LIPASE CATALYZED MODIFICATION OF MILKFAT

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ABSTRACT

Decreasing consumption of high fat milk and dairy products is driving the dairy industry to seek other uses for increasing surplus of milkfat. Enzyme catalyzed modification of milkfat using lipases is receiving particular attention. This review examines lipase-mediated modification of milkfat. Especial attention is given to industrial applications of lipases for producing structured and modified milkfat for improved physical properties and digestibility, reduced caloric value, and flavor enhancement. Features associated with reactions such as hydrolysis, transesterification, alcoholysis and acidolysis are presented with emphasis on industrial feasibility, marketability and environmental concerns. Future prospects for enzyme catalyzed modification of milk fat are discussed.

Key words: Milkfat; butterfat; lipases; interesterification.

INTRODUCTION

Enzyme catalysis is now widely used commercially [119]. One area of application of enzymes is in the chemical redesign of milkfat for improving physical, chemical and/or nutritional properties. Lipases are the enzymes that are principally used. Lipase-mediated technology has the advantages of requiring only mild reaction conditions, displaying high selectivity and mimicking natural pathways for controlled hydrolysis, (inter- and intra-) transesterification, acidolysis and alcoholysis of milkfat.

Convenience, safety, nutritional balance and sensory satisfaction are the basic driving forces behind the modification of existing food products and the development of new ones [83]. Growing consumer demand for healthier fat spreads that are palatable, can be used for cooking and possess good textural properties are the impetus for the production of modified butters and butter-based spreads. Milkfat currently ranks third in the worldwide production of edible fats and oils [76], but

there has been an overall decline in the *per capita* consumption of butter in most European countries [49]. In addition, demand for milks of lower fat content and concomitant increase of the global surplus of milkfat [49] have promoted research into developing innovative uses of this surplus fat.

As an essential component of the human diet, fats provide high levels of palatability (tenderness and flakiness), flavor (fat-soluble flavors), satiety and mouthfeel (lubricity and moistness) to the foods in which they are incorporated [44]. The rich creaminess of ice cream and the crispness of potato chips are but two examples of these attributes. Fats are broadly classified into three chemical groups based on the degree of saturation of their fatty acids: saturated, monounsaturated and polyunsaturated fats. Saturated fats are mainly derived from foods of animal origin (e.g., butterfat, tallow and lard) but can also be found in large amounts in some vegetable products such as coconut oil (92% saturated), palm kernel oil (84%) and palm oil (50%). They are normally solid at room temperature and play a key role in providing structure to foods. Monounsaturated fats, such as high-oleic-acid safflower oil (80% monounsaturated), olive oil (72%) and canola oil (60%), are found primarily in plants; they are generally liquid at room temperature and used for frying and seasoning of foods. Polyunsaturated fats are found mostly in plants such as safflower (77% polyunsaturated), sunflower (70%), soybean (60%) and corn (57%), as well as in fish oils (from e.g. anchovy, codfish, mackerel, herring and sardine) (1-18%) [48]. Polyunsaturated fats are also liquid at room temperature and have been claimed to possess pharmaceutical activity [48]. In general, unsaturated fats are healthier than saturated ones and the former have been associated with decreases in human serum cholesterol levels and risk for coronary heart disease, as well as decreased probability of tumor development [44]. Physical properties influenced by fat include structure (e.g., in chocolate and margarine), body (e.g., in mayonnaise and salad dressings), aeration (e.g., in icings and cakes), barrier properties (e.g., cereals) and preservation (e.g., immersion of Feta cheese in olive oil).

Although milkfat possesses a unique, universal appeal that is well entrenched in the history of culinary science and has yet to be completely matched by any other food component, increasing public awareness of negative health effects of fat ingestion has driven the food industry to reformulate products that would otherwise contain high levels of (saturated) fat. Several 'healthier' fats have thus been obtained via a number of approaches: (i) *biotechnology*, including strains modified by genetic engineering that produce desired fats and enzymatic techniques for downstream modification of fats using lipases; (ii) *chemical fat substitutes*, which can directly replace conventional fats and provide nearly all their functionality [13,33], e.g. Olestra™ (Procter & Gamble, U.K.), Simplesse™ (NutraSweet, U.S.A.), Trailblazer™ (Kraft General Foods, U.S.A.), Finesse™ (Reach Associates, U.S.A.), Lita™ (Opta Foods, U.S.A.), Bindex™ (Sanofi, France), Litesse™ (Pfizer, U.S.A.), Stellar™ (Staley Man. Corp., U.S.A.), Slendid™ (Copenhagen Pectin, Denmark), Oatrim™ (Rhone Poulenc, France & Quaker Oats, U.S.A.), Lycadex™ (Roquette, France), Maltrin™ (Grain Processing, U.S.A.), Paselli SA2™ (Avebe, Holland), Tapiocaline™ (Tipiak, France), Jojoba Oil™ (Nestlé, Switzerland & Lever Bros., U.K.), Caprenin™ (Procter & Gamble, U.K.) and Salatrim™ (Nabisco, U.S.A.); and (iii) *nonconventional plant crops*, e.g.

purslane (rich in ω-3 fatty acids), *Lesquerella fendleri* (a plant belonging to the mustard family, whose seed contains ca. 25% oil, 55% of which is lesquerolic acid and the remaining 45% is accounted for by oleic, linoleic and linolenic acids) and *Limnanthes alba* (a low-growing herbaceous winter annual wildflower that produces meadowfoam oil with more than 95% C20 and higher monounsaturated fatty acids) [3,44].

Enzymatic routes are clearly some of the most promising ways to modify fats. Enzymes are biological catalysts that selectively lower the activation energies of chemical reactions [113]; therefore, very high specificities and accelerations can be achieved over nonenzymatic rates [25]. The favorable activation energies, the high catalytic efficiency and selectivity of enzymes are associated with their structure. Enzymes are polymers of tens of thousands of amino acid residues, but only a small fraction of the enzyme linear amino acid sequence is actually involved in catalysis. The long amino acid chain can bend, twist and fold back upon itself so that the relative positions of those few amino acid residues that constitute the catalytic site can be precisely arranged three-dimensionally to allow unusually efficient specific interactions with complementary groups on the substrate [80,113].

The enzymes designed by nature to hydrolyze fats and oils are termed lipases. The industrial versatility and unique catalytic performance of lipases have attracted much attention. In 1989 lipases represented less than 4% of the overall enzyme market (75,000 tons) [73], but that figure has been steadily climbing [7]. Current annual sales of lipases amount to US$ 20 million [124]. Although this figure excludes several newly developed processes, it is still moderate compared with the market for other hydrolytic enzymes, which is about ten times larger [124]. Improving extraction and purification of lipases, as well as production via genetic engineering and cloning, are expected to enhance the acceptability of lipase-catalyzed processes as viable alternatives to bulk oleochemical processes. Lipases have been extensively investigated for novel biotransformations and hundreds of elegant bio-organic syntheses based on lipases have been described; furthermore, the diversity of the current and proposed applications of lipases greatly exceeds that of proteases or carbohydrases [124].

CHARACTERISTICS OF MILKFAT

Lipids consist of a broad group of compounds that are generally soluble in organic solvents but are only sparingly soluble in water. Glycerol esters of fatty acids, which constitute ca. 99% of the lipids of plant and animal origin, have traditionally been called fats and oils, and their low solubility in water arises from their hydrophobic fatty acid residues. The distinction between a fat and an oil is made on the basis of whether the material in question is solid or liquid at room temperature. The predominant fatty acids in the lipids of natural origin possess an even number of carbon atoms because of their biosynthetic pathway [79]. Neutral fats are mono-, di- and triesters of glycerol with fatty acids and are thus systematically termed monoacylglycerols, diacylglycerols, and

triacylglycerols, respectively. Although glycerol by itself is a symmetrical molecule, the central carbon acquires chirality if one of the primary hydroxyl groups (on carbons 1 and 3) is esterified, or if these two primary hydroxyls are esterified with different acids [93].

Milk has been described as nature's most nearly perfect food due to its particularly high nutrient density including protein, fat, sugar, minerals and vitamins. In addition to being an important source of dietary fat, milkfat imparts excellent flavor and superior mouthfeel to dairy products. The various components of milkfat perform many desirable and critical functions such as organoleptic (buttery aroma, creamy mouthfeel, overall sensory satisfaction and satiety effects), physical (buttery structure and texture) and nutritional (source of energy and essential fatty acids and facilitators of fat-soluble vitamin absorption). For a number of years, however, questions have been raised as to the health value of milkfat [27] and it has been often claimed as hypercholesterolemic [96]. Because cholesterol has consistently been implicated in coronary heart disease [109], the World Health Organization has recommended reduction in consumption of milkfat. The hypercholesterolemic effect of milkfat in human diets is associated mainly with lauric, myristic and palmitic fatty acid residues [96,123]. Recent work [27,31] has provided evidence that stearic acid, as well as oleic acid, are conversely effective in lowering plasma cholesterol levels when either replaces palmitic acid in the human diet. Short-chain fatty acids (containing fewer than 12 carbon atoms) do not apparently raise the cholesterol level [42]. Polyunsaturated fatty acids lower the levels of high density lipoprotein (HDL) and low density lipoprotein (LDL) cholesterol, while monounsaturated fatty acids lower only the LDL cholesterol level [42,123]. Therefore, selective modification of the fatty acid residue structure of the triacylglycerol molecules of milkfat is a potentially suitable approach for developing a healthier milkfat.

Milkfat is probably the most complex of all natural fats [16], being a mixture of more than 100,000 different triacylglycerols which possess a wide range of molecular weights (470-890 g/mol) and correspond to acyl carbon numbers ranging from 24 to 54 [78]. The fatty acid profile of milkfat is unique in that (i) it is primarily constituted by ca. 50 mol% long-chain saturated fatty acid residues and ca. 15 mol% short- and medium-chain fatty acid residues [16] and (ii) the distribution of fatty acid residues bound to the glycerol moiety is nonrandom (e.g. butyric and caproic acids are found almost exclusively at the *sn*-3 position and at the *sn*-1 and *sn*-3 positions, respectively). As many as ca. 400 different fatty acid moieties have been reported to exist in milkfat [52], 25% of which are accounted for by short-chain saturated residues and 45% by long-chain saturated residues [28]. Nutritionally, milkfat contains a high percentage of hypercholesterolemic saturated fatty acids, located predominantly at the *sn*-2 position [72]. This myriad of triacylglycerols and fatty acid moieties is responsible for the unique flavor and physical properties of butterfat [111]. The medium- and long-chain saturated fatty acids and their stereochemical distribution among the glycerol backbones [111] gives rise to relatively high melting points [98]: at room temperature milkfat is a mixture of oil, especially the triacylglycerols with 26-40 acyl carbon atoms, and semihard and hard fat, especially the triacylglycerols with 42-54 acyl carbon atoms [11]. The melting range of these

components extends from -30 °C to 37 °C [16]. Saturated fatty acids like C16 and longer are in general not as well absorbed as their unsaturated counterparts, particularly when the former are esterified to the *sn*-1,3 positions. Saturated fatty acids in the *sn*-1,3 positions are released by pancreatic lipase during the digestive process and tend to form poorly absorbed insoluble soaps with calcium [26]. The same fatty acids esterified in the *sn*-2 position of triacylglycerol are efficiently absorbed as a *sn*-2-monoglyceride, partly because soap formation is prevented. This is widely believed to be one of the major reasons why human milkfat, which contains a large proportion of major saturated fatty acids (e.g., C16) in the *sn*-2 position, is so well absorbed by infants when compared with plant fats of similar fatty acid composition where the saturated fatty acids are mainly esterified to the *sn*-1,3 positions [26].

CHARACTERISTICS OF LIPASES

Lipases, also known as glycerol ester hydrolases (EC 3.1.1.3), belong to the hydrolase enzyme class. These enzymes apparently evolved for hydrolyzing ester bonds in substrates insoluble in water. In addition to plants and animals, many natural or genetically engineered microorganisms produce such enzymes both in endogenous and exogenous forms. Lipases also have the ability to catalyze ester synthesis reactions—i.e., the reverse of hydrolysis —under microaqueous conditions. These two basic processes can be combined in a sequential fashion to generate a set of reactions termed interesterifications. A range of such lipase-catalyzed reactions have been comprehensively reviewed elsewhere [10]. The mechanisms of lipase-catalyzed reactions resemble closely the natural metabolic pathways; hence, lipase-based processes may be viewed as more environment-friendly than some bulk chemical syntheses. Owing to their chemical- and stereo-selectivity, lipases can produce high-added value products. Because of low activation energies, lipase-mediated processes require mild temperature and pH, thus energy consumption is small and there is little thermal damage to reactants and products.

Microbial lipases can be divided into two major groups according to their specificity: (i) non-specific, which act independently of the position esterified in the glyceride molecules (e.g., lipases produced by *Candida cylindracea*, *Staphylococcus aureus*, *Chromobacterium viscosum* and *Pseudomonas* spp.); and (ii) 1,3-specific, which catalyze reactions only at the outer positions (i.e. *sn*-1 and *sn*-3) of the glycerol backbone (e.g., lipases from *Aspergillus niger*, *Mucor javanicus*, *Humicola lanuginosa*, *Rhizopus delemar*, *Rhizopus oryzae*, *Candida lipolytica*, *Rhizopus niveus* and *Penicillium roquefortii*).

In the hydrolysis of an ester bond by a lipase several steps take place in a sequential fashion [50]: first, a nucleophilic attack by the oxygen of the serine side chain on the carbonyl carbon atom of the ester bond leads to the formation of a tetrahedral intermediate, which is assisted by a histidine residue; the tetrahedral intermediate decomposes into an acyl-enzyme complex as the alcohol moiety

leaves and the free lipase is regenerated by a reaction mediated by a water molecule, thus releasing a free fatty acid moiety [50].

Several theories have been postulated to explain the observed enhancement of lipase activity upon formation of an interface [10]. Theoretical interpretations have been aided by X-ray crystallographic determination of the 3D structure and characterization of the kinetic behavior and stereoselectivity of lipases from *Rhizomucor miehei*, human pancreas, *Candida geotrichum*, *Candida rugosa*, *Pseudomonas glumae*, *Candida antarctica*, *Humicola lanuginosa*, *Pseudomonas putida*, *Fusarium solani* and *Penicillium camembertii* [118]. It is now well established that all lipases share primary sequence homologies including significant regions His-X-Y-Gly-Z-Ser-W-Gly or Y-Gly-His-Ser-W-Gly (where X, Y, Z, and W denote generic amino acid residues) [6]. The structures of lipases from *Rhizomucor miehei*, *Humicola lanuginosa* and *Penicillium camembertii* are in fact structurally homologous [118] and their active site is accounted for by a catalytic triad of three residues: a serine residue that hydrogen-binds to a histidine residue which in turn hydrogen-binds to a carboxylic acid residue; the carboxylic acid may be either an aspartic or a glutamic acid residue [50,118]. The nature and architecture of the catalytic triad of lipases is similar to that found in serine proteases [50]. Most lipases and esterases so far examined share common features, including a socalled α/β-hydrolase fold consisting of a central 8-member β-sheet with several α-helices packed at both sites [105] and their catalytically active serine located in a turn connecting one of the α-helices with the β-strand [50,105]. Such Ser residue is, in the native structure of the enzyme, occluded by a polypeptide flap (or α-helical lid), thus resulting in inaccessibility of the active site to substrate in aqueous media. Interfacial activation occurs when such flap (or lid) opens upon contact of the lipase with an ordered interface. Such opening leads to restructuring of the lipase by creating an electrophilic region (the socalled oxyanion hole) around the aforementioned serine residue, by exposing hydrophobic residues and by burying hydrophilic ones, all of which increase the affinity of the complex for lipid substrates and help stabilize the negatively charged transition state intermediate generated during the nucleophilic attack of the carbonyl bond of the substrate during catalysis [120,121,125]. Structure resolution of inhibited lipases has shown that the oxyanion hole is formed by two main polypeptide chains [17,97,120]. The topological location of the lipase flap varies among lipases and its length and complexity increases with the size of the molecule. The presence and composition of the lid covering the active site, the geometry of the catalytic triad, the structure of the oxyanion hole and the dynamics of the lid opening have now been established as the features that impart lipases their unique structure-function characteristics and their specific activation in the presence of oil/water interfaces. The requirement for an interface (irrespective of its nature) is critical; even if one uses a hydrophobic solvent as the sole, homogeneous reaction medium, there are small local pools of water entrapped within the folded structure of the lipase which may provide the local interface necessary for enzyme activation.

One of the intriguing questions within enzyme research today is the different behavior for hydrolysis in aqueous solutions versus synthesis or interesterification reactions in organic solvents.

Enzymes such as esterases, phospholipases, cholesterolesterases, thioesterases and cutinases can also act as lipases if appropriate conditions are provided. The spectrum of side activities for lipases are broad, but although these enzymes have their highest specific activity on actual triacylglycerols, selectivity and/or specific activity can be improved by protein engineering [118]. Most industrially relevant protein engineering has focused on improving hydrolytic efficiency, peracid generation (perhydrolysis) and protease stability (especially for applications in the detergent industry); protein engineering for improvement of specificity is still a relatively minor research area because, in terms of commerce, the fine chemical syntheses continue to lag behind manufacture of detergents. Nonetheless, better processes based on engineered lipases will eventually be developed.

MILKFAT ENGINEERING

A dairy herd can be schematically viewed as a factory that takes food (feedstock) and produces milkfat (product). Hence, three types of modifications of milkfat are possible: those taking place upstream (i.e., changes of the feeding regime), in the process (i.e., genetic engineering of the cow's mammary gland) and downstream (i.e., physical fractionation and chemical modification). Genetic engineering is a long term effort; how exactly the bovine genetic code affects production and characteristics of milkfat is not entirely clear [30]. On the other hand, feeding efforts aimed at obtaining a fat with desired properties will also affect the protein content and quality of the milk [92]. Therefore, in the short term, the best results are likely to accrue from focusing on downstream processing of milkfat. An area of considerable interest is the use of butterfat in confectionery as a replacement for cocoa butter [18]. In the U.S.A. and several other countries milkfat is a legally recognized ingredient in milk and dark chocolates and is less expensive than cocoa butter. Unfortunately, milkfat cannot find unlimited use in chocolate manufacture because excessive incorporation causes undesirable softening as well as changes in tempering conditions, gloss and contraction [18]. Physical modification of milkfat can be brought about via fractionation, whereas milkfat chemical modification can be effected via hydrogenation, interesterification (chemical or enzymatic), hydrolysis and alcoholysis. Chemical modification of the fatty acid profile of milkfat can be effectively and efficiently brought about by lipases: whereas in the presence of excess water these enzymes catalyze the cleavage of the ester bonds of glycerides thus releasing free fatty acids, in microaqueous systems the hydrolysis and ester synthesis can occur sequentially in a process known as interesterification. Interesterification is currently receiving much attention. A literature search has indicated that interesterification accounts for ca. 36%, hydrolysis for 34%, transesterification for 12%, acidolysis for 10% and alcoholysis for 8% of the numerous references available on lipase-catalyzed modification of milkfat.

Physical Modification

Fractionation. The melting and crystallization patterns of milkfat offer interesting possibilities for fractionation based on a wide range of melting points of their constituent triacylglycerols. Methods available for this type of modification encompass vacuum distillation and crystallization at different temperatures with or without solvents [5]. The fractionation of milkfat using solvents is a commonly used method, especially at the laboratory scale. Crystallization of fat occurs in the fat-solvent mixture at a certain temperature and for a certain time [5]. In solventless fractionation, crystallization and separation of crystals from the supernatant melted fat are technically more difficult because of viscosity problems. The lack of functionality for the intermediate consumer (e.g., poor plasticity and hardness for puff pastry) and for the final consumer (e.g., poor spreadability after refrigerated storage) restricts potential uses of plain milkfat in the food industry [28]. Many such functionality constraints and seasonal variation of milkfat may be completely, or at least largely, overcome by fractional crystallization.

Single-step fractionation yields a hard fraction (called stearin) and a soft or liquid fraction (called olein) [28]. Over 800 tons per day of milkfat are currently fractionated in Europe via the Tirtiaux™ dry fractionation process [28]. On an industrial scale, milkfat is heated and then cooled in stainless-steel crystallizers equipped with a cooling coil and a variable-speed agitator; the crystals are recovered either by horizontal filtration under vacuum or by membrane filtration under pressure [28]. The liquid fraction contains more short chain saturated and unsaturated acids and less long chain saturated acids than the solid fraction [5]: the former fraction possesses a concentrated butter aroma and is appropriate for specialty bakery products [18,122]. Although the hard fraction can be used in the manufacture of extra hard butter, milk powder and ice cream as well as in the confectionery, bakery and chocolate industry [5], the hard fractions are especially suitable for manufacture of margarine, in which case the hydrogenation step could be skipped. Fractionation results in intensification of existing properties of milkfat rather than introduction of any novel properties; the milkfat remains chemically unchanged.

Chemical Modification

Hydrogenation. Approximately one third of all edible fats and oils produced in the world are hydrogenated, and of this only ca. 10% are interesterified [47]. Hydrogenation is largely used in the U.S.A.; the European companies prefer to use interesterification for a wider variety of products including margarines [47]. Hydrogenation is aimed at increasing the hardness of an oil, although the resulting product also gains oxidative stability and functionality that permit use in margarines, solid cooking fats and baker shortenings [47]. Hydrogenation, a chemical process that reduces the degree of unsaturation of fat [49], is based on reduction with gaseous hydrogen. Hydrogenation is seldom applied to milkfat because milkfat is expensive relative to other fat feedstocks. Furthermore,

monoenes form in a *cis* or *trans* geometrical configuration during hydrogenation. The *trans* isomer is more stable [46] and helpful in ensuring that fats remain firm below body temperature and melt easily and rapidly above it; hydrogenation is used to improve such products by maximizing the concentration of this *trans* component via nickel-based catalysts. However, *trans* fatty acids have been reported to play an active role in increasing cholesterol [46], therefore alternative catalysts that reduce production of *trans* isomer are being investigated. During hydrogenation, molecular hydrogen is intimately mixed with the solubilized oil in the presence of catalyst particles. Relatively high temperature and pressure are required. After hydrogenation, the slurry is filtered at elevated temperature to remove residual catalyst. The solvent is removed, and the oil is bleached with adsorbent clays; traces of the catalyst are chelated with citric acid [47].

Hydrolysis. The triacylglycerols of milkfat possess an unusually large proportion of short-chain fatty acid residues. Lipase-catalyzed release of these moieties as free fatty acids can impart sensations of richness, creaminess, buttery flavor and a variety of cheese aromas to the product. Controlled enzymatic hydrolysis of milkfat has been used in the dairy industry to produce butter-like or cheese-like flavor products and additives [8,73]. Lipolyzed milkfat has been extensively used in oils, fats, cereals, snacks and baked goods. One classic example is the oil used to pop corn, or to cover popped corn. Flavor profiles associated with lipolyzed milkfat can impart a range of effects on organoleptic character of food: at very low addition levels, a sensation of richness is imparted without any detectable free fatty acid flavor; as addition levels are increased, the flavors imparted resemble cream or butter. When addition levels are relatively high, the flavor imparted suggests cheese.

The essential manufacturing steps of lipolyzed milkfat include: (i) preparation of condensed milk or butteroil substrate; (ii) preparation of standardized lipase system in water; (iii) combination of milkfat substrate and lipase system; (iv) homogenization to form a stable emulsion (thereby promoting the maximum rate of enzyme activity); (v) incubation at a controlled temperature (until a specified degree of hydrolysis has been achieved); (vi) pasteurization (to completely inactivate residual lipase); and (vii) final standardization, spray-drying (or alternative formulation) and packaging. Lipase-modified milkfat products include [45,95]: (i) chocolate products, e.g. milk chocolate, compound coatings and chocolate flavor syrups and beverages; (ii) butter flavors, e.g. margarine, butter creams and butter sauces; (iii) milk and cream flavors, e.g. coffee whiteners, imitation sour cream and imitation milks; and (iv) cheese flavor additives, e.g. Italian cheese flavor. Lipolyzed milkfat emulsions are also very effective carriers for such flavor adjuncts as synthetic fatty acids, diacetyl, butter esters and lactones. The addition of lipase-treated milkfat to bakery/cereal products (cakes and cookie mixes, chemically leavened bakery formulations, sweet doughs, cheese cake mixes, pancake mixes and cereal flakes), candy/confectionery products (milk chocolate, creams, toffee and caramel fudges), dairy products (cheese dips and coffee whiteners) and other miscellaneous products (margarines, popcorn, popcorn oils, salad dressings, sauces, snack foods,

soups and cooking oils and fats) results in clear buttery flavors [32]. Enzyme-modified milk creams and cultured creams can also be incorporated as dairy flavor enhancers in coffee whiteners, candies, cheese cakes, dips, sauces, sweet doughs, soups and baked goods [32]. Lipolyzed milkfat emulsions, which may vary in fat content from 25 to 95% [45] with an average of 50% [95], are the most usual commercial form of lipase-treated milkfat. Whole lipolyzed milk powder, analogous in gross composition to unmodified whole milk powder, and spray-dried lipolyzed emulsion on a carrier such as plain whey solids or added milk solids have been also advertised [95]. Arnold *et al.* have shown that butterfat, modified by lipases from different sources, improved the flavor of bread after 24 h of storage if 35-40% of the shortening was replaced by enzyme-modified butterfat [8].

Recognition of the potential of milkfat in terms of production of flavors for use within the food industry has prompted numerous applications of lipase-catalyzed hydrolyses and several patents have been issued on enzyme-modified milkfat products and additives for use in baked products and other foods [73]. Major examples of commercial lipolyzed products are noted in Table 1. Suitable lipases for modification of milkfat emulsions for incorporation into baked goods are produced by a range of sources: milk (lipoprotein lipase), pancreas (pancreatic lipase), molds (*Aspergillus niger*, *Geotrichum candidum* and *Penicillium roquefortii*), bacteria (*Achromobacter lipolyticum* and *Pseudomonas fluorescens*) and gastrointestinal tract (kid and lamb pregastric esterases) [73]. Milkfat modified by the action of these enzymes possessed better flavor than when modification was carried out with enzymes from other sources. However, modification of milkfat for the purpose of incorporation into bread formulations should not use *Achromobacter lipolyticum*, *Penicillium roquefortii* and *Geotrichum candidum* lipases because soapy and sometimes musty flavors develop in bread [73]. In addition, kid and lamb esterases should not be used because they impart a rancid flavor [73]. A process for preparation of a cheese-like product used a lipase preparation from *Penicillium roquefortii* that was used to lipolyze milkfat previously emulsified with fermented condensed skim milk [114]. A lipase from *Rhizopus delemar* has also been industrially used for enhancing flavors of such dairy products as milk, butter and cheese [114].

Otting [100,101] used steapsin to modify milkfat in milk, whereas Kempf *et al.* [69] used milk lipase to prepare lipolyzed milk products, in which part of the volatile fatty acids released were removed by steam distillation. Farnham *et al.* [36,37] have described use of a pregastric esterase in the preparation of modified whole milk powder. The specificity of pregastric esterase for short-chain fatty acid residues is especially useful in exploiting the high content of short-chain fatty acid residues in milkfat [41] for flavor development and possible interesterification purposes. This approach was used by Garcia *et al.* (1991) with a lipase from *Aspergillus niger* acting on butteroil emulsions [39] and with a lipase from *Candida cylindracea* immobilized onto a spiral wound membrane reactor for treating plain butterfat [40]. Malcata [80] and Malcata and Hill [83,84] have assessed the technical feasibility of employing an immobilized lipase from *Aspergillus niger* to effect the controlled hydrolysis of melted butterfat in a hollow fiber reactor, whereas Claus [22] described a process for producing low moisture modified fats via lipase extracted from papaya. Furthermore, Pangier [103]

Table 1. Examples of commercial modified milkfat products.

Trade name	Manufacturer	Description	Major application
Marstar L-33	Miles Laboratories	Lipolyzed cultured milk or cream	Caramel candy
DAC-1600	Dairyland Food Laboratories	Lipolyzed cultured milk or cream	Caramel candy
Marstar L-60, L-80, L-95	Miles Laboratories	Lipolyzed milkfat	Butterscotch hard candy
LBO	Dairyland Food Laboratories	Lipolyzed milkfat	Butterscotch hard candy
Butter Buds	Morton-Norwich	Lipolyzed milkfat	Butterscotch hard candy
MIL LAIT	Dairyland Food Laboratories	Modified whole milk powder	-
Marstar L-50, L-55	Miles Laboratories	Imitation blue cheese flavor	-
BETAPOL	Unilever	Infant formula fat, mimicking the fatty acid structure of human milkfat	Incorporation into infant formulas
CPS 7305	Dairyland Food Laboratories	Provolone cheese flavor	Snacks and crackers
CPF 7405 and CPF 74205	Dairyland Food Laboratories	Romano cheese flavor	Snacks and crackers
CPF 7505 and CPF 75205	Dairyland Food Laboratories	Parmesan cheese flavor	Snacks and crackers
CPF 7605	Dairyland Food Laboratories	Mozzarella cheese flavor	Snacks and crackers
Dariteen 310	Miles Laboratories	Romano cheese flavor	Tomato sauce
Dariteen 245	Miles Laboratories	Cheddar cheese flavor	Cheese analogues, spaghetti sauces, cheese sauces
Dariteen L-22	Miles Laboratories	Lipolyzed cream flavor	Cheddar cheese soup, caramel candy
Dariteen L-95	Miles Laboratories	Lipolyzed butteroil	Butterscotch hard candy
Cremoral	Land O'Lakes & Amerchol	Fractionated milkfat	Cosmetics

described the consecutive use of lactic cultures and lipolytic enzymes to produce modified milkfat products. Milkfat, after having been subject to lipolysis catalyzed by microbial lipases, yields different types and amounts of fatty acids, as seen in Table 2. The lipase from *Achromobacter lipolyticum* releases linoleic acid very selectively. The lipase of *Geotrichum candidum* liberates linoleic or oleic acid to a greater extent than does *Achromobacter lipolyticum* lipase; while lipase from *Aspergillus niger* preferentially hydrolyzes stearic acid. Because the free fatty acid profiles vary significantly with the type of lipase used, different keynotes can be imparted to the flavor of the final product.

If milkfat in cream is pretreated with lipases and then subject to thermal treatment prior to inoculation with *Lactobacillus bulgaricus* to develop further acidity (arising mainly from formation of lactic acid), then alternative products, generally termed lipolyzed cultured cream products, can be generated. These products can enhance dairy flavors in candies, cheesecakes, sauces, dips, salad dressings, sweet doughs, soups and baked goods. Typical ratios of addition are 0.05-0.10% for subtle flavorings and 0.1-0.5% for more pronounced flavors, based on weight of finished product.

Finally, it should be noted that lipolysis may be an essential preceding step in other important flavor development pathways. For example, free fatty acids generated by the lipase of *Penicillium roquefortii* from milkfat, serve as precursors for additional flavor compounds, including methyl ketones and secondary alcohols [32], both of which are essential to the typical blue cheese flavor [54,115].

Intramolecular interesterification. Interesterification using plain milkfat (also known as ester interchange or randomization) involves the exchange and redistribution of acyl groups among triacylglycerols of milkfat. This technology, which was initially developed as high-temperature interesterification [49] and normally took advantage of such chemical catalysts as sodium methoxide, now frequently uses lipases. The modified milkfat produced exhibits the same total fatty acid residue composition as the starting material, but different triacylglycerol stereochemical composition (see Table 3) and hence distinct physical properties. Such interesterification is employed for the manufacture of margarines, shortenings and confectionery fats. Unlike happens with lipase-mediated processes, when chemical catalysts are used to promote random interesterification the final product needs usually to be bleached and deodorized, which impairs nutritional safety and may destroy the fine buttery flavor that is so much appreciated by the consumer [60].

Better spreadability was reported after random interesterification of milkfat by *Chromobacterium viscosum* lipase [45], but this process led to development of a wax-like mouthfeel. Lipase-catalyzed randomization of milkfat has also been suggested [18] to yield a substitute of cocoa butter for manufacture of chocolate. Randomized milkfat has been claimed to have a beneficial effect upon blood serum cholesterol levels: as much as 12% reduction in plasma cholesterol level has been reported [23] after replacement of native milkfat by chemically randomized

Table 2. Free fatty acid profiles in milkfat after treatment by several lipases [21,45,73,95].

		Free fatty acid profiles (mol%) released from milkfat by lipases from different origins											
Fatty acid chain length	Fatty acids in native milkfat (mol%)	Milk lipase	Steapsin	Pancreatic lipase	Calf pregastric esterase	Pregastric esterase & pancreatin	*Mucor javanicus* lipase	*Candida cylindracea* lipase	*Rhizopus arrhizus* lipase	*Achromobacter lipolyticum* lipase	*Geotrichum candidum* lipase	*Aspergillus niger* lipase	*Penicillium roquefortii* lipase
4:0	5.3-4.1	13.9	10.7	14.4	35.0	15.85	4.9	2.4-4.4 (27.1)	1.5-4.3 (13.2)	Trace	Trace	Trace	Trace
6:0	3.1-3.7	2.13	2.89	2.05	2.52	3.60	1.1	0.2-0.8 (2.0)	1.3-1.4 (6.3)	1.2	Trace	Trace	Trace
8:0	1.8-2.9	1.82	1.52	1.39	1.31	3.03	1.4	0.2-1.3 (2.5)	1.4-1.5 (2.8)	1.9	1.3	1.0	3.1
10:0	3.1-4.2	2.99	3.74	3.26	3.14	5.48	2.0	2.9-3.2 (3.5)	1.6-2.0 (3.6)	2.2	1.1	2.1	4.4
12:0	3.4-4.7	2.74	4.00	3.82	5.08	4.35	2.7	3.6-8.2 (5.2)	3.0-3.7 (4.2)	1.0	1.9	3.2	3.2
14:0 B	n.a.	0.78	0.55	0.24	0.83	0.84	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
14:0	9.5-14.0	7.67	10.70	10.10	13.16	8.53	9.3	3.4-6.9 (10.1)	9.5-13.7 (8.7)	4.2	2.4	11.0	7.6
14:1	3.6	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	-	1.0	2.8	2.2
15:0 B	n.a.	0.86	1.05	0.82	0.97	0.84	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
15:0	n.a.	1.28	2.60	1.35	1.23	1.21	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
16:1 B	1.6	1.73	1.50	1.22	2.01	1.68	1.9	3.7	1.4	n.a.	n.a.	n.a.	n.a.
16:0	25.1-30.0	21.60	21.60	23.99	15.93	19.30	27.7	25.5-26.7 (24.7)	29.7-30.0 (20.1)	12.9	15.7	33.7	17.8
16:1	3.0	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	-	3.3	1.2	2.1
17:0 B	n.a.	1.20	1.34	1.28	0.73	1.20	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
17:0	n.a.	0.72	0.87	0.70	0.40	0.84	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

(Table continued)

Table 2 continued

		Free fatty acid profiles (mol%) released from milkfat by lipases from different origins											
Fatty acid chain length	Fatty acids in native milkfat (mol%)	Milk lipase	Steapsin	Pancreatic lipase	Calf pregastric esterase	Pregastric esterase & pancreatin	*Mucor javanicus* lipase	*Candida cylindracea* lipase	*Rhizopus arrhizus* lipase	*Achromobacter lipolyticum* lipase	*Geotrichum candidum* lipase	*Aspergillus niger* lipase	*Penicillium roquefortii* lipase
18:0	7.3-12.3	10.53	13.38	9.66	3.19	10.06	14.3	1.7-4.0 (6.0)	13.6-16.4 (10.1)	8.0	1.1	14.0	9.0
18:1	15.0-17.2	29.18	24.32	25.48	14.24	21.06	28.0	42.3-43.5 (15.3)	26.4-30.5 (27.1)	47.4	61.0	28.7	47.9
18:2	1.5-2.0	29.18	24.32	25.48	14.24	21.06	1.9	2.5-5.9 (3.6)	1.0-2.7 (3.9)	6.5	5.3	1.6	2.1
18:3	0.4-0.5	-	-	-	-	-	1.8	3.4	0.4	8.1	5.9	0.8	1.2

n.a.: not available.
values in parentheses refer to the lipase forms immobilized in ENTP-4000 gel.
B: branched-chain.

Table 3. Triacylglycerol composition (mol%) of native milkfat [49], chemically interesterified milkfat [49] and lipase-interesterified milkfat [11,59, 60].

Triacylglycerol carbon number	Native milkfat	Chemically interesterified milkfat	Lipase-interesterified milkfat						
			Lipase from *Mucor javanicus*	Lipase from *Pseudomonas fluorescens*					
				Absence of solvent			In isooctane		
			40 °C	40 °C	50 °C	60 °C	40 °C	50 °C	60 °C
22	-	0.1	-	-	-	-	-	-	-
24	0.3	0.8	-	-	-	-	-	-	-
26	0.1-0.3	1.4	0.6	2.6	1.8	2.1	1.7	2.1	2.2
28	0.6-0.8	1.5	1.0	2.1	1.7	1.8	2.3	1.9	2.0
30	0.9-1.54	1.3	1.9	1.9	2.0	1.9	1.9	2.1	2.1
32	1.9-3.0	2.0	3.2	2.8	2.8	3.3	2.3	3.1	3.2
34	4.4-6.2	3.0	6.4	4.0	4.3	4.5	4.4	4.5	4.6
36	9.5-12.2	5.9	12.1	7.3	7.6	8.2	8.1	7.8	8.0
38	13.1-15.0	9.1	14.6	9.8	10.5	10.9	10.7	10.5	10.5
40	11.8-12.1	9.9	11.1	8.3	9.3	8.7	8.8	8.3	8.3
42	4.9-7.7	7.6	4.1	5.7	6.1	6.0	5.6	6.1	6.2
44	6.2-6.8	8.3	6.2	6.5	7.0	7.0	7.1	6.9	7.0
46	6.4-7.5	10.7	6.9	10.4	10.5	10.2	10.2	10.4	10.1
48	7.3-8.8	12.8	8.4	14.1	13.1	12.4	13.4	13.5	12.5
50	9.8-11.2	14.2	11.0	13.7	12.3	12.5	12.9	12.7	12.5
52	10.8	10.9	9.2	8.9	8.6	8.5	8.7	8.4	8.7
54	3.8-4.6	0.4	3.4	2.1	2.2	1.9	1.8	1.7	2.0

milkfat in the human diet, but other researchers [45] could not provide evidence for any reduction in such level following long term ingestion of enzymatically randomized milkfat.

Numerous other examples of modification of plain butterfat have been reported, including modification by an *sn*-1,3-specific lipase from *Mucor javanicus* immobilized by adsorption onto hydrophobic hollow-fibers [11], by a (non-specific) lipase from *Candida cylindracea* immobilized on Celite [56,64,71] using hexane as solvent; by a (non-specific) lipase from *Pseudomonas fluorescens* immobilized on Celite in the presence or absence of solvents [58,59,60]; by a lipase from *Pseudomonas fragi* suspended in phosphate buffer and acting on a microemulsion system [102]; and by lipases from *Rhizopus niveus*, *Rhizopus delemar*, *Mucor javanicus* and *Mucor miehei* [112]. As can be concluded from Table 4, in dairy-oriented work both specific and non specific (microbial) lipases are used to similar extents.

Acidolysis. In acidolysis, an acyl moiety is displaced between an acylglycerol and a carboxylic acid [10]. Because the physical properties of milkfat depend directly on the types of fatty acid residues, changing such structure in a controlled fashion may lead to tailor-made fats with added value [107].

Research efforts pertaining to acidolysis include the solvent-free interesterification of milkfat catalyzed by a (non-specific) lipase from *Pseudomonas fluorescens* immobilized on Celite [55,60,62] and by a (*sn*-1,3-specific) lipase from *Mucor miehei* immobilized on a macroporous anion exchange resin [14]. Interesterification of milkfat with oleic acid has been reported. In separate studies, the reaction was catalyzed by a lipase from *Rhizopus oryzae* immobilized on controlled pore glass particles [98] and by a lipase from *Mucor javanicus* immobilized by physical adsorption onto a bundle of hydrophobic hollow-fibers [12]. Interesterification with caprylic acid was catalyzed by a (specific) lipase from *Pseudomonas cepacia* immobilized onto microporous polypropylene powder [110]. Similarly, interesterification with free undecanoic acid has been reported [76]. It has been claimed that during the initial stages of acidolysis a portion of the triacylglycerols will be actually hydrolyzed, thus consuming water and releasing free fatty acids [43]; however, after this transient step of hydrolysis, interesterification can proceed smoothly because the pool of water molecules available has been lowered and maintained as such in a balanced fashion.

Lipids are the major source of energy for food in infants fed on human milk or infant formulas [68]. Hence, modification of fats and oils for infant formulas in order to obtain not only the correct fatty acid composition but also the same positional distribution as in human milkfat via interesterification is being investigated. In recent work [24,26] in this area, milkfat was interesterified with concentrates of unsaturated fatty acids using an immobilized *sn*-1,3-specific lipase from *Rhizomucor miehei*, thus a milkfat analogue could be prepared for inclusion in infant formulas. The total content of short- and medium-chain fatty acids was reduced from 23.1% to 9.5% whereas the linoleic acid content increased from 2.0% to 11.3% and long-chain polyunsaturated fatty acids could be easily introduced. Diets enriched in monounsaturated fatty acids are advantageous in infant feeding: unsaturated fatty acids are absorbed better than saturated ones of the same chain length

Table 4. Main features of reactors employing lipases for the purpose of engineering milkfat.

Reaction performed [solvent(s)]	Substrate (in addition to milkfat)	Reactor configuration	Source of lipase	Method of immobilization	Support for immobilization	Reference
Transesterification [Isooctane]	-	BSTR	*Aspergillus niger*	Adsorption	Celite	65
Transesterification [Isooctane]	-	BSTR	*Mucor miehei*	Adsorption	Synthetic resin	65
Transesterification [Isooctane]	-	BSTR	*Candida cylindracea*	Adsorption	Celite	71
Interesterification [Hexane, Isooctane]	-	BSTR	*Pseudomonas fluorescens*	Adsorption	Celite	58, 59, 61
Interesterification	-	BSTR	*Pseudomonas fluorescens*	Adsorption	Celite	57
Interesterification	-	BSTR	*Pseudomonas fluorescens*	Adsorption	Celite	60
Hydrolysis	Water	BSTR	*Aspergillus niger*	-	-	39
Hydrolysis	Water	FSMR	*Aspergillus niger*	Adsorption	Polypropylene	85
Alcoholysis	Glycerol	BSTR	*Pseudomonas fluorescens*	-	-	94
Hydrolysis	Water	CSTR	*Rhizopus delemar*	Containment	BSP, polyurethane	20
Hydrolysis	Water	BSTR	*Candida cylindracea*, *Rhizopus arrhizus*	Entrapment	ENT, ENTP	21
Interesterification	-	BSTR	n.a.	Precipitation & Adsorption	Celite	29
Hydrolysis	Water	FSMR	*Candida rugosa*	Adsorption	Polypropylene	40

(Table continued)

Table 4 continued

Reaction performed [solvent(s)]	Substrate (in addition to milkfat)	Reactor configuration	Source of lipase	Method of immobilization	Support for immobilization	Reference
Hydrolysis	Water	HFMR	*Aspergillus niger*	Adsorption	Polypropylene	82, 86-88
Transesterification	-	BSTR	*Pseudomonas fluorescens*	Adsorption	Celite	62, 66, 70
Hydrolysis	Water	HFMR	*Aspergillus niger*	Adsorption	Polypropylene	83, 89
Interesterification [Hexane]	Polyunsaturated fatty acid concentrate with 39% EPA and 28% DHA	BSTR	*Rhizomucor miehei*	Ion exchange	Synthetic resin	24
Interesterification	High-oleic sunflower oil and Soybean oil	BSTR	*Mucor miehei*	Ion exchange	Synthetic resin	38
Hydrolysis	-	HFMR	*Aspergillus niger*	Adsorption	Polypropylene	90
Interesterification	-	BSTR	*Candida rugosa*	Adsorption	Silica gel	18
Acidolysis [Isooctane]	Oleic acid	BSTR	*Rhizopus oryzae*	Adsorption	Glass	98
Acidolysis	Octanoic acid, Linolenic acid	BSTR	*Pseudomonas cepacia*	Precipitation & Adsorption	Polypropylene	110
Hydrolysis	-	BSTR	*Candida cylindracea, Rhizopus arrhizus, Mucor javanicus*	n.a.	n.a.	45
Random interesterification	-	BSTR	*Chromobacterium viscosum*	n.a.	n.a.	45

(Table continued)

Table 4 continued

Reaction performed [solvent(s)]	Substrate (in addition to milkfat)	Reactor configuration	Source of lipase	Method of immobilization	Support for immobilization	Reference
Interesterification [Hexane with sodium xylene sulfonate]	-	BSTR	*Pseudomonas fragi CRDA323*	Containment	Reversed micelles	102
Interesterification [Hexane with sodium xylene sulfonate]	-	BSTR	*Rhizopus niveus, Rhizopus delemar, Mucor javanicus*	Containment	Reversed micelles	112
Interesterification [Hexane with sodium xylene sulfonate]	-	BSTR	*Mucor miehei*	Ion exchange	Synthetic resin	112
Hydrolysis	Water	HFMR	*Aspergillus niger*	Adsorption	Polypropylene	84
Interesterification	-	BSTR	*Candida cylindracea*	Adsorption	Celite	63
Interesterification [Hexane]	-	BSTR	*Candida cylindracea*	Adsorption	Celite	64
Interesterification	-	BSTR	*Candida cylindracea*	Adsorption	Celite	56
Acidolysis	Oleic acid	HFMR	*Mucor javanicus*	Adsorption	Polypropylene	9, 12
Interesterification	-	BSTR	*Mucor miehei*	Ion exchange	Synthetic resin	14
Hydrolysis	-	BSTR	*Rhizopus javanicus, Candida rugosa*	Containment	Reversed micelles	104
Hydrolysis	-	BSTR	*Candida cylindracea*	Containment	Reversed micelles	19

(Table continued)

Table 4 continued

Reaction performed [solvent(s)]	Substrate (in addition to milkfat)	Reactor configuration	Source of lipase	Method of immobilization	Support for immobilization	Reference
Alcoholysis	Ethanol, 1-butanol, 1-octanol, 1-dodecanol, 2-octanol, *t*-butanol, linalool, undecanol, pentadecanol	BSTR	*Mucor miehei* Pancreatic lipase	Ion exchange	Synthetic resin	76
Acidolysis	Pentanoic, nonanoic, tridecanoic, heptadecanoic acids	BSTR	*Mucor miehei* Pancreatic lipase	Ion exchange	Synthetic resin	76
Alcoholysis	Butanol, methanol, ethanol, 1-propanol, 2-propanol, 1-dodecanol, cyclohexyl methanol, butane-1,4-diol, 2-fluoroethanol	BSTR	*Mucor miehei*	Ion exchange	Synthetic resin	116
Transesterification	Rapeseed oil	BSTR	*Candida cylindracea*	Precipitation & Adsorption	Celite	67

(Table continued)

Table 4 continued

Reaction performed [solvent(s)]	Substrate (in addition to milkfat)	Reactor configuration	Source of lipase	Method of immobilization	Support for immobilization	Reference
Alcoholysis [Presence or absence of *ter*-butanol]	Glycerol	BSTR	Porcine pancreatic lipase, *Candida cylindracea*, *Rhizopus arrhizus*, *Pseudomonas* spp., *Rhizopus javanicus*, *Rhizopus delemar*, *Geotrichum candidum*, *Mucor javanicus*	-	-	126

BSP biomass support particles
BSTR batch stirred tank reactor
CSTR continuous flow stirred tank reactor
ENT cross-linkable resin prepolymer containing polyethylene glycol
ENTP cross-linkable resin prepolymer containing polypropylene glycol
FSMR flat sheet membrane reactor
HFMR hollow fiber membrane reactor
n.a. not available

because monounsaturates are less prone to soap formation with calcium. In addition, because of a lower degree of unsaturation, monounsaturates are less susceptible to peroxidation than polyunsaturated fatty acids [24]. Although acidolysis may be used to lower the saturated fatty acid content of milkfat, it can also, depending on the reaction conditions and the selectivity characteristics of the lipase used, produce glycerides with such a fatty acid profile that the base milky flavor is eliminated; hence, special care is necessary during processing [9,12,98].

Alcoholysis. In alcoholysis, an acyl moiety is displaced between an acylglycerol and an alcohol [10]. Although this kind of reaction is not often reported for milkfat, a few examples exist: the modification of milkfat via lipase-mediated alcoholysis reactions with primary alcohols (ethanol, 1-butanol, 1-octanol, 1-undecanol, 1-dodecanol and pentadecanol), secondary alcohols (*sec*-butanol and 2-octanol) and tertiary alcohols (*t*-butanol and linalool) [76]. Similarly, alcoholysis with butanol, methanol, ethanol, 1-propanol, 2-propanol, 1-dodecanol, cyclohexyl methanol, butane-1,4-diol and 2-fluoroethanol catalyzed by a lipase from *Mucor miehei* immobilized on an ion exchange resin has been reported [116].

Monoacylglycerols (MAG) and diacylglycerols (DAG) are widely used as emulsifiers in food systems [126], where they account for ca. 75% of the world production of emulsifiers [75], as well as in the pharmaceutical and cosmetic industries [15]. MAG and DAG can be formed by controlled hydrolysis of triacylglycerols or controlled esterification of glycerol and fatty acids, or alternatively, via acyl exchange between excess glycerol and triacylglycerols (an alcoholysis process frequently termed glycerolysis) [126]. Yang *et al.* [126] have screened several commercial lipase preparations (porcine pancreatic lipase, *Candida cylindracea*, *Rhizopus arrhizus*, *Pseudomonas* spp., *Rhizopus javanicus*, *Rhizopus delemar*, *Geotrichum candidum* and *Mucor javanicus*) for alcoholysis of milkfat in the presence and absence of solvent with the purpose of producing MAG and DAG from this resource.

Transesterification. In transesterification, two acyl moieties are exchanged between two acylglycerols [10]. To perform this kind of reaction, two fats or oils are usually blended and added with a lipase. Therefore, controlled changes in the structure of milkfat can be achieved by interchange of given fatty acid moieties with another fat or oil. Transesterification of the solid fraction of milkfat with rapeseed oil using *Candida cylindracea* lipase has been reported [67].

One major disadvantage of transesterification is the difficult downstream separation: the modified milkfat and the remaining oil are more alike than for example modified milkfat and free fatty acids or modified milkfat and alcohols. Separation problems prevent widespread use of this technique.

Reduction of cholesterol level. Milkfat contains, on average, 7.3 $mg_{cholesterol}/g_{milkfat}$ [33]. Biological procedures for cholesterol removal utilize microorganisms that convert cholesterol into

harmless compounds. Enzymes responsible for cholesterol removal are being investigated. For example, attempts are underway to identify the best sources of cholesterol reductase [2], which converts cholesterol to coprostanol. The latter is poorly absorbed as it passes through the digestive tract. Such enzymatic methods can remove cholesterol without disrupting the milkfat globular membrane; however, the scale-up of this technology to the industrial level is at present both complicated and expensive [16,49].

INDUSTRIAL FEASIBILITY

Enzymes have several well-known advantages in industrial processing [106]. Lipases, in particular, have a number of potential industrial applications: in the production of esters and specialty fats, in the acceleration of cheese ripening, in the fermentation of vegetables, in the curing of meat products, in the processing of fish, in the refinement of rice flavor, in the modification of soybean milk, in the treatment of carp prior to smoking, in flavor improvement of alcoholic beverages, in improvement of whipping quality of egg whites, in the cosmetic industry, in the pharmaceutical industry, in the treatment of leathers, in the preparation of aliphatic acids from dark and highly acid oils and fats, in chewing gums and tooth pastes, in sewage treatment, in flavor enhancement and in improvement of detergents [106,114]. However, industrial use of lipases as alternatives to bulk chemical or physical processes for modification of edible fats (including milkfat) and oils is relatively recent. Wider use of lipases hinges upon their stability [34]. Lipases need to be stable against proteolytic action, against thermal processing and also against oxidative compounds and detergent ingredients [34]. Although lipases have been improved by genetic engineering, site directed mutagenesis and random mutagenesis [1,34,99,118], more effort is needed to provide more capable lipases.

Prospects for use of lipases are greatest in food and pharmaceutical processing. Improved understanding is needed concerning the stereopreference of lipases acting on triacylglycerols from milkfat or on compounds of pharmaceutical interest. Such understanding may eventually lead to deliberate specific modification of the active site and the binding site of lipases [34] through protein engineering techniques. In addition, the economic and efficient use of lipases as industrial catalysts also requires development of suitable systems for catalyst-reactant contacting that permit recovery and subsequent reuse of the catalyst [81]. These goals can be reached either by (i) attaching the enzyme to a support that is immiscible with the reaction medium; (ii) changing the nature of the reaction medium in such a way as to permit precipitation of the enzyme; or (iii) confining the enzyme to a localized region in space using a mechanical barrier [10,81].

ENVIRONMENTAL CONCERNS

Enzyme technology offers industry and consumers the opportunity to replace classical polluting processes by milder, non-toxic enzymatic processes. Enzyme-mediated processes have a minimal

impact on the environment [35] and resemble more closely the natural metabolic pathways. Compared to many traditional chemical syntheses, enzymatic processes are more environment-friendly. The discriminating abilities (e.g., stereospecificity, selectivity and substrate specificity) of enzymes are much greater than those of other catalysts. Thus, enzymes can be used to produce high-added value products at higher levels of purity. Catalytic efficiency (a result of much lower activation energies) of enzymes reduces energy requirements from non-renewable sources.

In addition, within the edible oils and fats industry, enzyme technology offers several opportunities for reducing the high BOD effluent streams that pollute water bodies. One approach is to reduce the fat load in effluent streams by diverting the fat to lipase-catalyzed processing [80,83,84]. Also, lipases can be employed for improving degradation of fat-containing waste. For example, lipases have been used in combination with a microbial cocktail (trade name Combizyme™) designed by Biocatalysts (U.K.) for treating fat-rich effluent from an ice-cream plant [124].

MARKETABILITY

Over the last two decades, world consumption of butter has steadily declined [49] because of factors such as health impact and functional limitations [16,111]. The major barriers to increased butter sales [49] include price, health image, poor spreadability, absence of product innovation, and legislation and regulatory restrictions (e.g., legislated amounts of fat in commercial milk). On the other hand, increasing demand for milk with higher protein contents but lower fat contents is promoting increasing surplus of milkfat. Improved feeding of the cows aimed at obtaining milk with higher protein contents also leads to higher fat contents, more extensive defatting requirements, and greater surpluses of milkfat. To counteract growing stocks, dumping has been a common practice in the more developed countries. Between 300,000 and 400,000 tons of butter have been sold annually for several years in the U.S.A. at reduced prices to the pastry, ice cream and chocolate industries in attempts to outgrow competition with vegetable fats [4].

Although the flavor and mouth feel of milkfat are greatly superior to those of other fats, poor spreadability of refrigerated butter make it unattractive to many consumers. Also, the margarine and spread industry can easily tailor a low-price competitor product despite the many advances in the ability to alter the texture and rheology (spreadability) of butter via lipase-mediated pathways. Hence, it is doubtful that a single market for milkfat will be found that will compensate for the decrease in butter sales. Therefore, it will be necessary to look for a large number of relatively small outlets (some of which have been discussed above).

FUTURE PROSPECTS

Severe compulsive cuts in the production of milk are not a reasonable solution to the ever increasing surplus of milkfat. Similarly, relying upon aggressive advertisement of existing dairy products is

insufficient. Any workable solution must involve a two pronged approach: (i) creating technology for tailor-made products derived from milkfat, and (ii) promoting such new products. Conventional glyceride modification in the fat and oil industry is based on chemical catalysis, but in many instances use of lipases is technically feasible and preferable from an environmental point of view. Improved quality and a more natural image are other benefits of enzyme catalyzed modification of milkfat. Interesterification mediated by lipases has become a powerful tool for the modification and optimization of both the structure and properties of milkfat. With the advent of fatty acid-specific lipases (e.g., that produced by *Geotrichum candidum* which is specific for hydrolysis of fatty acids such as oleic, linoleic and linolenic acids [51,53,74,77,117] that contain *cis* Δ9 double bonds), exciting developments such as the use of unsaturated fatty acid residues in production of more spreadable milkfat will be possible [91,108]. The incorporation of ω-3 fatty acids into milkfat—a long-time goal of the dairy industry—will also be feasible via enzymatic interesterification of milkfat with fish oils.

The lower processing temperatures and the absence of harsh chemicals in lipase-based processing should improve both the shelf life and the quality of the final product. As less expensive and more stable lipases become available and improved large-scale lipase bioreactors are developed, many fat and oil processes should switch from bulk chemical to lipase-mediated technology. However, even though the catalytic efficiency of lipases surpasses that of chemical catalysts and cost of producing lipases is currently dropping, enzymatic processes will always be more expensive than the conventional ones. Hence, extensive industrial use will be feasible provided that the new products developed possess a higher added value because of improved or unique nutritional and/or functional properties. Thus, accordingly, randomization of milkfat using lipases seems economically unfeasible at present, but transesterification of milkfat with polyunsaturated fatty acid concentrates via catalysis by *sn*-1,3-specific lipases seems to have excellent prospects. Lipase catalyzed interesterification is currently being used as a research tool by food technologists and nutritionists to explore the relationship between structure and function of triacylglycerols, with the ultimate aim of developing new products. Many challenges remain to be overcome, but the possible impact on the dairy industry is likely to be great. With accumulation of knowledge about the relationships between the structure of lipases and their catalytic and stability properties, new substantial improvements in lipase-mediated biotransformation of milkfat should be possible.

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