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Evidence of Enzymatic and Chemical Interconversions of Barley Malt 3-Sulfanylhexanol Conjugates during Mashing

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ABSTRACT: Recent studies have highlighted in malt the occurrence of the glutathionylated precursor of 3-sulfanylhexanol (G-3SHol) at concentrations reaching hundreds of μg/kg. Here, SIDA-LC-MS/MS was used to investigate the potential conversion of G-3SHol to its dipeptide and cysteinyl analogues during mashing. At 45 and 55 °C, malt γGT and carboxypeptidase activities quickly degrade G-3SHol (up to 90% loss), first to the cysteinylglycine conjugate and then to the cysteine conjugate (up to 205% increase). No γ-glutamylcysteine *S*-conjugate formation is observed. At 80 °C, despite enzyme inactivation, the G-3SHol level decreases steadily because of suspected imine formation with wort aldehydes at pH 5.5. More surprisingly, CysGly-3SHol is still generated at 80 °C. This indicates the presence in the wort of as yet unidentified precursors.

KEYWORDS: *polyfunctional thiols, glutathione, S-conjugates, barley malt, γGT*

INTRODUCTION

Over the past 10 years, a dozen glutathionyl and cysteinyl *S*-conjugates of 5−7 C polyfunctional thiols (PFTs) have been evidenced in various hop varieties[1−5] and some malts.[6,7] These nonvolatile compounds have real aroma potential, as the PFTs that can be released from them are often very potent (sensory thresholds at ng/L level) and pleasant (descriptors such as exotic fruit, grapefruit, ...).[6,8−10] Their fates during malting,[11] hop processing,[12] wort boiling, and fermentation[6,7,13−16] have been the focus of much recent research.

It is suspected that in most plants these compounds arise through a detoxification pathway involving addition of a glutathione onto an α,β-unsaturated carbonyl (toxic for plants). The resulting glutathionylated (G-) conjugate can be further metabolized through γ-glutamyltransferase (γGT) or carboxypeptidase activity to two intermediate dipeptide conjugates, the cysteinylglycine (CysGly-) and γ-glutamylcysteine (γGluCys-) conjugates (routes A and B in Figure 1, respectively), and ultimately to the cysteinylated (Cys-) conjugate. Although it was initially suggested that plant cells tend to use route B and animal and yeast cells essentially route A,[17−19] both dipeptides have been identified in plants such as grapes and hops.[20−22] Depending on the efficiency of the corresponding enzymatic activities, different proportions of all four *S*-conjugates are found in these matrices.[20]

Chemical degradation of *S*-conjugates after heating[23] or a pH increase[24] has also been mentioned. More recently, chemical breaking of the Cys−Gly bond at high temperature was evidenced in roasted malts, leading to the occurrence of the γGluCys dipeptide (absent from pale malts) and to an unexpected correlation between the CysGly- and Cys-*S*-conjugates.[11]

The biosynthesis of each glutathionylated precursor requires the presence of the corresponding α,β-unsaturated aldehyde (*trans*-2-hexenal for G-3-sulfanylhexanol/G-3SHol, 4-methyl-2-pentenal for G-3-sulfanyl-4-methylpentanol/G-3S4MPol, 2-pentenal for G-3-sulfanylpentanol/G-3SPol, ...). Chenot et al.[25] have explored the potential formation pathways of these aldehydes. Since *trans*-2-hexenal is the major oxidation product of linolenic acid (through lipoxygenase/LOX activity, extensively studied in the brewing field to understand the origin of *trans*-2-nonenal, responsible for the stale cardboard flavor),[26] the 3SHol *S*-conjugates are quite logically ubiquitous and usually the most abundant ones found in the different studied matrices.[25] Malt is no exception, with G-3SHol found at up to 700 μg/kg in pale malt.[6] Yet other *S*-conjugates have also occasionally been identified (up to 15 and 200 μg/kg Cys- and G-3SPol; up to 8 and 35000 μg/kg Cys- and G-3S4MPol; 10 μg/kg G-3SHptol, glutathionylated 3-sulfanylheptanol, found only in one green malt).[7,25]

In the wake of the discovery of this significant aromatic potential in malt but mostly in hops, strategies for optimizing free thiol release from the *S*-conjugate pool have been studied, based on enzymatic interconversions during the brewing process. These investigations have highlighted the β-lyase activity of *Saccharomyces cerevisiae* yeasts on Cys-conjugates during primary fermentation. To a lesser extent, ale yeasts are also able to release free thiols from *G*-conjugates, but there is no information regarding the catabolic pathway involved.[14] More recently, some *S. pastorianus* yeasts have emerged as even more active on *G*-conjugates.[7] The use of exogenous enzymes was also considered. A combination of bovine γGT (optimal incubation parameters: 37 °C, pH 7, in the presence

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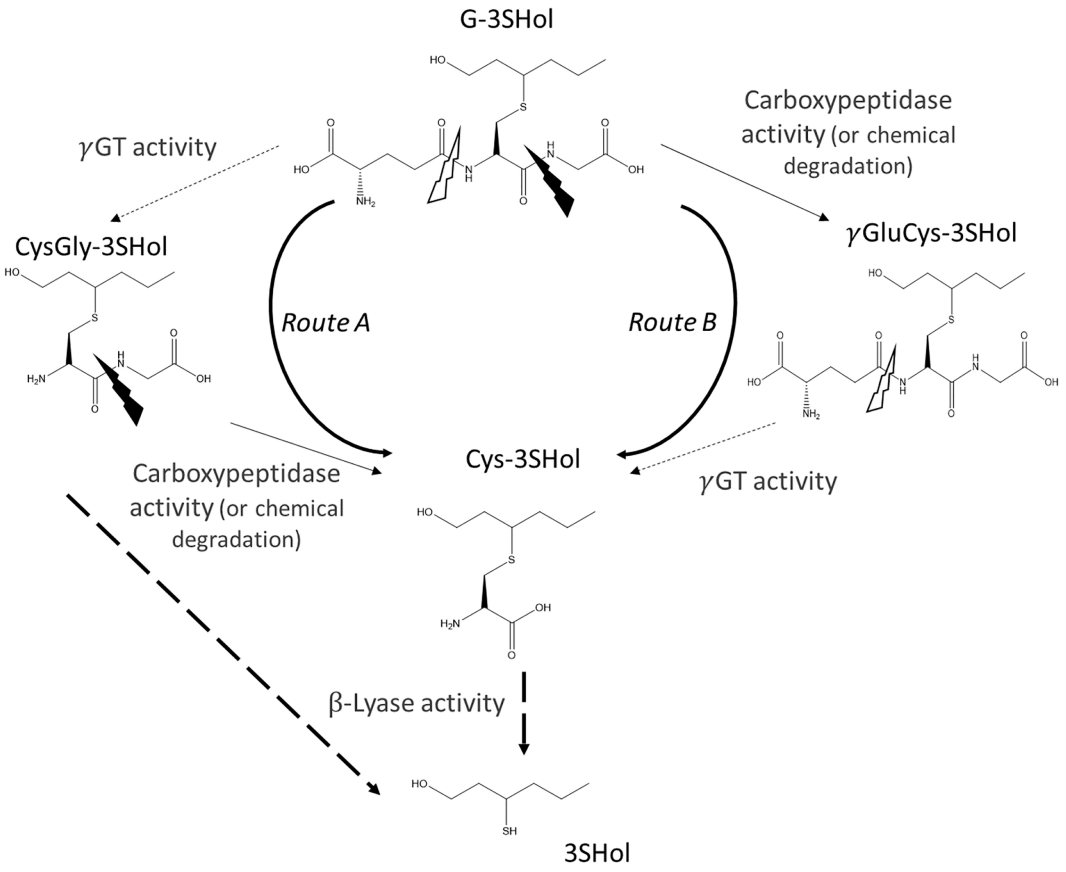

Figure 1. Enzymatic and chemical interconversions between the four 3SHol *S*-conjugates.

of amino acids as acceptors) and apotryptophanase (*β*-lyase activity from *E. coli*) has been shown to release free thiols from *G*-conjugates in model media, proving the *β*-lyase activity of apotryptophanase on CysGly-conjugates.[24] Purified tomato and tobacco *γ*GTs also show good activity at temperatures close to 30 °C.[27,28] In milk, 15 s at 77−80 °C inactivates *γ*GT.[29] In the oral cavity, microbiota carbon−sulfur lyases appeared to be involved in the release of the odorant allyl thiol from its cysteine conjugate.[30]

In a recent investigation of the 3SHol *S*-conjugate profile of 30 pale malts, the level of the CysGly-form was shown to be proportional to that of G-3SHol. This might be the first evidence of *in situ* *γ*GT acting during the malting process.[11] The high CysGly-3SHol content observed in wort by Molitor et al. (109 *μ*g/L in a 12°P wort) further suggests that *γ*GT is still active during the mashing step.[21]

In order to evidence potential actors capable of converting the different bound forms of PFTs to better yeast nutrients, the aim here was to identify all enzymatic and chemical interconversions of malt 3-sulfanylhexanol *S*-conjugates during mashing (these compounds having been identified as the most abundant and ubiquitous thiol precursors in all the studied malts).[11] In lab-scale mashing trials, the influences of the malt/water ratio, the temperature applied, the sample color (as a result of the kilning process), and acidity were studied.

MATERIALS AND METHODS

Chemicals. Formic acid (99%), methanol (99.98%), and water (99.98%) (UPLC/MS-CC/SFC grade), used for UPLC analyses, were purchased from Biosolve Chemicals (Holland). Methanol (>99.99%) (optima LC/MS grade), used for malt extract preparation, was purchased from Fisher Chemical (USA).

Malt Samples. All barley malt samples (listed in Table 1 with the code, name, producer, and color) were purchased from Rolling Beers (France).

Table 1. Barley Malt Samples

code	color (°EBC)	commercial name	producer
A	3	Pilsen	Soufflet
A′	3	Pilsen	Soufflet
A″	3	Diastatic	Weyermann
B	15	Munich	Soufflet
C	45	Abbaye	Weyermann
D	9	Acid	Weyermann

Model Wort Preparation and Sampling for PFT Precursor Analysis. Malt (10 g) was ground in a mixer. 1 g, accurately weighed, was further added to 5, 10, or 20 mL of demineralized water. The mixture was stirred and incubated at room temperature (25 °C, RT) or at 45, 55, or 80 °C for various times (up to 24 h for trials at RT, 3 h for trials at higher temperature).

At times of 15 min and 1, 2, 3, 8, and 24 h, a 150 *μ*L sample was taken from the aqueous mixture and diluted with the same volume of methanol (to inhibit enzymatic activities) for LC-MS/MS analysis.

Each trial was performed in triplicate.

Previously Synthesized Natural and Deuterated Thiol Precursors. Natural and deuterated G-3SHol/G-3SHol-d_2,[31] CysGly-3SHol/CysGly-3SHol-d_2,[32,33] *γ*GluCys-3SHol/*γ*GluCys-3SHol-d_2[32,33] and Cys-3SHol/Cys-3SHol-d_2[34] were synthesized prior to this work according to published methods. The purity of the natural and deuterated synthetic compounds was assessed by ^{1}H NMR[35] and

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found to be above 87%, except for Cys-conjugates, whose purity was only about 77% because of trifluoroacetic salt formation.

Analysis of *S*-Conjugates in Malt by Ultra-High-Performance Liquid Chromatography−Mass Spectrometry (UPLC-MS/MS). Analyses were performed on a 100 × 2.1 mm, 1.9 μm Hypersil GOLD aQ column, a polar end-capped C18 phase offering superior retention of polar compounds (ThermoFisher, Waltham, MA, USA). The elution solvents were water (solvent A) and methanol (solvent B), both containing 0.1% (v/v) formic acid. The flow rate was set at 0.6 mL/min. The elution gradient was as follows: 95% A for 1 min, from 95 to 65% in 9 min, from 65 to 2% in 3 min, held for 2 min, then back to the original conditions in 10 s and held for 2 min.

The analytical system consisted of a 1290 Infinity II UHPLC instrument (Agilent Technologies, Santa Clara, CA, USA) attached to a 6470B Agilent Triple Quadrupole mass spectrometer. Source parameters were as follows: gas temperature, 230 °C; gas flow, 4 L/min; nebulizer at 3.79 bar; sheath gas temperature, 400 °C; sheath gas flow, 11 L/min; capillary voltage, 3000 V in positive mode; nozzle voltage, 0 V; positive Delta EMV set at 400 V. Ionization was carried out by positive electrospray (ESI+), and detection was done by multiple reaction monitoring (MRM) as described by Chenot et al.[11]

Stable Isotope Dilution Assay (SIDA) for Quantitation of *S*-Conjugates in Malt. A calibration curve of each natural precursor (X) relative to its deuterated counterpart (X_{deut}) was recorded. Solutions at various concentrations (adapted for each precursor to the range usually found in samples) were used to plot straight lines (area ratio versus concentration ratio). The slope gave the natural conjugate-to-deuterated conjugate response coefficient (RC) ratio (R^2 > 0.99). The following equation was used for conjugate quantitation:

$$\text{conc(X)}\ (\mu\text{g/L}) = \left(\frac{\text{area(X)}}{\text{area}(X_{deut})} - y\text{-intercept}\right) \times \frac{\text{RC}(X_{deut})}{\text{RC(X)}} \times \text{conc}(X_{deut})\ (\mu\text{g/L})$$

***S*-Conjugate Contents.** In all cases, wort *S*-conjugate concentrations are given for a 1/10 malt/water ratio of wort (μg/L). Considering the different extraction volumes used for some trials, the following corrections were applied to the results obtained for the injected water−methanol extract: division by 2 for the 1/5 malt-water ratio worts; multiplication by 2 for the 1/20 malt-water ratio worts.

The contents presented in the figures are by means of triplicate experiments (error bars are shown).

RESULTS AND DISCUSSION

Enzymatic Interconversions of Thiol Conjugates. *Extraction Volume Effect in Pure Pilsen Malt Wort.* As already mentioned, many 3SHol *S*-conjugates have been identified in malt.[21] First, their fates at room temperature (25 °C) were investigated here for 24 h in water. After only 1 h, the Pilsen malt wort (sample A, 3°EBC; malt/water ratio 1/10) already displayed a loss of 28% of the G-3SHol and an increase in CysGly-3SHol from 0.3 to 14.3 μg/L. The malt γGT activity inducing this *S*-conjugate interconversion over time is illustrated in Figure 2a. G-3SHol continued to decrease through the first 8 h, finally reaching a stable low level (2.2 μg/L after 24 h). The level of CysGly-3SHol first increased over a period of 3 h and then began to decrease simultaneously with the appearance of a significant amount of Cys-3SHol. After 24 h, Cys-3SHol was significantly more abundant than CysGly-3SHol. Our data evidence a two-step catabolic pathway from G-3SHol to Cys-3SHol: release of CysGly-3SHol by γGT, followed by carboxypeptidase-catalyzed hydrolysis of the dipeptide, resulting in more than 10 μg/L Cys-3SHol.

As no trace of γGluCys-3SHol was found, it seems that malt carboxypeptidase was unable to remove the glycine moiety from glutathione. These results are not in line with previous work in which a carboxypeptidase able to act on glutathione *S*-conjugates was evidenced in barley.[36] However, in this former study, the chemical structure of the considered *S*-conjugate (glutathione linked to the herbicide alachlor) is far from that of G-3SHol, which could justify a complete change of carboxypeptidase activity. Moreover, the malting process could have induced changes of barley enzymatic activities, possibly due to inhibitors released.

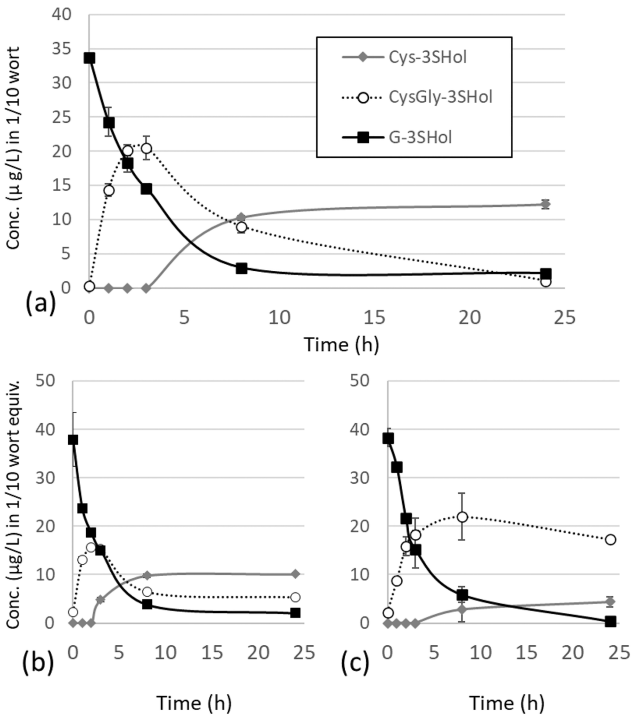

Figure 2. Pilsen malt (sample A, 3°EBC). Evolution over time, at 25 °C, of the 3SHol *S*-conjugate content of an aqueous medium with a malt/water ratio of 1/10 (a), 1/5 (b), or 1/20 (c). Error bars show standard deviation between triplicates.

Similar interconversion kinetics were observed in a more concentrated wort (malt/water ratio only 1/5), with Cys-3SHol appearing already at 3 h (Figure 2b). In a more diluted wort (malt/water ratio 1/20), the carboxypeptidase step appeared delayed. This led to a higher CysGly-3SHol concentration at 24 h (Figure 2c).

Figure 3 shows how the total *S*-conjugate molar concentration varied over time. It was found to increase slightly over the first 3 h, most probably because of slow malt constituent solubilization, and then to decrease. The fact that the total molar content was lower after 24 h than at the start (0.064 mol/L vs 0.084 mol/L) might be due to Cys-3SHol breakdown (by β-lyase) to free 3SHol (not easily measured in such experiments because of rapid oxidation[37] and/or adsorption).

Impact of Temperature and Malt. A malt-water ratio of 1/10 (easier sampling) was chosen to investigate the fate of the Pilsen malt A wort *S*-conjugates over a 3 h period at three classical mashing temperatures: 45, 55, and 80 °C (Figure 4, including also the first 3 h of the experiment discussed above conducted at room temperature). Investigations of an additional Pilsen malt (A′) and a diastatic 3°EBC malt (A″) are given in Supporting Information S1. Other darker malts

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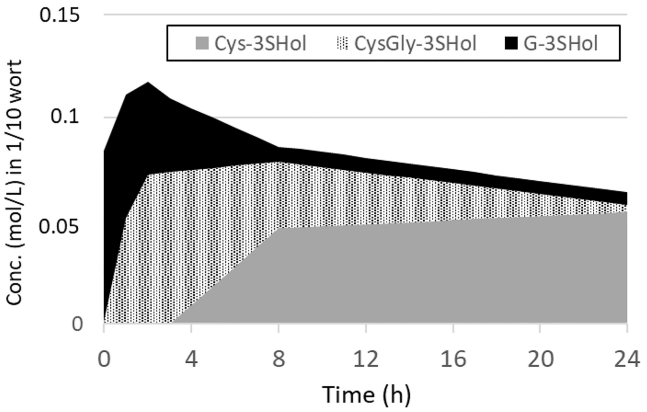

Figure 3. Evolution over time, at 25 °C, of the total 3SHol *S*-conjugate content (expressed in molar equivalents) of a Pilsen malt (sample A, 3 EBC; 1/10 malt-water ratio wort), with the individual contributions of Cys-, CysGly-, and Gly-3SHol.

(Munich malt sample B, Abbaye malt sample C, and acid malt sample D in Figure 4) were also investigated at 55 °C.

At 45 °C, the two Pilsen malt worts (samples A and A′) displayed similar behavior: a linear decrease in G-3SHol content, an increase in CysGly-3SHol for only 2 h, followed by its degradation, and a linear increase in Cys-3SHol. As compared to the same trial at RT, heating at 45 °C improved both γGT activity (greater loss of G-3SHol) and carboxypeptidase activity (earlier appearance of Cys-3SHol). At 55 °C, synthesis of CysGly-3SHol was even more pronounced with both traditional pale malts (A and A′′) and also with the diastatic 3°EBC malt sample (A″), suggesting a relatively high optimal temperature for malt γGT. This is not true for malt carboxypeptidases, usually classified as highly heat-sensitive.[26] Accordingly, degradation of the dipeptide to Cys-3SHol was less at 55 °C than at 45 °C.

When mashed at 55 °C, Munich malt (sample B, 15°EBC) displayed much lower carboxypeptidase activity, resulting in a continuous increase in CysGly-3SHol and little Cys-3SHol. As for special Abbaye malt (sample C, 35°EBC), its even higher kilning temperature explains why no increase at all in Cys-3SHol was observed (carboxypeptidases totally degraded). Yet γGluCys-3SHol was found at trace levels (3.7 μg/L), arising through thermal conversion of G-3SHol during malting, and remained stable over time.[11] When the acidic 9°EBC malt (sample D) was brewed at 55 °C, no bioconversion was observed. This highlights the key role of pH in optimizing γGT activity (congress wort at pH 3.4 vs 5.4 for all other samples).

Nonenzymatic Interconversions of Thiol Conjugates. At 80 °C, most enzymes are totally inactivated. Hence, only chemical reactions can occur. As expected, Cys-3SHol was not formed in pale malt (Figure 4 and Figure S1). Yet, significant degradation of G-3SHol was found to occur (not in pure water at 80 °C; data not shown). We hypothesize that a Schiff base could be produced between G-3SHol (containing a primary amine) and wort aldehydes (glucose, maltose, or Strecker and lipid derived aldehydes). Accordingly, imine synthesis in wort (evidenced at 80 °C by UV absorbance) was proposed long ago to explain the emergence of the cardboard off-flavor in aged beer.[38] Similar UV experiments, conducted in a pH 5.5 model medium, enabled us to visualize the Schiff base (absorption up to 0.38 a.u. at 265 nm) created at 80 °C between G-3SHol (only slightly degraded after 2 h when alone, Figure 5a) and *trans*-2-hexenal (Figure 5b). On the other hand, pH 3 prevented production of the Schiff base (Figure 5c).

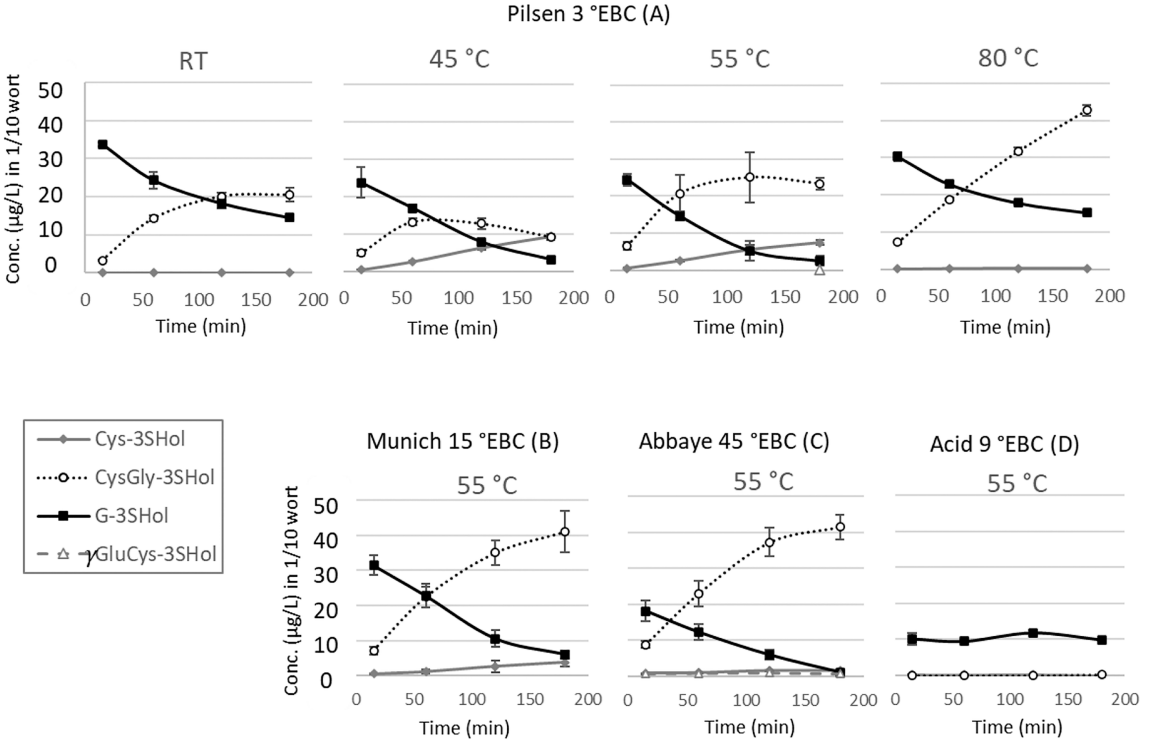

Figure 4. Interconversions of 3SHol *S*-conjugates in worts (malt/water ratio: 1/10) during a 3 h mashing of various malts (A−D) at room temperature (RT) or at 45, 55, or 80 °C. Error bars show standard deviation between triplicates.

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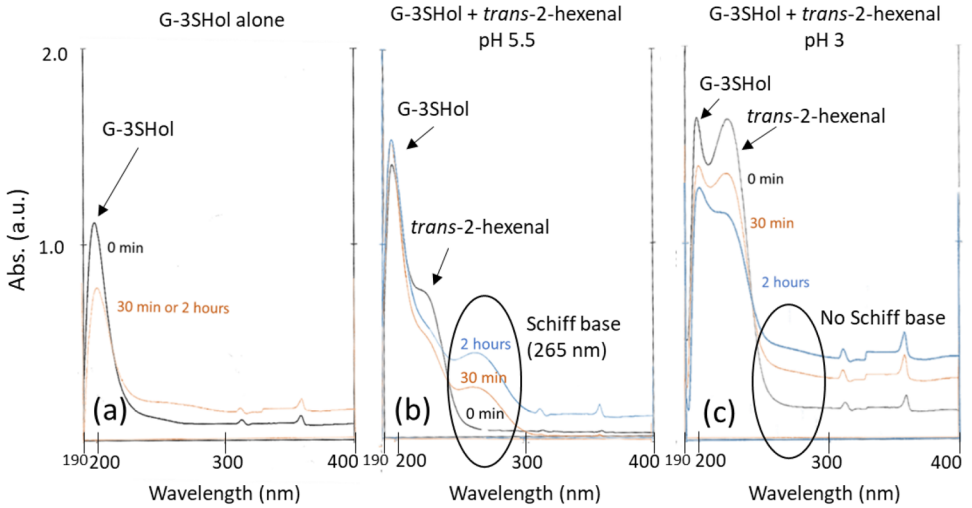

Figure 5. UV spectra obtained after heat treatment (80 °C) of G-3SHol (50 mg/L) (a) alone, (b) with *trans*-2-hexenal (5 mg/L) in aqueous medium at pH 5.5, or (c) with *trans*-2-hexenal in aqueous medium at pH 3.

Surprisingly, despite the absence of active enzymes, a steady increase in CysGly-3SHol was also observed at 80 °C (after 3 h, 42.7 μg/L in malt A and 45.1 μg/L in malt A′). This could not be totally explained by interconversion of G-3SHol (which decreased by only 10.7 μg/L in malt A and 15.4 μg/L in malt A′). Additional experiments are needed to assess if there might occur in wort an *in situ* addition of cysteinylglycine (a dipeptide present in malt) onto *trans*-2-hexenal (a well-known wort C18:3 LOX oxidation product). Other potential pro-precursors should also be investigated in worts, including thiazepine and thiazolidine derivatives, both previously evidenced as *S*-conjugate byproducts in model media.[39] A similar increase in CysGly-3SHol was also observed at 55 °C with malt C (45°EBC) at which higher temperatures were already applied during roasting.

CONCLUSION

Considering both the greater ability of *S. cerevisiae* to metabolize shorter conjugates (Cys- or CysGly-) and brew-masters' conviction that most PFTs from malt are lost in the spent grain (as no PFT flavors are found in unhopped beers),[13] several conclusions might be drawn from the present work. First, PFT release from hop *S*-conjugates during fermentation could be enhanced by adding to the usual brewing process a preincubation of hop with malt γGT enzymes. Next, given the Schiff base formation evidenced here between G-3SHol and wort aldehydes, a too-long boiling step is most probably detrimental to keeping unbound *S*-conjugates issued from hops. Lastly, since malt enzymes do not allow interconversions between G- and γGluCys-conjugates, the occurrence of the latter in a wort could be a marker proving that special malts have been used.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.3c03640.

Interconversions of 3SHol *S*-conjugates in worts (malt/water ratio: 1/10) during a 3 h mashing of Pilsen malt (A′) at 45, 55, or 80 °C and diastatic malt (A″) at 55 °C (PDF)

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

Cys, cysteinylated; CysGly, cysteinylglycinylated; γGluCys, γ-glutamylcysteinylated; G, glutathionylated; γGT, γ-glutamyltransferase; 3SHol, 3-sulfanylhexanol

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