Enzymatic Glycerolysis and Transesterification of Vegetable Oil for Enhanced Production of Feruloylated Glycerols

Joseph A. Laszlo* and David L. Compton

New Crops and Processing Technology Research, National Center for Agricultural Utilization Research, ARS, USDA, Peoria, Illinois 61604

ABSTRACT: Novel functional groups can be introduced into vegetable oils using enzymes, resulting in value-added products. The transesterification kinetics of ethyl ferulate with MAG, DAG, and TAG were examined. Transesterification was catalyzed by immobilized *Candida antarctica* lipase B in solventless batch and packed-bed reactors. Initial reaction rates with TAG were slightly sensitive to water activity, whereas rates with MAG and DAG were water activity independent. Transesterification was also three- to sixfold faster with MAG and DAG. These observations indicate that the reaction is rate limited by the acyl acceptor, and that oils with free hydroxyl groups are preferred acyl acceptors in comparison with TAG, which must undergo partial hydrolysis before becoming reactive.

Paper no. J11297 in *JAOCS 83*, 765–770 (September 2006).

KEY WORDS: Enzymatic transesterification, ferulic acid, lipase, triglyceride.

Biocatalytic transformation of lipids using immobilized lipases has produced commercially successful products for food and skin-care applications (1). Other large-volume products, such as biodiesel, await development of lower-cost biocatalytic systems (2-4). The enzyme-catalyzed synthesis of food and nonfood products from oilseed lipids has been recently reviewed (5). Realizing enhanced product quality and potential energy/environmental savings *via* immobilized lipase technology is contingent on keeping product throughput and enzyme stability high, as substrate costs are usually quite low in comparison. Thus, optimization of biocatalytic processes for throughput and enzyme stability is paramount.

Candida antarctica lipase B (CALB), when appropriately immobilized, is a stable, versatile biocatalyst particularly suited for nonaqueous reaction conditions. An examination of CALB's reaction kinetics indicates that it proceeds through an acylated enzyme intermediate (6). In organic solvents, the nature of the acyl donor/acceptor couple and solvent polarity interact to influence CALB reaction kinetics (6,7). Water activity (a_w) is another known key influence on the behavior of lipases in nonaqueous environments (8). Adjustment and control of all these factors needs to be considered for successful commercialization of a nonaqueous biocatalytic process.

The present study examines the role of water in the transesterification of ethyl ferulate (EF) with soybean oil (SBO). If the kinetics of the reaction are limited by the enzyme's acylation (feruloylation) step, then the nature of the deacylating agent is unimportant. This circumstance would indicate that a degree of enzyme (CALB) hydration is critical. Alternatively, if deacylation is the rate-controlling step, then water may serve its role by altering the acyl acceptor to create a better nucleophile. Determination of water's role in this reaction will guide efforts to intensify the process of manufacturing feruloylated MAG and DAG in a packed-bed bioreactor.

EXPERIMENTAL PROCEDURES

Materials. Ethyl ferulate (EF; ethyl 4-hydroxy-3-methoxycinnamate; m.p. 57–60°C; purity >98%) was purchased from Shanghai OSD (Shanghai, China). Novozym 435 (*C. antarctica* lipase B immobilized on acrylic beads) was obtained from Novozymes North America (Franklinton, NC). Soybean oil (SBO) and Enova DAG oil (EDO; a product of ADM Kao LLC, Decatur, IL) were purchased at a local grocery. All other reagents were from Sigma-Aldrich (St. Louis, MO) and Fisher Scientific (Pittsburgh, PA). Glycerol was spectroscopic grade (<0.1% w/w water). Silica gel (70–325 mesh) and 3-Å molecular sieves were dried at 110°C under vacuum.

Partially deacylated SBO (PD-SBO) was prepared by treatment of a 2:5 (mol/mol) mixture of 1-propanol and SBO with Novozym 435 (1 g of enzyme per 25 g of substrate) for 2 d at 60°C on an orbital shaker (200 rpm). Product was filtered through 20-μm nylon mesh (Spectrum Laboratories, Rancho Dominguez, CA) to remove enzyme and molecular-distilled (120°C, 16 mTorr) to remove FA propyl esters, FFA, and residual propanol. The distillation residue fraction (PD-SBO) had a TAG content that was 50% (w/w) of the original SBO and a very low MAG (<5% w/w) content.

Preparation of *sn*-2-monoacylglycerols (2-MAG) from SBO was conducted following the method of Irimescu *et al.* (9). SBO was emulsified with ethanol at a 1:4 w/w ratio and then treated with Novozym 435 (10:1 w/w substrate to enzyme ratio) at room temperature for 4 h. Enzyme was separated from the product by filtration, and excess ethanol was removed by

*To whom correspondence should be addressed at New Crops and Processing Technology Research, NCAUR, ARS, USDA, 1815 N. University St., Peoria, IL 61604. E-mail: laszloja@ncaur.usda.gov

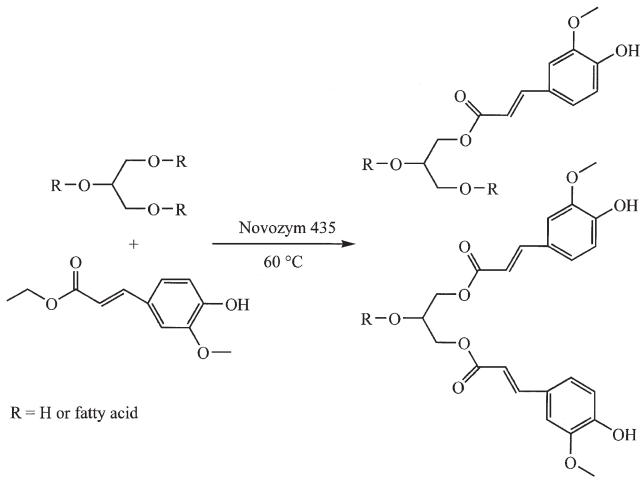
rotary evaporation at room temperature. 2-MAG were separated from ethyl FA esters by molecular distillation (120°C, 16 mTorr). The positional purity of the 2-MAG were confirmed to be 90% by ^{1}H NMR based on glyceryl C-2 β-proton integration. The ratio of the 2-MAG β-proton (4.95 ppm) to the 1-MAG β-proton (3.95 ppm) was used to determine the 2-MAG/1-MAG ratio after molecular distillation and was found to be 9:1. 2-MAG glyceroyl protons ($CDCl_3$, 500 MHz) are: δ 4.95 (1 H, *m*, $HOCH_2$–C*H*(OR)–CH_2OH) and 3.85 ppm (4 H, pseudo *dd*, $HOCH_2$–CH(OR)–CH_2OH). 1-MAG glyceroyl protons ($CDCl_3$, 500 MHz) are: δ 4.21 (2 H, *dd*, $ROCH_2$–CH(OH)–CH_2OH), 3.95 (1 H, *m*, $ROCH_2$–C*H*(OH)–CH_2OH), and 3.66 ppm (2 H, *dd*, $ROCH_2$–CH(OH)–CH_2OH). These assignments are in agreement with those obtained for monostearin.

HPLC analysis. Lipids and ferulate species were determined following previously described procedures (10). Analysis was conducted with a Thermo Separations Products (San Jose, CA) HPLC system consisting of a AS3000 autosampler, P4000 pump, SCM1000 solvent degasser, UV6000LP diode array detector, an ELSD, and a Prodigy C8 column (5 μm, 250 × 4.6 mm; Phenomenex, Torrance, CA).

For the determination of TAG concentration, the column was developed isocratically at 1.5 mL/min with 40:60 (vol/vol) acetone/acetonitrile (containing 1% glacial acetic acid). Samples were prepared by 200-fold dilution into acetone and then passage through a Gelman 0.45 μm 13LC syringe filter prior to injection. The injection volume was 10 μL. The column eluate was monitored at 340 nm. The ELSD (2.0 slpm N_2, 70°C) TAG response was calibrated using dilutions of SBO. A linear response was found for the most prominent species (eluting at 9.7 min) over a 1.0 to 5.0 mg SBO/mL concentration range.

For the quantification of FA and EF, a water/methanol/butanol gradient elution regime was used as detailed previously (10), with detection at 325 nm and with the acetone-diluted samples further diluted 20-fold with methanol. Detector response (325 nm, 7 nm bandpass) was calibrated with FA and EF. Responses were linear for both species in the range used (EF: 50 to 250 μM; FA: 2 to 20 μM). The sample injection volume was 10 μL.

Batch enzyme reactions. Transesterification batch reactions were conducted in 50-mL capped, plastic conical tubes containing EF (1.0 g), oil (4.0 g), and Novozym 435 (0.25 g). Tubes were placed upright into an orbital shaker (200 rpm) and incubated at 60°C (a temperature at which EF dissolves completely in all oils examined). Aliquots (100 μL) were removed at timed intervals for analysis by HPLC and UV spectroscopy (325 nm). UV spectroscopy was used to determine the total concentration of feruloyl species in the oil, which was necessary to correct for a small amount of EF absorption to the enzyme support (11). Details concerning reaction product analysis and the calculation of EF conversion to product percentage have been given previously (11). For this purpose, conversion percentage is based on a definition of product as comprising all feruloylated glycerol species containing 0–2 fatty acid acyl groups (Scheme 1). FA, an unwanted by-product, is not included as product in the conversion to product calculation.



SCHEME 1

Batch glycerolysis reactions were conducted in 50-mL glass round-bottomed Schlenk flasks. Silica (200 mg) and glycerol were mixed together, then oil, EF (if needed), and Novozym 435 were added. Flasks were evacuated and sealed under vacuum. Reactions were conducted as described for the transesterification procedure, but with the additional step of re-evacuating the flasks for 5 min following each sampling.

Controlled a_w reactions. Substrates (i.e., the various oils and EF) and enzyme were equilibrated for at least 7 d in separate sealed containers enclosed with saturated salt solutions or solid adsorbents to establish fixed water activities for transesterification reactions (12,13). The corresponding water concentrations in the equilibrated oils are given in Table 1. Water was measured using coulometric Karl Fischer analysis with 70:30 (vol/vol) Hydranal AG-H/chloroform as the analyte. Reaction components were combined in capped conical tubes and subjected to batch reaction conditions (*vide supra*).

Packed-bed reactions. Novozym 435 (32 g) was solvated in SBO under reduced pressure for 30 min, incubated overnight at room temperature, and then transferred to a jacketed chromatography column (2.5 × 30 cm, 147 mL nominal internal volume). Using β-carotene as a marker, the bed included volume was estimated to be 80 mL. The enzyme bed was conditioned overnight by recirculating SBO at 2 mL/min. The reactor was maintained at 60°C using a circulating bath. Reactants (EF and vegetable oil) were fed into the top of the reactor using a peristaltic pump at 2 mL/min. Reactor effluent was collected in a small reservoir (30 mL), which was kept under a slow stream of N_2 with the contents magnetically stirred, and recirculated back into the packed-bed bioreactor. The reaction mixture was prepared by combining 40 g of EF with 160 g of oil at 60°C. While retaining 18 mL of this solution for the reservoir, the reaction mixture was passed onto the column, discarding the displaced SBO to waste, then directing the reactants back to the reservoir once the reactor was entirely loaded. For SBO glycerolysis, a syringe pump delivered glycerol to the reservoir at fixed rates.

TABLE 1
Water Concentrations of Oils Equilibrated with Saturated Salt Solutions and Dry Adsorbents

Salt/adsorbent	Water content (mg/g)			Water activity
	SBO	PD-SBO	EDO	
3-Å Sieves	10 ± 2[a]	45 ± 1	94 ± 8	ND[b]
Silica gel	64 ± 17	179 ± 54	331 ± 90	<0.01
LiBr	87 ± 4	237 ± 4	435 ± 13	0.06
LiCl	126 ± 3	325 ± 6	609 ± 13	0.11
$MgCl_2$	320 ± 4	897 ± 3	1756 ± 3	0.3
$Mg(NO_3)_2$	525 ± 6	1535 ± 19	3070 ± 70	0.53

[a]$n = 3$.
[b]ND, not defined; SBO, soybean oil; PD-SBO, partially deacylated SBO; EDO, Enova (ADM Kao LLC, Decatur, IL) diacylglycerol.

RESULTS AND DISCUSSION

Influence of a_w on feruloylation kinetics. Efficiency of enzyme-catalyzed reactions in nonaqueous media is under the control of a_w rather than water concentration (8). By equilibrating enzyme and substrates with saturated salt solutions or solid adsorbents (Table 1), the initial a_w for EF transesterification with various acylglycerols (Scheme 1) can be fixed over a broad range. It was observed that a_w had minimal impact on transesterification efficacy (Fig. 1). For SBO, reaction under the lowest a_w condition (equilibrated with molecular sieves) produced the lowest amount of product formation in the first 24 h of reaction, whereas reactions performed at the highest a_w (0.53) were slightly faster ($P < 0.05$). However, for EDO and PD-SBO there was no statistically significant reactivity change over the a_w range examined. These findings indicate that the extent of enzyme hydration does not directly influence the transesterification reaction involving EDO or PD-SBO, but may impact the reaction with TAG.

At $a_w \geq 0.06$, detectable quantities of ferulic acid were produced during EF transesterification with the various oils (SBO, EDO, and PD-SBO), although the amount of EF hydrolysis was less than 2% within the time allotted (24 h). EF hydrolysis generally increased with a_w (data not shown), but there were no differences among the oils at a given a_w despite their enormous water content differences (Table 1). Consistent with these observations is the finding that, with the exception of molecular sieve-equilibrated reactants, water content values of the reaction media after 24 h were all lower than initially, suggesting that hydrolysis was responsible for lower water concentrations. EF hydrolysis is effectively irreversible under these conditions (10); however a steady-state (i.e., reversible) level of FFA may be produced during the reaction. A SBO transesterification rate increasing with a_w may arise from the generation of small amounts of MAG and DAG by hydrolysis, if the transesterification reaction is rate limited by the acyl acceptor. Therefore, these findings are consistent with EF/SBO transesterification proceeding in a sequential process of triglyceride hydrolysis followed by a feruloylation reaction *via* a Ping-Pong Bi-Bi mechanism (14), while hydrolysis is not required for EF reactions with EDO and PD-SBO.

Scheme 2 proposes a reaction sequence consistent with the suggested sequential process. TAG cannot serve as an acyl acceptor directly. Therefore, TAG must first undergo hydrolysis to generate a DAG containing a free hydroxyl group (step 1,

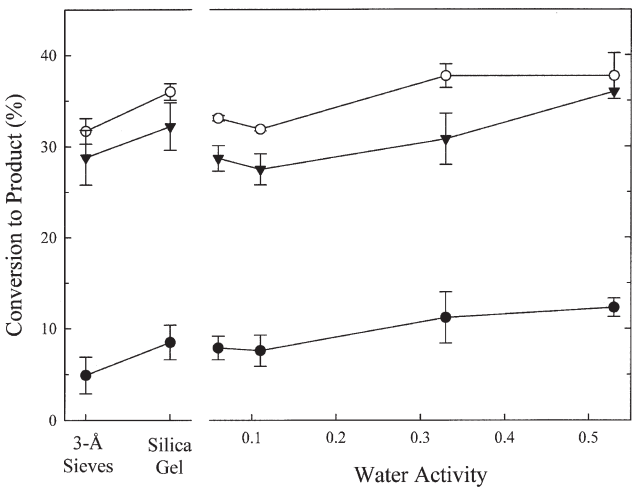
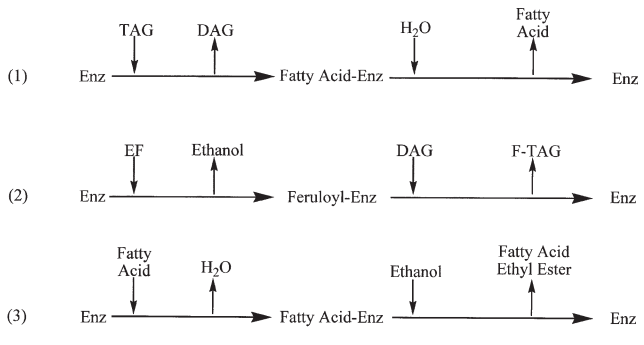


FIG. 1. Influence of water activity and nature of acyl acceptor on *Candida antarctica* lipase B reactivity with ethyl ferule (EF). Substrates and enzyme equilibrated to a fixed water activity were combined [1 g EF, 4 g oil, and 0.25 g Novozym 435 (Novozymes North American, Franklinton, NC)] and incubated at 60°C for 24 h. The oils used were soybean oil (SBO; ●), partially deacylated SBO (▼), and Enova ADM Kao LLC, Decatur, IL) diacylglycerol (EDO; ○). Experiments were performed in triplicate (error bars represent 1 SD from the mean).



SCHEME 2

Scheme 2). The generated DAG then reacts with EF to produce a product (F-TAG) containing one feruloyl group and two FA groups (step 2, Scheme 2). Finally, FFA generated in step 1 react with ethanol generated in step 2, producing FA ethyl esters (step 3, Scheme 2). Water is ideally conserved in the given overall reaction scheme, but in actuality is consumed to a small degree with the generation of ferulic acid and FFA. The EF reactions with EDO and PD-SBO proceeded only through step 2, bypassing water as a co-reactant, and were therefore insensitive to a_w.

The insensitivity of *C. antarctica* lipase B, free or immobilized, to very low a_w conditions has been noted previously (13,15). However, its kinetic response to changing a_w varies considerably. TAG ethanolysis rates were shown to drop with increased reaction water content (15). With gas-phase transesterification (methyl propionate with 1-propanol), optimal conditions are reached at $a_w = 0.1$, with a decreased activity at higher a_w attributed to formation of a water layer barrier on the enzyme (16). Solvent-free transesterification of ethyl lactate with 1-butanol is best with $a_w = 0.06$, as higher a_w values lower reaction rates (13). Thus, the slight rise in reactivity with increasing a_w observed here with the transesterification of EF with SBO, and the complete insensitivity to a_w of the reaction using partially deacylated oils (EDO and PD-SBO) as the acyl acceptors, is a novel finding.

Influence of acyl acceptor on feruloylation kinetics. Transesterification rates with EF were profoundly affected by the structure of the oil. Reactions with EDO or PD-SBO were three- to sixfold faster than with SBO (Fig. 1). Feruloylation of 2-MAG proceeded even faster, achieving 46 ± 1% conversion in 24 h (*cf.* 38% for EDO). These observations indicate that the reaction was rate-limited by the acyl acceptor and that oils with free hydroxyl groups were preferred acyl acceptors (better nucleophiles) in comparison with TAG.

In these reactions the weight of oil reactants was identical, but their molar concentrations differed slightly. Based on FA composition and acylglycerol distribution (17), average M.W. of 751 and 660 were estimated for PD-SBO and EDO, respectively. Therefore, the EDO concentration was 14% higher than that of PD-SBO. For observations pooled across all a_w values, EDO rates were 13% higher than those of PD-SBO (34.6 ± 2.7 and 30.5 ±3.4, respectively; $P < 0.05$). The small difference in reactivity observed between EDO and PD-SBO could thus be attributed to differing molar concentrations of the acyl acceptor.

However, the higher reactivity of EDO compared with PD-SBO is somewhat counterintuitive as there is the expectation that a free 1(3)-position alcohol would be more reactive than a *sn*-2-position alcohol in the acylglycerols. EDO consists approximately of 14 wt% TAG, 57 wt% 1,3-DAG, and 28 wt% 1(3),2-DAG (17). Therefore, EDO has a 1(3),2-DAG content comparable with PD-SBO (an approximately 50:50 mol% mixture of TAG and DAG, with an unknown distribution between 1,3-DAG and 1(3),2-DAG). A possible explanation for this circumstance is that an enzyme-catalyzed interesterification of EDO to produce free 1(3)-position alcohols occurs rapidly within the time frame of the EF transesterification reaction. Novozym 435 was observed to produce TAG from EDO under these reaction conditions, which could result from either enzyme-catalyzed *sn*-2-position acylation or nonenzymatic acyl migration to the 2-position followed by enzymatic 1(3)-position acylation.

Deacylation is the rate-determining step for acyl transfer with small alcohols as acyl acceptors (6). However, arylaliphatic acids, particularly aromatic ring-substituted cinnamates, are notoriously poor acyl donors (18–20). Steric hinderance and/or electrostatic interactions between the bulky phenyl ring of the acid and the enzyme active site result in low or nonreactivity of ferulic acid (21). Chemical esterification of cinnamic acids provides some activation so that they may serve as acyl donors in enzymatic transesterifications (10,22). Consequently, it was unanticipated that the reaction of EF with SBO would be deacylation-rate determined. Scheme 2 provides an alternative explanation for the reaction of EF with TAG, indicating the rate of formation of partially deacylated glycerols may control the reaction.

SBO glycerolysis. The improvement shown in EF transesterification kinetics by using partially deacylated oils in place of SBO suggested that MAG and DAG would be preferable substrates. For commercial considerations, introducing additional processing steps to generate and isolate MAG and DAG has negative consequences. Therefore, *in situ* MAG and DAG generation from SBO *via* glycerolysis was examined.

Figure 2 displays the time course for SBO glycerolysis at several glycerol/SBO molar ratios. Initial glycerolysis rates were similar for glycerol/SBO molar ratios between 1:4 and 1:1. The residual TAG content at completion was reduced to 64% after 4 h for 1:4 glycerol/SBO, to 44% after 8 h for 1:2 glycerol/SBO, and to 31% after 24 h for 1:1 glycerol/SBO. The

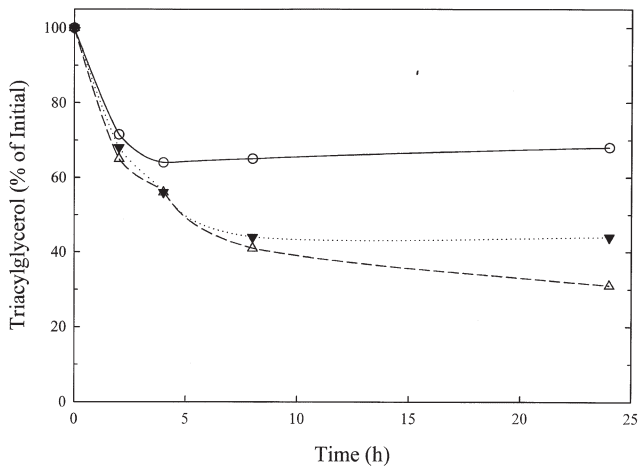


FIG. 2. Time course for SBO glycerolysis by Novozym 435. Glycerol/SBO molar ratios were 1:4 (○), 1:2 (▼), and 1:1 (△). Glycerol (92, 184, or 368 mg) was admixed with 200 mg of silica in a flask, and then SBO (4 mL) and Novozym 435 (200 mg) were added. Reactions proceeded under partial vacuum at 60°C. Data are mean values of duplicate experiments. For abbreviation and manufacturers see Figure 1.

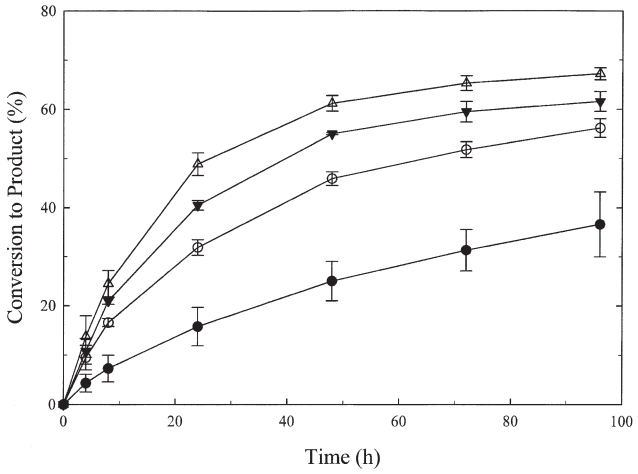


FIG. 3. Time course for transesterification of SBO with ethyl ferulate (EF) in the absence or presence of glycerol. The molar ratio of EF/SBO was 1:1, and the enzyme/substrate ratio (EF + SBO + glycerol) was 1:20 w/w. The reaction contained 200 mg of silica and no glycerol (●), or silica with glycerol at glycerol/SBO molar ratios of 1:4 (○), 1:2 (▼) and 1:1 (△). Glycerolysis was conducted for 24 h prior to the addition of EF. Reactions proceeded under partial vacuum at 60°C and were performed in triplicate (error bars represent 1 SD from the mean). For abbreviation see Figure 1.

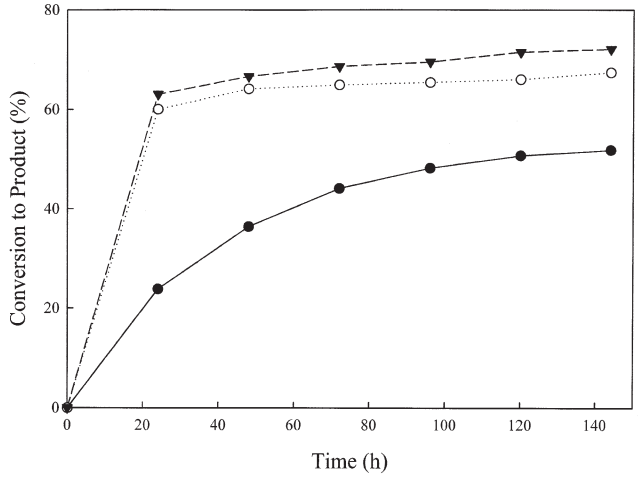


FIG. 4. Kinetics of packed-bed transesterification. EF and oil (1:4 w/w) were continuously recirculated through a column, maintained at 60°C, containing 32 g of Novozym 435 (1:4 w/w enzyme/substrate ratio). Substrate oil was either SBO (●), EDO (○), or SBO that had been subjected to glycerolysis (1:1 molar ratio) prior to combination with EF (▼). Experiments were performed in duplicate. For abbreviations see Figures 1 and 3; for manufacturers see Figure 1.

percentage of TAG remaining in each case was consistent with that expected for a treatment with a 1,3-specific lipase, i.e., 64.0, 44.4, and 25.0% for 1:4, 1:2, and 1:1 glycerol/SBO (14), respectively, although CALB is not strictly 1,3-specific.

In these experiments, glycerol was adsorbed to silica prior to the introduction of enzyme to avoid fouling the enzyme and its support with excess glycerol (23), a common practice in the enzymatic production of MAG and DAG from TAG (24,25). Keeping the reaction under vacuum minimizes the production of FFA (25). Operating under these conditions with a 1:1 glycerol/rapeseed oil mole ratio and Novozym 435 (at a 1:10 enzyme/substrate ratio instead of the 1:20 enzyme/substrate ratio used herein with SBO), Weber and Mukherjee (25) found an equilibrium mixture of 50 mol% DAG was produced in 6 h along with approximately 25 mol% each of MAG and TAG. Thus, rapeseed oil and SBO glycerolysis kinetics and product distribution are comparable.

Glycerol effects on feruloylation kinetics. The inclusion of silica-adsorbed glycerol in the transesterification reaction of EF with SBO produced enhanced product formation rates that increased with the amount of added glycerol (Fig. 3). In this experiment, SBO glycerolysis was conducted first for 24 h before the addition of EF, which allowed glycerolysis to be complete prior to the start of the EF transesterification (Fig. 2). During the early phase of EF transesterification (up to 8 h after EF addition), product formation rates with 1:2 and 1:1 glycerol/SBO were the same, approximately fourfold faster than in the absence of glycerol, but there was greater product formation with 1:1 glycerol/SBO than with 1:2 glycerol/SBO after this initial period. The enhanced product formation found using SBO glycerolysis prior to EF transesterification is consistent with the above findings that partially deacylated MAG and DAG (PD-SBO and EDO) react with EF more quickly than SBO.

Attempts to react glycerol, EF, and SBO synchronously produced inconsistent results. Trials using 1:4 glycerol/SBO produced an EF transesterification rate comparable with that depicted in Figure 3, but at higher glycerol/SBO ratios the enzyme support frequently agglomerated, resulting in significant activity loss. This may have resulted from EF precipitation induced by excess solubilized glycerol. Adding more silica to the reaction did not completely eliminate the agglomeration problem, whereas the inclusion of such large amounts of silica introduced mixing and sampling problems. Furthermore, it has been pointed out that silica-adsorbed glycerol has drawbacks when attempting to implement large-scale processes (24). Consequently, stepwise glycerolysis and transesterification were implemented on scaleup.

Packed-bed production. A packed-bed bioreactor represents a practical alternative to batch reactions that use expensive immobilized enzyme. Previous work has shown that EF transesterification with SBO in a packed-bed reaction provides long-term stability for the enzyme (11). Figure 4 compares product yields over time with SBO, EDO, and glycerolyzed SBO (1:1 glycerol/SBO mol ratio) using a packed-bed reactor. As expected, compared with TAG there was a pronounced increase in reactor performance using the partially deacylated oils. Owing to a lower substrate/enzyme ratio, packed-bed conversions were faster than those observed in batch reactions (*cf.* Fig. 3). Glycerolyzed SBO was conveniently generated using Novozym 435 in a packed bed by slowly metering in glycerol over a 24-h period. By this method the same extent of SBO TAG conversion (70%) to MAG and DAG was achieved as that obtained in a batch reactor (Fig. 2, 1:1 glycerol/SBO mol ratio).

These observations indicate that a facile two-step process, SBO glycerolysis followed by EF transesterification, can be implemented in a packed-bed reactor without relying on glycerol-adsorbed silica. The mixture of MAG and DAG from SBO glycerolysis is much more reactive than SBO during EF transesterification, which leads to greater reactor productivity.

ACKNOWLEDGMENTS

We thank Leslie Smith and Kendra Brandon for their valuable technical assistance.

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[Received December 20, 2005; accepted June 19, 2006]