Continuous Enzymatic Esterification of Glycerol with (Poly)Unsaturated Fatty Acids in a Packed-Bed Reactor

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Abstract: Enzymatic synthesis of mono-, di-, and triacyglycerols from (poly)unsaturated fatty acids (linoleic, oleic, and conjugated linoleic acids) has been studied as a solvent-free reaction in a packed-bed reactor containing an immobilized lipase from *Mucor miehei*. The extents of the esterification reactions of interest are primarily determined by the molar ratio of glycerol to fatty acid because the presence of excess glycerol as a immiscible phase is responsible for reducing the activity of the water produced by the esterification reactions. For molar ratios of fatty acid to glycerol of less than 1.5, the percentage of the fatty acid esterified decreases quasi-linearly with an increase in this molar ratio.

By appropriate manipulation of the fluid-residence time, one can control the relative proportions of the various acylglycerols in the effluent stream. At the outlet of the reactor, one observes excellent spontaneous separation of the glycerol and acylglycerol/fatty acid phases. At 50°C and a fluid residence time of 1 hour, as much as 90% of the fatty acid can be esterified when the molar ratio of fatty acid to glycerol is 0.33 or less. *Biotechnol Bioeng* **68**: 563–570, 2000.
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INTRODUCTION

The ability of lipases to catalyze esterification reactions has been known for nearly a century (Jalander, 1911), and the concomitant ability of these enzymes to bring about the (reverse) hydrolysis reactions has long been utilized by food manufacturers to generate flavoring agents (e.g., in production of lipolyzed butteroils). As epidemiological evidence for the role played by triacylglycerols and their associated fatty acid residues in the incidence of certain disease conditions (e.g., atherosclerosis and coronary heart disease) has become available in recent decades, food manufacturers have become increasingly interested in modifying naturally occurring oils and fats to eliminate undesirable fatty acid residues and to replace them with residues which are beneficial to human health. In this respect, saturated fatty acid residues such as myristic (C14:0) and palmitic (C16:0) are regarded as detrimental to human health and polyunsaturated fatty acid residues are looked on favorably. Consequently, researchers in the food industry are currently expressing increased interest in the possibility of employing lipases as biocatalysts for the synthesis and/or modification of fats and oils. The mild conditions characteristic of enzymatic processes offer an attractive alternative to the high temperatures (220–250°C) and pressures characteristic of classic industrial steam-splitting processes that can lead to discoloration and or degradation of the products. Those fats and oils containing highly unsaturated fatty acid residues are of particular interest (McNeill et al., 1991).

Economic considerations, usually related to the high cost of enzymes, have prevented more widespread commercial adoption of enzymatic synthesis procedures (Macrae and Hammond, 1985; Vulfson, 1994). One approach to circumventing this limitation is a low-cost enzymatic process that minimizes the problem of enzyme inactivation and optimizes both yield and reaction selectivity. Recent advances in the commercial development of immobilized lipases have led to biocatalysts that could be employed in bioprocesses for manipulation of the fatty acid composition of triacylglycerols (fats and oils) to obtain products with properties that are more advantageous from the standpoint of human health and/or functionality. To this end, we have explored the use of packed-bed reactors containing immobilized lipases to carry out several reactions for the synthesis of acylglycerols containing residues of unsaturated fatty acids, namely, linoleic, oleic, and conjugated linoleic acids.

In esterification reactions, it is desirable to shift the position of thermodynamic equilibrium by removal of the water produced by the reaction. Several approaches to removing this water, e.g., evaporation (Ergan et al., 1990), pervaporation (Keurentjes et al., 1994), and use of molecular

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sieves (Gancet, 1990) have been successfully employed. Although all these procedures are able to remove the water produced by esterification reactions in batch reactors, this task is more difficult to accomplish when packed-bed reactors are used for ester-synthesis reactions, especially when only food-grade materials can be employed.

Consequently, the present article focuses on a procedure for the continuous enzymatic esterification of glycerol with (poly)unsaturated fatty acids. An immobilized form of a lipase from *Mucor miehei* is packed in tygon tubing and a mixture formed exclusively by the fatty acid of interest and glycerol is fed to this reactor at an appropriate flow rate. The water produced by the esterification reaction is removed by employing an excess of glycerol. The reaction mixture consists of two liquid phases, both at the inlet and at the outlet of the reactor. The fact that the effluent mixture spontaneously separates rapidly into a lipophilic (acylglycerol/fatty acid) phase and a glycerol/water phase facilitates recovery of the excess glycerol for recycle (after removal of its water content).

In the present context, our primary interest in linoleic acid is that it represents a useful (and economic) surrogate for conjugated linoleic acid. Conjugated linoleic acid (CLA) is a generic term given to the mixtures of isomers of linoleic acid containing conjugated double bonds. CLA is of particular interest to manufacturers of nutraceuticals, i.e., foods or parts of foods that when ingested confer either therapeutic or prophylactic medicinal benefits in addition to providing nutritional value.

Several studies based on animal models demonstrate that ingestion of conjugated linoleic acid (CLA) has physiological benefits, e.g., protection against certain types of cancer, antiatherogenic effects, beneficial effects in the cardiovascular system, enhancement of immunological functions and alteration of metabolic processes to reduce the percent fatty tissue and increase the percent muscle tissue (Doyle, 1998). Ip et al. (1994) have suggested that relatively low consumption of CLA (ca. 3.5 g/d for a 70-kg person) could provide protection from certain forms of cancer. A Surgeon General's report indicated that 68% of all deaths in the United States occur as a direct result of coronary heart disease, arteriosclerosis, cancer, diabetes, and high blood pressure (U.S. Department of Health and Human Services, 1988). In this context, CLA is a very promising substance for inclusion in nutraceuticals.

Because the esterified form of CLA has greater oxidative stability in comparison to the free acid form, and because acylglycerols have desirable functional properties, nutraceuticals that incorporate CLA in foods for human consumption should employ acylglycerols enriched in CLA residues rather than free CLA. Potential products include a wide variety of dairy products containing milkfat (dairy spreads, frozen desserts, fluid products, etc.) that could be fortified in CLA. Potential routes to such products include direct polyesterification of glycerol with CLA (Arcos et al., 1998a) and enrichment of naturally occurring fats and oils with CLA via acidolysis reactions (Garcia et al., 1998). The work described below represents an extension of earlier batch-reactor studies involving the polyesterification of glycerol with CLA (Arcos et al., 1998a).

MATERIALS AND METHODS

Materials

An immobilized form of a *Mucor miehei* lipase (Chirazyme L-9) was kindly provided by Boeringer-Mannheim Biochemicals (Indianapolis, IN). The biocatalyst has a particle size in the range from 0.2 to 0.8 mm. Linoleic acid (65%) was obtained from ICN Biomedical (Costa Mesa, CA), while oleic (Class III B) was purchased from Fisher Scientific (Hanover, IL). Conjugated linoleic acid (60% purity) was produced by chemical isomerization of pure linoleic acid following the procedure developed by Chin et al. (1992). This acid was kindly provided by Dr. Michael Pariza of the Food Research Institute of the University of Wisconsin–Madison. Glycerol (99% pure), 1(3)-monolinoleylglycerol, 1,2 (2,3)-dilinoleylglycerol, 1,3-dilinoleylglycerol, and trilinoleylglycerol were purchased from Sigma Chemical Company (St. Louis, MO).

The solvents employed in the analytical work (hexane, 2-propanol, ethyl acetate, and formic acid) were all HPLC grade and were obtained from Fisher Scientific.

Apparatus

The packed-bed reactor consisted of a length of tygon tubing sufficient to contain the weight (ca. 560 mg) of immobilized enzyme employed (20 cm for an internal diameter [ID] of 0.32 cm, 8.9 cm for an ID of 0.47 cm, 5 cm for an ID of 0.63 cm, and 3.2 cm for an ID of 0.79 cm). The tygon tubing was packed manually with the dry immobilized enzyme, which was then fixed in place using plugs of glass wool. This tubing was coiled into a single loop and submerged in a constant temperature water bath. The glycerol and the fatty acid of interest were pumped individually using syringe pumps (Kd Scientific, model 220). Prior to entering the packed bed, the feed streams were mixed using a Y-shaped connector and pumped through 33 cm of a 0.16 cm (ID) stainless steel coil submerged in the water bath. Samples were collected manually at the outlet of the reactor. A schematic drawing of the process is shown in Figure 1.

All experimental runs were conducted in duplicate.

Operating Protocol

Each experiment was initiated by flushing the reactor with a total volume of the mixture of substrates equal to at least twice the void volume of the reactor. The mixture was fed at a rate corresponding to a reactor space-time of 15 min. [For a liquid-phase system the reactor space-time is equal to the mean residence time of the fluid in the reactor (Hill, 1977).] Next, the flow rate was adjusted to produce the

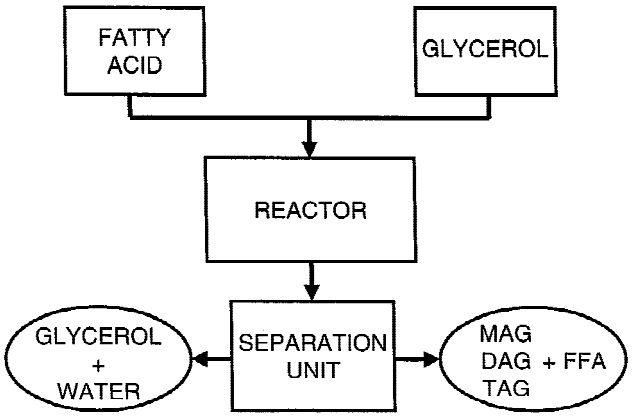

Figure 1. Schematic representation of the esterification process. MAG, DAG, and TAG represent mono-, di-, and triacylglycerols respectively, FFA represents free fatty acids.

desired space-time. After quasi-steady-state conditions were achieved, several samples of the effluent stream were taken over a time frame corresponding to at least four reactor space-times. Because all experiments were performed in duplicate, the experimental data points correspond to the average of no less than four measurements of the effluent composition.

Calculation of the Void Volume

To calculate the void volume of a packed-bed reactor, a length of tygon tubing was packed with 2 g of the immobilized enzyme. The void volume was calculated using the difference in the weight of the packed-bed reactor filled with a fatty acid of known density, and the weight of the packed-bed reactor in the absence of this fluid. No significance differences in the void volume per mg of enzyme were observed when tygon tubes of different diameters were utilized. There was no indication that there was significant swelling of the support when contacted with the fatty acid or glycerol. Void volumes ranged from 1.83 to 1.89 cm^3/g of catalyst. Reactor space-times were calculated as the ratio of the void volume of the reactor to the total volumetric flow rate of feedstock.

The porosity of the particle of the immobilized enzyme is 0.53 ± 0.02 (Mensah et al., 1998). The observed rates of reaction are sufficiently low that external and intraparticle mass transfer limitations are absent.

Analytical Methods

Samples (ca. 0.4 mL) of the reaction mixture were taken at the reactor outlet. Although excellent spontaneous separation of the glycerol and acylglycerol/fatty acid phases was observed at the exit from the reactor, the mixture was centrifuged at 2819*g* for 5 min. Then, 25 μL of the acylglycerol/fatty acid phase was dissolved in 3.5 mL of a mixture of chloroform and methanol (2:1, v/v). The percentages of mono- di- and triacylglycerols and unreacted fatty acid in the acylglycerol/fatty acid phase were determined by HPLC. Analysis of the glycerol phase indicated that the concentrations of both acylglycerols and fatty acids were negligible.

The HPLC analysis of the reaction mixture employed an Econosil-Silica 5U column (250 mm by 4.6 mm from Alltech). The HPLC system was equipped with a light-scattering detector. A modified version of the method described by Liu (1993) was used. Two mobile phases were employed: Phase A consisted of hexane, 2-propanol, ethylacetate, and formic acid in the respective proportions (v/v) 80:10:10:0.1, while phase B was pure hexane. The flow rate of the mobile phase was 2 mL/min. A splitter valve was used after the column, and only 50% of the mobile phase was directed through the detector.

The column was first eluted for 6 min with a 15 /85 (v/v) mixture of phase A and phase B and then with a 98/2 mixture for an additional 7 min. Next, the system was restored to its initial conditions by passing an 85/15 mixture of phases A and B through the column for 6 min. The retention times for the triester, fatty acid, 1,3-diester, 1,2-diester, 1(3) monoester and 2-monoester were 1.7, 3, 4.7, 7.9, 13, and 14 min, respectively. High-purity standards of these types were purchased from vendors and used to calibrate the response of the HPLC detector. No significant amount of the 2-monoester was detected in any experiment.

RESULTS AND DISCUSION

Linoleic acid with a purity of ca. 65% was used for the bulk of the experiments for economic reasons. A conjugated linoleic acid of approximately this purity is envisioned as the feedstock for a component of a process which we are developing. Subsequent experiments extended the methodology to synthesis reactions involving conjugated linoleic acid and oleic acid.

Effect of the Inner Diameter of the Reactor Tube

The effect of the inner diameter of the reactor tube on the percentage of fatty acid esterified at the reactor outlet was studied for a range of diameters from 0.32 to 0.79 cm (⅛ to 5⁄16 inch). Chirazyme L-9 (560 mg) was packed in all reactors. The corresponding-void volume of the packed bed is ca. 1.86 cm^3/g of catalyst. Next, the reactor was coiled to form a single loop, except for the reactor with an inner diameter of 5⁄16 in, which was installed as a straight tube in a vertical position and fed from the bottom.

A molar ratio of linoleic acid to glycerol 1.5 to 1 was used in this series of experiments because this ratio corresponds to the highest reaction rate observed in earlier experiments in batch reactors (Arcos et al., 1998a). A flow rate that provides a space-time of 1 h was employed in this series of experiments. For an ideal plug-flow reactor, the ratio of the length of the bed to its diameter should not have a significant influence on the extent of the reaction (Hill, 1977). However, the experimental data shown in Figure 2 indicate

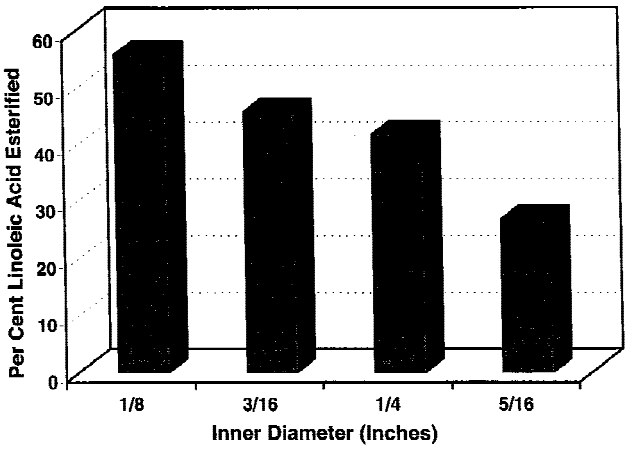

Figure 2. Extent of esterification (expressed as a mole percentage of the linoleic acid fed to the reactor) as a function of the inner diameter of the reactor. Conditions: 50°C; molar ratio of fatty acid to glycerol = 1.5; space-time = 1 h.

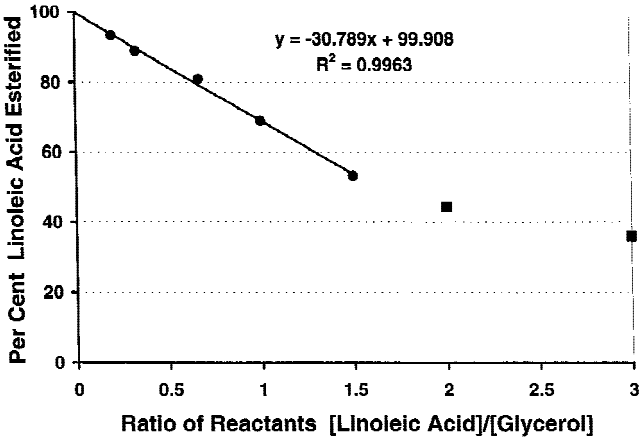

Figure 3. Dependence of the extent of esterification (expressed as a mole percentage of the linoleic acid fed to the reactor) on the molar ratio of linoleic acid to glycerol at the reactor inlet. Conditions: 50°C; space-time = 1 h.

that higher conversion levels were obtained in those experiments carried out in reactors with smaller inner diameters.

Because the reactions of interest involve reactants which are immiscible, the feed mixture consists of two liquid phases. Visual observations indicated that the glycerol was present as small droplets dispersed in the lipophilic phase (i.e., the fatty acid/acylglycerol phase). Use of reactors with smaller diameters appears to increase the interfacial area between the fatty acid and the glycerol, thereby facilitating mass transfer between the two phases and enhancing the reaction rate. The attendant increase in the surface area of the disperse (glycerol) phase facilitates dissolution of the glycerol in the continuous fatty acid/acylglycerol phase to replace the glycerol consumed by the reaction at the surface of the immobilized enzyme. Because visual observations indicate that (at least in the region near the reactor inlet) the glycerol phase remains in the center of the tube, it is possible that inefficient contacting of this phase with the portion of the enzyme located at the periphery of the tube is responsible for the lower conversions in the larger diameter reactors. Consequently, in subsequent experiments, the reactors were all constructed using a tygon tube with an inner diameter of 1/8 in.

Effect of the Ratio of Fatty Acid to Glycerol

The dependence of the effluent composition on the molar ratio of fatty acid to glycerol at the reactor inlet was studied in the range from 0.2 to 3.0 by changing the flow rates of both reactant streams to obtain the desired molar ratio while maintaining the overall flow rate at 1 mL/min.

Inspection of Figure 3 reveals that the percentage of fatty esterified at a fixed space-time decreases as the molar ratio of fatty acid to glycerol increases. For ratios from 0.2 to 1.5, a quasi-linear relationship exists between the percentage of fatty acid esterified and the ratio of fatty acid to glycerol at the reactor inlet. Although one cannot achieve quantitative conversion of all the fatty acid employed, a molar ratio of fatty acid to glycerol of 0.33 (corresponding to a volumetric ratio of fatty acid to glycerol of 1.45/1) permits one to continuously esterify ca. 90% of the fatty acid fed to the reactor.

This high degree of esterification at low ratios of acid to glycerol can be attributed not only to the influence of the species concentrations on the equilibrium yield, but also to the desiccant effect of the excess glycerol. Because excess glycerol is employed, a glycerol phase is present throughout the reactor. This phase dissolves the water produced by the esterification reaction, thereby reducing its activity in the fatty acid/acylglycerol phase and preventing accumulation of water on the enzyme support. This hypothesis was tested by using a Karl-Fischer titration to determine the amounts of water contained in each phase of the reaction mixture at both the inlet and the outlet of the reactor. For a molar ratio of glycerol to fatty acid in the feed of three, an operating temperature of 50°C, and a space-time of 1 h, the water content of the glycerol phase typically increased from 0.2% (w/w) at the inlet to 6% (w/w) at the outlet. The water content of the corresponding effluent fatty acid/acylglycerol phase was 0.4%, and the water content of the fatty acid at the reactor inlet was 0.2%.

Colombie et al. (1998) have previously studied the esterification of oleic acid and ethanol in a packed-bed reactor containing an immobilized enzyme. These investigators suggested that the ethanol could remove some of the water produced by the esterification reaction. However, once steady state was reached, the percentage of fatty acid esterified decreased to 50%. The higher polarity of the glycerol employed in our work seems to be responsible for the high efficiency of this compound in removing the water produced by the esterification reaction.

The percentage of triester in the product mixture increased as the ratio of fatty acid to glycerol increased (see Fig. 4). By contrast, the percentage of monoester was larger

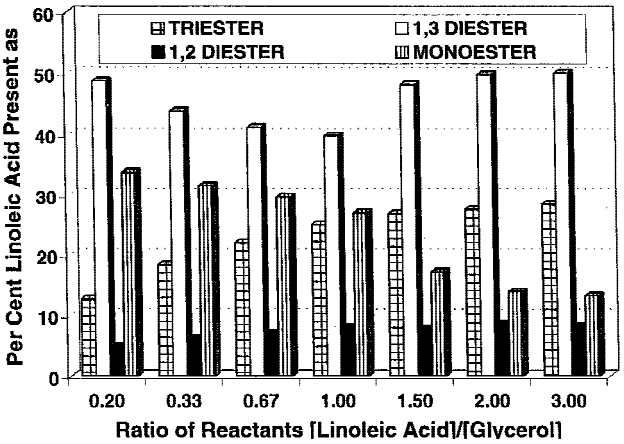

Figure 4. Dependence of the composition at the reactor outlet on the molar ratio of linoleic acid to glycerol at the reactor inlet. Conditions: 50°C; space-time = 1 h.

in those experiments involving a higher ratio of glycerol to fatty acid.

Effect of the Reaction Temperature

The dependence of the effluent composition on the reaction temperature was studied in the range from 20 to 60°C. A molar ratio of fatty acid to glycerol of 0.33 and a space-time of 1 h were employed in this series of experiments.

Examination of Figure 5 reveals that for temperatures from 20 to 50°C, the percentage of unreacted free fatty acid in the effluent decreased as the temperature increased. This result is a consequence of the increase in the reaction rate as the temperature increases. Kinetic effects also explain why the experiments conducted at higher temperatures produced larger amounts of the triester.

For reaction at 60°C, according to the results of batch-reactor studies, the final mixture contained greater proportions of the fatty acid and monoester but less of the triester. The difference in behavior of the experiments carried out at higher temperatures can be attributed to more rapid deactivation of the Chirazyme L-9 at this temperature.

Enzyme Stability at Reaction Conditions

The results of an experiment to ascertain the stability of the enzyme under reaction conditions at 50°C are shown in Figure 6. A molar ratio of fatty acid to glycerol of 0.33 and a space-time of 1 h were used in this experiment.

The total percentage of fatty acid esterified remained essentially constant for 12 days. However, examination of the data reveals an initial decrease in the percentage of triester formed, followed by a much slower decrease in the triester concentration. These results agree with an experiment (data not shown) designed to assess the effect of the enzyme loading on the effluent composition. The data indicate that for ratios of enzyme to feed flow rate (W/F) from 150 to 560 mg of enzyme h/mL, the percentage of fatty acid esterified lies in the range from 80 to 90%, and that increases in W/F are primarily responsible for the increase in the percentage triester at the reactor exit.

The decrease in the triester concentration can be attributed to the deactivation pattern of this enzyme. This biocatalyst is an enzyme adsorbed on a macroporous acrylic resin in the absence of any crosslinking agent. A portion of the lipase may be adsorbed on the exterior surface of the resin beads, while the remainder is adsorbed on the surfaces of the micropores within the support. The portion of the lipase located within the micropores would be better protected against fluid-shear forces than that portion of the enzyme located at the exterior surface. Moreover, because of the geometries of the pores and the enzyme, the portion of the enzyme adsorbed within the pore structure is less susceptible to loss via desorption processes. Recently, Arroyo et al. (1998) reported similar behavior for another li-

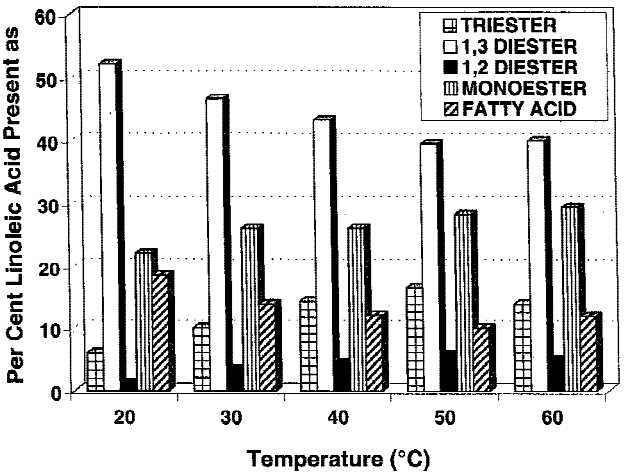

Figure 5. Effect of temperature on the effluent composition for esterification of linoleic acid and glycerol. Conditions: Molar ratio of fatty acid to glycerol = 0.33; space-time = 1 h.

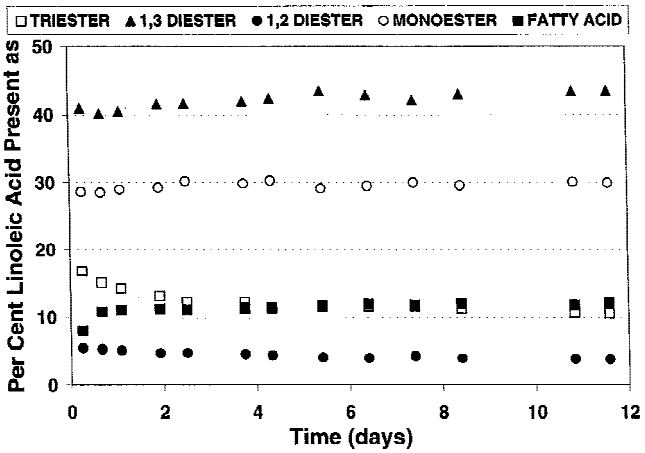

Figure 6. Effluent composition as a function of time on stream for the esterification of linoleic acid and glycerol. Conditions: 50°C; molar ratio of fatty acid to glycerol = 0.33; space-time = 1 h.

pase adsorbed on a macroporous resin. Alternatively, the deactivation of the enzyme could also be explained as one-step transition between native and denatured forms as proposed by Henley and Sadana (1985).

Study of the Reaction at Different Space-Times

Data characterizing the dependence of the effluent composition on the reactor space-time in the range from 5 min to 12 h are shown in Fig. 7 for a reaction conducted at 50°C with a molar ratio of fatty acid to glycerol of 0.33. Inspection of the speciation plots reveals that for space-times between 15 min and 12 h, the percentage of fatty acid consumed lies in the range from 80 to 90%. However, the acylglycerol composition at the reactor outlet is significantly affected by this parameter.

Formation of the monoesters of glycerol occurs very rapidly and the extent of these and subsequent reactions is limited primarily by thermodynamic constraints imposed by the water content of the acylglycerol/fatty acid phase. This constraint can be partially circumvented by utilizing the ability of the glycerol phase to function as a desiccant. Transfer of water from the acylglycerol/fatty acid phase to the glycerol phase reduces the activity of the water in the former phase, thereby influencing the thermodynamic driving force for the reaction. Once the glycerol phase is saturated with water, the amount of fatty acid esterified does not increase even if the space-time is increased from 30 min to 12 h.

The slow increase in the percentage triester present in the acylglycerol phase with increasing space-time reflects the influence of several reactions occurring in this phase. Reaction of the hydroxyl groups at the *sn-2* position is slow, relative to the rates of esterification at the *sn-1* and *sn-3* positions. Hence, formation of the triester may be limited by the necessity for acyl migration from the *sn-1* or *sn-3* position to the *sn-2* position. In addition, the slow increase in triester content may reflect the influence of alcoholysis reactions involving different acylglycerol species contained in the lipophilic phase. Moreover, the observed increase in triester content could also be affected by the rates of hydrolysis and re-esterification reactions occurring within the lipophilic phase.

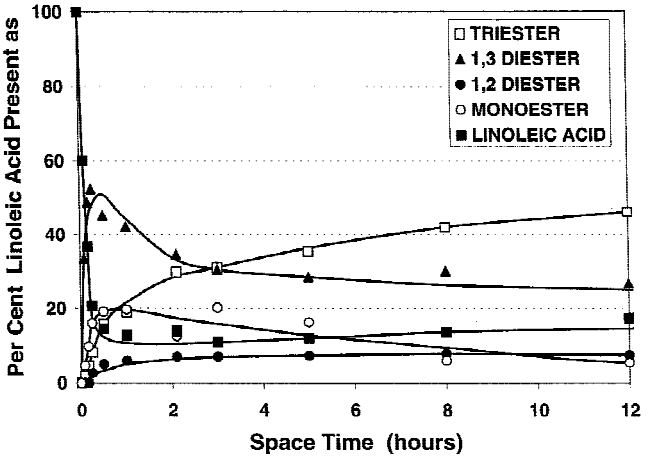

Figure 7. Dependence of effluent composition (speciation) for the reaction of linoleic acid and glycerol on the reactor space-time. Conditions: 50°C; molar ratio of linoleic acid to glycerol = 0.33.

Another interesting phenomenon is that when very short space-times (5 or 10 min) are employed, the molar percentage of diester at the reaction outlet is much larger than the molar percentage of the monoester. For example, at a space-time of 5 min, the relative yields of the various product acylglycerols (in mole percentages) are 3% triester, 76% diester, and 21% monoester; at a space-time of 10 min the corresponding values are 4, 68 and 28%, respectively. Similar behavior was observed in previous studies by Arcos et al. (1998b, 1998c) of the acylation of fructose and sorbitol in acetone. These results are readily explained if one assumes that the glycerol that is being esterified is only that portion dissolved in the lipophilic phase. Once the monoacylglycerol is synthesized, it will remain dissolved in the lipophilic (fatty acid) phase, and it will be rapidly esterified again to form a diester. The formation of the triester, takes place more slowly, because this step involves esterification of a secondary hydroxyl group.

In the experiments conducted at higher temperatures (see Fig. 5), the product contained larger amounts of monoacylglycerols. Because the solubility of glycerol in the fatty acid/acylglycerol phase (the lipophilic phase) increases with increasing temperature, those experiments carried out at higher temperatures will lead to higher percentages of the monoester in the effluent stream because of the concomitant shift in the ratio of glycerol to fatty acid in the lipophilic phase. The high concentrations of the diesters may reflect the fact that the large majority of the enzyme is contained within the pores of the support. Once a glycerol molecule reacts with the acylated form of the enzyme within the pore structure, the resulting monoester must diffuse out of the pore to the external surface of the support and then undergo mass transfer to return to the bulk fluid. During the diffusion process, the monoacylglycerols are confined in a region containing an abundance of adsorbed enzyme molecules, thereby facilitating conversion of the monoacylglycerol to the diacylglycerol.

Two Reactors in a Series Flow Configuration

Examination of Figure 3 reveals the impracticality of achieving quantitative conversion of all the fatty acid fed to the reactor. However, the fatty acid is the most expensive reactant in this synthesis. Because nearly perfect phase separation is observed at the reactor exit, one can readily recycle the glycerol stream after it has been dried to remove water.

In a effort to reduce the amount of glycerol required to esterify a fixed amount of fatty acid, an experiment was designed to simulate the operation of two packed bed reactors in a series configuration with removal of the glycerol phase between reactors (Fig. 8).

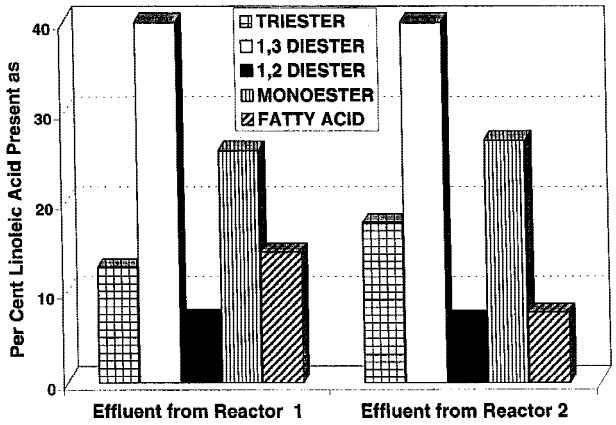

Figure 8. Simulation of two reactors in series for esterification of linoleic acid and glycerol. Conditions: 50°C; space-time = 30 min.

Effluents collected from several reactors operated at a space-time of 1 h were mixed and placed in a separatory funnel. The glycerol phase was discarded, and the upper phase (containing 12.7% triester, 40.1% 1,3 diester, 7% 1,2 diester, 25.8% monoester and 14.4% free fatty acid) was used as the feedstock for a reactor packed in the usual manner with Chirazyme L-9. This feedstock was pumped with a syringe pump through a Y-shaped connector where it was mixed with fresh glycerol (containing 0.2 weight percent water). The relative flow rates of the two syringe pumps were set to provide a space-time of 0.1 h and a molar ratio of free fatty acid to glycerol of 0.33.

Inspection of the data in Figure 8 reveals that at the outlet of the second reactor, the percentage of free fatty acid decreased by 50%. The corresponding global conversion after a total space-time of 1.1 h was 92.3%. The free fatty acid esterified in the second reactor was primarily incorporated in triester molecules. No significant increase was observed in the percentage of monoester. This result indicates that glycerolysis reactions do not occur to a significant extent in the second reactor and that the reactions of the free fatty acids occur primarily in the lipophilic phase.

Use of the equation shown in Figure 3 indicates that the feedstock to the second reactor would be equivalent (in terms of its percentage of fatty acid esterified) to the mixture obtained by feeding a reactor with a mixture containing a molar ratio of fatty acid to glycerol of 0.47 for a space-time of 1 h. One could also obtain the same overall conversion level (92.3%) in a single reaction by operating with a ratio of fatty acid to glycerol of 0.25.

The calculated amount of glycerol required to esterify 100 g of fatty acid to a final conversion of 92.3%, would be 84.5 g when two packed-bed reactors are employed in a series configuration, but 132.3 g would be required if one used a smaller molar ratio of fatty acid to glycerol. The global space-time increased by only 10% when a series configuration was employed, yet the total glycerol requirement is reduced by approximately 36% relative to the single reactor situation.

Reactions with Different Fatty Acids

The ultimate purpose of the present project is to develop a process for the continuous production of acylglycerols enriched in CLA. Hence, once an appropriate methodology had been developed for linoleic acid, it was extended to the analogous reactions of conjugated linoleic acid. The viability of the method was also confirmed using oleic acid (data not shown) as a second unsaturated fatty acid of potential interest. Both sets of experiments were conducted using a molar ratio of fatty acid to glycerol of 0.33.

Examination of the data in Figure 9 indicates that although the syntheses with conjugated linoleic acid do not exhibit exactly the same pattern as the esterification of linoleic acid with glycerol, both reactions are characterized by very similar behavior. The reactor space-time appears to be the variable primarily responsible for the distribution of acylglycerols at the reactor outlet. The results of the esterification reactions of oleic acid and glycerol very closely resembled those for the esterification of linoleic acid with glycerol.

CONCLUSIONS

A new strategy for the continuous enzymatic synthesis of acylglycerols from fatty acids and glycerol has been described. The procedure is simple and rapid and is especially appropriate for use in the synthesis of acylglycerols enriched in (poly)unsaturated fatty acids which are intended for use as food additives and/or food ingredients.

The percentage of fatty acid esterified is largely determined by the molar ratio of glycerol to fatty acid and the residence time of the two-phase mixture in the reactor. By appropriate manipulation of the flow rate, one can control the product distribution so as to synthesize reaction mixtures that on the one hand, could be employed as food emulsifiers or on the other hand, as fat substitutes. The high stability of the immobilized enzyme permits one to operate

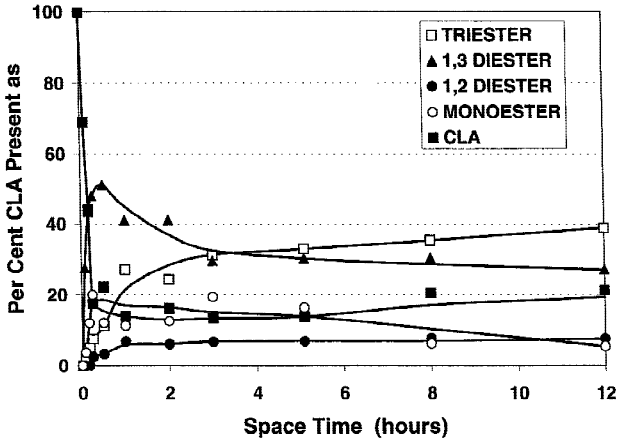

Figure 9. Dependence of effluent composition (speciation) for the reaction of conjugated linoleic acid and glycerol on the reactor space-time. Conditions: 50°C; molar ratio of oleic acid to glycerol = 0.33.

the reactor continuously for 12 days without observing significant deterioration in the percentage of free fatty acid esterified. The esterification reactions of glycerol with linoleic, conjugated linoleic, and oleic acid are characterized by very similar behavior. Consequently, the same strategy can be employed for the synthesis of nutraceuticals enriched in these (poly)unsaturated fatty acids.

Use of a packed-bed reactor is recommended for the continuous production of acylglycerols enriched in CLA intended for use as food emulsifiers (reaction mixtures containing mainly mono- and diesters). The approach described above permits one to continuously synthesize appropriate product mixtures using space-times as short as 15 minutes. However, for applications involving the production of mixtures suitable for use as fat substitutes, use of a batch reactor is recommended. Long residence times in a packed-bed reactor are required to produce mixtures rich in triacylglycerols. Combinations of packed-bed reactors in a series-flow configuration, together with removal of the glycerol phase between stages, could be utilized to reduce the total residence time required to synthesize these products.

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