Hydrolysis Characteristics of Bovine Milk Fat and Monoacid Triglycerides Mediated by Pregastric Lipase from Goats and Kids

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ABSTRACT

Commercial extracts from oro-pharyngeal tissues of goats and kids have been used as the source of pregastric lipase and have been processed to yield partially purified samples of the primary pregastric lipase. The activity of these lipases against tributyrylglycerol has been determined over a range of pH and temperatures. Optimum pH conditions for pregastric lipase ranged from pH 5.6 to 6.5 for goats and from pH 5.5 to 6.2 for kids, respectively; the optimum temperature ranged from 43 to 60°C. Optima for kid lipase extended slightly below pH 5.5 and higher than 60°C; which were the limits of the test conditions. The enzymes were also used as catalysts for the hydrolysis of monoacid triglycerides ($C_{4:0}$ to $C_{12:0}$) at 40°C and pH 6.5; activity was maximum against tributyrylglycerol ($C_{4:0}$). Values for the Michaelis-Menten constant, increased as carbon chain length of the carboxylic moiety on the triglycerides increased, but values were identical for pregastric lipases of both goats and kids. Anhydrous milk fat was hydrolyzed by the commercial extracts of pregastric lipases of goats and kids, and the resulting profiles for free fatty acids were very similar to one another and to the corresponding profile for a commercial sample of Parmesan cheese. There appear to be no significant differences in activity between the enzyme preparations from goats and kids.

(**Key words**: caprine pregastric lipase, milk fat, tributyrylglycerol, monoacid triglycerides)

Abbreviation key: K_{cat} = apparent turnover number, K_m = Michaelis-Menten substrate affinity constant, **TBG** = tributyrylglycerol.

INTRODUCTION

Many enzymes are involved in the manufacture and ripening of cheese, including exogenous enzymes and indigenous milk enzymes. Lipases have been used extensively in the dairy industry to enhance the flavor of cheeses, to accelerate cheese ripening, to lipolyze milk fat and cream, and to manufacture cheese-flavored products (3, 4, 5, 16, 17, 26, 27, 29). Commercially produced pregastric lipases were originally made of pastes of stomach and stomach contents that possessed milk-clotting activity as well as lipolytic activity. Increasingly, these lipases are being further processed to produce pastes, vacuum-dried powders, or freeze-dried powders. Each type of pregastric lipase (calf, kid, or lamb) has its own characteristic flavor profile, which is due to the fatty acid selectivity of the corresponding enzyme among other factors. Fox and Stepaniak (15) have reviewed the use of the liquid extract of oral tissue from calves as an alternative source of pregastric enzyme for the manufacture of Italian cheese. Extensive studies have shown that pregastric lipase, either as paste or extract, is essential for the development of the characteristic flavor of Romano and Provolone cheeses; a combination of calf gastric lipase and goat pregastric lipase has produced Cheddar or Provolone cheese that was superior in quality to cheese made with pregastric lipase alone (7). However, very little is known about the activity of pregastric lipases against individual substrates.

In an early study (24), we identified differences in physiochemical parameters when calf and lamb extracts were used as catalysts to hydrolyze a short-chain lipid (tributyrylglycerol, **TBG**; $C_{4:0}$), a long-chain lipid (trioleoylglycerol, $C_{18:1}$), and an ester (4-nitrophenylacetate). At that time, we commented that it would be important to identify the characteristics for activity of enzyme preparations obtained from different gene stock in different countries.

More recent studies have used the partially purified pregastric enzymes of lambs to hydrolyze a series

Received July 26, 1996.
Accepted April 15, 1997.
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of 4-nitrophenylalkanoates (carbon chain length ranging between 2 and 12) and monoacid triglycerides of differing carboxylic acid carbon chain length ranging between 4 and 10 (20, 21, 22) and triolein (23) and to make a detailed study of activity against TBG (1, 2). Similar studies are being conducted using partially purified calf pregastric enzymes.

Calves are generally slaughtered for veal during the suckling period, but lambs are generally slaughtered when they are approximately 6 mo old and their primary mode of feeding is by grazing (i.e., their ruminant digestive system is becoming dominant). It is unknown whether the differences between the calf and lamb in enzyme activity partly reflect this difference or are solely related to species.

In this study, therefore, we have compared the activity of extracts from goats and kids against a range of monoacid triglycerides and against anhydrous milk fat. The results show that the performance characteristics of both extracts are almost indistinguishable. Traditionally, the kid extract has been the preferred source of enzyme, but restrictions on its export from the US into countries that are free of Scrapie and other endemic diseases are now widespread. However, countries such as New Zealand that prohibit import of this enzyme may well be able to produce quantities of the goat extract that are sufficient not only to meet the demands of their own cheese industry but also to satisfy the organoleptic demands of clients in other countries.

MATERIALS AND METHODS

Lipase Preparations

The pregastric lipolytic extract from goats was prepared from the epiglottal regions (tongue roots) of 18-mo-old goats presented for commercial slaughter in New Zealand. Frozen tongue roots were flaked into water at alkaline pH. After 90 min of stirring, the fat and solids in suspension were removed by rotary vacuum filtration, and the resultant liquid extract was concentrated 10-fold by ultrafiltration. The concentrate was dried by freeze-drying. In excess of 90% of the apparent lipolytic activity of tongue roots was retained in the dried powder.

The extract from kids was obtained by dissolving a commercial preparation of enzyme (Rhône-Poulenc Marschall Products, Madison, WI) at alkaline pH, followed by rotary vacuum filtration, ultrafiltration concentration, and freeze-drying.

Chemicals and Reagents

The substrates used for lipase assay were TBG (96 to 98% grade II), trihexanoylglycerol, (tricaproin, $C_{6:0}$); trioctanoylglycerol, (tricaprylin, $C_{8:0}$); tridecanoylglycerol, (tricaprin, $C_{10:0}$); tridodecanoylglycerol (trilaurin, $C_{12:0}$) (from Sigma Chemical Co., St. Louis, MO), and anhydrous milk fat (New Zealand Research Institute, Palmerston North, New Zealand). Internal gas chromatographic standards *n*-valeric acid ($C_{5:0}$) and tridecanoic acid ($C_{13:0}$), reagent grade buffers Tris, and bis Tris propane [(1,3 bis[tris-(hydroxymethyl)methylamino]-propane)], and 1,2-diacyl-*sn*-glycero-3-phosphocholine (L-α-lecithin) were from Sigma Chemical Co. Color key pH calibration buffers were supplied by BDH Chemicals (Poole, United Kingdom), and the protein assay reagent was from Bio-Rad Laboratories, Inc. (Hercules, CA). Sodium caseinate (Alanate 180) was supplied by the New Zealand Dairy Board (Wellington, New Zealand). Fast flow Q-Sepharose was from Pharmacia LKB Biotechnology AB (Uppsala, Sweden) and Amberlyst 26 ion-exchange resin was from Acros Organics (Janssen Pharmaceuticalaan 3a, Geel, Belgium). All water was Milli-Q grade, and resistance was typically 1.8×10^7 ohm/cm; water was processed with equipment from Millipore Corp (Bedford, MA). Gases for gas chromatography were all instrument-grade: oxygen-free nitrogen, helium, hydrogen, and air (BOC Gases NZ Ltd., Auckland, New Zealand). Parmesan cheese, purchased from a local supermarket, was manufactured by Kraft Inc. (Port Melbourne, Victoria, Australia).

Equipment

Solutions were pumped with a microtube pump (Eyela MP-3, Rikadenki Co., Tokyo, Japan). The column used for enzyme purification (Amicon-Wright, Beverley, MA) was approximately 20 ml of internal volume. Dialysis was carried out in dialysis tubing (20DM; Union Carbide, Chicago, IL). Spectral analyses were made on a minicomputer (series HP9000; Hewlett Packard Co., Int., Palo Alto, CA) run with an external water bath temperature controller (Grant, Cambridge, United Kingdom). The pH-stat titrations were carried out in temperature-jacketed vessels with a programmable autotitrator (Mettler DL21; Mettler Instruments AG, Greifensee, Switzerland), and pH was measured with a Mettler DG 111-SC (on the autotitrator) or an Ingold 104023365 combined pH microelectrode (Ingold Messtachnik AG, Switzerland) on a pH meter (Orion SA 520; Orion Research AG, Kiismacht, Switzerland). An ultrasonicator bath (Elma T460; Hans Schmidtbauer KG, West Germany) was used for the emulsification of anhydrous milk fat; a micro ultrasonic cell disruptor (KT 50;

Kontes, Vineland, NJ) with 3-mm semimicro probe was used for emulsifcation of monoacid triglycerides. Samples were analyzed by gas chromatography (Hewlett Packard 5890 Series II Gas Chromatograph), using a J&W DB-225 column (15 m × 0.2433 mm; J&W Scientific (Fisons), Folsom, CA) with 0.25-μm film thickness. The temperature was programmed for 40°C with a 3-min hold, rising at 8°C/min to 220°C. The horizontal mechanical shaker was from Ratek Instruments (model SWB20; Boronia, Australia).

Mathematical data processing was predominantly carried out using Sigmaplot™ Version 1.0 (Jandel Scientific, Carle Madera, CA) on IBM personal computers; contour plots were generated from the Sigmaplot™ interpolated data matrix using MathCad™ 4.0 for Windows (Math Soft Inc., Cambridge, MA).

Enzyme Processing and Protein Assay

The crude enzyme extracts were partially purified as has been described previously for lamb pregastric lipase (2). The crude extract of goat enzyme (2.0 g) was dissolved in 50 m*M* Tris·HCl buffer (40 ml; pH 8.0 at 4°C) and centrifuged at 10,000 rpm for 10 min at 4°C to remove insoluble particles. The clear supernatant was applied onto an ion-exchange column of Q-Sepharose (1.0 cm × 18 cm) with a flow rate of 1.2 ml/min and was washed with a suspension buffer until no more protein was eluted. The column was then washed with 50 m*M* acetate·HCl buffer (pH 4.6 at 4°C), and the collected fraction contained a mixture of proteins, including serum albumin and components with activity against 4-nitrophenylacetate but little activity against TBG. The fraction that contained the active lipase was then eluted with 1 *M* NaCl/50 m*M* Tris·HCl buffer, dialyzed, lyophilized, and stored at –20°C until required for use. The mass recovered in this lipase fraction was 14%, specific activity was 13.2 μmol/min per mg (8.3 m*M* TBG at pH 6.5 for 35°C), and the purification factor was 9.1.

The crude kid enzyme (4.0 g) was dissolved in 50 m*M* Tris·HCl buffer (40 ml; pH 8.0 at 4°C), dialyzed to remove the lactose extender, and lyophilized. The enzyme extract (1.4 g) was collected, redissolved in buffer, centrifuged, and purified as just described for the goat extract. The mass recovered as the lipase fraction was 5.4%; the specific activity was 6.5 μmol/min per mg (8.3 m*M* TBG at pH 6.5 for 35°C), and the purification factor was 7.1.

The protein content of the partially purified lipase preparations (Bio-Rad protein assay kit) was 98 and 88% (±3) for goat and kid extracts, respectively.

TBG Assay

The technique used for kinetics characterization of lipase activity across a range of pH values and temperatures has been described previously (2). The activity was measured in a sodium caseinate (0.5 mg/ml)/L-α-lecithin (6 mg/ml) emulsion using pH-stat. The emulsion base (40 ml), composed of sodium caseinate and L-α-lecithin, was added to TBG that had been weighed into a dry, plastic titrator cup; the mixture was sonicated until it became monodispersed. The enzyme solution (variable volume, 5 mg/ml; 20 to 50 μl for goat extract; 50 to 100 μl for kid extract) was added, and the release of butyric acid from TBG was determined.

The activity of the partially purified enzyme extracts was measured at pH 5.5, 6.0, 6.5, 7.0, and 7.5 and at temperatures starting at 30°C; measured incrementally by 5° C up to 60°C; or measured until the initial rate of reaction was nonlinear (i.e., the enzyme was inactivated). For each assay condition, at least five different concentrations (0.2 to 9.0 m*M*) of TBG were used. The plots of initial rate of hydrolysis (the linear region at the beginning of the 6 min of pH-stat assay) were fitted by the Michaelis-Menten substrate affinity constant ($\boldsymbol{K_m}$) and the apparent turnover numbers ($\boldsymbol{K_{cat}}$) were thus obtained.

The plot of K_{cat} versus pH and temperature was fitted with a third-order polynomial against pH and temperature, respectively. The surface net was then generated with unit intervals of 0.1 pH and 1°C, and the contour plot of K_{cat} versus pH and temperature was subsequently constructed from the surface net values.

Measurement of the K_{cat} for Hydrolysis of Monoacid Triglycerides

The emulsion base (1 mg/ml of L-α-lecithin and 6 mg/ml of sodium caseinate) was prepared by the addition of 200 mg of L-α-lecithin and 1.2 g of sodium caseinate to 200 ml of Milli-Q water. The solution was stirred for at least 1 h until the caseinate was dispersed homogeneously. The substrate was added to a 40-ml emulsion base and sonicated at 40°C (using a Kontes ultrasonicator 3-mm probe) until the mixture was monodispersed, and the pH was then adjusted to pH 6.5. Enzyme solution (5 mg/ml for both partially purified goat and kid lipases) was added into the reaction vessel, and the reaction was monitored by titration of released carboxylic acids with standardized NaOH (typically 0.01 *M*) for 6 min at 40°C and pH 6.5.

The initial rate of hydrolysis of each lipid substrate (TBG, tricaproin, tricaprylin, tricaprin, and trilaurin) was assessed over a range of at least five concen-

trations and up to 5 to 10 times the value of K_m. These data were then used to generate Michaelis-Menten plots from which values of K_{cat} and K_m were evaluated.

Preparation of Milk Fat Emulsion

An emulsion of bis-tris propane buffer (200 ml, 0.1 *M* at pH 6.5) added to 1.2 g of sodium caseinate and 100 mg of L-α-lecithin was prepared by stirring; to this emulsion was added 90 mg of anhydrous milk fat. The mixture was stirred and sonicated until the fat was emulsified. The enzyme solution (5 ml, 3 mg/ml of crude goat extract, 10 mg/ml of crude kid extract) was added to 50 ml of this emulsion at 35°C to start the hydrolysis reaction. Samples (10 ml) were taken for analysis after 1, 2, 4, 8, and 24 h. The enzyme extracts were used directly as supplied by the manufacturers without further purification.

The amount of free fatty acids that were released by the hydrolysis of milk fat was analyzed using a method modified from that described by Needs et al. (19).

Extraction of Free Fatty Acids and Unreacted Lipid

A sample of reaction emulsion (10 ml) was added to an ice-cold mixture consisting of 3 ml of HCl (35%, vol/vol aqueous HCl), 20 ml of diethyl ether, and 500 μl of internal standards of *n*-valeric acid (1 mg/ml) and tridecanoic acid (1 mg/ml). The use of these short-chain internal standards allowed correction to be made for the unavoidable loss of methyl esters caused by diethyl ether evaporation from vials. The mixture was shaken for 3 min and centrifuged at 3332 rpm for 10 min. An aliquot (8 ml) of the ether layer was dried over 400 mg of anhydrous Na_2SO_4 for a minimum of 20 min. Then 2 ml were transferred to a 10-ml screw-top bottle for analysis of total fatty acids, and 5 ml was transferred to a 15-ml stoppered test tube containing 200 mg of Amberlyst 26 resin and 2.5 ml of methanol to analyze free fatty acids. The test tube was shaken for 1 h on an horizontal mechanical shaker (120 oscillations/min). The solvent was removed, and the resin was washed five times with 7 ml of a mixture of ether and methanol (2:1, vol/vol) and dried at room temperature (15 to 23°C) under a gentle stream of nitrogen to obtain the separated free fatty acids.

Methylation of Fatty Acids and Analysis by GLC

The methylating agent (5%, vol/vol, HCl in methanol) was prepared by addition of 1 ml of acetyl chloride by drop to 10 ml of ice-cold methanol (6), and 1 ml was added to the separated free fatty acids in the test tube and left at 30°C overnight. The ether was removed from the lipid sample in the screw-top bottle under a gentle stream of nitrogen at room temperature, 0.5 ml of hexane and 1 ml of methylating agent were added, and the mixture was left at 30°C overnight. Following methylation, 1 ml of hexane was added to each container, which was shaken gently for 2 min. Saturated NaCl (1 ml) was added, and the mixture was again shaken gently for 20 s. The hexane layer was removed with a Pasteur pipette and placed in a vial that was ready for gas chromatographic analysis.

A sample of Parmesan cheese was also analyzed for free fatty acid composition using the procedure described.

RESULTS AND DISCUSSION

TBG Activity

Figure 1 shows the contour plots for activity of the partially purified extracts of goat and kid lipase against TBG. The shaded areas identify regions of optimal activity (i.e., ≥90% of the maximum). The temperature range within these regions was 43 to 60°C for both enzymes, and pH values ranged from 5.6 to 6.5 for the goat enzyme and from pH 5.5 to 6.2 for the kid enzyme. The surface plots of K_{cat} for the experimental range of pH values and temperatures are shown in Figure 2 for the lipase extracts from goats and kids. The points of maximum activity on the smoothed plots are at 52°C and pH 6.0 for the goat enzyme and 56°C and pH 5.5 for the kid enzyme. The pH value for the kid extract lies on the edge of the surface plot, and the possibility therefore exists that the pH optimum may be pH <5.5. However, under the present experimental conditions, the pH could not be decreased further without precipitating sodium caseinate. In a separate set of experiments using the goat enzyme and excluding sodium caseinate from the emulsion, the enzyme was first allowed to remain in contact with the emulsion for 15 min; after centrifugation, the supernatant was then tested for activity against TBG. As the pH was lowered from pH 5.5 to 5.0, activity decreased 55%, a loss that was attributed largely to precipitation of the enzyme. Others (28) have noted precipitation of a pregastric enzyme at pH 5 and were able to use that property as the first step in separation and purification of the calf enzyme from calf gullet tissues. In light of those results, further attempts to identify the

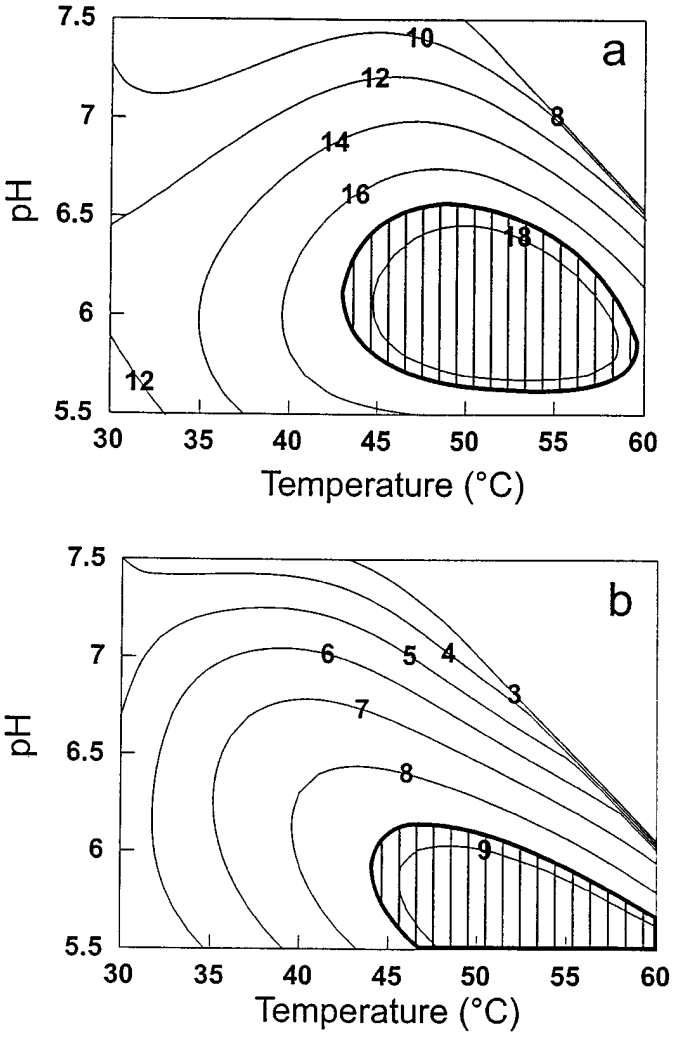

Figure 1. Contour plot for constant apparent turnover number against pH and temperature for the hydrolysis of tributyrylglycerol that was catalyzed by pregastric lipase of goat (a; upper plot) and kid (b; lower plot). The shaded area represents activity within 10% of the optimum value.

precise pH optimum for the kid enzyme were not warranted.

At pH 7.5, inactivation occurred within 6 min of reaction time at 50 and 45°C for goat and kid enzymes, respectively. As the pH was lowered, both enzymes exhibited more tolerance to thermoinactivation, as illustrated by the data shown in the contour levels plots (Figure 1). This thermostability may be affected by the proteins that were coeluted in our partial purification, and we are currently investigating possible interactions of protein and enzymes and of protein and micelles.

The K_m were generally low, frequently <0.15 m*M*, and some were low as 0.07 m*M*, indicating that both enzymes have a very high affinity for TBG.

The maximum values of K_{cat} were 20.3 and 10.0 μmol/min per mg for the goat and kid extracts, respectively; values of 1.46 and 0.92 μmol/min per mg were obtained at pH 6.5 and 35°C for the corresponding unprocessed commercial products. These values may be compared with the maximum value for activity of partially purified lamb lipase equal to about 2.6 μmol/min per mg at 43°C and pH 6.4 or to 0.7 μmol/min per mg obtained at pH 6.6 and 37.5°C for the corresponding unprocessed commercial product (2).

De Caro et al. (11) recently purified lamb pregastric lipase and obtained 3% recovery of a single protein with a molecular mass of 50 kDa on SDS-PAGE; activity was maximal against a TBG emulsion at pH 5.5. Those researchers (11) found, and we have also reported (22), that lamb pregastric lipase has a very marked specificity for short-chain lipids, and this specificity is likely to be advantageous in the dairy industry, which prefers that short- and medium-chain fatty acids from milk triglycerides be released during cheese ripening.

We have not attempted to compare the kinetics data obtained from tributyrin with those from natural substrate fats. Tributyrin is an artificial substrate and, although insoluble, interacts at a phospholipid interface quite differently from the normal form of lipase substrate, namely emulsified insoluble triglyceride. However, tributyrin is used as a standard substrate in the dairy industry (8) for monitoring the activity of pregastric lipase before adding it to the vats in the manufacture of cheese. It is therefore important to develop precisely defined parameters on reactivity in order to facilitate the further application of pregastric lipase. The present study demonstrates that the reactivity parameters for the commercially available sources of pregastric lipases of goat and kid are very similar, which is significant because the sources of the enzymes were likely to have been from different gene stock and originally processed in different countries.

Hydrolysis of Monoacid Triglycerides

Figure 3a shows percentage values of the derived values of K_{cat} (relative to the value for TBG) against carbon chain length of the carboxylic acid moieties of the monoacid triglycerides. Figure 3b shows the corresponding derived absolute values of K_m plotted against carbon chain length. The values for K_{cat} decreased as the acid chain length increased. For each additional pair of carbon atoms in the carboxylic acid chain, the K_{cat} value decreased by at least twofold. The shape of this profile was superimposable on that

from a similar study carried out on partially purified lamb pregastric lipase (22), except that we extended the range of lipid substrates in this study by inclusion of the longer chain lipid, trilaurin. A study by D'Souza and Oriel (14) on lamb pregastric lipase showed a similar trend, but their study used only a single concentration of substrate that was considerably below the K_m values for tricaprylin and tricaprin, for which activity was not detected.

Interestingly, Figure 3b shows that the K_m values for the goat and kid extracts are the same within experimental error. For the short-chain substrates, $C_{4:0}$ and $C_{6:0}$, the values for K_m are equal to 0.15 ± 0.05 m*M*, but, as the carbon chain length increases, so too does the value for K_m, reaching 8.20 ± 0.06 m*M* for $C_{12:0}$.

Thus, the preferred selectivity of these enzymes for short-chain fatty acids is a combination of both en-

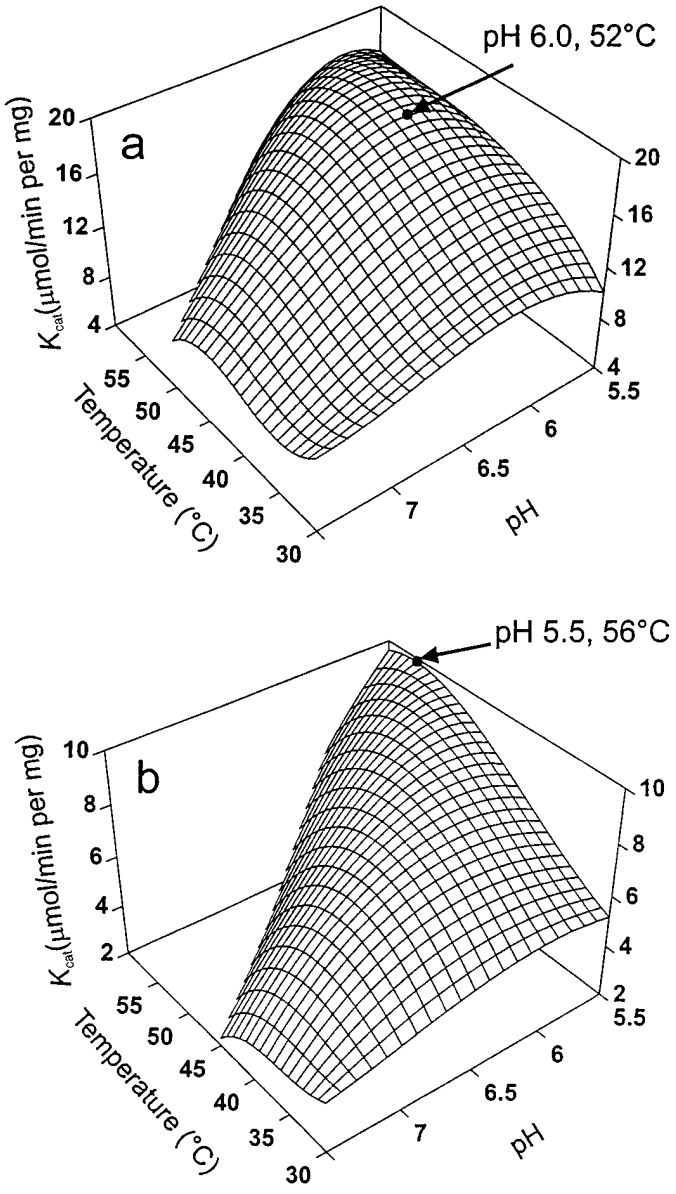

Figure 2. Surface plots for the kinetics of apparent turnover number (K_{cat}) against pH and temperature for the hydrolysis of tributyrylglycerol that was catalyzed by pregastric lipase of goat (a) and kid (b).

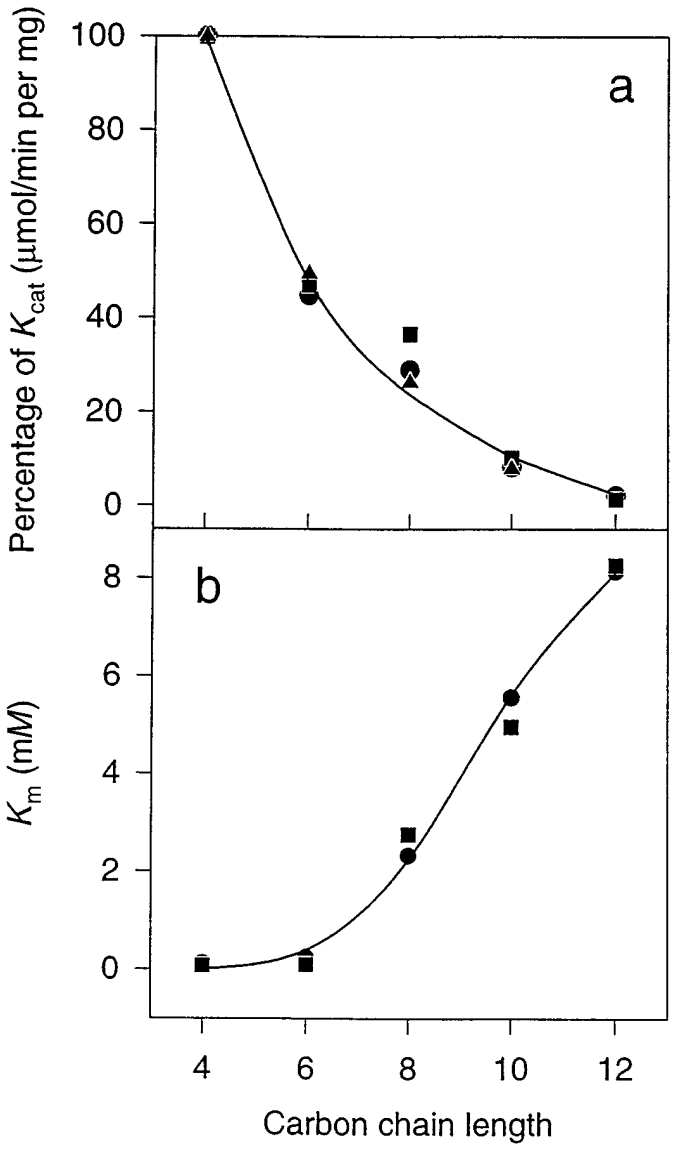

Figure 3. Activity of pregastric lipase of goats (●) and kids (■) toward monoacid triglycerides at 40°C and pH 6.5. Apparent turnover number (K_{cat}; a) and the relevant values of K_{cat} for lamb pregastric lipase (▲) were obtained at 37°C and pH 7.2 (22). Michaelis constant (K_m) values (b) are also given.

TABLE 1. Free fatty acid composition of anhydrous milk fat after hydrolysis catalyzed by commercially available goat and kid pregastric enzyme extracts, of commercially available Parmesan cheese, and of fully hydrolyzed anhydrous bovine milk fat.

Sample	Free fatty acid composition								
	$C_{4:0}$	$C_{6:0}$	$C_{8:0}$	$C_{10:0}$	$C_{12:0}$	$C_{14:0}$	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$
	(mol/100 mol)								
Goat[1]	40.0	15.3	4.9	8.3	5.5	6.8	10.9	3.5	4.9
Kid[1]	42.5	16.3	5.3	8.6	4.8	5.6	9.5	3.9	3.6
Parmesan[2] cheese	39.6	13.2	3.7	6.9	5.3	6.7	11.8	3.1	9.6
Milk fat[3]	13.6	5.1	2.3	4.6	4.5	12.4	30.2	9.7	17.6
sn-3 Fatty acids[4]	28.4	15.9	4.7	7.2	4.3	5.3	6.6	7.2	20.4

[1]The values for goat and kid pregastric enzyme catalyzed hydrolysis of anhydrous milk fat were interpolated at 17 mol/100 mol of free fatty acid from experimental results obtained for hydrolysis of fatty acids of goats (12.9 and 21.6) and kids (13.3 and 32.4) in moles/100 mol, respectively.

[2]17 mol/100 mol of free fatty acid.

[3]Total fatty acid composition of bovine milk fat. Random selectivity would result in these proportions of free fatty acids at any degree of hydrolysis.

[4]Fatty acid composition at the *sn*-3 position of bovine milk fat (25).

hanced K_{cat} and a preference for active site saturation. The absolute values for K_m are smaller than the corresponding values determined in a study using partially purified lamb pregastric lipase (22), but the two studies used a different emulsion system, and the temperature of assay was also different. Further comparison therefore is not justified.

Importantly, the detailed study of the kinetics study against TBG (Figures 1 and 2) and extension of this study into longer chain lipids (Figure 3) showed no difference other than specific activity in the practical behavior of the goat and kid lipases. Temperature optima (≥52°C) for hydrolysis of TBG, values of K_m over a range of lipid substrates, and rates of hydrolysis of these lipids relative to that of TBG were identical. The only small difference in behavior of the two extracts that we noted was that the pH optimum for activity against TBG was pH 6.0 for the goat extract and pH 5.5 for the kid extract.

Hydrolysis of Milk Fat

It should be noted that the similar reactivity of the goat and kid enzymes with the synthetic triglycerides may not necessarily indicate a similar reactivity with natural milk fat triglycerides. However, even when the substrate was much more complex (anhydrous milk fat), these two enzyme extracts continued to exhibit similar reactivity. We tested the extracts, as provided by the manufacturers, without further purification for ability to hydrolyze the anhydrous fat in bovine milk. The fat was emulsified with lecithin and sodium caseinate.

An emulsion of milk fat, lecithin, and casein may not necessarily be an exact mimic of fresh cream, which is the substrate for pregastric lipase in the manufacture of cheese. However, emulsions containing lecithin and casein are used as an industrial assay (8) to monitor lipase activity, and we have used this method as our standard to monitor the activity for a range of pregastric enzymes (1, 2, 21, 22, 23, 24, 25). Anhydrous milk fat is a suitable substrate to use with this emulsion and is free from contaminants such as bacterial lipases. Subsequently, we have extended this method for monitoring the activity against the lipids in fresh cream. However, the focus of this present investigation was a comparison of the activities of the lipases from goats and kids under identical experimental conditions, and anhydrous milk fat was a suitable substrate.

There is a substantial body of literature on the physicochemistry of milk fat emulsions formed with various coatings. The work of Dalgleish (9), Dalgleish and Sharma (10), and Dickinson (12, 13) has clarified the nature of competitive absorption of various materials such as caseins and lower molecular mass emulsifiers on milk fat during emulsification. The system is very complicated, and the nature of the emulsifiers used have an impact on the surface coating of the emulsified milk fat. This coating is likely to be different from the membrane that encloses an intact milk fat globule or the coating formed by the naturally occurring emulsifiers, including caseins, and peptides on milk fat from ruptured globules.

These factors, however, were not of primary importance to this investigation, which sought to highlight the similarities and differences between pregastric lipase from goats and kids and to compare quantitatively the fatty acid profiles of anhydrous milk fat hydrolyzed in the presence of these enzymes with a commercial sample of Parmesan cheese.

Results for hydrolysis of anhydrous bovine milk fat by the commercially available extracts of pregastric enzyme from the tongues of goat and kid are given in Table 1. These data were normalized to 17 mol/100 mol fatty acid hydrolysis to permit comparison with the data for composition of a commercially available sample of Parmesan cheese, which also showed the presence of 17 mol/100 mol free fatty acid. The proportion of free fatty acids in each sample may be related back to the data for total fatty acids that are available in anhydrous bovine milk fat (Table 1). Although palmitic acid ($C_{16:0}$) is the most abundant fatty acid available (30%), it constituted only 10.9 and 9.5% of the free fatty acid in the samples hydrolyzed by the extracts of goats and kids, respectively. By contrast, butyric acid, which constituted only 13.6% of the total available fatty acid, constitutes 40.0 and 42.5% of the free fatty acid product that was hydrolyzed by the goat and kid extracts, respectively. Pregastric lipase of both goats and kids clearly had marked selectivity for the hydrolysis of the short-chain acyl moieties of the lipids that were present in anhydrous milk; this selectivity decreased about 10-fold as the carbon chain length increased from to $C_{4:0}$ to $C_{18:0}$. The short-chain fatty acids are available in the highest proportions at the *sn*-3 position of bovine milk fat (Table 1). Pregastric lipases have been shown to hydrolyze triglycerides selectively at this position (1), but positional selectivity alone cannot account for the high concentrations of butyric acid that were observed with hydrolysis by the kid and goat pregastric lipases.

The data that were obtained for hydrolysis of the milk fat by goat and kid extracts were very similar; each pair of values generally fell within a range of <1% free fatty acid composition. This close similarity was observed at all levels of hydrolysis (data not given). This result is important because it indicates that goat extract could be used as a viable commercial alternative to kid extract to manufacture Italian-style cheeses. Previous studies (3, 4, 5) have compared the free fatty acid profiles resulting from the use of pregastric lipases from calves, kids, and lambs, but this study is the first record of a qualitative comparison between extracts from kids and extracts from goats.

The profiles of free fatty acid composition for both data files also closely resembled that for the commercial sample of Parmesan cheese, except for the datum point for $C_{18:1}$, which was twice as great in the cheese sample as it was in the samples of hydrolyzed milk fat. However, this comparison was qualitative at best because the source of the enzyme was not known for the cheese sample. Also, the milk fat emulsion and substrate concentration was not the same in the two systems. However, for all three hydrolyzed substrates, the amount of free butyric acid, which was responsible for the characteristic picante of Parmesan cheese, was almost invariant. A similar study (18), which measured the concentrations of volatile free fatty acids released from ruminant milk fats by calf, lamb, and kid pregastric lipases, showed that all three lipases exhibited selectivity for hydrolysis of volatile branched-chain and short n-chain fatty acids from each milk fat and for *sn*-1 and *sn*-3 positions on glycerides. Some of the differences in the ability of the lipases to release major n-chain fatty acids from various milk fats appeared to reside in the selectivities of pregastric lip-ases for certain glyceride structures. Ruiz-Sala et al. (26) recently published a comprehensive summary of the composition of fat from ovine, bovine, and caprine milks showing that ovine milk was richer in short- and medium-chain triglycerides, bovine milk fat was richer in long-chain and unsaturated triglycerides, and caprine milk fat was richest in polyunsaturated medium-chain triglycerides. Full-flavored, aged Par-mesan cheeses that had been produced using a rennet paste contained moderate amounts of major free fatty acids, which increased little during aging (29). However, the use of a commercial pregastric lipase resulted in a Parmesan cheese that lacked a balanced flavor and that contained higher amounts of short-chain free fatty acids (29).

Our earlier study (20) compared the percentage composition of free fatty acids of anhydrous milk fat hydrolyzed by pregastric extracts from calves, lambs, and goats; significant differences were noted. We concluded that the use of each of these extracts would produce Italian-style cheeses of different piquancy. However, the present results indicate that milk fat that was treated with goat extract under the same conditions as have been traditionally used for kid extract should produce a product with similar organoleptic attributes.

ACKNOWLEDGMENTS

The financial assistance of the University of Auckland Research Committee and New Zealand Lottery Science and the award of a doctoral scholarship from the New Zealand Agriculture and Marketing Research Development Trust are gratefully acknowledged. We are grateful to Paul Butler for technical assistance and to David Stevenson and Roger Stanley, Industrial Research Ltd. Lower Hutt, New Zealand for advice. The contribution of kid lipase by

Rhône-Poulenc Marschall Products, Madison, Wisconsin is gratefully acknowledged.

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