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Evaluation of lysozyme stability in young red wine and model systems by a validated HPLC method

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Abstract

Hen's egg lysozyme (LYZ) is added to red wine for preventing malolactic activity. Sulphur dioxide and polyphenolic compounds can depress LYZ solubility and effectiveness, likely by sulphonation of enzyme disulphide bonds or protein binding, respectively. These phenomena were evaluated in either young red wine or model wine systems. A reliable and validated HPLC method was first developed in order to quantify soluble LYZ in wine ($r_{95\%} = 10.7$ mg l^{-1}). The amount of insolubilized LYZ in red wine was related to the content of non-anthocyan flavonoids of low molecular weight and enzyme precipitation occurred in few minutes when flavonoid concentration exceeded 50 mg l^{-1}. Interaction between sulphur dioxide and LYZ occurred in polyphenol-free model wine systems and for the first time the formation of mono-thiosulphonated LYZ was demonstrated. This reaction was favoured by increasing pH value and sulphur dioxide concentration. Nevertheless, sulphonation alone did not fully explain LYZ instability in model wine systems.

Keywords: HPLC; Lysozyme; Sulphur dioxide; Polyphenols; Sulphonation

1. Introduction

Hen's egg lysozyme (LYZ) is used in winemaking in order to prevent the growth of *O. oeni* and alterative lactic acid bacteria. Concentrations exceeding 100 mg l^{-1} are effective against *O. oeni* (Gerbaux, Villa, Monamy, & Bertrand, 1997; Gerbaux et al., 1999) but higher concentrations may be required since the antibacterial activity is affected by a number of factors. Accordingly, the Regulation EC Nr. 2066 (2001) allows up to 500 mg l^{-1} of LYZ to be added to wine or must.

Free sulphur dioxide (SO_2) concentrations exceeding 10 mg l^{-1} decrease antimicrobial activity of LYZ (Amati, Chinnici, & Piva, 1994; Boschelle & Pitotti, 1988), probably upon interaction with disulphide bonds of the enzyme molecule (Cecil & Wake, 1962). Nevertheless, strong denaturing conditions and high SO_2 concentrations are needed to cleave cystine bonds of LYZ (Cecil & Wake, 1962). To date, there is no experimental evidence supporting LYZ thiosulphonation in wine or model wine systems by SO_2 and disulphide bond cleavage should be confirmed for such an enzyme. Additionally, little is known about how oenological pH values affect SO_2 reactivity with the LYZ cystine residues.

Some authors suggest the addition of LYZ to red wine after alcoholic fermentation in order to delay the malolactic activity (Gerbaux et al., 1997) and to perform the micro-oxygenation before the biological deacidification occurs. This is supposed to allow both a less astringent taste and a more intense red colour to be obtained, owing to a more effective procyanidin–anthocyan polymerization. Nevertheless, polyphenols can depress the stability of LYZ in wine and the enzyme precipitation, upon interaction with the tannin fraction (Amati, Chinnici, Piva, & Riponi, 1996; Green & Daeschel, 1994), can affect both the muramidase activity and the composition of the tannin fraction. Little is known about factors affecting this interaction

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and LYZ stability and activity in red wine. Moreover, the experimental criteria described in the literature for evaluating LYZ activity against lactic acid bacteria are a further source of uncertainty. Some authors report the LYZ effectiveness in wine in terms of time needed for the malolactic fermentation to start (Amati et al., 1994; Gerbaux et al., 1999) or as viable cell count after weeks or months from the alcoholic fermentation (Gerbaux et al., 1997). Such criteria do not allow a reliable evaluation of LYZ effectiveness since pH value and SO_2 concentration can modify enzyme activity and affect bacterial growth.

Evaluation of the phenomena involving LYZ, SO_2, pH and tannins requires reliable analytical methods for determining soluble LYZ in wine or must. The enzymatic methods usually applied are less accurate and repeatable in comparison to the chromatographic ones and the native soluble LYZ fraction separated by HPLC seems to correspond to the active enzyme (Daeschel, Musafija-Jeknic, Wu, Bizzarri, & Villa, 2002; Pellegrino & Tirelli, 2000). HPLC methods were proposed for LYZ determination in Champagne wine and must, but precision and accuracy parameters were not provided (Delfini et al., 2004; Marchal et al., 2000). The HPLC procedure proposed by Pellegrino and Tirelli (2000) proved to be interference-free and accurate when complex food matrices like ripened cheeses were analysed. A chromatographic method for LYZ determination in wine is under evaluation by the International Organization of Vine and Wine (Organization Internationale de la Vigne et du Vin, 2001) but the analytical procedure has been not published so far and the features of the method are not available.

In this work the HPLC method proposed by Pellegrino and Tirelli (2000) was modified in order to quantify soluble LYZ in either wine samples or model wine systems. The final goal was to elucidate some of the molecular mechanisms involved in LYZ insolubilization in wine and related to pH value, polyphenol and SO_2 contents.

2. Materials and methods

2.1. Chemicals

All reagents were at least of analytical grade unless otherwise specified. HPLC water was produced by a laboratory scale Milli-Q purification system (Millipore, Billerica, MA). HPLC grade acetonitrile was from Merck (Darmstadt, Germany) and HPLC grade trifluoroacetic acid was from Pierce (Rockford, IL).

2.2. Wine samples

Seven white wine samples and 20 red wine samples from different grape cultivars, winemaking processes and age were collected in an Italian market. These samples were submitted to HPLC determination for evaluating whether the chromatographic separation was interference-free.

Trebbiano white wine was used for the validation of the HPLC method. Two commercial samples of red wine younger than 8 months, from Sangiovese and Nebbiolo grape cultivars, were used to evaluate the effect of flavonoid concentration on LYZ solubility. Two French and 14 Italian *primeur* wine samples (1 month aged), produced from different grape cultivars, were used for evaluating the effect of proanthocyanidins on the solubility of LYZ.

2.3. Model wine systems

A 12% (v/v) hydro-alcoholic solution containing 7 g l^{-1} tartaric acid was brought to pH values in the range 2.9–3.5 with 3 M KOH. The precipitated potassium hydrogen tartrate was removed by filtration.

2.4. Validation of HPLC method

The calibration curve was obtained by analysing 0.5, 1, 2, 4, 10, 20 and 40 mg l^{-1} LYZ standard solutions prepared by diluting a 400 mg l^{-1} water solution of LYZ with 1 M NaCl. Duplicate determinations of each standard solution were performed in 10 days in order to determine linearity and repeatability.

Precision parameters were determined by analysing daily-prepared samples of Trebbiano wine spiked with 80 mg l^{-1}, 200 mg l^{-1} and 400 mg l^{-1} of LYZ and diluted 10 times with 1 M NaCl. The analytical data obtained from duplicate determinations, performed in 8–10 days, were used for the evaluation of day-to-day repeatability and sensitivity of the method.

2.5. Effect of flavonoid concentration on LYZ solubility in wine

Red wine samples from Sangiovese and Nebbiolo grape cultivars were measured for pH value and flavonoid or non-anthocyan flavonoid contents. Then, 0.5 ml of a 25 g l^{-1} LYZ solution were added to 50 ml of wine under magnetic stirring. Aliquots were taken after 0 to 21 h and submitted to HPLC analysis. Additional trials were carried out using wine samples diluted before LYZ addition in order to modify the flavonoid content. To this end, Sangiovese and Nebbiolo wine samples were diluted with a model wine system having the same pH values of the two wines used: 3.42 and 3.25, respectively.

2.6. Solubility of LYZ in young red wines

Fifty millilitres of *primeur* wine samples were slowly treated with 1 ml of a 50 g l^{-1} LYZ solution. After 10 min stirring, the sample was left standing for 24 h at 10 °C an then stirred again for 10 min. Precipitated LYZ was removed by centrifugation at 1000*g* for 15 min. The supernatant was filtered on 0.22 μm pore size filters (Millipore, Billerica, MA) and submitted to HPLC and chemical determinations.

2.7. Effect of SO_2 and pH value on LYZ stability in model wine systems

Model wine systems, at pH 2.9, 3.2 and 3.5, were used to prepare two stock solutions containing 500 mg l^{-1} of LYZ and 100 mg l^{-1} of SO_2, respectively. Fifty millilitres of 250 mg l^{-1} LYZ solutions were prepared by diluting 25 ml of LYZ stock solution with 22.5, 20, 15 and 5 ml of each model wine system. Finally 2.5, 5, 10 and 20 ml of the SO_2 stock solution were added in order to obtain 5, 10, 20 and 40 mg l^{-1} SO_2 solutions. Aliquots of these samples were taken after 1 h and 24 h at 10 °C and diluted with 1 M NaCl solution just before the HPLC separation.

2.8. Determination of total flavonoid and non-anthocyan flavonoid contents

Concentrations of flavonoid fractions were evaluated by means of the spectrophotometric method described by Di Stefano, Cravero, and Gentilini (1989). Wine samples were diluted 1 to 25 with a solution H_2O:ethanol:HCl solution (70:30:1, v/v/v) and the absorbance spectrum was collected in the wavelength range 230–700 nm using a Lambda 15 Perkin Elmer spectrophotometer (Wellesley, MA). The spectral data were used to calculate the concentration of the flavonoid fractions expressed as mg catechin l^{-1}.

2.9. Determination of proanthocyanidin content and index of vanillin

Proanthocyanidins content was determined according to Rigo et al. (2000). Results were expressed as mg cyanidin l^{-1}. The level of vanillin (as mg l^{-1} of catechin) was determined according to Di Stefano et al. (1989) and described by Rigo et al. (2000). The index of vanillin was expressed as vanillin value per proanthocyanidin content (w/w).

2.10. HPLC determination of LYZ

Wine samples were tenfold diluted with 1 M NaCl and filtered through 0.22 μm pore size filters before injection.

HPLC separation of LYZ was performed with an Alliance 2695 system (Waters, Milford, MA) equipped with a PLRP-S 250 × 4.6 mm, 5μm particle size, 300 Å pore size column (Polymer Laboratories, Shropshire, UK) kept at 45 °C. Detection was carried out by a FP-920 Jasco spectrofluorimeter (Tokyo, Japan) at 280 nm excitation wavelength and 340 nm emission wavelength. The chromatographic signal was acquired and reprocessed by Millennium 32 software (Waters, Milford, MA).

The eluting solvent was prepared by mixing 100 g of H_2O/trifluoroacetic acid (0.1% v/v) with 38.4 g of acetonitrile/trifluoroacetic acid (0.1% v/v). The HPLC run was performed at 1 ml min^{-1} flow under isocratic conditions for 25 min, followed by 2 min of column rinsing with acetonitrile/trifluoroacetic acid (0.1% v/v) and 10 min of column equilibration with the eluting solvent.

2.11. Electrospray ionisation-mass spectrometry conditions

Two fractions were collected in correspondence with the chromatographic peaks of interest during the HPLC analysis. The sampled fractions were concentrated under vacuum to about 100 μl, diluted to 500 μl with a methanol/water solution (1:1, v/v) and then infused at 10 μl min^{-1} into the mass spectrometer. Data were acquired in positive mode using a LCQ Deca XP (Thermo Finnigan, San Jose, CA) ion trap equipment. Nitrogen was used as nebulisation gas (relative flow value = 12). Capillary temperature was set at 300 °C, the potential applied was 4.5 kV and the cone voltage was 32 V. Full scan mass spectra were acquired, scanning the range m/z 1500–1900. Deconvoluted mass spectra were obtained using the measured protonated ($[M + nH^+]^{n+}$) molecules with the Bioworks software (Thermo Finnigan, San Jose, CA). Mass accuracy was ensured by calibration with a mixture of reserpine and PFK (in methanol:water 1:1, 0.1% acetic acid), infused separately. All solvents were of HPLC grade.

2.12. Statistics and evaluation of results

A calibration curve was calculated by the least-square regression method. Response factors were calculated by peak area/LYZ concentration ratio and compared by single factor ANOVA. Between days repeatability was calculated according to International Standard Organization (1994). Calculations were carried out using Excel® (Microsoft Corp., Redmond, WA).

3. Results and discussion

3.1. Evaluation of the method performance

The HPLC separation procedure described by Pellegrino and Tirelli (2000) was applied to wine to separate and quantitate soluble LYZ. Before injection, wine samples were diluted with 1 M NaCl; this resulted in decreased electrostatic interactions of LYZ with wine constituents, so preventing enzyme insolubilization. Sodium chloride also increased the analytical response when compared to tartaric or citric acid solutions (data not shown). Due to the high sensitivity of spectrofluorometric detection, dilution was also needed when LYZ concentrations exceeded 50 mg l^{-1} in order to avoid detector signal off-scaling. The analytical method was applied to 27 commercial wine samples from different grape cultivars, country and ageing conditions. No interferences were detected in the related chromatograms, even when undiluted wine samples were analysed (Fig. 1). On this basis, linearity and precision of the HPLC method were evaluated by analysing water solutions containing 0.5–40 mg l^{-1} of LYZ. A linear calibration curve was obtained (Fig. 2) and the response factor (peak area/concentration) showed no significant difference ($p > 0.05$) in the evaluated range, even though precision

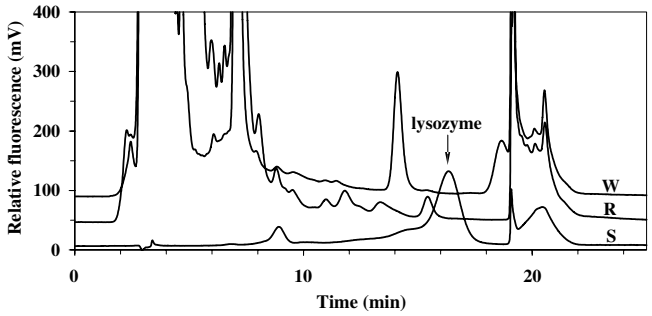

Fig. 1. HPLC patterns of water standard solution (10 mg l^{-1}) (S) of lysozyme and undiluted white (W) and red (R) wine samples.

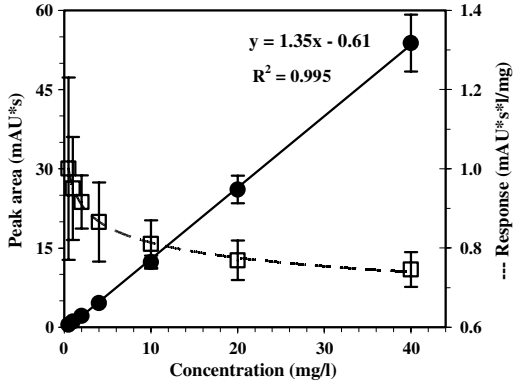

Fig. 2. Calibration curve (●) and response factor (□) for lysozyme concentrations in the range 0.5–40 mg l^{-1}. Data reported are means ± relative (calibration line) or absolute (response factor) standard deviation.

decreased at the lowest tested concentrations (i.e. <2 mg l^{-1}).

In order to evaluate precision paramenters the HPLC method was applied to white wine (Trebbiano grape) spiked with 80 mg l^{-1}, 200 mg l^{-1} and 400 mg l^{-1} of LYZ. The enzyme was dissolved in white wine in order to minimize the occurrence of tannic compounds which are detrimental for LYZ solubility. LYZ precipitation was not related to the added amount. Additions lower than 50 mg l^{-1} were completely insolubilized and about 20–40 mg $l^{-1}$of LYZ were precipitated when amounts exceeding 80 mg l^{-1} were dissolved (Table 1). Such behaviour did not allow the accuracy of the method to be evaluated and the comparison of the method linearity in wine and in standard solution could not be performed as well. For the concentration range considered here, a repeatability mean value of 10.7 mg l^{-1} (reliability 95%) was calculated for undiluted wine. Injected amounts as low as 13 ng (0.25 mg l^{-1}) could be detected when a signal-to-noise ratio higher than 3 was assumed.

Table 1
Precision parameters of the HPLC method calculated for different levels of lysozyme added to wine

	LYZ (mg/l)		
	80	200	400
Duplicates	9	10	8
Mean values (mg l^{-1})	62.7	160.4	357.1
SD (mg l^{-1})	4.5	4.7	5.5
RSD (%)	7.2	2.7	1.6
$r_{95\%}$ (mg l^{-1})	10.1	9.6	12.5
$r_{rel95\%}$ (%)	16.1	6.0	3.5

3.2. Stability of lysozyme in wine and model wine systems

The precipitation of LYZ in red wine is known (Amati et al., 1996; Green & Daeschel, 1994) but quantitative data have been not reported. In order to evaluate the stability of LYZ in young red wine, 250 mg l^{-1} of LYZ were dissolved in either Sangiovese or Nebbiolo wine samples. Precipitation of the enzyme rapidly occurred in the former, just after the addition, so bringing the final concentration to levels (97 mg l^{-1}) reported as ineffective for controlling the activity of lactic acid bacteria (Table 2). Faster and more severe precipitation was recorded in Nebbiolo wine owing to a higher flavonoid content and soluble LYZ was not detectable 30 min after the addition. The two wine samples were then diluted in order to evaluate the effect of non-anthocyan flavonoid level on LYZ solubility. For this purpose, model wine systems having the same pH value and alcohol content of the tested wines were used for dilution. As shown in Fig. 3, lowering the non-anthocyan flavonoid concentration produced only minor changes in LYZ solubility, which exponentially decreased with the tannin level. Enzyme concentrations unable to affect LAB activity were found in wine when flavonoid content exceeded 50 mg l^{-1}. Lower flavonoid levels can hardly be expected in red wine as several hundred milligrams of tannins per litre or more are commonly present. Since the protein-binding capability of tannins can be affected by their molecular structure, the effect of polymerization degree of tannins was investigated. Indices of vanillin, procyanidin and flavonoid contents of 16 commercial samples of *primeur* wine were evaluated before and 24 h after the addition of 1 g l^{-1} of LYZ. The amount of insolubilized non-anthocyan flavonoid was the only parameter significantly ($r^2 = 0.73$) related to the level of insolubilized LYZ (Fig. 4). Over 240 mg l^{-1} of LYZ were precipitated and less than

Table 2
Amount of soluble lysozyme detected in two different red wine samples (250 mg l^{-1}enzyme added) at different times

Time (h)	LYZ (mg/l)	
	Sangiovese	Nebbiolo
0	97	22
0.5	72	tr
1	76	tr
2	71	tr
3.5	39	tr
5	36	tr
21	39	tr

tr: traces.

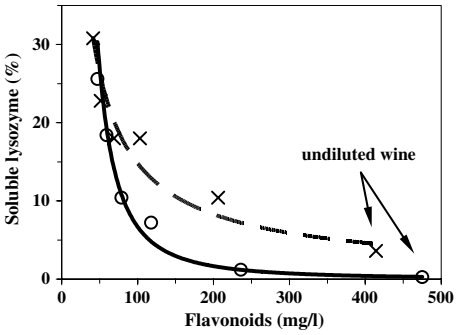

Fig. 3. Levels of soluble lysozyme detected in Sangiovese (X) and Nebbiolo (○) red wine samples (250 mg $l^{-1}$of enzyme added) after dilution with model wine systems.

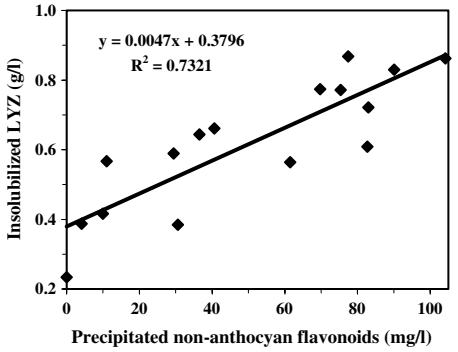

Fig. 4. Amount of lysozyme precipitated by non-anthocyan flavonoids in young red wine samples treated with 1 g l^{-1} of lysozyme.

600 mg l^{-1} of soluble LYZ were detected in 15 out of 16 *primeur* wine samples due to co-precipitation with the flavonoid fraction. Stronger LYZ precipitation could be expected if red wine aimed for ageing is considered, since *primeur* wine is manufactured preventing the extraction of the most astringent tannins or by decreasing the procyanidin content by addition of oenologic coadjuvants. The amount of soluble LYZ remaining in red wine depends on the amount of flavonoids if the permitted amounts are added to red wine. Nevertheless, the flavonoid content alone can not fully explain LYZ instability, since similar amounts of LYZ can be precipitated by very different amounts of non-anthocyan flavonoids. The degree of procyanidin polymerization affects LYZ stability as well. The index of vanillin of the insoluble procyanidin fraction was higher than that of the soluble fraction in 14 out of the 16 samples tested (Table 3), showing that low molecular weight procyanidins are involved in the insolubilization. According to these results, the addition of LYZ to red wine could directly modify the astringent taste of red wine by modifying the qualitative and quantitative profile of the flavonoid fraction. Moreover, LYZ addition could indirectly affect the chemical properties of the tannin fraction following to micro-oxygenation.

As previously shown, the amount of the insolubilized LYZ can be considered to be well correlated with the precipitated non-anthocyan flavonoids, since the *primeur* wine

Table 3
Vanillin indices (as catechin/cyanidin, w/w) detected in *primeur* wine samples before and after the addition of 1 g l^{-1} of lysozyme

Sample	Indices of vanillin of procyanidin fractions (% catechin, w/w)		
	Total	Soluble	Insoluble
1	0.31	0.33	0.28
2	0.40	0.45	0.31
3	0.32	0.31	0.33
4	0.35	0.34	0.37
5	0.41	0.40	0.44
6	0.32	0.25	0.49
7	0.42	0.39	0.50
8	0.33	0.26	0.50
9	0.44	0.41	0.55
10	0.40	0.33	0.55
11	0.42	0.36	0.60
12	0.44	0.39	0.67
13	0.28	0.18	0.72
14	0.32	0.27	0.72
15	0.36	0.30	0.75
16	0.42	0.30	1.00

samples were characterized by either different SO_2 contents or pH values. These two parameters can greatly affect the LYZ stability; nevertheless, data concerning the combined effect of SO_2 and pH value have not been reported in the literature. We investigated these aspects using model wine systems having pH values of 2.9, 3.2 or 3.5 and SO_2 contents ranging from 5 to 40 mg l^{-1}.

Decrease of LYZ stability occurred at all the pH values and SO_2 concentration tested. Moreover, a new peak was observed in the related HPLC chromatograms about 2 min before the peak of soluble LYZ (Fig. 5). The new peak was submitted to mass spectrometry (MS) after collection of the fraction eluted during HPLC separation. According to the mass spectrum, a M_r of 14378.0 Da was assigned to the new peak, 14307.0 Da being that observed and expected for the eluted fraction of native LYZ (Fig. 6). The shift in M_r agreed with the monosulphonation of LYZ. Anyway, the concentration of the thiosulphonate derivative was lower than expected if the residual amount of native LYZ was considered and a

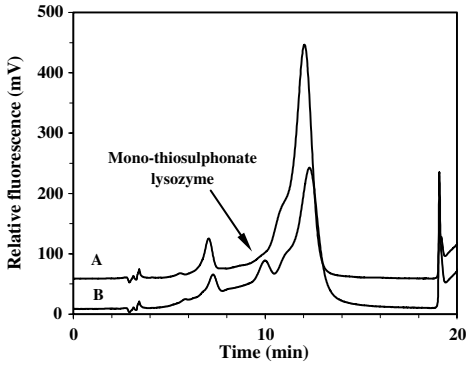

Fig. 5. HPLC patterns of model wine system containing 250 mg l^{-1} of lysozyme at pH 3.5 without (A) and with (B) 40 mg l^{-1} of SO_2 after 1 h standing.

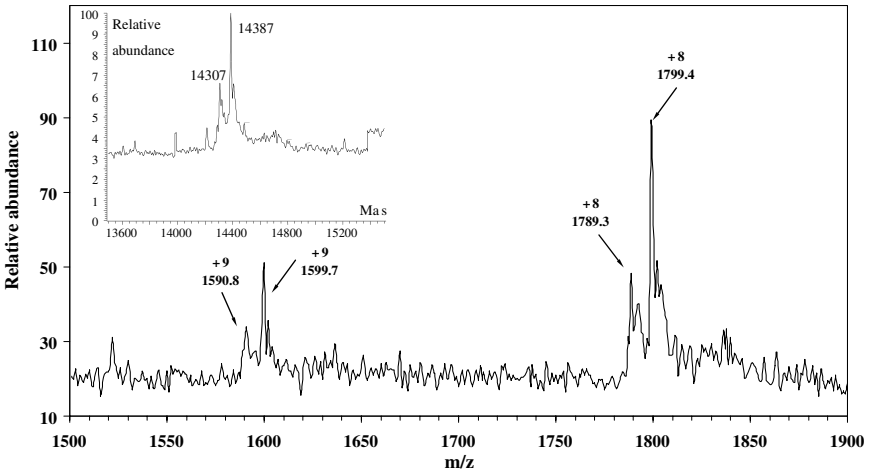

Fig. 6. Full scan mass spectrum of peaks eluting from 8 to 12 min in the chromatogram of Fig. 5. The inner box shows the deconvoluted spectra.

fluorimetric response factor similar to the native LYZ was assumed for the derivative. Such a discrepancy was particularly evident when a 1 h reaction in the model wine system at pH 3.5 with 40 mg l^{-1} of SO_2 was considered (Fig. 7). Under the same conditions, no poly-thiosulphonate forms of LYZ were detected by means of MS experiments, suggesting that stronger denaturating conditions are likely needed to allow the HSO_3^- to reach the cystine residues hidden inside the protein structure (Cecil & Wake, 1962). The additional effect of pH should be considered as well, since the concentration of the more reactive SO_3^{2-} decreased with the pH value. In the same model wine system, the amount of detectable LYZ increased from 93 mg l^{-1} to 168 mg l^{-1} during the 24 h reaction (Fig. 7) while the amount of the mono-thiosulphonate form did not change significantly. These data seem to show that a molecular modification other than disulphide splitting was likely involved in the LYZ–SO_2 interaction. The LYZ modifications induced by SO_2 observed in model wine systems are likely to occur in red wine but simultaneous LYZ–flavonoid interaction prevents a more comprehensive evaluation of all the phenomena involving LYZ insolubilization.

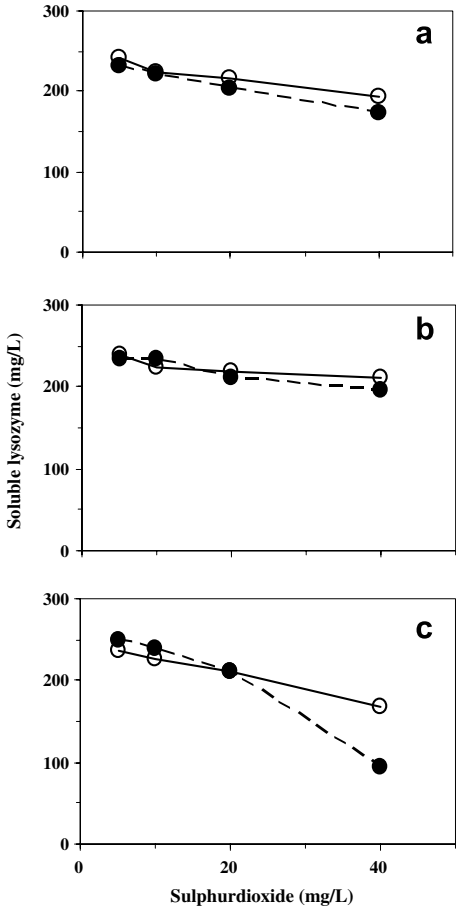

Fig. 7. Variation of native lysozyme level (initial 250 mg l^{-1}) in model wine systems containing different amounts of SO_2 at pH 2.9 (a), 3.2 (b) and 3.5 (c) after 1 h (●) and 24 h (○) standing.

4. Conclusions

LYZ proves to be unstable in young red wine, particularly when both high pH value and high SO_2 concentration occur. This raises some doubts about the effectiveness of this muramidase to control the malolactic fermentation and to modulate the effects of micro-oxygenation during fining. The native form of the enzyme disappears, mainly because of precipitation with low molecular weight tannins but, also, other interactions, including sulphonation are likely involved. In this regard, mono-sulphonation of LYZ was demonstrated in a model wine system. On the basis of our data, the effectiveness of LYZ in red wine should be evaluated more rigorously than previously reported in the literature since enzyme insolubilization may occur.

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