In Vitro Hydrolysis of Fungal Oils: Distribution of Arachidonic Acid-Containing Triacylglycerol Molecular Species

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ABSTRACT: Four commercially prepared arachidonic acid-rich oils from the fungus *Mortierella alpina* were analyzed by high-performance liquid chromatography and gas chromatography. The levels of arachidonic acid and the distribution of triacylglycerol (TG) molecular species varied significantly among these oils. The major arachidonate-containing TG species were AAA, LAA, DAA, OAA, PAA, SAA, OLA, PGA, PLA, POA, and SOA where the abbreviations A, D, G, L, O, P, and S represent arachidonic (20:4n-6), dihomo-γ-linolenic (20:3n-6), γ-linolenic (18:3n-6), linoleic (18:2n-6), oleic (18:1n-9), palmitic (16:0), and stearic (18:0) acids, respectively. *In vitro* incubation of the TG fractions, purified from these oils with porcine pancreatic lipase for 5 min, yields a mixture of intermediate products, such as 1,2- and 2,3-diacylglycerols (1,2- and 2,3-DG), 2-monoacylglycerol (2-MG) and free fatty acids (FFA), as well as residual TG. The degrees of hydrolysis varied significantly among the four oil preparations, ranging from 35 to 57%. The levels of arachidonic acid in the residual TG and 1,2(2,3)-DG were significantly higher than those in the original TG, whereas those in the FFA fraction were significantly lower than those in 1,2(2,3)-DG and 2-MG. Results from this study suggest that the bioavailability of arachidonic acid differs among fungal oils prepared by different suppliers. These differences could be attributed to the arachidonic acid content of the oil as well as to the association of arachidonic acid with other fatty acids in the same TG molecule.

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KEY WORDS: Fungal oils, HPLC separation, hydrolysis, molecular species, pancreatic lipase, triacylglycerol.

Long-chain polyunsaturated fatty acids (LC-PUFA), mainly arachidonic acid (20:4n-6) and docosahexaenoic acid (22:6n-3), play important roles in the growth and development of the nervous system in the fetus and in the newborn infant (1,2). Normally, newborn infants can continue to obtain LC-PUFA from breast milk (3). However, when those infants are fed with formula that contains no LC-PUFA, they must rely on endogenous synthesis from precursors (linoleic acid, 18:2n-6, and α-linolenic acid, 18:3n-3). There are concerns about premature infants who are born before sufficient amounts of LC-PUFA can be accumulated and yet lack the ability to metabolize and convert adequately the precursor fatty acids to LC-PUFA (4). Owing to these concerns, many international health organizations have urged that both arachidonic and docosahexaenoic acids be added to infant formulas (5,6).

With this in mind, the formula industries are seeking arachidonic acid-rich oils that are suitable for infant formula supplementation. Recently, storage oils that are rich in arachidonic acid have been extracted from the fungus *Mortierella,* and these are commercially available for supplementation. The present study examined the effects of arachidonic acid content and its distribution in triacylglycerols (TG) on the bioavailability of arachidonic acid in these oils by *in vitro* pancreatic lipase hydrolysis.

MATERIALS AND METHODS

The fungal oils, prepared from *M. alpina,* were obtained from four different suppliers (S1, S2, S3, and S4). The oils were first purified in an open column, packed with silicic acid, followed by high-performance thin-layer chromatography (HPTLC). The purified TG was separated into various subfractions in a Hewlett-Packard 1090 high-performance liquid chromatograph (HPLC) (Palo Alto, CA) equipped with two Supelcosil C-18 (25 cm × 0.46 mm, i.d., 5 μm) (Supelco, Bellefonte, PA) columns connected in series (7). The TG subfractions were eluted isocratically with acetonitrile/isopropanol (65:35, vol/vol) at 1 mL/min for 100 min and monitored with an ultraviolet detector at 210 nm or an evaporative light scattering detector. The separated TG subfractions were collected, methylated, and analyzed for fatty acid composition with a Hewlett-Packard 5890 II plus GC, equipped with a Suplecomega column (50 m × 0.25 mm i.d) (Supelco) as described previously (7).

The procedures to examine the *in vitro* TG hydrolysis were as described previously (8). Basically, the purified TG fraction (100 mg) was dissolved in 0.2 mL dimethyl sulfoxide and mixed with 3 mL tris buffer (1 M, pH 7.7) and 0.1 mL of 0.2% (wt/vol) bile salts in a 30-mL test tube. After mixing with 0.2 mL of 22% (wt/vol) calcium chloride, the mixture was pre-incubated at 40°C for 5 min. The enzymatic hydrolysis was initiated by the addition of 1000 IU pancreatic lipase

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(Sigma Chemical Co., St. Louis, MO) and carried out at 37°C. The reaction was terminated at different time intervals (0, 1, 2, 3, 4, 5, and 10 min) by adding 1 mL of 6 N HCl and 2 mL ethanol. Thereafter, the lipids were extracted and separated into 2-monoacylglycerol (2-MG), free fatty acids (FFA), 1,2- and 2,3-diacylglycerols (1,2- and 2,3-DG), and TG fractions on high-performance thin-layer silica plates that were impregnated with 5% boric acid. The plates were developed with a solvent mixture of chloroform/acetone (85:15, vol/vol) and sprayed with 2,7-dichlorofluorescein. Each lipid fraction was saponified, methylated, and analyzed by gas chromatography (GC) for fatty acid composition. Triheptadecanoin was used as the internal standard for quantitation.

RESULTS AND DISCUSSION

Fatty acid composition of fungal oils. Table 1 shows the fatty acid compositions of the purified TG fractions from fungal oils prepared by four different suppliers. In these oils, palmitic (P, 16:0), stearic (S, 18:0), oleic (O, 18:1n-9), linoleic (L, 18:2n-6), and arachidonic (A, 20:4n-6) acids were the major fatty acids. Minor fatty acids included γ-linolenic (18:3n-6), dihomo-γ-linolenic (20:3n-6), behenic (22:0), and lignoceric (24:0) acids. The arachidonic acid contents of the four oils ranged from 29.2% in S4 oil to 46.3% in S1 oil. Based on arachidonic acid alone, it appears that the S1 oil is the best among the four oils. However, as shown by others (9,10), LC-PUFA, such as arachidonic acid, are generally difficult to cleave from the *sn*-1 and *sn*-2 positions by pancreatic lipase. Therefore, it is important to determine (i) the TG molecular species in these oils, (ii) the distribution of arachidonic acid on TG molecules, and (iii) the bioavailability of these arachidonic acid-containing TG molecules.

Distribution of arachidonic acid-containing TG molecular species. Approximately 40 peaks were separated by HPLC (Fig. 1). Among them, 25 major TG molecular species were identified by GC. The predominant arachidonic acid-containing TG species included those containing: (i) two or three molecules of arachidonic acid, i.e., AAA, LAA, DAA, OAA, PAA, SAA, and (ii) only one molecule of arachidonic acid, i.e., OLA, PGA, PLA, POA, and SOA. In addition to the definitions of P, S, O, L, and A (above) the abbreviations D and G represent dihomo-γ-linolenic (20:3n-6) and γ-linolenic (18:3n-6) acids, respectively. The distribution of different arachidonic acid-containing TG species varied significantly between suppliers (Table 2). The highest AAA content (24.0%) was found in the S1 oil. This is significantly higher than the values of 8.0, 5.9, and 10.8% in the S2, S3, and S4 oils, respectively. PAA was highest (24.7%) in the S4 oil, while SAA was highest (23.0%) in the S1 oil. SLA (containing some POA) was highest (17.8%) in the S3 oil. A significant proportion of TG species contained one molecule of either saturated fatty acids (palmitic or stearic) or monounsaturated fatty acid

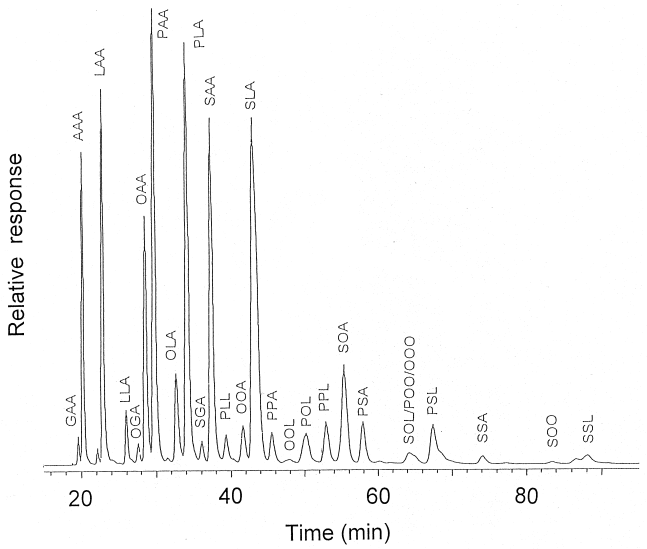

FIG. 1. A typical high-performance liquid chromatogram of triacylglycerol (TG) fractions in a fungal oil, where A = arachidonic acid, G = γ-linolenic acid; L = linoleic acid; O = oleic acid; S = stearic acid; and P = palmitic acid.

TABLE 1
Composition (%, w/w) of Major Fatty Acids in the Triacylglycerol Fraction of Four Fungal Oils

Fatty acid	S1	S2	S3	S4
16:0	8.4	12.9	13.6	12.7
18:0	10.7	10.3	12.9	6.4
18:1n-9	15.1	13.3	12.7	14.2
18:2n-6	7.1	8.2	14	19.8
18:3n-6	2.9	2.9	3	2.1
20:3n-6	2.2	3	3	3.4
20:4n-6	46.3	40	34.1	29.2
22:0	1.7	2	1.5	2.1
24:0	1.3	1.6	1.4	4.5

TABLE 2
Distribution (%, w/w) of Triacylglycerol (TG) Molecular Species in Four Fungal Oils

TG species	S1	S2	S3	S4
AAA	24	8	5.9	10.8
GAA	1.6	0.7	0.9	0.7
LAA	7	13.3	12.7	14.2
OAA	6.2	8.5	5.9	3
PAA	13.3	16.7	11.3	24.7
SAA	23	12.3	9.7	15.7
OGA	0.6	0.9	0.6	0.3
SGA	0.9	0.8	0.9	0.3
LLA	0.2	0.9	1.3	0.4
OLA	0.8	3.2	3	1.1
PLA	2.4	8.4	11.5	5.1
SLA	7	13.9	17.8	4.3
OOA	0.4	1.9	2.3	0.6
PPA	0.3	1.4	1.5	1
SOA	2.5	4.5	5.1	0.8
PSA	0.7	1.4	1.9	0.8
SSA	0.2	0.4	0.5	0.3
Others[a]	2.4	6.1	10.4	17.3

[a]Nonarachidonic acid-containing TG.

(i.e., oleic). The S1 and S4 oils were relatively richer in TG species that contained two and three molecules of arachidonic acid (e.g., OAA, PAA, SAA and AAA), while the S2 and S3 oils were richer in species containing one molecule of arachidonic acid (e.g., OLA, PLA, and SLA).

In vitro *hydrolysis of TG*. Pancreatic lipase is an enzyme that cleaves the fatty acyl groups from the *sn*-1 and *sn*-3 positions of the TG moiety, leaving one molecule of 2-MG. In this hydrolysis experiment, increasing the incubation time decreased the proportions of residual TG, while it increased those of 1,2(2,3)-DG and FFA progressively. At the end of 5-min incubation, approximately 34.6, 45.7, 49.7, and 57% of the initial TG in the S1, S2, S3 and S4 oils, respectively, were hydrolyzed. The levels of 1,2(2,3)-DG and FFA were increased to 16 and 15%, 21.4 and 17.9%, 22.6 and 21.2%, and 21.7 and 26.4% in the S1, S2, S3 and S4 oils, respectively (Fig. 2A). The levels of 2-MG were low for all four oils, which suggests that the pancreatic lipase did not effectively hydrolyze 1,2(2,3)-DG to 2-MG. This is probably due to the presence of arachidonic acid on the *sn*-1 and *sn*-3 positions of DG molecules.

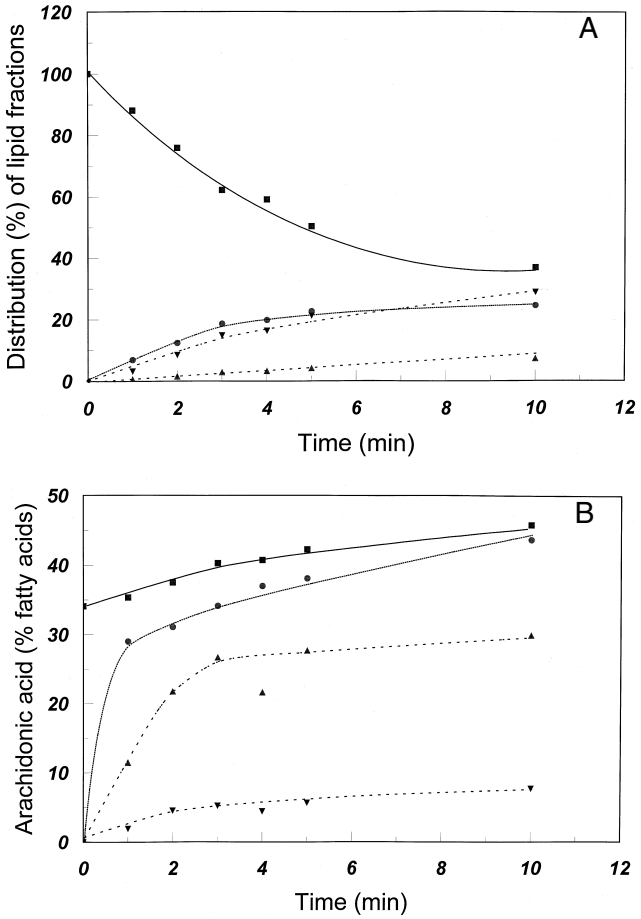

FIG. 2. A typical time course of hydrolysis of a fungal oil, where ■, residual TG; ●, 1,2(2,3)-diacylglycerols; ▲, 2-monoacylglycerol; ▼, free fatty acids. (A) Percentage of different lipid fractions; (B): arachidonic acid content of different lipid fractions. For abbreviations see Figure 1.

The arachidonic acid contents of various lipid fractions [unhydrolyzed TG, 1,2(2,3)-DG, 2-MG, and FFA] increased with the length of incubation time. After 5 min of incubation, the arachidonic acid content of the residual TG rose from 46.3 to 61.7% in the S1 oil, from 40 to 51.3% in the S3 oil, from 34.1 to 45.8% in S2 oil, and from 29.2 to 53.1% in the S4 oil. The increase was a result of the rapid hydrolysis of the non-arachidonic acid-containing TG, as opposed to the slower hydrolysis of the arachidonic acid-containing TG (Fig. 2B). The arachidonic acid contents of the 1,2(2,3)-DG fraction also increased to the same extent as in the residual TG. The arachidonic acid contents of the 2-MG were also increased but were consistently lower than those of the original TG. Throughout the incubation, the arachidonic acid contents of the FFA fraction were low. This may be explained by (i) the failure of the pancreatic lipase to hydrolyze the TG with arachidonic acid located at the *sn*-1 (or *sn*-3) position, and (ii) the initial proportions of arachidonic acid located at these positions were low. Figure 3 shows the amount of TG species in the oil before and after pancreatic lipase digestion. Except in the S1 oil, there was no difference in the amount of AAA present in the reaction mixture before and after lipase treatment. This indicates that AAA was not hydrolyzed by the porcine pancreatic lipase under the prescribed experimental condition. On the other hand, the TG species which contained two arachidonic acids showed various degrees of hydrolysis. Among them, those with another unsaturated fatty acid, such as LAA and OAA, decreased only slightly after lipase digestion, while those with a saturated fatty acid, such as PAA and SAA, decreased by 50% or more. The TG species that contained only one arachidonic acid, such as OLA, OOA, PLA, SLA, SOA, PPA and PSA, decreased substantially. Others (9) have reported that TG with LC-PUFA present at all three (*sn*-1, *sn*-2, and *sn*-3) positions were hydrolyzed slower than those with only an LC-PUFA located at the *sn*-2 position. Further studies on the hydrolysis of TG with arachidonic acids at known stereospecific positions are needed to help explain these differences in degree of hydrolysis among the various TG molecular species.

Results from this study suggest that the bioavailability of arachidonic acid differs among fungal oils prepared by different suppliers. These differences could be attributed to the arachidonic acid content of the oil as well as to the association of arachidonic acid with other fatty acids in the same TG molecule.

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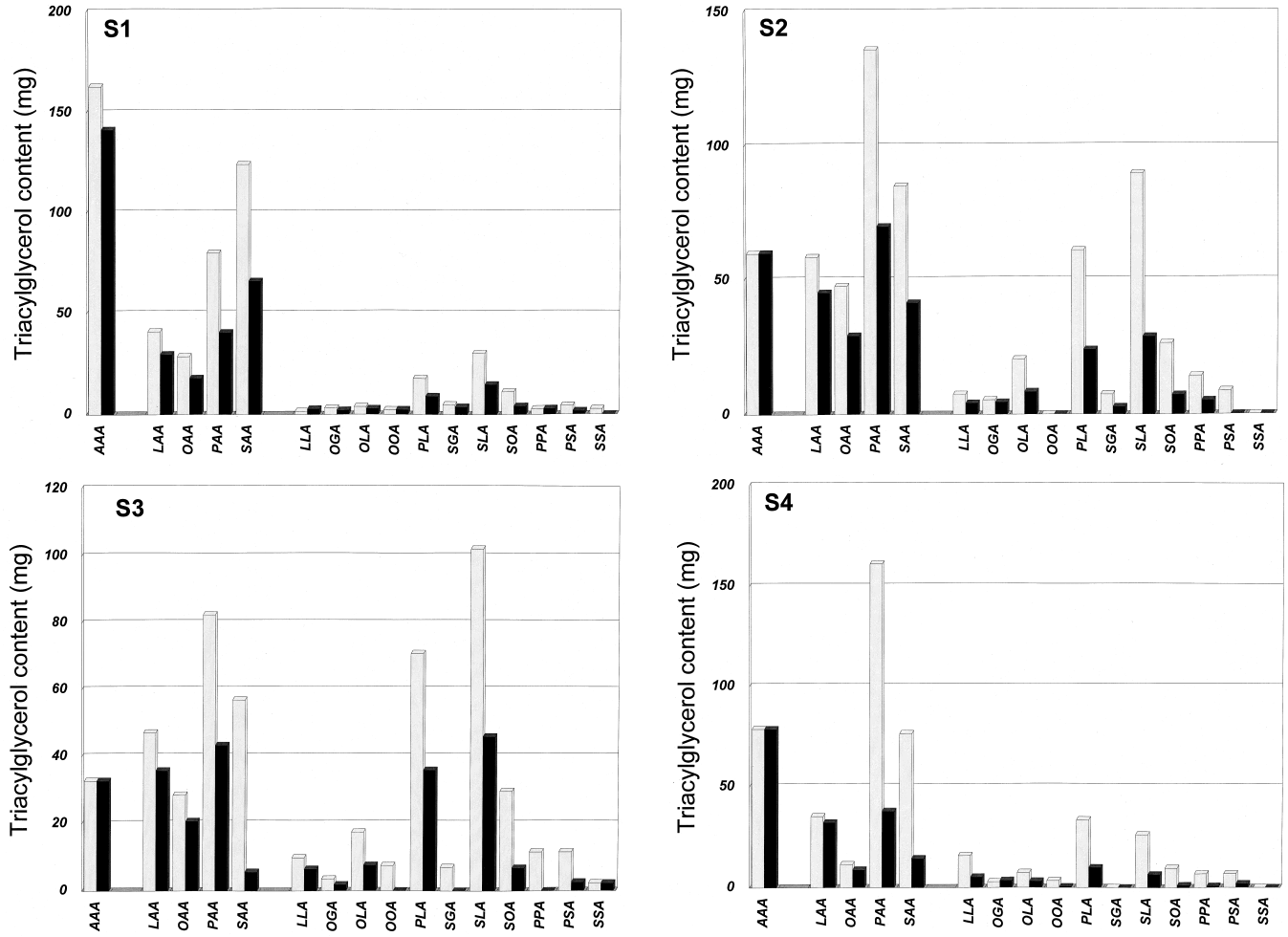

FIG. 3. The amount (mg) of various arachidonic acid-containing TG species in a reaction mixture that contained 600 mg of S1, S2, S3, and S4 fungal oils before (□) and after (■) pancreatic lipase hydrolysis. See Figure 1 for abbreviations.

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