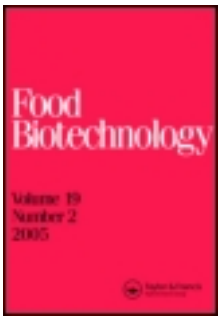

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Lipase-catalyzed interesterification (acidolysis) of corn oil and conjugated linoleic acid in organic solvents

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ABSTRACT

Lipase mediated interesterification of acylglycerols from corn oil with conjugated linoleic acid (CLA) was studied in several organic solvents. Two commercially available lipases, IM-60 from *Mucor miehei* and Chirazyme L-2 from *Candida antarctica* brought about greatest extents of interesterification. Hexane was the best solvent from the standpoint of both maximizing the reaction rate and the extent of interesterification.

INTRODUCTION

Conjugated linoleic acid (CLA) is a mixture of positional and geometric isomers of linoleic acid characterized by double bonds at positions 8 and 10, 9 and 11, 10 and 12, or 11 and 13. Geometric isomers exist for each positional isomer (*cis-cis, cis-trans, trans-cis or trans-trans)*. The *cis*-9, *trans*-11 and *trans*-9, *cis*-11 isomers have been associated with important biological activities, including anticarcinogenic activity (Belury, 1996; Ha *et al.*, 1990; Ip *et al.*, 1990, 1991, 1994a,b,c; Parodi, 1994,1996; Shultz *et al.*, 1992) and inhibition of development of atherosclerosis in animals (Lee *et al.*, 1994). CLA may also act as a growth-promoting agent (Chin *et al.*, 1994). Since preparative procedures for purification of the individual isomers have not been reported, it has been difficult to determine the biological significance of each isomer independently. Kramer et al. (1998) have determined the

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distributions of CLA isomers in tissue lipid classes of pigs fed with a commercial CLA mixture using gas chromatography (GC) and Silver Ion-High-Performance Liquid Chromatography (Ag^+–HPLC). They reported the presence of ten and twelve CLA isomers in the diet and in pig tissue lipids, respectively.

In general usage, the term CLA normally refers to an isomeric mixture containing the two most important isomers of octadecadienoic acid; the *cis*-9, *trans*-11 and *trans*-9, *cis*-11 isomers. This usage will be employed throughout this article. The *cis*-9, *trans*-11-octadecadienoic acid isomer is produced in ruminant animals as a first intermediate in the biohydrogenation of dietary linoleic acid by a linoleic acid isomerase from the rumen bacteria *Butyrivibrio fibrisolvens* (Kepler *et al.*, 1966). Dairy products are the richest dietary sources of CLA. Depending upon pasture conditions, milk fat may contain up to 30 mg CLA/g fat (predominantly in the form of the biologically active *cis*-9, *trans*-11 isomer) (Parodi, 1994). In general, fat from meat of ruminant animals contains more CLA than fat from non-ruminants, which may obtain their CLA by dietary means, combined with some bacterial isomerization of linoleic acid. Vegetable oils contain only small amounts of CLA (Chin et al., 1992). On the basis of several animal model studies, Ip et al. (1994b), suggested that for a 70 kg individual, consumption of ca. 3.5 g of CLA per day could provide protection from certain forms of cancer. One mechanism by which incorporation of CLA into human diets could be accomplished is via foods enriched in CLA, e.g., modified oils or fats. Creation of a new generation of novel foods via this approach may produce foods (nutraceuticals) which are particularly appealing to individuals who are unwilling to change their primary eating habits but still desire alternative choices of foods offering both nutritional and preventative medicinal benefits.

Modification of fats and oils with lipases as biocatalysts in the presence or absence of organic solvents has attracted interest from lipid researchers (Li and Ward., 1993; Garcia et al., 1998; Arcos et al., 1998). Lipases are known to catalyze esterification reactions under mild conditions with high specificities. There appears to be great potential for using lipases to effect the synthesis of acylglycerols of interest to a variety of manufacturers in the food, nutritional supplement, and pharmaceutical industries.

Data concerning the lipase-mediated interesterification reaction of corn oil and a CLA concentrate (acidolysis) in several organic solvents are reported below. The thrust of this research was to identify how the rate and extent of incorporation of CLA depended on the source of lipase, the polarity of the solvent and the ratio of enzyme to substrate.

MATERIALS AND METHODS

Materials: Lipases PS-30 and AY-30 were obtained from Amano, Lipase IM-60 (immobilized form of a lipase from *Rhizomucor miehei*) was purchased from Novo Nordisk (Mexico City). An immobilized lipase from *Candida antarctica*, Chirazyme L-2 carried-fixed (CHI L-2), was kindly provided by Boëhringer-Mannheim (Indianapolis, IN). Sunflower and corn oil were purchased at the local market.

All solvents (reagent grade or better) were obtained from Baker. Molecular sieves (3 Å) were obtained from Sigma. Coommassie Brilliant Blue reagent was obtained from Pierce Chemical, Rockford, IL.

Methanolic HCl (3M) was purchased from Supelco and 0.1N methanolic NaOH was prepared from its components.

Methods:

Alkali isomerization of linoleic acid to CLA: CLA was prepared as previously described by Chin et al. (1992), with some modifications. Sunflower oil was used as the starting material because of its high linoleic acid content (see Table 1). The resulting mixture of isomerized fatty acids was employed to prepare the CLA concentrate.

Preparation of CLA concentrate: Purification of CLA was accomplished by diluting 10 g of the mixture of isomerized fatty acids with 70 ml acetone in a round-bottom flask. This solution was cooled to –15 °C in a circulating bath and held at this temperature overnight. The mixture was then quickly paper-filtered inside a freezer. The filtrate was poured into another round-bottom flask and the solvent was evaporated under a stream of N_2 gas through it. In a separate 125 ml Erlenmeyer flask, 11 g urea were dissolved in 50 ml of warm (50 °C) methanol. The urea solution was then added to the round-bottom flask containing the isomerized fatty acids. This mixture was first heated to 60 °C to dissolve all solutes and this hot solution was transferred to a 125 ml Erlenmeyer flask, together with a rinse of 5 ml of hot methanol. The hot urea-methanol solution was cooled until a large crop of crystals was formed; then cooled further in a freezer with occasional swirling. The urea precipitate was collected by paper filtration and the clear filtrate was transferred to a 125 ml separation funnel. The filtrate was then mixed with 50 ml water, and the CLA was extracted following the method reported by Chin *et al.* (1992). The product thus prepared contained ca. 95% (w/w) CLA.

Interesterification reactions: In the first group of experiments, the reaction mixtures consisted of 1750 mg corn oil, 250 mg CLA concentrate (weight ratio = 7:1), and 30 ml hexane. These solutions, together with a free or immobilized enzyme (50 or 80 mg, respectively), were placed in 50 ml Erlenmeyer flasks fitted with septa-capped stoppers (to prevent evaporation of the solvent). Each enzyme preparation had previously been dried under vacuum for 20 minutes. The headspace in each flask was purged with nitrogen. The suspensions were then incubated in an orbital shaker (set at 200 rpm and 30 °C for the free enzymes, or at 50 °C for the immobilized lipases). At various times during incubation, two 0.2 ml samples of the reaction mixture were withdrawn for analysis by GC. The immobilized enzymes were immediately removed by filtering the suspensions through 0.2 μm nylon membranes.

The second group of experiments involved the same quantities of substrates and hexane, but different concentrations of the immobilized enzyme (2%, 4%, 8% and 16% based on the weight of the substrate mixture). The suspensions were then processed in the same manner as those in the first group.

The third group of experiments employed the same quantities of substrates, and the concentration of the immobilized enzyme (IM-60 or Chirazyme L-2) was 4% (based on the weight of the substrate mixture). The remainder of the reaction medium consisted of 30 ml of one of the following solvents: hexane, tetrahrydofuran, isooctane, dioxane, *t*-amyl alcohol, and *tert*-butanol. All Solvents were dried through molecular sieves before use. The experiments were carried out as previously described.

Determination of the extent of interesterification: The analyses for esterified fatty acids were conducted by adding one 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, then 200 µl distilled water was added. The resulting mixture was extracted with one ml hexane. The analyses for total fatty acids were conducted by adding one ml of 0.2 M methanolic HCl to a second 200 µl aliquot of each sample. The sample vial was then flushed with N_2 and held overnight in a heating block at 80 °C. Then 200 µl water were added, and the mixture was extracted with one ml hexane. For each aliquot, heptadecanoic acid methyl ester (C17) was added to serve as an internal standard. One µl of each of the paired samples was analyzed by gas chromatography.

Analyses:

Analyses for fatty acid methyl esters (FAMEs) were conducted by gas chromatography following the method of Chin *et al.* (1992). Separation of the FAMEs was performed using a Hewlett Packard HP 6890 GC fitted with a flame ionization detector (FID). Samples were injected to a Supelcowax-10 capillary column (Supelco, Inc., 60 m x 0.32 mm i.d., 0.25 µm film thickness) using a splitless injector with nitrogen as the carrier gas. Protein contents of the enzyme preparations were determined using the method of Bradford (Friedenauer and Berlet, 1989).

RESULTS AND DISCUSSION

Selection of the source of linoleic acid. The compositions of the oils used to select the source of linoleic acid (C18:2) for the preparation of the CLA concentrate showed that sunflower oil contained the highest natural concentration of linoleic acid, because of, it was used for the preparation of the CLA concentrate.

Esterification activities of commercially available lipases: Lipases from different sources exhibit characteristic specific activities. On the basis of results reported by Li and Ward (1993), the temperatures selected for investigation were 30 °C for the free enzymes and 50 °C for the immobilized lipases. These researchers reported that the free enzymes gave highest yields of esters when utilized at 30 °C, whereas the immobilized forms gave better yields at higher temperatures. This result can be attributed to differences in the optimum temperature associated with the balance between the effects of temperature

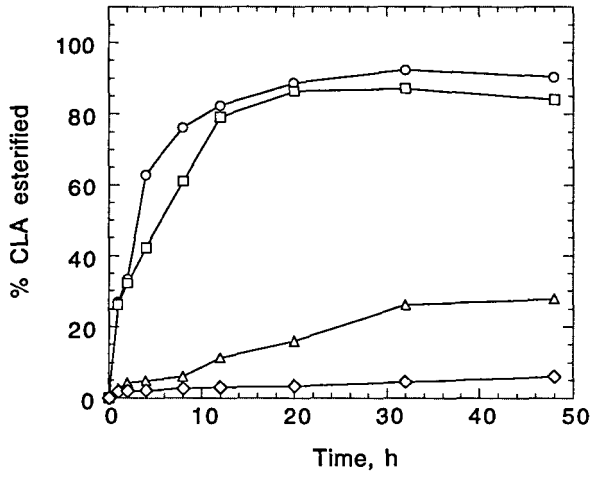

FIGURE 1
Esterification activities of several lipases. Reaction mixtures consisted of 1750 mg corn oil, 250 mg CLA concentrate, and 30 ml hexane. Orbital shaker set at 200 rpm. Temperature: 30 °C and 50 °C for free and immobilized enzymes respectively: IM–60 (circles); CHI L-2 (squares); PS-30 (triangles) and AY-30 (diamonds).

on the enzyme-mediated reaction and the kinetics of enzyme deactivation processes. The free enzymes are more labile and rapidly deactivate when employed at 50 °C.

Inspection of the data in Figure 1 permits one to compare the relative activities of two free and two immobilized lipases for acidolysis of corn oil with CLA. Of the commercial lipases tested, the two immobilized forms [IM–60 (*sn-1,3* specific) and CHI L-2 (non-specific)] produced the largest extents of incorporation of CLA in the corn oil. This result is consistent with those reported by Garcia and co-workers (1998) who reported that immobilized lipases were able to interesterify large percentages of CLA with butteroil. Arcos, et al. (1998), obtained complementary results for the immobilized lipase-mediated (poly)esterification reactions of CLA and glycerol in a solvent-free system. Lipase IM-60 was the most effective biocatalyst for these sequential esterification reactions.

The protein content for each enzyme was analyzed using the method of Bradford (Friedenauer and Berlet, 1989). The contents (mg of enzyme protein/g of product) determined for the four enzymes were as follows: AY-30 (111.18), PS-30 (81.35), IM-60 (22.65) and CHI L-2 (38.03). Although the protein contents were lower for the immobilized preparations, these lipases produced the best yields and possessed high specific activities. Based on these results, immobilized forms were used in subsequent experiments.

Effect of enzyme concentration: The effect of the enzyme/substrate ratio on the interesterification reaction was investigated for the two immobilized lipase preparations (see Figures 2 and 3). When CHI

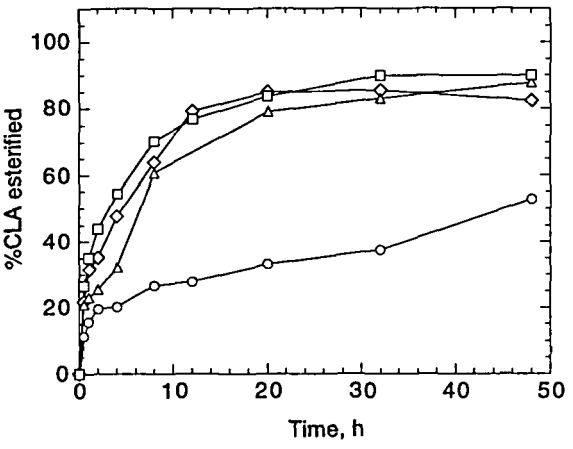

FIGURE 2
Effect of enzyme CHI L-2 concentration on interesterification Reaction mixtures consisted of 1750 mg corn oil, 250 mg CLA concentrate, and 30 ml hexane. Orbital shaker set at 200 rpm and 50 °C. Enzyme concentration (w/w): 2% (circles); 4% (diamonds), 8% (triangles) and 16% (squares)

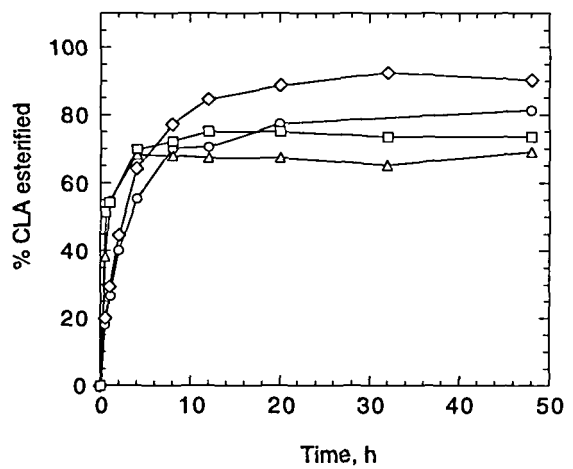

FIGURE 3
Effect of enzyme IM-60 concentration on interesterification. Reaction mixtures consisted of 1750 mg corn oil, 250 mg CLA concentrate, and 30 ml hexane. Orbital shaker set at 200 rpm and 50 °C. Enzyme concentration (w/w) 2% (circles), 4% (diamonds); 8% (triangles) and 16% (squares).

TABLE 1. Log P* values of the organic solvents tested (Laane, et al., 1987)

Solvents	log P
Isooctane	4.5
Hexane	3.5
t-amyl alcohol	2.0
tert-butanol	0.8
Tetrahydrofuran	0.49
Dioxane	-1.1

* P is defined as the partition of a given solvent between a two-phase system consisting of 1-octanol and water.

L-2 was employed, the percentage of the CLA esterified increased when the enzyme concentration was increased from 2 to 4% (based on the weight of the substrate mixture). However, comparable extents of reaction were obtained at enzyme levels of 4, 8, and 16% (Figure 2). After 12 h of reaction, the maximum extent of CLA esterified (ca. 80%) was accomplished when the (CHI L-2) lipase was present at the 4% level. When the IM-60 lipase was used, comparable extents of interesterification were achieved at all four percentages tested (Figure 3). However, after ca. 12 h the maximum extent of interesterification (more than 80%) was reached when the IM-60 lipase was present at the 4% level. As reported by Arcos *et al.* (1998), these results may be a consequence of a variety of factors, e.g., failure of the shaker to maintain all the immobilized enzyme in suspension, agglomeration of suspended particles, or mass transfer limitations on adsorption or reaction rates. To ensure maximum extent of esterification, subsequent experiments were conducted using 4% enzyme.

Effect of the organic solvent: To carry out the bioconversion of lipophilic compounds effectively, it may often be desirable to employ organic solvents. The use of organic solvents can enhance the solubility of nonpolar substrates or other hydrophobic components of the reaction mixture. Conformational changes in enzymes suspended in organic solvents have resulted in alterations of substrate specificity and the affinity of enzymes for substrates (Kvittingen, 1994). To select the most suitable solvent for interesterification over a range of log P values (Table 1, the effect of the solvent on the catalytic activity of immobilized lipase was examined (see Figures 4 and 5). For both IM-60 and CHI L-2, greater extents of interesterification were observed in hexane and isooctane as compared to several solvents with higher polarities. More polar solvents were not appropriate media for carrying out the synthetic reaction.

As Yang et al. (1994) have observed for lipase-mediated reactions, the solvents most suitable for use as the bulk phase are those with log P values above 2, and preferably less than 4. Their conclusion was based partially on the general observation that solvents with log P values below 2 are able to distort the molecular configuration of the lipase by stripping away enzyme-associated water. This hypothesis is

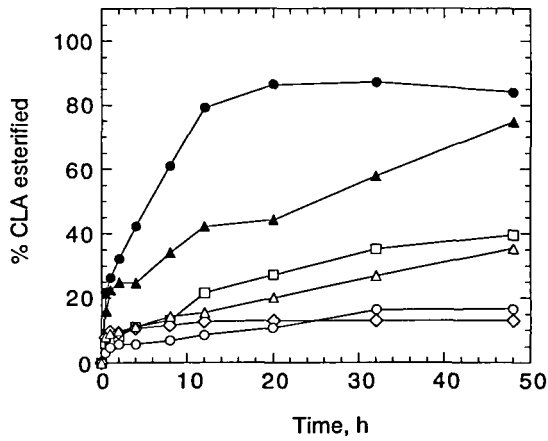

FIGURE 4

Effect of organic solvent on the interesterification reaction catalyzed by enzyme CHI L-2 Reaction mixtures consisted of 1750 mg corn oil, 250 mg CLA concentrate, and 30 ml of one of the following solvents: *tert*-butanol (open squares); hexane (filled circles); isooctane (filled triangles); tetrahydrofuran (open diamonds), *t-amyl* alcohol (open circles) and dioxane (open triangles). Orbital shaker set at 200 rpm and 50 °C.

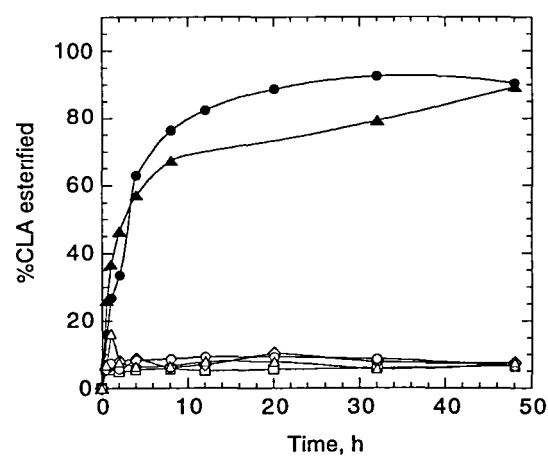

FIGURE 5

Effect of organic solvent on the interesterification reaction catalyzed by enzyme IM-60. Reaction mixtures consisted of 1750 mg corn oil, 250 mg CLA concentrate, and 30 ml of one of the following solvents: *tert*-butanol (open squares); hexane (filled circles), isooctane (filled triangles); tetrahydrofuran (open diamonds); *t*-amyl alcohol (open circles) and dioxane (open triangles). Orbital shaker set at 200 rpm and 50 °C.

in agreement with the results found in our experiments. According to the study reported by Kvittingen (1994), the ideal solvent should dissolve substrates and should not desorb or distort enzyme-associated water that is essential for catalytic activity. Since hexane is the only organic solvent accepted by health and food authorities for use in processing foods, it is the solvent most appropriate for use in the acidolysis of corn oil with CLA.

CONCLUSIONS

Immobilized lipase preparations IM-60 from *Mucor miehei* and Chirazyme L-2 from *Candida antarctica* produced substantial (>80%) extents of interesterification of CLA and corn oil at 50 °C in 12 h or less. Of the organic solvents tested, hexane was particularly suitable for this application although isooctane was also appropriate for use, but the former is the only solvent that meets the constraints imposed by regulatory agencies for food applications. Interesterification (acidolysis) can be employed to prepare edible oils rich in CLA residues, although additional work is required to assess the efficacy and economic viability of this approach.

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