Enzymic Enhancement of n-3 Fatty Acid Content in Fish Oils[1]

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Commercially available fish oils with n-3 fatty acid contents ranging from 29 to 34% were converted enzymically, with Amano P lipase, to mixtures of glycerides with n-3 fatty acid contents of *ca.* 50%, in weight recovery yields of 23–50%, depending upon extraction procedures. Glyceride mixtures with n-3 fatty acid contents above 70% were obtained in yields of 14–21%. The processes are based on the relative stability of the ester linkages that involve n-3-fatty acyl groups and the regioselectivity of the enzyme toward acyl groups at the 1,3-positions of glycerol.

KEY WORDS: n-3 Fatty acids, fish oils, lipase, mono-, di- and triglycerides.

The beneficial health effects of fish oils (1–5), especially in occlusive vascular and atherosclerotic diseases (6–8), have been ascribed to their n-3 fatty acid contents, notably 5,8,11, 14,17-eicosapentaenoic acid (EPA), 7,10,13,16,19-docosapentaenoic acid (DPA) and 4,7,10,13,16,19-docosahexaenoic acid (DHA). As close relatives of arachidonic acid (AA), these n-3 fatty acids compete with AA for residency in phospholipids and as substrates for cyclooxygenase and lipoxygenase enzyme systems. Although AA is a much better substrate for cyclooxygenase than EPA, DHA actually inhibits this enzyme. Different reaction rates in prostaglandin (PG)- and thromboxane (TX)-catalyzed reactions can lead to an n-3 fatty acid-induced change of the PG/TX ratio, but the more pronounced generation of those hydroperoxy products, which are followed by the synthesis of leukotriene A_5 (LTA_5) and its metabolites, can give rise to a different prostanoid/leukotriene ratio. An increased intake of n-3 fatty acids can thus change the chemistry of the eicosanoids as well as their ratios and may be responsible for changes of cellular membrane function due to n-3 fatty acid-modified phospholipids. Taken together, these changes are suspected to be responsible for endothelial protection and antiatheromatous effects, including reduced monocyte adherence and migration, anti-aggregatory effects on blood platelets in general, reduction of blood viscosity and arterial blood pressure, and increase of fibrinolytic activity. The decrease of plasma lipids, especially triglycerides, and the improvements in the lipoprotein–cholesterol profile, as a result of dietary n-3 fatty acid intake, are well established (9). In addition to the abovementioned anti-thrombotic and hypolipidemic effects, n-3 fatty acids have been linked to anti-inflammatory and immunological parameters of white cells, especially monocytes. Further effects of n-3 fatty acids on the conditions of the immunologic and autoimmune status, such as asthma, lupus erythematosus, arthritis, ulcerative colitis and Crohn's disease, are suspected (10–12). Reduced growth and incidences of certain tumors in animal models have been observed in connection with fish oil-enriched diets; for example, the growth rate of a human mammary carcinoma, MX-I, was significantly reduced in a mouse model (13), and low breast cancer rates in Greenland Eskimos and Japanese are ascribed to relatively high fish consumption. There are indications that n-3 fatty acids are essential for normal growth, development and visual function (10). n-3 Fatty acids have also been implicated in the prevention of psoriasis, a condition virtually unknown among Greenland Eskimos (14). Increases of cerebral infractions, cardiovascular diseases and malignant neoplasma in the Japanese population during the past 20 years have been attributed to an increased ratio of animal fat/fish oil in the diet, concomitant with decreased rice consumption (15).

Although certain therapeutic responses can be observed upon fish oil consumption, others do not appear to be immediately attainable by simple dietary adjustment. The expression of some of the fish oil's beneficial health effects may be overinterpreted in favor of n-3 fatty acids (16) or may require an intake of fish oils against a background of a high dihomo-γ-linolenic acid (DGLA)/AA (C20:3n-6/C20:4n-6) ratio in phospholipids, which, as in the Greenland Eskimo population, may be the result of a genetic disposition (17).

"Fish oil" is a term for a commercial triglyceride product containing many types of fatty acids. Depending upon the species and the harvest time, the total n-3 content in terms of EPA, DPA and DHA varies considerably (18,19).

A number of methods have been employed with the aim of preparing n-3 fatty acid-enriched products from natural fish oils. Perhaps the simplest process consists of the transesterification of fish oils with methanol or ethanol and purification of the resulting simple alkyl esters. To reconstitute the fats, these esters were interesterified with groundnut oil by catalysis with a 1,3-specific lipase from *Mucor miehei* (20). Cod liver and sardine oils were tested as substrates for six lipases. Those enzymes derived from *Candida cylindracea* and *Aspergillus niger* gave glycerides with a better than twofold increase in n-3 fatty acid content (21). In a similar study, six microbial lipases were screened for their activity to concentrate the DHA content in fish oil glycerides. The lipase of *C. cylindracea* exhibited the most pronounced DHA specificity (22). Homogeneous triglycerides that contained either EPA or DHA were prepared by direct esterification or interesterification methods with an immobilized lipase derived from *C. antarctica* (23).

We began our studies in 1988 with the intent of developing methods that would generate modified fish oils with enhanced n-3 fatty acid contents by a "nature-like" process conducted with lipases. Success for such an approach was envisioned by the fish oil's high population of triglycerides, containing two n-3 fatty acyl residues in 1,2-glyceryl positions. Esters with "saturated" acyl groups and ester linkages at the 1,3-positions are hydrolyzed selectively by most lipases, so that three product types could be expected purely by manipulation of the extent of the enzymic digestion and the nature of the extraction: Product *type 1* consists mostly of triglycerides with moderately enhanced n-3 fatty acid content (*ca.* 50%); mixtures of mono-, di- and triglycerides with either moderate (*ca.* 50%) or high (*ca.* 70%) n-3 fatty acid contents are representative of product *type 2* and *type 3*, respectively.

EXPERIMENTAL PROCEDURES

Starting materials. Winterized menhaden (lot 1338) and anchovy oils (lot 1561) with n-3 fatty acid contents of 29.3

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and 34.0%, respectively, were provided by Zapata Haynie (Reedville, VA).

Separation of mono-, di- and triglycerides. Glyceride mixtures were separated on a column (3.5 × 25 cm) of Florisil (100 g, 100–200 mesh) deactivated with water (7%) and slurried in hexane. Development with 3:17 ether/hexane eluted triglycerides, while diglycerides and monoglycerides were eluted with 1:1 ether/hexane and diethyl ether, respectively. Aliquots of the reaction mixture were acidified with phosphoric acid and partitioned between 1:1 ether/water. The ether phase was washed with brine, dried (sodium sulfate) and evaporated, and 1 g of the residual oil was redissolved in hexane and applied to the column. The chromatographic separation was monitored by thin-layer chromatography (silica gel G, 1:1 ether/hexane). The spots were visualized by a sequence of two sprays consisting of 7% phosphomolybdic acid in ethanol, followed by 10% $Ce(HSO_4)_4$ in 1:9 sulfuric acid/water. The plates were then heated until the blue color developed. The pure glyceride fractions were obtained after pooling of fractions and evaporation of solvent. Approximate R_f values were monoglycerides, 0.05; 2,3-diglycerides, 0.4; 1,3-diglycerides, 0.48; triglycerides, 0.95. Fatty acids streak in this system and overlap with the diglycerides.

Fatty acid analysis. Fatty acids are expressed in weight percent and were determined (24) on an absolute basis by capillary gas chromatography with internal standards of methyl esters (0.25 mm × 30 m, 0.25 m fused silica; Carbowax 20M; Supelco, Inc., Bellefonte, PA). Glycerides were converted to the methyl esters by transesterification with trimethylanilinium hydroxide directly in the injection port (B. Buglio, private communication).

Experiment A. A 5-L, three-neck round-bottom flask, equipped with a mechanical stirrer, pH electrode, argon inlet and addition tube for sodium hydroxide solution, was charged with deionized water (1 L) and calcium chloride (44 g, 0.4 mol), hexane (2 L), triton X-100 (50 drops) and anchovy oil (200 g, *ca.* 0.2 mol). The mixture was stirred vigorously at ambient temperature, and Amano P lipase (Amano International Enzyme, Co., Inc., Troy, VA) (5 g) was added to the emulsion. As the digestion progressed, 1 N sodium hydroxide solution was automatically added to maintain a pH range of 7.2–7.3 (Fig. 1). The base was added by a peristaltic pump, activated *via* a pH controller (Model 5997-30, Cole Parmer Instrument Company, Niles, IL). The reaction mixture was quenched by dilution with diethyl ether to a volume of 4.5 L and addition of phosphoric acid to a pH of 1.5. The phases were separated, the upper layer was washed twice with brine, dried (sodium sulfate) and evaporated to an oil (198 g) consisting of mono-, di- and triglycerides and fatty acids. This concentrate was processed as illustrated in Scheme 1.

Experiment B. A mixture of menhaden oil (200 g, *ca.* 0.2 mol), water (1 L) and calcium chloride (88 g, 0.79 mol), hexane (3 L), Triton X-100 (60 drops) and Amano P lipase (1.5 g) was digested at ambient temperature, as described previously, within a pH range of 7.5–7.6 (2 N NaOH). After 4 h, 180 mL of the alkali solution were consumed. The reaction mixture was quenched by pH adjustment to 2 (phosphoric acid) and diluted with *t*-butyl methyl ether (150 mL). The organic phase was washed with brine (2 × 250 mL) and processed as illustrated in Scheme 2. The product profile is shown in Figure 2.

Experiment C. Anchovy oil (20 g, *ca.* 0.02 mol), water (100 mL), hexane (300 mL) Triton X-100 (5 drops), calcium chloride (8.88 g, 0.08 mol) and Amano P lipase (0.5 g) were stirred for 6.75 h at 19–22°C at pH 7.4–7.6 (1 N NaOH), as described. Aliquots were analyzed by extractive isolation with ether after acidification with phosphoric acid to pH 1.8, followed by chromatography on deactivated Florisil after 3, 4.5 and 6.75 h as summarized in Figure 3.

Experiment D. A mixture of menhaden oil (200 g, *ca.* 0.2 mol), water (1 L), calcium chloride (88 g, 0.793 mol), hexane (3 L), Triton X-100 (1 mL) and Amano P lipase (1.1 g) was treated as described at ambient temperature for 20 h at pH 7.45–7.65 (2 N NaOH). The resulting emulsion was acidified with phosphoric acid to pH 1.5, and the hexane phase was washed with brine (3 × 200 mL). The subsequent extraction protocol is illustrated in Scheme 3.

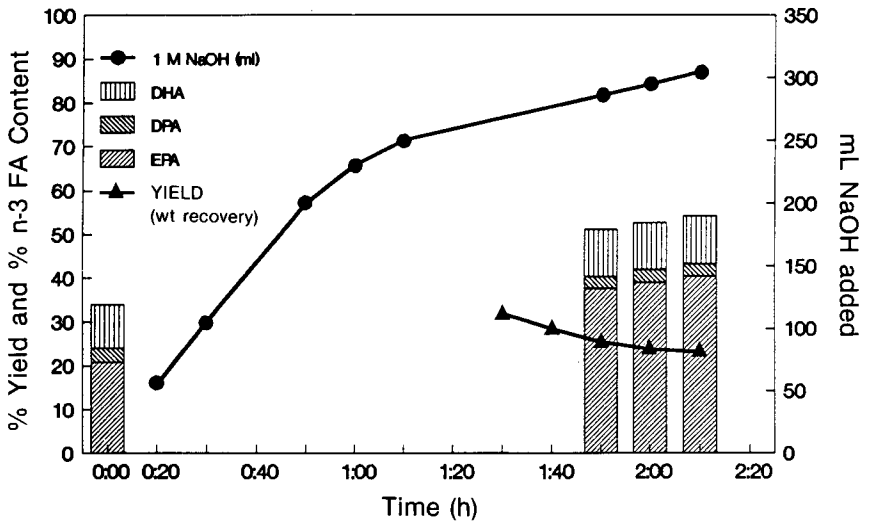
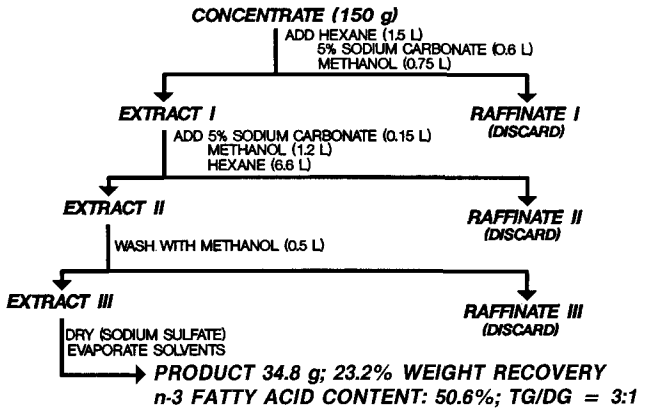
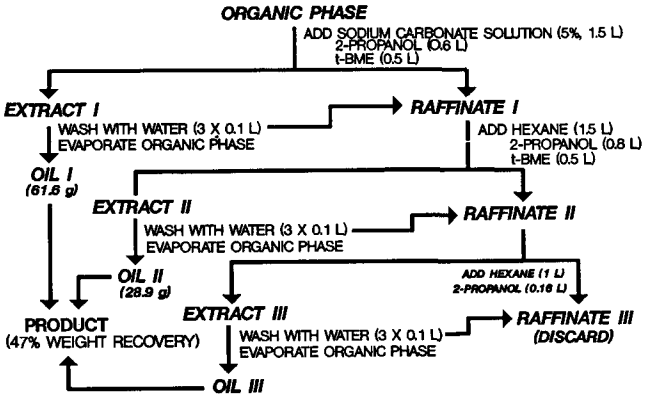


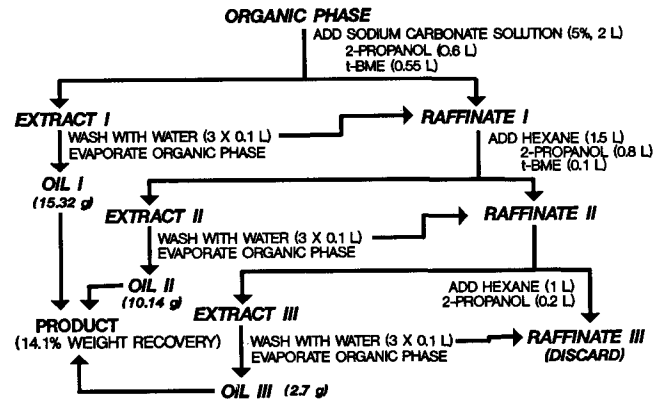
FIG. 1. Alkali consumption and progress of hydrolysis. FA, fatty acid; DPA; docosapentaenoic acid; DHA, decosahexaenoic acid; EPA, eicosapentaenoic acid.



SCHEME 1



SCHEME 2



SCHEME 3

RESULTS AND DISCUSSION

To produce product *type 1*, one needs to aim at the destruction of glycerides with "saturated" (non-n-3) fatty acids and at the hydrolysis of those triglycerides with only one "saturated" fatty acyl group. A simple extraction protocol with hexane/methanol, as illustrated in Scheme 1, is selective for tri- and diglycerides. As exemplified by Experiment A, the n-3 fatty acid content of the product increases gradually to 54.2%. After 130 min, a product with a tri/diglyceride ratio of 3:1 is obtained with a yield of 23.2 weight percentage (Fig. 1). The advantage of extraction simplicity is offset by a relatively low yield due to incomplete diglyceride extraction and disregard of the produced monoglyceride fraction, which usually exhibits a high n-3 fatty acid content. Decreased enzyme concentrations require longer reaction times with little change in product composition.

Product *type 2* is prepared in a similar fashion, but without attempts to optimize the residual triglyceride content by selective extraction. Thus, the subsequent product recovery is necessarily aimed at all surviving glycerides, including mono-, di- and triglycerides. The extraction scheme shown in Scheme 2 affords a product recovery of *ca.* 45–50 weight percent; three consecutive extractions recovered 31, 15 and 2% of the product weight (Fig. 2). Assuming a quantitative product recovery by chromatography suggests the discontinuous extraction scheme to be 87% efficient.

If the digestion is permitted to progress so far that only *ca.* 20–25% of glycerides remain, exhaustive extraction yields product *type 3*. Menhaden and anchovy oils gave glyceride mixtures with n-3 fatty acid content of at least 70%, with 14 and 22% weight recovery, respectively. Experiment C (Fig. 3) illustrates the gradual build-up of a glyceride mixture obtained in 21.4 weight percentage upon chromatographic (Florisil) isolation. The experiment was repeated under essentially the same conditions with analysis of the glyceride fractions after 6.75 h. Figure 4 shows that the monoglyceride fraction plays a predominant role, both in terms of high n-3 fatty acid content as well as a major contributor to the bulk of the product.

Hydrolysis of menhaden oil (Experiment D) proceeded similarly. The selected extraction protocol (Scheme 3) led to a product containing 71% n-3 fatty acids and was obtained in 14% weight recovery. The lower n-3 fatty acid content of menhaden oil is reflected in a lower product recovery yield when compared to anchovy oil (Fig. 5).

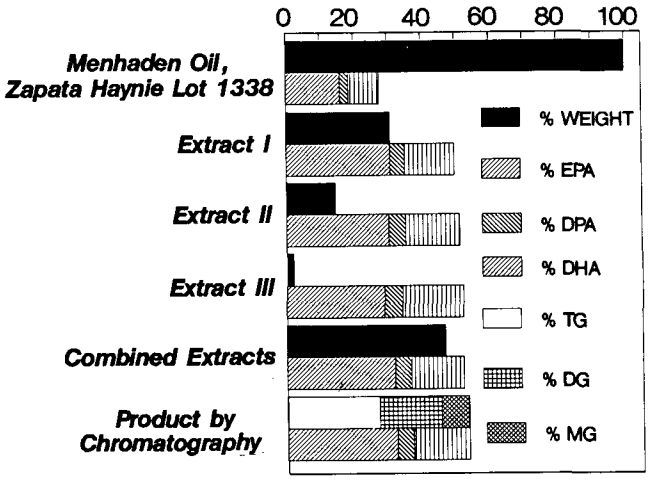


FIG. 2. Comparison of extractive and chromatographic product isolation. Abbreviations as in Figure 1; TG, triglyceride; DG, diglyceride; MG, monoglyceride. Zapata Hayne Corp., Reedville, VA.

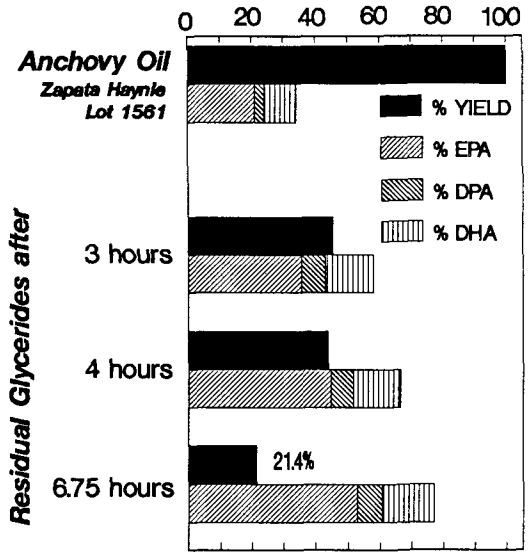


FIG. 3. Progress of hydrolysis (product isolated by chromatography). Abbreviations and company source as in Figures 1 and 2.

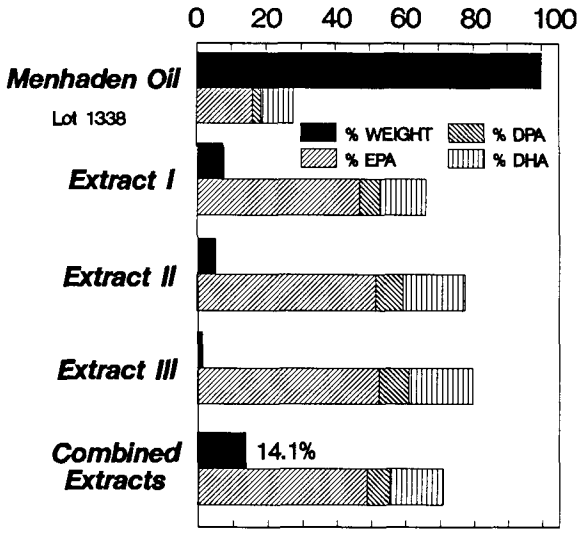


FIG. 5. Comparison of extracts. Abbreviations as in Figure 1.

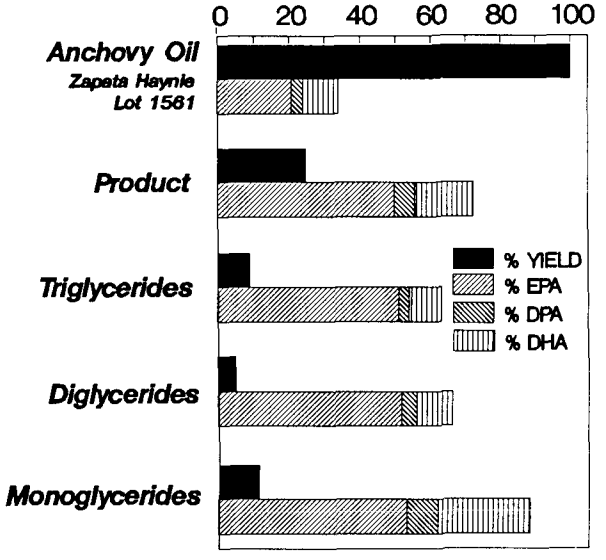


FIG. 4. Survey of glyceride contributions to the overall n-3 fatty acid content of product. Abbreviations and company source as in Figures 1 and 2.

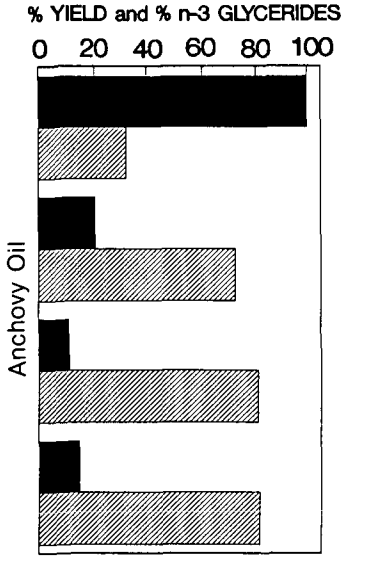


FIG. 6. Effect of enzyme concentration on reaction time. Enzyme concentrations (%) were 2.6, 0.875 and 0.32 for reaction times of 6, 18 and 42 h, respectively. Closed bars, yield (%); hatched bars, n-3 glycerides (%).

Varying the enzyme concentation and reaction temperature provides tools for the adjustment of the digestion time required. The relatively high enzyme concentrations used in the experiments described here were dictated by the desire for reaction times that did not exceed overnight periods. Figure 6 illustrates similar results obtained with different enzyme concentrations after arbitrary adjustment of reaction times. Optimum reaction temperatures were not established, and ambient temperatures were chosen for the sake of simplicity. The linearity of alkali consumption in the later stage of hydrolysis rendered the experiments entirely reproducible.

A comparison between a pancreatic (porcine, Steapsin; Sigma Chemical Co., St. Louis, MO) and a bacterial enzyme (*Pseudomonas*, Amano P) did not reveal significant differences in the residual glycerides in terms of mono-, di- and triglyceride ratios. Increased reaction times generally decreased the residual triglyceride fraction in favor of relatively higher di- and monoglyceride concentrations, and this shift was concomitant with an increase in the relative amounts of DPA and DHA in the remaining glycerides. Because DPA and DHA are predominantly esterified at glycerol's position 2, it is not surprising that monoglycerides, and, hence, product *type 3*, are particularly rich sources of these two fatty acids (Fig. 4).

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