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Comparison of free fatty acid concentrations from the action of Pseudomonas fluorescens and milk lipases on butteroil

Carol J. Breeding

To the Graduate Council:

I am submitting herewith a thesis written by Carol J. Breeding entitled "Comparison of free fatty acid concentrations from the action of Pseudomonas fluorescens and milk lipases on butteroil." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Science and Technology.

Genevieve Christen, Major Professor

We have read this thesis and recommend its acceptance:

J.L. Collins, B.J. Demott

Accepted for the Council:
Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Accepted for the Council:

Lee Minkel

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Date _____

COMPARISON OF FREE FATTY ACID CONCENTRATIONS FROM THE ACTION OF *PSEUDOMONAS FLUORESCENS* AND MILK LIPASES ON BUTTEROIL

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Carol J. Breeding

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ABSTRACT

The purpose of this research was to determine fatty acid profiles of P. fluorescens 32A and 22F and milk lipases by hydrolysis of butteroil. The lipases were extracted from the microorganisms and from milk and were purified. Fatty acid profiles were determined by gas chromatography.

Mean concentrations of fatty acids from all enzyme sources were significantly higher for 32A lipase at $p \leq 05$. Milk lipase yielded mean fatty acid concentrations that were significantly different from 22F and the control. The mean fatty acid concentrations from the combined data for all enzyme sources, pH values and replications showed that butyric (C4), myristic (C14), palmitic (C16), stearic (C18), and oleic (C18:1) acids were present in the largest concentrations when compared to total fatty acids released. The lipase from 32A released more butyric (C4), caproic (C6), caprylic (C8), capric (C10), and oleic (C18:1) acids at pH 6.5 than at pH 8.8. It also released more fatty acids than any other lipase at either pH. Few differences were found among the other lipases.

Based on this research the lipase from 32A could have a significant effect on the quality and shelf life of milk and milk products since it released both short chain fatty acids which affect flavor and long chain fatty acids which affect acid degree values.

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CHAPTER I

INTRODUCTION

Quality and acceptability of milk are dependent upon its clean, fresh flavor and excellent keeping ability. With the decrease in the number of dairy farms, especially in the southeast, the route of transport for raw bulk milk is long and time consuming. Usually, milk is collected on alternate days and may be stored at low temperatures (4 C) for up to 4 days before being processed, especially during weekend periods (Cousin, 1982). This long storage period before pasteurization has resulted in quality problems for the dairy industry as a result of growth and metabolic activities of microorganisms. Microorganisms capable of activity at 7 C or less are classified as psychrotrophs. During raw milk storage, heat-stable extracellular enzymes may be synthesized which may result in spoilage later. Rancid flavors caused by release of free fatty acids (FFA) from triglycerides by lipase create an unpleasant bitter, soapy taste that undermines the shelf life of milk (Brockerhoff and Jensen, 1974). Since 1949, measurement of acid degree values (ADV) has been used to indicate level of rancidity and to predict expected shelf life. According to Standard Methods for the Examination of Dairy

Products (Richardson, 1985), milk exhibiting an ADV of 1.5 or greater is considered rancid and unacceptable. The ADV procedure does not consider the fact that short chain fatty acids (C4-C14) are partially soluble in skimmilk. These free fatty acids responsible mostly for producing lipolyzed flavors are distributed partially in the skim phase and are not completely measured by ADV (Connolly et al., 1979). Previous research has shown that long chain fatty acids produce large increases in ADV but little sensory response while short chain fatty acids have the reverse effect (Duncan and Christen, 1988). Randolph and Erwin (1974) discussed the difficulties in relating ADV to organoleptic flavor since a milk sample with a high proportion of short chain FFA but low ADV could have a more rancid flavor than a sample containing a high number of long chain FFA and a high ADV. Scanlan et al. (1965) reported that short chain FFA were responsible for producing off- and rancid flavors in milk.

The ADV detects long chain fatty acids that have been released as a result of lipolysis by microbial enzymes, especially those from psychrotrophic bacteria. Khan et al. (1967) compared the FFA released by milk lipase with those released by lipase from Achromobacter lipolyticum and found that the distribution of FFA liberated by milk lipase closely resembled that of the original milk fat.

Furthermore, Khan et al. (1967) found that the FFA released by milk lipase consisted of 23% oleic acid, 2.2% linoleic acid and 1.0% linolenic acid, whereas, the free fatty acids released by A. lipolyticum lipase consisted of 47.4% oleic acid, 6.5% linoleic acid and 8.1% linolenic acid. These data showed that Achromobacter lipase released a greater percentage of C18 unsaturated fatty acids than did milk lipase. Khan et al. (1967) reported that fungal and microbial lipases released 66-99% C18 acids. The objective of this research was to isolate and purify lipase from milk and from two strains of P. fluorescens (32A, 22F) and to quantitate the free fatty acid concentrations released from a butteroil emulsion.

CHAPTER II

LITERATURE REVIEW

Microorganism

Psychrotrophs are defined as those microorganisms capable of growth at or below 7 C regardless of the optimum growth temperature (Cousin, 1982). Psychrotrophs found in refrigerated bulk milk are mainly gram-negative, rod-shaped bacteria of the genera Pseudomonas, Achromobacter, Alcaligenes, and Enterobacter although the most lipolytic microorganisms were of the Pseudomonas genus (Thomas et al., 1966). Alford et al. (1964) reported that several Pseudomonas species hydrolyzed the primary ester of triglycerides. Strains of Pseudomonas have gained recognition because of their ability to produce heat resistant lipases that can survive pasteurization.

Heating at 71 C for 180 min destroyed only 47% of the lipolytic activity of Achromobacter lipolyticum. However, the enzyme lost all of its activity when heated at 99 C for 40 min or when autoclaved at 121 C for 15 min (Khan et al., 1967). Christen and Marshall (1984) found that Pseudomonas fluorescens 27 produced a lipase also capable of surviving heating at 70 C for 30 min when in crude

extract form but did not survive at 40 C for 30 min. A survey of 12 psychrotrophs commonly found in raw milk indicated that all strains produced lipases that survived 63 C for 30 min to varying degrees (Law et al., 1976). Furthermore, P. fluorescens and P. fragi produced lipases that partially survived 100 C for 30 min, and cheese made from milks containing high counts of these organisms developed rancidity. P. fluorescens strains 32A and 22F were the two most lipolytic among the ten strains studied by McKellar and Cholette (1986).

Lipase Characteristics

Wills (1965) reported that the optimum pH for milk lipase was in the range of 8.0 to 9.0. According to Deeth and Fitz-Gerald (1976) milk lipase is most active at alkaline pH and is almost completely inactive below pH 6.0. Khan et al. (1967) found that lipase activity from A. lipolyticum was optimum at pH 7.0 and 37 C. Stepaniak et al. (1987) found maximum lipolytic activity for P. fluorescens B52 to be pH 7.2 and at 35 C. Ren et al. (1988) discovered that maximum lipase activity occurred at 37 C with a tributyrin assay at pH 6.5 but showed 15% of maximum activity at 10 C.

Tarassuk and Frankel (1955) reported that lipase was associated with casein and treatments that cause agitation

such as homogenization, foaming, and sloshing can disturb the fat globular membrane and expose the fat to the action of enzymes. Lipase in raw bulk milk can also be activated by changes in temperatures such as cooling, warming, and then recooling (Groves, 1971).

Microbial Growth and Enzyme Production

Variations in optimum pH and temperature for production of lipase occur, depending upon species or strain of psychrotroph. Cousin (1982) reported that maximum lipase for P. fragi was produced at 15 C or lower after three or more days of incubation. Lawrence (1967) reported that production was greatest when the organism was grown at temperatures less than optimum growth temperature.

Alford and Elliott (1960) found that lipase activity was low if cultures had been shaken. However, according to Lawrence (1967) when a preparation of P. fragi was shaken, there was an increase of cell growth and initial lipase production although activity decreased upon further agitation. In contrast, aeration of Staphylococcus aureus and Micrococcus freudenreichii produced higher concentrations of lipase in less time, and the activity did not decrease with continued shaking (Lawrence, 1967). Lawrence (1967) reported that aeration was important but

that the amount necessary seemed to vary from species to species. Asher and Cardwell (1979) used samples that were shaken gently three times per day as a means to imitate the movement of milk from farm through the pasteurization process. Fox and Stepaniak (1983) found that aeration increased growth and lipolytic activity in milk containing P. fluorescens AFT 36 as isolated from raw bulk milk. Griffiths and Phillips (1984) found that aerating milk decreased proteolysis but increased lipolytic activity during storage at 6 C.

Purification Techniques

The isolation of an enzyme depends upon the pH, the net charge of the molecule, the size, and the method and material used for the separations. It is important to stabilize the enzyme to preserve the activity that would otherwise be lost rapidly. By holding in a 50% glycerol solution, enzymes can remain active in frozen condition for weeks because the solution does not freeze. With the concentrated glycerol-solute mixture containing the enzyme, storage at very low temperatures is rarely detrimental (Scopes, 1982).

The removal of the effective water activity seems to be as beneficial as lyophilization. High ammonium sulfate concentrations are very stabilizing, and usually most

enzymes sold commercially are prepared as suspensions in 2-3 M ammonium sulfate (Scopes, 1982). If an ammonium sulfate fractionation is being conducted, the enzyme should be held in a solution containing as high a concentration of ammonium sulfate as possible.

The degree of precipitation of an individual protein at its point of lowest solubility depends on its concentration and solubility at its isoelectric point (pI) (where the net charge is zero). The charge density of a protein can be affected by changing the pH. At a low pH, the prototrophic groups will be protonated and the protein will have a net positive charge. As the pH is increased from the acid state more and more carboxyl group protons become neutralized until, at a pI unique to each protein, equal numbers of positive and negative charges are developed. Thus, the protein has a net charge of zero. Although proteins are least soluble at their isoelectric point, solubility increases as the pH varies above and below the isoelectric point. To produce protein precipitation several molecules of that protein must come close enough together to form an aggregate with a combined solubility is less than that of the individual molecules because of mass size and the masking of charged groups. Aggregation can be accomplished by adjusting the pH of the

solution to the isoelectric point of the protein (Whitaker, 1972).

Solubility is also determined by the extent of hydration of the molecule. The more charged the group on a protein molecule, the more hydrated it will be. The usual way to decrease the degree of hydration, thus allowing the protein to precipitate, is to add compounds such as ammonium sulfate, sodium chloride, sodium sulfate, and magnesium chloride, which disrupt the water of hydration around the protein. Polyvalent salts such as ammonium sulfate are effective because of high solubility in water, and in general, they do not denature proteins (Whitaker, 1972).

Ion-exchange chromatography is based upon isolation by ion effect. An insoluble material with some hydrophobic properties and ionizable groups of opposite charges to that of the net charge on the protein is needed. The protein must also be capable of being readily and selectively removed from the material. This can be done by varying the salt concentration or the pH of the solution. This is shown schematically with an anion-exchange resin where R+ is that resin.

$$(R^+\ Cl^-) + \text{Protein} \longrightarrow (R^+\ \text{protein}^-)^\circ + Cl^-$$

Since Cl^- and other anions can interact electrostatically with the anion-exchange material, it is in competition with the protein for binding sites. To insure that all of the proteins are adsorbed, the Cl^- concentration should be low. Proteins are then selectively eluted from the exchange material by a systematic increase in the salt concentration. A protein with a net charge of 4^- requires a higher concentration of Cl^- to cause an exchange of ions because more Cl^- are available for exchange at binding sites than a protein having a net charge of 2^-, all other things being equal (Whitaker, 1972).

In 1956, protein separation was revolutionized by the introduction of cellulose ion-exchange derivatives (Whitaker, 1972). The materials are harmless toward proteins, can be used under a wide variety of conditions, have a high capacity for proteins, and can be used repeatedly after regeneration. Under ideal conditions, one gram of dry cellulose ion-exchange material is capable of binding about one gram of protein. About 100 to 500 mg of protein may be bound to a 2-cm diameter column.

The diameter and length of a column determines the capacity. The more protein that is added to the column, the larger the diameter of the column must be (Whitaker, 1972). A long narrow column may yield considerably less resolution than a column with a higher diameter to height

ratio. Therefore, it is better to increase the volume of the column by increasing the diameter (Cooper, 1977).

Both cation and anion exchange cellulose resins can be used. The most frequently used materials are carboxy methyl (CM-)- cellulose and diethylaminoethyl (DEAE)-cellulose. Both of these cellulose materials can be bound covalently to Sephadex which is a cross-linked dextran. Sephadex ion-exchange materials are very useful for protein separation. These materials have several advantages over cellulose derivatives in that very few enzymes can degrade the support material, the particles are more uniform in size, and they have a higher capacity for proteins (Whitaker, 1972). Sephadex ion exchange resins also have some disadvantages in that they swell and shrink depending upon the ionic strength of the eluting material. These materials cling to unsilanized glassware very tightly and loss of material occurs (Whitaker, 1972).

A linear gradient is recommended with ion-exchange columns because of the large volume of information it will provide with the smallest amount of research effort. To achieve a linear gradient, two identical vessels are connected through an opening at the bottom of each or by a siphon arrangement between the two. When the containers are left open at the top, the levels in both fall at the

same rate, thus producing the linear gradient (Cooper, 1977).

Proteins can also be separated based on their differential sedimentation in a gravitational field which is related to their molecular weight. Sephadex can be used to separate proteins on the basis of their size as well as give good molecular weight determination. Sephadex gels consist of cross-linked dextran molecules that are long polymers of glucose linked 1-6 (95%) and 1-3 (5%) with epichlorohydrin. The degree of cross-linking can be controlled by the amount of epichlorohydrin added to provide different grades of Sephadex. The lower the number of the Sephadex type, the more extensive the degree of cross-linking and the lower the molecular weight of the compounds that can be separated (Johnson and Stevenson, 1978). An advantage of using Sephadex is that the material placed on the column does not have to be pure if the elution volume of the protein can be determined by biological activity (Whitaker, 1972). The largest proteins are eluted from the column first because the large molecules cannot diffuse into the gel matrix to the extent of the smaller molecules. The maximum flow rate through Sephadex G-100, G-150, and G-200 is inversely proportional to the bed height (Scopes, 1982).

Isolation of Enzyme

Precipitation of enzymes is dependent upon the size or the net charge of the molecule. Lawrence et al. (1967b) passed a two-thirds saturated ammonium sulfate supernatant containing lipase from P. fragi over Sephadex G-100. Then they pooled the active portion over Sephadex G-200 column to remove low molecular weight material, thus providing more sensitive separation. Ion-exchange, separation by net charge using DEAE-cellulose with dimethylformamide as dissociating agent, was used to isolate milk components (Yaguchi and Rose, 1971). Davies and Law (1977) used ion-exchange chromatography at 4 C on DEAE-cellulose with a tris-chloride-urea buffer and a NaCl gradient. Lipase purified from P. fluorescens with ammonium sulfate precipation and separated by Sephadex G-75 and DEAE-cellulose represented a 500-fold increase in activity (Sugiura and Oikawa, 1977).

Lipase Activity and FFA Determination

Law et al. (1976) measured lipase activity using radial diffusion assay. Lawrence et al. (1967a) considered that the sensitivity of the tributyrin emulsion assay made it especially suitable for the screening of microbial lipolytic activity. The assay depends on the solubility of the mono- and di-glycerides and on the

solubility of the calcium salts of the fatty acids produced by hydrolysis. For triglycerides containing fatty acids with 12 or less carbons, hydrolysis was indicated by the clearing of the triglyceride emulsion (Lawrence et al, 1967b). For triglycerides containing high molecular weight fatty acids, the zone of hydrolysis appeared as increased opaque zones against the background. Presumably this finding occurred from the precipitation of the calcium salts of the liberated fatty acids since agar is known to contain exchangeable calcium (Cooper, 1963). Incorporating a fat-soluble dye into the medium facilitates the detection of the lipolytic activity in butterfat.

Lipids were extracted from milk in ether, and FFA were recovered by shaking the extract with anion exchange resin Amberlyst 26. The resin-bound FFA were methylated and individual acids were separated by gas-liquid chromatography, using internal standards (Needs et al., 1983). Concentration of individual fatty acids was determined with a high degree of reproducibility over a wide range of concentrations. The values agreed closely with the actual concentrations when measured directly. Butyric acid varied by 4% from the mean concentration and was within 4.5% of the known concentration. This method was reported to possess the capability of determining

accurately individual FFA's in milk over a wide range of concentrations. This method also possessed freedom from disadvantages such as loss of short-chain acids either due to evaporation or poor recovery from anion exchange resin or by hydrolysis of other milk lipid by adsorption medium (Needs et al., 1983).

The extraction of free fatty acids has been performed by adsorption chromatography. The common absorbents can be divided into two groups based on type: polar adsorbents such as silica and alumina and nonpolar adsorbents such as charcoal. The polar adsorbents interact with adsorbed molecules through specific forces such as electrostatic attraction and hydrogen bonding. Thus, adsorption energy, chromatographic retention, and solvent strength tend to increase with polarity such that saturated hydrocarbons possess the weakest adsorption energy. Acids and bases possess the strongest energy (Karger et al., 1973).

The polar adsorbents can be further differentiated as acidic or basic. Silica is weakly acidic because of its surface Si-OH groups, whereas, alumina is strongly basic because of its surface O^{2-} groups. Basic adsorbents such as alumina preferentially retain acidic compounds and are simple to use (Karger et al., 1973).

The addition of water to polar adsorbents leads to a selective covering or blocking of the most active sites of the adsorbent surface. Generally, it is preferable to work with adsorbents that have been deactivated by at least half a monolayer of adsorbed water (i.e., 0.02g water/100 m^2 of adsorbent surface); water-deactivated adsorbents are superior in yielding more efficient beds and in reducing the danger of adsorbent-catalyzed sample.

Sample oxidation seems to be promoted by adsorbents, in general, so that rigorous exclusion of oxygen is required when dealing with oxidizable samples (Karger et al., 1973). The adsorbed solute can only be eluted with a substance that is more polar than the solute.

FFA's were determined using a gas chromatographic method in milk and milkfat (Deeth et al., 1983). The procedure called for a hexane-ether extract of the product which was dried with anhydrous sodium sulfate. Other glyceride forms were removed by several washings with clean hexane-ether solution. The fatty acids adsorbed onto neutral deactivated alumina were eluted with a 6% formic acid in diisopropyl ether. An aliquot of this solution was chromatographed and all major fatty acids were quantified. The method was simple, convenient and displayed a high degree of repeatability; therefore, this

method was selected for the determination of FFA in the following research.

CHAPTER III

MATERIALS AND METHODS

Production and Isolation of Microbial Lipase

Microorganisms studied were Pseudomonas fluorescens 32A and 22F and were isolated by Dr. R. C. McKellar and Dr. F. Driessen, respectively (McKellar and Cholette, 1986). Lyophilized ampules of both strains were kindly provided by Dr. R. C. McKellar (McKellar, 1988). Strains of the microorganisms were rehydrated in sterile 10% (w/v) reconstituted nonfat dry milk (RNDM), incubated 24 h at 21 C, and transferred to trypticase soy broth for 24 h and to trypticase soy slants for storage at 4 C.

One hundred ml of RNDM were heated at 110 C for 10 min, cooled, inoculated with each strain, and incubated at 21 C for 24 h in quiescent state. Four hundred ml RNDM were stirred with a magnetic stirring bar, sterilized by autoclaving, cooled, and inoculated with 4 ml of subculture. Erlenmeyer flasks containing inoculated milk were incubated at 10 C for 9 days under aerated conditions using the stirring bar. The decrease in pH initially coagulated the milk and the bacteria digested the coagulum to yield a yellowish, semi-transparent culture. Proteins were separated by centrifugation (IEC B-20A Centrifuge,

Needham Heights, Mass.) at 20,000 x g for 30 min at 0-4 C; the clear supernatant was sterilized by filtration through a 0.45 μm Nalgene sterile disposable membrane filter system (Nalge Company, Rochester, NY). The supernatant was dialyzed using cellulose dialysis tubing 45 mm x 28.6 mm diameter x 22 cm length with a molecular weight cut off: 12,000 - 14,000 (Spectrapor Membrane Tubing, Spectra/Por 4) in 4 L 0.01 M Na-phosphate buffer for 24 h with buffer being changed every 12 h (Appendix A). Dialysis facilitated the removal of nitrogenous material with very little loss of activity (Fox and Stepaniak, 1983). Following dialysis, the crude enzyme was frozen at -70 C, lyophilized and labeled as concentrated crude enzyme (CCE).

Isolation of Milk Lipase

Fresh milk was drawn by hand from individual mastitis-free Holstein cows at the University dairy farm and processed into skim milk within 2 h by centrifugation at 1000 x g for 30 min at 20 C. Skim milk was distributed into 200-ml portions in centrifuge bottles. Two-tenths ml of commercial rennet extract was added to each 200-ml sample, mixed thoroughly, and held at 37 C for 15 min. Curd and whey were separated by centrifugation at 10,000 x g for 15 min. Whey volume was measured and discarded (Fox

and Tarassuk, 1968). An equal volume of 1.0 M NaCl in 0.02 M Na-phosphate buffer, pH 7.0 was added to the curd to replace the discarded whey and dispersed using the Stomacher Lab-Blender 400 (Seward Laboratory, Blackfriars Road, London). The supernatant was decanted and stored at 4 C. The chopped curd was mixed with a measured volume of 1.0 M NaCl solution, and the suspension was stirred gently overnight at 4 C with a magnetic stirring bar. The remaining lipase was removed from the curd by centrifugation at 0-4 C at 10,000 x g for 15 min. The curd was discarded (Fox and Tarassuk, 1968).

The supernatant (869 ml) was sterilized with a 0.45 µm Nalgene sterile disposable filter system and was then dialyzed against 0.01 M Na-phosphate buffer, pH 7.0 at 4 C for 24 h. Four L of buffer were replaced every 12 h. The dialysed supernatant was frozen at -70 C and lyophilized (CCE).

Purification of Crude Enzyme

The lyophilized CCE which contains the lipase from milk and the two strains of *P. fluorescens* were rehydrated in a DEAE-cellulose (diethylaminoethyl-cellulose) ion exchange slurry containing 0.01 M Na-phosphate buffer, pH 7.0 for milk lipase and pH 6.5 for microbial lipase, at 8 C (Appendix B). Protein was eluted using a NaCl gradient

(0-1.0 M). Fractions were collected, and lipase-rich fractions were dialyzed, frozen at -70 C, and lyophilized.

The partially purified enzyme (PPE) from the DEAE-cellulose column was rehydrated in phosphate buffer at appropriate pH and applied to Sephadex G-150 gel filtration column (Appendix D). Lipase-rich fractions were collected, frozen, and lyophilized. The resulting purified enzyme (PE) was used in subsequent experiments.

Protein Concentration

Protein concentrations of the initial supernatant, CCE, PPE, and PE were determined using a dye-binding procedure. Bovine serum albumin (BSA) (Bio-Rad Laboratories, Richmond, CA) served as the protein standard (Brockerhoff and Jensen, 1974). Using dilutions of BSA contained from 0.2 to 1.4 mg protein per ml, a standard curve was prepared. One-tenth ml aliquots of the standards and appropriately diluted samples were placed into a clean dry test tube. A blank was prepared using 0.1 ml of 0.01 M Na-phosphate buffer. Five ml of diluted dye reagent were added to tubes containing standards and samples, and both were thoroughly mixed by vortex. After a time of 5 min to 1 h, absorbance was measured at 595 nm with a Shimadzu Double-Beam Spectrophotometer UV-190.

Lipolytic Activity

Lipolytic activity was determined using the radial diffusion method (Christen and Marshall, 1984). Spirit Blue agar (Difco Laboratories, Detroit, MI) was prepared according to manufacturer's instructions. Three percent lipase reagent (tributyrin, Difco), and 0.05% thimersol were added to the medium after sterilization and temperature reduction to 50-55 C. The medium was sonicated at 80% of maximum setting for 20 min using a 19 mm probe (Sonic 300 Dismembrator, Artek Systems Corp. Farmingdale, NY). The medium was dispensed into 100 mm petri plates (12-15 ml/plate) and solidified. Wells were punched into medium using a sterilized cork borer (7 mm diameter) with the center well serving as the negative control. Wells were filled to the surface with enzyme solution using a micro-pipettor. Plates were incubated upright at 35 C for 24 h. Following incubation, the diameters of a clear zone or a dark blue zone were measured using calipers. Diameters were used to determine the $\log_{10}$ adjusted zone area, using the following formula (Christen and Marshall, 1984):

$$\log_{10} \text{adjusted zone area} = \log_{10} \pi \left[(D/2)^2 - (d/2)^2 \right],$$

where D = diameter of the zone and d = diameter of the well. All results were recorded and referred to as the zone area ($\log_{10}$ mm^2).

The fractions collected from ion exchange and gel filtration columns were subjected to Spirit Blue lipolytic test to determine which fractions were lipase-rich and capable of hydrolyzing the tributyrin. The lipase-rich fractions were pooled.

Determination of Purity of the Lipases

The presence and activity of protease were determined using agar diffusion in skim milk agar (Difco) prepared according to the manufacturer's instructions and Standard Methods for the Examination of Dairy Products (Richardson, 1985). Seven mm wells were punched into agar and filled to the surface with rehydrated PE solution. Plates were incubated at 21 C for 72 h and flooded with 1% HCl solution for 1 min. Excess acid solution was decanted and the clear zones around wells were measured and recorded using the same formula as used for the measurement of lipolytic activity.

Disc gel electrophoresis followed the purification procedures (Cooper, 1977). Stacking gel consisted of 7.1% acrylamide having a pH of 6.6. The separating gel consisted of 20% acrylamide and had a pH of 8.8 (Appendix F).

Preparation of Substrate

Unsalted sweet butter (Land-O-Lakes, Inc., Arden Hills, MN) was melted and centrifuged at 20 C. The butteroil was decanted (Iyer et al., 1967). When heated, the butteroil was a very clear bright yellow.

The substrate consisted of an emulsion prepared by dispersing 10% (v/v) butteroil in a 10% aqueous solution of gum arabic (Sigma) at 50 C by sonicating with a 19 mm diameter probe for 30 min on 80% of maximum setting while using a stirring bar and gentle heating (Parry et al., 1966).

Titration by pH-stat

The pH-stat operates on the principle of maintaining a constant specific pH by automatic titration. In a lipase assay, the FFA's caused a slight depression in the pH which is automatically corrected by the addition of standardized basic solution. The volume of NaOH was added for each decrease in pH as the fatty acids were released

until a previously specified volume was met (Parry et al., 1966). The pH-stat used in these titrations was manually operated.

Ten ml of butteroil emulsion and 0.4 ml 2.85 M NaCl were combined and adjusted to pH 6.5 or 8.8 with 0.1 N NaOH. Lyophilized enzyme was rehydrated with 1 ml distilled water and added to the substrate maintained at 37 C. Controls were made by the addition of distilled water (1 ml) instead of enzyme. The complete substrate was adjusted to the appropriate pH, followed by titration with 2 ml of 0.01 N NaOH while maintaining the pH at 6.5 or 8.8 (Ha and Lindsay, 1988).

Ten ml of the titrated lipolysed substrate were pipetted into a 50-ml screw-capped Teflon FEP Oak Ridge centrifuge tube (American Scientific) containing 3 ml HCl (36.5-38.0 %); the stopped further hydrolysis (Deeth et al., 1983). After mixing with HCl, the extraction procedure was halted until the following day. The tubes were stored at 10 C.

Extraction and Adsorption of FFA

Twenty-three ml of ice-cold (-15 C) diethyl ether (ether) containing 100 µg of C5 as recovery indicator was added to each previously described sample (Deeth et al., 1983). Extraction was performed by centrifugation at 0 C

for 15 min at 15,000 x g. The ether layer (15 ml) was transferred by pipetting to an Erlenmeyer flask containing hexane (15 ml) and anhydrous Na_2SO_4 (1 g) (Sigma). After drying for about 20 min with the anhydrous Na_2SO_4, the liquid was decanted onto an alumina chromatography column (Appendix E).

The eluant was collected and poured over the column again to insure that all free fatty acids were adsorbed. The second eluant was discarded. Two washes of 5 ml each of 1:1 (v/v) ether-hexane served to wash any monoglycerides, diglycerides or any unreacted triglycerides from the column. The total eluant was discarded. The alumina with the adsorbed FFA was dried with a vacuum applied to the column outlet at room temperature for approximately 5 min and was transferred to a 15 ml disposable centrifuge tube (Kimble, Division of Owens, Illinois) with screw cap. The alumina was flushed with nitrogen and stored at 10 C until the next day.

For the recovery of the FFA, 1 ml diisopropyl ether containing 6% formic acid was added and mixed thoroughly with the alumina using a vortex mixer followed by centrifugation (2000 x g for 5 min). The supernatant (approximately 450 µl) was removed using a micro-pipettor.

Gas Chromatographic Conditions For Separation of FFA

A 4 µl aliquot of the recovered FFA was injected into a Shimadzu Mini GC-2 gas chromatograph equipped with a six foot column packed with a 10% SP-216 PS on 100/120 Supelcoport (Supelco Inc., Bellefonte, PA) and a flame ionization detector. A Shimadzu Chromatopak E-1A data printer and an Omni Scribe Recorder recorded chromatograms, peak retention times, and peak relative concentrations. The temperature program was: initial temperature - 110 C; heating rate - 8 C/min to 195 C with a holding time of 22 min for final temperature of 195 C. The injector/detector temperature was 230 C and the carrier gas (N_2) had a flow rate of 55 ml/min.

All chromatograms were quantified by relating the peak areas to the peak area of the C5 recovery indicator and converted to normalized concentrations (µmole/ml) with following formulas.

1. Relative area = area / area of C5 per chromatogram;
2. Relative concentration = relative area x µmole of C5 added per sample;
3. Concentration = relative concentration / 250;
4. Normalized concentration = concentration x (10 / concentration of C5).

All results presented are means of 3 replications.

Statistical Analysis

Fatty acid profiles were analyzed using an 18 x 4 x 3 x 2 factorial design (SAS, 1985). Significance was preestablished at α = 0.05. Variations among each fatty acid with different enzyme source and pH value were determined with analysis of variance (ANOVA) by fatty acid. Means were separated using the Least Square Means (LSMeans) procedure with $p \leq 0.05$.

CHAPTER IV

RESULTS AND DISCUSSION

Purification of Lipase

Partially purified milk lipase was eluted from the ion-exchange column at a lower overall salt concentration than P. fluorescens 32A or 22F as shown on Table 1. Also, all of the lipases were eluted from the Sephadex column within 100 ml eluant.

By the Spirit Blue agar diffusion method, zone areas for the partially purified and the purified lipases were almost identical, indicating that lipolytic activity was not lost during the purification procedures. Purified milk lipase displayed a blue zone with a clear zone inside next to the well. 32A lipase displayed a dark blue zone with no clear zone while 22F lipase displayed a clear zone only. The dark blue zone displayed by 32A lipase indicated strong lipolytic activity to the point of changing the pH, while 22F lipase demonstrated activity only sufficient to solubilize the substrate.

Table 2 contains data obtained from the final purification step of 32A lipase. As the lipase was eluted from Sephadex column, the absorbance was read, values were plotted on a standard curve, and protein concentrations

Table 1. NaCl concentration, zone diameters and adjusted zone areas for *P. fluorescens* and milk lipases.

	P. fluorescens 32A	*P. fluorescens* 22F	Milk
Ion-Exchange Column - Partially Purified			
[NaCl]	0.34-.66	0.30-.63	0.25-.45
Zone Area (mm^2)	1.95	1.89	2.05
Sephadex Column - Purified			
Eluted within (ml)	70	65	100
Zone Area (mm^2)	1.65	1.59	1.82

Table 2. Protein concentration and lipase activity of *P. fluorescens* 32A fractions following elution from Sephadex G-150.

Fraction Number	Absorbance (595 nm)	Protein (mg/ml)	Lipolytic Assay Zone Area (mm^2)
1	0.010	0.00	0.00
2	0.012	0.00	0.00
3	0.348	0.40	1.60
4	1.272	1.40	1.95
5	0.775	0.89	1.89
6	0.440	0.50	1.82
7	0.529	0.61	1.71
8	0.720	0.82	1.57
9	0.623	0.71	1.07
10	0.262	0.30	0.76
11	0.127	0.15	0.00
12	0.088	0.10	0.00

were determined and ranged from 0.10 to approximately 1.40 mg/ml. Zone area from each fraction ranged from 0.76 to 1.95 mm^2. Purification of the other lipases yielded similar results.

Purity was confirmed by assaying for protease activity and by disc gel electrophoresis. Purified enzymes from all lipase-rich fractions were checked for activity of protease and none was found. Gels of 32A and 22F lipases from electrophoresis had only one band while milk had about nine bands, although four bands were faintly visible (Figure 1). In as much as fresh milk contains many enzymes and other proteins, it was more difficult to purify than the microbial lipases. However, since lipase is substrate specific other proteins which may be present would not interfere with the results obtained.

Titration by pH-stat

As shown on Table 3 the titration times were lengthy at pH 6.5 as compared to those at pH 8.8, even though equal quantities of enzyme material were used. Had more enzyme material been available, a greater quantity could have been utilized for an increase in the reaction rate. During the release of fatty acids at pH 8.8, the pH decreased steadily, and the emulsion was adjusted with the necessary volume of NaOH. However, this steady lowering of the pH did not occur

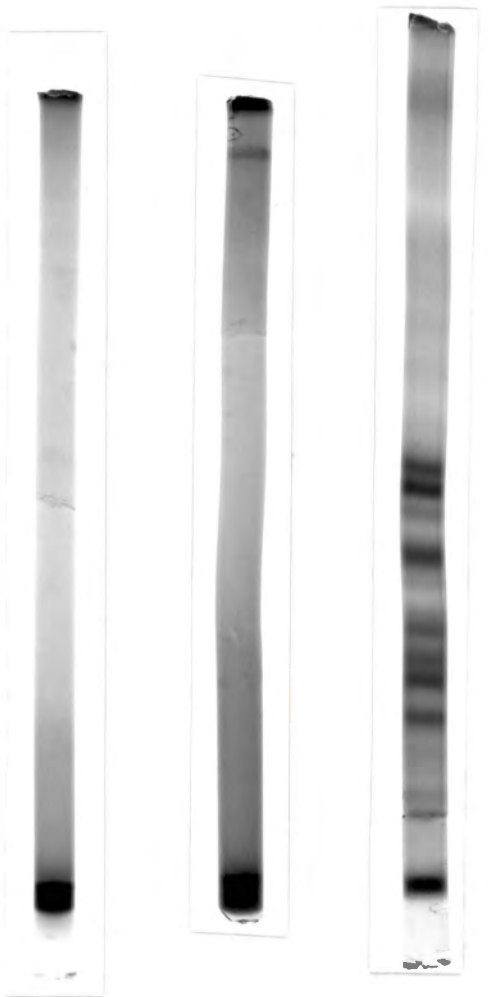

Figure 1. Disc gel electrophoretogram of P. fluorescens 32A and 22F and milk lipases eluted from Sephadex G-150 gel filtration column (left to right).

Table 3. Weight of purified enzyme material and time required for titration with two ml of 0.01 N NaOH at 37 C and constant pH.

Enzyme Source	pH	Rep	Wt (g)	Time (h:min)	Rep	Wt (g)	Time (h:min)	Rep	Wt (g)	Time (h:min)
Control	6.5	1	0.00	0:00	2	0.00	0:20	3	0.00	0:15
Control	8.8	1	0.00	0:00	2	0.00	0:20	3	0.00	0:15
Milk	6.5	1	0.20	1:20	2	0.13	4:45	3	0.10	4:10
Milk	8.8	1	0.13	1:50	2	0.13	0:40	3	0.12	2:15
32A	6.5	1	0.39	0:20	2	0.18	1:10	3	0.13	3:20
32A	8.8	1	0.14	0:45	2	0.14	0:15	3	0.14	0:15
22F	6.5	1	0.18	1:15	2	0.14	2:00	3	0.14	4:15
22F	8.8	1	0.14	0:15	2	0.15	0:50	3	0.10	1:15

at pH 6.5. Following the stabilization of the emulsion containing the enzyme, the pH dropped a few tenths and was adjusted. However, following this initial reaction, the pH did not change even one-tenth for several minutes and sometimes for hours. Finally, the pH value would decrease several tenths. Approximately six-tenths ml of NaOH was needed to adjust the pH. This cycle was repeated until two ml of titrant were added. The difference in the reactions at pH 8.8 and 6.5 raised the question of the validity of the pH-stat. The dissociation constants for butyric and octanoic acids are 4.82 and 4.89, respectively (Gordon and Ford, 1972). Both pH 6.5 and 8.8 are sufficiently above these dissociation constants to produce equivalent results by titration.

Quantification of FFA

The preparation of the alumina for extraction improved when the total batch of alumina was neutralized instead of by individual sample, resulting in more consistent repetitions (data not presented). A difference in weight of alumina neutralized by sample and by batch was evident (data not presented).

Tentative free fatty acid identifications were made by comparing the retention times of newly purchased standards that were run identically to the samples from

the gas chromatographic separation procedure. Table 4 lists the retention times of the standard free fatty acids and the retention time range obtained under the experimental conditions. The range of retention times nearly always bracketed that of the standard and always fell close to the standard. Identities were not assigned for two fatty acids that were eluted between C4 and C5, and C5 and C6 but were assigned U1 and U2, respectively. A fatty acid appearing just after nonanoic acid (C9) (RT range = 6.84-7.42) was tentatively identified as a branched chain isomer of C9 (Webb et al., 1974). A fatty acid appearing after lauric acid (C12) (RT range = 10.58-10.97) was tentatively identified as an unsaturated form of C12.

The peak areas from the integrator were converted to normalized concentrations. The normalized concentrations of valeric (C5) acid were deleted from analysis since the valeric acid concentration was a constant and would therefore skew the results. Analysis of variance (ANOVA) of the combined data revealed that all effects were significant except for the interaction between replication, enzyme source and fatty acid, and between enzyme source, pH and fatty acid (Appendix G-1). LSMeans were calculated to determine difference in replications. There was not a significant difference between replication

Table 4. Retention times of standard fatty acids and tentatively identified sample fatty acids run on same gas chromatography column under experimental conditions.

Standards	Retention Time (min.)	Retention Time Range (min.)
Formic	1.62	1.58- 1.66
C_4	2.16	2.05- 2.27
C_5	2.93	2.87- 3.14
C_6	3.75	3.77- 4.03
C_7	4.55	4.39- 4.89
C_8	5.52	5.60- 5.90
C_9	6.54	6.16- 7.20
C_{10}	7.44	7.56- 7.65
C_{11}	8.50	8.28- 8.91
C_{12}	9.35	9.40-10.60
C_{14}	11.30	11.25-11.40
C_{15}	12.18	12.25-12.81
C_{16}	13.30	13.31-13.90
$C_{16:1}$	14.55	14.54-15.50
C_{18}	16.45	16.44-17.47
$C_{18:1}$ (cis)	20.97	
$C_{18:1}$ (trans, cis)	17.71	17.94-19.57
$C_{18:2}$	18.10	

1 and 3, although both were different from replication 2 (Table 5). This difference was attributed to the centrifugation at 20 C during the extraction procedure of replication 2 rather than at 0 C as with replication 1 and 3. Therefore, the data from replication 2 was removed since it was not a true replication, and the analysis was repeated (Appendix G-2). The LSMean analysis on fatty acid indicated that seven fatty acids were not present in concentrations significantly different from zero (Table 6). Therefore, heptanoic (C7), nonanoic (C9), branched isomer of nonanoic (C9-B), unknown between C4 and C5 (U1), unknown between C5 and C6 (U2), undecanoic (C11), and an isomer of lauric (C12:1) acid were deleted from the data set and the analysis was repeated (Appendix G-3). From this analysis over all replications, pH values and enzyme sources, a significant difference between mean concentrations was found between replication 1 and 3, although the reason for this difference could not be determined (Table 7). A difference was found between pH 6.5 and 8.8 with the mean concentration being significantly higher at pH 6.5. Significant differences were found between concentrations due to the enzyme source. The concentrations of the control and 22F were not different; however, both were different from 32A and milk. The mean concentration from 32A and milk were

Table 5. Effect of replication on mean concentration of fatty acid over all treatments.

Replication	Mean Concentration
1	1.37 a
2	5.25 b
3	0.75 a

a,b - means followed by the same letter are not significantly different at $p > 0.05$.
[1]N=144.

Table 6. Mean fatty acid concentration and probability that the concentration is equal to zero.

Fatty Acid	Mean Concentration	Prob > |T| HO:LSMean=0
C4	1.18	0.0001
C6	0.66	0.0001
C7	0.01	0.8935*
C8	0.32	0.0012
C9	0.00	0.9812*
C9-B	0.00	0.9694*
U1	9.91	0.8894*
U2	0.00	1.0000*
C10	0.54	0.0001
C11	0.09	0.3668*
C12	0.60	0.0001
C12:1	0.12	0.2180*
C14	1.53	0.0001
C15	0.70	0.0001
C16	4.78	0.0001
C16:1	1.06	0.0001
C18	2.75	0.0001
C18:1	4.76	0.0001

* - Means which were not significantly different from zero thus were eliminated from further analysis.

Table 7. Mean concentration of fatty acids calculated over all replications, pH values or enzyme source.

Source of Variation	Mean Concentration
Rep[1]	
1	2.21 b
3	1.21 a
pH[2]	
6.5	1.95 a
8.8	1.48 b
Enzyme Source[3]	
Control	1.19 a
P. fluorescens 22F	1.37 a
P. fluorescens 32A	2.45 c
Milk	1.85 b

a,b,c - Mean concentration followed by the same letter in each group are not significantly different at $p > 0.05$.

[1]N=88.
[2]N=88.
[3]N=44.

significantly different with 32A releasing a higher quantity of fatty acids. Table 8 contains the mean fatty acid concentrations for all enzyme sources, pH values and replications 1 and 3. As expected, differences were found in the quantity of free fatty acids released. Butyric (C4), myristic (C14), palmitic (C16), stearic (C18), and oleic (C18:1) were present over all treatments in the largest concentrations. This was not unexpected since these five fatty acids constitute approximately 74% of the total fatty acids in the milk triglyceride (Table 9).

Variation in the concentration of free fatty acids was expected because fatty acids occur in unequal concentrations in the milk triglyceride. Therefore, the data were analyzed by fatty acid to determine whether the enzyme source and/or pH of the assay had an effect on the free fatty acid concentration. In order to separate the means for the interaction effect, a new variable was created which combined pH and enzyme source. Appendix G-4 through G-14 contains each ANOVA table for this analysis. LSMeans were used to determine significance between concentration of each fatty acid for each pH and treatment.

Analysis of variance by each fatty acid revealed that there was a significant effect of treatment on the concentration of butyric (C4) through myristic (C14) acid

Table 8. Mean fatty acid concentrations from the combined data for all enzyme sources, pH values and replications.

Fatty Acid	Mean[1] Concentration
C4	1.18 c
C6	0.66 b
C8	0.32 a
C10	0.54 a,b
C12	0.60 a,b
C14	1.53 d
C15	0.70 b
C16	4.78 f
C16:1	1.06 c
C18	2.75 e
C18:1	4.76 f

a,b,c,d,e,f - Mean concentrations followed by the same letter are not significant at $p > 0.05$.

[1]N=16.

Table 9. Mean concentration of fatty acids released from butteroil by P. fluorescens 32A and milk lipases at pH 6.5 compared to intact milk fat.

Fatty Acid	32A	Milk	Intact Milk Triglyceride[1]
C4	11.08	1.49	2.79
C6	5.68	1.68	2.34
C8	3.24	0.72	1.06
C10	4.22	2.49	4.10
C12	3.34	3.45	2.87
C14	6.39	9.58	8.94
C15	2.88	4.26	0.79
C16	20.55	29.76	23.80
C16:1	4.12	5.80	1.46
C18	10.59	16.34	13.20
C18:1	27.90	24.44	25.50

[1](Webb et al., 1974)

but no differences were found from pentadecanoic (C15) to oleic (C18:1) acid (Appendix G-4 through G-14). However, when the means were separated using LSMeans procedure, differences were evident for all acids except palmitoleic (C16:1) acid (Table 10).

The lipase from 32A released more butyric (C4), caproic (C6), caprylic (C8), capric (C10) and oleic (C18:1) acids at pH 6.5 than at pH 8.8, and larger concentrations of fatty acids than the other two lipases at either pH. It also released more myristic (C12), pentadecanoic (C15), palmitic (C16), and stearic (C18) at pH 6.5 than at pH 8.8. However, other treatments released similar concentrations of these fatty acids. At pH 6.5, mean concentrations of fatty acids released by the lipase of 22F were different from the control for caproic (C6) and caprylic (C8). At pH 8.8, no differences between the lipase of 22F and the control were found. At pH 6.5, milk lipase released higher quantities of myristic (C14) when compared to the control, although at pH 8.8 no significant differences were noted. At pH 6.5, the lipase from 32A released more butyric (C4), caproic (C6), caprylic (C8), capric (C10), lauric (C12) and oleic (C18:1) acid when compared to milk lipase at the same pH. Lipase from 32A released more caproic (C6) than milk at pH 8.8; no other differences

Table 10. Mean[1] fatty acid concentrations released from butteroil at 37 C.

	pH=6.5				pH=8.8			
FAID[2]	CONTROL	22F	32A	MILK	CONTROL	22F	32A	MILK
C_4	0.77^{a}	1.19^{a}	4.04^{b}	0.31^{a}	0.96^{a}	0.60^{a}	1.23^{a}	0.34^{a}
C_6	0.25^{a}	0.74^{b}	2.07^{c}	0.35^{a}	0.29^{a}	0.47^{ab}	0.74^{b}	0.37^{a}
C_8	0.04^{a}	0.37^{b}	1.18^{c}	0.15^{ab}	0.03^{a}	0.19^{ab}	0.48^{b}	0.15^{ab}
C_{10}	0.11^{a}	0.56^{ab}	1.54^{c}	0.52^{ab}	0.18^{a}	0.29^{ab}	0.69^{b}	0.44^{ab}
C_{12}	0.25^{a}	0.57^{ab}	1.22^{b}	0.72^{a}	0.33^{a}	0.39^{a}	0.61^{a}	0.67^{a}
C_{14}	0.94^{a}	1.23^{ab}	2.33^{b}	2.00^{b}	1.63^{ab}	1.03^{a}	1.23^{ab}	1.85^{b}
C_{15}	0.52^{a}	0.61^{ab}	1.05^{b}	0.89^{ab}	0.57^{a}	0.52^{a}	0.58^{a}	0.77^{ab}
C_{16}	2.99^{a}	3.97^{a}	7.49^{b}	6.21^{ab}	3.93^{a}	3.25^{a}	4.09^{a}	6.30^{ab}
$C_{16:1}$	1.12^{a}	1.10^{a}	1.50^{a}	1.21^{a}	1.04^{a}	0.98^{a}	0.73^{a}	0.83^{a}
C_{18}	2.11^{a}	2.47^{ab}	3.86^{b}	3.41^{ab}	2.70^{ab}	1.99^{a}	2.05^{a}	3.40^{ab}
$C_{18:1}$	2.29^{a}	4.23^{a}	10.17^{b}	5.10^{a}	3.21^{a}	3.34^{a}	4.91^{a}	4.79^{a}

a,b,c - Mean concentrations in the same row followed by the same letter are not significantly different at $p > 0.05$.

[1]N=2.

[2]FAID=Fatty acid identification.

were noted. No differences were found between milk lipase at pH 6.5 and 8.8.

Since the majority of differences in fatty acid concentration occurred with the lipases from 32A and milk at pH 6.5, these concentrations were converted to relative percentages and compared to the values found in the literature for intact milk triglyceride (Webb et al., 1974). It is apparent from the data in Table 9 that the lipase of 32A increased the concentration of butyric (C4), caproic (C6), and caprylic (C8), pentadecanoic (C15), palmitoleic (C16:1) and oleic (C18:1) acids while releasing lesser amounts of myristic (C14), palmitic (C16) and stearic (C18) acids. The concentrations of capric (C10) and lauric (C12) acids were similar to those found in the milk triglyceride.

At pH 6.5, milk lipase released fatty acids in concentrations similar to those found in the milk triglyceride, except that relatively large increases in concentration of pentadecanoic (C15) and palmitoleic (C16:1) acids occurred. Palmitic (C16) and stearic (C18) acids were present in slightly larger quantities than the intact triglyceride. Milk and 32A lipases appeared to readily liberate pentadecanoic (C15) acid. However, the extraction and separation procedure used in this research produced a much larger concentration of pentadecanoic

(C15) acid in the untreated control than was reported by Webb et al. (1974) (data not presented).

In conclusion Khan et al. (1967) reported that the microbial lipase from Achromobacter concentrated long-chain fatty acids while milk lipase released fatty acids in a ratio similar to those found in the milk triglyceride. The lipase of Pseudomonas fluorescens 32A did not behave in a similar manner. Therefore, this research did not completely support the original hypothesis that increased ADV may be a result of microbial lipolysis which preferentially liberated long-chain fatty acids. However, only two microbial lipases were examined here and they differed significantly. Since there are many strains of psychrotrophic bacteria present in raw milk, it is not possible to conclude that the hypothesis is completely false based on the data. It is apparent from these data, however, that microbial and milk lipases hydrolyze milk triglycerides differently and the effect is pH dependent.

CHAPTER V

SUMMARY

The purpose of this research was to determine the fatty acid profiles of butteroil when hydrolyzed by P. fluorescens 32A and 22F and milk lipases. The lipases were separated from the microorganisms by centrifugation and from milk by a series of separation techniques. The lipases were purified by ion-exchange and gel filtration chromatography. Purity was determined by protease assay and disc gel electrophoresis. The lipases reacted with a butteroil emulsion and concentrations of fatty acids released were standardized by the pH-stat procedure. The free fatty acids were extracted and adsorbed to an alumina chromatographic column. Free fatty acids were removed by formic acid and diisopropyl ether and 4 µl aliquots were injected into gas chromatograph for analysis. Peak areas, converted to normalized concentrations using a C5 internal standard were statistically analyzed.

Mean concentrations of fatty acids over all data were significantly higher for 32A lipase. Milk lipase yielded free fatty acid mean concentrations that were higher than concentrations released by the lipase from 22F and the control. The mean fatty acid concentrations from the

combined data for all enzyme sources, pH values and replications showed that butyric (C4), myristic (C14), palmitic (C16), stearic (C18), and oleic (C18:1) were present in the largest concentrations when compared to the total free fatty acid concentrations released. The lipase from 32A released significantly more butyric (C4), caproic (C6), caprylic (C8), capric (C10), and oleic (C18:1) acids at pH 6.5 than at pH 8.8, and more than the other two lipases at either pH.

At pH 6.5, mean concentrations of fatty acids released by 22F lipase were different from the control for caproic (C6) and caprylic (C8) acids.

No differences were found between milk lipase at pH 6.5 and 8.8. At pH 6.5, milk lipase released higher quantities of myristic (C14) when compared to the control. No differences were noted between milk lipase and the control at pH 8.8. At pH 6.5 milk lipase released quantities of fatty acids similar to those found in the milk triglyceride except that relatively large increases in concentrations of pentadecanoic (C15) and palmitoleic (C16:1) acids occurred. The extraction and separation procedure used here yielded higher concentrations of pentadecanoic (C15) acid than the literature reported.

Lipase from 32A is worthy of further investigation to determine its activity in milk. This research demonstrated that the lipase of 32A has strong lipolytic activity. This ability to release high concentrations of short chain fatty acids suggests that P. fluorescens 32A could have a significant effect on the quality and shelf life of milk and milk products.

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APPENDIXES

APPENDIX A

Dialysis Tube Preparation

Tubing was cut to desired lengths and boiled in 0.001 M EDTA (ethylenediaminetetraacetic acid) for 30 min in a 4 L beaker. Following this treatment, the tubing was handled only with disposable rubber gloves since the fingers are a source of degradative enzymes. The tubing was rinsed well 2-3 times with distilled water and stored in distilled water or dilute phosphate buffer containing 0.02 % (w/v) sodium azide (Sigma) at 4 C. Before immediate use, the tubing was rinsed well.

APPENDIX B

Ion Exchange Chromatography

Preparation

A fully rehydrated anion exchange gel DEAE-cellulose (Bio-Rad) was washed with 0.01 M Na-phosphate buffer, pH 6.5 or 7.0 in preparation for use in purification of microbial and milk lipases.

The lyophilized, concentrated crude enzyme (CCE) was rehydrated within the DEAE-cellulose/buffer slurry. The slurry was stirred intermittently for about 15 min to allow for the binding of the proteins and was filtered through a large Buchner funnel using Whatman 2 filter paper. The slurry was washed with 0.01 M Na-phosphate buffer to remove any excess material that was not bound to the gel. The resin cake was removed from the filter paper, mixed with buffer, stirred, and poured into a 2.0 X 50 cm glass silanized (Appendix C) column. About five layers of nylon netting served as support for the bed.

After 2-3 cm of the bed had settled, the liquid was allowed to drain until the surface of the liquid reached the packed bed level. At this point (height about 10 cm) the outlet was closed, and the column was filled with 0.01 M Na-phosphate buffer. With the aid of a long glass rod the top of the bed remained undisturbed during the filling of the column. The gradient mixer was filled with 1.0 M

NaCl solution in the unstirred vessel and 0.01 M Na-phosphate buffer in the stirred vessel. The gradient mixer was allowed to operate for about 30 min before being connected to column. Small plastic tubing connected the gradient mixer to the column. A stream of liquid was allowed to flow thus insuring that the line contained no air bubbles that would prevent the flow of liquid from the gradient mixer. The tubing was attached to an 18 gauge needle protruding through a stopper fitted into the top of the column. A small amount of buffer was allowed to exit the stirred vessel through the tube while it was being connected to the needle. The stopcock at the bottom of the column and the connector between the two vessels of the gradient mixer were opened upon ascertaining that the system was closed and no air was entering.

The column was run at 8 C and fractions were collected in 6-8 ml volumes. Approximately 125 ml/h was the flow rate. NaCl concentration at the point where the enzyme eluted was calculated according to the following formula (Cooper, 1977):

$$c = [(C_a - C_b)/V]\ (v) + C_b$$

where c = concentration of eluant passing over the column at any given time, v = volume of solution that had already

passed from gradient mixer over the column, C_a and C_b are the concentrations of solute in the unstirred and stirred vessels of the gradient maker, respectively. V = the total volume of the gradient.

Regeneration

The bed was poured into a Buchner funnel and washed with 0.1 N NaOH. The adsorbent was washed with large volumes of distilled water to remove the NaOH and with several volumes of 0.01 M Na-phosphate buffer to equilibrate to the appropriate pH. Intermittent checks were made to determine if the pH was decreasing to the desired level. Regeneration was performed each time before addition of a fresh sample.

APPENDIX C

Column Silanization

Silanization was carried out in a fume hood and rubber gloves were worn for protection against the dimethyldichlorosilane which is volatile and extremely toxic. The column was rinsed twice with toluene, drained and secured to a ring stand. A 50 ml, 5 % dimethyldichlorosilane solution was prepared by thoroughly mixing 2.5 ml of this compound with 47.5 ml toluene in an Erlenmeyer flask. The mixture was poured into the column and filled completely to the top. The column stood undisturbed for at least 2 hours in the hood and was then emptied. The column was rinsed again twice with toluene and several times with water. The column was washed using a mild detergent and rinsed with glass-distilled water (Cooper, 1977).

APPENDIX D

Gel Filtration Chromatography

Preparation

A 2.5 X 50 cm silanized column was used. The gel was prepared according to Cooper (1977). The slurry equilibrated with 0.01 M Na-phosphate buffer was poured into the column with the aid of a long glass rod. The bed was allowed to settle to 27 cm length until the excess buffer drained to the settled bed level. Rehydrated sample was pipetted to the top of the bed so that the bed surface remained undisturbed. The sample was allowed to seep into the bed until the sample drained even with the bed surface. Buffer was pipetted onto the bed until the column was filled. Fractions were collected in 6-8 ml volumes.

Regeneration

Upon completion of the run, the slurry was poured into a Buchner funnel, washed several times with distilled water, and washed with several volumes of 0.01 M Na-phosphate buffer.

APPENDIX E

Alumina Chromatography Column Preparation

Chromatographic alumina (neutral Type WN3 Activity Grade I, Sigma) for one replication was measured. To deactivate the total weight of the alumina, 4 % water per sample was also measured, and the volume of water was allowed to adhere to the sides of a clean dry test tube. The total weight of the alumina was poured into the test tube thus absorbing the water. The tube was gently tapped to insure that all water was absorbed and no clumps were apparent. The end of the tube was covered with plastic wrap to exclude air and prevent further hydrolysis. The tube was turned upside down several times until the alumina flowed easily. This procedure was performed at least 2 hours before adsorption of the free fatty acids to give a Brockman Activity Grade II-III.

One gram of deactivated alumina was added to a small glass chromatography column with a small glass-wool plug acting as support for the alumina.

APPENDIX F

Disc Gel Electrophoresis Preparation

Tubes were filled within 2 cm of top with separating gel that was allowed to polymerize. One-tenth ml of stacking gel was applied to the top of the separating gel and polymerization occurred in the presence of fluorescent light for about 30 min. Concentrated, purified enzyme (0.1 g) was dissolved in 1 ml of distilled water and 5 µl were applied to the gel followed by two drops of tracking dye. One-tenth ml of stacking gel was added immediately. The tubes were covered with parafilm and inverted several times and allowed to polymerize again. Before each polymerization step, a drop of water was added to the top of the gel to produce a flat surface on the gels. The gels polymerized (12.5 cm tube lengths) and were attached to an electrophoresis cell (Bio-Rad Model 150A). Electrophoresis buffer was dispensed into the top and bottom reservoirs. Current was applied at 3 mA/tube and the proteins traveled downward until the tracking dye was within 10 mm of the end of the tube; then the current was turned off. It was important to keep a close watch on the unit since the voltage dropped and had to be adjusted to its initial power several times. Otherwise, the proteins would migrate very slowly.

Gels were removed from the tubes and placed into screw-cap tubes and stained with a solution containing glacial acetic acid (46 ml), 50% methanol (454 ml), 1.25 g Coomassie Blue G250 (Alexander et al., 1985). Following the staining procedure, the gels were destained with 75 ml acetic acid, 50 ml methanol and 875 ml distilled water at 37 C overnight. The destaining solution was changed several times over the next 3-5 days. The gels were photographed, stored, and purities of the enzymes were determined. Migration of a protein as a single band at two pH values and over a range of gel concentrations sufficient to cause separation on the basis of size as well as charge was considered to be evidence of purity. Purity was also determined by showing that all other enzymatic activity was absent. An advantage to this latter method was that very low concentrations of the enzyme were necessary (Whitaker, 1972).

APPENDIX G

G-1. Analysis of variance over all pH values, enzyme sources, replications, fatty acids, and interactions.

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: CONC[1]

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE
MODEL	295	14474.67345043	49.06668966
ERROR	136	1475.70803230	10.85079436
CORRECTED TOTAL	431	15950.38148273	
MODEL F =	4.52		PR > F = 0.0001

R-SQUARE	C.V.	ROOT MSE	CONC MEAN
0.907481	133.9686	3.29405439	2.45882488

SOURCE	DF	TYPE III SS	F VALUE	PR > F
FAID[2]	17	4813.20983376	26.09	0.0001
TRT[3]	3	435.13591783	13.37	0.0001
REP[4]	2	1713.72578745	78.97	0.0001
PH[5]	1	301.55833510	27.79	0.0001
TRTxPH	3	167.83567107	5.16	0.0021
REPxPH	2	410.66185829	18.92	0.0001
REPxTRT	6	442.80388939	6.80	0.0001
PHxFAID	17	542.07893740	2.94	0.0003
TRTxFAID	51	980.42244389	1.77	0.0048
REPxFAID	34	3054.03184302	8.28	0.0001
TRTxPHxFAID	51	418.57541053	0.76	0.8723
REPxTRTxPH	6	241.91480044	3.72	0.0019
REPxTRTxFAID	102	952.71872226	0.86	0.7868

[1]Concentration of fatty acid (µmole/ml).
[2]Fatty acid identification.
[3]Enzyme source.
[4]Replication = 3.
[5]pH = 6.5 and 8.8.

G-2. Analysis of variance for data excluding replication 2.

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: CONC[1]

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE
MODEL	219	902.86205970	4.12265781
ERROR	68	10.00535270	0.14713754
CORRECTED TOTAL	287	912.86741240	

MODEL F =	28.02	PR > F = 0.0001

R-SQUARE	C.V.	ROOT MSE	CONC MEAN
0.989040	36.1211	0.38358511	1.06194073

SOURCE	DF	TYPE III SS	F VALUE	PR > F
FAID[2]	17	628.67473249	251.34	0.0001
TRT[3]	3	26.18941860	59.33	0.0001
REP[4]	1	27.81525132	189.04	0.0001
PH[5]	1	6.16719785	41.91	0.0001
TRTxPH	3	16.55012061	37.49	0.0001
REPxPH	1	0.31232854	2.12	0.1497
REPxTRT	3	7.37538720	16.71	0.0001
PHxFAID	17	9.14147873	3.65	0.0001
TRTxFAID	51	75.33570318	10.04	0.0001
REPxFAID	17	45.54210935	18.21	0.0001
TRTxPHxFAID	51	29.12290238	3.88	0.0001
REPxTRTxPH	3	1.48011454	3.35	0.0239
REPxTRTxFAID	51	29.15531492	3.89	0.0001

[1]Concentration of fatty acid (μmole/ml).
[2]FAID = Fatty acid identification.
[3]TRT = Enzyme source.
[4]Replication = 2.
[5]pH = 6.5 and 8.8.

G-3. Analysis of variance including selected fatty acids for replication 1 and 3.

GENERAL LINEAR MODELS PROCEDURE

DEPENDENT VARIABLE: CONC[1]

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE
MODEL	135	709.73506453	5.25729677
ERROR	40	8.92280597	0.22307015
CORRECTED TOTAL	175	718.65787050	
MODEL F =	23.57		PR > F = 0.0001
R-SQUARE	C.V.	ROOT MSE	CONC MEAN
0.987584	27.5230	0.47230303	1.71603216

SOURCE	DF	TYPE III SS	F VALUE	PR > F
REP[2]	1	44.65902942	200.20	0.0001
TRT[3]	3	41.58232191	62.14	0.0001
PH[4]	1	9.66119181	43.31	0.0001
FAID[5]	10	434.82396235	194.93	0.0001
TRTxPH	3	26.27481655	39.26	0.0001
REPxPH	1	0.48018800	2.15	0.1501
REPxTRT	3	11.60688927	17.34	0.0001
FAIDxPH	10	5.62813135	2.52	0.0185
TRTxFAID	30	59.78539490	8.93	0.0001
REPxFAID	10	28.68155269	12.86	0.0001
TRTxFAIDxPH	30	19.36713977	2.89	0.0009
REPxTRTxPH	3	2.37460965	3.55	0.0228
REPxTRTxFAID	30	24.80983687	3.71	0.0001

[1]Concentration of fatty acid (µmole/ml).
[2]Replication = 2.
[3]TRT = Enzyme source.
[4]pH = 6.5 and 8.8.
[5]FAID = Fatty acid identification.

G-4. Analysis of variance for butyric acid.

GENERAL LINEAR MODELS PROCEDURE - FAID=C4

DEPENDENT VARIABLE: CONC[1]

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE
MODEL	8	23.54446766	2.94305846
ERROR	7	1.95338047	0.27905435
CORRECTED TOTAL	15	25.49784814	

MODEL F = 10.55 PR > F = 0.0028

R-SQUARE	C.V.	ROOT MSE	CONC MEAN
0.923390	44.6880	0.52825595	1.18209687

SOURCE	DF	TYPE III SS	F VALUE	PR > F
REP[2]	1	3.09785521	11.10	0.0126
TRTNEW[3]	7	20.44661246	10.47	0.0031

[1]Concentration of fatty acid (μmole/ml).
[2]Replication = 2.
[3]TRTNEW = Enzyme source by pH.

G-5. Analysis of variance for caproic acid.

GENERAL LINEAR MODELS PROCEDURE - FAID=C6

DEPENDENT VARIABLE: CONC[1]

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE
MODEL	8	5.89242697	0.73655337
ERROR	7	0.15763171	0.02251882
CORRECTED TOTAL	15	6.05005868	

MODEL F =	32.71	PR > F =	0.0001

R-SQUARE	C.V.	ROOT MSE	CONC MEAN
0.973945	22.6800	0.15006271	0.66165125

SOURCE	DF	TYPE III SS	F VALUE	PR > F
REP[2]	1	0.83372422	37.02	0.0005
TRTNEW[3]	7	5.05870275	32.09	0.0001

[1]Concentration of fatty acid (μmole/ml).
[2]Replication = 2.
[3]TRTNEW = Enzyme source by pH.

G-6. Analysis of variance for caprylic acid.

GENERAL LINEAR MODELS PROCEDURE - FAID=C8

DEPENDENT VARIABLE: CONC[1]

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE
MODEL	8	2.09533246	0.26191656
ERROR	7	0.14052262	0.02007466
CORRECTED TOTAL	15	2.23585508	

MODEL F = 13.05 PR > F = 0.0014

R-SQUARE	C.V.	ROOT MSE	CONC MEAN
0.937150	43.6935	0.14168507	0.32427000

SOURCE	DF	TYPE III SS	F VALUE	PR > F
REP[2]	1	0.07605185	3.79	0.0927
TRTNEW[3]	7	2.01928061	14.37	0.0011

[1]Concentration of fatty acid (µmole/ml).
[2]Replication = 2.
[3]TRTNEW = Enzyme source by pH.

G-7. Analysis of variance for capric acid.

GENERAL LINEAR MODELS PROCEDURE - FAID=C10

DEPENDENT VARIABLE: CONC[1]

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE
MODEL	8	3.01447784	0.37680973
ERROR	7	0.34199890	0.04885699
CORRECTED TOTAL	15	3.35647674	

MODEL F =	7.71		PR > F = 0.0071
R-SQUARE	C.V.	ROOT MSE	CONC MEAN
0.647146	40.7771	0.22103616	0.54206000

SOURCE	DF	TYPE III SS	F VALUE	PR > F
REP[2]	1	0.20005045	4.09	0.0827
TRTNEW[3]	7	2.81442739	8.23	0.0063

[1]Concentration of fatty acid (µmole/ml).
[2]Replication = 2.
[3]TRTNEW = Enzyme source by pH.

G-8. Analysis of variance for lauric acid.

GENERAL LINEAR MODELS PROCEDURE - FAID=C12

DEPENDENT VARIABLE: CONC[1]

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE
MODEL	8	1.68022940	0.21002868
ERROR	7	0.32566601	0.04652372
CORRECTED TOTAL	15	2.00589541	

MODEL F = 4.51 PR > F = 0.0309

R-SQUARE	C.V.	ROOT MSE	CONC MEAN
0.837646	36.1976	0.21569357	0.59587750

SOURCE	DF	TYPE III SS	F VALUE	PR > F
REP[2]	1	0.38249277	8.22	0.0241
TRTNEW[3]	7	1.29773663	3.98	0.0442

[1]Concentration of fatty acid (μmole/ml).
[2]Replication = 2.
[3]TRTNEW = Enzyme source by pH.

G-9. Analysis of variance for myristic acid.

GENERAL LINEAR MODELS PROCEDURE - FAID=C14

DEPENDENT VARIABLE: CONC[1]

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE
MODEL	8	6.74894406	0.84361801
ERROR	7	0.89120026	0.12731432
CORRECTED TOTAL	15	7.64014431	

MODEL F = 6.63 PR > F = 0.0109

R-SQUARE	C.V.	ROOT MSE	CONC MEAN
0.883353	23.3134	0.35681133	1.53049750

SOURCE	DF	TYPE III SS	F VALUE	PR > F
REP[2]	1	3.23458427	25.41	0.0015
TRTNEW[3]	7	3.51435979	3.94	0.0454

[1]Concentration of fatty acid (µmole/ml).
[2]Replication = 2.
[3]TRTNEW = Enzyme source by pH.

G-10. Analysis of variance for pentadecanoic acid.

GENERAL LINEAR MODELS PROCEDURE - FAID=C15

DEPENDENT VARIABLE: CONC[1]

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE
MODEL	8	1.42338639	0.17792330
ERROR	7	0.29248258	0.04178323
CORRECTED TOTAL	15	1.71586897	

MODEL F =	4.26		PR > F = 0.0359
R-SQUARE	C.V.	ROOT MSE	CONC MEAN
0.829543	29.5683	0.20440946	0.69131375

SOURCE	DF	TYPE III SS	F VALUE	PR > F
REP[2]	1	0.88692132	21.23	0.0025
TRTNEW[3]	7	0.53646508	1.83	0.2210

[1]Concentration of fatty acid (µmole/ml).
[2]Replication = 2.
[3]TRTNEW = Enzyme source by pH.

G-11. Analysis of variance for palmitic acid.

GENERAL LINEAR MODELS PROCEDURE - FAID=C16

DEPENDENT VARIABLE: CONC[1]

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE
MODEL	8	66.21802314	8.27725289
ERROR	7	16.06196247	2.29456607
CORRECTED TOTAL	15	82.27998561	

MODEL F =	3.61	PR > F = 0.0540

R-SQUARE	C.V.	ROOT MSE	CONC MEAN
0.804789	31.6953	1.51478251	4.77920500

SOURCE	DF	TYPE III SS	F VALUE	PR > F
REP[2]	1	28.06488362	12.23	0.0100
TRTNEW[3]	7	38.15313952	2.38	0.1382

[1]Concentration of fatty acid (µmole/ml).
[2]Replication = 2.
[3]TRTNEW = Enzyme source by pH.

G-12. Analysis of variance for palmitoleic acid.

GENERAL LINEAR MODELS PROCEDURE - FAID=C16:1

DEPENDENT VARIABLE: CONC[1]

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE
MODEL	8	5.14774224	0.64346778
ERROR	7	1.42942247	0.20420321
CORRECTED TOTAL	15	6.57716471	

MODEL F =	3.15	PR > F = 0.0741	
R-SQUARE	C.V.	ROOT MSE	CONC MEAN
0.782669	42.5058	0.45188849	1.06312312

SOURCE	DF	TYPE III SS	F VALUE	PR > F
REP[2]	1	4.36854936	21.39	0.0024
TRTNEW[3]	7	0.77919288	0.55	0.7791

[1]Concentration of fatty acid (µmole/ml).
[2]Replication = 2.
[3]TRTNEW = Enzyme source by pH.

G-13. Analysis of variance for stearic acid.

GENERAL LINEAR MODELS PROCEDURE - FAID=C18

DEPENDENT VARIABLE: CONC[1]

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE
MODEL	8	24.27070356	3.03383795
ERROR	7	3.39620346	0.48517192
CORRECTED TOTAL	15	27.66690702	

MODEL F =	6.25	PR > F =	0.0129

R-SQUARE	C.V.	ROOT MSE	CONC MEAN
0.877247	25.3377	0.69654284	2.74903437

SOURCE	DF	TYPE III SS	F VALUE	PR > F
REP[2]	1	16.96461641	34.97	0.0006
TRTNEW[3]	7	7.30608715	2.15	0.1668

[1]Concentration of fatty acid (µmole/ml).
[2]Replication = 2.
[3]TRTNEW = Enzyme source by pH.

G-14. Analysis of variance for oleic acid.

GENERAL LINEAR MODELS PROCEDURE - FAID=C18:1

DEPENDENT VARIABLE: CONC[1]

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE
MODEL	8	95603844670	11.95048058
ERROR	7	23.20385880	3.31483697
CORRECTED TOTAL	15	118.80770347	

MODEL F = 3.61 PR > F = 0.0541

R-SQUARE	C.V.	ROOT MSE	CONC MEAN
0.804694	38.2717	1.82066937	4.75722437

SOURCE	DF	TYPE III SS	F VALUE	PR > F
REP[2]	1	15.23085264	4.59	0.0693
TRTNEW[3]	7	80.37299203	3.46	0.0617

[1]Concentration of fatty acid (µmole/ml).
[2]Replication = 2.
[3]TRTNEW = Enzyme source by pH.

VITA

Carol J. Breeding was born on July 25, 1958 in Bulls Gap, Tennessee to Gale and June Jones. She married Wade C. Breeding in January of 1984. Following graduation from Rogersville High School in 1976, she entered Walters State Community College. She later transferred to East Tennessee State University and Lincoln Memorial University where she majored in Biology and was awarded a Bachelor's Degree in Science in June, 1986. In September 1987, she was given the opportunity to pursue a Master of Science degree in Food Technology and Science, which was awarded in August 1989.

She is a member of the Institute of Food Technologists, Phi Kappa Phi, Alpha Chi, and Gamma Sigma Delta.