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FACTORS INFLUENCING ACTIVITY OF MICROBIAL LIPASES

by

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INTRODUCTION

Many microorganisms are capable of breaking down fats by hydrolytic action. Some have been studied extensively both as to conditions under which the lipolytic enzymes are produced and the conditions under which they act. Considerable differences in the lipolytic abilities of various strains of the same species exist, as well as differences between the various lipolytic species. Both medium composition and time and temperature of incubation play important roles in the amount of lipase activity produced. Similarly, the ability of these enzymes to produce their characteristic reaction(s) is dependent on many of these same factors.

Because of the complexity and variety of the different fat molecules in any natural fat, analytical procedures have been limited to a considerable degree to measuring the total amount of released fatty acids. While this gives much information as to optimum conditions under which the lipases are produced and react, little information is available as to the extent to which any individual acid is released. This has been due in a considerable degree to the lack of adequate analytical procedures for the quantitative extraction and separation of fatty acids. Recent developments in partition chromatography have provided a tool by which this information may be obtained.

Three species of microorganisms were selected for this

study. They are Pseudomonas fragi, Geotrichum candidum and Candida lipolytica. All show considerable lipolytic activity and have been shown to be common to dairy products, causing considerable economic loss, largely because of the changes they produce in the butterfat fraction.

STATEMENT OF PROBLEM

This study was made with the twofold purpose of finding a more suitable procedure for the isolation and quantitative measurement of some of the free fatty acids in dairy products and to show by means of this procedure some of the differences between the lipolytic enzyme systems of *Ps. fragi*, *G. candidum* and *C. lipolytica*.

REVIEW OF LITERATURE

Determination of Lipase Activity

Early investigators (38) determined lipase activity merely by titrating the glyceride or ester containing substrate in order to detect the release of fatty acids. The activity of the lipase of Penicillium glaucum was determined by Gerard (15) by using a two per cent monobutyrin substrate and titrating with 0.1N NaOH after various incubation intervals. Camus (4) and Garnier (14) also used 0.1N NaOH for measuring lipolytic activity by direct titration.

Kendal et al. (28) adjusted 0.25 per cent triacetin and ethyl butyrate substrates to the phenolphthalein end point and then retitrated after the reaction period. Wells and Corper (68) extracted the fatty acids from olive oil, ethyl butyrate and triacetin emulsions with ether and titrated this with alcoholic NaOH. They found that aqueous alkaline solutions do not extract all of the free fatty acids held in the emulsion. Wilstätter et al. (70), rather than perform an extraction, added a mixture of ether and 95 per cent ethyl alcohol to the olive oil substrate and titrated this with 0.2N alcoholic NaOH to the thymolphthalein end point.

Fleming and Neill (12) observed lipase activity on a tributyrin substrate by following changes in pH. Lubert et al. (35) used tributyrin emulsified in skim milk as his substrate.

After incubation, this was acidified with phosphoric acid and the butyric acid extracted with ether and titrated.

Breazeale and Bird (1) studied various methods for determining acidity in butterfat in order to reduce the obscuring of the end point due to clouding of the solvent mixture and end point fading. Their procedure is to weigh 10 g. of fat into 125 ml. Erlenmeyer flasks. The fat is dissolved in 25 ml. petroleum ether and 10 ml. absolute alcohol. Ten drops of 0.1 per cent solution of phenolphthalein in absolute alcohol are added and the mixture is titrated using 0.05N KOH in absolute alcohol. Tucker and Bird (66) modified this procedure. Ten g. of melted, filtered fat is weighed into 125 ml. Erlenmeyer flasks and dissolved in 25 ml. solvent mixture (800 ml. petroleum ether and 200 ml. n-propanol). Ten drops of one per cent phenolphthalein in absolute ethanol or absolute methanol are added and the mixture then titrated with 0.05N KOH in absolute methanol.

The effect of the lipase of Mycotorula lipolytica (Candida lipolytica) on a variety of fat-agar emulsions was studied by Peters and Nelson (51). They analyzed for fatty acid release by adding 50 ml. of a 50:50 mixture of diethyl ether and 95 per cent ethanol to 10 g. of substrate and titrating with 0.05N KOH in absolute ethanol to a phenolphthalein end point.

Johnson and Gould (26) examined two methods for studying fatty acid release. In the first, 125 g. of cream is mixed

with 100 ml. ethanol. This is then mixed well with 80 ml. ethyl ether and 120 ml. petroleum ether. The ethereal layer is separated by centrifugation and removed. The fat-ether mixture is then heated to remove the solvents. For complete extraction, the cream must be acidified to pH 2, as the acids were bound in the aqueous phase as salts. They also tried drying milk in plaster of Paris and using a continuous extraction procedure, but found that this procedure did not remove fatty acids efficiently.

An emulsion of olive oil and agar was subjected to the lipase of Geotrichum candidum by Nelson (47). This was analyzed by adding 25 ml. of a 1:1 mixture of diethyl ether and neutralized 95 per cent ethanol and titrating with 0.1N alcoholic KOH to a phenolphthalein end point.

Nashif and Nelson (41) measured the activity of the lipase of Pseudomonas fragi on a sodium taurocholate emulsion of coconut oil, using a modification of the Johnson and Gould (26) method. For this method, 10 g. of this emulsion is weighed into Mojonnier butterfat extraction flasks. This is acidified with 25 per cent H_2SO_4 to a definite pink color of thymol blue. This is shaken well with 10 ml. 95 per cent ethanol and extracted twice using 10 ml. portions of an ether mixture (two volumes of ethyl ether and three volumes of petroleum ether) and the ether is decanted into a 125 ml. Erlenmeyer flask. It is then titrated to a phenolphthalein end point, using

0.05N KOH in absolute methanol. They noted that on checks for recovery of butyric and caproic acids, extraction was poor but that these acids are lacking in coconut oil and so this deficiency was of little importance. Recoveries of higher molecular weight fatty acids were excellent.

Harper and Armstrong (20) measured total free fatty acids in fat, using a silica gel column procedure. Phosphate buffer (pH 6.4) is adsorbed onto silicic acid and slurried with chloroform. This is packed into a specially formed column. The fat sample is acidified with 20 per cent H_2SO_4 and adsorbed onto silicic acid. This is transferred to the column as the upper section. The acids are extracted by forcing through the column 100 ml. 0.75 per cent n-butanol in chloroform, followed by 100 ml. 5.0 per cent n-butanol in chloroform. The solvent is collected in fractions, the first 150 ml. containing all of the acids except butyric acid, the next 10 ml. containing no acids and the remainder containing the butyric acid. To each fraction is added 1.0 ml. neutral absolute alcohol. These are then titrated to a phenol red end point using 0.01N base in absolute alcohol. This procedure has the advantage of separating butyric acid from the rest of the fatty acids and thus affording a means of detecting substitute fats in dairy products.

A faster procedure for measuring total free fatty acids in dairy products using silica gel was developed by Harper

et al. (21). As in the previous work, the lower section is buffered to pH 6.5 and slurried in chloroform. The sample is acidified to pH 1.8 to 2.0 with 20 per cent H_2SO_4 and adsorbed onto silicic acid. This is transferred to the column as the upper section and developed with 150 ml. of 5 per cent n-butanol in chloroform. The entire 150 ml. of developing solvent is collected in one fraction. Before titration, 15 ml. neutral absolute alcohol is added and the total material then titrated to a phenol red end point with 0.01N base in absolute alcohol.

Fatty Acid Separation by Partition Chromatography

Partition chromatography for separating mixtures of organic acids is a relatively new analytical procedure. It was developed from a counter current distribution method used for separating mixtures of amino acids and mixtures of polypeptides by Martin and Synge (36). They adsorbed water on silica gel, packed this in a glass tube, placed the sample on top of the packed silica gel and forced through a 1.0 per cent n-butanol in chloroform mixture. Methyl orange had been mixed with the water adsorbed on the silica gel and thus they were able to follow visually the course of the amino acids as they proceeded through the column. As a result of this original procedure, many new procedures based on the same principle have been worked out for other types of organic acids.

Smith (63), working with the procedure of Martin and

Synge, could not reproduce the sharp separation with mixtures of other organic acids. He found that rather than being a simple partition between two solvents, there was some adsorption of the acids on the silica gel. This supposedly was due to a temporary neutralization of the acids by the base used to adjust the indicator to the proper color. A change in the partition coefficient was correlated with changes in the concentration of any one acid. Larger quantities would travel faster and push forward the next lower band. As an example, if propionic acid came off the column first with acetic acid following it, 1.0 per cent acetic acid in propionic acid was detectable, but 1.0 per cent propionic acid in acetic acid would not be. They also noted that while the front of the moving band was sharp, the rear portion tailed out and would mix with the band behind if it was close enough. An interesting result of this study was the relative ease of distinguishing butter from margarine by the butyric acid band of butter.

Many factors will determine whether a column is suitable for separating any given mixture of organic acids. The solvents used probably play the most important role. Ramsey and Patterson (57) adsorbed water onto silicic acid[1] and extracted with 1.0 per cent n-butanol in chloroform and 10 per cent

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[1]Mallinckrodt #2844

n-butanol in chloroform for separation of the volatile saturated fatty acids C_1-C_4. They (58) adsorbed methanol on the silicic acid and extracted with isooctane[1] for separating the straightchain saturated fatty acids C_5-C_{10}. They (59) adsorbed furfuryl alcohol-2-aminopyridine on silicic acid and extracted with n-hexane for separating the saturated straightchain fatty acids C_{11}-C_{19}.

For separation of the acids commonly associated with the citric acid cycle (fermentation acids), mixtures of n-butanol in chloroform have been used most commonly as the developing solvent (3, 8, 24, 36, 37, 46, 65). However, no one proportion has been found suitable for separating all of these acids. Using more than one solvent mixture complicates the procedure. Donaldson et al. (8) developed a procedure for progressively changing the proportion of n-butanol to chloroform to give good separation of mixtures of large numbers of acids. Modifications of this procedure have been developed by Steele et al. (65) and Naguib and Beerbom (40).

Another procedure also has been used successfully for separating large mixtures. Fairbairn and Harpur (10) used two columns, one above the other, but used only a 1:199 concentration of n-butanol in chloroform.

Other solvent mixtures that have been used are n-butanol

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[1] 2, 2, 4-trimethyl pentane

in benzene (3), benzene-ether (30), tertiary amyl alcohol in chloroform (13, 24, 32) and 4-methyl-2-pentanone in methylene chloride (61). The use of tertiary amyl alcohol was introduced to prevent any possible esterification between n-butanol and the acids in the mixture.

Various indicators have been used to provide a visible means of taking fractions. In order to have the pH of the adsorbed solvent at a proper level, the pH must be adjusted using either an inorganic acid or an inorganic base. This apparently does not affect recovery of the acids but does change the partition coefficient of the acids.

Ramsey and Patterson (57, 58) used NH_4OH to adjust pH for columns using bromocresol green as indicator. Elsden (9), Fairbairn and Harpur (10) and Vandenheuvel and Hayes (67) used NaOH to adjust the pH of the column using bromocresol green indicator. Others (4, 13, 30, 32, 40, 55, 61) have used H_2SO_4 to acidify the columns, presumably to prevent adsorption of the acids onto the silica gel. No indicator was used for these columns. Other indicators such as methyl orange (36, 17) and alphamine red R (10, 11) have been used. An external indicator such as thymol blue (24) or phenol red (46) may be used with acidified columns. Hydrochloric acid may also be used for acidifying the column (46).

The amount of solvent that the silicic acid is capable of adsorbing seems to play an important role in the resolving power

of the system. The reproducibility of any procedure for preparing the silicic acid appears to be poor considering the many different procedures reported in the literature (9, 10, 11, 24, 36, 46, 65). Only one procedure seems to have been used by others and found reproducible (24). The Association of Official Agricultural Chemists recognizes a commercial[1] preparation which is used quite widely (27, 37, 57, 58, 59, 61, 67) although some have even developed procedures for modifying this silicic acid so that it will adsorb more of the internal phase solvent (3, 30).

The identification of the acids after they have been separated has been done in several ways. Threshold volumes (the amount of solvent required to elute an acid from the start of the developing until the acid is completely eluted) have been used when working with known mixtures (9, 10, 27, 58). The formulation of a typical chromatogram also is used (40). Other means such as the crystallographic identification of Ramsey and Patterson (57) for formic, acetic, propionic and butyric acids, molar extinction coefficients as used by Donaldson et al. (8), absorption spectra in the ultra violet range as used by Frohman (13) and two-way paper chromatography as used by Ladd and Nossal (32) have been used.

There are three principal means of separating the acids

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[1] Mallinckrodt's #2844

as they come off the column. The simplest is by merely separating the fractions as they come off a column containing an acid-base indicator (58). When many acids are to be collected, an automatic fraction collector may be used (40) each fraction then being titrated. Manual collection of fractions may be used when acids with known threshhold volumes are being separated (27). The use of indicators for collecting fractions is not always reliable, as some acids are so weakly acidic they will not appear as well-defined bands and some acids will elute the indicator as they proceeded through the column (9).

Pseudomonas fragi, Its Lipase and Economic Importance

Hussong et al. (23) isolated and identified Ps. fragi as the organism causing most rancidity in dairy products. They noted its sensitivity to heat showing that only one of five strains survived a temperature of 62.2° C. for 10 minutes and that most strains would not survive an even shorter exposure than this. The organism characteristically produced a small ring of acid curd at the surface of litmus milk and had an odor resembling that of the May apple. They also noted that it would grow slowly at a temperature of from 3° to 5° C. Long (34) observed that the acid ring was produced only by freshly isolated cultures.

Nashif and Nelson (42) reported that the lipase of Ps.

fragi is primarily extracellular. The optimum temperature for lipase production was 15° C. when incubated for 3 days. If time was extended, lower temperatures were preferred. The growth medium for optimum lipase production was nutrient broth buffered to a pH of 6.5 to 7.5 with phosphate buffer. Increased counts were associated with high lipase production but the two were not proportional. If glucose (6) was added to the growth medium, the growth rate was accelerated but lipase production decreased. Lipase production was inhibited by 2 per cent NaCl while growth was retarded very slightly. Small amounts of caprylic acid or tricaprylin tended to stimulate lipase production. While aeration or agitation of the growth medium retarded lipase production, a large surface area in proportion to the volume of medium used was necessary, so medium depth was kept shallow.

Activity of the lipase of Ps. fragi, according to Nashif and Nelson (41), was optimum at pH 7.2 when using coconut oil as substrate, although activity was detected over a wide range (pH 6.0 to 9.0). In contrast to lipase production, the lipase itself was very stable to heat, requiring a temperature of 99° C. for 20 minutes for complete inactivation. Considerable lipase activity was detectable after exposure to temperatures of 61.6° C. and 71.6° C. for 30 minutes. The greatest loss of activity was during the time required to bring the lipase preparation up to the desired temperature. Various attempts

were made to purify the enzyme but purification beyond precipitation with $(NH_4)_2SO_4$ was not achieved. It was noted that the enzyme was sensitive to oxidation.

Of economic importance is the effect of the enzyme in dairy products (43). It is active in cream between pH 4.9 to 8.2 with an optimum at pH 5.7 to 6.6 when incubated at 36° C. for 24 hours. Growth of the organism and resultant lipase production in cream at 15° C. for 3 days is rather slow; however, enough lipase is produced under these conditions to cause extensive fat breakdown in the cream and the butter made therefrom. Over 50 per cent of the lipase remains active after pasteurizing the cream at 71.5° C. for 30 minutes. Extensive growth of Streptococcus lactis in cream, resulting in a lowering of the pH, does not cause any appreciable inhibition of the growth of Ps. fragi, but markedly reduces lipase production and activity. Butter containing residual lipase undergoes considerable fat degradation during storage, even at a temperature of -10° C., developing a pronounced rancid flavor; this is especially true at temperatures of 5° C. and higher.

Candida lipolytica, Its Lipase and Economic Importance

Of five media tested, Peters and Nelson (52) found that nutrient broth containing 0.05 per cent glucose was most suitable for lipase production by M. lipolytica (C. lipolytica), and glucose concentrations over 0.25 per cent were inhibitory

(50). Lipase production was influenced importantly by pH (51). While acetate buffers were inhibitory, phosphate buffers were satisfactory when the pH was adjusted to 5.5. If the pH was adjusted with citric acid in combination with phosphate salts, a pH of 4.5 was preferred.

Optimum temperature for growth of the organism was 25° C. A temperature of 30° C. proved to be optimum for lipase formation. Maximum temperature for production was 36° C. and minimum temperature was 21° C. Aeration of the medium restricted lipase production, but addition of cysteine reversed this and was actually somewhat stimulatory.

An association between cell morphology and lipase production was noted. Long-oval or slender cells and a somewhat smaller population of cells were associated with high lipase production. High populations and short-oval cells were associated with low lipase production. Thus the addition of substances stimulatory to growth, such as glucose at levels over 0.25 per cent, reduced lipase production.

Peters and Nelson (54) found that thiamine or its pyrimidine component was necessary for lipase production. Also, if both the peptone and beef extract components of nutrient broth were replaced by peptone, beef extract, yeast extract or gelatin singly, lipase production was either lowered considerably or was absent.

In a defined medium (54) using glucose as sole carbon

source, growth was limited and lipase production was absent. Growth was considerably increased by the addition of biotin, thiamine hydrochloride, nicotinic acid or pantothenic acid. However, lipase was produced only in the presence of thiamine hydrochloride. Addition of 2-methyl-5-bromomethyl-6-amino-pyrimidine dihydrobromide also stimulated lipase production.

The activity of the lipase of M. lipolytica (C. lipolytica) was studied by Peters and Nelson (52). The enzyme is active over a pH range of 4.0 to 8.0 with the greatest activity at pH 6.2 to 6.5, the decrease in activity being more abrupt on the alkaline side. Optimum temperature for activity was 28 to 33° C. The enzyme was inactivated after 8 hours at a temperature of 52° C. Activity was quite high at 10° C. Concentration by lyophilization was possible, but about 50 per cent of the lipolytic activity was lost. Concentration by salting out with $(NH_4)_2SO_4$ yielded only about 3 per cent of the original activity.

The influence of M. lipolytica (C. lipolytica) lipase on blue cheese ripening was studied by Peters and Nelson (53) in an attempt to make the cheese from pasteurized homogenized milk in which the natural milk lipase had been inactivated by pasteurization. Addition of this lipase resulted in increases in total volatile acidities in proportion to the amount of lipase added. The flavor score and to a degree the score for freedom from defects showed good correlation with total volatile acidity.

No indication was obtained that mold growth was affected by the different amounts of volatile fatty acids present. This lipase might be of considerable value in the proper ripening of blue cheese made from pasteurized milk, although too much of the lipase resulted in a soapy flavor.

Geotrichum candidum, Its Lipase and Economic Importance

As with C. lipolytica lipase production, a high growth rate by G. candidum is not associated with high lipase production. Nelson (48) has shown that while monosaccharides enhanced growth rate, lipase production was reduced. Addition of fats or fatty acids had little effect and by themselves would not support growth. Formic acid actually slowed growth. Growth factors also had no effect. Optimum pH for lipase production was 5.35 to 5.85 in a carbohydrate medium and 6.70 to 6.85 in a glycerol medium at a temperature of 20° C.

Nelson (47) found that filtrates of the medium were more active lipolytically than mycelial preparations, indicating that the lipase is primarily extracellular. It is active over a pH range of 5.0 to 8.0, with an optimum at pH 6.0. The enzyme is active from 20° to 37° C., with the optimum at 30° C. Naturally occurring fats are more readily hydrolyzed than are synthetic triglycerides. The lipase is quite sensitive to acid production, being inactivated at pH 4.0 and destroyed at pH 3.0. It is partially and irreversibly inactivated by oxygen.

Bichloride of mercury inhibition is partially reversed by glutathione and other reducing agents.

Purko and Nelson (56), studying the liberation of water-insoluble acids (WIA) in cream by G. candidum, inoculated cream with cultures of the organism. After 9 days at 4° C., there was no increase in WIA values. As temperatures increased up to 37° C. the rate of WIA formation increased. After formation of WIA at a high temperature, storage at 4° C. brought about no further increase. The rate of WIA formation decreased in proportion to the amounts of lactic acid added to the cream. Associative growth with lactic acid bacteria also reduced the rate of lipolysis. Added glucose markedly reduced the rate of WIA formation in cream, as well as in the studies using artificial substrates.

Specificity of Lipolytic Enzymes

Lipase specificity has been examined from several viewpoints. First, emulsions of pure triglycerides have been used, following the reaction by means of simple titration procedures. By this means, a general idea may be obtained as to which triglycerides are attacked and to what degree. However, this information may not hold true for specificity in mixtures of pure triglycerides or natural fats. Most of the present data on specificity, nevertheless, are from studies using pure triglycerides.

Activity on a variety of pure triglycerides by the lipase of Ps. fragi was determined by Nashif and Nelson (41). Tricaprylin was hydrolyzed to a greater degree than any of the others tested with 15.85 per cent of the one per cent emulsion being hydrolyzed. Trilaurin and tricaproin were also hydrolyzed fairly actively (7.98 and 7.63 per cent respectively). Activity also was determined on a variety of natural fats and oils. Coconut oil, which has a relatively high proportion of caprylic acid, was hydrolyzed to the greatest degree, with activity on butterfat being somewhat lower. Net activity (expressed as ml. of 0.05N base to titrate) with the fat concentration at 10 per cent was 1.80 for coconut oil and 1.38 for butterfat.

Wilcox et al. (69) found that C. lipolytica lipase attacked triglycerides which contained low molecular weight fatty acids (butyric and caproic) as substrates in comparison to higher molecular weight fatty acids. They reported that G. candidum lipase attacked long chain acids, while Nelson (47) reported that G. candidum attacked lower triglycerides more readily. Purko and Nelson (56) have reported that considerable amounts of WIA are produced in cream by G. candidum.

Richards and El-Sadek (60) compared fatty acid release from butterfat by three species of molds (Aspergillus glaucus, Cladosporium herbarum and Penicillium cyclopium) and three species of bacteria (Achromobacterium liquidum, Micrococcus aureus and Pseudomonas fluorescens). The fatty acids released

were classified into three main groups, the volatile group (butyric and caproic acids), the solid group (stearic and palmitic acids) and the liquid group (oleic and linoleic acids). Molds produced a greater quantity of total acids but a lesser proportion of volatile acids than bacteria. The bacteria produced more of the solid acids than volatile acids, while the liquid acids made up the greatest portion of acids released by either bacteria or molds.

The lipases of Penicillium roqueforti and Aspergillus niger were investigated by Shipe (62). Tributyrin was hydrolyzed to the greatest extent by P. roqueforti and tricaprylin by A. niger. Others (39, 69) also reported P. roqueforti and Achromobacter lipolyticum released more of the low molecular weight fatty acids. Kester et al. (29) showed that A. lipolyticum was almost selective in preference for butyric acid in butter in that the amount of butyric acid released was large in respect to total free fatty acids.

A comparison of fatty acid release in cream and relative activity on a variety of triglycerides has been reported by Harper (18, 19), using a series of animal and microbial lipases. Comparisons between the fatty acids released from cream and activity on the synthetic triglycerides by calf rennet paste, kid oral glandular preparations and lamb oral glandular preparations showed little agreement. For example, tricaprin was the synthetic triglyceride preferentially attacked by calf

rennet paste, while the activity of this enzyme in cream was predominantly towards the higher molecular weight acids (lauric and above). Activity by kid rennet paste and calf oral glandular preparations, however, was quite similar for the two types of substrates, the low molecular weight fatty acids being preferentially released in both cases. Butyric acid was preferentially released by hog pancreatic, Aspergillus and milk lipases.

EXPERIMENTAL METHODS

Isolation of Lipolytic Microorganisms

The microorganisms used in this study were isolated using the lipolysis detection procedure of Knaysi (31). Coconut oil, which had been neutralized and washed, was mixed thoroughly with a saturated solution of Nile blue sulfate (10 ml. oil to 1 ml. dye solution). The fat layer was separated from the aqueous layer in a separatory funnel with the fat now being red colored. This was washed twice with distilled water and sterilized by autoclaving (121° C. for 15 minutes). The sterile fat was mixed with nutrient agar at a temperature of 55° C., 1 ml. of the fat being enough for 100 ml. of the agar medium.

For the isolation of Ps. fragi, a water sample was taken from Lake LaVerne on the Iowa State University campus. Pour plates using dilutions of 1:100, 1:1000, 1:10,000 and 1:100,000 were made in order to get single isolated colonies. Several of these colonies were picked and streaked on Knaysi's agar for further characterization. Gram stains were made of all isolates. Three of those which conformed morphologically were characterized physiologically and lipase preparations were made. These were then tested for ability to hydrolyze butterfat by direct titration of the acetone-chloroform extract of the lipolysis test substrate used in this study.

The isolate showing the greatest activity was chosen for this study. Morphological and physiological characteristics of this organism were in accordance with those found in Bergey's Manual (2) except that the typical May apple aroma was lacking, being covered up by the strong ammoniacal odor caused by the proteotytic activity of the organism. The organism was then carried on nutrient agar slants, incubation for these being for 24 hours at 21° C.

Isolates of G. candidum were obtained from plates poured by students in an advanced class in Dairy Microbiology and were tested for lipolytic ability in the same manner as was Ps. fragi. The isolate chosen for this study agreed morphologically and physiologically with the description as given by Gilman (15). Cultures were carried on unacidified potato dextrose agar slants (7).

The strain of C. lipolytica was obtained from Dr. E. R. Garrison, Department of Animal Industry and Veterinary Science, University of Arkansas, Fayetteville, Arkansas, with a group of other yeasts. All were tested for lipolytic ability and this species showed more lipolysis under the conditions used for this study. Morphological and physiological characters agreed with those given for C. lipolytica by Lodder and Kreger-Van Rij (33). Cultures were carried on unacidified potato dextrose agar slants (7).

Media for Lipase Production

For lipase production by Ps. fragi, phosphate buffered (0.05M) nutrient broth was used (42). The reaction was adjusted to pH 7.5 with NaOH. For C. lipolytica, nutrient broth containing glucose (0.4 g./l.), citric acid (17 g./l.) and $Na_2HPO_4 \cdot 7H_2O$ (37 g./l.) and adjusted to pH 4.5 with NaOH was used. For G. candidum, one per cent tryptone broth containing KH_2PO_4 at a concentration of 0.2M and adjusted to pH 5.4 with NaOH was used.

In all cases, 100 ml. amounts of these media were dispensed in 1000 ml. Erlenmeyer flasks. These were stoppered with cotton and sterilized by autoclaving at 121° C. for 15 minutes.

Lipase Preparations

All three lipase preparations were treated in much the same manner, with the exceptions being noted. After the cultures had grown out on the slants, they were washed free with 3 ml. of the proper lipase production medium, using a pipette to help free the cells from the slants. A 0.1 ml. amount was then used as inoculum. Ps. fragi was incubated at a temperature of 10° C. for 72 hours, C. lipolytica at 30° C. for 90 hours and G. candidum at 21° C. for 96 hours. Following the incubation period, C. lipolytica and G. candidum cultures were filtered through pyrex wool into 100 ml. special centrifuge

tubes. The Ps. fragi culture was decanted directly into the centrifuge tubes as filtering was unnecessary. All cultures were centrifuged at a speed of 4000 R.P.M. for 30 minutes in an International Model U centrifuge using a number 840, 100 ml. special head. The clear supernatant fluid was decanted into 500 ml. Erlenmeyer flasks, being careful not to disturb the packed cells and then mixed gently with 0.1 per cent merthiolate[1] solution (1 ml. merthiolate solution to 100 ml. medium) to prevent further growth. This preparation was immediately placed at 4° C. for storage.

Lipase Test Substrates

The same lipase test substrate was used for all three lipase preparations. Butter was heated to about 60° C. and the aqueous and fat layers separated. The resulting butteroil was washed with hot distilled water until the wash water came off clear. It was then washed twice with 0.5M $NaHCO_3$ and washed twice more with hot distilled water. The thus washed and neutralized butteroil was stored at a temperature of 55° C. until ready for use (any extended holding resulted in the butteroil becoming oxidized). Sodium taurocholate (0.8 g.) was mixed thoroughly with 80 ml. distilled water, 20 ml. 0.5M KH_2PO_4 and 4.0 ml. merthiolate solution (0.1 g./100 ml.). The

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[1] Sodium ethyl mercurithiosalicylate

reaction was adjusted to pH 7.2 for Ps. fragi, pH 7.3 for C. lipolytica and pH 7.3 for G. candidum, using 1.0N NaOH. The butteroil was then added and the final volume made to 150 ml. This was tempered to 55° C. and homogenized twice with a hand homogenizer and stored overnight at a temperature of 35° C. The emulsion was mixed with the lipase preparation at a rate of 3 ml. emulsion to 1 ml. lipase preparation. This preparation varied from that of Nashif and Nelson (41) only in the substitution of butteroil for coconut oil and the use of merthiolate instead of formaldehyde as preservative.

Extraction of the Fatty Acids

For studying the release of any given fatty acid from butterfat by a lipase preparation, it is first necessary to extract the acids from the product in which the acids are contained. The extraction procedure used by Nashif and Nelson (41) was satisfactory for extracting the fatty acids found in coconut oil but was not efficient in extracting the lower molecular weight fatty acids which are more prevalent in butterfat. Both Harper et al. (21) and Kemp and Hetrick (27) devised extraction procedures which are supposed to extract the lower molecular weight fatty acids. For their recovery studies, the fatty acids were merely added to the products. Since the acids are more likely to be dissolved in the fat portion of the product, the acids should be dissolved in

butteroil and this used for reconstitution with a non-fat product. This procedure was used for examining both extraction methods.

The Rapid Silica Gel Method of Harper et al. (21) required the use of a special pyrex chromatographic tube.[1] It measured 38 mm. O.D. by 230 mm. in length above the joint. It was fitted with a standard taper 34/28 joint. The receiving tube was fitted with a fritted glass disc at the upper end of a standard taper joint with the lower tube from this measuring 10 mm. by 185 mm. Both outer joint and inner joint have hooks for fastening the joint together so that pressure may be used when taking fractions.

The column was made up in two sections, the lower section consisting of 5 g. silicic acid[2] ground thoroughly with 3 ml. 0.2M KH_2PO_4-K_2HPO_4 buffer at a pH of 6.5. This was slurried with 20 ml. U.S.P. chloroform and poured into the chromatographic tube onto a filter paper disc. For the top section, the milk sample was acidified to pH 1.8-2.0 (predetermined) with 20 per cent H_2SO_4 (V:V). Silicic acid was ground thoroughly with the milk sample (5 g. silicic acid to 3 ml. milk) and slurried with 5 per cent n-butanol in chloroform. This was transferred quantitatively to the tube. Before the transfer, the chloroform was allowed to drain just to the surface of the

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[1]Corning #98770

[2]Mallinckrodt #2847

previously placed silicic acid and a filter paper disc placed on the top. The top section was then added and collection of 10 ml. fractions started. After the butanol-chloroform mixture had sunk just to the top of the silicic acid, a filter paper disc was placed on the top and 150 ml. of 5 per cent n-butanol chloroform added. Light air pressure was now applied and the remainder of the solution collected in 10 ml. fractions. One ml. neutral absolute alcohol was added to each fraction before titration to a phenol red end point with 0.01N KOH in absolute alcohol.

The simplified chromatographic method of Kemp and Hetrick (26) required the use of a pyrex extraction thimble[1] measuring 35 mm. O.D., 90 mm. in overall height and fitted with a coarse fritted glass disc. A filter paper disc was placed over the fritted glass disc to prevent clogging. A 2 g. milk sample was ground thoroughly with 4 g. silicic acid[2] and transferred to the extraction thimble. A filter paper disc was placed on the surface. The extraction solvent was a 50:50 mixture of acetone and chloroform. The mortar, pestle and spatula needed for the mixing and transfer were washed four times with extraction solvent (not to exceed 20 ml. total solvent) and this poured into the thimble and allowed to drain through the column

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[1]Corning #33950

[2]Mallinckrodt #2847

and collected. Extraction was then continued under light air pressure until a total of 60 ml. extraction solvent was collected in 10 ml. fractions. The fractions were titrated to a phenolphthalein end point, using specially prepared indicator solution and base solution.

The phenolphthalein solution consists of 1 g. phenolphthalein, 50 ml. 95 per cent ethyl alcohol and made to a final volume of 100 ml. with isopropyl alcohol.

A stock solution for the base was made up by mixing 25 g. of 85 per cent KOH pellets with 400 ml. isopropyl alcohol. This was warmed to dissolve the KOH and then decanted from the resulting aqueous fraction. A 10 ml. portion of this was mixed with 240 ml. isopropyl alcohol and made to 500 ml. with absolute methanol. The normality of this KOH solution was determined by titrating to a phenolphthalein end point in 0.05N benzoic acid. The benzoic acid was made by adding 500 ml. absolute methanol to 6.1059 g. benzoic acid and making to 1000 ml. with 95 per cent isopropyl alcohol. The normality of the benzoic acid solution remained constant at 0.0500N for several months when kept under refrigeration. The normality of the base was determined daily when used.

For the first two studies on fatty acid release by the lipase of Ps. fragi, the enzyme substrate mixture was incubated in 6 oz. prescription bottles with the 2.0 g. samples being weighed directly into the mortar. In all trials

following this, the mixture was incubated in individual 2.0 g. quantities in small test tubes. This mixture was poured directly into the mortar. The mixture remaining in the tubes was then extracted twice by rubbing with silicic acid and twice with extraction solvent. The silicic acid was ground directly with the main portion and the solvent extracts were poured into the extraction thimble at the start of the extraction.

Preparation of the Sample for Chromatographic Analysis

The developing solvent used for separating the fatty acids C_4-C_{12} by partition chromatography is generally isooctane. Since this solvent is not suited for the extraction procedures, other solvents must be used and the acids then transferred to isooctane. The distillation procedures of Kemp and Hetrick (27) or Ramsey and Patterson (58) have been used and for this study a special modification[1] of the Kemp and Hetrick procedure was employed. The acetone-chloroform solvent of the Kemp and Hetrick extraction procedure was collected in a 125 ml. Erlenmeyer flask[2] fitted for a ground glass stopper. A glass bead and 15 ml. isooctane[3] were added to the flask before extraction began.

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[1]Kemp, A. R. Dean Milk Company, Rockford, Illinois Private communication, 1959

[2]Corning #5020

[3]Phillips Petroleum Company, pure grade 2,2,4-trimethylpentane

The distillation apparatus consisted of a Vigreaux distilling tube[1] 470 mm. in length and fitted with a standard taper 24/40 joint at the lower end. The side arm was lengthened to 270 mm. with the last 70 mm. bent perpendicularly downward. This goes directly to a Liebig condenser[2] with a jacket 300 mm. in length. The receiving flask was a 250 ml. filtering flask[3] with tubulation. This was placed in an ice water bath. The 125 ml. Erlenmeyer flask was used for a boiling flask and was joined to the Vigreaux column with a Teflon standard taper 24/40 sleeve[4] at the joint. The flask was heated by placing an electric hot plate[5] directly in contact with the base of the flask, the heat being controlled with a voltage regulator[6]. A thermometer was not necessary, since the distillation has been found to be complete when a certain residual volume remains. The top of the Vigreaux column was stoppered with a rubber stopper completely covered with aluminum foil.

Distillation was started with the voltage set at 75 volts

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[1]Corning #9400

[2]Corning #2340

[3]Corning #5430

[4]Arthur S. LaPine and Company

[5]Fisher #11-467-1A

[6]Powerstat type 116

and continued until less than 10 ml. of solvent remained in the boiling flask. The apparatus was allowed to cool and 5 ml. acetone and 4 ml. isooctane added through the top of the Vigreaux column. Distillation was again started with the voltage regulator set at 85 volts and continued until no more distillate came over. The residual volume will be between 7 and 10 ml. but should not be more than 10 ml. The apparatus was allowed to cool once more and the boiling flask removed and stoppered. The distillate flask was removed and 5 ml. isooctane washed through the Vigreaux column into it. The distillate was then titrated to a phenolphthalein end point. A titration of less than 0.20 ml. 0.015N base is ignored. If titration exceeds this, it is assumed to be butyric acid and is calculated as such. Titration rarely exceeded 0.16 ml. of base.

Chromatographic Analysis

Ramsey and Patterson (58) and Kemp and Hetrick (27) both developed chromatographic procedures for the separation of mixtures of fatty acids. The main differences between the two procedures are in the way the columns are prepared and the method in which the fractions are taken. The procedure used here is similar in some respects to both, but the column preparation is quite different from either in that no acid-base indicator is used. Fractions are taken in a manner similar to

that of Kemp and Hetrick.

The packing material consisted of 16 g. silicic acid[1] and a predetermined amount of absolute methanol. To determine the amount of methanol a certain lot of silicic acid would adsorb, 10 ml. methanol was added to 16 g. silicic acid in a 500 ml. Erlenmeyer flask. This was mixed by shaking until the mixture was free from lumps. Methanol was then added in 0.5 ml. amounts with thorough mixing after each addition until the mixture became slightly sticky. This mixture must be free-flowing and dustless so the proper amount of methanol to add is 0.5 ml. less than the amount causing the stickiness. This was sufficient packing material for one column, but enough for several was made at one time in separate flasks and stored under refrigeration.

To pack the column, 50 ml. isooctance saturated with 90 per cent methanol was added to the packing material and swirled until uniform. A portion of this (45 ml.) was transferred to a chromatographic tube measuring 13 mm. I.D. by 600 mm. in length. The tube was made from Pyrex glass tubing in which a constriction had been formed 50 mm. from the lower end for holding a small wad of Pyrex glass wool. A filter paper disc was placed on the wadding and the packing material added. The silicic acid settled considerably as the isooctane slowly

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[1]Mallinckrodt #2847

drained through. Air pressure occasionally was applied to speed draining. After the isooctane had drained just to the surface of the silicic acid, a filter paper disc was pressed onto the surface by means of a glass rod fitted with a small cork at its end. It was found necessary to apply as much pressure on the glass rod as possible as the silicic acid must be packed tightly. The isooctane was drained to the surface again, air pressure being necessary this time. The sample was placed on the column by means of a volumetric pipette, 2 ml. being the optimum sample size. Collection of fractions was now started. Various fractions of 1 ml., 2 ml., 5 ml. and 10 ml. were taken during the development. Test tubes marked at the proper level with a diamond marking pencil were found adequate. The order of the fractions taken was dependent on column size and the product being analyzed. Thus a trial column was run in order to determine this. The first two fractions taken were 5 ml. fractions, the first serving as a column blank. The 2 ml. sample was drained just to the surface of the column, 3 ml. of isooctane saturated with 90 per cent methanol was then added to wash down the sides of the tube and this drained to the surface. The tube was now filled with isooctane saturated with 90 per cent methanol and development continued. The liquid level of the column was never allowed to drop below the surface of the silicic acid, as this caused the column to crack and form

bubbles. The fractions were titrated as soon as they were removed from the column, using the method described for the Kemp and Hetrick extraction procedure (27).

Calculations for milligrams acid released per 10 g. fat are as follows:

$$\frac{100}{2.0} \times \frac{\text{total extract titration}}{\text{total column titration}} \times \frac{\text{normality of base} \times 1000}{1000}$$

$$= k$$

k x ml. base (individual acid) x molecular weight of the acid = milligrams acid per 10 g. fat.

The total extract titration is the sum of the titrations of a. the total titration from the several acids separated on the column, b. the residue of the extracted acids which were not placed on the column and c. the butyric acid which distilled over during solvent displacement. The fraction 100/2.0 is used for increasing the sample size from 2.0 g. (0.2 g. fat) to a 100 g. (10 g. fat) basis. The final result less the control value, calculated in the same manner, gives the milligrams acid released by lipolysis. The milligrams of acid calculated for lauric acids and above are calculated from the average of molecular weights and molar per cent figures given on Table 1 obtained from Hilditch (22). These figures are not precise but merely an estimation, since the composition of butterfat is not constant and also the proportion of each fatty acid released during lipolysis may not be the same among a number of runs. Total acid titrations for the extracts from which the

samples for determination of individual fatty acids were taken are given in the tables as footnotes.

The values in the tables for milliliters of base to titrate the acids released by lipolysis are the actual values from the columns and include residual acids in the butteroil as well. All other values are computed less the values for zero time, so that they express only the acids released by lipolysis. While the usual method of expressing fat acidity is in milliliters of 1N base for 10 g. fat, for purposes of calculating molar percentages and per cent of each acid released, the fat acidity is expressed in milligrams of acid per 10 g. fat. The percentage values used for computing the

Table 1. Molar per cents and percentage by weights of fatty acids in butterfat as given by Hilditch (22).

Acids	Molecular weights	Molar per cent	Per cent by weight
Butyric	88.10	10	4
Caproic	116.16	4	2
Caprylic	144.21	2	1
Capric	172.26	3	2
Lauric and above	268.02	81	87
Glycerol	92.09		4
Totals		100	100

amounts of each fatty acid originally present in the fat are those given by Hilditch (22), as shown on Table 1.

Heat Inactivation Studies

The lipase preparation from Ps. fragi was pipetted into screw-capped test tubes[1] measuring 16 by 125 mm. (10 ml. per tube). These were capped tightly and placed in a water bath previously adjusted to a temperature of 62.1±0.1° C. The temperature of the lipase preparation was followed by inserting a thermometer into a tube containing the same amount of lipase preparation. A rubber stopper was used to seal this tube. All tubes were submerged so that the tops were at least 1 inch below the surface.

After the tubes had been in the bath for the desired period of time, they were removed and immediately 1 ml. of the lipase preparation was pipetted into a tube containing 3 ml. of the lipase test substrate. The temperature of the test substrate must be no lower than room temperature or the emulsion will break and not significantly higher or the lipase preparation will not be cooled sufficiently after the heat treatment. The contents of the tube were mixed by tipping the tube slowly so that aeration was held to a minimum.

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[1]Corning #9825

EXPERIMENTAL RESULTS

The Rapid Silica Gel Method of Harper *et al.* (21)

Washed and neutralized butteroil was mixed with a series of fatty acids as shown in Table 2. This was reconstituted with skim milk to make a final product containing 10 per cent by weight of the fatty acid-butteroil mixture. This was mixed in a Waring blendor by adding the butteroil mixture dropwise as mixing took place. All ingredients were at a temperature of 45° C. Samples of 2.00 g. were immediately taken and weighed on an analytical balance. In order to compare this procedure more critically with the procedure of Kemp and

Table 2. The acids added to butteroil for reconstituting with skim milk and their titration values in the final product.

Acids Added	Amount (g.) added to butteroil to make 10 g. mixture	Ml. of 0.0118N base to titrate the acids in 2 g. of product
Butyric	1.2775	24.57
Caproic	0.4870	7.10
Caprylic	0.3165	3.71
Capric	0.4960	4.88
Lauric	0.7501	6.34
Myristic	1.5908	11.80
Butteroil	5.0813	
Totals	9.9992	58.40

Hetrick, two of the samples were adjusted to pH 1.8-2.0 as is normal and two were left unadjusted. Table 3 shows the results of these experiments.

One sample on which the pH was not adjusted gave a notably lower recovery than did the other unacidified sample and the two acidified samples were more constant with 0.83 ml. of base difference as contrasted with a 4.45 ml. difference between the unacidified samples. It is apparent from the control titrations that the neutralized butteroil used here contained some residual acidity. This was probably made up of higher molecular weight fatty acids which do not combine as readily with the base used for the neutralization.

The Silica Gel Method of Kemp and Hetrick (27)

Washed and neutralized butteroil was mixed with a series of fatty acids as shown in Table 4. This was reconstituted with skim milk in the same manner as for the previous trial, except that none of the samples was acidified prior to extraction. Table 5 shows the results of this trial.

As was noted before, the controls contained some residual acidity, it being extracted with very little of the acetone-chloroform solvent. The amount of solvent required for extraction was considerably less with this method, 40 ml. being sufficient, as indicated by Kemp and Hetrick (27).

Recovery of the added acids by this method was virtually

Table 3. Fatty acid recovery by the method of Harper *et al.* (21) expressed as ml. 0.0118N base.

Fraction	Control* unadjusted	Control* pH 1.8-2.0	I unadjusted	II unadjusted	III pH 1.8-2.0	IV pH 1.8-2.0
10	0.42	0.74	9.06	36.80	40.00	38.62
20	0.12	0.43	11.89	3.62	2.04	4.17
30	0.11	0.45	14.23	0.28	1.05	0.73
40	0.11	0.38	1.15	0.16	0.55	0.65
50	0.11	0.41	0.57	0.15	0.58	0.59
60	0.12	0.44	0.20	0.13	0.69	0.57
70	0.08	0.50	0.10	0.20	0.75	0.65
80	0.11	0.62	0.10	0.20	0.61	0.81
90	0.07	0.70	0.11	0.14	0.55	0.64
100	0.09	0.73	0.08	0.14	0.47	0.50
110	0.07	0.81	0.11	0.13	0.44	0.48
120	0.07	0.82	0.09	0.09	0.40	0.36
130	0.07	0.72	0.09	0.11	0.41	0.34
140	0.07	0.69	0.07	0.10	0.26	0.35
150	0.06	0.65	0.10	0.15	0.21	0.28
Totals	1.70	9.09	37.95	42.40	49.01	49.74
Less Controls			36.25	40.70	39.88	40.65
Per Cent recovery			62.07	69.69	68.29	69.61

*Distilled water replaced the acids in both controls.

complete with 99.21 per cent recovery being the lowest obtained. Compared with the less than 70 per cent recoveries of the method of Harper et al., this is far more efficient. In order to test this procedure more fully, a second trial was performed. Table 6 shows the amounts of acids added and the extraction results. Recoveries of not less than 96 per cent again show that this method is highly effective for extracting the free fatty acids from products containing butteroil.

Table 4. The acids added to butteroil for reconstituting with skim milk and their titration values in the final product.

Acids	Amount (g.) added to butteroil to make 10 g. mixture	Ml. of 0.0126N base calculated to titrate the acids in 2 g. of product
Butyric	0.3208	5.78
Caproic	0.4950	6.76
Caprylic	0.4331	4.77
Capric	0.5246	4.83
Lauric	0.7462	5.91
Myristic	0.8637	6.00
Totals	3.3834	34.05
Butteroil	6.6219	
Total	10.0053 g.	

Table 5. Fatty acid recovery by the method of Kemp and Hetrick (27) expressed as ml. 0.0126N base.

Fraction	Control* I	Control* II	Recovery I	Recovery II
2	0.20	0.23	27.71	24.07
4	0.09	0.09	5.41	6.90
6	0.06	0.07	0.73	1.79
8	0.05	0.06	0.34	0.59
10	0.05	0.05	0.14	0.27
15	0.08	0.08	0.14	0.36
20	0.06	0.07	0.10	0.19
30	0.09	0.08	0.11	0.19
40	0.07	0.08	0.10	0.13
50	0.07	0.07	0.09	0.09
60	0.07	0.06	0.10	0.09
70	0.08	0.07	0.08	0.10
Totals	0.97	1.01 (0.99 avg.)	35.05	34.77
Less Control			34.06	33.78
Per cent recovery			100.03	99.21

*Distilled water replaced the acids in the controls.

A further trial was performed to test the efficiency of the method for extracting the acids from the sodium taurocholate emulsion of Nashif and Nelson (41). The acids were added to washed and neutralized butteroil as shown in Table 7. Mixing was performed with a hand homogenizer as would be normal for this emulsion. Results of this trial are also shown in Table 7. Recovery values again were high, with the lowest recovery being 96.94 per cent. All succeeding extractions were by the method of Kemp and Hetrick.

Table 6. The acids added to butteroil for reconstituting and their recovery by the method of Kemp and Hetrick (27).

Acids	Amount (g.) added to butteroil to make 10 g. mixture	Ml. 0.0615N base calculated to neutralize the acids in 2 g. of 10% product	Results (ml. 0.0615N base)
Butyric	0.4232	1.56	I 5.12 ml.
Caproic	0.4286	1.20	II 5.09 ml.
Caprylic	0.3453	0.78	Control 0.31 ml.
Capric	0.2920	0.55	% recovery
Lauric	0.2918	0.47	I 96.59
Myristic	0.2916	0.42	II 95.98
Total	2.0725	4.98	
Butteroil	7.9288		
Total	10.0013		

Sample Preparation by the Modified Kemp and Hetrick Distillation Technique

Since recovery data were not available for this modification of the Kemp and Hetrick distillation procedure, recovery procedures were performed as used previously in this study. Table 8 shows the amounts of acids added to washed and neutralized butteroil. A 0.2134 g. portion of this was weighed into a 250 ml. Erlenmeyer flask fitted with a standard taper ground glass joint. To this were added 15 ml. pure isooctane

Table 7. The acids added to butteroil for reconstituting and their recovery from the lipase test substrate by the method of Kemp and Hetrick (27).

Acids	Amount (g.) added to butteroil to make 10 g. mixture	Ml. 0.0119N base calculated to neutralize the acids in 2 g. of substrate	Results (ml. 0.0119N base)
Butyric	0.2537	4.84	I 14.46
Caproic	0.1545	2.24	II 14.21
Caprylic	0.1478	1.72	Control 0.59
Capric	0.1686	1.64	% recovery
Lauric	0.2105	1.77	I 98.72
Myristic	0.2501	1.84	II 96.94
Total	1.1852	14.05	
Butteroil	8.8189		
Total	10.0041		

and 70 ml. of the mixture of 50 per cent acetone in chloroform so as to reproduce actual extraction conditions as closely as possible. Distillation was then performed as previously described.

Recovery of the individual fatty acids was determined by the Kemp and Hetrick chromatographic technique. For this method, 16 g. of silicic acid was ground thoroughly with 11.0 ml. of a solution consisting of 100 mg. bromocresol green, 0.35 ml. concentrated ammonium hydroxide reagent and made to

Table 8. Recovery of fatty acids by the modified distillation procedure.

Acids	Amount (g.) added to butteroil to make 10 g. mixture	Ml. 0.0127N base to titrate the added acids	Ml. 0.0127N base recovered from column	Per cent recovery
Myristic	0.2555	0.590	1.203	103.7
Lauric	0.2167	0.579		
Capric	0.4018	1.230	1.266	102.9
Caprylic	0.3110	1.137	1.150	101.1
Caproic	0.3015	1.369	1.415	103.4
Butyric	0.3065	1.833	1.819	99.2
Butteroil	8.3048			
Totals	10.0975	6.729	6.853	101.7

a final volume of 250 ml. with absolute methanol. The resulting dry powder was then slurried with 45 ml. isooctane saturated with 90 per cent methanol. This was poured into a chromatographic tube measuring 18 by 500 mm. with a small wad of pyrex wool at the constriction and a filter paper disc covering this. All isooctane going into the column must be saturated with 90 per cent methanol to prevent running of the indicator. The silicic acid was allowed to settle and the isooctane then pressed through with air pressure. After the isooctane level had almost reached the surface of the silicic acid column, pressure was released and a filter paper disc was then placed

on the surface. The column was packed firmly with a cork plunger. The sides of the tube were washed down with 3 to 4 ml. of isooctane and this was then drained just to the surface of the column.

The sample was then placed on the column, using a volumetric pipette and fraction collection started immediately. Sample size for this column was 2 ml. The sample was allowed to drain just to the column surface and the sides of the tube again washed down with 3 ml. isooctane, this being drained just to the column surface before filling the tube with isooctane (about 50 ml. required). Fraction sizes used are shown on Table 9 and were titrated as previously described immediately after removal from the column. The first 10 ml. fraction will contain no acidity and is used as the column control. Normality of the base for all titrations was 0.0127N.

The distillate titrated 0.780 ml. base and the control 0.200 ml. base, leaving a net titration of 0.580 ml. base. The cumulative total from the column was 6.669 ml. base and the titration of the extract remaining in the flask was 14.355 ml. base. The overall total extract titration plus distillate is 21.604 ml. The amount from the distillate which should have been on the column was:

$$\frac{6.669}{21.024} \times 0.580 = 0.184 \text{ ml.}$$

This makes the total corrected column titration 6.853 ml. base.

Table 9. Separation of the acids recovered from the modified distillation procedure by the Kemp and Hetrick chromatographic technique (27).

Fraction size (ml.)	Cumulative (ml.)	Titration (ml.)	Control (ml.)	Net titration (ml.)	Cumulative (ml.)	Ind. (ml.)	Acids
10	10	0.030	0.030				
5	15	0.030	0.015	0.015	0.015		
5	20	0.265	0.015	0.250	0.265		
2	22	0.300	0.006	0.294	0.559		
2	24	0.265	0.006	0.259	0.818		
2	26	0.240	0.006	0.234	1.052		
2	28	0.120	0.006	0.114	1.166		
2	30	0.080	0.006	0.074	1.240	1.203	C_{14}+C_{12}
2	32	0.135	0.006	0.129	1.369		
2	34	0.265	0.006	0.259	1.628		
2	36	0.370	0.006	0.364	1.992		
2	38	0.270	0.006	0.264	2.256		
2	40	0.130	0.006	0.124	2.380		
2	42	0.070	0.006	0.064	2.444		
5	47	0.065	0.015	0.050	2.494	1.266	C_{10}
10	57	0.840	0.030	0.810	3.304		
5	62	0.250	0.015	0.235	3.539		
5	67	0.080	0.015	0.065	3.604		
10	77	0.060	0.030	0.030	3.634	1.150	C_8
10	87	0.485	0.030	0.455	4.089		
10	97	0.715	0.030	0.685	4.774		
10	107	0.250	0.030	0.220	4.994		
10	117	0.070	0.030	0.040	5.034		
10	127	0.030	0.030	0.000	5.034	1.415	C_6
20	147	0.150	0.090	0.090	5.124		
20	167	0.870	0.060	0.810	5.934		
20	187	0.665	0.060	0.605	6.539		
10	197	0.095	0.030	0.065	6.604		
10	207	0.055	0.030	0.025	6.629		
10	217	0.050	0.030	0.020	6.649		
10	227	0.050	0.030	0.020	6.669		
10	237	0.030	0.030	0.000	6.669	1.635	C_4
Distillate						0.184	
Total						1.819	C_4

Calculations for amounts which should be on the column are:

$$\underset{\text{I}}{\frac{0.2134}{10.0975}} \times \underset{\text{II}}{\frac{6.853}{21.604}} = 0.006704$$

$$0.006704 \times \frac{\text{amount of acid added}}{\text{molecular weight of the acid}} \times \frac{1000}{\text{normality of base}} = \text{ml. base for each acid}$$

Fraction I represents the corrected portion of the sample distilled. Fraction II represents the corrected portion of the distilled material placed on the column.

It can be seen from the results shown on Table 8 that recovery of all acids was quite complete. Again, there was some evidence for residual acids being present in the butteroil to give the high figures noted with the higher molecular weight acids. Since these acids were expected to be in only the lauric acid and above fraction, no control was used for the butteroil and this can only be speculated on at this point. Controls were used in all experiments performed where the amount of free fatty acids was unknown and it will be shown from these that there were small amounts of free fatty acids present in the butteroil starting with caprylic acid and increasing slightly as the molecular weight of the acids increased.

The Modified Chromatographic Technique

The column used in the previous experiment required over 230 ml. developing solvent to elute all of the acids placed on

the column. This is both expensive and time consuming. Also, if there are traces of chloroform remaining in the sample after the distillation step, the bromocresol green indicator would be eluted and would mask the phenolphthalein end points when titrating the fractions. Since the indicator is of little value when so many fractions are collected, columns were tried using absolute methanol alone for the internal phase. The ammonium hydroxide was also omitted since its principal function was to raise the pH of the column above the end point of the indicator. This also could cause errors in titration if eluted with the indicator.

A series of acids was weighed out and dissolved in isooctane as shown on Table 10, 2 ml. of this being placed on the column. The column was freshly prepared as in the procedures section of this paper. The fractions were titrated with 0.0133N base as shown on Table 11.

The reduction to 85 ml. developing solvent required for complete elution on this column is partly due to column size and partly to other undetermined factors. The break between lauric and caproic acids is not sharp enough for accurate estimation of either acid. Since the difference in column size was not enough to cause a two-thirds reduction in eluting solvent, it could be caused by the absence of a factor influencing the retention of the acids on the column. Butteroil, which was previously used for dissolving the acids was missing

Table 10. Recovery of fatty acids from the modified chromatographic technique.

Acids	Amount (g.) added to 100 ml. isooctane	Ml. 0.0133N base to titrate the acids in 3 ml. isooctane	Ml. 0.0133N base recovered from column	Per cent recovery
Myristic	0.1713	1.128	2.274	100.4
Lauric	0.1514	1.137		
Capric	0.0472	0.412	0.407	98.8
Caprylic	0.0241	0.251	0.246	98.0
Caproic	0.0295	0.382	0.383	100.3
Butyric	0.0124	0.212	0.209	98.6
Butteroil	None			
Totals	0.4359	3.522	3.519	99.9

in this case but would not be in the actual experimental trials, since it is extracted with the acids and then concentrated with the acids when the solvent was distilled off. For this reason, a further trial was made to see what effect butteroil might have on the threshhold volumes of the acids.

The acids were dissolved in butteroil as shown on Table 12. This was made to a final volume of 250 ml. with pure isooctane and 2 ml. of this placed on a freshly packed column. The results of this trial are shown on Table 13. Both this column and the previous column were prepared in the same way, yet the

Table 11. Fatty acid separation by the modified chromatographic technique.

Fraction (ml.)	Cumulative (ml.)	Titration (ml.)	Control (ml.)	Net Titration (ml.)	Cumulative (ml.)	Ind. (ml.)
5	5	0.015	0.015	0.000	0.000	
5	10	0.015	0.015	0.000	0.000	
2	12	0.060	0.006	0.054	0.054	
2	14	0.495	0.006	0.489	0.543	
2	16	0.810	0.006	0.804	1.347	
2	18	0.680	0.006	0.674	2.021	
1	19	0.200	0.003	0.197	2.218	2.274
1	20	0.115	0.003	0.112	2.330	
1	21	0.150	0.003	0.147	2.477	
1	22	0.075	0.003	0.072	2.549	
1	23	0.055	0.003	0.052	2.601	
1	24	0.055	0.003	0.052	2.653	0.407
1	25	0.050	0.003	0.047	2.700	
1	26	0.075	0.003	0.072	2.772	
1	27	0.065	0.003	0.062	2.834	
1	28	0.040	0.003	0.037	2.871	
1	29	0.025	0.003	0.022	2.893	
1	30	0.020	0.003	0.017	2.910	
1	31	0.020	0.003	0.017	2.927	0.246
1	32	0.030	0.003	0.027	2.954	
2	34	0.090	0.006	0.084	3.038	
2	36	0.100	0.006	0.094	3.132	
2	38	0.080	0.006	0.074	3.206	
2	40	0.050	0.006	0.044	3.250	
2	42	0.030	0.006	0.024	3.274	
2	44	0.020	0.006	0.014	3.288	
2	46	0.017	0.006	0.011	3.299	
2	48	0.017	0.006	0.011	3.310	0.383
2	50	0.020	0.006	0.014	3.324	
5	55	0.115	0.015	0.100	3.424	
5	60	0.075	0.015	0.060	3.484	
5	65	0.035	0.015	0.020	3.504	
5	70	0.025	0.015	0.010	3.514	
5	75	0.020	0.015	0.005	3.519	0.209
5	80	0.015	0.015	0.000		

butteroil apparently caused a 30 per cent increase in effluent volume. This was sufficient to considerably sharpen the break between lauric and capric acids and still retain good recovery values.

Table 12. Effect of butteroil on recovery of fatty acids by the modified chromatographic technique.

Acids	Amount (g.) added to 250 ml. isooctane	Ml. 0.0132N base calculated	Ml. 0.0132N base recovered from column	Per cent recovery
Myristic	0.4883	1.296		
Lauric	0.4406	1.333		
Sub-totals		2.629	2.713	103.2
Capric	0.0498	0.175	0.180	102.8
Caprylic	0.0689	0.290	0.290	101.4
Caproic	0.0301	0.157	0.154	98.1
Butyric	0.0243	0.167	0.165	98.8
Butteroil	5.1458			
Totals	6.2478	3.418	3.506	102.6

Lipase Activity by Ps. fragi

For the initial study with the lipase of Ps. fragi, the substrate and enzyme preparation were mixed as previously

Table 13. Effect of butteroil on separation of fatty acids by the modified chromatographic technique.

Fraction (ml.)	Cumulative (ml.)	Titration (ml.)	Control (ml.)	Actual (ml.)	Cumulative (ml.)	Ind. (ml.)
5	5	0.015	0.015			
5	10	0.015	0.015			
2	12	0.010	0.006			
2	14	0.020	0.006	0.014	0.014	
1	15	0.130	0.003	0.127	0.141	
1	16	0.430	0.003	0.427	0.568	
1	17	0.490	0.003	0.487	1.055	
1	18	0.515	0.003	0.512	1.567	
1	19	0.450	0.003	0.447	2.014	
1	20	0.370	0.003	0.367	2.381	
1	21	0.180	0.003	0.177	2.558	
1	22	0.125	0.003	0.122	2.680	2.713
1	23	0.070	0.003	0.067	2.747	
1	24	0.090	0.003	0.087	2.834	
1	25	0.040	0.003	0.037	2.871	
1	26	0.025	0.003	0.022	2.893	0.180
1	27	0.030	0.003	0.027	2.920	
1	28	0.040	0.003	0.037	2.957	
1	29	0.050	0.003	0.047	3.004	
1	30	0.060	0.003	0.057	3.061	
2	32	0.075	0.006	0.069	3.130	
2	34	0.035	0.006	0.029	3.159	
2	36	0.025	0.006	0.019	3.178	0.294
2	38	0.015	0.006	0.009	3.187	
2	40	0.020	0.006	0.014	3.201	
2	42	0.035	0.006	0.029	3.230	
2	44	0.040	0.006	0.034	3.264	
2	46	0.040	0.006	0.034	3.298	
2	48	0.025	0.006	0.019	3.317	
2	50	0.020	0.006	0.014	3.331	
5	55	0.025	0.015	0.010	3.341	
5	60	0.015	0.015	0.000	3.341	0.154
5	65	0.020	0.015	0.005	3.346	
5	70	0.030	0.015	0.015	3.361	
5	75	0.050	0.015	0.035	3.396	
5	80	0.070	0.015	0.055	3.451	
10	90	0.075	0.030	0.045	3.496	
10	100	0.040	0.030	0.010	3.506	
10	110	0.030	0.030	0.000	3.506	0.165

described and a zero time analysis made. Further analyses were made after incubation for 48 and 96 hours. Incubation temperature was 32° C. Results of these analyses are shown on Table 14.

A second trial was then made under the same conditions, but using another lipase preparation from the same organism and fresh butteroil. Three individual trials using the same lot of lipase were performed simultaneously so that conditions would be as constant as possible. Results of these trials are shown in Tables 15, 16 and 17.

Due to a tendancy for fat separation in the test substrate, individual 2.0 g. portions were incubated in small test tubes. Table 18, shows the results of a single trial using this method.

The Effect of Heat Treatment on the Lipase of Ps. fragi.

Nashif and Nelson (41) previously reported that the lipase of Ps. fragi retained considerable activity after being exposed to pasteurization. Since activity is altered by this treatment, the proportions of the acids released could be altered as well.

The lipase preparation was treated as previously described for periods of 0, 5, 10, 15, 20 and 30 minutes at 62.1° C. The time required for the samples to reach 62.1° C.

Table 14. The fatty acids released from butterfat by the lipase of Ps. fragi.

Acids	Ml. 0.0122N base from column	Mg. of acid released per 10 g. fat	Molar per cent released	Per cent acid released
Control				
Lauric & above	0.259	178.1		
Capric	0.028	12.4		
Caprylic	0.038	14.1		
Caproic				
Butyric				
Totals	0.325[a]	204.6		
48 Hours				
Lauric & above	1.847	821.4	72.7	9.4
Capric	0.159	42.9	6.0	21.5
Caprylic	0.179	38.0	6.5	38.0
Caproic	0.110	25.8	5.0	12.9
Butyric	0.215	38.2	9.8	9.6
Totals	2.510[a]	966.3	100.0	10.1
96 Hours				
Lauric & above	3.316	1266.1	75.6	14.6
Capric	0.301	71.9	6.8	36.0
Caprylic	0.239	40.5	5.0	40.5
Caproic	0.155	29.3	3.8	14.7
Butyric	0.355	50.8	8.8	12.7
Totals	4.366[a]	1458.6	100.0	15.2

[a]Total acid titrations of the extracts were:

Zero time	1.390
48 hour	8.240
96 hour	11.630

Table 15. The fatty acids released from butterfat by the lipase of Ps. fragi (trial 1).

Acids	Ml. 0.0139N base from column	Mg. of acid released per 10 g. fat	Molar per cent released	Per cent acid released
Zero time				
Lauric & above	0.238	172.3		
Capric				
Caprylic				
Caproic				
Butyric				
Totals	0.238[a]	172.3		
48 Hours				
Lauric & above	1.745	835.1	66.8	9.6
Capric	0.278	103.2	12.8	51.6
Caprylic	0.217	67.4	10.0	67.4
Caproic	0.117	29.3	5.4	14.6
Butyric	0.108	20.5	5.0	5.1
Totals	2.465[a]	1055.5	100.0	11.0
96 Hours				
Lauric and above	1.439	871.3	63.7	10.0
Capric	0.217	101.2	11.5	50.6
Caprylic	0.222	86.6	11.8	86.6
Caproic	0.114	35.8	6.0	17.9
Butyric	0.132	31.5	7.0	7.9
Totals	2.124[a]	1126.4	100.0	11.7

[a]Total acid titration of the extracts were:

Zero time	0.925
48 hours	7.640
96 hours	8.270

Table 16. The fatty acids released from butterfat by the lipase of Ps. fragi (trial 2).

Acids	Ml. 0.0139N base from column	Mg. of acid released per 10 g. fat	Molar per cent released	Per cent acid released
Zero time				
Lauric & above	0.238	172.3		
Capric				
Caprylic				
Caproic				
Butyric				
Totals	0.238[a]	172.3		
48 Hours				
Lauric & above	2.018	905.1	70.9	10.4
Capric	0.202	69.3	8.4	34.6
Caprylic	0.270	77.6	11.3	77.6
Caproic	0.090	20.8	3.8	10.4
Butyric	0.135	23.7	5.6	5.9
Totals	2.715[a]	1069.5	100.0	11.1
96 Hours				
Lauric & above	3.506	1213.7	73.1	14.0
Capric	0.378	96.0	9.0	48.0
Caprylic	0.272	57.9	6.5	57.9
Caproic	0.218	37.4	5.2	18.7
Butyric	0.260	33.8	6.2	8.4
Totals	4.634[a]	1438.8	100.0	15.0

[a]Total acid titrations of the extracts were:

Zero time	0.925
48 hours	7.780
96 hours	9.835

Table 17. The fatty acids released from butterfat by the lipase of Ps. fragi (trial 3).

Acids	Ml. 0.0139N base from column	Mg. of acid released per 10 g. fat	Molar per cent released	Per cent acid released
Zero time				
Lauric & above	0.238	172.3		
Capric				
Caprylic				
Caproic				
Butyric				
Totals	0.238[a]	172.3		
48 Hours				
Lauric & above	2.101	932.5	75.2	10.7
Capric	0.137	46.3	5.8	23.2
Caprylic	0.187	52.9	7.9	52.9
Caproic	0.126	28.7	5.3	14.4
Butyric	0.136	23.5	5.8	5.9
Totals	2.687[a]	1083.9	100.0	11.3
96 Hours				
Lauric & above	3.324	1066.0	72.8	12.2
Capric	0.356	85.2	9.1	42.6
Caprylic	0.284	56.9	7.2	56.9
Caproic	0.235	37.9	6.0	19.0
Butyric	0.192	23.5	4.9	5.9
Totals	4.391[a]	1269.5	99.9	13.2

[a]Total acid titrations of the extracts were:

Zero time	0.925
48 hours	7.585
96 hours	8.783

Table 18. Values received from lipolysis of butterfat by the lipase of Ps. fragi when individual 2 g. portions of substrate were employed.

Acids	Ml. 0.0132N base from column	Mg. released per 10 g. fat	Molar per cent released	Per cent acid released
Zero time				
Lauric & above	0.307	237.5		
Capric	0.071	35.2		
Caprylic	0.037	15.4		
Caproic				
Butyric				
Totals	0.415[a]	288.1		
48 Hours				
Lauric & above	2.337	1269.5	74.6	14.6
Capric	0.207	50.6	5.0	25.3
Caprylic	0.246	70.0	7.7	70.0
Caproic	0.125	34.9	4.6	17.5
Butyric	0.220	46.6	8.1	11.7
Totals	3.135[a]	1471.6	100.0	15.3
96 Hours				
Lauric & above	3.920	1840.7	81.5	21.2
Capric	0.268	56.1	4.4	28.1
Caprylic	0.267	60.8	5.2	60.8
Caproic	0.200	45.9	4.5	23.0
Butyric	0.195	34.0	4.4	8.5
Totals	4.850[a]	2037.5	100.0	21.2

[a]Total acid titrations of the extracts were:

Zero time	1.815
48 hours	11.430
96 hours	15.985

was 2.5 minutes. Following heating, the enzyme preparations were immediately mixed with the substrate and a zero time sample analyzed. Results are shown on Table 19. Analyses on the 5 and 15 minutes treated samples did not give distinct separation of the individual fatty acids and only the total values are given.

A second trial was performed so as to include the effect of the time required to reach 62.1° C. Times included in this trial were 0, 3 minutes after zero time and 5, 15 and 30 minutes at 62.1° C. Results of this trial are shown on Table 20.

Lipase Activity by C. lipolytica

For studying fatty acid release by the lipase of C. lipolytica, the enzyme-substrate mixture was incubated in individual 2.0 g. portions for 72 hours at 35° C. Results of analyses are shown on Table 21.

Poor separation of the several acids on the columns made it necessary to perform a second experiment. The conditions used were the same as for the first trial. Results of this second series are shown in Table 22. The threshhold volumes from these analyses were used as guides for computing separations on trials reported in Table 21.

Table 19. Effect of heat treatment on lipolysis of butterfat by the lipase of Ps. fragi.

Acids	Ml. 0.0120N base from column	Mg. of acid released per 10 g. fat	Molar per cent released	Per cent acid released
Zero time				
Lauric & above	0.284	223.6		
Capric				
Caprylic				
Caproic				
Butyric				
Totals	0.284[a]	223.6		
Untreated				
Lauric & above	2.270	1255.4	75.3	14.4
Capric	0.290	121.4	11.0	60.7
Caprylic	0.171	59.9	6.5	59.9
Caproic	0.091	25.7	3.4	12.8
Butyric	0.100	21.4	3.8	5.4
Totals	2.922[a]	1483.8	100.0	15.4
5 Minutes				
Totals	10.670[a]	1099.2		11.4
10 Minutes				
Lauric & above	2.416	880.4	71.8	10.1
Capric	0.255	74.9	8.6	37.5
Caprylic	0.169	41.6	5.7	41.6
Caproic	0.180	35.6	6.1	17.8
Butyric	0.235	35.3	7.9	8.8
Totals	3.255[a]	1067.8	100.0	11.1
20 Minutes				
Lauric & above	2.550	699.1	72.7	8.0
Capric	0.220	51.2	7.1	25.6
Caprylic	0.265	51.6	8.5	51.6
Caproic	0.125	19.6	4.0	9.8
Butyric	0.240	28.5	7.7	7.1
Totals	3.400[a]	850.0	100.0	8.9

Table 19. (Continued)

Acids	Ml. 0.0120N base from column	Mg. of acid released per 10 g. fat	Molar per cent released	Per cent acid released
30 Minutes				
Lauric & above	1.592	694.2	77.9	8.0
Capric	0.141	52.2	8.4	26.1
Caprylic	0.139	43.1	8.3	43.1
Caproic	0.040	10.0	2.4	5.0
Butyric	0.050	9.5	3.0	2.4
Totals	1.962[a]	809.0	100.0	8.4

[a]Total acid titrations of the extracts were:

Zero time	1.390 ml.
Untreated	11.190
5 minutes	10.670
10 minutes	9.250
15 minutes	8.290
20 minutes	7.650
30 minutes	7.035

Table 20. Effect of heat treatment on lipolysis of butterfat by the lipase of Ps. fragi.

Acids	Ml. 0.0159N base from column	Mg. of acid released per 10 g. fat	Molar per cent released	Per cent acid released
Zero time				
Lauric & above	0.163	200.3		
Capric				
Caprylic				
Caproic				
Butyric				
Totals	0.163[a]	200.3		
Untreated				
Lauric & above	2.068	1373.3	81.0	15.8
Capric	0.172	84.1	7.3	42.0
Caprylic	0.108	44.2	4.6	44.2
Caproic	0.098	32.3	4.2	16.2
Butyric	0.070	17.5	3.0	4.4
Totals	2.516[a]	1551.4	100.1	16.2
3 Minutes				
Lauric & above	2.213	1102.2	74.9	12.7
Capric	0.238	90.0	8.7	45.0
Caprylic	0.205	64.9	7.5	64.9
Caproic	0.090	23.0	3.3	11.5
Butyric	0.155	30.0	5.7	7.5
Totals	2.901[a]	1310.1	100.1	13.6
5 Minutes				
Lauric & above	1.843	1146.7	77.9	13.2
Capric	0.142	66.7	6.6	33.4
Caprylic	0.160	62.9	7.4	62.9
Caproic	0.060	19.0	2.8	9.5
Butyric	0.115	27.6	5.3	6.9
Totals	2.320[a]	1322.9	100.0	13.8

Table 20 (Continued).

Acids	Ml. 0.0159N base from column	Mg. of acid released per 10 g. fat	Molar per cent released	Per cent acid released
15 Minutes				
Lauric & above	1.270	958.0	79.8	11.0
Capric	0.126	73.9	9.1	36.9
Caprylic	0.100	19.1	7.2	19.1
Caproic	0.025	9.9	1.8	5.0
Butyric	0.030	9.0	2.2	2.2
Totals	1.551[a]	1069.9	100.1	11.1
30 Minutes				
Lauric & above	1.812	805.5	78.4	9.3
Capric	0.208	74.2	9.9	37.1
Caprylic	0.140	41.8	6.7	41.8
Caproic	0.055	13.2	2.6	6.6
Butyric	0.050	9.1	2.4	2.3
Totals	2.265[a]	943.8	100.0	9.8

[a]Total acid titrations of the extracts were:

Zero time	0.940
Untreated	8.984
3 minutes after zero time	8.015
5 minutes	7.960
15 minutes	6.640
30 minutes	5.900

Table 21. The fatty acids released from butterfat by the lipase of *C. lipolytica*.

Acids	Ml. 0.0145N base from column	Mg. of acid released per 10 g. fat	Molar per cent released	Per cent acid released
Zero time				
Lauric & above	0.262	276.0		
Capric	0.046	31.1		
Caprylic	0.012	6.8		
Caproic				
Butyric				
Totals	0.320[a]	313.9		
Trial 1				
Lauric & above	2.315	1137.4	82.0	13.1
Capric	0.300	86.6	10.1	43.3
Caprylic	0.130	35.9	4.7	35.9
Caproic	0.065	17.2	2.6	8.6
Butyric	0.015	3.0	0.6	0.8
Totals	2.825[a]	1280.1	100.0	13.3
Trial 2				
Lauric & above	2.220	1206.7	82.9	13.9
Capric	0.335	108.4	11.8	54.2
Caprylic	0.145	45.3	4.2	45.3
Caproic	0.025	7.0	1.1	3.6
Butyric				
Totals	2.725[a]	1367.6	100.0	14.2

[a]Total acid titrations of the extracts were:

Zero time	1.735
Trial 1	8.875
Trial 2	9.365

Table 22. The fatty acids released from butterfat by the lipase of C. lipolytica.

Acids	Ml. 0.0127N base from column	Mg. of acid released per 10 g. fat	Molar per cent released	Per cent acid released
Zero time				
Lauric & above	0.508	359.6		
Capric	0.086	31.1		
Caprylic	0.078	29.7		
Caproic				
Butyric				
Totals	0.672[a]	428.4		
Trial 1				
Lauric & above	1.889	670.7	78.7	7.7
Capric	0.257	51.0	9.7	25.5
Caprylic	0.209	31.6	7.5	31.6
Caproic	0.056	13.2	3.2	6.6
Butyric	0.015	2.7	0.9	0.7
Totals	2.426[a]	769.2	100.0	8.0
Trial 2				
Lauric & above	2.231	742.4	83.7	8.5
Capric	0.210	27.6	6.0	13.8
Caprylic	0.179	17.9	4.9	17.9
Caproic	0.040	8.6	1.9	4.3
Butyric	0.070	11.4	3.4	2.8
Totals	2.730[a]	807.9	99.9	8.4

[a]Total acid titrations of the extracts were:

Zero time	2.795
Trial 1	7.775
Trial 2	7.925

Lipase Activity by G. candidum

For studying fatty acid release by the lipase of G. candidum, incubation of the individual 2.0 g. portions was for 96 hours at 30° C. Results of these analyses are shown in Table 23.

Table 23. The fatty acids released from butterfat by the lipase of G. candidum.

Acids	Ml. 0.0145N base from column	Mg. of acid released per 10 g. fat	Molar per cent released	Per cent acid released
Zero time				
Lauric & above	0.260	164.0		
Capric	0.070	28..4		
Caprylic	0.060	20.4		
Caproic	0.035	9.6		
Butyric				
Totals	0.425[a]	222.4		
Trial 1				
Lauric & above	1.488	873.7	76.3	9.1
Capric	0.342	124.9	16.9	62.5
Caprylic	0.150	35.9	5.6	35.9
Caproic	0.055	7.0	1.2	3.5
Butyric				
Totals	2.035[a]	1041.5	100.0	10.8
Trial 2				
Lauric & above	1.759	960.9	80.8	11.0
Capric	0.331	107.6	14.1	53.8
Caprylic	0.135	25.6	4.0	25.6
Caproic	0.055	5.6	1.1	2.8
Butyric				
Totals	2.280[a]	1099.7	100.0	11.5

[a]Total acid titrations of the extracts were:

Zero time	1.380
Trial 1	7.305
Trial 2	7.505

DISCUSSION

Extraction and Separation of Fatty Acids from Butterfat

In an attempt to find a suitable means for studying lipolysis of butterfat by microbial lipases, two essentially similar methods were evaluated. The method of Harper et al. (21) for fatty acid extraction and the subsequent separation of these acids by the partition chromatography method of Ramsey and Patterson (58) were tried first.

When known amounts of acids were added to milk, the silica gel extraction method of Harper et al. appeared to be adequate. However, in the natural state, the free fatty acids are dissolved to a greater degree in the fat portion rather than in the aqueous portion of the milk. In order to evaluate the extraction procedure from this viewpoint, known amounts of acids were dissolved in butteroil and this used to reconstitute skim milk to a product containing 10 per cent fat. When the fatty acids were then extracted by the method of Harper et al., the efficiency of the method was low, with less than 70 per cent of the added acids being recovered.

Transferring the acids to a solvent suitable for separation by partition chromatography was the next step. Evaporation of the extract to dryness, as suggested by Ramsey and Patterson (58) and later used by Harper et al. (21) was thought to be too harsh a treatment, since the lower molecular weight

fatty acids found in butterfat are quite volatile and could well be lost in the evaporation procedure. The fat extracted with the acids was also found to adhere firmly to the boiling flask and did not dissolve completely in the solvent.

The partition chromatography method of Ramsey and Patterson (58) was found inadequate for separating the acids found in butterfat. When a mixture of lauric, capric, caprylic, caproic and butyric acids were placed on this column, the first three acids were eluted as a single band. Butyric acid came off very slowly and when the column was lengthened to afford better separation of the first three, butyric acid was very difficult to elute without using extremely large volumes of developing solvent. Accurate separation of the acids also was difficult because the bands were difficult to follow by visual means.

The chromatographic technique of Kemp and Hetrick (27) was next to be evaluated. Again the acids were dissolved in butteroil and this used for reconstitution with skim milk to a product containing 10 per cent fat. Recoveries of not less than 95 per cent for the extraction procedure indicated that the method was suitable for this study.

The procedure for distilling off excess solvent was then evaluated. Several equipment modifications were proposed by Dr. A. R. Kemp to afford better temperature control in the boiling flask. An electric hot plate, controlled by a voltage regulator, was placed in direct contact with the boiling flask to

accomplish this. All ground glass joints were sealed with standard-taper Teflon sleeves. A thermometer was found unnecessary as a result of these changes and the thermometer insertion joint was sealed with a rubber stopper covered with aluminum foil.

Since the distillation procedure is carried on in an essentially closed system, there would be little chance for losses of fatty acids due to volatility. The one point where losses could occur would be into the distillate. Some acid does distill over and can be accounted for as butyric acid. When calculated with the butyric acid eluted from the column, the two quantities account for more than 99 per cent of this acid as originally added.

The recovery values for the acids of higher molecular weight than butyric acid were all in excess of 100 per cent. Free fatty acids not removed from the butteroil in the purification process probably were responsible for this. However, no controls were analyzed in the early phases of the study to account for this. Later studies on controls indicated that these acids were sometimes present. Butyric acid was never detected in the controls.

Recovery values for the added acids were determined by the partition chromatography method of Kemp and Hetrick (27). With this method, visual separation is used only as a supplement, since small fractions of the eluate are titrated

individually as they come off the column. Separation of lauric acid from capric acid and capric acid from caprylic acid was more distinct than with the method of Ramsey and Patterson. The elution of butyric acid without the need for excessive amounts of developing solvent is also possible. The proper ratio of column height to column diameter is only a partial explanation for this. A more important factor, according to Kemp and Hetrick (27), is the necessity for a firmly compacted column, which results in an increased partitioning between the two solvents used.

The removal of the chloroform from the extraction solvent during the distillation procedure is very critical. Chloroform causes elution of the bromocresol green indicator, methanol and, very possibly, small amounts of ammonium hydroxide. Elution of the indicator will cause the end points of the fraction titrations to be obscured. If the methanol is eluted, the acids will not be partitioned and will come off in a single band. Columns using an internal phase consisting only of absolute methanol with neither indicator nor ammonium hydroxide added tend to overcome this problem. When these columns are very carefully compacted, they may also be of smaller diameter and somewhat shorter in length and still provide good separation of the desired fatty acids.

When fatty acids are dissolved in isooctane with no butteroil added, the threshhold volumes of each acid will be

considerably less than when butteroil is used. Since fatty acids are soluble in butteroil, the addition of a third solvent, so to speak, will have an effect on the partition coefficient between the methanol and the isooctane. The effect of the concentration of one of the acids in the sample would be related to this also. The rate of flow of any acid through the column depends on the partition ratio and the rate of flow of the solvent where $\frac{C \text{ movable phase}}{C \text{ stationary phase}}$ = partition ratio where "C" stands for the concentration of the fatty acid in the solvent. The effect of the butteroil in this case would be to make the acids either somewhat less soluble in the isooctane or more soluble in the methanol.

Lipolysis of Butterfat by Ps. fragi

In order to determine the quantities of lower molecular weight fatty acids released by the lipase of Ps. fragi, a sodium taurocholate emulsion of butteroil was subjected to its action. After 48 hours incubation at a temperature of 32° C., considerable amounts of the acids had been released. Butyric, caproic, caprylic and capric acids accounted for 20 to 30 molar per cent of the total acids released or from 145 to 220 mg. of these acids released from 10 g. of fat. According to Jenness and Patton (25), the flavor threshhold for butyric acid is 25 mg. per liter of milk and for caproic acid is 14 mg. per liter of milk. These figures are representative

for inexperienced persons and it is likely experienced judges of milk products could detect considerably lower levels of these fatty acids. Comparisons of the threshhold values with those of the acids released by lipolysis show that the acids released would certainly be detectable by taste alone. Nashif and Nelson (43) have shown that the lipase of Ps. fragi is capable of producing considerable rancidity in butterfat even to the extent of the production of a peppery flavor.

The unofficial limit for water-insoluble acids in butter is 400 mg. per 100 g. fat. If only lauric acid and those of higher molecular weight than lauric acid are considered, the 820 mg. and 930 mg. amounts released during these experiments would be far in excess of this limit.

A preferential release of certain acids in the substrate also is evident. After 96 hours incubation, over 40 per cent of the caprylic acid and 36 per cent of the capric acid had been released. This could be termed a preference when it is considered that these acids comprised 6 molar per cent of the total acids in butterfat (22) and they comprise over 11.8 molar per cent of the acids released by lipolysis. At the 48 hour level, a preference for release of caprylic acid is shown by the data on molar per cent release in four of the five trials. At the 96 hour level, this preferential release is shown in only two of the trials. This probably is due to the continuing release of the other acids and a lesser release of

caprylic acid after the 48 hour level had been reached. This is made increasingly apparent in some of the trials because of the slightly different degrees of enzyme activity. Correlated with this is the apparent lack of preferential release of capric acid in all but one of the analyses at 48 hours with three of the 96 hour analyses showing the preference. This relatively greater release of capric acid is also noticeable after incubation for 72 hours in both of the untreated controls of the studies on heat resistance.

Evidence exists for more than one lipolytic enzyme being present in the preparation. Nashif and Nelson (41) found that heat inactivation of the lipase of Ps. fragi did not proceed at a uniform rate. Part of the activity of the preparation was destroyed during the time required to bring the preparation to a temperature of 61.6° C. The remainder of the activity was destroyed very slowly at this temperature. Previous attempts to concentrate enzyme activity by salting out resulted in two separate fractions (41). Activity was detected both in a fluffy white material formed on the surface of the lipase preparation and in a brownish precipitate which sunk to the bottom, although no difference in type of activity or other characteristics of the two preparations was established. The interaction between these possibly multiple lipolytic enzymes in the preparation and the complex nature of the butterfat substrate might account for some of the

variations between trials in amounts of fatty acids released. The values given by Hilditch (22) on Table 1 are averages of analyses of many different lots of butterfat. The actual amount of any individual acid present in butter will vary from sample to sample. This would be even more important when comparing trials of different preparations, even though the preparations were from the same organism and produced, as closely as possible, under the same conditions.

A wide range of activity on both natural and synthetic substrates has been shown by Nashif and Nelson (41). Certain preferences were found for action on triglycerides of different fatty acid compositions. However, activity was demonstrated for all of the triglyceride-type lipid substrates tested. Activity by the lipase of Ps. fragi towards ester linkages other than those of glycerol were not tried. Nelson (47), however, found that the lipase of G. candidum would hydrolyze the ester linkages of diglycol oleate, diglycol laurate and propylene glycol monolaurate.

When the sampling procedure was changed to carry out the enzyme activity in individual 2 ml. quantities of reaction system, an increase in overall activity was obtained. This increase in activity could be due to the improvement in the procedure; however, total activity would still be expected to fall within the ranges obtained in previous trials. The same lipase preparation used in the previous trial also was used

in this trial. The only marked change in reaction environment was a reduction in the ratio of the surface area to the volume of reaction mixture. This suggests that the lipase may be sensitive to oxidation. Nashif and Nelson (42) found that total enzyme production by Ps. fragi was reduced when the surface area was reduced, but lipase production per unit of cells was increased. Further study concerning the effect of aeration both on production and action of this lipase would be necessary before a positive explanation could be made.

Effect of Heat Treatment on the Lipase of Ps. fragi

Most enzymes, according to Neilands and Stumpf (45), are denatured at temperatures above 60° C. With very few exceptions, complete denaturation following brief exposures is to be expected in the range from 80° to 100° C.

One exception to these generalities is the lipase of Ps. fragi. Nashif and Nelson (41) have shown that at a temperature of 99° C., complete inactivation requires an exposure of at least 20 minutes. Considerable activity remains after exposure to temperatures of 61.6° C. and 71.6° C. for 30 minutes and is still detectable after 60 minutes at 61.6° C.

Heat resistance of lipolytic enzymes is not peculiar to those produced by Ps. fragi. Stadhouders (64) has shown varying levels of heat resistance of lipases produced by other representatives of the genus Pseudomonas, as well as by those

of the Achromobacteriaceae. Members of the genera Pseudomonas, Alcaligenes and Serratia were cultured in sterile cream. This was then pasteurized and cheese made from it. Increased fat acidity in the cheese was shown for members of all three genera. A further study showed that the lipase of the genus Achromobacter also was heat resistant.

Nashif and Nelson (44) also found that heat resistance by lipolytic enzymes was rather widespread. The lipases of Achromobacter lipolyticum, Ps. fluorescens and Pseudomonas synxantha were still active after 30 minutes at 71.6° C. The lipases of Pseudomonas aeruginosa and Alcaligines viscosus were still active after 30 minutes at 61.6° C.

In the present studies, a trend for a decrease in the molar per cent of lower molecular weight fatty acids released after come-up time was followed by a slow increase in these acids as the heat treatment became longer. Changes in the proportions of individual fatty acids released due to the exposure of the lipase preparation to heat were inconsistant. If a mixture of lipolytic enzymes is involved, new patterns would be likely to emerge due to different preferences of the different enzymes. In order to establish these patterns, many additional trials would be necessary.

A large portion of the activity loss in these trials was during the time required to bring the preparation up to 62.1° C. However, where Nashif and Nelson (41) found losses of 40

to 60 per cent with the strains of Ps. fragi and the test conditions used by them, only 15 to 25 per cent of the activity was lost during come-up time by the strain used in this study. Again, where Nashif and Nelson reported losses of 68 per cent after 30 minutes at 61.6° C., only 32 to 45 per cent of the activity was lost in the present studies. This could be explained on the basis of strain variation. Csiszar and Romlehner-Bakos (5) studying the heat resistance of the lipase of Pseudomonas fluorescens, found considerable differences in both heat resistance and rates of activity between lipases of different strains. Stadhouders (64) found differences in activity and heat resistance between members of the same genus, as well as between members of different genera.

A difference in the reaction conditions following the heat treatment also could play an important role. If the heat treatment made the enzyme more sensitive to oxidation, the reduced ratio of surface area to volume in the reaction environment used in this study could play a protective role during the course of the reaction period.

It would seem that if only one enzyme were involved, the rate of reduction in activity due to exposure to heat would be a logarithmic function since heat inactivation is a first order reaction. The rapid rate of reduction in activity during the time the lipase preparation takes to reach 62.1° C.

and the slower rate of reduction following this period tends to support the conclusion that there is more than one enzyme. A second rapid decrease in activity occurs between the 10 minute and 20 minute exposures, this being followed again by a slower rate of decrease. This trend in overall activity, although at a slightly different level of activity, is the same in both trials.

Lipolysis of Butterfat by the Lipase of *C. lipolytica*

The fatty acids released from butterfat by the lipase of *C. lipolytica* were predominantly the higher molecular weight fatty acids. The quantities of the lower molecular weight acids released would probably be detectable organoleptically, but not to the degree that was associated with the action of the lipase of *Ps. fragi*. There was a slight preferential release of caprylic acid, but capric acid was preferentially released to an even greater degree. While Wilcox *et al.* (69) found that tributyrin and tricaproin were preferred synthetic substrates, it could well be that their substrate preparations and conditions were not suited for release of the higher molecular weight triglycerides such as trilaurin or trimyristin, especially. Nashif and Nelson (41), using sodium taurocholate as an emulsifying agent, found that some of the higher molecular weight triglycerides did not make stable emulsions. Only those which were liquid at the

incubation temperature used were suitable as substrates.

There was a noticeable variation in the amount of total acids released between the two series. Lipase production is very sensitive to both medium composition and aerobic conditions (6). While the lipase preparations used in the two series were individually produced, medium composition and growth conditions were as nearly alike as possible. If conditions did vary slightly, it would be very likely that the activity level both in total activity and with individual acids released would vary accordingly. Since activity levels within a series are nearly alike, the variation between series is probably due to lipase concentration. Differences between molar percentages of individual fatty acids released again is possibly indicative of the non-homogeneity of the lipase preparations. As with the lipase of Ps. fragi, the crude material obtained from the culture was used without purification.

Lipolysis of Butterfat by the Lipase of G. candidum

The lipase of G. candidum released even less of the lower molecular weight fatty acids than did the lipase of C. lipolytica. Butyric acid was never detected and caproic acid was detected in very small amounts. Capric acid was released in very large quantities, exceeding even that released by the lipase of Ps. fragi, with more than 60 per cent of the capric acid being released in one trial.

Previous findings on preferences for certain acids in the substrate are not in total agreement. Nelson (47) has found that low molecular weight synthetic triglycerides were hydrolyzed to a greater degree than are those of higher molecular weight. Wilcox et al. (69) found just the reverse of this. Nelson (47) also stated that natural fats and oils were the preferred substrates in comparison to the synthetic triglycerides. The natural fats and oils would be composed of a large proportion of high molecular weight fatty acids.

Some of the differences between results of this study and of preceding studies might be on the basis of relative availability of the substrate. The relative availability as substrate of synthetic triglycerides containing only a single species of fatty acid of high molecular weight and of a natural triglyceride in which this same fatty acid of high molecular weight is combined in the triglyceride with other fatty acids, especially those of low molecular weight or appreciable unsaturation, may be quite different due to differences in the chemical and physical properties of the triglycerides. A means of studying the high molecular weight triglycerides should be found whereby the physical state of the substrate would not be affected as strongly by temperature conditions. It might also be of value to study the melting points of mono- and diglycerides in mixtures since availability of these undoubtedly is of importance during the course of lipolysis.

SUMMARY AND CONCLUSIONS

Two methods were evaluated for their efficiency in extracting mixtures of known quantities of fatty acids from aqueous mixtures containing butterfat. The fatty acids were dissolved in the butterfat and this solution used for reconstituting with skim milk to a 10 per cent butterfat concentration. The extraction method of Kemp and Hetrick (27) proved to be satisfactory under the conditions used.

An unpublished modification proposed by Dr. A. R. Kemp for the Kemp and Hetrick distillation technique for selective solvent removal was examined for losses of fatty acids during the distillation procedure. The technique was found to be satisfactory under the stated conditions.

A partition chromatography method for the separation of some of the free fatty acids found in butterfat which had been subjected to lipase activity was proposed. Recovery of added fatty acids was found to be satisfactory. When the fatty acids were dissolved in butteroil before addition to the extracting solvent, a slight increase in the threshhold volumes and a more distinct separation of the acids was observed than when the acids had not been dissolved in butteroil.

The release of fatty acids from butterfat by enzyme preparations of three basically different types of microorganisms has been studied by means of partition chromatography. Basic differences in the amounts of lower molecular weight fatty

acids released from the butterfat by these microorganisms have been shown to exist.

The lipase of Ps. fragi released considerable quantities of butyric, caproic, caprylic and capric acids from butterfat. After 48 hours incubation at 32° C., a high percentage of the caprylic acid had been released. On continued incubation, the amount of caprylic acid released increased very slowly, while other acids, especially capric acid, were released to a greater degree.

The lipase of C. lipolytica preferentially released capric acid from the butterfat substrate. Caprylic acid, while released to a somewhat lesser degree, still could be considered to be released preferentially.

The lipase of G. candidum released only very small amounts of caproic acid from butterfat. Butyric acid, if released, was not detectable. Capric acid was released in very large quantities exceeding even that released by the lipase of Ps. fragi.

Evidence was presented to indicate the possible presence of more than one lipolytic enzyme in the lipase preparation of Ps. fragi. The interaction of these lipolytic enzymes and the complex nature of the butterfat substrate probably were responsible for the observed differences in the amounts of individual acids released between trials.

An increase both in total activity and in rate of activity

was associated with a reduction in the ratio of surface area to volume in the enzyme-substrate reaction environment. This suggested that the enzyme might be sensitive to oxidation.

A trend for a decrease in the amount of lower molecular weight fatty acids released by the lipase of Ps. fragi following the come-up time in heating experiments was observed. This was followed by a gradual increase in release of these acids in proportion to the high molecular weight acids (lauric and above) as exposure to heat was extended. Differences in the amounts of any individual acid released as the heating time was extended prevented the establishment of more definite patterns without data from additional trials.

A large portion of the loss in activity occurred during the time required to bring the lipase preparation up to 62.1° C. This loss, however, was less than previously reported. This was attributed to strain variation and possibly to the difference in the reaction environment used in this study. The non-uniform rate of heat inactivation by this enzyme suggests the presence of more than one lipolytic enzyme.

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