Chapter 10
Lipases from Milk and Other Sources

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10.1 Introduction

This chapter focusses on the lipases in bovine and caprine milk, and lipolysis caused by the main lipase, lipoprotein lipase (LPL). It also discusses the use of lipases for modifying milk fat for various applications.

Bovine milk lipase and its effects in milk were widely studied in the 1970s and 1980s (e.g. Castberg and Solberg 1974; IDF 1975, 1980, 1987), but relatively little has been published since then. There has been considerable interest in the biochemical aspects of LPL because of its similarity with other LPLs, including human LPL, and its ready availability from milk for lipid metabolic studies. These studies have been reviewed by Olivecrona and Olivecrona (1999) and Olivecrona et al. (2003).

Lipolysis caused by LPL has attracted, and continues to attract, considerable interest from the dairy industry because of its practical consequences for the quality of milk and milk products. It has been studied in detail in both bovine and caprine milk and extensively reviewed (IDF 1980, 1987; Chilliard et al. 1984, 2003, 2014; Deeth 2006; Deeth and Fitz-Gerald 2006; Ray et al. 2013).

Lipolysis caused by lipases involves the transfer of an acyl group to water with formation of a carboxylic acid. Under water-limiting conditions, lipases can cause other acyl transfer reactions, which include: interesterification, in which acyl groups are interchanged between an introduced ester and the original ester such, as a triglyceride (TG); acidolysis, in which an introduced carboxylic acid replaces one of the original esterified acids and a new free acid is formed; and alcoholysis, in which new esters are formed by transfer of an acyl group to an alcohol. The lipases involved in these biotechnological modifications of milk fat are mostly of microbial origin.

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Lipase-catalysed modifications now have several commercial applications (Bourlieu et al. 2009).

10.2 The Milk Enzymes

10.2.1 Lipoprotein Lipase

Lipoprotein lipase (LPL) has been established as the only lipase in bovine milk, and is present in the milk of virtually all mammals. However, in human milk it occurs with another carboxyl ester lipase, commonly known as bile-salt-stimulated lipase (Hui and Howles 2002), which is not present in bovine milk (Castberg and Solberg 1974). Milk LPL is synthesised in the mammary gland, where it hydrolyses circulating lipoproteins from the blood into free fatty acids and 2-monoglycerides (2-MGs). These products are absorbed by the mammary gland and used in the synthesis of milk glycerides, mainly TGs.

The presence of LPL in bovine milk was first reported by Quigley et al. (1958) and confirmed by Korn (1962). The former identified the enzyme in milk fat globule membrane (MFGM) material (which they called lipoprotein microsomes) from washed cream buttermilk but Korn (1962) found that, although some of the enzyme was associated with the MFGM, most was associated with the casein in the skim milk. It was not until the 1970s that it was established that LPL was responsible for virtually all of the lipase activity in normal mature bovine milk (Egelrud and Olivecrona 1973; Castberg and Solberg 1974; Castberg et al. 1975; Olivecrona et al. 1975). Before that, there had been several reports of two or more different lipases in milk, some of which had been separated by gel filtration (e.g., Downey and Andrews 1969). The early reports of these enzymes were reviewed by Schwartz and Parks (1974). Interestingly, these authors made only passing reference to LPL in the statement "*Milk also contains a lipoprotein lipase similar to that found in mammalian tissue and heart tissue (*Korn 1962*). The enzyme is highly specific for lipoproteins and probably is not involved in rancidity development to any great extent*". As discussed below, this has turned out to be untrue.

Bovine milk LPL has been extensively studied as it has been used as a model enzyme for studies on lipoprotein metabolism, where it is the major lipase involved (Olivecrona et al. 2003). In blood, it hydrolyses TGs in chylomicrons and very-low-density lipoproteins into free fatty acids and 2-MGs. These products are absorbed by tissues and used for energy production and, in the case of the mammary gland, for the synthesis of lipids, mainly TGs.

Milk LPL has some unique features; for example, its full activity is realised only when a specific lipoprotein activator is present. Hence, LPL is assayed against TGs in systems containing blood serum, serum lipoproteins or the specific activator, apolipoprotein C-II. It is believed that the activator helps to orient the enzyme favourably towards the substrate at the oil-water interface. However, LPL is active against

tributyrin in the absence of these activators, although in such cases the total activity is ≤50% of the activated LPL activity (Castberg et al. 1975).

In normal raw bovine milk, LPL is mostly associated with the casein fraction, with only a small proportion being soluble or attached to the MFGM (Deeth and Fitz-Gerald 1975; Anderson 1982). However, this does not apply in caprine milk, where a substantial proportion of the LPL is normally associated with the MFGM. Chilliard et al. (1984) reported that the percentage distribution of the enzyme between the cream, serum and caseins in caprine milk was 46, 46 and 8, respectively, while the corresponding figures for bovine milk was 6, 17 and 78 respectively. Those authors suggested that the difference may relate to different LPL secretion processes in the mammary gland, differences in casein and fat globule composition, structure or physicochemical properties, or the presence of an LPL inhibitor in the casein fraction of caprine milk.

The association with the casein micelles is mostly electrostatic, as most of it can be released by 0.75-1 M NaCl into a soluble form. The electrostatic binding is through positive charges on the enzyme to negative charges on the caseins such as κ-casein. An important feature of LPL is that it binds strongly to heparin, through what is also an electrostatic interaction as heparin is negatively charged. Thus, heparin, at ~5 μg/mL, can also be used to release LPL from casein micelles (Hoynes and Downey 1973); in fact, the method of choice for isolating LPL from milk is by affinity chromatography on immobilised heparin (Egelrud and Olivecrona 1972; Castberg et al. 1975; Kinnunen et al. 1976). Hydrophobic bonding may also be involved in the attachment of lipase to casein micelles, as the enzyme can be released by dimethylformamide.

LPL exists predominantly as a dimer of two identical monomers. According to Olivecrona et al. (1982), the molecular weight of each monomer is 41.7 kDa, of which 8% is carbohydrate. However, Senda et al. (1987) calculated the molecular weight of the unglycosylated form from the cDNA coding for it to be 50.548 kDa, and Hayashi et al. (1986) reported a molecular weight of 56 kDa as determined by SDS-PAGE. The LPL molecule has a heparin binding site in the groove between the two monomers and a surface loop known as a lid which shields the hydrophobic active site from a hydrophilic environment (van Tilbeurgh et al. 1994). The lid opens to expose the active site when the lipase adsorbs to a lipid-water interface.

The enzyme has a reactive serine in an active-site peptide which is located in a β-turn, a structure similar to that at the active sites on other serine hydrolases (Reddy et al. 1986). In addition to the active site and the heparin-binding region, LPL also has an apo-lipoprotein interaction region and a lipid-binding or interface-recognition site. The apo-lipoprotein interacting region is independent of the heparin-binding region and the active site (Matsuoka et al. 1980; Olivecrona et al. 2003).

LPL is relatively unstable to heat as well as UV light, acid and oxidising agents. In milk, it is largely inactivated by normal pasteurisation conditions, involving heating at 72 °C for 15 s (Farkye et al. 1995). This means that adequately pasteurised milk and products made therefrom do not contain active lipase. As discussed below, if this was not the case, most (homogenised) pasteurised milk would develop a rancid flavour. However, some workers have maintained that more severe heating

conditions such as 79 °C for 20 s (Shipe and Senyk 1981) or 85 °C for 10 s (Driessen 1987) are required to completely inactivate LPL in milk.

LPL has an optimum pH of approximately 8.0 under ideal (activated) conditions; however, in the absence of activating cofactors, the optimum is 8.8–9.0. LPL is inactivated by acid conditions such as at pH 4.6 (Driessen 1976), and thus it is highly unlikely that it is involved in digestion of fat by the newborn, as it would be inactivated by the acidic gastric juice in the stomach. This has led researchers to suggest its presence in milk is due to spill-over from the mammary gland and has no useful function in milk.

TGs are the normal substrates for LPL. If TGs contain long-chain fatty acids, lipoproteins or apolipoproteins, especially apo-LP CII, are required to activate the enzyme (Östlund-Lindqvist and Iverius 1975). Blood serum, either bovine or human, supplies such activating factors and is used in assays of LPL using emulsified long-chain TG substrates (Egelrud and Olivecrona 1973). LPL is usually assayed in the presence of bovine serum albumin, which absorbs the released free, fatty acids and reduces product inhibition. One reason why FFAs cause inhibition is that they block the effect of activator lipoproteins (Bengtsson and Olivecrona 1980; Olivecrona et al. 2003). LPL can also be assayed against tributyrin; in this case, neither serum cofactors nor fatty acid acceptors are required (Rapp and Olivecrona 1978).

Normal unprocessed milk contains an abundance both of LPL and the ideal TG substrate. At the maximum activity in milk, of around 5 μmoles of FFA/min/mL (Castberg et al. 1975), milk would develop a very high level of FFAs in a very short time. This, however, does not happen because the LPL is physically separated from the TG substrate in the fat globules by the MFGM which it cannot penetrate; in this respect, LPL differs from bacterial lipases and pancreatic lipase which can hydrolyse the fat in intact fat globules. This difference was neatly demonstrated by Danthine and Blecker (2014) with MFGM monolayers using a Langmuir film balance. Pancreatic and bacterial lipase penetrated the monolayer but LPL did not. If blood serum, which contains activating cofactors, is added to milk, the interaction between the enzyme and the TG substrate is facilitated and lipolysis ensues (Castberg and Solberg 1974; Jellema and Schipper 1975). This serum-mediated induction of lipolysis is believed to be largely due to enhancement of binding of the LPL to the fat globule by the activating cofactors (Bengtsson and Olivecrona 1982).

In hydrolysis of mixed TGs, such as occur in milk fat, LPL does not show fatty acid specificity. However, it does have strong positional specificity, hydrolysing the fatty acids in the primary positions, *sn*-1 and *sn*-3, of TGs (Morley and Kuksis 1977). Because short-chain fatty acids such as butyric acid are mostly positioned in the primary positions, it has sometimes been interpreted that LPL preferentially releases short-chain fatty acids. When LPL hydrolyses a fatty acid from a primary position, *sn*-1,2- and *sn*-2,3-diglycerides (DGs) are formed. These are then hydrolysed to 2-MGs (see Fig. 10.1). These can only be hydrolysed to a free fatty acid and glycerol if they are converted to the *sn*-1- or *sn*-3-MGs (Nilsson-Ehle et al. 1973).

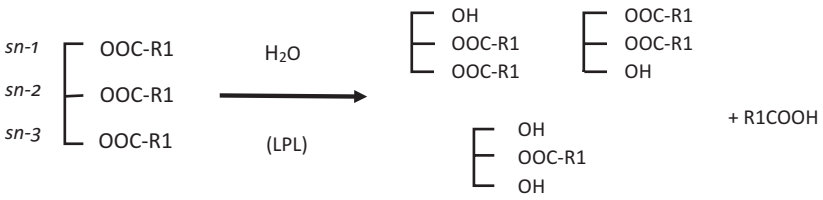

Fig. 10.1 Hydrolysis (lipolysis) of TG catalysed by LPL

LPL also hydrolyses phospholipids such as phosphatidyl choline. In this case, it hydrolyses only the primary ester bond at the *sn*-1 position to produce a 2-lysophospholipid, i.e., LPL has phospholipase A_1 activity. Interestingly, most phospholipases, such as those in snake venom, exhibit A_2 activity.

10.2.2 Somatic Cell Lipase

Chandan and Shahani (1963a, b) reported the purification and characterization of what they called "milk lipase". The enzyme, which was isolated from clarifier sediment, had a molecular weight of ~7000, determined by sedimentation-diffusion and osmotic pressure methods (Chandan et al. 1963). They considered that it was not from somatic cells as they (mistakenly) believed that leucocytes did not possess lipase activity. Gaffney and Harper (1965) isolated somatic cells from separator slime and showed that they contained a lipase which was different from the majority of the lipase in skim milk. They also showed that it was an intracellular lipase as the activity expressed was much greater after the cells had been disrupted by a ball mill. The specific lipase activity (per mg protein) against 3% homogenised milk fat at pH 8.6 was around four times greater than that of skim milk. The lipase was also active against tributyrin and dibutylfluorescein. When the somatic cell extract and skim milk were run on polyacrylamide gel electrophoresis, the band of the cell extract containing lipase activity (against dibutylfluorescein) did not match any of the four lipase-active bands in skim milk.

Azzara and Dimick (1985) isolated somatic cells from pooled raw milk with somatic cell count (SCC) of $<5 \times 10^5$/mL. The cells consisted of macrophages, polymorphonuclear leucocytes and lymphocytes. Of these, only macrophages possessed lipase activity. The total activity in the milk from the macrophages was calculated to be only about 0.1% of the total activity in skim milk. Curiously, when a concentrate of macrophages was added to milk, the total milk lipase activity after 48 h was 11.6% higher than in control milk to which cells had not been added.

The assay system used by Azzara and Dimick (1985) was one for lipoprotein lipase but the authors did not claim that the lipolytic activity present was LPL; they

commented that further investigations were required to determine if it was an LPL. However, they pointed out that LPL activity had been reported in other macrophages. Since that time, there have been many reports, particularly in the medical field, indicating that LPL is a major secretory product of macrophages (e.g., Sartippour et al. 1998; Takahashi et al. 2013). Further research on milk macrophage lipase does not seem to have been published; a review of the role of somatic cells in dairy processes and products by Li et al. (2014) only lists the lipase activity reported by Azzara and Dimick (1985).

10.2.3 Colostral Lipase

Colostrum has been shown to contain a lipase which differs from lipoprotein lipase (Driessen 1976). Its activity decreases during the first four milkings, whereas LPL increases from virtually zero in the same time period. It is present in the milk serum and is stable at pH 4.6; LPL is mostly associated with casein micelles and is unstable at pH 4.6, and so clearly the colostral lipase differs from LPL.

10.2.4 Milk Esterases

There have been several reports of esterases in bovine milk. However, little detailed information on them is available. By definition, esterases differ from lipases in that they act on ester substrates in solution rather than emulsified lipid substrates (Desneulle 1972; Jaeger et al. 1994) or show a preference for esters of short-chain acids over esters of long-chain acids (Okuda and Fujii 1968).

Aryl- or A-esterase (EC 3.1.1.2) (Forster et al. 1961; Kitchen 1971), carboxyl- or B-esterase (EC 3.1.1.1) (Jensen et al. 1961; Montgomery and Forster 1961; Deeth 1978) and cholin- or C-esterase (EC 3.1.1.7; EC 3.1.1.8) (Forster et al. 1961; Kitchen 1971) have been reported. Arylesterase activity has attracted interest because it is elevated in mastitic milk; it is also present in colostrum (Forster et al. 1959; Marquardt and Forster 1965). Its activity is well correlated with other indices of mastitis (Luedecke 1964) and has been suggested as a sensitive indicator of the disease (Forster et al. 1961).

Of particular interest in this chapter is carboxylesterase (B-esterase) activity. Its presence in milk was reported (Jensen et al. 1961; Montgomery and Forster 1961) before it was established that LPL was the only lipase in normal mature milk and was responsible for lipolysis in raw milk (Castberg and Solberg 1974). It was identified as a carboxylesterase because it hydrolysed tributyrin; it appears that the enzyme concerned was LPL.

It has now been shown that a carboxylesterase which differs from LPL exists in milk. It was identified as a carboxylesterase through inhibitor studies where it was not affected by inhibitors of arylesterase (EDTA, La^{3+}) or of cholinesterase (eserine

sulfate) but was almost completely inhibited by diethyl-*p*-nitrophenyl phosphate (an inhibitor of carboxylesterase). Its activity is low in normal mature milk but is elevated is colostrum and mastitic milk. In mastitic milk, it is well correlated with other indices of mastitis (Deeth 1978; Fitz-Gerald et al. 1981), and is present in both the milk serum and the cellular fractions of mastitic milk but not in the casein fraction (where most of the LPL resides). It has been assayed using 4-methylumbelliferyl heptanoate, a substrate against which LPL is not active (Deeth 1978). Its activity in non-mastitic milk was found to be around 0.004 μmoles of FFA/min/mL of milk while, in mastitic milk (SCC > 2×10^6/mL) and colostrum, it was up to 0.15 and 0.04 μmoles of FFA/min/mL respectively. Given that the LPL activity is around 5 μmoles of FFA/min/mL (Castberg et al. 1975), the carboxylesterase activity represents a small proportion of the carboxyl ester hydrolase activity in bovine milk, even in abnormal milks.

It seems highly likely that the carboxylesterase activity in mastitic milk corresponds to the reported lipase(s) of somatic cells (Gaffney and Harper 1965; Azzara and Dimick 1985) and colostrum (Driessen 1976). Furthermore, human milk shows very high carboxylesterase activity against 4-methylumbelliferyl heptanoate (~18 μmoles of FFA/min/mL, Deeth 1978) which is presumably due to the bile-salt-stimulated lipase (Hernell and Olivecrona 1974) which has been shown to be identical to the non-colipase-dependent pancreatic carboxylesterase/lipase (Falt et al. 2001) and cholesterol esterase (Hui and Howles 2002).

10.3 Lipolysis Due to Milk Lipase in Milk

Lipolysis by milk lipase occurs in milk before heat treatments equivalent to HTST pasteurisation. It is traditionally divided into two categories, spontaneous and induced lipolysis, as their mechanisms of action are quite different. The former is mainly related to factors associated with the cow, e.g., stage of lactation, nutritional status, while the latter is related to milk handling.

The main reason for the interest in lipolysis in milk is the fact that the free fatty acids formed, particularly the short-chain acids such as C4 (butyric) and C6 (caproic), impart an unpleasant flavour to milk. A second reason for the interest is that the lipolysis products, particularly the partial glycerides, are surface-active and reduce the foaming capacity of milk, an important consideration for making cappuccino coffee, for example.

The FFA level of milk as it leaves the cow is of the order of 0.5 mmol/L. When this level increases to around 1.5 mmol/L, many consumers detect an unpleasant taste variously described as rancid, butyric, chemical or bitter. As the FFA level increases further, more consumers will find the milk unacceptable. It should be noted that the literature varies in reports on the FFA levels deemed to make milk unacceptable. This is attributable to the different methodologies used for both analysis of the FFAs and the sensory evaluation methodology used.

Similarly, the foaming capacity declines with increasing FFA level. At around 1.5 m mol/L, the foaming capacity is usually impaired and, at 2.0 m mol/L, most milk shows little foaming. As for the sensory perception of lipolyzed milk, the reported FFA levels for impaired foaming also vary.

10.3.1 Spontaneous Lipolysis

Under normal circumstances, the milk fat globule membrane in bovine milk acts an effective barrier between the LPL in the milk serum and its TG substrate contained within the milk fat globule. In this case, when milk is extracted from the cow and cooled, no lipolysis ensues, that is, the FFA level remains the same as when the milk first leaves the cow. If the milk fat was not protected in this manner, the LPL in the milk serum would rapidly act on the fat and cause extensive lipolysis. Under ideal conditions for the enzyme action, it can be calculated that milk would become rancid within a few minutes (Olivecrona et al. 2003).

While "normal" milks do not lipolyse after leaving the cow, there are some milks in which lipolysis proceeds after they are cooled to <~10 °C; this is referred to as spontaneous lipolysis. For the sake of brevity, milks which undergo spontaneous lipolysis will be referred to as "spontaneous" milks in this chapter. There has been conjecture about the difference between non-spontaneous and spontaneous milks and the factors which influence the extent of spontaneous lipolysis. A summary of extrinsic and intrinsic factors with an assessment of their significances is given in Table 10.1. These factors are discussed below.

The situation in a herd is that the milk of some cows will always be non-spontaneous, milk of some will always be spontaneous, and the milk of others will be spontaneous under certain conditions. These conditions include late lactation and poor-quality feed. The extent of lipolysis in spontaneous milk can vary from <1 mmol/L up to 5 mmol/L or more.

Table 10.1 Factors influencing spontaneous lipolysis in raw milk

Factor	Significance
Extrinsic	
Late lactation	Medium
Poor quality feed	Medium
Individuality (genetics?)	High
Breed	Low
Mastitic infection	Medium
Intrinsic	
LPL activity	Very low
MFGM integrity	Medium
Activator-inhibitor balance	High
MFGM-associated LPL	High

A priori, the LPL activity in the milk and/or the integrity of the MFGM could be expected to account for much of the difference. Because there is ample lipase activity in all raw milk to cause extensive lipolysis under the right conditions, differences in lipase activity cannot account for the difference between the milks. Furthermore, through the use of mixing experiments where creams from spontaneous milks are mixed with skim milk from non-spontaneous milk, the nature of the MFGM does not appear to be a major factor in most cases.

What has been shown to be a major difference between non-spontaneous and spontaneous milks is the balance between inhibiting and activating factors in milks. This is indicated in the results shown in Table 10.2 of an experiment in which a non-spontaneous milk was mixed with spontaneous milks. It has long been recognised that, when non-spontaneous milk is mixed with spontaneous milk soon after milking and before cooling, the FFA level formed during low-temperature storage is less than the expected average of the FFA levels of the two milks when kept separate. In other words, the non-spontaneous milk has an inhibitory effect on the lipolysis in the spontaneous milk. This inhibition is shown in the first data row in Table 10.2. However, it can also be seen from Table 10.2 that mixing a very spontaneous milk (FFA of 4.8 mmol/L) with two other spontaneous milks (FFAs of 3.1 and 3.15 mmol/L) resulted in little change in one and a substantial (35%) activation in the other. It is generally accepted that this inhibitor-activator balance is most significant and that the nature of the MFGM is also significant in some cases (Deeth and Fitz-Gerald 1975; Sundheim and Bengtsson-Olivecrona 1987c; Cartier and Chilliard 1990).

The major activators of LPL are lipoproteins and apolipoprotein C-II, so it is logical to expect such factors to be present in spontaneous milks. In fact, milk does contain material which is immunologically cross-reactive with bovine serum lipoproteins (Anderson 1979). In support of the supposition that such lipoproteins are the activating factors is the fact that adding blood serum lipoproteins to milk initiates lipolysis. In doing so, they facilitate the movement of the LPL from the skim phase to the MFGM (Bachman and Wilcox 1990); the same occurs in spontaneous lipolysis (Sundheim and Bengtsson-Olivecrona 1985). The extent of spontaneous lipolysis correlates positively with the amount of LPL attaching to the MFGM (Ahrné and Björck 1985; Sundheim and Bengtsson-Olivecrona 1985).

Table 10.2 Inhibition and activation of spontaneous lipolysis in mixed individual bovine milk samples (data based on Deeth and Fitz-Gerald 1975)

Added milk	FFA[a]	FFA[a] of mixed milk (1:1 with Spontaneous milk 3, FFA = 4.8)		
		Observed FFA	Calculated FFA	% inhibition
Non-spontaneous	0.5	0.9	2.65	81
Spontaneous 1	3.15	4.1	4.2	2
Spontaneous 2	3.1	5.15	4.1	−35
Spontaneous 3	4.8			

[a]All FFA values were determined after overnight storage of milks at 5 °C

The attachment of LPL to the MFGM in spontaneous milk does not occur or is reduced if: the milk is not cooled; cooling is delayed; or NaCl or other inhibiting factors are added. However, it is not reduced if the milk is rewarmed after being cooled (Sundheim and Bengtsson-Olivecrona 1987a).

An effective inhibitor of spontaneous lipolysis is proteose-peptone fraction 3 (Anderson 1981; Cartier et al. 1990) which is antigenically similar to a soluble glycoprotein in the MFGM which may also act as an inhibitor (Shimizu et al. 1982). Vanbergue et al. (2018) have recently suggested that proteose-peptone fraction 5 may be an inhibitor of spontaneous lipolysis. They found a negative correlation between the level of spontaneous lipolysis and the concentration of proteose-peptone fraction 5 which appeared to be related to forage type. In addition, a caprine milk proteose peptone fraction inhibits spontaneous lipolysis in caprine milk (Chilliard et al. 1984). Interestingly, Danthine and Blecker (2014) reported that proteose peptone strengthened MFGM monolayers against penetration by both LPL and pancreatic lipase, suggesting that the mechanism of inhibition of spontaneous lipolysis may be due to strengthening of the MFGM.

The MFGM contains both activating lipoproteins (Castberg and Solberg 1974) and inhibiting proteins (Shimizu et al. 1982; Sundheim and Bengtsson-Olivecrona 1987b). In addition, LPL attaches to the MFGM of both non-spontaneous and spontaneous milk when milk fat globules are cooled in the absence of skim milk, which suggests the membrane changes its conformation to facilitate attachment of LPL, if not prevented by inhibitors (Sundheim and Bengtsson-Olivecrona 1987d). The MFGM in the milk of some cows becomes more permeable in late lactation, which may partially explain the increased susceptibility of late-lactation milk to spontaneous lipolysis.

A possible role of genetics in the susceptibility of the milk of some cows to spontaneous lipolysis has been indicated in studies on both cows and goats. Vanbergue et al. (2016) investigated the relationship between diacylglycerol acyltransferase 1 (DGAT1), an enzyme linked to milk fat synthesis, and spontaneous lipolysis through identifying cows of three different DGAT1 genotypes, KK, KA and AA. Significantly higher levels of spontaneous lipolysis were observed in the milk of the KK genotype than in the milk of either the KA or AA genotype.

In goats, the α_{S1}-casein (CSN1S1) gene polymorphism has been shown to be related to spontaneous lipolysis. More spontaneous lipolysis was observed in the milk of goats of the CSN1S1–FF genotype (sometimes referred to as the low genotype as it is associated with production of a low concentration of α_{S1}-casein in milk) than in milk from goats of the (high) AA genotype (Chilliard et al. 2003, 2013, 2014).

Breed of cow may also be a factor in spontaneous lipolysis in bovine milk. Vanbergue et al. (2017) reported that milk from Holstein cows was more susceptible than milk from Normande cows, while Bachman et al. (1988) found the milk of Jerseys to be more susceptible than that of Holsteins. However, other authors have reported no differences between breeds in relation to spontaneous lipolysis (Chilliard 1982); Chazal and Chilliard (1987) found no differences between the milk of Friesian and Montbéliarde cows.

Several authors have reported a relationship between feed and spontaneous lipolysis (reviewed by Jellema 1980). The influence of feed involves the availability, type and nutritive value. For example, Stobbs et al. (1973) found that adding 6 kg/d of a balanced concentrate to the diet of late-lactation cows fed a low quality tropical pasture significantly reduced spontaneous lipolysis in the milk of those cows (average FFA decreased from 2.09 to 1.47 mmol/L). Vanbergue et al. (2018) concluded that the higher level of spontaneous lipolysis associated with feed restriction is probably due to higher tissue lipid mobilization.

There have been several reports on the effect of mastitic infection on lipolysis in milk. Most reports show that the level of free fatty acids in raw milk increases with somatic cell count (SCC) (Gargouri et al. 2008). Mastitic milk as it leaves the udder usually has a higher FFA level than non-spontaneous milk, and this level increases during storage (Gargouri et al. 2013). However, Jurczak and Sciubisz (1981) reported that milks with different SCCs analysed immediately after milking had similar FFA levels to non-mastitic milks. Gargouri et al. (2008) analysed the bulk milk from 80 farm supplies to investigate the relationship between the total SCC, counts of different types of somatic cells (macrophages, lymphocytes and polymorphonuclear leukocytes (PMN)) and FFA level. They found a significant positive correlation between the total SCC, the PMN counts and FFA level in milk, and concluded that the PMN leucocytes could be involved in the lipolysis of mastitic milk. This suggests that the lipase in the cells, as discussed in Sect. 2.2, may play a role in elevating the FFA in mastitic milk. This is consistent with the conclusion of Jurczak and Sciubisz (1981) that lipolysis of mastitic milk was due to both spontaneous lipolysis involving LPL and lipolysis due to the activity of lipases of somatic cells, with the former being the most significant. This is despite the fact that LPL levels generally decrease with increasing somatic cell count, largely due to to impaired biosynthetic capability of the mammary tissues caused by the infection (Fitz-Gerald et al. 1981; Murphy et al. 1989). However, this decrease would not be expected to affect the level of lipolysis, as the residual LPL activity would be more than sufficient to cause considerable lipolysis.

Caprine milk differs from bovine milk in that its total LPL activity is lower. Furthermore, for spontaneous lipolysis in caprine milk, there is a reasonable correlation between LPL activity and level of spontaneous lipolysis, whereas, in bovine milk, the total LPL has little bearing on the level of spontaneous lipolysis. This may relate to the fact that a much larger proportion of the LPL is naturally associated with the MFGM in caprine milk than in bovine milk (Chilliard et al. 2003).

10.3.2 Induced Lipolysis

The major form of induced lipolysis involves the physical disruption of the MFGM allowing access of LPL to its substrate in the damaged fat globule. This type of lipolysis is equivalent to the action of the lipase on an emulsified TG substrate and does not require activating factors.

Table 10.3 Processes which cause induced lipolysis in raw milk

Location	Process
Farm	Air leaks at teat cup cluster
	Milk surging in vertical risers
	Temperature activation
	High milking frequency
	Milk transport with foaming
Factory	Homogenisation
	Mixing homogenised milk with raw milk
	Centrifugal pumping, especially with air intake
	Pumping milk over long distances, especially with sharp pipe bends
	Gas agitation of silos
	Membrane processing (UF, RO)
Laboratory	Mechanical agitation (e.g., with blenders such as Silverson and Sorvall Omni Mixer)
	Gas bubbling
	Mixing homogenised milk with raw milk

The physical disruption to induce lipolysis can take different forms. Table 10.3 summarises ways in which lipolysis can be induced in the laboratory, on the farm and in the factory. In the laboratory, agitation in a blender is an effective way of producing the effect. The temperature of the milk treated in this way has a major effect on the amount of lipolysis induced. In general, the greatest level lipolysis is induced at 37–40 °C and the least at <~5 °C. However, under certain conditions, the amount of induced lipolysis shows maxima at 12–15 °C and 30–40 °C, with a minimum at 20 °C. Furthermore, there appears to be a threshold mechanical stress intensity at each temperature above which lipolysis can be induced (Deeth and Fitz-Gerald 1977; Hisserich and Reuter 1984).

One effect of agitation is an increase in lipase activity associated with the fat portion of milk. As a result, cream separated from milk agitated at 5–10 °C or 37 °C has a several-fold higher lipase activity than cream from milk which has not been agitated (Deeth and Fitz-Gerald 1977). The lipase in the cream from the agitated milk appears to have enhanced heat stability due to protection afforded by the fat.

In industrial processing, the most effective means of inducing lipolysis is homogenisation. Homogenisation of raw milk causes rapid lipolysis, particularly at the warm temperature commonly used in commercial processing. Because of this, homogenisation is usually carried out either immediately before or immediately after pasteurisation (in which LPL is inactivated).

A related issue is the mixing of homogenised (pasteurised) milk with raw milk. In this case, rapid lipolysis occurs because the active lipase in the raw milk can act on the fine TG emulsion in the homogenised milk. This issue is of constant concern to the dairy industry, as personnel unaware of the consequences can add homogenised milk to raw milk in the misguided belief that it will prevent wastage of left-over homogenised milk.

Perhaps the most common cause of induced lipolysis in the dairy industry is through the intensive mechanical handling of raw milk, particularly if accompanied by incorporation of air with formation of foam. This can occur in milking machines at the farm and during pumping of milk where air is drawn into the milk flow through damaged or worn seals. The shear forces at the air-liquid interface disrupt the membranes of the fat globules adhering to the interface and expose the fat to the lipase.

Other industrial processes which have been implicated in inducing lipolysis in raw milk are pumping milk over long distances, especially if the pipe includes sharp bends, tanker transport, especially when the milk has a large headspace and foam is generated, and during some membrane processing where rapid changes of pressure occur (Barbano et al. 1983). It has been noted that lipolysis has increased of an issue for the dairy industry since the introduction of mechanical means of handling raw milk. A recent detailed study of on-farm factors which may affect lipolysis, as measured by FFA levels, in bulk milk supports this contention (Wiking et al. 2019); that study showed that an automated milking system contributed significantly more to FFA levels than other milking systems. Furthermore, the high milking frequency of the automated system was a major factor contributing to higher FFA levels.

Another form of induced lipolysis is what is termed "temperature activation". This involves cooling raw milk or cream to ≤5 °C, warming to 25–35 °C, and re-cooling to <10 °C. Lipolysis occurs during subsequent low temperature storage. In this way, this form of induced lipolysis resembles spontaneous lipolysis; in fact, spontaneous milks are more likely to be affected. The susceptibility of a milk to temperature-activated lipolysis is related to the MFGM which is essential for the phenomenon. The temperature manipulation causes a conformational change of the membrane which allows the lipase to attach to the membrane and gain access to the fat. The lipase–MFGM attachment is reversed on rewarming; this reversal of attachment does not occur in spontaneous or agitation-induced lipolysis (Claypool 1965; Cartier and Chillard 1989; Kon and Saito 1997).

10.4 Lipase-Catalysed Acyl-Transfer Reactions on Milk Fat

Acyl-transfer reactions occur in organic solvents containing just enough water to maintain the activity of the enzyme but minimising the hydrolysis reaction. The most common enzymic transfer reactions for modifying milk fat discussed in recent publications are, in decreasing order of number of publications, interesterification, acidolysis and alcoholysis (particularly glycerolysis) (Bourlieu et al. 2009).

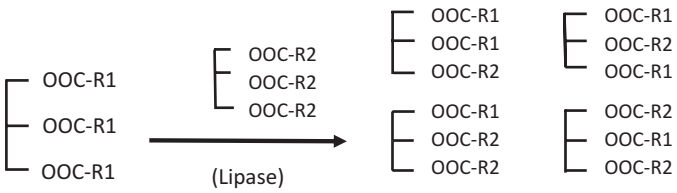

Fig. 10.2 Interesterification of two TGs catalysed by a non-sterospecific lipase

10.4.1 Interesterification

There are basically two forms of interesterification of milk fat, one in which milk fat is the only fat reacted with the lipase and another where TGs which differ from those in milk fat are incorporated (see Fig. 10.2 for generic interesterification reaction). If carried out with just milk fat, a modified milk fat is the major product, that is, a mixture of TGs with the same overall fatty acid composition as the original milk fat but with the fatty acids in different relative positions. The changes in TG structure are determined by the positional specificity of the lipase used. Therefore, to change the fatty acids at the *sn*-1 and *sn*-3 positions, a lipase which specifically attacks these positions, such as *Rhizomucor miehei* lipase, is used. If the lipase used does not have any positional specificity, a random exchange of all fatty acids present will result. If milk fat alone is reacted with a lipase, the product has different melting characteristics from the original fat. For example, interesterification of milk fat with the *sn*-1,3-specific lipase of *Rhizomucor miehei* decreased the proportion of TG molecules containing 34 to 42 carbon atoms and increased the content of those containing 44 to 52 carbons; it also resulted in an increase in hardness at 5 °C (Rousseau et al. 1996a).

The lack of solid content in milk fat at high temperatures limits its application in bakery and confectionery. One way of overcoming this limitation is to interesterify milk fat with another fat such as palm oil stearin. Nor Hayati et al. (2000) interesterified binary mixtures of milk fat and soft and hard palm oil fractions using Lipozyme IM (*Mucor miehe*i lipase) and found the interesterified blend had a higher maximum peak temperature (Tp) than the untreated mixture. Furthermore, the interesterified blends of milk fat and 25–75% soft palm stearin or milk fat and 25% hard palm stearin had similar melting behaviour to that of high melting fraction milk fat. Thus, such blends have commercial potential in pastry and confectionery.

A limitation of milk fat from a nutritional perspective is its low percentage of unsaturated fatty acids. Again, this limitation can be solved by interesterification of milk fat with unsaturated oils such as canola (Rousseau et al. 1996b) and linseed (flaxseed) (Aguedo et al. 2008: Shin et al. 2010). Another nutritional benefit

to be gained by lipase-catalysed interesterification is in producing a low-*trans* fat suitable for spreads (Shin et al. 2010). Unlike chemical reactions such as hydrogenation, enzymic interesterification does not cause the production of *trans* double bonds because of the milder conditions used (Aguedo et al. 2008). For example, De et al. (2007) performed chemical inter-esterification at 180 °C for 4 h while inter-esterification using a *Mucor miehei* lipase was performed at 60 °C for 4 h.

10.4.2 Acidolysis

Another acyl-transfer approach to altering the fatty acid composition of milk fat for nutritional and functional properties is acidolysis in which a lipase is used to incorporate a fatty acid or fatty acids (see Fig. 10.3) This was used by Balcão and colleagues (Balcão and Malcata 1997, 1998a, 1998b; Balcão et al. 1998) to incorporate oleic acid into milk fat using lipases from *Mucor circinelloides* and *M. javanicus.* The acidolysis reaction increased the oleic acid content of the milk fat by 27–30% and reduced the contents of lauric and myristic acids by 8% and 2–6%, respectively. The milk fat modified by the *Mucor circinelloides* lipase contained 83% less high-melting fraction and 19% more low-melting fraction than the original milk fat.

Improved spreadability was the aim of Kim et al. (2002) who used *Rhizopus arrhizus* lipase to incorporate α-linolenic acid from perilla oil into milk fat. Their modified milk fat, which contained ~24% (w/w) α-linolenic acid, had better spreadability than the original milk fat as measured by a texture analyser after the fats had been held at 4 °C for 24 h.

In addition to increasing the content of oleic acid, in line with the current nutritional advice of increasing monounsaturated fatty acids in the diet, there has also been research on incorporating fatty acids with particular therapeutic properties, such as conjugated linoleic acid (CLA). Using *Candida antarctica* lipase in a solvent-free system at 50 °C, Garcia et al. (2000) successfully incorporated CLA into milk fat. Sehanputri and Hill (2003) achieved the same result using the same enzyme in a packed bed reactor.

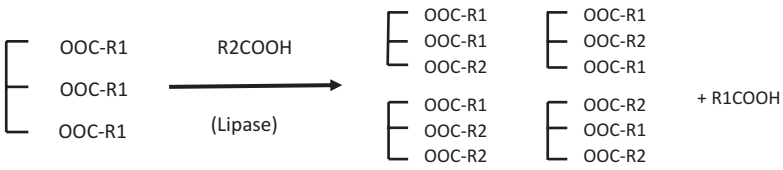

Fig. 10.3 Acidolysis of TG catalysed by a non-sterospecific lipase

The holy grail of lipase-catalysed acyl-transfer reactions on (bovine) milk fat is to modify its structure to resemble that of human milk fat for use in infant formula. To produce human milk fat substitute (HMFS), the 2 position of the TG needs to be modified to contain mostly saturated fatty acids such as palmitic acid, and the *sn*-1 and *sn*-3 positions need to be modified to contain mostly unsaturated fatty acids. This is not easy using bovine milk fat as the starting material and, for this reason, it is more common to interesterify unsaturated marine or vegetable oils with fats containing a high percentage of palmitic acid in the *sn*-2 position, even tripalmitin, to produce HMFSs (Karabulut et al. 2007; Soumanou et al. 2013). Two approaches have been used to produce HMFSs from bovine milk fat. The first is to use an *sn*-1,3-regiospecific lipase (e.g., from *Rhizomucor miehei*) to produce a fat enriched in unsaturated fatty acids in the *sn*-1 and *sn*-3 positions, and then fractionate the interesterified fat and retain a solid fraction as a HMFS. Using this approach, Sorensen et al. (2010) obtained a fat which contained 56% palmitic acid in the *sn*-2 position; human milk fat contains ~72%. Alternatively, a solid milk fat fraction can be interesterified with unsaturated oils using the same enzyme (Sorensen et al. 2010).

10.4.3 Alcoholysis

Alcoholysis of milk fat involves the transfer of an acyl group to an alcohol with the formation of an ester (see Fig. 10.4). A commercial example is glycerolysis, in which the lipase-catalysed reaction forms diglycerides (DGs) and monoglycerides (MGs) from milk fat in the presence of glycerol. These compounds are used widely as emulsifiers in the food industry (Balcão and Malcata 2002). Lipases vary in their ability to catalyse glycerolysis of milk fat; Yang et al. (1994) found two lipases from *Pseudomonas* species which produced mixtures of 55–60% of MG and 24–39% of DG.

Since DG-rich oils are physiologically superior to TG-rich oils in relation to obesity management (Rudkowska et al. 2005), ways of producing them *via* lipase catalysis have been sought. One approach is ethanolysis using a *Pseudomonas*

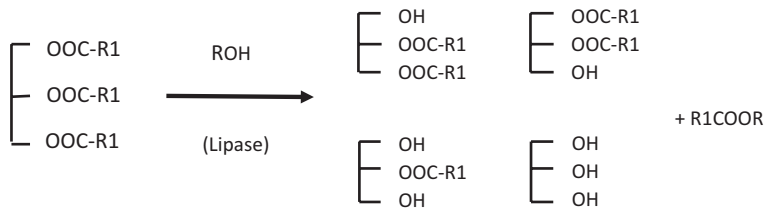

Fig. 10.4 Alcoholysis of TG catalysed by a non-sterospecific lipase

fluorescens lipase. This produces a mixture of 1,2-DGs and fatty acid ethyl esters. If pure DGs are required, the ethyl esters can be removed by supercritical carbon dioxide. Furthermore, if 1,3-DGs are required, they can be obtained by isomerisation of the 1,2-DGs by the *P. fluorescens* lipase (Lubary et al. 2011).

Lipase-catalysed ethanolysis is also used in synthesising structured TGs with palmitic acid in the 2 position, the predominant form in human milk fat. The alcoholysis reaction is carried out on tripalmitin (PPP) with anhydrous ethanol and a 1,3-regiospecific lipase such as that from *Rhizopus delemar.* The 2-monopalmitin formed can then be esterified with oleic acid to produce the TG, OPO, by acidolysis with oleic acid using the same lipase (Schmid et al. 1998).

Andrewes (2018) recently reported the use of the lipase-catalysed alcoholysis reaction for the analysis of residual bacterial lipase activity in UHT milk. Using methanol as the acyl acceptor, the amount of fatty acid methyl esters formed was quantified as a measure of the lipase activity in the milk.

10.5 Conclusion

Lipases are ubiquitous in nature and, in dairy science, are recognised for both their detrimental and beneficial effects. This chapter focuses on the detrimental lipolytic effects of the native LPL and on some beneficial acyl-transferase effects of microbial lipases in modifying milk fat. LPL is present in all raw milk in relative high activities. Under ideal circumstances, it could cause hydrolysis of the milk triglycerides to such an extent that the milk would be unacceptable in a short time. However, lipolysis only occurs under certain circumstances which can be categorised as spontaneous or induced. The reason why spontaneous lipolysis only occurs in the milk of some animals has been a puzzle for some time, but recent research indicating a genetic involvement is an exciting development. If this is confirmed in future research, it could lead to the inclusion of susceptibility of the milk to spontaneous lipolysis as a selection trait in future breeding programs. The causes of induced lipolysis are now quite well known, but knowledge of the causes and vigilance of dairy factory operatives are on-going challenges in the dairy industry. The composition and structure of milk fat do not fit every purpose in the food industry. Lipases used under restricted water conditions are now being used to effect modifications of milk fat to meet a range of needs. Commercial applications of such modifications are expected to increase and make use of new enzymes with improved or new specificities.

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