

# A small HSP, Lo18, interacts with the cell membrane and modulates lipid physical state under heat shock conditions in a lactic acid bacterium

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## Abstract

The small heat shock proteins (sHSP) are characterized by a chaperone activity to prevent irreversible protein denaturation. This study deals with the sHSP Lo18 induced by multiple stresses in *Oenococcus oeni*, a lactic acid bacterium. Using in situ immunocytochemistry and cellular fractionation experiments, we demonstrated the association of Lo18 with the membrane in *O. oeni* cells submitted to heat shock. The same result was obtained after exposure of cells to ethanol or benzyl alcohol, agents known to have an influence on membranes. For the different stresses, the protein was located on the periphery of the cell at membrane level and was also found within the cytoplasm. In order to determine if Lo18 could interact with the phospholipids, we used model membranes made of lipids extracted from *O. oeni* cells. Using fluorescence anisotropy of diphenylhexatriene (DPH) and generalized polarization of Laurdan, we showed that purified Lo18 interacts with these liposomes, and increases the molecular order of the lipid bilayer in these membranes when the temperature reaches 33.8 °C. All these data suggest that Lo18 could be involved in an adaptive response allowing the maintenance of membrane integrity during stress conditions in *O. oeni* cells.

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## 1. Introduction

All organisms respond to environmental stress by synthesizing heat shock proteins, including small heat shock proteins (sHSP) with molecular masses ranging from 15 to 42 kDa. This is a ubiquitous class of molecular chaperones, which are sequence related to the vertebrate eye lens  $\alpha$ -crystallins. Sequence similarities among sHSP are restricted to short domains on 100 amino acids at the C-terminal region referred to as the  $\alpha$ -crystallin domain. In vitro, sHSP can bind to denatured proteins, prevent their irreversible aggregation, and maintain them in a refolding state [1]. This pool of sHSP/denatured protein complex can then interact with ATPase chaperones, such as the DnaK system or GroEL/ES, in order to

restore their functional structure [2,3]. In their native state, sHSP form oligomers of 9–32 subunits or display a continuum of variable and dynamic quaternary structures.

The synthesis of sHSP is induced by various stresses, such as heat, ethanol and low pH, in prokaryotes and plays a role in the acquisition of thermotolerance in many organisms [4]. During stress, the plasma membrane is the first sensitive target of damage in cells, as demonstrated by the leakage of intracellular substances and the variation in membrane fluidity [5,6]. Temperature is an important factor in the environment of biological organisms, in particular for bacteria. It has two main consequences on the plasma membrane; it both increases fluidity, and denatures proteins. Recently, a new function of certain sHSP on cell membranes has been discovered. Indeed,  $\alpha$ -crystallin, a major component of the lens in vertebrate eyes, has been shown to bind to ocular plasma membranes and synthetic phospholipid vesicles [7]. Moreover, part of the protein HSP17 is associated with thylakoid membranes in

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*Synechocystis* PCC 6803 [8]. Both these sHSP stabilize heat-stressed membranes by increasing membrane physical order [9]. This ability to interact with model lipid membranes and to increase lipid order in the liquid crystalline state was described for the GroEL from *Escherichia coli*, and so designated lipochaperonin [10].

*Oenococcus oeni* is a lactic acid bacterium mostly responsible for malolactic fermentation in wine [11]. An 18 kDa sHSP, called Lo18, is induced by multiple stresses, such as heat, ethanol, sulphites, low pH, and during the stationary growth phase in *O. oeni* cells [12,13]. The protein Lo18 was found to be partly peripherally associated with the cytoplasmic membrane of *O. oeni* after a temperature upshift (30 to 42 °C) [14]. Membrane association of Lo18 depends on the temperature. Up to 44 °C, the partition between cytoplasmic and membrane fractions appeared to be equivalent. After heat shock at 46 °C, Lo18 was associated with the membrane fraction to a greater extent [15].

Moreover, expression of this sHSP is induced by the addition of a membrane fluidizer, benzyl alcohol, suggesting that Lo18 expression could be regulated by the level of membrane fluidity [15].

The aim of this work is to highlight the lipochaperone activity of Lo18, a sHSP induced in *O. oeni* cells by multiple stresses. Using in situ immunocytochemistry and cellular fractionation experiments, we pinpointed the localization of Lo18 in *O. oeni* cells submitted to heat shock and exposure to ethanol or benzyl alcohol, all of which have an influence on membranes. Moreover, using fluorescence anisotropy of diphenylhexatriene (DPH) and generalized polarization of Laurdan, we showed that purified Lo18 interacts with liposomes formed by lipids extracted from *O. oeni* cells, and increases the molecular order of the lipid bilayer in these membranes as soon as the temperature reaches 33.8 °C.

## 2. Materials and methods

### 2.1. Bacterial strains and media

*O. oeni* ATCC BAA-1163 (formerly IOB 8413) was grown at 30 °C in FT80 medium (pH 5.3) [16] modified by the addition of meat extract instead of casamino acids. *Escherichia coli* BL21 Star (DE3) (Invitrogen) was grown in Luria–Bertani medium at 37 °C supplemented with kanamycin (50 µg/mL) under aerobic conditions.

### 2.2. Shock experiments

Exponential growth phase *O. oeni* cells were shocked for 1 h at 30 °C by adding absolute ethanol (10 and 11% v/v) or benzyl alcohol (60 mM), or by heat shock alone at 42 °C and 46 °C. Samples were then prepared for immunocytochemistry or for cellular fractionation.

### 2.3. Cellular fractionation

All samples were washed with 10 mM Tris–HCl buffer pH 8. Then cells were broken by ultrasonic treatment (4×45 s, spaced out one min on ice, power 5) (Vibra Cell, Sonics and Materials INC, Danbury, USA) and fractionated as previously described by Jobin et al. [14]. Briefly, after centrifugation to remove unbroken cells, the supernatant was ultracentrifuged at 68,600×g for 1 h. The supernatant, containing cytoplasmic proteins, was

collected, and the pellet, containing the membranes, was resuspended in 10 mM Tris–HCl buffer pH 8.

### 2.4. Immunoblotting

For each sample, 15 µg proteins of crude extract, cytoplasmic extract and membrane were loaded on 12% SDS-PAGE performed by the method of Laemmli [17]. Protein concentration was determined using a Biorad protein assay method [18] with bovine serum albumin (BSA) (Euromedex) as a standard. Immunoblot was carried out as previously described by Guzzo et al. [13], with Lo18 rabbit polyclonal antibody diluted at 1:1000 as primary antibody. The peroxidase coupled to anti IgG antibody was detected using chemiluminescence (ECL; Amersham).

### 2.5. Immunolabelling and TEM

*O. oeni* cells were washed with 0.1 M sodium phosphate buffer pH 7.2 and suspended in the same buffer containing 0.1% or 0.5% (v/v) glutaraldehyde and 4% (w/v) paraformaldehyde for 3 h at 4 °C. After fixation, the cells were washed in the same buffer over a period of 1 h. To block the non-specific attachment of antibodies to free aldehyde groups for indirect immunocytochemical labelling, the cells were treated with 50 mM ammonium chloride in phosphate buffer 30 min at 4 °C. After dehydration at 4 °C by the incremental addition of ethanol to 30%, and at –30 °C to 100%, the cells were progressively infiltrated in Lowicryl K4M in a Reichert AFS (Automatic Freeze Substitution System) according to a progressive low-temperature protocol at –30 °C [19]. K4M resin was polymerized for 48 h at –30 °C, then for 1 h at –18 °C and 1 h at +10 °C with UV light. Alternatively, dehydration and infiltration were performed in AFS at –20 °C in LR White Medium Grade resin (Oxford Instruments, Orsay, France) [20]. Resin, to which 0.5% benzoin methyl ether (Sigma) was added, was polymerized for 48 h at –20 °C, then 3 h at 10 °C with UV light.

Ultrathin sections were collected on formvar-coated nickel grids and then successively incubated at room temperature as follows for Lo18 localization: 30 min with 10 mM Tris–HCl buffer pH 7.2 with 0.9% (w/v) NaCl (TBS), containing 0.5% (w/v) dried milk, 10% (v/v) normal goat serum and 100 mM glycine, then 30 min with purified polyclonal rabbit anti-Lo18 antibody diluted 1/30 in TBS–0.5% dried milk. The antibodies were purified on a HiTrap rProteinA affinity column (Amersham Pharmacia Biotech) as described by the manufacturer. After washes in the same buffer, the grids were incubated for 1 h with a goat anti–rabbit immunoglobulin G conjugate (EM GAR-15 Biocell Research Laboratory) labelled to 15 nm colloidal gold particles and diluted at 1/100 in TBS. The grids were washed twice for 5 min in TBS–0.05% Tween 20, then twice in TBS and in distilled water before being counterstained with 1% (w/v) aqueous uranyl acetate for 10 min. Sections were visualized with a Hitachi 7500 transmission electron microscope operating at 80 kV. Specificity of labelling was assessed through the following control experiments: (i) omission of the primary antibody incubation step, (ii) incubation with the pre-immune serum instead of primary antibody (iii) incubation with the immunodepleted antibody.

### 2.6. Expression and purification of *O. oeni* Lo18

The *hsp18* gene was generated from the plasmid pJMP2 [14] by PCR amplification using two primers: Lo18N-*Nco* 5'-GACGTGCCATGGCAAATGAATTAATGGATAGA-3' (forward) and Lo18C-*Xho*-SSH 5'-GCTCCGCTC-GAGTTATTGGATTTC AATATGATGAG-3' (reverse), respectively. The coding region of the *hsp18* gene was then cloned into the *Nco*I and *Xho*I sites of the expression vector pET28a (Invitrogen). The resultant plasmid, designated pET-*hsp18*, contained the unmodified complete *hsp18* coding region without the hexahistidine tag, under control of T7 inducible promoter. *E. coli* BL21 Star (DE3) was transformed with the plasmid pET-*hsp18* and was grown at 37 °C to an OD<sub>600</sub> of 0.8. Expression was induced by addition of isopropyl-β-D-thiogalactoside (50 µM) at 15 °C overnight. After centrifugation, the cells were resuspended in a buffer containing 20 mM Tris, 250 mM NaCl, pH 8, and then disrupted by sonication. The suspension was centrifuged at 10,000×g for 15 min at 4 °C to remove unbroken cells. After a second centrifugation at 10,000×g for 15 min, the supernatant was filtered through 0.22 µm filter and loaded onto a HIC-

Phe 1 mL column (Amersham Pharmacia Biotech) equilibrated with the same buffer. The protein Lo18 was eluted with 20 mM Tris pH 8 buffer and then dialysed against a buffer containing 20 mM sodium phosphate, 150 mM NaCl, pH 7. The size exclusion chromatography was carried out on HiLoad 16/60 Superdex 200 pg (Amersham Pharmacia Biotech) equilibrated in 20 mM phosphate sodium, 150 mM NaCl, pH 7 at 4 °C, and loaded with 800  $\mu$ L of Lo18 at 5 mg/mL.

### 2.7. Lipid extraction

To extract the cellular lipids of exponential growth phase *O. oeni* cells, the modified Bligh and Dyer method [21] was used after washing and resuspension of cells in NaCl 9%. After centrifugation at 3000 $\times$ g, the chloroformic phase was recovered and stored at –20 °C. Lipids were quantified by measuring the amount of inorganic phosphorus using the kit Phosphore UV (Biomérieux).

### 2.8. Preparation of liposomes

A film of extracted lipids was obtained by evaporation of chloroform with rotavapor at 65 °C under reduced pressure for 45 min followed by rehydration with pre-warmed buffer 20 mM sodium phosphate, 150 mM NaCl, pH 7 at 65 °C. The liposome suspension was gently mixed and sonicated 2 $\times$ 30 s. The average size of the liposomes in the suspension, determined by the laser scattering method with the granulometer Mastersizer 2000 (Malvern Instrument, Malvern, UK), was 100 nm.

### 2.9. Steady-state fluorescence anisotropy measurements with DPH

Measurements were done using a Fluorolog 3 spectrofluorimeter (Jobin Yvon Horiba, USA) equipped with excitation and two emission polarizers and a thermoelectric peltier junction (Wavelength electronics Inc., USA) for the temperature. Experiments were done using a cuvette filled with 2.5 mL of liposome suspension (12 nmol phospholipids) labelled by 4  $\mu$ L of 50  $\mu$ M DPH (1,6-diphenyl-1,3,5-hexatriene, Molecular Probes) (excitation 360 nm; emission 430 nm). After probe insertion at 30 °C, the protein was either added or not and the temperature was modified. The temperature was controlled inside the cuvette using a thermocouple ( $\pm$ 0.2 °C) and modified at a rate of 1 °C/min. Anisotropy values were calculated according to Shinitzky et al. [22], with the *G* value fixed after measurement on this instrument (*G*=1.042). BSA (67 kDa) was used as a control as cytoplasmic protein for unspecific protein effects.

### 2.10. Laurdan fluorescence measurements

Measurements were done in a Fluorolog 3 spectrofluorimeter without the polarizers. 2.5 mL of liposomes suspension (12 nmol phospholipids) were equilibrated in the fluorometer sample cell with 4  $\mu$ L of 60  $\mu$ M Laurdan (2-dimethylamino-6-lauroyl-naphthalene, Molecular Probes) at 30 °C until the stabilization of fluorescence emission (generally about 45 min). Emission spectra were then acquired from 400 to 520 nm (excitation=365 nm, 2 nm bandpass) at different temperatures as previously done for anisotropy measurement. Generalized polarization (GP) was calculated from the emission intensities using the following equation adapted from the work of Parasassi et al. [23]:  $GP = (I_{435} - I_{485}) / (I_{435} + I_{485})$ , where  $I_{435}$  and  $I_{485}$  refer to the emission intensities at those wavelengths.

## 3. Results

### 3.1. Immunolocalization of the Lo18 protein in *O. oeni* cells

To elucidate if Lo18 location could be influenced by other agents than heat, that have an action on the membrane fluidity, *O. oeni* cultures were sampled in the exponential growth phase and were submitted to different treatments at 30 °C: ethanol 10% v/v or benzyl alcohol 60 mM, a membrane fluidizer. They were then fractionated to separate membrane and cytoplasmic

fractions, as described in materials and methods. All the tested concentrations of agents neither interfered with the growth of the bacteria nor induced lethality (data not shown). As shown in Fig. 1, Lo18 was found to be associated with the membranes and within the cytoplasm.

Immunoelectron microscopy with specific purified antibodies against Lo18 was used to localize the protein in situ and to determine its pattern of distribution in *O. oeni* cells after heat shock or membrane fluidizer treatments. First, the appropriate fixation and embedding conditions preserving antigenicity had to be determined. The polyclonal antibody gave specific response only with 0.5% glutaraldehyde–4% paraformaldehyde as a fixative mixture and when embedding was done with LK4M. This technique is sufficiently resolvent to determine with precision the protein localization, even if the ultrastructure was not well preserved.

Exponential growth phase *O. oeni* cells were free of labelling (Fig. 2A). However, in some cells, one or a maximum of two gold particles could appear in the cytoplasm. This is in accordance with previous work [12] that showed low expression during this growth phase of the *hsp18* gene encoding Lo18. After heat shock at 42 °C, *O. oeni* cells induced Lo18 synthesis. Most of the labelling was found to be evenly distributed within the cytoplasm and some of the gold particles were located at the periphery of the cell, most frequently on the cytoplasmic membrane level (Fig. 2B). After heat shock at 46 °C (Fig. 2C), cells exhibited a specific labelling localized at the periphery of the cell and gold particles were found to be associated with the cytoplasmic membrane (inset, Fig. 2C). A specific labelling was also visualized when cells were treated with ethanol 11% (v/v). For approximately 70% of cells observed under the microscope (the estimation was done on 300 cells observed on three different embedded samples), gold particles were detected in the cytoplasm and along the plasma membrane (Fig. 2D). In some cells treated with ethanol, gold particles were mainly detected along the plasma membrane (Fig. 2E). The cytoplasm of 60 mM benzyl alcohol treated cells, displayed irregular labelling and a few gold particles could be observed along the cytoplasmic membrane (Fig. 2F). Negative results (data not shown) were obtained for immunological controls.

### 3.2. Purification of Lo18 in one step

We overproduced the recombinant protein using the pET system without any additional amino acids in *E. coli* BL21 Star.

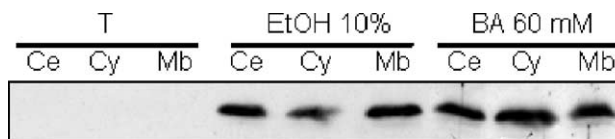


Fig. 1. Immunoblot analysis of Lo18 in *O. oeni* fractionated cells. Fractionation was performed on exponential growth phase cells after 1 h at 30 °C in the absence (T) or in the presence of ethanol 10% v/v or benzyl alcohol 60 mM. Ce, Cy and Mb correspond to crude extract, cytoplasm and membranes, respectively, separated on a 12% SDS gel.



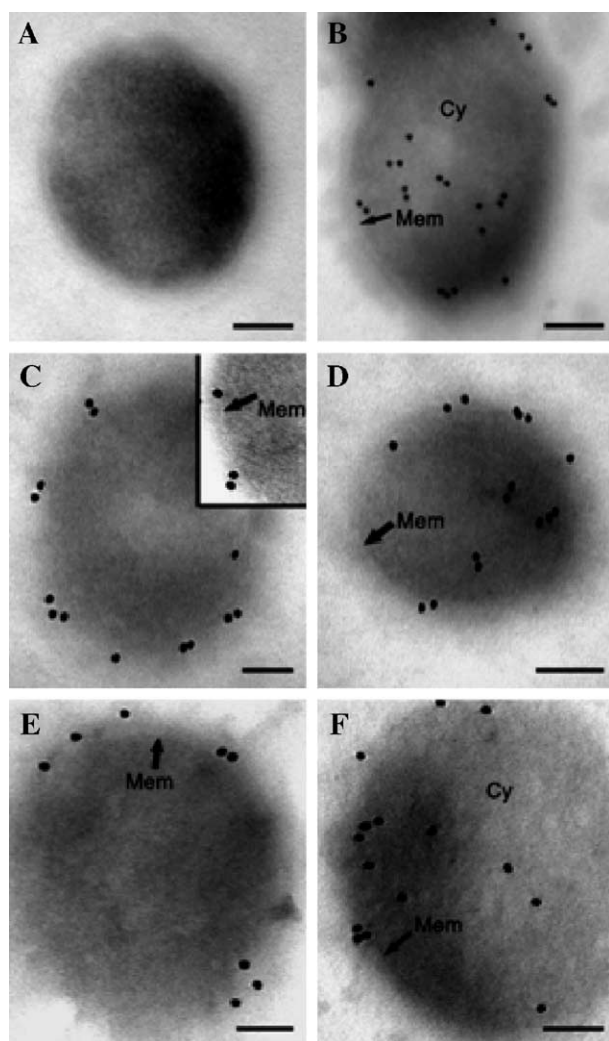


Fig. 2. Immunolocalization of Lo18 in *O. oeni* cells using a polyclonal antibody against Lo18 followed by GAR-15 secondary antibodies conjugated to 15 nm gold particles. (A) *O. oeni* cells sampled at 30 °C in the exponential growth phase do not exhibit gold labelling. (B) Heat shocked cells at 42 °C: gold targets are dispersed in the cytoplasm and are also located specifically in the cytoplasmic membrane. (C) Heat shocked cells at 46 °C: the antigen is then associated mainly with the cytoplasmic membrane, in the inset enlargement of the membrane. (D and F) Antigen distribution within a cell after membrane fluidizers: ethanol (11% v/v) (D and E) or benzyl alcohol (60 mM) (F): for both treatments, gold labelling occurs within the cytoplasm and on the cytoplasmic membrane. (E) For other ethanol treated cells, the labelling was mainly detected along the cytoplasmic membrane. Scale bars, 100 nm.

The purification was performed in one step using hydrophobic interaction chromatography. Lo18 was eluted as one homogeneous peak with buffer without NaCl. After migration on SDS PAGE (Fig. 3A), we detected weakly two other bands that correspond to the tryptophanase of *E. coli* (55 kDa) and the protein Lo18 in which about twenty amino acids in the N terminal region were missing, according to the results of mass spectrometry and N-terminal sequencing. The quantity of these proteins was negligible in relation to the recombinant protein Lo18. The purified protein displayed a chaperone activity on the citrate synthase in vitro at 45 °C as described by Delmas et al. [15] (data not shown). The apparent molecular mass of the

recombinant Lo18 protein was estimated to 380 kDa by size exclusion chromatography (Fig. 3B). As deduced from the sequence, Lo18 has a theoretical mass of 16,930 Da. Therefore, we can estimate that, in our experimental conditions, Lo18 forms a 22 to 24 subunits oligomer.

### 3.3. Influence of Lo18 on the membrane fluidity of liposomes prepared from *O. oeni* cells

To determine if Lo18 could interact with phospholipids, we tested the impact of protein on liposomes formed of lipids extracted from *O. oeni* cells. The aim of these experiments was to determine the variation of membrane fluidity, measured by steady-state fluorescence anisotropy of DPH, during temperature ramping in presence of Lo18. The steady-state fluorescence anisotropy of DPH labelled liposomes, a value inversely related to the fluidity of the bilayer, was used to monitor the dynamic properties of membranes. The motional order within the hydrophobic core of the membrane was measured using the hydrophobic probe DPH. Fig. 4 shows the temperature-dependent profiles of DPH anisotropy in liposomes within a temperature range from 15 to 70 °C. The increasing temperature produced a gradual decrease in the anisotropy values indicating a rise in fluidity. The absence of an abrupt change in the slopes of these profiles indicates that no abrupt phase transition of bulk lipids occurred within the tested temperature range. Nevertheless, several phase transitions could have occurred progressively according to the nature of phospholipids mixture and would explain the gradual decrease in the anisotropy values. A statistical analysis after the linearization of curves with a logarithmic transformation allowed to calculate hyperboles of confidence at 95%. It appears that the curves with or without Lo18 are different. There is no significant difference below 33.8 °C and a significant difference above 33.8 °C, between these curves.

In the presence of Lo18 incubated with liposomes, there is no effect on the order of the membrane below the temperature of 33.8 °C. However, above 33.8 °C, the increase in acyl chain motion caused by the rise in temperature was significantly reduced in the presence of Lo18, indicating greater lipid order. Therefore, Lo18 insertion caused a higher degree of membrane order (or decrease in fluidity) in liposomes prepared from lipid membranes. This phenomenon of fluidity at high temperatures is obtained for the liposomes in the presence of the sHSP Lo18. As a control experiment, we used the BSA in the same molar ratio instead of Lo18 and such an effect on the membrane above 33.8 °C was not observed.

### 3.4. Effect of Lo18 on the membrane physical state

To study the influence of Lo18 on the phase behaviour of phospholipids and their structural properties, fluorescence of Laurdan on liposomes was measured under different temperatures. Laurdan spectral shifts are usually quantified in the form of generalized polarization (GP). The emission spectrum obtained from liposomes of *O. oeni* labelled by a Laurdan probe is

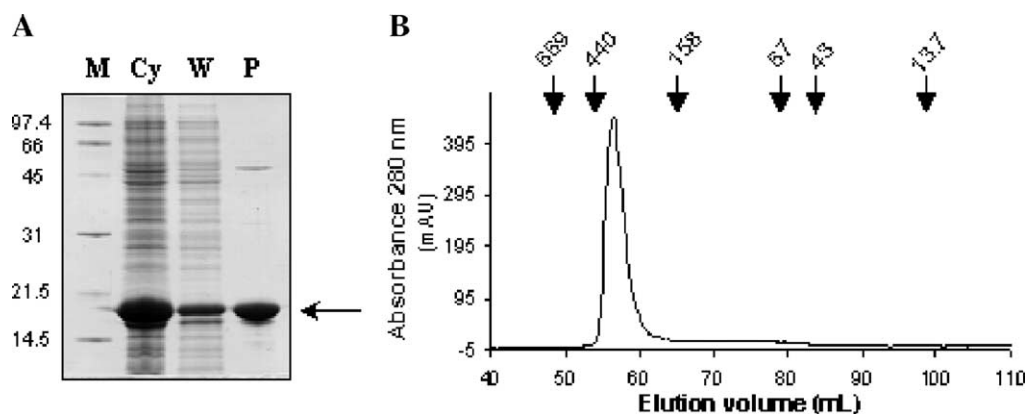


Fig. 3. (A) Lo18 purification on a 12% SDS gel stained with Coomassie blue. M, Cy, W, P correspond to molecular weight markers (kDa), cytoplasmic fraction, wash and purified fraction, respectively. Arrow indicates the location of Lo18. (B) Elution profile of purified Lo18 on HiLoad 16/60 Superdex 200 pg size exclusion column at 4 °C monitored by absorption at 280 nm. Lo18 elutes as one homogenous peak corresponding to 380 kDa. The elution positions of the molecular mass standards (kDa) are indicated at the top.

characterized by two maxima, at 435 nm (blue band) and 485 nm (red band). This predominantly blue band at 30 °C is a characteristic of a lipid bilayer in the gel state. The GP value decreased gradually with temperature showing a progressive phospholipid shift from the gel state to the liquid crystalline state (Fig. 5).

The comparison of thermal profiles of Laurdan GP inserted into liposomes in presence or not of Lo18 reveals a more evident ordering effect of the protein on the membrane at elevated temperature in the tested temperature range. The decrease in the GP slope in the presence of Lo18 is in the same temperature range as observed with DPH probe.

These results suggest that Lo18 has a direct effect on the phospholipids and so modulates the membrane properties of *O. oeni* phospholipids by increasing their molecular order, which leads to a decrease in membrane fluidity in *O. oeni* membranes.

#### 4. Discussion

The 18-kDa sHSP Lo18 identified in the lactic acid bacterium *O. oeni*, mainly responsible for the malolactic fermentation in wines, is induced by multiple stresses: exposure to heat, ethanol and benzyl alcohol, acidity and sulphites, during the stationary growth phase [12,13,15]. Preliminary studies have shown that the localization of this protein was at once cytoplasmic and associated with membrane during heat shock at 42 °C, and that this association was due to weak bonds [14]. In this work, immunogold electron microscopy showed that Lo18 has a double localization and is mostly associated with the membrane, when cells are heat-shocked or incubated with ethanol or benzyl alcohol, which have an action on the membrane. Several prokaryote and eukaryote sHSP are well known to have different localizations. Six families of plant sHSP can be distinguished based on their sequence similarities and their cellular location. Three families (I, II and III) reside in

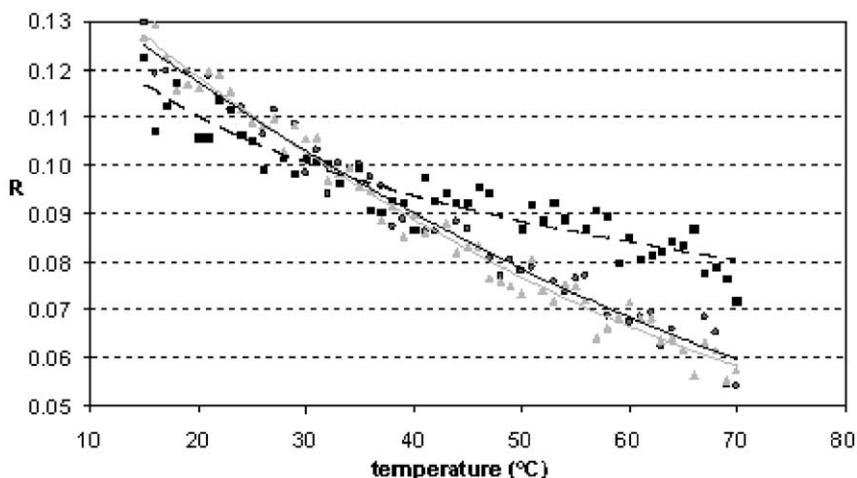


Fig. 4. Effect of Lo18 on membrane fluidity of lipid vesicles extracted from *O. oeni* cells. Fluorescence anisotropy of DPH probe (excitation 360 nm; emission 430 nm) inserted in vesicles according to the temperature, in the presence of Lo18 (■) or BSA (▲) with a protein:lipid molar ratio of 1:40 and without protein (●). The experiments were done in triplicate. The results of one experiment are shown.

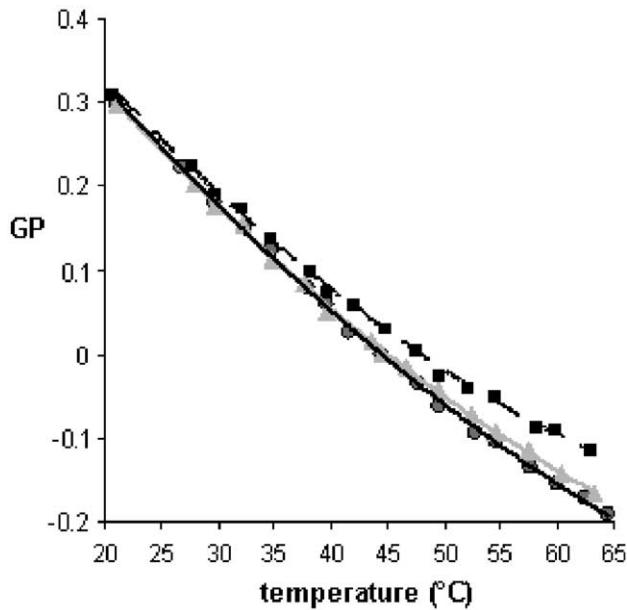


Fig. 5. Effect of Lo18 on the structural properties of phospholipids. Generalized polarization of Laurdan (excitation = 365 nm) in lipid vesicles extracted from *O. oeni* cells was measured according to the temperature in the absence (●) or in presence of Lo18 (■) or BSA (▲) with a protein:lipid molar ratio of 1:40. The experiments were duplicated.

the cytosol; one is located in the chloroplasts, one is located in the mitochondria and one is located in the endoplasmic reticulum [24]. In contrast to plant sHSP, the mammalian family members of sHSP, including  $\alpha$ A- and  $\alpha$ B-crystallin, seem to be mainly restricted to the cytosol and to the nucleus [25]. However, association of HspB2 with the outer membrane components of mitochondria was shown under heat stress and linked to the adaptive stress response on C2C12 cells [26].  $\alpha$ -Crystallin has been shown to bind to ocular plasma membranes and synthetic phospholipid vesicles [7,27]. *Synechocystis* HSP17 has been shown to be in part associated with thylakoid membranes [8]. The sHSP SP21 of *Stigmatella aurantiaca* [28] and HSP 12 of *Saccharomyces cerevisiae* [29] are located on the external side of cytoplasmic membrane.

Heat and ethanol stress cause similar changes to the composition of plasma membrane proteins in yeast [30]. Both heat shock and ethanol exposure will cause membrane-disruptive effects and membrane–lipid changes in addition to protein denaturation. Benzyl alcohol is known to have a strong fluidizing effect [31]. In comparison, ethanol acts through a more complex mechanism and has a deleterious effect on cellular proteins. Alcohols increase membrane fluidity mainly in the hydrophobic core of the lipid bilayer [32]. Our results show clearly that, when *O. oeni* cells are incubated in the presence of ethanol or benzyl alcohol, Lo18 synthesis is induced and the protein is associated with the membrane. These results are similar to those obtained on heat-stressed cells, indicating that Lo18 should help to maintain cell integrity at the membrane level in stress conditions.

sHSP can be found in various locations in the cell, that may determine their functions. A potential link between sHSP and membrane function has emerged over the last years. Moreover,

it seems that Lo18 interacts with phospholipids and our results showed that the association of Lo18 with liposomes formed from purified total lipids of *O. oeni* membranes reduced membrane fluidity at elevated temperatures. Indeed by fluorescence anisotropy of DPH, the membrane rigidifying effect of Lo18 is observable above 33.8 °C. This phenomenon was explained by GP of Laurdan which showed that Lo18 maintained phospholipids in a higher lipid order. Lo18 can modulate membrane lipid properties by direct action on phospholipids. Nevertheless, we do not exclude that the protein could interact with the membranes at all the tested temperatures, but the effect on the phospholipids is only detectable for temperatures superior to 33.8 °C with this technique. A similar activity has been demonstrated for *Synechocystis* HSP17, i.e., an amphitropic protein that can stabilize heat-stressed membranes [9], but, like  $\alpha$ -crystallin, can regulate membrane lipid polymorphism [33]. Both of the latter sHSP have a rigidifying action on highly unsaturated membranes and, in addition, HSP17 increases the fluidity in the gel state [33]. On the contrary, our results showed that Lo18 acts on membrane fluidity only at high temperatures and has no detectable effect at low temperatures, between 15 to 33.8 °C.

Several hypotheses can be suggested to explain the interaction of Lo18 with membranes when the temperature reaches a value of about 34 °C. First, significant rearrangement of acyl chain packing can occur allowing recognition and interaction with Lo18. Indeed, HSP17 and  $\alpha$ -crystallin strongly modulate the membrane properties of anionic phospholipids [33], and *S. cerevisiae* HSP12 protects the membrane of positively charged liposomes against desiccation with electrostatic interactions [29]. It could be interesting to analyse the Lo18/membrane interaction on liposomes with different lipid compositions, for example sphingomyelin (SM), phosphatidylethanolamine (PE) or phosphatidylglycerol (PG) that were the most abundant lipids identified in the plasma membrane of *O. oeni* accounted for about 70% of the total phospholipids [34]. Second, conformational changes of Lo18 can occur thus allowing membrane interaction. Different studies have shown that increase in temperature induced exposure of hydrophobic surfaces of sHSP [9,35]. Moreover, as demonstrated for rhizobial HspB and HspH, the oligomeric structure of sHSP can dissociate reversibly with elevated temperatures [36]. The apparent molecular masses of HspB were superior to 1.5 MDa at 4 °C, 229 kDa at 30 °C, 75 kDa at 40 °C and 43 kDa at 45 °C. This kind of phenomenon could explain the interaction of Lo18 on membranes for temperatures above 33.8 °C.

Taken together, these data reinforce the idea that a lipid associated pool of sHSP may play a role in membrane protection. The rigidification of the membrane by sHSP during heat stress in the initial phases of stress conditions could be a component of cell adaptation involving the readjustment of lipids.

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