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Abstract

The molecules that elicit taste sensation are perceived by interacting with the taste receptors located in the taste buds. Enzymes involved in the detoxification processes are found in saliva as well as in type II cells, where taste receptors, including bitter taste receptors, are located. These enzymes are known to interact with a large panel of molecules. To explore a possible link between these enzymes and bitter taste perception, we demonstrate that salivary glutathione transferases (GSTA1 and GSTP1) can metabolize bitter molecules. To support these abilities, we solve three X-ray structures of these enzymes in complexes with isothiocyanates. Salivary GSTA1 and GSTP1 are expressed in a large panel of subjects. Additionally, GSTA1 levels in the saliva of people suffering from taste disorders are significantly lower than those in the saliva of the control group.

Keywords
Saliva, taste, bitter, enzymes, glutathione transferases

Introduction

The first step of conscious taste perception is the activation of taste receptors located on the tongue by tastant molecules. Saliva contributes to this perception by dissolving taste substances, allowing their diffusion to taste receptors. Additionally, saliva protects taste receptors from numerous types of damage, contributing to the maintenance of taste perception capacity. The protective effect of salivary proteins is due to their capacity to regulate multiple functions, including microbial homeostasis and redox balance buffering (Schwartz, Neiers, Feron, & Canon, 2020). Flavour perception is the combination of three main modalities: taste, smell and trigeminal sensations. Salivary proteins are proposed to play a role in each of these three modalities. Proline-rich proteins (PRPs), found at high levels in the saliva, are directly involved in the astringency sensation by aggregation on the surface of the buccal epithelium after binding with tannins and indirectly by interacting with the mucins after binding with tannins (Canon, Belloir, Bourillot, Brignot, Briand, Feron, et al., 2021).

Oral processing of food plays a role in retronasal aroma perception. Different phenomena contribute to the modulation of aroma perception, including saliva flow and composition, the chewing process and oral proteins that encounter these odorants. Indeed, aroma compound composition and concentration are modulated by protein trapping, such as mucins that interact with specific aromas (Pages-Helary, Andriot, Guichard, & Canon, 2014). Additionally, these aromas are enzymatically converted in the nasal mucus (Heydel, Menetrier, Belloir, Canon, Faure, Lirussi, et al., 2019; Ijichi, Wakabayashi, Sugiyama, Ihara, Nogi, Nagashima, et al., 2019; Neiers, Jarriault, Menetrier, Briand, & Heydel, 2021; Schwartz, Menetrier, Heydel, Chavanne, Faure, Labrousse, et al., 2020) as well as in the human saliva (Buettner, 2002a, 2002b; Munoz-Gonzalez, Feron, & Canon, 2018), affecting aroma persistence (Munoz-Gonzalez, Feron, & Canon, 2021). Enzymatic conversions can lead to the termination of their perception as well as the perception of these metabolites as additional aromas (Mayr et al., 2014).

With respect to taste detection, some studies support an important role for saliva (Guerreiro, Brandao, de Jesus, Goncalves, Perez-Gregorio, Mateus, et al., 2021). Indeed, saliva flow modulates the ability to dissolve taste molecules, such as salt (NaCl), and consequently its perception (Heinzerling, Stieger, Bult, & Smit, 2011). Unstimulated saliva composition, particularly ion levels, can also impact taste sensitivity. The role of salivary proteins in taste perception is supported by only a few studies focusing on salivary proteins in a general manner (Martin, Kay, & Torregrossa, 2019; Martin, Nikonova, Kay, & Torregrossa, 2019) or more specifically on some proteins. This link can be direct, for example, human lipocalin (LCN1) is proposed to solubilize fatty acids to facilitate their detection (Gilbertson, 1998), or it can be indirect, for example, human anhydrase carbonic VI (gustin) is proposed to impact the number of fungiform papillae (Barbarossa, Melis, Mattes, Calo, Muroni, Crnjar, et al., 2015). To date, the only protein suggested to play a role in bitter molecule detection is PRP, mainly for tannins perceived as bitter in addition to their astringency (Ployon, Morzel, Belloir, Bonnotte, Bourillot, Briand, et al.,

2018). Bitter taste perception allows the detection of harmful compounds, leading to aversive reactions. The 25 bitter taste receptors allow humans to detect a large panel of molecules, including plant metabolites, synthetic molecules and fatty acids or inorganic ions (Dagan-Wiener, Di Pizio, Nissim, Bahia, Dubovski, Margulis, et al., 2019; Meyerhof, Batram, Kuhn, Brockhoff, Chudoba, Bufe, et al., 2010). PRPs have been proposed to facilitate or reduce bitter perception, due to their ability to interact with bitter substances (Matsuo, 2000).

The buccal cavity and saliva contain numerous xenobiotic metabolizing enzymes (Schwartz, Neiers, Charles, Heydel, Munoz-Gonzalez, Feron, et al., 2021). Whether these enzymes metabolize taste molecules remains an open question. Glutathione transferase (GST) is an important superfamily of xenobiotic metabolizing enzymes that appears to be a candidate to metabolize taste molecules. Indeed, GST activity is increased in the saliva of people fed a diet rich in broccoli or coffee as mRNA coding bitter taste receptors are also increased by a coffee diet (Lipchock, Spielman, Mennella, Mansfield, Hwang, Douglas, et al., 2017). The GST activity increase indicates increasing expression of salivary GSTs with a potential link to the richness of this food in bitter molecules (Sreerama, Hedge, & Sladek, 1995). GST catalyzes the conjugation of glutathione to various substrates, in addition to its isomerase activity or simple binding to ligandin. Additionally, GSTs are located in type II cells of rat taste buds (Nishino, Kudo, Doi, Maeda, Hamasaki, Morita, et al., 2001). Type II cells express receptors involved in bitter, umami and sweet molecule detection (Roper & Chaudhari, 2017). This localization, in addition to the detection of GST isoforms in human saliva using mass spectrometry analysis (Sivadasan, Gupta, Sathe, Balakrishnan, Palit, Gowda, et al., 2015) and glutathione transferase activity detection (Fabrini, Bocedi, Camerini, Fusetti, Ottaviani, Passali, et al., 2014; Sreerama, Hedge, & Sladek, 1995), supports a potential role for GSTs in taste detection.

To explore this hypothesis, this study measured levels of expression of two GST isoforms in saliva in a panel of people. This measurement was also performed in a panel of people suffering from taste disorders (ageusic and dysgeusic) to evaluate a potential link between these enzyme expression levels and taste problems. Then, using heterologous expression, the ability of GSTs to interact with bitter compounds was analyzed using biochemical methods measuring enzymatic activity and X-ray experiments determining the structure of human salivary GST isoforms in complexes with bitter molecules.

Materials and methods

Saliva collection, GST titration and statistical analysis

For this study, a cohort of 104 people (males and females aged from 24 to 82) participated in saliva collection. Saliva samples were collected as previously described (Walliczek-Dworschak, Schops,

Feron, Brignot, Hahner, & Hummel, 2017; Zhu, Feron, Von Koskull, Neiers, Brignot, & Hummel, 2021). Briefly, subjects were asked to chew a piece of Parafilm® laboratory film (American National Can, Chicago, IL) for 5 min to stimulate saliva secretion. Saliva samples were stored at –80 °C. After thawing, samples were centrifuged for 15 min at 15,000 *g* for simultaneous analysis. Thirty-two saliva samples from the control group were available for analysis, 72 from the group possessing taste disorders (54 suffering from dysgeusia and 18 suffering from ageusia). The gustatory function was screened *via* "taste strip" and the results compared to sex and age differentiated normative data. The diagnosis of qualitative taste disorders such as salty, bitter or metallic taste disorder was based on patient's self-report. To avoid conditions which could create temporary or permanent impairment of gustatory function, patients presenting untreated hypothyroidism, inflammation of the oral cavity or renal disorder were excluded. At least 30 min before the test volunteers were asked to drink only water and to not smoke. GSTA1 and GSTP1 were titrated following an ELISA protocol using commercial kits (Abbexa, Cambridge, UK). Descriptive analysis of the GSTA1 and GSTP1 ELISA data in each group was performed. However, the small number of subjects belonging to the ageusia group did not allow us to perform a robust comparison test considering all 3 groups. Therefore, analyses were performed considering 2 groups: the control group and a combined group including saliva from people suffering from either ageusia or dysgeusia. The data did not follow a normal distribution, and the samples were independent; thus, a nonparametric Mann–Whitney test was used to compare the means for GSTA1 and GSTP1 between the 2 groups.

GSTs production and purification

The DNA sequences encoding *Homo sapiens* GSTA1 (GSTA1, UniProt code P08263) and GSTP1 (UniProt code P09211) were optimized and synthesized using DNA2.0 for expression in *E. coli*. After subcloning into a pET22b vector (Novagen, Darmstadt, Germany), plasmids were used for transformation of the *E. coli* BL21 (DE3) strain (Novagen, Darmstadt, Germany). Expression and purification of the recombinant GSTs was performed as previously described (Schwartz, et al., 2020). Briefly, the transformed cells were grown at 37 °C in LB medium (containing 100 µg mL^{-1} ampicillin) and induced by the addition of 0.1 mM isopropyl β-D-1-thiogalactopyranoside when the cell culture reached an OD of 0.7 measured at 600 nm. Twelve hours later, the cells were harvested by centrifugation, suspended in PBS pH 7.0 buffer, and disrupted at 4 °C using two cycles of sonication. After centrifugation at 20,000 *g* for 45 min at 4 °C, the supernatant was loaded onto a GSTrap™ Fast Flow 5-mL column (GE Healthcare, Chicago, IL). GSTA1 and GSTP1 were eluted using 50 mM Tris-HCl, pH 7.4 buffer supplemented with 10 mM glutathione. After pooling the fractions containing the pure protein, samples were concentrated and stored at –20 °C. Protein concentrations were determined spectrophotometrically at 280 nm using an extinction coefficient of 20,400 M^{-1} cm^{-1} and 29,130 M^{-1} cm^{-1} for GSTA1 and GSTP1, respectively.

Enzymatic assays

A screening strategy based on the ability of taste compounds to inhibit the glutathione transfer reaction was performed as previously described (Gonzalez, Fraichard, Grassein, Delarue, Senet, Nicolai, et al., 2018). GST activity was monitored using glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB) as substrates, which produce a conjugate absorbing at 340 nm as previously described (Habig, Pabst, & Jakoby, 1974). In brief, the reactions were performed at 20 °C in 1 mL 0.1 M potassium phosphate buffer pH 7.4 with 1 mM GSH and 1 mM CDNB. GST concentrations were 5 nM (GSTA1) and 25 nM (GSTP1). Each taste compound was tested at final concentrations of 100 µM and 10 µM. Reactions were triggered by adding GSH to the reaction volume after a 2-min preincubation of the GSTs with the taste compound or with methanol as a control. The absorbance increase was monitored on a Jasco V-730 spectrophotometer (Jasco, Tokyo, Japan). For each experiment, three independent measurements were performed. The inhibition percentages were calculated using the following equation:

inhibition percentage = 100 × average slope of the initial rate (with tastant)/average slope of the initial rate (without tastant) ,

where the slope of the initial rate was obtained from the average of the 3 measurements. Statistical significance was assessed using Student's *t*-test comparing the activity with and without the bitter compounds.

Bitter compounds

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Chemicals used for the screening were apigenin, diosmetin, fisetin, 3-hydroxyflavone, kaempferol, luteolin, naringenin, quercetin, tangeritin, (–)-nicotine, berberine, caffeine, papaverine, quinin, strychnine, theobromine, theophylline, digitonin, glycyrrhizin, naringin, sinigrin hydrate, coumarin, limonin, L-canavanine, taurine chloramphenicol, denatonium benzoate, piperidine, pyridine, thiamine, thiazolidine, allyl isothiocyanate, propyl isothiocyanate, butyl isothiocyanate, hexyl isothiocyanate, benzyl isothiocyanate, phenethyl isothiocyanate, erucin, iberin, sulphoraphane.

Salivary glutathione transferase activity measurement in saliva with isothiocyanates

Saliva from three healthy subjects without taste disorders (men, age 29–41 years) was collected for this experiment. Salivary glutathione transferase activity is rapidly inactivated due to hypothiocyanite ions and needs to be reactivated (Fabrini, et al., 2014). For this purpose, saliva samples were incubated with 10 mM DTT and heated at 37 °C for 45 min. Glutathione transferase activity with isothiocyanates was spectrophotometrically assessed by following the absorbance at 274 nm, which corresponds to the formation of glutathionyl-dithiocarbamate conjugates (Kolm, Danielson, Zhang, Talalay, & Mannervik, 1995). Assays were performed at 25 °C in a 1-mL reaction volume containing 100 µL reactivated saliva in 100 mM potassium phosphate buffer at pH 6.5. Controls were assessed using the same conditions without saliva and by adding 100 µL of solution containing 10 mM DTT that was subject to the same

reactivation protocol. In each case, 500 µM isothiocyanate was added except for allyl-ITC, benzyl-ITC, sulforaphane, iberin and erucin (100 µM of substrate). The reaction was triggered by adding 1 mM glutathione. Values are shown as the mean of three measurements with standard deviations.

HPLC analysis

To detect the glutathione metabolite of hexyl isothiocyanate (hexyl-ITC) formed *in vitro* after saliva addition, an HPLC–HRMS approach was used. Reaction volumes (200 µL) contained 500 µM GSH, 500 µM hexyl-ITC, and 20 µL saliva in 100 mM potassium phosphate buffer at pH 6.5. The enzymatic control contained 10 µM recombinant GSTP1 instead of saliva. After one hour of incubation at room temperature, 5 volumes of methanol were added to the reaction volumes, and tubes were centrifuged at 20,000 *g* for 30 min. Supernatants were retrieved separately using a Vanquish UHPLC system (Thermo Fisher Scientific, San Jose, CA) consisting of a degasser, a quaternary pump, a thermostated autosampler, and a column oven. Chromatographic separation was performed on an XSelect CSH C18 column (150 × 2.1 mm, 2.5 µm) from Waters. The mobile phase consisting of (A) 0.1% formic acid in water and (B) acetonitrile with 0.1% formic acid was delivered at a flow rate of 0.5 mL min^{-1}. A gradient program was used as follows: 2% of mobile phase (B) was used from 0.00 to 2.50 min, 2% (B) to 95% (B) from 2.50 to 5.50 min, maintained at 95% (B) from 5.50 to 7.00 min, then decreased back to 2% (B) from 7.00 to 7.25 min and finally the column was re-equilibrated with 2% (B) from 7.25 to 9.00 min. A 4-µL aliquot of the extract was injected. The column and autosampler were maintained at 40 °C and 10 °C, respectively. The UHPLC system was coupled to a Q-Orbitrap-HRMS mass spectrometer (Thermo Fisher Scientific, Waltham, MA) equipped with a heated electrospray ionization probe (HESI II) operating in positive ionization mode. Nitrogen was used for spray stabilization, and collision-induced dissociation experiments were performed in the higher energy collision dissociation (HCD) cell and as the damping gas in the C-trap. The following ionization parameters were applied: electrospray voltage 3 kV, heater temperature 350 °C, capillary temperature 350 °C, sheath gas (N_2) 60 arbitrary units (arb), auxiliary gas (N_2) 20 (arb), and S-lens RF level at 70 (arb). Full scan data were acquired at a mass resolving power of 60,000 FWHM. The *m/z* scan range was 100–1000.

X-ray crystallography

Before crystallization, GSTs were dialyzed twice against 20 mM pH 8.0 Tris-HCl buffer at 4 °C. Crystals of GSTA1 and GSTP1 without ligands were obtained in Linbro plates with hanging drop vapor diffusion at 20 °C starting from previously published crystallization conditions (Oakley, Lo Bello, Battistoni, Ricci, Rossjohn, Villar, et al., 1997; Schwartz, et al., 2020). GSTA1 (18 mg/mL) was crystallized by mixing 1 µL of protein with 3 µL of solution containing 18% PEG 4000 in 0.1 M pH 7.5 Tris-HCl buffer. GSTP1 (10 mg/ml) was crystallized by mixing 2 µL of protein with 2 µL of solution containing 25% PEG 8000, 20 mM $CaCl_2$ and 5 mM DTT in 0.1 M pH 6.0 MES buffer. In each case,

the reservoirs were filled with 1 mL of the same crystallization solutions. Crystals appeared usually within one week. To obtain complexes of GSTs with taste compounds, a soaking strategy was adopted since cocrystallization assays often lead to damaged crystals with poor diffraction power. GST crystals were soaked a few minutes in their mother liquor supplemented with isothiocyanate (allyl isothiocyanate, hexyl isothiocyanate or iberin) at high concentration (approximately 20 mM) with or without 5 mM glutathione. To minimize crystal handling, cryoprotection was performed during the same soaking time by adding 20% glycerol to the soaking drop. Crystals were flash frozen in liquid nitrogen and shipped to SOLEIL synchrotron.

Diffraction experiments were performed on the SOLEIL synchrotron beamline PROXIMA1. The crystals of GSTP1:iberin, GSTA1:hexyl isothiocyanate and GSTA1:allyl isothiocyanate diffracted to 1.73 Å, 2.03 Å and 2.46 Å, respectively. Datasets were indexed and integrated with XDS and scaled with pointless. The three structures were solved by molecular replacement with the coordinates of the previously solved GSTA1 (PDB 6YAW) and GSTP1 (PDB 5X79) as search models. The structures were manually adjusted using COOT and refined using PHENIX. Careful inspection of electron density maps allowed identification and building of ligands into their binding sites. Restraint files were generated for iberin, allyl isothiocyanate and the glutathione adduct of hexyl isothiocyanate with the GRADE webserver (http://grade.globalphasing.org). Structures were validated using Molprobity (Chen, Arendall, Headd, Keedy, Immormino, Kapral, et al., 2010). Coordinates and structure factors were deposited in the Protein Data Bank under accession codes 7BIA (GSTP1:iberin), 7BIB (GSTA1:hexyl isothiocyanate) and 7BIC (GSTA1:allyl isothiocyanate).

Results

Glutathione transferases GSTA1 and GSTP1 were found in all tested saliva samples

Glutathione transferase activity has been previously reported in human saliva (Fabrini, et al., 2014; Sreerama, Hedge, & Sladek, 1995) and is supported by identification using mass spectrometry of the two isoforms GSTA1 and GSTP1 (Sivadasan, et al., 2015). To confirm their expression in a large panel of subjects with no taste perception issues and to specifically quantify each isoform, samples were measured using ELISA on each saliva sample from a cohort of 32 people. It was previously demonstrated that salivary GST activity is quickly inhibited after saliva sampling due to their inactivation by an oxidizing salivary component, the hypothiocyanite ion. ELISA has the advantage of allowing the measurement of each isoform specifically and independently from inactivation (Fabrini, et al., 2014). Table 1 shows that all 32 tested people expressed the two isoforms GSTA1 and GSTP1. Of the 32 saliva samples, average concentrations of 5.4 ng/mL and 9.9 ng/mL were measured for GSTA1 and GSTP1, respectively. All tested subjects expressed both isoforms with interindividual differences

in concentration: from 1.1 ng/mL to 14.2 ng/mL for GSTA1 and from 2.9 ng/mL to 49.2 ng/mL for GSTP1.

Table 1: Salivary GST isoform concentrations in 32 saliva samples from people with no taste disorders

	GSTA1	GSTP1
Number of saliva samples	32	32
Minimum concentration (ng/mL)	1.09	2.90
Maximum concentration (ng/mL)	14.22	49.21
Average (ng/mL)	5.37	9.85
Standard deviation (ng/mL)	3.11	9.49

Screening of bitter compounds interacting with recombinant GSTA1 and GSTP1

A library of bitter compounds was designed, comprised of approximately 40 molecules of different chemical families, including flavonoids, isothiocyanates, heterosides, alkaloids and *N*-heterocycles. GSTs bind molecules in their active site to either catalyze an enzymatic reaction or not. When an enzymatic reaction occurs, the molecule can be considered a substrate; when not, it is a nonsubstrate. Additionally, molecules can also bind in another site called ligandin and be consequently considered as a nonsubstrate. To demonstrate these interactions, a competitive experiment using the enzymatic activity of these enzymes on a chromophoric substrate (CDNB) was performed. Based on the ability of GSTs to conjugate GSH with the chemical substrate CDNB, we performed a screening test to identify bitter compounds that interact with GSTA1 and GSTP1. The enzymatic reaction with CDNB and GSH was monitored at 340 nm following the formation of the conjugation product GS-DNB. Each bitter compound was assayed at 10 or 100 µM (Figure 1 and Supplementary Figure 1).

From this screen, two families of bitter compounds showed strong inhibition potential: flavonoids and isothiocyanates (Figure 1). In addition to these two compound families, other compounds were identified in additional chemical families, including compounds such as berberin, strychnine, quinine and limonin, which induce >20% GSTP1 inhibition at 10 µM. Flavonoids are polyphenols commonly found in fruits and vegetables. Some of the tested flavonoids strongly inhibited only GSTA1 (e.g., naringenin) or only GSTP1 (e.g., fisetin or apigenin), while others significantly inhibited both salivary isoforms, including diosmetin, quercetin, kaempferol and luteolin. Quercetin was the strongest inhibitor of both GSTA1 and GSTP1, with more than 50% inhibition at 10 µM. These compounds bind different taste receptors with different affinities summarized in Supplementary Table 1. The molecules interacting with GSTs are recognized by different bitter taste receptors. The second family of compounds that

showed significant inhibition of GST activity was isothiocyanates. Isothiocyanates are found in numerous vegetables from the Brassica family, such as broccoli, cabbage or mustard, and have a pungent and bitter taste *via* their binding to the TAS2R38 receptor (Meyerhof, et al., 2010). All tested isothiocyanates interacted with both salivary GSTs. At 10 µM, the bulkiest isothiocyanates (namely, phenethyl, benzyl and hexyl isothiocyanate) inhibited nearly 50% of the GSH-transfer reaction of GSTA1 but to a lesser extent for GSTP1.

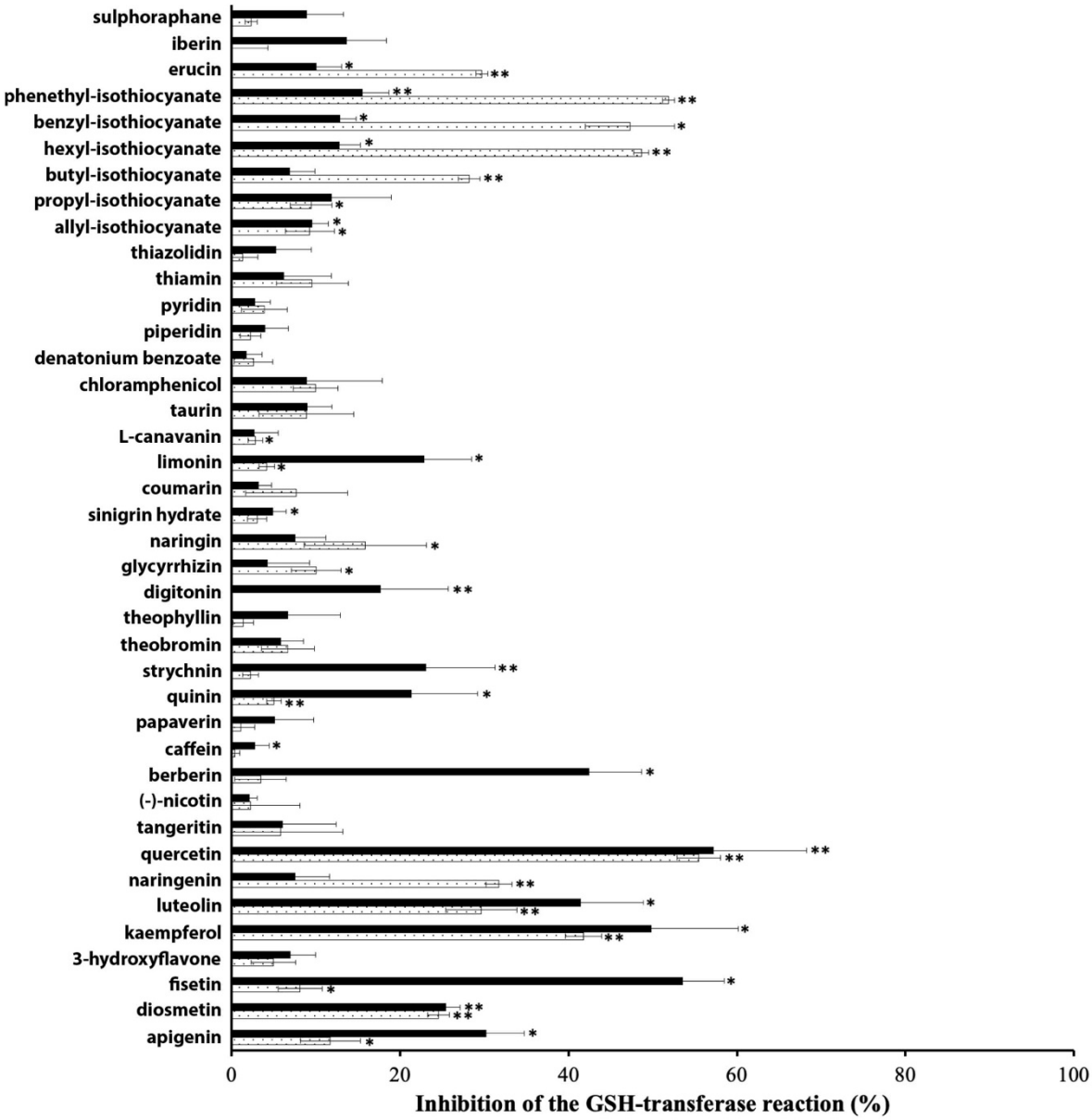

Figure 1*:* **Bitter compound screening using the CDNB inhibition test on recombinant GSTA1 and GSTP1.**

Compounds were screened at a 10 µM concentration (white dotted bars, GSTA1; black bars, GSTP1) for their potential to inhibit the GSH-transferase reaction. The results are the mean of three independent measurements. Statistical significance was assessed using Student's *t*-test comparing the activity with and without the bitter compounds ($p < 0.05$ *, $p < 0.01$ **, no sign indicates not significant).

Interactions between salivary GSTs and bitter molecules studied by X-ray crystallography

To decipher the interaction of bitter molecules and GST, we solved X-ray structures of the enzymes in complexes with bitter tasting molecules. This approach has some technical limitations, primarily the difficulty of obtaining crystals of complexes and their quality compatible with diffraction experiments. Cocrystallization of flavonoids (naringenin, quercetin and diosmetin) or soaking with GST crystals failed to result in GST-ligand structures. Success was achieved with the second compound family, isothiocyanates. Three dietary isothiocyanates, hexyl isothiocyanate, allyl isothiocyanate and iberin, were successfully soaked in GST crystals, allowing us to solve the three X-ray structures of GST in complexes with isothiocyanates (Supplementary Table 2 and 3 and Figure 2).

GSTA1 and GSTP1 are both homodimers, and their active site constitutes a glutathione site (G site) and the hydrophobic site involved in substrate recognition (H site). Moreover, molecules can bind outside to the active site in the ligandin site (L site). Electron density map analysis revealed the presence of bitter compounds in different binding pockets of GSTA1 and GSTP1. The three solved structures included GSTP1 bound to iberin and the cosubstrate glutathione, GSTA1 bound to hexyl isothiocyanate-glutathione and GSTA1 bound to allyl isothiocyanate. These three solved structures support (i) the ability of these salivary enzymes to catalyze the glutathione conjugation of isothiocyanates, (ii) their ability to bind isothiocyanates in the absence of reaction within the active site and (iii) and additionally for GSTA1 the ability to bind covalently allyl sothiocyanate in the ligandin site.

For the GSTP1:iberin complex, soaking experiments yielded a structure where the active sites of the two monomers are occupied by a glutathione molecule in the G site with one iberin molecule in the H site of only one monomer (Figure 2, panel A and B). Interestingly, no covalent adduct resulting from the enzymatic reaction was found between the two cosubstrates, possibly due to experimental conditions (constraints resulting from the crystal packing, pH of the crystallization solution of 6.0). Glutathione is stabilized in the G site by residues through several hydrogen bonds (Y8, K45, Q52, L53, Q65, S66 and D99 from adjacent monomers). The iberin molecule is stabilized by hydrophobic interactions with side chains of residues I105 and Y109 of the H site. Moreover, the GSTP1 R14 NHε group interacts through H-bonds with the sulfur atom of the iberin isothiocyanate group, thus improving the electropositive character of the adjacent carbon atom. Additionally, this isothiocyanate carbon atom is in close proximity to the sulfur of glutathione, favoring its attack through a Michael addition reaction.

In the case of GSTA1:hexyl isothiocyanate, soaking experiments resulted in a structure where both active sites are occupied by the glutathione adduct of hexyl isothiocyanate, which likely formed within the crystal (mother liquor at pH 7.5) (Figure 2, panel C and D). Residues from the G site (Y9, R45, Q54, V55, P56, Q67, R131 and D101 from the adjacent monomer) interact through H-bonds with the glutathionyl group, whereas residues from the helical domain that form the H site interact with the hexyl isothiocyanate group. These residues include V111, R217 and F220, which form a hydrophobic cleft

that stabilizes the hexyl isothiocyanate moiety. The GSTA1 H site appears more buried than the GSTP1 H site, which is more exposed to the solvent. These structural features could explain the better inhibition potential of bulky isothiocyanates on GSTA1 compared to GSTP1 observed from enzymatic inhibition experiments.

For the GSTA1:allyl-isothiocyanate complex, no glutathione was added in this case during soaking experiments, avoiding any enzymatic reaction to favor the production of a complex between GST and the free ligand (Figure 2, panel E and F). Interestingly, no binding of molecules was observed in the active site. However, electron density indicating the presence of the allyl isothiocyanate molecule was observed near the Cys112 residue from monomer B at the interface between the two subunits. Cys112 is located on the *C*-terminal end of helix alpha 4 and is particularly solvent-exposed. Cys112 was previously shown to be subject to alkylation by reactive compounds such as isothiocyanates (Kumari, Dyba, Holland, Liang, Singh, & Ji, 2016). According to our X-ray data, we demonstrated that Cys112 is covalently bound to allyl isothiocyanate. The allyl isothiocyanate moiety is accommodated in a hydrophobic groove made of the side chains of Val111, Pro113 and Lys120. This pocket (L-site) could accommodate diverse substrates without enzymatic activity; however, substrate binding in this pocket can modulate the enzymatic activity towards other substrates (Ketley, Habig, & Jakoby, 1975).

Consequently, based on these structural observations, we propose that salivary GST catalyzes glutathione conjugation with isothiocyanate but also simply binds isothiocyanates, possibly leading to catalytic inhibition. In saliva, it cannot be excluded that these reactions or interactions might be modulated due to additional diverse interactions between salivary GSTs and salivary compounds.

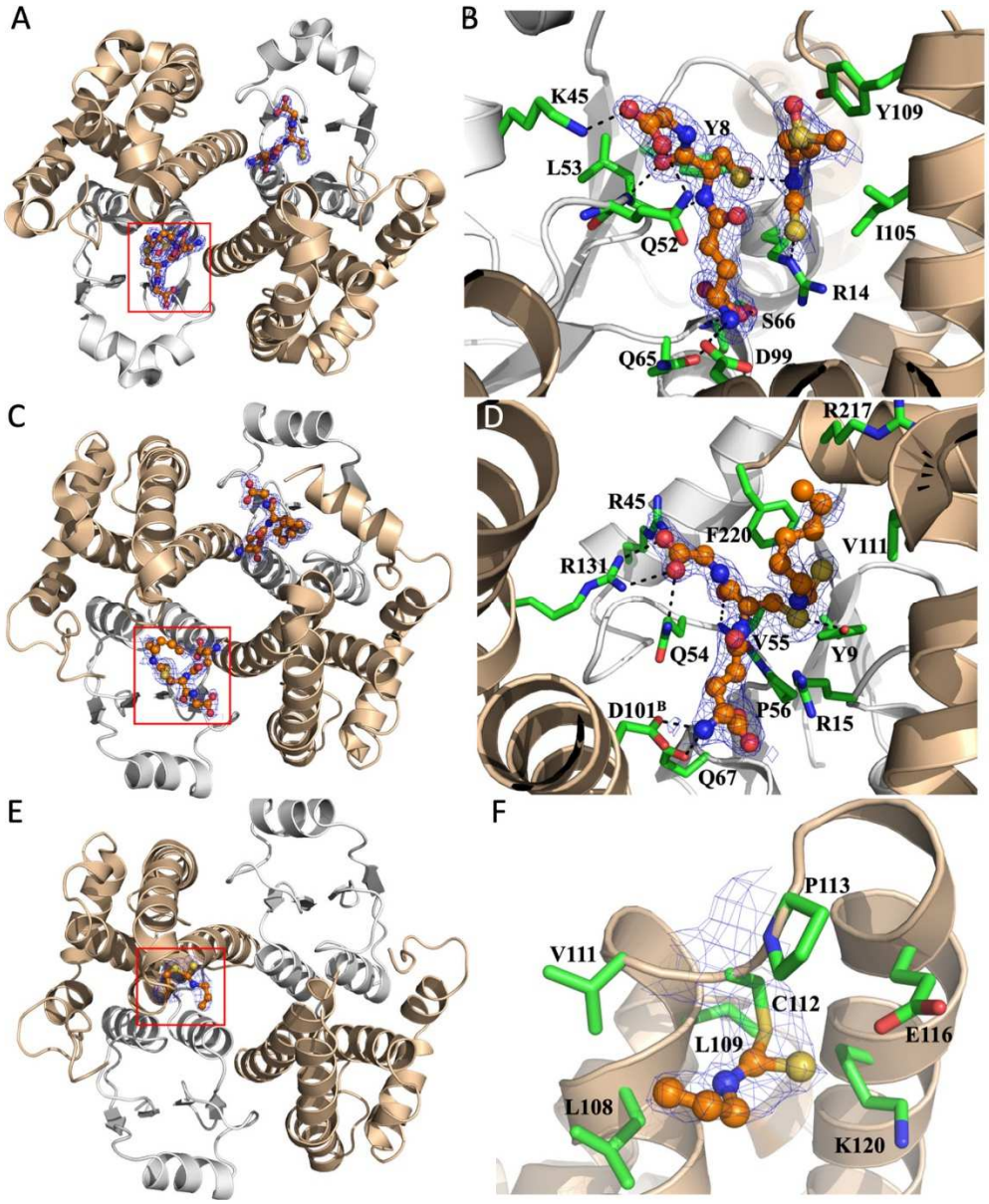

Figure 2. X-ray structures of glutathione transferases alpha 1 and pi 1 bound to bitter compounds isothiocyanates

A and **B**, GSTP1 bound to glutathione and iberin. One glutathione molecule is present in the G site of both monomers and one iberin molecule in the H site of only one monomer. **C** and **D**, GSTA1 bound to the glutathione adduct of hexyl isothiocyanate. The conjugate is present in both monomers. **E** and **F**, GSTA1 bound covalently to allyl isothiocyanate on Cys112 from one monomer. GST structures are represented as cartoons with *N*-terminal domains in white and *C*-terminal domains in wheat. On the left panels (A, C and E) are shown global views of GST dimers with bound ligands. On the right panels are shown enhanced views of the corresponding red squares from the left panel. Ligands are represented as orange sticks and spheres, and surrounding binding residues are represented as green sticks and labelled. Polar contacts are represented as dashed lines. 2mFo-DFc composite omit maps around ligands were calculated with PHENIX and are represented at 1.2 σ.

Glutathione conjugation of isothiocyanates catalyzed by saliva

To determine the glutathione transfer of isothiocyanates in saliva, saliva samples from three subjects without pathologies in taste perception (S1, S2 and S3) were collected and assayed with the nine selected

isothiocyanate compounds (Figure 3). The three saliva samples allowed inter-individual variation of the GST activity to be observed. As salivary glutathione transferase activity is hampered by hypothiocyanite ions (Fabrini, et al., 2014), saliva samples were reactivated using the procedure previously described in the same study. As isothiocyanates can be spontaneously glutathionylated, a control without saliva and the same reactivation procedure was included. Isothiocyanate conjugation can be directly followed at 274 nm; indeed, isothiocyanate conjugation leads to compounds absorbing light at this wavelength (Gonzalez, et al., 2018; Habig, Pabst, & Jakoby, 1974). Saliva addition to reaction volumes resulted in increased reaction rates compared to samples without saliva. Interindividual variations in activity rates were observed between the three subjects. This result supports enzymatic conjugation activity towards isothiocyanates within the saliva samples as well as interindividual variations. For example, rates obtained with saliva S1 were smaller with only three compounds (allyl-ITC, hexyl-ITC and erucin), which were better conjugated than with the control. Sulforaphane and iberin did not exert any significant effect after treatment with any of the three tested saliva samples. Interestingly, significant decreases in reaction velocities after adding saliva were observed in response to butyl-ITC, propyl-ITC and benzyl-ITC. As isothiocyanates are reactive compounds that can alkylate protein cysteine residues, a possible explanation for this rate decrease is the depletion of isothiocyanates available for spontaneous chemical GSH transfer due to their reaction with some salivary proteins. Salivary protein concentrations may vary among individuals. The most dramatic increase was observed with saliva from S3 with hexyl isothiocyanate, yielding a near 3-fold improvement in reaction velocity. Taking advantage of the ability of the three tested saliva samples to significantly increase the conjugation of hexyl isothiocyanate, we used them after incubation with hexyl isothiocyanate to detect the corresponding conjugates. After extracting the saliva content, the metabolite resulting from hexyl isothiocyanate conjugation, which corresponds to glutathionyl-hexyl-dithiocarbamate, was detected using high-resolution mass spectrometry (m/z = 450.15965) (Supplementary Figure 2). This direct detection in saliva of a conjugated isothiocyanate supports the possibility of generating glutathione conjugates within the saliva.

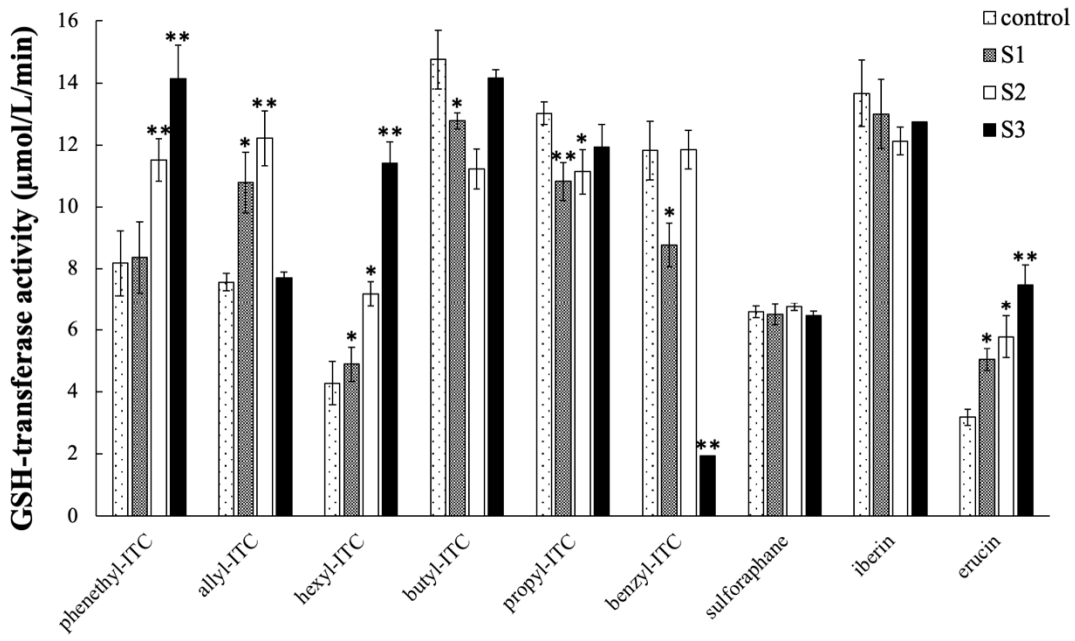

Figure 3. Conjugation of glutathione to dietary isothiocyanates catalyzed by saliva from three subjects.

Assays were performed at 25 °C in a 1-mL reaction volume containing 100 µL reactivated saliva (10 mM DTT, heated at 37 °C for 45 min; (Fabrini, et al., 2014) from three subjects (S1, S2 and S3) in 100 mM potassium phosphate buffer at pH 6.5. To measure the spontaneous chemical reaction, a control was treated using the same conditions without saliva and the addition of 100 µL solution containing 10 mM DTT that was subject to the same reactivation protocol. In each case, 500 µM isothiocyanate was added except for allyl-ITC, benzyl-ITC, sulforaphane, iberin and erucin (100 µM of substrate). The reaction was triggered by adding 1 mM glutathione, and GSH transfer was assessed by measuring the absorbance of the conjugated products at 274 nm (Kolm, Danielson, Zhang, Talalay, & Mannervik, 1995). Values are the mean of three measurements with standard deviations. Statistical significance compared to controls was assessed using Student's *t*-test ($p < 0.05$ *, $p < 0.01$ **, no sign indicates not significant).

Glutathione transferase GSTA1 levels are lower in saliva from people suffering from taste disorders

In addition to the 32 saliva samples collected from subjects without taste disorder, GST levels were measured in saliva from subjects suffering from taste disorder (ageusic and dysgeusic). GSTA1 levels in the saliva of people suffering from taste disorders (mean: 4.9 ± 4.8 ng/mL) were significantly lower than that in the saliva of the control group (mean: 5.4 ± 3.1 ng/mL), with a *p*-value of 0.05 (Figure 4 and Table 2). However, GSTP1 was higher in the ageusic and dysgeusic groups than in the control group, but the difference was not significant (mean: 19.8 ± 25.7 ng/ml and 9.8 ± 9.5 ng/ml, respectively) (Figure 4 and Table 2). Any correlation was found for GSTP1 due to the high standard deviation, leading to a *p*-value of 0.09. Interestingly, we observed that GST concentration variations were larger in the group of people suffering from taste disorders than in the control group. This could be explained by

numerous hypotheses, such as variations due to patient pathologies or increases due to drug intake, which may boost detoxification enzyme expression, as well as a perturbation of the buccal microbiota.

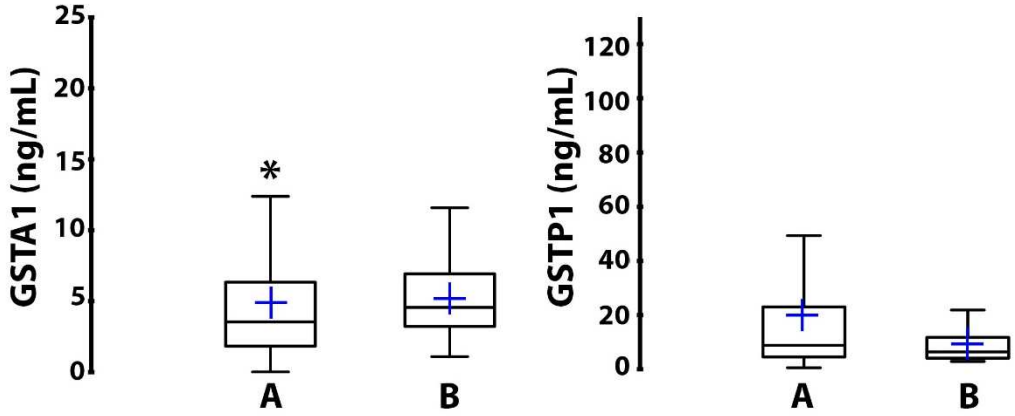

Figure 4: Titration of salivary glutathione transferase isoforms.
GST isoform concentrations were measured using a specific immunoenzymatic assay (ELISA). GSTA1 and GSTP1, the two main salivary GST isoforms, were quantified in saliva from 72 people presenting taste disorder (A) and without any issues for the 32 subjects in the control group (B). Boxes represent interquartile ranges, middle horizontal lines represent medians, error bars represent 1.5-fold the interquartile range, and blue crosses represent mean values.

Table 2: Salivary GST isoform concentrations in 32 saliva samples from people without taste disorders and 72 people suffering from taste disorders.

	Ageusic and dysgeusic GSTA1 (ng/mL)	Controls GSTA1 (ng/mL)	Ageusic and dysgeusic GSTP1 (ng/mL)	Controls GSTP1 ng/mL
Subjects	72	32	72	32
Minimum	0.01	1.09	0.56	2.90
Maximum	22.02	14.22	121.55	49.21
1st Quartile	1.83	3.23	4.54	4.13
Median	3.54	4.55	8.84	6.42
3rd Quartile	6.34	6.93	23.03	11.76
Mean	4.94	5.37	19.78	9.85
Variance (n–1)	22.56	9.68	661.10	89.97
Standard-deviation (n–1)	4.75	3.11	25.71	9.49

Discussion

This relationship should be investigated to support our hypothesis of a potential role for oral enzymes in taste perception and a link to taste disorder. However, future studies will be needed to definitively demonstrate this hypothesis in accord with the new results presented here. We detected expression of a family of salivary enzymes (GST) in a large panel of subjects, assessed their ability to interact specifically with bitter compounds, and tested their ability to be metabolized in saliva. Lower expression of GSTA1 was observed in the saliva of people suffering from taste disorders (ageusic and dysgeusic). Even if no significant variation was observed with respect to GSTP1, for both groups, the variance was exacerbated in the ageusic and dysgeusic groups. This observation can suggest that taste disorders impact salivary GST expression in a direct or indirect manner, leading to values far from the mean. This could be explained by different hypotheses, including metabolic pathway imbalances due to pathologies as well as drug intake or disturbed diet, which can lead to modified expression of glutathione transferases. However, we can also hypothesize that low GST levels predispose individuals to gustatory dysfunction, leading to reduced GSTA1 levels in the saliva of people suffering from taste disorder. Our X-ray structure data support different modes of interaction in addition to metabolization, opening new concepts. Among tastants, bitter molecules appear to be the most diverse and can be potentially toxic (Di Pizio, Ben Shoshan-Galeczki, Hayes, & Niv, 2019; Laffitte, Neiers, & Briand, 2016). Saliva enzymatic activity is involved in the detoxification process, consequently protecting the body from harmful compound ingestion. In this context, we tested the ability of the two main isoforms of a major detoxifying family of enzymes, GST, to interact with bitter compounds. GST is a phase II detoxifying enzyme; the detoxification process is divided into three phases: phase I involving oxidizing enzymes, then phase II including transferases such as GST and phase III consisting of cellular excretion when the process occurs within the cells. However, GST can act directly on exogenous molecules without the first phase and exert functions in addition to its well-known transferase activity. In a previous study, Sreerama et al. showed an increase in the expression of salivary GSTs in response to specific diets, such as coffee- or broccoli-rich diets (Sreerama, Hedge, & Sladek, 1995). These plant-based foods contain many bioactive compounds, including bitter tasting compounds. Moreover, bitter molecules are the most chemically diverse family of taste molecules.

Our results support the ability of the two primary salivary isoforms to glutathione-conjugated bitter tasting molecules, particularly isothiocyanates. Our *in vitro* observation confirms a previous study showing the ability of GSTs to glutathione conjugate isothiocyanates into the corresponding dithiocarbamates (Kolm, Danielson, Zhang, Talalay, & Mannervik, 1995). Additionally, with respect to the potential of these salivary enzymes to interact with a large panel of bitter molecules revealed in this study, we showed the capacity of GST to glutathione conjugate isothiocyanate in saliva. Isothiocyanates can spontaneously react with glutathione to form conjugates at the pH of saliva (between 6.2 and 7.4), but our results show that salivary glutathione transferase activity significantly increases this

metabolization. Indeed, *ex vivo* measurements (on saliva) have demonstrated the ability of GSTs to metabolize hexyl isothiocyanate within the saliva. The two GST isoforms present a different spectrum of interaction towards the diverse bitter molecules tested within this study, supporting, on the one hand, the large panel of molecules with which these enzymes interact and, on the other hand, the specificity of each isoform to a different panel of molecules. The X-ray structures of GSTA1 and GSTP1 were previously reported in complex with glutathione-conjugated phenethyl isothiocyanate (Kumari, Dyba, Holland, Liang, Singh, & Ji, 2016). In our study, we reported structures in complex with three new isothiocyanate molecules, iberin, hexyl isothiocyanate and allyl isothiocyanate. These compounds display hydrophobic chains of various lengths that can be favorably accommodated in GST binding pockets. GSTA1 and GSTP1 both exhibit a hydrophobic site adapted for isothiocyanate binding near the glutathione site. Interestingly, the X-ray structure determination of both GST isoforms in complexes with tasting molecules supports their ability to interact with bitter molecules without catalysis within the active site under certain conditions as well as outside the active site.

Moreover, as observed for phenethyl isothiocyanate (Kumari, Dyba, Holland, Liang, Singh, & Ji, 2016), a covalent adduct between the GSTA1 Cys112 residue was observed with allyl isothiocyanate. It was shown that the covalent adduct of GSTA1 with phenethyl isothiocyanate irreversibly inhibits the catalytic activities of the enzyme. Based on the new structure showing a covalent adduct at the same position as allyl isothiocyanate, we hypothesize that the first observation is more general to isothiocyanate. It is likely that isothiocyanates generally suppress their own metabolism, consequently modulating the metabolism of other bitter molecules taken in charge by the same salivary GSTs. A food product is a complex mixture of diverse molecules, including bitter molecules; consequently, the interaction of the different molecules with GSTs will also be dependent on the content of the mixture. This observation opens the possibility of formulating the bitter molecule content of food to modulate their perception as bitter. It opens new questions on their potential role in the dynamics of bitter tasting perception. When bitter molecules are metabolized, their concentration will be quickly decreased in saliva and possibly change our ability to perceive them. Indeed, it is unknown from this study or any other to date if the conjugation of a bitter molecule removes its bitterness and consequently increases or decreases its perception. Enzymatic processes were demonstrated to be sufficiently fast *in vivo* to contribute to the modulation of the perception of aroma compounds (Robert-Hazotte, Schoumacker, Semon, Briand, Guichard, Le Quere, et al., 2019). Indeed, a dynamic real-time *in vivo* study coupling sensory evaluation demonstrated the quick metabolization of aroma compounds in the nose, consequently modifying their perception. It is likely based on the proof of concept in this paper to extend the fact that metabolization is also fast enough to impact bitter taste perception. This relationship should be investigated in the future using biochemistry coupled with sensory evaluation and more particularly to link the perception threshold of a particular bitter molecule taken in charge by GSTs with their level of expression.

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