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Immunodetection of Proteins from Grapes and Yeast in a White Wine

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The objective of this study was to analyze the origin of proteins of a Chardonnay wine. Three various polyclonal antibodies raised against must, yeast, and bacteria proteins were produced. For microorganisms, only the secreted macromolecules were used. To this end, yeast and bacteria were cultured in a model medium under conditions close to those of winemaking. Results obtained using these specific antibodies indicate that most of the wine proteins came from grapes and many of them were glycoproteins. Some proteins of this Chardonnay wine came from the yeast; they were released during the alcoholic fermentation and consisted of high molecular weight mannoproteins. In contrast, no bacteria proteins were detected in this Chardonnay wine.

KEYWORDS: Antibodies; must; yeast; wine; immunoblotting; proteins

INTRODUCTION

Proteins are quantitatively minor constituents of wine, but they are of technological and enological interests because they are responsible for many phenomena. Specifically, they have positive effects such as the stabilization of foam in sparkling wines (*1*−*4*); the reduction of haze formation in white wines, due to the presence of yeast mannoproteins (*5*−*7*); the interaction with aroma compounds (*8*); and the protection of wine against tartaric salt precipitation (*9*−*11*). However, proteins originating from the grape can have negative effects such as the formation of haze in wines, which causes depreciation of the finished product (*12*−*15*).

Numerous investigations, using classical techniques, have been conducted in order to study the wine protein fraction (for a review, see *16*) and especially the origin of these proteins in wine. However, these works were contradictory in their conclusions. Ruiz-Larrea et al. (*17*) compared, by using SDS−PAGE, the patterns of white musts and wines and concluded that soluble proteins present in Viura wines exclusively originated from grapes. Using anion exchange fast performance liquid chromatography (FPLC), Lugera et al. (*18*) observed, in a Chardonnay wine, that the alcoholic fermentation and the stabilization process decrease the protein content of wine. These authors found that proteins released by yeast did not occur during the alcoholic fermentation but only after 18 months of aging.

Similar results were obtained by Ferreira et al. (*19*). By using polyclonal antibodies raised against the total wine protein fraction or individual proteins isolated from a Portuguese Assario white wine, they suggested that wine proteins came entirely from the grape and especially from the pulp of the fruit.

On the other hand, Yokotsuka and Singleton (*20*), using Sephadex G-100 and anion exchange high-performance liquid chromatography (HPLC), showed that some glycoproteins of the red wine came from the yeast and appeared during the initial fermentation process or during the secondary fermentation (i.e., malolactic fermentation). In a Chardonnay wine, Marchal et al. (*21*) isolated, by Con A affinity chromatography, seven glycoproteins. By comparing the must and the corresponding wine protein fractions isolated by Con A and submitted to SDS−PAGE, they showed that some of these glycoproteins probably originate from the grape berry while the others originate from the yeast. Waters et al. (*6*) isolated two mannoproteins from both a white and a red wine fermented with a *Saccharomyces cerevisiae* strain. Dupin et al. (*22*) observed that these mannoproteins were released during fermentation as well as during storage on yeast lees. Finally, Monteiro et al. (*23*) showed, by sequencing of the N-terminal extremity, that some proteins purified from a Moscatel wine have a great homology with some microbial proteins originating from the yeasts or bacteria.

Proteins are subjected to important changes during the winemaking process. Some grape proteins become insoluble and are removed later during wine clarification treatments (*20*, *24*) or hydrolyzed by yeast proteases (*25*) or bacterial proteases (*26*). In addition, proteins could be released to the wine by various microorganisms during and after the fermentative stage of vinification, e.g., yeast (*Saccharomyces bayanus*) (*5*, *22*) and bacteria (*Oenococcus oeni*).

Many of these studies used classical but nonspecific techniques. To have a better knowledge of the nature of wine proteins, we have used an immunotechnique to specifically detect proteins originating from the must, yeast, or bacteria. In this way, soluble proteins were obtained from various organisms (grape juice, yeasts, and bacteria). For microorganism culture,

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we used a model juice system; its composition is close to that of grape juice without grape proteins in order to specifically obtain the compounds released by the yeast or by bacteria during the fermentation process and to carry out this study in conditions close to those of winemaking.

The aim of this work is to determine the origin of the soluble proteins from a Chardonnay wine, using various polyclonal antibodies raised against must, yeast, or bacterial proteins. Because wine proteins are present in very low amounts (*27*, *28*), the high sensitivity and specificity of the antibody−antigen binding proved to be a good means to determine the origin of wine proteins.

MATERIALS AND METHODS

Must. Grape berries of the Chardonnay variety were collected from the Champagne area (France). Grapes were hand-harvested in mid-October 1998 at common commercial maturity [sugar (g/L)/acid (g/L tartaric acid) = 12]. Grapes were collected and pressed with a laboratory pneumatic press (pressure between 1.5 and 2 bar). SO_2 (150 mg/L) was added to the free-run juice. After static settling (24 h at 12 °C), the must (10 L) was centrifuged (10 min at 8000*g*). Supernatants were separated, filtered through a 0.45 μm membrane, and stored at 4 °C.

Wine. The settled Chardonnay must was racked and chaptalized with sucrose (35 g/L). The alcoholic fermentation was done by *Saccharomyces bayanus* at 18 °C. After malolactic fermentation, the wine was filtered through diatomaceous earth and then through a 0.45 μm membrane (HA Millipore, Saint-Quentin Yvelines, France). The wine protein concentration was 7.1 mg/L, as determined by the direct Bradford method (*29*) using a bovine serum albumin (fraction V powder, Sigma-Aldrich, Saint-Quentin Fallavier, France) standard curve. The blank contained the same alcohol concentration (11% v/v) as the studied wine.

Yeast Growth. The strain of yeast used in this study was *S. bayanus*. Yeasts were rehydrated following the manufacturer's instructions. A 3.2 g amount of active dry yeasts was rehydrated in 500 mL of a model juice buffer diluted with 500 mL of distilled water at 32 °C during 15 min. After rehydration, the yeast suspension was subsequently added to the buffered model juice (160 L). Composition of the juice buffer per liter was as follows: KH_2PO_4, 935 mg; $NH_4H_2PO_4$, 561 mg; $(NH_4)_2SO_4$, 187 mg; $MgSO_4$, 467 mg; NaCl, 94 mg; $CaCl_2$, 94 mg; biotin, 187 μg; inositol, 1.87 mg; pyridoxal, 1.87 mg; pantothenate Ca, 1.87 mg; thiamine chlorhydrate, 1.87 mg; nicotinic acid, 0.468 mg; H_3BO_3, 0.47 mg; KI, 0.094 mg; $FeCl_3$, 0.752 mg; Zn SO_4, 0.188 mg; $CuSO_4$, 0.0376 mg; $MnSO_4$, 0.376 mg; $(NH_4)_6Mo_7O_{24}$, 0.188 mg; D-glucose, 187.5 g; citric acid, 0.5 g; tartaric acid, 3 g; malic acid, 6 g; Tween 80, 13.5 mg; and ergosterol, 0.3 mg, in distilled water.

Bacterial Growth. The *O. oeni* strain used in this study was *O. oeni* BL 01 (Station Oenotechnique de Champagne Martin Vialatte, Epernay, France). The synthetic medium consisted of the following components in deionized water (L^{-1}): D-glucose, 8 g; L-malic acid, 5 g; sodium acetate, 1 g; Tween 80, 1 mg; K_2HPO_4, 1 g; KH_2PO_4, 1 g; $FeSO_4$, 10 mg; $MnSO_4$, 10 mg; $MgSO_4$, 100 mg; adenine, 5 mg; cytosine, 5 mg; guanine, 5 mg; thymidine, 5 mg; ascorbic acid, 200 mg; pyridoxal, 15 mg; pantothenic acid, 7.5 mg; nicotinic acid, 0.75 mg; folic acid, 0.75 mg; and all amino acids at 0.3 mmol. The pH of the medium was adjusted to 4.5 with NaOH. The medium (2 L) was then inoculated at a concentration of 10^7 cells mL^{-1} and incubated at 28 °C.

Macromolecule Isolation. Must, wine, yeast, and bacterial cultures were centrifuged (10 min at 8000*g* for must, wine, and yeast culture and 30 min at 10000*g* for bacterial culture) and filtered through a 0.45 μm membrane (HA Millipore). The different liquids were concentrated (10 times) and then four times dialyzed against distilled water. A hydrophilic polysulfone membrane of 10 000 MW cutoff was used (Minisette Omega, screen channel membrane, PALL-FILTRON, France). The cross-flow filtration module (1.2 m^2) was connected to the Hi-Flow system (pumping system plus glass tank). Ultrafiltration was carried out at 4 °C under a stream of nitrogen to avoid oxidation. The ultrafiltrate flow was 40 mL/min. For the concentration step, the must and the wine were laced with distilled water (v/v) to reduce viscosity and to avoid complexation between proteins and polyphenolic compounds. The dialyzed retentates were freeze-dried (Serail CS 5L) and conserved at −20 °C. Lyophilysates of must, yeast, and bacteria macromolecules were used as antigens.

Analytical SDS−PAGE and Western Blotting. Discontinuous SDS−PAGE was performed according to the method of Laemmli (*30*) using slab gels (0.75 mm thick). The stacking gels consisted of T = 5% and C = 2.7%, and the separating gels consisted of T =12% and C = 2.7% (where T is the total percentage concentration of acrylamide and *N*,*N*′-methylenebisacrylamide in the gel (g/100 mL gel) and C is the concentration of *N*,*N*′-methylenebisacrylamide as a weight percentage of acrylamide and *N*,*N*′-methylenebisacrylamide). A vertical electrophoresis apparatus (Mini-Protean, Bio-Rad) was used to run the gel at a constant voltage setting of 150 V until the bromophenol blue tracker dye reached the bottom of the gel (usually 65 min at room temperature). Standard proteins from 14 to 94 kDa were used as MW markers (LMW Pharmacia). These proteins were treated as the protein samples (Laemmli buffer v/v), and 24 μL was loaded in the wells for each analysis. The MWs of unknown molecules were calculated from the linear regression equation of log MW vs mobility. After electrophoresis, the separated proteins were either transferred at 4 °C to a nitrocellulose membrane using a Bio-Rad electroblotting apparatus (*31*) or stained with 1.5% CBB in 50% (v/v) methanol and destained in acetic acid/methanol/water (1:2:7) or stained by the PAS (*32*) to characterize the presence of sugars. Composition of the transfer buffer was 25 mM Tris, 190 mM glycine, and 20% (v/v) methanol. During Western blotting, a constant current of 100 V was applied for 1 h. Bio-Rad low MW prestained markers (19.4−104 kDa) were employed for Western blotting studies and PAS staining.

Production of Polyclonal Antisera. Preimmune sera were obtained from three rabbits (New Zealand white females) before immunization. Soluble antigens (must proteins, yeast, or bacteria-secreted proteins) were incorporated in 0.3 mL of 3% polyacrylamide and mixed with 0.9 mL of Freund's complete adjuvant (Sigma-Aldrich) (*33*) to give a final protein concentration of approximately 1 mg/mL. The three rabbits were intradermally immunized on the back at six sites, one rabbit with must proteins, one rabbit with yeast-secreted proteins, and another one with bacteria-secreted proteins (6 × 0.15 mL of the immunogen). Subsequent injections with the same antigen preparation were made at weeks 3 and 6 in Freund's incomplete adjuvant. Each rabbit was bled at week 8. Blood samples were left for 1 h at 30 °C before being centrifuged (10 min at 6000*g*). The antisera supernatants containing polyclonal antibodies were taken and stored at −20 °C.

Cross-Reactivity and Western Blotting Staining. The specificity of the antibodies raised against proteins secreted by yeasts, bacteria, and must proteins was tested using a noncompetitive dot-blot technique. Must, wine, yeast, and bacteria proteins and nonimmune sera were spotted (5 μL) in triplicate onto nitrocellulose membranes (Sartorius, Göttingen, Germany) and air-dried at room temperature. Nonspecific binding was blocked with TBS (Tris buffer saline: 25 mM Tris, 0.5 M NaCl, adjusted to pH 5 with HCl) plus 2% nonfat dry milk. The membranes were rinsed three times in TBS and incubated for 3 h with the appropriate antibodies (antisera) diluted 1/1500 in TBS containing 1% (w/v) nonfat dry milk. The membranes were successively washed with TBS, TBS + 0.05% Tween 20, and TBS, before being incubated with goat antirabbit IgG (1/1500 dilution in TBS plus 1% nonfat dry milk) during 3 h at room temperature and washed again with TBS, TBS + 0.05% Tween 20, and TBS, successively. Goat antirabbit horseradish peroxidase-conjugated polyclonal antibodies were obtained from Sigma-Aldrich. Membranes were further washed in phosphate buffer saline (PBS); peroxidase activity was stained using 4-chloro-1-naphthol (1.2 g/L) in ice-cold methanol and 0.4% H_2O_2 in PBS. The ability of antisera to bind to each macromolecules spot was assessed by visually comparing the color of each spot stained with the antiserum test with that of the nonimmune serum. Western blot membranes were stained as dot-blots.

Affinity Chromatography with Con A. The Con A-Sepharose (Sigma-Aldrich) column (10 mm × 150 mm) was equilibrated in 100 mM sodium acetate buffer, pH 5, containing 0.3 M NaCl, $CaCl_2$, $MnCl_2$, $MgCl_2$ (1 mM of each), and 0.02% NaN_3. Elution was carried out with

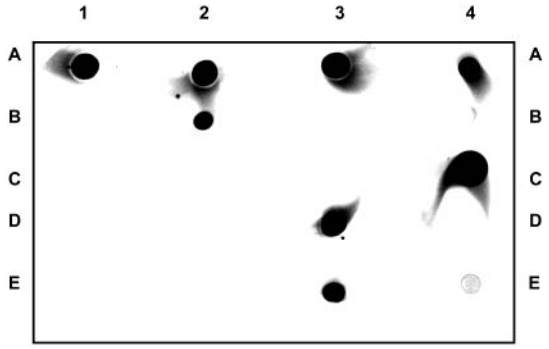

Figure 1. Dot-Blot on nitrocellulose strips with color developed using 4-chloronaphthol/peroxidase-labeled goat IgG in PBS buffer: strip 1, rabbit preimmune serum; strip 2, rabbit antibacteria-secreted proteins polyclonal antibodies; strip 3, rabbit antimust proteins polyclonal antibodies; strip 4, rabbit antiyeast-secreted proteins polyclonal antibodies; spot A, preimmune serum; spot B, bacteria-secreted proteins; spot C, yeast-secreted proteins; spot D, must proteins; spot E, wine proteins.

the above buffer containing 0.1 M methyl α-D-mannopyranoside (Sigma-Aldrich). The flow rate was 0.16 mL/min. Detection of proteins was monitored continuously at 280 nm using a UV detector (Shimadzu SPD 2A, Japan).

RESULTS AND DISCUSSION

Specificity of Antigen−Antibody Recognition. A control experiment using preimmune antisera yielded no positive cross-reactions with any of the protein fractions (must, yeast, and bacteria) (**Figure 1**, strip 1).

Figure 1 shows that all of the antibodies obtained were able to specifically recognize their antigens. The antimust antibodies specifically recognized must proteins (strip 3, spot D); they also recognized some wine proteins (strip 3, spot E). This first observation confirmed the presence of grape proteins in wine.

Yeast or bacterial proteins were not recognized (strip 3, spots B and C). Antibodies raised against proteins secreted by bacteria (strip 2, spot B) recognized their antigens but not other antigens such as must proteins or yeast-secreted proteins (strip 2, spots C and D). The response is also negative with the wine protein fraction (strip 2, spot E). Then, proteins secreted by bacteria were probably absent or in too low amounts in wine to be detected.

Antiyeast-secreted proteins antibodies were able to recognize their antigens (strip 4, spot C); there were no positive responses with must proteins or bacteria-secreted proteins (strip 4, spots B and D, respectively), but a slight response was observed with wine proteins (strip 4, spot E). These discrepancies can be explained by the different protein concentrations between each spot, e.g., yeast-secreted proteins and wine protein spots. It thus appears that some yeast proteins are secreted into the wine.

Comparison between the Must and the Wine Protein Fraction Using SDS−PAGE and CBB or PAS Staining. **Figure 2**, lane 2, shows the protein pattern of the must after staining with CBB. The must protein fraction contains six major proteins with MW ranging from 14 000 to 64 000. The most intense bands have MWs ranging from 25 000 to 30 000. Numerous minor proteins appeared with MW between 14 000 and 20 000. Four proteins with MW of, respectively, 14 000, 20 000, 32 000, and 60 000/64 000 were stained.

The electrophoregram of the wine (**Figure 2**, lane 3) originating from the same must was almost similar; meanwhile, some bands disappeared (i.e., bands at 20 000 and 30 000) or their intensity diminished (i.e., the band at 25 000). A decrease

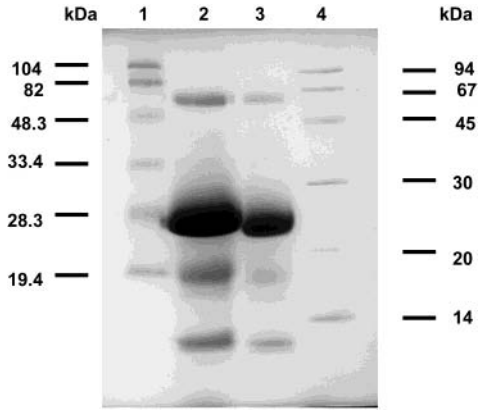

Figure 2. SDS–PAGE analysis of a Chardonnay must and the corresponding wine total proteins isolated by a 10 kDa ultrafiltration and stained with CBB: lane 1, Bio-Rad low MW prestained markers; lane 2, must total proteins; lane 3, wine total proteins; lane 4, MW markers. Relative MWs ($\times 10^{-3}$) of protein standards are given at the left and the right side of the gel.

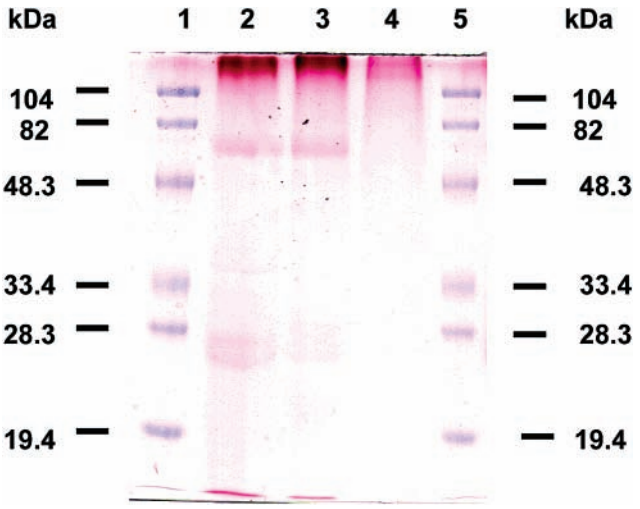

Figure 3. SDS–PAGE analysis of a Chardonnay must, the corresponding wine, and total proteins secreted by the yeast, isolated by a 10 kDa ultrafiltration and stained with the PAS staining: lanes 1 and 5, MW prestained markers; lane 2, must total proteins; lane 3, wine total proteins; lane 4, total proteins secreted by yeast in a model juice. Relative MWs ($\times 10^{-3}$) of protein standards are given at the left and the right side of the gel.

in the relative concentration of proteins during the wine making process is thus observed, confirming previous data (*21*, *34*, *35*).

By comparing SDS−PAGE patterns of the must and the wine, no changes were observed in the electrophoretic mobilities of proteins. Using this technique, no major proteins released in the wine during the alcoholic fermentation and the malolactic fermentation were detected.

These results are in good agreement with the fact that yeast mannoproteins, which are expected to be secreted to the wine by yeast, are not stained with CBB or the more sensitive silver stain procedure (*5*, *36*), but mannoproteins can be stained by the PAS staining.

The PAS staining revealed the presence of numerous proteins associated with sugars (**Figure 3**). In the must, four molecules giving a fuchsia coloration were observed as follows: two large colored areas, one at 20 000 and another between 25 000 and 30 000; one band at 60 000; and finally, an intensively colored area in the upper part of the separating gel (**Figure 3**, lane 2),

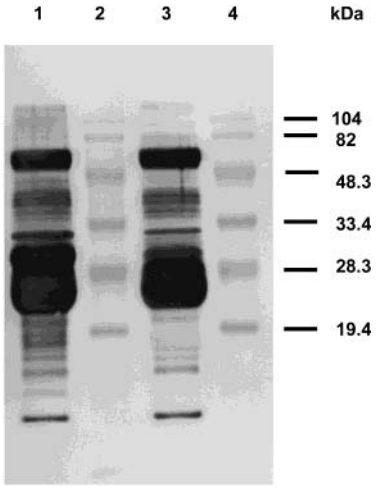

Figure 4. Detection of must proteins in a Chardonnay must and the corresponding wine by SDS–PAGE and immunoblotting using rabbit antimust polyclonal antibodies as described under Materials and Methods: lane 1, must total proteins; lanes 2 and 4, MW prestained markers; lane 3, wine total proteins. Relative MWs ($\times 10^{-3}$) of protein standards are given at the right side of the gel.

corresponding to macromolecules with high MW. We highly supposed this area is linked to the presence of arabinogalactan proteins (*37*).

In the wine, a similar profile was observed (**Figure 3**, lane 3), with an increase in the intensity of the deep spot linked to the arabinogalactan proteins originating from the must (*7*, *38*) and the presence of yeast mannoproteins, which are released during the alcoholic fermentation (*5*, *21*, *22*).

Immunospecific Recognition of Must Proteins in Wine. When the SDS−PAGE gels of must and wine proteins are blotted onto a nitrocellulose membrane and probed with must protein polyclonal antibodies (**Figure 4**), globally the same pattern was observed as compared with the CBB staining gel (see **Figure 2**), with some differences; i.e., better resolution and an increase in the number of the bands, due to the high specificity and sensitivity of this technique. Antibodies recognized the six major proteins of the must (**Figure 4**, lane 1) with a MW ranging from 14 000 to 60 000/64 000 and also minor proteins of various MW. In addition, proteins with a MW higher than 70 000 became visible with this technique. For this wine (**Figure 4**, lane 3), the protein pattern resembled that of the must and once again a decrease in the intensity of some proteins was observed although no band disappeared. This result showed that most of the wine proteins with MW lower than 70 000 originated from the grape berry.

Wine Proteins Isolated by Con A. To obtain further knowledge of the wine protein fraction, and especially the wine proteins originating from the must, which constitutes heat unstable proteins responsible for haze (*14*), wine proteins were submitted to an affinity chromatography with Con A. The different fractions retained by Con A were separated by SDS−PAGE, blotted onto a nitrocellulose membrane, and probed with must protein antibodies (**Figure 5**).

The protein fraction isolated by Con A chromatography showed compounds of MWs ranging from 25 000 to more than 70 000, with four major proteins with MW of 24/25 000, 30 000, 32 000, and 60/64 000, respectively (**Figure 5**, lane 5). Numerous minor proteins appeared with MW between 32 000 and 44 000. Finally, proteins with MW higher than 70 000 were weakly stained.

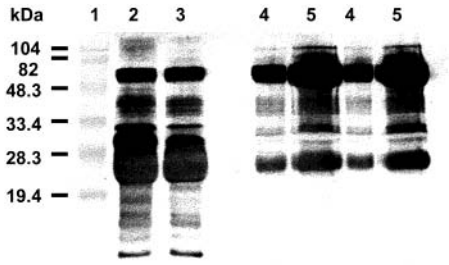

Figure 5. SDS–PAGE analysis of the wine protein fraction isolated by Con A chromatography and immunoblotting using rabbit antimust polyclonal antibodies as described under Materials and Methods: lane 1, MW prestained markers; lane 2, must total proteins; lane 3, wine total proteins; lanes 4 and 5, wine proteins isolated by Con A (quantities of deposits are single and double, respectively). Relative MWs ($\times 10^{-3}$) of protein standards are given at the left side of the gel.

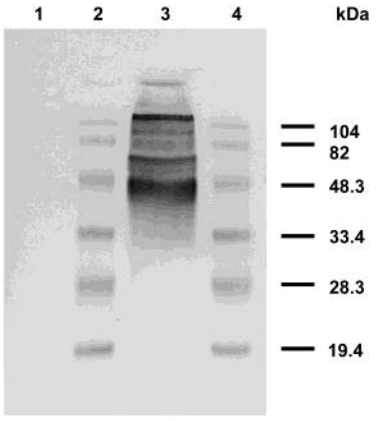

Figure 6. Detection of bacterial proteins in a Chardonnay wine by SDS–PAGE and immunoblotting using rabbit antibacteria polyclonal antibodies: lane 1, wine total proteins; lanes 2 and 4, MW prestained markers; lane 3, bacteria-secreted proteins. Relative MWs ($\times 10^{-3}$) of protein standards are given at the right side of the gel.

These observations suggest that wine proteins originating from grapes are composed of a huge number of compounds, covering a wide range of MW between 14 000 and more than 60 000. Furthermore, proteins of MW ranging from 25 000 to 60 000 are shown to be essentially glycoproteins. Similar results were reported by others (*17*, *21*). However, unlike previous studies, we showed, using a specific immunomethod, the origin of these wine glycoproteins.

Immunodetection of Bacteria Proteins in Wine. To search whether bacteria release some proteins in wine, one aliquot of wine proteins was submitted to a SDS−PAGE, blotted on nitrocellulose, and probed with antibacteria-secreted proteins antibodies (**Figure 6**). The resulting immunoblot showed no positive reaction with the total protein pattern of wine (**Figure 6**, lane 1). It thus seems that there was no release of bacterial proteins in wine. It is also possible that the sensitivity of this technique is insufficient to reveal trace amounts of bacterial proteins.

Immunodetection of Yeast Proteins in Wine. Proteins secreted by yeast in a model juice were analyzed by SDS−PAGE and were visualized with both the protein staining and the PAS carbohydrate staining. These macromolecules gave a very poor and smeary response to protein staining (CBB or silver stain) (data not shown). In contrast, they responded increasingly to the PAS staining (**Figure 3**, lane 3), showing a large and smeary band at the upper part of the gel, indicating that these bands contained a large amount of polysaccharide material.

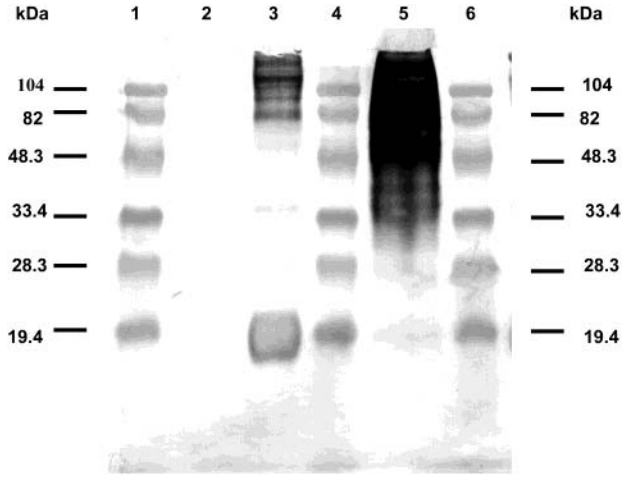

Figure 7. Detection of yeast proteins in a Chardonnay wine by SDS–PAGE and immunoblotting using rabbit antiyeast polyclonal antibodies: lanes 1, 4, and 6, MW prestained markers; lane 2, must total proteins; lane 3, wine total proteins; lane 5, yeast-secreted proteins. Relative MWs ($\times 10^{-3}$) of protein standards are given at the left and the right side of the gel.

These results are consistent with the fact that macromolecules secreted in the medium correspond to the high MW mannoprotein fraction (*22*, *39*).

These macromolecules secreted by yeast were separated by SDS−PAGE and blotted. Antiyeast-secreted protein antibodies recognized a large and smeary band at the upper part of the membrane (**Figure 7**, lane 5), similar to the smearing of the PAS staining (**Figure 3**, lane 3).

The western blotting of wine was probed with antiyeast-secreted protein antibodies. Wine proteins yielded a positive reaction in three distinct regions (**Figure 7**, lane 3) with yeast-secreted protein polyclonal antibodies. The staining pattern obtained with these antibodies showed three bands, a wide band around 20 000, a minor band at 38 000, and a third area of wide MWs between 80 000 and more than 100 000. No response was obtained with must macromolecules (**Figure 7**, lane 2).

Conclusion. Our results seem to be somewhat in contradiction with those obtained by Ferreira et al. (*19*), who used a similar immunological method. They concluded that wine proteins exclusively originate from the grape fruit flesh. These discrepancies can be explained by the different experimental conditions used in these two studies. In their work, Ferreira et al. (*19*) produced antibodies raised only against wine proteins. They further used these antibodies to detect the origin of the wine proteins in extracts of grape and yeast (total yeast soluble proteins). These conditions were very different from the actual conditions of winemaking (fermentation or crushing).

In contrast, in our study, antibodies against must, yeast-secreted, and bacteria-secreted proteins were prepared and probed against wine proteins. Our results confirm that proteins of a wine mainly originate from the must, with most of them being glycoproteins. However, unlike data obtained by Ferreira et al. (*19*), our study demonstrates that yeast secreted some macromolecules into the wine and thus confirm numerous studies in which immunological methods were not used. These yeast macromolecules mainly consist of high MW mannoproteins. In contrast, no bacterial proteins are detected in the wine.

ABBREVIATIONS USED

MW, molecular weight; Con A, concanavalin A; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; CBB, Coomassie brilliant blue; PAS, periodic acid-Schiff method.

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