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ARTICLE

# Plasma Membrane Sterols Are Involved in Yeast's Ability To Adsorb Polyphenolic Compounds Resulting from Wine Model Solution Browning

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The aim of this work was to demonstrate the direct interaction between membrane sterols of yeast lees and some polymerized phenolic compounds resulting from wine model solution browning. For this purpose, we first demonstrated by measurement of steady-state fluorescence anisotropy of the cationic fluorescent TMA-DPH probe the effect of polymerized compounds from the model reactions of (+)-catechin/acetaldehyde and (+)-catechin/glyoxylic acid on the plasma membrane order of *Saccharomyces cerevisiae* yeast lees enriched with different sterols. In a second set of experiments, we used *S. cerevisiae* plasma membrane vesicles spiked with different sources of sterol (ergosterol, cholesterol or a mix of grape phytosterols) to assess the effect of the same polymerized compounds on both vesicle integrity and membrane leakiness to protons by ACMA fluorescence. All the obtained results prove that yeast membrane sterols are able to strongly interact with some polymerized compounds resulting from the browning of model solutions, likely explaining the yeast ability to adsorb polyphenolic compounds and mainly the colorless intermediate compounds of the browning reactions.

**KEYWORDS: Yeast lees; wine browning; sterols**

## INTRODUCTION

The absence of browning in pale sherry wines during biological aging has been ascribed to a protective effect of the wine against browning by the flor yeasts growing on its surface (*1*, *2*). Although flor yeasts differ notably from fermentative yeasts in their oxidative metabolism, no scientific work has been dedicated to the evaluation of yeast growth conditions (aerobiosis or anaerobiosis) on their ability to prevent wine browning.

In a recent study (*3*), we observed that different yeast species grown under aerobiosis or anaerobiosis exhibited different capacities for delaying the browning of simulated wine solutions. We discarded rapidly the involvement of oxygen consumption by yeast lees as a competitor for brown polymeric compounds formation in the case of glyoxylic acid/(+)-catechin model solutions, in which oxygen plays an important active role in the oxidation reaction. Moreover, we showed that the observed lowering of browning could not be attributed to a larger absorption of brown compounds by the yeast lees. As a matter of fact, we demonstrated that a focused adsorption of the colorless intermediate compounds, mainly (+)-catechin and primary dimers, occurred in the case of acetaldehyde/catechin model solutions. Because the role of these colorless compounds as intermediates in the reaction pathways leading to brown pigments has been previously demonstrated (*4*–*6*), the observed inhibition of browning by yeast lees could be attributed to the strong decrease of these compounds, which are indeed necessary to develop the polymerization reactions (*3*).

Therefore, all of these results raised new questions on the nature of the component(s) of the yeast lees responsible for the strong adsorption of the colorless intermediate compounds. In a preliminary study, we checked the in vitro direct interaction between ergosterol (the main yeast membrane sterol) in a solubilized form and these compounds (*3*). We proved that membrane sterols could be involved in the yeast's ability to adsorb brown phenolic compounds, as already hypothesized previously (*7*, *8*). On this basis, we dedicated the present work to demonstrate clearly the existence of such interactions between membrane sterols and polymeric phenolic compounds. We checked first the effect of polymeric compounds resulting from the browning of wine model solutions on the plasma membrane order of *Saccharomyces cerevisiae* yeast lees enriched with different sterols. Second, the reactivity of different sterols trapped in reconstituted plasma membrane vesicles against brown phenolic compounds was investigated.

We demonstrate here that polymeric compounds resulting from the oxidation of glyoxylic acid/(+)-catechin model solution increased strongly the membrane fluidity of yeast lees whatever the sterol enrichment and that polymeric compounds resulting from both model solutions strongly altered the physical and chemical properties of functional reconstituted plasma membrane vesicles, mainly in vesicles enriched with grape phytosterols.

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## MATERIALS AND METHODS

**Yeast Strains.** The commercial enological yeast strain *S. cerevisiae* K1 (ICV-Lallemand, Montréal, Canada) was used.

**Culture Media and Growth Conditions.** All precultures were performed in a standard nutrient medium YPD containing yeast extract (Difco, Detroit, MI) 10 g $L^{-1}$, bactopeptone (Difco) 20 g $L^{-1}$, and glucose 20 g $L^{-1}$) for 24 h at 28 °C in Erlenmeyer flasks.

Anaerobic cultures were grown in a synthetic medium MS300 strongly buffered to pH 3.3 (*9*). This medium contained (per liter) 200 g of glucose, 6 g of citric acid, 6 g of DL-malic acid, 750 mg of $KH_2PO_4$, 500 mg of $K_2SO_4$, 250 mg of $MgSO_4 \cdot 7H_2O$, 155 mg of $CaCl_2 \cdot 2H_2O$, 200 mg of NaCl, 4 mg of $MnSO_4 \cdot H_2O$, 4 mg of $ZnSO_4$, 1 mg of $CuSO_4 \cdot 5H_2O$, 1 mg of KI, 400 $\mu$g of $CoCl_2 \cdot 6H_2O$, 1 mg of $H_3BO_3$, 1 mg of $(NH_4)_6Mo_7O_{24} \cdot 2H_2O$, 20 mg of *myo*-inositol, 2 mg of nicotinic acid, 1.5 mg of calcium pantothenate, 250 $\mu$g of thiamin-HCl, 0.25 mg of pyridoxine-HCl, 3 $\mu$g of biotin, and 300 mg of $NH_4Cl$. Ergosterol, cholesterol, or a mixture of grape phytosterols ($\beta$-sitosterol 90%, stigmasterol 10%) (15 mg $L^{-1}$) and oleic acid (5 mg $L^{-1}$) dissolved in 1 mL of Tween 80/pure ethanol (50:50, v/v) was also added to the medium after sterilization. The fermentation medium was inoculated with $10^6$ yeast cells $mL^{-1}$. Filling conditions were controlled, and fermentations were carried out under anaerobic conditions under isothermal conditions (28 °C) with continuous magnetic stirring (500 rpm). All fermentors (working volume = 1.1 L) were fitted with fermentation locks ($CO_2$ bubbling outlets filled with water). Fermentation media were strongly deoxygenated by bubbling pure sterile argon for 30 min before inoculation. Fermentation was considered to be complete when the medium contained < 2 g $L^{-1}$ of glucose. None of the fermentations were stopped if they were < 99% complete.

Cell number was determined after sonication (30 s, 10 W) using an electronic particle counter (model ZB2, Beckman-Coulter, Margency, France) fitted with a 100 $\mu$m aperture probe.

Unless otherwise stated, anaerobically grown yeasts were harvested by centrifugation (1000*g*, 5 min), rinsed twice with water, and frozen at −20 °C. The corresponding biomass was then lyophilized (Christ Alpha I-5, Bioblock Scientific, Illkirch, France) and stored before use at ambient temperature under argon gas, to protect biomass from oxidation.

**Simulation of Wine Browning with Model Solutions.** Two hydroalcoholic solutions containing 12 g $L^{-1}$ (+)-catechin (Sigma-Aldrich Chemicals, St. Louis, MO) in 10 and 14% (v/v) ethanol were prepared. The first one was supplied with acetaldehyde (21.2 mM), adjusted to pH 3.2 with concentrated acetic acid, and incubated at 20 °C for 48 h. The second solution was supplied with glyoxylic acid (20.4 mM), adjusted to pH 3.2 with NaOH 4 N, and incubated at 20 °C for 25 days. All reagents were provided by Merck (Darmstadt, Germany). For the determination of plasma membrane order by steady-state fluorescence anisotropy, the two model solutions were diluted before use to 5% (v/v) ethanol with pure water to impede a deleterious effect of ethanol at high concentration on the plasmatic membrane fluidity of yeast cells (*10*, *11*).

**Purification of Plasma Membranes.** All of the purification steps were performed at 4 °C, as previously described (*12*). Fresh yeast cells grown on MS300-ergosterol synthetic medium were harvested by centrifugation at the end of the exponential growth phase and washed twice in buffer A [50 mM bis-tris-propane/2-[*N*-morpholino]ethanesulfonic acid (BTP/MES), pH 7.5, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethanesulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT)]. About 10 g fresh-weight was mixed with 25 mL of buffer A and 15 g of glass beads (0.5 mm diameter), and the mixture was shaken in an MSK cell homogenizer (Braun Biotec International, Melsungen, Germany) for 3 min under liquid $CO_2$ cooling. Glass beads were removed by filtration under vacuum through a nylon mesh and washed with buffer A. The homogenate was centrifuged twice for 5 min at 1000*g* and once for 10 min at 3000*g*. The resulting pellets were discarded, and the supernatant was further centrifuged for 40 min at 15000*g*. The pellet containing the yeast membranes was then suspended in 16 mL of buffer B [10 mM BTP/MES, pH 6.5, 1 mM DTT, 1 mM PMSF, 20% (w/v) glycerol]. This membrane suspension (8 mL) was applied to a 43/53% (w/w) discontinuous sucrose gradient and centrifuged for 2 h at 170000*g*. The membrane fraction located at the sucrose interface was collected, suspended in 4 mL of buffer B, and washed by centrifugation (30 min, 150000*g*). To complete the purification, a second 43/53% (w/w) discontinuous sucrose gradient step was performed under the same conditions. The final plasma membrane pellet was suspended in 4 mL of buffer B without PMSF and stored in liquid nitrogen until use.

**Reconstitution of Plasma Membrane Vesicles.** Plasma membrane vesicles were reconstituted according to Camarasa et al. (*12*). This protocol of vesicle reconstitution by a solubilization/gel filtration reconstitution procedure was used because it was previously shown that at least 80% of the obtained vesicles were sealed with an inside-out orientation. The reconstitution protocol was designed to enrich specifically membranes in ergosterol, cholesterol, or grape phytosterols. Soybean phospholipids (L-$\alpha$-phosphatidylcholine type II-S, 60 mg) and pure sterols [6 mg of ergosterol, cholesterol, or of a mixture of grape phytosterols ($\beta$-sitosterol 90%, stigmasterol 10%), all purchased from Sigma Chemicals, L'Isle-d'Abeau, France] were dissolved in 1 mL of chloroform. This solution was brought to dryness under argon gas to impede lipid oxidation. The resulting pellets were dispersed under argon by vigorous mixing on a vortex mixer in the presence of glass beads (2 mm diameter) in 1 mL of reconstitution buffer [10 mM BTP/MES, pH 7.5, 7.5% (w/v) glycerol, 25 mM $K_2SO_4$]. To obtain small unilamellar vesicles, the suspension of phospholipid/sterol vesicles was sonicated for 15 min to clarity in a bath sonicator. Octylglucoside 50 mM and the equivalent of 0.1 mg of membrane proteins (20 $\mu$L) were added to 11.3 $\mu$L of the phospholipids/sterols in a final volume of 246 $\mu$L containing 10 mM BTP/MES pH 7.5, 10% (w/v) glycerol (lipid/protein weight ratio = 20). The mixture was applied to the top of a Sephadex G50 column (3.5 cm × 0.5 cm disposable syringes fitted with porous glass filters), pre-equilibrated with reconstitution buffer. The column was centrifuged at 150*g* for 5 min. Octylglucoside was removed during this chromatographic step, and sealed plasma membrane vesicles were directly eluted. Vesicles were used immediately or frozen and stored in liquid nitrogen. As previously calculated (*12*), such plasma membrane vesicles exhibit lipids/proteins and lipids/sterols ratios of 10 and 100 (w/w), respectively.

**Analytical Methods.** *Protein Determination*. Protein concentrations were determined, after precipitation of the different fractions with 5% trichloroacetic acid, by the BCA proteic reagent method (Pierce Chemicals, Rockford, IL). Bovine serum albumin was used as standard.

*Fluorescence Depolarization Determination of Plasma Membrane Order*. Plasma membrane lipid order was determined by measuring steady-state fluorescence anisotropy in yeast lees labeled with 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene-*p*-toluenesulfonate (TMA-DPH; Molecular Probes, Eugene, OR) as previously described (*13*). This cationic probe anchors primarily in the plasma membrane of yeast cells (*14*). Yeast lees (200 mg) were let in contact with both wine browning model solutions diluted to 5% (v/v) ethanol at the end of incubation for 90 min. As blank experiments, yeast lees were let in contact for the same duration with either 10.6 mM acetaldehyde in 5% (v/v) ethanol adjusted to pH 3.2 with concentrated acetic acid, 7.3 mM glyoxylic acid in 5% (v/v) ethanol adjusted to pH 3.2 with NaOH 4 N, or 14.7 mM (+)-catechin in 5% (v/v) ethanol adjusted to pH 3.2. One hundred microliters of the yeast lees suspension was then added to 1.8 mL of 0.9% (w/v) NaCl solution and equilibrated with continuous stirring at 30 °C for 30 min in a 1 cm path length quartz cuvette. TMA-DPH (20 $\mu$L) from a 0.6 mM stock solution in methanol was then added to the suspension. After stabilization of the suspension, fluorescence measurements were made every 10 s on a Perkin-Elmer LS50-B fluorescence spectrometer with polarization turrets (Perkin-Elmer, Courtaboeuf, France). The TMA-DPH probe was excited with vertically polarized light at 360 nm (3 nm bandwidth), and the vertical and horizontal components of the emitted light were measured each 12 s at 450 nm (3 nm bandwidth). The fluorescence intensities were corrected for background fluorescence and light scattering from an unlabeled sample. Membrane order was expressed as the order parameter *S*, which gives a measure of the order of the membrane phospholipids: $S = (r/r_0)^{0.5}$ (*14*), where $r_0$ is the theoretical limiting anisotropy (0.395 for TMA-DPH) in the absence of rotational motion and *r* is the steady-state anisotropy measured in the membrane.

*Fluorescence Quenching of ACMA*. Fluorescence quenching of 9-amino-6-chloro-2-methoxy-acridine (ACMA, Molecular Probes) was used to monitor the formation and dissipation of pH gradients (acid inside) across reconstituted plasma membrane vesicles (*15*). Fluorescence intensities of the probe were recorded with a Perkin-Elmer LS50-B

(Perkin-Elmer, Courtaboeuf, France) photon counting spectrofluorometer. Plasma membrane vesicles (50 $\mu$L or 26 $\mu$g of proteins) were added to a cuvette (2 mL total working volume) containing 10 mM BTP/MES, pH 7.5, 10% (w/v) glycerol, 1 mM $KNO_3$, 25 mM $K_2SO_4$, 300 mM $MgSO_4$, 250 nM valinomycin, 4 $\mu$M ACMA, and either 714 $\mu$L of the glyoxylic acid/(+)-catechin or 1000 $\mu$L of the acetaldehyde/(+)-catechin wine browning model solutions. As blank experiments, the cuvettes contained either 10.6 mM acetaldehyde in 5% (v/v) ethanol adjusted to pH 3.2 with concentrated acetic acid, 7.3 mM glyoxylic acid in 5% (v/v) ethanol adjusted to pH 3.2 with NaOH 4 N, or 14.7 mM (+)-catechin in 5% (v/v) ethanol adjusted to pH 3.2.

The cuvette was stirred and maintained at 30 °C for at least 30 min. Emission signals were recorded at 480 nm after excitation at 412 nm. Once a stable fluorescence signal was observed, proton pumping was induced by the addition of 5 mM ATP-BTP, pH 7.5. $(NH_4)_2SO_4$ 10 mM was added after stabilization of the signal to abolish the quenching of ACMA fluorescence.

*Mathematical Treatment of ACMA Fluorescence Quenching Kinetics*. Fluorescence quenching kinetics were fitted by a two-parameter single-exponential rise to a maximum function with the help of Sigmaplot (v. 9.0) software (Systat, Chicago, IL), allowing the determination of initial velocities and maximal amplitude of ACMA fluorescence quenching for each experiment.

*Statistical Analysis*. The statistical KyPlot (v. 2.0 b.15) software (KyensLab, Tokyo, Japan) was used to perform ANOVA and Tukey tests (pairwise comparisons for one-way layout design) to classify the data into homogeneous groups.

## RESULTS

**Effect of Polymeric Compounds Resulting from the Browning Reaction of Wine Model Solutions on the Plasma Membrane Order of Yeast Lees Enriched with Different Sterols during Anaerobic Growth.** Under anaerobic conditions, yeast growth normally requires added oxygen to synthesize lipids (*16*, *17*), which are essential for plasma membrane integrity. In strict anaerobic conditions, *S. cerevisiae* is therefore able to incorporate a wide variety of exogenous sterols (*18*, *19*), whereas this is not the case in aerobic conditions. We use this property to specifically enrich *S. cerevisiae* cells in different sterols: ergosterol (the main sterol in viable *S. cerevisiae* cells), cholesterol (the main human sterol), or a mix of grape phytosterols ($\beta$-sitosterol 90%, stigmasterol 10%). A wide variety of sterols can indeed efficiently play a "bulk" role in yeast membranes grown under anaerobiosis, acting as architectural components of membranes (*18*). We previously demonstrated that, in the absence of added oxygen, grape phytosterols quickly perturb the yeast membrane properties of viable yeasts by being the predominant sterols, leading to sluggish alcoholic fermentations (*20*). In the conditions used in the present experiments, yeast lees contained in their plasma membranes 7.72 $\mu$g of ergosterol $10^{-9}$ cells, 7.72 $\mu$g $10^{-9}$ cells cholesterol, or 4.19 $\mu$g $10^{-9}$ cells $\beta$-sitosterol and 1.61 $\mu$g $10^{-9}$ cells stigmasterol, respectively. In yeast lees, whatever the type of sterol used for the enrichment of the cells during their anaerobic growth, the values of plasma membrane order were almost identical (**Table 1**). Blank experiments performed with the addition of either (+)-catechin, acetaldehyde, or glyoxylic acid did not alter significantly the plasma membrane order of the cells. No changes were observed when polymeric phenolic compounds resulting from the browning reaction of (+)-catechin with acetaldehyde in model solution were added. On the contrary, addition of polymeric phenolic compounds resulting from the oxidation of (+)-catechin/glyoxylic acid model solutions led to a significant increase of plasma membrane order in ergosterol- and cholesterol-enriched yeast lees (**Table 1**). This modification of the orderliness of membrane phospholipids, as measured by fluorescence anisotropy of the TMA-DPH probe within lipid bilayers, was consistent with the angular reorientation of the lipid acyl chains (*21*) and, thus, indicated that changes in the conformation of membrane lipids occurred.

**Table 1.** Effect of the Addition of Polymeric Compounds Resulting from the Oxidation of the Wine Browning Model Solutions on Plasma Membrane Order (*s*) of Yeast Lees Enriched in Different Types of Sterol[a]

| | *s* of yeast lees enriched in | | |
|---|---|---|---|
| | ergosterol | phytosterols | cholesterol |
| control | 0.90 ± 0.08 | 0.90 ± 0.01 | 0.90 ± 0.01 |
| acetaldehyde | 0.90 ± 0.08 (NS) | 0.85 ± 0.02 (NS) | 0.89 ± 0.06 (NS) |
| glyoxylic acid | 0.89 ± 0.00 (NS) | 0.96 ± 0.00 (NS) | 0.95 ± 0.00 (NS) |
| catechin | 0.92 ± 0.00 (NS) | 0.91 ± 0.00 (NS) | 0.90 ± 0.00 (NS) |
| catechin/acetaldehyde | **0.83 ± 0.00 (NS)** | **0.86 ± 0.02 (NS)** | **0.86 ± 0.00 (NS)** |
| catechin/glyoxylic acid | **1.03 ± 0.02 (*)** | **0.99 ± 0.02 (NS)** | **1.04 ± 0.01 (***)** |

[a] Mean and standard deviation of duplicates. The differences between each modality and the control were tested by Tukey's statistical test. NS, not significant; *, significant differences at a level of $P \leq 0.05$; ***, significant differences at a level of $P \leq 0.001$. By comparison with the three blank experiments, only the modalities written in bold letters were taken into account for discussion.

**Alteration of the Functional Properties of Reconstituted Plasma Membrane Vesicles by the Polymeric Compounds Resulting from the Browning Reaction of Wine Model Solutions.** To verify the reactivity of different sterols within their lipidic environment in cell membranes against the polymeric oxidized phenolic compounds, we used an improved protocol of reconstitution of plasma membrane vesicles, which preserves membrane functional properties and produces a high percentage of inside-out tightly sealed plasma membrane vesicles (*15*, *12*). The generation of a pH gradient through these vesicles, measured by ACMA quenching, was used to determine the integrity of the obtained vesicles. As a result of plasma membrane $H^+$-ATPase activation by ATP addition, vesicle acidification was observed (**Figure 1**). On the first part of the experiment, two parameters were calculated: the initial velocity of vesicle acidification ($V_{max}$ $H^+$ pumping), which corresponds to the activity of plasma membrane $H^+$-ATPase, and the absolute value of the maximum quenching obtained ($Q_{max}$ $H^+$ pumping), which is roughly proportional to the membrane tonicity or rigidity toward the establishment of a proton gradient (*15*, *12*). In a second part of the experiment, addition of $(NH_4)_2SO_4$, which equilibrates protons across the membrane (by neutral diffusion of $NH_3$), reversed the fluorescence quenching, indicating that the first signal is effectively due to proton transport. The complete collapsing of the proton gradient across the vesicle confirms the tightly sealed nature of plasma membrane vesicles because they could accumulate protons without leakage. In this second part of the experiment, two other parameters were calculated: the initial velocity of gradient collapsing by ammonium ($V_{max}$ $NH_4^+$), which corresponds to the permeability of the plasma membrane to protons and $NH_3$ by neutral diffusion, and the absolute value of fluorescence obtained after stabilization of the signal ($Q_{max}$ $NH_4^+$). In completely tightly sealed plasma membrane vesicles, the absolute values of $Q_{max}$ $NH_4^+$ and $Q_{max}$ $H^+$ pumping should be almost equal, because tightly sealed vesicles should accumulate protons without leakage (**Figure 1**).

When a similar experiment was performed with the same vesicles in the presence of the polymeric compounds resulting from the browning reaction of the wine model solutions, it could be seen that the value of $Q_{max}$ $H^+$ pumping was very often superior to the value of $Q_{max}$ $NH_4^+$ within the same experiment, indicating that leakage of protons from the vesicles occurred during the time frame of the experiment (**Figure 2** and **Table 5**). As stated before, we used the $Q_{max}$ $H^+$ pumping, $Q_{max}$ $NH_4^+$, $V_{max}$ $H^+$ pumping, and $V_{max}$ $NH_4^+$ parameters to finely characterize

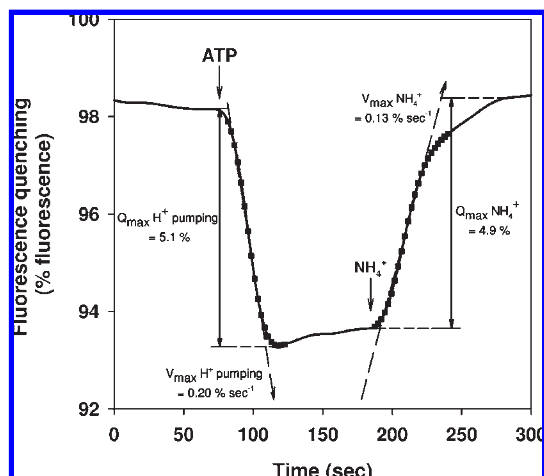


**Figure 1.** ACMA fluorescence quenching produced by activation of the $H_+$-ATPase contained in plasma membrane vesicles reconstituted with ergosterol: (■) two-parameter single-exponential rise to a maximum function curve fitting; $V_{max}$ $H_+$ pumping, initial velocity of vesicle acidification; $Q_{max}$ $H_+$ pumping, absolute value of the maximum quenching obtained; $V_{max}$ $NH_4^+$, initial velocity of gradient collapsing by ammonium; $Q_{max}$ $NH_4^+$, absolute value of fluorescence obtained after stabilization of the signal.Vesicles were incubated until a stable fluorescence signal was observed. Proton pumping was induced by addition of 5 mM ATP-BTP (pH 7.5) as substrate. $(NH_4)_2SO_4$ (10 mM) was added after stabilization of the signal.

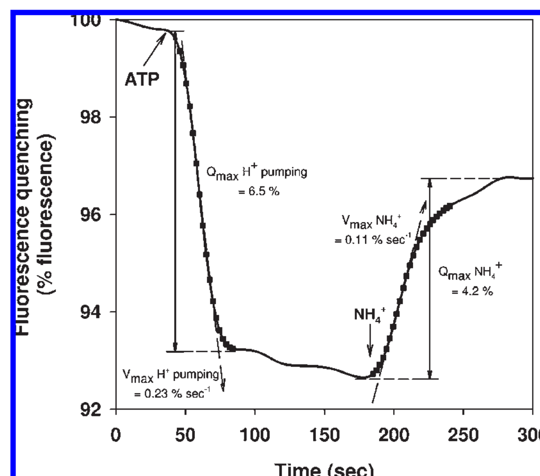


**Figure 2.** ACMA fluorescence quenching produced by activation of the $H_+$-ATPase contained in plasma membrane vesicles reconstituted with ergosterol. Vesicles were incubated in the presence of 1000 $\mu$L of the acetaldehyde/(+)-catechin wine browning model solution [diluted to 5% (v/v) ethanol] until a stable fluorescence signal was observed. Proton pumping was induced by addition of 5 mM ATP-BTP (pH 7.5) as substrate. $(NH_4)_2SO_4$ (10 mM) was added after stabilization of the signal. Legends are the same as in **Figure 1**.

the behavior of plasma membrane vesicles specifically enriched in different types of sterol (ergosterol, phytosterols, or cholesterol) in the presence of the added polymeric compounds (**Tables 2– 5**).

Addition of polymeric compounds resulting from both wine browning model solutions led to a significant increase of the activity of the plasma membrane $H^+$-ATPase, as revealed by the high values of the $V_{max}$ $H^+$ pumping parameter in the case of plasma membrane vesicles enriched in grape phytosterols by comparison with blank experiments (**Table 2**). It is noteworthy that modification of the lipidic (lipids and sterol) environment of the plasma membrane-bound ATPases could affect their specific activities (*22*, *23*).

In a similar manner, the same vesicles exhibited a higher permeability to $NH_3$ by simple diffusion, because they exhibited significantly higher values of the $V_{max}$ $NH_4^+$ parameter in the presence of polymeric compounds resulting from the oxidation of (+)-catechin/glyoxylic acid model solution (**Table 3**). All of these results were consistent with a modification of the lipidic environment of the vesicle membrane enriched in grape phytosterols upon addition of polymeric compounds resulting from the browning reaction of the wine model solutions.

Addition of both types of polymeric compounds led also to a significant increase of the value of the maximum quenching obtained upon ATPase activation ($Q_{max}$ $H^+$ pumping) in vesicles enriched in phytosterols by comparison with the blank experiments (**Table 4**). This parameter is roughly proportional to the membrane tonicity or rigidity toward the establishment of a proton gradient (*15*, *12*). Therefore, it could be hypothesized that a specific interaction between phytosterols and some phenolic polymeric products occurred within the vesicle membrane leading to a strong modification of its permeability.

Finally, the leakage of protons during the time frame of the experiment from the vesicles was monitored by calculating the differences between $Q_{max}$ $H^+$ and $Q_{max}$ $NH_4^+$ parameters (**Table 5**). In the case of addition of polymeric compounds resulting from the browning reaction of (+)-catechin/acetaldehyde

**Table 2.** Effect of the Addition of Polymeric Compounds Resulting from the Oxidation of the Wine Browning Model Solutions on the $V_{max}$ $H^+$ Pumping Parameter of Plasma Membrane Vesicles Specifically Enriched in Different Types of Sterol[a]

| | $V_{max}$ $H^+$ pumping (% quenching $s^{-1}$) of vesicules enriched in | | |
|---|---|---|---|
| | ergosterol | phytosterols | cholesterol |
| control | 0.27 ± 0.06 | 0.34 ± 0.08 | 0.40 ± 0.09 |
| acetaldehyde | 0.21 ± 0.01 (NS) | 0.34 ± 0.06 (NS) | 0.47 ± 0.04 (NS) |
| glyoxylic acid | 0.82 ± 0.03 (***) | 0.31 ± 0.09 (NS) | 0.35 ± 0.04 (NS) |
| catechin | 0.20 ± 0.00 (NS) | 0.34 ± 0.07 (NS) | 0.44 ± 0.14 (NS) |
| catechin/acetaldehyde | **0.28 ± 0.00 (NS)** | **0.66 ± 0.17 (***)** | **0.42 ± 0.03 (NS)** |
| catechin/glyoxylic acid | 0.63 ± 0.07 (***) | **0.52 ± 0.25 (***)** | **0.35 ± 0.08 (NS)** |

[a] Mean and standard deviation of triplicates. The differences between each modality and the control were tested by Tukey's statistical test. NS, no significant; *, significant differences at a level of $P \leq 0.05$; ***, significant differences at a level of $P \leq 0.001$. By comparison with the three blank experiments, only the modalities written in bold letters were taken into account for discussion.

model solutions, the vesicles enriched in ergosterol and grape phytosterols exhibited a strong leakiness toward protons by comparison to blank experiments, revealing a strong modification of their permeability to protons.

## DISCUSSION

In a recent work (*3*), we demonstrated that membrane sterols could be involved in the yeast's ability to adsorb brown phenolic compounds from oxidized wine model solutions, as already hypothesized previously (*7*, *8*). Moreover, in the same work, we proved that a direct interaction between the main yeast membrane sterol—ergosterol—in a solubilized form and the phenolic brown compounds could occur in vitro. On this basis, we dedicated the present study to check the reactivity of different sterols in entire yeast cell membranes or trapped in artificial membranes against brown phenolic compounds.

First, a modification of the orderliness of membrane phospholipids was observed in the presence of polymeric phenolic compounds resulting from the oxidation of (+)-catechin/glyoxylic

**Table 3.** Effect of the Addition of Polymeric Compounds Resulting from the Oxidation of the Wine Browning Model Solutions on the $V_{max}$ $NH_4^+$ Parameter of Plasma Membrane Vesicles Specifically Enriched in Different Types of Sterol[a]

| | $V_{max}$ $NH_4^+$ (% quenching $s^{-1}$) of vesicules enriched in | | |
|---|---|---|---|
| | ergosterol | phytosterols | cholesterol |
| control | 0.17 ± 0.01 | 0.29 ± 0.00 | 0.24 ± 0.00 |
| acetaldehyde | 0.15 ± 0.09 (NS) | 0.29 ± 0.07 (NS) | 0.49 ± 0.05 (***) |
| glyoxylic acid | 0.32 ± 0.01 (***) | 0.27 ± 0.04 (NS) | 0.34 ± 0.02 (***) |
| catechin | 0.13 ± 0.06 (NS) | 0.28 ± 0.07 (NS) | 0.40 ± 0.04 (***) |
| catechin/acetaldehyde | **0.11 ± 0.01 (NS)** | **0.24 ± 0.07 (NS)** | 0.30 ± 0.03 (***) |
| catechin/glyoxylic acid | 0.24 ± 0.03 (***) | **0.36 ± 0.03 (***)** | 0.32 ± 0.03 (***) |

[a] Mean and standard deviation of triplicates. The differences between each modality and the control were tested by Tukey's statistical test. NS, not significant; *, significant differences at a level of $P \leq 0.05$; ***, significant differences at a level of $P \leq 0.001$. By comparison with the three blank experiments, only the modalities written in bold letters were taken into account for discussion.

**Table 4.** Effect of the Addition of Polymeric Compounds Resulting from the Oxidation of the Wine Browning Model Solutions on the $Q_{max}$ $H^+$ Pumping Parameter of Plasma Membrane Vesicles Specifically Enriched in Different Types of Sterol[a]

| | $Q_{max}$ $H^+$ (% quenching) of vesicules enriched in | | |
|---|---|---|---|
| | ergosterol | phytosterols | cholesterol |
| control | 6.36 ± 1.75 | 8.45 ± 1.78 | 13.26 ± 1.30 |
| acetaldehyde | 5.11 ± 0.18 (NS) | 9.07 ± 1.21 (NS) | 12.6 ± 0.70 (NS) |
| glyoxylic acid | 19.3 ± 0.25 (***) | 7.73 ± 1.66 (NS) | 9.13 ± 1.27 (***) |
| catechin | 5.13 ± 0.12 (NS) | 10.03 ± 2.47 (NS) | 15.53 ± 2.20 (NS) |
| catechin/acetaldehyde | **6.84 ± 0.06 (NS)** | **18.03 ± 4.53 (***)** | **11.73 ± 0.67 (NS)** |
| catechin/glyoxylic acid | 15.83 ± 2.49 (***) | **13.45 ± 5.73 (***)** | 8.53 ± 1.72 (***) |

[a] Mean and standard deviation of triplicates. The differences between each modality and the control were tested by Tukey's statistical test. NS, not significant; *, significant differences at a level of $P \leq 0.05$; ***, significant differences at a level of $P \leq 0.001$. By comparison with the three blank experiments, only the modalities written in bold letters were taken into account for discussion.

acid model solutions, when entire yeast cells were previously enriched in ergosterol or cholesterol. This strengthening of the plasma membrane is therefore linked to an angular reorientation of the lipid acyl chains within the membrane. We had previously shown that the inhibition of wine model solution browning by the yeast lees could be attributed to a focused adsorption of the colorless intermediates compounds, mainly (+)-catechin and primary dimers (*3*). Because addition of (+)-catechin did not alter the orderliness of plasma membrane, it could be hypothesized that primary dimers resulting from the oxidation of (+)-catechin/glyoxylic model solutions are the main protagonists of such membrane fluidity decrease.

In a second set of experiments, yeast plasma membrane vesicles reconstituted with different types of sterol were used. ACMA quenching experiments upon plasma membrane $H^+$-ATPase activation by ATP addition revealed that the functional properties of the reconstituted vesicles in the presence of grape phytosterols were severely affected by contact with polymeric compounds resulting from the wine model solutions. This alteration of their functional properties led to activation of the plasma membrane-bound ATPase and to a strong modification of their passive permeability toward protons. Such an activation may be attributed to the fact that membrane phytosterols, sphingolipids, and phenolic polymeric compounds aggregated into membrane microdomains (or lipid rafts), which provide more stable membrane surface to the plasma membrane-bound ATPase but also increase the permeability barrier of the membrane, by similarity

**Table 5.** Effect of the Addition of Polymeric Compounds Resulting from the Oxidation of the Wine Browning Model Solutions on the Differences between $Q_{max}$ $H^+$ Pumping and $Q_{max}$ $NH_4^+$ Parameters of Plasma Membrane Vesicles Specifically Enriched in Different Types of Sterol[a]

| | $[Q_{max}\ H^+ - Q_{max}\ NH_4^+]$ (% quenching) of vesicules enriched in | | |
|---|---|---|---|
| | ergosterol | phytosterols | cholesterol |
| control | 0.85 ± 1.65 | 0.00 | 0.00 |
| acetaldehyde | 0.00 ± 0.34 (NS) | 0.00 ± 2.80 (NS) | 0.00 ± 1.70 (NS) |
| glyoxylic acid | 0.00 ± 3.68 (***) | 0.00 ± 0.89 (NS) | 0.00 ± 2.28 (NS) |
| catechin | 0.00 ± 0.09 (NS) | 0.00 ± 2.88 (NS) | 0.83 ± 2.54 (NS) |
| catechin/acetaldehyde | **2.99 ± 0.09 (***)** | **7.80 ± 3.8 (***)** | **0.00 ± 1.46 (NS)** |
| catechin/glyoxylic acid | 1.58 ± 5.22 (NS) | **0.00 ± 3.99 (NS)** | **0.00 ± 7.53 (NS)** |

[a] Mean and standard deviation of triplicates. The differences between each modality and the control were tested by Tukey's statistical test. NS, not significant; *, significant differences at a level of $P \leq 0.05$; ***, significant differences at a level of $P \leq 0.001$. By comparison with the three blank experiments, only the modalities written in bold letters were taken into account for discussion.

with the complexation of lipids (sphingolipids and sterols) and plasma membrane-bound ATPase during the biogenesis of the membrane (*24*).

These results raised two different types of questions that needed to be resolved: (1) what is the role of the yeast cell wall as a mechanical riddle in the selection of polymeric phenolic compounds that could really reach the yeast cellular membrane, and (2) what are the chemical mechanisms underlying the strong reactivity between these polymeric compounds and the membrane sterols (and mainly phytosterols) within the lipidic membrane environment?

A preliminary answer to the first question could be proposed. Water-soluble phenolic compounds exhibit molecular weights ranging between 500 and 3000 as follows: catechin, MW 280; dimers, MW about 572; trimers, MW about 858; and highly polymerized polymers, MW ranging from 950 to 3000 (the limit of water solubility was reached for oligomers over six units). On the other hand, only a few works have dealt with the "porosity" of the yeast cell wall: the threshold for exclusion from the yeast cell wall was calculated to be between 620 and 780 (*25*) and up to 4500 (*26*). Therefore, it could be hypothesized that some of the highly polymerized phenolic compounds may be excluded from the yeast cell wall, but because yeast cell wall is plastic in many ways (*27*) (modified during cell growth and after cell death), it is difficult to draw any conclusions on this fact.

The second question could find part of an answer in the known reactivity of polyphenols. It is known that precipitation of polyphenols is linked to intermolecular binding dominated by stacking of polyphenol rings onto planar hydrophobic surfaces and is strengthened by multiple cooperative binding of polyphenolic rings (*28*). Sterols play major roles in the building and maintenance of eukaryotic membranes: they mainly regulate membrane fluidity and permeability, ethanol resistance, and plasmic $H^+$-ATPase activity as well as the cell cycle and uptake of exogenous sterols (for a review, see ref *29*). The three sterols used in the present study to enrich yeast cells or yeast plasma membranes belong to the class of the "promoter" or "membrane active" sterols, which all exhibited flat, fused rings, a hydroxyl headgroup, an alkyl tail, and a small molecular area (*30*). As recently demonstrated, the three tested sterols exhibit smooth α-faces that imply that the ring part of the molecules pack more closely to the lipid chains of phospholipids within the membrane (*31*). Therefore, these sterols were inserted on the outer and inner faces of the membranes exposing their hydroxyl headgroups toward the exterior of the membrane. Besides their ring part, the

main structural differences between these three sterols are the result of the fact that ergosterol differs structurally from cholesterol having a methyl group on its side chain and that $\beta$-sitosterol exhibits a longer substitution on the side chain than ergosterol. These subtle structural variations give rise to marked alterations in membrane properties, modifying mainly lipid packing within the membrane (*20*, *31*, *32*). Several works have demonstrated that the repartition of these sterols within the biological membranes is not homogeneous, forming clusters (*33*), the sterol structure influencing also phase properties of the membranes (*34*). Quite recently, it was demonstrated that the lipidic environment of the membrane does not influence the side-chain flexibility and that only the straightening of the side chain influences the formation of hydrogen bonding between the phosphate group of phospholipids and the hydroxyl group of sterols, with an average of one hydrogen bond per phospholipid (*35*). However, no data are available on the behavior of $\beta$-sitosterol in such systems. It can only be hypothesized that the longer substituted side chain of $\beta$-sitosterol guarantees a more favorable location of the hydroxyl group within the more polar and hydrated region of the lipid bilayer, leading to a substantial exposure of the sterol hydrophobic surface to water. Because of the presence of multiple hydroxyl groups, phenolic compounds can form extensive hydrogen bonding. Such binding becomes very strong as a result of multisite binding, and this explains the complexation of polyphenols with various substrates capable of making hydrogen bonds (*36*). Moreover, conformation mobility also contributes to a great extent, which facilitates multiple site binding. It could then be hypothesized that some polymeric phenolic compounds (likely dimers and trimers mainly resulting from the oxidation of (+)-catechin/glyoxylic model solutions) could engage multiple hydrogen bonding with $\beta$-sitosterol clusters at the surface of cellular membranes as planar hydrophobic surfaces. It is noteworthy that xanthen and xanthylium dimers (formed after dehydration and oxidation of the first condensation dimer) exhibit rather planar structures, with three hydroxyl groups surrounding the empty core of the molecule. Such non-covalent association between $\beta$-sitosterol clusters and phenolic dimers could be stabilized by intermolecular binding dominated by stacking of polyphenol rings and strengthened by multiple cooperative bindings of polyphenolic rings, as observed for the precipitation mechanism of polyphenols (*28*).

To prove that polymerized phenolic compounds could interact with yeast plasma membrane sterols in a conclusive way is very difficult due to the complexity of both the support (yeast membranes) and the reaction products. On this basis, further studies dedicated to check at the molecular level the reactivity of different sterols trapped in artificial membranes against some purified polymeric phenolic compounds and to demonstrate effective adsorption of these compounds on cellular membranes are already in progress. A future prospect in this research area could be the utilization of slightly modified sterols in order to interfere with the presumed interactions between sterol headgroups and the polymeric phenolic dimers.

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