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Present and Future Applications of Lipases

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Introduction

Lipases (glycerol ester hydrolases EC 3.1.1.3) comprise a group of enzymes of widespread occurrence throughout the animal and plant kingdoms. Their biological function is to catalyse the hydrolysis of triacylglycerols to give free fatty acids, diacylglycerols, monoacylglycerols and glycerol. This reaction is reversible, so that the enzymes also catalyse the formation of acylglycerols from glycerol and free fatty acid (*Figure 1*). Although lipases have been studied for many years and can be produced on a large scale by growth of micro-organisms in fermenters, in contrast to the other major groups of hydrolytic enzymes, the proteases and carbohydrases, lipases have so far found few industrial uses. However at present there is an increasing interest in the development of new applications for these enzymes in products and processes, particularly in the detergents, oils and fats, and dairy industries.

The biochemical properties of lipases have been reviewed by Brockerhoff and Jensen (1974), Semeriva and Desnuelle (1979) and Borgstrom and Brockman (1984). In this chapter we shall discuss the present and potential applications of lipases for analysis and industrial processes and products, and describe briefly the biochemical properties of the enzymes which are relevant to their industrial uses. Because extracellular microbial lipases can be produced relatively cheaply by fermentation and are available in large quantities for industrial use, we shall concentrate on the applications and properties of these enzymes but, when appropriate, lipases from animal and plant sources will also be mentioned.

Properties of lipases

CHARACTERISTICS OF THE LIPASE REACTION

The natural substrates of lipases are triacylglycerols of long-chain fatty acids. These compounds have a very low solubility in water, and lipases are

Biotechnology and Genetic Engineering Reviews — Vol. 3, September 1985
0264–8725/85/03/193–25$10.00 + $0.00 © Intercept Ltd

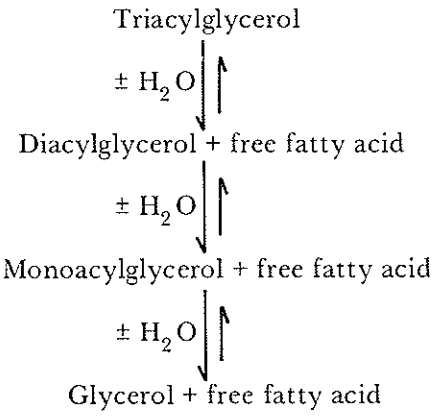

Figure 1. The lipase reaction.

characterized by the ability rapidly to catalyse the hydrolysis of ester bonds at the interface between an insoluble substrate phase and an aqueous phase in which the enzyme is dissolved. Lipases catalyse the hydrolysis of a wide range of essentially water-insoluble fatty-acid esters, although acylglycerols are normally the best substrates, but the hydrolysis of water-soluble carboxylic-acid esters by many lipases is very slow. The ability of lipases to catalyse hydrolysis of insoluble fatty-acid esters distinguishes them from other esterases which catalyse the hydrolysis of water-soluble esters in preference to insoluble esters.

The requirement by lipases for a non-aqueous substrate phase to provide an interface at which rapid catalysis can be expressed has been clearly illustrated by studies of the effect of substrate concentration on the lipolysis of partially soluble substrates using pancreatic and microbial lipases. With these substrates, appreciable lipolysis was observed only above their critical micellar concentration, i.e. the concentration above which micelles, small aggregates or emulsion particles of the substrates were present (Semeriva and Dufour, 1972; Brockerhoff and Jensen, 1974).

The occurrence of the lipase reaction at an interface between the substrate and aqueous phases causes difficulties in the assay and kinetic analysis of the reaction. In lipase reaction systems an emulsion of the substrate in a continuous aqueous phase is normally used, and factors affecting the amount or properties of the interface between the two phases of the emulsion will affect the observed reaction rate. Partial glycerides and soaps of free fatty acids, which are formed during lipase reactions, are surface active and tend to accumulate at the interface. This may prevent access of the lipase to further substrate, and consequently it is often difficult to devise reaction conditions under which the reaction rate is constant over a reasonable period of time, and extensive hydrolysis of the substrate is obtained. If accurate and reproducible results are to be obtained in the assay of lipases, great care must

be taken to ensure that the emulsion used in the incubation mixture contains the required amount and type of interface and that true reaction rates are measured. The various techniques used for determining lipase activity have recently been reviewed by Jensen (1983).

Conventional enzyme kinetics have been developed for reaction mixtures in which both the enzyme and substrates are dissolved. Kinetic treatment of lipase-catalysed reactions has to differ from conventional methods in many respects in order to take account of the interaction between enzyme and insoluble substrate at an interface. For example, determination of the effective concentration of an insoluble substrate presents problems. At best only those substrate molecules which are at the interface on the surface of emulsion droplets are available to the enzyme, and surface area of substrate per unit volume should be used in place of the usual concentration term. Benzonana and Desnuelle (1965) have shown that hydrolysis of triacylglycerols catalysed by pancreatic lipase will apparently conform to the Michaelis–Menten equation if the substrate concentration is expressed in units of interfacial area per unit volume. The kinetics of lipolysis reactions have been discussed in some detail by Brockerhoff and Jensen (1974).

STRUCTURE

Lipases from several sources have been purified, and some of their properties investigated (Brockerhoff and Jensen, 1974; Macrae, 1983b). Generally they are acidic glycoproteins of molecular weights ranging from 20 000 to 60 000. The specific activities of the pure proteins vary from 1000 to 10 000 units per milligram protein (1 unit of lipase releases 1 μmole of fatty acid per minute from triacylglycerol). Some lipases are known to form aggregates in solution, and this may account for the high molecular weights reported for some partially purified lipases. The majority of purified lipases have been shown to contain between 2% and 15% carbohydrates, the major glycoside residue in all cases being mannose. The carbohydrate side-chains of the lipases are probably not associated with the enzymes' catalytic activity. For example, autolysis of *Rhizopus arrhizus* lipase gives a low-molecular-weight glycopeptide and a carbohydrate-free protein with high lipase activity (Semeriva, Benzonana and Desnuelle, 1969). The multiple forms of lipases apparently produced by some animal tissue and micro-organisms may result from self association of enzyme molecules or variation in the carbohydrate side-chains.

The amino-acid compositions of a number of purified lipases have been published. Some workers have suggested that lipases contain an exceptionally high proportion of hydrophobic amino acids and that this characteristic is responsible for their interaction with hydrophobic substrates. However, examination of the published amino-acid compositions clearly shows that, as a group, lipases are not more hydrophobic than other groups of enzymes (Macrae, 1983b). Because lipases do not contain an exceptionally high proportion of hydrophobic residues, the strong interaction with hydrophobic substrates at an interface is probably caused by hydrophobic patches on the

lipase surfaces. Such patches may also be responsible for the self-association behaviour shown by the enzymes in aqueous solution. To date, porcine pancreatic lipase is the only lipase whose primary structure has been determined by protein sequencing (De Caro *et al.*, 1981). The sequence contains two segments especially rich in hydrophobic amino acids and these regions may be associated with substrate recognition and binding. Detailed information on the secondary structure of lipases is required to determine the mechanism by which these enzymes catalyse reactions at the substrate–water interface. At present, high-resolution X-ray crystal structural data on lipases are not available, although a low-resolution structure for *Geotrichum candidum* lipase has been published (Hata *et al.*, 1979).

PH OPTIMA, STABILITIES AND COFACTOR REQUIREMENTS

Lipases are usually reasonably stable in neutral aqueous solutions at room temperature. Solutions of pancreatic and many extracellular microbial lipases lose activity on storage at temperatures above 40°C, but some microbial lipases are more resistant to heat inactivation. Thus the enzymes produced by *Aspergillus niger* (Fukumoto, Iwai and Tsujisaka, 1963), *Rhizopus japonicus* (Aisaka and Terada, 1980) and *Chromobacterium viscosum* (Yamaguchi *et al.*, 1973) are stable in solution at 50°C and the thermotolerant fungus *Humicola lanuginosa* excretes a lipase which is stable at 60°C (Liu, Beppu and Arima, 1973a). A strain of *Pseudomonas nitroreducans* gives a lipase which is stable at 70°C (Watanabe *et al.*, 1977), and the *Pseudomonas fluorescens* enzyme, which can be responsible for spoilage of heat-treated dairy products, is only partially irreversibly inactivated at high temperatures (Adams and Brawley, 1981). To date, lipases produced by thermophiles have not been isolated on a significant scale or studied in detail. Lipases with extreme heat stability would be particularly useful for some industrial applications.

In general, lipases have a broad pH-activity profile, showing high activity between pH 5 and 9 with a maximum between pH 6 and 8. The extracellular lipases produced by *Aspergillus niger, Chromobacterium viscosum* and *Rhizopus arrhizus* are particularly active at low pH (Fukumoto, Iwai and Tsujisaka, 1963; Laboureur and Labrousse, 1966; Yamaguchi *et al.*, 1973), and an alkaline lipase active at pH 11 has been isolated from *Pseudomonas nitroreducans* (Watanabe *et al.*, 1977).

Cofactors are not required for expression of lipase activity, although substances which affect the amount or properties of the interface between the substrate and the aqueous phase have an effect on the reaction rate. For example, high concentrations of surfactants can inhibit the reaction by preventing access of the enzyme to its substrate. Pancreatic lipase is inhibited by physiological concentrations of bile salts, but this inhibition is reversed by a low-molecular-weight co-lipase produced by the pancreas. The co-lipase enables the lipase to bind to its substrate in the presence of high concentrations of bile salts and other surfactants. There is no evidence for the production of co-lipases by micro-organisms and the pancreatic co-lipase does not reverse the inhibition of microbial lipases by bile salts (Canioni *et al.*,

1977). Salts can have a pronounced effect on the lipase reaction by influencing the ionization of the fatty-acid product. In particular, calcium ions often stimulate the reaction by removing, as calcium soaps, fatty-acid anions which are inhibitory.

SUBSTRATE SPECIFICITY

The substrate specificity of lipases is often crucial to their application for analytical and industrial purposes. The enzymes can show specificity with respect to either the fatty acyl or alcohol parts of their substrates. The methods of determining lipase specificity have recently been reviewed by Jensen, deJong and Clark (1983).

During investigations of the fatty-acid specificity of lipases, appropriate substrates and conditions must be used to ensure that observed changes in reaction rates result from alterations in the structure of the fatty acyl group and not other effects. In many studies mono-acid triacylglycerols are used. The results obtained are difficult to interpret because the alcohol product of the reaction (e.g. diacylglycerol) varies together with the fatty-acid product, and the triacylglycerols of long-chain saturated fatty acids are solids at normal assay temperatures, whereas those of unsaturated fatty acids and short- or medium-chain saturated fatty acids are liquids. Solid triacylglycerols are only slowly hydrolysed by lipases (Brockerhoff and Jensen, 1974; Sugiura and Isobe, 1975a). Simple alkyl esters of fatty acids are useful substrates for investigating fatty-acid specificity, because they are liquids at normal assay temperatures and the alcohol group can be kept constant as the fatty acyl group is altered.

The fatty-acid specificities of pancreatic lipase (Brockerhoff and Jensen, 1974) and the extracellular microbial lipases from *Candida cylindracea* (Benzonana and Esposito, 1971), *Candida paralipolytica* (Ota, Nakamiya and Yamada, 1972), *Humicola lanuginosa* (Liu, Beppu and Arima, 1973b), *Chromobacterium viscosum* (Sugiura and Isobe, 1975b) and *Penicillium cyclopium* (Iwai, Okumura and Tsujisaka, 1975) have been studied using alkyl esters. In each case, only moderate differences in the rates of hydrolysis of the various long-chain saturated and unsaturated fatty-acid esters were observed. Similar results have been obtained from studies of the hydrolysis of triacylglycerols using the lipases from *Rhizopus japonicus* (Aisaka and Terada, 1980), *Pseudomonas fluorescens* (Sugiura and Isobe, 1975a) and *Staphylococcus aureus* (Vadehra, 1974). Therefore, in general, lipases show little fatty-acid specificity when incubated with most natural oils and fats. Exceptions occur when fish oils and milk fat are used as substrates. The very-long-chain polyunsaturated fatty acids found in fish oils are only slowly released by pancreatic and *Candida cylindracea* lipases (Brockerhoff and Jensen, 1974; Asahi Denka Co., 1983). The presence of a double bond close to the carboxyl group in these acids probably makes their esters resistant to attack by the enzymes. For steric reasons, esters of fatty acids containing a double bond or a bulky substituent close to the carboxyl group are poor substrates for pancreatic lipase. Tributyrin is hydrolysed slowly by some

microbial lipases (Liu, Beppu and Arima, 1973b; Sugiura and Isobe, 1975b; Aisaka and Terada, 1980), and therefore butyric acid and other short-chain acids may not readily be released from milk fat by these enzymes. In contrast, *Mucor miehei* lipase preferentially releases butyric acid from milk fat, especially at low pH (Moskowitz *et al.*, 1977). The fungus *Mucor lipolyticus* produces two extracellular lipases, which differ both in their fatty-acid specificities and in their physical properties (Nagaoka and Yamada, 1973). One enzyme catalyses the hydrolysis of triacylglycerols of medium- and long-chain fatty acids, but shows no activity with tributyrin, while the other enzyme catalyses the hydrolysis of tributyrin, medium- and long-chain fatty acid triacylglycerols. Similar results have been obtained with two lipases produced by *Penicillium cyclopium* (Iwai, Okumura and Tsujisaka, 1975). Many micro-organisms may produce two or more extracellular lipases with differing fatty-acid specificities especially with respect to short-chain fatty acids.

The only lipase which has been shown to have a very pronounced specificity for the hydrolysis of esters of a particular type of long-chain fatty acid is an extracellular enzyme produced by the mould *Geotrichum candidum*. The substrate specificity of this lipase has been studied in some detail, and the results are summarized in three review articles (Jensen, 1974; Jensen and Pitas, 1976; Macrae, 1983b). The lipase catalyses the hydrolysis of esters of long-chain unsaturated fatty acids with a double bond in the 9 position. Esters of saturated fatty acids and unsaturated fatty acids without a double bond in the 9 position are only slowly attacked. Generally *cis*-Δ9 fatty acid esters are hydrolysed more rapidly than their *trans*-Δ9 isomers. The presence of an additional double bond between the carboxyl group and the double bond in the 9 positions makes unsaturated fatty-acid esters resistant to hydrolysis by the lipase. The specificity of the enzyme is such that it catalyses the preferential release of oleic, palmitoleic, linoleic and α-linolenic acids from oils and fats leaving acylglycerols enriched in saturated fatty acids and unsaturated fatty acids without a double bond in the 9 position. The unique specificity of *Geotrichum candidum* lipase has been exploited in the analysis of triacylglycerols (Sampugna and Jensen, 1969), and may also be useful for the production of special fatty-acid fractions. The discovery of lipases showing other types of pronounced fatty-acid specificity would be of considerable commercial interest.

As mentioned previously, acylglycerols are the natural substrates of lipases, but the enzymes will catalyse the hydrolysis of a wide range of other types of fatty-acid esters although the reaction rates are generally substantially lower than those observed with acylglycerols. Lipases show both regio- and stereospecificity with respect to the alcohol moiety of their substrates (*see* Chapman, 1969).

The enzymes can be divided into two groups according to the regiospecificity exhibited with acylglycerol substrates. Lipases in the first group show no regiospecificity and release fatty acids from all three positions of glycerol (*Figure 2*). These lipases catalyse complete breakdown of triacylglycerols to glycerol and free fatty acid. Diacylglycerols and monoacylglycerols are

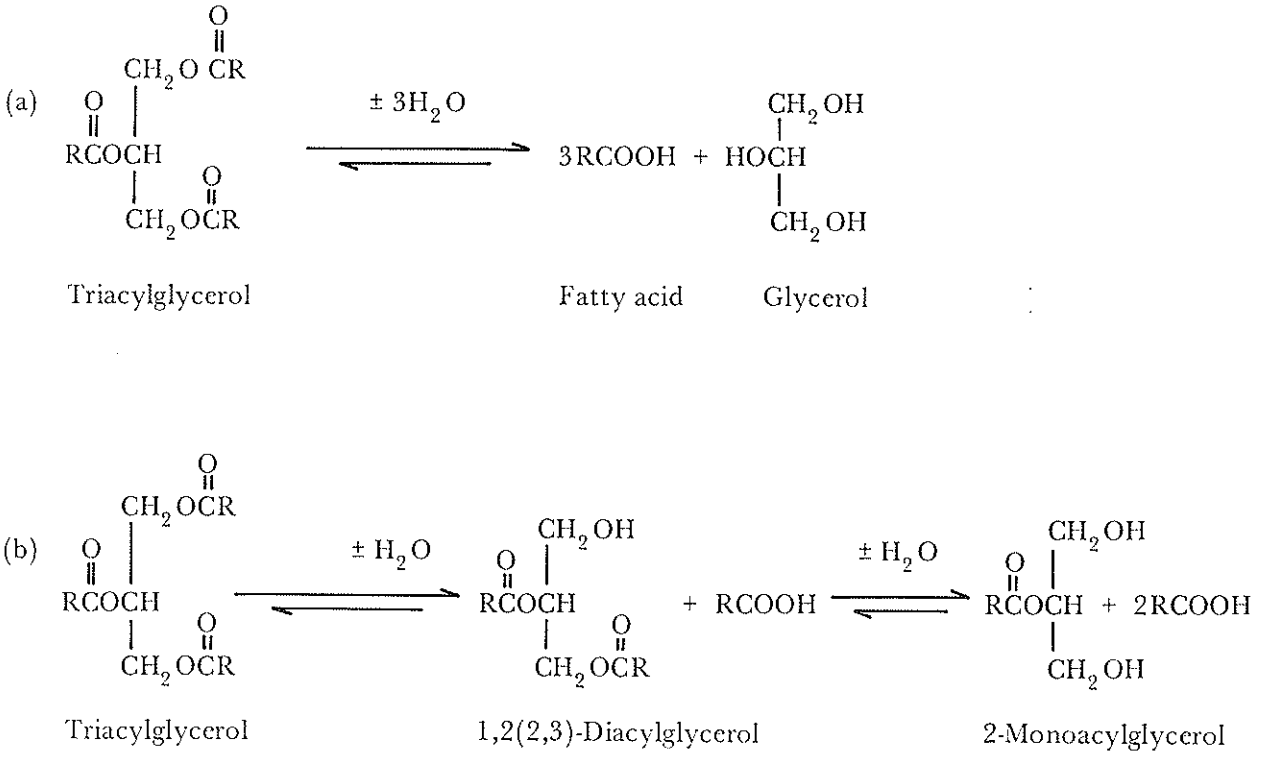

Figure 2. Regiospecificity of lipases. (a) Reaction catalysed by non-specific lipases. (b) Reaction catalysed by regiospecific lipases.

intermediates in the reaction, but do not normally accumulate to a high level during the reaction, presumably because they are hydrolysed more rapidly than triacylglycerols. Castor-bean lipase (Noma and Borgstrom, 1971) and the extracellular microbial lipases produced by *Candida cylindracea* (Benzonana and Esposito, 1971), *Corynebacterium acnes* (Hassing, 1971), *Propionibacterium acnes* (Ingham *et al.*, 1981), *Staphylococcus aureus* (Vadehra, 1974), and *Geotrichum candidum* (Jensen, 1974) show no significant regiospecificity. The mould *Penicillium cyclopium* produces extracellular lipases with no regiospecificity and an enzyme which catalyses hydrolysis of diolein and mono-olein much more rapidly than triolein. Combination of this partial acylglycerol hydrolase with the conventional lipases gives a very efficient system for the total hydrolysis of oils and fats (Okumura, Iwai and Tsujisaka, 1980). Monoacylglycerol hydrolase activity is found in many animal tissues (Brockerhoff and Jensen, 1974), and it will be of interest to determine whether partial acylglycerol hydrolases are commonly produced by lipolytic micro-organisms.

The second group of lipases release fatty acids regiospecifically from the outer 1- and 3-positions of acylglycerols (*Figure 2*). With these lipases, triacylglycerols are hydrolysed to give free fatty acids, 1,2(2,3)-diacylglycerols and 2-monoacylglycerols. The rate of hydrolysis of triacylglycerols is normally faster than that of diacylglycerols, and consequently substantial quantities of both diacylglycerols and monoacylglycerols accumulate during the reaction. Because 1,2(2,3)-diacylglycerols and 2-monoacylglycerols are chemically unstable and undergo acyl migration to give 1,3-diacylglycerols and 1-monoacylglycerols respectively, prolonged incubation of an oil with these enzymes will give complete breakdown of some of the acylglycerols with the formation of glycerol. 1,3-Regiospecificity is exhibited by pancreatic lipase (Brockerhoff and Jensen, 1974) and by many extracellular microbial lipases including those produced by *Aspergillus niger* (Okumura, Iwai and Tsujisaka, 1976), *Mucor javanicus* (Ishihara *et al.*, 1975), *Rhizopus arrhizus* (Semeriva, Benzonana and Desnuelle, 1967) and an *Alcaligenes* species (Kokusho, Machida and Iwasaki, 1982b). We have shown that the lipases excreted by *Rhizopus japonicus, Mucor miehei, Humicola lanuginosa, Chromobacterium viscosum* and *Pseudomonas fluorescens* are also 1,3-regiospecific (A. R. Macrae and R. C. Hammond, unpublished results). In general, the regiospecificity of the enzymes listed above is almost absolute, and probably results from an inability of sterically hindered esters of secondary alcohols (e.g. those of the 2-position of glycerol) to enter the active sites of the enzymes. To date there are no authenticated reports of lipases which catalyse release of fatty acids selectively from the central 2-position of acylglycerols.

Partial stereospecificity in the hydrolysis of triacylglycerols has been observed for some animal lipases. For example, the lipoprotein lipases (EC 3.1.1.34) from plasma, milk and adipose tissues hydrolyse the *sn*-1 position of triacylglycerols at approximately twice the rate of the *sn*-3 position, while lingual and insect lipases preferentially release fatty acids from the *sn*-3 position (Jensen, deJong and Clark, 1983). The stereospecificities of pancreatic lipase and of the extracellular lipases produced by *Rhizopus*

arrhizus and *Pseudomonas fluorescens* have been investigated by Akesson *et al.* (1983). The enzymes do not show any stereospecificity with triacylglycerols and alkyldiacylglycerols, but 2-acyl-3-alkyl-*sn*-glycerol is hydrolysed more rapidly than its stereoisomer 1-alkyl-2-acyl-*sn*-glycerol by the three lipases. Partial stereospecificity with other fatty-acid monoesters has been observed for pancreatic, *Rhizopus arrhizus*, *Rhizopus delemar*, *Candida cylindracea* and *Pseudomonas aeruginosa* lipases, and it has been suggested that these enzymes could be used to isolate optically pure esters and alcohols (Iriuchijima and Kojima, 1982; Lavayre, Verrier and Baratti, 1982; Cambou and Klibanov, 1984). Isolation of an extracellular microbial lipase possessing pronounced stereospecificity in the hydrolysis of triacylglycerols would be of considerable commercial interest.

Fat splitting

Oils and fats are hydrolysed industrially to produce free fatty acids, soaps and glycerol. For production of fatty acids the oils and fats are split with water in a countercurrent continuous process operated at a high temperature and pressure. This process is very efficient, giving an aqueous stream containing about 10% glycerol and a fat stream containing 97% free fatty acids. Fatty acids are isolated from the fat stream by distillation and, after removal of impurities, concentrated glycerol is produced from the aqueous stream by evaporation of water. Because of the high temperature used in the process, discolouration and some degradation of the fatty acids can occur during splitting and this may cause a loss of product quality and yield, particularly in the case of highly unsaturated and hydroxy fatty acids. Soaps can be produced from fatty acids, but alternative procedures use direct saponification of the oils and fats with sodium hydroxide. Brine is used to separate the soap from the aqueous phase, and glycerol is recovered from solution in brine.

Historically, castor-bean lipase has been used to split castor oil, but with the development of more efficient chemical methods this enzyme-catalysed process fell into disuse (Sonntag, 1979). However, the availability of comparatively cheap extracellular microbial lipases has led to a renewed interest in the use of lipases as fat-splitting catalysts. Lipases with no regiospecificity are particularly suitable because they catalyse the complete hydrolysis of triacylglycerol to fatty acid and glycerol. For example, Linfield *et al.* (1984a) have shown that *Candida rugosa* (formerly known as *Candida cylindracea*) lipase can be used to give 95–98% hydrolysis of tallow, coconut oil and olive oil. The Myoshi Oil Company of Japan have reported operation of a process catalysed by this lipase for production of fatty acids to be used for soap manufacture. They claim that use of enzyme catalysis in place of conventional fat splitting yields a fatty acid of better quality for conversion into soap and facilitates glycerol recovery, giving a cheaper overall process (Anonymous, 1981).

The usual procedure for enzyme-catalysed fat hydrolysis is to mix the molten fat with lipase dissolved in water or buffer in a stirred-tank reactor for up to 72 hours. If the pH of the reaction mixture is low (<6·5), ionization of free fatty acids will not occur but, at higher pH, addition of alkali to

neutralize released fatty acids is necessary. Calcium ions should also be added to remove inhibitory fatty-acid anions as their insoluble calcium soaps. At the end of the reaction period, the reaction mixture is acidified, if necessary, to convert soaps into free fatty acid, and the fat and aqueous phases are separated and processed for fatty-acid and glycerol recovery respectively. If the extent of hydrolysis achieved in the reaction is insufficient, the fat phase can be reacted with a further batch of lipase solution before the fatty-acid recovery step. Active lipase cannot normally be recovered from the aqueous phase for reuse. An alternative continuous process, in which droplets of fats are passed through the lipase solution, has been described in a recent patent (Nippon Oils and Fats Co., 1983). Some fats are not molten at normal enzyme reaction temperatures, and development of enzyme-catalysed splitting processes for these fats will be dependent on the availability of suitable thermostable lipases.

Because of the expense of the lipase catalyst and the necessity to use long reaction times, enzyme-catalysed splitting processes are more costly to operate than the conventional highly developed and efficient chemical process. Accordingly, enzyme processes will be used in preference to the chemical method, only if improvements in the quality or yield of the reaction products can be achieved. Improvements in reaction-product quality may reduce downstream processing costs as, for example, in the Myoshi process. Higher yields of fatty acids, which are subject to degradation by side reactions in the high-temperature chemical process, may be achievable by use of lipase catalysts, and enzyme-catalysed fat splitting has been suggested for the preparation of hydroxyfatty acids from castor oil and polyunsaturated fatty acids from fish oils (Stansby, 1979).

Partial hydrolysis of oils and fats using fatty-acid-specific lipases as catalysts can be exploited to produce fractions enriched in a particular type of fatty acid. For example, *Geotrichum candidum* lipase specifically releases, from triacylglycerols, unsaturated fatty acids with a *cis* double bond in the 9 position, and Jensen and Pitas (1976) have suggested that this lipase could be used to obtain pure oleic acid from partially hydrogenated fats containing oleate and its isomers. The inability of some lipases to catalyse the release from triacylglycerols of long-chain polyunsaturated fatty acids, such as eicosapentaenoic acid, has been exploited to prepare glyceride or ester fractions enriched in these acids from fish oils (Asahi Denka Co., 1983; Nippon Oils and Fats Co., 1984). The discovery of microbial lipases with other types of fatty-acid specificity could lead to the development of new commercially viable enzyme processes for the production of high-value fatty-acid fractions.

Lipases with 1,3-regiospecificity can, in principle, be used to produce monoacylglycerols from oils and fats. However, it is unlikely that this type of enzyme-catalysed reaction will displace the chemical glycerolysis processes currently used for monoacylglycerol manufacture.

As mentioned previously, the cost of lipase is a major component in the total cost of operating enzyme-catalysed fat-splitting processes. Use of immobilized lipases could permit easy recovery and reuse of the enzyme after

fat splitting, thereby reducing processing costs. Various techniques for lipase immobilization have been developed although none have, as yet, been utilized for commercial fat-hydrolysis processes.

An effective method of lipase immobilization is by entrapment in hydrophobic photo-crosslinked resin gels. *Candida cylindracea* lipase immobilized in these gels showed good activity and stability for the hydrolysis of olive oil in oil–water mixtures and a laboratory-scale reactor for the semi-continuous hydrolysis of olive oil was successfully operated (Kimura *et al.*, 1983). Lipases have also been immobilized by adsorption on to ion-exchange resins or hydrophobic materials such as iodopropyl spherosil and octyl- or phenylsepharose (Lavayre and Baratti, 1982; Yamane, Funada and Ishida, 1982; Yokozeki *et al.*, 1982a), and by covalent attachment to sepharose, polyacrylamide or porous silica beads (Liebermann and Ollis, 1975; Kilara, Shahani and Wagner, 1977; Kimura *et al.*, 1983). Generally, the activity of these immobilized lipases was low and the preparations were not particularly effective as catalysts for the hydrolysis of triacylglycerols in dispersions of oil in water. With oil–water dispersions the reaction rate may be depressed by an inability of oil droplets to penetrate into the pores of the catalysts and gain access to a substantial proportion of the immobilized lipase (Lavayre and Baratti, 1982). These immobilized-lipase catalysts may be more effective when a limited hydrolysis of acylglycerols is required. In these cases a continuous oil phase can be used and water for the hydrolysis can be supplied either by dissolution in the oil phase or from the catalyst particles themselves (Yamane, Funada and Ishida, 1982). A proportion of the lipase produced by lipolytic microbes often remains attached to the cells and it has been suggested that these cells can be used as a source of immobilized lipase. For example, laboratory-scale reactors containing *Rhizopus arrhizus* mycelium have been used for the continuous partial hydrolysis of olive oil dissolved in diisopropyl ether containing a small amount of water (Bell *et al.*, 1981).

Modification of oils and fats

The hydrolysis of triacylglycerol by lipase is an equilibrium reaction, as discussed above (*see Figure 1*). The equilibrium may be perturbed by altering the concentration of reactants and/or products and it has proved possible to shift the equilibrium in the direction of ester synthesis. Exploitation of various aspects of lipase specificity allows the synthesis of compounds that are difficult to prepare by chemical routes and some examples are described later. The application which is likely to be commercialized soon involves the interesterification reaction discussed below.

THE INTERESTERIFICATION REACTION

Natural triacylglycerol mixtures may be directly suitable for some applications but it is frequently necessary to modify their properties, particularly melting characteristics, beyond the point achievable by blending different mixtures. To this end, the oils and fats industry has developed chemically

catalysed hydrogenation (saturation of double bonds) and interesterification processes. Interesterification catalysed by sodium metal or sodium alkoxide results in the random migration of the fatty-acyl residues among the acylglycerol molecules (Sreenivasan, 1978) (*Figure 3*). By carrying out the reaction with a mixture of low- and high-melting triacylglycerols, a product can be obtained with melting and crystallization behaviour significantly better than either component or the blend.

It has proved possible to use lipases to catalyse interesterification by restricting the amount of water in the reaction system whereupon the interesterification reaction predominates over hydrolysis (Unilever, 1980; Fuji Oil Company, 1980, 1981). This has generally been achieved by reaction in organic solvents which also help to dissolve the reactants. It is, however, insufficient merely to lower the water concentration: reversal of the hydrolytic reaction requires operation at a water activity (a_w) below 1 (Halling, 1984); not surprisingly, attempts at interesterification in predominantly aqueous systems resulted in poor yield (Stevenson, Luddy and Rothbart, 1979).

Lipase-catalysed interesterification has been applied particularly to the production of cocoa-butter-type triacylglycerols and these processes exploit the 1,3-regiospecificity common among microbial lipases. The types of triacylglycerol products obtainable by lipase-catalysed interesterification depend on the specificity of the lipase used. The various possibilities have been widely discussed (Jensen *et al.*, 1978; Macrae, 1983a). *Figure 4* illustrates the reactions catalysed by a 1,3-regiospecific lipase involving (a) triacylglycerols only, and (b) triacylglycerol with added fatty acid. The interesterification reaction takes place between residues in the 1 and 3 positions in the case of triacylglycerol/triacylglycerol reactions or between these residues and added free fatty acids in the case of triacylglycerol/fatty-acid reactions. Such reactions have been used to enrich cheap fats such as palm-oil fractions in the

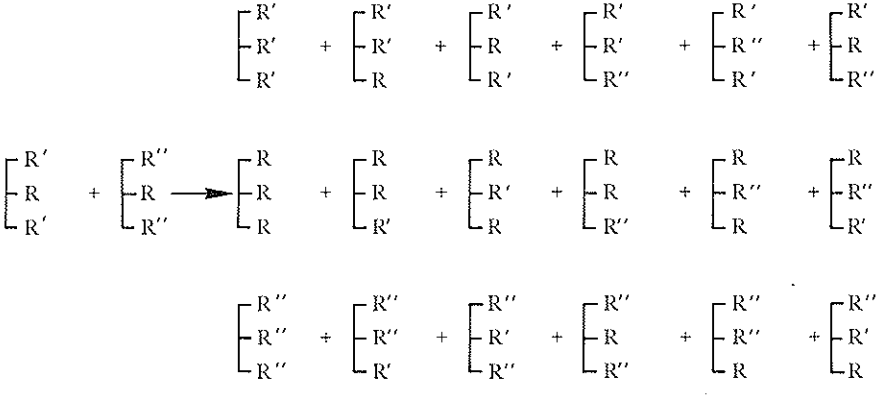

Figure 3. Random interesterification of a triacylglycerol mixture. R, R′ and R″ represent different fatty acyl groups.

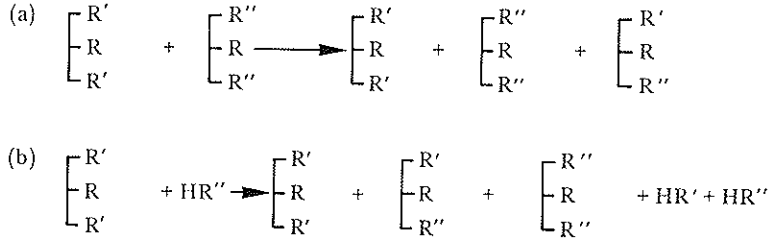

Figure 4. Interesterification with a 1,3-specific lipase. (a) Interesterification with two triacylglycerols. (b) Interesterification with a triacylglycerol and a fatty acid. R, R′ and R″ represent different fatty acid groups.

POSt* and StOSt† triacylglycerols important in confectionery fats (Unilever, 1980; Macrae, 1983a,b, 1984). Microbial lipases particularly suitable for such reactions are those from *Aspergillus niger*, *Mucor javanicus* and several *Rhizopus* species.

Different products are, of course, obtained with random lipases such as those from *Candida cylindracea* and some bacteria. In this case the reaction product is that obtained by the chemically catalysed route (*Figure 3*) ignoring any side reactions. Despite the lipase-catalysed process operating at lower temperatures, it would seem unlikely that the random enzymic process could compete with the chemical route on the basis of energy-cost savings alone.

Fatty-acid-specific lipases such as that from *Geotrichum candidum*, specific for unsaturated residues with a *cis*-Δ9 double bond, can also act as interesterification catalysts (Macrae, 1983a). In this case, there is a relatively minor change in functional properties of the reactants because only unsaturated residues are subject to exchange, although such alterations may be sufficient to 'fine tune' a product to a particular application.

Although triacylglycerol or triacylglycerol/fatty-acid reaction systems would appear to be the simplest basic substrates for lipase-catalysed interesterification, other processes have claimed the use of methyl or ethyl esters of fatty acids (Fuji Oil Company, 1980, 1981). It has also been reported that fatty-acid anhydrides are lipase substrates and could be used in interesterification reactions (Aneja, 1984).

INTERESTERIFICATION CATALYSTS

For industrial exploitation of interesterification reactions, reuse of the lipase is almost certainly necessary. To this end, lipases have been immobilized in a variety of ways, depending partly on the end use of the catalyst. Some of the methods have been discussed above in relation to fat splitting. In studies of esterification reactions, mycelial-bound enzymes have been used as catalysts

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* POSt: 1(3)-palmitoyl, 2-oleoyl, 3(1)-stearoyl glycerol. Fatty acyl residues designated thus: P: palmitoyl; O: oleoyl; St: stearoyl.

†StOSt: 1(3)-stearoyl, 2-oleoyl, 3(1)-stearoylglycerol.

(Bell *et al.*, 1978; Patterson *et al.*, 1979; Knox and Cliffe, 1984). These reactions are discussed further below.

Most work on interesterification has used biomass-free extracellular microbial lipases entrapped in a matrix or coated on a particulate support. Diatomaceous earth has been successfully used as a support in several investigations (Unilever, 1980; Tanaka *et al.*, 1981; Yokozeki *et al.*, 1982a; Kimura *et al.*, 1983). The lipase may be immobilized on the diatomaceous earth by simple adsorption of a lipase solution (Yokozeki *et al.*, 1982a) or by acetone precipitation (Unilever, 1980; Macrae, 1983a). The importance of the nature of the diatomaceous earth has been highlighted by the detailed study of Wisdom *et al.* (1984) who showed that Hyflo Supercel from Johns-Manville offered the appropriate surface properties for an interesterification catalyst support. Catalysts may be stored for considerable periods in a dry state.

Lipases are effectively insoluble in the organic phase and entrapment is unnecessary for retention of the enzyme on a support. Nevertheless, Yokozeki *et al.* (1982a) studied catalysts prepared by adsorbing lipase on to diatomaceous earth and then entrapping the enzyme using various resins prepared from photocrosslinkable pre-polymers. Such entrapment methods offer the possibility of varying the hydrophilicity/hydrophobicity of the matrix and could be used to improve diffusion of various hydrophobic substrates into the gel (Fukui and Tanaka, 1984). In the interesterification reaction, lipase adsorbed on to diatomaceous earth gave the best activity compared with entrapment of free enzyme in various resins or with attachment to Spherosil by covalent binding, ionic interaction or adsorption (Yokozeki *et al.*, 1982a). For entrapment in gels, use of hydrophobic matrices such as ENTP-2000 (Fukui and Tanaka, 1984) generally gave improved catalysts compared with hydrophilic gels, although dispersion of the lipase on diatomaceous earth before entrapment was required for reproducible activity. When used repeatedly in batch interesterification reactions, however, the ENTP-2000-entrapped catalyst was apparently more stable than that prepared by simple adsorption on diatomaceous earth (Yokozeki *et al.*, 1982a,b).

INTERESTERIFICATION REACTION SYSTEMS

In developing experimental reactor systems for enzymic interesterification, the reactants have generally been dissolved in an organic solvent to maintain the reactant/product stream in the liquid state and to limit the amount of water in the system. Although lipases have been studied in a variety of organic solvents (Blain, Akhtar and Patterson, 1976), water-immiscible solvents appear to be preferable, if not essential, for these reactions and for transformations of other lipophilic compounds (Fukui *et al.*, 1980). The catalyst may be stirred with the reactants dissolved in an alkane such as 100–120°C petroleum ether (a stirred-tank reactor, STR) or may be passed through a packed bed of the catalyst (packed-bed reactor, PBR). Productivity in a continuously operated PBR is likely to be greater than that in an STR,

even though the same catalyst batch may be used up to 10 times in repeated STR operations (Macrae, 1983a).

When used in either STR or PBR, interesterification catalysts require a small amount of water to activate them from the dry state and to maintain their activity. Hence, inactive dried catalysts are hydrated with up to 10% (w/w) water before use and the feedstream is maintained partly saturated with water during operation of PBR (Macrae, 1983a). During operation, an equilibrium is rapidly established between triacylglycerol, water, free fatty acids and diacylglycerol. At steady state in a PBR operated with a water-saturated feed, by-product formation due to hydrolysis resulted in a loss of only about 14% (w/w) of input triacylglycerol (data of Macrae, 1983a).

Although hydrolysis may be restricted to low levels, even when water-saturated feedstreams are used, other investigations have been directed towards suppressing hydrolysis further. Workers at the Fuji Oil Company (Fuji Oil Company, 1981) employed a controlled drying process to prepare interesterification catalysts consisting of microbial lipases supported on diatomaceous earth. When dried slowly for an initial period, the catalyst apparently retained interesterification activity in otherwise dry fatty acid/triacylglycerol or fatty-acid lower alcohol ester/triacylglycerol reaction systems. Another approach was used by a Japanese group (Ajinomoto Company, 1981) who replaced the water usually required for catalyst activation by diols or triols such as ethylene glycol, propylene glycol or glycerol. It is claimed that the yield of interesterified triacylglycerol product was increased, presumably because of a reduction in hydrolysis during the reaction. It is not clear, however, what effect such a treatment had on reaction rate: it has been long known that lipase-catalysed esterification activity is undetectable in 100% glycerol (Iwai, Tsujisaka and Fukumoto, 1964).

There is considerable effort, notably in Japan and Europe, to commercialize lipase-catalysed interesterification for the production of relatively valuable triacylglycerol mixtures such as cocoa-butter equivalents. It is likely that pre-production or production plants operating such processes will come on stream in the next few years. Future exploitation of this technology for larger-tonnage, lower-added-value products such as margarine fats will necessitate cheaper processes. The onus will be on enzyme technologists to provide catalysts showing greater productivity attributable to longer operating life and/or greater interesterification activity. In this respect, thermophilic lipases might be exploited in order to operate processes at higher temperatures and to prolong the life of the catalyst, because thermophile enzymes may be more stable to denaturation than are mesophilic enzymes (Zale and Klibanov, 1983; Wasserman, 1984).

Synthesis of organic compounds

The broad substrate specificity of lipases has been employed in studies of synthesis of various compounds other than triacylglycerols. While a considerable range of compounds of diverse chemical structure may be acted upon,

the enzymes retain regio- and/or stereospecificity, allowing the preparation of compounds difficult to obtain by chemical routes. The reactions catalysed may be hydrolyses, ester syntheses or ester-exchange reactions. Ester synthesis reactions make use of the law of mass action to drive the equilibrium in the direction of synthesis by removing water generated during the reaction. Ester-exchange reactions take place at low water activity. In these respects, these two reactions have much in common with the technology described above for lipase-catalysed interesterification reactions.

HYDROLYSIS

While fat splitting has been discussed above in relation to fatty-acid and glycerol production from triacylglycerols, hydrolyses of other esters might become important steps in future synthetic routes. The early work of Oritani and Yamashita (1973) indicated the potential of microbial lipases and esterases for the resolution of racemic mixtures of terpene alcohol esters, by exploiting the stereospecificity of such enzymes. Several examples of reactions of potential interest in natural-product chemistry recently have been reviewed by Sariaslani and Rosazza (1984). Resolution of isomeric alcohols, notably terpene alcohols, by chemical esterification to yield acetate or chloroacetate esters, followed by exploitation of lipase stereospecificity giving asymmetric hydrolysis of the esters, may be commercially attractive for higher-value products. The enzymes may be commercially available (e.g. *Candida cylindracea* lipase) or prepared from a variety of microbial sources, both bacterial (e.g. *Brevibacterium ammoniagenes, Acinetobacter calcoaceticus, Pseudomonas aeruginosa*) and fungal (including *Mucor, Streptomyces, Fusarium* as well as the *Rhizopus* species mentioned earlier). The *Pseudomonas aeruginosa* lipase which has been used (Iriuchijima and Kojima, 1982) is often described as a lipoprotein lipase. The application of regiospecificity is well shown in an example cited by Sariaslani and Rosazza (1984). *Candida cylindracea* lipase was able to hydrolyse only the ester in the 2-position of boldine diacetate (a partially aromatic, nitrogen-containing polycyclic compound); the 9-acetate residue remained unaffected.

Reaction systems suitable for hydrolytic transformations of this type have not been optimized. Many of the potential products are low-tonnage high-value compounds of importance in flavour, fragrance and drug industries where processing cost is of lesser or minor importance. Adequate conversions may be achieved by simple STR technology, either treating the lipase as disposable or making use of suitably immobilized enzyme (Lavayre and Baratti, 1982) as previously discussed.

ESTER-EXCHANGE REACTIONS

In reactions analogous to the interesterification reactions discussed above, the stereospecificity of lipases may be employed to prepare optically active esters from racemic mixtures. Cambou and Klibanov (1984) used *Candida cylindracea* lipase immobilized on Chromosorb (diatomaceous earth) in the

presence of only a small amount of water to carry out alcoholysis reactions between a racemic alcohol and an appropriate ester such as tributyrin. The products were the optically active butyrate ester of the alcohol and unreacted alcohol enantiomer. The other enantiomer might be obtained by subsequent alkaline hydrolysis of the ester. Regiospecificity was also retained, primary alcohols reacting more readily than secondary alcohols with this enzyme. Because the lipase immobilization, reaction conditions and potential reactor configuration are essentially the same as those described for triacylglycerol interesterification, this process offers potential for scaling up to a level suitable for synthesis or optical resolution of medium-value compounds.

Workers in Klibanov's group have taken these alcoholysis reactions a stage further with the observation (Zaks and Klibanov, 1984) that the reaction can take place in almost completely dry systems, even at temperatures up to 100°C. Activity at such temperatures would not normally be expected of the mesophile enzymes used. In addition, the stability of the enzymes under such conditions was good. However, the absence of water does affect the lipase: porcine pancreatic lipase under dry conditions was inactive towards tertiary alcohols, perhaps because of restricted mobility of the lipase active-site area resulting in the exclusion of bulky substrates (Zaks and Klibanov, 1984). It might be possible in the future to take advantage of these observations to avoid hydrolytic side reactions in lipase-catalysed processes.

ESTER SYNTHESIS

The potential for net synthesis of esters by reversal of lipase hydrolysis has long been recognized (Iwai, Tsujisaka and Fukumoto, 1964) but high yields require removal of the water formed (Unilever, 1982). This may be accomplished either by direct removal of water by some separation or drying means, or possibly by use of agents such as glycerol or polymers to maintain the a_w below 1. Glycerol is also, of course, a reactant in triacylglycerol synthesis and high concentrations will tend to shift the equilibrium towards synthesis. Thus Iwai, Tsujisaka and Fukumoto (1964) carried out the synthesis of acylglycerols from glycerol and oleic acid in the presence of polyvinyl alcohol. Later, Tsujisaka, Okumura and Iwai (1977) investigated various additives to improve acylglycerol synthesis. These authors noted that high glycerol concentrations were required for synthesis but, in 95% glycerol, activity was undetectable. Lipases from *Aspergillus niger*, *Rhizopus delemar*, *Geotrichum candidum* and *Penicillium cyclopium* were all active in synthesis and their fatty-acid and positional specificities appeared to be the same in the synthetic direction as in hydrolysis. These four lipases could be used to synthesize esters from a variety of alcohols and acids, according to their specificity (Okumura, Iwai and Tsujisaka, 1979). Terpene alcohol esters, of potential use as fragrances, could also be synthesized (Iwai, Okumura and Tsujisaka, 1980) although the authors apparently did not investigate the stereochemistry of the products.

Water removal during esterification reactions between oleic acid and glycerol has been carried out by room-temperature distillation under vacuum

(Linfield *et al.*, 1984b). Removal of too much water stopped the reaction, presumably because the dry precipitated lipase was inactivated. Knox and Cliffe (1984) used a packed bed of *Rhizopus arrhizus* mycelia to catalyse the esterification of long-chain acids and alcohols to wax esters. The reaction took place in solvent (hexane) using a recycle loop including a bed of molecular sieve to remove the water formed in the esterification reaction. It has been claimed that esterification reactions can take place in the virtual absence of water (Haarman and Reimer, 1982). However, as water is generated during the reaction, it would be necessary to remove this water to maximize the yield of ester.

Recently, Seino *et al.* (1984) have reported the esterification of carbohydrates and fatty acids using microbial lipases from a variety of sources, although *Candida cylindracea* lipase was the most active. This report is in apparent contradiction to the finding of Okumura, Iwai and Tsujisaka (1979) who were unable to detect synthesis of sugar alcohol esters despite using lipases from similar sources, although *Candida* lipase was not tested. Sugar esters, notably sucrose esters, have a relatively large market as emulsifiers in foods, pharmaceuticals and cosmetics. Some esters may also have important medicinal properties, including antitumour activity (Nishikawa *et al.*, 1981). Enzymic synthesis would be attractive, relative to chemically catalysed routes, in terms of energy saving and other reduced processing costs, as well as in avoiding side reactions that result in colouring of the chemically synthesized product. In addition, the exploitation of lipase regiospecificity could give products unobtainable by the chemical route. This area is likely to receive increased attention in future years.

Two potentially interesting developments in reaction systems for ester synthesis have recently been published. Hoq *et al.* (1984) reported on the construction of a membrane bioreactor in which an aqueous glycerol/lipase solution was separated from fatty acid by a hydrophobic microporous polypropylene membrane. The reaction took place presumably at the membrane interface and the acylglycerol product was obtained in the fatty-acid stream. The aqueous glycerol/lipase phase was recycled (1) to replenish glycerol used and (2) to remove water formed by passage through a bed of molecular sieve. The reactor could be run for extended periods: the lipase half-life was 52 days in the presence of stabilizing Ca^{2+} ions. Morita *et al.* (1984) have employed reverse micelles incorporating lipase to synthesize triacylglycerol from 1,2-diacylglycerol and fatty acid in an organic solvent. In this system the amount of water in the micelles was restricted by using a particular H_2O/surfactant (phosphatidylcholine) ratio. In their experimental system, the amount of water liberated during the esterification reaction represented only about 2% of that in the original system. Scale-up of such a reaction system would require a means of removing the liberated water and also of retaining the lipase-containing micelles for reuse.

Although considerable effort has been devoted to the synthesis of triacylglycerols by esterification reactions, yields are generally less than 70%. It is likely that esterification will be limited in any future application to synthesis of products of value greater than that of triacylglycerol, especially as

lipase-catalysed interesterification can give triacylglycerols of required quality at suitable cost.

Detergent products

Lipases are not only attractive as catalysts for the modification and synthesis of useful compounds as discussed above, but are of potential use as functional components of mixtures. For example, a large potential market for lipolytic enzymes is in detergent formulations where they could be effective in removing fatty soil, particularly at low washing temperatures.

Use of hydrolytic enzymes in household detergent formulation has been restricted mainly to the inclusion of proteases (Starace, 1983). Removal of fatty deposits by lipase is attractive if suitable enzymes can be obtained which are active under washing conditions. Bacterial proteases used in washing detergents show maximal activity at about pH 11 and 60°C. It could be advantageous for lipases to be able to function under similar conditions, although the increasing use of lower washing temperatures could allow the application of lipases with lower temperature optima. Lipases would also need to be stable to protease attack and to function in the presence of components of washing formulations such as surfactants, which are known lipase inhibitors (Wills, 1955; Treves *et al.*, 1984). Although it may be possible to select for organisms producing lipases with acceptable pH optima and thermostability (Watanabe *et al.*, 1977; Kokusho, Machida and Iwasaki, 1982a,b), studies of application of lipases in washing formulations have, perhaps, been hampered by lack of enzyme samples to test. Literature reports of evaluation of lipases in washing formulations are sparse. A Japanese study is referred to by Tatara *et al.* (1983) but the most detailed work in the open literature is that of Andree, Müller and Schmid (1980) who also reviewed earlier patent literature. The latter authors tested several commercially available microbial lipases and pancreatic lipase in various standard washing formulations. The lipases were optimally active at neutral pH and in the 35–45°C range. Washing tests indicated that, with the lipases tested, improved efficiency could be attained at low temperatures (20–30°C) but required a high lipase concentration and was not seen with all types of fabric. The magnitude of the lipase effect was not great and similar improvements could be obtained by increasing the concentration of non-ionic surfactants in the formulation.

Lipases are unable to digest most fatty deposits to fully water-soluble products but their action may improve soil removal by the surfactants present in the formulation. Indeed, some improvement in detergent performance might well be evident as a result of improved surfactant properties of lipolysis products themselves, as noted when phospholipase A-2 acts on lecithin (Starace, 1983). Despite the rather unpromising results reported, that there is continued interest in the use of lipases in detergent formulations is shown by recent patents in the area (Showa-Denko, 1982; Lion, 1983). Detergent applications represent a potential high-tonnage use of lipases in the future.

Analytical applications

Many enzymes find a market in basic research and in analysis, and lipases are no exception. Often, the requirements for stability and acceptable activity under apparently adverse operating conditions, which are generally important in other applications, are replaced for analytical use by the need for high enzyme-specific activity and minimal side reactions. This sets a challenge for enzyme manufacturers to produce relatively pure, highly active enzyme preparations with good batch-to-batch consistency. As the amounts of enzymes needed for analytical uses are small, satisfactory preparations can generally be obtained.

The specificity of lipases has been exploited for many years in determining the structure of triacylglycerols (Coleman, 1963). In addition to the use of the fatty-acid-specific lipase from *Geotrichum candidum* (Sampugna and Jensen, 1969), the almost absolute 1,3-regiospecificity of pancreatic lipase has been widely used to determine the fatty-acid distribution in many natural triacylglycerols. Such lipase applications will continue while other basic investigations involving lipase-catalysed hydrolysis could become important. For example, Morii (1983) used a limited digestion of liver tissue by *Rhizopus arrhizus* lipase and phospholipase A_2 to investigate intracellular lipid distribution.

An important current analytical use of lipase enzymes is in determination of lipids, notably for clinical purposes. The basic concept is to utilize a lipase to generate glycerol from the triacylglycerol in the sample and to quantify the released glycerol by subsequent chemical or enzymic methods (Bucolo and David, 1973). Such procedures have been automated (Megraw, Dunn and Biggs, 1979). *Pseudomonas aeruginosa* lipase has been used in such applications. Uwajima and Terada (1981) have recently discussed the analysis of serum phospholipids and triacylglycerols. In both cases, the products of hydrolysis of either phospholipid using phospholipase D (EC 3.1.4.4), or of triacylglycerol using lipase, serve as substrates for further enzymes generating easily quantifiable products. Thus choline released by the action of phospholipase D is oxidized by choline oxidase (EC 1.1.3.17), and glycerol from the action of lipoprotein lipase is oxidized by glycerol oxidase (EC 1.1.3.21). Both of these secondary reactions liberate hydrogen peroxide which may be quantitatively detected by a colour reaction or electrochemically. Matsumoto *et al.* (1980) have immobilized choline oxidase at an amperometric H_2O_2 electrode by glutaraldehyde cross-linking. Such technology might be applicable to immobilizing glycerol oxidase for the determination of glycerol produced in lipolysis of triacylglycerol; alternatively, the reaction might be linked to a sensor via glycerol kinase (EC 2.7.1.30) or glycerol dehydrogenase (EC 1.1.1.6). Enzymic methods for the detection of fatty acid released during lipolysis have been suggested (Toyo Jozo Company, 1983; Sherwood-Medical Industries, 1983) and these could also be used in appropriate sensors. The range of options in the construction of biosensors has been reviewed recently by Aston and Turner (1984). A practical, reusable biosensor for triacylglycerol will require immobilization of the lipase as well as of the enzymes involved in the sensing reaction. The reports of immobilized lipases active

and stable in hydrolysis and interesterification reactions (*see above*) suggest that a triacylglycerol biosensor might be a practical proposition, provided that the substrate can be presented in a manner which meets the interfacial requirements of lipase activity (Brockerhoff and Jensen, 1974).

References

ADAMS, D. M. AND BRAWLEY, T. G. (1981). Factors influencing the heat resistance of a heat-resistant lipase of *Pseudomonas*. *Journal of Food Science* **81**, 673–676.

AISAKA, K. AND TERADA, O. (1980). Purification and properties of lipoprotein lipase from *Rhizopus japonicus*. *Agricultural and Biological Chemistry* **44**, 799–805.

AJINOMOTO COMPANY (1981). US Patent 4275011.

AKESSON, B., GRONOWITZ, S., HERSLOF, B., MICHELSON, P. AND OLIVECRONA, T. (1983). Stereospecificity of different lipases. *Lipids* **18**, 313–318.

ANONYMOUS (1981). Untitled article. *Japan Chemical Week*, 14 May, p. 2.

ANDREE, H., MÜLLER, W.-R. AND SCHMID, R. D. (1980). Lipases as detergent components. *Journal of Applied Biochemistry* **2**, 218–229.

ANEJA, R. (1984). Enzymic esterification and interesterification. *Journal of the American Oil Chemists' Society* **61**, 661.

ASAHI DENKA CO. (1983). Japanese Patent 58165796.

ASTON, W. J. AND TURNER, A. P. F. (1984). Biosensors and biofuel cells. In *Biotechnology and Genetic Engineering Reviews* (G. E. Russell, Ed.), volume 1, pp. 89–120. Intercept, Ponteland, Newcastle upon Tyne.

BELL, G., BLAIN, J. A., PATTERSON, J. D. E., SHAW, C. E. L. AND TODD, R. (1978). Ester and glyceride synthesis by *Rhizopus arrhizus* mycelia. *FEMS Microbiology Letters* **3**, 223–225.

BELL, G., TODD, J. R., BLAIN, J. A., PATTERSON, J. D. E. AND SHAW, C. E. L. (1981). Hydrolysis of triglyceride by solid phase lipolytic enzymes of *Rhizopus arrhizus* in continuous reactor systems. *Biotechnology and Bioengineering* **23**, 1703–1719.

BENZONANA, G. AND DESNUELLE, P. (1965). Étude cinétique de l'action de la lipase pancréatique sur des triglycérides en emulsion. Essai d'use enzymologie en milieu hétérogène. *Biochimica et biophysica acta* **105**, 121–136.

BENZONANA, G. AND ESPOSITO, S. (1971). On the positional and chain specificities of *Candida cylindracea* lipase. *Biochimica et biophysica acta* **231**, 15–22.

BLAIN, J. A., AKHTAR, M. W. AND PATTERSON, J. D. E. (1976). Study of lipase activities using organic solvent systems. *Pakistan Journal of Biochemistry* **10**, 41–53.

BORGSTROM, B. AND BROCKMAN, H. L. (1984). *Lipases*. Elsevier, Amsterdam.

BROCKERHOFF, H. AND JENSEN, R. G. (1974). Lipases. In *Lipolytic Enzymes*, pp. 25–175. Academic Press, New York.

BUCOLO, G. AND DAVID, H. (1973). Quantitative determination of serum triglycerides by the use of enzymes. *Clinical Chemistry* **19**, 476–482.

CAMBOU, B. AND KLIBANOV, A. M. (1984). Preparative production of optically active esters and alcohols using esterase-catalysed stereospecific transesterification in organic media. *Journal of the American Oil Chemists' Society* **106**, 2687–2692.

CANIONI, P., JULIEN, R., RATHELOT, J. AND SARDA, L. (1977). Pancreatic and microbial lipases: A comparison of the interaction of pancreatic colipase with lipases of various origins. *Lipids* **12**, 393–397.

CHAPMAN, D. (1969). *Introduction to Lipids*. McGraw-Hill, London.

COLEMAN, M. H. (1963). The structural investigation of natural fats. *Advances in Lipid Research* **1**, 1–64.

DE CARO, J., BOUDOUARD, M., BONICEL, J., GUIDONI, A., DESNUELLE, P. AND ROVERY, M. (1981). Porcine pancreatic lipase: completion of the primary structure. *Biochimica et biophysica acta* **671**, 129–138.

FUJI OIL COMPANY (1980). UK Patent Application 2,035,359A.

FUJI OIL COMPANY (1981). European Patent Application 0,035,883.

FUKUI, S. AND TANAKA, A. (1984). Application of biocatalysts immobilized by prepolymer methods. *Advances in Biochemical Engineering/Biotechnology* **29**, 1–33.

FUKUI, S., AHMED, S. A., OMATA, T. AND TANAKA, A. (1980). Bioconversion of lipophilic compounds in non-aqueous solvent. Effect of gel hydrophobicity on diverse conversions of testosterone by gel-entrapped *Nocardia rhodocrous*. *European Journal of Applied Microbiology and Biotechnology* **10**, 289–301.

FUKUMOTO, J., IWAI, M. AND TSUJISAKA, Y. (1963). Studies on lipase. I. Purification and crystallisation of a lipase secreted by *Aspergillus niger*. *Journal of General and Applied Microbiology* **9**, 353–361.

HAARMAN AND REIMER (1982). European Patent Application 0061023.

HALLING, P. J. (1984). Effects of water on equilibria catalysed by hydrolytic enzymes in biphasic reaction systems. *Enzyme and Microbial Technology* **6**, 513–516.

HASSING, G. S. (1971). Partial purification and some properties of a lipase from *Corynebacterium acnes*. *Biochimica et biophysica acta* **242**, 381–394.

HATA, Y., MATSUURA, K., TANAKA, N., KAKUDO, M., SUGIHARA, A., IWAI, H. AND TSUJISAKA, K. (1979). Low resolution crystal structure of lipase from *Geotrichum candidum* (ATCC 34614). *Journal of Biochemistry* **86**, 1821–1827.

HOQ, M. M., YAMANE, T., SHIMIZU, S., FUNADA, T. AND ISHIDA, S. (1984). Continuous synthesis of glycerides by lipase in a microporous membrane reactor. *Journal of the American Oil Chemists' Society* **61**, 776–781.

INGHAM, E., HOLLAND, K. T., GOWLAND, G. AND CUNLIFFE, W. J. (1981). Partial purification and characterisation of lipase from *Propionibacterium acnes*. *Journal of General Microbiology* **124**, 393–401.

IRIUCHIJIMA, S. AND KOJIMA, W. (1982). Asymmetric hydrolysis of (±)-1,2-diacetoxy-3-chloropropane and its related compounds with lipase. Synthesis of optically pure (*s*)-propanolol. *Agricultural and Biological Chemistry* **46**, 1153–1157.

ISHIHARA, I., OKUYAMA, H., IKEZAWA, H. AND TEJIMA, S. (1975). Studies on lipase from *Mucor javanicus*. I. Purification and properties. *Biochimica et biophysica acta* **388**, 413–422.

IWAI, M., OKUMURA, S. AND TSUJISAKA, Y. (1975). The comparison of the properties of two lipases from *Penicillium cyclopium* Westring. *Agricultüral and Biological Chemistry* **39**, 1063–1070.

IWAI, M., OKUMURA, S. AND TSUJISAKA, Y. (1980). Synthesis of terpene alcohol esters by lipase. *Agricultural and Biological Chemistry* **44**, 2731–2732.

IWAI, M., TSUJISAKA, Y. AND FUKUMOTO, J. (1964). Studies on lipase. II. Hydrolytic and esterifying actions of crystalline lipase of *Aspergillus niger*. *Journal of General and Applied Microbiology* **10**, 13–22.

JENSEN, R. G. (1974). Characteristics of the lipase from the mold, *Geotrichum candidum*: a review. *Lipids* **9**, 143–157.

JENSEN, R. G. (1983). Detection and determination of lipase (acylglycerol hydrolase) activity from various sources. *Lipids* **18**, 650–657.

JENSEN, R. G. AND PITAS, R. E. (1976). Specificity of the lipase from *Geotrichum candidum*. In *Lipids* (R. Paoletti, G. Porcellati and G. Jacini, Eds), pp. 141–146. Raven Press, New York.

JENSEN, R. G., DEJONG, F. A. AND CLARK, R. N. (1983). Determination of lipase specificity. *Lipids* **18**, 239–252.

JENSEN, R. G., GERRIOR, S. A., HAGERTY, M. M. AND MCMAHON, K. E. (1978). Preparation of acylglycerols and phospholipids with the aid of lipolytic enzymes. *Journal of the American Oil Chemists' Society* **55**, 422–427.

KILARA, A., SHAHANI, K. M. AND WAGNER, F. W. (1977). Preparation and properties of immobilised papain and lipase. *Biotechnology and Bioengineering* **19**, 1703–1714.

KIMURA, Y., TANAKA, A., SONOMITO, K., NIHARA, T. AND FUKUI, S. (1983). Applica-

tion of immobilised lipase to hydrolysis of triacylglyceride. *European Journal of Applied Microbiology and Biotechnology* **17**, 107–112.

KNOX, T. AND CLIFFE, K. R. (1984). Synthesis of long-chain esters in a loop reactor system using a fungal cell bound enzyme. *Process Biochemistry* **19**, 188–192.

KOKUSHO, Y., MACHIDA, H. AND IWASAKI, S. (1982a). Studies on alkaline lipase: isolation and identification of lipase producing organisms. *Agricultural and Biological Chemistry* **46**, 1159–1164.

KOKUSHO, Y., MACHIDA, H. AND IWASAKI, S. (1982b). Production and properties of alkaline lipase from *Alcaligenes* sp. strain No. 679. *Agricultural and Biological Chemistry* **46**, 1743–1750.

LABOUREUR, P. AND LABROUSSE, M. (1966). Isolation, purification and properties of the lipase of *Rhizopus arrhizus* var. delemar. *Bulletin de la Société de Chimie Biologique* **48**, 747–769.

LAVAYRE, J. AND BARATTI, J. (1982). Preparation and properties of immobilised lipases. *Biotechnology and Bioengineering* **14**, 1007–1013.

LAVAYRE, J., VERRIER, J. AND BARATTI, J. (1982). Stereospecific hydrolysis by soluble and immobilised lipases. *Biotechnology and Bioengineering* **24**, 2175–2187.

LIEBERMAN, R. B. AND OLLIS, D. F. (1975). Hydrolysis of tributyrin in a fluidised lipase reactor. *Biotechnology and Bioengineering* **17**, 1401–1419.

LINFIELD, W. M., O'BRIEN, D. J., SEROTA, S. AND BARAUSKAS, R. A. (1984a). Lipid–lipase interactions. I. Fat splitting with lipase from *Candida rugosa*. *Journal of the American Oil Chemists' Society* **61**, 1067–1071.

LINFIELD, W. M., BARAUSKAS, R. A., SIVIERI, L., SEROTA, L. AND STEVENSON, R. W. (1984b). Enzymatic fat hydrolysis and synthesis. *Journal of the American Oil Chemists' Society* **61**, 191–195.

LION (1983). Japanese patent 58045300.

LIU, W. H., BEPPU, T. AND ARIMA, K. (1973a). Purification and general properties of the lipase of thermophilic fungus *Humicola lanuginosa* S-38. *Agricultural and Biological Chemistry* **37**, 157–163.

LIU, W. H., BEPPU, T. AND ARIMA, K. (1973b). Substrate specificity and mode of action of the lipase of thermophilic fungus *Humicola lanuginosa* S-38. *Agricultural and Biological Chemistry* **37**, 1349–1355.

MACRAE, A. R. (1983a). Lipase-catalysed interesterification of oils and fats. *Journal of the American Oil Chemists' Society* **60**, 243A–246A.

MACRAE, A. R. (1983b). Extracellular microbial lipases. In *Microbial Enzymes and Technology* (W. M. Fogarty, Ed.), pp. 225–250. Applied Science, London.

MACRAE, A. R. (1984). Microbial lipases as catalysts for the interesterification of oils and fats. In *Biotechnology for the Oils and Fats Industry: American Oil Chemists' Society, Monograph No. 11*. (C. Ratledge, P. S. S. Dawson and J. B. M. Rattray, Eds), pp. 189–198. AOCS, Illinois.

MATSUMOTO, K., SEIJO, H., KARUBE, I. AND SUZUKI, S. (1980). Amperometric determination of choline with use of immobilised choline oxidase. *Biotechnology and Bioengineering* **22**, 1071–1086.

MEGRAW, R. E., DUNN, D. E. AND BIGGS, H. G. (1979). Manual and continuous-flow colorimetry of triacylglycerols by a fully automatic method. *Clinical Chemistry* **25**, 273–278.

MORII, S. (1983). Ultracytochemical observations on intracytoplasmic lipids by enzymic digestion methods. *Journal of Histochemistry and Cytochemistry* **31**, 150–153.

MORITA, S., NARITA, H., MATOBA, T. AND KOTO, M. (1984). Synthesis of triacylglycerol by lipase in phosphatidylcholine reverse micellar system. *Journal of the American Oil Chemists' Society* **61**, 1571–1574.

MOSKOWITZ, G. J., CASSAIGNE, R., WEST, I. R., SHEN, T. AND FELDMAN, L. I. (1977). Hydrolysis of animal fat and vegetable oil with *Mucor miehei* esterase. Properties of the enzyme. *Journal of Agricultural and Food Chemistry* **25**, 1146–1150.

NAGAOKA, K. AND YAMADA, Y. (1973). Purification of *Mucor* lipases and their properties. *Agricultural and Biological Chemistry* **37**, 2791–2796.

NIPPON OILS AND FATS Co. (1983). Japanese Patent 58126794.

NIPPON OILS AND FATS Co. (1984). Japanese Patent 59014793.

NISHIKAWA, Y., YOSHIMOTO, K., NISHIJIMA, M., FUKUOKA, F. AND IKEKAWA, T. (1981). Chemical and biochemical studies on carbohydrate esters. IX Antitumour effects of selectively fatty acylated products of maltose. *Chemical & Pharmaceutical Bulletin* **29**, 505–513.

NOMA, A. AND BORGSTROM, B. (1971). The acid lipase of castor beans: positional specificity and reaction mechanisms. *Biochimica et biophysica acta* **227**, 106–115.

OKUMURA, S., IWAI, M. AND TSUJISAKA, Y. (1976). Positional specificities of four kinds of microbial lipases. *Agricultural and Biological Chemistry* **40**, 655–660.

OKUMURA, S., IWAI, M. AND TSUJISAKA, Y. (1979). Synthesis of various kinds of esters by four microbial lipases. *Biochimica et biophysica acta* **575**, 156–165.

OKUMURA, S., IWAI, M. AND TSUJISAKA, Y. (1980). Purification and properties of partial glyceride hydrolase of *Penicillium cyclopium* M1. *Journal of Biochemistry* **87**, 205–211.

ORITANI, T. AND YAMASHITA, K. (1973). Microbial resolution of racemic carvomenthols. *Agricultural and Biological Chemistry* **37**, 1691–1694.

OTA, Y., NAKAMIYA, T. AND YAMADA, K. (1972). On the substrate specificity of the lipase produced by *Candida paralipolytica*. *Agricultural and Biological Chemistry* **36**, 1895–1898.

PATTERSON, J. D. E., BLAIN, J. A., SHAW, L. E. L., TODD, R. AND BELL, G. (1979). Synthesis of glycerides and esters by fungal cell-bound enzymes in continuous reactor systems. *Biotechnology Letters* **1**, 211–216.

SAMPUGNA, J. AND JENSEN, R. G. (1969). Stereospecific analysis of the major triglyceride species in the monounsaturated fraction of cocoa butter. *Lipids* **4**, 444–449.

SARIASLANI, F. S. AND ROSAZZA, J. P. N. (1984). Biocatalysis in natural products chemistry. *Enzyme and Microbial Technology* **6**, 242–253.

SEINO, H., UCHIBORI, T., NISHITANI, T. AND INAMASU, S. (1984). Enzymatic synthesis of carbohydrate esters of fatty acid (I). Esterification of sucrose, glucose, fructose and sorbitol. *Journal of the American Oil Chemists' Society* **61**, 1761–1765.

SEMERIVA, M. AND DESNUELLE, P. (1979). Pancreatic lipase and colipase. An example of heterogeneous biocatalysis. *Advances in Enzymology* **48**, 319–370.

SEMERIVA, M. AND DUFOUR, C. (1972). Further studies on the extracellular lipase of *Rhizopus arrhizus*. *Biochimica et biophysica acta* **260**, 393–400.

SEMERIVA, M., BENZONANA, G. AND DESNUELLE, P. (1967). Lipase of *Rhizopus arrhizus* I. Positional specificity. *Bulletin de la Société de Chimie Biologique* **49**, 71–81.

SEMERIVA, M., BENZONANA, G. AND DESNUELLE, P. (1969). Some properties of a lipase from *Rhizopus arrhizus*: separation of a glycopeptide bound to the enzyme. *Biochimica et biophysica acta* **191**, 598–610.

SHERWOOD-MEDICAL INDUSTRIES (1983). US Patent 4369250.

SHOWA-DENKO (1982). Japanese Patent 57137400.

SONNTAG, N. O. V. (1979). Fat splitting. *Journal of the American Oil Chemists' Society* **56**, 729A–732A.

SREENIVASAN, V. (1978). Interesterification of fats. *Journal of the American Oil Chemists' Society* **55**, 796–805.

STANSBY, M. E. (1979). Marine-derived fatty acids or fish oils as raw materials for fatty acids manufacture. *Journal of the American Oil Chemists' Society* **56**, 793A–796A.

STARACE, C. A. (1983). Detergent enzymes—past, present and future. *Soap Cosmetics Chemical Specialities* **59**, 48–50F.

STEVENSON, R. W., LUDDY, F. E. AND ROTHBART, H. L. (1979). Enzymatic acyl exchange to vary saturation in di- and triglycerides. *Journal of the American Oil Chemists' Society* **56**, 676–680.

SUGIURA, M. AND ISOBE, M. (1975a). Effects of temperature and state of substrate on the rate of hydrolysis of glycerides by lipase. *Chemical & Pharmaceutical Bulletin* **23**, 681–682.

SUGIURA, M. AND ISOBE, M. (1975b). Studies on the lipase of *Chromobacterium viscosum*. IV. Substrate specificity of a low molecular weight lipase. *Chemical & Pharmaceutical Bulletin* **23**, 1226–1230.

TANAKA, T., ONO, E., ISHIKARA, M., YAMANAKA, S. AND TAKINAMI, K. (1981). Enzymatic acyl exchange of triglyceride in *n*-hexane. *Agricultural and Biological Chemistry* **45**, 2387–2389.

TATARA, T., FUJII, T., KAWASE, T. AND MINAGAWA, M. (1983). Quantitative determination of tri-, di-, monooleins and free oleic acid by the thin layer chromatography–flame ionization detector system using internal standards and boric acid impregnated chromarod. *Lipids* **18**, 732–736.

TOYO JOZO COMPANY (1983). German Patent, 3,223,874.

TREVES, C., VINCENZINI, M. T., FAVILLI, F., VANNI, P. AND BACCARI, V. (1984). On the interaction between synthetic detergents and enzymatic proteins. *Canadian Journal of Biochemistry and Cell Biology* **62**, 55–59.

TSUJISAKA, Y., OKUMURA, S. AND IWAI, M. (1977). Glyceride synthesis by four kinds of microbial lipase. *Biochimica et biophysica acta* **489**, 415–422.

UNILEVER (1980). UK Patent 1,577,933.

UNILEVER (1982). European Patent Application 64855.

UWAJIMA, T. AND TERADA, O. (1981). Clinical analysis of lipids using microbial enzymes. *Trends in Analytical Chemistry* **1**, 55–58.

VADEHRA, D. V. (1974). Staphylococcal lipases. *Lipids* **9**, 158–165.

WASSERMAN, B. P. (1984). Thermostable enzyme production. *Food Technology* **38**, 78–98.

WATANABE, N., OTA, Y., MINODA, Y. AND YAMADA, K. (1977). Isolation and identification of alkaline lipase producing micro-organisms; cultural conditions and some properties of crude enzymes. *Agricultural and Biological Chemistry* **41**, 1353–1358.

WILLS, E. D. (1955). Effect of surface-active agents on pancreatic lipase. *Biochemical Journal* **60**, 529–534.

WISDOM, R. A., DUNNILL, P., LILLY, M. D. AND MACRAE, A. (1984). Enzymic interesterification of fats: factors influencing the choice of support for immobilized lipase. *Enzyme and Microbial Technology* **6**, 443–446.

YAMAGUCHI, T., MUROYA, N., ISOBE, M. AND SUGIURA, H. (1973). Production and properties of lipase from a newly isolated *Chromobacterium*. *Agricultural and Biological Chemistry* **37**, 999–1005.

YAMANE, T., FUNADA, T. AND ISHIDA, S. (1982). Repeated use of lipase immobilised on amphiphilic gels for hydrolysis of a small amount of glycerides included in liquid crude fatty acid. *Journal of Fermentation Technology* **60**, 517–523.

YOKOZEKI, K., YAMANAKA, S., TAKINAMI, K., HIROSE, Y., TANAKA, A., SONOMOTO, K. AND FUKUI, S. (1982a). Application of immobilised lipase to regio-specific interesterification of triglyceride in organic solvent. *European Journal of Applied Microbiology and Biotechnology* **14**, 1–5.

YOKOZEKI, K., TANAKA, T., YAMANAKA, S., TAKINAMI, T., HIROSE, Y., SONOMOTO, K., TANAKA, A. AND FUKUI, S. (1982b). Ester exchange of triglyceride by entrapped lipase in organic solvent. In *Enzyme Engineering, Volume 6* (I. Chibata, S. Fukui and L. B. Wingard, Eds), pp. 151–152. Plenum Press, New York.

ZAKS, A. AND KLIBANOV, A. M. (1984). Enzymatic catalysis in organic media at 100°C. *Science* **224**, 1249–1251.

ZALE, S. E. AND KLIBANOV, A. M. (1983). On the role of reversible denaturation (unfolding) in the irreversible thermal inactivation of enzymes. *Biotechnology and Bioengineering* **25**, 2221–2230.