Esterification of Glycerol with Conjugated Linoleic Acid and Long-Chain Fatty Acids from Fish Oil

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ABSTRACT: Free fatty acids from fish oil were prepared by saponification of menhaden oil. The resulting mixture of fatty acids contained *ca.* 15% eicosapentaenoic acid (EPA) and 10% docosahexaenoic acid (DHA), together with other saturated and monounsaturated fatty acids. Four commercial lipases (PS from *Pseudomonas cepacia*, G from *Penicillium camemberti*, L2 from *Candida antarctica* fraction B, and L9 from *Mucor miehei*) were tested for their ability to catalyze the esterification of glycerol with a mixture of free fatty acids derived from saponified menhaden oil, to which 20% (w/w) conjugated linoleic acid had been added. The mixtures were incubated at 40°C for 48 h. The ultimate extent of the esterification reaction (60%) was similar for three of the four lipases studied. Lipase PS produced triacylglycerols at the fastest rate. Lipase G differed from the other three lipases in terms of effecting a much slower reaction rate. In addition, the rate of incorporation of omega-3 fatty acids when mediated by lipase G was slower than the rates of incorporation of other fatty acids present in the reaction mixture. With respect to fatty acid specificities, lipases PS and L9 showed appreciable discrimination against esterification of EPA and DHA, respectively, while lipase L2 exhibited similar activity for all fatty acids present in the reaction mixture. The positional distribution of the various fatty acids between the *sn*-1,3 and *sn*-2 positions on the glycerol backbone was also determined.

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KEY WORDS: Acylglycerol, conjugated linoleic acid, esterification, fish oil, lipase, menhaden.

Polyunsaturated fatty acids (PUFA) have been the subject of much attention in recent years because of their special physiological functions in humans (1). Fish oil is an important natural source for PUFA, especially 5,8,11,14,17-eicosapentaenoic (C20:5) (EPA) and 4,7,10,13,16,19-docosahexaenoic (C22:6) (DHA) acids. The health-promoting effects of EPA and DHA in human subjects have been reviewed (2,3). These fatty acids are representatives of a general class of beneficial fatty acids known as n-3 fatty acids, commonly referred to as omega-3 fatty acids. Various methods are available for obtaining concentrated EPA and DHA from fish oil, for example, formation of urea adducts (4), iodolactonization (5), separation *via* high-performance liquid chromatography (HPLC) (6), supercritical fluid extraction (7), extraction by silver nitrate (8), and solvent extraction (9).

The ability of some lipases to discriminate among different types of fatty acids has been utilized for the enrichment of DHA and EPA from precursor mixtures. Esterification (10), hydrolysis (11,12), transesterification (13), and acidolysis (14) reactions have all been employed for selective enrichment of these n-3 PUFA. The four commercial lipases employed in this work were selected on the basis of literature reports indicating their ability to catalyze esterification reactions involving fatty acids other than those involved in the present study.

"Conjugated linoleic acid (CLA)" is a collective term for the mixture of isomers of octadecadienoic acid containing conjugated double bonds. Incorporation of CLA in the diets of animals produces several prophylactic benefits (15), in particular, anticarcinogenic, antiatherogenic, and fat-partitioning effects.

Here we report the synthesis of acylglycerols containing both CLA and fatty acids derived from saponified fish oil, with particular emphasis on incorporation of EPA and DHA residues in the product acylglycerols. Four commercial lipases were evaluated in terms of the relative values of their activity, fatty acid specificity, acylglycerol specificity, and 1,3-regioselectivity toward the fatty acids of interest. Data concerning the selectivities of these lipases in esterification reactions permit one to develop new strategies for the synthesis of tailored fats and oils rich in both n-3 and CLA residues.

MATERIALS AND METHODS

Materials. Menhaden oil, type II porcine pancreatic lipase (L-3126), sodium borate, bile salts, and molecular sieves with a pore size of 4 Å were purchased from Sigma (St. Louis, MO). CLA (C18:2) was kindly provided by Natural Asa (Hovdebygda, Norway). As reported by the vendor, the area percentages of the different isomers present in the CLA were: 0.42% *c*9,*c*12, 42.73% *c*9,*t*11, 44.24% *t*10,*c*12, 1.33% *c*9,*c*11, 1.35% *c*10,*c*12, 1.19% (*t*9,*t*11 + *t*10,*t*12). The balance of the CLA consisted of other fatty acids. Lipases from *Pseudomonas cepacia* (PS) and *Penicillium camemberti* (G) were obtained from Amano (Lombard, IL). The immobilized lipases L2 (lipase from *Candida antarctica* fraction B) and L9 (lipase from *Mucor miehei*) were obtained from Boëringer-Mannheim (Mannheim, Germany). All solvents used were HPLC grade from Fisher (Chicago, IL.

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Methods. (i) Saponification of menhaden oil. Fish oil (167 g) was added to 500 mL of a 2.7 N NaOH solution prepared by dissolving 55.52 g of NaOH in 450 mL of ethanol and 50 mL of water. The solution was held for 30 min at 60°C while being stirred by a magnet (500 rpm). The mixture was then neutralized with HCl and extracted with 250 mL of hexane. The hexane phase was evaporated under nitrogen to produce a mixture of free fatty acids (FFA). HPLC analysis indicated that essentially complete saponification (>96%) was achieved. No acylglycerols were observed, and only small quantities of other esters (2–4% in total) were present in the hydrolyzed product. Of the total fatty acids present in the saponified product, EPA and DHA were present at levels of *ca.* 15 and 10 mol%, respectively.

(ii) Reaction protocol. FFA from fish oil (8 g) and CLA (2 g) were added to a 60-mL flask containing 1.1 g of glycerol and 0.7 g of hexadecane (used as an internal standard) and mixed by swirling. Then 2 g of the lipase preparation of interest was added. The flasks were flushed with nitrogen, stoppered, and placed in an orbital shaker (300 rpm) at 40°C. After 5 min, 2 g molecular sieves was added to remove the water released by esterification. Samples (200 μL) were withdrawn periodically and the flasks flushed with nitrogen and resealed after each sampling. All trials were allowed to proceed for 48 h.

(iii) Analysis of reaction products—gas chromatography (GC). The following analytical procedure was based on that of Williams *et al.* (16) to effect methylation of the esterified fatty acid residues present in the samples. The samples (200 μL) were mixed with 1800 μL of a mixture of chloroform and methanol (volume ratio = 2 to 1) and immediately filtered with a 0.45 μm Whatman (Clifton, NJ) nylon syringe filter. Aliquots of the final transparent solution (400 μL) were methylated by addition of 1 mL of 0.1 M methanolic NaOH. This mixture was allowed to stand for 30 min at 60°C. Then, 200 μL of water was added. The resulting mixture was extracted with two 1-mL portions of *n*-hexane. Samples were dried with sodium sulfate and centrifuged for 2 min at 5035 × *g*.

To prepare methyl esters of free and esterified fatty acids, 400 μL of sample was methylated by addition of 1 mL of 0.2 M methanolic HCl. This mixture was allowed to stand for 4 h at 60°C. After addition of 200 μL of water, the mixture was subjected to the extraction, drying, and centrifugation procedures noted in the previous paragraph.

Sample (1 μL) was injected into a Hewlett-Packard (Avondale, PA) gas chromatograph (Model 5890 series II) fitted with a 60-m HP Supelcowax 10 column (Supelco, Bellefonte, PA) (0.32 mm i.d.). Injector and detector temperatures were set at 220 and 230°C, respectively. The temperature program was as follows: starting at 100°C and then heating to 180°C at 20°C/min; followed by heating from 180 to 220°C at 15°C/min. The final temperature (220°C) was held for 30 min. Identification of the various FFA was based on a menhaden oil fish standard (#4-7085) obtained from Supelco. Identification of CLA and the associated retention time were accomplished by direct injection of the methyl esters of CLA obtained by reaction in methanol containing 0.2 M HCl.

(iv) Analysis of reaction products—HPLC. The HPLC analysis of reaction mixtures employed an Econosil-Silica 5U column (250 by 4.6 mm; Alltech, Deerfield, IL) with detection *via* evaporative light scattering (Alltech). A modified version of the method of Liu *et al.* (17) was used as the analytical protocol. Two mobile phases were employed: phase A consisted of hexane, 2-propanol, ethyl acetate, and formic acid (80:10: 10:0.1, by vol) while phase B was 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 (vol/vol) mixture of phases A and B and then with a 98:2 mixture of these phases for an additional 7 min. Next, the system was restored to its initial conditions by passing a 15:85 mixture of phases A and B through the column for 6 min. The retention times for the triacylglycerol (TAG), fatty acid, 1,3-diacylglycerol, 1,2-diacylglycerol, 1(3)-monoacylglycerol, and 2-monoacylglycerol were 1.7, 3, 4.7, 7.9, 13, and 14 min, respectively. Standards for these analyses were obtained from Sigma.

Purification of TAG from the reaction mixture. Separation and recovery of the TAG were accomplished *via* solid phase extraction on silica gel columns (10 g, grade 60, 70–230 mesh) (Aldrich, Milwaukee, WI) (18). The columns were conditioned by washing with 25 mL of hexane, taking care to prevent them from becoming dry. The sample (10 mL containing TAG at a concentration of *ca.* 100 mg/mL as determined by stoichiometric calculations) was applied to a column and then eluted under vacuum (5 mm Hg) with solvent mixtures of increasing polarity: first, 30 mL of hexane/diethyl ether (200:3, vol/vol); second, 100 mL of hexane/diethyl ether (96:4, vol/vol). The eluate from the second elution (containing the TAG) was collected and evaporated for subsequent analysis of the distribution of fatty acid residues along the glycerol backbone.

Positional distribution of fatty acid residues in TAG. A modified version of the method of Williams *et al.* (16) and Luddy *et al.* (19) was employed to release fatty acids from the *sn*-1,3 positions of acylglycerols. This modification consists in the use of longer reaction times (7 min) to overcome the possible lower activity of porcine pancreatic lipase toward n-3 fatty acids and the use of borax to minimize the possibility of migration of residues from position 2 to positions 1 and 3 of the glycerol backbone. A known weight of TAG and an appropriate (*ca.* 20–50 mg) weight of porcine pancreatic lipase were added to a 60-mL stoppered flask. Next, 0.65 mL of Tris-HCl buffer (1 M, sodium salt, pH 8.0), 0.35 mL of sodium borate (0.19 M), 0.1 mL of $CaCl_2$ (22%, w/w), and 0.25 mL of bile salts (0.1%, w/w) were added. The resulting mixture (pH = 7.91) was maintained at 40°C for 1 min without shaking, then shaken at 300 rpm at 40°C for 7 min. The reaction was stopped by addition of 1 mL of acetic acid (0.1 M). The mixture was extracted three times with 1 mL of chloroform/methanol (2:1, vol/vol). The pooled organic phases were passed through a 0.45-μm syringe filter and then methy-

lated with 0.1 M of methanolic NaOH as described above in the "Analysis of reaction products—gas chromatography" section. This protocol provides information concerning the distribution of fatty acid residues at the *sn*-2 position. The distribution of fatty acid residues at the *sn*-1,3 positions was then calculated by subtracting the amount of a fatty acid residue at the *sn*-2 position from the total quantity of this fatty acid present in the corresponding unhydrolyzed TAG as determined by GC.

RESULTS AND DISCUSSION

Acylglycerol selectivity. The rates of esterification were comparable for three of the four lipases studied (PS, L2, and L9) (see Fig. 1). After 12 h of reaction, total acylglycerol concentrations were *ca.* 55–60%. However, more careful analysis of these data indicates the presence of differences in the catalytic properties of these three lipases, particularly with respect to their activity for formation of TAG. Lipase PS gave the highest rate of production of TAG, yielding 22% TAG after only 4 h. By contrast, after 24 h of incubation with lipase G, the TAG content was less than 5%.

Fatty acid specificity. Lipases PS, L2, L9, and G were also evaluated regarding their substrate specificities. The specificity constants, calculated according to the method of Rangheard *et al.* (20), are shown in Table 1, as is the fatty acid composition (in mol%) of the starting mixture of CLA and the fatty acids from saponified fish oil. Competitive factors (α) for the fatty acids of interest were calculated from Equation 1 using the initial substrate concentration and the substrate concentrations after 0.5 and 4 h

$$\alpha = [\log (FA1_0/FA1_t)]/\log(FA2_0/FA2_t) \quad [1]$$

FA1 and FA2 are the concentrations of the fatty acid of interest and the reference fatty acid (in this case CLA), respectively. The subscripts 0 and *t* refer to the initial value and the value at time *t*, respectively. The specificity constant was calculated as ($1/\alpha$) and expressed relative to an assigned specificity constant of 1.0 for the fatty acid present in the highest concentration (CLA).

The lipases tested exhibited similar substrate specificity constants, although at 0.5 h, PS and L9 strongly discriminate against esterification by EPA and DHA (*cf.* Table 1). The selectivity of the esterification reaction network changed with incubation time. After 4 h of reaction, all lipases were characterized by comparable fatty acid specificity constants, although PS and L9 discriminate to some extent against esteri-

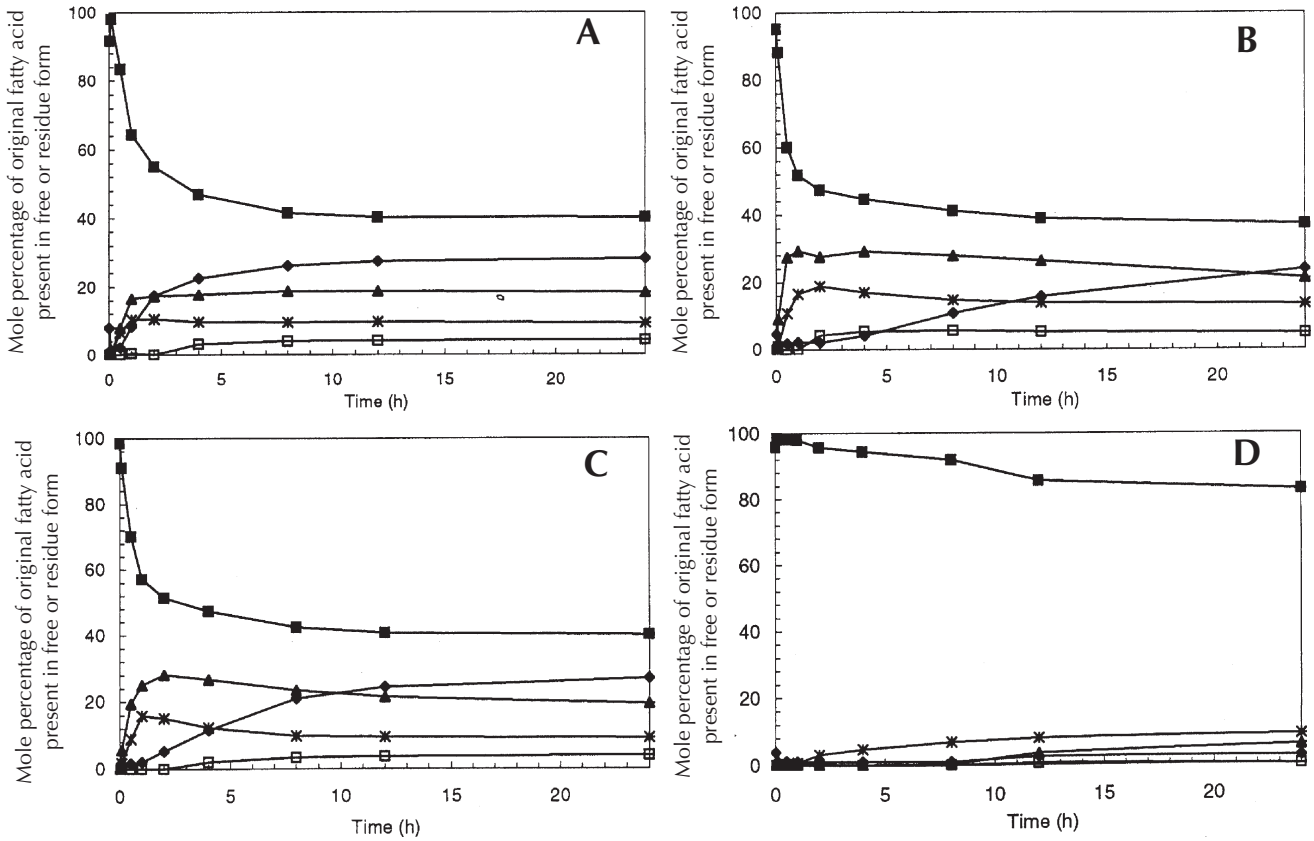

FIG. 1. Distribution of acylglycerols in the lipase-mediated esterification of glycerol with conjugated linoleic acid (CLA) and free fatty acids (FFA) from saponified menhaden oil. Conditions: 8 g free fatty acids from menhaden oil, 2 g of CLA, 1.1 g of glycerol, 2 g of lipase, 2 g of molecular sieves, 40°C, shaken at 300 rpm. (A) Lipase PS, (B) Lipase L2, (C) Lipase L9, and (D) Lipase G. ◆ Triacylglycerol , ■ FFA, ▲ 1,3-diacylglycerols (DAG), □ 1,2-DAG, * monoacylglycerols.

TABLE 1
Specificity Constants for the Esterification of Glycerol with Conjugated Linoleic Acid and Free Fatty Acids from Menhaden Oil. Values Correspond to 0.5 and 4 h of Reaction at 40°C.

	Initial composition* (%)	Lipase PS		Lipase L2		Lipase L9		Lipase G	
		0.5 h	4 h	0.5 h	4 h	0.5 h	4 h	0.5 h	4 h
C14	8.48	1.12	0.90	1.12	0.89	1.29	0.87	0.88	0.93
C16	9.06	1.89	0.93	1.20	0.95	1.33	0.91	0.93	1.08
C16:1	12.02	0.97	0.90	1.07	0.90	1.27	0.88	0.85	1.10
C18	3.42	1.10	0.85	1.14	0.93	0.88	0.85	0.84	0.79
C18:1	11.76	0.76	0.93	1.15	0.94	1.32	0.93	0.92	1.02
CLA	22.45	1	1	1	1	1	1	1	1
EPA	11.81	0.28	0.60	1.06	0.95	0.66	0.90	0.79	0.64
DHA	8.16	0.39	1.01	0.99	0.96	0.27	0.76	0.94	0.70

[a,]*Mole percentages of major species in the initial pool of free fatty acids. The specificity constant for conjugated linoleic acid (CLA) was defined as unity. Tabular entries correspond to the average of three independent determinations of the composition of the residues. At the 95% confidence level, differences of 5% or greater can be regarded as statistically significant. DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid.

fication with EPA and DHA, respectively. At 0.5 h, lipase G discriminated against esterification with not only EPA but also stearic (C18), myristic (C14), and palmitoleic (C16:1) acids. At 0.5 h, lipase PS also exhibited a high specificity constant for esterification with palmitic acid (C16). For esterification reactions, lipase L9 has previously been reported to discriminate against DHA (21). Lipase G has been reported to discriminate strongly against unsaturated fatty acids (22). These reports are not inconsistent with the results reported in Table 1 if one recognizes that the tabulated specificity constants reflect the interactions of two factors: (i) the intrinsic specificities of the four lipases and (ii) the composition of the pool of fatty acids that participate in the esterification reactions. As time elapses, the composition of the pool changes to reflect the different rates at which individual FFA are consumed by esterification reactions. These changes in turn are reflected in changes in the distribution of acid residues contained in the product acylglycerols with concomitant implications for the measured specificity constants.

The time-courses for incorporation of individual fatty acids in the presence of the different lipases are displayed in Figure 2. A stoichiometric ratio of fatty acids and glycerol (3:1) was employed for the experiments in question. Examination of Figure 2 indicates that at long times for the reactions mediated by lipases PS, L2, and L9, CLA and n-3 each constitute *ca.* 15% of the total fatty acid residues (sum = *ca.* 30%). Because *ca.* 40% of the original fatty acids remains unreacted (Fig. 1), 60% of the sites on the glycerol backbone contain fatty acid residues. Thus in the product acylglycerols, *ca.* 30:60, or 50% of the total fatty acid residues, is either CLA or n-3 residues.

Positional distribution of fatty acids in TAG. Table 2 shows the positional distribution of fatty acids in the TAG produced by lipases PS, L2, and L9 after 12 h of reaction. The entries represent the average of three independent determinations. At the 95% confidence level, differences of 5% or more can be regarded as statistically significant. For myristic (C14) and palmitoleic (C16) acids, all three lipases produce percentages of the corresponding residues at the *sn*-1,3 positions that are slightly (20% or less) greater than the percentage at the *sn*-2 position. For palmitic (C16) acid, lipase PS has no positional selectivity within the uncertainty limits of the analytical results. All three lipases incorporate n-3 fatty acids at the *sn*-2 position to an extent that (in percentage terms) exceeds that at the *sn*-1,3 positions (by up to 34%). For CLA, no positional selectivity was found for the three lipases investigated. All three lipases marginally discriminate against introduction of C18:0, and lipases PS and L2 slightly favor formation of C18:1 residues at the *sn*-2 position. However, it should be remembered that these results reflect the interactions of two factors, namely the specificities of the enzymes and the initial composition of the pool of fatty acids. If fatty acids other than CLA and n-3 react more rapidly and preferentially at the 1 and 3 positions, the pool of fatty acids will become depleted in these species. Moreover, in this situation during the later stages of the reaction, the majority of the pool of fatty acids will consist largely of CLA and n-3 species, and the open sites on the glycerol backbone will be primarily those at the *sn*-2 position. Hence the apparent selectivity of the reaction network for formation of n-3 and CLA residues at the *sn*-2 position will differ from that which would be observed if only CLA and n-3 species were present initially.

Assessment of the effects of the various lipases. Lipase PS gave the highest rate of production of TAG species. It also slightly discriminated against EPA. The latter property could possibly be used to concentrate EPA from reaction mixtures, although the effect is small. Lipase L2 gave a lower rate of production of TAG species although the initial rates of incorporation of CLA and n-3 residues were faster than those obtained with the other three lipases investigated. Moreover, lipase L2 showed no discrimination against either EPA or DHA. Lipase L9 discriminated against esterification by DHA. The final level of conversion was similar to those obtained with PS and L2 although in this case the rate of production of TAG was slower than that obtained for lipase PS. Use of lipase G produced lower reaction rates than the other three

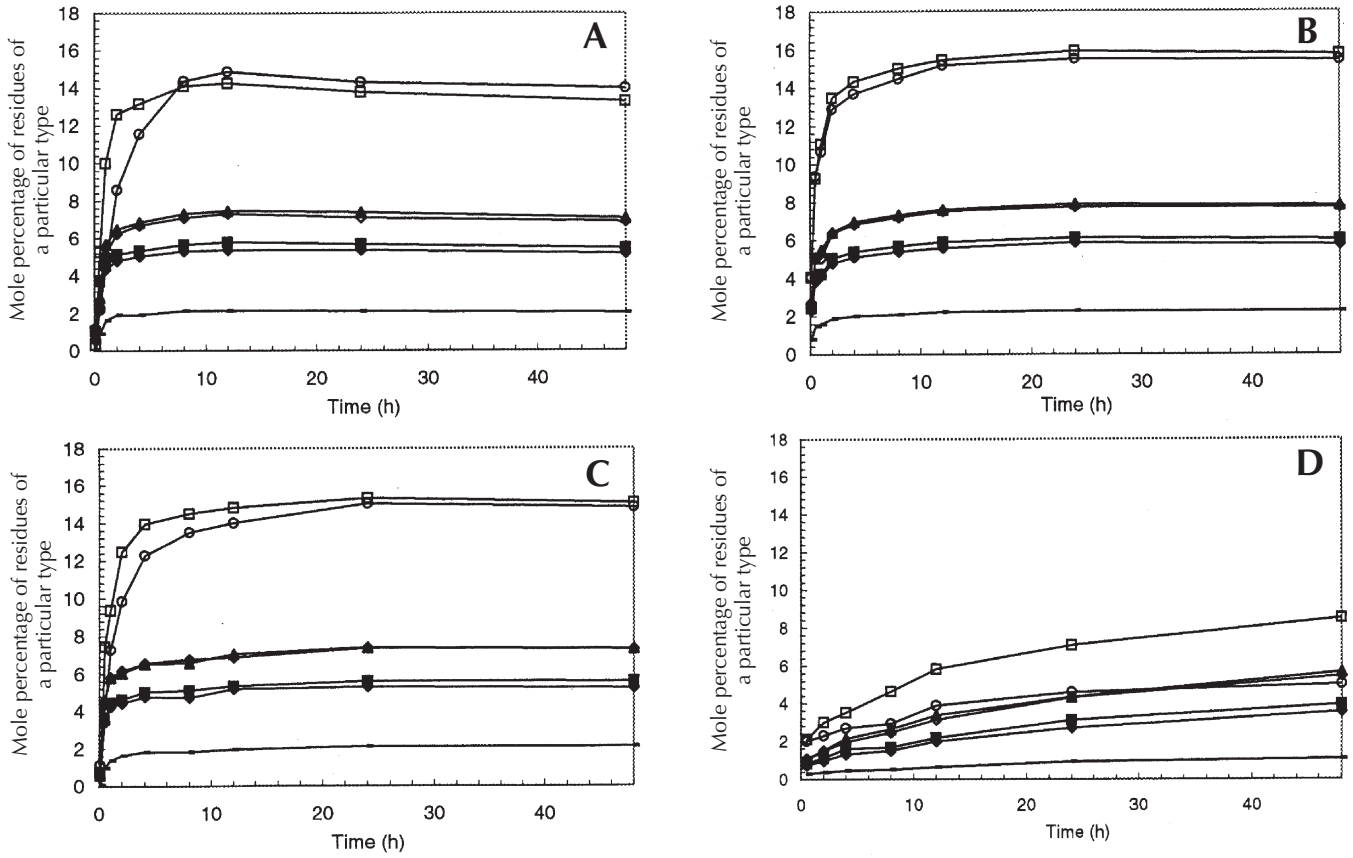

FIG. 2. Time course of the lipase-mediated esterification of glycerol with CLA and FFA from saponified menhaden oil. Ordinate values are the mole percentages of the acylglycerol residues corresponding to a particular fatty acid. Conditions: 8 g of FFA from menhaden oil, 2 g of CLA, 2 g of lipase, 1.1 g of glycerol, 2 g of molecular sieves, 40 °C, shaken at 300 rpm. (A) Lipase PS, (B) Lipase L2, (C) Lipase L9, and (D) Lipase G. ◆ C14, ■ C16, ▲ C16:1, ◇ C18, * C18:1, ○ n-3, □ CLA.

lipases under study and also discriminated against esterification by both EPA and DHA.

The differences exhibited by the four lipases investigated may provide a basis for developing useful strategies for the selective production of acylglycerols containing substantial proportions of both n-3 and CLA residues. Further optimization of the synthesis procedure will permit the production of tailored fats that are both rich in CLA and contain different proportions of EPA and DHA residues.

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TABLE 2
Positional Distributions of Fatty Acids (in mol%) in the Triacylglycerol Products of the Esterification of Glycerol with CLA and Free Fatty Acids from Menhaden Oil After 12 h of Reaction[a]

	Lipase PS (%)			Lipase L2 (%)			Lipase L9 (%)		
	Total	*sn*-1,3	*sn*-2	Total	*sn*-1,3	*sn*-2	Total	*sn*-1,3	*sn*-2
C14	7.95	8.24	7.73	8.43	9.43	7.89	8.20	8.95	7.60
C16	9.25	9.19	9.29	9.44	9.64	9.33	10.01	10.36	9.74
C16:1	11.61	12.61	10.84	11.44	12.08	11.10	11.77	12.62	11.08
C18	2.25	3.04	1.64	2.05	2.98	1.55	2.58	3.25	2.04
C18:1	10.51	8.91	11.75	11.46	10.39	12.03	13.28	13.64	12.99
CLA	23.19	22.53	23.70	24.29	24.43	24.22	22.32	21.28	23.15
n-3	25.00	23.20	26.40	23.05	19.04	25.20	22.34	18.83	25.17

[a]See Table 1 for abbreviations.

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