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Lipase catalyzed modification of fish oil to incorporate capric acid

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Abstract

Immobilized lipase, IM60, from *Rhizomucor miehei*, was used as a biocatalyst for the incorporation of capric acid (C10:0) into menhaden fish oil concentrate containing 34.7 mol% eicosapentaenoic acid (20:5n-3) and 34.4 mol% docosahexaenoic acid (22:6n-3). Transesterification (acidolysis) was performed in hexane and solvent-free media. Tocopherol content was analyzed before and after enzymatic modification. Products were analyzed by gas liquid chromatography. After 24 h incubation in hexane, there was an average of 31.1±4.6 mol% incorporation of C10:0 into fish oil, while 20:5 and 22:6 were reduced to 12.6±3.1 and 13.7±4.4, respectively. The solvent-free reaction produced an average of 28.8±4.7 mol% capric acid incorporation; 20:5 and 22:6 decreased to 16.1±5.7 and 13.5±3.0 mol%. The effect of incubation time, substrate mole ratio, enzyme load, and added water were also studied. Generally, as enzyme load, mole ratio, and incubation time increased, mol% capric acid incorporation also increased. Time course of reaction indicated that the highest C10:0 incorporation occurred at 72 h, for both the reaction in hexane (33.5 mol%) and the solvent-free reaction (36.0 mol%). The highest C10:0 incorporation for the substrate mole ratio reaction occurred at a mole ratio of 1:8 in hexane (50.7 mol%) and the solvent-free reaction (36.7 mol%). Although the highest C10:0 incorporation (31.8 and 48.6 mol%) occurred at an enzyme load of 15% in hexane and 20% for the solvent-free reaction respectively, the values were not significantly different ($P<0.05$) after 5% enzyme load. Mol% incorporation of C10:0 declined with increasing amounts of water. At 1% added water, high C10:0 incorporation was achieved for the reaction in hexane (39.3 mol%) and the solvent-free reaction (26.0 mol%). Pancreatic lipase catalyzed *sn*-2 positional analysis was performed on the fish oil before and after enzymatic modification. Fish oil containing capric acid was successfully produced and may be beneficial in certain food and nutritional applications. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: Acidolysis; Capric acid; Immobilized lipase; IM60; Rhizomucor miehei; Fish oil; Structured lipid; Transesterification

1. Introduction

The health benefits of the n-3 polyunsaturated fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) found in fish oils are well known (Kinsella, 1988). Therefore, their presence in the diet is desirable. Menhaden oil has a high content of EPA and DHA located mainly at the *sn*-2 position.

Medium-chain triacylglycerols (MCT) also offer numerous health benefits and have been studied extensively for medical, nutritional, and food applications. MCT have been used to treat fat absorption abnormalities that occur in premature infants and in patients with cystic fibrosis (Kennedy, 1991). MCT are burned quickly for energy and not deposited in the adipose tissue (Megremis, 1991). However, physical mixtures of MCT and long-chain triacylglycerols (LCT) retain their original individual absorption rates. Structured lipids (SL) containing medium-chain fatty acids (MCFA) at *sn*-1,3 positions and long-chain fatty acids at *sn*-2 position are more readily absorbed and oxidized for energy compared to LCT (Jandacek, Whiteside, Holcombe, Volpenhein and Taulbee, 1987). A structured lipid, containing both, fish oil EPA and DHA and MCFA, would have the combined effects of quick energy and other health benefits. In this study, the fatty acid composition of menhaden fish oil was modified to contain MCFA, capric acid (C10:0), by using an immobilized lipase, IM60, from *Rhizomucor miehei* as the biocatalyst. The effects of added water, enzyme load, substrate mole ratio, and reaction time were also studied. Pancreatic lipase catalyzed *sn*-2 positional analysis and vitamin E analysis were performed on the fish oils before and after modification.

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2. Materials and methods

2.1. Materials

Menhaden fish oil concentrate, high in EPA and DHA, was provided by National Marine Fisheries Service (NMFS, Charleston, SC). Immobilized 1,3 specific lipase IM60 (Lipozyme IM) was provided by Novo Nordisk, Biochem North America, Inc. (Franklinton, NC). Capric acid (C10:0) was obtained from Sigma Chemical Co. (St. Louis, MO). Organic solvents were purchased from J.T. Baker Chemical Co. (Phillipsburg, NJ) or Fisher Scientific (Norcross, GA).

2.2. Methods

2.2.1. Enzymatic modification reaction

The general synthesis of structured lipids with menhaden fish oil and C10:0 as substrates was performed as previously described (Jennings & Akoh, 1999). The mole ratio was 1:2 fish oil triacylglycerol (TAG) to C10:0 free fatty acid (FFA) in 3 ml of hexane for reactions conducted in an organic solvent. All reactions were performed in duplicate and average values were reported.

2.2.2. Analysis of product

The enzyme was removed by passing the reaction mixture through an anhydrous sodium sulfate column. Fifty microliters of the reaction product was analyzed by thin-layer chromatography (TLC) on silica gel 60 plates and developed with petroleum ether/ethyl ether/acetic acid (80:20:0.5, v/v/v). The bands were visualized under ultraviolet light after being sprayed with 0.2% dichlorofluorescein in methanol. The bands corresponding to TAG were scraped from the TLC plate and methylated with 3 ml of 6% HCl in methanol at 70–80°C for 2 h. The fatty acid methyl esters (FAME) were extracted and analyzed by gas-liquid chromatography (GLC) as described previously (Jennings & Akoh, 1999). The relative content of FAME as mol% was calculated by computer with 17:0 as the internal standard.

2.2.3. Lipase catalyzed sn-2 positional analysis

Pancreatic lipase-catalyzed *sn*-2 positional analysis was performed as previously outlined (Luddy, Barford, Herb, Magidman & Riemenschneider, 1963; Jennings & Akoh, 1999). The band corresponding to *sn*-2 monoacylglycerol (2-monoolein was used as a standard) was scraped after the TLC plate was developed in hexane/diethyl ether/acetic acid (50:50:1.0, v/v/v). The *sn*-2 monoacylglycerol was then methylated and analyzed by GLC.

2.2.4. Tocopherol analysis

Tocopherol analysis was performed on the menhaden fish oil before and after enzymatic modification following the method described by Lee, Landen, Phillips and Eitenmiller (1998). The oil samples were analyzed with a normal phase high-performance chromatography column (Jennings & Akoh, 1999). Tocopherol standards were obtained from Sigma (St. Louis, MO). Purity and stability of standards were monitored by specific absorption coefficients ($E^{1\%}_{1cm}$) values, measured by using a DU-64 Spectrophotometer (Beckman Instruments Inc., Fullerton, CA). Specific $E^{1\%}_{1cm}$ and maximum wavelengths (λmax) for tocopherols were 71.0 and 294, respectively, for α-tocopherol; 86.4 and 297, respectively, for β-tocopherol; 92.8 and 298, for γ-tocopherol; and 91.2 and 298 for δ-tocopherol, respectively (Scott, 1978).

2.2.5. Statistical analysis

The Statistical Analysis System was used to analyze data (SAS, 1989–1996). Analysis of variance was used to compare means among treatments and mean separation tests were used to identify which treatment means were different. Values were considered significant at $P < 0.05$ level.

3. Results and discussion

The menhaden fish oil concentrate used for this study contained 13 fatty acids, with 20:5 and 22:6 being the major ones. After modification, 10:0, 20:5, and 22:6 were the major fatty acids present, followed by 16:0, 16:1, 18:0 and 18:1. Table 1 shows the relative fatty acid composition of the oils. Relative fatty acid composition of fish oil before modification was significantly different from fatty acid composition after modification

Table 1
Relative fatty acid composition (mol%) in National Marine Fisheries Service menhaden fish oil before and after modification with and without hexane[a]

Fatty acid	Before modification	After modification	
		Hexane	Solvent-free
10:0	0.0±0.0	31.1±4.6	28.8±4.7
14:0	1.9±0.6	9.5±1.2	9.7±6.1
14:1	1.2±0.1	ND[b]	ND
16:0	2.5±0.9	13.6±1.0	15.1±2.0
16:1n-7	2.7±0.1	11.2±0.6	9.4±2.2
18:0	3.5±0.3	1.6±1.1	1.2±1.3
18:1n-9	9.9±1.7	6.7±3.1	6.3±1.4
18:2n-6	1.8±0.6	ND	ND
18:3n-3	1.5±0.4	ND	ND
20:0	0.4±0.1	ND	ND
20:1n-9	5.0±2.2	ND	ND
20:5n-3	34.7±3.0	12.6±3.1	16.1±5.7
22:6n-3	34.4±3.1	13.7±4.4	13.5±3.0

[a] Values are average of two determinations with standard deviations.

[b] ND, not detected.

($P<0.05$). After 24 h incubation in hexane, there was an average of 31.1±4.6 mol% incorporation of C10:0 into fish oil; 20:5 and 22:6 declined to 12.6±3.1 and 13.7±4.4, respectively. The solvent-free reaction produced an average of 28.8±4.7 mol% C10:0 incorporation, and 20:5 and 22:6 decreased to 16.1±5.7 and 13.5±3.0. These values for the hexane and solvent-free reactions were not significant at $P<0.05$, indicating that both processes are equally good. However, for food use of the SL, the solvent-free process is more desirable. Immobilized lipase IM60, from *R. miehei*, was used as a biocatalyst for the incorporation of C10:0 into fish oil because its 1,3 positional specificity would place C10:0 at these positions on the TAG molecule for maximum metabolic benefit. The menhaden oil supplier (NMFS, Charleston, SC) indicated that 20:5 and 22:6 concentrations were intentionally increased during processing. Their values after modification (Table 1) were closer to the natural concentrations (Uauy-Degach & Valenzula, 1992). In general, as the enzyme load, substrate mole ratio, and incubation time increased, mol% incorporation of C10:0 also increased (Figs. 1–4). These findings are consistent with other studies involving the enzymatic modification of TAG performed in our laboratory (Akoh, Jennings & Lillard, 1995, 1996; Jennings & Akoh, 1999).

3.1. Time course

Fig. 1 shows that the time courses with and without hexane exhibited very similar pattern and were not significantly different ($P<0.05$). C10:0 incorporation increased throughout the reaction up to 72 h to 33.5 and 36.0 mol% for the reactions with and without hexane, respectively. Other studies have reported that longer reaction times result in increased acyl migration in a laboratory-scale continuous reactor when MCFA were incorporated into TAG (Mu, Xu & Høy, 1998). The highest mol% C10:0 incorporation at 72 h was similar to the values obtained at 48, 24, and 20 h, but was significantly different than the incorporation achieved at 12 h or less ($P<0.05$) for both solvent and solvent-free reactions. This means that prolonging the reaction beyond 20 h will not lead to a significant increase in C10:0 incorporation.

3.2. Mole ratio

The highest overall incorporation of C10:0 (50.7 mol%) occurred at a 1:8 substrate mole ratio (fish oil to C10:0) in hexane and 37.7 mol% for the solvent-free reaction (Fig. 2). This value was significantly higher than the C10:0 incorporation at 1:6 or lower mole ratio ($P<0.05$). Mol% C10:0 incorporation was significantly higher for reactions in hexane compared to solvent-free. The lower C10:0 incorporation in the absence of hexane could be due to mass transfer limitations in the solvent-free system. Previous studies have shown that a high mole ratio required a shorter reaction time or improved reaction rate and resulted in less acyl migration (Mu et al., 1998). Although the substrate mole ratio can be manipulated to achieve the desired level of C10:0 incorporation, economically there is no advantage in using excess capric acid, which is expensive. High C10:0 concentration in the reaction may indeed result in substrate inhibition. In addition, the overall product yield may

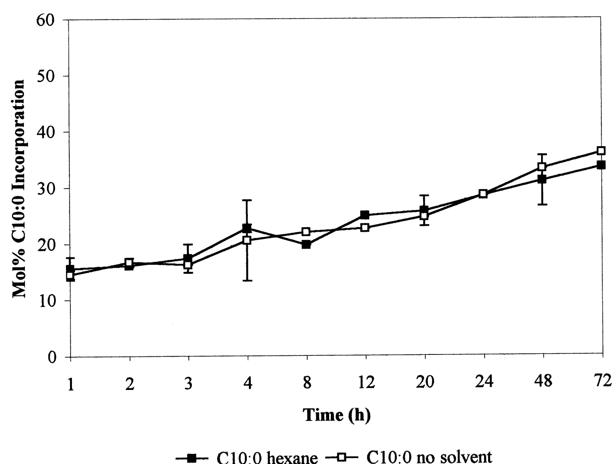

Fig. 1. Time course of IM60 lipase catalyzed modification of National Marine Fisheries Service, (NMFS, Charleston, SC) menhaden fish oil with and without hexane to incorporate capric acid (C10:0) as determined by gas liquid-chromatography. Samples were analyzed at 1, 2, 3, 4, 8, 12, 20, 24, 48, and 72 h (x-axis). All reactions were in duplicate. Enzyme amount was 10% by weight of reactants. Incubation was at 55°C and 200 rpm with and without hexane. The y-axis represents mol% C10:0 incorporation.

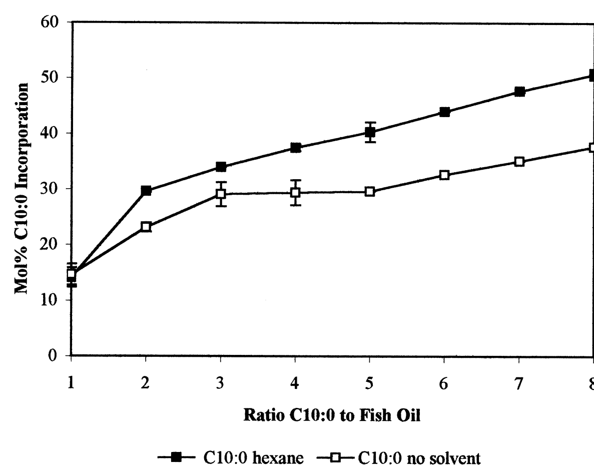

Fig. 2. Effect of mole ratio of substrates (fish oil to C10:0) on C10:0 incorporation with IM60 lipase as the biocatalyst. The numbers 1–8 on the x-axis correspond to mole ratios of 1:1, 1:2, 1:3, 1:4, 1:5, 1:6, 1:7, and 1:8, respectively. All reactions were in duplicate. Enzyme amount was 10% by weight of reactants. Incubation was at 55°C and 200 rpm with and without hexane. The y-axis represents mol% C10:0 incorporation.

decrease during downstream processing to remove excess C10:0.

3.3. Enzyme load

Incorporation of C10:0 reached its maximum (31.8 mol%) with hexane at 15% enzyme load; in solvent-free reactions, maximum C10:0 incorporation (48.6 mol%) occurred at 20% enzyme load (Fig. 3). Others have reported increased incorporation of capric acid into rapeseed oil with increasing enzyme load which also resulted in increased acyl migration (Xu, Skands, Adler-Nissen & Høy, 1998). Analysis of variance indicated that the mol% C10:0 incorporation, achieved at 5% enzyme load, was not significantly different from 10, 15, and 20% enzyme load, except for the slight increase observed at 20% enzyme in solvent-free reaction. An increase in enzyme concentration will only speed up the reaction. The best cost-cutting approach, based on the costs of the substrates, enzymes, energy (power), manpower time, and the particular product characteristics desired, will have to be decided by industry.

3.4. Effect of added water

C10:0 incorporation was highest at 1% added water, reaching 39.3 mol% for the reaction in hexane and 26.0 mol% for the solvent-free reaction (Fig. 4). Mol% C10:0 incorporation was almost constant up to 12% added water, but decreased significantly at 24 and 48% water for both solvent and solvent-free reactions. In general, mol% C10:0 incorporation was significantly higher for the reaction in hexane than without ($P < 0.05$). For most reactions, a water content of less than 1% is required, but the optimum water content varies between 0.04 and 11% w/v for different lipases (Li & Ward, 1993; Malcata, Reyes, Garcia, Hill & Amundson, 1992).

3.5. Solvent versus solvent-free reactions

Organic solvents, such as hexane, have several functions, including increasing the solubility of nonpolar substrates, and shifting thermodynamic equilibria to synthesis rather than hydrolysis (Klibanov, 1986). Concerns for hexane toxicity, flammability, cost, and additional time associated with the purification process, have led to lipase-catalyzed modification of TAG being performed without organic solvent. Mol% incorporation increased with increasing enzyme load, substrate mole ratio, and incubation time for both solvent and solvent-free reactions (Figs. 1–4). In general, the solvent-free mole ratio and effect of water reactions showed lower mol% C10:0 incorporation than the reactions in hexane. For the time course and enzyme load reactions experiment, mol% C10:0 incorporation was nearly equal and higher than the reactions in hexane respectively (Figs. 1 and 3). For food applications, enzymatic lipid modifications are preferred over chemical modification or reactions in organic solvents.

3.6. Pancreatic lipase catalyzed *sn*-2 positional analysis

Pancreatic lipase catalyzed *sn*-2 positional analysis of the menhaden fish oil, before and after modification,

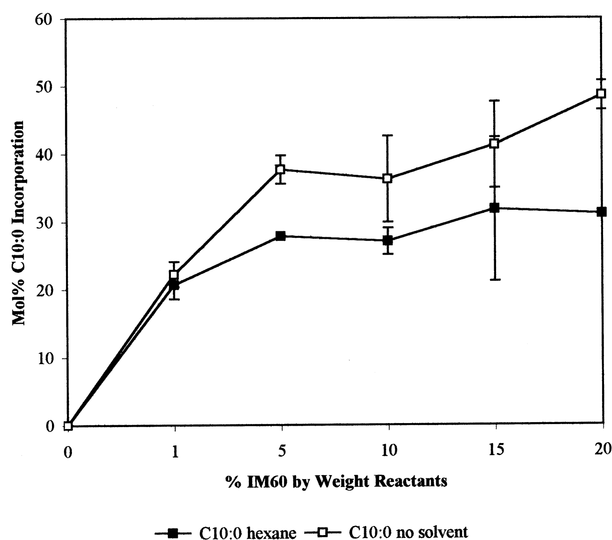

Fig. 3. Effect of enzyme load on C10:0 incorporation into NMFS fish oil with IM60 as a biocatalyst. Amount of enzyme was based on weight of reactants (0, 5, 10, 15, and 20%, respectively). All reactions were in duplicate. Incubation was at 55°C and 200 rpm, with and without hexane. The *y*-axis represents mol% C10:0 incorporation.

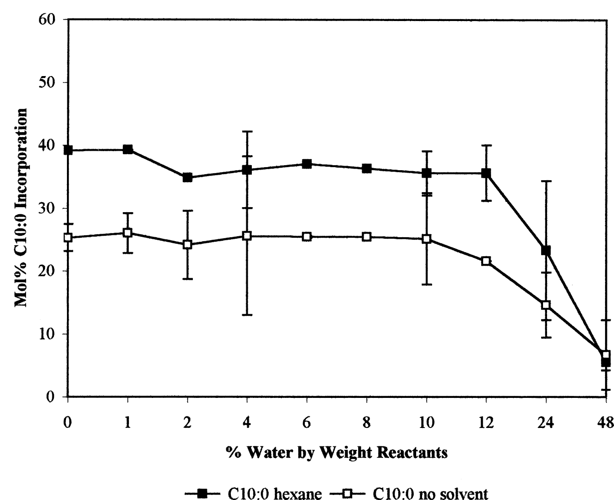

Fig. 4. Effect of added water on C10:0 incorporation into NMFS fish oil with IM60 lipase as a biocatalyst. Amount of water added was based on the weight of the reactants (0, 1, 2, 4, 6, 8, 10, 12, 24, and 48%, respectively). All reactions were in duplicate. Incubation was at 55°C and 200 rpm, with and without hexane. The *y*-axis represents mol% C10:0 incorporation.

showed the following relative fatty acid composition at the 2-position, respectively: 14:0, 9.5, and 7.9 mol%; 16:0, 13.7, and 11.0 mol%; 16:1, 10.7, and 7.4 mol%; 18:1, 5.9, and 6.3 mol%; 18:2, 2.5, and 4.6 mol%; 20:5, 12.3, and 14.1 mol%; 22:6, 45.3, and 48.8 mol%. The predominant fatty acids at the 2-position before modification were 16:0, 20:5, and 22:6. After modification, the major fatty acids at the 2-position were 20:5 and 22:6. Fatty acids esterified at the 2-position are easily absorbed, regardless of the type of fatty acid esterified at that position (Haumann, 1997; Jandacek et al., 1987). No C10:0 was found at the *sn*-2 position indicating that no acyl migration took place in this assay. However, in our previous study we observed acyl migration to the 2-position even though a 1,3 specific lipase was used (Jennings & Akoh, 1999). However, a different source of fish oil was used in the present study. The variation in results could have been due to differences in sources and processing of the two fish oils. The menhaden oil used in the current study was concentrated and rich in 20:5 and 22:6.

3.7. Tocopherol analysis

Tocopherols are found naturally in oils and function as antioxidants; they may offer several health benefits. Table 2 shows the effect of enzymatic modification on tocopherol content of fish oil. The unmodified fish oil contained predominantly α- and γ-tocopherols with a total tocopherol content of 2.4 mg/g. Menhaden fish oil contains approximately 0.066 mg/g tocopherol (Uauy-Degach & Valenzula, 1992). National Marine Fisheries Service indicated that tocopherol was added (1.1 mg/ml α-, 1.0 mg/ml γ-tocopherol) as antioxidant, which would explain the elevated levels of tocopherols observed in this study. The tocopherol content values of modified fish oil were close to those obtained for unmodified fish oil for the α-, β-, γ-, and δ-tocopherols (Table 2). Pronova fish oil, from a previous study showed a decline in α-, β-, and γ-tocopherols with δ-tocopherol remaining unchanged (Jennings & Akoh, 1999). Again, the differences in results could have been due to differences in sources and processing of the oils.

Table 2
Tocopherol content of National Marine Fisheries Service menhaden fish oil before and after enzymatic modification

Fish oil	Tocopherol content (mg/g)				
	α	β	γ	δ	Total
Unmodified	1.07	0.0201	0.903	0.386	2.37
Modified	1.01	0.0210	0.882	0.387	2.30

This study has shown that a structured lipid, containing fish oil and C10:0, can be successfully produced with and without the addition of organic solvent. One potential problem with the modified oil reported herein is the relative increase in C14:0 content which may increase serum total cholesterol concentration in man (Zock, de Vries & Katan, 1994). The benefits of a fish oil structured lipid containing capric acid may be in disease prevention but need to be studied. It is known that SL are more readily absorbed than their component physical mixtures. The conservation of EPA and DHA at the *sn*-2 position of this SL may enhance their absorption and use as nutraceutical lipids.

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