Lipase-Catalyzed Production of Wax Esters

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The lipase (triacylglycerol acylhydrolase, E.C. 3.1.1.3) catalyzed synthesis of wax esters has been investigated via two different approaches. All studies were performed using an immobilized 1,3-specific lipase [Lipozyme from Novo Industries (Montréal, Québec, Canada)]. The first approach involves reacting stoichiometric amounts of a fatty acid and stearyl alcohol in the presence of lipase. The medium is solvent-free, which allows for high substrate concentrations (1.55 M) and use of 5% (w/w) Lipozyme. In this reaction, maximum wax ester synthesis was found to be dependent upon the efficient removal of the water produced by the reaction. Under optimal conditions, yields of 100% were routinely reached after only 2 hr. The medium was then exclusively composed of the wax and the enzyme, no purification was required. The second method involves alcoholysis of a triglyceride, in this case triolein, with stearyl alcohol to produce 1,2-diolein, 2-monoolein and the wax ester of oleic acid. Again, no organic solvent was used. The wax ester yield was found to be directly dependent upon the alcohol concentration that was used to modulate the outcome of the reaction towards either the wax or the partial glycerides. The process was applied to the synthesis of waxes from high erucic acid rapeseed oil.

KEY WORDS: Alcoholysis, biocatalysis, lipase, synthesis, wax ester.

Research on the manufacture of esters through lipase (triacylglycerol acylhydrolase, E.C. 3.1.1.3) catalyzed synthesis and alcoholysis reactions has been steadily developing in the past few years (1). The driving force for this research has been the possibility of preparing esters which resemble naturally occurring waxes of commercial interest (2).

Esters of short-chain acids are important in the food industry as flavor and aroma constituents (3–5). Methyl and ethyl esters of long-chain acids provide valuable oleochemical species that may function as Diesel fuels (6). On the other hand, long chain esters (those derived from alcohols and acids both possessing chain lengths of 12 carbons or more), which are typically referred to as waxes, have potential applications from lubricants to cosmetics. A naturally occuring wax ester presently in use is the well known jojoba oil obtained from *Simmondsia chinensis.*

In this paper, the synthesis of waxes using lipase-catalyzed synthesis and alcoholysis is investigated using pure substrates and an immobilized enzyme under solvent-free, anhydrous conditions. Except for Mukherjee and Kiewit (2) and Macrae (7), workers have used organic solvents in these reactions. As was shown by us previously (8), the absence of water in the reaction medium does not imply the use of an organic solvent. The advantages of a reaction medium composed solely of the substrates and enzyme are obvious—high substrate concentration reduces downstream processing. Here, we apply these conditions to the synthesis of a jojoba oil substitute derived from high erucic acid rapeseed (HEAR) oil and erucyl alcohol.

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MATERIALS AND METHODS

All reactions investigated were catalyzed by Lipozyme IM-20, a 1,3-specific lipase derived from *Mucor miehei* immobilized on a weak anion exchange resin from Novo Industries (Montréal, Québec, Canada). High erucic acid rapeseed (HEAR) oil (unrefined) was supplied by CSP Foods (Saskatoon, Canada). All other fatty materials were purchased from Sigma Chemical Co. (St. Louis, MO).

In order to measure the percentage of esterification of oleic acid (OA) to the oleic acid stearyl ester, 885 μmoles each of oleic acid and stearyl alcohol were reacted in the presence of 25 mg of Lipozyme (5.1% w/w) at 60°C (the temperature used for all reactions unless otherwise stated), as reported previously (8). Aliquots (5 μL) were withdrawn at 10 min intervals and diluted with 800 μL of acetone. A 15 μL sample of this solution was used for high performance liquid chromatography (HPLC) analysis. Oleic acid consumption and ester production were detected and quantified using our method for mixtures of free fatty acids (FFA), monoglycerides (MG), diglycerides (DG) and triglycerides (TG) previously described (9). The amounts of oleic acid and ester were calculated as percentages of the initial amount of oleic acid, in oleic acid equivalents (OA equivalents).

In an Eppendorf vial, 0.522 g (590 μmoles) of triolein plus 50 mg of Lipozyme were mixed and varying amounts of stearyl alcohol added. When the reaction volume exceeded the working volume of an Eppendorf vial, the reactions were scaled up in thermostated beakers while maintaining the enzyme to triolein ratio constant. The amounts of each component (except for stearyl alcohol, which could not be detected) were calculated as percentages of the initial amount of oleic acid equivalents present in the triolein, taking into account that diolein and triolein each contain 2 and 3 oleic acids moieties, respectively.

For HPLC standards, erucyl esters of linolenic, linoleic, oleic, 11-eicosenoic and erucic acid (the five major fatty acids present in HEAR oil) were synthesized using the above conditions except that the reaction temperature used 40°C. HEAR oil (0.15 g) was reacted with 0.1457 g of erucyl alcohol (the stoichiometric amount corresponding to the total content of fatty acids in the oil) and 30 mg of Lipozyme (10.1% w/w). The HPLC method used for monitoring this reaction was the same as previously published (9), except the linear flow gradient was modified as follows: 0.0, 6.0, 10.0, 35.0, 39.0, and 40.0 min had flow times (mL/min) of 0.8, 0.8, 4.0, 4.0, 0.8, and 0.8, respectively.

RESULTS AND DISCUSSION

Efficient removal of water is essential for maximal synthesis to occur (8,10,11). In our procedure, the water by-product was removed by simple evaporation because the reaction was carried out at 60°C in an open vial. As shown in Figure 1, 100% conversion of oleic acid to the ester was obtained within 2 hr. Nonenzymatic reaction accounted for only 1% of the total conversion. The use of

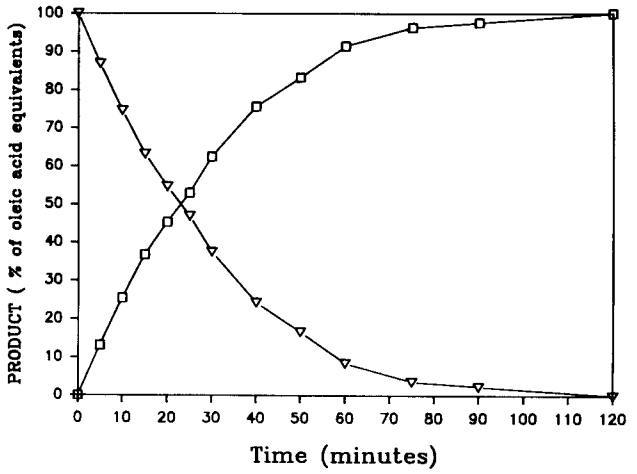

FIG. 1. Time course of the synthesis of oleic acid stearyl ester from oleic acid (885 μmoles) and stearyl alcohol (885 μmoles) using 25 mg of Lipozyme; (▽) oleic acid, and (□) oleic acid stearyl ester.

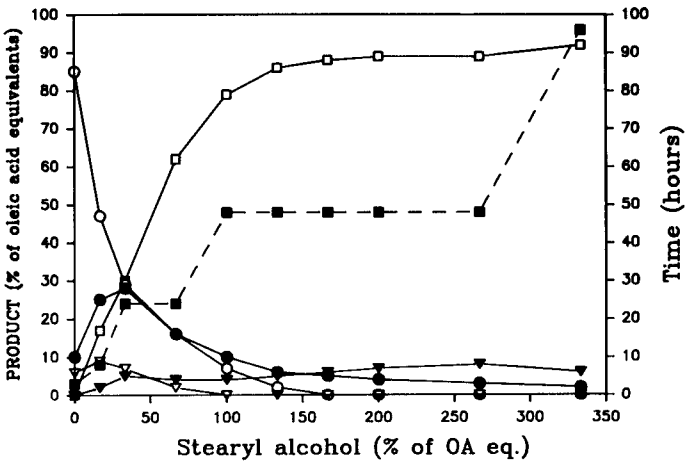

FIG. 3. Medium composition at equilibrium for the alcoholysis of triolein with different amounts of stearyl alcohol varying from 17% to 333% of oleic acid equivalents. Symbols are kept the same as in Figure 2; (---■---) approximate time to reach equilibrium.

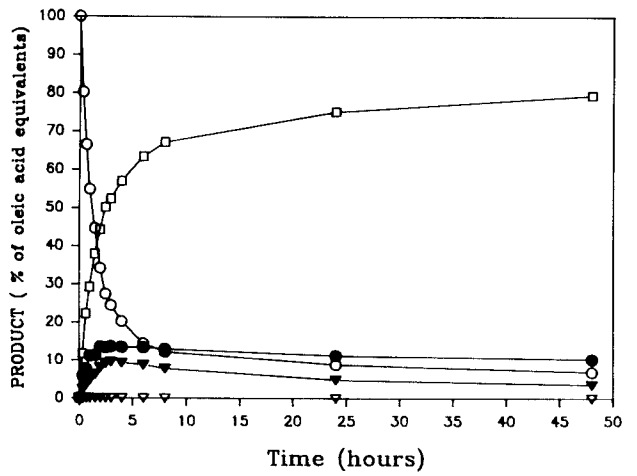

FIG. 2. Time course of the alcoholysis of triolein (590 μmoles) with stoichiometric amounts of stearyl alcohol (1770 μmoles) using 50 mg of Lipozyme; (▽) oleic acid (▼) 2-monoolein, (●) 1,2-diolein, (○) triolein, and (□) oleic acid stearyl ester.

stoichiometric amounts of substrates provides an efficient means of producing wax esters that requires only filtration for product purification.

A comparison can be made between the present work and that of Mukherjee and Kiewitt (2), who used stoichiometric amounts of oleyl alcohol and oleic acid (1 mmol), Lipozyme and no solvents. After 2 hr, these authors obtained a rate of 15 μmoles of ester per mg of enzyme while our experiments gave 35 μmoles. This difference may be due to the nature of the alcohols used, but is more likely due to the presence of water in their reaction medium.

In the alcoholysis of triolein, not only was wax ester production evident but also production of 1,2-diolein and 2-monoolein was observed. Varying amounts of triolein and stearyl alcohol were reacted without the addition of organic solvent in the presence of lipase. As water is not a by-product in alcoholysis reactions, evaporation is not required and capped reaction vessels were used. Figure 2 shows the time course of one of these reactions (stoichiometric amounts of triacylglycerol and alcohol, in OA equivalents). The rate of formation of wax esters by alcoholysis (Fig. 2) is lower than the rate observed in the esterification (Fig. 1) reaction. The initial rate of alcoholysis reaction was found to be 0.2 μmoles of ester formed per mg of lipase per min of reaction while the esterification rate was 1. Comparing the average rate of the reactions after 2 hr confirms that direct esterification synthesizes 35 μmoles of esters/mg of enzyme while alcoholysis produces 15.

As shown in Figure 3, increasing the stearyl alcohol concentration up to 1.5 OA equivalents led to an increase in wax ester production that leveled off at about 86% (based on initial OA equivalents). An excess of stearyl alcohol above 1.5 equivalents brought little change in the equilibrium concentrations of wax ester, 1,2-diolein and 2-monoolein. At stearyl alcohol concentrations below 1.5 equivalents, a linear increase in conversion to wax ester was observed. At very low stearyl alcohol concentrations, some free oleic acid was produced, because hydrolysis was then the dominant reaction due to the 10% water (w/w), which is bound to Lipozyme. Above 1 equivalent of stearyl alcohol, all of the oleic acid released from triolein was esterified. At a stearyl alcohol concentration of 0.45 OA equivalents, maximum 1,2-diolein concentration (28%) was produced. The equilibrium concentration of 2-monoolein remained unchanged throughout the whole range of alcohol concentrations studied, hovering between 4 and 8% OA equivalents. This series of experiments led to the conclusion that when pure substrates are concerned, the composition of the alcoholysis reaction medium essentially varies with the concentration of alcohol used. Concentrations of alcohol equivalents above half of the stoichiometric amount formed wax esters as the primary product.

Finally, because erucyl erucate is one of the main constituents of jojoba oil, the feasibility of producing a jojoba oil substitute from HEAR oil was attempted by reacting HEAR oil and a stoichiometric amount of erucyl alcohol (relative to the fatty acid composition of the oil).

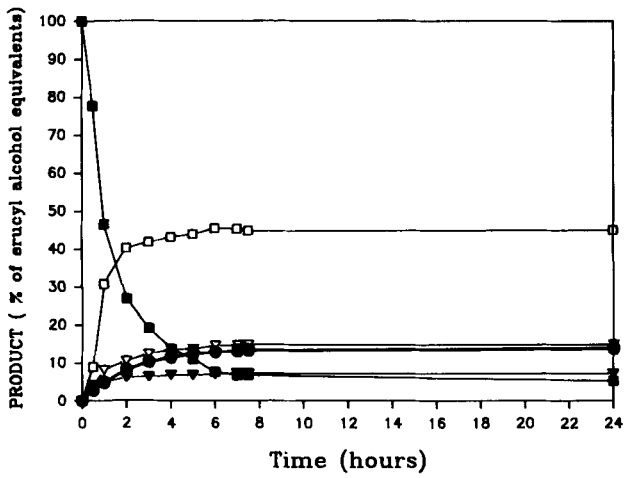

FIG. 4. Time course of alcoholysis of HEAR oil with stoichiometric amount of erucyl alcohol (see details in text). (○) Linolenic acid wax ester, (●) linoleic acid wax ester, (▽) oleic acid wax ester, (▼) 11-eicosenoic acid wax ester, (□) erucic acid wax ester, and (■) erucyl alcohol.

TABLE 1

Comparison of Alcoholysis of Different Oils with Erucyl Alcohol, in Different Conditions

Workers	Oil	Alcohol[a] oil	Wax esters formed (mole.mg^{-1} lipase) 2 hr	4 hr	6 hr
Mukherjee and Kiewitt (ref. 2)	*L. annua*	0.66	7.5	9.3	9.3
This work	HEAR	1	10.9	12.9	13.8

[a]Expressed in total fatty acid equivalents.

The waxes synthesized had the following retention times: erucyl esters of linolenic acid, linoleic acid, oleic acid, 11-eicosenoic acid and erucic acid had retention times (min) of 10.98, 12.09, 13.90, 15.95 and 18.70, respectively. The retention time of erucyl alcohol was 7.47 min.

Alcoholysis of HEAR oil was found to occur very quickly, as is seen from the time course of the reaction (Fig. 4). The reaction yield after 2 hr was calculated to be 11 μmoles of esters/mg of enzyme, while the alcoholysis of triolein with stearyl alcohol gave a yield of 15. It is clear that equilibrium was attained after only 8 hr of reaction with only traces of erucyl alcohol left.

A comparison between our work and that of Mukherjee and Kiewitt (2), where alcoholysis of *L. annua* oil with erucyl alcohol was performed, shows results in the same order of magnitude (Table 1). The presence of water [Mukherjee and Kiewitt (2) added 25 μL of water to the medium] and differences in substrate nature and concentrations probably account for the differences in yields. Reactions of HEAR oil with excess erucyl alcohol gave results similar to those observed in the alcoholysis of triolein. Those data on the decrease on reaction rate with increasing alcohol concentrations corroborate those of Knox and Cliffe (12).

REFERENCES

1. Lazar, G., *Fette Seifen Anstrichm. 87*:394 (1987).
2. Mukherjee, K.D., and I. Kiewitt, *J. Agric. Food Chem. 36*:1333 (1988).
3. Gillies, B., H. Yamazaki and D.W. Armstrong, *Biotechnol. Lett. 9*:709 (1987).
4. Langrand, G., C. Triantaphylides and J. Baratti, *Ibid. 10*:549 (1988).
5. Welsh, F.W., and R.E. Williams, *J. Food Sci. 54*:1565 (1989).
6. Mittelbach, M., *J. Am. Oil Chem. Soc. 67*:168 (1990).
7. Macrae, A.R., *Biochem. Soc. Trans. 17*:1146 (1989).
8. Ergan, F., M. Trani and G. André, *Biotechnol. Bioeng. 35*:195 (1990).
9. Ergan, F., and G. André, *Lipids 24*:76 (1989).
10. Ergan, F., M. Trani and G. André, *Biotechnol. Lett. 10*:629 (1988).
11. Miller, C., H. Austin, L. Posorske and J. Gonzlez, *J. Am. Oil Chem. Soc. 65*:927 (1988).
12. Knox, T., and K.R. Cliffe, *Process Biochemistry 19*:188 (1984).

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