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Preparation of Mono- and Diacylglycerols by Enzymatic Esterification of Glycerol With Conjugated Linoleic Acid in Hexane

C. E. Martinez,[1] J. C. Vinay,[2] R. Brieva,[3] C. G. Hill, Jr.,[4] and H. S. Garcia*,[2,4]

[1]Coordinación de Posgrado e Investigación, Instituto Tecnológico de Tuxtepec, Tuxtepec, Oax. 68300, Calz. Dr. Víctor Bravo Ahuja s/n, Mexico;
[2]UNIDA-Instituto Tecnológico de Veracruz, M.A. de Quevedo # 2779, Veracruz, Ver. 91897, Mexico, E-mail: hsgarcia@itver.edu.mx;
[3]Facultad de Química, Universidad de Oviedo, 33071 Oviedo, Spain; and [4]Department of Chemical and Biological Engineering, University of Wisconsin–Madison, 1415 Engineering Drive, Madison, WI 53706

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Abstract

Esterification of glycerol with conjugated linoleic acid (CLA) was carried out in hexane. Lipase from *Rhizomucor miehei* provided a high degree of esterification (80%) in 8 h at 50°C when used at 15% (w/w) in a system containing a 1:2 molar ratio of glycerol to free fatty acids. Esterification levels >80% were obtained in 8 h at 40°C with 15% (w/w) lipase from *Candida antarctica* at the same molar ratio of reactants. The extent of esterification of CLA was >90% after 4 h of reaction at 50°C with a 5% (w/w) loading of either *R. miehei* or *C. antarctica* lipase, together with a 1:1 molar ratio of substrates. Both enzymes incorporated the original CLA as acylglycerol residues in primarily 1,3-diacylglycerol and 1-monoacylglycerol. The CLA-rich acylglycerols can be employed as emulsifiers or as substitutes for natural fats and oils.

Index Entries: Conjugated linoleic acid; lipases; polyesterification; nutraceuticals; structured lipids.

*Author to whom all correspondence and reprint requests should be addressed.

Introduction

Conjugated linoleic acid (CLA) is a class of positional and geometric conjugated dienoic isomers of linoleic acid. This terminology originated in 1985 when Pariza and Hargraves *(1)* reported biologic activity of CLA produced by alkali isomerization of linoleic acid. Further studies demonstrated that synthetically prepared CLA was both an effective inhibitor of benzo[*a*]pyrene-initiated mouse epidermal neoplasia and an effective agent in inhibiting the development of mammary tumors induced by dimethylbenzo[*a*]anthracene *(2–7)*. These reports were followed by studies demonstrating that feeding CLA (0.5% by weight of diet) to rodents and chickens protected them from the catabolic effects of immune stimulation *(8,9)*. CLA may also act as a growth-promoting agent *(10)*, exert a hypocholesterolemic effect *(11)*, and play a role in mobilization of body fat *(12)*. Ip et al. *(6)* suggested that a relatively low consumption of CLA (3.5 g/d) would provide a protective effect for a person weighing 70 kg. For all these reasons, CLA is an impressive addition to the category of nutraceuticals.

One route by which CLA might be incorporated into human diets involves the production of fats and oils enriched in CLA residues. These materials can be used to manufacture nutraceutical dairy spreads and frozen desserts designed for consumption by individuals who are at risk for those disease conditions against which CLA has demonstrated bioactivity.

Lipases have been used with increasing frequency as biocatalysts for the synthesis and/or modification of fats and oils, as well as for their lipolysis. The mild conditions of enzymatic processes offer an attractive alternative to the high temperatures (220–250°C) and pressures of classic industrial steam-splitting processes, which can lead to discoloration and degradation of the product. Fats and oils containing highly unsaturated fatty acid residues are of particular interest *(13)*. Preparation of structured lipids as a technology for modification of oils to improve their nutritional and health benefits is currently attracting worldwide attention *(14)*.

In this article, we report protocols for the synthesis of acylglycerols containing CLA and the fatty acids generated as byproducts in commercial-scale processes for the production of CLA. These protocols involve the use of immobilized lipases in hexane to achieve nearly quantitative incorporation of CLA and other associated (present as benign contaminants) fatty acids in acylglycerols to produce mixtures of monoacylglycerols (MAGs) and diacylglycerols (DAGs) that can be employed as food-grade emulsifiers.

Materials and Methods

Lipases and Chemicals

The immobilized lipases Chirazyme L-2 from *Candida antarctica* fraction B (10,000 PLU/g) and Chirazyme L-9 from *Rhizomucor miehei* (minimum of 150 IEU/g) were obtained from Boehringer-Mannheim (Indiana-

polis, IN). CLA concentrate containing about 57% CLA was prepared by chemical isomerization of sunflower oil as reported previously *(15)*. The primary impurity in this preparation is oleic acid (C18:1). The fatty acid composition of the CLA concentrate was 0.24 wt% C14:0, 7.85 wt% C16:0, 0.22 wt% C16:1, 4.32 wt% C18:0, 27.53 wt% C18:1, 1.88 wt% C18:2, and 57.96 wt% CLA. All solvents were reagent grade or better as procured from Baker (Mexico City). Molecular sieves (3 Å) were purchased from Sigma (Mexico City), methanolic HCl (3 *M*) was obtained from Supelco (Mexico City), and 0.1 *N* methanolic NaOH was prepared from its components.

Esterification Reactions

Reactions were carried out by mixing 644 mg (7 mmol) of glycerol adsorbed on silica gel (weight ratio of adsorbate to adsorbent = 1:1) according to the approach of Berger et al. *(16)* and 1.946 g (7 mmol) of the CLA mixture (for a molar ratio of glycerol to CLA of 1:1). The molar ratios of the CLA mixture to adsorbed glycerol employed were 1:1 and 1:2. (The amount of glycerol was fixed and the amount of the CLA mixture was varied as appropriate.) These substrates were mixed with 20 mL of hexane and 3 g of molecular sieves and placed in 50-mL Erlenmeyer flasks fitted with septa-capped stoppers. Reaction temperatures ranged from 30 to 50°C. Lipase loadings were 5, 10, 15, and 20% (w/w) of the combined weight of the substrates. Each enzyme preparation had previously been dried under vacuum for 20 min, and the headspace in each flask was purged with nitrogen. The suspensions were incubated in an orbital shaker set at 150 rpm. At various times during incubation, two 0.2-mL samples were withdrawn from each flask and mixed with 0.8 mL of 2:1 (v/v) chloroform/methanol. The extent of esterification of CLA was determined by gas chromatography (GC) analysis, and the various acylglycerols were analyzed by high-performance liquid chromatography (HPLC).

Determination of Extent of Esterification

Analyses for esterified fatty acids were conducted by adding 1 mL of 0.1 *N* methanolic NaOH to a 200-µL aliquot of each sample. The mixture was allowed to stand at room temperature for 30 min, and then 200 µL of distilled water was added. The resulting mixture was extracted with 1 mL of hexane. Analysis of total fatty acids was conducted by adding 1 mL of 0.2 *N* methanolic HCl to a second 200-µL aliquot of each sample *(17)*. The sample vial was then flushed with N_2 and held overnight in a heating block at 80°C. Then 200 µL of water was added, and the mixture was extracted with 1 mL of hexane. For each aliquot, heptadecanoic acid methyl ester (C17) was added as an internal standard. Samples (1 µL) were then injected into the gas chromatograph.

GC Analysis of Fatty Acid Methyl Esters

Analyses for fatty acid methyl esters (FAMEs) were conducted by GC following the method of Chin et al. *(18)*. Separation of the FAMEs was

accomplished using a Hewlett-Packard HP 6890 GC fitted with a flame ionization detector set at 250°C, and a Supelcowax-10 capillary column (60 m × 0.32 mm id, 0.25-μm film thickness; Supelco, Bellefonte, PA). A splitless injector (set at 250°C) was employed and nitrogen was the carrier gas (4 mL/min). The temperature program used was from 60 to 200°C at 20°C/min and held for 43 min. An internal standard (C17) was employed.

HPLC Analysis of Acylglycerols

The lipid classes were separated using solid-phase extraction on Bond-Elut (3 g) silica prepacked columns (Supelco). Mobile phases used for selective elution of acylglycerol fractions were those reported by Prieto et al. *(19)*. The protocol for the HPLC analyses of acylglycerols in the reaction mixture utilized an Econosil-Silica 5U column (250 × 4.6 mm; Alltech, Deerfield, IL) in a Waters (Milford, MA) HPLC system equipped with an Alltech 500 evaporative light-scattering detector. The modified version of the method of Liu et al. *(20)* used in these analyses is described elsewhere *(15,21)*.

Two mobile phases were employed: phase A consisted of pure hexane, and phase B consisted of hexane, 2-propanol, ethyl acetate, and formic acid in the respective volumetric proportions of 80:10:10:0.1. 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 an 85/15 (v/v) mixture of phase A and phase B. Then a gradient was programmed to go from this eluent composition to a 2/98 (v/v) A/B mixture. The latter mixture was then employed for an additional 7 min. Next, the system was restored to its initial conditions by passing an 85/15 (v/v) mixture of phases A and B through the column for at least 6 min prior to the next injection. Retention times were 1.72 min (triacylglycerol [TAG]), 3.03 min (free fatty acids), 4.56 min (1,3-DAG), 7.31 min (1,2-DAG), 11.39 min (1-MAG), and 11.69 min (2-MAG).

Results and Discussion

Use of Molecular Sieves

Molecular sieves were employed throughout each trial to sequester the water produced by esterification. No trials were carried out without molecular sieves because other researchers have clearly demonstrated the benefits of their incorporation into the reaction mixture. Robles-Medina et al. *(22)* suggested that molecular sieves should be added at the beginning of the reaction to attain high rates of esterification, whereas Ergan et al. *(23)* recommend their addition at a stage close to equilibrium.

Effects of Enzyme Loading

The effects of loading the immobilized enzyme were determined for each molar ratio of substrates. Lipase L-9 was used at 5% of the combined weight of substrates with a molar ratio of glycerol to CLA of 1:1.

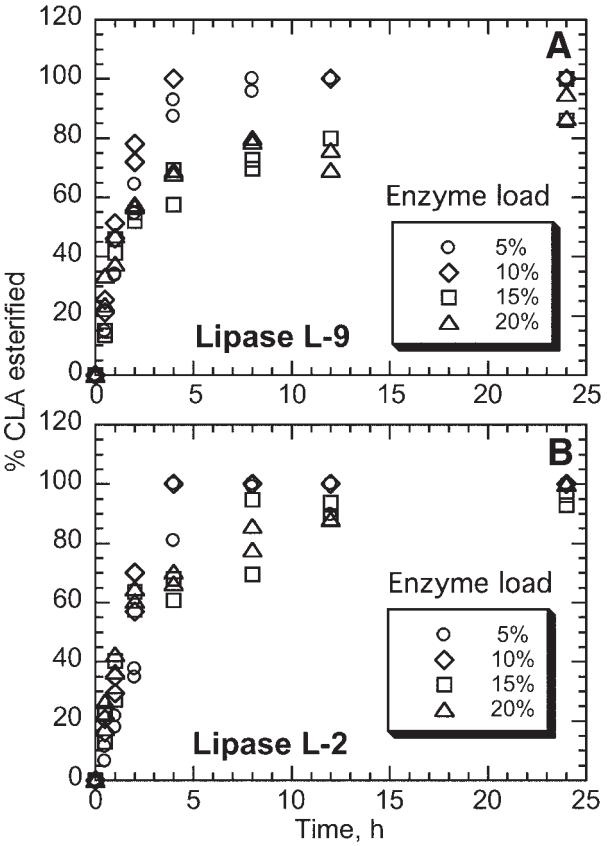

Fig. 1. Effect of enzyme loading (in weight percent) on extent of esterification of CLA: **(A)** lipase L-9; **(B)** lipase L-2. Reaction conditions were as follows: 644 mg of glycerol, 1.946 g of CLA, molar ratio of CLA to glycerol of 1:1, 3 g of molecular sieves, 20 mL of hexane, and orbital shaker set at 150 rpm and 50°C.

These conditions produced nearly 100% esterification in 8 h of reaction at 50°C. By contrast, only 4 h at the same temperature was required to achieve 100% esterification when an enzyme loading of 10% was used (Fig. 1A). For enzyme loadings of 5 and 10%, the higher loading led to the same degree of esterification in a shorter time; however, for higher enzyme loadings, no significant increase in the rate of esterification of CLA was observed. This result is consistent with data reported by Arcos et al. *(13)*, who found that for enzyme loadings between 0 and 7.5%, reaction rates were roughly linear in the loading, but at higher loadings the extent of esterification of CLA was independent of the enzyme loading employed. These researchers concluded that the observed results could be a consequence of a variety of factors, such as failure of the shaker to maintain all of the immobilized enzyme in the suspension, agglomeration of suspended particles, or mass transfer limitations on the rates of adsorption or reaction, among others. To ensure that mass transfer limitations would be minimized, we employed a 5% loading of lipase L-9 in subsequent experiments.

Data obtained for reactions catalyzed by Lipase L-2 indicated that essentially 100% esterification was achieved in 4 h, regardless of whether an enzyme loading of 5 or 10% was used (Fig. 1B). In experiments in which enzyme loadings of 15 and 20% were employed, the rate and extent of esterification of CLA were essentially independent of the amount of enzyme used.

Ratio of Glycerol to Fatty Acids

Molar ratios of glycerol to fatty acids of 1:1 and 1:2 were employed to ascertain the effects of this ratio on the esterification reaction. The amount of glycerol was fixed and the weight of CLA was adjusted to obtain the desired ratio. Since the purpose of the present study was to prepare MAGs and DAGs rich in CLA residues, the 1:3 ratio required by stoichiometric considerations for the synthesis of TAGs was not employed. This approach is consistent with the results reported by Robles-Medina et al. *(22)*, who recommend using a molar ratio of glycerol to fatty acid of 1.2:3 for the preparation of lower acylglycerols from glycerol and polyunsaturated fatty acids. Moreover, Arcos et al. *(13)* suggested using a molar ratio of 1:2 for the same purpose. Inspection of Fig. 1 indicates that reactions conducted using a 5% (w/w) loading of either immobilized lipase (L-2 or L-9) for a 1:1 molar ratio of reactants gave essentially 100% esterification in 8 h of reaction.

Reactions conducted using a 1:2 molar ratio of glycerol to fatty acid took longer to achieve high degrees of esterification of the CLA, but it must be remembered that in these systems twice as much fatty acid is present as in systems with a 1:1 molar ratio. In the systems with a molar ratio of glycerol to fatty acid of 1:2, complete esterification (100%) of the fatty acid was not achieved in 24 h (Fig. 2). Lipase L-9 effected a high degree of esterification (about 80%) in 8 h when the enzyme loading was 15% (w/w) in a system containing a 1:2 molar ratio of glycerol to fatty acid (Fig. 2A). Esterification levels >80% were obtained after 24 h of reaction when a loading of 15% (w/w) lipase L-2 was used together with the same 1:2 molar ratio of glycerol to fatty acid (Fig. 2B).

The dependence of lipase activity on the interfacial surface area was demonstrated by Akoh and Min *(24)*, who measured the rates of hydrolysis in coarse and fine emulsions. They also observed that the reaction rate depends on the concentration of substrates in the reaction medium. Cerdán et al. *(25)* reported that a stoichiometric ratio of 1:3 (glycerol:fatty acid) is optimal for production of high proportions of TAG. The use of an excess of glycerol increased the degree of esterification of CLA because the equilibrium extent of reaction shifts toward the product ester. However, under these conditions the yield of TAG is reduced, and greater proportions of MAG and DAG are formed. Similar results were obtained by Ergan et al. *(26)*, who utilized an immobilized *Mucor miehei* lipase to catalyze synthesis reactions between glycerol and oleic acid. Our approach was intended to shift the reaction equilibrium toward the formation of lower acylglycerols by using an excess of glycerol relative to that required for synthesis of TAG.

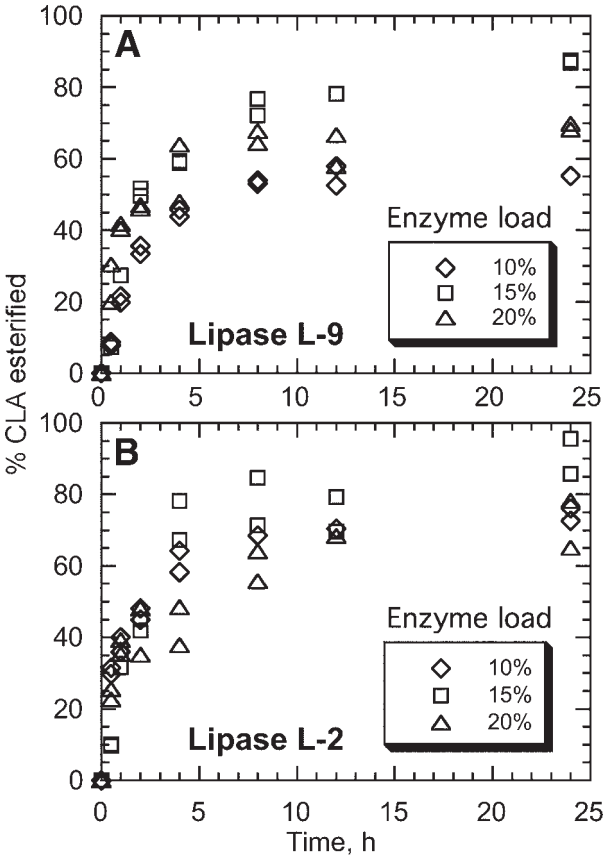

Fig. 2. Effect of enzyme loading on extent of esterification of CLA: **(A)** lipase L-9; **(B)** lipase L-2. Reaction conditions were as follows: 644 mg of glycerol, 3.892 g of CLA, molar ratio of CLA to glycerol of 1:2, 3 g of molecular sieves, 20 mL of hexane, and orbital shaker set at 150 rpm and 50°C.

Fewer TAGs were obtained with the 1:1 molar ratio of substrates. The results of the analysis for the lower acylglycerols are discussed in the section Composition of DAG and MAG Species.

For subsequent experiments, we employed a loading of 5% (w/w) of one of the two immobilized enzymes and a 1:1 molar ratio of substrates.

Effects of Temperature

The effects of temperature on the time course of esterification of CLA with glycerol are shown in Fig. 3A,B for lipases L-9 and L-2, respectively. For lipase L-9, a slightly greater extent of esterification was observed at 50°C than at lower temperatures, but the differences in the extent of reaction between 30 and 40°C were small. The effect of temperature was most evident between 40 and 50°C. The use of lipase L-9 at 50°C produced an approach to equilibrium in 8 h. Although the conversions measured at 30 and 40°C were similar, a much greater extent of esterification was observed at 50°C. This trend may reflect not only the exponential (Arrhenius)

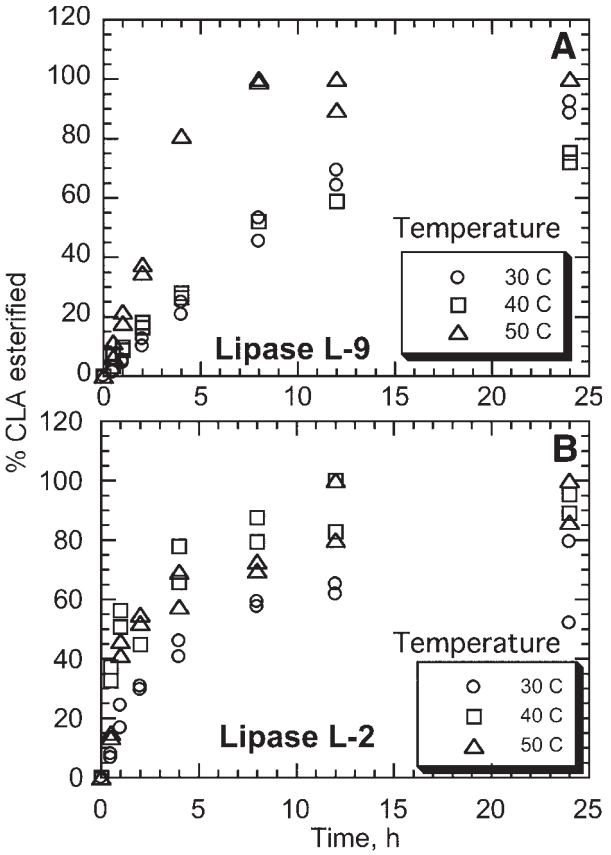

Fig. 3. Effect of temperature on extent of esterification of CLA: **(A)** lipase L-9; **(B)** lipase L-2. Reaction conditions were as follows: 644 mg of glycerol, 1.946 g of CLA, molar ratio of CLA to glycerol of 1:1, 3 g of molecular sieves, 20 mL of hexane, and orbital shaker set at 150 rpm and 50°C.

dependence of intrinsic rate constants on temperature, but also the effect of temperature on the inherent activity of the immobilized preparation and on its activation in the presence of the organic solvent *(27)*. This hypothesis is consistent with the reports by Li and Ward *(28)*, who found similar effects when they esterified an omega-3 fatty acid concentrate with glycerol in an organic solvent. In their studies of the effect of temperature on the acidolysis of tristearin with oleic and lauric acids, Sellappan and Akoh *(29)* reported increased enzyme activity with increasing temperature for temperatures up to 55°C. Our experiments at 50°C with both immobilized enzymes and a 1:1 molar ratio of glycerol to fatty acids produced higher degrees of esterification of CLA than were obtained at lower temperatures.

Compositions of Acylglycerol Mixtures

The distribution of acylglycerols during the course of the reaction are shown in Fig. 4A,B for reactions mediated by lipases L-9 and L-2, respec-

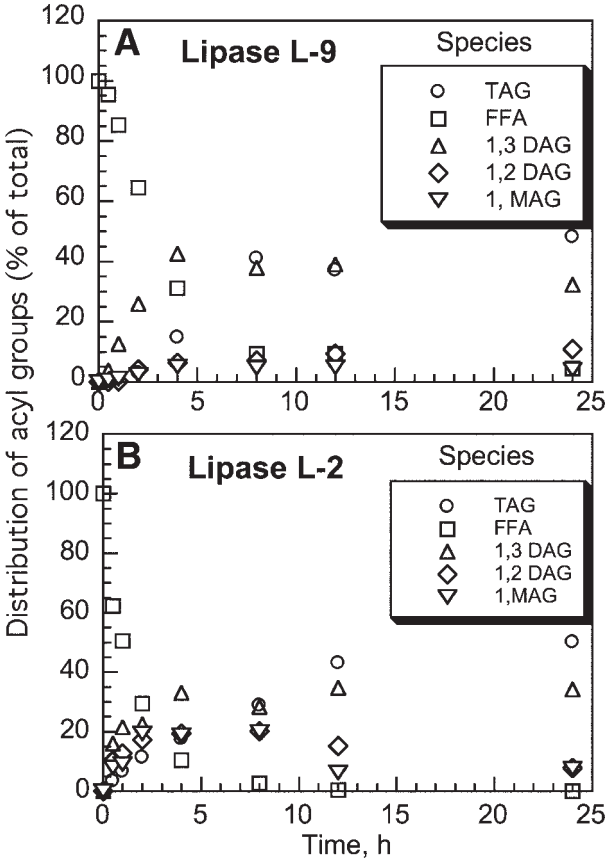

Fig. 4. Distribution of acyl groups: **(A)** lipase L-9; **(B)** lipase L-2. Reaction conditions were as follows: 644 mg of glycerol, 1.946 g of CLA, 5% (w/w) enzyme loading, 3 g of molecular sieves, 20 mL of hexane, and orbital shaker set at 150 rpm and 50°C.

tively. For both immobilized enzymes, the free fatty acid (FFA) fraction diminished continuously during the course of the esterification reaction. When lipase L-9 was used as the biocatalyst (Fig. 4A), the product contained substantial amounts of DAG after 4 h. At this time, the MAG fraction was low. In a study of the kinetics of the lipase-catalyzed synthesis of triolein, Lortie et al. *(30)* observed that a 1,3-specific lipase can apparently catalyze the synthesis of triolein because the intermediates 1-MAG and 1,3-DAG can be isomerized, via acyl migration, to form an ester link with the hydroxyl group bound to the secondary carbon atom on the glycerol backbone. The free hydroxyl group thus formed on a primary carbon atom may then undergo further enzyme-mediated esterification. Lortie et al. *(30)* concluded that the rate of isomerization depends on the concentration of FFAs. This result is also consistent with data reported by Li and Ward *(28)*. For the reactions mediated by the L-9 lipase, the composition of lipid constituents at 4 h was 14.8 wt% TAG, 31.2 wt% FFA, 42.5 wt% 1,3-DAG, 6.4 wt% 1,2-DAG, and 5.1 wt% 1-MAG. 2-MAG was not detected.

After 12 h of reaction, the DAG fraction comprised 49% of the lipid fraction, the 1-MAG only 5%, the FFA 9%, and the TAG 37%.

The reactions mediated by lipase L-2 produced a greater proportion of 1,2-DAG at 4 h, probably because this lipase is generally regarded as nonspecific. Examination of Fig. 3B reveals the presence of larger amounts (25.1%) of TAG at 8 h, whereas the FFAs represented only 4.2% of the lipid fraction. The product contained 46.2% 1,3-DAG and 14.5% 1,2-DAG. Arcos et al. *(13)* reported similar proportions of the various acylglycerol fractions in a study conducted in a solvent-free system using lipase L-2 as the biocatalyst. They suggested that this composition may meet Food and Drug Administration requirements relative to the fatty acid and glycerol content of a mixture of MAGs and DAGs intended for use as a food-grade emulsifier. Even though a 3:1 molar ratio of CLA to glycerol would be required for stoichiometric synthesis of the triester, the necessity for migration of an acyl group to the *sn*-2 position appeared to limit the rate of formation of the triester in this system. The compositions of the lipids obtained were as follows: 37.2% (w/w) TAG, 9.4% FFA, 48.5% DAG, and 4.9% MAG for the product obtained at 8 h using lipase L-9. The product obtained in 12 h with lipase L-2 contained 43.1% TAG, 0.5% FFA, 50.0% DAG, and 6.4% MAG. These concentrations are similar to those reported by Arcos et al. *(13)* and suggest the potential for using products of this work as emulsifiers.

Composition of DAG and MAG Species

Inspection of Table 1 reveals that the use of each immobilized enzyme at a loading of 5% (w/w) led to similar product compositions when the starting material contained equal moles of glycerol and fatty acids. The crude preparation of CLA used as the starting material has oleic acid as its primary impurity. The percentages of saturated fatty acid residues in both the MAG and TAG fractions and in the mixtures produced using both enzymes were low (<13.5%). The major fatty acid residues were those of oleic acid and CLA. CLA residues comprised in excess of 55% of the total. Both MAG and DAG fractions were obtained from the samples using solid-phase extraction with prepacked silica columns. Homogeneity was tested by analyzing each fraction using HPLC, and the corresponding fatty acid content was determined by GC. Reported fatty acid contents correspond to those of the indicated fraction. These results are consistent with those for acidolysis of corn oil with CLA reported by Martínez et al. *(21)*, who found that incorporation of CLA into TAG preferentially displaced the C18:2 and C16:0 fatty acid residues. Other fatty acid residues did not undergo significant release. Both lipases released C18:2 to the same extent (11% [w/w]), a result suggesting similar specificities for this fatty acid.

Results from the present study show rather low incorporation of saturated fatty acids in the MAG and DAG fractions, although these fatty acids are originally present at relatively low levels in the CLA preparation (<13%). The L-2 and L-9 lipases exhibit high selectivities for incorporation of fatty acids with the same chain length, regardless of their degree of unsaturation,

Table 1
Composition of Acylglycerols Produced Using 1:1 Molar Ratio of CLA to Glycerol and Immobilized Enzyme Loading of 5% by Weight

	Residue (wt%) in 1,3-DAG fraction[a]		Residue (wt%) in 1-MAG fraction[a]	
Fatty acid residue	L-9	L-2	L-9	L-2
16:0	7.7	6.7	7.35	7.4
18:0	5.1	4.97	5.61	6.05
18:1	27.67	27.6	28.47	30.61
18:2	1.74	1.58	1.74	0
CLA	57.69	59.09	56.73	55.91

[a]The ratios of MAG to DAG were 1:6 for L-9 and 1:8 for L-2.

because about 95% of both 18:1 and 18:2 fatty acids were incorporated into the acylglycerols in approximately the same proportion.

Food-Related Applications of MAG and DAG

The production of surfactants and emulsifiers suitable for use by the food industry is about 200,000 t/yr *(31)*. This demand is increasing in response to the growing use of fast and convenience foods and demands for increased shelf life. MAGs constitute by far the largest component of the emulsifier market (about 70%). Typically, these compounds are used to stabilize water in oil emulsions such as margarines, to promote aeration of emulsions such as in ice cream, and to extend the shelf life of bread and other cereal products *(32)*.

Considering the potentially labile nature of MAGs and DAGs containing unsaturated fatty acid residues such as those of CLA, we suggest that food manufacturers interested in such emulsifiers focus on their use in formulating frozen desserts and dairy spreads and as substitutes for natural fats and oils, all targeted at the nutraceuticals/health foods market.

The product mixture can be used directly, or it can be subjected to high-vacuum thin-film molecular distillation to obtain an MAG product containing only low levels of DAGs, TAGs, and FFAs (<5% total). Often the properties desired in an MAG for specific uses may be improved by acylation of one of the free hydroxyl groups. This acylation can be achieved by reaction with lactic or citric acid or with an acid anhydride (acetic, succinic, and diacetyltartaric) *(31)*.

These compounds, the so-called acetoacylglycerols, possess interesting properties that may be useful in specific applications: they are stable in α-crystal form, they exhibit unusual flexibility and film-forming properties, and they can be stretched to eight times their original length *(33)*. The reaction conditions described herein represent an attractive alternative means of preparing specific acylglycerols for use as emulsifiers with special properties. Future studies can focus on preparing a CLA-rich MAG

fraction by using molecular distillation to separate the corresponding product mixture of MAG and DAG species and on determining the functional properties of the various products.

Conclusion

Lipase-catalyzed esterification reactions can be employed to obtain products suitable for use as emulsifiers or as substitutes for naturally occurring fats and oils. Further studies of the reaction products are needed to assess the functional properties of these acylglycerols and their potential use in formulating food spreads to be marketed as nutraceuticals.

Acknowledgments

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