Journal of the American Society of Brewing Chemists
The Science of Beer

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/ujbc20

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To cite this article: Cécile Chenot, Guillaume Willemart, Jacques Gros & Sonia Collin (2022): Ability of Exogenous or Wort Endogenous Enzymes to Release Free Thiols from Hop Cysteinylated and Glutathionylated *S*-Conjugates, Journal of the American Society of Brewing Chemists, DOI: 10.1080/03610470.2021.2021766

To link to this article: https://doi.org/10.1080/03610470.2021.2021766

Published online: 26 Jan 2022.

JOURNAL OF THE AMERICAN SOCIETY OF BREWING CHEMISTS
https://doi.org/10.1080/03610470.2021.2021766

Ability of Exogenous or Wort Endogenous Enzymes to Release Free Thiols from Hop Cysteinylated and Glutathionylated *S*-Conjugates

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ABSTRACT

Following identification of the substantial pool of aromas represented by cysteinylated and glutathionylated precursors in hop, many studies have focused on developing methods for releasing volatile thiols based on chemical hydrolysis or yeast activity. The present work aimed to evaluate the release activity of either commercially available exogenous enzymes or endogenous wort enzymes (issued from hop or malt) on synthetic Cys- or G-precursors of 3SHol, 3SPol, 3SHptol, 3S4MPol and 4S4M2Pone. In a model medium at pH 7.7, both apotryptophanase and cystathionine β-lyase appeared very efficient on Cys-adducts (up to 77.9% release). Interestingly, a slight proportion of this release (up to 0.9%) appeared to result from chemical hydrolysis. In polyphenols rich media such as hop and beer, total release was much lower (under 0.2%). For release from G-adducts, a combination of γGT and apotryptophanase proved to be effective, highlighting for the first time the ability of β-lyase activity to release free PFTs also from CysGly-adducts. The best release rates (above 2% for all thiols) were obtained with a two-step incubation: 75 units γGT with synthetic glutathionylated precursors for 16 h at 37 °C in the presence of alanine followed by apotryptophanase for 7 h at room temperature. The alternative combination of γGT with *S. cerevisiae* was found to release free thiols more efficiently than yeast activity alone. Compared to commercially available enzymes, endogenous enzymes extracted from hop or malt displayed lower efficiency (below 0.3% for γGT activity and below 0.002% for β-lyase).

Abbreviations: Apo: apotryptophanase; Cys-: cysteinylated; CysGly-: cysteinylglycine; Cysta: cystathionine β-lyase; EDTA: ethylenediaminetetraacetic acid; EST: external standard; F3: Flavorpro™ 373MDP; F8: Flavorpro™ 852MDP; G-: glutathionylated; GC: gas chromatography; PFPD: pulsed flame photometric detector; γ: Bovine γGT; γGT: γ-glutamyl transpeptidase; GluCys: γ-glutamylcysteine; GSH: glutathione; GST: glutathione-S-transferase; IST: internal standard; PLP: pyridoxal-5′-phosphate; PMSF: phenylmethylsulfonyl fluoride; PFTs: polyfunctional thiols; PVPP: polyvinyl polypyrrolidone; TRIS: tris(hydroxymethyl)aminomethane; 3SHol: 3-sulfanylhexan-1-ol; 3SHptol: 3-sulfanylheptan-1-ol; 3SPol: 3-sulfanylpentan-1-ol; 3S4MPol: 3-sulfanyl-4-methylpentan-1-ol; 4S4M2Pone: 4-sulfanyl-4-methylpentan-2-one

KEYWORDS

β-lyase;
Hop (*Humulus lupulus* L.);
γGT;
polyfunctional thiols;
S-conjugates

Introduction

Among the components of hop essential oils,[1] polyfunctional thiols (PFTs) have been identified as key contributors to the hoppy aroma of beer (mostly thanks to their very low thresholds, at the ng/L level).[2–4] In beer, PFTs have been found at concentrations higher than expected on the basis of the free thiol content and the hopping rate.[5] To explain this unexpected increase, the existence of cysteinylated and glutathionylated precursors, already evidenced in other plant matrices such as grapes,[6] was suspected and therefore investigated in hop.

As a result, S-3-(1-hydroxyhexyl)-cysteine (Cys-3SHol) has been identified at concentrations up to 1 mg/kg in the Cascade hop cultivar and up to 0.7 mg/kg in malt.[5,7] S-3-(1-hydroxypentyl)-cysteine (Cys-3SPol) has been found at much lower concentration: up to 0.16 mg/kg in the Polaris cultivar.[8] The compound S-4-(4-methylpentan-2-one)-cysteine (Cys-4S4M2Pone) has been detected at even lower concentrations in most varieties (up to 0.04 mg/kg in Cascade).[9] As for glutathione *S*-conjugates, S-3-(1-hydroxyhexyl)-glutathione (G-3SHol) and S-3-(1-hydroxypentyl)-glutathione (G-3SPol) have been found in all studied varieties at levels ranging respectively from 20 to 118 mg/kg and from 1 to 18 mg/kg.[8] Like its cysteinylated analogs, S-4-(4-methylpentan-2-one)-glutathione (G-4S4M2Pone) has been found only in low quantity (0.01 mg/kg in Chinook hop).[9] In contrast, the cysteinylated and glutathionylated precursors of 3-sulfanyl-4-methylpentanol (Cys- and G-3S4MPol), like the free form, seem more variety-specific, the highest concentration (3.6 mg/kg of G-3S4MPol) having been detected in Polaris hop.[8] Very

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recently, traces of S-3-(1-hydroxyheptyl)-glutathione (G-3SHptol) were also found in green malt.[10]

Originally, cysteine and glutathione *S*-conjugates arise directly from glutathione (GSH), an essential tripeptide (γ-glutamyl-cysteinyl-glycine) known in the plant kingdom for its role in sulfur metabolism and for its antioxidant and detoxifying properties.[11–17] In the case of thiol precursor biosynthesis in hop (Figure 1), glutathione *S*-conjugates result directly from binding of GSH to an α,β-unsaturated carbonyl, catalyzed by a cytosolic glutathione S-transferase (GST) (1). G-adducts can thus be viewed as pro-precursors of Cys-adducts, themselves precursors of free forms.

In plants, the vacuolar enzymes known to be involved in G-adducts catabolism to Cys-adducts are γ-glutamyl transpeptidase (γGT) (2) and carboxypeptidase (hydrolysis of a C-terminal peptide) (3).[18–20] As depicted in Figure 1, two routes can occur: route A to B, in which a carboxypeptidase (3) first removes the glycine moiety to yield a γ-GluCys-intermediate, and route C to D, in which γGT (2) first removes the γ-glutamyl moiety to yield a CysGly *S*-conjugate.[21,22] Both dipeptides have been identified in Sauvignon Blanc grapes but appear rather unstable, constituting only a small part of the whole *S*-conjugate pool.[23] In barley, the carboxypeptidase was evidenced with the formation of γ-GluCys *S*-conjugates from glutathione *S*-conjugates.[24] Note that in the transpeptidation reaction catalyzed by γGT, the γ-glutamyl group is transferred to an "acceptor" molecule such as an amino acid or a peptide.

Finally, an enzyme with β-lyase activity (4) releases free thiols. Note that since β-lyase activity requires only a free amino terminus and not a free carboxy terminus, direct release of PFTs from CysGly-adducts may also occur.[24,25] The β-lyase additionally requires pyridoxal-5′-phosphate (PLP), a derivative of the B6 vitamin, as a cofactor at its

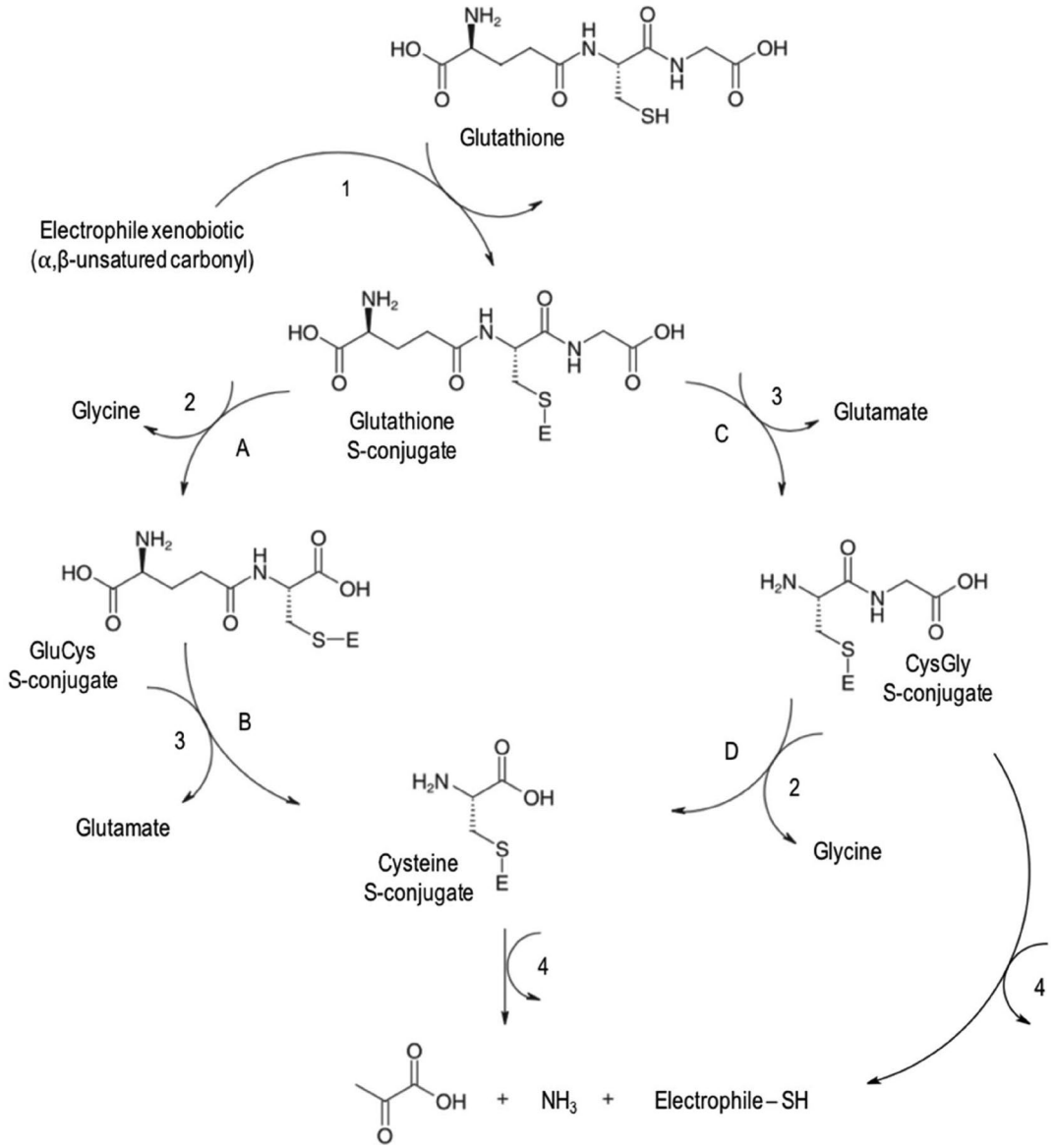

Figure 1. Catabolism of glutathione *S*-conjugates (modified from Starkenmann et al.[26]).

active site.[26] Chemical degradation of cysteine adducts to free PFTs has also been evidenced during beer storage (spiked beer samples). It seems that beer co-constituents such as dicarbonyls can participate in this release. A modified Strecker degradation pathway has been proposed.[27]

The ability of yeast to degrade glutathione pro-precursors and cysteine precursors to free PFTs has also been highlighted. It was first observed during bottle refermentation of media spiked with cysteine adducts, especially after 3 weeks.[28] More recently, it was evidenced during beer fermentation (10 days at 24 °C and 1 day at 4 °C) with a top-fermentation yeast (*S. cerevisiae* K-97) after spiking of unhopped wort (with synthetic cysteine and glutathione precursors).[8] After optimization (wort density, fermentation temperature, number of days at low temperature, yeast strain…), degradation to free PFTs was observed from Cys-3SHol, -3SPol, and -3S4MPol in the 0.35–0.45% range and from G-3SHol, -3SPol, and -3S4MPol in the 0.02–0.08% range.[29]

Many authors have investigated, initially for quantitation purposes (as an alternative to HPLC), how efficiently some commercially available exogenous enzymes (especially apotryptophanase from *Escherichia coli*) release free PFTs from Cys-adducts (and presumably from CysGly analogs) present in several hop varieties.[30,31] Because of the variable efficiency of apotryptophanase (4 to 50% of 3SHol released from Cys-3SHol according to the supplier), an internal standard (S-benzyl-L-cysteine) was used for recovery factor correction. A commercial γ-glutamyl transpeptidase (purified from bovine kidney) was further shown to yield cysteinylglycine *S*-conjugates from glutathionylated adducts.[8,32]

The aim of the present work was to investigate some unexplored enzymatic approaches, potentially promising under brewing conditions, to achieve the release of free PFTs from hop precursors. Hop and malt contain many enzymes involved in degrading their own adducts to free PFTs, but unfortunately also many enzyme inhibitors such as polyphenols. The pH could be another limiting factor, as most of the enzymes concerned exhibit optimal activity at a pH much higher than that of wort or beer.

Here we have used GC–PFPD to quantitate thiol release from adducts in spiked media, after incubation with either exogenous commercial enzymes or natural enzymatic extracts (of hop and malt). Most enzyme activities (just defined here by the level of free PFTs measured) were assessed both in model biochemical media and under conditions closer to the brewing process.

Experimental

Chemicals

Acetonitrile, dichloromethane, 37% hydrochloric acid, and sodium chloride were purchased from VWR (Leuven, Belgium). The 2-acetylthiophene, 4-methoxy-2-methylbutane-2-thiol, 6-mL Discovery® Ag-ion SPE tubes, 98% L-cysteine hydrochloride monohydrate, β-mercaptoethanol, phenylmethylsulfonyl fluoride (PMSF), S-benzyl-L-cysteine and TRIS were purchased from Sigma-Aldrich (Bornem, Belgium). Alanine, anhydrous sodium sulfate (Na_2SO_4), glycylglycine and sodium hydroxide were purchased from Acros Organics (Geel, Belgium). Polyvinyl polypyrrolidone (PVPP) was supplied by Spindal (Gretz-Armainvilliers, France). Pyridoxal-5-phosphate (PLP) was purchased from Alfa Aesar (Haverhill, MA, U.S.A.). Ethylenediaminetetraacetic acid (EDTA) was purchased from JT Baker Chemicals (Radnor Township, PA, U.S.A.). Miracloth filter was purchased from Merck Millipore (MA, U.S.A.). Tripotassium phosphate (K_3PO_4) was purchased from Merck (Darmstadt, Germany). Milli-Q water was used (Millipore, Bedford, MA, U.S.A.). Amarillo (U.S.A., harvest 2015; α acids: 9.2%; oil content: 1.7%) was provided by Yakima Chief (Belgium). Polaris (Germany, harvest 2019; α acids: 19.1%; oil content: 3.6%) was provided by Hopsteiner (Germany). Nelson Sauvin (New Zealand, harvest 2018; α acids: 12.4%; oil content: 1.1%) was provided by Brouwerij Anders!.

Synthesis of previously investigated reference conjugates

Cys-3SPol, Cys-3S4MPol, Cys-3SHol, Cys-3SHptol, G-3SPol, G-3S4MPol, G-3SHol and G-3SHptol were synthesized prior to this work according to the methods of Chenot et al., Gros et al., and Kankolongo et al.[5,8,33] Cys-4S4M2Pone was purchased from AromaLAB (Germany).

Exogenous commercial enzymes

The γ-glutamyl transpeptidase (γGT) purified from bovine kidney was purchased from MyBioSource (San Diego, CA, U.S.A.). Recombinant cystathionine β-lyase was purchased from Creative Enzyme (Kampenhout, Belgium). Apotryptophanase was purchased from Sigma-Aldrich (Bornem, Belgium). Flavorpro™ 373MDP (F373, an endoprotease preparation derived from *B. subtilis* that contains a glutaminase side activity) and Flavorpro™ 852MDP (F852, an exopeptidase preparation derived from *Aspergillus sp.* that contains endopeptidase and glutaminase side activities) were provided by Biocatalysts Limited (Cardiff, Wales, UK), and are enzymes originally designed for food industry flavor enhancement.

Yeast

SafAle™ K-97 dry yeast (*Saccharomyces cerevisiae*) was purchased from Fermentis (Marcq-en-Baroeul, France).

Preparation of incubation media

Three model media, one beer medium and two hop media were prepared for incubation trials. One model medium was prepared as follows: mixing K_3PO_4 (0.1 M) with pyridoxal 5-phosphate (PLP, 0.1 mM) and ethylenediaminetetraacetic acid (EDTA, 1 mM) in Milli-Q water and increasing the pH to 7.7 by addition of 2 M NaOH. A

second medium was prepared similarly, but without PLP and EDTA. A third contained only Milli-Q water adjusted to pH 4.2 with HCl. The beer medium was a commercial Belgian lager beer (pH 4.2) that had simply been degassed by passage through a paper filter. The hop medium was prepared by mixing Amarillo hop pellets with Milli-Q water (hop/H_2O ratio: 1/30) before stirring for 1 h, centrifuging at 9000 rpm for 30 min, recovering the supernatant, and increasing the pH to 7.7 by addition of 2 M NaOH. Removal of polyphenols from this medium was also tested, by passing it through a column filled with PVPP.

Incubation of synthetic glutathionylated and cysteinylated precursors in incubation media with exogeneous enzymes

Incubations were conducted as follows: incubation of cysteine adducts (Cys-4S4M2Pone, Cys-3SPol, Cys-3SHol, Cys-3S4MPol, Cys-3SHptol and S-benzyl-L-cysteine, see Table 1) or glutathione adducts (G-3SPol, G-3SHol, G-3S4MPol and G-3SHptol, see Table 2) with enzymes in different media, slow stirring. All experiments were performed in duplicate.

Preparation of hop and malt enzymatic extracts

Enzymatic extracts of hop and malt were obtained as follows by differential centrifugation (three different enzymatic extract fractions obtained): (i) grinding of 10 g hop or malt (Nelson Sauvin and Polaris hop varieties; pale and green malts); (ii) dilution in 100 mL of the corresponding extraction buffer (80 mM Tris-HCl pH 9 with 1 mM EDTA, 10 mM β-mercaptoethanol, and 1 mM PMSF for hop; 50 mM tris-HCl pH 8 with 1 mM EDTA, 10 mM β-mercaptoethanol, and 1 mM PMSF for malt), addition of 1.5 g PVPP powder (for hop extract only), stirring for 10 min, filtration on a Miracloth filter, centrifugation at 1000 g for 10 min, and recovery of 5 mL supernatant (recovered as the first enzymatic extract called SN_1); (iii) centrifugation of the remaining supernatant at 100,000 g for 30 min and recovery of this second enzymatic extract, called SN_2 (soluble enzymes), and (iv) suspension of the pellet in 20 mL of the corresponding extraction buffer (recovered as the third enzyme extract called SP, with mitochondria, chloroplasts, and the microsomal fraction).

Incubation of synthetic glutathionylated precursors in hop and malt enzymatic extracts

For each hop or malt enzymatic extract (SN_1, SN_2, and SP), incubation trials were conducted as follows: incubation of the enzymatic extract with G-3SPol, G-3S4MPol, and G-3SHol (4000 mg/L each) and 0.1 M glycylglycine. Two blanks were prepared as follows: incubation of G-3SPol, G-3S4MPol, and G-3SHol (4000 mg/L each) in extraction buffer (no enzymatic extract) and incubation of enzymatic extract without precursors. In all trials, incubation was carried out in a water bath at 30 °C for 1 h. All experiments were performed in duplicate.

Free thiol extraction from incubation media and enzymatic extracts

Free PFTs were extracted from the incubation media and enzymatic extracts as follows: liquid/liquid extraction with bidistilled dichloromethane under strong stirring for 15 min, recovery of the organic phase, drying with anhydrous Na_2SO_4, concentration to 250 µL in a Danish-Kuderna apparatus and to 70 µL with a Dufton column (immersion of the vessel in a water bath at 45 °C). The 4-methoxy-2-methylbutan-2-thiol was added as an internal standard (IST, at 20 µg/L to 500 µg/L according to the experiment) before liquid/liquid extraction.

Unhopped wort mashing

Wort was produced from pale malt (Boortmalt) in a 50-liter-scale pilot plant (Coenco, Oostkamp, Belgium). The 17°Plato

Table 1. Trials with exogeneous enzymes in various media spiked with cysteinylated adducts and incubated under various conditions.

Trial denomination		Precursor	Medium			Enzyme		Timing and temperature (Room temperature = RT)
		Cys-	Made of	pH	Cofactor (PLP)	Cystathionine β-lyase (Cysta)	Apotryptophanase (Apo)	
pH effect	pH7	5 mg/L	Model	7.7	/	/	/	20 h at RT
	pH4	5 mg/L	Model	4.2	/	/	/	20 h at RT
Enzyme nature and cofactor effect	Cysta + PLP	5 mg/L	Model	7.7	0.1 mM	600 units/mL	/	20 h at RT
	Cysta	5 mg/L	Model	7.7	/	600 units/mL	/	20 h at RT
	Apo + PLP	5 mg/L	Model	7.7	0.1 mM	/	25 units/mL	20 h at RT
	Apo	5 mg/L	Model	7.7	/	/	25 units/mL	20 h at RT
Medium effect	Beer Cysta + PLP	5 mg/L	Beer	4.2	0.1 mM	30 units/mL	/	20 h at RT
	Beer Cysta	5 mg/L	Beer	4.2	/	30 units/mL	/	20 h at RT
	Hop Apo	5 mg/L	Hop	7.7	/	/	25 units/mL	20 h at RT
	Hop + PVPP	5 mg/L	Hop + PVPP	7.7	/	/	/	20 h at RT
	Hop + PVPP Apo	5 mg/L	Hop + PVPP	7.7	/	/	25 units/mL	20 h at RT

Table 2. Trials with exogeneous enzymes in model media spiked with glutathionylated adducts and incubated at pH 7.7 with PLP 0.1 mM and 115 units of apotryptophanase.

		Precursor	Medium		Enzyme + Apo (115 units)				Timing and temperature	
Trial denomination		G-	Made of	Acceptor for γGT activity (100 mg/L) (Alanine = ala, Glycyl-glycine = gg)	Cofactor (for Apo)	Bovine γGT (γ)	F373 (F3)	F852 (F8)	Temperature (room temperature = RT, 37 °C = T37)	Time
Enzyme nature and dosage effect	γ15 + gg	50 mg/L	Model pH7.7	gg	PLP	15 U	/	/	RT	20 h
	γ75 + gg	50 mg/L	Model pH7.7	gg	PLP	75 U	/	/	RT	20 h
	F3-10 + gg	50 mg/L	Model pH7.7	gg	PLP	/	10 mg (900 U)	/	RT	20 h
	F3-50 + gg	50 mg/L	Model pH7.7	gg	PLP	/	50 mg (4500 U)	/	RT	20 h
	F8-5 + gg	50 mg/L	Model pH7.7	gg	PLP	/	/	5 mg (1650 U)	RT	20 h
	F8-10 + gg	50 mg/L	Model pH7.7	gg	PLP	/	/	10 mg (3300 U)	RT	20 h
	F8-50 + gg	50 mg/L	Model pH7.7	gg	PLP	/	/	50 mg (16,500 U)	RT	20 h
Acceptor and temperature effect	γ15	50 mg/L	Model pH7.7	/	PLP	15 U	/	/	RT	20 h
	γ75 + ala	50 mg/L	Model pH7.7	ala	PLP	75 U	/	/	RT	20 h
	γ75 + gg T37	50 mg/L	Model pH7.7	gg	PLP	75 U	/	/	37 °C for both enzymes	20 h
	γ75 + gg T37/RT	50 mg/L	Model pH7.7	gg	PLP	75 U	/	/	37 °C for γGT RT for Apo	16 h for γGT 7 h for Apo
	γ75 + ala T37/RT	50 mg/L	Model pH7.7	ala	PLP	75 U	/	/	37 °C for γGT RT for Apo	16 h for γGT 7 h for Apo

unhopped wort was obtained after 90 min of boiling. After clarification, it was diluted to 15°Plato with sterile water and frozen until the fermentation trials.

Incubation of synthetic glutathionylated precursors with exogenous enzymes and yeast in unhopped wort

The 15°Plato unhopped wort was first spiked with G-3SPol, G-3SHol, G-3S4MPol, and G-3SHptol (15 mg/L each). Bovine γGT (750 units) and SafAle K-97 yeast (final concentration: 0.46 g/L) were pitched into 250 mL wort, either simultaneously (trial: Wort γ + K97) or 24 h apart (bovine γGT first, trial: Wort γ/K97). Fermentation was conducted for 7 days at 24 °C under shaking at 80 rpm (Labwit ZWY-240 incubator shaker). The fermented wort was then kept at 4 °C for three days before extraction of specific free thiols with a silver cartridge (see next section). The experiment was performed in duplicate.

Specific extraction of free thiols from fermented spiked medium

As fermented spiked medium contained lower amounts of PFTs, a more efficient procedure using a silver cartridge was used for extraction, as described by Chenot et al.[29]: (i) stirring 150 mL medium for 15 min with 50 mL dichloromethane; (ii) centrifuging at 4500 rpm for 20 min; (iii) recovering the organic phase and loading it on a Discovery® Ag-ion SPE cartridge conditioned beforehand with 10 mL dichloromethane; (iv) rinsing the cartridge first with 10 mL dichloromethane, then with 20 mL acetonitrile, and finally with 10 mL Milli-Q water (reversed cartridge in this last case); (v) releasing free PFTs from the Ag cartridge by percolating 20 mL washed cysteine solution (4 × 20 mL dichloromethane for washing 215 mg cysteine in 20 mL water); (vi) double extraction of the eluent with bidistilled dichloromethane (5 mL for 5 min and 10 mL for 10 min), drying the resulting organic phase with anhydrous Na_2SO_4, and concentrating to 250 µL in a Danish-Kuderna distillation apparatus and to 70 µL with a Dufton column (immersion of the vessel in a water bath at 45 °C). The 4-methoxy-2-methylbutane-2-thiol was added as an internal standard (IST, 2 µg/L in 150 mL sample) and the 2-acetylthiophene as an external standard (EST, 0.5 mL of a 200 mg/L solution added before concentration).

Quantitation by GC–PFPD of released free thiols

One microliter of free thiol extract was analyzed with an Agilent 5973 N gas chromatograph equipped with a splitless injector maintained at 250 °C. Compounds were separated with a wall-coated open tubular (WCOT) apolar CP-Sil 5 CB capillary column (50 m, 0.32 mm inner diameter, and 1.2 µm film thickness). The carrier gas was helium and the pressure was set at 50 kPa. The oven temperature was programmed to rise from 36 to 85 °C at 20 °C/min, then to

145 °C at 1 °C/min, finally to 250 °C at 3 °C/min, and held for 30 min. The following parameters were selected for the PFPD detector: temperature, 220 °C; voltage, 590 V; gate width, 18 ms; gate delay, 6 ms; trigger level, 400 mV; pulse frequency, 3.33 Hz. PFPD chromatograms were recorded throughout elution. ChemStation software was used to process the resulting data. The following general equation was used for compound X quantitation:

$$\mu g\,L^{-1}\text{ of }\mathbf{X} = \mu g\,L^{-1}\text{ of IST} \times \frac{\mathbf{X}\text{ area}}{\text{IST area}} \times \frac{\text{IST molar response coefficient}}{\mathbf{X}\text{ molar response coefficient}} \times \frac{\mathbf{X}\text{ molar weight}}{\text{IST molar weight}} \times \frac{\text{IST recovery factor}}{\mathbf{X}\text{ recovery factor}}$$

For all thiols, the IST-relative recovery factor was set at 1 (experimental values from 0.8 to 1.2, previously determined by standard addition). The good equimolarity of the PFPD detector allowed setting the IST-relative molar response coefficients at 1.

Release efficiency determination

The efficiency of release of free thiol **X** from bound synthetic precursors (cysteinylated and glutathionylated) was calculated with the following equation (given as means of duplicates):

$$\mathbf{X}\text{ release efficiency }(\%) = \frac{\mu g\,L^{-1}\,\boldsymbol{X}}{\mu g\,L^{-1}\text{ added bound }\boldsymbol{X}} \times \frac{\textit{bound }\boldsymbol{X}\textit{ molar weight}}{\textit{free }\boldsymbol{X}\textit{ molar weight}} \times 100$$

Note that since we did not carry out any precursor quantitation after incubation, we do not refer here to the amount of precursor removed through incubation, but to the total concentration spiked in the experiment. Therefore, enzymatic efficiencies are underestimated and should be carefully handled when compared to other data available in the literature.

Quantitation by HPLC-ESI(+)-MS/MS of Cys-adducts produced from glutathionylated precursors in presence of endogenous enzymes

Analyses were performed on a 100 × 2.1 mm, 3 µm Hypersil GOLD aQ column, a polar end-capped C18 phase offering superior retention of polar compounds (Thermo Fisher Scientific). A system equipped with an autosampler and a quaternary pump (Agilent Technologies, 1200 Series) and controlled with Agilent ChemStation software was used. The elution solvents were water (solvent A) and acetonitrile (solvent B), both containing 0.1% v/v formic acid. The Cys-adducts were eluted and quantitated using the elution program and the mass spectrometer parameters described by Chenot et al.[8]

Results and discussion

The release of free thiols from Cys-adducts has been evaluated according to various parameters such as medium or enzyme nature and cofactor effect (detailed in Table 1).

Chemical release of free thiols from Cys-adducts at different pH values

The single effect of pH on free thiol chemical release from Cys-adducts was assessed in a model medium, at room temperature, in the absence of enzymes (results in Table 3). The pH was found to influence significantly the release of free thiols from cysteinylated precursors: no release was detected at pH 4.2, close to the pH of beer, but release from all four precursors was observed at pH 7.7 (up to 0.9%). These results are surprisingly promising, especially as compared to the β-lyase activity of *S. cerevisiae* yeast under fermentation conditions (only up to 0.4% from the same precursors).[8] Some adducts appeared more sensitive to chemical hydrolysis than others (twice as much 3S4MPol as 3SPol was released).

Enzymatic release of free thiols from Cys-adducts according to the exogenous enzyme nature and cofactor presence in model media

In our investigations conducted to establish the ability of cystathionine β-lyase (Cysta) and apotryptophanase (Apo) to release PFTs from cysteinylated *S*-conjugates, tests were performed in model media at pH 7.7, at room temperature, with and without PLP (Table 1).

As illustrated in Figure 2, with these purified enzymes used here under their optimal conditions, the release rates were highly improved compared to chemical hydrolysis, ranging from 3.7% to 77.9%. Except for 3S4MPol (for which Apo + PLP was more efficient), the best release rates were obtained with Cysta + PLP. In most cases, both enzymes appeared PLP-dependent.

Results also depended strongly on thiol structure (e.g.,: 3SHol badly released compared to 4S4M2Pone).

Good news for indirect quantitation of cysteine adducts by GC–PFPD, the Apo + PLP trial proved to be less structure-selective (13.9% to 45.7%) than Cysta + PLP (26.3% to 77.9%). Furthermore, a similar efficiency was achieved with fewer enzyme units of apotryptophanase than cystathionine β-lyase (25 units/mL versus 600 units/mL, respectively).

Table 3. Rates of free thiol release in model media at different pHs spiked with cysteinylated adducts.

	Free thiol release [%]			
Trial denomination	3SPol	3S4MPol	3SHol	3SHptol
pH7	0.41[b]	0.91[a]	0.85[a]	0.63[a,b]
pH4	n.d.[a]	n.d.[a]	n.d.[a]	n.d.[a]

Variation coefficients are under 15%. Standard deviations have been considered in the Tukey test. Values in the same row that do not share a common letter (a or b) are significantly different ($p > 0.05$).

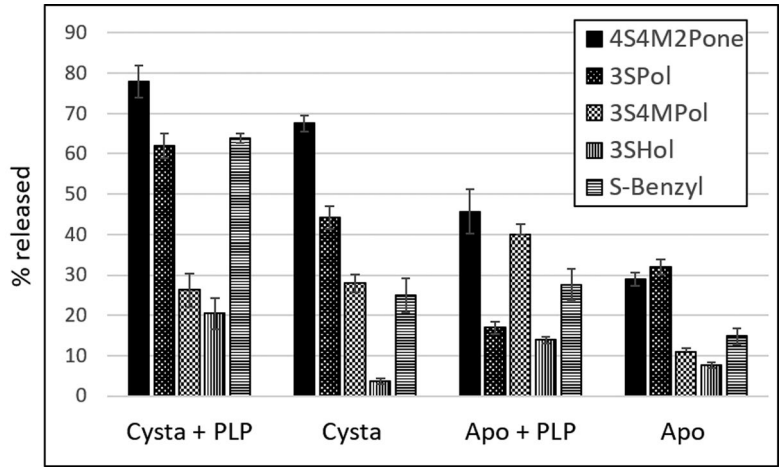

Figure 2. Release rates (%) of free thiols from spiked cysteinylated adducts in model medium with cystathionine β-lyase (Cysta) or apotryptophanase (Apo) in presence or absence of cofactor (PLP). Error bars indicate duplicates variation (under 15%).

Enzymatic release of free thiols from Cys-adducts by cystathionine β-lyase in beer media, cofactor effect

As it would be interesting to release PFTs directly in beer medium, trials were performed with 30 units/mL cystathionine β-lyase in a degassed lager beer at pH 4.2 at room temperature in the presence or absence of PLP. The release rates ranged from 0.1% to 0.8% (Beer Cysta + PLP and Beer Cysta in Figure 3a). These values, much lower than those obtained in model media with the same enzymes, are closer to the values obtained with yeast under fermentation conditions. This is not surprising, as the pH of the medium is outside the range of the cystathionine β-lyase enzymatic activity (pH range from 7 to 9). Moreover, beer is a more complex medium and might contain inhibitory compounds. No PLP-dependence was obvious for any compound, the release rates seeming even slightly higher for 3SPol, 3S4MPol, and 3SHol in the tests without PLP. Yet PLP being a derivative of vitamin B6, beer may already contain some. The thiols 3SPol and 3S4MPol were the most efficiently released in both trials (0.5–0.8% against 0.1–0.4% for the two others).

Enzymatic release of free thiols from Cys-adducts by apotryptophanase in hop media, polyphenols removal effect

In order to assess how brewers might use apotryptophanase at its optimized pH (pH 7.7) but directly on aqueous hop medium, two additional trials were compared, one with polyphenol removal before adding the enzyme and synthetic precursors (Hop Apo and Hop + PVPP Apo in Figure 3b). Note that, at this early lab stage, the apotryptophanase used was not food grade (the implementation at an industrial scale would require another quality).

As depicted in Figure 3(b), no free thiol at all was observed in untreated hop medium at pH 7.7 (Hop Apo). This highlights the suspected inhibitory action of polyphenols on apotryptophanase activity. Polyphenols have been already shown to inhibit the activity of a wide range of enzymes, including hydrolases, oxygenases, etc.,[34] Besides enzyme inhibition, polyphenols and derived quinones could also react with the released thiols (nucleophilic addition). In the oenological field, it has been shown that both (+)-catechin and (−)-epicatechin significantly decrease the level of 3SHol in the absence of sulfur dioxide.[35]

Even after PVPP treatment (Hop + PVPP Apo), few thiols were evidenced (0.05%–0.2% released, Figure 3(b), values under the chemical hydrolysis at pH 7.7 in model medium). This suggests that other hop constituents, in addition to polyphenols, are probably either inhibiting the hydrolysis or reacting with the released thiols (e.g., carbonyls working like acetaldehyde in wine,[36] iron or copper leading to disulfides,…[7,35]). As depicted in Figure 4, the chromatogram corresponding to the PVPP-pre-treated solution (Hop + PVPP Apo) showed many more peaks than that obtained for the untreated hop solution (Hop Apo), confirming that removal of polyphenols with PVPP improves the general recovery of sulfur-containing compounds (here by dichloromethane liquid/liquid extraction).

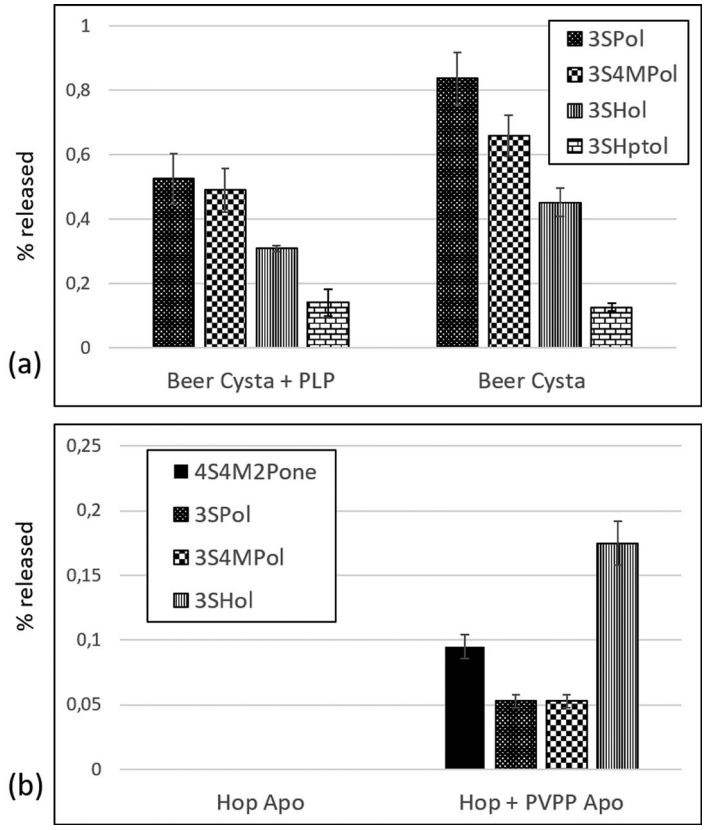

Figure 3. Release rates (%) of free thiols from spiked cysteinylated adducts in (a) beer or (b) hop (treated with PVPP) medium with cystathionine β-lyase (Cysta) or apotryptophanase (Apo) in presence or absence of cofactor (PLP). Error bars indicate duplicates variation (under 15%).

Enzymatic release of free thiols from G-adducts according to enzyme nature and dosage

The release of free thiols from G-adducts was further investigated (all trials are detailed in Table 2). The following idea was explored: combining γGT (known to remove the γ-glutamyl part of a G-precursor to yield a CysGly-intermediate) with β-lyase (known to release free thiols from Cys-adducts but supposedly also from CysGly-adducts, since its activity requires only a free ammonia function on the Cys-moiety). Since apotryptophanase showed more consistent release of free thiols from Cys-adducts in model media (trial Apo + PLP, in Figure 2), it was selected instead of cystathionine β-lyase.

The occurrence of free thiols in our trials confirmed, for the first time, the ability of apotryptophanase to release free PFTs from CysGly-intermediates. This discovery has two main implications. First, any CysGly-conjugates present in hops will contribute to the pool of β-lyase substrates. Second, the indirect quantitation method involving apotryptophanase treatment is liable to overestimate levels of Cys-*S*-conjugates. This highlights the importance of using the direct HPLC method for accurate quantitation.

The first trials focused on comparing γGT candidates, namely bovine γGT (γ), F373 (F3), and F852 (F8) at various dosage, in model media at pH 7.7 containing glycylglycine (gg) as an acceptor for transpeptidation (Figure 5a). The total "G-adducts to free thiols" release rates were highly variable according to γGT candidates, ranging from 0 to 1% release.

Trials carried out in the presence of 75 units bovine γGT (γ75 + gg) and 10 mg F852 (F8-10 + gg) showed the best release from G-adducts, especially for G-3SHol. With bovine γGT, the precursors were preferentially degraded at higher dosage (75 units versus 15 units). With F852, the best release was obtained with the intermediate dosage (10 mg versus 5 and 50 mg). Besides the γGT-like enzyme, the F852 powder might contain unknown compounds which, if present at sufficient concentration, could inhibit its activity. The dosage of F373 was not a significant parameter, as both trials exhibited lower release rates.

In order to establish the activity of γGT alone, the release rates obtained earlier for Cys-adducts in the presence of apotryptophanase (Apo + PLP) were removed from the total "G-adducts to free thiols" release rates here determined (Apo + PLP efficiency set at 100%). This was performed on the assumption that the efficiency was the same for CysGly-adducts and Cys-adducts. As shown in Figure 5(b), this γGT activity reached 7% for G-3SHol (against < 1% for G-3S4MPol).

With the highest release rates, the combination of bovine γGT and apotryptophanase was selected for further optimization trials (Figure 6a).

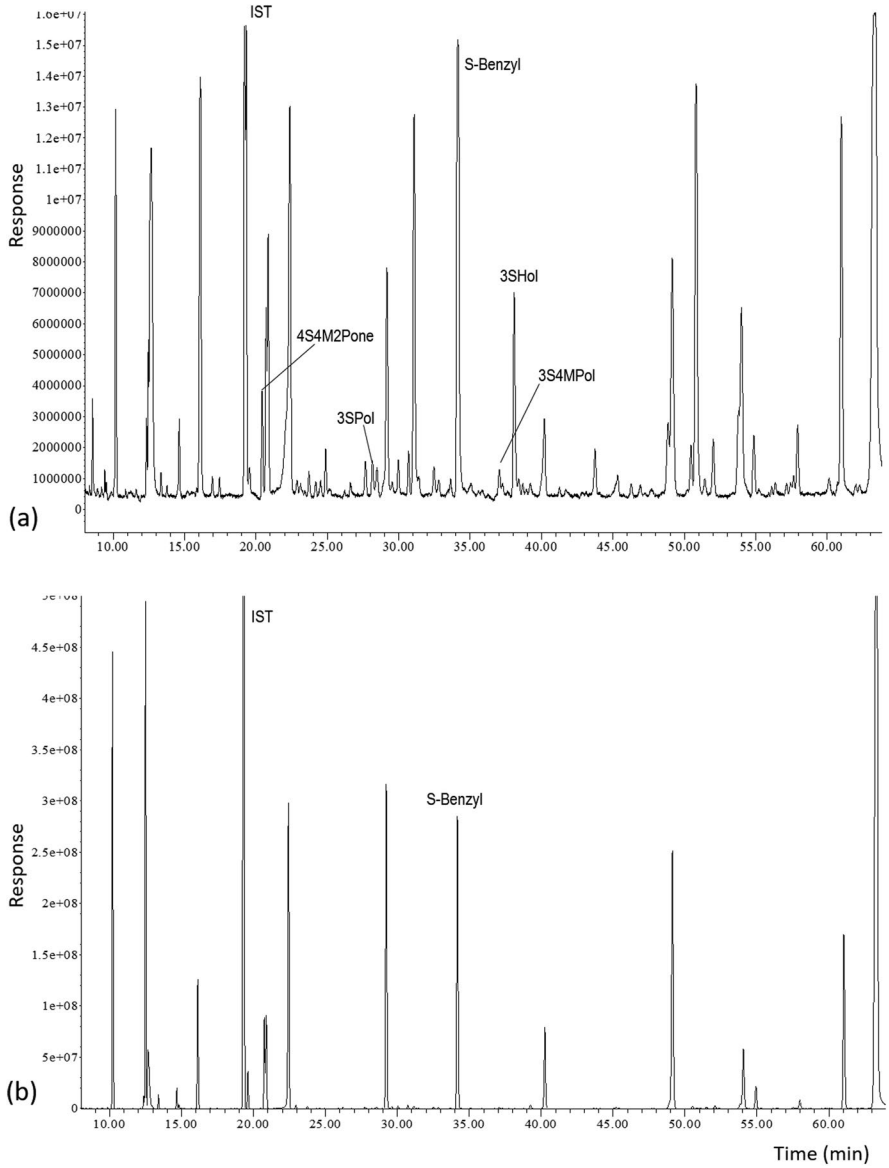

Figure 4. PFPD chromatograms of compounds extracted in trials (a) Hop + PVPP Apo (PVPP-treated hop medium at pH 7.7) and (b) Hop Apo (untreated hop medium at pH 7.7) conducted in the presence of Cys-*S*-conjugates and apotryptophanase.

Enzymatic release of free thiols from G-adducts according to γGT acceptor presence and nature

To assess the impact of having an acceptor (e.g., glycylglycine, gg) for γGT transpeptidation, a trial at low level of bovine γGT and without acceptor was conducted (γ15 in Figure 6 to be compared to γ15 + gg). As expected, the presence of glycylglycine did improve release from G-adducts (factor above 2 except for G-3S4MPol).

The effect of the acceptor nature was, then, also studied. The presence of alanine instead of glycylglycine favored even higher release (γ75 + ala and 75 + gg in Figure 6b). Previous studies have shown a weak correlation between the amino acids level as a whole in grape juices and thiols in wines but better relationships with glutamic acid or proline,[37] suggesting our release rates could still be improved by testing other amino acids.

In all of these trials, whatever the acceptor, the same trend was observed with a strong preference for G-3SHol.

Enzymatic release of free thiols from G-adducts according to incubation temperature

Lastly, since the optimal temperature of bovine γGT (a purified enzyme of animal origin) was known to be around 37 °C, different incubation temperature combinations were investigated to optimize PFT release. As shown in Figure 6, the best results (G-adducts to free thiols release rates above 1% for all compounds) were obtained with a two-step incubation: first the incubation of the synthetic glutathionylated precursors for 16 h at 37 °C in the presence of γGT, followed by incubation with apotryptophanase for 7 h at room temperature (γ75 + gg T37/

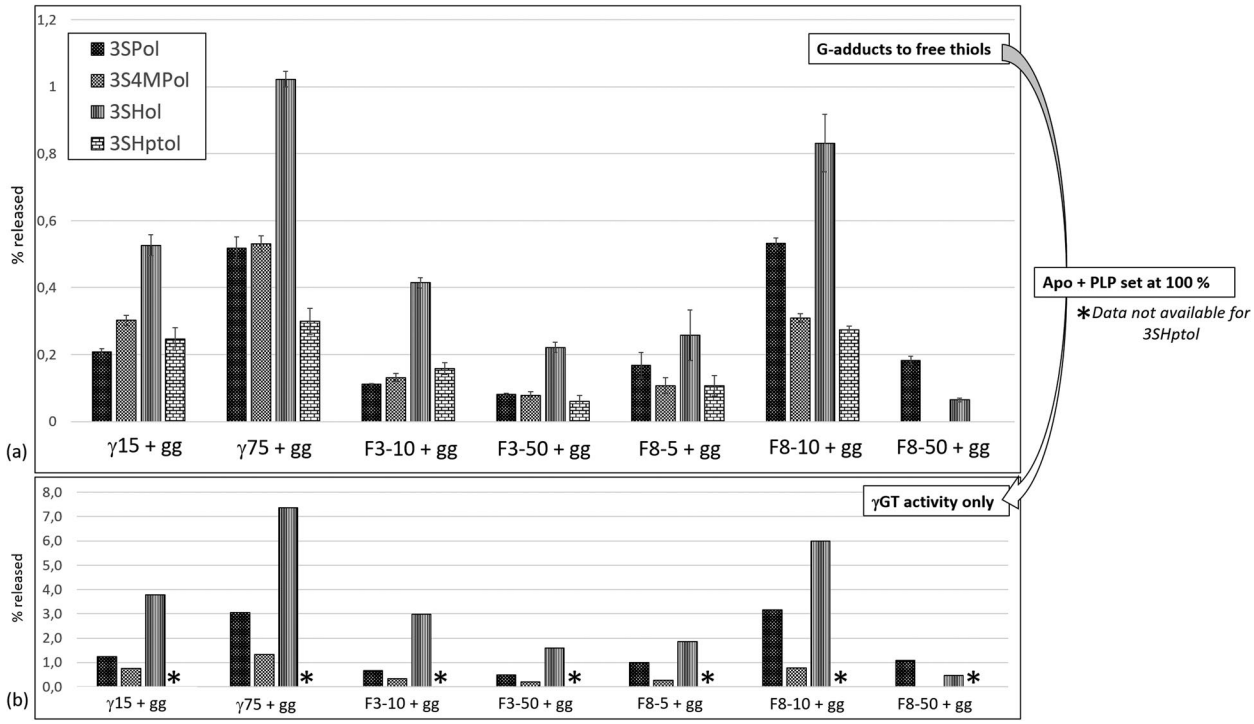

Figure 5. (a) G-adducts to free thiols release rates (%) and (b) γGT activity only in presence of apotryptophanase and bovine γGT (γ15 and γ75 for 15 or 75 units/mL, respectively), F373 (F3-10 and F3-50 for 10 or 50 mg, respectively) or F852 (F8-5, F8-10 and F8-50 for 5, 10 or 50 mg, respectively) and gg indicates the use of glycylglycine as acceptor. Error bars indicate duplicates variation (under 15%).

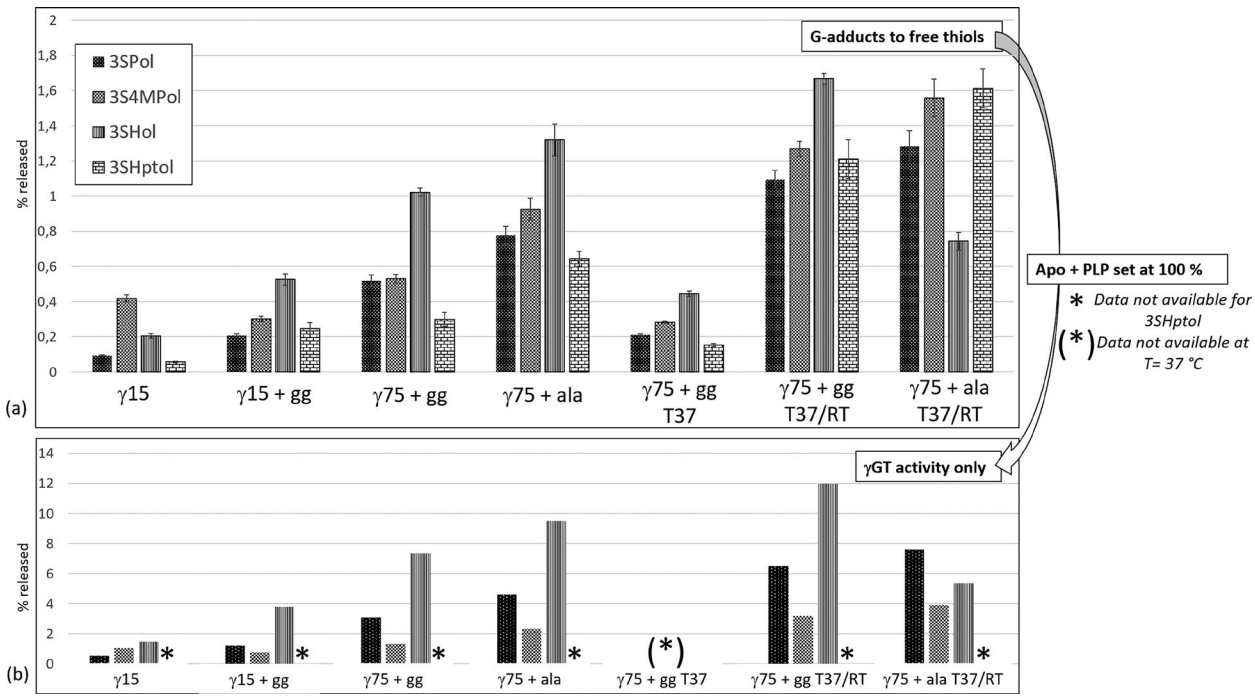

Figure 6. (a) G-adducts to free thiols release rates (%) and (b) γGT activity only in presence of apotryptophanase and bovine γGT (γ15 and γ75 for 15 or 75 units/mL, respectively) and gg and ala indicate the use of glycylglycine or alanine as acceptor. Incubation at 37 °C (T37) or first at 37 °C, then at room temperature (T37/RT). Error bars indicate duplicates variation (under 15%).

RT). Similarly to single incubation at room temperature, alanine as an acceptor (γ75 + ala T37/RT) slightly favored even higher release in the two-step incubation except for G-3SHol.

Release rates were below 0.5% when a single incubation with both γGT and apotryptophanase at 37 °C was applied (γ75 + gg T37, where apotryptophanase activity is the suspected limiting factor).

Table 4. Rates of free thiol release from added glutathionylated adducts in fermentation medium (15°P wort, for 7 days at 24 °C and 3 days at 4 °C), with γGT and SafAle K-97 yeast added simultaneously (γ + K97) or successively (γ/K97).

	Free thiol release [%]			
Trial denomination	3SPol	3S4MPol	3SHol	3SHptol
γ + K97	0.06[a]	0.09[a]	0.10[a]	0.06[a]
γ/K97	0.05[a]	0.06[a]	0.06[a]	0.04[a]

Variation coefficients are under 15%. Standard deviations have been considered in the Tukey test. Values in the same row that do not share a common letter (a) are significantly different ($p > 0.05$).

Compared to trials with yeasts under fermentation conditions, the G-adducts to free thiols release was much higher here with up to 1.6% by combining γGT activity at 37 °C and apotryptophanase at room temperature (versus only 0.1% with yeast).

Release of Cys-adducts and free thiols from G-adducts with endogenous enzymatic extracts

As it would be very interesting and advantageous to isolate directly from hop and malt the enzymes involved in glutathione *S*-conjugate catabolism, several enzymatic extracts (SN_1, SN_2, and SP) were obtained by differential centrifugation and used as incubation media spiked with G-adducts.

The fact that Cys-adducts, arising from G-adducts, were found after incubation, confirmed the presence of γGT and carboxypeptidase enzymes in the hop enzymatic extracts, albeit with a very low release activity (0.01% for enzymatic extract made of Polaris). Since the SP fraction gave the best results, the enzymes involved are believed to be membrane proteins. However, Cys-adducts formation was up to 30 times as high in enzymatic extracts made of pale malt as the ones made of hop (0.3% of G-3SHol degraded into Cys-3SHol). We suspect in that case that the enzymes involved are soluble, as fraction SN_2 worked best.

The action of β-lyase on Cys-adducts was also only slightly detected in hop and malt enzymatic extracts (0.0002% thiols released in enzymatic extract made of Nelson Sauvin and 0.002% in enzymatic extract made of green malt). The enzymes involved are more likely soluble (SN_2 was the best fraction for both hop and malt enzymatic extracts).

Release of free thiols from G-adducts by combining γGT activity and yeast

As some yeasts (e.g., SafAle K-97) proved to release PFTs very efficiently from cysteinylated adducts (0.4–0.5%),[8,29] but much less from glutathionylated ones (0.02–0.1%), trials were performed in which K-97 yeast was combined with bovine γGT under ale fermentation conditions. As depicted in Table 4, this synergetic combination proved able to release slightly more free PFTs than K-97 alone (0.1% against 0.04% for G-3SHol, and 0.06% against 0.02% for G-3SPol). The timing of γGT and yeast addition, γGT first (γ/K97), or both together (γ + K97), did not appear to affect 3SPol or 3SHptol release, but addition of both enzymes simultaneously slightly yielded more 3S4MPol and 3SHol.

Conclusion

Various exogenous enzymes (especially apotryptophanase and cystathionine β-lyase) can release free thiols efficiently from Cys-adducts at room temperature. Unfortunately, all trials confirmed the importance of a basic pH (7.7) for β-lyase activity (as well as for chemical hydrolysis). In addition, hop polyphenols and other co-constituents could limit their implementation in the brewing process.

At 37 °C in presence of an acceptor (alanine), bovine γGT was revealed to be very efficient to bring new substrates, either to this β-lyase activity or to yeast. Using a combination of γGT and apotryptophanase, we have also demonstrated, for the first time, the ability of β-lyase activity to release free thiols from CysGly-adducts. Endogenous hop and malt enzymes appear less promising, as they were found to release significantly lower amounts of free thiols from G-adducts.

Addition of low-polyphenol hop extracts pretreated with γGT (instead of hop pellets) could be an interesting new dry-hopping strategy for increasing the utilization of the huge amount of G-adducts present in hop.

Acknowledgments

We are indebted to AB-InBev Belgium for kindly providing the enzymatic preparations of Cystathionine β-Lyase Recombinant, Flavorpro™ 373MDP and Flavorpro™ 852MDP.

Disclosure statement

No potential conflict of interest was reported by the authors.

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