Detection and Quantification of Lysozyme in Champagne Wines

R. Marchal,*,† D. Chaboche,† L. Marchal-Delahaut,† C. Gerland,‡ J. P. Gandon,§ and P. Jeandet†

Laboratoire d'Oenologie, URVVC, UPRES EA 2069, Faculté des Sciences, Université de Reims, BP 1039, 51687 Reims Cédex 2, France, Station Oenotechnique de Champagne-Martin Vialatte, 79 av. Thévenet, BP 1031, 51319 Epernay Cédex, France, and Champagne Lanson, 12 Boulevard Lundy, BP 163, 51056 Reims Cédex, France

We describe here techniques to detect and quantify lysozyme in Pinot noir and Chardonnay Champagne wines. Using a dot-blot technique, lysozyme antibodies were able to recognize their antigens even when the concentration of lysozyme in wine was 75 mg/L. SDS−PAGE was the second technique used. After Coomassie Brilliant Blue (CBB) staining or antibody immunostaining was performed, the wine originating from the lysozyme-treated must gave only one band corresponding to the lysozyme. It is then possible to precisely determine the concentration of lysozyme in a must or a wine by densitometric measurement of this band. The control wine gave no band with the CBB staining, such as with the immunostaining. The quantification of lysozyme with HPLC is another useable technique because the lysozyme elution time is largely superior to that of all of the wine compounds. In wines, losses of lysozyme were higher when the enzyme was added at one time to the must (−34% for the Pinot noir and −37% for the Chardonnay) than when lysozyme is added in 2-fold both in the must and in the wine (around −26% for the two wines). The lowest diminution is observed when lysozyme was added to the wine only (−18%) in comparison to the addition to the must at 300 mg/L (−43%).

Keywords: *Lysozyme; wine; Champagne; immunodetection; SDS−PAGE; quantification; must; HPLC*

INTRODUCTION

Lysozyme (EC 3.2.1.17) has long been used as a natural antimicrobial agent in food preservation for its muramidase action (Proctor and Cunningham, 1988; Ibrahim et al., 1996b). In the field of enology, studies addressing the use of lysozyme to prevent malolactic fermentation have shown that *Oenococcus oeni* species are sensitive to the action of lysozyme (Amati et al., 1994; Green et al., 1994, 1995; Gerbaux et al., 1997). This enzyme has also been investigated to reduce the lactic bacteria flora in musts and wines after completion of malolactic fermentation. Quantities added to musts and wines ranged from 250 to 1000 mg/L (Pittoti et al., 1991; Amati et al., 1994; Gerbaux et al., 1997). These studies have focused mainly on the enzymatic stability of lysozyme in wines at various concentrations, in the presence or absence of sulfur dioxide (Boschelle and Pittoti, 1988) and after addition at different stages of the winemaking process (i.e., before or after alcoholic and malolactic fermentations). Nevertheless, lysozyme was never directly quantified in these studies. Moreover, no relation was found between the lysozyme enzymatic activity and the concentration of soluble lysozyme in a medium (Ibrahim et al., 1996a).

The first investigation of this paper was designed to precisely quantify residual lysozyme in wines by using two different techniques. Indeed, lysozyme concentration in wines can be lowered through insolubilization resulting from protein−polyphenol interactions. Crude tannins caused significant loss of activity (Green and Daeschel, 1994), lysozyme probably being partially out of solution. It can also be removed by using common fining agents (gelatine, caseinate) and active carbon or bentonite treatments (Amati et al., 1996). Also, wine components such as organic acids and ethanol depressed lysozyme activity (Green and Daeschel, 1994), but we do not know if this protein was partially eliminated or was only affected by conformational modifications (lowering the activity of the enzyme that remained soluble).

The second investigation focused on detecting the presence of lysozyme in wines of the Champagne area by specific or nonspecific techniques. In France at present, lysozyme is only authorized for experimental treatments. In this way, the high specificity of antibody−antigen binding was assayed to visualize lysozyme originating from enological treatments.

MATERIALS AND METHODS

Production of Musts and Wines. Grape berries of the Pinot noir and the Chardonnay varieties were collected from the Champagne area (France). Grapes were hand-harvested in September 1998 and pressed with a Cocquart 4000-kg press (pressure between 1.5 and 2 bar). Conditions under which musts were obtained and wines produced in Lanson winery (Reims, France) are described in Figures 1 and 2 for the Chardonnay and the Pinot noir, respectively. Treatments done in duplicate are noted on these figures. Lysozyme was added after static settling (24 h at 15 °C) and/or after alcoholic fermentation. Lysozyme concentrations decreased by 20% when lysozyme was added directly in the musts and by 12% when lysozyme was added both in musts and wines, because of chaptalization and topping up.

* To whom correspondence should be addressed [fax: 33 (0)-326 91.33.40; e-mail: richard.marchal@univ-reims.fr].
† Université de Reims.
‡ Station Oenotechnique de Champagne-Martin Vialatte.
§ Champagne Lanson.

10.1021/jf990848a CCC: $19.00
Published on Web 07/01/2000

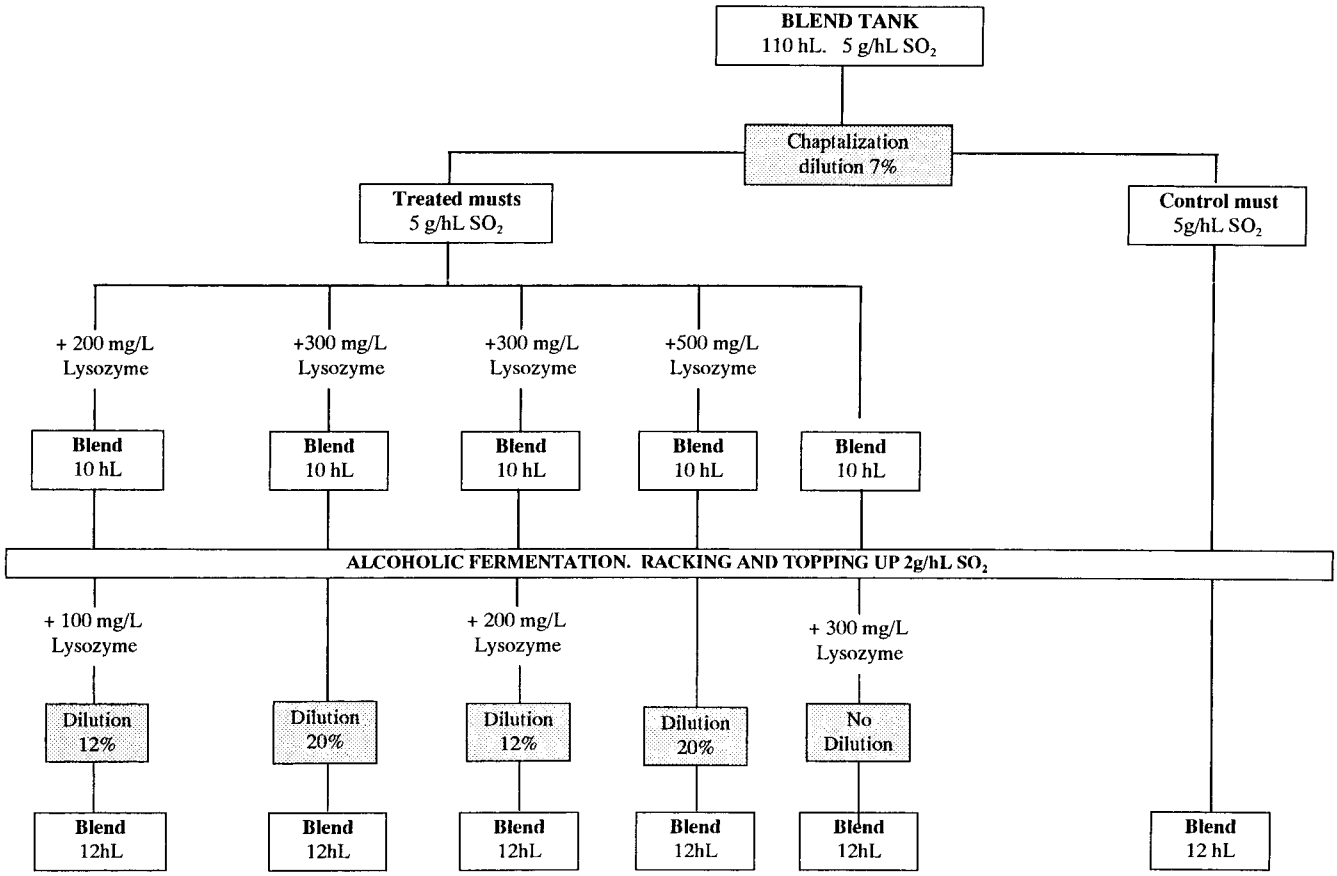

Figure 1. Description of the Chardonnay wine processing and the lysozyme treatment.

Must and Wine Standard Chemical Analyses. Enzymatic kits of Boehringer Mannheim (France) were used to measure concentrations of lactic acid. The pH was determined using an Orion 420A pH meter. Tartaric acid was measured according to a modified Rebelein method, using ammonium monovanadate (Blouin, 1977) and a SAFAS spectrophotometer (France). Total acidity was determined by M/64 NaOH additions using blue bromothymol as a colorimetric indicator (end point titration is approximately pH 7.0); results are expressed in g/L of tartaric acid. l-Malic acid was quantified using an automatic enzymatic method (Kone Progress apparatus).

Lysozyme. Enological lysozyme was furnished by Fordras (Lugano, Switzerland). Its purity was compared by SDS−PAGE (T = 13.5%; C = 3%) with a highly purified lysozyme purchased from Boehringer Mannheim (Germany). Protein solutions were 1 g/L, treated with Laemmli buffer (v/v) (62.5 mM Tris-HCl, pH 6.8; 10% glycerol; 2% SDS; 5% β-mercaptoethanol; and 0.005% bromophenol blue); a total of 4 μL were loaded in each well.

Chromatography Quantification. Wine lysozyme concentrations were determined 4 months after the addition. A Waters (Milford, MA) HPLC system comprising a Waters 600 pump and a model W 490E UV detector, interfaced with a Millennium ^{32} chromatography manager (Waters), was used. Lysozyme was analyzed by reversed phase chromatography on a TSK-gel 5PW-phenyl column (4.6 × 75 mm, Tosohaas). A linear gradient elution from 100% A and 0% B to 0% A and 100% B within 44 min was used. A second linear gradient elution from 100% B and 0% A to 100% A and 0% B between 44 and 52 min was used. This was followed by a 10-min equilibrium period with 100% A at room temperature. Solvents used were as follows: A, 1% CH_3CN + 0.2% TFA + 98.8% H_2O MilliQ; B, 70% CH_3CN + 0.2% TFA + 29.8% H_2O MilliQ. The flow rate was 0.8 mL/min. The absorbance was measured at 225 nm. All standards and wines were injected (20 μL) in triplicate. Concentrations of residual lysozyme in wines are calculated with regard to the standard curve ($y = 23239x$ and $R^2 = 0.9989$). Standard deviations are indicated on the graph for each calibration point ($P = 0.95$; $n = 3$).

Analytical SDS−PAGE and Western Blotting. Discontinuous SDS−PAGE was performed according to the method of Laemmli (1970) using slab gels (0.75 mm thick). The stacking gels consist of T = 5% and C = 2.7%, and the separating gels consist of T = 12% and C = 2.7%. A vertical electrophoresis apparatus (Mini-Protean, Bio-Rad, Richmond, CA) 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 molecular weight markers (LMW Pharmacia). Wines and standard proteins were treated with the Laemmli buffer (v/v); a total of 10 μL was loaded in the wells for each analysis. The molecular weight (MW) of bands 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 (Sartorius, Göttingen, Germany) using a Bio-Rad electroblotting apparatus or stained with 1.5% Coomassie Brilliant Blue (CBB) in 50% (v/v) methanol and destained in acetic acid/methanol/water (1:2:7). The 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 molecular weight prestained markers (21−112 kDa) were employed during western blotting studies. After SDS−PAGE, lysozyme was quantified by densitometric measurements with a laser densitometer (LKB2202 Ultroscan, Sweden) connected to an Intersmat ICR-1B integrator (Shimadzu, Japan).

Production of Polyclonal Antisera. Preimmune sera were obtained from a New Zealand white female rabbit before immunization. Soluble lysozyme (antigen) was incorporated in 0.3 mL of 3% polyacrylamide and mixed with 0.8 mL of Freund's complete adjuvant (Sigma-Aldrich, France) (Freund, 1956) to give a final protein concentration of approximately 1 mg/mL. The rabbit was intradermally immunizated on the

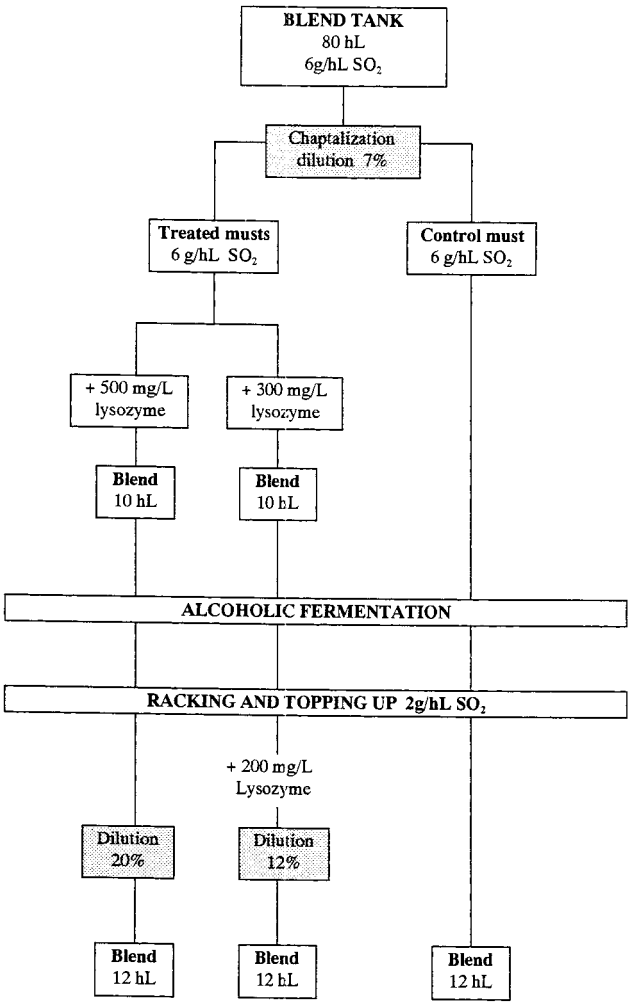

Figure 2. Description of the Pinot noir wine processing and the lysozyme treatment.

back at six sites (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. The rabbit was bled at week 8. Blood was left 1 h at 30 °C before being centrifugated (10 min at 6000*g*). The antisera containing polyclonal antibodies were stored at −20 °C.

Cross-Reactivity and Western Blotting Staining. The specificity of the antibodies raised against the enological lysozyme was tested using a noncompetitive dot-blot technique. Wines and nonimmune sera were spotted (3 μL) in duplicate onto nitrocellulose membranes (Sartorius, Göttingen, Germany) and air-dried at room temperature. Nonspecific binding was blocked with Tris buffer saline (TBS: 25mM Tris and 0.5M NaCl, adjusted to pH = 7.5 with HCl) plus 2% nonfat dry milk. The membranes were rinsed 3 times in TBS and incubated 3 h in the presence of lysozyme antibodies. The antisera dilutions were 1/1500 for 4-chloro-1-naphthol staining and 1/15000 for fluorescent staining. The membranes were successively washed with TBS, TBS + 0.05% Tween 20, and TBS before being incubated with goat anti-rabbit 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 anti-rabbit horseradish peroxidase-conjugated polyclonal antibodies were obtained from Sigma-Aldrich (France). After further washing in PBS, peroxidase activity was stained using two methods differing by their sensitivity. Western blotted membranes were incubated with 4-chloro-1-naphthol (1.2 g/L) in ice-cold methanol + 0.4% H_2O_2 in phosphate buffer saline (PBS: 8 g/L NaCl + 1.15 g/L Na_2HPO_4 + 0.2 g/L KCl + 0.2 g/L K_2HPO_4 , pH not adjusted) and gave a blue-mauve coloration when the antigen was recognized. For dot-blots, lysozyme was detected using a chemiluminescent substrate (Supersignal Western Blotting, Pierce): the nitrocellulose membrane was incubated with equal parts luminol/enhancer solution and peroxide solution for 8 min with a sufficient volume to ensure that the blot was completely wetted with the substrate. The membrane was then placed in a plastic sheet protector and exposed against a standard autoradiographic film (Biomax ML, Kodak) for 30 s. Finally, the film was developed and fixed using GBX solutions (Kodak, France). The ability of the antisera to bind to a wine spot (after dot-blots) was assessed by visually comparing the color of each spot stained with the antiserum test with that of the nonimmune serum.

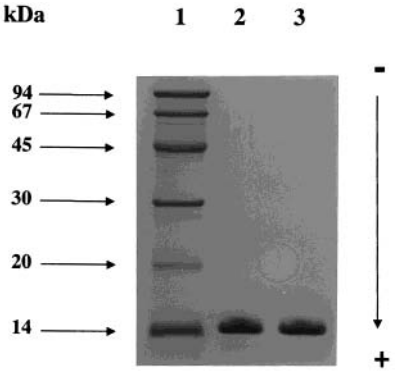

Figure 3. SDS−PAGE analysis of lysozyme stained with Coomassie Brilliant Blue: lane 1, MW markers; lane 2, enological lysozyme; and lane 3, highly purified lysozyme (Boehringer fine chemical). Relative molecular weights ($\times 10^{-3}$) of protein standards are given at the left side of the gel.

RESULTS AND DISCUSSION

Enological Lysozyme. The protein composition of enological lysozyme is compared with that of a pure standard of lysozyme (Figure 3). The two electrophoretic profiles obtained present similar patterns, with only one band at 14.5 kDa. This very high electrophoretic purity (absence of proteic contaminants) authorizes the use of commercial antibodies for lysozyme immunodetection.

Current Analysis. Enological current analysis are noted in Table 1. The Pinot noir and the Chardonnay musts have similar sugar concentrations. The Pinot noir must came from the blend of second pressings and presented a total acidity of 10.4 g/L expressed in tartaric acid. The Chardonnay must corresponded to a blend of top growths. Its total acidity (14.2 g/L) was higher than the Pinot noir must, because of higher concentrations of malic and tartaric acids principally. The two musts present a pH difference of 0.28 unit. All these differences were still observed for the corresponding wines. However, the Chardonnay and the Pinot noir wines originating from lysozyme-treated musts showed higher concentrations of tartaric acid. We have also observed that tartaric stabilization was more difficult than for control wines. Time necessary to stabilize lysozyme-treated wines were longer than for the corresponding control wines. Lysozyme seems to play a protective action in relation to tartaric stabilization, even if we make an enrichment with cream of tartar (Gandon, personal communication).

Specificity of Antigen−Antibody Recognition in Wines. By using the dot-blot technique, lysozyme antibodies obtained are able to recognize their antigens when present in a wine originating from a must treated with 500 mg/L of lysozyme (Figure 4). This quantity is currently added to the musts to prevent malolactic fermentation. The concentration of residual lysozyme

Table 1. Current Analysis of the Pinot Noir and the Chardonnay Musts and Wines

process stage	musts		wines			
grape variety	Pinot noir	Chardonnay	Pinot noir		Chardonnay	
lysozyme			nontreated	treatment	nontreated	treatment
sugar (g/L)	159.3	155				
maturity index (sugar/total acidity)	15.3	10.9				
total acidity (g/L tartaric acid)	10.4	14.2	9.7	9.7	13.4	13.4
pH	3.24	2.96	3.33	3.33	2.97	2.97
alcohol (% v/v)			11.1	10.8	11.1	10.8
malic acid (g/L)	6.4	7.2	5.0	4.6	6.3	6.1
tartaric acid (g/L)	4.88	5.87	2.3	3.5	4.63	4.96

Table 2. Residual Lysozyme Concentrations (mg/L) in Wines[a]

lysozyme (mg/L) (must + wine)	500 + 0	300 + 200	300 + 0	0 + 300
dil fact (chaptal + top)	× 0.80	× 0.88	× 0.80	× 1 (no dil)
Pinot noir (mg/L lyso)	TC: 400 AC: 264[b] loss: −34%	TC: 440 AC: 319[b] loss: −27%		
Chardonnay (mg/L lyso)	TC: 400 AC: 252[b] loss: −37%	TC: 440 AC: 327[b] loss: −26%	TC: 240 AC: 137 loss: −43%	TC: 300 AC: 245 loss: −18%

[a] Measurements were made 4 months after treatment for the wine and 4 months + 3 weeks for the must. [b] Treatments done in duplicate; TC, theoretical concentrations; AC, actual concentrations.

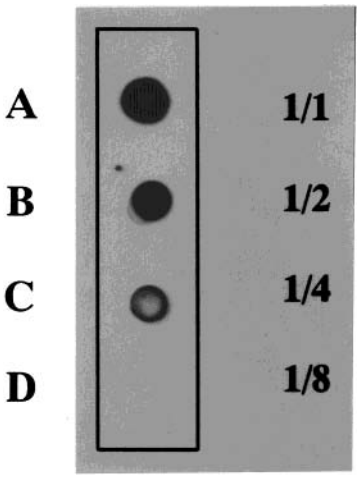

Figure 4. Dot-blot on nitrocellulose strips with color developed using chemiluminescent Supersignal substrate. The first antibody was rabbit antilysozyme polyclonal antibodies; spot A, nondiluted wine; spot B, 1/2 diluted wine; spot C, 1/4 diluted wine; and spot D, 1/8 diluted wine.

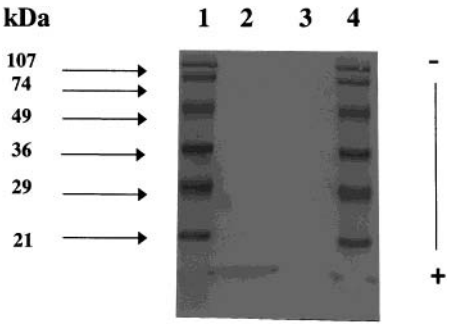

Figure 5. Detection of lysozyme in a Pinot noir wine by SDS−PAGE, immunoblotting using rabbit anti-lysozyme polyclonal antibodies and color developed using 4-chloro-1-naphthol: lanes 1 and 4, MW prestained markers; lane 2, wine treated with lysozyme added to the must; and lane 3, control wine. Relative molecular weights ($\times 10^{-3}$) of protein standards are given at the left side of the gel.

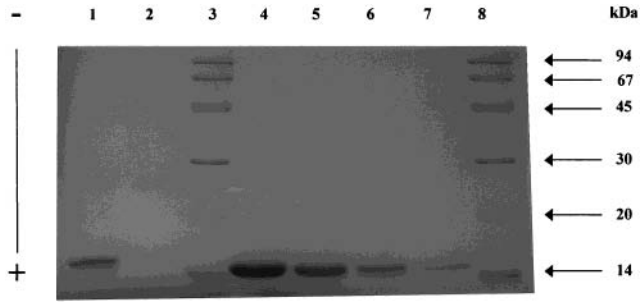

Figure 6. SDS−PAGE analysis stained with Coomassie Brilliant Blue: lane 1, wine treated with 500 mg/L lysozyme added to the must; lane 2, control wine (nontreated with lysozyme); lanes 3 and 8, MW markers; and lanes 4−7, lysozyme in aqueous solutions at 500, 250, 125, and 62.5 mg/L, respectively. Relative molecular weights ($\times 10^{-3}$) of protein standards are given at the right side of the gel.

was approximately 250−260 mg/L (see section HPLC Quantification of Lysozyme and Table 2). The detection is still correct even when the wine is diluted 4 times (Figure 4, spot C): the concentration of lysozyme was then 75 mg/L. This result clearly indicates the presence of residual lysozyme in wine. A control experiment using lysozyme antisera yields no positive cross-reaction with four nontreated French wines, even when the wines are not diluted.

It is also possible to detect lysozyme in wines after SDS−PAGE, Western blotting onto a nitrocellulose membrane, and immunostaining using 4-chloro-1-naphthol. The filtered wine was only treated with Laemmli buffer (v/v) before being loaded in the well (20 μL). The unique protein stained in these conditions is the lysozyme (Figure 5, lane 2). One can also observe the absence of a band for the control wine (Figure 5, lane 3). SDS−PAGE is more time-consuming than the dot-blot technique, but it provides further data such as the molecular weight of the protein and its eventual degradation during wine aging. Sometimes, dot-blot cross-reactivity gives positive coloration due and non-protein compounds of low molecular weights. Then, immunostaining after SDS−PAGE/Western blotting remains the only suitable technique to detect lysozyme in wines. Lysozyme can also be directly detected in wines after separation by SDS−PAGE and Coomassie Brilliant Blue (CBB) staining. The wine was treated with only Laemmli buffer (v/v) before being loaded in the well (20 μL). Since proteins are present in very low amounts in wines of the Champagne area (Maujean et al., 1990; Marchal et al., 1997), no band can be detected

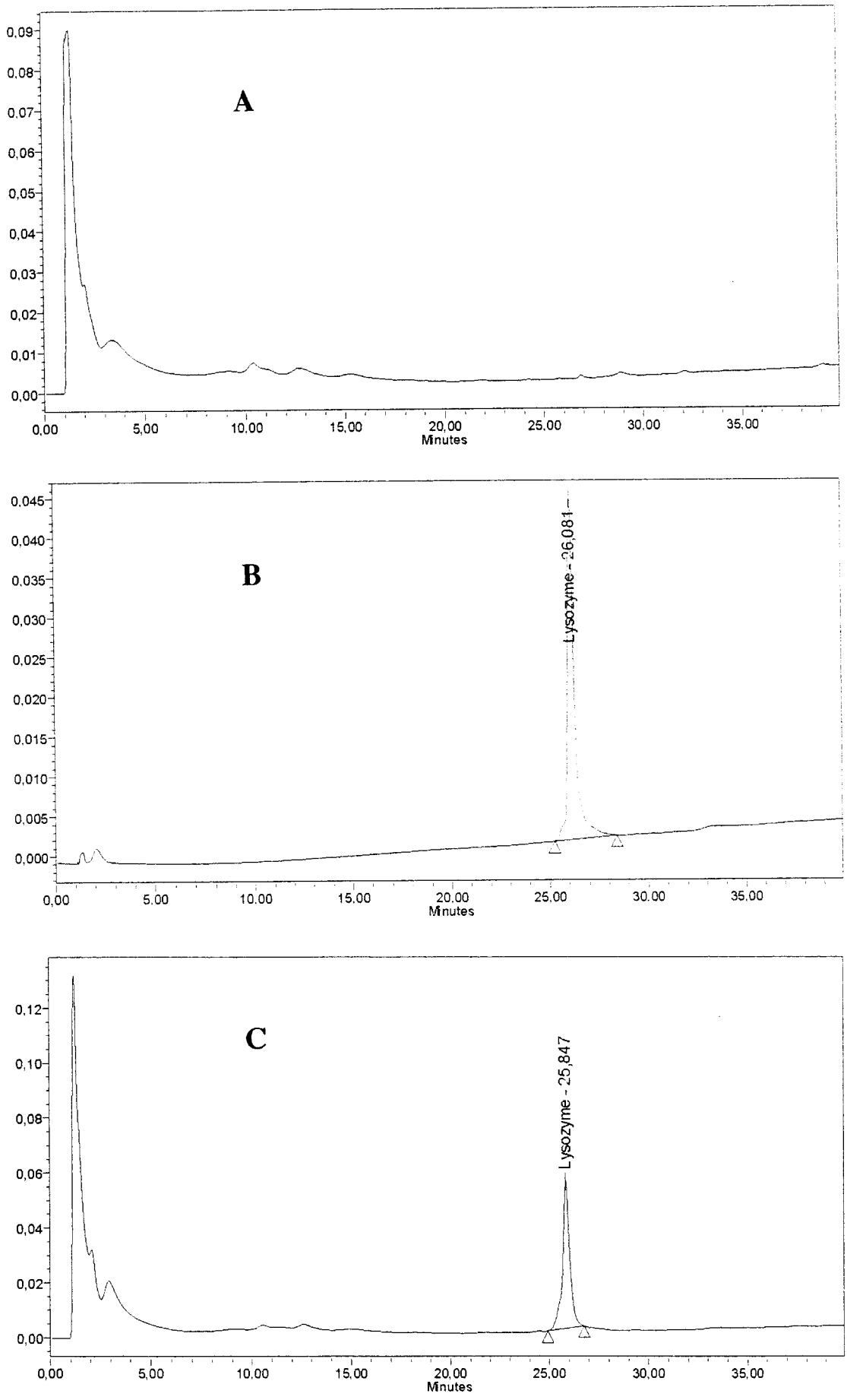

Figure 7. HPLC analysis of lysozyme in a Pinot noir wine. (A) nontreated wine; (B) synthetic wine + lysozyme; and (C) Pinot noir wine + lysozyme. For chromatographic conditions, see Materials and Methods.

in the control Chardonnay wine. The pattern of lysozyme-treated wine stained with CBB (Figure 1, lane 1) and by immunostaining is identical; only one band appears. Nevertheless, CBB coloration is not a specific technique and could reveal another 14 kDa protein (with an extremely low probability).

Densitometric Quantification Lysozyme. SDS−PAGE is a technique allowing lysozyme quantification after CBB coloration. Decreasing concentrations of lysozyme (dissolved in water) show bands with decreasing blue intensity (Figure 6, lanes 4−7). Densitometric measurements of these bands give a calibration curve with a very high correlation coefficient ($R^2 = 0.998$). It is then possible to precisely determine the concentration of lysozyme in a must or a wine during conservation or aging. Once again, the control wine gives no band (Figure 6, lane 2), such as in Figure 5 for the immunostaining. The wine originating from the lysozyme-treated must gives only one band corresponding to the lysozyme. Its concentration is 187 mg/L. As previously stated, CBB coloration is not a specific method, and all the proteins present in sufficient quantities can be stained. Champagne wines never contain more than 20−50 mg/L total proteins, as estimated by the Bradford method (Maujean et al., 1990; Marchal et al., 1997). According to this, a maximum of 0.5 μg of total proteins was loaded in the well. A band is correctly visualized with the Bio-Rad apparatus when it contains a minimum of 0.5 μg of protein. Champagne wine proteins essentially range between 20 and 30 kDa, with a major glycoprotein at 62/64 kDa (Brissonnet and Maujean, 1993; Marchal et al., 1996). In contrast, they contain only minor proteins at about 14 kDa. Finally, if a wine contains only 100 mg/L lysozyme, the CBB coloration stains 1 μg of lysozyme for a deposit of 20 μL (the wine was treated v/v with the Laemmli buffer). So, the probability of staining another protein than lysozyme is extremely low.

HPLC Quantification of Lysozyme. The elution of a nontreated wine by reversed phase HPLC shows that major peaks were completely eluted during the first 20 minutes (Figure 7A). Lysozyme dissolved in a synthetic wine is eluted at 26 min. (Figure 7B). No peak can be observed in the control wine profile at this retention time. This result thus allows the quantification of lysozyme though HPLC. The technique (Figure 7C) is not as specific as immunodetection methods. Table 2 gives lysozyme concentrations in wines 4 months after the treatment. Lysozyme was added before (first number in brackets) or after (second number in brackets) the alcoholic fermentation. Concentrations always diminish because of chaptalization and topping up. It is also due to the insolubilization of lysozyme. For Green and Daeschel (1994), tannins may bind to lysozyme and cause it to partially fall out of solution. Losses of lysozyme are higher when the enzyme was added at one time to the must (−34% for the Pinot noir and −37% for the Chardonnay) than when lysozyme is added in 2-fold both in the must and in the wine (−27% for the Pinot noir and −26% for the Chardonnay). The lowest diminution is observed when lysozyme was added to the wine only (−18%) in comparison with the addition to the must at 300 mg/L (−43%).

CONCLUSION

We have described here a highly sensitive immunochemical technique to specifically detect lysozyme in Champagne wines after SDS−PAGE and blotting or by dot-blot. Residual lysozyme can also be quantified by RP-HPLC or by densitometry after SDS−PAGE.

We are now studying the relationship between the presence of lysozyme and the foam properties of Champagne wines. The modification of the protein composition of a wine when adding lysozyme is of a particular interest when considering that the proteins present in wine exhibit foaming properties (Maujean et al., 1990; Brissonnet and Maujean, 1991; Malvy et al., 1994).

ACKNOWLEDGMENT

We thank Fordras S.A (Lugano, Switzerland) for providing enological lysozyme.

LITERATURE CITED

Amati, A.; Chinnici, F.; Piva, A. Il lisozima in Enologia per il controllo della fermentazione malolattica. *Ind. Bevande* **1994**, *23*, 215−22.

Amati, A.; Chinnici, F.; Piva, A.; Arfelli, G.; Riponi C. Influence of enological operations on lysozyme activity in winemaking. *Vitic. Enol. Sci.* **1996**, *51*, 59−92.

Blouin, J. Manuel pratique d'analyse des moûts et des vins. Chambre d'agriculture de la Gironde, 1977.

Bocshelle, O.; Pitotti, A. Utilizzo del lisozimo come stabilizzante: verifica di applicabilita in mezzo acido (Utilization of the lysozyme as stabilization agent. Verification of the useability in acid medium). *Ind. Aliment.* **1988**, *27*, 337−344.

Brissonnet, F.; Maujean A. Identification of some foam-active compounds in champagne base wines. *Am. J. Enol. Vitic.* **1991**, *42*, 97−102.

Freund, J. The mode of action of immunologic adjuvants. *Adv. Tuberc. Res.* **1956**, *7*, 130−148.

Gerbaux, V.; Villa, A.; Monamy, C.; Bertrand, A. Use of lysozyme to inhibit malolactic fermentation and to stabilize wine after malolactic fermentation. *Am. J. Enol. Vitic.* **1997**, *48*, 49−53.

Green, J. L.; Daeschel, M. A. The effects of wine components on the activity of lysozyme in a model wine system. *Am. J. Enol. Vitic.* **1994**, *45*, 355.

Green, J. L.; Watson, B. T.; Daeschel, M. A. Efficiency of lysozyme in preventing malolactic fermentation in Pinot noir wines. *Am. J. Enol. Vitic.* **1994**, *45*, 355−356.

Green, J. L.; Watson, B. T.; Daeschel, M. A. Efficiency of lysozyme in preventing malolactic fermentation in Oregon Chardonnay and Pinot noir wines (1993 and 1994 vintages). *Am. J. Enol. Vitic.* **1995**, *46*, 410−411.

Ibrahim, H. R.; Higashiguchi, S.; Juneja, L. R.; Kim, M.; Yamamoto, T. A. Structural Phase of Heat-Denatured Lysozyme with Novel Antimicrobial Action. *J. Agric. Food Chem.* **1996a**, *44*, 1416−1423.

Ibrahim, H. R.; Higashiguchi, S.; Koketsu, M.; Juneja, L. R.; Kim, M.; Yamamoto, T.; Sugimoto, Y.; Aoki, T. Partially unfolded lysozyme at neutral pH agglutinates and kills Gram-negative and Gram-positive bacteria through membrane damage mechanism. *J. Agric. Food Chem.* **1996b**, *44*, 3799−3806.

Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680−685.

Malvy, J.; Robillard, B.; Duteurtre, B. Influence des protéines sur le comportement de la mousse des vins de Champagne. (Influence of proteins on Champagne wine foaming behavior). *Sci. Aliments* **1994**, *14*, 88−98.

Marchal, R.; Bouquelet, S.; Maujean, A. Purification and partial biochemical characterization of glycoproteins in a champenois Chardonnay wine. *J. Agric. Food Chem.* **1996**, *44*, 1716−1722.

Marchal, R.; Seguin, V.; Maujean, A. Quantification of interferences in the direct measurement of proteins in wine from the Champagne region using the Bradford method. *Am. J. Enol. Vitic.* **1997**, *48*, 303−309.

Maujean, A.; Poinsaut, P.; Dantan, H.; Brissonnet, F.; Cossiez, E. Etude de la tenue et de la qualité de mousse des vins effervescents. II: Mise au point d'une technique de mesure de la moussabilité, de la tenue et de la stabilité de mousse des vins effervescents. (Study of the performance and quality of the foam in sparkling wines. II: Perfecting of a measuring technique for foamability, performance and stability of the foam in sparkling wines). *Bull. O.I.V.* **1990**, *63*, 405−427.

Pitotti, A.; Zironi, R.; Dal Bo, A.; Amati, A. Possible Application of Lysozyme in Wine Technology. *Med. Fac. Landbouww. Rijksuniv. Gent.* **1991**, *56*, 1697−1699.

Proctor, V. A.; Cunningham, F. E. The chemisttry of lysozyme and its use as a food preservative and a pharmaceutical. *Food Sci. Nutr.* **1988**, *26*, 4.

Received for review July 29, 1999. Revised manuscript received April 7, 2000. Accepted April 26, 2000. We express our gratitude to Europol'Agro and the Association Recherche Oenologie Champagne et Université for financial support.

JF990848A