Increasing Short-Chain Fatty Acid Yield During Lipase Hydrolysis of a Butterfat Fraction with Periodic Aqueous Extraction

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ABSTRACT: Factors affecting the release of short-chain fatty acids during hydrolysis of a butterfat fraction with a 1,3-positional and short-chain-specific *Penicillium roqueforti* lipase were investigated. When a short-chain triglyceride fraction was used as substrate, as opposed to whole butterfat, the ratio of desirable flavor short-chain free fatty acids (FFA) to undesirable medium-chain FFA in the FFA fraction increased from 0.75 to 1.80. However, with both substrates, FFA accumulation eventually led to lipase inhibition and limited the total amount of triglyceride hydrolysis. This inhibition phenomenon was principally due to product inhibition. Periodically extracting the FFA with a buffer solution minimized this inhibition phenomenon, thereby significantly increasing lipase activity and the degree of triglyceride hydrolysis. Thus, on-line extraction of FFA in lipase reactors has the potential of greatly increasing system productivity.
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KEY WORDS: Butterfat fraction, extraction, fatty acid, inhibition, lipase.

The main advantage butter has over other edible fats and oils is its highly desirable flavor. Many of butter's desirable flavor notes result from the presence of short-chain ($C_{4:0}$ to $C_{8:0}$) free fatty acids (SCFFA). These compounds are found in substantial quantities, both as free fatty acids (FFA) and as part of triacylglycerols (TAG) in butterfat. Lipases that hydrolyze TAG to produce elevated levels of SCFFA are often added to cheese to enhance flavor development (1). Enzymatically lipolyzed butterfat can also serve as a base for the production of food flavor ingredients (2). However, to obtain the most appealing flavor, the production of $C_{10:0}$ and $C_{12:0}$ FFA—compounds that produce an unpleasant, soapy note—must be minimized (2,3).

With butterfat, several strategies have been used to maximize the yield of SCFFA per total amount of FFA. First, because the short chains tend to reside on the 1 and 3 positions on the TAG molecule, 1,3-specific lipases can be used to selectively hydrolyze these compounds (4). Lipases, such as those from *Candida cylindracea* that are not 1,3-specific but tend to show a preference for short- vs. longer-chain fatty acids, have also been used (5). In this study, we chose to use the lipase derived from *Penicillium roqueforti*, an enzyme that is both 1,3- and short-chain-specific (6).

In certain situations, it would also be desirable to have as high a concentration of SCFFA as possible in the hydrolyzed butterfat; for example, when producing low-fat cheese or flavor bases with a high flavor impact. One potential method of increasing the SCFFA concentration would be to raise the amount of short-chain, low-molecular-weight compounds in the TAG substrate. Such a substrate can be produced from butterfat by short-path distillation (7). Thus, one of the goals of this work is to determine if substrate alterations can increase the total concentration of desirable SCFFA in hydrolyzed butterfat.

Another problem that can limit the amount of SCFFA produced is product inhibition (8). With free-enzyme systems, the buildup of FFA will reduce lipase activity until eventually the enzyme loses its catalytic ability (9). Product inhibition can be minimized by immobilizing the lipase on a solid support, but at the cost of a large loss in activity (10). Therefore, the second goal of this study was to better understand the underlying mechanisms of this inhibition phenomenon.

MATERIALS AND METHODS

Materials. A short-chain fraction of cow's butterfat was produced on a pilot-scale short-path distillation unit at Université Laval (Ste. Foy, Québec, Canada). The fraction was obtained at a temperature and pressure of 225°C and 100 µm Hg, respectively. The fatty acid compositions of the short-chain fraction and whole butterfat used in this study are given in Table 1. *Penicillium roqueforti* lipase (Amano R: 900–1,000 U g^{-1}) was purchased from Amano Enzyme USA (Lombard, IL). All solvents and chemicals used were of the highest purity and, if not mentioned otherwise, were obtained from Fisher Scientific (Toronto, Canada).

Butter oil hydrolysis and FFA extraction. A 638-µL sample of a 2 mg/mL lipase solution in 0.05, 0.20, or 0.40 M phosphate buffer (pH 7.0) was first added to 10 g of butterfat or the short-chain fraction and then emulsified with a Polytron Tissumizer (Trekmar, Cincinnati, OH) for 2 min. The pH and water concentration used were the optimal conditions

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TABLE 1
Free Fatty Acid Composition of the Short-Chain Fraction and Whole Butterfat

Fatty acid	Short-chain fraction (wt%)	Whole butterfat (wt%)
$C_{4:0}$	8.25	3.10
$C_{6:0}$	4.20	1.97
$C_{8:0}$	2.77	1.10
$C_{10:0}$	5.71	2.27
$C_{12:0}$	6.42	3.24
$C_{13:0}$	0.21	0.28
$C_{14:0}$	16.2	11.1
$C_{14:1}$	1.02	1.06
$C_{15:0}$	1.49	2.88
$C_{16:0}$	31.7	28.6
$C_{16:1}$	1.24	1.30
$C_{17:0}$	0.48	2.76
$C_{18:0}$	6.72	12.8
$C_{18:1}$	11.8	24.2
$C_{18:2}$	1.31	2.39
$C_{18:3}$	0.42	0.95

suggested by the enzyme manufacturer. After placing the test tubes with the emulsions in a 30°C heated recirculating water bath (Caron, Marietta, OH), samples were taken at various times and analyzed for FFA by gas chromatography. For enzyme activity initial rate experiments, samples were taken every 10 min for a period of 1 h. With the extraction experiments, sampling rates were typically once every 2 h.

To extract the FFA produced by the lipase reaction, the hydrolyzed oil sample was mixed with a 0.40 M sodium phosphate buffer solution (pH = 7.0) in an oil-to-buffer ratio of 1:4 and vortexed for 1 min. Phase separation was accelerated by ultracentrifuging (Beckman model L8-70M; Fullerton, CA) the sample for 10 min at 90,000 × *g* and 25°C. After ultracentrifugation, the top oil layer was decanted from the emulsion and water fractions, and a 30-mg sample of the oil was weighed into a 10-mL vial. This sample was then analyzed for fatty acid concentration. From the difference between the total amounts of each FFA before and after reaction, the amount of hydrolyzed FFA could be compared with the initial amounts present in the TAG solution to determine the percentage hydrolysis of the various FFA.

After separation and analysis, the remaining oil was weighed, and fresh enzyme solution was added to restart the hydrolysis reaction. Care was taken to compensate for any butter oil losses that occurred during the extraction process by maintaining the same oil-to-enzyme-solution ratio throughout the extraction experiment.

Partition coefficient measurements. Butter oil with known quantities of butyric, caproic, caprylic, or oleic acid was mixed with 0.40 M sodium phosphate buffer, pH 7.0, in an oil-to-buffer ratio of 1:4. The mixture was incubated for 1 h in a 30°C recirculating water bath (Caron). After vortexing the sample for 1 min, the buffer containing the FFA was separated in an ultracentrifuge (Beckman L8-70M; 10 min, 90,000 × *g*, 25°C). The top oil phase was then analyzed by gas chromatography.

Gas-chromatographic analysis of FFA. Gas-chromatographic analysis was performed on a Varian 6000 gas chromatograph (Georgetown, Ontario, Canada), connected to a Vista 401 data system. The 2.2 m × 4 mm i.d. coiled glass column was packed with 10% Silar 9CP on Chromosorb W, AW 80/100 mesh (Chromatographic Specialties Ltd., Brockville, Ontario, Canada). The carrier gas was high-purity N_2 with a flow rate of 20 mL min^{-1} (BOC gases, Guelph, Ontario, Canada). The flame-ionization detector and injector were held at 235 and 190°C, respectively. The oven was programmed as follows: initial temperature, 60°C; 15°C min^{-1} to a final temperature of 210°C; 210°C for 8 min.

The amount of FFA liberated by the lipase was measured according to the method of Williams and Macgee (11) with some modifications. About 30 mg oil, prepared as described under "Butter oil hydrolysis and FFA extraction," was weighed into a 10-mL glass centrifuge tube and then brought up to a volume of 5 mL with hexane that contained 18 μg mL^{-1} margaric acid ($C_{17:0}$) as an internal standard. After the mixture was vortexed for 15 s, 200 μL of trimethylphenylammonium hydroxide (TMPH) solution was added, and the sample was again vortexed for 15 s, then centrifuged at 7000 × *g* for 1 min. After the hexane phase was removed and discarded, 5 mL diethyl ether was added to remove any remaining glycerides. After centrifugation, the ether was removed, and 5 mL hexane was added and mixed on a vortex for 10–15 s. After centrifugation at 7000 × *g* for 1 min, the lower phase was ready for injection into the gas chromatograph. A 0.5–2-μL sample, followed by 0.5 μL methyl acetate, was drawn up into the syringe and then injected into the gas chromatograph. AOCS No. 5 (Sigma Chemical Co., St. Louis, MO) was used as an external standard.

RESULTS

Figure 1 shows the production time course of the principal fatty acids during short-chain butterfat fraction hydrolysis. For all FFA, a concentration plateau was reached at approximately 2 to 6 h. With regular butterfat, concentration plateaus were also observed after a comparable length of time (results not shown). However, the final plateau concentrations of the various fatty acids were different for the short-chain and whole butterfat fractions (Table 2). In addition, on a weight basis, more fatty acids were produced with the short-chain butterfat fraction. But perhaps more importantly, the ratio of desirable ($C_{4:0}$ to $C_{8:0}$) to undesirable ($C_{10:0}$ and $C_{12:0}$) fatty acids was much higher in the short-chain fraction (1.80) as compared to whole butterfat (0.75). However, high levels of oleic acid ($C_{18:1}$) were produced in both samples. Because 28% of oleic acid is found on the *sn*-2 position of butterfat (12), it is unlikely that the high levels of this acid produced were simply due to the enzyme's 1-3-specificity. It is more likely that *P. roqueforti* lipase has an affinity for this fatty acid.

To better understand the reasons why these concentration plateaus were observed, the experiment in Figure 1 was repeated with different buffer types (carbonate and TRIS) and

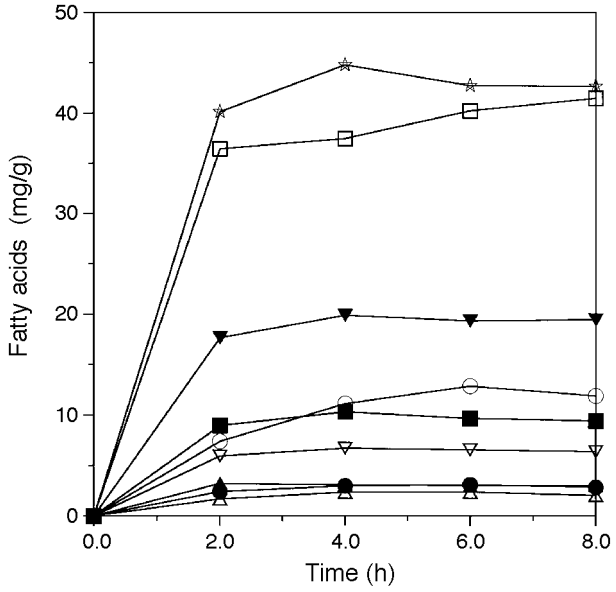

FIG. 1. Free fatty acid concentration as a function of time during hydrolysis of the short-chain butterfat fraction: ○, $C_{4:0}$; ●, $C_{6:0}$; △, $C_{8:0}$; ▲, $C_{10:0}$; ▽, $C_{12:0}$; ▼, $C_{14:0}$; □, $C_{16:0}$; ■, $C_{18:0}$; ☆, $C_{18:1}$.

strengths (0.05, 0.2, and 0.4 M). In all experiments, results were similar to what was observed in Figure 1. Short-chain FFA, such as butyric acid, are water-soluble and will partition into the aqueous phase, causing the pH to drop. This effect was quite significant with the 0.05 M buffer solution, leading to a final aqueous pH of 4.36. Nevertheless, the enzyme was determined to still have an initial rate that was 22% of its value at pH 7.0. Consequently, not all of this activity loss could be attributed to decreasing pH. With a 0.40 M phosphate buffer, the pH only dropped to 5.70 at the activity plateau. At this pH, initial activity experiments indicate that the lipase should have 91% of the activity observed at pH 7.0. Our initial activity results at pH 5.70 were similar to the data supplied by the enzyme manufacturer (6). Therefore, it appears that, at high buffer strength, the pH shift caused by the presence of SCFFA only plays a minor role in lipase activity loss.

Another potential explanation for the concentration plateaus is that high levels of FFA destabilize the *P. roqueforti* lipase, leading to denaturation. A dilution experiment with 0.40 M phosphate buffer (pH = 7.0) was performed to determine if denaturation played a role. Once the FFA concentrations plateaued, an additional 10 g of butterfat was added to the 10 g already present. Results indicated that, for all FFA examined (Fig. 2 gives representative curves for butyric and caprylic acid), this dilution almost immediately restored enzymatic activity; however, the rate of hydrolysis was approximately one-half of the initial activity (probably a direct result of the doubling of the reaction volume with the additional substrate, and the attendant halving of product concentrations, rather than a real halving of enzyme). This indicates that the enzyme was still quite active. Nevertheless, hydrolysis ceased upon achieving the same FFA concentrations as shown in Figure 1.

Product inhibition could also be causing the dramatic decrease in lipase activity. To test this hypothesis, different levels of various FFA were introduced into the short-chain fraction before the 0.40 M phosphate-buffered (pH = 7.0) enzyme solution was added. The resulting effect of added FFA concentrations, relative to that observed without FFA addition, is plotted in Figure 3. Low concentrations of butyric acid (<20 mg g^{-1}) actually caused a slight increase in lipase activity. This activation by $C_{4:0}$ has been observed previously by Elliot and Parkin (9) with lipase-catalyzed acyl-exchange reactions. However, activation was not observed with the other FFA examined, which was also true in the Elliot and Parkin study (9). On a weight basis, both caproic and caprylic acids inhibited lipase activity to a similar degree, whereas oleic acid much more strongly inhibited lipase activity. Because oleic acid has a much higher molecular weight than caproic or caprylic acids, on a molar basis this difference in inhibitory

TABLE 2
Plateau Concentrations and Percentage Hydrolysis of Principal Fatty Acids After Eight Hours of Lipolysis

Fatty acids	Short-chain fraction		Whole butterfat	
	(mg/g)	(%hydrolysis)	(mg/g)	(%hydrolysis)
$C_{4:0}$	12.5	20.9	3.15	11.2
$C_{6:0}$	3.21	10.2	1.23	6.86
$C_{8:0}$	2.38	11.2	0.95	9.48
$C_{10:0}$	3.31	3.31	1.67	8.09
$C_{12:0}$	6.72	6.72	5.43	18.5
$C_{14:0}$	19.6	19.6	9.53	9.48
$C_{16:0}$	40.9	40.9	30.8	11.9
$C_{18:0}$	9.72	9.72	13.5	11.6
$C_{18:1}$	42.9	42.9	37.1	16.9

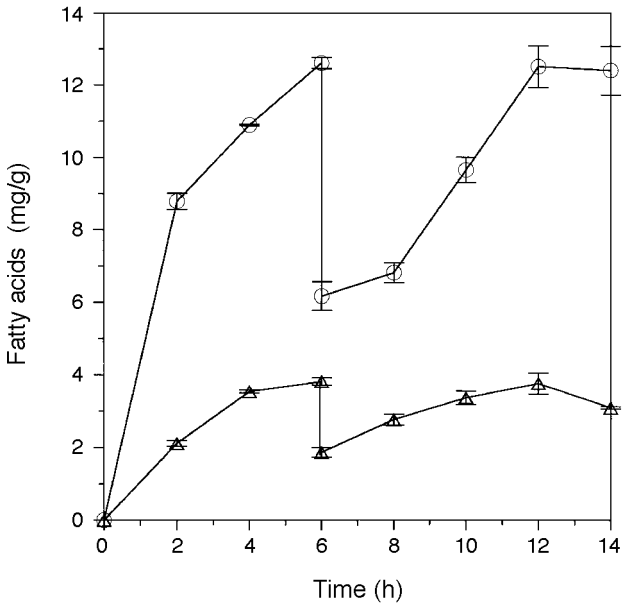

FIG. 2. Free fatty acid concentration as a function of time during hydrolysis of the short chain butterfat fraction with dilution after 6 h: ○, $C_{4:0}$; △, $C_{8:0}$.

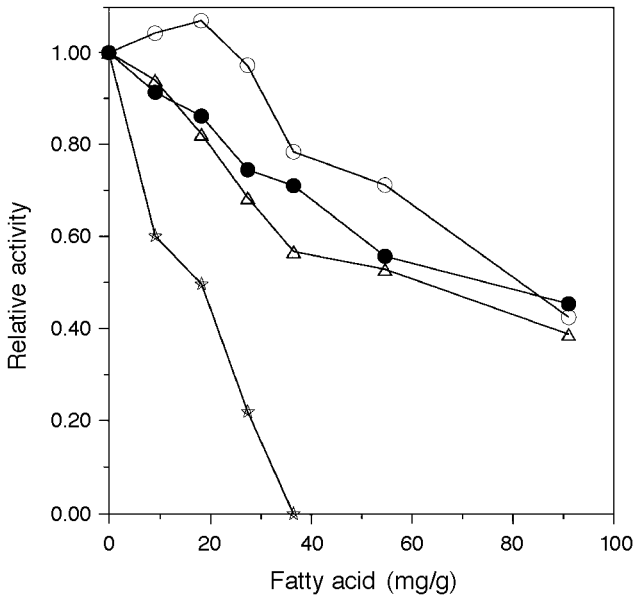

FIG. 3. Relative initial activity of lipase as a function of added fatty acid concentration: ○, $C_{4:0}$; ●, $C_{6:0}$; △, $C_{8:0}$; ☆, $C_{18:1}$.

effect would be more significant. Inhibition of lipase hydrolysis by FFA has been previously reported in the literature. For example, Dunhaupt *et al.* (13) studied the inhibitory effect of $C_{8:0}$ to $C_{18:1}$ FFA on the hydrolytic activity of a *Pseudomonas cepacia* lipase in olive oil. With this lipase, inhibition was also fatty acid-specific, with $C_{8:0}$ being less inhibitory than $C_{18:1}$ on a weight basis. The concentration at which oleic acid completely inhibited *P. roqueforti* lipase activity (37 mg g^{-1}) was slightly lower than the oleic acid plateau level (42.9 mg g^{-1}) observed in Figure 1. Perhaps the activation effect that would be present with butyric acid at the plateau concentration of 12.5 mg g^{-1} partially counterbalanced the inhibitory effect of the $C_{18:1}$ FFA.

One method for counteracting the effect of product inhibition is to remove the inhibiting species as they are being produced. This approach has proven effective in increasing the productivity of product-inhibited enzymatic (14) and fermentation (15) reactors. A simple method for removing FFA from the short-chain fraction would be to wash the fat with buffer solution. To test if this method is effective, a range of concentrations of various FFA was first added to samples of the short-chain fraction. Samples were first taken to obtain precise initial concentration values. These butter oil solutions were then contacted with a known quantity of 0.40 M phosphate buffer (pH = 7.0). After ultracentrifugation, three layers were formed: a top oil phase, a middle emulsion phase, and a bottom aqueous phase. The middle emulsion phase was typically less than 10 vol% of the fat phase. By measuring the FFA concentration in the butterfat phase before and after being contacted with the buffer solution, the concentration of FFA in the buffer + emulsion phases can be determined by a mass balance. A partition coefficient ([butterfat]/[buffer + emulsion]) can then be calculated from these two concentrations.

The partition coefficient was a function of concentration for all FFA examined (Fig. 4). However, at concentrations above 15 mg g^{-1}, the partition coefficient tended to plateau to a constant value. With butyric, caproic, caprylic, and oleic acid, plateau partition coefficient values of approximately 0.012, 0.018, 0.042, and 0.057, respectively, were observed. Thus, the vast majority of the FFA were found in the buffer + emulsion water phase. Because of the low solubility of many of the longer-chain FFA in water, it is most likely that the majority of these molecules were concentrated in the emulsion phase. Nevertheless, these results indicate that extracting 10 g of the short-chain fraction with 40 g of water would reduce the amount of FFA by over 98%.

Figures 5 and 6 illustrate the effect of periodic extraction of FFA on the degree of $C_{4:0}$ and $C_{8:0}$ hydrolysis, respectively, in the short-chain fraction (similar results were observed for all FFA). As observed in Figure 1, with no extraction, all FFA concentrations reached a constant level after about 4 h; the resulting percentage total hydrolysis was relatively low. However, if FFA were extracted once every 2 h, a substantial improvement in total FFA hydrolysis was noted (Figs. 5 and 6). After 8 h, a doubling of the percentage hydrolysis was observed with $C_{4:0}$, whereas with $C_{8:0}$, the percentage hydrolysis increased by a factor of 3.8.

Even greater improvement was observed when extractions were performed once every hour. With both $C_{4:0}$ and $C_{8:0}$, almost 40% hydrolysis could be obtained in 4 h. Beyond 4 h, the reaction began to slow significantly, perhaps due to the accumulation of mono- and diglycerides that also could inhibit lipase activity. Unfortunately, beyond 40% conversion, emulsion formation during extraction became significant, making it difficult to continue the experiment to higher degrees of conversion.

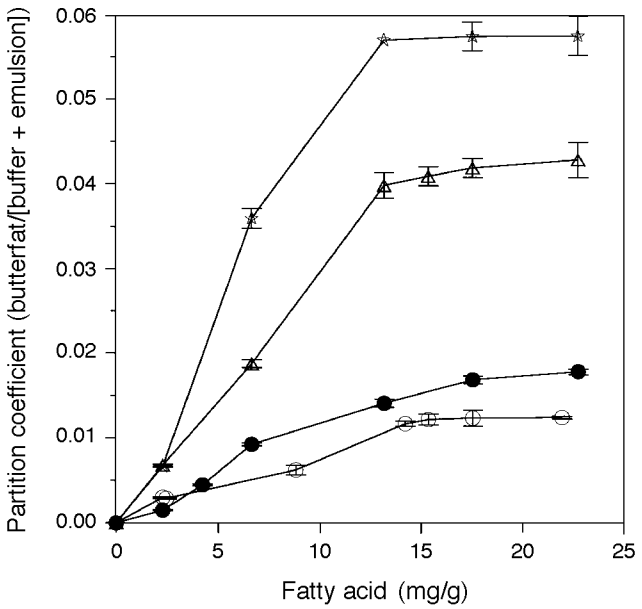

FIG. 4. Free fatty acid partition coefficient as a function of fatty acid concentration: ○, $C_{4:0}$; ●, $C_{6:0}$; △, $C_{8:0}$; ☆, $C_{18:1}$.

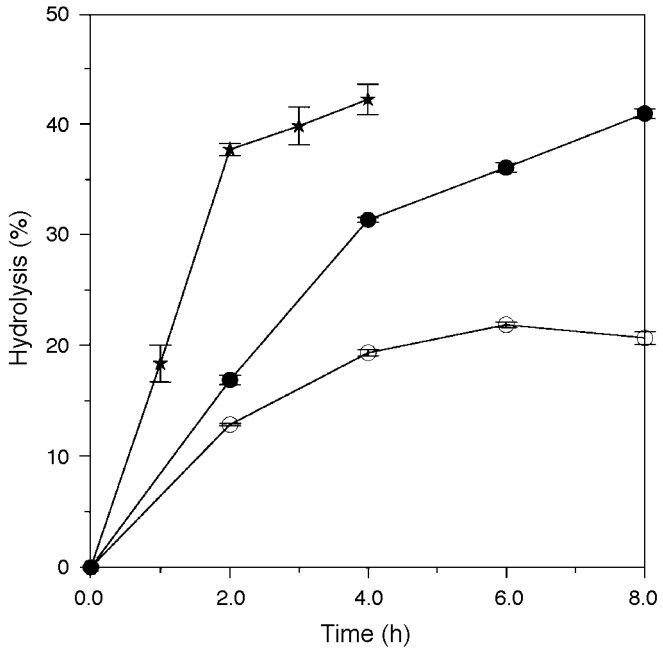

FIG. 5. The production of $C_{4:0}$ free fatty acid as a function of time: ○, no extraction; ●, extraction every 2 h; ★, extraction every hour.

DISCUSSION

Results indicate that altering the fatty acid composition of the butterfat substrate has a significant effect on the total concentration of FFA produced. This is a direct result of the fact that, on a weight or molar basis, SCFFA are less inhibitory to *P. roqueforti* lipase than their long-chain counterparts. In fact, butyric acid appears to have an activating effect on this enzyme at low concentrations. However, not only is the total concentration of FFA higher when the short-chain fraction is hydrolyzed, but the percentage of total FFA that contribute to taste (i.e., $C_{4:0}$ to $C_{8:0}$) is also higher. Therefore, short-chain butterfat fractions could potentially be used as substrates for improved flavor development during *in-situ* lipase hydrolysis in dairy products, such as cheese.

It would appear that, no matter what the substrate, the total FFA concentration will eventually reach a plateau concentration, a result mostly due to product inhibition of the lipase enzyme. Consequently, if short-chain FFA are to be produced *ex-situ* in a lipase reactor, our initial experiments indicate that on-line extraction of the FFA, as they are produced, could significantly increase system productivity. However, the method used in this work to remove the FFA—buffer extraction—would not be practical due to emulsion formation problems. The loss of lipase to the aqueous phase during this extraction also would necessitate the addition of more enzyme after each FFA removal step, further increasing the cost of operation. A more ingenious method would be required before a combined hydrolysis/on-line extraction system could be implemented on an industrial scale.

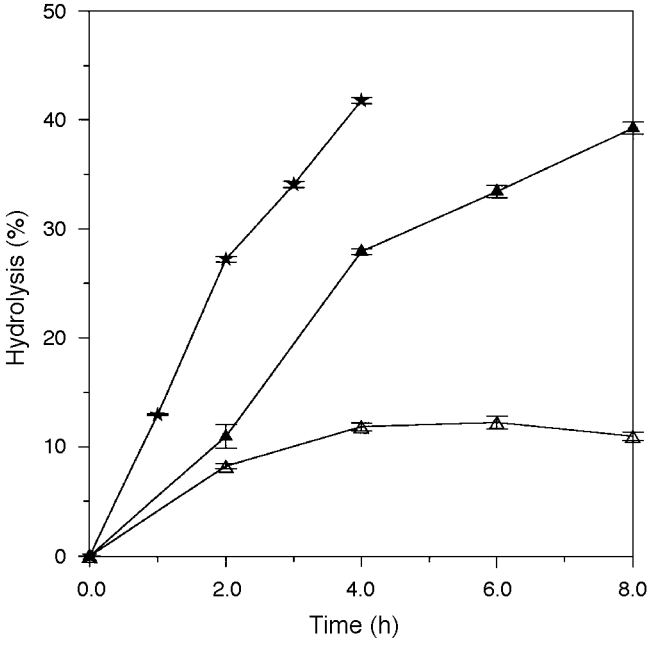

FIG. 6. The production of $C_{8:0}$ free fatty acid as a function of time: △, no extraction; ▲, extraction every two hours; ★, extraction every hour.

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REFERENCES

1. Shahani, K.M., R.G. Arnold, A. Kilara, and B.K. Dwivedi, Role of Microbial Enzymes in Flavor Development in Foods, *Biotechnol. Bioeng. 18:*891–907 (1976).
2. Stead, D., Microbial Lipases: Their Characteristics, Role in Food Spoilage and Industrial Uses, *J. Dairy Res. 53:*481–505 (1986).
3. Chen, J., and B. Yang, Enhancement of Short Chain Fatty Acids from Milkfat with Immobilized Microbial Lipase, *J. Food Sci. 57:*781–782 (1992).
4. Kilara, A., Enzyme-Modified Lipid Food Ingredients, *Process Biochem. 20:*33–42 (1985).
5. Marangoni, G.A., *Candida* and *Pseudomonas* Lipase-Catalyzed Hydrolysis of Butteroil in the Absence of Organic Solvents, *J. Food Sci. 59:*1096–1099 (1994).
6. *Enzyme Specifications,* Amano Enzyme USA, Lombard, IL, 1996.
7. Arul, J., A. Boudreau, J. Makhlouf, R. Tardif, and T. Bellavia, Fractionation of Anhydrous Milk Fat by Shortpath Distillation, *J. Am. Oil Chem. Soc. 65:*1642–1646 (1988).
8. Garcia, H.S., C.H. Amundson, and C.G. Hill, Partial Characterization of the Action of an *A. niger* Lipase on Butteroil Emulsions, *J. Food Sci. 56:*1233–1237 (1991).
9. Elliot, J.M., and K.L. Parkin, Lipase-Mediated Acyl-Exchange Reactions with Butteroil in Anhydrous Media, *J. Am. Oil Chem. Soc. 68:*171–175 (1991).
10. Kosugi, Y., and H. Suzuki, Functional Immobilization of Lipase Eliminating Lipolysis Product Inhibition, *Biotechnol. Bioeng. 40:*369–374 (1992).
11. Williams, M.G., and J. Macgee, Rapid Determination of Free Fatty Acids in Vegetable Oils by Gas Liquid Chromatography, *J. Am. Oil Chem. Soc. 60:*1507–1509 (1983).

12. Nawar, W.W., Lipids, in *Food Chemistry,* 2nd edn., edited by O.R. Fennema, Marcel Dekker, New York, 1985, p. 150.
13. Dunhaupt, A., S. Lang, and F. Wagner, *Pseudomonas cepacia* Lipase: Studies on Aggregation, Purification and the Cleavage of Olive Oil, *Biotechnol. Lett. 14:*953–958 (1992).
14. Andersson, E., B. Mattiasson, and B. Hahn-Hägerdal, Enzymatic Conversion in Aqueous Two-Phase Systems: Deacylation of Benzylpenicillin to 6-Aminopenicillanic Acid with Penicillin Acylase, *Enzyme Microb. Technol. 6:*301–306 (1984).
15. Kwong, Y.J., R. Kaul, and B. Mattiasson, Extractive Lactic Acid Fermentation Poly(ethyleneimine)-Based Aqueous Two-Phase System, *Biotechnol. Bioeng. 50:*280–290 (1996).

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