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Exogenous C–S Lyase Enzyme, a Potential Tool To Release Aromas in Wine or Beer?

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KEYWORDS:

varietal thiol precursors, wine aroma, cysteine S-conjugates

ABSTRACT:

Varietal thiols have an impact on the overall aroma of many white, rosé, and red wines and beers. They originate from the metabolism of non-odorant aroma precursors by yeast during the fermentation step, via an intrinsic enzyme, the carbon–sulfur β -lyase (CSL, EC 4.4.1.13). However, this metabolism is directly dependent upon efficient internalization of aroma precursors and intracellular CSL activity. Consequently, the overall CSL activity converts on average only 1% of the total precursors available. To improve the conversion of thiol precursors during winemaking or brewing, we investigated the possibility of using an exogenous CSL enzyme from *Lactobacillus delbrueckii* subsp. *bulgaricus* produced in *Escherichia coli*. We first implemented a reliable spectrophotometric method to monitor its activity on different related aroma precursors and studied its activity in the presence of various competing analogues and at different pH values. This study allowed us to highlight the parameters to define CSL activity and structural insights for the recognition of the substrate, which paved the way for the use of exogenous CSL for the release of aromas in beer and wine.

INTRODUCTION

In recent years, consumer preferences have been strongly directed toward wines and beers with a high content of varietal thiols, providing the fresh, citrus, and fruity profile of these beverages. These compounds are important in the aroma of Sauvignon blanc¹ and could contribute in the global aroma of several Provence rosé² and many other red, white, and rosé wine varieties,^{3–5} in which they have been identified. These varietal thiols have also been found in craft beer as India pale ale (IPA) products and were identified especially in different hop cultivars used in brewing, like Simcoe, Summit, Apollo, and others.⁶ The three main studied varietal thiols are 4-mercapto-4-methylpentan-2-one (4MMP) reminiscent of boxwood and blackcurrant bud^{7,8} and 3-mercaptohexan-1-ol (3MH) and its acetate form (3MHA) described with passion fruit and grapefruit aromas.⁹ These molecules are of great interest from an organoleptic point of view because of their very low thresholds of perception, which are, for instance, in wine, 0.8, 60, and 4 ng L⁻¹, respectively. The study of the origin of varietal thiols in wine began with the demonstration of the presence of an odorless aroma precursor of 3MH cysteine *S*-conjugate.⁹ Since 2009, several 3MH precursors have been identified to be associated with peptide-based scaffolds, such as glutathione (GSH), cysteinylglycine, and γ -glutamic acid-cysteine.^{10–13} These discoveries represented an important advance in knowledge on their metabolic cascade, called the mercapturate pathway, presumed to be a mechanism of detoxification of electrophilic compounds in plants. First, hexenal, the electrophilic compound, is conjugated to GSH by a glutathione *S*-transferase. Second, the contribution of peptidase toward a glutathionylated precursor leads to a cysteinylated *S*-conjugate through a dipeptide conjugate as an intermediate. Finally, *N*-acetylation affords the corresponding *N*-acetylated cysteine *S*-conjugate (mercapturate), which is then excreted thanks to its higher water solubility than the original electrophilic compounds. This mercapturate could also be one of the hypothetical thiol precursors.¹⁴ This pathway may explain the presence of these different precursors in grapes and musts. The conversion into thiols of these precursors occurs during the alcoholic fermentation step by the yeasts. It has been demonstrated by gene depletion that yeasts internalize aroma precursors through different amino acid transporters: *OPT1* to internalize the GSH *S*-conjugate¹⁵ and *GAP1* for

the cysteinylated form.¹⁶ In the medium, amino acids are usually a nitrogen source for the yeast development, and at a high concentration of ammonium, the expression of these transporters will be downregulated.¹⁶ Once the precursors are internalized by the yeasts, genes coding for the carbon–sulfur β -lyase (CSL) enzyme, such as *STR3*¹⁷ and *IRC7*,¹⁸ are activated to mediate the release of 4MMP and 3MH from cysteinylated precursors. CSL (EC 4.4.1.13) is a pyridoxal 5'-phosphate (PLP)-dependent enzyme and is structured as a homotetramer, with each monomer binding a PLP molecule.¹⁹ CSL is involved in methionine biosynthesis in plants and microorganisms but has also been identified to catalyze β -elimination from *S*-conjugate compounds to release their corresponding thiols.²⁰ It has been demonstrated that capacities of yeast to internalize and form aroma volatiles resulted in an average conversion yield of 1%.³ To improve the low rate of internalization of thiol precursors and their intracellular conversion, one solution could be the direct use of exogenous CSL on must, wine, or wort and beer. For the moment and from our best knowledge, none of the national or international regulation for food production is mentioning the use of CSL. Consequently, extensive studies on the technical interest of those enzymes especially in wine and beer production are necessary to support any evolution of the related regulations.

A previous study showed the effectiveness of CSL in an alkaline medium, with an optimal pH between 8 and 9.²⁰ On the other hand, a first demonstration recorded CSL activity in bacterial lysate of several strains of *Lactobacillus casei* under acidic conditions (pH 5.5) but a lower activity at higher pH.²¹ Likewise, it has also been shown that *Shewanella putrefaciens*, *Saccharomyces cerevisiae*, and *Kluyveromyces lactis* lysate presented CSL activity at acidic and neutral pH, with thiol release from Cys(4MMP) after 30 min of incubation in media at pH 3.5, 4.5, 5.5, and 7.²² Because wine and beer are acidic matrices with a pH range between 2.7 and 3.5 and between 4.5 and 5.5, respectively, these insights confirm a real potential benefit to directly use exogenous CSL in winemaking or brewing. In the present study, the functional characterization of CSL from *Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC BAA-365 was undertaken to monitor its abilities to convert thiol at different pH from different aroma precursor structures.

MATERIALS AND METHODS

Chemicals.

L-Cysteine-*S*-4-nitrophenyl [Cys(4NP)] and *N*-acetyl- L-cysteine-*S*-4-nitrophenyl [*N*-Ac-Cys(4NP)] were purchased from TCI Europe.

Pyridoxal 5'-phosphate hydrate, sodium hydroxide, sodium phosphate monobasic, sodium phosphate dibasic, citric acid, 5,5'- dithiobis(2-nitrobenzoic acid) (DTNB), L-cysteine, *N*-acetyl-L-cysteine (*N*-Ac-L-Cys), L-cysteine methyl ester (L-Cys-OMe), L-serine, *S*-benzyl-L-cysteine, *O*-benzyl-L-serine, L-cystine, L-cystine-di-*tert*-butyl ester, glycine, di-*tert*-butyl dicarbonate (Boc2O), sodium borohydride (NaBH₄), 4-nitrothiophenol, dithioerythritol (DTE), (*E*)-2-hexenal, *N,N*-diisopropylethylamine (DIEA), and benzotriazole-1-yl-oxy-tris-(dimethylamino)-phosphonium (BOP) were purchased from Merck- France.

Cysteine *S*-conjugate β -lyase from *L. delbrueckii* subsp. *Bulgaricus* was produced as a recombinant protein in *Escherichia coli*²⁰ and was provided by Prof. Alessandra Astegno from the Department of Biotechnology of the University of Verona (Italy).

Nuclear Magnetic Resonance (NMR) and Liquid Chromatography/ Mass Spectrometry (LC/MS) Analysis.

NMR spectra were recorded on a Bruker Avance III spectrometer 400 (400 MHz). LC/MS analyses were recorded on a Quattro micro electrospray ionization (ESI) triple quadrupole mass spectrometer (Micromass, Manchester, U.K.) equipped with a Merck Chromolith High-Resolution RP-I8e column (25 × 4.6 mm) coupled to an Alliance 2695 HPLC system (Waters, Milford, MA, U.S.A.) with a gradient from 100% [H₂O/0.1% formic acid (HCO₂H)] to 100% [acetonitrile (CH₃CN)/0.1% HCO₂H] in 2.5 min, a flow rate of 3 mL/min, and ultraviolet (UV) detection at 214 nm. All chromatographic solvents were of chromatographic-grade quality and purchased from Merck- France.

Enzymatic Assays.

The assay was performed in 400 μL of sodium phosphate/citric acid buffer at pH 7.6 containing 5 μM PLP. The reaction was initiated with the addition of 100 μL of 3 μM CSL and then stopped after 1 min with 100 μL of 0.1 M NaOH. The β- elimination from the Cys(4NP) substrate by CSL was monitored on a Nanodrop ultraviolet–visible (UV–vis) spectrophotometer by measuring the absorbance at 416 nm of produced 4-nitrothiophenol ($\epsilon_{4NTP} = 10\,000\text{ M}^{-1}\text{ cm}^{-1}$; see [Figure S1](#) of the Supporting Information). The assay was performed at 25 °C. Absorbance was measured against the blank with the same composition with prior addition of NaOH to inhibit enzyme activity.

When assays were conducted with substrates, which did not generate a molecule with spectrophotometric properties, Ellman's assay²³ was performed to quantify free sulfhydryl groups ($\epsilon_{\text{TNB}} = 13\,600\text{ M}^{-1}\text{ cm}^{-1}$ at 412 nm). Absorbance was measured against the blank with the same composition with prior addition of NaOH to inhibit enzyme activity.

Determination of CSL Activity at Different pH.

Enzymatic assay at 25 °C in sodium phosphate/citric acid buffer at different pH values, including 3.2, 4.1, 4.9, 5.6, 6, 7.2, and 8, were performed for 5 min, as described above in the [Enzymatic Assays](#) section.

Determination of the Michaelis Constant (*K_m*).

In the enzymatic assay at 25 °C in sodium phosphate/citric acid buffer at pH 7.6, 5 μM PLP was performed with different concentrations of substrate [Cys(4NP) or Cys(3MH)]: 20, 40, 60, and 80 μM. For each concentration, an enzymatic kinetic was followed for 2 min with readings every 3 s. The Michaelis constant (*K_m*) was graphically extracted from Lineweaver–Burk representation.

Competition Method for the Determination of Recognition Criteria of the CSL Active Site.

Enzymatic assays were conducted with model substrate [Cys(4NP)] in competition with different compounds, including Cys(3MH), *N*-Ac-Cys(4NP), *N*-Ac-L-Cys, Lcysteine, L-Cys-OMe, *S*-benzyl-L-cysteine, *O*-benzyl-L-serine, and Lcystine. To quantify the impact of the competition, several substrate/ competitor ratios, such as 1:1, 1:10, and 1:100, were used. These assays were performed at 25 °C at pH 7.6 as described above.

Synthesis of Cysteine-3-mercaptohexan-1-ol.

This synthesis was inspired from two previous works.^{13,24} Triethylamine (840 μL , 5.8 mmol, 2.5 equiv) and Boc₂O (1.13 g, 5.17 mmol, 2.2 equiv) were added to cystine di-*tert*-butyl ester dihydrochloride (1 g, 2.35 mmol) in 10 mL of dichloromethane (DCM). The reaction was stirred overnight at room temperature, and the crude mixture was then washed with 4 \times 20 mL of KHSO₄ (1 M), 10 mL of DCM, and 30 mL of NaCl saturated solution, dried over MgSO₄ powder, and finally evaporated to afford *N,N'*-di-Boc-cystine-di-*tert*-butyl ester (600 mg, 1.09 mmol, 46% yield). Triethylamine (450 μL , 3.16 mmol, 2.9 equiv) and DTE (255 mg, 1.64 mmol, 1.5 equiv) were then added to this compound solubilized in 10 mL of DCM, and the mixture was stirred under a nitrogen atmosphere for 50 min. The reaction was quenched with 20 mL of 5% (m/v) citric acid solution and then washed and dried again as reported above. The organic phase was then evaporated to afford crude *N*-Boc-cysteine-*tert*-butyl ester with solvent traces (Boc-Cys-*Ot*Bu) (315 mg, 1.14 mmol, quantitative yield). Boc-Cys-*Ot*Bu and Cs₂CO₃ (222 mg, 681 μmol) were mixed in 18 mL of dioxane/H₂O (1:1), and then a solution of (*E*)-2-hexenal (162 μL , 1.37 mmol, 1.2 equiv) in 4 mL of dioxane was added dropwise for 8 h, with this mixture stirred overnight under a nitrogen atmosphere. NaBH₄ (130 mg, 3.4 mmol) in 6 mL of methanol was slowly added to the mixture and stirred for 3 h before being quenched with ammonium chloride solution. The crude sample was then purified on a silica gel column eluted with 95:5, 9:1 and then 8:2 cyclohexane/ Et Ac solvent mixtures to obtain the pure protected compound (80 mg, 0.212 mmol, 19% yield). To a solution of the purified product in DCM (0.6 mL) was added trifluoroacetic acid (TFA, 850 μL) in the presence of triisopropyl silane (TIS, 0.1 mol %) to remove both Boc and *tert*-butyl ester. It was washed with 3 \times 5 mL diethyl ether and then lyophilized to obtain pure Cys(3MH) as an oil (45 mg, 0.203 mmol, 96% yield). LC-ESI-MS: [M + H]⁺ 222.2 Da, [M + Na]⁺ 244.5 Da. ¹H NMR (400 MHz, D₂O): δ (ppm) 0.80 (t, *J* = 7.3 Hz, 3H, CH₃), 1.29–1.35 (m, 2H, CH₂CH₃), 1.47–1.50 (m, 2H, CH₂CH₂CH₃), 1.62–1.82 (m, 2H, CH₂CH₂OH), 2.78–2.82 (m, 1H, CHS), 3.00–3.07 (m, 2H, CH₂S), 3.63–3.64 (m, 2H, CH₂OH), 4.08–4.12 (m, 1H, CH α).

Synthesis of Cysteine-S-4-nitrophenyl-glycine.

Because of the photosensitivity of Cys(4NP), all reaction and purification steps were performed in the dark. *N*-Boc-cysteine-S-4-nitrophenyl (A) was synthesized from Cys(4NP).¹¹ Then compound A was coupled to glycine methyl ester¹³ (Gly-OMe) to obtain *N*-Boc-cysteine-S-4-nitrophenyl-glycine methyl ester (B), which was then deprotected with KOH (2 M) in MeOH to afford *N*-Boc-cysteine-S-4-nitrophenylglycine (C). This latter product was then treated with TFA in DCM in the presence of TIS to remove the Boc protecting group. Then, it was concentrated under vacuum and lyophilized to obtain Cys(4NP)-Gly (D) as a final product. Analytical high-performance liquid chromatography (HPLC) and LC/MS analyses were in full agreement with the expected quality and characterization of the product.

Synthesis of Glutathione-S-4-nitrophenyl.

N-Boc-L-glutamic acid α -*tert*-butyl ester (Boc-Glu-*Ot*Bu) was obtained from *N*-Boc-L-glutamic acid γ -benzyl ester.¹³ Compound B was treated with TFA in the presence of TIS to obtain *H*-Cys(4NP)-Gly-OMe (E). Boc-Glu-*Ot*Bu (18 mg, 58 μmol) was then coupled to compound E (26 mg, 68 μmol) in 630 μL of DCM with BOP (61 mg, 138 μmol) and DIEA (77 μL , 442 μmol). This mixture was stirred overnight at room temperature and was then concentrated under vacuum. The obtained compound was dissolved in 25 mL of EtOAc and then washed with 3 \times 20 mL of KHSO₄ (1 M), 3 \times 20 mL of NaHCO₃ saturated solution, and

20 mL of NaCl saturated solution. The solution was dried over MgSO₄ and concentrated under vacuum, and then the crude mixture was purified on a silica gel column eluted with a 8:2 cyclohexane/EtOAc solvent mixture. The purified compound was deprotected as reported above to finally obtain G(4NP) (G). Analytical HPLC and LC/MS analyses were in full agreement with the expected quality and characterization of the product.

Statistical Analysis.

All experiments were performed at least in triplicate, and values are reported as the mean \pm standard error of the mean (SEM). Statistical analyses using Kruskal–Wallis followed by Dunnett’s multiple comparison test were performed using GraphPad Prism version 8.0.1 for Windows, GraphPad Software, San Diego, CA, U.S.A. (www.graphpad.com).

RESULTS AND DISCUSSION

CSL has been described to release varietal thiols, such as, for instance, 3MH, upon β -elimination of the odorless Sconjugated precursor (Figure 1).^{9,13} To monitor the conversion rate, quantification of 3MH was usually based on ultra-high-performance liquid chromatography–mass spectrometry (UHPLC–MS) or gas chromatography–mass spectrometry (GC–MS) analysis. Unfortunately, these methods are restrictive for a routine use. As an alternative, Ellman’s assay could be used to monitor CSL activity throughout the quantification of sulfhydryl groups of 3MH digested from Cys(3MH).²³ The main expected problem could be the strict control of the oxidoreductive state of the experimental media to obtain accurate and reproducible values. We then decided to use a commercially available S-4-nitrophenyl cysteine conjugate as a CSL substrate to obtain a direct reading of CSL activity upon the release of a chromophore easily quantifiable by spectrophotometry at 416 nm.

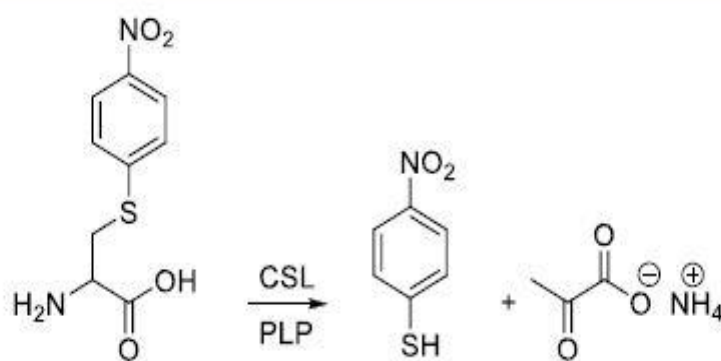


Figure 1. Model reaction for the enzymatic method of CSL activity quantification. β -Elimination of Cys(4NP) produces 4NTP, pyruvate, and ammonia.

To ensure the pertinence of the method, we synthesized native cysteinylated 3MH [Cys(3MH)] and compared this compound to the model substrate [Cys(4NP)]. The strategy to synthesize this non-commercial compound was very challenging with a rather time-consuming process and an overall very low yield. However, we obtained enough substrate to compare the ability of CSL to cleave Cys(4NP) by measuring the release of 4NTP and to cleave Cys(3MH) by measuring the release of 3MH using Ellman's assay. Interestingly, CSL from *L. delbrueckii* subsp. *bulgaricus* at pH 7.6 showed equivalent abilities to perform the β -elimination reaction toward Cys(4NP) and Cys(3MH) with $K_m = 76.9 \pm 18.6$ and $93.1 \pm 5.2 \mu\text{M}$, respectively, with no significant difference (Table 1).

Table 1. Kinetic Parameters for β -Elimination of Different Substrates at pH 7.6^a

substrate	K_m (μM)
Cys(4NP)	76.9 ± 18.6 a
Cys(3MH)	93.1 ± 5.2 a
S-benzyl-L-cysteine	520 ± 120 b
L-cystine	3950 c

^aDifferent letters (a, b, and c) highlight significant differences ($p < 0.1$) according to the Kruskal–Wallis test. Results are presented as the mean \pm SEM.

Furthermore, in a competition assay, the release of 4NTP from Cys(4NP) was about 50% lower when competed with an equivalent concentration of Cys(3MH) (Figure 2). When using a 10-fold excess of Cys(3MH), 10% of 4NTP only was released. These results can be linked to the comparable affinity of the enzyme for both substrates. Thus, Cys(4NP) is a reliable alternative substrate to study CSL activity toward aroma precursors.

Because the main constraint for the application of the enzyme in wine and beer is the pH of the product (beer and wine present $\text{pH } 5 \pm 0.5$ and 3.5 ± 0.5 , respectively), we investigated the activity of CSL at different pH values (Figure 3). Because this CSL activity showed no significant difference between pH 8 and 9,²⁰ we established the upper limit of the pH range of our study at pH 8. We also considered pH 3 as the lower limit value to mimic acidic conditions of winemaking processes.

PLP-dependent enzymes were reported to be strongly dependent upon alkaline pH.²⁰ Under our experimental conditions, CSL from *L. delbrueckii* sp. has its highest activity at pH 8 and showed only 6% of this activity at pH 5, but at lower pH (pH 3.2 and 4.1), CSL showed no apparent activity (Figure 3), even for an incubation duration up to 24 h. Therefore, this CSL could be potentially used in brewing but not during winemaking.

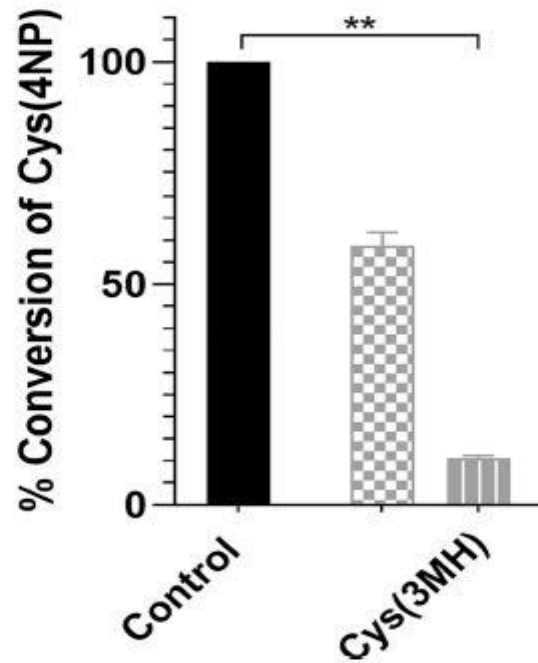


Figure 2. Enzymatic activity of CSL toward Cys(4NP) in competition with Cys(3MH). The black box represents CSL activity with Cys(4NP) without a competitor as a control; the checkboard shape represents a 1:1 ratio [Cys(4NP)/competitor]; and the vertical line represents a 1:10 ratio ($n = 3$). The error bars represent standard deviation (SD). Values are statistically different in the Kruskal–Wallis test with (*) $p < 0.05$, (**) $p < 0.01$, and (***) $p < 0.001$ compared to the control.

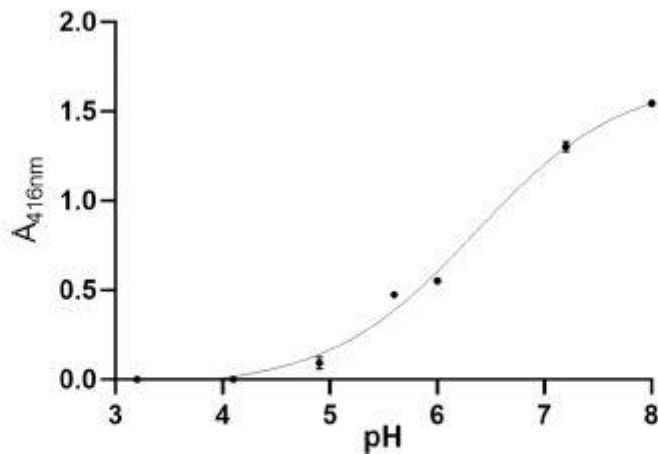


Figure 3. Evaluation of CSL activity at different pH values. Results are shown as the mean ($n = 3$). The error bars represent SD. At pH 3.2, 4.1, 6, or 8, error bars could not be distinguished.

To further study the specificity of this CSL, we performed competition experiments using different related substrates as competitors. These investigations allowed us to obtain insights about the specificity of the enzyme for different competing substrates, upon monitoring the production of 4NTP. In the presence of 10 and 100 molar excess of *S*-benzyl-L-cysteine, Lcystine, or *O*-benzyl-L-serine, conversion of Cys(4NP) was slightly reduced, as recorded with a lower release of 4NTP compared to the control (Figure 4). This suggested that this CSL was able to recognize a broad diversity of substrate with few changes on the conjugated part of the structure. *S*-Benzyl- L-cysteine and L-cystine could be considered as competitive inhibitors of CSL because, when we used Ellman’s assay to monitor the direct release of sulfhydryl groups, we clearly showed that *S*-benzyl-L-cysteine and L-cystine were CSL substrates but with a higher K_m , as reported in Table 1.

Furthermore, the presence of L-cysteine resulted in an inhibition of $20 \pm 5\%$ of 4NTP release by CSL when used at an equimolar ratio (ratio of 1:1) and $90 \pm 4\%$ inhibition with a 10 times excess of the competitor (ratio of 1:10). From a technical point of view, L-cysteine is present at a low amount in must and beer wort and is then almost completely consumed by yeast as the sulfur source during fermentation. In the case of grape must, if we consider that the cysteine level is between 1 and 8.2 mg/L,²⁵ and the mean level of Cys(3MH) is 22.3, 22.7, and 57.1 $\mu\text{g/L}$ in samples of Sauvignon blanc, Riesling, and Gewurztraminer, respectively,¹¹ and the corresponding ratio ranges from 1:40 and 1:350, thus resulting in possible total inhibition of Cys(3MH) conversion. In addition, as previously reported,²⁰ CSL showed cysteine desulhydrase activity, thus producing hydrogen sulfide (H₂S) known for its unpleasant smell, that could have technological consequences in beer wort and grape juice/must containing a high L-cysteine concentration. This could limit the use of CSL to the postfermentation phase when yeast has consumed all cysteine.²⁶ Potential inhibition by other amino acids is under investigation.

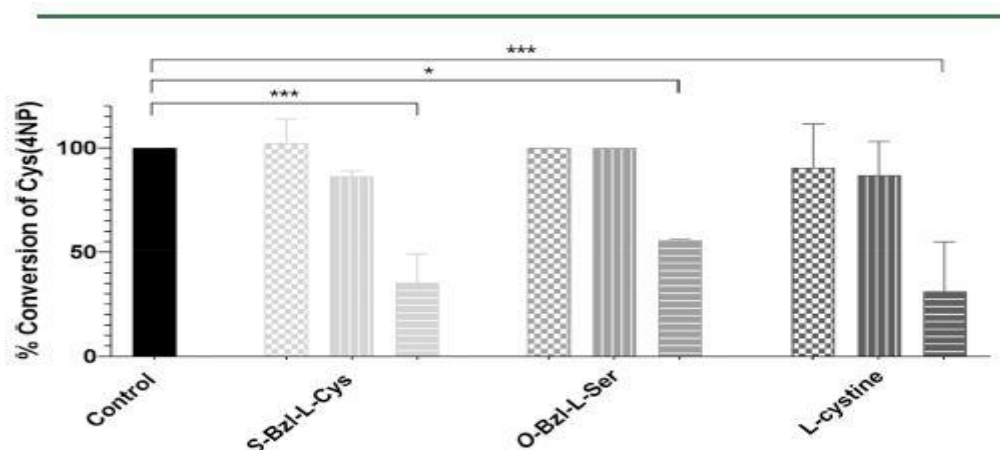


Figure 4. Enzymatic activity of CSL toward Cys(4NP) in competition with different substrates. The black box represents CSL activity for Cys(4NP) without a competitor as a control; the checkerboard shape represents a 1:1 ratio [Cys(4NP)/competitor]; the vertical line represents a 1:10 ratio; and the horizontal line represents a 1:100 ratio, at pH 7.6. From left to right, *S*-benzyl-L-cysteine, *O*-benzyl-L-serine, and L-cystine are presented as competitors ($n = 6$). The error bars represent SD. Values are statistically different in the Kruskal–Wallis test with (*) $p < 0.05$, (**) $p < 0.01$, and (***) $p < 0.001$ compared to the control.

On the other hand, *N*-acetylated forms of cysteine competitors, such as *N*-Ac-Cys(4NP) and *N*-Ac-L-cysteine, did not induce any significant decrease of Cys(4NP) conversion (Figure 5). This lack of competition probably reflects the inability of these substrates to enter the binding site of CSL when the amino group is masked because, under our experimental conditions, we did not observe any release of 4NTP from *N*-Ac-Cys(4NP) (data not shown).

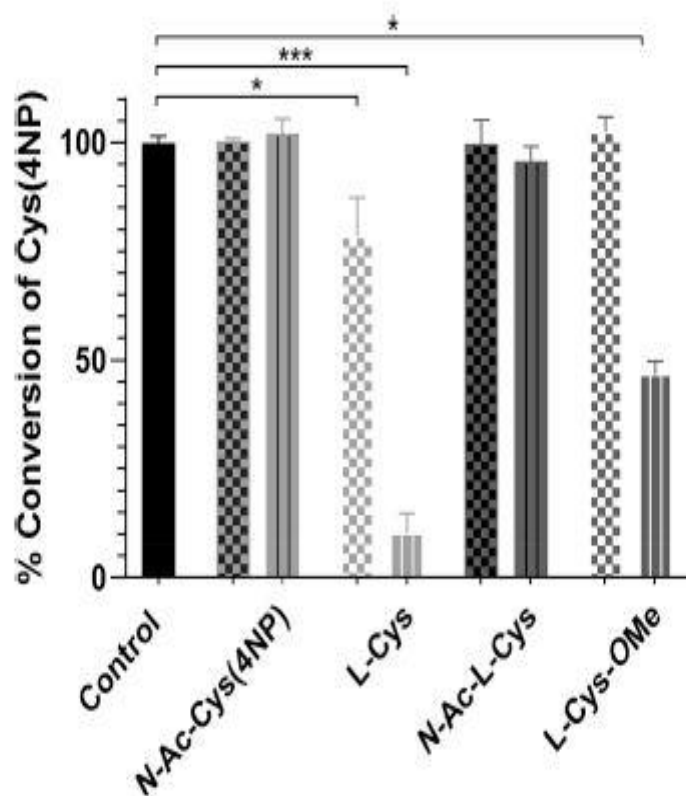
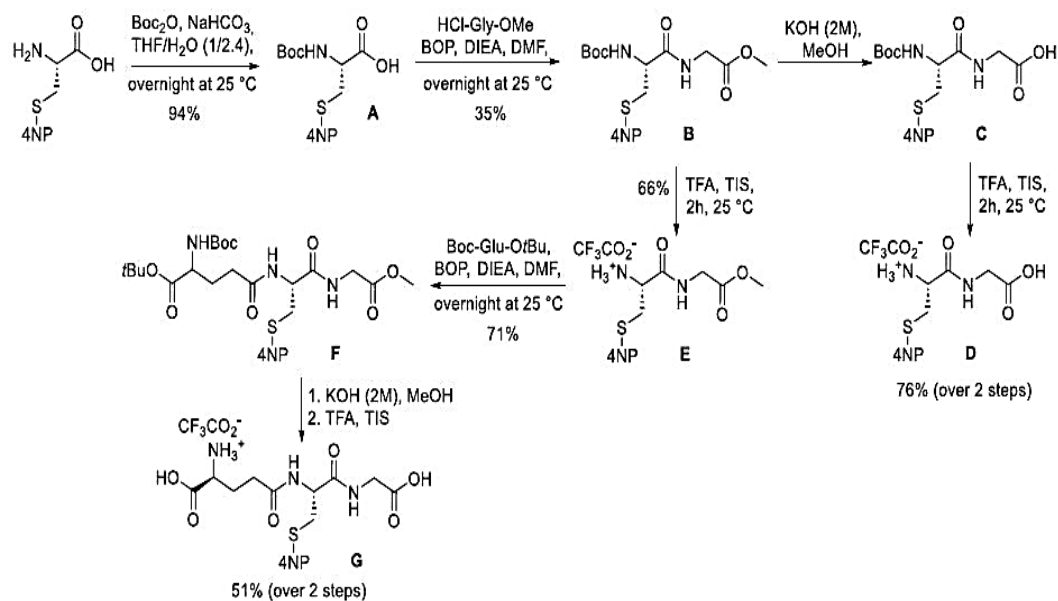


Figure 5. Enzymatic activity of CSL toward Cys(4NP) in competition with different cysteine *S*-conjugates. The black box represents CSL activity with Cys(4NP) without a competitor as a control; the checkboard shape represents a 1:1 ratio [Cys(4NP)/competitor]; and the vertical line represents a 1:10 ratio, at pH 7.6. From left to right, *N*-Ac-Cys(4NP), *L*-cysteine, *N*-Ac-*L*-cysteine, and *L*-cysteine-OMe are presented as competitors ($n = 6$). The error bars represent SD. Values are statistically different in the Kruskal–Wallis test with (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$ compared to the control.

Scheme 1. Synthesis of Cys(4NP)-Gly (D) and γ -Glu-Cys(4NP)-Gly (G)



The absence of competition of these acetylated substrates indeed corroborates the β -elimination reaction mechanism involving an internal aldimine form between PLP and ϵ -NH₃⁺ of the lysine residue of the active site conducting a transaldimination reaction between catalytic lysine bound to PLP and the α -amino group of the substrate. This transaldimination resulted in the formation of an external aldimine;²⁰ likewise, a proton abstraction from this α -amino group was required for the transaldimination.¹⁹

We also investigated the importance of the acid function of cysteine using L-Cys-OMe as a competitor of the Cys(4NP) substrate. We clearly showed for the first time that L-Cys-OMe also impacted CSL activity when used at a 10 molar excess but less than native L-cysteine (Figure 5). Methyl ester modification of the C-terminal position of the cysteine residue suppresses the deprotonation of the acidic function but also increased steric hindrance of this substrate. Both the absence of anionic charge and the increase in size of the C terminus could explain the reduced competitive character of L-Cys-OMe compared to L-cysteine.

In addition to cysteinylated conjugates, different aroma precursors naturally present in winemaking and brewing processes were conjugated to a dipeptide (Cys-Gly or γ Glu- Cys) or glutathione. In these structures, the amine and/or acid function of cysteine are indeed involved in an amide bond. Therefore, we synthesized Cys(4NP)-Gly (D) and G(4NP) (G), using conventional peptide synthesis methods (Scheme 1), to test whether these cysteine derivatives would be substrates of CSL. Under our experimental conditions, we did not observe any release of 4NTP from these two precursors (data not shown). We then performed competition experiments using these substrates Cys(4NP)-Gly and G- (4NP) (Figure 6). The absence of competition recorded for G(4NP) may support the hypothesis that the free α -amine function of the cysteine residue is mandatory for recognition of the competing substrate, as observed when the N-terminal amino group of cysteine was acetylated (see Figure 5). On the other hand, despite restoration of the anionic C terminus upon glycine grafting, the complete absence of competition recorded with a 20-fold excess of competing Cys(4NP)-Gly dipeptide confirms the idea of steric hindrance as the key determinant already supported by the partial decrease of CSL activity when using Cys-OMe as a competitor (see Figure 5).

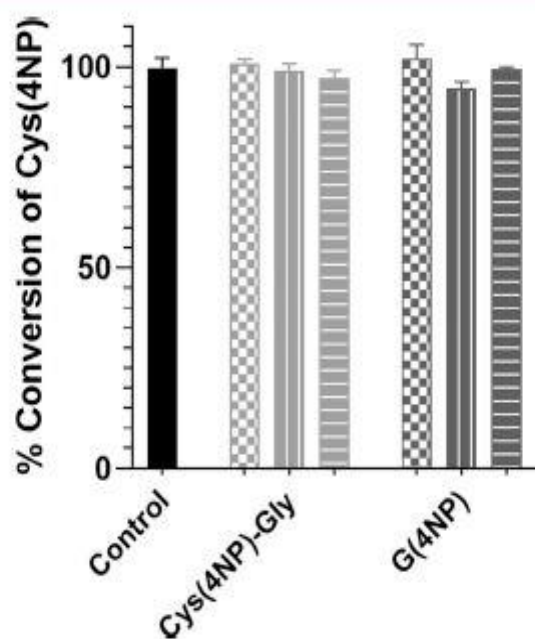


Figure 6. Enzymatic activity of CSL toward Cys(4NP) in competition with different 4NP *S*-conjugate aroma precursors. The black box represents CSL activity with Cys(4NP) without a competitor as a control; the checkboard shape represents a 1:1 ratio [Cys(4NP)/competitor]; the vertical line represents a 1:10 ratio; and the horizontal line represents a 1:20 ratio. From left to right, Cys(4NP)-Gly and G(4NP) are presented as competitors ($n = 3$). The error bars represent SD. Values are statistically different in the Kruskal–Wallis test with (*) $p < 0.05$, (**) $p < 0.01$, and (***) $p < 0.001$ compared to the control.

In addition, even if the Cys-Gly *S*-conjugate has a completely free α -amino group required for a good binding of the substrate (see [Figure 5](#)), this is not a sufficient reason to access the binding site of CSL. This confirms that steric hindrance localized at the C-terminal position of cysteine prevents the recognition of the substrate. Moreover, the presence in the enzymatic assay of competing molecules, such as Cys(4NP)-Gly or G(4NP) ([Figure 6](#)), does not induce noncompetitive inhibition by allosteric regulation. CSL can only recognize and transform the cysteine *S*-conjugate form of 3MH from other *S*-conjugate precursors, with the upstream involvement of peptidases leading to the cysteine *S*-conjugate from the glutathione *S*-conjugate.¹³ With the reminder that G(3MH) is a major precursor and the only one present in wine after fermentation,²⁷ a previous or simultaneous use of peptidases to convert G(3MH) into the cysteine *S*-conjugate could be a promising solution to release thiol with the subsequent action of CSL during the winemaking process.

Finally, CSL from *L. delbrueckii* sp. could be considered as a good thiol enhancer in beer but not in wine as a result of constraints of the pH. To go further on the potential application of CSL during the brewing process, we developed in this study a new and convenient

spectrophotometric method to monitor CSL activity under different conditions. This analytical tool will allow us to compare the level of substrate metabolized by various microorganisms containing other types of CSL. The objective will be to identify CSL with different selectivity for 3MH substrates and/or their ability to metabolize them at different pH values compatible with the wine industry.

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Notes

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ABBREVIATIONS USED

Cys(3MH), cysteine-3-mercaptohexan-1-ol; Cys(4NP), cysteine- *S*-4-nitrophenyl; 4NTP, 4-nitrothiophenol; Cys(4NP)- Gly, cysteine-*S*-4-nitrophenyl-glycine; G(4NP), glutathione-*S*- 4-nitrophenyl; G(3MH), glutathione-3-mercaptohexan-1-ol; DTE, dithioerythritol; DTNB, 5,5'-dithiobis-2-nitrobenzoic acid; Boc2O, di-*tert*-butyl dicarbonate; TIS, triisopropyl silane; TFA, trifluoroacetic acid; CSL, carbon-sulfur β -lyase; EtOAc, ethyl acetate

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