JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. 2004, 52, 1861−1866 1861

Resistance Screening Essay of Wine Lactic Acid Bacteria on Lysozyme: Efficacy of Lysozyme in Unclarified Grape Musts

CLAUDIO DELFINI,* MANUELA CERSOSIMO, VINCENZO DEL PRETE, MORELA STRANO, GIUSEPPE GAETANO, ADOLFO PAGLIARA, AND STEFANO AMBRÒ

Istituto Sperimentale per l'Enologia, via P. Micca, 35-14100 Asti, Italia

In wine making, the bacteriolytic activity of lysozyme has primarily been used to control the malolactic fermentation in wines. The use of lysozyme in musts before settling and the beginning of the alcoholic fermentation to inhibit the growth of lactic acid bacteria could be very beneficial. In a resistance test carried out in MT/b broth, lysozyme had greater antimicrobial activity toward *Oenococcus oeni* than *Lactobacillus* species. Several strains of wine bacteria belonging to *Oenococcus* proved sensitive to the bacteriolytic activity of lysozyme at low concentrations in both synthetic medium (MT/b) (50 mg/L), white must, or red must made with or without the skins (100 mg/L). *Lactobacillus* and *Pediococcus* strains survived at lysozyme concentrations of 200−500 and 500 mg/L, respectively, in MT/b and musts. Suspended solids in unclarified musts may strongly bind to lysozyme thereby causing its removal by filtration or centrifugation. One hour after lysozyme was added to musts, it was quantified by HPLC and found after centrifugation to be 40−50% and only 10% in musts made with or without the skins, respectively. Although appreciable amounts of lysozyme were bound to wine components, this did not appear to be a serious hindrance to lysozyme activity.

KEYWORDS: **Lysozyme; winegrape must; malolactic bacteria resistance; lysozyme determination**

INTRODUCTION

The bacterial activity in musts and wines is traditionally controlled through the use of sulfur dioxide, a compound giving multiple functionality (antioxidant, antimicrobial, extractive solvent) along with some undesirable effects such as negative sensory characteristics and eliciting allergic responses in sensitive people. Other antimicrobials such as sorbic acid and dimethyl carbonate are active against yeasts but have limited activity against bacteria (*1*, *2*).

Lysozyme is an enzyme with bactericidal activity that is isolated from hen egg albumen and has recently found to be useful in controlling bacterial activity in wines. The use of lysozyme has been shown to be an efficacious antimicrobial in many foods, most notably in cheeses.

Lysozyme is a protein with an isoelectric pH of 10.5−11.0 and a molecular mass of about 14 500 Da. Its maximum stability and activity is found at pH values lower than 7.0, namely, in the range of 2.8−4.2, which is the pH range for most wines (*7*). Lysozyme is available in hydrochloride form, which permits rapid solubility. Its chitinolytic activity causes the degradation of the bacterial cell wall thus accelerating cell lysis.

Bacterial sensitivity to lysozyme depends on the peptidoglycan structure in the cell wall. The antimicrobial activity of lysozyme toward lactic bacteria was reviewed by Cunningham et al. (*3*) and has been since shown to depend on both the cell physiological state and the lysozyme structure in the medium (H^+ concentration, reacting compounds) (*4*, *5*).

As an enzyme, lysozyme shows a specific action toward both species and strain. Its efficacy toward Gram-negative bacteria (i.e., acetic bacteria) is much less and is more bacteriostatic than bactericidal (*3*, *6*), presumably because the outer membrane acts as a barrier.

From the point of view of practical applications in winemaking, it would be instructive to understand the variability of lysozyme sensitivity in strains of wine spoilage bacteria such as *Lactobacillus*, which is usually blamed for serious defects in musts and wines (stuck wines, ferocious lactic acid spoilage, tartaric acid degradation, etc.). Some studies (*7*, *8*) have shown a general sensitivity of wine lactic bacteria to lysozyme at concentrations of 250−500 mg/L.

The primary factor that has been observed to affect the bacteriolytic action of lysozyme in wines is believed to be the polyphenolic components, which may bind to lysozyme. It has been observed that lysozyme is more active in white wines than red, which reflects the polyphenolic content. As wine pH becomes lower, the antimicrobial activity of sulfur dioxide increases. The opposite is true for lysozyme, which makes it an attractive candidate to prevent spoilage in high pH wines. Amati et al. (*9*) highlighted a decrease in the lysozyme activity as the SO_2 concentration and the temperature increased. According to these authors, the lysozyme activity in SO_2-containing wines reached a minimum value after a contact time of approximately 15 days, at which point thereafter it remained

* To whom correspondence should be addressed. Tel: +39-0141-433817-593476. Fax: +39-0141-436429-593476. E.mail: cldelfini@libero.it.

10.1021/jf034824m CCC: $27.50 © 2004 American Chemical Society
Published on Web 03/05/2004

approximately constant. This result would be important if lysozyme needs time to destroy bacterial cells, but it would be meaningless if the bacteriolytic activity occurs quickly soon after the lysozyme addition and SO_2 can be added some hours later, or when lysozyme is added some hours before bottling or in musts soon after crushing before the beginning of alcoholic fermentation, in other words, when the wine needs no special protection against further contamination.

Pitotti et al. (*7*) obtained very good results by adding lysozyme at a concentration of over 100 mg/L immediately after crushing and cold juice clarification, while Castino et al. (*10*) prevented the malolactic activity in a fermenting Moscato juice contaminated with commercial malolactic bacteria, with a concentration of 500 mg/L (*1*). Gerbeaux et al. (*11*) and Gerland (*12*) found lysozyme advantageous, at a concentration of 250−500 mg/L, in sluggishly fermenting musts due to growth by spoilage lactic acid bacteria.

Bentonite, charcoal, and silica gel (when used in musts) decreased significantly the content of free lysozyme and, accordingly, the antibacterial activity, while casein, gelatine, PVPP, diatomaceous, cellulose, and pectolytic enzymes had no effect (*9*). Moreover, no significant changes in both chemical and organoleptic features of wine were observed after the addition of lysozyme, but a slight increase in turbidity occurred on the addition of 1000 mg/L lysozyme (*7*). However, it may be desirable to have any residual lysozyme in wines because of possible allergenic responses by sensitive individuals. Residual lysozyme in wine can be determined by high-performance liquid chromatography (HPLC) according to Marchal et al. (*13*) and Daeschel et al. (*14*). No methods were proposed so far to evaluate lysozyme in musts.

Lysozyme has been widely reported to be effective against undesirable malolactic fermentation during wine processing and/or aging in bottle, during grape crushing, to prevent musts from bacterial contamination and/or before and during alcoholic fermentation to avoid bacterial spoilage and stuck fermentations.

The goal of this research is (i) to verify the stability of lysozyme in must, (ii) to study the sensitivity (or resistance) in some lactic acid bacteria strains of different species isolated from grapes, juice, or wine, and (iii) to check the efficacy of lysozyme in preventing the growth of lactic bacteria in musts just crushed with or without the skins, prior to clarification.

MATERIAL AND METHODS

Bacterial Strains. Strains were used as follows: 35 *Oenococcus oeni* (CNLBSV-ISEAT 5001−5006*, 5008, 5010−5013*, 5015*, 5019, 5026, 5028, 5030, 5031*, 5038*, 5041*, 5043*, 5051*, 5056*, 5058*, 5060*, 5062*, 5063*, 5071*, 5081*, 5084*, 5089*, 5091*, 5097*, 5102*, 5106*, and 5117* from the Istituto Sperimentale per l'Enologia-Asti, ISEAT, Italy), four *Lactobacillus brevis* (CNLBSV-ISEAT 5199, from Oregon State University and CNLBSV-ISEAT 5212−5219 from Gutenberg Universitat, Germany), two *buchneri* (CNLBSV-ISEAT 5196* from CNLBSV-ISEAT-Asti and CNLBSV-ISEAT 5204 from UCD, Davis, CA), five *casei* (CNLBSV-ISEAT 5200, 5209, 5210 from UCD, Davis, CA and CNLBSV-ISEAT 5220*−5229* from Gutenberg Universitat), eight *hilgardii* (CNLBSV-ISEAT 5032*−5034*, 5201*−5203*, 5205*, 5206* from CNLBSV-ISEAT-Asti and CNLBSV-ISEAT 5211* from Sigmo Laboratory, Chateau de la Frémoire, France), two *plantarum* (CNLBSV-ISEAT 5197*, 5198* from Oregon State University), one *sakei* (CNLBSV-ISEAT 5208* from UCD, Davis, CA), and one *Pediococcus parvulus* (CNLBSV-ISEAT 5177* from CNLBSV-ISEAT). All strains were grown in MT/b broth medium at 25 °C and inoculated in the experimental samples during exponential growth. Unclassified strains (*) were identified by taxonomic analyses at the Istituto Sperimentale Lattiero Caseario di Lodi (MI), Italy.

Table 1. Results of HPLC Analysis of Residual Lysozyme (Average of Two Replicates) in Barbera Musts Containing 500 mg/L Lysozyme after Filtration, Centrifugation, and/or Acidification at pH 2.0

filtration without acidification		filtration after acidification		centrifugation without acidification		centrifugation and acidification	
mg/L	loss %	mg/L	loss %	mg/L	loss %	mg/L	loss %
47.90	−90.42	244.00	51.20	257.50	−48.50	244.20	−51.16

Media. (a) Synthetic broth medium at pH 5.0 [D-(+)-glucose, 15 g/L; tryptone, 8 g/L; yeast extract, 5 g/L; casamino acids, 1 g/L; sodium acetate, 3 g/L; L-(−)-malic acid, 6 g/L; triammonium citrate, 2 g/L; magnesium sulfate heptahydrate, 0.2 g/L; manganese sulfate hydrate, 0.035 g/L; and Tween 80, 1 mL; reach pH 5.00 with potassium hydroxyde; vitamin solution 1% was added after sterilization at 120 °C/20 min; vitamin solution: pyridoxine hydrochloride, 400 mg/L; thiamine hydrochloride, 400 mg/L; inosine, 2000 mg/L; biotin, 20 mg/L; calcium pantothenate, 400 mg/L; nicotinamide, 400 mg/L; and *p*-aminobenzoic acid, 200 mg/L] without (MT/b) and with actidione (50 mg/L) and potassium sorbate (400 mg/L) (MT/bs) to become selective against molds and yeasts according to Delfini et al. (*1*) were used. (b) Unclarified Chardonnay white must (pH 3.80; total phenols, 127 mg/L) with no skins. (c) Unclarified Barbera red must (pH 3.30; total phenols, 298 mg/L). (d) Unclarified Barbera red must with skins (pH 3.2; total polyphenols, 227 mg/L). All musts were obtained from frozen grapes at −20 °C after thawing at 4 °C while the juices of b and c were obtained by gentle pressure after 4 h of contact on skins.

Reagents. Lysozyme hydrochloride Muramidase (E.C.3.2.1.17; CAS No. 9066-59-5), granular food grade (Fordras, S.a. Lugano, Switzerland) 95% pure, soluble in water. The product was used after filter sterilization through a 0.22 μm membrane.

Analytical Methods. *Lysozyme Determination by HPLC.* The analyses were carried out on a Varian Liquid Chromatograph mounted with a column Supelco Progel-TSK, Phenyl-5PW-RP; 75 mm × 4.6 mm and linked to a Varian 9050 UV detector. Varian Star 4.5 software was used for the integration of peak areas and data handling.

Chromatographic Conditions. Taking into account the methods described in the literature (*13*, *14*) and the results of our preliminary trials, the following HPLC method was chosen. Eluent A: 1% acetonitrile; 0.2% TFA; 98.8% water. Eluent B: 70% acetonitrile, 0.2% TFA; 29.8% water.

Working Conditions. Gradient program: from 100% A to 100% B in 36 min; from 100% B to 100% A in 5 min; equilibration time, 9 min; flow, 1 mL/min; injection, 20 μL; λ, 281 nm [a spectrum of a lysozyme solution in methanol (1.0 g/L), carried out prior to fix the working conditions for the detector, showed a maximum at 281 nm instead of 280].

Stability of Lysozyme in Must. *Sample Preparation for the Analysis in HPLC. Free and Bound Lysozyme in Must.* Trials were initially performed in order to find the best procedure for the preparation of must for the evaluation of residual free lysozyme at the sampling time by HPLC. Daeschel et al. (*14*) acidified the must at pH 2.0 with HCl soon after the sampling before HPLC injection. The scientific reason and mechanism of this stabilizing treatment were not explained by the cited authors, but probably, it was adopted trying to stop any running binding process or to avoid precipitation during the storage as well as to free lysozyme from phenols or other combining compounds. The efficacy of this treatment in order to the above-hypothesized purposes was checked along with the use of a membrane filtration and/or centrifugation before the HPLC analysis, as follows.

Trial No. 1. Lysozyme was added to 150 mL of red must c to give a concentration of 500 mg/L. The samples obtained were acidified and/or centrifuged and/or filtered as shown in **Table 1**.

Trial No. 2. Lysozyme was added to 100 mL of must c so as to obtain a concentration of 500 mg/L. After 4 h, the must was centrifuged at 5000 rpm for 10 min. Aliquots sampled after 0, 1, 2, 3, and 4 h, respectively, were analyzed by HPLC to determine free lysozyme (**Table 2**).

Table 2. Results of HPLC Analysis of Residual Lysozyme vs Time in a Sample of Barbera Must Added with 500 mg/L Lysozyme after Centrifugation (Trial 2)

hours	lysozyme (mg/L)	lysozyme loss %
0	179	−64
1	177	−65
2	180	−63
3	179	−64
4	178	−64

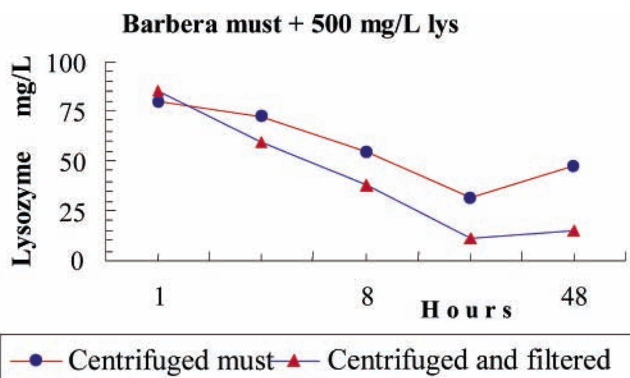

Figure 1. Loss of free lysozyme vs time in centrifuged must and in centrifuged and filtered must.

Table 3. Enological Parameters and Viable Bacteria (CFU) in the Control Sample and in Samples of Wines Affected by Lactic Acid Stuck Fermentation Treated with Lysozyme (Cellar Preliminary Experiments)

wines	ethanol (%)	sugars (g/L)	pH	volatile acidity (g/L)	CFU/mL control sample	CFU/mL treated sample
Chardonnay	13.79	13.00	3.51	1.11	$1.28 \cdot 10^6$	18
Barbera	14.52	24.90	3.34	0.90	$1.98 \cdot 10^6$	0

Trial No. 3. Lysozyme was added to 375 mL of must c to give concentrations of 500 mg/L. The sample was divided in five subaliquots, which were centrifuged after 1, 4, 8, 24, and 48 h, respectively. For each sample, free lysozyme was determined by HPLC in the supernatant before and after filtration (**Figure 1**).

Method of Analysis. The samples of winegrape must spiked with lysozyme after centrifugation were injected directly into the HPLC and analyzed at room temperature.

Calibration curves were constructed both in water and in centrifuged must. Each concentration was analyzed in duplicate. The values related to 6.25 and 12.5 mg/L were obtained by injecting 50 μL. Control tests were carried out with solutions of lysozyme of known concentrations.

Resistance Screening Essay in MT/b Broth. A population of 10^7 CFU was introduced into four 10 mL MT/b aliquots with the addition of lysozyme to obtain concentrations of 0, 50, 100, and 200 mg/L. The more lysozyme resistant bacteria *Lactobacillus* and *Pediococcus* were treated with lysozyme at the concentration of 500 mg/L. After a contact time of 16 h (usually needed in practice to obtain a clarification at room temperature favorable to bacterial growth) at 25 °C, 1 mL of each aliquot was transferred into a 15 mL centrifuge tube and centrifuged at 5000 rpm/10 min. After the supernatant was discarded, 10 mL of MT/b was added to the residue and the solution was vortexed and incubated at 25 °C for 20 days. The inhibition of growth was verified daily by visual observation (turbidity).

Efficacy of Lysozyme in Musts. *Preliminary Cellar Trials.* After attempts to eliminate microbial contamination by both settling and SO_2 addition, two quantities of 400 L of Chardonnay and Barbera wines both stopped fermentation (see enological parameters in **Table 3**), following a strong contamination by *O. oeni*, were divided into two subaliquots. Both wines were very rich in suspended solids. To one of them lysozyme (previously dissolved in water during 5 h) was added to obtain a concentration of 500 mg/L. CFU microbial populations were determined on MTb/s agar at 20 °C in both the control and the treated aliquots either before the lysozyme addition or after 20 days of growth.

Laboratory Tests in Must. Strains 5026 (*O. oeni*) and 5032 (*L. hilgardii*), which proved strongly resistant to lysozyme in MT/b, were inoculated in duplicate in 75 mL of musts a and b in the ratio of 10^6 CFU/mL. The red juice (b) was separated from skins by settling and then treated with both lysozyme and bacterial cells. After treatment, it was mixed again with skins to ensure a distribution of lysozyme and bacterial cells as homogeneous as possible. The above treatment was repeated three times, according to the "delastage" maceration technique. The same procedure of delastage was carried out for the aliquots sampled after 16 h of contact (the same used in the resistance screening essay in MT/b broth). This methodology was chosen to reproduce real conditions similar to those used in wine processing, although the presence of skins might not guarantee quite a good distribution of bacterial cells during the sampling. That is the reason only the total absence of growth was considered as a positive result for bacteriolysis, while any quantitative survival (growth) was taken as a negative result.

To check for bacteria survival, two aliquots of 2.5 and 5.0 mL of the inoculated sample and after 16 h of lysozyme−bacterial cell contact were filtered through a 0.2 μm membrane to remove any residual lysozyme from the bacterial cells. The membranes were transferred to a Petri disk containing two porous septa soaked with 3.5 mL of MT/bs and enumerated for bacteria after 12 days of incubation at 25 °C. Furthermore, a 50 mL aliquot of each inoculated sample was centrifuged after 16 h of lysozyme−bacterial cell contact and the supernatant was analyzed for free lysozyme.

RESULTS AND DISCUSSION

Analytical Method. The analytical method proved reliable showing under our working conditions (centrifuged musts) with a minimum detectable level (MDL) of 6.25 mg/L obtained by injecting 50 μL volumes. However, in routine HPLC analysis, an injection of a volume lower than 20 μL is preferable, to prevent the column from being stressed, and eventually, the analysis with higher volumes could be repeated in the case of very low concentrations.

The gradient used proved effective for both red and white products, always giving a very good repeatability of the RT for the lysozyme peak. Lysozyme appears on the chromatogram as a peak at a constant average RT of 19.5 min.

The coefficients of regression calculated for both lysozyme solutions in water and in must proved very similar (0.9979 and 0.9976, respectively). The calibrations in water and in must proved satisfactory and overlapped each other.

Stability of Lysozyme in Must. *Sample Preparation for the Analysis in HPLC. Free and Combined Lysozyme in Must.* The HPLC working conditions chosen allow one to detect and evaluate the actual free lysozyme. The results of the first trial allow us to conclude that (i) filtration caused a 90% decrease in free lysozyme. In acidified samples, a decrease of 51% was observed (**Table 1**). (ii) Centrifugation caused a 48% loss of free lysozyme that proved to keep constant also after acidification (**Table 1**). Thus, in an unclarified must, the centrifugation seems to eliminate the same quantity of combined lysozyme as filtration does after acidification. The above, along with the incomplete liberation of lysozyme in the acidified and filtered samples, cannot be easily explained so far and warrants further investigation.

Thus, filtration applied to unclarified musts soon after the lysozyme addition has the strong effect to eliminate lysozyme from the filtrate. The above would prove a quick combination of lysozyme with colloids and solids suspended. The effect of centrifugation, instead, proved less dramatic causing a loss of

Table 4. Sensitivity (Days of Growth Delay) of Several *O. oeni* Strains in MT/b Containing Increasing Concentration of Lysozyme after 16 h of Contact

	Oenococcus oeni								
	lysozyme (mg/L)					lysozyme (mg/L)			
strains	50	100	200	500	strains	50	100	200	500
5001	20	20	20	20	5041	**9**	20	20	20
5002	20	20	20	20	5043	**11**	18	20	20
5003	20	20	20	20	5051	20	20	20	20
5004	**10**	20	20	20	5056	**14**	20	20	20
5005	20	20	20	20	5058	**10**	20	20	20
5006	20	20	20	20	5060	18	20	20	20
5008	20	20	20	20	5062	20	20	20	20
5010	18	20	20	20	5063	19	19	20	20
5011	18	20	20	20	5071	20	20	20	20
5012	20	20	20	20	5081	20	20	20	20
5013	20	20	20	20	5084	20	20	20	20
5015	20	20	20	20	5089	20	20	20	20
5019	20	20	20	20	5091	**10**	**11**	**13**	20
5026	**11**	**12**	20	20	5097	**1**	**4**	**4**	20
5028	19	20	20	20	5102	**5**	**20**	**20**	20
5030	20	20	20	20	5106	**5**	**20**	**20**	20
5031	20	20	20	20	5117	**11**	**11**	**11**	20
5038	**11**	20	20	20					

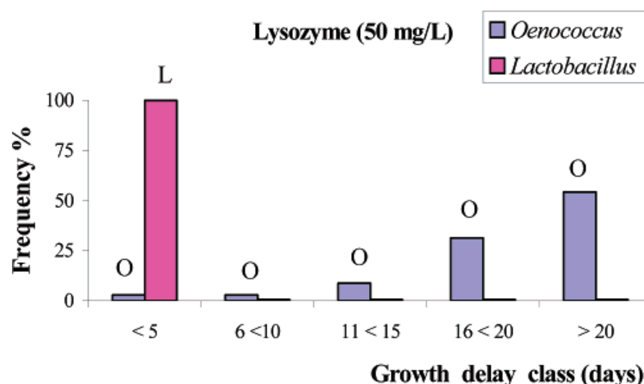

Figure 2. Frequency % distribution of the *O. oeni* and *Lactobacillus* spp. strains in classes of growth delay in MT/b containing increasing concentrations of lysozyme. The major the frequency and growth delay class are, the minor the resistance is. L = *Lactobacillus*; O = *Oenococcus*.

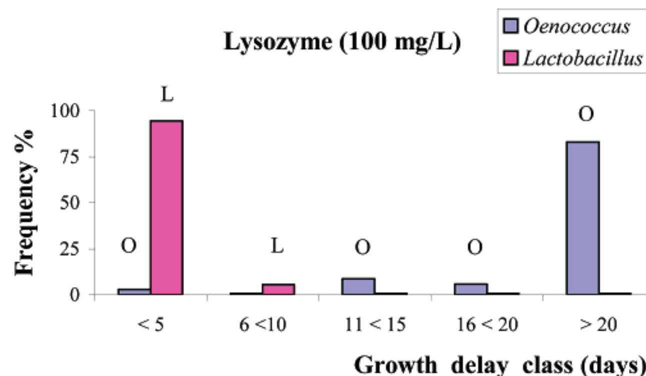

Figure 3. Frequency % distribution of the *O. oeni* and *Lactobacillus* spp. strains in classes of growth delay in MT/b containing increasing concentrations of lysozyme. The major the frequency and growth delay class are, the minor the resistance is. L = *Lactobacillus*; O = *Oenococcus*.

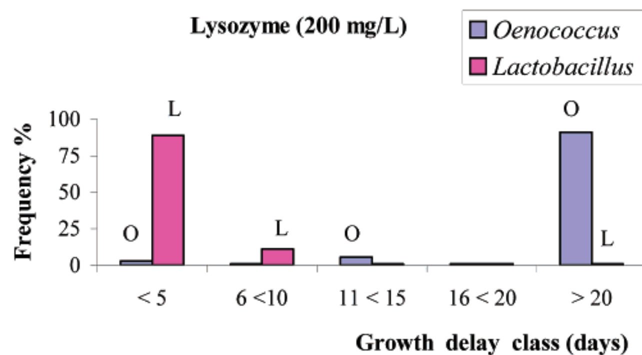

Figure 4. See **Figures 2** and **3**. L = *Lactobacillus*; O = *Oenococcus*.

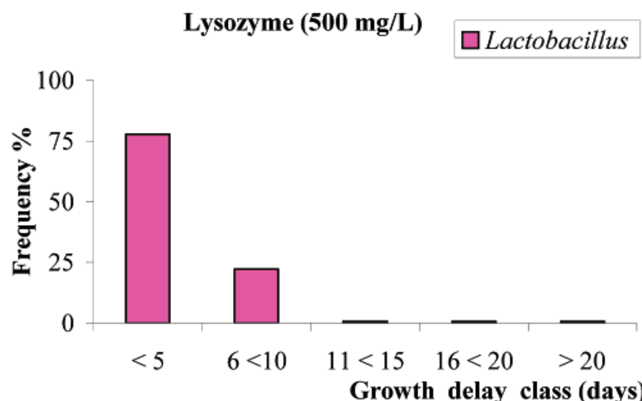

Figure 5. See **Figures 2** and **3**. L = *Lactobacillus*; O = *Oenococcus*.

lysozyme of only about 50%. Furthermore, the concentration of free lysozyme in the supernatant keeps almost constant with time as indicated by the results of trial no. 2 (**Table 2**). These results indicate that musts can be analyzed some hours after centrifugation with no significant decrease in free lysozyme concentration.

Trial no. 3 showed for both centrifugation and filtration a percentage loss of free lysozyme increasing with time (**Figure 1**). Filtration through 0.22 μm membranes eliminates much more lysozyme than centrifugation. A complete removal of colloids takes place so avoiding a possible colloidal aggregation that might slowly continue in the must even after centrifugation. It should be emphasized that in a must very rich in colloids and suspended solids, it can be assumed that a system continuously featuring new aggregation steps neither centrifugation nor other clarification techniques meet at the moment the practical needs to separate free and combined lysozyme during sampling. On the other hand, untreated musts, quite rich in solids, cannot be directly injected in a HPLC column and that supports the use of centrifugation in the sample preparation for the analysis of lysozyme in musts by HPLC.

Finally, the speed at which suspended solids of a must react to bind lysozyme must be taken into consideration. In the sample of **Figure 1** (Barbera red must without skins prepared as described for medium c), the HPLC analysis carried out within an hour after centrifugation found only 10−20% of "free" lysozyme while in other Barbera musts a loss of 60−70% was observed (data not reported).

The results strongly depend on both the crushing conditions and the time of addition of lysozyme to must; the centrifugation and analysis times also seem to play a major role. In conclusion, it is worth noting that winegrape must is such a complex and varying medium that makes reproducibility, even in rigorous experimental conditions, difficult and that the amount of suspended solids is extremely variable.

Resistance Screening Essay in MT/b Broth. *Lactobacillus* and *Pediococcus* proved more resistant to lysozyme than *O. oeni* along with other sensitive strains (e.g., 5201, 5211, 5032−5034) (**Tables 4** and **5**). A growth of 100% was observed within 5 days for *Lactobacillus* strains at a lysozyme concentration of

Table 5. Sensitivity (Days of Growth Delay) of *Lactobacillus* spp. and *P. parvulus* in MT/b Containing Increasing Concentration Lysozyme after 16 h of Contact

species	strains	lysozyme (mg/L) 50	100	200	500
	Lactobacillus spp.				
hilgardii	5032	2	1	3	4
hilgardii	5033	3	5	5	6
hilgardii	5034	3	4	5	6
hilgardii	5201	3	4	4	7
hilgardii	5202	0	0	1	2
hilgardii	5203	0	0	0	0
hilgardii	5205	0	0	0	0
hilgardii	5206	0	0	1	2
hilgardii	5211	3	3	3	4
brevis	5199	0	0	0	0
buchneri	5196	1	1	3	4
buchneri	5204	0	0	1	2
casei	5200	0	0	0	0
casei	5209	0	0	0	0
casei	5210	0	0	0	0
plantarum	5197	0	0	0	0
plantarum	5198	0	0	0	0
sakei	5208	0	0	0	0
	Pediococcus				
parvulus	5177	0	0	2	2

50 mg/L. A growth of 33% was observed within 5 days for *O. oeni* at 100 mg/L of lysozyme, while 31.4% developed within 16−20 days and 54.3% showed no growth within 20 days. *Lactobacillus*, instead, proved to grow at a high percentage within 5 days even at high lysozyme concentrations, namely, 94.4% at 100 mg/L, 88.9% at 200 mg/L, and 77.8% at 500 mg/L.

Considering the frequency % obtained for each lysozyme concentration, it is suggested that all strains of *Oenococus oeni* were strongly inhibited in MT/b (**Figures 2** and **3**). Only five strains survived in the presence of 200 mg/L lysozyme after 16 h of contact, and none grew in the presence of 500 mg/L. By contrast, *P. parvulus* (**Table 5**) and all species of *Lactobacillus* (**Figures 4** and **5**) were very resistant and grew also at a concentration of 500 mg/L. However, these results must be evaluated taking into consideration the fact that the favorable growth medium and exponentially growing cells may compromise the efficacy of lysozyme under these conditions.

Efficacy of Lysozyme in Must. *Preliminary Cellar Trials.* The results reported in **Table 3** prove a strong efficacy of lysozyme in both white and red wines. While the control samples of Chardonnay and Barbera showed, respectively, 1.28×10^6 and 1.98×10^6 CFU/mL, no CFU/mL but only 18 were respectively detected in the Chardonnay and Barbera aliquots treated with lysozyme. Considering that both wines, particularly the red one, were very turbid and rich in phenols, the above results could be viewed as very significant, from a technological point of view, and then worthy of further research.

Laboratory Tests in Must. Results concerning the bacterial survival confirmed the efficacy of 100 mg/L lysozyme to control, during 16 h of exposure, the development of *O. oeni* resistant strain 5026 in white must without skins, and of 200 mg/L lysozyme in red must with skins. By contrast, the *L. hilgardii* resistant strain 5032 survived in any experimental condition, even in the presence of 500 mg/L lysozyme (**Table 6**). However, it must emphasized that these results were obtained in musts that had a strong affinity for binding lysozyme. After 16 h from lysozyme addition, HPLC analyses revealed amounts of “free” lysozyme from 28−50% in white off skins must and from 3−6% in red on skins must for the corresponding concentrations of lysozyme of 100, 200, and 500 mg/L (**Table 7**), suggesting that the combined lysozyme might have not lost completely its bacteriolytic activity. Weak binding interactions with the must compounds probably not involving the biological active site of lysozyme may be an explanation.

This idea seems to be supported by the positive results obtained in the preliminary trials on Chardonnay and Barbera stuck wines contaminated by *O. oeni* (**Table 3**). Furthermore, the relative lack of antimicrobial activity of lysozyme on

Table 6. Survival of Strains *O. oeni* 5026 and *L. hilgardii* 5032, Observed in Both a White Must Off Skins and a Red Must with Skins, Inoculated at Time = 0 (T_0) and after 16 h (T_{16}) of Contact with Increasing Concentrations of Lysozyme[a]

		samples added with 100 mg/L lysozyme			samples added with 200 and 500 mg/L lysozyme			
		control sample		+100	control sample		+200	+500
must	bacterial strain	T_0	T_{16}	T_{16}	T_0	T_{16}	T_{16}	T_{16}
white off skins	*O. oeni*	10^6	++++	0	10^6	++++	0	0
	L. hilgardii	ND	ND	ND	10^6	++++	++++	++++
red on skins	*O. oeni*	ND	ND	ND	10^6	++++	0	0
	L. hilgardii	ND	ND	ND	10^6	++++	++++	++++

[a] ND = not determined; ++++ = strong growth; 0 = no growth.

Table 7. Results of HPLC Analysis of Residual Lysozyme (mg/L) in the Musts of **Table 6** at the Sampling Time T_{16}[a]

			lysozyme added (mg/L)								
			single data (mg/L)			average data (mg/L)					
must	bacterial strains		+100	+200	+500	+100	loss %	+200	loss %	+500	loss %
white off skins	*O. oeni* 5026	a	29	44	222	28.0	−72.0	44.0	−78.0	223.0	−55.4
		b	27	44	224						
	L. hilgardii 5032	a	ND	50	209			50.5	−74.8	208.5	−58.3
		b	ND	51	208						
red on skins	*O. oeni* 5026	a	ND	12	12			12.0	−94.0	13.0	−97.4
		b	ND	12	14						
	L. hilgardii 5032	a	ND	6	12			7.5	−96.3	13.5	−97.3
		b	ND	9	15						

[a] a,b = replicates; ND = not determined.

Lactobacillus strain must be evaluated taking into consideration the challenging experimental conditions chosen. Specifically, these are as follows: (i) the strains used in this experiment were both very resistant to lysozyme, (ii) the exponential phase of growth of the inoculated cells increased the resistance to lysozyme, and (iii) the large amount of cells inoculated (10^6/mL CFU), much larger than those usually found in musts, freshly pressed. Undoubtedly, the lysozyme activity in unclarified musts is not so compromised that the existence of other factors may warrant further research to fully explain precipitation phenomena involving lysozyme and must/wine components.

ACKNOWLEDGMENT

We thank Helmut König (Gutemberg University of Mainz, Germany), Ralph Kunkee (University of California, Davis), Mark Daeschel, Oregon State University, and Sigmo Laboratory (Chateau de la Frémoire, France) for providing lactic acid bacteria strains and Giorgio Giraffa and Cristina Rossetti at the Istituto Sperimentale Lattiero Casueario di Lodi (Italy) for the taxonomic determination of the unclassified lactic acid strains. We also thank Fordras for the support and for supplying lysozyme.

LITERATURE CITED

(1) Delfini, C.; Formica, J. V. *Wine Microbiology. Science and Technology*; Marcel Dekker: New York, 2001; pp 67, 149.

(2) Delfini, C.; Gaia, P.; Schellino, R.; Strano, M.; Pagliara, A.; Ambrò, S. Fermentability of grape must after inhibition with Dimethyl Dicarbonate (DMDC). *J. Agric. Food Chem.* **2002**, *50*, 5605−5611.

(3) Cunningham, F. E.; Proctor, V. A.; Goetsch, S. J. Egg-white lysozime as a food preservative: an overview. *World's Poult. Sci. J.* **1991**, *47*, 141−163.

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

(5) Ibrahim, H. R.; Higashiguchi, S.; Koketsu, M.; Juneja, L. R.; Kim, M.; Yamamoto, T.; Sugimoto, Y.; Aoki, T. Partially unfolfed 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.

(6) Hughey, V. L.; Johnson, E. A. Antimicrobial activity of lysozyme against bacteria involved in food spoilage and food-borne disease. *Appl. Environ. Microbiol.* **1987**, *53* (9), 2165−2170.

(7) Pitotti, A.; Zironi, R.; Dal Bo, A.; Boschelle, O. Assay of lysozyme by its lytic action on *Leuconostoc oenos*: a suitable substrate at acidic pH. *J. Food Biochem.* **1991**, *15*, 393−403.

(8) 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* (1), 49−54.

(9) Amati, A.; Chinnici, F.; Piva, A. Il lisozima in enologia per il controllo della fermentazione malolattica. *Ind. Bevande XXIII* **1994**, *3*, 215−221.

(10) Castino, M. In *Wine Microbiology. Science and Technology*; Delfini, C., Formica, J. F., Eds.; Marcel Dekker: New York, 2001; p 149.

(11) Gerbaux, V.; Meistermann, E.; Cottereau, Ph.; Barrière, C.; Cuinier, C.; Berger, J. L.; Villa, A. Use of lysozyme in enology. *Bull. l'O.I.V* **1999**, *819*−*820*, 348−373.

(12) Gerland, C. Applications of lysozyme in enology−Technical report. *Mitt. Klosterneuburg* **2002**, *52*, 116−121.

(13) Marchal, R.; Chaboche, D.; Marchal-Delahaut, L.; Gerland, C.; Gandon, J. P.; Jeandet, P. Detection and quantification of lysozyme in Champagne wines. *J. Agric. Food. Chem.* **2000**, *48*, 3225−3231.

(14) Daeschel, M. A.; Musafija-Jeknic, T.; Wu, Y.; Bizzarri, D.; Villa, A. High-performance liquid chromatography analysis of lysozyme in wine. *Am. J. Enol. Vitic.* **2002**, *53* (2), 154−157.

Received for review July 23, 2003. Revised manuscript received December 29, 2003. Accepted January 23, 2004.

JF034824M