Research Article

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Inhibition and inactivation evaluation of exogenous glycosidases in wines using *p*-nitrophenylglycosides

Alicia G Cid* and Guillermo Ellenrieder

Abstract

BACKGROUND: Glycosidases are often used to improve the aroma of wines via hydrolysis of glycosidic precursors. The aim of this work was to develop a method to test activity and stability of these enzymes in wine environments using *p*-nitrophenylglycosides, avoiding interference by colored substances.

RESULTS: The proposed procedure for determination of glycosidase activity in wines involves quantification of the hydrolysis product *p*-nitrophenol by HPLC. The method was applied to compare the inhibition of some commercial glycosidases by red and white wines. It was found that inhibition of a β-D-glucosidase by ethanol, glucose and gluconolactone was smaller than that produced by wine. This enzyme was also inhibited by wine fractions rich in phenolic compounds and glycosides. When glycoside fractions were first hydrolyzed, their inhibition was strongly reduced. The stability of glycosidases in red wines was tested, showing a destabilizing effect of β-glucosidases that was stronger than in white wine.

CONCLUSION: The method developed allows measurement of glycosidase activity on *p*-nitrophenylglycosides in the presence of colored wine compounds. Comparison of relative glycosidase activity in wines to that in buffer solution under the same conditions can assist in the selection of appropriate aroma-enhancing enzymes.

Keywords: glycosidase inhibition; wine aroma; stability; β-glucosidase

INTRODUCTION

Grape juices and musts for wine production contain small quantities of a wide variety of glycosides.[1] During alcoholic fermentation they are partially hydrolyzed by yeast enzymes, liberating free aglycones;[2] these include monoterpenes, which are responsible for the varietal aromas of wines.[3] The most important glycosides showing this aroma precursor characteristic are *O*-β-D-glucosides and the *O*-diglycosides: α-L-arabino-β-D-glucosides, α-L-rhamno-β-D-glucosides or β-D-apio-β-D-glucosides. Aglycones, which are always directly bound to glucose, are mainly monoterpenes, C13-norisoprenoids and benzene derivatives.[4–7] Most of the enzymes hydrolyzing diglycosides act sequentially; in the first step α-L-rhamnosidase (EC 3.2.1.40), α-L-arabinosidase (EC 3.2.1.55) or β-D-apiosidase liberate the corresponding sugar and a glucoside, which is subsequently hydrolyzed by β-D-glucosidase (EC 3.2.1.21).[1] As hydrolysis is partial, a large proportion of the glycosides remain in the wines and their aroma can even be enhanced by adding exogenous enzymes.[8] Plant, yeast and fungal glycosidases have been used for this purpose.[9,10] Enzymes of fungal origin, in most cases produced by *Aspergillus niger,* are often used due to their high activity, stability, availability and low cost.[11] Independently of the addition of exogenous glycosidases for enhancing aroma, fungal pectinases are used in order to support the process of extraction and clarification of musts. As these pectinases also contain glycosidases such as β-D-glucosidases, α-L-arabinosidases and generally lower levels of α-L-rhamnosidase and β-D-apiosidases, aroma compounds can be liberated in this step of wine elaboration.[12]

The effects of addition of exogenous glycosidases on the liberation of aroma compounds and the simultaneous hydrolysis of glycosides have been well tested in several wines.[2,9,13,14] The methods used permit measurement of the actual effect of added enzymes on specific aroma precursors and their rate of action in wines.[15] However, in spite of possible differences compared to natural glycosides, chromogenic reference substrates of the type of *p*-nitrophenylglycosides are frequently used to characterize these enzymes, to determine their activities in buffer solutions under conditions of the beverages and their inhibition produced by ethanol, glucose and other wine components, in order to predict their behavior in wines. This is due to the simplicity and accessibility of these procedures compared with those of the above-mentioned methods, which often include laborious steps for extraction, identification and quantification of glycosides and aglycones. Besides possible differences with natural glycosides, *p*-nitrophenylglycosides also show limitations when colored substances of wines interfere with the usual colorimetric determination, especially in red wines. Due to this interference, few data exist on the influence of the whole wine components

* *Correspondence to: Alicia G Cid, INIQUI, Universidad Nacional de Salta, Buenos Aires 177, 4400 Salta, Argentina. E-mail: agcid@unsa.edu.ar*

INIQUI, Universidad Nacional de Salta, Buenos Aires 177, 4400 Salta, Argentina

on enzyme activity on *p*-nitrophenylglycosides. The stability of glycosidases in white wines was determined in a few cases,[16,17] but we did not find this type of data for red wines.

In this paper a procedure for determination of the activity of exogenous glycosidases in the presence of any amount of white or red wines, which eliminates the interference of colored substances, is described. Its application to determination of enzyme inhibition and long-term inactivation in wines, and measures related to the effect of other wine compounds on the activity of a β-glucosidase are also reported.

MATERIAL AND METHODS

Materials

Torrontés white and Tannat red wine were produced by a local winery in Cafayate, Salta, Argentina. *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG), *p*-nitrophenyl-α-L-rhamnopyranoside (*p*NPR), *p*-nitrophenyl-α-L-arabinofuranoside (*p*NPA) and gluconolactone were supplied by Sigma (St. Louis, MO, USA), *p*-nitrophenol (*p*NP) by Merck (Darmstadt, Germany) and Amberlite XAD-2 by Supelco (Bellefonte, PA, USA). The enzymatic kit for glucose determination was purchased from Wiener Laboratorios (Rosario, Argentina).

Endozym Rouge of Pascal Biotech was a commercial enzyme used in the winemaking industry. Cellulase 5000 of Biocom was a preparation of high β-1,4-D-glucosidase activity, and α-rhamnosidase HPS 11 518 was from Tanabe, Japan. All enzymes were produced by *Aspergillus niger*.

Wine phenolic compounds

Red and white wine phenolic compounds were extracted according to the procedure of Fernández de Simón *et al*.[18] Fifty milliliters of wine were concentrated under vacuum at 35 °C to about 13 mL. The resulting liquid was extracted three times with 15 mL diethyl ether and three times with 15 mL ethyl acetate. Organic layers were pooled and evaporated at 32 °C under vacuum and the resulting extract was dissolved in 50 mL of 60 mmol L^{-1} tartaric acid/Na tartrate buffer at the pH of each wine.

Wine glycosides

Red and white wine glycosides were extracted by a procedure based on those of Gunata *et al*.[5] and Williams *et al*.,[19] by adsorbing them selectively on Amberlite XAD-2. The column (15 mm × 0.9 mm i.d.) was pre-treated six times with 25 mL methanol and six times with 25 mL diethyl ether, and then washed six times with 25 mL water. After loading 50 mL of wine, resin was washed with 50 mL water and eluted with 50 mL methanol. Glycoside fractions, after solvent evaporation, were re-dissolved in 25 mL of 60 mmol L^{-1} tartaric acid/Na tartrate buffer at the pH of each wine. To reconstitute the original concentration in wine in inhibition experiments, each fraction was diluted with the same volume of a solution containing the other substances of interest. Hydrolyzed white wine glycosides were prepared by incubating a sample containing 0.84 IU mL^{-1} Cellulase 5000 β-glucosidase, 0.16 IU mL^{-1} Endozym Rouge α-arabinosidase and 0.14 IU mL^{-1} HPS 11 518 α-rhamnosidase at 20 °C for 72 h. The hydrolyzed solution was then separated from the enzymes by ultrafiltration in an AMICON cell provided with a PM10 membrane.

Red and white wine glycosides were determined according to Arévalo Villena *et al*.[20] One milliliter of H_2SO_4 (2.25 mol L^{-1}) was added to 0.5 mL glycoside fractions to give solutions for hydrolysis containing 1.5 mol L^{-1} H_2SO_4. A control solution for determination of free glucose concentration was prepared by adding water instead of H_2SO_4. The samples were boiled for 1 h, while controls were held at room temperature. Released glucose was measured using a glucose oxidase assay kit after neutralizing the acid with an equal volume of 3 mol L^{-1} NaOH.

Enzyme assays

Spectrophotometric determination of p-nitrophenylglycoside hydrolyzing activity

In some cases when interference by colored substances was low or absent, the performance of the HPLC method described below was compared with traditional spectrophotometric procedures.[21,22] The activity of β-D-glucopyranosidase, α-L-rhamnopyranosidase and α-L-arabinofuranosidase was determined by measuring the absorbance of the produced *p*-nitrophenol as usual in basic media at its absorbance maxima, 400 nm. The enzymatic reaction mixture contained 0.2 mL of either 1.9 mmol L^{-1} *p*NPG, 2.4 mmol L^{-1} *p*NPR or 1 mmol L^{-1} *p*NPA, with selected buffers and pHs, and the appropriate enzyme amount (generally four parts of a water solution of substrate were mixed with six parts of a buffer solution of enzyme and other eventual additives). These mixtures were incubated for 10 min at the specified pH and temperature (according to the different selected conditions), taking out samples of 50 µL at different time intervals, adding them to 1 mL or 1.5 mL 0.5 mol L^{-1} NaOH in a cold bath and measuring absorbance at 400 nm. A blank without *p*NP-glycoside and another without enzyme were run in each determination. Activity expressed as µmol min^{-1} was calculated from the slope, taking a molar absorptivity, ε, of 16.44 mmol L^{-1}. The spectrophotometric method was also used for standard determination of activities of buffer enzyme solutions at their optimum conditions.

HPLC determination of p-nitrophenylglycoside hydrolyzing activity

When interference from wine colored substances was present in the spectrophotometric test, enzymatic activity was determined by measuring the concentration of the product *p*NP in acid media by HPLC chromatography. Advantage was taken of differences between the retention times of the product and interfering substances. The first part of the method was in this case similar to that of the preceding section, but the 50 µL samples of enzymatic reaction were added to 0.75 mL acetonitrile to stop the reaction and were analyzed by HPLC chromatography. The HPLC system consisted of a Gilson 307 pump, a Gilson 234 autoinjector, a 20 µL sample loop, a Shimadzu C-R8A Chromatopac integrator (Kyoto, Japan), a Gilson 118 UV/Vis detector (Middleton, WI, USA) and a GraceSmart RP 18 5u column (4.6 × 250 mm) (Deerfield, IL, USA). The mobile phase was acetonitrile/water/acetic acid (32 : 67.5 : 0.5, v/v/v) at a flow rate of 0.8 mL min^{-1}. The *p*NP was detected at 316 nm, the wavelength of maximum absorbance in acidic media. Calibration curves were constructed by adding *p*-nitrophenol at different concentrations to the appropriate wine-containing blanks. The analytical performance of this method was tested and compared with that of the spectrophotometric procedure.

Determination of cellobiase activity

Endozym Rouge and Cellulase 5000 β-glucosidase activity was also tested using cellobiose as the substrate. Fifteen microliters of enzyme solution were added to 1 mL of cellobiase 1 g L^{-1} in 60 mmol L^{-1} tartaric acid/Na tartrate buffer pH 3.25. Samples of this mixture were incubated at 50 °C for different time periods up

to 20 min. The reaction was stopped by boiling the samples for 5 min and then glucose, the reaction product, was determined using a glucose oxidase assay kit.

Exogenous glycosidase inhibition by wines and their components

Enzymatic activity on p-nitrophenylglycosides in the presence of wine

The influence of wine on the hydrolysis of *p*-nitrophenylglucoside by Endozym Rouge β-glucosidase was determined at 50 °C by adding an increasing proportion of freeze-dried wine to the reaction mixture for the measurement of activity as described above.

Endozym Rouge α-arabinosidase and β-glucosidase, HPS 11 518 α-rhamnosidase and Cellulase 5000 β-glucosidase activity in 100% wines under the conditions of aroma-enhancing treatments was also measured. To avoid dilution, beverages were reconstituted from freeze-dried wine samples with an appropriate water/ethanol solution plus the corresponding *p*-nitrophenylglycoside and enzyme. Samples were incubated at 20 °C and from time to time the *p*-nitrophenol liberated was measured by the HPLC procedure.

Reversibility of the inhibition of p-nitrophenylglucoside hydrolysis

This was tested by adding Endozym Rouge β-glucosidase to the beverage and determining *p*NP-glucoside hydrolyzing activity. Similar samples were dia-filtrated in an AMICON ultrafiltration cell provided with a PM10 membrane (W.R. Grace & Co., Beverly, MA, USA). The volume was reduced to 10% followed by dilution to its original value with 60 mmol L^{-1} tartaric acid/Na tartrate buffer pH 4. This process was repeated three times and activity was measured again.

Inhibition of Endozym Rouge β-glucosidase

Endozym Rouge β-glucosidase inhibition produced by several compounds present in wines was studied. Enzyme activity was measured spectrophotometrically at pH 3.25 and 3.6 in the presence of ethanol (10 and 15%), glucose (0.43 and 2.13 g L^{-1}), gluconolactone (0.02 and 0.1 mmol L^{-1}) and phenolic compounds extracted from white and red wines; and by HPLC in the presence of glycosidic extracts of both wines and hydrolyzed white wine glycosidic extract.

Enzyme stability in wines

In order to determine their stability in Torrontés white and Tannat red wines, glycosidases were added to the beverages and incubated at 20 °C at levels recommended for aroma enhancement.[23] At selected times samples were taken out and always added to the same volume of *p*-nitrophenylglycoside solution to determine residual activity at 50 °C. The linearity of activity versus fresh enzyme concentration was first tested in samples without incubation. Comparative enzyme stability was also tested in 60 mmol L^{-1} tartaric acid/Na tartrate buffer pH 3.6 under the same conditions used to measure stability in wines.

At least two replicates were measured in each determination. Statistical analyses were carried out using the PRISM software of GraphPad (San Diego, CA, USA).

RESULTS AND DISCUSSION

The inhibition and stability of exogenous glycosidases in wine are important properties to take into account in order to guarantee an effective aroma-enhancing process. Studies presented in this paper aimed to obtain general information on methods to estimate inhibition and inactivation of enzymes inside wines using *p*-nitrophenylglycosides as substrates. Catalysts were selected to include common aroma-liberating fungal glycosidases with different degrees of stability and inhibition to test the effect of two wines on these properties. They were not necessarily the optimum for the final aroma enhancement process of these wines, but these methods can obviously be applied to catalysts from other promising sources.

Measurement of glycosidase activity on *p*-nitrophenylglycosides in the presence of wines

Wine aroma-liberating exogenous enzymes are frequently characterized by means of their hydrolyzing activity on chromogenic substrates such as *p*-nitrophenylglycosides. However, interference in measurements often occurs when samples of wines are introduced to the system. This is due to the presence of colored substances in the beverages that have high absorbance at 400 nm, the maximum wavelength of *p*NP in the test basic media. Interference is thus higher in red than in white wines. In order to avoid this problem we determined the concentration of *p*-nitrophenol (*p*NP) produced by *p*-nitrophenylglycoside hydrolysis chromatographically by HPLC according to the method described above. The product was in its non-ionized form due to the acidic pH of the medium, and it could be separated satisfactorily from other absorbing substances by the C18 column. As the product was also chromatographically separated from *p*NPG substrates (retention times between 4 and 6 min) it was not necessary to make the medium basic for its quantification. Absorbance was then recorded at the maximum wavelength for *p*NP under these conditions (316 nm). After complete elution of several peaks, *p*NP emerged with a retention time of 14.8 min. By adding different *p*NP quantities to the wine sample, a linear absorbance (peak area) versus concentration relationship was obtained which could be used as a calibration curve (Fig. 1). Measurements of enzyme activities on *p*NP-glycosides in wines carried out with this technique yielded values with an average coefficient of variation (standard deviation divided by the mean) of about 5% (see Table 1). Comparison of the HPLC chromatographic method with the conventional spectrophotometric one indicated no statistically significant differences when activity was measured in buffer solution. A value of $P = 0.23$ was observed using a paired *t*-test for eight activity determinations of this type. However, comparing both methods in a clear Torrontés white wine, the spectrophotometric determinations yielded values up to about 20% higher than those using HPLC. This difference was statistically significant ($P = 0.013$) when the same test was applied. Given these differences, the colorimetric technique was used in only a few cases in which no colored substances were present.

Inhibition of enzymatic *p*-nitrophenylglycoside hydrolysis by wines and their components

It is well known that several wine components inhibit wine aroma-enhancing glycosidases;[7] *p*NP-glycosides are generally used to quantify this inhibition. These determinations are usually carried out in buffer solutions at wine pH (and sometimes wine processing temperature), but they do not include the whole wine components. Measurement of activity on these substrates carried out in wine could provide the necessary information to determine whether the inhibition is produced only by the known components or if other substances participate in the reduction

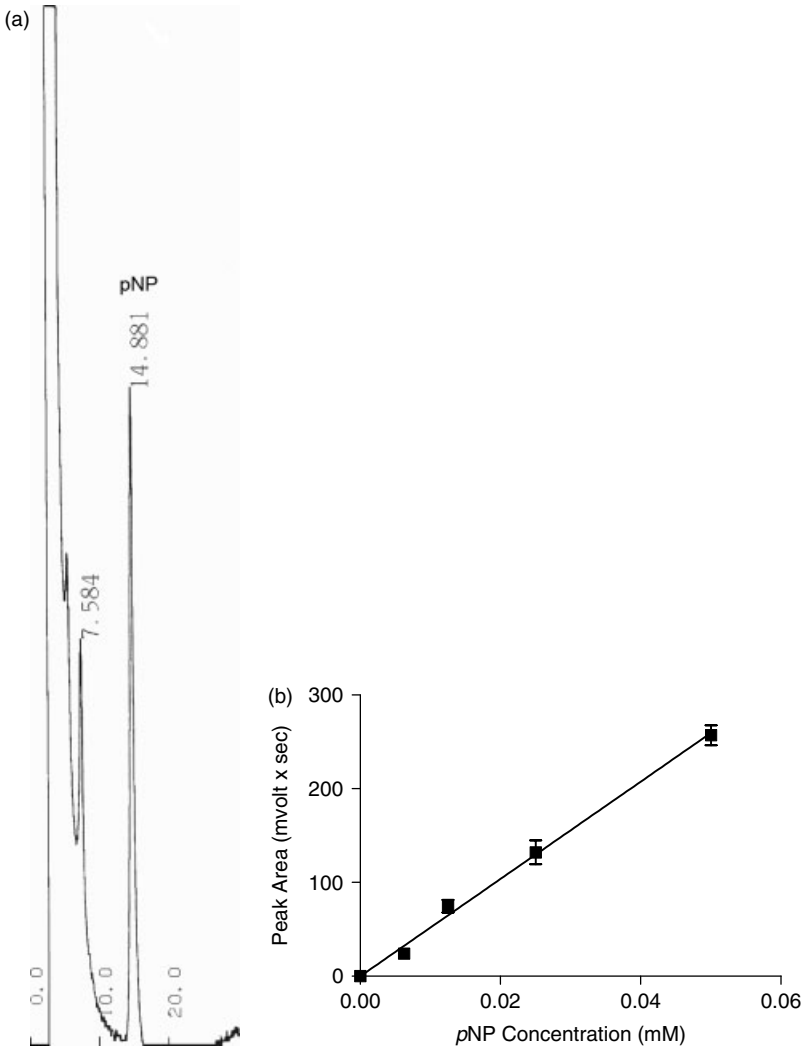

Figure 1. (a) HPLC chromatogram of *p*-nitrophenol in Tannat red wine. (b) Calibration curve of *p*-nitrophenol in Tannat red wine.

Table 1. Activity of several glycosidases in Torrontés white and Tannat red wines at 20 °C

Enzyme	Optimum pH	A_{opt} (IU mg^{-1})	pH 3.25 Activity (U mL^{-1})	pH 3.25 Relative activity (%)	pH 3.6 Activity (U mL^{-1})	pH 3.6 Relative activity (%)
			Torrontés		Tannat	
β-Glucosidase Cellulase 5000	4.5	0.31 ± 0.01	$(4.14 \pm 0.18) \times 10^{-4}$	3.4 ± 0.3	$(5.02 \pm 0.2) \times 10^{-4}$	5.3 ± 0.4
β-Glucosidase Endozym Rouge	4	0.077 ± 0.001	$(1.14 \pm 0.07) \times 10^{-4}$	1.81 ± 0.14	$(5.8 \pm 0.3) \times 10^{-4}$	0.99 ± 0.07
α-Arabinosidase Endozym Rouge	4	0.080 ± 0.001	$(2.17 \pm 0.08) \times 10^{-3}$	72 ± 5	$(1.21 \pm 0.04) \times 10^{-3}$	37 ± 3
α-Rhamnosidase HPS 11 518	3.25	20.9 ± 0.1	$(1.37 \pm 0.04) \times 10^{-3}$	35 ± 3	$(1.4 \pm 0.2) \times 10^{-3}$	40 ± 6

A_{opt}, activity at the optimum pH and 50 °C.
Relative activity is (activity in wine/activity in buffer under the same conditions) × 100. Buffer: 60 mmol L^{-1} tartaric acid/Na tartrate
Enzyme added to samples: β-glucosidase, 0.16 U mL^{-1}; α-Arabinosidase, 0.025 U mL^{-1}; α-rhamnosidase, 0.027 U mL^{-1}.

of activity. The activity of Endozym Rouge β-glucosidase on *p*NP-glucoside was determined in the presence of increasing proportions of wines according to the method described above. This enzyme is used in the wine elaboration process as a pectinase to help the process of extraction and clarification of must, but its utility in aroma liberation is also mentioned by its suppliers. It is employed locally in the elaboration of Tannat red wine. The results of the effect of Torrontés white and Tannat red wine on the activity of β-glucosidase are shown in Fig. 2. It can be seen that both wines produced strong inhibition of the hydrolysis of *p*-nitrophenylglucoside, that by the red one being the greatest.

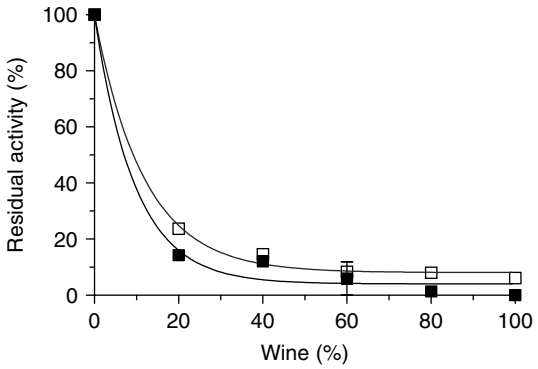

Figure 2. Effect of addition of wines to a buffer solution of Endozym Rouge β-D-glucosidase on *p*NPG hydrolysis. Residual activity: (activity/activity in buffer at same pH) $\times$ 100. The reaction mixture consisted of 2 mmol L^{-1} *p*-nitrophenyl-β-glucoside; buffer: 60 mmol L^{-1} tartaric acid/Na tartrate; temperature: 50 °C; added enzyme: 0.05 IU mL^{-1}. (□) Torrontés white wine: pH 3.25; 100% activity: 0.046 $\pm$ 0.001 U mL^{-1}. (■) Tannat red wine: pH 3.6; 100% activity: 0.027 $\pm$ 0.002 U mL^{-1}.

The activity of some potential aroma-enhancing glycosidases on *p*-nitrophenylglycosides in a 100% wine environment is shown in Table 1; activity relative to that of the same enzyme in buffer solutions under similar pH (3.25 and 3.6) and temperature (20 °C) is also indicated. The glycosidases correspond to three commercial *A. niger* preparations: Endozyme Rouge, Cellulase 5000 and HPS 11 518.

Endozym Rouge had an activity as shown in Fig. 2 and as mentioned above. Cellulase 5000 was a preparation rich in cellobiase (4-*O*-β-D-glucopyranosyl-D-glucose hydrolase) activity. Le Traon-Masson and Pellerin[24] found that a β-D-glucosidase from *A. niger* showing this type of activity is also very active toward glycoside precursors and contains low levels of discoloring activity. Activity on cellobiose of Cellulase 5000 and Endozym Rouge preparation was 218 $\pm$ 8 and 37 $\pm$ 2 IU g^{-1}, respectively; that on *p*NP-glucoside is indicated in Table 1. HPS 11 518 had high α-rhamnosidase and very low β-glucosidase activity.[25]

The β-glucosidase activity of these enzymes was much more reduced in wines than α-arabinosidase and α-rhamnosidase activities. Given that the action of β-glucosidase is necessary for the hydrolysis of all the glycosides and the consequent liberation of aroma compounds, this result is important for the selection of appropriate enzymes. It can also be seen in Table 1 that the β-glucosidase of Cellulase 5000 was more inhibited in Torrontés than in Tannat, whereas the reverse was true for the β-glucosidase and α-arabinosidase of Endozym Rouge, showing that inhibition of potential aroma-liberating enzymes depends on the characteristics of the wine. This property should thus be tested individually for each enzyme–wine system.

Reversibility of enzyme inhibition by wines

The reduction in activity of fresh solutions of enzymes in wines with respect to that in buffer under the same conditions is a fast effect that may be due either to reversible binding of inhibitors or to an irreversible chemical modification of the enzymes. Inhibition of Endozym Rouge β-glucosidase by white wine was demonstrated to be totally reversible by removing the low molecular weight components of the beverage by ultrafiltration. After that, almost 100% of β-glucosidase activity was recovered (0.853 $\pm$ 0.028 U against 0.845 $\pm$ 0.007 U of the original activity). Reversibility of β-glucosidase inhibition by Tannat red wine could not be demonstrated by this procedure because high molecular weight components of the wine either inhibited the enzyme or retained inhibitors during the ultrafiltration process. The high molecular weight fraction of the red wine, isolated in the absence of the enzyme by the dia-filtration procedure, caused appreciable inhibition when added to buffer solutions of the enzyme.

Table 2. Endozym Rouge β-glucosidase activity (%) on *p*NPG in the presence of several wine components in buffer solution

Inhibitor	pH 3.6	pH 3.25
Ethanol		
10%	100.5 $\pm$ 0.9	97.8 $\pm$ 0.9
15%	94.3 $\pm$ 2.9	95.7 $\pm$ 4.3
Glucose		
0.43 g L^{-1}	85.1 $\pm$ 0.6	93.5 $\pm$ 1.6
2.13 g L^{-1}	59.2 $\pm$ 1.7	69.0 $\pm$ 2.1
Gluconolactone		
0.02 mmol L^{-1}	89.9 $\pm$ 9.2	96.7 $\pm$ 3.0
0.10 mmol L^{-1}	63.5 $\pm$ 4.7	64.7 $\pm$ 2.1
Phenolic compounds		
Tannat red wine	87.4 $\pm$ 5.7	–
Torrontés white wine	–	94.0 $\pm$ 5.0
Glycosides		
Tannat red wine	38.7 $\pm$ 0.3	–
Torrontés white wine	–	64.1 $\pm$ 1.0
Hydrolyzed Torrontés white wine glycosides	–	92.8 $\pm$ 1.6
Whole wine		
Tannat red wine	5.2 $\pm$ 0.3	–
Torrontés white wine	–	6.1 $\pm$ 0.3

Temperature, 50 °C; *p*NPG, 2 mmol L^{-1}; 100% activity, 0.05 U mL^{-1}.

Inhibition of β-glucosidase in buffer solution by individual wine components

The high level of inhibition of β-glucosidases observed in Table 1 could be due to the simultaneous effect of several known inhibitors such as ethanol, glucose and gluconolactone.[7] Their effect was tested on Endozym Rouge β-glucosidase up to levels corresponding to the highest concentration expected for these inhibitors in wines.[26,27] Inhibition in the whole wines was in both cases larger than the additive effects of the mentioned inhibitors in the most unfavorable conditions (Table 2). To determine the eventual presence of other inhibitors, phenolic substances and glycoside fractions of white and red wines were prepared and tested (the total amount of glycosides of the red and white wines was 128 $\pm$ 17 and 118 $\pm$ 7 nmol mL^{-1} respectively). The results shown in Table 2 indicated that both wine components produced appreciable inhibition. It is also possible that other substances or impurities present in the glycoside fractions could also cause the observed inhibition because they were not extensively purified. However, fractionation procedures effectively remove hydrophilic inhibitors such as ethanol and glucose. The level of glucose measured in the glycoside fractions was about 0.01 g L^{-1}, a value appreciably smaller than those in Table 2. Furthermore, a blank fractionation test carried out with a gluconolactone solution in buffer, indicated that it was eluted in the first water fraction and that it was completely absent in the methanol fraction from

the XAD-2 column (corresponding to a glycoside fraction). To estimate the actual inhibition by glycosides, samples of the white wine fraction were hydrolyzed as described previously and their effect on Endozym Rouge β-glucosidase activity on *p*NPG was determined (this procedure was not applied to the red wine glycoside fraction because some of its inhibitors are retained by the ultrafiltration membrane). After this treatment the inhibition was substantially reduced (Table 2), showing that glycosides at least partially inhibit the enzyme. This result can be explained by the fact that the enzymatic systems consist of several β-glucoside substrates, which are hydrolyzed by only one enzyme (or a few enzymes showing this activity). All the β-glucosides present in the system will then compete with *p*NPG for the binding site in the enzyme, acting as competitive inhibitors. As only a few of the whole wine glycosides are responsible for the aroma enhancement of these beverages, the eventual inhibition of their hydrolysis by the other glycosides will probably be caused by a group of compounds very similar to those causing *p*NPG inhibition.

Inhibition of *p*NP-glycoside hydrolysis by whole wines may be a useful method for detecting the joint inhibitory effects by all of their components. Although these chromogenic substrates can be hydrolyzed at a rate that differs from that of natural substrates responsible for the aroma liberation, it is probable that both are inhibited by the same substances in a more or less similar proportion. Consideration of the most common theoretical cases of multiple competitive, uncompetitive or non-competitive inhibitors indicates that, unlike inhibited absolute enzyme activity, activity relative to that of enzymes in buffer under the same conditions does not depend on the maximum rate kinetic constant. It is therefore reasonable to include measures of whole wine relative inhibition in the characterization of enzymes when using *p*NP -glycoside reference substrates.

Stability of exogenous glycosidases in wines

Measurements of glycosidase stability using *p*-nitrophenyl-glycosides to test residual activity in wine during long-term enzymatic treatments of white wines were reported by Günata *et al*.[17] However, for evaluation of red wine stability, interference is too high to use the colorimetric method, and generally this enzyme property is determined only in buffer solution or model wines.[28] The effects of some components on red wine stability can therefore be missed. The chromatographic technique described above thus becomes appropriate for this determination because all destabilizing wine components can act during the experiment. In the case of Torrontés and Tannat wines some enzyme activities of added exogenous glycosidases were very low due to the strong inhibition in samples taken from beverages and the additional inactivation of the catalyst. Therefore, the residual activity of glycosidases during incubation at 20 °C was measured at 50 °C because it was about 10 times higher at this temperature. In order to avoid pitfalls caused by the unknown behavior of inhibitors, the linearity of activity on *p*-nitrophenylglucoside versus enzyme concentration was tested using fresh enzyme solutions in the presence of wine aliquots of constant volume. Such linearity was indeed observed in all cases.

The stability of β-D-glucosidases in the wines studied is shown in Fig. 3. By comparing this with the observed behavior of the enzymes in buffer solution under the same conditions, it can be seen that both β-D-glucosidases were destabilized by wine components, the effect being stronger in red wine than in white. Cellulase 5000 β-glucosidase showed very good stability in buffer. In white wine the residual activity after 15 days incubation was about 77%; this value was similar to that previously found for the

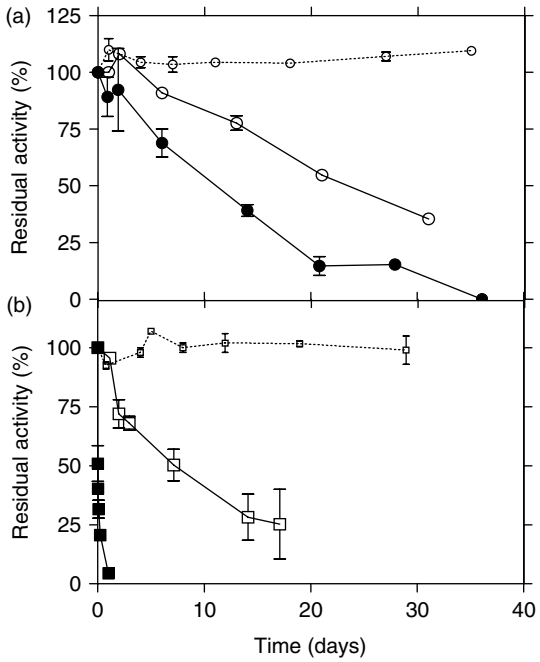

Figure 3. Stability of β-glucosidases in Torrontés white and Tannat red wines. The incubations were carried out at a temperature of 20 °C, and 0.15 IU mL^{-1} enzyme was added to the samples. Broken lines: 60 mmol L^{-1} tartaric acid/Na tartrate buffer pH. Open symbols and solid lines: Torrontés (pH = 3.25). Solid symbols and lines: Tannat (pH = 3.6). (a) Cellulase 5000 β-glucosidase, 100% activity: 0.019 ± 0.002 U mL^{-1}. (b) Endozym Rouge β-glucosidase, 100% activity: 0.012 ± 0.001 U mL^{-1}.

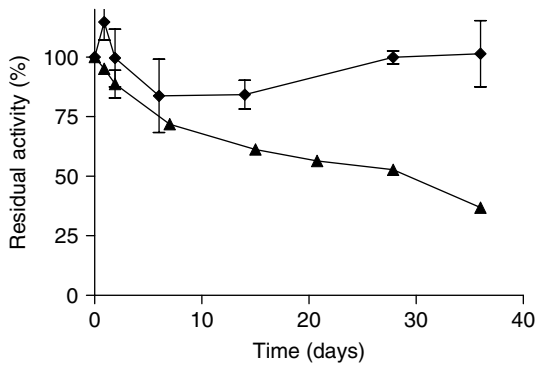

Figure 4. Stability of α-L-rhamnosidase and α-L-arabinosidase in Tannat red wine at 20 °C. (▲) HPS 11 518 α-rhamnosidase (added enzyme: 0.04 U mL^{-1}); (♦) Endozym Rouge α-arabinosidase (added enzyme: 0.02 U mL^{-1}).

Klerzyme 200 enzyme in a Muscat grape juice,[17] but in Tannat wine after the same treatment the value was about 35%. β-Glucosidase of Endozym Rouge was also stable in buffer at wine pH and temperature, but activity decreased continuously in Torrontés and was completely lost during the first day of incubation in Tannat, showing that this enzyme was not appropriate for aroma enhancement of these wines. These results showed that measures of stability of enzymes in buffer at wine pH and temperature conditions could be insufficient, and appreciable differences could exist between different wines. Figure 4 shows the stability of Endozym Rouge α-arabinosidase and HPS 11 518 α-rhamnosidase in Tannat red wine; the former was the most stable, and the latter

showed intermediate behavior, both glycosidases being more resistant than the tested β-glucosidases.

Unlike the inhibition experiments of the previous section, differences between activity on *p*-nitrophenylglycosides and natural glycosides do not affect this type of measurement because data on activity referred to the initial activity and are taken in these determinations as a measure of the remaining intact enzyme concentration. Stability data are very important for the selection of exogenous enzymes for use as immobilized catalysts. Working with a free glycosidase, activity must be sufficient only during the period of aroma liberation, but if it is immobilized, stability must be the highest possible to permit its prolonged use, in this way decreasing its effective cost.

CONCLUSIONS

The described method of testing *p*NP-glycosides hydrolysis by HPLC is appropriate to determine glycosidase activity in 100% wines. Activity in wines, expressed in relation to that of the enzyme in buffer solution under the same conditions, can be used as a reference property accounting for inhibition produced by wine components. Comparison of the effect of wine with that of the common inhibitors can reveal the presence of other inhibitory compounds. The described method also makes it possible to measure the slow inactivation of glycosidases in red wines. The high level of inhibition observed for the studied β-glucosidase of fungal origin in wines suggests that these methods must be applied to test more appropriate catalysts coming from other promising sources, such as wine yeast.[2,29,30]

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