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Impact of *Lactobacillus plantarum* on thiol precursor biotransformation leading to production of 3-sulfanylhexan-1-ol

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ABSTRACT

3-Sulfanylhexan-1-ol (3SH) is an important contributor to the fruity notes of wine. 3SH exists as odorless precursors in grape and its release from the precursors is generally mediated by yeast during alcoholic fermentation. Here, the impact of lactic acid bacteria on 3SH production was investigated. Among the species tested, only *Lactobacillus plantarum* released 3SH from *S*-3-(hexan-1-ol)-L-cysteine (3SH-*S*-cys) and *S*-3-(hexan-1-ol)-L-cysteinylglycine (3SH-*S*-cysgly) in the whole-cell biotransformation assay. The conversion yields of 3SH from 3SH-*S*-cysgly by *L. plantarum* were always higher than those from 3SH-*S*-cys, suggesting that the direct cleavage of 3SH-*S*-cysgly to yield 3SH predominantly occurred. *L. plantarum* biotransformed the 3SH precursors, including 3SH-*S*-glut, to release 3SH in fermented grape juice. The results indicate that *L. plantarum* induces the release of 3SH from the 3SH precursors. To the best of our knowledge, this is the first study showing the impact of *L. plantarum* on thiol precursor biotransformation.

1. Introduction

Volatile thiols are the major contributors to the organoleptic properties of wine. As examples of volatile thiols, 3-sulfanylhexan-1-ol (3SH) and 3-sulfanylhexyl acetate (3SHA) have been identified in Sauvignon Blanc wine (Tominaga, Darriet, & Dubourdieu, 1996; Tominaga, Furrer, Henry, & Dubourdieu, 1998). These compounds play an important role in conferring aromatic complexity and varietal typicity to wine. 3SH produces a grapefruit-like odor and 3SHA, a passion fruit like odor in Sauvignon Blanc wine. 3SH is present as odorless precursors in the grape berry, such as glutathione *S*-conjugate (*S*-3-(hexan-1-ol)-glutathione; 3SH-*S*-glut) (Gachons, 2002), cysteinyl-glycine *S*-conjugate (*S*-3-(hexan-1-ol)-L-cysteinylglycine; 3SH-*S*-cysgly) (Capone, Pardon, Cordente, & Jeffery, 2011), and cysteine *S*-conjugate (*S*-3-(hexan-1-ol)-L-cysteine; 3SH-*S*-cys) (Tominaga, Peyrot des Gachons, & Dubourdieu, 1998). Furthermore, *S*-3-(hexan-1-ol)-γ-glutamyl-cysteine was recently identified as a potential precursor of 3SH in Sauvignon Blanc juice (Bonnaffoux et al., 2017). Although the metabolic pathway of 3SH *S*-conjugates is not fully understood in the grapevine, it is considered that 3SH-*S*-glut is the primary precursor of 3SH in Sauvignon Blanc juice. The first step for 3SH release is the cleavage of the γ-glutamyl moiety by a γ-glutamyl trans-peptide to produce 3SH-*S*-cysgly, followed by the reaction of carboxypeptidase to generate 3SH-*S*-cys in the grapevine (Kobayashi, Takase, Suzuki, et al., 2010; Thibon, 2011).

3SH release from its precursors can occur via an enzymatic reaction of *Saccharomyces cerevisiae* yeast, including the carbon-sulfur β-lyase activity, during alcoholic fermentation (Thibon et al., 2008). Furthermore, 3SHA is generated together with released 3SH by alcohol acetyltransferase from *S. cerevisiae* (Swiegers et al., 2006). However, the conversion yield of 3SH from each precursor by *S. cerevisiae* is low, although the conversion of 3SH-*S*-cys to 3SH occurs significantly more efficiently than that of 3SH-*S*-glut (Kobayashi, Takase, Kaneko, et al., 2010; Winter, Van Der Westhuizen, Higgins, Curtin, & Ugliano, 2011). Therefore, to enhance 3SH release from its precursors, many researchers have investigated the optimum fermentation conditions, the breeding of *S. cerevisiae*, and the utilization of non-*Saccharomyces* yeast species, such as *Torulaspora delbrueckii* and *Pichia kluyveri* (Anfang, Brajkovich, & Goddard, 2009; Renault, Coulon, Moine, Thibon, & Bely, 2016; Swiegers & Pretorius, 2007). In addition, it is reported that some bacterial species, such as *Eubacterium limosum* and *Fusobacterium nucleatum*, have the ability to release 3SH-*S*-cys (Starkenmann et al., 2008; Tominaga & Dubourdieu, 2000).

Lactic acid bacteria (LAB) are one of the most important microorganisms in the food and beverage industry, including the winemaking industry. LAB are responsible for malolactic fermentation (MLF), which

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is the decarboxylation of malic acid to produce lactic acid during the winemaking process. MLF is necessary for the aging of red wine and certain white wines because it ensures a decrease in acidity and provides additional advantages, such as microbial stability and improved aroma complexity. Four genera, *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Oenococcus*, are the principal LABs in winemaking. Among these LAB, *Oenococcus oeni* is the main species used in MLF and commercially available as a starter culture. Studies have shown that some *Lactobacillus* species, particularly *Lactobacillus plantarum*, are also suitable for MLF (du Toit, Engelbrecht, Lerm, & Krieger-Weber, 2011; Miller, Franz, Cho, & du Toit, 2011). In addition to the decarboxylation of malic acid as the primary metabolic reaction, various secondary metabolic reactions occur during MLF, producing an impact on the aromatic characteristics of wine (Antalick, Perello, & de Revel, 2012; de Revel, Martin, Pripis-Nicolau, Lonvaud-Funel, & Bertrand, 1999). Citrate degradation to produce diacetyl, ester biosynthesis, methionine/cysteine metabolism to give volatile sulfur compounds with an unpleasant odor, and the hydrolysis of glycosylated derivatives to release aromatic compounds, such as monoterpenoids, into wine were demonstrated (Liu, 2002; Matthews et al., 2004). However, it remains unknown whether LAB are involved in the biotransformation of thiol precursors to produce volatile thiols, such as 3SH and 3SHA.

The objective of this study was to assess the impact of LAB on the production of 3SH and to acquire a better understanding of 3SH-*S*-conjugate biotransformation mediated by LAB. We propose a biotransformation profile of 3SH-*S*-conjugates occurring in several species, based on the results of the whole-cell biotransformation assay, and monitored the kinetics of 3SH and 3SH-*S*-conjugates in grape juice during lactic fermentation.

2. Materials and methods

2.1. Chemicals

Analytical reagents were purchased from Sigma-Aldrich Japan (Osaka, Japan). All chromatographic solvents were high-performance liquid chromatography (HPLC) grade. Milli-Q water was obtained from a Milli-Q purification system (Merck Millipore, Tokyo, Japan). *S*-3-(hexan-1-ol)-L-cysteine (3SH-*S*-cys), *S*-3-(hexan-1-ol)-L-cysteinylglycine (3SH-*S*-cysgly), and *S*-3-(hexan-1-ol)-glutathione (3SH-*S*-glut) were purchased from Wako Pure Chemical Industries (Osaka, Japan). These synthetic thiol precursors were dissolved in sterile 100 mM citrate buffer (pH 6.0) and stored at −30 °C until use. The stock concentrations of 3SH-*S*-cys, 3SH-*S*-cysgly, and 3SH-*S*-glut were 1.8, 1.4, and 1.1 mM, respectively.

2.2. Bacterial strains and growth conditions

All strains used in this study were listed in the Table 1. Two commercial strains, *Oenococcus oeni* Lalvin VP41 and *Lactobacillus plantarum* V22, were purchased from Lallemand (Montréal, Canada). Other strains were provided by the National Institute of Technology and Evaluation (Chiba, Japan). These strains were grown at 30 °C in MRS broth (Difco Laboratories, Detroit, MI, USA). pH of MRS broth was adjusted to 4.8 for V22 and 6.5 for others.

Table 1
Lactic acid bacteria species and strains used in this study.

Code name	Scientific name	Strain name
L. mesenteroides	*Leuconostoc mesenteroides*	NBRC100496
P. pentosaceus	*Pediococcus pentosaceus*	NBRC107768
O. oeni	*Oenococcus oeni*	Lalvin VP41
L. plantarum_1	*Lactobacillus plantarum*	MBR V22
L. plantarum_2	*Lactobacillus plantarum*	NBRC101978
L. plantarum_3	*Lactobacillus plantarum*	NBRC15891
L. plantarum_4	*Lactobacillus plantarum*	NBRC109604

2.3. Microbial counts

LAB population was determined by plate counting on MRS agar. LAB counts were taken after anaerobic incubation at 30 °C for 2–3 days.

2.4. Whole-cell biotransformation assay for conversion of 3SH S-conjugates into 3SH

Whole-cell biotransformation assay was carried out according to a previous report with modifications (Starkenmann, Niclass, Troccaz, & Clark, 2005). LAB strains were grown in 10–50 mL of MRS broth at 30 °C. Cells were harvested by centrifugation at 3000*g* for 10 min, washed once with sterile 100 mM citrate buffer (pH 6.0), and resuspended in fresh buffer. Neither 3SH nor 3SHA was detected in the cell suspension. Into a 1.5 mL sterile vial, the following were added: bacterial cells to an optical density (OD_{600}) of 2.0, synthetic 3SH-*S*-cys, 3SH-*S*-cysgly or 3SH-*S*-glut substrate dissolved in 100 mM citrate buffer and 100 mM citrate buffer to adjust the volume to 0.5 mL. The mixture was incubated at 30 °C for up to 24 h under the hermetically sealed condition to minimize the risk of oxidation. The final concentrations of 3SH-*S*-cys, 3SH-*S*-cysgly, and 3SH-*S*-glut were 36, 28, and 22 μM, respectively. For the time-course study of 3SH release, *L. plantarum*-2 was used and samples were taken at appropriate time points. Assays without bacterial cells were also carried out as control for individual experiments. After incubation, the cells were separated by centrifugation at 15,000*g* for 5 min and the supernatant was used for the quantification of 3SH-*S*-conjugate by using a liquid chromatography-tandem mass spectrometer (LC–MS/MS) and of 3SH by using a GC–MS. All experiments were performed in triplicate.

2.5. Preparation of fermented grape juice

Concentrated Chardonnay grape juice from Australia was diluted with water and the diluted grape juice was used to prepare the fermented grape juice. The general composition of the diluted grape juice was as follows: pH: 3.51, total soluble solids: 21 °Brix, titratable acidity: 5.1 g/L as tartaric acid, and available nitrogen concentration: 140 mg/L. Prior to inoculation with LAB, the grape juice was sterilized by filtration (0.22 μm, Merck Japan, Tokyo, Japan) and carefully added into sterile glass bottles (720 mL) with screw caps to minimize the risk of oxidation. For lactic fermentation, 500 mL of grape juice was inoculated with *L. plantarum*-2 at OD600 = 0.26 (6.6×10^8 viable cells/mL) on average. Lactic fermentation was carried out at 30 °C for 5 days. Sampling was performed every day to monitor 3SH release and the biotransformation of 3SH-*S*-conjugates during lactic fermentation. Grape juice without bacterial cells was also prepared as control. All experiments were performed in triplicate.

2.6. LC–MS/MS analysis of 3SH-S-conjugates

The supernatant from the biotransformation assays and the fermented grape juice were diluted with 0.1% (v/v) formic acid to an appropriate concentration of 3SH-*S*-conjugates and filtered through 0.45 μm cellulose acetate filter (Advantec Toyo, Tokyo, Japan). The filtrate was used for the quantification of 3SH-*S*-conjugates in a LC–MS/MS system (API 3200 QTRAP; AB SCIEX, Tokyo, Japan). The quantification of 3SH-*S*-conjugates was carried out as previously reported (Kobayashi et al., 2012). In the present study, the gross weights of *R*- and *S*-configuration products were measured for all the 3SH-*S*-conjugates.

2.7. Extraction of 3SH and 3SHA from samples

For the whole-cell biotransformation assay, clear supernatant