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Comparative characterization of baking lipase substrate specificities using emulsions and the *p*-nitrophenyl assay

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ABSTRACT

For use in cakes, lipases with suitable substrate specificity are needed to minimize the release of short-chain fatty acids that cause undesirable off-flavors. We analyzed the substrate specificities of 17 lipases using the *p*-nitrophenyl (PNP)-assay. These results were compared to the reactions of the lipases with the baking fats rapeseed oil, margarine and butter in a new model emulsion. The free fatty acids (FFA) released from this model emulsion were quantitated by gas chromatography. The broad spectrum of lipase specificities seen in the PNP-assay was not apparent in the patterns of FFA released from the model emulsions. There, all lipases released similar percentages of FFA depending on the fat and emulsifier used. The prediction of lipase reactions via the PNP-assay should therefore be critically reevaluated for real food systems such as cakes.

1. Introduction

Lipases are commonly used as additives for bakery goods and are well-known for improving dough and product characteristics of bread (Gerits et al., 2014). They catalyze the hydrolysis of the ester bond(s) of triacylglycerols (TAG) and release diacylglycerols, monoacylglycerols and free fatty acids (FFA). By interacting with the gluten network, FFA and polar lipids lead to enhanced dough consistency and eased machinability. Lipase-treated breads have higher product volumes, improved texture and delayed staling (Olesen et al., 2000). Therefore, lipases are considered as suitable replacements for traditional emulsifiers like mono- and diacetyl tartaric acid esters of mono- and diglycerides of fatty acids (DATEM, E472e) (Colakoglu & Özkaya, 2012).

Little is known about the effects of lipases in bakery goods which contain fat in the recipe. A first study showed a potential use for lipases in cake manufacturing to increase batter viscosity and reduce its aeration time (Guy & Sahi, 2006). However, a deeper understanding of the underlying reactions is missing and lipases are not yet applied. This is mostly due to the rancidity caused by lipases when interacting with baking fats (Gerits et al., 2014). Especially the released short-chain fatty acids (SCFA, less than 7 carbon atoms) as well as caprylic acid and capric acid among medium-chain fatty acids (MCFA, 8 to 12 carbon atoms) lead to undesirable off-flavors. The odors of butyric acid (C4:0) and caproic acid (C6:0), e.g., are described as cheesy, sweaty and sour (Wang & Xu, 2009).

This problem could be overcome by using lipases which selectively do not release odor-active FA according to their substrate specificity. A specificity mainly for long-chain fatty acids (LCFA, more than 12 carbon atoms) could therefore lead to the desired improvement of cake dough and product characteristics while maintaining the natural flavor of cakes.

The most common way to determine the substrate specificity of lipases is the *p*-nitrophenyl (PNP)-assay (Beisson et al., 2000; Song et al., 2008; Vanleeuw et al., 2019), although the use of PNP-substrates has been criticized, mostly due to lack of comparability to natural substrates like TAG in foods.

Lipases are water-soluble enzymes, while their substrates are fat-soluble. An emulsion of both phases is therefore needed for the reaction to take place, but there is no standard procedure for emulsion preparation to analyze lipase reactions. Ultrasonic treatment has been used (Carpen et al., 2019), as well as stirring (Avelar et al., 2013), shaking (Omar et al., 2016) or homogenization (Bourlieu et al., 2012; Byun et al., 2007). The resulting droplet size of the emulsion directly affects lipase activity with increasing interfacial areas leading to higher lipase activities (Saktaweewong et al., 2004). Incubation times range from 30 min to 48 h (Bourlieu et al., 2012; Byun et al., 2007). Further, the emulsifier and the viscosity of the resulting emulsion affects lipase activity either positively or negatively, depending on the specific setup

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of the emulsion (Dikeman & Fahey, 2006; Palacios et al., 2014).

After the reaction, FFA need to be extracted from the emulsion and quantitated. Gas chromatography (GC) is the method of choice for FFA analysis (Mannion et al., 2016b) and it has been used for FFA from milk and rapeseed oil (Amores & Virto, 2019; Zadernowski & Sosulski, 1978). FFA can either be analyzed directly or after derivatization (Amores & Virto, 2019; Jong & Badings, 1990). Although the reaction to methyl esters has been widely used, it is not suitable for the analysis of SCFA, because butyric acid methyl ester coelutes with the solvents (Mannion et al., 2016b, 2019). However, since butyric acid in particular is associated with rancid off-flavors, its release should be included in the analysis. Butyl esters were suitable for this purpose (Mannion et al., 2019).

We hypothesize that fat structure and the reaction matrix influence the substrate specificity of lipases. Therefore, our aim was to create a model emulsion for the reaction of lipases with naturally occurring fats to analyze substrate specificity in comparison to the PNP-assay. The released FFA were quantitated as butyl esters via GC and the resulting FFA distribution was compared to the results of the PNP-assay. A deeper understanding of the relationship between lipase specificities depending on the food matrix would ease the prediction of lipase reactions in different media especially concerning the development of off-flavors through SCFA.

2. Materials and methods

2.1. Chemicals and enzymes

All chemicals were of analytical grade or higher. PNP-butyrate, PNP-caprate, PNP-laurate, PNP-myristate, PNP-palmitate and PNP-stearate, boron trifluoride-butanol and all fatty acid standards were from Merck (Darmstadt, Germany). PNP-caproate was from TCI (Eschborn, Germany) and PNP-caprylate from Alfa Aesar (Karlsruhe, Germany). *p*-Nitrophenol, Triton X-100 and gum arabic (GA) were from Carl Roth (Karlsruhe, Germany). Egg yolk lecithin (EL) was from VWR (Darmstadt, Germany). Lipases (randomly named A to Q) were kindly donated by ABEnzymes (Darmstadt, Germany), DSM (Heerlen, Netherlands), Kuchenmeister (Soest, Germany), Novozymes (Bagsvaerd, Denmark) and Backaldrin (Asten, Austria). All lipases were used before as baking lipases in bread. Rapeseed oil, margarine and butter were bought at local supermarkets.

2.2. Determination of lipase substrate specificity using the PNP-assay

Lipase substrate specificity was determined using the PNP-assay according to Glogauer et al. (2011) with small modifications. Stock solutions (17.5 mmol/L) of all PNP-derivatives were prepared in acetonitrile:2-propanol (1:4, v:v) and stored frozen. These solutions were stable for at least 2 months. The stock solutions were diluted with assay buffer (50 mmol/L Tris-HCl, 1 mmol/L $CaCl_2$, 0.3% Triton X-100, pH 7.5) directly before use. Lipases were dissolved at 1 mg/mL in lipase buffer (50 mmol/L Tris-HCl, 1 mmol/L $CaCl_2$, pH 7.5). For calibration, *p*-nitrophenol solutions from 0.005 mmol/L to 0.175 mmol/L were prepared using assay buffer. The analysis was carried out in 96-well plates using a Tecan multiplate reader (Tecan, Männedorf, Switzerland). The substrate specificity of one lipase towards all substrates was determined in sextuplicate on a single plate. Lipase solution (20 μL) was added to 230 μL of substrate working solution or 230 μL of assay buffer (lipase control) or 230 μL of calibration solutions (calibration). Additionally, 20 μL of lipase buffer were added to 230 μL of substrate working solution (substrate control). The absorbance at 410 nm was recorded at 30 °C for 60 min.

The absorbance of the released *p*-nitrophenol was corrected by subtracting both lipase control and substrate control at the corresponding time. The enzymatic activities were expressed as mmol *p*-nitrophenol/min and checked for linearity using linear regression ($R^2 > 0.98$). To ensure comparability, all activities of one lipase were recalculated as percentage of the total activity (sum of all activities) of the corresponding lipase.

2.3. Characterization of a model emulsion for the reaction with natural substrates

2.3.1. Preparation of emulsions

To ease comparability, the preparation of emulsions was chosen to be as close to the PNP-assay as possible, including Triton X-100 as emulsifier. Rapeseed oil, margarine or butter (50 g) were weighed into a beaker, mixed with 150 mL of emulsifying solution (50 mmol/L Tris-HCl pH 7.5, 1 mmol/L $CaCl_2$, 2.5% Triton X-100) heated to 50 °C and stirred thoroughly for 20 min at 22 °C. The emulsion was dispersed with an Ultra-Turrax (IKA, Staufen im Breisgau, Germany) with a stator diameter of 18 mm for 2.5 min at 12500 rpm. For incubation, the emulsions were mixed (1:1, m:v) with 20 mg/mL of lipase solution in lipase buffer (50 mmol/L Tris-HCl pH 7.5, 1 mmol/L $CaCl_2$) or lipase buffer (control) and shaken in an overhead shaker for 16 h at 22 °C. The enzyme:substrate ratio had been optimized in preliminary experiments so that the amount of FFA released was within the range of quantitation.

2.3.2. Particle size distribution

The particle size distribution of the emulsions was determined in triplicate 3 h after preparation by laser diffraction using a LS13320 XR (Beckman-Coulter, Brea, CA, United States). Three more samples of each fat were incubated in an overhead shaker for 24 h prior to analysis.

2.3.3. Petrographic microscopy

The samples (see 2.3.2) were also analyzed using an Eclipse LV100 ND microscope (Nikon, Minato, Japan) and the software NIS Elements BR (Nikon) by polarized light microscopy. This makes optically anisotropic materials like fat crystals visible without staining agents.

2.4. Analysis of fatty acids released from the model emulsion

The method includes the FA butyric acid (C4:0), caproic acid (C6:0), caprylic acid (C8:0), capric acid (C10:0), lauric acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2) and α-linolenic acid (C18:3) as well as valeric acid (C5:0), undecanoic acid (C11:0) and nonadecanoic acid (C19:0) to be used as internal standards (IS). The solvent for all standards was 2% formic acid in diethyl ether. Unless indicated otherwise, all determinations were carried out in triplicate.

2.4.1. Extraction, purification and derivatization of FFA

The extraction and clean-up of FFA was performed following Mannion et al. (2016a) with minor modifications. To 5 mL of the emulsions described above, 10 g of anhydrous sodium sulfate, 0.3 mL of 2.5 mol/L sulfuric acid and 1 mL of IS solution (4 mg/mL of each compound) were added and extracted three times with 15 mL of diethyl ether:n-heptane (1:1, v:v) by shaking for 3 min followed by centrifugation. The supernatants were combined and purified via SPE: Aminopropyl columns (500 mg, Macherey-Nagel, Düren, Germany) were conditioned twice with 5 mL n-heptane. After applying the lipid extracts to the column, neutral lipids were removed using twice 5 mL of 20% diethyl ether in n-heptane (v:v). FFA were then eluted from the column with 4 mL of 2% formic acid in diethyl ether (v:v).

Derivatization of FFA to butyl esters was based on the method by Mannion et al. (2019). The purified extract (750 μL) was transferred into an amber glass tube with 750 μL of a 10% BF_3 solution in 1-butanol and 500 μL of n-heptane and vigorously shaken for 1 min. The mixture was heated to 80 °C for 60 min with gentle shaking. After cooling to 22 °C, the derivatized FFA were washed three times with 10 mL of distilled water following the procedure by Iverson and Sheppard (1997) to remove 1-butanol. The organic supernatant was used for further

analysis.

2.4.2. Gas chromatography with flame ionization detection

GC was carried out on a Shimadzu GC 2010-Plus (Shimadzu, Duisburg, Germany) with a flame ionization detector and a DB-FATWAX Ultra Inert column (30 m × 0.25 mm, film thickness 0.25 µm, Agilent Technologies, Waldbronn, Germany) with pre-column (deactivated fused silica, 5 m × 0.25 mm). The injection volume was 1 µL, the inlet temperature 220 °C and a split ratio of 10:1 up to 100:1 was applied, depending on the concentration of the sample. The carrier gas was helium at a constant linear velocity of 40.0 cm/s. The detector temperature was 240 °C. The oven temperature program started at 40 °C and increased at a rate of 5 °C/min to 60 °C. This temperature was held for 2 min and then further increased at a rate of 10 °C/min to 220 °C. The final temperature was held for another 10 min. The total runtime was 32 min.

2.4.3. Quantitation of released FA and calculation of specificity factors

Quantitation of the released FA was performed using external calibrations of the ratio of analyte peak area to IS peak area. An IS with similar chain length was chosen for each FA (IS C5:0 for C4:0 and C6:0, IS C11:0 for C8:0, C10:0, C12:0 and C14:0, IS C19:0 for C16:0, C18:0, C18:1, C18:2 and C18:3). The calibrations were linear in a range from 0.001 to 1.7 (C6:0, C8:0, C10:0 and C12:0), 0.001 to 15 (C4:0, C14:0, C18:0 and C18:3) and 0.001 to 100 (C16:0, C18:1 and C18:2). To determine the intermediate precision of the experiment (same person, same instrument), emulsions of all fat types were prepared in triplicate on three consecutive days and incubated with lipase A overnight, worked up and the FFA were quantitated.

To enable comparison between the lipases considering the natural distribution of FFA of rapeseed oil, margarine and butter, specificity factors were calculated. They describe the distribution of FFA of a lipase-treated emulsion divided by the distribution of an emulsion of the same fat without lipase. The higher the specificity factor of a lipase towards a certain FA, the more of this FA is preferably released by the lipase.

2.4.4. Time course of the reaction

The time course of the reaction was recorded by stopping the reaction of samples of a butter emulsion incubated with lipase A at different time points (1 h, 2 h, 4 h, 6 h, 8 h) by adding the extraction solutions.

2.4.5. Impact of the emulsifier

To further investigate the influence of the emulsifier used, comparative studies were performed on three lipases A, J and Q using GA (20%, w:v, in water) and EL (2.5%, w:v, in water) as emulsifiers instead of Triton X-100.

2.5. Statistical analysis

Statistical analysis was performed using Microsoft Excel built-in functions (means and standard deviations) and Origin 2021b for analysis of variance (ANOVA) with Tukey's test ($p < 0.05$) (OriginLab Corporation, Northampton, MA, USA).

3. Results and discussion

3.1. Determination of lipase substrate specificity using the PNP-assay

Under the conditions of the assay, 16 of the 17 lipases hydrolyzed the PNP-substrates from C4:0 to C18:0 to a measurable amount of *p*-nitrophenol (Fig. 1). Lipase D showed no reaction. According to the manufacturer, the optimal conditions for this lipase are 45 °C–70 °C at a pH from 4.0 to 6.5. Therefore, the lack of effect was probably due to the reaction conditions, because the PNP-assay is carried out at 22 °C and a pH of 7.5.

The highest reaction rates were measured towards PNP-caprylate. The overall activities of the 16 lipases towards the substrate ranged

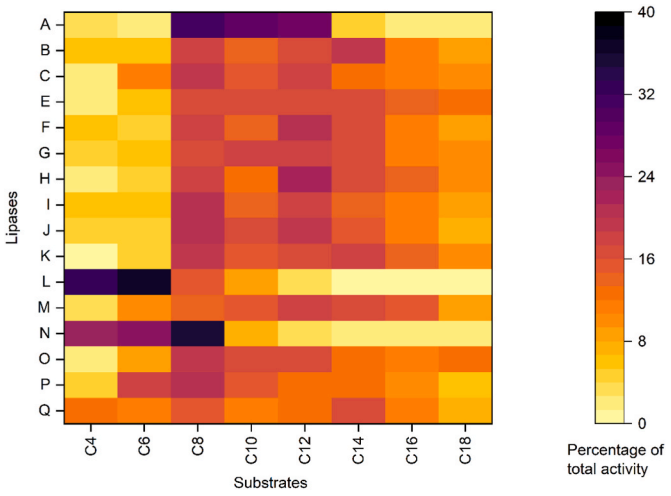

Fig. 1. Activity of different lipases A to Q towards the *p*-nitrophenyl-derivatives of different fatty acids from C4:0 to C18:0. All results (means, n = 3) are shown as percentage of the total activity (sum of all activities) of the respective lipase for better comparability (Table S1). Lipase D showed no reaction under the conditions of the assay.

from 4.1 ± 0.3 $g^{-1}s^{-1}$ to 99.4 ± 4.1 $mg^{-1}s^{-1}$ (Table S1), showing a more than 24,000-fold range of reaction rates. Twelve of the 16 lipases released mostly MCFA and LCFA, showing similar reaction patterns. Only four lipases did not match this pattern: Lipase A preferred MCFA, showing fast reactions with PNP-caprylate, PNP-caprate and PNP-laurate. Lipases L and N reacted exclusively with PNP-butyrate and PNP-caproate, therefore resembling more to esterases than to classical lipases. Lipase Q cleaved all PNP-substrates with approximately equal percentages of total activity. Still, except for lipases L and N, all lipases exhibited the highest reactivity towards MCFA. Studies of other lipases from the literature showed no comparable preference for those chain lengths (Cai et al., 2021; Noro et al., 2020). Instead, specificities for different FA chain lengths were found (Albayati et al., 2020). It is possible that the specificity for MCFA is a property specific to the baking lipases studied here. According to the PNP-assay, the lipases thus differ in both their substrate specificity and their overall activity. No clear trend for all lipases emerged in the substrate specificity patterns.

3.2. Characterization of a model emulsion for the reaction with natural substrates

The model emulsion used for the analysis of lipase reactions with naturally occurring fats has to fulfill three requirements. First, to ensure similar reaction conditions as in the PNP-assay, the emulsions were prepared with Triton X-100 as emulsifier at a pH of 7.5 and a $CaCl_2$ concentration of 1 mmol/L. Second, to ensure reproducibility, the emulsions have to be stable throughout the whole incubation process. The particle size distributions of the emulsions were monitored by their Sauter diameter, which describes the specific surface area, based on a mean spherical size of the disperse phase. The Sauter diameters of the samples ranged from 1.45 ± 0.09 to 4.22 ± 0.16 µm (Table 1), depending on the emulsified fat. There were no significant changes in the Sauter diameters of rapeseed oil and margarine emulsions over time. The interfacial surface of the butter emulsion increased over time as the Sauter diameter decreased by about 20% after 24 h compared to 3 h after preparation. Third, the model emulsion should be suitable for butter, margarine and rapeseed oil. Because butter and margarine are solid at 22 °C, melt emulsions have to be prepared, by heating the emulsifying solution to the melting point of butter and margarine before mixing. Petrographic microscopy was used to check for recrystallization. No crystals were formed in rapeseed oil emulsions (Fig. S1). In

Table 1
Sauter diameter of the different fats in emulsion measured 3 h and 24 h after preparation.

Fat	Sauter diameter [μm]	
	After 3 h	After 24 h
Rapeseed oil	4.16 ± 0.15^{a}	4.12 ± 0.15^{a}
Margarine	1.45 ± 0.09^{b}	1.47 ± 0.23^{b}
Butter	4.22 ± 0.16^{a}	3.34 ± 0.12^{c}

Results are indicated as mean values ± standard deviation (n = 3). Numbers with different superscript letters are significantly different (ANOVA with Tukey's test, $p < 0.05$).

margarine, crystals were formed in both the lipase-treated and the control samples (Fig. S2). Butter emulsions without lipase recrystallized over time. The addition of lipase inhibited the formation of crystals in butter emulsions (Fig. S3).

All in all, the developed model emulsion fulfilled all requirements. It was stable with unaltered particle size distribution for rapeseed oil and margarine and only small changes for butter. The melting process for solid fats led to reliable results, although partial recrystallization processes took place over time. The conditions in the emulsions were thus similar to the ones in the PNP-assay.

3.3. Analysis of fatty acids released from the model emulsion

A GC method was established for C4:0 to C18:3 and IS (Fig. 2). The test for intermediate precision showed that the relative standard deviation within one day (n = 3, mean of all analytes: 6.82%) was not significantly different from the one between three different approaches on consecutive days (n = 9, mean of all analytes: 7.80%) (Tukey's test, $p \leq 0.05$). Thus, the procedure was reproducible and suitable for further analyses.

The substrate specificities of the different lipases are reported as specificity factors in the following. They are based on the FFA distribution of an emulsion incubated without lipase addition (control, Tables S2–S7, Figs. S4–S6). The FA distributions of the fats were also considered. However, to account for the different behavior of FFA compared to FA, e.g., in terms of volatility and oxidative degradation, the FFA fractions were used for the calculation of specificity factors.

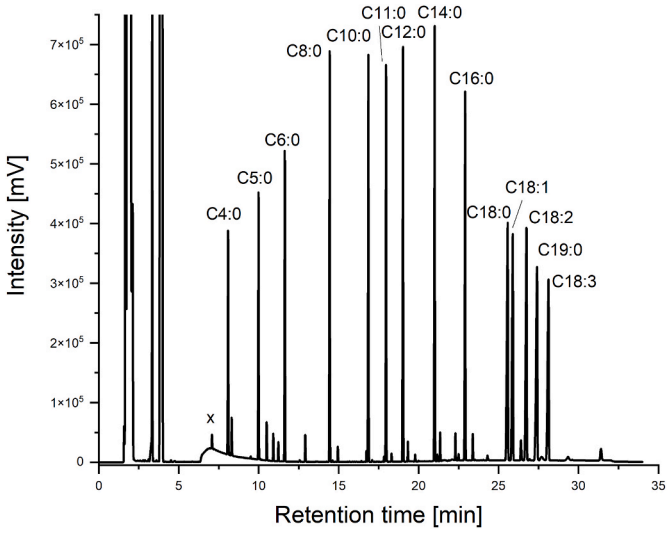

Fig. 2. Gas chromatographic separation of the free fatty acid butyl esters. x butanol, C4:0 butyric acid, C5:0 valeric acid, C6:0 caproic acid, C8:0 caprylic acid, C10:0 capric acid, C12:0 lauric acid, C14:0 myristic acid, C16:0 palmitic acid, C18:0 stearic acid, C18:1 oleic acid, C18:2 linoleic acid, C19:0 nonadecanoic acid, C18:3 α-linolenic acid.

Because the calculated specificity thus depends on the control sample, the FFA distribution of the control sample was analyzed carefully and compared to the FA distribution of each fat. Additionally, we considered that the total amounts of FFA released from the emulsified fats are notably lower than the amounts of FFA known from the analysis of pure fats. While rapeseed oil contains 0.5–3% FFA according to literature (Kusdiana & Saka, 2001; Zadernowski & Sosulski, 1978), only 0.1% were quantitated after emulsification and incubation. Similar effects also occurred with butter, which was found to contain 0.14–0.19% FFA (Mannion et al., 2016a, 2019), but only 0.1% were identified out of the model emulsion. This is probably due to losses, e.g., due to evaporation or oxidative degradation during the comparatively long incubation process that is required for the lipases to act.

3.3.1. Rapeseed oil

Rapeseed oil emulsions without lipase addition contained 0.1% FFA based on rapeseed oil content (Table S2). The distribution of FFA was as follows: 45.9% C18:1, 21.0% C16:0, 13.7% C18:2, 14.0% C18:0 and 5.4% C18:3 (Table S3). Lipase-treated samples contained 0.1–12.9% FFA (mean: 4.3%). The lipases D, F, L and N, which had shown low reaction rates in the PNP-assay, also released only low amounts of FFA. The highest percentages of FFA were released by the lipases C, O and P (11.3%, 12.9% and 9.9%, respectively). The specificity factors for the lipases in rapeseed oil emulsions showed a clear trend towards the preferred release of unsaturated FFA for most lipases (Fig. 3A). Only the lipases D, L, and N resulted in FFA distributions similar to those of the control.

For rapeseed oil emulsions without lipase addition (control), the distribution of FFA was similar to that known from literature (Zadernowski & Sosulski, 1978). Compared to the FA in the TAG fraction (Souci & Kraut, 2011), less unsaturated FFA were found. The FFA distribution after treatment with lipases resembled the one known from the TAG fraction (Table S3).

3.3.2. Margarine

The margarine emulsions without lipase (controls) contained 0.1% FFA based on margarine content (Table S4). Their FFA distribution included about one third unsaturated FFA (17.4% C18:1, 10.7% C18:2, 1.2% C18:3). Additionally, it consisted of high levels of saturated FFA (35.1% C16:0, 28.6% C18:0, 5.9% C12:0 and 1.1% C14:0) (Table S5). The lipase-treated margarine emulsions contained 0.1–39.8% FFA (mean: 21.7%). The highest concentrations of FFA in margarine emulsions were released by lipases C, E and M (39.8%, 39.2% and 38.0%, respectively). Lipases O and P, which were also highly active in rapeseed oil emulsions, resulted in comparatively high concentrations of FFA (30.9% and 26.9%). The lipases D, L and N again showed low activity.

The lipases released preferentially unsaturated FFA (Fig. 4A) from margarine. Only the FFA distribution of the three least reactive lipases D, L and N was similar to that of the control. Lipase F showed a specificity for C18:1. The remaining 13 lipases preferentially released C18:3 (specificity factor 4.8 to 7.2), less specifically also C18:1 (2.7–3.4) and C18:2 (1.3–2.0). The MCFA C12:0, which was amongst the substrates released with the highest reaction rates by all lipases in the PNP-assay, was only released with a specificity factor of a maximum of 0.8. The distribution of FFA of the untreated sample corresponds to the FA distribution known for TAG (Astiasarán et al., 2017; Anwar et al., 2006; Brát & Pokorný, 2000).

High concentrations of unsaturated FFA were found in all samples. 13 of 17 lipases had high specificities for C18:3, indicating a preferred release of this FFA. The other unsaturated FFA also were released preferentially by most lipases. Although differences in substrate specificities concerning the reaction with MCFA and LCFA were found in the PNP-assay, 13 of 17 lipases showed similar substrate specificities when reacting with margarine. Those results show that probably other factors than lipase substrate specificity influence the choice of substrate in this model emulsion. The results from the PNP-assay could not be confirmed.

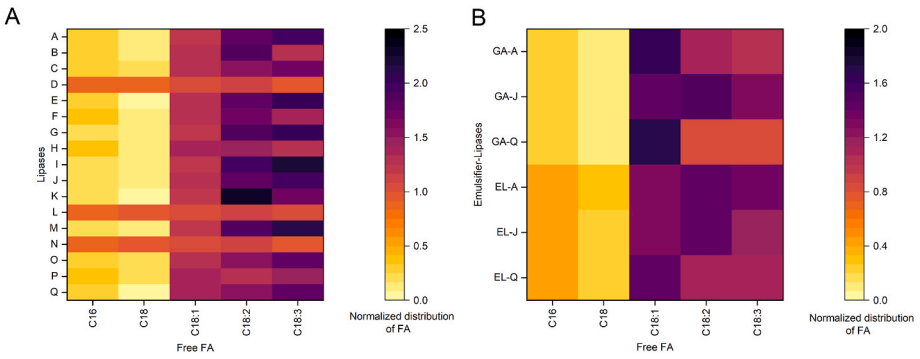

Fig. 3. Distribution of free fatty acids (FFA) released by 17 different lipases A to Q from rapeseed oil emulsified with Triton X-100 in water (**A**); or by lipases A, J and Q from rapeseed oil emulsified with gum arabic (GA) or egg yolk lecithin (EL) in water (**B**). All means (n = 3) were normalized to the results of a control sample without lipase to account for the natural occurrence of the different FFA in the rapeseed oil emulsions, respectively.

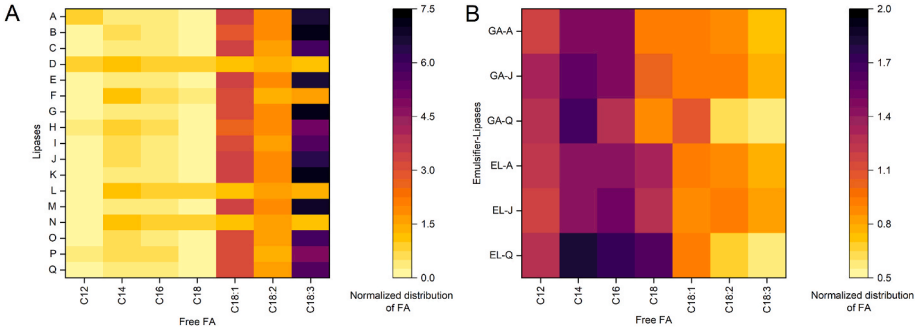

Fig. 4. Distribution of free fatty acids (FFA) released by 17 different lipases A to Q from margarine emulsified with Triton X-100 in water (**A**); or by lipases A, J and Q from margarine emulsified with gum arabic (GA) or egg yolk lecithin (EL) in water (**B**). All means (n = 3) were normalized to the results of a control sample without lipase to account for the natural occurrence of the different FFA in the margarine emulsions, respectively.

The decisive factor could possibly be the accessibility of substrates at the oil-water-interface.

3.3.3. Butter

Butter emulsions without lipase addition contained 0.1% FFA (based on butter content) (Table S6). They consisted of 39.8% C16:0, 22.0% C18:1, 17.1% C18:0, 7.8% C14:0, 4.5% C4:0, 3.4% C12:0, 2.3% C18:2, 1.6% C10:0, 0.9% C6:0 and 0.6% C8:0 (Table S7). The lipases released 0.1–25.9% FFA (mean: 13.2%). The highest amounts of FFA were found after incubation with the lipases C, I and O (25.9%, 25.4% and 25.1%, respectively). The lowest amounts were released by the lipases D, F, L and N (0.1%, 0.4%, 0.2% and 0.2%, respectively). Besides the lipases D, F and N, all lipases released preferentially the FFA C6:0 and C8:0 (specificity factors 2.6 to 3.8) and high amounts of C4:0 and C10:0 as well as unsaturated FFA (specificity factors 0.9 to 1.9) (Fig. 5A). The FFA distribution of the untreated butter sample corresponded to the distribution for FFA known from literature (Mannion et al., 2016a, 2019). Compared to the TAG fraction (Souci & Kraut, 2011), less C6:0 and MCFA were detected, probably due to their higher volatility. The amount of C4:0 found in free form, however, was not reduced compared to the TAG fraction (Table S7).

Similar to the margarine and rapeseed oil emulsions, most lipases showed very similar reactivity patterns, with a preferential release of unsaturated FFA. However, the lipases showed even higher specificities for C4:0, C6:0 and C8:0. The FA distribution of the TAG fraction contained higher amounts of C6:0 and C8:0 than the amounts found in the control sample that was used for normalization. Therefore, the alleged specificity for C6:0 and C8:0 was somewhat expectable and probably only due to the normalization. C4:0 was also released specifically, although this cannot be due to the normalization effect, because the

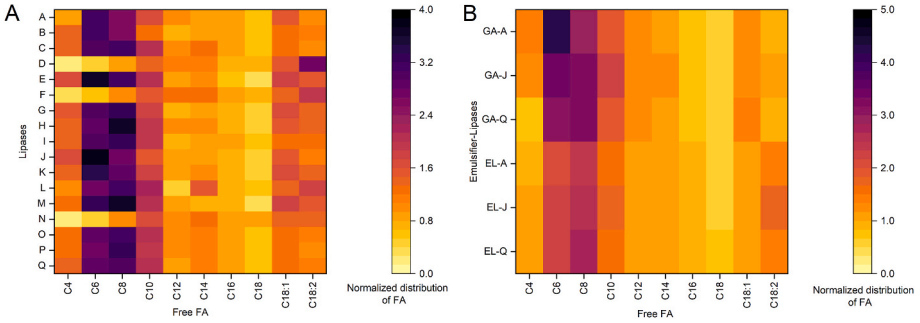

Fig. 5. Distribution of free fatty acids (FFA) released by 17 different lipases A to Q from butter emulsified with Triton X-100 in water (**A**); or by lipases A, J and Q from butter emulsified with gum arabic (GA) or egg yolk lecithin (EL) in water (**B**). All means (n = 3) were normalized to the results of a control sample without lipase to account for the natural occurrence of the different FFA in the butter emulsions, respectively.

amount of C4:0 used for normalization corresponded to the one known for TAG. In this case, the lipases showed a true preference for its release. This is in contrast to the results of the PNP-assay, where mostly C8:0 to C12:0 were released. The lipase substrate specificity as defined in the PNP-assay was therefore not reproducible for the reaction with butter. Similar to the reaction with margarine, specificity might not be the crucial factor for the reaction after all. Instead, the accessibility of FA could decide about their release. As C4:0 is more polar than longer chain FA, it is most likely located at the surface of the micelles, and therefore easily accessible for the water-soluble lipases.

3.3.4. Time course of the reaction

As the incubation time may be another factor that influences lipase-induced release of FFA from the model emulsion, shorter incubation times from 60 to 480 min were tested instead of the standard 960 min (16 h). The total amount of FFA released after incubation with lipase A increased from 0.1% to 6.0% within the first hour and another 2% were released in the next hour (Table S8). Thereafter, the total amount of released FFA decreased, presumably due to volatile FFA or degradation reactions. After 4 h, 6.0% FFA were found, after 6 h 5.5%, after 8 h 4.6%. As the reaction proceeded further, 23.6% FFA were identified after 16 h. In contrast, the composition of the released FA, as represented by the substrate specificity factors (Fig. 6), remained largely unchanged over the entire reaction period. For LCFA, the factors fluctuated by a maximum of 23% without a clear trend. The SCFA and some MCFA were released most strongly at 240 min (C4:0) or 120 min (C6:0 and C8:0). The specificity for C4:0 varied by a maximum of 17% during the course of the reaction. In sum, the reaction time had a minor influence on the distribution of FFA (Table S9), indicating that the type of emulsion itself is likely to be important to explain the similar FFA release pattern of the different lipases.

3.3.5. Impact of the emulsifier

If the type of emulsion and its associated micelle surface is indeed the decisive factor for the reaction, then its modification should lead to different lipase reactivity patterns, because the micelle surface is mainly determined by the chosen emulsifier (Berton-Carabin et al., 2014). This hypothesis was tested for three lipases in emulsions with GA and EL instead of Triton X-100. Triton X-100 (2-[4-(2,4,4-trimethylpentan-2-yl) phenoxy]ethanol) is a nonionic surfactant for laboratory applications and cleaning products. We used it in the model emulsions, because the model was originally designed to be as closely comparable to the PNP-assay as possible, only with natural fats as substrates instead of PNP-esters of FA. One drawback is that Triton X-100 is not used in real food systems. This is why we selected two different emulsifiers commonly used in foods, but with different molecular structures and emulsifying properties. GA is a mixture of arabinose- and galactose-based polysaccharides and glycoproteins, whereas EL is primarily composed of phospholipids.

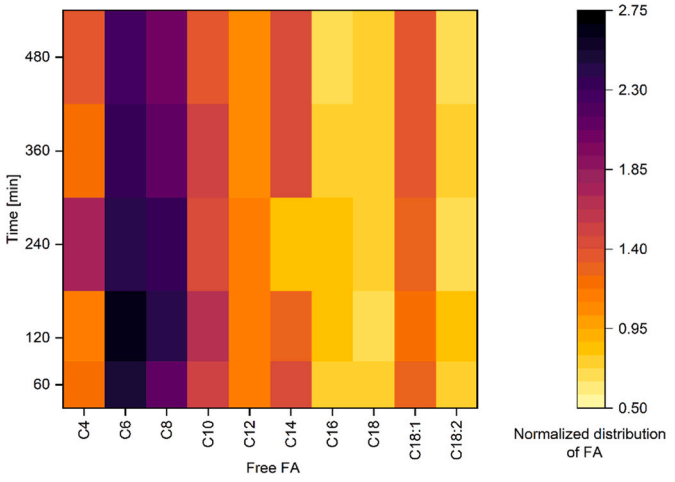

Fig. 6. Distribution of free fatty acids (FFA) released by lipase A within several time periods (60 min–480 min) from butter emulsified with Triton X-100 in water, calculated as mean (n = 2). All amounts were normalized with the results of a control sample without lipase to account for the natural occurrence of the different FFA in butter.

Because of their different reactivity patterns in the PNP-assay, the lipases A, J and Q were chosen. A change of the emulsifier also led to changes of overall reactivity rates as measured by the total amount of FFA after lipase treatment (Table S10). The change depended on the lipase, the emulsifier and the fat analyzed. In all fats, either increase or inhibition of lipase activity occurred, compared to the activity in the Triton-X emulsion: e.g., in rapeseed oil, the activity of lipase J was enhanced in GA and EL emulsions, while for the lipases A and Q this depended on the emulsifier. In margarine, the activities of the lipases A and Q were both inhibited when the emulsifier was changed, whereas that of lipase J was positively affected in EL emulsions, but negatively with GA. In butter, the activity of lipase A was increased by both emulsifiers while the activities of lipases J and Q were inhibited. Besides the direct effects of the emulsifier on the micelle surface, lipase activity could also be affected by the concomitant change of viscosity or droplet size of the emulsion. Another aspect is the availability of additional substrates for lipase reactions from EL which consists of phospholipids.

Additionally, both enhancement and inhibition of the release of single FFA occurred when GA and EL were used as emulsifiers. In rapeseed oil, e.g., the release of C16:0 and C18:0 by lipase A was highly affected: in combination with GA as emulsifier, it was reduced by 70% and 64%, while in the EL emulsion, 199% and 311% of C16:0 and C18:0 more than in the Triton X-100 emulsion were released, respectively. In margarine, especially the release of unsaturated FA was influenced by the change of the emulsifier. For lipase J, compared to the Triton X-100 emulsion, GA as the emulsifier lead to smaller releases of C18:1, C18:2 and C18:3 (by 35.7%, 35.5% and 50.7%) while the use of EL lead to considerably higher releases (by 71.4%, 72.8% and 45.2%, respectively). In butter, both new emulsifiers had similar impacts depending on the lipase, but to different extents. An example for this is the release of C4:0, which was reduced in case of lipase A by 67.2% (GA) and 46.4% (EL) and increased in case of lipase J and Q (35.9% and 203.8% for lipase J and 4.4% and 352.6% for lipase Q by GA and EL, respectively). Partly and for LCFA only, the increases can be explained by the addition of lipids from EL with a different FA distribution. However, those constitute only about 9% of total lipids and thus cannot explain the changes completely.

The results demonstrate the importance of the emulsifier for the reaction, also highlighting the dependence on the chosen lipase. The chosen lipase, however, seemed to be influenced mostly in its total activity, because similar patterns were again found for substrate specificities (Table S11).

Concerning substrate specificities in rapeseed oil, mostly unsaturated FFA were released in both emulsions as already seen for the Triton X-100 emulsion (Fig. 3B). In the EL emulsion, C16:0 and C18:0 were set free with slightly higher specificity factors than in the GA emulsion and the Triton X-100 emulsion (0.2–0.5 instead of 0.1–0.2 and 0.1–0.3). In margarine, all three studied lipases released preferentially C12:0, C14:0 and C16:0 from GA emulsions. In EL emulsions, all of them also showed substrate specificities for C18:0 (Fig. 4B), while in the Triton X-100 emulsion, they had mostly released unsaturated FFA. In butter, mostly C6:0, C8:0 and C10:0 were released from both emulsions with slightly lower specificity factors in the EL emulsion than in the ones with GA and Triton X-100 (Fig. 5B).

Within one type of emulsion (same fat and same emulsifier), the lipases again showed similar reactivity patterns. Similarities between all emulsions were visible, probably because the same sample was used for normalization. Still, substrate specificity factors depended on the chosen emulsifier, as is, e.g., clearly visible for the substrate specificity factors

towards C18:0 (margarine).

These findings support the hypothesis that a modification of the emulsion should lead to different lipase reactivity patterns. Fat structure and matrix, especially the surface of the fat micelles where the hydrolysis reaction takes place, influence the substrate specificity of lipases. One limitation is that our current approach did not account for potential positional preferences of lipases. FA are not randomly distributed over the TAG backbone, but with positional preferences in the outer sn1/3 vs. the inner sn2 position. It is expected that lipases are more likely to hydrolyze the outer FA due to better accessibility. Further work will include not only FFA analyses, but a more comprehensive lipidomics workflow to obtain in-depth insights into both substrate and positional specificities.

4. Conclusion

When reacting with baking fats in emulsions, 17 baking lipases released similar FA patterns, although their substrate specificities were characterized differently in the PNP-assay. Lipase activity seemed to influence the resulting pattern, as seen for the less active lipases D, F, L and N which mostly did not fit the trend observed for all lipases with higher activities. The similarities between the lipases are probably due to the different accessibilities of FA in fat micelles. Therefore, the reaction seems to be not only influenced, but decided by fat structure and surface properties of the micelles. This effect was further proven by showing that a change of emulsifier led to considerable changes in substrate specificities. The PNP-assay is hence not suitable to predict lipase reactions in complex media such as emulsions. Its use to identify lipases with certain substrate specificities should be critically reviewed.

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CRediT authorship contribution statement

Charlotte Dorothea Stemler: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. **Katharina Anne Scherf:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare no competing financial interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2022.113914.

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