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Lipase-Assisted Generation of 2-Methyl-3-furanthiol and 2-Furfurylthiol from Thioacetates

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Enzymatic hydrolysis of *S*-3-(2-methylfuryl) thioacetate and *S*-2-furfuryl thioacetate using lipase from *Candida rugosa* produced 2-methyl-3-furanthiol and 2-furfurylthiol, respectively. When reactions were carried out at room temperature and pH 5.8, 2-methyl-3-furanthiol was produced in a optimal yield of 88% after 15 min of reaction, whereas 2-furfurylthiol was obtained in a yield of 80% after 1 h of reaction time. Enzymatic hydrolysis was also performed in *n*-hexane, *n*-pentane, and water/propylene glycol mixture. The reaction rates in these media were slower as compared to those in aqueous medium; however, the reaction yields were quite similar. As expected, the stability of the generated 2-methyl-3-furanthiol and 2-furfurylthiol was better in *n*-hexane, *n*-pentane, and the water/propylene glycol mixture as compared to that in water or phosphate buffer.

KEYWORDS: Thiols; thioacetates; 2-methyl-3-furanthiol; 2-furfurylthiol; lipase; hydrolysis; flavor

INTRODUCTION

Natural flavors are defined as biologically derived aroma chemicals generated by microbial fermentation and/or by the action of endogenous or technical enzymes (*1*). Among these biocatalysts, lipases and esterases have received special attention because of their effectiveness in regio- and enantioselective esterification and transesterification of organic acids and alcohols (*2*). Moreover, the use of lipases and esterases in different media, such as organic solvents and supercritical fluids, has been extensively studied (*3*−*5*). From an industrial point of view, the organic media exhibit many advantages as compared to aqueous systems: a decrease in microbial contamination, the possibility of dissolving apolar solutes, a reduction of water-dependent side reactions, the enhancement of the thermal stability of enzymes, and finally the easy recovery of products from low-boiling solvents. Because of their stability, effectiveness, and ease to use, lipases and esterases have been considered in many industrial applications such as dairy products (*6*).

These enzymes are also the main biocatalysts used in the production of numerous flavoring compounds, particularly esters that are impact aroma compounds in fruit flavors (*7*−*9*). However, only a few studies deal with thioesters, which are used as aroma molecules in savory, baked, and dairy goods (*10*−*12*). Among these thioester derivatives, thioacetates have received little attention, and very few studies deal with the generation and enzymatic hydrolysis of thioacetates to produce thiols (*13*). These thiols are important constituents of food aroma, and in many cases they have been proven to be impact odorants (*14*, *15*). They especially seem to impart the characteristic cooked and roasted notes to meats (*16*) and to roasted coffee (*17*). However, thiols are very unstable molecules and easily oxidize to the corresponding disulfides upon storage, even at low temperature (*18*, *19*). To overcome this drawback, enzyme-assisted formation of thiols from thioacetates may be an interesting approach if the reaction rate and yield are high. In fact, thiols could be stored in their more stable thioacetate form, and enzymatic hydrolysis could be achieved just before their use.

In this study, thiols were generated by enzymatic hydrolysis of thioacetates in aqueous and organic media using lipase from *Candida rugosa* as biocatalyst and *S*-3-(2-methylfuryl) thioacetate and *S*-2-furfuryl thioacetate as model substrates.

MATERIALS AND METHODS

Materials. All chemicals were of analytical grade purchased from Aldrich (Buchs, Switzerland; *S*-2-furfuryl thioacetate), from Oxford Chemicals [Hartlepool, U.K.; 2-methyl-3-furanthiol and *S*-3-(2-methylfuryl) thioacetate], or from Merck (Darmstadt, Germany; sodium chloride, sodium dihydrogen phosphate, disodium hydrogen phosphate, and anhydrous sodium sulfate). Lipase from *C. rugosa* (EC 3.1.1.4, type VII) was purchased from Sigma (Buchs, Switzerland). Immobilized lipase from *C. rugosa* (30 units/g of solid) on Sol-Gel-AK support was purchased from Fluka (Buchs, Switzerland). Organic solvents were of analytical grade purified by distillation using a Vigreux column (1 m × 1 cm).

Enzymatic Hydrolysis of Thioacetates. Different amounts of enzyme were added to 10 mL of a solution of thioacetate (0.064 mmol) in distilled water or phosphate buffer (0.2 M). Enzymatic reactions were performed at different temperatures (4, 23, and 37 °C) with gentle magnetic stirring. Samples were withdrawn at various time intervals (from 1 min to 72 h). A solution of benzyl mercaptan in diethyl ether

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(500 μL, 2000 ppm) was then added as internal standard for quantification. The mixture was extracted with diethyl ether, and the ether extracts were dried over sodium sulfate and concentrated to a volume of 2 mL using a Vigreux column (30 × 1 cm). The concentrated solution was then analyzed by various chromatographic techniques. The respective concentrations of thioacetates, thiols, and disulfides were determined by gas chromatography. The influence of the following parameters was studied: pH (6.0, 7.0, and 8.0), temperature (4, 23, and 37 °C), and the ratio of enzyme (units) to substrate (millimoles) (13/0.064, 65.5/0.064, 77/0.064, and 65/0.64).

Hydrolysis of *S*-3-(2-Methylfuryl) Thioacetate in *n*-Hexane and *n*-Pentane. Different amounts of immobilized lipase from *C. rugosa* were added to 10 mL of a solution of *S*-3-(2-methylfuryl) thioacetate (0.064 mmol) in *n*-hexane or *n*-pentane. Reactions were performed at room temperature with magnetic stirring. Samples were withdrawn at various time intervals and then filtered, and a solution of benzyl mercaptan in *n*-hexane (500 μL, 2000 ppm) was added as internal standard. Samples were then analyzed by different chromatographic techniques.

Hydrolysis of *S*-3-(2-Methylfuryl) Thioacetate in Water/Propylene Glycol Media. To a solution of thioacetate (10 mL, 0.064 mmol) containing different ratios of water and propylene glycol (90/10, 70/30, 50/50) was added lipase from *C. rugosa* (26 units). Reactions were performed at room temperature under magnetic stirring. Samples were withdrawn at various time intervals, and a solution of benzyl mercaptan (500 μL, 2000 ppm) in diethyl ether was added as internal standard. Samples were then analyzed by different chromatography techniques.

Gas Chromatography. Gas chromatography−olfactometry (GC-O) analyses were performed using a Carlo Erba gas chromatograph (Mega 2, Fisons Instruments, via Brechbühler, Schlieren, Switzerland) equipped with an automatic cold on-column injector, a flame ionization detector (FID), a flame photometric detector (FPD), and a sniffing port (*20*). Fused silica capillary columns (DB-1701 and DB-FFAP) were used, both 30 m × 0.32 mm i.d., with a film thickness of 0.25 μm (J&W Scientific, Folsom, CA). The temperature program for the DB-1701 was as follows: 35 °C (2 min), 40 °C/min to 50 °C (1 min), 6 °C/min to 240 °C (10 min). The temperature program for the FFAP was as follows: 50 °C (2 min), 6 °C/min to 180 °C, 10 °C/min to 240 °C (10 min). Linear retention indices (RI) were calculated according to the method of van den Dool and Kratz (*21*).

Mass Spectrometry. Gas chromatography−mass spectrometry (GC-MS) analyses were carried out using a MAT-8430 mass spectrometer (Finnigan, Bremen, Germany) using the same GC conditions as described above. The MS-EI spectra were generated at 70 eV and MS-CI at 150 eV with ammonia as the reagent gas.

*Bis(2-furfuryl) disulfide (**3**):* MS-EI, *m/z* (relative intensity) 226 (20), 161 (10), 113 (5), 85 (10), 82 (20), 81 (100), 53 (75), 51 (10), 45 (20).

*Bis(2-methyl-3-furyl) disulfide (**6**):* MS-EI, *m/z* (relative intensity) 226 (100), 183 (5), 146 (5), 114 (40), 113 (95), 86 (5), 85 (5), 73 (10), 72 (15), 61 (15), 51 (25), 45 (20).

RESULTS AND DISCUSSION

Thiols are potent odorants that play a key role in many food flavors. We investigated the feasibility of enzymatic hydrolysis of thioacetates to produce thiols as an approach to overcome the problem of chemical instability, which limits their application. In this study, *S*-2-furfuryl thioacetate (**1**) and *S*-3-(2-methylfuryl) thioacetate (**4**) were chosen as model substrates. Enzymatic reactions were performed in water, in phosphate buffer, or in nonconventional media such as *n*-hexane and *n*-pentane. As shown in **Figure 1**, enzymatic hydrolysis of compounds **1** and **4** using lipase from *C. rugosa* as biocatalyst generated 2-furfurylthiol (**2**) and 2-methyl-3-furanthiol (**5**), respectively. By chemical oxidation, parts of these thiols were transformed into the corresponding dimers: bis(2-furfuryl) disulfide (**3**) and bis(2-methyl-3-furyl) disulfide (**6**). The yield of thiols and related disulfides were determined after quantification using benzyl mercaptan as an internal standard. For all of the parameters studied, reaction controls were performed under the same conditions but without enzyme. No hydrolysis of thioacetates was observed in any of the control samples.

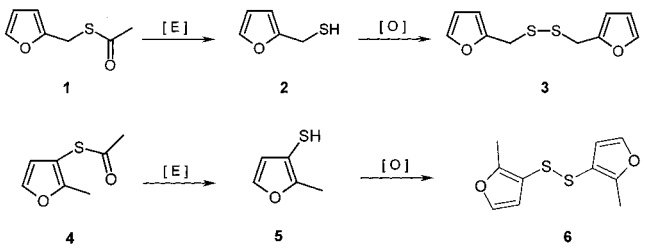

Figure 1. Generation of 2-furfurylthiol (**2**), 2-methyl-3-furanthiol (**5**), and related disulfides.

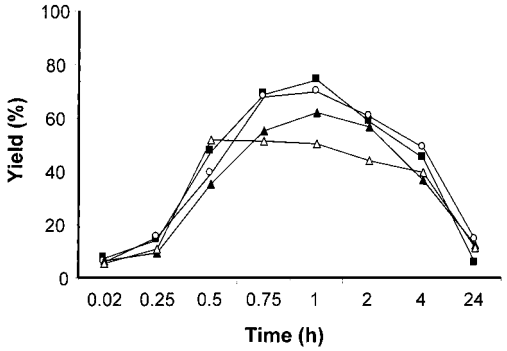

Figure 2. Influence of pH on enzymatic hydrolysis of *S*-2-furfuryl thioacetate at pH 5.8 (■), pH 6.0 (○), pH 7.0 (▲), and pH 8.0 (△): substrate, 0.064 mmol; temperature, 23 °C; enzyme, 65 units; solvent, phosphate buffer.

Enzymatic Hydrolysis of *S*-2-Furfuryl Thioacetate. *Influence of pH.* The study of the influence of the pH on the enzymatic hydrolysis and on the product stability was performed in phosphate buffer and in water. **Figure 2** shows the generation of 2-furfurylthiol at the pH values of 5.8, 6.0, 7.0, and 8.0. The substrate was completely transformed after 1 h of incubation. The reaction rates were similar at all pH values studied. However, the yields were different. A maximum yield of 74% was obtained at pH 5.8, whereas the maximum yield at pH 8.0 was only 50%. Part of the 2-furfurylthiol was chemically oxidized to the dimer: up to ∼10% of compound **3** was obtained at all pH values studied after 24 h of reaction time.

Influence of Enzyme Amount. Only a few examples of enzymatic hydrolysis using thioesters as substrates have been reported (*22*). The ability of common hydrolytic enzymes to transform thioesters into thiols is not well documented. Therefore, we studied the effect of the concentration of enzyme on the reaction rate and on the overall yield. Trials were performed in water, at pH 5.8 and room temperature. The range of enzyme quantity varied from 13 to 262 units for the same substrate concentration (0.064 mmol). As shown in **Figure 3**, the quantity of enzyme influenced the reaction rate but had no significant effect on the final yield of 2-furfurylthiol generated. For all trials, a maximum yield of 70−80% of aroma compound **2** was obtained.

The degradation of this volatile molecule was much higher when the amount of biocatalyst was increased. However, this degradation was insignificant when the concentration of the substrate was increased 10-fold for the same quantity of enzyme (enzyme, 13 units; substrate, 0.64 mmol; data not shown). This phenomenon could be explained by the presence of side activities due to the presence of impurities in the commercial crude enzyme preparation.

Influence of Temperature. To study the influence of the temperature on the reaction rate and on the stability of the

Table 1. Aroma Qualities and Thresholds of the Odorants 2-Furfurylthiol, 2-Methyl-3-furanthiol, *S*-3-(2-Methylfuryl) Thioacetate, and *S*-2-Furfuryl Thioacetate

odorant	aroma quality	odor threshold in air (ng/L)	odor threshold in water (μg/L)
2-furfurylthiol	sulfury, coffee-like, roasty, fresh note	0.01–0.02 (*17*)[a]	0.01 (*24*)
2-methyl-3-furanthiol	sulfury, meat-like, sweet, boiled note	0.005–0.01 (*23*)	0.007 (*24*)
bis(2-furfuryl) disulfide	sulfury, roasty	0.00015–0.0006 (*25*)	
bis(2-methyl-3-furyl) disulfide	sulfury, meat-like, boiled note	0.0006–0.0024 (*25*)	0.00002 (*26*)

[a] Literature given in parentheses.

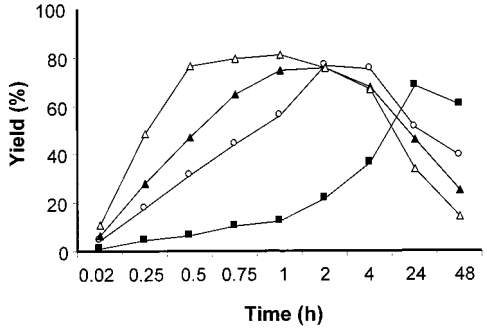

Figure 3. Effect of enzyme amount on hydrolysis of *S*-2-furfuryl thioacetate using 262 units (△), 77 units (▲), 65 units (○), and 13 units (■): substrate, 0.064 mmol; temperature, 23 °C; pH, 5.8; solvent, phosphate buffer.

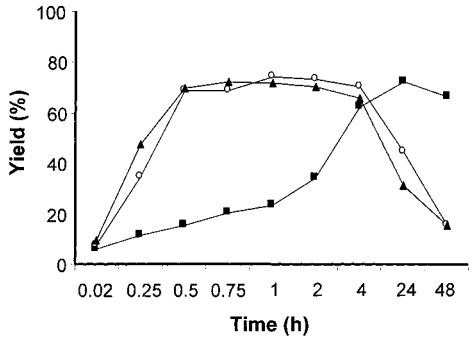

Figure 4. Effect of temperature on formation of 2-furfurylthiol at 37 °C (▲), 23 °C (○), and 4 °C (■): substrate, 0.064 mmol; enzyme, 65 units; pH, 5.8; solvent, phosphate buffer.

product, trials were performed at 4, 23, and 37 °C using the same enzyme-to-substrate ratio (65 units/0.064 mmol). **Figure 4** shows the generation of 2-furfurylthiol and its stability as functions of the temperature. As expected, no significant differences were observed when the reaction took place at 23 or 37 °C, whereas at 4 °C the reaction rate was much slower.

A maximum yield of ~70% of 2-furfurylthiol was obtained after 24 h at 4 °C and after 1 h and 45 min at 23 and 37 °C, respectively. Moreover, 2-furfurylthiol was much more stable at 4 °C as compared to 23 and 37 °C, leading mainly to the dimer **3**. Reaction controls were performed without enzyme at all temperatures and pH values studied, and no hydrolysis of thioacetate was observed.

Enzymatic Hydrolysis of *S*-3-(2-Methylfuryl) Thioacetate. *Effect of the Quantity of Enzyme.* The study of the influence of the enzyme amount on the hydrolysis of *S*-3-(2-methylfuryl) thioacetate was performed at room temperature in a phosphate buffer (pH 5.8) containing 0.064 mmol of substrate. The concentration range of the lipase from *C. rugosa* varied from 0 to 65 units. **Figure 5** shows the formation and degradation of 2-methyl-3-furanthiol as a function of the enzyme concentration. The quantity of enzyme had an influence not only on the reaction rate but also on the yield of the target compound **5**. Indeed, a

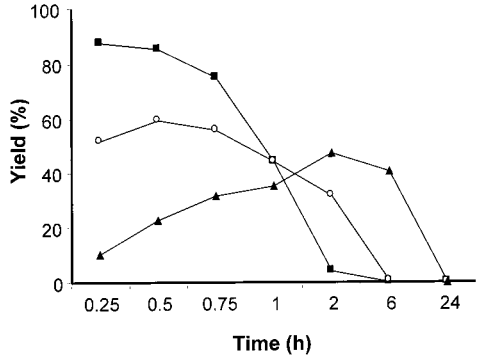

Figure 5. Influence of quantity of enzyme on hydrolysis of *S*-3-(2-methylfuryl) thioacetate using 65 units (■), 26 units (○), and 6.5 units (▲): substrate, 0.064 mmol; temperature, 23 °C; pH, 5.8; solvent, phosphate buffer.

yield of 88% was obtained after 15 min of reaction time when 65 units of enzyme was used for the hydrolysis, whereas the yields were only 50 and 10% when 26 and 6.5 units of enzyme were used, respectively. However, the degradation rate of aroma compound **5** was proportional to the quantity of enzyme. This phenomenon was similar to that discussed previously for 2-furfurylthiol.

The data also show that hydrolysis of *S*-3-(2-methylfuryl) thioacetate (**4**) is much more rapid as compared to that of *S*-2-furfuryl thioacetate (**1**). For example, with 65 units of lipase about 15% of **2** and 90% of **5** were released after 15 min of hydrolytic reaction. Furthermore, these results show that odorant **5** is easily consumed as only 50% was left after an incubation period of 1 h. This is well in line with the higher oxidative instability of **5** as compared to **2**, mainly leading to the dimer **6** (*18*). The loss of odorants **2** and **5** may affect the overall aroma of the sample as the dimers formed show different aroma qualities and thresholds levels (**Table 1**).

Enzymatic Hydrolysis in n-Hexane and n-Pentane. As reported above, enzymatic hydrolysis of *S*-3-(2-methylfuryl) thioacetate in water resulted in 2-methyl-3-furanthiol in good yields (~90%) and with high reaction rate. However, thiol **5** was completely transformed into the corresponding disulfide **6** after 2 h of reaction time. It has been reported that 2-methyl-3-furanthiol is more stable in *n*-pentane and in dichloromethane than in diethyl ether or in water (*18*). Thus, enzymatic hydrolysis was performed in *n*-hexane and in *n*-pentane using immobilized lipase from *C. rugosa*. The reaction rates in *n*-hexane and *n*-pentane were slightly slower in comparison to those in water or phosphate buffer; however, the yields of 2-methyl-3-furanthiol generated were similar (85%). As expected, the stability of aroma compound **5** was better in the organic solvents as compared to water.

Enzymatic Hydrolysis in Water/Propylene Glycol Mixture. Propylene glycol is a food grade solvent used particularly in the flavor industry as most of the aroma compounds are stable

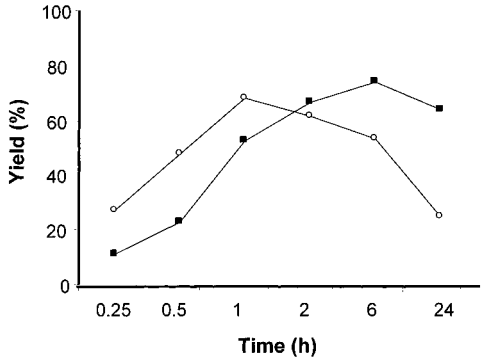

Figure 6. Enzymatic hydrolysis of *S*-3-(2-methylfuryl) thioacetate in water/propylene glycol mixtures with propylene glycol of 30% (○) and 50% (■): substrate, 0.064 mmol; temperature, 23 °C; enzyme, 65 units.

in this organic solvent. Enzymatic hydrolysis of *S*-3-(2-methylfuryl) thioacetate was performed in a mixture of water and propylene glycol. Kinetic studies were carried out to evaluate the influence of the solvent on the reaction rate and on the stability of 2-methyl-3-furanthiol. Propylene glycol concentrations of ≤10% in water as reaction medium did not significantly change the reaction rate, stability, or yield of odorant **5** (data not shown). However, the increase of propylene glycol concentration to 30 and 50% lowered markedly the degradation rate of 2-methyl-3-furanthiol. As shown in **Figure 6**, maximum yields of ∼70% were obtained after 6 h of incubation in an aqueous reaction medium containing 50% propylene glycol.

In conclusion, enzymatic hydrolysis of *S*-3-(2-methylfuryl) thioacetate and *S*-2-furfuryl thioacetate was performed in water and in organic solvents, yielding the corresponding thiols. This enzymatic hydrolysis was achieved in good yield and with high reaction rate. The optimum pH was ∼5.5−6.5. The reaction performed at room temperature was found to be a good compromise among reaction rate, cost, and energy saving.

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