THE SELECTIVE RELEASE OF VOLATILE ACIDS FROM BUTTERFAT BY MICROBIAL LIPASES

J. C. WILCOX, W. O. NELSON, AND W. A. WOOD
Laboratory of Bacteriology, Department of Dairy Science
University of Illinois, Urbana

The lipases of microorganisms have been studied extensively from the aspects of occurrence in individual species and factors affecting enzyme production and activity. These lipases exhibit different patterns of substrate hydrolysis which can be attributed in part to the source of the enzyme and the nature of the substrate (4, 6, 13, 14).

Specifically, the lipases of Penicillium roqueforti and Aspergillus niger showed distinct differences in cleavage products when an equimolar mixture of tributyrin and tricaprylin was used as substrate. The P. roqueforti lipase released butyric and caprylic acids in the ratio of 3:1, whereas the same acids released by A. niger lipase were in a 1:4 ratio (13). The lipases of P. roqueforti also exhibited greater relative specificity for triglycerides than for ethyl esters, and tributyrin was more readily hydrolyzed than butterfat (4). Geotrichum candidum lipase hydrolyzed natural fats, such as olive oil and butterfat, more readily than synthetic triglycerides, monoglycerides, and fatty acid esters of glycols (6).

Richards and El-Sadek (12) reported that molds produce a greater total acidity in butterfat than bacteria under identical conditions. Lipolytic bacteria produced relatively less volatile acids than molds but slightly more solid fatty acids. These investigators also observed that of the volatile acids produced, the bacteria produced a greater portion of butyric and caproic acids than the molds. Fouts (2) reported that rancidity and the acid value of butterfat in commercial butter were not correlated and that fat in cream which had supported the growth of Oospora lactis (G. candidum) for 6 days contained only 1.9% of the total fat acidity as volatile acids. In contrast, other microorganisms, including Mycotorula lipolytica (Candida lipolytica) produced appreciable amounts of volatile acids, which ranged from 8.4 to 14.7% of the total fat acidity.

Although relative substrate specificities or preferences of some microbial lipases have been described, little is known of the specificity of these lipases in relation to the selective release of the component fatty acids of complex natural fats. The purpose of this report is to present data relative to the identity of the C₄-C₈ fatty acids released from butterfat by cell-free lipases from G. candidum and other lipolytic species. The data indicate that microbial lipases differ in their capacity to free butyric, caproic, and caprylic acids from butterfat.

METHODS AND MATERIALS

Bacteriological. The identity of the cultures and the sources from which they were obtained are shown in Table 1. The media used for lipase production included Protolysate broth (6) for G. candidum and the Penicillium species, pep...
TABLE 1
Sources of experimental cultures

<table>
<thead>
<tr>
<th>Culture</th>
<th>Identification</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Candida lipolytica</em></td>
<td>I</td>
<td>Dept. of Dairy Science, Univ. of Ill., Urbana.</td>
</tr>
<tr>
<td><em>Penicillium roqueforti</em></td>
<td>P-108-1, P-1IM</td>
<td>J. J. Jezeski, Dept. of Dairy Husbandry, Univ. of Minn., St. Paul.</td>
</tr>
<tr>
<td><em>Achromobacter lipolyticum</em></td>
<td>1XB-1</td>
<td>R. V. Hussong, Research Lab., Kraft Foods Co., Glenview, Ill.</td>
</tr>
</tbody>
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**tone-beef extract broth (9)** for *C. lipolytica*, and a phosphate-buffered peptone solution (5) for *Achromobacter lipolyticum*.

**Lipase preparations.** Cell-free filtrates from cultures incubated at 26° C. for 3 days were used as the sources of lipase. *G. candidum* lipase was obtained by filtering the cultures through asbestos-matted Gooch crucibles to remove the mycelia. The supernatant liquid of centrifuged cultures of *C. lipolytica* and *A. lipolyticum* was the source of lipases from these species. The lipases of the *Penicillium* species were prepared by homogenizing the mycelia in a cold Pyrex tissue homogenizer and recombining the filtrate and homogenate.

**Lipase assay.** Lipase activity was estimated by titrating the fatty acids liberated at 32.6° C. from a buffered substrate prepared and handled as previously described (6). *G. candidum* reaction mixtures were buffered at pH 6.0 (6), those of *C. lipolytica* at pH 6.5 (9), and those of the other test organisms at pH 7.2 (3, 5).

**Chemical.** The volatile acids were separated from the reaction mixtures by the steam distillation procedure proposed by McAnnally (3). A 10-g. aliquot was diluted with 50 ml. of a saturated magnesium sulfate solution containing 25% concentrated sulfuric acid, and the total volume was made up to 100 ml. with distilled water. This mixture was shaken, allowed to stand for 5 minutes, and filtered. A 50-ml. aliquot of filtrate was subjected to constant volume steam distillation, and 50 ml. of distillate was collected. Preliminary trials indicated an 89.79 ± 2% recovery of known concentrations of butyric acid added to reaction mixtures.

The volatile acidity of the distillate was measured by titrating a 10-g. aliquot to a phenolphthalein end point with 0.1 N alcoholic KOH delivered by a micro-burette. Excess ammonium hydroxide was added to the remaining distillate, which was then placed on a steam bath and concentrated under alkaline conditions to volume of approximately 1 ml.

The separation and identification of the free saturated C₄-C₈ fatty acids in 0.05-ml. aliquots of concentrated distillates was accomplished by paper partition
chromatography with redistilled n-butanol saturated with an equal volume of 1.5 N ammonia (11). The indicator solution was 0.04% (w/v) bromocresol purple in a 1:5 (v/v) dilution of formalin in ethanol adjusted to pH 5.0 with 0.1 N sodium hydroxide. The Rf values of control acids were essentially those reported by Reid and Lederer (11). Preliminary trials demonstrated that a concentration of acid as low as 4.8γ on the paper was detected by this procedure.

All trials and all analyses were run at least in duplicate and mean values are shown. Titration values of controls containing heated lipase did not change during incubation.

RESULTS

The data in Table 2 show the relative activity of the lipases of C. lipolytica and G. candidum on three widely different substrates. Tributyrin was more readily hydrolyzed than olive oil and butter oil by the C. lipolytica lipase. In contrast, olive oil and butter oil were hydrolyzed by the G. candidum lipase, whereas tributyrin was not appreciably attacked. It has been shown in previous work (6) that tributyrin may be hydrolyzed by G. candidum lipase but to a smaller degree than either olive oil or butter oil. These data suggest that, although there may be quantitative differences in the extent of lipolysis between experiments, qualitatively olive oil is more readily hydrolyzed than butter oil by G. candidum lipase and that these substrates support greater lipolysis than tributyrin.

The wide differences in the extent of lipolysis of olive oil, butter oil, and tributyrin by G. candidum lipase suggest a preference of this enzyme for long-chain fatty acid linkages of butterfat and olive oil, and that the short-chain fatty acid esters may be less susceptible to hydrolysis. Thus, the relative amount of specific fatty acid esters may be a limiting factor in the lipolysis of butter fat by G. candidum lipase.

A series of experiments was conducted to test the effects of the addition of butter oil, lipase, and buffer added separately to reaction mixtures in which lipolysis had approached a maximum. Double volume reaction mixtures were

| TABLE 2 | Lipolysis of selected substrates by cell-free culture filtrates from C. lipolytica and G. candidum |
|---------|-------------------------------------------------|-------------------------------------------------|-------------------------------------------------|
| Substrate titration values | Olive oil | Butter oil | Tributyrin |
| Culture | (6 hr.) | (24 hr.) | (6 hr.) | (24 hr.) | (6 hr.) | (24 hr.) |
| C. lipolytica I | 1.48 | 2.56 | 0.89 | 1.51 | 2.05 | 4.29 |
| C. lipolytica 846 | 1.65 | 2.69 | 0.86 | 1.60 | 4.28 | 5.85 |
| C. lipolytica 848 | 1.26 | 2.53 | 1.47 | 2.32 | 5.50 | 7.81 |
| G. candidum 77E | 0.29 | 0.83 | 0.20 | 0.36 | 0.00 | 0.07 |
| G. candidum 78F | 0.21 | 0.65 | 0.10 | 0.27 | 0.00 | 0.00 |
| G. candidum 79G | 0.32 | 0.93 | 0.10 | 0.29 | 0.00 | 0.05 |

*Net mean duplicate values of duplicate reaction mixtures expressed as ml. of 0.1 N KOH per 10 g. aliquot after 6- and 24-hour incubation. Reaction mixtures contained 44 g. buffered agar, 1 g. substrate, 5 g. culture filtrate, and 0.3 ml. toluene.
preparation and incubation, and aliquots were titrated at 24 and 48 hours. After
48 hours the additions indicated were made, and the course of lipolysis was
followed for an additional 48 hours.

The data from these experiments, represented in Figure 1, show that supple-
mentation of the *G. candidum* reaction mixture with additional enzyme did not
appreciably alter the course of lipolysis. However, the supplementary addition
of butter oil resulted in increased titration values during the second incubation
period. The addition of more substrate to *C. lipolytica* reaction mixtures was
without marked effect, whereas the addition of more enzyme resulted in resump-
tion of lipolysis of the substrate already present. These data further suggest
that the relative amounts of specific susceptible fatty acid linkages may be a
factor limiting the extent to which butter oil is hydrolyzed by *G. candidum* lipase
and suggest differences in R-group specificity of microbial lipases.

The concentrations of titratable acidity in distillates of reaction mixtures
containing lipases from different species also suggest that these lipases differ in
their ability to cleave volatile fatty acids from butter oil. In *C. lipolytica* lipase
reaction mixtures prepared from six strains the titratable volatile acidity of
distillates ranged from 0.05 to 0.22 ml. of 0.1 N KOH per 10 ml. aliquot, whereas
those from nine cultures of *G. candidum* ranged from 0.01 to 0.04 ml. Total fat
acidity in 10-g. aliquots of reaction mixtures ranged from 1.48 to 6.79, and 2.63
to 6.13 ml. of 0.1 N KOH for the six *C. lipolyticum* and nine *G. candidum*
cultures, respectively.

The chromatographic separation and identification of the volatile C₄-C₈ fatty
acids contained in concentrated steam distillates of butter-oil-lipase reaction
mixtures are summarized in Figure 2. As can be seen, volatile fatty acids were

![Fig. 1. Limiting factors in lipase assay. Assay emulsions contained 80 g. buffered agar (pH 6.0 and pH 6.5 for *G. candidum* and *C. lipolytica* lipases, respectively), 10 g. butter oil, and 10 g. lipase solution. After 2 days at 32.5°C, 10 g. butter oil, 10 g. enzyme solution, or 10 g. buffer solution was added as indicated and emulsion reincubated. Data are calculated from mean titration values for 10 ml. aliquot.](image-url)
not detected in seven *G. candidum* reaction mixture distillates, and only butyric acid was detected in distillates from *G. candidum* 74-B and 75-C reaction mixtures.

Butyric acid was present in all trials with *C. lipolytica* lipase, and caproic acid also was found in distillates from all *C. lipolytica* reaction mixtures except that containing the lipase from strain 840.

The lipases of the *P. roqueforti* and *A. lipolyticum* cultures tested liberated butyric, caproic, and caprylic acids from the butter oil substrate. Butyric acid and an unidentified acid, which consistently migrated to an Rf value midway between the C₆ and C₇ controls, were encountered in *P. camemberti* reaction mixtures.

**DISCUSSION**

The absence of appreciable volatile acidity in butterfat which has undergone extensive lipolysis has led to the belief that certain lipolytic molds consume the volatile fatty acids as rapidly as they are released (2, 8).

Fouts (1, 2) reported that 1.5-2.4% of the total fat acidity of cream cultures of *O. lactis* was volatile acidity, as compared to values of 8.1-8.9, 13.1-18.9, 10.6-12.5, and 10.7-12.0 for *M. lipolytica*, *Pseudomonas fluorescens*, *A. lipolyticum*, and *Alcaligenes lipoyticus*, respectively. In this connection, Purko et al. (10) observed that the volatile acidity of cream supporting pure cultures of *G. candidum* did not change during an incubation period in which the water insoluble free fatty acids increased from a value of 200 mg. to 30,000 mg. per 100 g. of fat.

Salts of some volatile fatty acids in artificial media are utilized by cultures of *G. candidum* (2, 7). However, the release of such acids from butterfat by cell-free lipase of *G. candidum* should be demonstrable if fatty acid utilization is a valid explanation of the conspicuously low volatile acidities encountered in butterfat hydrolyzed by growing cultures of *G. candidum*. 

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**Fig. 2. Chromatogram—steam distillates.** A schematic representation of fatty acids detected in concentrated steam distillates from lipase-butterfat assay emulsions incubated 3 days at 32.5°C. Figures in parentheses under each species denote number of strains tested.
The experimental data presented in this report indicate that differences in microbial lipase specificities may account for the differences in volatile acidity encountered in butterfat that has been hydrolyzed by *G. candidum* and other lipolytic organisms.

During the course of this investigation several categories of data pertaining to the question of cell-free lipase specificity have been obtained. The relative substrate specificity patterns in Table 2 suggest that the *G. candidum* lipase has a predilection for long-chain fatty acid esters, such as those in olive oil, whereas the short-chain esters may not be attacked. In contrast, the *C. lipolytica* lipase hydrolyzes short-chain triglycerides more readily than higher fatty acid triglycerides.

The concentrations of volatile acidity in distillates of reaction mixtures containing cell-free lipases also suggest that the above lipases differ in their ability to cleave volatile fatty acids from butterfat. Similarly, the data shown in Figure 1 suggest that the relative amount of specific susceptible fatty acid esters may be one of the factors limiting the extent to which butterfat is hydrolyzed by *G. candidum* lipase. The *C. lipolytica* lipase was not limited in the same manner.

The chromatographic inventory and identification of volatile fatty acids released from butterfat by cell-free microbial lipases also indicate that these lipases possess certain fatty-acid R-group specificities. These data, summarized in Figure 2, show that lipases of seven of nine *G. candidum* cultures did not release detectable quantities of volatile fatty acids from butterfat. The lipases of the two other cultures liberated butyric acid as the only detectable acid. All of the *C. lipolytica* lipases tested released butyric acid, and five of the six cultures also released caproic acid. The lipases of the *P. roqueforti* and *A. lipolyticum* cultures tested hydrolyzed butyric, caproic, and caprylic acids from the substrate.

**SUMMARY**

Relative substrate specificity patterns, differences in volatile fat acidity of hydrolyzed fat, substrate limitations in the course of the lipolytic reaction, and differences in chromatographic analysis of distillates from hydrolyzed fat indicate that cell-free microbial lipases differ in their ability to release volatile fatty acids from butterfat. Some strain variations were evident.

The lipases of seven of nine cultures of *G. candidum* did not release detectable quantities of volatile fatty acids from butterfat. Two cultures freed butyric acid only.

*C. lipolytica* lipases hydrolyzed butyric acid from the butterfat and five of the six culture lipases also released caproic acid.

Butyric, caproic, and caprylic acids were found in distillates of reaction mixtures containing butterfat and the lipases of either *P. roqueforti* or *A. lipolyticum*.

Butyric acid and an unidentified acid which consistently migrated to an Rf value midway between the caproic and caprylic controls were encountered in *P. camemberti* lipase-butterfat reaction distillates.
REFERENCES


