Enzymes-Assisted Generation of Thiols from Thioacetates

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Abstract
Thiols are important constituents of the flavour of many foods and beverages. They were produced efficiently by enzymatic hydrolysis of thioacetates, which are commercially available or easily accessible, by reaction of thioacetic acid with alkenes. Enzymatic reactions were performed in water or phosphate buffer resulting in good overall yields of the target thiols.

Introduction
Natural flavours are defined as biologically derived aroma chemicals generated by microbial fermentation and/or by the action of endogenous or technical enzymes (Bakker et al., 1994). Lipases and esterases are the main biocatalysts used in the production of flavouring compounds, particularly esters that are widely present in fruit flavours. Among these esters, thioacetates have received little attention and very few studies deal with the generation and enzymatic hydrolysis of thioacetates to produce thiols (Sproull et al., 1997). These thiols play, as potent odorants, a key role in many food flavours. They occur in low concentrations and contribute significantly to characteristic aroma notes due to their low odour thresholds (Blank, 2002). However, thiols are very unstable and easily oxidise and polymerise upon storage, even at low temperature (Hofmann et al., 1996). To overcome this drawback, we investigated the feasibility of enzymatic hydrolysis of thioacetates, which are much more stable, into the corresponding thiols. This could be an interesting approach if the reaction rate and yield are satisfactory. The thiols could be stored in their stable thioacetate before release by enzymatic hydrolysis. In this study, we report on the generation of 2-methyl-3-furanthiol and 2-furfurylthiol by enzymatic hydrolysis of their thioacetates. In addition, we describe a chemoenzymatic approach to produce 4-mercapto-4-methyl-2-pentanone and 3-mercaptohexanal.

Experimental
Chemicals and enzymes
The chemicals were of analytical grade. S-2-Furfuryl thioacetate, trans-2-hexenal and 4-methyl-3-penten-2-one were purchased from Aldrich. Thioacetic acid was from Fluka and S-3-(2-methylfuryl) thioacetate from Oxford chemicals. Lipase from Candida rugosa and esterase from porcine liver were purchased from Sigma.

Synthesis of thioacetates
10 mmol of trans-2-hexenal or 4-methyl-3-penten-2-one were added to a solution of thioacetic acid in n-hexane (11 mmol, 30 mL). Reactions were performed at room temperature under stirring. Samples were withdrawn at various time intervals and analysed by gas chromatography (FID, FPD, MS). After 48 h reaction time, the solvent was evaporated and samples were used for enzymatic hydrolysis.

Enzymatic hydrolysis of thioacetates in water
Various 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
under gentle magnetic stirring. Samples were withdrawn at various time intervals (from 1 min to 72 h). A solution of benzyl mercaptan in diethyl ether (500 μL, 2000 ppm) was then added as internal standard for quantification. The mixture was extracted with diethyl ether and the extracts were dried over sodium sulphate and concentrated to a volume of 2 ml using a Vigreux column (30 x 1 cm). The concentrated solution was then analysed by various chromatographic techniques. 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 enzyme (units) to substrate (mmol) (13/0.064, 65/0.064, 77/0.064, and 65/0.64).

Gas Chromatography and Mass Spectrometry

Gas chromatography analyses were performed using a Carlo Erba gas chromatograph (Mega 2) equipped with an automatic cold on-column injector, flame ionisation detector (FID), flame photometry detector (FPD), and a sniffing port. Fused silica capillary columns (DB-1701 and DB-FFAP) were used, both 30 m x 0.32 mm with a film thickness of 0.25 μm. GC-MS analyses were carried out using a Finnigan 8430 mass spectrometer. The MS-EI spectra were generated at 70 eV and MS-CI at 150 eV with ammonia as the reagent gas.

Results and discussions

Enzymatic hydrolysis of S-2-furfuryl thioacetate

As shown in figure 1, hydrolysis of S-2-furfuryl thioacetate (1) using lipase from Candida rugosa allowed producing 2-furfurylthiol (2). Enzymatic reactions were performed in water or in phosphate buffer. The influence of pH on the reaction rate and yield and on the product stability was studied. Figure 2 shows the generation of 2-furfurylthiol at pH 5.8, 6.0, 7.0, and 8.0. After 1 h of reaction, the substrate was completely transformed. While the reaction rates were similar at all pH values, the yields were different. A maximum yield of 74% was obtained at pH 5.8 while this yield was only 50% at pH 8.0.

Figure 1. Enzymatic hydrolysis of S-2-furfuryl thioacetate.

Figure 2. Influence of pH on the enzymatic hydrolysis of S-2-furfuryl thioacetate at pH 5.8 (■), pH 6.0 (○), pH 7.0 (■) and pH 8.0 (■).
The influence of the amount of enzyme on the reaction rate and on the overall yield was performed in water, at pH 5.8 and at room temperature. The range of enzyme quantity varied from 13 to 262 units for the same substrate concentration (0.064 mmol). The quantity of enzyme influenced the reaction rate but had no significant effect on the yield of 2-furfurylthiol generated.

The influence of temperature on the enzymatic hydrolysis was also studied. Trials were performed at 4°C, 23°C and 37°C using the same enzyme to substrate ratio (65 units / 0.064 mmol). As expected, no significant differences were observed when the reaction took place at 23°C or 37°C, while at 4°C the reaction rate was much slower and the product more stable. Room temperature was found to be a good compromise between reaction rate, cost, and energy saving.

Enzymatic hydrolysis of S-3-(2-methylfuryl) thioacetate

As shown in figure 3, enzymatic hydrolysis of S-3-(2-methylfuryl) thioacetate (3) resulted in 2-methyl-3-furanthiol (4). The reaction was performed at room temperature, in water (pH 5.8) containing 0.064 mmol of substrate. The concentration range of the lipase from *Candida rugosa* varied from 0 to 65 units.

Enzymatic hydrolysis of S-3-(2-methylfuryl) thioacetate.

Figure 3 shows the formation and degradation of 2-methyl-3-furanthiol from S-3-(2-methylfuryl) thioacetate as function of the amount of enzyme. An optimal yield of 88% was obtained after 15 min of incubation when 65 units of enzyme were used for the hydrolysis. This yield was only 50% and 10% when 26 and 6.5 units of enzyme were used, respectively. However, the degradation rate of 2-methyl-3-furanthiol into dimer was higher when 65 units of enzyme were used comparatively to 26 and 6.5 units.

Enzymatic hydrolysis of S-3-(2-methylfuryl) thioacetate.

![Figure 3. Enzymatic hydrolysis of S-3-(2-methylfuryl) thioacetate.](image)

Figure 4 shows the formation and degradation of 2-methyl-3-furanthiol from S-3-(2-methylfuryl) thioacetate as function of the amount of enzyme. An optimal yield of 88% was obtained after 15 min of incubation when 65 units of enzyme were used for the hydrolysis. This yield was only 50% and 10% when 26 and 6.5 units of enzyme were used, respectively. However, the degradation rate of 2-methyl-3-furanthiol into dimer was higher when 65 units of enzyme were used comparatively to 26 and 6.5 units.

Enzymatic hydrolysis of S-3-(2-methylfuryl) thioacetate.

![Figure 4. Influence of the quantity of enzyme on the hydrolysis of S-3-(2-methylfuryl) thioacetate. Enzyme units: 65 (■), 26 (○), 6.5 (▲).](image)

Chemoenzymatic synthesis of 4-mercapto-4-methyl-2-pentanone

Recently, the blackcurrant-like smelling odorant 4-mercapto-4-methyl-2-pentanone (4-MMP) (8) was reported as key constituent of grapefruit juice (Buettner et al., 1999) and identified in Sauvignon Blanc (Darriet et al., 1995). As shown in figure 5, we produced this aroma compound in two steps. Reaction of mesityl oxide (5) with thioacetic acid (6) in n-hexane resulted in S-(1,1-dimethyl-3-oxobutyl) thioacetate (7) with a yield of 75%. Thioacetate 7 was characterised on the basis of GC-MS data analysis and by comparison of
its NMR data with those reported in the literature (Trost et al., 1971). Enzymatic hydrolysis of compound 7 using porcine liver esterase (PLE) allowed producing 4-MMP in 83% yield. This aroma compound was characterised by comparison of its chromatographic properties with those of the reference compound.

![Figure 5. Chemoenzymatic synthesis of 3-mercapto-3-methyl-2-pentanone.](image)

**Chemoenzymatic synthesis of 3-mercaptohexanal**

As shown in figure 6, reaction between 2-hexenal (9) and thioacetic acid (6) resulted in thioacetate (10) with a yield of 78%. This thioacetate was characterised on the basis of GC-MS and NMR data. Enzymatic hydrolysis of compound 10 using PLE as biocatalyst allowed producing 3-mercaptohexanal (11) in 47% yield. Compound 11 was characterised by comparison of its chromatographic properties and odour characteristic with those reported in the literature (Werkhoff et al., 1996).

![Figure 6. Chemoenzymatic synthesis of 3-mercaptohexanal.](image)

**References**


