Influence of Yeast Macromolecules on Sweetness in Dry Wines: Role of the Saccharomyces cerevisiae Protein Hsp12

Axel Marchal[*†], Philippe Marullo[†‡], Virginie Moine[‡], and Denis Dubourdieu[†]

[†] UMR 1219 Oenologie, Institut des Sciences de la Vigne et du Vin, Université Victor Segalen Bordeaux 2, 210 chemin de Leysotte CS 50008, 33882 Villenave d'Ornon Cedex, France

[‡] Laffort group, BP 17, 33015 Bordeaux, France

Corresponding author:

Axel Marchal

axel.marchal@u-bordeaux.fr

Abstract:

Yeast autolysis during lees contact influences the organoleptic properties of wines especially by increasing their sweet taste. Although observed by winemakers, this phenomenon is poorly explained in enology. Moreover, the compounds responsible for sweetness in wine remain unidentified. This work provides new insights in this way by combining sensorial, biochemical and genetic approaches. First, we verified by sensory analysis that yeast autolysis in red wine has a significant effect on sweetness. Moderate additions of ethanol or glycerol did not have the same effect. Second, a sapid fraction was isolated from lees extracts by successive ultrafiltrations and HPLC purifications. Using nano-LC-MS/MS, peptides released by the yeast heat shock protein Hsp12p were distinctly identified in this sample. Third, we confirmed the sweet contribution of this protein by sensorial comparison of red wines incubated with two kinds of yeast strains: a wild-type strain containing the native Hsp12p and a deletion mutant strain that lacks the Hsp12p protein (Δ°*HSP12* strain). Red wines incubated with wild-type strain showed a significantly higher sweetness than control wines incubated with Δ°*HSP12* strains. These results demonstrated the contribution of protein Hsp12p in the sweet perception consecutive to yeast autolysis in wine.

Keywords: wine, sweetness, yeast lees, autolysis, ethanol, glycerol, peptide, *HSP12*

INTRODUCTION

Most of fermented beverages and wine in particular are kept in contact with their lees after the end of alcoholic fermentation. During this time, which can vary from a few days to several months depending on the type of wine, yeasts undergo autolysis. Consequently a large number of biochemical and physicochemical changes take place in the wine leading to major modifications of its organoleptic properties.

For example, “aging on lees” is an elaboration technique of some white wines historically used in Burgundy but now developed all over the world. During this aging, the interactions between the wine, the lees and possibly the wood lead to a final improvement of aroma, taste and physicochemical stability of the wines that were studied. In particular, the lees modify the woody flavor of white wines by reducing the vanillin[1] and producing furanmethanethiol from the furfural of heated wood,[2] which respectively decreases the vanilla flavor and increases the toasted flavors.

Aging on lees also raises the parietal polysaccharides content of wine, and particularly the levels of mannoproteins.[3] These molecules contribute toward enhancing the protein[4] and tartaric[5] stability of wines. They also prevent wine from pinking on oxidation[6] and from developing bitterness; polysaccharides can combine with phenolic compounds of the wine and of the barrel.[1] Moreover, because they are able to bind dissolved oxygen[7] and to release glutathione,[8, 9] lees protect certain fruity aromas from oxidation and prevent the development of defective aging aromas. At the same time, the cysteine residues within the mannoproteins released from the lees can combine with thiols to form disulfide. This reduces the levels of compounds (such as methanethiol or ethanethiol) that would otherwise impart unpleasant odors.[10]

In red wines, yeast autolysis occurs at a higher temperature (about 30 degrees) and in a shorter time (about ten days) than in white wines and corresponds to the phase called “post-fermentative maceration”. During this time, the physicochemical and organoleptic properties of red wines are also significantly modified, and it has been reported[11] that glycoproteins of parietal origin provided by yeast autolysis seem to interact with polyphenolic compounds, resulting in a decrease in the astringency sensations attributed to these compounds.

In addition to these benefits, winemakers also note empirically a gain in sweetness of both white and red wines during the period of contact with their lees. This perceived sweetness

might be due to compounds exhibiting a genuine sweet taste as well as by a masking effect on sourness and bitterness.

However, to our knowledge no work providing a chemical or biochemical explanation of this phenomenon has been published to date.

Ethanol[12] and glycerol[13] exhibit a sweet taste in aqueous solutions that is also slightly perceptible in wines,[14] but recent works have shown that they have no significant impact on sweetness in dry wines.[15, 16] Aside from the controversial status of ethanol and glycerol, they are of little consequence in perceived sweetness of most of dry wines.

These observations suggest the existence of other sweet compounds in dry wines, potentially originating from yeast autolysis as suggested previously. It has been shown that neither polysaccharides nor proteins (Jones et al.[16]) nor nucleotides (Charpentier et al.[17]) affect sweetness at conventional concentrations.

Even if many peptides such as aspartame[18, 19] are well-known to be highly sweet, no study demonstrating the role of a peptidic wine fraction on sweetness has been published until now. Owing to the difficulty and lack of specific techniques of their analysis,[20, 21] peptides remain indeed the least known nitrogenated substances in wine. However, these compounds exhibit several properties, such as gustatory activity,[22] tensioactivity[23] and antihypertensive activity.[24, 25]

The aims of the present investigation were first to validate the role of yeast lees on the increase of sweetness empirically observed during the autolysis process, and then to identify the chemical or biochemical origin of this phenomenon. To assay the impact of lees on the sweet taste of wine, autolysis conditions in red wines were reproduced and the resulting wine was submitted to sensorial analysis. To elucidate the origin of the proteins responsible for sweetness, a fraction was isolated from yeast extracts and analyzed by LC−MS. The role of the identified protein was confirmed by sensorial analysis. To our knowledge, it is the first identification of a protein from *Saccharomyces cerevisae* generating sweet peptides during the autolysis process. In addition to these findings, new insights to understanding the sweet taste of dry wines are provided that establish and explain the gustatory impact of yeast lees.

MATERIALS AND METHODS

Biological Material. *Strain and Culture Conditions.* *Escherichia coli* DH5α was used for cloning experiments. *E. Coli* cells were grown as described previously.[26] The

Saccharomyces cerevisiae strains used are listed in Table 1. The strain Zymaflore Fx10 (Laffort, France) is a homothallic, fully homozygous strain referenced as H4-1D in a previous work.[27] Yeast strains were propagated at 28 °C on YPD medium (1% yeast extract, 2% peptone, dextrose 2%) complemented with 2% agar to prepare a solid medium. For pZEO induction, dextrose was replaced by maltose 2%. For the selection of genetic markers KANMx, BLER, and HYGR the drugs G418, phleomycin and hygromycin B (Sigma, France) were used at final concentrations of 1 μg/mL, 1 μg/mL and 50 μg/mL, respectively.

Construction of Δ°hsp12 Null Mutant. Yeast transformations were carried out by chemical permeabilization according to Gietz protocols.[28] The Δ°*hsp12* null mutant was constructed using short-flanking homology PCR of *HSP12* According to Guldener et al.,[29] this strategy allows the deletion of the *HSP12* gene by the *loxP::kanMX::loxP* cassette. This cassette can be then excised using the Cre recombinase by transforming deleted strains with the pZEO plasmid. As the Fx10 is diploid, a haploid derivative (YPM32) was constructed to easily carry out the *loxP::kanMX::loxP*/Cre recombinase system. The *ho::HYG*^{R} cassette was obtained by PCR using the p26 (5′AAATCGAAGACCCATCTGCT) and p36 (5′GTCACGAAAAGTGATGTAAC) primers and the genomic DNA of RG1 strain as template. The YPM33 strain was obtained transforming YPM32 with the *loxP::kanMX::loxP* cassette containing flanking regions of *HSP12* gene. This cassette was obtained by using p484 **gataatctcaaacaaacaactcaaaacaaaaaaaactaaatacaacaGCATAGGCCACTAGTGGATCT G**; p485 **tcacacatcataaagaaaaaaccatgtaactacaaagagttccgaaagatCAGCTGAAGCTTCGTACGC** primers (*HSP12* homology sequence in lowercase, sequence for *loxP::kanMX::loxP* in upper case) and the pUG6 plasmid as template. The YPM34 strain was then obtained transforming YPM33 with pZEO allowing the excision of *loxP::kanMX::loxP* cassette at *HSP12* locus. After *loxP::kanMX::loxP* cassette excision the pZEO plasmid was removed by successive subculture in nonselective media. The YPM35 hybrid was obtained by crossing YPM34 with a spore of Fx10 by using a micromanipulator. This hybrid is heterozygous for *HO* locus (*HO/ho::HYG*) and for *HSP12* locus (Δ°*hsp12/HSP12*); the Δ°*hsp12* null mutant strain is an appropriate YMP35 meiotic segregant containing the *HO* gene and the deleted copy of *HSP12*. This spore clone undergoes a self-diploidization due to its functional endonuclease *HO*.[30] The genetic construction of all strains was verified by PCR. The diploid status of Δ°*hsp12* was verified by its positive sporulation of ACK medium. The genetic information of strains and plasmids is shown Table 1.

Sensorial Analysis. *Materials and Sample Preparation.* The term "model solution" used in this article describes a hydroalcoholic solution with 12% ethanol (v/v) and 3 g/L of tartaric acid and readjusted at pH 3.4 with NaOH (1 mol/L). Fractions tasted in synthetic solution were added in quantity corresponding to the same volume before freeze-drying and dissolved prior to the sensorial test (Table 2).

The wine used in this study was a red wine from AOC Bordeaux 2006 (12.2% ethanol, 6.9 g/L glycerol, 0.37 g/L glucose + fructose). The different modalities used are summarized in Table 2.

For ethanol and glycerol (Sigma, St. Louis, MO, USA) tasting assays, these compounds were added to red wines in defined concentrations after bottle opening, one hour before the tasting session. For yeast lees tasting assays, wines were prepared as described as follows.

Yeast cultures obtained from YPD medium were harvested by centrifugation (4000 rpm, 10 min), and the pellet was washed twice. Yeast concentration was estimated by OD_{600nm}, and increasing quantities (see Table 2) were added in 1 L of red wine into a sterilized glass flask. Yeast autolysis was carried out, and this medium was left at 32 °C during 10 days without light or stirring. At the end of this period, the wines were centrifuged (4000 rpm, 10 min) to eliminate yeast lees, and kept at 4 °C for 24 h in sterilized glass bottles inerted with CO_2 until tasting.

General Tasting Conditions and Panel. All tasting sessions took place in a dedicated room equipped with individual booths and air-conditioned at 20 °C. The samples (20 mL) were presented in normalized dark glasses coded with random numbers.

All the panelists (22−59 years) were wine-tasting specialists or wine-making professionals and had been previously informed of the nature and risks associated with the present investigation. The number of panelists (*n*), indicated in Table 2, varied between the different tests depending on the type of test and on their personal availability.

Tests Used for Sensorial Analysis. Two kinds of sensorial tests were used in this study.

Test 1 (ranking test): To evaluate the influence of a given factor on perceived sweetness in wine, four glasses corresponding to modalities 1 to 4 as described in Table 2 were presented in randomized relative positions to the assessors, who were asked to classify the samples from less to more sweet.[31]

Test 2 (triangular test): To assess the taste properties of given samples, triangular tests were used. Three glasses filled with 20 mL were indeed presented to the panelists in randomized and equilibrated positions. Two of these three glasses contained the same solution, and the assessors were asked to choose the different one.[32]

Statistical Analysis. Results obtained from sensorial tests were statistically interpreted following the norms published by the international organization for standardization (ISO). Test 1 consists of a ranking test with a previewed order;[31] therefore the Page test was used.[33] For each assessor, a value between 1 and 4 was attributed to each sample, depending on the response of the assessor (1 for the sample designated as less intense, 4 for the more intense). The sums of the ranks were obtained for each sample, then the parameters L and L' were calculated using Page test specifications,[31, 33] and L' was compared to reported values in order to determine if the result of the test is significant or not for the factor concerned. If the test was significant ($P < 0.05$), a Mann−Whitney test[34] was applied to determine the groups of samples significantly different.

Test 2 was a triangular test,[32] therefore a binomial law with $p0 = 1/3$ was used and the limit values of correct answers were read in a table for different thresholds of significance.

Purification of a Sapid Fraction from Yeast Autolysate in Model Medium.

Preparation of Yeast Lees Autolysis Medium. Yeast lees autolysis medium (YLAM) was prepared from fermented model medium as follows. The active dry yeast Actiflore C (Laffort, France) was inoculated (100 mg/L) in a synthetic grape juice. The chemical composition of the model synthetic medium (MSM) was exhaustively described in a previous work[35]. Before yeast inoculation, the medium was sterilized by filtration (nitrate cellulose membrane, 0.45 μm, Millipore, France) and supplemented with sulfur dioxide (20 mg/L) in accordance with enological treatments. A fatty acid mixture was prepared in ethanol solution and fixed by drying on cellulose (0.5 g/L) in order to obtain 200 NTU (nephelometric turbidity units).[35] Fermentation took place in 6 L round-bottom flasks. The yeasts were regularly oxygenated by rapid bubbling with compressed air. The end of alcoholic fermentation was assessed by quantification of residual sugars by infrared reflectance (Infra-Analyzer 450, Technicon, France).

After fermentation, yeast lees were put back into suspension allowing the autolysis to take place at 32 °C, in the dark, for 10 days; during this time, lees were homogenized twice. Finally, the medium was freed from the yeast lees by centrifuging (4000 rpm, 10 min) and kept at 4 °C in sterilized glass bottles inerted with CO_2.

Multiple-Step Ultrafiltration (UF). The freshly prepared autolysis model medium was fractionated by means of tangential ultrafiltrations on a Minitan (Millipore) module following a procedure described by Humbert (2003). Three successive steps were applied using polysulfone filters with cutoffs of 10 kDa (Minitan-S 10,000NMWL, Millipore), 3 kDa (Iris

3028, Orelis) and 0.5 kDa (Nadir filtration). The product to be filtered was forced into the tangential ultrafiltration module using a type 112 Beckman pump. The filtrations were carried out at laboratory temperature (23 °C). The retentates were dialyzed at 500 Da (Spectra/Por CE [cellulose ester]) to desalinate them and eliminate the smallest molecules, then freeze-dried and stored at −18 °C.

Enzymatic Digestion of the Sapid Fraction Using a Nonspecific Protease: Proteinase K. The freeze-dried retentate corresponding to 500 mL of the sapid fraction (between 0.5 and 3 kDa) was dissolved in 200 mL of a buffer Tris-HCl 50 mmol/L; pH = 8; with 0.2% of SDS (w/v). Proteinase K (Roche, France) was added at the concentration of 100 mg/L. The digestion occurred at 37 °C during 24 h. After the end of this period, the medium was submitted to ultrafiltration with a cutoff of 10 kDa in order to eliminate proteinase K (28 kDa). The filtrate was ultrafiltered at 0.5 kDa in order to eliminate amino acids liberated during digestion and salts from buffer. Cleaning of the retentate obtained is optimized by dialyzing it through a membrane of 0.5 kDa. The retentate after dialysis was freeze-dried and kept at −18 °C for tasting.

HPLC−UV. The freeze-dried retentate corresponding to 10 mL of the sapid fraction (0.5 − 3 kDa) was dissolved in water (1 mL) and membrane-filtered. Aliquots (50 μL) were then separated according to the molecular weight on a column Superdex Peptide 10/300 300 mm × 100 mm (Pharmacia), by high performance liquid chromatography (Spectra System Thermo Fisher Scientific Inc., Waltham, MA, USA). This column has a fractioning zone comprised from 0.1 to 7 kDa and was previously calibrated with a mixture of peptides and proteins (glutathione, 0.3 kDa; insulin fragment, 3.5 kDa; aprotinin, 6.5 kDa; cytochrome C, 12 kDa). The solvent used for separation was an aqueous solution of NaCl (0.1 mol/L) in an isocratic mode at a flow rate of 0.5 mL/min. Detection was carried out at 220 nm with an UV-detector (Spectra System UV 1000). The predominant peak (eluted at a retention time of 34 min) was collected in ice-cooled glass vials. The corresponding fractions obtained from 40 HPLC runs were combined, dialyzed at 0.5 kDa to desalinate them, and freeze-dried.

Following this, the collected freeze-dried fraction was dissolved in 1 mL of water and membrane-filtered, and aliquots (20 μL) were purified again by HPLC on a 250 mm × 4.6 mm RP-18 column, Lichrospher, 5 μm (Supelco). Solvents used for separation were mixtures of water, formic acid and acetonitrile: eluent A (0.1% formic acid in water; 8% acetonitrile) and eluent B (0.1% formic acid in water; 80% acetonitrile). The following gradient of solvents was employed at a flow rate of 1 mL/min: 0 min, 15% B; 10 min, 40% B; 15 min, 81% B; 18 min, 81% B; 20 min, 17% B; 22 min, 17% B. Detection was carried out at 220 nm.

The apexes of the predominant peak were collected, and the corresponding fractions obtained from 50 HPLC runs were combined, freed from solvents under vacuum, and freeze-dried.

Identification of the Origin of the Sapid Fraction Using Nano-LC−MS/MS. Powder was suspended in 200 μL of water/acetonitrile 50/50; the suspension was concentrated in a vacuum centrifuge to reach a volume of 100 μL. Supernatant was directly injected in LC−MS/MS (supernatant A). The pellet was again solubilized in 100 μL of 0.1% formic acid and submitted to an ultrasonic bath for 15 min. Supernatant was injected in LC−MS/MS (supernatant B).

Online Capillary HPLC Nanospray Ion Trap MS/MS Analyses. Peptide mixture was analyzed by online capillary HPLC (LC Packings, Amsterdam, The Netherlands) coupled to a nanospray LCQ ion trap mass spectrometer (ThermoFinnigan, San Jose, CA). Ten microliters of peptide were loaded onto a 300 μm inner diameter × 5 mm C18 PepMap trap column (LC Packings, Amsterdam, The Netherlands) at a flow rate of 30 μL/min. The peptides were eluted from the trap column onto an analytical 75 μm inner diameter × 15 cm C18 PepMap column (LC Packings, Amsterdam, The Netherlands) with a 5−50% linear gradient of solvent B in 30 min (solvent A was 0.1% formic acid in 5% acetontrile, and solvent B was 0.1% formic acid in 80% acetonitrile). The separation flow rate was set at 200 nL/min. The mass spectrometer operated in positive ion mode at a 2 kV needle voltage and a 45 V capillary voltage. Data were acquired in a data-dependent mode alternating a MS scan survey over the range *m/z* 300−2000 and 3 MS/MS scans in an exclusion dynamic mode. MS/MS spectra were acquired using a 2 *m/z* unit ion isolation window, a 35% relative collision energy, and a 30 s dynamic exclusion duration.

Database Search. +Data were searched by SEQUEST through Bioworks 3.3.1 SP1 interface (ThermoFinnigan, San Jose, CA) against a subset of the SwissProt database (release 57.3) restricted to *Saccharomyces cerevisiae* (7114 entries). DTA files were generated for MS/MS spectra that both reach a minimal intensity (5×10^4) and a sufficient number of ions.[15] The DTA generation authorized the averaging of several MS/MS spectra corresponding to the same precursor ion with a tolerance of 1.4 Da. Spectra from precursor ion higher than 3500 Da or lower than 600 Da were rejected. Data were searched with no enzyme specificity. Search parameters were as follows: mass accuracy of the peptide precursor and peptide fragments was set to 2 and 1 Da respectively. Only b and y ions were considered for mass calculation. Oxidation of methionines (+16) was considered as differential modifications. Only peptides

with Xcorr higher than 1.9 (single charge), 2.2 (double charge) and 3.75 (triple charge) were retained. In all cases, ΔC_n must be superior to 0.1 and peptide p value lower than 10^{-3}. All protein identifications were based on a minimum of two-peptide assignments.

RESULTS AND DISCUSSION

Effect of Ethanol, Glycerol, and Yeast Lees on Perceived Sweetness of Red Wines. To evaluate the influence of some components of wine on the perceived sweetness, various quantities of yeasts were added ten days before a tasting session in bottled red wine. The wine used in this study contained less than 1 g/L of residual sugars, so yeasts were not able to develop and were degraded following autolysis mechanisms. Parallel to these samples and one hour before tasting, different concentrations of ethanol and glycerol were added in the same wine after opening (Table 2). Due to the small quantities added, the dilution effects (less than 2%) were considered as negligible.

Data presented in Table 3 showed that, for both ethanol and glycerol, panelists were not able to distinguish modalities with different concentrations of these compounds. These results demonstrated that glycerol and ethanol do not influence the sweet taste of wine when they are added in quantities generally encountered in red wines. This confirms previous studies published by Gawel et al.[15] and carried out on Riesling white wines. On the other hand, two groups of modalities (1, 2) and (3, 4) were judged as significantly different for samples obtained by yeast lees addition. More interestingly, such lees addition affected therefore the sweetness of the wines: modalities containing more lees were considered as sweeter by the panelists.

This experiment demonstrated the sweetening effect of lees after they have been subjected to autolysis conditions of red winemaking (10 days at 32 °C). This result suggested that some sapid compounds must be released in wine during this period. The wines used for this experiment were bottled, and therefore they previously underwent such a treatment during their elaboration. This may explain why the panelists were not able to distinguish modalities 1 from 2 and 3 from 4. The molecular origin of these supposed sapid compounds has to be determined in order to examine more accurately this hypothesis and to estimate their sensorial role in wine.

Isolation and Purification of a Sapid-Peptidic Fraction from Yeast Lees Autolysate. After having established the role of yeast lees on perceived sweetness in wine,

identification of the molecular origin of this phenomenon was begun. Consequently, a purification strategy was formulated from yeast autolysates in model solution rather than in wine to avoid the presence of grape compounds. This synthetic medium obtained after yeast lees autolysis (YLAM) was considered as different and more sapid by the panelists in comparison with a hydroalcoholic solution of same composition without lees contact (P = 0.006, Table 4). This result confirmed the pertinence of the medium chosen for purification. The YLAM was then submitted to successive ultrafiltration steps, and each fraction was tasted by the panel following the same procedure (Table 2).

These results showed that only the fraction between 0.5 and 3 kDa (YLAM 0.5−3) was detected as different by the tasters who described it as sweeter (Table 4). Consequently the size of the sapid compounds liberated during yeast autolysis was determined approximately between 0.5 and 3 kDa, considering the relative precision of the ultrafiltration threshold cutoffs. In 2008, Jones et al.[16] already showed that yeast proteins with a molecular weight higher than 3 kDa have no direct effect on wine sweetness.

The YLAM 0.5−3 fraction was then submitted to a proteic digestion using proteinase K. The resulting medium obtained after treatment was not differentiated from blank by the panelists (triangular test, P = 0.67, Table 4). Proteinase K is a nonspecific protease, and therefore the loss of taste induced by digestion demonstrated the peptidic nature of the sapid compounds present in the YLAM 0.5−3 fraction. Prior to this work, several gustatory peptides were identified in wine, exhibiting bitterness, sourness and umami, but the presence of sweet peptides has never been described until now.[22]

Molecular Characterization of the Sapid Faction Using LC−MS. In order to identify the origin of the sapid peptidic fraction, a peptide analysis method for molecular screening by HPLC was used. Following the results of the ultrafiltration experiments, the column chosen for separation by HPLC was Superdex Peptide HR 10/300 with a fractioning zone from 100 to 7000 Da. The characteristic retention time of the major signal of the YLAM 0.5−3 fraction was 34 min, corresponding to a molecular weight of about 2.750 Da given by the calibration line (Figure 1). This result confirmed those obtained from ultrafiltration. This signal was collected from 20 HPLC runs and tasted by a reduced panel (4 expert tasters) who testified to its sapid properties. The fraction obtained was then submitted to HPLC on a C18 column. The major signal of the chromatogram was poorly retained (2.1 min), and the reduced panel confirmed its gustatory role after collection of 50 runs. Despite the fact that this step was poorly separative, it allowed some impurities to be eliminated while keeping the sapid

compounds. The freeze-dried gustatory fraction collected from signal at 2.1 min of the C18 HPLC runs was then analyzed by liquid chromatography coupled with tandem mass spectrometry.

Both supernantant A and supernatant B were injected in nano-LC−MS/MS. Datafiles were searched against a *Saccharomyces cerevisiae* database without enzyme specificity. These analyses led in both fractions to the unambiguous identification of Hsp12p.

HSP12 is a small heat shock gene of *Saccharomyces cerevisae*.[36] It encodes a plasma membrane localized protein of 12 kDa.[37] The expression of HSP12 is notably induced by temperature, oxidative stress and high concentrations of alcohol and glycerol,[38] which are conditions obviously encountered during the winemaking process. Indeed, a recent study described the presence of Hsp12p among the proteins extracted from yeast lees in wine.[39]

Lees Sapidity Is in Part Determined by the Plasma Membrane Protein Hsp12p. In order to verify the hypothesis of a contribution of Hsp12p to the sweetness increase observed during yeast lees autolysis, we compared the sensorial impact of lees containing or not this protein. By using a molecular genetics approach, we constructed a yeast strain deleted for both copies of *HSP12* gene. The protein impact was then evaluated by a comparative sensorial test between deleted and nondeleted strain. In order to safely validate the gustatory effect of Hsp12p, sensorial analyses were carried out with wines that do not present recombinant proteins of bacterial origin. Therefore a Δ°hsp12 null mutant strain was constructed and compared to wild-type strain Fx10. Both strains are diploid and only differ by the presence of *HSP12* gene in their genome. They are both exempt of recombinant DNA as verified by PCR and antibiotic resistance analyses (Figure 2).

The sensory test used was a triangular test presenting two modalities: yeast autolysis (10 days, 32 °C) carried out in a red wine with the two strains Fx10 and Δ°hsp12 introduced in the same quantity (2×10^8 cells/mL) in wine (Table 2). Tasters were asked to distinguish the different wine, but also to compare their sweetness. The results obtained for this test are statistically significant ($P = 0.019$, Table 4) indicating that the samples were judged as different by tasters. Moreover, all tasters who were able to correctly detect the difference described the modality Fx10 as sweeter than the Δ°hsp12. Consequently, sensorial analysis confirmed the role of Hsp12p in the perceived sweetness afforded by yeast lees autolysis. To our knowledge, it is the first time that an organoleptic property of wine has been related specifically to a yeast protein.

Regarding only this experiment, the nature of the role of Hsp12p remains uncertain. It could be indirect, since the lack of Hsp12p might involve other modifications in the molecular composition of the yeast and in its metabolism. Nevertheless, the fact that peptides coming from Hsp12p were identified in a sapid fraction corroborates the results of sensorial analysis after genetic modification. The presence of peptides liberated from Hsp12p during yeast autolysis and increasing wine sweetness is a strong hypothesis. Further work concerning the molecular identification of these peptides, their sensorial characterization and the conditions of their liberation in wine are in progress.

It is also possible that other molecules from *Saccharomyces cerevisae* exhibit gustatory properties increasing the sweet perception of dry wines. This possibility may be the subject of further studies.

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Figures

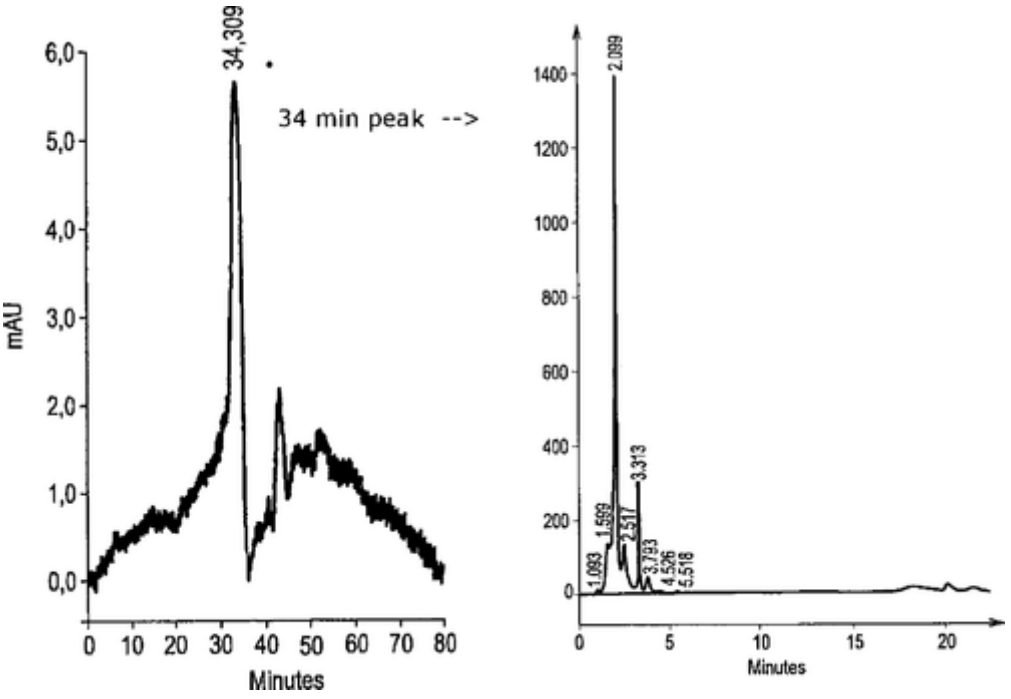

Figure 1. Chromatographic purification of sapid fraction. Chromatograms HPLC with UV detection at 220 nm of (a) YLAM 0.5−3 on Superdex Peptide HR column and (b) collected 34 min peak on RP-18 column.

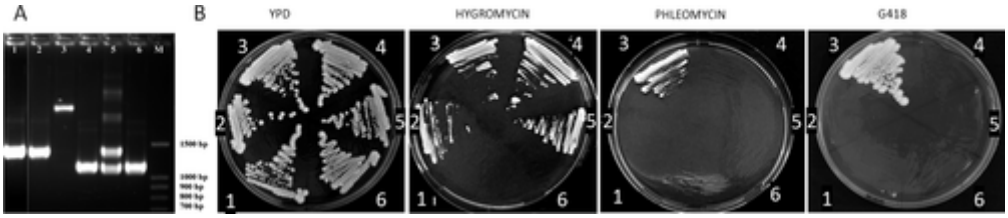

Figure 2. PCR and antibiotic resistance test for Δ°*hsp12* construction. (A) The genomic region containing the *HSP12* gene was amplified by PCR by primers flanking the 500 bp upstream and downstream of *HSP12*. The genomic DNA of Fx10 (1) and YPM32 (2) amplified a band of 1316 bp. The genomic DNA of YPM33 (3) containing the *loxP::kanMX::loxP* cassette at the *HSP12* locus amplified a band of 2014 bp. Once transformed by pZEO, the YPM34 (4) the cassette was excised generating a PCR fragment that contained neither the HSP12 gene nor the KanMx4 gene. The YPM34 was then crossed with Fx10 in order to obtain a diploid strain YPM35 (5) containing the HSP12 and the Δ°HSP12 alleles. The Δ°hsp12 (6) was then obtained by meiotic segregation screening for a diploid homothallic strain showing the HSP12 deleted gene. (B) Antibiotic resistance of strains. The resistance Fx10 (1), YPM32 (2), YPM33 (3), YPM34 (4), YPM35 (5), and Δ°hsp12 (6) strains were tested for hygromycin, phleomycin and G418 respectively.

Tables

Table 1. Yeast Strains and Plasmids Used

biological material	description	origin
	Yeast Strains	
Actiflore C	commercial starter	Laffort Inc.
Fx10	commercial starter *HO/HO* fully homozygous strain (Zymaflore Fx10, Laffort)	referenced as H4-1D 27
RG1	F10 *ho::HYG^R*, *Mat a*	kind gift of Pr. Richard Gardner
YPM32	haploid derivate of Fx10, *ho::HYG^R*, *MATa*	this study
YPM33	YPM32, *hsp12::LoxP::KANMx::LoxP*, *ho::HYG^R*, *MATa*	this study
YPM34	YPM33, *Δ°hsp12, HO::HYG^R*, *MATa*	this study
YPM35	YPM34 x Fx10 spore, *HO/ho::HYG^R*, *HSP12/Δ°hsp12*	this study
Δ°hsp12	meiotic segregant of YPM35, *HO/HO*, *Δ°hsp12/Δ°hsp12*	this study
	Plasmid	
pUG6		kindly donated by Pr. Bruno Blondin
pZEO		kindly donated by Pr. Bruno Blondin

Table 2. Modalities Used for Sensorial Tests

factor studied	test	modality 1	modality 2	modality 3	modality 4
			Effect on Sweetness		
ethanol effect	ranking (n = 38)	red wine	red wine + 0.5% (v/v)	red wine + 1% (v/v)	red wine + 1.5% (v/v)
glycerol effect	ranking (n = 38)	red wine	red wine + 1 g/L	red wine + 3 g/L	red wine + 5 g/L
yeast lees effect	ranking (n = 38)	red wine[a]	red wine + 2×10^8 cells/mL[a]	red wine + 4×10^8 cells/mL[a]	red wine + 8×10^8 cells/mL[a]
			Purification of Sapid Fraction		
autolysis	triangular (n = 23)	synthetic soln[a]	YLAM[a]		
mol wt	triangular (n = 23)	synthetic soln	synthetic soln + UF fractions		
biochemical nature	triangular (n = 23)	synthetic soln	synthetic soln + retentate after digestion		
Hsp12 effect	triangular (n = 23)	red wine + Fx10 (2×10^8 cells/mL)[a]	red wine + Δ°hsp12 (2×10^8 cells/mL)[a]		

[a]These wines and solutions were kept at 32 °C for 10 days before sensory analysis was performed.

Table 3. Ethanol, Glycerol, and Yeast Lees Effect on Perceived Sweetness

factor studied	R_1[a]	R_2[a]	R_3[a]	R_4[a]	***L***	***L′*** **[b,c]**
ethanol	98	88	94	100	956	0.34 ns
glycerol	89	93	99	99	968	1.01 ns
yeast lees	67	71	106	123	1019	3.87**

[a] R_1, R_2, R_3 and R_4 are the sums of ranks for modalities 1 to 4.

[b] L and L' were calculated as described in ISO 8587:2006: (31)

$L = \sum_{i=1}^{p} \mathrm{iR_i}$ and $L' = \frac{12L-3np(p+1)}{p(p+1)\sqrt{n(p-1)}}$

(n is the number of panelists and p the number of modalities).

[c] Significativity: ns, nonsignificant; (*) significant at 5%; (**) significant at 1%.

Table 4. Evaluation of Molecular Weight and Biochemical Nature of Sapid Fractions. Confirmation of the Role of Hsp12 Protein

modality	fraction name	no. of "correct" answers[a] (n = 23)	P[b]
autolysis medium before UF	YLAM	14	0.006**
retentate after UF 10 kDA	YLAM > 10	4	0.974 ns
retentate after UF 3 kDA	YLAM 3−10	9	0.349 ns
retentate after UF 0.5 kDa	YLAM 0.5−3	14	0.006**
filtrate after UF 0.5 kDa	YLAM < 0.5	8	0.519 ns
enzymatic digestion of YLAM 0.5−3	D-YLAM 0.5−3	7	0.670 ns
autolysis of Fx10 and Δ°hsp12 yeast strains in red wine (Hsp12 effect)		13	0.019*

[a]The expression "correct answers" designates the expected answer, i.e. when the taster has chosen the sample of different composition.

[b]P was calculated using binomial law. Significativity: ns, nonsignificant; (*) significant at 5%; (**) significant at 1%.