13 were cell wall mannoproteins, and four were glycolytic enzymes. Most cytosolic proteins were not detected after 6 months. Native mannosylated yeast invertase was assayed for binding to wine tannin and was found to have a 10-fold lower affinity than nonglycosylated bovine serum albumin. Enrichment of mannoproteins in the aged model wines implies greater solution stability than other yeast proteins and the possibility that their contributions to wine quality may persist long after bottling.

KEYWORDS: Yeast; *Saccharomyces cerevisiae*; wine protein; mannoprotein; HPLC-MS; tryptic peptides; wine; protein−tannin binding; aging on yeast lees; protein; tannin; cell wall

INTRODUCTION

The process of aging wine on the yeast lees refers to the postfermentation aging of wine in a barrel, tank, or bottle in the presence of the yeast biomass produced during vinification (*1*). The process involves simultaneous extraction of yeast components into wine and adsorption of grape constituents onto insoluble yeast cell wall fragments. The adsorbed grape constituents are eventually removed by clarification of the wine prior to bottling. The process of aging on the lees is believed to result in a net improvement in wine quality. The duration of commercial aging on the lees varies, but in a barrel, it typically lasts about 9 months. Wine aged on the lees in a barrel is generally subjected to a mild stirring regimen that varies in timing and duration but is meant to increase the surface contact between the wine and the yeast sediment, hastening both extraction of yeast components and adsorption of grape constituents. Yeast lees recovered from red wines are typically pigmented because the yeast cell wall adsorbs grape pigments, among other constituents (*2−4*). Traditional sparkling wines represent an extreme case of aging on the lees where wine may be kept in a bottle for years in contact with the lees following completion of the secondary fermentation (*1*).

Several benefits of aging wine on the lees have been ascribed specifically to the extraction of yeast mannoproteins (*5*). Specific yeast mannoproteins, or fragments thereof, have been shown to enhance protein stability in white wines presumably by interfering with aggregation of grape proteins and other wine constituents that would otherwise form undesirable hazes (*6−10*). Mannoproteins have been shown to be responsible for enhanced tartrate stability that prevents undesirable precipitation of potassium bitartrate in bottled wine (*11*). They have also been shown to influence wine aroma (*12*, *13*) and to increase foam stability in sparkling wines (*14*, *15*). There is great interest in correlating potential interactions between mannoproteins and tannins in red wines with desirable changes in sensory characteristics, for example, astringency, texture, and mouthfeel (*16−21*). Genetic interventions have also been reported that increase the release of mannoproteins in laboratory and industrial strains of *S. cerevisiae* (*9*, *22−24*).

The present study determined changes in protein and protein-associated mannan concentrations in model wines aged on the yeast lees over a 9 month time course and identified individual extracted proteins. To our knowledge, a systematic accounting of protein extraction and fate in wine during aging on the yeast lees has not been described previously. Relative to sampling at the end of the 2 week fermentation, cell wall mannoproteins were enriched with respect to proteins associated with other cellular compartments in the 9 month samples, and the ratio of protein-associated mannan to total protein was significantly greater in wines made by three of the seven yeast strains.

MATERIALS AND METHODS

Yeast Strains and Starter Culture Preparation. Wine strains of *Saccharomyces cerevisiae* were provided by Lallemand, Inc. (www.lallemandwine.com). Strains were transferred from isolated colonies on fresh

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Table 1. Composition of Model Must, pH 3.5

sugars and salts	g/L
glucose	200
potassium hydroxide	1.17
potassium tartrate	6
$CaCl_2$ dihydrate	0.44
K_2HPO_4	1.14
nitrogen	**g/L**
diammonium phosphate	1.5
vitamins	**mg/L**
myo-inositol	100
pyridoxine HCl	2
nicotinic acid	2
calcium pantothenate	0.25
thiamin HCl	0.5
p-amino benzoic acid	0.2
riboflavin	0.2
folic acid	0.2
biotin	0.01
minerals	**mg/L**
$MnCl_2$ tetrahydrate	0.198
$ZnCl_2$	0.136
$FeCl_2$	0.050
$CuCl_2$	0.014
boric acid	0.006
$CoCl_2$ hexahydrate	0.001
$NaMoO_4$ dihydrate	0.024
KI	0.010

YEPD plates (2% w/v glucose, 2% w/v peptone, and 1% w/v yeast extract) to 100 mL of broth containing 10% w/v glucose, 1% w/v peptone, and 1% w/v yeast extract and were incubated on an incubator-shaker at 25 °C for 48 h. After the 48 h incubation, cells were concentrated 10-fold by centrifugation at 2000*g* for 10 min to yield 10^9 cfu/mL.

Fermentations and Sampling. Duplicate 3 L model wine fermentations were performed at 25 °C in presterilized 1 gallon glass jugs fitted with fermentation locks. A sterile synthetic must (*25*) was modified slightly (**Table 1**) and inoculated to an initial yeast cell count of approximately 10^6 cfu/mL. The must was sterilized prior to inoculation by filtration through a 0.45 μm filter (Ultipor N66 filter device, Paul Co., East Hills, NY). As a fermentation aid, 3 g of sterile cellulose powder suspended in 25 mL of sterile distilled water was added to each fermentation prior to inoculation. The fermenting musts were stirred daily (10 min at midrange speed, setting #5) on a magnetic stirrer (Nuova II, Thermolyne, Dubuque, IA). After 14 days of fermentation when glucose levels were $<0.75\%$ (Clinitest tablets, Bayer, Elkhart, IN), the fermenters were transferred to 15 °C storage for periodic postfermentation sampling and were stirred once a month for 10 min as described above. Samples (10 mL) were removed every other day until the fermentations were complete using sterile-filtered N_2 gas to force samples through an aseptic sampling device and into sterile 15 mL screw-capped polypropylene tubes, which were immediately placed on ice. The fermentations were stirred just prior to sampling. The zero time point samples were taken immediately after yeast inoculation. Viable cell counts were determined by plating duplicate dilutions that yielded 50−400 colonies per YEPD plate. Colonies were counted after 48 h of incubation of the plates at 30 °C. Following the removal of samples needed to determine viable cell counts, the remaining sample was centrifuged (2000*g* for 10 min), and the supernatant was filtered through a sterile 0.45 μm PVDF filter (Fisher Scientific, Ireland). Filtrates were stored at −20 °C in sterile 15 mL screw-capped polypropylene tubes until chemical analyses were performed.

Chemical Analyses. Glucose and ethanol in the filtrates were separated by high-performance liquid chromatography (HPLC) (HP-1047A HP, Hewlett-Packard, Palo Alto, CA) and detected by refractometry at 35 °C. The analytical conditions included a Bio-Rad HPX-87C (Bio-Rad Laboratories, Hercules, CA) column, a mobile phase consisting of H_2O with 200 mg/L $Ca(NO_3)_2$ at 85 °C, a flow rate of 0.7 mL/min, and an injection volume of 15 μL. Protein was isolated by the KDS method and assayed as described (*26*). Briefly, 10.1 μL of a 10% sodium dodecyl sulfate (SDS) solution was added to 1 mL of prefiltered model wine sample (0.45 μm PVDF membrane filter) in a 1.7 mL screw-capped microfuge tube, which was vortexed vigorously and placed in a 100 °C water bath for 5 min. Tubes were cooled to room temperature, and to each, 252.2 μL of 1 M KCl was added. The tubes were then mixed gently for 30 min at room temperature, and the resulting precipitate was centrifuged at 16000*g* at 4 °C for 15−20 min. The pellet was washed twice with 1 M KCl by centrifugation, solubilized in 1 mL of distilled water, diluted 2-fold in distilled water, and filtered through a 0.45 μm PVDF filter. The protein was measured in the filtrates as described (*27*) using a commercial kit (Pierce Laboratories, Rockford, IL). The protein recovered by this precipitation method based on spiking wine with bovine serum albumin (BSA, Fraction V, lyophilized powder) was typically $\geq 90\%$ (data not shown). BSA, invertase (Grade VII, lyophilized powder, a mannosylated *S. cerevisiae* protein), SDS, triethanolamine (TEA), ferric chloride hexahydrate, (+)-catechin hydrate, urea, and caffeine were from Sigma Chemical Co. (St. Louis, MO).

Immunoblotting. Protein-associated mannan was quantified by immunoblotting performed according to the manufacturer's instructions (Bio-Dot SF microfiltration apparatus, Bio-Rad Laboratories). Briefly, 50 μL samples containing 1−500 ng of protein-associated mannan (isolated by KDS extraction from the model wines), yeast mannan (#M7504, Sigma-Aldrich), BSA, or nisin (Aplin & Barrett Ltd., Beaminster, United Kingdom) as a nonmannosylated control were blotted onto nitrocellulose membranes in a 48-well slot blot apparatus. Preparations were typically diluted 5−20-fold prior to blotting. After the samples and standards were loaded onto the membrane by gravity flow, 250 μL per well of Tris-buffered saline (20 mM Tris, pH 7.5, 500 mM NaCl, TBS) was added under gentle vacuum. The membrane was removed from the apparatus, placed in a plastic box, and rinsed with a blocking/wash solution of TBS containing Tween 20 (Tris-buffered saline + 0.1% Tween 20, TBST) for 5 min. This rinse was repeated once with fresh TBST. The membrane was then incubated for 30 min at room temperature with continuous mixing with 9.5 μL/mL (final concentration) of the mannose-specific primary antibody, biotinylated *Narcissus pseudonarcissus* lectin (Vector Laboratories, Burlingame, CA) in TBST. *N. pseudonarcissus* lectin has a similar affinity for mannose as Con A but does not bind glucose (*28*). The membrane was washed twice with TBST and subsequently placed in TBST containing 1 μg/mL of streptadivin-conjugated alkaline phosphatase (Vector Laboratories) for 30 min at room temperature with continuous mixing. The membrane was washed twice in TBST and rinsed once in TBS for 5 min to remove residual Tween 20 and was then equilibrated in 100 mM Tris pH 9.5 for 5 min. After equilibration, the membrane was removed, shaken to remove excess liquid, and placed—blotted side up—on top of plastic wrap within a dry plastic box under subdued light. The chemiluminescent/fluorescent alkaline phosphatase substrate phosDuoLux (Vector Laboratories) was added at a rate of 50 mL/cm^2 (4.45 mL per membrane) onto the membrane, which was covered with plastic wrap to uniformly spread the substrate under subdued light for 5 min. The membrane was removed and rinsed in 100 mM Tris, pH 9.5, for an additional 2−3 min. This rinse step was found to minimize background on the X-ray film. The membrane was then stored in a plastic Ziploc bag and kept in a light-proof and sealed container at 4 °C overnight. Exposure to X-ray film (Hyperfilm, Amersham Biosciences, Buckinghamshire, United Kingdom) was done the next day in a darkroom with the membrane sandwiched between new or clean used 8.5″ × 11″ plastic sheets to protect the film from moisture on the membrane. After the film was developed, it was scanned as a transparency (to make the light background dark and the dark bands light) into a data file, and the integrated densities were determined using ImageJ image analysis software (Rasband, W.S., ImageJ, NIH, Bethesda, MD, http://rsb.info.nih.gov/ij/, 1997−2007).

Processing of Protein Samples for Identification of Tryptic Peptides. Model fermentation and postfermentation samples (100 mL) were collected to assay total and mannan-associated protein and to identify individual proteins. To identify individual proteins, these samples were concentrated about 150-fold by ultrafiltration using a centrifugal

filter cartridge (5 kDa regenerated cellulose membrane, Millipore, Billerica, MA) following initial removal of suspended solids by centrifugation (2000*g* for 10 min) and subsequent filtration through a 0.45 μm PVDF filter. To dilute low molecular weight wine solutes (< 5 kDa), the concentrated samples were diluted with about 50 mL of distilled water and reconcentrated 100−200-fold by ultrafiltration. Aliquots of the concentrated samples generated by ultrafiltration were adjusted to 50 mM ammonium bicarbonate, pH 8.5, and 0.1% acid-labile surfactant, a long chain derivative of 1,3-dioxolane sodium propyloxy sulfate (RapiGest, Waters Co., Milford, MA), in a final volume of 200 μL and were sequentially reduced and alkylated at final concentrations of 5 mM DTT and 15 mM iodoacetamide, respectively (RapiGest protocol, Waters Co.). Enzymatic deglycosylation of N-linked glycan was subsequently performed overnight with PNGase F according to the manufacturer's protocol (*N*-glycanase, Prozyme, San Leandro, CA). Following deglycosylation, the ammonium bicarbonate concentration was increased to 200 mM, and trypsin was added to a final concentration of about 1 μg/mL. The mixture was then incubated at 37 °C for 8−12 h. Aliquots (10 μL) were subjected to subsequent HPLC-MS analysis. Samples subjected to HPLC-MS analysis were concentrated approximately 100-fold relative to the original wine as the deglycosylation and trypsin treatments involved minor dilution steps.

HPLC-MS Identification of Tryptic Peptides. Samples were subjected to HPLC/MS-MS analysis using a Waters nano Acquity HPLC connected to a Waters Q-Tof Ultima Global. One microliter of sample (about 1 μg of total protein) was loaded onto a Waters Symmetry C-18 180 μm × 20 mm trap at 6 μL/min for 3 min. Peptides were then eluted from the trap onto a 15 or 20 cm × 75 μm Waters BEH analytical column at 260 nL/min. The HPLC gradient went from 2 to 40% B in 60 min and then to 93% B in 5 min and was held for 12 min. Solvent A was 0.1% formic acid in water, and B was 0.1% formic acid in acetonitrile. Data were acquired for 80 min. Peptide precursor ions were monitored as they eluted from the analytical column with 0.6 s survey scans from *m*/*z* 500 to 1990. Up to three parent ions per scan that had sufficient intensity and two or three positive charges were chosen for MS/MS. The MS/MS scans were 2.4 s from *m*/*z* 50 to 1990. The mass spectrometer was calibrated using the MS/MS spectrum from glu-fibrinopeptide. Masses were corrected during each run using a lock mass scan every 60 s of glu-fibrinopeptide. The raw data were processed with Waters Protein Lynx Global Server 2.3 software to produce pkl files, which are a set of smoothed and centroided parent ion masses with the associated fragment ion masses. The pkl files were searched with Mascot 2.2 (Matrix Science Ltd., London, United Kingdom) database searching software, using mass tolerances of 35 ppm for the precursors and 0.1 Da for fragments. The Mascot results were combined and reviewed using Scaffold_2_05_01 software (Proteome Software, Inc., Portland, OR). The only database searched to identify proteins was that of the *S. cerevisiae* proteome, and thus, the search was more limited than a standard Mascot search, which further increased the probability of a match. Mascot provides a probability estimate based on database size but not on sample characteristics. By incorporating the sample-specific distribution, Scaffold provides better estimates of the probability of a correct identification. In this study, 80% probability was used as a cutoff for all time points except for the 285 day sample for which the cutoff was lowered to 65%. The lower cutoff was used because a few additional proteins were identified at that probability. At earlier time points, no new proteins were detected when the probability cutoff was lowered to 65%. The probability of correct protein identifications was assigned by the ProteinProphet algorithm (*29*).

Protein−Tannin Binding Assays. A fixed volume (0.2 mL) of red wine (Columbia Valley, Merlot, 2003) was added to various amounts of BSA or invertase to determine the tannin binding capacity of these two proteins. The amount of protein added varied from 0.1 to 4 mg of invertase and from 0.1 to 1 mg of BSA. The pH was varied using 0.2 mM acetic acid with 0.2 mM sodium chloride buffered to pH 3, 5, 6, and 7 by adjustment with NaOH or HCl. The final reaction volume was 1.2 mL. Each reaction was performed in triplicate ($n = 3$) and was incubated for 15 min and then centrifuged at 13500*g* for 5 min to facilitate pellet formation. The tannin content of each pellet was determined (*30*) as modified for wine (*31*). Briefly, each supernatant was discarded, and the remaining pellet was incubated for 10 min in TEA buffer containing 5% TEA (v/v) and 5%

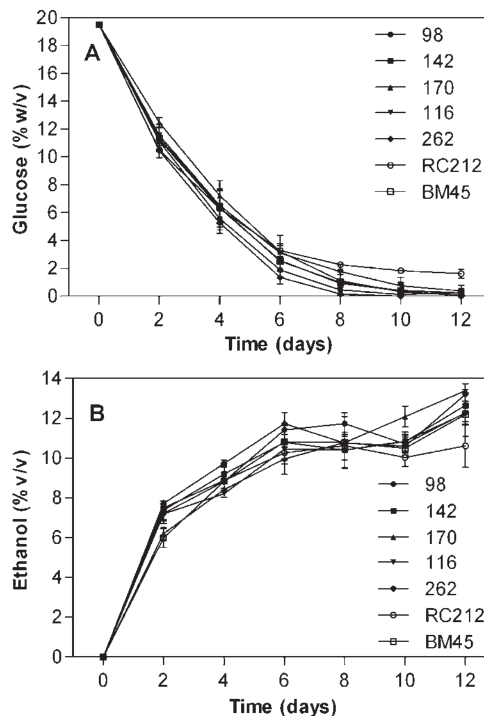

Figure 1. Model wine fermentations. (**A**) Change in glucose concentration during fermentation. (**B**) Change in ethanol concentration during fermentation. Data are means of two replicates, and error bars are standard deviations.

SDS (w/v) adjusted to pH 9.4 with HCl. Once the incubation period ended, samples were mixed to dissolve the tannin−protein pellet. An aliquot (125 μL) of 10 mM $FeCl_3$ and 0.01 N HCl were added to all samples which were incubated at room temperature for 10 min. A_{510} values were then determined in a Beckman DU 640 spectrophotometer (Fullerton, CA) using the TEA buffer as a blank.

Hydrogen Bond Disruption Experiments. A fixed amount (0.2 mL) of red wine (Columbia Valley, Cabernet Sauvignon, 2003) was added to 1 mL of BSA (1 mg/mL, 0.2 mM NaCl, pH 4.9) or to 1 mL of invertase (4 mg/mL, pH 3.3). Immediately thereafter, 0.3 mL of caffeine (1.5 mg/mL), urea (1.5 mg/mL), or ethanol was added. Each reaction was performed in duplicate. The incubation period and determination of tannin content of the pellet are described above.

Catechin Standardization. (+)-Catechin (5 mg) was dissolved in 5 mL of an aqueous ethanol 10% (v/v) solution to make a 1 mg/mL stock solution. A standard curve was constructed by adding aliquots of the stock solution (50−300 μL) to a final volume of 875 μL using TEA buffer. As noted above, each sample was brought to 1 mL with a 125 μL aliquot of 10 mM $FeCl_3$ and 0.01 N HCl and incubated at room temperature for 10 min. A_{510} values were then measured in 1 cm path length disposable cuvettes.

Statistical Analysis. Multiple range tests on duplicate samples between time points and yeast strains were performed by the Fisher's least significance difference (LSD) procedure discriminating between means at $p < 0.05$. Alternatively, Student's *t* test (two-tailed) was used to assess significance of differences. For the assessment of the effect of ethanol, urea, and caffeine on tannin−protein binding, a one-way analysis of variance was performed ($p < 0.05$). Fisher's LSD test was used as a posthoc comparison of means ($p < 0.05$). All statistical analyses were performed using Statistica 8 (StatSoft, Inc., Tulsa, OK).

RESULTS AND DISCUSSION

Fermentations. The synthetic must was inoculated with starting yeast populations ranging from 2 to 6×10^6 cfu/mL, which increased about 10-fold within 2−4 days and decreased thereafter. The final populations for six fermentations were measured on day 12 when glucose concentrations were less than 0.5% and

Table 2. Change in Protein Concentration in Model Wines during Fermentation and Aging[a]

strain	2 days	7 days	14 days	45 days	105 days	195 days	285 days
98	$7.4^{a} \pm 0.5$	$14.8^{a} \pm 0.9$	$9.3^{ab} \pm 1.6$	$8.4^{a} \pm 1.0$	$18.8^{bc} \pm 0.1$	$16.2^{ab} \pm 5.7$	$20.2^{a} \pm 5.0$
142	$8.7^{a} \pm 5.9$	$14.4^{a} \pm 4.3$	$10.5^{b} \pm 1.3$	$10.5^{a} \pm 4.0$	$16.2^{bc} \pm 1.0$	$18.8^{ab} \pm 6.2$	$17.7^{a} \pm 1.3$
170	$5.4^{a} \pm 2.1$	$12.6^{a} \pm 0.5$	$8.2^{ab} \pm 0.6$	$6.5^{a} \pm 1.6$	$16.6^{bc} \pm 2.5$	$10.5^{a} \pm 1.1$	$14.2^{a} \pm 4.6$
116	$7.0^{a} \pm 0.5$	$16.3^{a} \pm 3.1$	$9.1^{ab} \pm 0.5$	$8.6^{a} \pm 1.6$	$9.3^{a} \pm 0.6$	$12.7^{ab} \pm 2.6$	$14.6^{a} \pm 5.4$
262	$4.3^{a} \pm 3.5$	$14.4^{a} \pm 4.5$	$7.1^{a} \pm 2.5$	$9.6^{a} \pm 2.1$	$14.6^{b} \pm 4.1$	$13.3^{ab} \pm 3.2$	$19.2^{a} \pm 0.4$
RC212	$7.1^{a} \pm 0.8$	$32.0^{b} \pm 0.5$	$7.7^{ab} \pm 0.6$	$18.1^{b} \pm 1.4$	$19.9^{c} \pm 0.0$	$20.6^{b} \pm 5.7$	$13.8^{a} \pm 1.9$
BM45	$6.5^{a} \pm 0.4$	$13.7^{a} \pm 2.2$	$9.3^{ab} \pm 0.9$	$7.5^{a} \pm 1.6$	$17.0^{bc} \pm 0.8$	$10.8^{ab} \pm 1.4$	$17.0^{a} \pm 1.0$

[a] Data are means ± standard deviations (μg/mL BSA equiv) for two replicates. Means within the same column with no common letters are significantly different (Fisher's LSD procedure, $p < 0.05$).

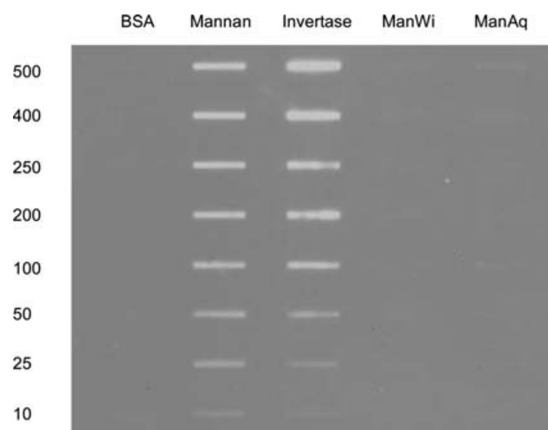

Figure 2. Immunoblot of protein-associated mannan probed with mannan-specific biotinylated lectin from *N. pseudonarcissus* as described in the Materials and Methods. BSA (negative control), mannan, and invertase (positive control) were dissolved in water, diluted in TBS, and spotted onto the dot blot in the amounts noted on the left (ng). ManWi is mannan (10 mg) dissolved in 1 mL of model wine (12.5% [v/v], 0.6% potassium bitartrate [w/v], pH 3.5), which was subjected to KDS precipitation and resolubilization in water and subsequently spotted onto the blot. ManAq is mannan (10 mg) dissolved in 1 mL of distilled water but otherwise treated as described for ManWi. The amounts of ManWi and ManAq spotted onto the blot would have corresponded to the amounts indicated had all of the mannan precipitated upon KDS treatment and been completely resolubilized.

ranged from 2×10^5 to 10^6 cfu/mL. The RC212 fermentation was slower than the others, with sugar (**Figure 1A**) and viable cell concentrations measuring 1.6% (w/v) and 10^4 cfu/mL on day 12, respectively. The sugar concentration in the RC212 fermentation dropped to less than 0.75% (w/v) by day 14 (Clinitest data not shown), at which time the fermentations were transferred to 15 °C storage where they were held for the remainder of the time course. As expected, ethanol concentrations (**Figure 1B**) increased in rough proportion to sugar consumed. On day 12, when the residual sugar concentrations indicated that the fermentations were essentially complete, except for that involving strain RC212, ethanol concentrations ranged from 10.6 to 13.4% (v/v).

Protein and Protein-Associated Mannan Concentrations. Protein levels ranged from about 4 to 9 μg/mL on day 2 of the fermentation for all strains and from 7 to 11 μg/mL by the end of the fermentation (14 day sample) (**Table 2**). After 9 months of aging on the yeast lees, levels ranged from about 14 to 20 μg/mL (285 day sample). While some statistically significant differences were observed between strains for intermediate time points, differences in protein concentrations between strains measured at day 2 and in the 9 month samples were not significant. Protein concentrations increased 1.8−2.7-fold between the 9 month and the 14 day samples.

Because protein-associated mannan was quantified as mannan, it was important to establish that protein-free mannan in the model wine was not precipitated by the KDS procedure used to isolate proteins. **Figure 2** is an inverted image of a western blot of BSA (negative control), mannan standard, mannosylated invertase from *S. cerevisiae* (positive control), and mannan solutions initially subjected to KDS precipitation, subsequent dissolution in water, dilution, and subsequent blotting. The blot was probed with biotinylated mannan-specific lectin from *N. pseudonarcissus*. The mannan standard spotted directly on the blot and yeast invertase both yielded positive signals as expected. Neither the mannan dissolved in a model wine (ManWi) consisting of 12.5% (v/v) alcohol and 0.6% (w/v) potassium tartrate, pH 3.5, nor the mannan dissolved in water (ManAq) yielded signals. Blotted samples prepared from these two solutions would have contained the amount of mannan indicated on the left side of the figure had it all precipitated and redissolved. No KDS-induced precipitate was visible during processing of the ManWi or ManAq samples as was routinely observed for protein-containing samples (data not shown). We interpret the lack of signal as indicating that the KDS protein isolation protocol did not precipitate protein-free mannan.

Protein-associated mannan concentrations quantified in mannan equivalents (**Table 3**) ranged from about 1 to 5 μg/mL in the initial 2 day sample and from 1 to about 6 μg/mL at the end of fermentation (14 day samples). While some differences between strains were significant at both of these early time points, differences were not statistically significant between strains in the 9 month samples, where concentrations ranged from about 11 to 16 μg/mL. Protein-associated mannan concentrations increased about 2−14-fold between the 9 month and the 14 day samples.

The fraction of total protein comprised of mannoproteins was estimated throughout the time course by quantifying the ratio of protein-associated mannan (mannan equivalents) to total protein (BSA equivalents). To determine whether aging the model wine on the yeast lees over 9 months affected this ratio, 14 day values measured immediately after the completion of fermentation were compared to values for the 285 day samples (**Table 4**). The ratios increased from about 1.2 at 14 days (strain 116) to 8.5 at 9 months (strain 142). The increase in ratio was statistically significant ($p < 0.01$) for wines made by three yeast strains, 142, 170, and RC212, and is consistent with at least two possibilities, which are not mutually exclusive. First, extraction of cytoplasmic proteins may be faster than extraction of mannoproteins. Extraction of mannoproteins, which are known to be largely associated with the cell wall, may be slow, particularly for those proteins known to be covalently linked to wall glycan. In contrast, extraction of cytoplasmic proteins is likely to be faster and dependent not on breakage of covalent bonds but rather on the rate of plasma membrane disorganization. The net result of this differential

Table 3. Change in Protein-Associated Mannan Concentration in Model Wines during Fermentation and Aging[a]

strain	2 days	7 days	14 days	45 days	105 days	195 days	285 days
98	$2.3^{ab} \pm 1.4$	$4.6^{ab} \pm 2.9$	$2.3^{ab} \pm 0.3$	$8.0^{c} \pm 0.5$	$9.9^{b} \pm 0.8$	$7.5^{a} \pm 0.4$	$15.6^{a} \pm 1.4$
142	$1.9^{ab} \pm 1.4$	$8.0^{b} \pm 1.3$	$1.1^{a} \pm 0.4$	$5.9^{bc} \pm 1.9$	$4.4^{a} \pm 0.1$	$14.6^{b} \pm 0.6$	$16.0^{a} \pm 0.1$
170	$2.0^{ab} \pm 0.5$	$3.1^{a} \pm 0.9$	$1.9^{ab} \pm 0.5$	$2.0^{a} \pm 0.4$	$4.3^{a} \pm 4.4$	$5.7^{a} \pm 0.5$	$13.5^{a} \pm 3.8$
116	$3.1^{ab} \pm 2.6$	$3.4^{a} \pm 1.7$	$6.3^{c} \pm 0.0$	$3.5^{abc} \pm 1.7$	$7.0^{ab} \pm 1.9$	$7.4^{a} \pm 0.3$	$12.3^{a} \pm 7.2$
262	$2.4^{ab} \pm 0.8$	$3.8^{ab} \pm 0.1$	$3.5^{ab} \pm 2.6$	$7.4^{c} \pm 1.7$	$10.2^{b} \pm 0.6$	$6.6^{a} \pm 0.7$	$14.1^{a} \pm 6.1$
RC212	$5.1^{b} \pm 0.6$	$8.0^{b} \pm 3.2$	$1.8^{ab} \pm 0.7$	$6.6^{c} \pm 2.7$	$8.2^{ab} \pm 0.9$	$8.4^{a} \pm 5.4$	$12.6^{a} \pm 2.6$
BM45	$1.4^{a} \pm 1.0$	$4.9^{ab} \pm 1.1$	$3.9^{bc} \pm 0.6$	$2.2^{ab} \pm 1.0$	$6.4^{ab} \pm 0.7$	$5.6^{a} \pm 0.6$	$10.9^{a} \pm 3.3$

[a] Data are means ± standard deviations (μg/mL mannan equiv) for two replicates. Means within the same column with no common letters are significantly different (Fisher's LSD procedure, $p < 0.05$).

Table 4. Ratios of Protein-Associated Mannan to Protein in Model Wines[a]

yeast strain	protein-associated mannan/protein day 14	day 285
98	0.249 ± 0.079	0.785 ± 0.127
142[b]	0.106 ± 0.049	0.904 ± 0.060
170[b]	0.225 ± 0.038	0.961 ± 0.042
116	0.693 ± 0.031	0.810 ± 0.194
262	0.458 ± 0.205	0.734 ± 0.332
RC212[b]	0.236 ± 0.073	0.905 ± 0.063
BM45	0.420 ± 0.021	0.640 ± 0.157
mean ± SD	**0.341 ± 0.197**	**0.820 ± 0.112**

[a] Data are means ± standard deviations for the ratio of protein-associated mannan (μg/mL mannan equiv) to protein (μg/mL BSA equiv) on day 14 (end of fermentation) and on day 285. [b] The ratio at day 285 is significantly greater than the ratio at day 14 by Student's *t* test ($p < 0.01$, dependent variable, two-tailed).

extraction would be expected to be an increase in mannoprotein content relative to total protein at 9 months as compared to 14 days. Alternatively, differential protein solubility in the wine matrix is also expected to have an important bearing on protein concentrations. Proteins with the greatest solution stability are more likely to be found in the 9 month samples regardless of when they were extracted, whereas proteins with poor solution stability are unlikely to be detected at all and certainly not in late samples. Qualitative data presented below suggest that as a class, mannoproteins have greater solution stability in the model wine matrix than other yeast proteins.

Identification of Proteins. A comprehensive compilation of all identified proteins found in the model wines as a function of yeast strain and sampling time is given in Table 1 of the Supporting Information. Predicted pI values, cellular localizations, number of identified peptides per protein, and the probabilities of correct protein identifications are also indicated. A total of 219 proteins were detected over the entire time course. This number is likely to be an underestimate as many proteins were detected on the basis of single diagnostic peptides. Fifteen proteins were detected at the time of inoculation, and four of these were detected in at least one replicate of four of the seven wine samples. Two were cell wall mannoproteins, Ecm33 and Pst1, and the other two were glycolytic enzymes, Tdh1 and Pgk1. Interestingly, these four proteins were detected at all subsequent time points including the final 285 day samples in all seven wines. Pst1 has previously been associated with heat-induced haze reduction in white wines (*10*). Between 110 and 140 proteins were detected in wines sampled at 7, 14, 45, and 105 days, and about half of these proteins were found in at least one replicate of four of the seven wine samples. Only about 50 proteins were detected in the 195 and 285 day wine samples, and among them, 50% were common to at least one replicate of four of the seven wines.

One striking finding is illustrated in **Figure 3**, which shows the total number of proteins detected at day 14 and day 285 as a function of cell compartment. The majority of the 140 proteins detected in wines sampled at day 14 were cytoplasmic (80) with representation from the cell wall (24), mitochondrion (14), vacuole (3), nucleus (3), ER (1), unknown, and multiple compartments. In contrast, among the 48 proteins detected in the samples taken at 285 days, 25 were associated with the cell wall, 19 with the cytoplasm, one with the mitochondrion, and no others with known or unique associations with specific cell components (**Table 5**). In other words, while 75% of the cytoplasmic proteins detected in samples taken at day 14 disappeared by the time the 285 day samples was taken, 100% of the cell wall proteins detected at day 14 was still present 9 months later. These observations indicate that while most proteins, including the cell wall mannoproteins, were already extracted into the wine by the end of the fermentation, the mannoproteins had greater solution stability in the wine matrix than the others. This is consistent with what is known about glycoproteins in general. For example, in a study that compared five different glycoproteins with their deglycosylated counterparts (*32*), it was reported that deglycosylation decreased thermal stability dependent on the amount of carbohydrate rather than specific linkage (e.g., N- or O-) or structure (branched vs unbranched). Furthermore, deglycosylated proteins were found to have poorer thermal reversibility of denaturation than their glycosylated counterparts and tended to aggregate during thermal inactivation at acidic pH. Similarly, while heat was found to irreversibly denature wild-type nonglycosylated lysozyme and induce precipitation, artificially mannosylated lysozyme variants underwent reversible denaturation, retaining both activity and solubility following the heat treatment (*33*). We speculate that yeast mannoproteins extracted into the model wines would have been less likely to aggregate even if

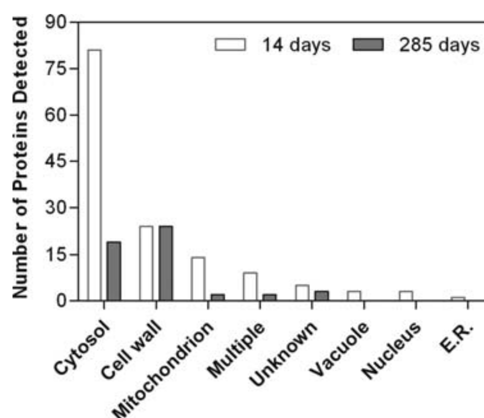

Figure 3. Number of proteins detected in 14 and 285 day samples as a function of cellular compartment. Of the 19 cytosolic proteins detected in at least one wine sample at 285 days, 18 were also detected at 14 days. Of the 25 wall proteins detected in at least one wine sample at 285 days, 17 were also detected at 14 days.

Table 5. Proteins Detected in Model Wines after 9 Months of Aging on the Yeast Lees

protein	pI[a]	prob[b] (%)	pept[b] (#)	mol/cell[c]	comp[d]	strain[e]	description[f]
Pgk1	7.77	100	3−8	3×10^5	**cyto**	all	phosphoglycerate kinase
Gpm1	7.23	100	3−11	2×10^5	**cyto**	all	phosphoglycerate mutase
Cdc19	7.66	100	3−9	3×10^5	**cyto**	all	pyruvate kinase
YJL171c	4.67	77−99	1−3	6×10^3	**wall**	all	GPI-anchored protein of unknown function
Ecm33	4.76	69−100	1−5	unk	**wall**	all	GPI-anchored protein of unknown function
Gas1	4.3	97−100	2−5	10^4	**wall**	all	GPI-anchored β-1,3-glucanosyltransferase, required for cell wall assembly
Pst1	9.91	100	13−17	10^4	**wall**	all	GPI-anchored cell wall protein of unknown function
Tdh1	8.59	100	3−11	10^5	**cyto**, **wall**	all	glyceraldehyde-3-P dehydrogenase
Pau17	5.04	100	3−9	unk	**unk**	all	putative protein of unknown function
Pau5	4.55	69−100	1−3	unk	**wall**[g]	all	active during fermentation, anaerobiosis
Bgl2	4.16	100	7−10	4×10^4	**wall**	all	endobeta-1,3-glucanase, major cell wall protein
Crh1	4.3	69−100	1−3	3×10^4	**wall**	all	putative chitin transglycosidase that functions in transfer of chitin to β(1-6)glucan
Dan3	5.04	95−100	1−3	unk	**wall**	all	cell wall mannoprotein expressed under anaerobic conditions
Exg1	4.42	100	6−13	4×10^3	**wall**	all	major exo-1,3-β-glucanase of cell wall
Scw4	4.52	99−100	2−6	6×10^3	**wall**	all	cell wall protein with similarity to glucanases
Gas5	4.3	65−100	1−6	10^4	**wall**	all	1,3-β-glucanosyltransferase
Ygp1	5.17	100	2−10	unk	**wall**	all	cell wall-related secretory glycoprotein
Sun4	4.02	65−100	1−3	2×10^4	wall	123456	cell wall glucanase related
Fba1	5.65	93−100	1−4	10^6	cyto	123467	fructose 1,6-bisphosphate aldolase
Ssa1	4.82	93−99	1−2	3×10^5	cyto, wall	123457	ATPase involved in protein folding
Plb2	4.35	66−100	1−3	6×10^2	wall	123457	phospholipase B involved in phospholipid metabolism
Tef1	9.72	69−100	1−4	4×10^2	cyto	123467	translational elongation factor EF-1 α; also encoded by TEF2; functions in the binding reaction of aminoacyl-tRNA (AA-tRNA) to ribosomes
Pfk2	6.65	65−99	1−2	9×10^4	cyto	12456	β-subunit of heterooctameric phosphofructokinase
Eno1	6.6	99−100	2−7	8×10^4	cyto	23467	phosphopyruvate hydratase
Ccw14	5.81	99−100	2−5	4×10^4	wall	12347	covalently linked cell wall glycoprotein
Hsp12	5.1	65−100	1−7	4×10^3	cyto	12347	induced by heat shock, oxidative stress, osmostress, stationary phase entry, glucose depletion, oleate and alcohol; regulated by the HOG and Ras-Pka pathways
Sec31	5.53	65−99	1−2	2×10^3	multi	23467	essential phosphoprotein component (p150) of the COPII coat of secretory pathway vesicles, in complex with Sec13p; required for ER-derived transport vesicle formation
Pdc1	6.12	77−100	1−5	9×10^3	cyto	13567	pyruvate decarboxylase
Pir3	5.32	65−98	1−2	unk	wall	12347	*O*-glycosylated covalently bound cell wall protein
Scw10	4.32	69−99	1−2	10^4	cyto, wall	12356	cell wall protein with similarity to glucanases
Vel1	4.16	77−100	1−5	unk	wall	13456	unknown function
Trx2	4.62	98−99	2	2×10^4	cyto	1237	cytoplasmic thioredoxin isoenzyme
Tos1	4.33	99−100	2−3	10^4	wall	2456	covalently bound cell wall protein of unknown function
Mic14	5.38	65−99	1−3	unk	mito	1234	14 kDa mitochondrial intermembrane space cysteine motif protein
Hsp26	5.22	80−100	1−4	2×10^4	cyto	247	small heat shock protein with chaperone activity expressed in stressed cells
Tpi1	5.86	75−100	1−3	2×10^5	cyto	247	triose phosphate isomerase
Sim1	4.31	77−99	1−2	2×10^3	wall	156	SUN family protein
YIL169c	4.21	94−97	1−2	unk	unk	36	Ser/thr-rich and highly similar to YOL155C, a putative glucan α-1,4-glucosidase
Zps1	4.72	100	5−6	unk	wall	56	putative GPI-anchored protein
Cwp1	4.32	99−100	2−3	2×10^3	wall	12	cell wall mannoprotein
Gas3	4.43	72−99	1−2	2×10^4	wall	12	putative 1,3-β-glucanosyltransferase
Tsl1	6.61	99	2	2×10^3	cyto	5	large subunit of trehalose 6-phosphate synthase
Atp1	9.85	100	3	4×10^4	mito	7	α-subunit of F1 sector of mitochondrial F1F0 ATP synthase
Pma1	4.81	99	2	10^6	multi	2	plasma membrane H^+-ATPase
Rps3	10.22	99	2	2×10^5	cyto	7	protein component of small (40S) ribosomal subunit
Pdc5	6.41	98	2	5×10^5	cyto	6	minor pyruvate decarboxylase
Nca3	4.09	99	2	unk	unk	3	regulates mitochondrial expression of subunits of the Fo-F1 ATP synthase
Gnd1	6.6	99	2	10^5	cyto	7	6-phosphogluconate dehydrogenase

[a] pI values are predicted based on amino acid composition and do not reflect post-translational contributions (e.g., phosphorylation). [b] Probabilities (prob) of a correct protein identification based on the ProteinProphet algorithm (Nesvizhskii et al., 2003) and number of peptides (pept) detected per protein are provided as a range when the same protein was detected in multiple samples and when the number of probabilities or peptides differed among samples. [c] These literature values are based on extracts obtained from log-phase YEPD-grown lab strains (ATCC 201388) carrying C terminally tagged ORFs (*36*). [d] Cellular compartment associations are based on *Saccharomyces* Genome Database (SGD) annotations as of 8/2/09 (http://www.yeastgenome.org/). "Cyto", cytoplasm including cytoplasmic ribosome but excluding membrane-bound organelles; "mito", mitochondrion. Association with one compartment does not preclude association with others. If multiple associations are known and include either the wall or the cytoplasm, these two components are specified; if not, "multi" is indicated. [e] Strains 1, 2, 3, 4, 5, 6, and 7 = 98, 142, 170, 116, 262, RC212, and BM45, respectively. Single digit strain numbers in the table are not separated by commas. The 17 proteins in **bold** were detected in all wines aged 9 months (285 day samples). Among them, Pgk1, Pyk1, Ecm33, Pst1, and Tdh1 were also detected at all other sampling times; Gpm1, Gas1, Pau5, Bgl2, Exg1, Scw4, Gas5, and Ygp1 were detected at all other sampling times except at 7 days; and YJL171c, Crh1, Dan3, and Pau17 were not detected at multiple sampling times. [f] SGD annotations as of 8/2/09 (http://www.yeastgenome.org/). [g] Wall association based on ref *38*.

they became denatured because the attached glycan presumably constrained unfolding, reducing the likelihood that exposed hydrophobic residues would interact with those of other denatured proteins and lead to formation of a particle large enough to precipitate.

Yeast Proteases. An alternative explanation for the disappearance of most nonmannoproteins in the 285 day samples is that they were hydrolyzed by yeast proteases, as glycoproteins are generally believed to be less prone to proteolysis than nonglycoproteins due to carbohydrate masking susceptible bonds (*34*). Five known yeast proteases (Ape3, Lap4, Hsp31, Prb1, and Yps3) were detected at various time points in wines made by the seven yeast strains (Supporting Information, Table 1). Pep4, which has been shown to be active intracellularly during yeast autolysis (*35*), was not detected. Ape3, a vacuolar aminopeptidase active toward peptide substrates at pH 7.5 (*36*), was found in all wines made by five of the seven yeast strains that were sampled from 7 days through 6 months. In the remaining two strains, Ape3 was found in all samples taken from 14 days through 6 months. Lap4 (also known as Ape1), a methionine aminopeptidase with a pH optimum of 7.0 (*37*), was detected in at least one replicate of wines made by five of the seven strains. The cysteine protease Hsp31, active at pH 7.5 (*38*), was detected in wines made by strains 142 and 262 but only at one time point. The serine protease Pbr1 (*39*) was only detected in the 14 day wine sample made by strain 142. Yps3 was also detected in wines made by all strains but not at as many time points. When detected, it was usually found within the 14 day to 3 month samples. Because Yps3 is an aspartic protease active at acidic pH (*40*), it has the greatest potential of any of these proteases of being active in wine. While one cannot rule out the possibility that Yps3 activity contributed to the disappearance of yeast proteins, it seems unlikely that it made a major contribution as it was detected in samples containing the greatest number of proteins (days 14−105) but not in samples following the major decrease in detected proteins (days 195 and 285).

Long-Lived Proteins. Proteins found in the 9 month samples are listed in **Table 5**. The table also lists predicted pI values, an estimate of intracellular protein concentrations based on analysis of a laboratory strain harvested in log phase in YEPD (*41*), cellular compartment with which the protein is associated, yeast strains used to make the wines, and a description of protein function (*Saccharomyces* Genome Database, http://www.yeastgenome.org/). All other factors being equal, one would predict that the most highly charged proteins at pH 3.5 might be the most readily extractable and soluble in the model wines. While potentially informative, the predicted pI values do not reflect post-translational modifications. As a case in point, yeast cell wall mannoproteins are variably phosphorylated, which gives the yeast cell surface a net negative charge (*42*). Among the 17 proteins found in at least one replicate of wines made by all yeast strains, 13 were associated with the cell wall or were cell-wall related [*Saccharomyces* Genome Database (*43*)], and four were glycolytic enzymes. Among the 16 proteins detected in 4−6 of the seven wine samples, 10 were also in these two categories. Assuming most if not all of the cell wall proteins are mannoproteins, it is likely that the their longevity is related to the folding constraints imposed by the glycan, which would tend to limit denaturation-induced unfolding and minimize aggregation leading to precipitation. The presence of the glycolytic enzymes is likely a direct reflection of their abundance in an organism that generates energy primarily by fermentation (**Table 5**).

The likelihood of detecting a yeast protein is dependent on a number of factors. The protein must be expressed, evade internal degradative pathways, be extracted and soluble in the wine, avoid

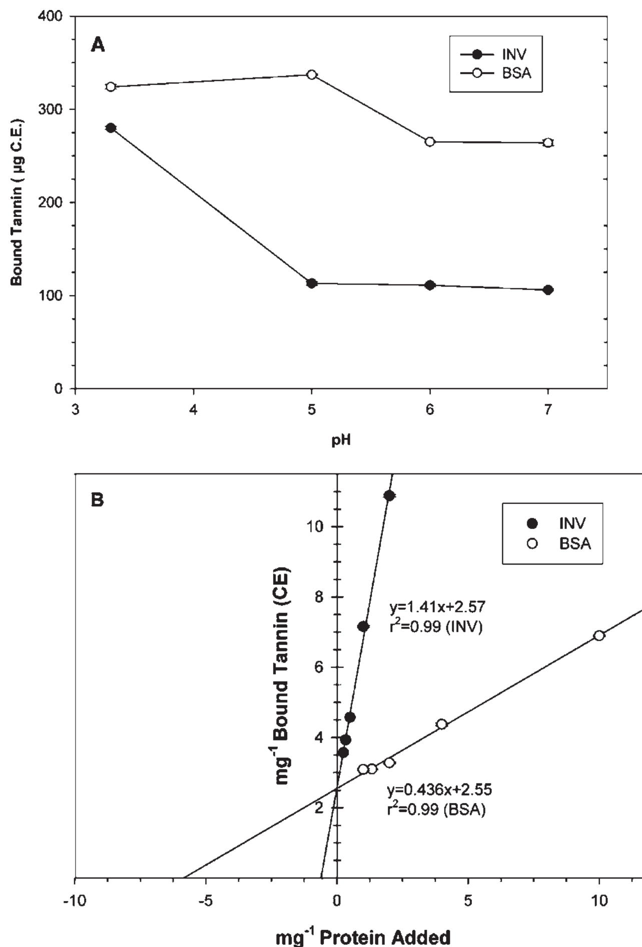

Figure 4. Tannin binding by nonmannosylated BSA and mannosylated yeast invertase (INV). (**A**) Effect of pH on tannin-binding capacity of BSA and INV. Tannin is expressed in (+)-catechin equivalents (CE). (**B**) Inverse plot of tannin bound by INV at pH 3.3 and by BSA at pH 5.0. Data are means of two replicates, and error bars are standard deviations. Error bars are mostly obscured by the data symbols.

proteolysis that might hydrolyze diagnostic peptides, and, finally, must be present at a concentration of at least 1 pmol/mL of wine, the approximate limit of detection for the MS system used. This concentration takes into account the approximate net 100-fold concentration step that was part of the protein isolation protocol. With respect to expression, it was reported that approximately 80% of the yeast proteome was detected and that protein concentrations varied enormously, from less than 50 to more than 10^6 molecules/cell in a laboratory strain harvested in log phase in YEPD (*41*). Among the 48 proteins detected in the 9 month wine samples, 37 were also detected and quantified in the laboratory study. Among these 37 proteins, 75% were found in the laboratory strain at relatively high concentrations >10000 molecules/cell and 25% at concentrations <10000 molecules/cell. Concerning protein solubility, the large differences in pH, ionic strength, and alcohol content between the intracellular yeast environment and the model wine were likely to have led to denaturation, aggregation, and precipitation of the most susceptible extracted proteins. This can explain in part why only about 200 proteins (about 3% of the known yeast proteome) were detected. Thus, while total protein concentration increased about 2-fold on average between the 285 day and the 14 day samples (**Table 2**), the majority of proteins detected at early time points were not detected in either the 195 day or the 285 day samples (Supporting Information, Table 1).

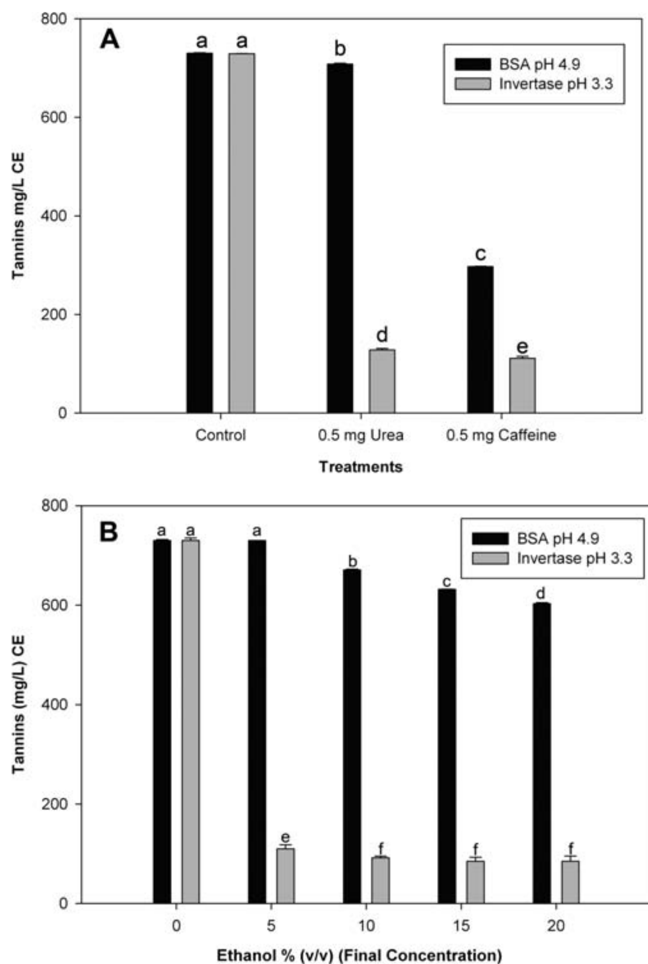

Figure 5. Effect of urea, caffeine, and ethanol on tannin binding by nonmannosylated BSA and mannosylated yeast invertase. The binding assays were performed at pH values determined to be optimal for binding by each protein (pH 3.3 for invertase and 4.9 for BSA). (**A**) The effect of two different hydrogen bond disruptors, urea and caffeine, on recovery of wine tannins by precipitation with protein. (**B**) The effect of ethanol on recovery of wine tannins by precipitation with protein. Data are means of two replicates, and error bars are standard deviations. Different letters per treatment indicate significant differences in tannin binding by the two proteins at $p < 0.05$.

Tannin−Protein Binding. The tannin-binding ability of native mannosylated yeast invertase was compared to that of BSA to assess the likelihood that tannin−mannoprotein interactions in red wines could explain indirect observations of complex formation. The formation of soluble tannin−mannoprotein complexes has been suggested by short-term observations of either increased tannin solubility or a decrease in tannin particle size upon addition of yeast-derived mannnoprotein extracts to tannin in a wine matrix (*17*, *19*, *20*). We found invertase−tannin binding to be maximal at pH 3.3 and less effective at pH 4.9, 6, or 7. The tannin-binding ability of BSA was also less effective at neutral pH values. However, for BSA, binding was maximal at pH 4.9 and only slightly diminished at pH 3.3 (**Figure 4A**). The binding reaction shown in **Figure 4B** was performed at pH values determined to be optimal for binding by each protein (pH 3.3 for invertase and 4.9 for BSA). At these pH values, the two proteins were found to have about the same tannin-binding capacity (*Y*-intercept). On a mass basis, native invertase required 1.63 mg of protein to reach 50% tannin binding saturation, whereas BSA required only 0.17 mg, a difference of about 10-fold (*X*-intercept, **Figure 4B**). In other words, BSA was found to have about a 10-fold greater binding affinity for tannin than invertase. This is consistent with previous observations of glycoproteins having less affinity for tannin than nonglycosylated proteins (*21*, *44*, *45*). Urea, caffeine, and ethanol were assessed for their ability to disrupt protein−tannin precipitate formation. On the basis of differential tannin binding by BSA and invertase, the invertase concentration was adjusted to 4 mg/mL so that similar amounts of tannin would be precipitated by the two proteins. Urea and caffeine were found to be significantly more effective at diminishing tannin binding by native invertase than by BSA, suggesting that hydrogen bonding is a greater contributor to the stability of the tannin−native invertase complex than to the BSA−tannin complex (**Figure 5A**). Similarly, the presence of ethanol was found to be significantly more disruptive of the interactions between tannin and native invertase than between tannin and BSA (**Figure 5B**). The disruption of insoluble invertase−tannin complex formation by ethanol at concentrations similar to those found in red wines (10−15%, [v/v]) suggests that mannoproteins may be recovered in red wines aged on yeast lees.

The present study determined changes in protein and protein-associated mannan concentrations in model wines aged on the yeast lees over a 9 month time course, identified individual proteins, and assessed tannin binding by invertase, a well-characterized yeast mannoprotein. To our knowledge, systematic documentation of extracted yeast proteins over a typical aging period has not previously been described. The finding that mannoproteins are enriched after 9 months implies the possibility that their contributions to wine quality may persist long after bottling. This includes interactions with wine tannin that generally have been assessed in short-term experiments. Although we found such interactions to be weak relative to BSA, they could be important, as we speculate that in a red wine, proteins with the greatest affinity for tannin would tend to bind it early in the winemaking process, leading to aggregate formation and early loss due to precipitation. In the absence of proteins with higher affinity for tannin, mannoproteins might then compete more effectively to form soluble complexes with tannin that may contribute to alterations in wine texture. Determining how long mannoproteins remain soluble in wine, as opposed to model wine, will help to clarify their long-term contributions to wine quality.

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Supporting Information Available: Table of a comprehensive compilation of all 219 identified proteins by gene name found in the model wines as a function of yeast strain and sampling time, including systematic names, numbers of matching peptides per protein, probabilities (%) of correct protein identifications, cellular localizations, and predicted pI values. This material is available free of charge via the Internet at http://pubs.acs.org. Additional data are available at http://www.cgrb.oregonstate.edu/faculty/bakalinsky.

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