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Influence of Human Salivary Enzymes on Odorant Concentration Changes Occurring in Vivo. 1. Esters and Thiols

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The influence of human whole saliva on odor-active esters and thiols was investigated. Special emphasis was placed on food-relevant concentrations of the odorants. It was found that the amounts of the esters were reduced during incubation with saliva to different extents according to their chemical structures. Considerable degradations were also observed for 2-furfurylthiol, 2-phenylethanethiol, and 3-mercapto-3-methyl-1-butanol, being reduced from ~70 to 20% of their initial concentrations within a period of 10 min. Decrease of the odorants did not occur after thermal treatment of the saliva. Generally, the enzymic processes were found to be dependent on the salivary activity of each individual panelist as well as on the odorant's concentrations applied. These investigations were aimed at finding an explanation for the persistence of aftertaste in humans, as it is induced by some odor-active compounds after the consumption of food materials.

KEYWORDS: 2-Furfurylthiol; 2-phenylethanethiol; 3-mercapto-3-methyl-1-butanol; ethyl butanoate; ethyl hexanoate; ethyl octanoate; aftertaste

INTRODUCTION

Saliva, a complex dilute aqueous solution containing numerous inorganic salts and a diversity of organic components such as enzymes or mucous glucoproteins, has been often thought to play an important role in retronasal aroma perception. With regard to the mastication process, authors have suggested that aroma release from foods is influenced, for example, by dilution during continuous salivation. Also, the effect of salivary constituents such as salts, sugars, amylase, or mucins on the volatile partitioning from solutions has been investigated using static headspace techniques (*1*, *2*). In these studies model saliva systems have been used. On the other hand, the influence of salivary enzymic activity on retronasal aroma perception has been discussed in terms of a degradation of food polymers such as starch, inducing, thereby, a release of odorants from inclusion complexes (*3*).

Odorous substances are found among very different substance classes such as esters, aldehydes, alcohols, thio compounds, heteroaromatic or terpenic compounds, and many more. Despite the evidence for a diversity of enzymes in human saliva, the possibility of a degradation or formation of odorous substances due to salivary enzymic activity has generally not been investigated. On the other hand, such enzymic reactions could be a possible explanation for differences in the persistence of odorous molecules after food consumption and for the development and/or duration of the so-called aftertaste, which should be better called "aftersmell" when talking of odorants. Up to now, the reasons for this phenomenon could not be elucidated. Some years ago, evidence had been found for the partial hydrolysis of several odor-active acetates, as well as the reduction of benzaldehyde and cinnamaldehyde to their corresponding alcohols when these compounds were rinsed in the oral cavity (*4*). An influence of human salivary enzymes on these reactions has been assumed. However, the degree as well as the speed of these degradations has not been studied by the authors in detail.

Moreover, numerous types of enzymes have been described as constituents of human saliva or were detected in salivary glands, respectively. Salivary amylase, for initial starch and glycogen breakdown, and salivary lipase are the dominating enzyme systems in human saliva (*5*). Apart from this, salivary esterases as well as enzymes with hydrogen-transferring properties have been described (*6*−*9*). Also, peroxidase activity has been studied in human saliva, being mostly associated with lactoperoxidase and thiocyanate-sensitive peroxidase, with only a minor contribution from leukocyte myeloperoxidase (*10*). Their influence on aroma modification is completely unknown.

Therefore, the present study is aimed at the investigation of the influence of human whole saliva on naturally occurring and highly odor-active esters and thiols. The focus of the present work was placed on concentration ranges that are relevant for real food materials.

MATERIALS AND METHODS

Chemicals. The following odorants were obtained from the suppliers shown: ethyl butanoate 99%, ethyl hexanoate 99+%, ethyl octanoate 99+%, 2-phenylethanethiol 99%, and 2-furfurylthiol 98% (Aldrich, Steinheim, Germany). The compounds were freshly distilled prior to

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Table 1. Selected Ions and Calibration Factors Used for Quantitation by Stable Isotope Dilution Assays

odorant[a]	ion (*m*/*z*)	internal standard	ion (*m*/*z*)	calibration factor[b]
ethyl butanoate	117	[2,2,2-$^2H_3$]ethyl butanoate	120	1.00
ethyl hexanoate	145	[2,2,2-$^2H_3$]ethyl hexanoate	148	1.00
ethyl octanoate	173	[2,2,2-$^2H_3$]ethyl octanoate	176	1.00
butanoic acid	89	[3,4-$^2H_2$]butanoic acid	91	0.95
hexanoic acid	117	[3,4-$^2H_2$]hexanoic acid	119	0.88
octanoic acid	145	[3,4-$^2H_2$]octanoic acid	147	0.84
2-phenylethanethiol	105	[1,1-$^2H_2$]-2-phenylethanethiol	107	0.95
2-furfurylthiol	115	2-[α-$^2H_2$]-2-furfuylthiol	117	1.00
3-mercapto-3-methyl-1-butanol	87	[$^2H_6$]-3-mercapto-3-methyl-1-butanol	92	1.00

[a] Compounds were analyzed by MS/CI using the ion trap detector ITD-800 (Finnigan, Bremen, Germany) and methanol as the reagent gas. [b] The calibration factor was determined as reported previously (*21*).

Table 2. Important Esters and Thiols in Foods

odorant	odor quality[a]	orthonasal odor threshold in water[a] (μg/L)	concn in food[b] (μg/kg)
ethyl butanoate	fruity	1	50–1000 (grapefruit juice, orange juice)
ethyl hexanoate	fruity	5	5–100 (orange juice, grapefruit juice)
ethyl octanoate	fruity	70	250–650 (white wine)
phenylethanethiol	sulfury, rubber-like	0.05 (sunflower oil)	5–50 (roasted sesame)
2-furfurylthiol	meatlike	0.01	50–2500 (roasted sesame, coffee)
3-mercapto-3-methyl-1-butanol	catty	2.1	50–1500 (coffee)

[a] Data from ref *48*. [b] Data from refs *22*, *49*, *12*, *15*, *50*, and *51*, respectively.

analysis. Chemical and sensory purity was checked by gas chromatography−olfactometry (GC-O) as well as gas chromatography−mass spectrometry (GC-MS).

Preparation of the Aqueous Odorant Solutions. Stock solutions (1%) of the single odorants in absolute ethanol were freshly prepared and diluted with deodorized water prior to enzymic analysis to obtain 500 mL of single aqueous solutions of each odorant (concentrations of 100 and 1000 μg/L of water, respectively).

Syntheses. The following labeled internal standards were synthesized according to the literature cited: [2,2,2-$^2H_3$]ethyl butanoate (*11*); [2,2,2-$^2H_3$]ethyl hexanoate and [2,2,2-$^2H_3$]ethyl octanoate (*12*); 2-[α-$^2H_2$]-2-furfuylthiol (*13*); [$^2H_6$]-3-mercapto-3-methyl-1-butanol (*14*); [1,1-$^2H_2$]-2-phenylethanethiol (*15*); [3,4-$^2H_2$]butanoic acid (*16*); [3,4-$^2H_2$]hexanoic acid (*17*); and [3,4-$^2H_2$]octanoic acid (*18*). The unlabeled 3-mercapto-3-methyl-1-butanol was synthesized according to the method in ref *19*.

The concentrations of the labeled internal standards and the response factors (FID) were determined gas chromatographically using methyl octanoate as the internal standard as described recently (*20*). The calibration factors for the labeled compounds were calculated as reported recently (*21*). The mass traces as well as the calibration factors used for quantitation are given in **Table 1**.

Enzymic Analysis. *Collection of Saliva.* Mixed whole saliva (10 mL) was collected from each panelist separately 2 h after breakfast and thorough cleaning of the teeth and oral cavity with a commercial toothpaste (5 min) and with a commercial alcohol-free, low-aromatized, and antimicrobial mouthwash. Before sampling, each panelist rinsed his mouth several times with tap water to avoid any contamination. The collected saliva was used immediately for analysis. Panelists were nonpregnant volunteers (nonsmokers) of the Technical University of Munich, exhibiting no known illnesses at the time of examination and with normal olfactory and gustatory function. Subjective aroma perception was normal in the past and at the time of examination. The panelists had a normal salivary flow and were selected for their excellent oral hygiene, thereby not suffering from oral diseases and nuisances, such as plaque, caries, tartar, gingivitis, and periodontis.

Enzyme Assay. Flavor Samples. Ten milliliters of each aqueous odorant solution was kept in a closed flask, sealed with a lid, and thermostated at 37 °C. After application of 1 mL of whole saliva, the solution was stirred at 37 °C for 1, 5, and 10 min, respectively. The pH of the odorant solution containing saliva was always between 7.5 and 8.

Then, 10 mL of a saturated $CaCl_2$ solution was immediately added to avoid further enzymic processes and immediately applied for quantitation by stable isotope dilution assays. Each experiment was performed four times.

To ensure that the observed degradations did not occur without addition of saliva, a reference analysis was performed in parallel, treating the reference sample (10 mL of odorant solution) in exactly the same way but without adding saliva to the sample. Furthermore, a blank (10 mL of water instead of odorant solution) was treated with saliva in exactly the same way as was done with the flavor samples. Therefore, contamination of the samples with odorants originating from the saliva or from the water could be excluded.

Inhibition of Enzymic Activity. The same experiments were performed after thermal treatment of the saliva samples in a closed vessel (100 °C, 10 min). The saliva was cooled to 37 °C and immediately applied for the enzyme assays as described above.

Quantitation of the Odorants by Stable Isotope Dilution Assays. After the enzyme assay had been performed, the solution was immediately spiked with known amounts of the labeled internal standards, stirred for equilibration (20 min), and extracted with dichloromethane (three times, total volume = 200 mL). The combined organic extracts were dried over anhydrous Na_2SO_4, then concentrated to a total volume of 200 μL, and subsequently analyzed by multidimensional GC-MS.

High-Resolution Gas Chromatography−Mass Spectrometry. The odorants were quantified by two-dimensional gas chromatography (TD-HRGC) using a Mega 2 gas chromatograph (Fisons Instruments, Mainz-Kastel, Germany) as the precolumn system in tandem with a Fisons GC 5160 as the main column system. MS analyses were performed with an ITD-800 (Fisons Instruments) running in the CI mode with methanol as the reagent gas. The following fused silica columns were used: DB-FFAP (30 m × 0.32 mm i.d., 0.25 μm FD, J&W Scientific, Folsom, CA) in combination with DB-5 (SE-54; 30 m × 0.32 mm i.d., 0.25 μm FD, J&W Scientific). The gas chromatographic conditions were the same as described previously (*22*).

RESULTS

The odor qualities and the orthonasal odor thresholds in water as well as an overview of concentration ranges in foods of the investigated compounds are given in **Table 2**. Care was taken, as already stated in the Introduction, to perform all experiments in concentrations comparable to those found in foods.

Carboxyl Esters. After incubation with saliva (1, 5, and 10 min, respectively), ethyl butanoate was steadily reduced by ~8−

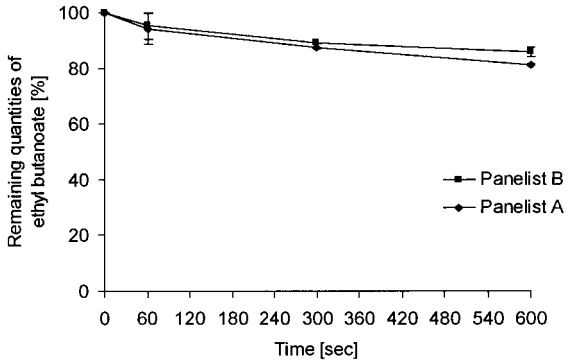

Figure 1. Remaining quantities of ethyl buanoate after treatment with saliva, comparison between two panelists (values are the means of four replicates; the error bars show the standard deviations). Initial concentration of ethyl butanoate was 100 μg/L of water.

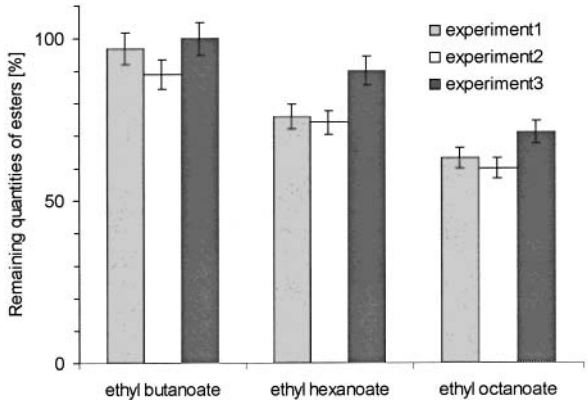

Figure 2. Remaining quantities of homologous esters after treatment with saliva for 10 min (one panelist), comparison of three different sampling days (experiments 1, 2, and 3). Values are the means of four replicates; the error bars show the standard deviations. Initial concentrations of the esters was 100 μg/L of water.

15% of the initial concentration (100 μg/L of water), following a similar pattern of degradation for the salivas of two panelists (**Figure 1**). Variation between saliva samples and panelists was found to be very low.

On the other hand, comparison of the enzymic degradation of ethyl butanoate, ethyl hexanoate, and ethyl octanoate, consistently using one sample of saliva on the same day, showed a decrease in their degradation going along with an increase in the chain length (100 μg/L) after an incubation of 10 min (**Figure 2**). The experiment was repeated three times with three different samples of saliva on three different days (experiments 1, 2, and 3). Generally, the enzymic degradations of the esters could vary by ~5−10% from one day to the other, indicating that salivary enzymic activity for one panelist is not fully consistent. However, similar degradation patterns were observed in all three experiments, with the least polar ester ethyl octanoate being the compound with the highest degradation after treatment with saliva.

After thermal treatment of the saliva (100 °C, 10 min), no degradation of the esters was observable, resulting in a 100% recovery of the odorants after incubation with saliva (data not shown).

Thiols. A considerable decrease was observed in the group of thiols. After an incubation period of 10 min, 2-phenylethanethiol was reduced by up to 35% of the initial concentration, whereas for 2-furfurylthiol and 3-mercapto-3-methyl-1-butanol reductions of even 75 and 80% were found. The

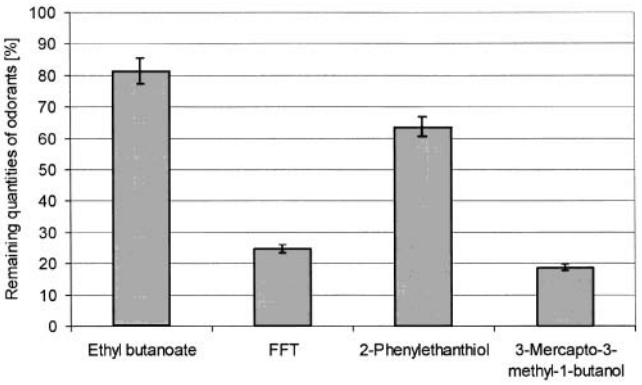

Figure 3. Remaining quantities of odorants after treatment with saliva for 10 min (one panelist, values are the means of four replicates; the error bars show the standard deviations). Initial concentration of the odorants was 100 μg/L.

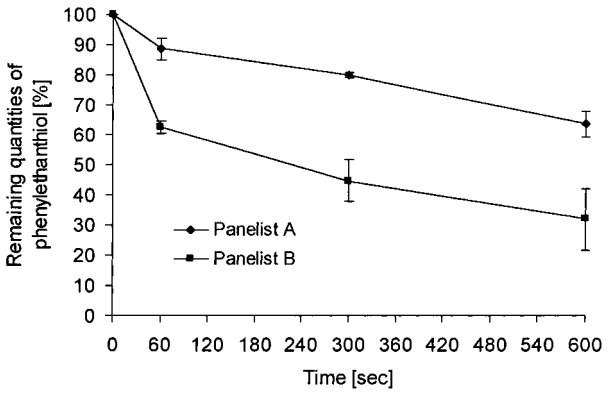

Figure 4. Remaining quantities of 2-phenylethanethiol after treatment with saliva, comparison between two panelists. Values are the means of four replicates; the error bars show the standard deviations. Initial concentration of 2-phenylethanthiol = 100 μg/L of water.

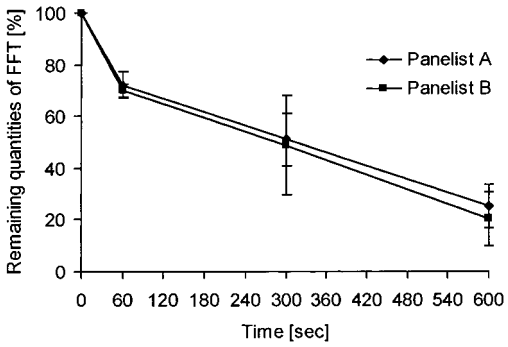

Figure 5. Remaining quantities of FFT after treatment with saliva, comparison between two panelists. Values are the means of four replicates; the error bars show the standard deviations. Initial concentration of FFT was 100 μg/L.

decrease of the thiols is displayed in comparison to that of ethyl butanoate in **Figure 3** (data for one panelist for better comparison). Again, variation between saliva samples from one panelist and each thiol was found to be very low.

Interestingly, a considerable difference in degradation could be observed for phenylethanethiol between two panelists, with ~35% for panelist A after 10 min and ~70% for panelist B (**Figure 4**). On the other hand, the decrease found for 2-furfurylthiol was very similar for the saliva of the two persons (~80%) (**Figure 5**).

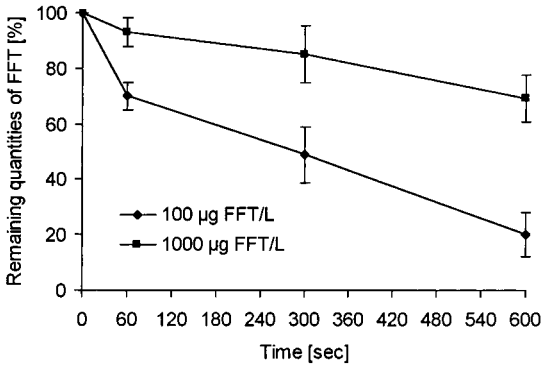

Figure 6. Remaining quantities of FFT after treatment with saliva, as dependent on the initial odorant's concentration (100 and 1000 μg/L, respectively). Values are the means of four replicates; the error bars show the standard deviations.

Moreover, the decrease of 2-furfurylthiol was shown to be related to the initial odorant concentration in the aqueous solution, resulting in a considerably higher (relative) decrease (80% after 10 min) when lower concentrations (100 μg/L) were applied (**Figure 6**). For a 1000 μg/L furfurylthiol solution a decrease of only ~30% was observed.

After thermal treatment of the saliva (100 °C, 10 min), no considerable degradation of the thiols was observable, with a recovery of ~90% of the initial amount for phenylethanethiol and a 90−100°% recovery of 2-furfurylthiol after incubation with saliva (data not shown).

DISCUSSION

Recent investigations showed that the amounts of odorants present in a food material or aroma solution can be considerably reduced in the food during mastication in the oral cavity (*23*). This reduction can still be observed when the oral cavity is closed off from the air flow in the air passages, as was shown in detail by our investigations on the influence of the human physiology on the transport of volatiles from the oral to the nasal cavity via the retronasal route (*24*). Therefore, losses of odorants, for example, due to the tidal air flow of breathing, could be excluded. This means that the observed reductions must have been induced by factors given in the oral cavity such as adsorptive or resorptive effects of the mouth mucosa or enzymic degradation of the odorants or, generally, interactions of odorants with salivary constituents.

In previous investigations, it has been shown that the observed losses of odorants during exposure to the oral cavity were clearly correlated to the chemical structures of the odorants (*23*, *24*). After rinsing of odorant solutions in the oral cavity for 10 min and following expectoration of the masticated material, for example, methionol, 3-isopropyl-2-methoxypyrazine, 3-isobutyl-2-methoxypyrazine, and 3-*sec*-butyl-2-methoxypyrazine were reduced by ~30−40%, whereas reductions of 70−80% were observed for ethyl butanoate and methional. Interestingly, these differences could not be simply explained by differences of the compounds' polarities; for example, they seem unrelated to their partition coefficients. From a sensory point of view, major differences in the duration of perception after food consumption were also described for several key food odorants from different substance groups (*25*). Both findings underscore the theory that odorants are adsorbed to different extents to the oral mucosa, forming a kind of aroma depot. The extent of adsorption directly depends on the odorants' chemical structures. The adsorbed residue, therefore, plays an important role in the persistance of (desirable or undesirable) aroma in the mouth. According to the present investigation, some of these adsorbed compounds can be degraded by enzymic activity of the saliva, thereby reducing the persistence as well as the overall intensity of the "aftersmell" of these compounds, whereas others might not be affected at all. The compounds that remain unmodified could be the ones being perceived for the longest time, as long as they are adsorbed to oral mucosa to a significant extent and are available for a prolonged delivery to the olfactory system. Moreover, it also seems likely that potent odorants are formed due to enzymic activity from odorless or only slightly odor-active compounds.

The variations observed for the reduction of the odorants between panelists indicate that differences in the salivary activity of different humans exist, possibly resulting in a varying aroma perception, for example, in the aftertaste. In the present investigation, the effect of differences in saliva composition due to, for example, variations in flow rate, stimulation, diurnal rhythms or differences in the physiological status of the panelists (*5*, *26*−*28*), have not been studied to full extent with regard to enzymic activity and aroma modification. However, it can be assumed that these variations influence the degree of the observed processes. These effects need to be investigated in more detail with a higher number of panelists also taking into account habits of consumption, smoking activities, etc.

On the other hand, it can, at present, not be completely excluded that the observed reactions are a result of bacterial activity. In the present study, considerable effort has been applied to oral hygiene prior to investigation. Therefore, microbial enzymic activity seems to me not to be the reason for the observed reactions. However, if the observed degradations were indeed induced by oral bacteria, it would, in my opinion, not reduce the importance of these processes in terms of retronasal aroma perception, as a microorganism-free oral cavity is unlikely to be found in normal human subjects. Undoubtedly, the exact origin of the salivary enzymic activity needs further clarification.

Carboxyl Esters. With regard to the ester degradation, hydrolysis can be assumed as the most probable mechanism as many esterolytic enzymes can be found in human saliva. The esterolytic activity of freeze-dried salivary gland tissue (parotid, submaxillary, and sublingual) isolated from mouse, rat, and human has been investigated by Burstone as early as 1956 (*29*), followed by the partial separation of these enzymes (*30*, *31*). However, only a few publications report the presence of esterases in saliva itself. Nonspecific esterase activity in human whole saliva has been described (*6*) but was then suggested to be derived from oral bacteria (*32*). Later, the esterase activity of human saliva was quantified using thiophenyl acetate, thiophenyl butyrate, and butyrylthiocholin iodide as the substrates, indicating that one of two esterase main groups originates from submandibular saliva (*7*). Due to their substrate and inhibitor properties, the authors discussed a classification of the main saliva esterases as carboxylesterases. This observation has also been supported by previous findings of Tan, who characterized carboxylic esterases on the basis of inhibition studies and attempted a first genetic classification of salivary esterases (*33*). The polymorphisms of the salivary esterases have been investigated by application of isoelectrofocusing, showing salivary esterases to be derived from cells in the tissue of the human oral cavity, but not from the submandibular saliva (*34*). Furthermore, it has been proven by the use of immunoelectro-osmophoresis that the observed esterase activity is not related

to material of some selected strains of oral bacteria. Three esterases were then characterized by means of gel chromatography, isoelectric focusing, and affinoelectrophoresis (*35*).

Apart from carboxylesterases, other enzymes such as acetylcholinesterase, trypsin, chymotrypsin, carbonic anhydrase, and pseudocholinesterase all can exhibit esterase activity (*36*). Therefore, the esterolytic activity reported here on odor-active substances has to be further investigated, for example, by application of specific inhibition studies.

On the other hand, observation of acid or ethanol liberation was not practicable under the given experimental conditions (low odorant concentrations), as all investigated acids (butanoic, hexanoic, and octanoic acid) and ethanol were found to some extent and in varying concentrations in human untreated saliva, making the determination of additional trace amounts very difficult. Therefore, further metabolic pathways such as transesterification can, at present, not be excluded and need additional consideration.

In the present study evidence was found for differences in specifity within the homologous series of the esters with the highest degradation observable for the longer chained esters. This observation could represent, at least partially, the explanation for the differences in reduction observed when model solutions of esters were rinsed within the oral cavity (*23*). The highest decrease has been found for rinsing of ethyl octanoate in the oral cavity. However, differences in adsorptive and even resorptive effects of the oral mucosa on the esters (according to their polarity) are further possible explanations for this effect.

Thiols. When the degradation of the thiols is considered, the extent and speed of this process are striking and suggest that it is induced by enzymic activity. This notion is further confirmed by the fact that the same degradations do not occur when the saliva is heated prior to enzyme assay. Obviously, there are differences in specifity against different types of thiols which need further investigation.

The ability of thiols to function as peroxidase substrates has been described several times but has been, up to now, mainly focused on compounds such as cysteine esters, cysteamine, glutathione, penicillamine, and many more (*37*, *38*). Interestingly, peroxidase activity assays have also been performed by use of guaiacol as the substrate, which is an important aroma compound in foods (*39*). Generally, the influence of pH and the presence of carboxy and amino groups as well as hydrogen peroxide and thiocyanate have been investigated, the two latter compounds being general constituents of human saliva (*37*, *38*, *40*). Thereby, the thiolate anion has been postulated as the key reactive species. Oxidation of thiols via peroxidase reaction has also been discussed to be induced by $OSCN^-$ (being produced from H_2O_2 and SCN^-) (*41*) or to take place in the presence of redox cosubstrates such as phenols and arylamines (*42*). Additionally, the ability of peroxidases for thiol and phenol oxidation has been studied by use of electron spin resonance measurements, indicating one- and two-electron peroxidase reactions as two possible mechanisms (*37*, *38*, *43*−*46*). Observed products have been thiyl radicals, disulfide radical anions with final formation of disulfides, and many more products.

It has been shown recently that 2-furfurylthiol (FFT) is considerably degraded in the presence of reagents of the Fenton reaction, involving hydrogen peroxide (H_2O_2), iron, and ascorbic acid (*47*). It has been found that after incubation for 1 h at 37 °C, ∼80% of FFT was lost and difurfuryl disulfide (besides furfural, difurfuryl monosulfide, and difurfuryl trisulfide) has been assigned as the major degradation product. However, the concentrations applied (3.3 mM) as well as the reaction times were considerably higher than in the present investigation, so that direct comparison of the data is not possible. Generally, the iron-containing peroxidase system, as it has been found to be present in saliva (*40*), in combination with an oxidizing cellular metabolite can also be regarded as a kind of Fenton system. This allows the assumption that the observed salivary thiol degradation might also or partially be induced by this mechanism (not only by involvement of enzymic activity) and could serve as an explanation that a minor thiol degradation was still observable when the enzyme assays were performed with heated saliva.

Most probably, the thiols applied in the present investigation lead not simply to one degradation product but to a range of compounds, depending also on the exact reaction conditions such as concentrations of the thiols applied, and therefore need further thorough elucidation. This is mainly valid with regard to intense odorants that might be formed during these reactions and could exhibit even higher aroma intensities than the original odorants. It also has to be clarified whether reactive intermediates form, for example, adducts with salivary constituents such as proteins.

Sensory Relevance. Generally, the sensory relevance of the observed reactions has to be investigated carefully, as each degradation of an odorant can lead at the same time to the formation of odoriferous reaction products, for example, liberation of butanoic acid from ethyl butanoate or formation of disulfides during thiol degradation. Therefore, sensory analyses have been undertaken with a sensory panel trained specifically for this purpose, bearing in mind the impact of each single reaction product under the respective odorant concentrations applied (*25*).

Conclusions. Enzymic degradation of odorants in the presence of saliva was shown to occur to a very high extent not only for thiols but also for esters. These results can represent the basis for the explanation of differences in the persistence of odorous substances in the oral cavity after consumption of food materials. Differences observed between panelists and between sampling days indicate varying enzymic activity between humans and their physiological status.

ABBREVIATIONS USED

GC-O, gas chromatography−olfactometry; GC-MS, gas chromatography−mass spectrometry; TD-HRGC, two-dimensional high-resolution gas chromatography; FFT, 2-furfurylthiol.

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