Salt induces biosynthesis of hemolytically active compounds in the xerotolerant food-borne fungus *Wallemia sebi*

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**Abstract**

*Wallemia sebi* is a xerotolerant, ubiquitous, food-borne, mycotoxigenic fungus. An ethanol extract of its mycelium demonstrated a strong hemolytic activity, which was further enhanced at high salt concentrations in the growth medium. Characterization of the extract using gas chromatography–mass spectrometry revealed a mixture of sterols and unsaturated fatty acids, indicating the latter as responsible for the hemolytic activity. The lytic activity of the extract is here studied using red blood cells and artificial small lipid vesicles with various lipid compositions. This shows concentration-dependent hemolysis and preferential activity toward lipid membranes with greater fluidity. The *W. sebi* lytic activity on mammalian erythrocytes shows its potential involvement in the formation of lesions in subcutaneous infections, in farmer’s lung disease, and in consumption of food and feed that are contaminated with food-borne *W. sebi*.

**Introduction**

Until 2005, the xerotolerant fungal genus *Wallemia* comprised of a single cosmopolitan species, *Wallemia sebi* (Zalar et al., 2005). *Wallemia sebi* is frequently involved in food spoilage of particularly sweet, salty, and dried food (Samson et al., 2002) and has also often been isolated from indoor and outdoor air (Takahashi, 1997), from soil (Domsch et al., 1990), and from sea salt (Das-Sarma et al., 2010). Its importance has been further emphasized by its ability to commonly cause allergy problems, which can result in farmer’s lung disease (Lappalainen et al., 1998; Roussel et al., 2004) and cutaneous and subcutaneous infections in humans (De Hoog & Guarro, 1996; Guarro et al., 2008). As a food-borne mycotoxigenic species, *W. sebi* isolated from spoiled sweet cake was shown to synthesize mycotoxins walleminol A (Wood et al., 1990) and walleminone (Frank et al., 1999), antitumor antibiotics UCA1064-A and UCA1064-B (Takahashi et al., 1993), and a related, but as yet unidentified, antifungal and cytotoxic metabolite (Mu et al., 2008).

To clarify the unresolved phylogenetic position of the genus *Wallemia* within the fungal kingdom (Wu et al., 2003) and to potentially describe new species within this genus, a large group of strains collected globally were studied. These were obtained from food preserved with low water activity (a_w), from different natural hypersaline ecological niches, and from some medically relevant samples. The morphological, physiological, and molecular characteristics analyzed resolved a new class, *Wallemiomyces*, which covers the order *Wallemiales* (Zalar et al., 2005; Matheny et al., 2006) and includes three species: *Wallemia ichthyophaga*, *W. muriae*, and *W. sebi*.

Tests of xerotolerance have shown that the *Wallemia* spp. represents one of the most xerophilic fungal taxa (Zalar et al., 2005). However, owing to the previous descriptions related to the complex of species described as *W. sebi*, the pathogenic and mycotoxin-producing potential of these individual species has remained unknown.

Our recent study on the production of bioactive metabolites by different fungal species that inhabit natural...
hypersaline environments revealed that organic extracts of all three newly described *Wallemia* species exert hemolytic activity (Sepčić et al., 2011), which was enhanced at increased salt concentrations.

Previous reports on the mycotoxigenic properties of food-borne *W. sebi* (Wood et al., 1990) and the new finding that an ethanol extract of *W. sebi* mycelia can induce concentration-dependent hemolysis of red blood cells, thus prompted the present study. As *W. sebi* can be classified as a serious threat for food safety, the aim here was to investigate hemolytically active extracts of *W. sebi* in relation to their composition and their specificity toward various lipid membranes and to the effects of external factors.

**Materials and methods**

**Culture**

*Wallemia sebi* EXF-958 (CBS 818.96) originally isolated from sunflower seeds (Zalar et al., 2005) was used. The strain was obtained from the Culture Collection of Extremophilic Fungi (EXF) of the Department of Biology, Biotechnical Faculty, University of Ljubljana.

**Culture growth conditions**

*Wallemia sebi* was grown in 500-mL Erlenmayer flasks in liquid culture on a rotary shaker at 28 °C and 180 r.p.m. for 14 days (until stationary growth phase). The medium (150 mL per flask) was composed of 0.8 g L\(^{-1}\) complete supplement mixture (CSM; Q-Biogene Bio-Systems, France), 1.7 g L\(^{-1}\) yeast nitrogen base (YNB; Q-Biogene Bio Systems), 20.0 g L\(^{-1}\) glucose (Chemica, Croatia), 5.0 g L\(^{-1}\) (NH\(_4\))\(_2\)SO\(_4\), and 200.0 g L\(^{-1}\) NaCl (Merck, Germany). The final concentration of NaCl in the growth medium was either 5% or 20%. After 15 days of incubation, the fermentation broth medium (1200 mL) was filtered (pore size, 0.8 μm). The separated mycelia were washed with 5% or 20% NaCl in distilled H\(_2\)O, to remove all traces of growth medium. These fungal mycelia were immediately freeze-dried and stored at −20 °C until the ethanol extraction.

**Preparation of *W. sebi* ethanolic extract**

Ten milliliters of 96% ethanol was added to 1 g lyophilized dry mycelia. The mixture was extracted overnight by orbital shaking at 25 °C and then centrifuged at 10 g for 40 min, followed by centrifugation at 21 500 g for 15 min. The obtained supernatant was used in all of the biological assays. The total solids (TS) in ethanolic extract were subsequently determined by gravimetry.

**Characterization of the *W. sebi* ethanolic extract**

The characterization of this ethanolic extract from *W. sebi* was performed using gas chromatography–mass spectrometry (GC/MS). This analysis by GC/MS was carried out using an Agilent 6890N/5973 GC/MSD system. The interface, source, and quadrupole temperatures were set to 280, 150, and 230 °C, respectively. An inert DB-5ms Agilent J&W column (30 m × 0.25 mm × 0.25 μm) was used, and 1 μL of the fraction to be analyzed was injected. Splitless injection was used, at a temperature of 280 °C. The oven temperature was set to 50 °C for 10 min, followed by step heating at 40 °C min\(^{-1}\), up to 200 °C. Acquisition was in the EI mode, with the mass range set for m/z 45–450. The identification of the compounds in the ethanolic extract was performed by comparison of peaks with the mass spectra of both the Wiley library and the NIST02 internal reference.

**Evaluation of hemolytic activity of the *W. sebi* ethanolic extract**

The hemolytic activity of the ethanolic extract from *W. sebi* was determined by combining 20 μL of the extract in various final concentrations with 80 μL of suspension of bovine erythrocytes in the erythrocyte buffer [140 mM NaCl, 20 mM tris(hydroxymethyl)aminomethane (TRIS) (Merck), pH 7.4], as described by Sepčić et al. (2003). The time necessary for 50% hemolysis (t\(_{50}\)) was determined at the end of each experiment. All experiments were performed at 25 °C and with three repeats. Twenty microliters of ethanol-dissolved oleic (C18:1), linoleic (C18:2), and palmitic acids (C16:0) in various final concentrations, both in pure solutions or combined in 1 : 1 : 1 molar ratio, was also assayed using the same procedure. All the used fatty acids were from Fluka (Germany). As a control, 20 μL of 96% ethanol was tested with 80 μL of the suspension of bovine erythrocytes, and it was shown to be hemolytically inactive.

To study the temperature stability of the ethanolic extract or of the fatty acid mixture, aliquots were either heated to 55, 75, or 100 °C for 30 min or frozen at −20 °C, and the residual hemolytic activities were measured as described earlier. The effects of different pH values on the hemolytic activity were determined following the pH adjustment of the erythrocyte buffer solution to pH 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, and 9.5. To study the effects of ionic strength on the hemolytic activity of the ethanolic extract, the ionic strength of the erythrocyte buffer was first adjusted to 100, 140, 180, 220, 260, 300, 340, 380, 420, 460, 500, and 540 mM NaCl.
Preparation of small unilamellar lipid vesicles

To study the interactions of the hemolytically active compounds in the ethanolic extract from *Wallemia sebi* with lipid vesicles, various small unilamellar vesicles (SUVs) at a final concentration of 2 mg mL\(^{-1}\) were prepared as described by Rebolj *et al.* (2006). The dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), dipalmitoylphosphatidylethanolamine (DPPE), dipalmitoylphosphatidylserine (DPPS), dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), sphingomyelin, and cholesterol that were used were from Avanti Polar Lipids and Merck. The permeabilization of SUVs was assayed as described by Rebolj *et al.* (2006).

Effects of lipid composition on membrane activity of the *Wallemia sebi* ethanolic extract

The membrane binding of hemolytically active compounds from the *Wallemia sebi* ethanolic extract was estimated by measuring the residual hemolytic activity of unbound compounds after a 30-min incubation period of SUVs with the extract at 25 °C (Sepčić *et al.*, 2003). Here, 80 μL SUVs (2 mg mL\(^{-1}\)) of various compositions were prepared in vesicle buffer and pipetted into multiwell plates, followed by the addition of 20 μL (TS = 0.54 mg mL\(^{-1}\)) of the ethanolic extract. The residual hemolytic activity of the extract was then assessed after this incubation period by adding 100 μL erythrocyte suspension to each well (Sepčić *et al.*, 2003).

Vesicle permeabilization by the ethanolic extract was determined by combining 20 μL of the extract (TS = 0.54 mg mL\(^{-1}\)) and 2 μL calcein-loaded SUVs in 1 mL of vesicle buffer (erythrocyte buffer supplemented with 1 mM EDTA), as described by Rebolj *et al.* (2006).

Statistical analysis

Significant differences among experimental groups were compared with one-way ANOVA, using least significant difference (LSD) post hoc tests (\(P < 0.05\)). All of the statistical tests were performed using SPSS for Windows, version 15.00 (SPSS Inc. 2006).

Results

Characterization of the ethanolic extract from *Wallemia sebi*

The composition of the ethanolic extract from the *Wallemia sebi* mycelia was determined by GC/MS analysis. Twenty-one compounds were identified in the extract, the majority of which were variously saturated and unsaturated fatty acids and sterols, as summarized in Table 1. The most intense chromatographic peaks were recorded at the retention times of 19.4, 20.8–21.2, and 29.8 min (Fig. 1), which correspond to palmitic acid (C16:0), a mixed peak of linoleic acid (C18:2) and oleic (C18:1) acids, and ergosterol, respectively.

### Table 1. Characterization of peaks obtained by GC/MS analysis of *Wallemia sebi* ethanolic extract

<table>
<thead>
<tr>
<th>Peak no.</th>
<th>Retention time (min)</th>
<th>Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>17.1</td>
<td>Myristic acid</td>
</tr>
<tr>
<td>2</td>
<td>18.2</td>
<td>Pentadecanoic acid</td>
</tr>
<tr>
<td>3</td>
<td>19.4</td>
<td>Palmitic acid</td>
</tr>
<tr>
<td>4</td>
<td>19.6</td>
<td>Ethylpalmitate</td>
</tr>
<tr>
<td>5</td>
<td>19.8</td>
<td>Acetylcarboline</td>
</tr>
<tr>
<td>6</td>
<td>20.8–21.2</td>
<td>Mixed peak of linoleic acid and oleic acid</td>
</tr>
<tr>
<td>7</td>
<td>21.3</td>
<td>Stearic acid</td>
</tr>
<tr>
<td>8</td>
<td>24.6</td>
<td>2-ethylhexylphthalate</td>
</tr>
<tr>
<td>9</td>
<td>25.7</td>
<td>Nonidentified phthalate</td>
</tr>
<tr>
<td>10</td>
<td>26.7</td>
<td>Squalene</td>
</tr>
<tr>
<td>11</td>
<td>27.8</td>
<td>Nonidentified sterol</td>
</tr>
<tr>
<td>12</td>
<td>29.4</td>
<td>Ergosta-5,7,9(11)tetraen-3β-ol (dehydroergosterol)</td>
</tr>
<tr>
<td>13</td>
<td>29.8</td>
<td>Ergosta-5,7,22-trien-3β-ol (ergosterol)</td>
</tr>
<tr>
<td>14</td>
<td>29.9</td>
<td>Ergosta-7,22-dien-3β-ol</td>
</tr>
<tr>
<td>15</td>
<td>30.1</td>
<td>3β,22E-19-norergosta-5,7,9,22-tetraen-3-ol (neoeergosterol)</td>
</tr>
<tr>
<td>16</td>
<td>30.3</td>
<td>Ergosta-5,8-dien-3β-ol</td>
</tr>
<tr>
<td>17</td>
<td>30.5</td>
<td>Ergosta-7-en-3β-ol</td>
</tr>
<tr>
<td>18</td>
<td>30.7</td>
<td>Nonidentified sterol</td>
</tr>
<tr>
<td>19</td>
<td>31.1</td>
<td>Lanosta-8,24-dien-3β-ol (lanosterol)</td>
</tr>
<tr>
<td>20</td>
<td>31.7</td>
<td>Methylidydrolanosterol</td>
</tr>
<tr>
<td>21</td>
<td>31.9</td>
<td>Nonidentified sterol</td>
</tr>
</tbody>
</table>
Ergosterol, which was detected in considerable amounts in the extract (Fig. 1), was also tested for hemolysis and found inactive.

Exposure to 100 °C significantly affected this ethanolic extract activity, as there was almost total loss of hemolytic activity in comparison with the control (Fig. 3a). The same loss of the activity after heating to 100 °C could be observed with the equimolar mixture of three tested fatty acids (Fig. 2b). A significant increase in hemolytic activity of the W. sebi extract was observed at pH above 8.5 (Fig. 3b), and the higher ionic strengths also induced significant increases, although small, in the hemolytic activity (Fig. 3c).

**Effects of lipid composition on hemolytic activity of the ethanolic extract from W. sebi**

As shown on Fig. 4a, the SUVs containing phosphocholine (i.e. those formed with DPPC, DOPC, and POPC) and/or sphingomyelin completely prevented lysis of the erythrocytes that otherwise occurred in first few minutes of assay. This suggests that the phospholipids with a choline headgroup in their structures can bind the hemolytically active compound(s) in the extract and thus diminished their activity toward the erythrocytes. Additionally, the fluorescence of the calcine released from the SUVs was measured after the addition of the extract. Here, the percentage of released calcine was highest in cholesterol-containing vesicles (Fig. 4b), indicating that membranes with a higher degree of fluidity are more susceptible to lysis induced by this W. sebi ethanolic extract.

**Discussion**

*Wallemia sebi* is an important pan-global contaminant of foods and feeds preserved with low aw. It can contaminate food not only as an airborne or soil-borne contaminant, but it can also be inoculated with the preservative itself (Butinar et al., 2011). *Wallemia sebi* can grow over a wide range of aw (0.997–0.690) in glucose/fructose media (Pitt & Hocking, 1997), but in media with NaCl as the major solute, the lowest aw for its growth was reported as 0.80 (Zalar et al., 2005; Plemenitaš et al., 2008), which corresponds to 4.5 M NaCl.

The importance of this fungus increased over the last decade, because of its unique phylogenetic position (Zalar et al., 2005; Matheny et al., 2006), its adaptive abilities under conditions that stress most organisms (Plemenitaš et al., 2008), and the increased awareness of its various health implications (De Hoog & Guarro, 1996; Lappalainen et al., 1998; Roussel et al., 2004; Guarro et al., 2008).

The great majority of food-borne fungi synthesize mycotoxins at high humidities of their substrate (aw = 1.0) and at mesophilic temperatures (20–25 °C) (Frisvad, 1995). With only in few exceptions, the synthesis of mycotoxins is stimulated by unfavorable conditions, from desiccation and/or high concentrations of solutes to low temperatures, which trigger common (osmotolerant) cellular responses (Kerzaon et al., 2008). In W. sebi, this...
trend was observed with wallemione, which was shown to be synthesized in higher quantities in media with low aw (Frank et al., 1999).

In the present study, we report for the first time that W. sebi has a concentration-dependent hemolytic activity toward bovine erythrocytes (Fig. 2a). Surprisingly, this hemolytic activity, which we detected only in ethanolic extracts of the mycelium and not in the culture filtrate or from the substrate, is considerably higher if the W. sebi is exposed to either high salinity or low temperatures (10 °C) during growth (Sepčić et al., 2011). GC/MS analysis of this ethanolic extract from W. sebi showed it to contain a complex mixture of various fatty acids and sterols. The most abundant of these are the unsaturated fatty acids, linoleic and oleic acids (C18:2 and C18:1), and amongst the sterols, ergosterol. As ergosterol did not show any hemolytic activity in our tests, the latter is most likely associated with the fatty acids in the extract. The hemolytic activity of the equimolar mixture of linoleic, oleic, and palmitic acids is indeed comparable with the activity of the W. sebi extract and is associated with the unsaturated fatty acids. It has already been reported that fatty acids extracted from marine fungi and from other marine organisms, such as algae (Ikawa, 2004), dinoflagellates (Dorantes-Aranda et al., 2009), seaweed (Gerasimenko et al., 2010), and fish (Mancini et al., 2011), have a role in the lysis of erythrocytes. Among nine fatty acids that were tested, the α-linoleic (C18:3), linoleic (C18:2), and oleic (C18:1) acids were the most potent algal growth inhibitors, as they can cause deleterious damage to the plasma membrane (Wu et al., 2006).

According to this last study, free fatty acids have important interactive cytotoxic roles in aquatic ecosystems, as they can affect the growth of phytoplankton, algae, and cyanobacteria.

Although higher concentrations of unsaturated fatty acids have also been found in membranes of halophilic fungi that inhabit marine solar salterns (Lesage et al., 1993; Turk et al., 2007), their relative increases were interpreted solely as adaptive responses to growth at high salinities. As the peak of fungal presence in salterns
immediately follows the peak of the main eukaryotic primary producer, the halophilic alga *Dunaliella salina* (Gunde-Cimerman *et al.*, 2000), the increase in fungal fatty acids at higher environmental salinities might also have ecological implications. When the *W. sebi* was grown at a higher salt concentration in the growth medium (20% vs. 5% NaCl), the hemolytic activity of the extracts increased. This is also probably because of the increased proportion of fatty acids, as an increase in the proportion of palmitic, margaric, stearic, and oleic acids was seen when this fungus was cultivated at higher (20%) NaCl concentrations (M. Spiteller, pers. commun.).

Although free fatty acids have been reported to interact nonspecifically with the erythrocyte membrane (Zavodnik *et al.*, 1997), lipid vesicles containing phospholipids with a choline headgroup effectively prevented the hemolysis induced by this *W. sebi* ethanolic extracts. Furthermore, membranes with a higher degree of fluidity were seen to be more sensitive to permeabilization by the *W. sebi* ethanolic extract, because the highest amount of calcein was released from SUVs that also contained cholesterol.

To study the influences on hemolytic activity linked to the compounds in the extract, the extract was exposed to different temperatures, pH values, and NaCl concentrations and then tested for its remaining hemolytic activity. Only heating of the extract to 100 °C for 30 min resulted in the loss of the hemolytic activity. The same effect could be observed when heating the mixture of three tested fatty acids, reinforcing the hypothesis that the latter were responsible for the hemolytic activity of *W. sebi* ethanolic extract. The erythrocyte buffer with high pH or ionic strength increased the hemolytic activity of this extract. As pH and ionic strength do not interfere with fatty acid conformations, these increases are most probably because of the altered erythrocyte susceptibility under these conditions.

In conclusion, our data indicate that mammalian erythrocytes, and eukaryotic membranes in general, are susceptible to the hemolytic activity of this *W. sebi* ethanolic extract. This xerotolerant fungus might have an interactive role in the complex microbial community of solar saltworks in new and as-yet-undescribed ways. However, these findings also indicate the potential involvement of *W. sebi* in the formation of lesions in subcutaneous infections and in the destruction of lung tissue in farmer’s lung disease, with the possibility of hemolytic diseases linked to consumption of food and feed that is contaminated with *W. sebi*.

**Acknowledgements**

We are grateful to Ladislav Kučan (Institute for Public Health, Maribor, Slovenia) for expert help and assistance with the GC/MS analysis. Additionally, we thank Nataša Pipenbaher (University of Maribor, Maribor, Slovenia) for help with the statistical analysis.

**References**


Lesage L, Genot C, Record E, Pouliquen C & Richard-Molard D (1993) Fatty acid composition and molecular order of...


