Model Studies on the Formation of Monochloropropanediols in the Presence of Lipase

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The formation of chloropropanols was investigated using model systems comprised of lipase, vegetable oil or fat, water, and sodium chloride. The results showed that measurable levels of the foodborne carcinogen 3-chloro-1,2-propanediol (3-MCPD) are formed in the presence of commercially available lipases of mammalian, vegetable, and fungal origins, incubated at temperatures of 40 °C. The highest yield of 3-MCPD was obtained in reaction mixtures containing lipase from *Rhizopus oryzae*, and all the lipases studied exhibited a high hydrolytic activity toward triglycerides from palm and peanut oil. In contrast, hydrolysis over time and the yield of 3-MCPD in olive and sunflower oils were significantly lower (up to 10-fold), possibly linked to the relatively lower amount (<18%) of saturated fatty acids in these oils. We provide here for the first time evidence that lipases are able to induce the formation of chloropropanols under model system conditions. However, the key intermediates and precise mechanistic aspects governing the formation of 3-MCPD in the presence of lipase still need to be elucidated.

INTRODUCTION

Chloropropanols are foodborne contaminants that may occur at low levels in many foods or food ingredients as a result of processing or storage conditions (*1*, *2*). Toxicological studies have shown that 3-chloro-1,2-propanediol (3-MCPD), the most familiar member of the group of chloropropanols, is an animal carcinogen, inducing tumors mainly in endocrine-sensitive organs in male rats and kidney tumors in both sexes (*3*, *4*). 3-MCPD was also shown to have genotoxic activity in vitro (*4*, *5*). However, the toxicological data have recently been reviewed by several committees (*5–8*), concluding that the genotoxic activity illicited in vitro was not expressed in vivo. Therefore, a provisional maximum tolerable daily intake of 2 μg (kg of body mass)$^{-1}$ has been set. The European Community (EC) has established a regulatory limit of 0.02 mg kg^{-1} for 3-MCPD in hydrolyzed vegetable proteins and soy sauce (*9*).

3-MCPD was originally identified as a processing contaminant of acid-hydrolyzed vegetable proteins, which are frequently used as ingredients of savory foods such as soups, snacks, gravy mixes, and bouillon cubes (*10–12*). Conventional acid-hydrolyzed vegetable protein (HVP) is produced using strong hydrochloric acid, and chloropropanols are formed as a result of the chlorination of glycerol at elevated temperatures present in fats and oils in the crude protein starting materials (*11*).

However, 3-MCPD has also been found in foods prepared without acid hydrolysis, such as grilled cheese or roasted cereals, as well as barley during the production of malt or domestically toasted bread (*13–15*). The formation of 3-MCPD was also found to be dependent on the cooking process (*16*).

Several mechanisms of formation and potential precursors of chloropropanols in foods have been proposed and discussed (*17*). Heat-induced reactions leading to the formation of chloropropanols include reactions of hydrochloric acid with glycerol, lipids, and carbohydrates. All reactions require prolonged heating at temperatures above 100 °C. Collier et al. (*18*) initially proposed a mechanism explaining the heat-induced formation of chloropropanols from triacylglycerols under acidic conditions. The key step involves the nucleophilic substitution of the acyl group by the chloride anion at positions activated by neighboring ester groups. The resulting intermediate is a chloropropanediol diester that under hydrolytic conditions leads to the formation of chloropropanol. A full review on the occurrence and the mechanisms governing the formation of 3-MCPD has recently been presented by Hamlet et al. (*17*).

However, not all chemical mechanisms necessitate triglycerides. An alternative route involving allyl alcohol (prop-2-en-1-ol) has been described that in the presence of hypochlorous acid (HOCl) may lead to the formation of chloropropanols (*17*). This reaction may be important in heat-treated garlic and onions, which harbor the precursor of allyl alcohol, namely, the cysteine amino acid aliin [(*S*)-allyl-L-cysteine sulfoxide].

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Surveys on the occurrence of chloropropanols have revealed that 3-MCPD is found in several foodstuffs that are fermented and/or not subjected to thermal treatment, such as meats (ham, salami), fish, and fermented cheese (*15*). The hitherto unexplained sources of traces of 3-MCPD in many of these foods led us to conduct fundamental model studies to assess the possible involvement of hydrolytic enzymes in the formation of chloropropanols. This study reveals for the first time an alternative pathway to the formation of these processing contaminants at relatively low temperatures, induced by lipase in the presence of oils, water, and sodium chloride.

MATERIALS AND METHODS

Reagents and Chemicals. All chemicals and solvents were of reagent grade. Sodium chloride, 2,2,4-trimethylpentane, *n*-hexane (Lichrosolv), acetonitrile (Uvasol), Hepes buffer, Extrelut NT, and glycerol were from Merck (Darmstadt, Germany). 4-Nitrophenol and the lipases (CAS no. 9001-62-1) from *Rhizopus oryzae* and porcine pancreas were from Fluka (Buchs, Switzerland). 4-Nitrophenyl acetate and the lipase from wheat germ were from Sigma (Buchs, Switzerland). Ethyl acetate was from SdS (Peypin, France). *N*-(Heptafluorobutyryl)-imidazole was from Pierce Biotechnology (Rockford, IL). ASE filters were from Dionex AG (Olten, Switzerland). Isotope-labeled d_5-3-MCPD was from CDN Isotopes (Augsburg, Germany). Florisil was from Aldrich (Buchs, Switzerland). 3-MCPD (CAS no. 96-24-2) was from Fluka, and 2-MCPD (CAS no. 497-04-1) was custom synthesized by TNO Nutrition and Food Research (Zeist, The Netherlands). Olive oil was purchased in a local retail outlet. Refined, bleached, and deodorized (RBDO) palm oil, sunflower oil, and peanut oil were provided by selected suppliers.

Incubation. Typically oil or fat (5 g), NaCl (100 mg), and distilled H_2O (1.5 g) were vortexed for 5 min to form an emulsion. If necessary, the fat was liquefied at 60 °C. Lipase was added to the emulsion, which was again vortexed and incubated in a convection oven at 40°C (Heraeus Serie 6000, Kendro Laboratory Products, Carouge, Switzerland).

Extraction. The reaction mixture (1 g)—as defined above in Incubation—was supplemented with 1—2 g of water and the d_5-3-MCPD spiking solution (10 μL of a 10 ng/μL solution). Hexane (3 mL) was added and the mixture vortexed for 30 s and subsequently centrifuged for 5 min at 2500 rpm. The supernatant (organic phase) was discarded and hexane added (3 mL). The mixture was again vortexed and centrifuged as previously described. The lower aqueous phase was carefully removed and extracted using accelerated solvent extraction (ASE) as described below.

ASE. Extrelut NT (3 g) was added to the prepared test sample (containing a maximum of 3 g of liquid) and mixed with a spatula. The ASE columns constituted deactivated (at 3%) Florisil (7 g), the sample (Extrelut NT and test sample, maximum 6 g), and additional Extrelut NT filling (~2 g), The column was firmly closed and placed in the holder of the ASE-200 apparatus (Dionex, Sunnyvale, CA). Operating conditions were the following: pressure, 200 psi; temperature, room temperature (oven off); no heating; purge time, 4 min; solvent, ethyl acetate; flush volume, 80%; number of static cycles, two; no preheating time. The extracts were collected automatically. The ASE extract was transferred to a flask and concentrated to about 1 mL under vacuum (Büchi Rotavapor, Flawil, Switzerland) at 40 °C. The concentrated extract was transferred to a 5 mL conical vial and the flask rinsed with ethyl acetate. The solution was evaporated to approximately 100 μL under a stream of nitrogen at 40 °C. 2,2,4-Trimethylpentane (900 μL) was added, and the samples were derivatized as described below.

Derivatization with Heptafluorobutyrylimidazole (HFBI). The reconstituted extracts and the calibration standards were supplemented with HFBI (50 μL) and the vials tightly closed. These were then agitated for a few seconds using a vortex mixer and heated at 70 °C for 20 min in a convection oven. The mixtures were allowed to cool to room temperature, and the solutions were then transferred to clean vials. Distilled water (4 mL) was added and the mixture vortexed for a few seconds. The vials were centrifuged at 2500 rpm for 5 min. The lower

aqueous phase was discarded and distilled water (4 mL) added. The sample was again agitated for a few seconds and centrifuged as described before. The upper organic phase was transferred to a sample vial for injection into the detection system.

Calibration Standards. Standard solutions of 3- and 2-MCPD were prepared in 2,2,4-trimethylpentane as described by Brereton et al. (*19*) at final concentrations of 3- and 2-MCPD of 0, 5, 10, 25, 50, 100, 150, and 200 μg/kg. The final concentration of d_5-MCPD in each calibration solution was 100 μg/kg. Pooled calibration standard solutions (1 mL each of 2-MCPD/3-MCPD) were transferred to a set of eight 5 mL conical vials, and derivatized as described above.

Gas Chromatography—Mass Spectrometry (GC—MS). The GC—MS system constituted a Trace 2000 GC instrument coupled to a PolarisQ (ion-trap) detector equipped with an AS 200 sampler (all instruments from ThermoFinnigan, San Jose, CA). The whole system was controlled by Xcalibur software (version 1.2). Chromatography was performed on a DB-XLB ITD column (length 30 m, i.d. 0.25 mm, fil 0.25 μm) from J & W Scientific, Agilent Technologies (Basel, Switzerland). The carrier gas was helium at a constant flow of 1 mL/min. The oven temperature gradient was as follows: from 0 to 1.0 min, hold at 50 °C, ramp to 110 °C at 7 °C/min, ramp to 325 °C at 40 °C/min, and keep the temperature constant at 325 °C for 10 min. The MS settings were as follows: source temperature, 220 °C; transfer line temperature, 280 °C. The MS system was operated in the EI (electron ionization)—MS full scan mode (m/z 70—460), collecting 0.45 scan/s. The ions monitored for 3- and 2-MCPD were m/z 75, 253, and 289 and 453 for 3-MCPD. The ion m/z 257 was recorded for the deuterated internal standard. Retention times for 3- and 2-MCPD and the deuterated standard were 8.23, 8.31, and 8.15 min, respectively.

Quantitation. All data evaluation was normalized to the area response of the analyte to the internal standard, and the amount of the compounds in the samples was extrapolated from the respective linear regression equation. For the final quantitation, the median of all ions in a given sample was taken into account. 3- and 2-MCPD are expressed as the amount formed per kilogram of oil unless otherwise specified.

Determination of Esterase Activity. The UV—vis spectrophotometer was an Agilent 8453 from Agilent Technologies (Palo Alto, CA). The procedure is a rapid spectrophotometric assay measuring the quantity of *p*-nitrophenol (pNP) released from *p*-nitrophenyl acetate. The assay was performed in disposable semi-microcuvettes from Sarstedt (Sevelen, Switzerland). A 937 μL sample of Hepes buffer, 50 mM, pH 5.5, was mixed with 10 μL of the enzymatic solution, 53 μL of *p*-nitrophenyl acetate solution (60 mM) in acetonitrile was added, and the resulting solution was mixed and incubated at 40 °C for 10 min. The absorbance at 401 nm was measured as a function of time (molar absorption coefficient $\epsilon = 10150$ M^{-1} cm^{-1}).

The esterase activity was calculated in units per milliliter, by applying the following formula:

$$\frac{v_{max}(A_{401})[\text{mOD/min}]V_{total}[\text{mL}]}{\epsilon[\text{L mol}^{-1}\text{ cm}^{-1}]d[\text{cm}]}\frac{\text{dilution}}{V_{enzyme}[\text{mL}]} \times 1000$$

where v_{max} is the initial velocity, V_{total} is the total volume of the reaction (1 mL), "dilution" represents the dilution factor of the enzymatic solution, ϵ is the molar absorption coefficient, d is the cuvette path length (1 cm), V_{enzyme} is the volume of the enzymatic solution (0.01 mL), and the factor 1000 is for unit harmonization.

The results are expressed in micromoles of *p*-nitrophenol formed per milliliter of enzyme solution per minute [U/mL].

RESULTS AND DISCUSSION

Commercial lipases of different sources were used in this study and display a wide range of different activities and sensitivities toward changes in pH. All experiments were conducted using natural vegetable oils as substrate and major component of the mixture. Vegetable oils display an acidic pH at around 5.5. However, the mold, animal, and vegetable lipases employed in this study exhibit different pH optima, but all residing at pH above 7. Furthermore, the lipases also differ in

Table 1. Summary of the Main Properties of Commercial Lipases Used in This Study

lipase origin	Sigma-Aldrich code	pH optimum	activity at optimal pH, 37 °C (U[1]/g)[a]	activity at pH 5.5, 40 °C (U[2]/g)[a]	temp optimum (°C)	substrate specificity (ref)
R. oryzae	80612	7.2	53000	23200	37	saturated fatty acid chains (C$_8$–C$_{18}$), but not triacylglycerols containing *n*–3 poly-unsaturated fatty acids (*21*)
wheat germ	L 3001	7.7	107	0.4	37	
porcine pancreas	62300	8.0	27000	3.4	37	short to medium fatty acid chains (*24*)

[a] U[1] corresponds to the amount of enzyme that liberates 1 μmol of fatty acid from triglycerides/min at optimal pH and 37 °C. U[2] corresponds to the amount of enzyme which liberates 1 μmol of *p*-nitrophenol from *p*-nitrophenyl acetate/min at 40 °C, pH 5.5.
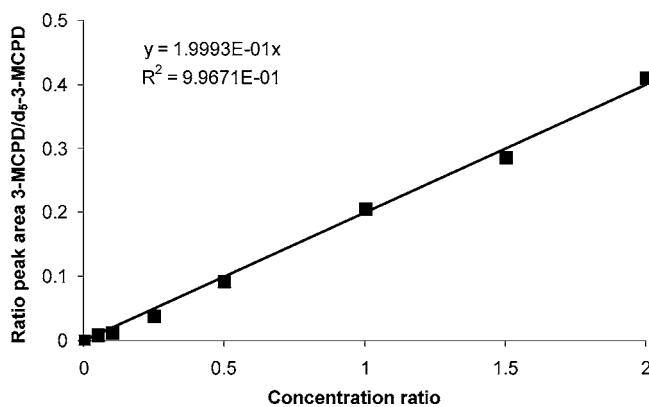

Figure 1. Typical calibration line for 3-MCPD plotting the peak area ratio of *m/z* 289/257 (*d$_5$*-3-MCPD) against the concentration ratio.

substrate specificity, and the most salient properties are summarized in **Table 1**.

Consequently, the optimal activity of the individual enzymes as determined by the suppliers on a triglyceride substrate is no longer valid under the experimental conditions used here, necessitating remeasurement of the activities under acidic conditions for better comparison. Furthermore, the lipase activity in our system is expressed as esterase activity using *p*-nitrophenyl acetate as substrate. Therefore, in all cases the more acidic environment will significantly affect (lower) the activity of the enzymes.

Method Performance. Detailed method performance characteristics will be communicated in a separate paper, and only salient features are highlighted here. The quantitation procedure uses eight calibration points over the calibration range of 0−200 pg/μL. Furthermore, we chose to calculate the median of all relevant ions, which provides more confidence in achieving a quantitative result compared to taking into account only one characteristic ion (e.g., *m/z* 253). The relationship between an analyte response and concentration was shown to be linear over this range, with correlation coefficients >0.99 in all cases, and is exemplified in **Figure 1** for *m/z* 289.

The limit of detection (LOD) was estimated at 5 μg/kg for 3-MCPD, S/N = 3, established by measuring the response of *m/z* 257 from *d$_5$*-3-MCPD. The limit of quantification (LOQ) was estimated at 10 μg/kg for 3-MCPD using the same ion trace (S/N = 10).

Impact of Lipase Activity on 3-MCPD Formation. In the model studies employed, chloropropanols were not detected or were present only in spurious amounts (<10 μg/kg) in the absence of lipase under comparative incubation conditions (**Figure 2**). Reaction mixtures without salt showed approximately 10% of the amount of 3-MCPD formed under the same experimental conditions (time, temperature) in the presence of salt. Experiments were done to determine the dependency of

the reaction on salt, and initially up to a certain level there is a nearly proportional increase in 3-MCPD with increasing salt concentration. At very high salt amounts (200 mg) in the reaction mixture, a drop in the 3-MCPD level is recorded, probably due to the inhibition of the enzyme at high salt concentrations. Furthermore, substitution of triacylglycerols with glycerol did not lead to the formation of 3-MCPD.
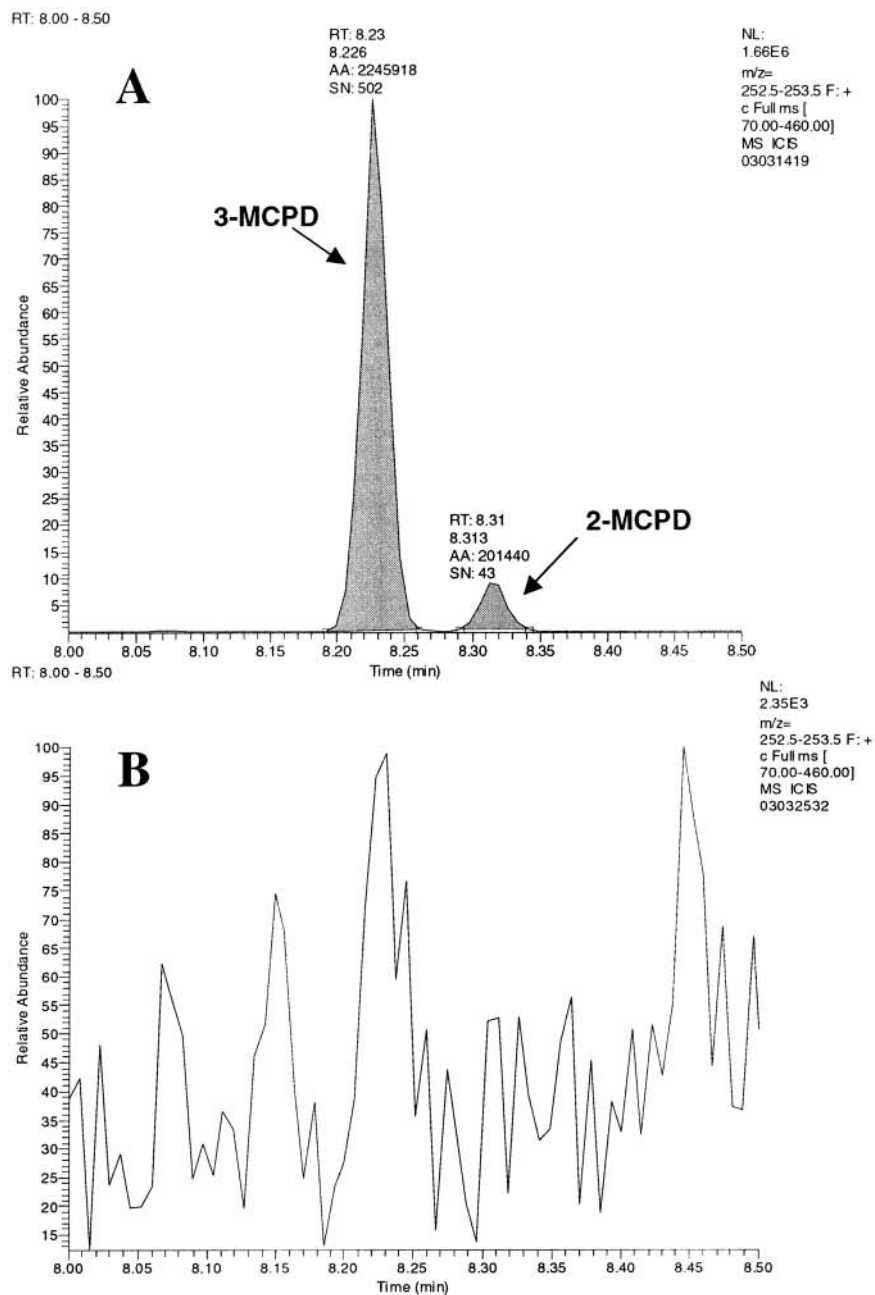
To assess whether the formation of 3-MCPD is correlated to lipase activity, a simple model experiment was conducted by incubating different activities of lipase from *R. oryzae* in a mixture of palm oil, water, and sodium chloride (at 40 °C). The formation of 3- and 2-MCPD was recorded after a period of 10 days.

Palm oil supplemented with 4.6 units of lipase/kg of oil (pH 5.5), and prepared as described in Incubation, induced the formation of 1290 and 170 μg/kg 3-MCPD and 2-MCPD, respectively (**Figure 3**). The generation of chloropropanols was proportional to the lipase activity in the mixture, leading to the formation of more than 1 mg/kg chloropropanols within 10 days. The analytical method also allowed the measurement of formation of the isomer 2-MCPD, the rate of formation of 2-MCPD being approximately 8 times lower than that recorded for 3-MCPD. This ratio is consistent with levels found in foods and in model studies (*18, 20*). Explanations for this observation may be (i) that the sn-2 position of the triglyceride may be less accessible for chlorination, (ii) a higher specificity of the lipase for the hydrolysis of fatty acids at the sn-3 position, and (iii) a lower stability of 2-MCPD versus 3-MCPD.

Formation of 3-MCPD in Different Vegetable Oils. *Lipase from R. oryzae.* Monochloropropanediols were formed from oils in the presence of lipase of fungal origin, namely, from *R. oryzae*, and a chloride source. This lipase has been characterized by Hiol et al. (*21*) and Razak et al. (*22*), and has a known preference for the hydrolysis of saturated fatty acid chains (C$_8$−C$_{18}$) and a specificity for the 1,3-positions. Moreover, the enzyme exhibited poor hydrolytic activity toward triacylglycerol containing *n* − 3 (or 18:3) polyunsaturated fatty acids (*21*) and, as most fungal lipases, optimal activity in the relatively lower pH range (*22*).

The formation of 3-MCPD over time in the presence of *R. oryzae* lipase (4.6 units/kg of oil) and different lipid substrates is depicted in **Figure 4**.

Particularly high levels of 3-MCPD were measured in the sample containing RBDO palm oil as a substrate. After an incubation period of 4 days, the 3-MCPD level reached 1370 μg/kg of oil, while all other lipid substrates showed amounts below 420 μg/kg of oil. On the 10th day of incubation, samples with peanut, sunflower, and olive oils had reached their apparent maximal level of 3-MCPD, i.e., 745, 140, and 15 μg/kg, respectively. In contrast, 3-MCPD was still generated in the sample with RBDO palm oil, reaching 1885 μg/kg on the 24th

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Figure 2. GC–MS chromatograms showing the formation after 10 days of incubation at 40 °C of 3- and 2-MCPD in reaction mixtures comprised of palm oil (5 g), water (1.5 g), and salt (100 mg), (**A**) in the presence of lipase from *R. oryzae* (0.5 g of sample analyzed; the final concentration of 3-MCPD is 1375 μg/kg) and (**B**) without enzyme (1 g of sample analyzed; the final concentration of 3-MCPD is <10 μg/kg).

day of storage. The control sample, RBDO palm oil without the addition of lipase, did not show any measurable formation of 3-MCPD after the same time period.

The specificity of the lipases is controlled by the molecular properties of the enzyme, structure of the substrate, and factors affecting binding of the enzyme to the substrate (*23*). The fatty acid composition of the oils may be an important determinant in the activity of lipase and thereby may indirectly play a role in the formation of chloropropanols. Therefore, the oils used in this study were characterized with regard to their fatty acid composition (**Table 2**). Palm oil was shown to contain the most saturated fatty acid chains, representing 50% of the total fatty acids in this oil. All other oils studied showed levels of saturated fatty acids below 18% (**Table 2**). The fungal lipase is thus expected to exhibit relatively higher hydrolytic activity toward palm oil. As shown in **Figure 4**, experimentally determined
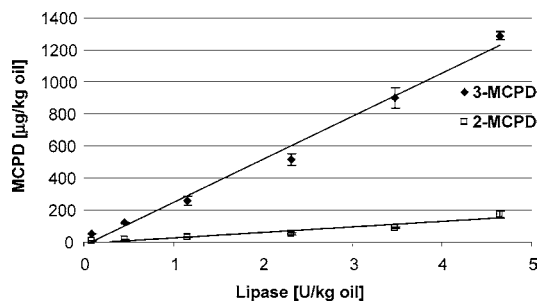
Figure 3. Formation of 3- and 2-MCPD as a function of lipase (*R. oryzae*) activity in palm oil (incubation for 10 days at 40 °C). All entries are averages of *n* = 3 independent determinations. One U corresponds to the amount of enzyme that liberates 1 μmol of *p*-nitrophenol from *p*-nitrophenyl acetate/min at 40 °C, pH 5.5.

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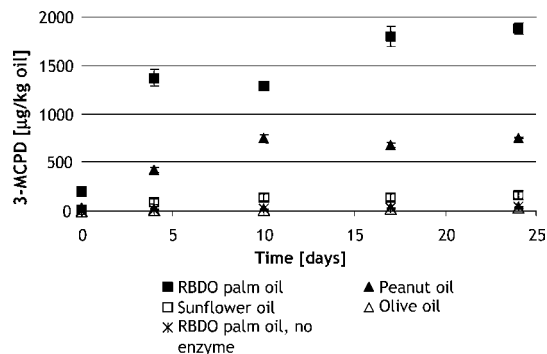
Figure 4. Time-dependent formation of 3-MCPD in oils in the presence of lipase from *R. oryzae.* Lipase activity: 4.6 units/kg of oil, incubated at 40 °C, pH 5.5. All entries are averages of $n = 3$ independent determinations.

Table 2. Fatty Acid (FA) Distribution [% (g of FA/100 g of total FA)] in the Different Vegetable Oils Used in This Study[a]

|	RBDO palm oil	peanut oil	sunflower oil	olive oil
C-12:0	0.16	0	0	0
C-14:0	0.54	0.04	0.08	0
C-16:0	36.54	9.8	6.42	11.55
C-16:1	0.12	0.07	0.08	0.74
C-17:0	0.09	0.08	0.05	0.09
C-17:1	0	0.06	0	0.16
C-18:0	5.44	2.32	4.36	2.8
C-18:1	45.99	43.68	20.59	75.3
C-18:2	10.14	35.57	66.95	7.62
C-18-3	0.28	0.44	0.07	0.75
C-20:0	0.38	1.09	0.27	0.43
C-20:1	0.16	1.62	0.13	0.3
C-20:2	0	0.04	0	0
C-22:0	0.05	2.99	0.71	0.11
C-22:1	0	0.2	0	0
C-24:0	0	1.88	0.16	0
C-24:1	0	0.03	0	0
OFAs	0.11	0.09	0.13	0.15

[a] OFAs = other fatty acids.
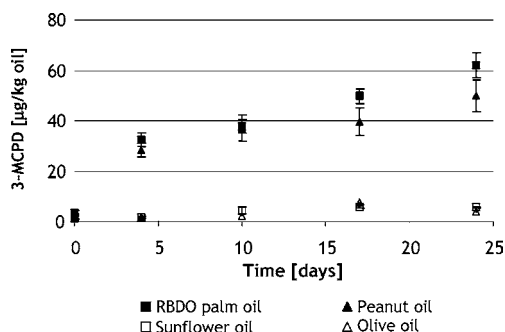
Figure 5. Time-dependent formation of 3-MCPD in oils in the presence of lipase from porcine pancreas. Lipase activity: 1.3 units/kg of oil, incubated at 40 °C, pH 5.5. All entries are averages of $n = 3$ independent determinations.

3-MCPD levels are indeed relatively higher in palm oil versus the other oils used in the study.

Lipase from Porcine Pancreas. The results obtained in the model on lipases of fungal origin prompted us to investigate also the potential involvement of lipases of plant and animal origin on the formation of 3-MCPD. **Figure 5** shows the influence of different vegetable oil types on the kinetics of 3-MCPD formation using porcine pancreas lipase at an activity
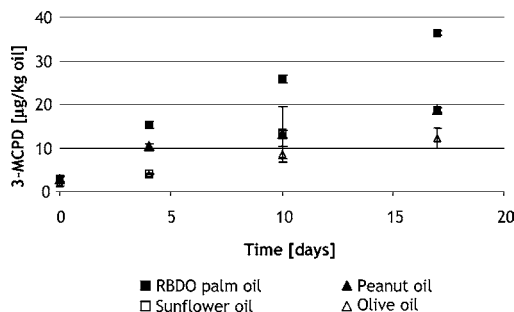
Figure 6. Time-dependent formation of 3-MCPD in oils in the presence of lipase from wheat germ. Lipase activity: 4.5 units/kg of oil, incubated at 40 °C, pH 5.5. All entries are averages of $n = 3$ independent determinations.

of 1.3 units/kg of oil. Even though the enzyme activity employed in this series of experiments was almost 4 times lower than that of the lipase from *R. oryzae*, a maximal level of 38 μg of 3-MCPD/kg of oil was reached after 10 days of incubation when RBDO palm oil was used as a substrate. In fact, as the lipase activity from *R. oryzae* has been shown to be linear (**Figure 3**), the formation of around 334 μg/kg 3-MCPD is estimated using lipase from *R. oryzae* at the same activity of 1.3 units/kg of oil.

Despite its known selectivity for short to medium chains of fatty acids (*24*), the porcine pancreas lipase displays less favorable chloropropanol formation kinetics than the lipase originating from *R. oryzae*. This feature may be attributed to a change of the shape and/or of the hydrophobicity of the acyl binding site due to a lower stability at acidic pH. Indeed, the pH optimum for porcine pancreatic lipase lies at 8.9 (*25*). However, the scissible fatty acid binding site is not the only determinant of chain length specificity. Mutations near the active site and the lid region could also mediate chain length specificity (*26*).

Lipase from Wheat Germ. A wheat germ lipase was incubated under the same conditions and also proved to catalyze the formation of measurable levels of chloropropanols (**Figure 6**). As previously observed for lipases of fungal and animal sources, the highest activity in terms of 3-MCPD formation was recorded with RBDO palm oil as substrate. The enzyme activity, measured at 4.5 units/kg of oil, was comparable to that employed in the fungal lipase experiments. A maximal level of 26 μg/kg 3-MCPD was attained after 10 days of incubation when RBDO palm oil was used as a substrate. This represents roughly only 2% of the formation of 3-MCPD that would have been expected if the lipase from *R. oryzae* had been used at the same esterase activity (4.5 units/kg of oil). When peanut, sunflower, or olive oil was used as a substrate, 3-MCPD formation hardly reached 20 μg/kg of oil, suggesting a lower affinity of the vegetable lipase for these substrates.

Proposed Mechanism of Formation of Chloropropanols. The hydrolysis of fatty acids from triacylglycerols by lipase occurs in different catalytic steps. First, a noncovalent Michaelis complex involving the enzyme and the substrate is formed. Then, the attack of the nucleophilic serine O$^{\gamma}$ will generate a tetrahedral, hemiacetal intermediate, the oxyanion being stabilized by two or three hydrogen bonds (the so-called oxyanion hole). Subsequently, the substrate ester bond is cleaved and the tetrahedral intermediate broken down to the acyl enzyme. Finally, the acyl enzyme is cleaved, the leaving group protonated, and the fatty acid dissociated.

Lipases catalyze the hydrolysis of acyl esters by a common mechanism that involves a serine protease like catalytic triad.
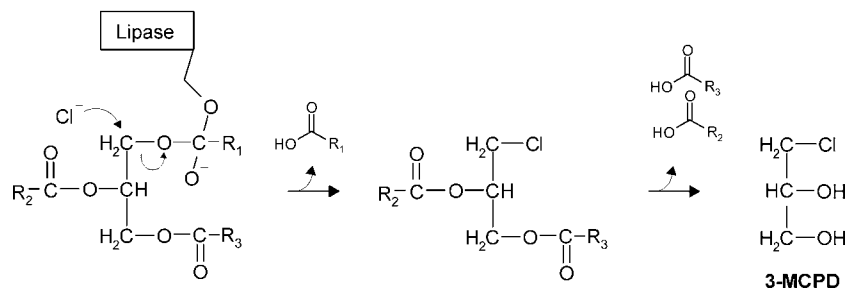
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Figure 7. Proposed hypothetical mechanism of formation of chloropropanols involving lipase.

Although the geometry of the catalytic site is similar for most lipases, even closely related lipases differ significantly with respect to regio- and stereoselectivity toward natural and synthetic substrates. Most lipases show regioselectivity toward the primary position of triacylglycerols, and for several lipases stereopreference for the sn-1 or sn-3 position of the prochiral substrate has been demonstrated (*27*). Within the frame of this study, we did not attempt to separate the (*R*)- and (*S*)-MCPD enantiomers, which would have allowed more insight into the involvement of the enzyme in the chloride-transfer reaction. For lipase-catalyzed triacylglycerol hydrolysis there is neither a simple empirical rule nor a structural model to describe the diversity of lipase stereoselectivity. According to Rogalska et al. (*28*, *29*), the widely differing substrate selectivity toward triacylglycerols, even of closely related enzymes, suggests that preferential attack at the sn-1 or sn-3 position resulted from specific interactions of the nonhydrolyzed sn-2 acyl residue with distinct regions distant from the catalytic triad.

It is feasible that mono- or diesters of chloropropanols are obtained as a side reaction of the classical lipolysis from triacylglycerols as a result of a nucleophilic substitution of the acyl group by a chloride anion (**Figure 7**). In fact, such chlorinated mono- and diacylglycerols have already been reported to occur in goat milk and milk fat (*30*−*32*) or in cooking oils believed to have been treated with HCl (*33*). In the latter work, the authors speculated that HCl may have been used to remove the aniline contained as a denaturant in the rapeseed oils used to adulterate the cooking oils. The milk fractions were however fresh and had not been exposed to HCl at any time. The presence of chloropropanediol diesters was explained by the esterification of either chloropropanediol or its monoester in the mammary gland. On the basis of this study, it is feasible that chloropropanediol diesters may have been formed as a side reaction of normal triacylglycerol lipolysis. Further reaction of these intermediates as depicted in **Figure 7** could then lead to the formation of chloropropanols.

The model experiments conducted here provide a new nonthermal pathway in the formation of chloropropanols in foodstuffs. In dried savory foods that contain salts and lipids, residual lipase activity of certain ingredients will be an important parameter to monitor, as it may reflect the formation of these storage and processing contaminants over time.

As far as the mechanistic aspects are concerned, more detailed work especially in identifying the chlorinated acyl intermediates and their role as possible substrates of hydrolytic enzymes is warranted. Further research addressing these intermediates is being conducted in our laboratory and will be reported in due course.

ABBREVIATIONS USED

ASE, accelerated solvent extraction; EC, European Community; EI, electron ionization; FAO, Food and Agriculture Organization; FAs, fatty acids; GC−MS, gas chromatography−mass spectrometry; HFBI, heptafluorobutyrylimidazole; HVPs, hydrolyzed vegetable proteins; MCPD, monochloropropanediol; OD, optical density; OFAs, other fatty acids; pNP, *p*-nitrophenol; RBDO, refined bleached and deodorized oil; U, unit; v_{max}, initial velocity; WHO, World Health Organization.

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