Technical News Feature

Characteristics of an Immobilized Lipase for the Commercial Synthesis of Esters[1]

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The lipase-catalyzed synthesis of ester bonds has been well-documented lately and is of much current commercial interest. Immobilization of a fungal lipase on a unique macroporous support allows not only the ability to operate in non-aqueous media but to catalyze ester synthesis in quantitative yields, employing attractive commercial conditions. Catalyst dose and process configurations will be illustrated. The capability of the catalyst to operate efficiently in reverse under a variety of unnatural, hostile, solvent-containing environments will be discussed. The range of substrates for this immobilized lipase, Lipozme, has been investigated. The enzyme will catalyze ester synthesis with saturated, unsaturated and a variety of branched carboxylic acids. The alcohol specificity for this enzyme also is equally broad. A wide variety of straight-chain, branched and polar alcohols can be substrates. In addition, some examples of alcohol specificity for kinetic isomer resolution will be cited.

The use of enzymes to catalyze the synthesis of ester bonds has become a commonplace practice recently. The literature is replete with a wide variety of different enzymes, substrates and conditions to accomplish such transformations (1-4).

Our work in this area originated several years ago (5). At that time, we were working with a cell-associated fungal enzyme that was immobilized simply by drying the mycelia. We since have expanded upon this basic concept and have developed new enzymes and immobilization supports specifically targeted for this application. A commercial product called Lipozyme, which is a fungal lipase from *Mucor miehei* immobilized on a macroporous synthetic resin, can be obtained from Novo Laboratories. This enzyme has been described by Hansen et al. and Eigtved et al. (6,7). In this study, we describe substrate specificity, solvents in which the reaction can be run, and how the enzyme can be used for the commercial synthesis of esters.

The generally accepted mechanism for lipase-catalyzed hydrolysis of an ester is shown in Figure 1. The reaction is believed to proceed via an acyl-enzyme intermediate much like a serine protease. The reaction is reversible, and under conditions of low water activity, the enzyme will function "in reverse," that is the synthesis of ester bonds rather than hydrolysis.

Even under conditions of low initial water concentration, during the course of the reaction 1 mole of water is formed for every mole of ester synthesized. Consequently, it becomes critical if a quantitative yield of reaction is required, then not only must the initial water level be low, but the formed water somehow must be removed from the reaction. A relatively easy method to remove the "formed water," particularly when dealing with higher boiling reactants, is to simply run the reaction under reduced pressure. As illustrated in Figure 2, the reaction of oleic acid with oleoyl alcohol tends to level off at an 85% yield when no provision is made to remove formed water. However, when the reaction is run under vacuum, the formed water is continually "pulled off" and the esterification driven to completion. The Lipozyme immobilization support has been specifically selected such that it will allow this removal of water from the reaction medium yet still enable the lipase itself to retain its own essential water. The ability to retain this essential water is a very critical feature of Lipozyme.

Enzymes have been described to function in non-aqueous media, although, in at least the majority of cases, a finite level of water associated with the protein must be present to retain conformational integrity, and thereby, activity (8-14). This hypothesis can be illustrated by our work in solvent. Our previous reported work regarding ester synthesis with Lipozyme all has been done using solvent-free conditions or reactions in hexane. Because many starting materials that may be desirable as ester synthesis substrates are neither liquids at reaction temperatures nor mutually soluble together, we investigated a wide variety of common solvents to see which would support enzyme-catalyzed ester synthesis. As shown in Figure 3, hex-

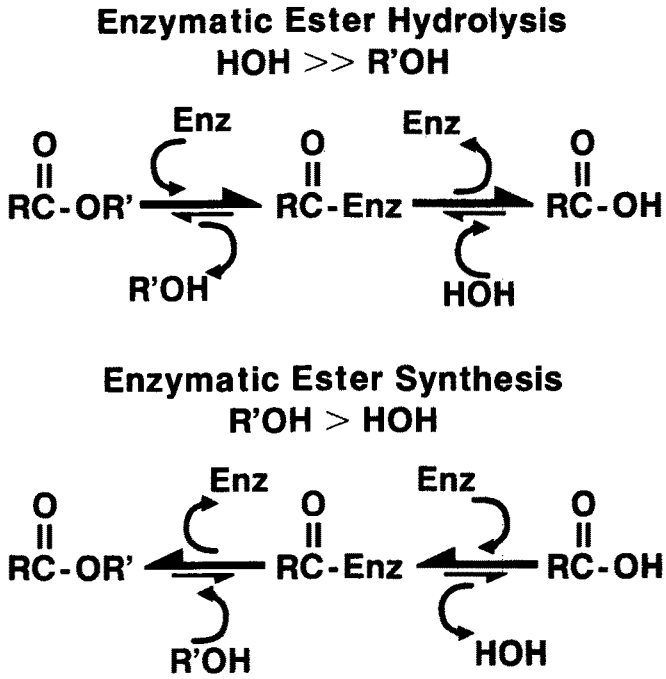

FIG. 1. Enzymatic ester hydrolysis.

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ane gave the highest reaction rate for a model esterification, but many other solvents also worked. In fact, it seems very much like an on-off type of situation. The enzyme was either fully on near-fully active, or it was not active at all. The solvents in which the lipase was active all were water-immiscible. Conversely, the solvents in which the enzyme was not active were all water-miscible.

Water certainly plays a very key role in maintaining conformational integrity of proteins. Forces that tend to hold a protein together, such as salt bridges and hydrophobic interactions, all are very much related to water. Consequently, it is hypothesized that the water-miscible solvents extract this essential water from the protein. A structural change in the protein accompanies water loss, resulting in a non-active conformation. This change, depending upon how disruptive it was, may be reversibe. For instance, Lipozyme did not support ester synthesis either in tetrahydrofuran (THF) or dimethyl formamide (DMF). The recovered catalyst from both reactions was washed well with hexane and reused in hexane-based esterifications. Neither catalyst was active. Addition of a minimal amount of water to the reactions resulted in a recovery of activity in the THF-exposed catalyst but no recovery in the DMF system. We would like to interpret this situation as meaning that the THF simply removed weakly bound water, resulting in a minor, yet critical to activity, conformational change that was readily reversible by the addition of water. The DMF extracted all water, resulting in catastrophic, irreversible loss of conformation.

The lipase enzyme was designed by nature to hydrolyze fatty acids from a glycerol backbone. Critical to the success for commercialization of enzymes as ester synthesis catalysts would be broad substrate specificity. One certainly would not want to employ a new enzyme for every different ester. The substrate specificity for Lipozyme was investigated and found to be very broad.

A series of esterifications with carboxylic acids of increasing chain length resulted in an increase in reaction rate up to heptanoic acid (Fig. 4). Further lengthening of the carbon chain had no increase on the rate. Apparently, the binding region on the enzyme is seven carbon atoms long, and any additional carbon atoms are hanging into space, not involved in the binding process.

It would be interesting if the Lipozyme active site could accommodate more than straight-chain carboxylic acids. Consequently, we looked at the effect of placing alkyl substituents on the carbon backbone of the fatty acid. "Moving" a methyl substituent out along the carbon chain from C_2-C_4 had a dramatic effect upon the rate of esterification as shown in Table 1. Branching decreased the rate in all cases, but suprisingly the greatest decrease was seen by branching at the 3-position.

A series of esterifications investigating the effect of increasing the size of the alpha-substituent then were performed (Table 2). Increasing the size from methyl to ethyl totally wiped out the ability to be a

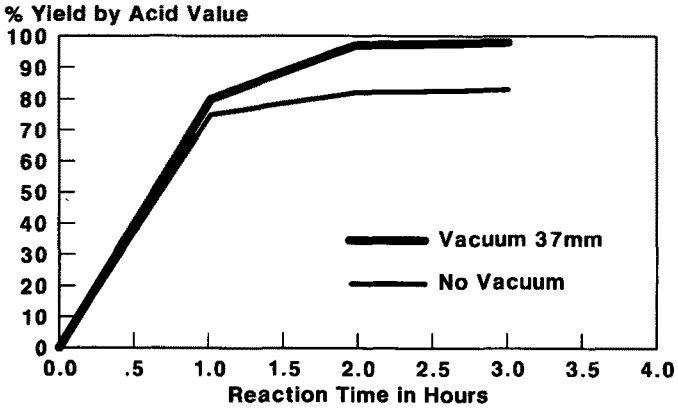


FIG. 2. Effect of vacuum on yield synthesis of oleyl oleate. Reaction Conditions: 70 C, 1:1 oleic acid-oleyl alcohol, 1.0 g Lipozyme/.05 moleolic acid. Analysis of percent reaction by acid value (AOCS Method [TE2a-64]).

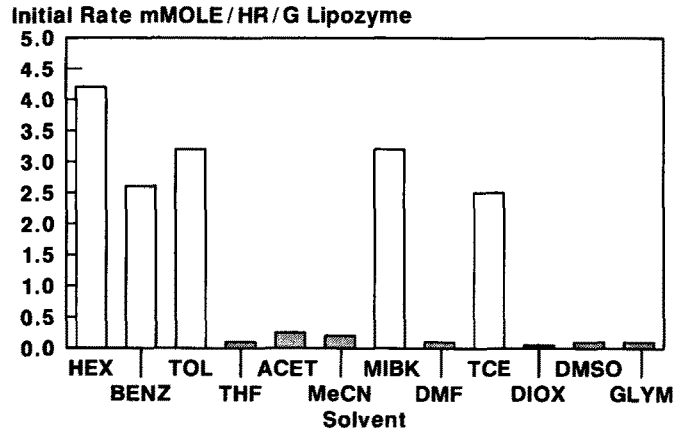


FIG. 3. Synthesis of propyl myristate in solvent. Reaction conditions: 60 C, 3:1 propanol-myristic acid, 0.8M myristic acid in stated solvent. Samples were taken at two-hr and four-hr time points. Analysis by acid value.

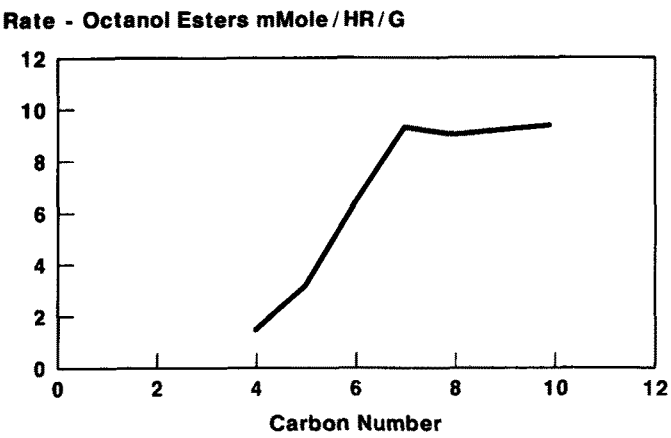


FIG. 4. Esterification rate vs carbon number. Reaction conditions: 60 C, 1:1 octanol acid, 1.0 g Lipozyme/0.5 mol acid. Analysis by acid value at four-hr time point.

TABLE 1

Effect of Methyl Branching on Esterification Rate

Acid	Relative Rate
R - CH_2 - CH_2 - CH_2 - COOH	1
R - CH_2 - CH_2 - CH(-CH_3) - COOH	0.42
R - CH_2 - CH(-CH_3) - CH_2 - COOH	0.21
R - CH(-CH_3) - CH_2 - CH_2 - COOH	0.84

Reaction conditions: 60 C, 1:1 octanol-acid (R=Me), 1.0 g Lipozyme/.05 mol acid. Analysis by acid value at four-hr time point.

TABLE 2

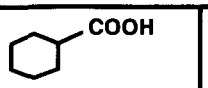
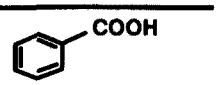
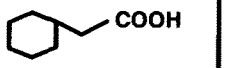
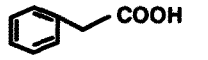
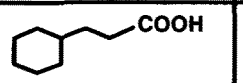
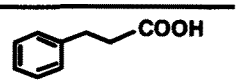
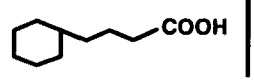
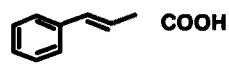
Substituent Effects at the Alpha Carbon

Acid	Relative Rate
R - CH_2 - CH_2 - CH_2 - COOH	1
R - CH_2 - CH_2 - CH(-CH_3) - COOH	0.42
R - CH_2 - CH_2 - CH(-CH_2-CH_3) - COOH	0
CH(-CH_2-CH_2-CH_2) - CH_2 - CH - COOH	0.22

Reaction conditions listed in Table 1.

TABLE 3

Esterification Rates of Cyclohexyl vs Phenyl-substituted Carboxylic Acids

mM ESTER/HR/G LIPOZYME - OCTANOL ESTERS			
	1.41	.15	
	.02	.29	
	4.88	7.95	
	1.42	-	

Reaction conditions: 60 C, 1:1 octanol-acid. 2.0 g Lipozyme/.05 mol acid. Analysis by acid value at four-hr time point.

TABLE 4

Effects of Unsaturation on Esterification Rate

Acid	Relative Rate
R - CH_2 - CH_2 - CH_2 - COOH	1
R - CH_2 - CH = CH - COOH	.05
R - CH = CH - CH_2 - COOH	.17

Reaction conditions listed in Table 1, R=ethyl.

TABLE 5

Stereoselectivity for "R" vs "S" Methylbutyric Acids

	mM Ester/hr/g Lipozyme	Relative Rate
butyric	2.05	1
± 2-methyl butyric	1.06	.52
S 2-methyl butyric	1.23	.60

Reaction conditions: 60 C, 1:1 octanol-acid. 2.0 g Lipozyme/.05 mol acid. Analysis by acid value at 5 1/2-hr time point.

substrate for the enzyme. Interestingly, if the ethyl group was "tied back," thereby making it part of a cyclohexane ring, substrate capability was regained.

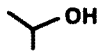
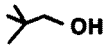
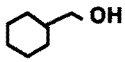
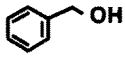
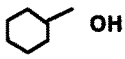
The issue of branching was further pursued by "moving" cyclohexyl or phenyl substituents along the backbone. As illustrated in Table 3, this series again demonstrated the damaging effect of branching at the beta-carbon. However, branching at the gamma-carbon was favorable. In fact, the phenyl propanoic (hydrocinnamic) acid rate was ca. 90% of the maximum caprylic acid rate under similar conditions.

The effect of unsaturation in the substrate was investigated in two separate experiments. One, hydrocinnamic vs cinnamic acid esterifications were run as described, except using benzene as solvent instead of methyl isobutyl ketone (MIBK). The cinnamic acid did not esterify at all under these condition. This is particularly striking because the saturated analog was an excellent substrate. Unsaturation in the carbon backbone of the straight-chain acids had the same effect as seen in the example in Table 4. The 2-hexenoic acid was esterified at a very low rate. Moving the double bond out along the chain to the 3-position resulted in an improvement, but still much less than the fully, saturated derivative.

As the enzyme was designed by nature to hydrolyze glycerides, one would not expect the enzyme to

TABLE 6

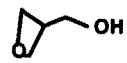
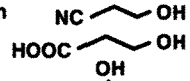
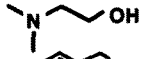
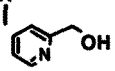
Synthesis of Myristate Esters in Hexane

Alcohol	Structure	Initial Rate mM/hr/g
n-propanol		10.3
isopropanol		0.42
neopentanol		4.45
cyclohexanemethanol		12.8
benzyl alcohol		3.34
cyclohexanol		0.70

Reaction conditions: 60 C, 2.5:1 alcohol-myristic acid. 1.0 g Lipozyme/.025 mol myristic acid. 0.8 M myristic acid in solvent listed. Analysis by acid value at two-hr time point.

TABLE 7

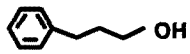
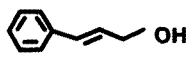
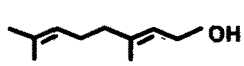
Synthesis of Myristate Esters

Alcohol	Structure	Initial Rate mM/hr/g	Comments
glycidol		2.92	toluene
methoxyethanol		7.78	hexane
ethylene cyanohydrin		6.53	MIBK
glycolic acid		2.36	MIBK
methoxypropanediol		4.17	hexane
dimethylamino ethanol		-	hexane
pyridine methanol		0.70	hexane

Reaction conditions listed in Table 6.

TABLE 8

Synthesis of Myristate Esters in Hexane

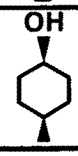
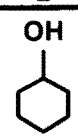
Alcohol	Structure	Initial Rate mM/hr/g
3-phenylpropanol		12.93
cinnamyl alcohol		11.53
geraniol		12.51
allyl alcohol		9.73

Reaction conditions listed in Table 6.

demonstrate any stereoselectivity about the alpha)carbon atom. Butyric acid substituted at the 2-position was utilized to discover whether any chirality was fortuitously built into the active site. As the data

TABLE 9

Resolution of Methylcyclohexanols Synthesis of Myristate Esters in Hexane at RT

	Relative Rates
trans 	1
cis 	0.4
	1.6

Reaction conditions: room temperature, 1:1 alcohol myristic acid. 0.5 M myristic acid in hexane. 1.0 g Lipozyme/.005 mol myristic acid: Analysis by acid value after five days reaction.

in Table 5 illustrate, only a very slight preference for the S-isomer of methylbutric acid was observed.

We have demonstrated by this work that Lipozyme can be employed to esterify a very broad range of carboxylic acids. The enzyme is definitely not limited to only straight-chain fatty acids. We next turned our attention to determine the accessibility of the Lipozyme active site to various hydroxy compounds. If one assumes an acyl enzyme intermediate, then ester synthesis within a class of acids will be dependent upon the accessibility of the reacting nucleophile (hydroxy compound) to the bound acyl moiety. In our first series of esterifications, we looked at the effect of branching of the alcohol. From this work, shown in Table 6, we can make several conclusions. One, primary alcohols, will react much better than secondary. However, alkyl branching at the beta-carbon was nowhere near as critical and indeed, cyclohexane methanol reacted at a better rate than propanol. The aromatic analog, benzyl alcohol, was a substrate but not as good as the saturated analog. We then decided to look at the effect of polar functionalities at the beta-carbon. As shown in Table 7, the active site again seems to be able to accomodate a broad range of vastly different species. Table 8 illustrates the last series we investigated, that is, the effect of phenyl substituents and unsaturation.

The range of alcohols that are substrates for Lipozyme is very broad. We believe it is safe to assume that the active site of the enzyme occupies, and is surrounded by, a very hydrophobic region. Therefore, it follows that the more hydrophobic substrate will have a greater affinity for this greasy region and hence undergo esterification reactions with ease. Alcohols with polar functionalities theoretically would have less affinity for the active site and would not work as well.

Enzymatic esterifications also can be used to resolve a mixture of isomers. For example, as shown in Table 9, the rate of esterification of myristic acid with

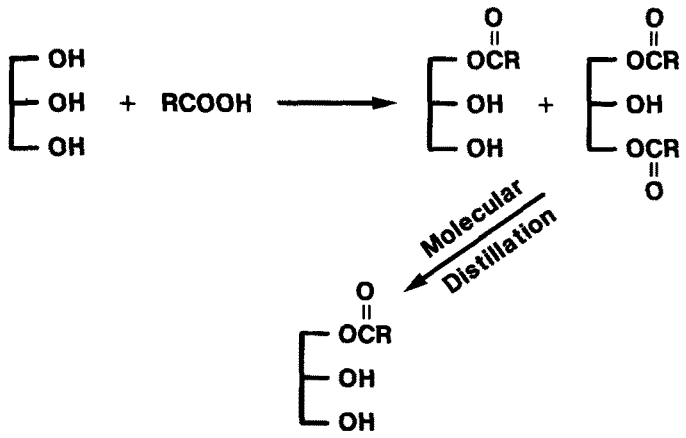


FIG. 5. Glyceride synthesis.

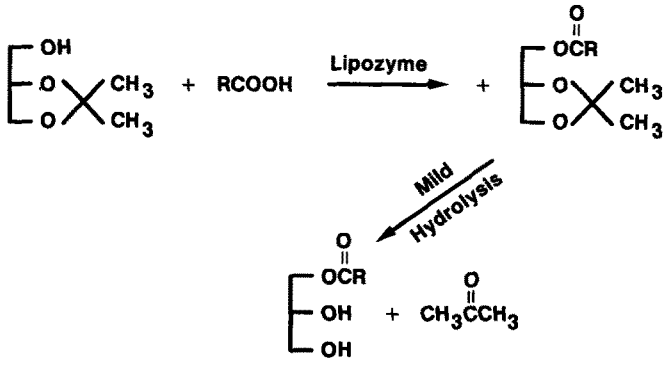


FIG. 6. 100% Monoglyceride process.

trans 4-methyl cyclohexanol is two times greater than that for the *cis* isomer. Elegant examples of enantioselective resolution catalyzed by lipases can be found in the work of Sonnet (15,16).

An important class of fatty acid derivatives are the monoglycerides. The product of commerce actually is a 1:1 mixture of mono and diglyceride. A 90% monoglyceride product then can be prepared from this mixture by molecular distillation as shown in Figure 5. Novo has developed an alternative proprietary technology that results in a 100% monoglyceride product without the distillation step (17). A fatty acid is enzymatically esterified with a derivatized glycerol moiety that has two of the three hydroxyl positions blocked. Once esterified, the blocking group then can be removed by mild acid treatment, resulting in a very clean 100% monoglyceride. The overall process is shown in Figure 6.

We have demonstrated that Lipozyme can be used to synthesize a very broad range of esters, albeit at an equally broad range of rates, but certainly, a key point is that other than simple carboxylic acids and alcohols can be used in such a process. We have shown how the catalyst can be used to effect quantitative yields by vaccum-stripping the water from the reaction as it is generated. The product quality of such enzyme-synthesized esters normally is better than the chemical-derived product due to the lower reaction temperature and avoidance of strong acid-catalyzed degradation products. The final point, and certainly a very critical factor, is that of productivity of the catalyst. The constraints and structure of the industry coupled with the need to remove water during the reaction has led us to perform productivity estimations in a batch reactor. These investigations, summarized in Table 10, have been run in a 3-l glass reactor at 60 C/.05 bar vaccum charged with 1 kg of reactants per batch. At the end of a 20-hr reaction period, the product was discharged and fresh reactants introduced. The acid values through 12 such runs reusing the original 10 g dose of catalyst all indicated a more than 96% yield of product, in this case, myristyl myristate. From these data, we can conclude that the productivity is at least 1.2 ton of ester per kg of Lipozyme.

TABLE 10

Productivity: Synthesis of Myristyl Myristate

- 3 Liter Stirred Reactor
- 60°C / .05 atm / 20 hr.
- 1 Kg Reactants - Equimolar
- 10 g Lipozyme Charge
- 12 Cycles of Catalyst at 96+% Yield

∴ Productivity Is at Least 1.2 Ton of Product Per Kg Catalyst

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