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Protein Haze Formation in White Wines: Effect of *Saccharomyces cerevisiae* Cell Wall Components Prepared with Different Procedures

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Cell wall material was extracted by five different methods from an oenological strain of *Saccharomyces cerevisiae*. Enzyme preparations containing beta-glucanase activity (Zymolyase, Glucanex, and Finizym 250L) allowed a better extraction yield compared to that of dithiothreitol (DTT) and EDTA. The yeast extracts were only soluble in part in wine. The wine-soluble fraction (WSF) of the five extracts, differing in both protein and sugar contents, when added in increasing amounts to white wine differently affected protein haze formation, as determined by the heat test, giving dose/response curves of different shapes. The curves obtained with the WSF derived from DDT and Zymolyase extracts showed a plateau value corresponding to 90% and 80% of wine haze reduction, respectively. In contrast, addition of the WSF derived from the other extracts resulted in increased turbidity with respect to the original wine. The mannoproteins (MP), isolated from each yeast extract by Concanavalin-A chromatography, gave dose/response curves showing shapes more similar among them than those obtained with the whole WSFs. The best wine stabilization was obtained with the MP of the DTT and Zymolyase extracts. The supernatants obtained after heating the MP of the different extracts were also tested. The stabilizing effect of the heat-stable MP (HSMP) was always larger than that of the corresponding total (un-heated) MP. The HSMP obtained starting from the DTT and Zymolyase extracts showed the best haze-protecting effect, which was, however, lower than that obtained with their corresponding WSF. This result suggests that wine protein stabilization by compounds of the yeast cell wall could be related, in addition to the action of the MP, also to the presence of other substances of different nature.

KEYWORDS: Wine; haze; mannoprotein; cell wall extraction; *Saccharomyces cerevisiae*

INTRODUCITON

Clarity is of great qualitative importance to white wines, whereas haziness makes them unattractive and can be perceived as spoilage by the consumer. One of the main causes of turbidity is due to instability of the grape proteins that occur naturally in wine (*1*), their denaturation and precipitation being influenced by intrinsic and extrinsic factors such as pH, ethanol, storage temperature, and reactions with other wine components (*2, 3*). To prevent haze formation in white wine, it is a common practice to lower its protein concentration by bentonite fining (*1*). However, this technique has some drawbacks, including the removal of aroma compounds (*4*) and the loss of wine in the sediment (*5*). Alternative methods for protein removal from wine, including proteolytic treatments (*6*) and ultrafiltration (*7*), are not used in practice, although they seem to have some potential in reducing the risk of haze formation.

As an alternative to these subtractive methods, the use of additive methods based on yeast cell extracts have been proposed (*8–11*). In particular, it was demonstrated that the yeast components able to prevent wine protein from heat-induced precipitation were the cell walls mannoproteins (MP), a class of high molecular weight compounds containing over 90% sugar, mainly mannose, which are bound to the cell wall glucans by covalent and noncovalent linkages, such as ionic interactions and disulfide bridges (*12*). These macromolecules, which are spontaneously released in wine during both alcoholic fermentation and storage on yeast lees (*10, 11*), can also be extracted from yeast cells and added to wine to improve protein stability, thus reducing the need for bentonite fining (*8, 9*). Several methods for MP extraction from yeast cells have been investigated, including chemical, enzymatic, and physical procedures (*8, 9*). Enzymatic extraction, on the basis of beta-glucanase activity, is one of the most successful practices to obtain MP for use in wine making (*8*). Other methods include high temperature treatments, reducing or chelating agents, and detergents to release yeast macromolecules with differing wine haze-preventing ability (*9*).

This article reports on the effect of yeast cell wall macromolecules in affecting protein stability in a white wine by

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studying five different methods (three enzymatic and two chemical) of extraction and some purification procedures. Moreover, for each sample derived from the different yeast extracts, the effect of the doses added to wine on its protein heat stability is described.

MATERIALS AND METHODS

Yeast Growth. The *Saccharomyces cerevisiae* strain 444 was obtained from the collection of "Istituto Agrario San Michele all'Adige", Italy.

A frozen cell suspension was inoculated into yeast medium containing 1% (w/v) yeast powder (type 1, Hi Media lab. Pvt., Fontenay, France), 2% (w/v) peptone, and 2% (w/v) glucose in distilled water. After 12 h at 30 °C, this pre-inoculum was used to inoculate 5 to 6% (v/v) of 4 L of the same medium. Cells were grown at 30 °C, collected to late exponential phase by centrifugation at 1100*g* at 4 °C for 10 min, washed in distilled water, and centrifuged again. This washing procedure was repeated 3 times. The pellet was weighted (9–11 g/L of medium) and immediately used for extraction.

Yeast Cells Extraction. For each extraction, 40 g of cells (wet weight) were used, and 1.5 M sorbitol was added as osmotic protector to the buffers. Extractions were performed as follows. Extractions with Glucanex and Finizym 250 L: yeast cells were re-suspended 1:3 (w/v) in 0.1 M Na acetate buffer, pH 5.1, containing 5% (w/w cells) Glucanex 200 G (Novozymes, Switzerland) or Finizym 250 L (Novozymes, Switzerland), both with or without 1 mM dithiothreitol (DTT). The resulting suspensions were shaken in a water bath for 3 h at 30 and 55 °C for Glucanex (*13*) and Finizym 250 L, respectively. Extraction with Zymolyase: cells were re-suspended 1:1 (w/v) in 5 mM Tris-HCl buffer, pH 7.5, containing 10 mM $MgCl_2$ and 30 mM DTT. After 1 h of incubation at 37 °C, the cell suspension was centrifuged at 1100*g* for 10 min at 4 °C. The pellet was re-suspended 1:3 (w/v) in the same buffer containing 1 mM DTT and 2 mg/mL Zymolyase 100T (ICN Biomedicals, Inc., Costa Mesa, CA, USA) and shaken in a water bath at 37 °C for 2 h (*14, 15*). Extractions with DTT and EDTA: cells were re-suspended 1:3 (w/v) in Tris-HCl buffer, pH 7.5, with different DTT or ethylenediaminetetracetic acid (EDTA) concentrations, (1, 5, 10, 20, and 30 mM) and shaken in a water bath at 37 °C for 1 h.

After extraction with the different procedures, the suspensions were centrifuged at 1100*g* for 15 min. The insoluble material was discarded and the supernatant collected. Supernatants were dialyzed with a 3.5 kDa cut-off membrane (Spectra/Por, Spectrum Laboratories, Breda, The Netherlands) against distilled water (5 L, 10 changes) for 36 h at 4° C, freeze dried, and weighted.

Protein and Sugar Quantification. Samples to be analyzed were solubilized in water, and the protein content was determined by the Bradford method (*16*), using bovine serum albumin (BSA) as the standard. Sugars were measured by the Periodic Acid Schiff (PAS) method (*17*), as follows. Fifty micrograms of protein were solubilized in 500 μL of phosphate buffered saline (PBS), pH 7.2, added to 200 μL of 10 mM $NaIO_4$, and kept at room temperature for 10 min. Two-hundred microliters of the Schiff's reagent fuchsin-sulfite (Sigma, Milan, Italy) was added to the samples. After 1 hour at room temperature, the reaction was stopped with 100 μL of 1 M NaOH. Calibration curves were obtained with yeast invertase (Sigma, Milan, Italy), assuming that invertase contains 50% sugar by weight (*18*). BSA was used as the negative control.

Preparation of the Wine-Soluble Fraction of the Extracts. The different yeast extracts (1 mg of protein) were added to 2 mL of Manzoni Bianco wine, previously ultrafiltered on a 5 kDa Amicon membrane (Millipore, Milan, Italy) to remove endogenous proteins (deproteinised wine, DpW), by shaking at room temperature for 30 min. Samples were centrifuged at 1100*g* for 5 min, and the protein content of both the supernatant and the pellet was measured. The sugar content was also measured, but only on the pellet because the wine strongly interfered with the Schiff's reagent used to this end. Before the analysis, pellets were washed 3 times with a model wine solution [12% EtOH (v/v) and 3 g/L of L(+)-tartaric acid; pH adjusted to 3.5 with NaOH 1 M]. The sugar content of each DpW-soluble cell wall fraction (WSF) was calculated as the difference between the sugar content of the total yeast extract and that measured in the pellet.

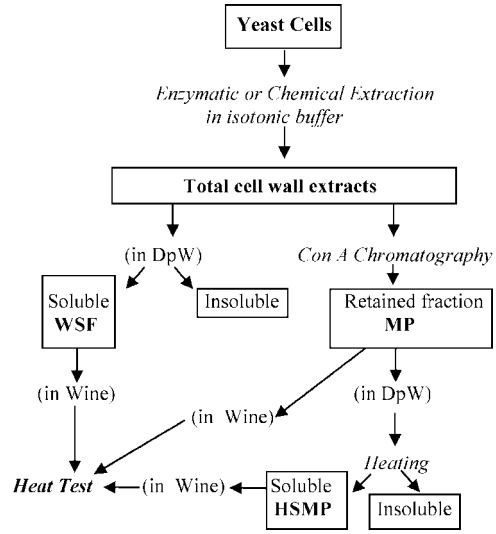

Figure 1. Scheme of the procedures used.

Preparation of the Mannoprotein Fractions. Mannoproteins were isolated from each yeast extract essentially as described by Dupin et al. (*9, 10*) with some modifications, as follows. The extracts were dialyzed with a 3.5 kDa cut-off membrane (Spectra/Por) against 5 L of binding buffer (BB) (20 mM Tris-HCl, pH 7.4, containing 0.5 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mM $MnCl_2$) for 18 h at 4° C with 2 changes. Dialyzed samples were 5-fold diluted with BB and loaded at a flow rate of 0.5 mL/min onto a Concanavalin-A Sepharose 4B column (26 × 1.8), previously equilibrated with BB. Unbound material was eluted with BB, while the fraction retained by the column was eluted with BB containing 0.3 M methyl-α-D-mannoside (Sigma). Eluted proteins were detected by monitoring the absorbance at 280 nm. Both the un-retained and the Con A-retained fractions (corresponding to the mannoproteins, MP) were dialyzed (3.5 kDa cut-off) against water (5 L, 10 changes) and freeze dried. Freeze dried fractions were weighted and analyzed for protein and sugar contents.

Preparation of the Heat-Stable Mannoproteins. Each MP sample (1 mg of protein) was dissolved in 2 mL of DpW. The resulting solutions were treated for 1 h at 90° C and kept for 18 h at 4° C. After centrifugation, the protein content of both the supernatant (containing the heat-stable mannoproteins, HSMP) and the pellet was quantified. The sugar content of the HSMP was calculated as the difference between the sugar content of the original MP preparation and that measured in the pellet resulting from the heat treatment.

Heat Test. Six white wines (cv. Prosecco, Manzoni Bianco, Bianchetta Trevigiana, Pinot Blanc, Malvasia, and Moscato) were subjected to the heat test according to Dupin et al. (*9, 10*), with some modifications. Each wine (2 mL) was kept shaking in a water bath at 90 °C for 1 h. After 18 h at 4 °C, the wine samples, left at room temperature for 30 min, were analyzed spectrophotometrically at 490 nm. Each test was carried out in triplicate considering the absorbance of the unheated wines as the blank. The various fractions (WSF, MP, and HSMP) derived from the different procedures were added in increasing quantities (1–120 μg of protein) to 2 mL of wine. The resulting solutions were heat tested, and the effect of the additions on wine protein stability was expressed as the percentage of variation in turbidity with respect to the original wine. For each measure, six replicates were used.

RESULTS AND DISCUSSION

Yeast Cell Extraction by Different Methods. Procedures based on the use of enzymatic preparations with glucanase activity as well as chemical treatments were used for the extraction of yeast cells. The scheme of the procedures is shown in **Figure 1**. Extractions were always performed in isotonic buffer (1.5 M sorbitol), thus allowing removal of the intact spheroplasts (i.e., the cytoplasm and the plasma membrane) (*15*), whose components, in this way, were eliminated. Therefore,

Table 1. Yield and Protein and Sugar Contents of the Yeast Crude Extracts Obtained with Different Treatments

treatment	yield[a] (g/100 g wet cells)	total protein[a] (mg/100 g wet cells)	protein[a] (mg/g dry extract)	total sugar[a] (mg/100 g wet cells)	sugar[a] (mg/g dry extract)	sugar %	sugar/protein
Zymolyase/DTT	1.43	265	183.3	489	341.9	65	1.8
Glucanex	0.20	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Glucanex/DTT	1.66	155	93.4	358	215.7	70	2.3
Finizym	0.30	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Finizym/DTT	1.16	194	167.2	360	310.3	65	1.9
DTT 30 mM	0.65	55	84.6	208	320.0	79	3.8
EDTA 30 mM	0.90	87	96.7	468	520.0	84	5.4

[a] The values are the averages of 3 replicates. Standard deviations were always < 5%. n.d.: not determined.

only material derived from the cell wall (and from the periplasmic space) was considered, different from what was previously done by others in similar studies (*9*). Besides glucanase preparations, whose action in releasing cell wall material is obviously related to their hydrolytic activity on cell wall glucans, DTE and EDTA were also shown previously to allow solubilization of yeast cell walls when used at concentrations of 5 mM (*9*). By increasing the concentration of both DTT (a reducing agent similar to DTE) and EDTA over 5 mM, an increasing quantity of cell wall material was released from yeasts. The dose/extraction curves obtained for DTT and EDTA showed similar shapes, approaching a plateau value for concentrations higher than 20 mM (not shown). However, the maximum quantity of cell wall material obtainable with EDTA ($\approx$ 900 mg of dry material/100 g of wet cells) was higher than that extracted with DTT ($\approx$ 650 mg of dry material/100 g of wet cells). According to these results, DTT and EDTA were used at a concentration of 30 mM.

Three enzymatic preparations of different origin (Zymolyase, Glucanex, and Finizym 250 L) were evaluated for their ability to extract yeast cell wall material. Zymolyase, which is a bacterial preparation for laboratory use containing both beta-glucanase and protease activity (*9*), was used on the cells pretreated with 1 mM DTT, according to a previously suggested procedure (*14*). The other two preparations (Glucanex and Finizym 250 L) are commercial enzymes of fungal origin. Glucanex, which is similar to Zymolyase, is industrially employed in wine making for improving the filtration of young wines containing *Botrytis* glucan but can also be used to extract cell wall material from yeast (*8*). Finizym 250 L is also a fungal beta-glucanase, which is utilized in the brewing industry to improve beer filtration and reduce glucan haze. Both preparations were used without pre-treating the yeast cells with DTT because this treatment did not improve the extraction, as assessed in preliminary experiments (not shown). When these enzymes were used as previously reported (*8, 13*) the extraction yield was very low compared to that obtained by Zymolyase/DTT (**Table 1**). However, by performing the enzymatic extraction in the presence of 1 mM DTT, as during the Zymolyase treatment (*14*), the effectiveness of the extraction was greatly improved (**Table 1**). The quantity of cell wall material recovered with the different treatments from 100 g of wet yeast cells are reported in **Table 1**, along with protein and sugar contents. Because both analyses were based on standards (BSA for protein and yeast invertase for sugars) that may not warrant a precise measure of the real protein and sugar contents, the obtained results were considered only to compare the different fractions and not to give their actual composition by weight. The enzymatic extractions generally resulted in the recovery of a total protein amount that was higher than that obtained with the two chemicals. This result could be explained by considering that treatments with beta-glucanases by degrading the glucan component of the cell wall release almost all of the proteins associated with the cell wall and also those entrapped in the periplasmic space, whereas DTT and EDTA may act more specifically, releasing only those proteins associated with the cell wall through interactions sensitive to these chemicals. The quantity of sugars measured in the different extracts did not parallel that of the protein content, resulting in different sugar/protein ratios. In particular, the DTT and EDTA extracts showed a ratio that was higher than that of the three enzymatic extracts, which were instead comparable (**Table 1**). These results suggest that different types of molecules are released with the different extraction systems and confirm that these systems break down the yeast cell wall structure with different mechanisms (*9*).

Fractionation of the Extracts by Concanavalin A Chromatography. Fractionation of the total yeast cell wall extracts by affinity chromatography on a Concanavalin A (ConA, a plant lectin specific for mannose and glucose) column allowed us to separate the fraction bound by the lectin, comprising the cell wall mannoproteins (MP), from that which was un-retained (UF) by the column matrix (*9*). Both fractions were dialyzed, freeze-dried, weighted, and analyzed by the Bradford method. This method may be unsuited for the quantification of the protein content of MP and was used only to compare the different samples. The sugar content of the MP fraction was also determined (**Table 2**). After ConA fractionation, the extracts gave different quantities of MP, indicating different effects of the extraction methods used. The MP yields of the DTT, EDTA, and Zymolyase/DTT extracts were in the range of those obtained previously by others (*9*). The DTT extract gave the best MP yield (about 80%), although the largest quantity of MP obtainable from 100 g of wet cells was that derived from the Zymolyase/DTT extract. This suggests that the extraction by DTT has the highest specificity in extracting cell wall MP from yeast. The EDTA extract gave the lowest MP amount, confirming the results obtained previously on fresh yeast cells (*9*). Finally, the fractionation of the Glucanex/DTT extract resulted in a relatively low quantity of MP, whereas that of the Finizym 250 L/DTT extract gave an average amount of MP, which was characterized by the largest protein content, as measured by the Bradford method. Indeed, the various MP fractions differed in both protein and sugar content, suggesting the presence of components with different composition. In particular, the sugar/protein ratio varied from more than 10 in the MP fraction derived from the Zymolyase/DTT extract down to 0.9 in the MP obtained after extraction with Finizym 250 L/DTT and EDTA (**Table 2**). On the basis of the percentage of sugars (calculated on the sum of the weight of sugars and proteins found for 1 gram of dry fraction), the obtained MP fraction could be divided in two groups: those showing a sugar content higher than 80% (MP of the DTT, Glucanex/DTT and Zymolyase/DTT, and extracts) and those having a sugar content of about 50% (MP of the EDTA and Finizym 250L/DTT extracts). These sugar contents are in the range of those reported for the

Table 2. Yield and Protein and Sugar Contents of the Retained (MP) and Unretained (UF) Fractions from Affinity Chromatography on Concanavalin A of the Yeast Extracts Obtained with the Different Treatments

treatment	yield [a] (g/g extract)	yield [a] (g/100 g wet cells)	total protein [a] (mg/100 g wet cells)	protein[a] (mg/g dry fraction)	total sugar[a] (mg/100 g wet cells)	sugar[a] (mg/g dry fraction)	sugar/protein	sugar[b] %
Zymolyase/DTT								
MP	0.54	0.776	24.0	30.9	246	317	10.3	91
UF	0.40	0.580	224.0	386.2	n.d.	n.d.	n.d.	n.d.
Glucanex/DTT								
MP	0.15	0.260	20.2	77.7.	127	488	6.3	86
UF	0.90	1.495	125.0	83.6	n.d.	n.d.	n.d.	n.d.
Finizym 250L/DTT								
MP	0.34	0.403	101.0	250.6	90	223	0.9	47
UF	0.61	0.710	75.0	105.6	n.d.	n.d.	n.d.	n.d.
DTT 30 mM								
MP	0.80	0.521	19.3	37.0	85	163	4.4	81
UF	0.29	0.189	27.0	142.9	n.d.	n.d.	n.d.	n.d.
EDTA 30 mM								
MP	0.11	0.096	13.3	138.5	12	125	0.9	47
UF	0.81	0.73	56.0	76.7	n.d.	n.d	n.d.	n.d.

[a] The values are averages of 3 replicates. Standard deviations were always < 5%. [b] Calculated on the sum of sugars and proteins found in 1 g of dry fraction. n.d.: not determined.

two types of yeast glycoproteins that can be found in wine, which differ for the degree of glycosylation and mode of release from the cell (*19*).

Heat Tests. The different yeast cell wall extracts and their corresponding MP fractions were studied for their effects on the protein stability of a white wine by the heat test (*10, 20*). In order to choose a wine for these experiments, six white wines (Malvasia passito, Manzoni Bianco, Bianchetta trevigiana, Prosecco, Moscato, and Pinot blanc), taken before bentonite fining, were examined for protein stability. A different reactivity to the heat test was detected for the different wines (not shown). Because of its high, but not excessive, protein instability, Incrocio Manzoni Bianco was chosen. The addition to this wine of the different yeast fractions was always performed on the basis of their protein content because, as observed in preliminary experiments, the reproducibility among similar experiments was much higher than that obtainable with additions based on weight, allowing a more reliable comparison of the effects of the various samples on wine protein stability. The addition to wine of increasing quantities of the various yeast fractions followed by the heat test (**Figure 1**) allowed one to construct, for each of them, a curve showing the variation of wine protein (heat) stability in relation to the quantity added (dose/response curves).

Effect of the Yeast Extracts on Wine Hazing. When the yeast crude extracts obtained with the different extraction systems were added to wine, it was observed that they did not dissolve completely, making the wine turbid. In order to quantify the extent of solubilization of each extract, a wine sample, deprived of protein by ultrafiltration on a 5 kDa membrane (deproteinized wine, DpW), was added to the extracts (0.5 mg of protein/mL), and the insoluble protein was measured after centrifugation of the suspension. The extracts gave different percentages of DpW-insoluble protein (**Table 3**), again indicating their different composition. The supernatant of the suspensions of the various crude extracts in DpW (i.e., the solutions containing only the wine-soluble components, WSF) were added in increasing amounts to wine (1–120 μg of protein/2 mL of wine). The resulting clear mixtures were heat tested, and the variation in haze formation with respect to the original wine (i.e., without addition) was measured. The dose/response curves obtained with the WSF of the different extracts showed different shapes (**Figure 2**). For the WSF obtained starting from the Glucanex/DTT and Finizym 250 L/DTT extracts, the largest obtainable reduction in heat-induced haze was observed at 1 μg of added protein followed by a gradual increase in turbidity with higher additions. However, these maxima of haze reduction were rather low for both of these WSFs, being ≈15% for the Glucanex/DTT-extracted WSF and even lower (≈10%) for the WSF derived from the Finizym 250 L/DTT extract.

Table 3. De-protenised wine (DpW)-insoluble protein (as % of the total protein) of the yeast crude extracts obtained with the different treatments

treatment	insoluble protein (%) [a]
Glucanex/DTT	39.8 (3.8)
Finizym 250L/DTT	4.2 (0.8)
Zymolyase/DTT	51.4 (4.3)
DTT 30 mM	13.8 (1.2)
EDTA 30 mM	36.8 (4.1)

[a] The values are averages of 3 replicates. Standard deviation is given in parentheses.

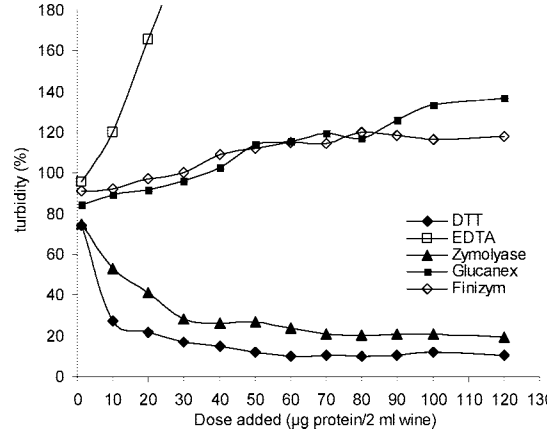

Figure 2. Effect on wine protein stability of the DpW-soluble fraction (WSF) of the yeast extracts obtained by treatments with 30 mM DTT (DTT), 30 mM EDTA (EDTA), Zymolyase/DTT (Zymolyase), Glucanex/DTT (Glucanex), and Finizym 250L/DTT (Finizym). Turbidity is expressed as the percent variation of OD at 490 nm after heat test with respect to the original wine (turbidity 100%). Each point is the average of six replicates. Standard deviations were always <5%.

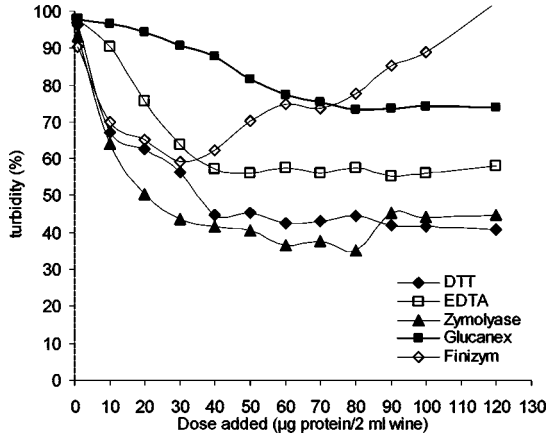

Figure 3. Effect on wine protein stability of the mannoproteins (MP) derived from the yeast extracts obtained by treatments with 30mM DTT (DTT), 30 mM EDTA (EDTA), Zymolyase/DTT (Zymolyase), Glucanex/DTT (Glucanex), and Finizym 250L/DTT (Finizym). Turbidity is expressed as described for **Figure 2**. Each point is the average of six replicates. Standard deviations were always <5%.

When the Glucanex/DTT WSF was added at doses higher than 40 μg of protein/2 mL of wine, the resulting heat-induced haze was even higher than that of the original wine. This effect on hazing was completely different compared to that previously reported by Moine-Ledoux and Dubourdieu (*8*), who obtained a significant haze reduction in wine after the addition of a yeast extract obtained by Glucanex extraction. Addition to the wine of the WSF of the EDTA extract caused an increase of the heat-induced haze at each of the doses tested, giving a very high turbidity at the highest dose (**Figure 2**). The increased haze formation observed in all these cases suggested the presence of some heat-unstable material in the tested solutions, which, above certain doses, could mask the haze-protective effects of other substances of the mixture. The presence of the heat-unstable material was confirmed by heating the WSFs in DpW, which became turbid (not shown). Compared to these results, a completely different behavior was observed for the WSF of both the DTT and the Zymolyase/DTT extracts. The dose/response curves, although showing slightly different shapes, indicated a continuous increase of the heat-stability of wine with increasing doses of addition up to a plateau value corresponding to a haze reduction of ≈90% and ≈80% for the DTT-and the Zymolyase/DTT-extracted WSF, respectively (**Figure 2**).

Taken together, these results indicate that the wine-soluble material extracted from yeast with the different treatments very differently affects the wine protein heat-stability and that some of these extracts are heat-unstable *per se*, contributing to haze formation after the heat test. These different effects are obviously related to a different composition of the various mixtures, as suggested above.

Effect of the Mannoprotein Fractions on Wine Hazing. The freeze-dried MP (obtained by ConA chromatography) was added to DpW as described above for the WSFs. In contrast to the behavior observed for the latter, the MP showed a rapid and complete solubilization in DpW, confirming the good solubility in wine of these yeast components (*9*). The solutions of MP in DpW were added in increasing amounts to wine, and the resulting mixtures were heat tested. The shapes of the obtained dose/response curves were more similar among them compared to those obtained with the WSFs. (**Figure 3**). The exception was the MP recovered from the Finizym 250 L/DTT extract, whose stabilizing effect reached a maximum (at about 30 μg of protein addition), then decreased, and completely disappeared at the highest dose used (**Figure 3**). All the other MP preparations, when added in increasing amounts, showed a stabilizing effect, which tended to reach a plateau value. The MP fractions derived from the DTT and Zymolyase/DTT extracts showed similar plateau values (≈60% of haze reduction), which were reached with additions of 40–50 μg of protein/2 mL of wine. The maximum effect of the MP of the EDTA extract was instead ≈45% of haze reduction starting from an addition of 40 μg of protein/2 mL of wine and that of Glucanex/DTT-extracted MP ≈25% of reduction starting from 80 μg of protein/2 mL of wine, which was even lower.

Table 4. Protein Content of the Insoluble (Pellet) and Soluble (Supernatant) Fractions derived from Heating (80 °C for 1 h) 1 mg of the Mannoproteins from the Yeast Extracts Obtained with the Different Treatments

	protein (mg)[a]	
treatment	pellet	supernatant
Glucanex/DTT	0.51 (0.12)	0.48 (0.08)
Finizym 250L/DTT	0.35 (0.07)	0.60 (0.08)
Zymoliase/DTT	0.06 (0.02)	0.92 (0.05)
DTT 30mM	0.18 (0.05)	0.78 (0.10)
EDTA 30mM	0.15 (0.04)	0.79 (0.09)

[a] The values are averages of 3 replicates. Standard deviation is given in parentheses.

In a previous work, Dupin et al. (*9*) reported that the yeast MP extracted from freeze-thawed cells by 5 mM DTE (a reducing agent with the same action as that of DTT) not only did not show any haze-protective effect but also dramatically increased the level of heat-induced turbidity. Moreover, the haze formed in the presence of Zymolyase-extracted MP was about 80% of that of the control wine (*9*). Our results do not confirm those findings because, in our case, the stabilizing effect of the MP extracted by 30 mM DTT from fresh yeast cells and added to wine in a quantity corresponding to that used in (*9*) is similar to that obtained by the use of Zymolyase/DTT, which is, among those tested, the treatment giving the best results (>60% of haze reduction). The extent of haze reduction caused by adding to wine the MP derived from the EDTA extract was similar to that reported previously after the addition of the MP extracted by 5 mM EDTA from freeze-thawed yeast cells but not from fresh cells (*9*). However, by using 30 mM EDTA, we were able to extract an MP fraction with haze-protecting activity starting from both fresh and freeze-thawed yeast cells (not shown).

Finally, it is difficult to compare the results obtained with the MP fraction of the Glucanex/DTT extract to those obtained by Moine-Ledoux and Dubourdieu with the same enzymatic preparation (*8*) because these authors considered a total extract derived from a preparation of yeast cell walls and a purified MP component, corresponding to the invertase fragment called MP 32.

In order to establish whether the MP fractions added to wine contributed to the residual heat-induced wine turbidity, these solutions were heated (as for the heat test) after being diluted at 1 mg of protein/mL of DpW. Heating resulted in protein precipitation in all of the MP solutions, although its extent was different (**Table 4**). A large proportion of protein resulted in being heat-unstable in the MP solutions derived from both the Glucanex/DTT and the Finizym 250 L/DTT extracts. The other MP preparations were more heat-stable, giving only a relatively low amount of protein precipitation after heating. The quantity of heat-stable proteins found in the different MP solutions showed a certain correspondence with the stabilizing effect observed in wine for each of them (compare the data of **Table**

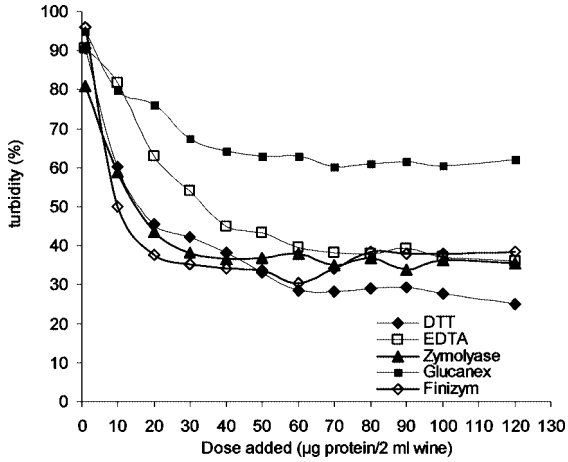

Figure 4. Effect on wine protein stability of the heat-stable mannoproteins (HSMP) derived from yeast extracts obtained by treatments with 30 mM DTT (DTT), 30 mM EDTA (EDTA), Zymolyase/DTT (Zymolyase), Glucanex/DTT (Glucanex), and Finizym 250L/DTT (Finizym). Turbidity is expressed as described for **Figure 2**. Each point is the average of six replicates. Standard deviations were always <5%.

4 with the results of **Figure 3**). In order to eliminate the interference of the heat-unstable material and to establish the effect of the heat-stable MP (HSMP) on hazing, the supernatants obtained after heating the total MP solutions were added to wine in increasing amounts (**Figure 4**). The heat-test results indicated that in general the stabilizing effect of the HSMP was higher than that of the corresponding total (unheated) MP fraction (compare **Figures 3** and **4**). In most of the cases, this increase in wine stabilization was proportional to the percentage of the heat-stable protein found in the total MP solution. For example, for the MP extracted by Zymolyase/DTT (containing only 6% of heat-unstable protein; **Table 4**), the maximum stabilizing effect varied only a little when HSMP were used. In contrast, when the HSMP of the Finizym 250 L/DTT extract (which contained 35% of heat-unstable proteins; **Table 4**) were added to wine, the effect increased from 40% of stabilization (given by the total MP fraction at an addition of 30 mg/2 mL of wine) up to 70%, suggesting that the heating step selects the MP with the highest stabilizing effect.

Among the various HSMP preparations, that giving the largest stabilization was the fraction derived from the 30 mM DTT extraction (**Figure 4**). This indicates that the treatment with DTT allows one to obtain an MP fraction that when deprived of the heat-unstable material contains molecules with a very high effectiveness in protecting wine from protein haze formation. However, it must be noted that the WSF of the yeast extract obtained with 30 mM DTT gave protection from haze formation that was even higher (≈90%, **Figure 2**) than that of its HSMP fraction (≈75%, **Figure 4**). This unexpected result suggests that the mechanism of wine protein stabilization could be related, in addition to the action of the MP (*9*), also to the presence of other substances of different nature, which are removed during the MP purification step.

An Interpretation of the Stabilizing Effect. In several cases, a hyperbolic relationship between the concentration of added yeast fractions and the haze-protecting effect was found (**Figure 5**). As a matter of fact, for most of the curves, good fittings of the data were obtained with $R^2 > 0.9$ for the hyperbolic equation describing monolayer adsorption on solid surfaces, i.e., the type I isotherm, also known as the Langmuir isotherm. The Langmuir equation [$q_s = K_L C_s/1 + a_L C_s$, where q_s is the adsorbate concentration per weight unit of adsorbent (solid phase), C_s is the concentration of adsorbate in solution (fluid phase) at equilibrium, and K_L and a_L are constants] was initially developed to describe the gas adsorption on solid surfaces (*21*) but can be successfully applied also to systems in which the Langmuir's underlying hypotheses are not verified (*22*), as, for example, in the case of procyanidins adsorption on solid polysaccharides (*23*). Therefore, we can speculate that the wine haze reduction noticed with the addition of increasing amounts of yeast cell wall fractions is related to an adsorption phenomenon involving molecules present in these fractions. If haze formation is the result of the interactions of the wine proteins with other wine compounds (*1–3*), then it is possible that the haze-reducing action of the yeast cell wall components is due to their capacity to prevent these interactions after being physically adsorbed on one of the interacting compounds. This is indicated by the fact that the reduction in haze formation with increasing quantities of yeast components follows a curve that is typical of an adsorption phenomenon. An adsorption mechanism, however, would imply the presence of a solid–liquid interface. Therefore, invisible particles in the colloidal state should be present in the system during the formation of the visible haze caused by the heat test. As previously reported (*20*), the particle size of wine haze samples decreased as a consequence of the addition of increasing amounts of a haze-protecting factor (which was similar to the MP of the yeast cell wall), suggesting that limpidity can be related to the low size of the particles ($<5\ \mu$m) rather than to their absence. The presence of these visually undetectable particles could explain the possibility of the occurrence of surface adsorption phenomena. In particular, the following sequence of events can be hypothesized: formation of small (undetectable) particles, adsorption of the stabilizing compounds of yeast origin on the surface of these small particles, and, consequently, the formation of a molecular layer acting as a barrier for the interactions leading to particle enlargement. In this way, the formation of particles with a size that is detectable would be prevented by the compounds present in the yeast extracts. This view is compatible with the previously reported hypothesis that the mechanism of action of the haze-protective factors of yeast origin is a competition with the denatured wine proteins for the other wine compounds required to build up large protein aggregates (*3, 11*). However, whether the initial small particles are formed during the heat test or whether they pre-exist in the wine remain to be established, and the nature of these particles also has to be defined. As previously shown, wine procyanidins are able to form colloidal particles in model wine, whose growth is inhibited by yeast MP (*24, 25*), and self association of procyanidins via their aromatic ring has been also reported (*26*). Moreover, the occurrence of adsorption phenomena, which follow the Langmuir behavior, between phenolics and polysaccharides has been demonstrated (*23*). Therefore, it is likely that procyanidins naturally present in wine in a colloidal state are the components involved in the physical adsorption of the yeast components, which in this way would prevent interactions with the wine proteins during the heat test (*3*). Another possibility is that the particles acting as adsorbents for the yeast compounds with haze-protecting ability are formed during the heat test. In this case, these particles would be originated by wine protein denaturation, a process accelerated by heating and induced by the so-called essential factors such as sulfate (*3*). Also in this case, further protein aggregation, possibly mediated by phenolic compounds (*3, 11*) and leading to visible haze formation, would be prevented.

In conclusion, the results obtained here confirm that the yeast cell wall contains molecules that are active in reducing heat-

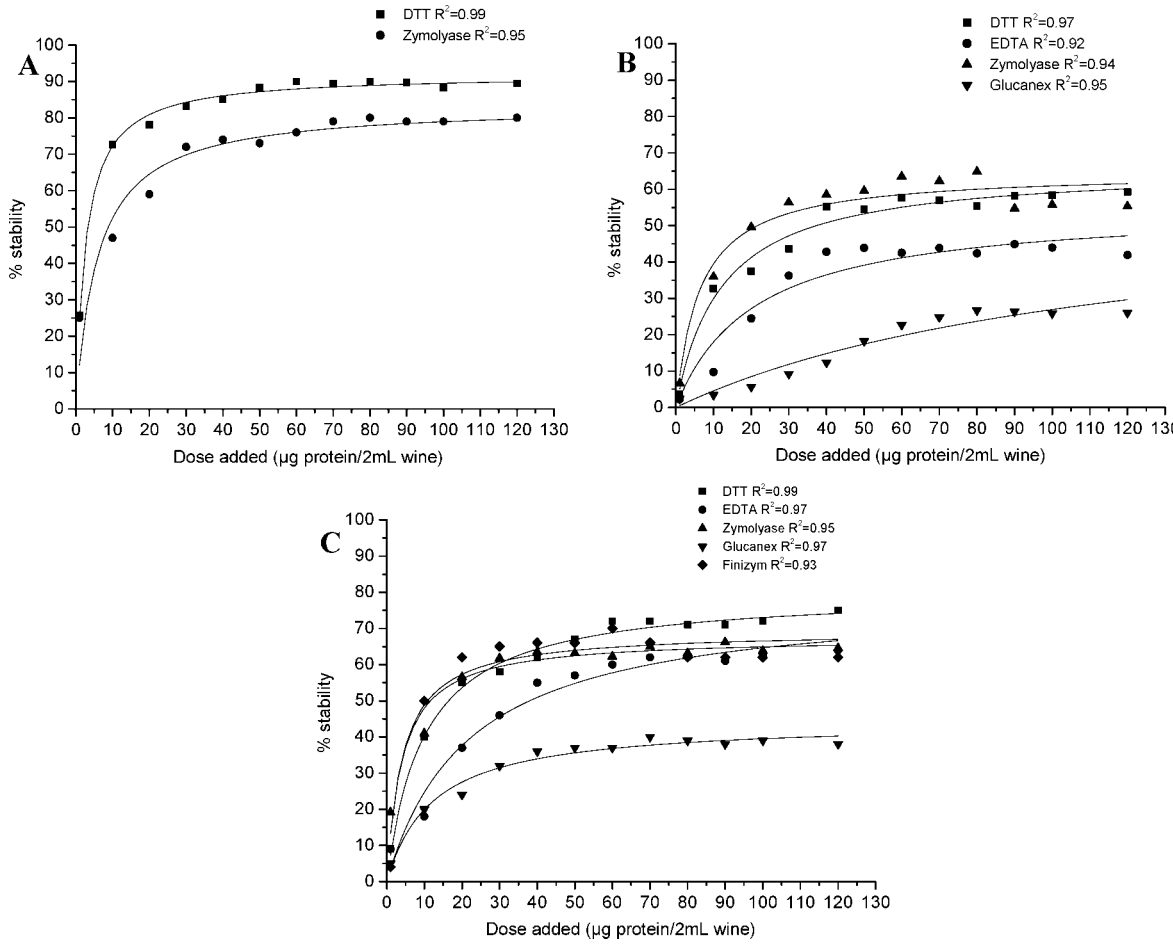

Figure 5. Hyperbolic relationships between haze protecting activity (% stability) and doses added to wine for some yeast preparations. **A**: wine-soluble fractions (WSF); **B**: mannoprotein fractions (MP); **C**: heat-stable mannoprotein fractions (HSMP). Yeast extractions with 30 mM DTT (DTT), 30 mM EDTA (EDTA) Zymolyase/DTT (Zymolyase), Glucanex/DTT (Glucanex), and Finizym 250L/DTT (Finizym). For each curve, R^2 is indicated.

induced protein haze formation in white wines. However, large differences in this action can be found among extracts obtained from yeast cells with different enzymatic and chemical methods, and these differences should be related to a different composition of the extracts. Some extraction methods (i.e., those using 30 mM DTT and Zymolyase) yielded a wine-soluble yeast extract with a very strong stabilizing effect, even higher than that of the corresponding MP fraction. Therefore, these extracts should contain a mixture of haze-protecting factors, some of which are lost during the MP purification step. The identification of the non-MP factors contributing to the stabilizing effect warrants further investigation.

In practical terms, the possibility of stabilizing white wines by using yeast extracts would be rather unrealistic because of the large quantity of fresh yeast cells that is necessary (*8*). However, on the basis of the results reported here, it appears that different procedures can be developed to this aim. For example, a simple reduction step (with DDT, but possibly with other methods) of the yeast cell wall components can yield an extract that when added at about 1 g/hL (starting from about 2 Kg of fresh cells) is able to reduce haze formation in wine to 20% or less of the original value. Once the effectiveness of this approach is established in the actual conditions of wine making, the simplicity of the procedure will warrant an evaluation of its economical convenience.

ABBREVIATIONS USED

BB, binding buffer; BSA, bovine serum albumin; ConA, Concanavalin A; DpW, de-proteinized wine; DTT, dithiothreitol; EDTA, ethylenediamine tetraacetic acid; HSMP, heat-stable mannoprotein; MP, mannoprotein; PAS, periodic acid Schiff; PBS, phosphate buffered saline; UF, unretained fraction; WSF, wine soluble fraction.

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