Fatty Acid Selectivity of Microbial Lipase and Lipolytic Enzymes from Salmonid Fish Intestines Toward Astaxanthin Diesters

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ABSTRACT: The objective of the work described in this paper was to study a possible FA selectivity of digestive lipolytic enzymes isolated from salmon and trout intestines toward astaxanthin diesters of various FA composition and compare it with the FA selectivity of microbial lipase. Astaxanthin diesters of varying FA composition were prepared in excellent yields (>90%) by chemical esterification using a carbodiimide coupling agent. The astaxanthin diesters were screened in a hydrolysis reaction by various commercially available lipases. The highest conversion rates were observed with the *Candida rugosa* lipase, which discriminated against n-3 PUFA. The rate of hydrolysis was determined by HPLC. Digestive lipolytic enzymes isolated from salmon and rainbow trout intestines displayed reversed FA selectivity. Thus, astaxanthin diesters highly enriched with n-3 PUFA including EPA and DHA were observed to be hydrolyzed at a considerably higher rate than the more saturated esters. Similar trends in FA selectivity were observed in the hydrolysis of fish oil TAG by the digestive lipolytic enzyme mixtures.

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KEY WORDS: Astaxanthin, astaxanthin diesters, DHA, EPA, lipase, lipolytic enzymes, n-3 PUFA, rainbow trout, salmon.

Astaxanthins (3,3′-dihydroxy-β,β′-carotene-4,4′-diones; Fig. 1) are carotenoid pigments of the xanthophyll type consisting of eight isoprene units. Astaxanthins are abundant in nature and can be found in fish, crustaceans, and birds, as well as in certain algae, plants, fungi, and bacteria (1–3). They are synthesized *de novo* by algae and plants as well as bacteria and fungi. In algae, phytoplankton, and plants, carotenoids, including astaxanthins, play key roles in photosynthesis and photoprotection. Astaxanthin is responsible for much of the red coloring in the animal kingdom, e.g., in various crustaceans such as lobster, shrimp, and crab and in the spawning array and flesh of many fish including salmon and trout. Astaxanthin is of great significance for both the health and reproductive capability of these fish.

Astaxanthin is the major carotenoid of wild salmon and the most commonly used carotenoid for pigmentation in salmonid fish farming (4–6). The most important commercial source of astaxanthin is a synthetic material comprising a rather complicated mixture of astaxanthin geometrical and optical isomers (7). It is supplied in an unesterified form, which is the deposited form of the pigment in the fish muscle (5). Natural sources of astaxanthin, such as krill or algae, yield astaxanthin predominantly in the esterified form (8). Free astaxanthin is deposited in the flesh of salmonids after hydrolysis of these esters (5,9).

Astaxanthin is by far the most expensive feed ingredient on a weight basis in salmonid fish farming (10), and normally only 10–15% of the ingested astaxanthin can be recovered from the muscle (5,6). There are several reasons for this very low biological retention of commercial astaxanthin, including various rather poorly understood processes related to digestion and absorption (11). In addition, astaxanthin is very unstable with regard to exposure to air and elevated temperatures as well as light. The pigment is therefore to a great extent degraded during feed processing and storage. Clearly, even a small improvement in bioavailability could result in substantial savings for the salmonid aquaculture industry.

Astaxanthin as the diester is more stable than free astaxanthin (12). In the literature, dipalmitate is the predominant diester studied, and it is reported to give less pigmentation than the diol in both Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhyncus mykiss*) (5,9,13). This is explained by a low degree of hydrolysis of the diester in the fish intestines. However, in nature astaxanthin is often present as diester. Crustaceans are the major source of carotenoids for salmonids (9).

The main objectives of the study described in the current paper were to investigate (i) whether astaxanthin diesters were selectively hydrolyzed by a digestive lipolytic enzyme mixture isolated from salmonid intestines and (ii) whether such a possible selectivity could be reflected by astaxanthin retention in salmon muscle. The answer to the first question is provided by the results described in this report, whereas the second question has been addressed in a related patent publication (14).

EXPERIMENTAL PROCEDURES

Instrumentation. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AC 250 NMR spectrometer in deuterated chloroform as a solvent. FA analyses were performed on methyl esters employing a PerkinElmer 8140 gas chromatograph using a 30-m capillary column, DB-225 30N 0.25 mm (J&W Scientific, Folsom, CA) with hydrogen as a carrier gas according to our previously described procedure (15). Analytical HPLC was performed on a constaMetric 3200 solvent delivery system with spectroMonitor 3200 variable wavelength detector (470 nm), both from LDC Analytical using a Nucleosil 50-5 column (Art. 721210) from Macherey-Nagel (Easton, PA). Electronic spectra were obtained on an Ultrospec 4000 UV/vis spectrophotometer from Pharmacia Biotech.

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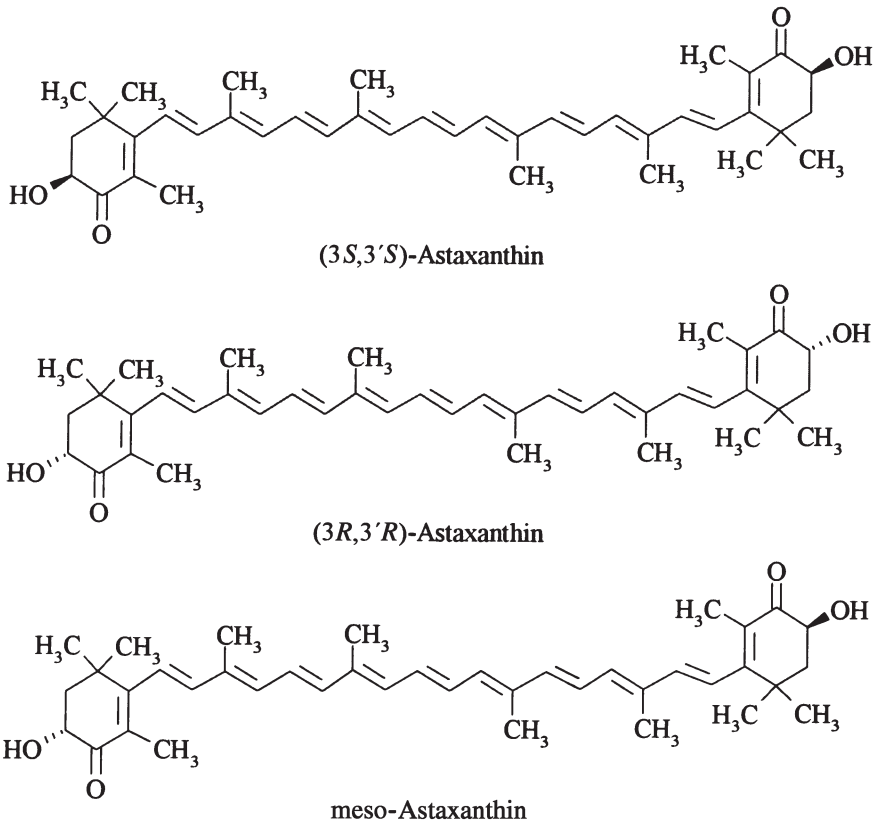


FIG. 1. Structures of the three stereoisomers of all-*E* astaxanthin: (3*S*,3′*S*), (3*R*,3′*R*), and meso.

Centrifugation was performed on a Sorval RC5C centrifuge and ultracentifugation on a Beckman L5-50E centrifuge. Agitation was performed on a VXR basic IKA-Vibrax unless otherwise stated.

Materials and solvents. The microbial lipases were supplied and employed as powder by Amano Enzyme Europe Ltd. (Milton Keynes, England). They include *Pseudomonas cepacia* (Lipase PS), *P. fluorescens* (Lipase AK), *Geotrichum candidum* (Lipase GC), *Penicillium roqueforti* (Lipase R), *Aspergillus niger* (Lipase A), *Candida rugosa* (Lipase AE), *Humicula lanuginosa* (Lipase CE), *Rhizopus delemar* (Lipase D), *R. oryzae* (Lipase F), *P. camembertii* (Lipase G), *C. lipolytica* (Lipase L), *Rhizomucor javanicus* (Amano M), and *R. niveus* lipase (Lipase N). Pig liver esterase was purchased from Sigma Chemical Company (St. Louis, MO). EPA (98%), DHA (>95%), astaxanthin dicaprate and dielaidate, and concentrates of various EPA/DHA composition, including 85% (50% EPA, 35% DHA), 55% (33% EPA, 22% DHA), and 30% (18% EPA, 12% DHA), were obtained as free acids from Norsk Hydro Research Centre (Porsgrunn, Norway). Reagent grade tris[hydroxymethyl]aminomethane (Trisma base, 99.9%) and palmitic acid (99%) were purchased from Sigma Chemicals. Silica gel (Silica gel 60), analytical TLC plates (DC Alufolien Kieselgel 60 F_{254}), 4-dimethylaminopyridine (DMAP, >99%) and hydrochloric acid (37% w/w) were obtained from Merck (Darmstadt, Germany), and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI, >98%) was obtained from Sigma-Aldrich Chemical Company (St. Louis, MO). They were all used without further purification. Solvents (*n*-hexane, dichloromethane, and acetone) were obtained from Acros Organics (Geel, Belgium) and were of analytical grade and used without further purification. Free astaxanthin, with an indicated extinction coefficient of 2070 in chloroform (1% solution, 1-cm path) at 492 nm, was obtained as a crystalline material from Alexis Biochemicals (San Diego, CA). The polyoxyethyleneglycol (PEG) emulsifier was supplied as Cremophor EL by BASF AG (Ludwigshafen, Germany). Herring oil was from Pronova Biocare AS (Sandefjord, Norway). Cod liver oil was obtained from Lysi hf (Reykjavik, Iceland). Farmed Atlantic salmon (*S. salar*) were obtained live (3–5 kg) from Islandslax hf (Grindavik, Iceland), and farmed rainbow trout (*O. mykiss*) were obtained live (1–1.5 kg) from Stofnfiskur hf (Hafnarfjordur, Iceland).

Preparation of astaxanthin diesters. The free astaxanthin starting material displayed the following NMR spectrum: ^{1}H NMR δ 6.70–6.61 (*m*, 4H, =CH), 6.48–6.18 (*m*, 10H, =CH), 4.33 (*dd*, *J* = 5.6 Hz, *J* = 13.8 Hz, 2H, C*H*OH), 3.63 (*br s,* 2H, OH), 2.16 (*dd*, 5.6 Hz, *J* = 12.7 Hz, 2H, CH_2CHOH), 2.00 (*s*, 6H, CH_3C=CH), 1.99 (*s*, 6H, CH_3C=CH), 1.95 (*s*, 6H, =C(CH_3)CO), 1.81 (*dd*, *J* = 12.7 Hz, *J* = 13.8 Hz, 2H, CH_2CHOH), 1.32 (*s*, 6H, C(CH_3)$_2$), and 1.21 (*s*, 6H, C(CH_3)$_2$) ppm.

Astaxanthin dipalmitate. To a mixture of pure astaxanthin (251 mg, 0.421 mmol) and palmitic acid (220 mg, 0.858

mmol) in dichloromethane (5 mL) was added DMAP (100 mg, 0.820 mmol) and EDCI (200 mg, 1.043 mmol), and the resulting solution was stirred on a magnetic stirrer at room temperature (r.t.) for 24 h. The reaction was monitored by TLC using 95:5 (vol/vol) dichloromethane/acetone as an eluent (diol: R_f 0.0–0.1, monoester: R_f 0.3–0.5, diester: R_f 0.8–0.9). The solvent was evaporated off *in vacuo* on a rotary evaporator and the crude reaction mixture run through a short silica gel column using dichloromethane as an eluent. Pure palmitate diesters were obtained as a deep red-purple powder (415 mg, 92% yield). ^{1}H NMR δ 6.71–6.60 (*m*, 4H, =CH), 6.48–6.17 (*m*, 10H, =CH), 5.53 (*dd*, *J* = 6.2 Hz, *J* = 13.0 Hz, 2H, CHOCO), 2.54–2.33 (*m*, 4H, CH_2COO), 2.18–1.94 (*m*, 4H, CH_2CHOCO), 2.00 (*s*, 6H, CH_3C=CH), 1.99 (*s*, 6H, CH_3C=CH), 1.90 (*s*, 6H, =C(CH_3)CO), 1.76–1.64 (*m*, 4H, CH_2CH_2COO), 1.38–1.20 (*m*, 48H, CH_2), 1.35 (*s*, 6H, C$(CH_3)_2$), 1.22 (*s*, 6H, C$(CH_3)_2$), and 0.88 (*t*, 6H, *J* = 6.6 Hz, C$H_3$$CH_2$) ppm.

Astaxanthin dieicosapentaenoate. An identical procedure was used as described for the palmitate adduct above using astaxanthin (250 mg, 0.419 mmol) and EPA as free acid (259 mg, 0.855 mmol) in dichloromethane (5 mL), DMAP (102 mg, 0.835 mmol) and EDCI (197 mg, 1.028 mmol). Pure EPA diesters were obtained as a deep red-purple syrupy material (449 mg, 92% yield). ^{1}H NMR δ 6.70–6.60 (*m*, 4H, =CH in astax.), 6.47–6.16 (*m*, 10H, =CH in astax.), 5.53 (*dd*, *J* = 6.2 Hz, *J* = 13.1 Hz, 2H, C*H*OCO), 5.43–5.28 (*m*, 20H, =CH in EPA), 2.86–2.78 (*m*, 16H, =CCH_2C=), 2.52–2.37 (*m*, 4H, CH_2COO), 2.20–1.94 (*m*, 12H, CH_2CH_2C=, CH_3CH_2C=, and CH_2CHOCO), 1.99 (*s*, 6H, CH_3C=CH), 1.98 (*s*, 6H, CH_3C=CH), 1.89 (*s*, 6H, =C(CH_3)CO), 1.83–1.72 (*m*, 4H, C$H_2$$CH_2$COO), 1.34 (*s*, 6H, C$(CH_3)_2$), 1.21 (*s*, 6H, C$(CH_3)_2$), and 0.97 (*t*, *J* = 7.5 Hz, 6H, C$H_3$$CH_2$C=) ppm.

Astaxanthin didocosahexaenoate. An identical procedure was used as described for the palmitate adduct above using astaxanthin (250 mg, 0.419 mmol) and DHA as free acid (282 mg, 0.858 mmol) in dichloromethane (5 mL), DMAP (103 mg, 0.840 mmol), and EDCI (200 mg, 1.043 mmol). Pure DHA diesters were obtained as a deep red-purple syrupy material (464 mg, 91% yield). ^{1}H NMR δ 6.70–6.61 (*m*, 4H, =CH in astax.), 6.47–6.17 (*m*, 10H, =CH in astax.), 5.53 (*dd*, *J* = 6.2 Hz, *J* = 13.0 Hz, 2H, CHOCO), 5.44–5.32 (*m*, 24H, =CH in DHA), 2.87–2.79 (*m*, 20H, =CCH_2C=), 2.52–2.43 (*m*, 8H, CH_2CH_2COO), 2.18–1.95 (*m*, 8H, $CH_3$$CH_2$C= and C$H_2$CHOCO), 2.00 (*s*, 6H, C$H_3$C=CH), 1.99 (*s*, 6H, C$H_3$C=CH), 1.90 (*s*, 6H, =C(CH_3)CO), 1.35 (*s*, 6H, C$(CH_3)_2$), 1.22 (*s*, 6H, C$(CH_3)_2$), and 0.97 (*t*, *J* = 7.5 Hz, 6H, C$H_3$$CH_2$C=) ppm.

Astaxanthin diesters of 85% EPA + DHA concentrate. An identical procedure was used as described for the DHA adduct above using astaxanthin (251 mg, 0.421 mmol) and the 85% EPA + DHA concentrate as free acids (264 mg, approximately 0.860 mmol) in dichloromethane (5 mL), DMAP (100 mg, 0.818 mmol), and EDCI (198 mg, 1.033 mmol). The 85% EPA + DHA diesters were obtained as a deep red-purple syrupy material (455 mg, 92% yield).

Astaxanthin diesters of 55% EPA + DHA concentrate. An identical procedure was used as described for the DHA adduct above using astaxanthin (250 mg, 0.419 mmol) and the 55% EPA + DHA concentrate as free acids (259 mg, approximately 0.863 mmol) in dichloromethane (5 mL), DMAP (101 mg, 0.823 mmol), and EDCI (199 mg, 1.038 mmol). The 55% EPA + DHA diesters were obtained as a deep red-purple syrupy material (457 mg, 94% yield).

Astaxanthin diesters of 30% EPA + DHA concentrate. An identical procedure was used as described for the DHA adduct above using astaxanthin (252 mg, 0.422 mmol) and the 30% EPA + DHA concentrate as free acids (251 mg, approximately 0.865 mmol) in dichloromethane (5 mL), DMAP (102 mg, 0.835 mmol), and EDCI (198 mg, 1.033 mmol). The 30% EPA + DHA diesters were obtained as a deep red-purple syrupy material (453 mg, 94% yield).

Astaxanthin diester stock solution. A Tris buffer (0.25 M) solution was prepared by dissolving Tris (4.54 g, 37.5 mmol) in 150 mL distilled water. The pH of the buffer solution was adjusted to 8.0 by adding 2 M hydrochloric acid. All the hydrolysis reactions were performed at r.t. so no special effort to adjust the pH was necessary. The astaxanthin diester was weighed accurately (approximately 40 mg, 33 mmol) along with Chremophor EL emulsifier (2.5 g) into a 100-mL Erlenmeyer flask. The solution was stirred vigorously for 20 min (until homogeneous), and then 20 mL of the Tris buffer was added and the mixture was stirred for an additional 10 min. The solution was suction-filtered, and the red filtrate was poured into a 25-mL volumetric flask and diluted to the mark with buffer. The concentration of all the solutions was determined spectrophotometrically (492 nm) to be about 1 mg/mL. All the solutions were stored at 4°C under nitrogen and protected from light.

Isolation of the lipolytic enzyme preparation. Fresh salmon and rainbow trout that had been fed in the last 12 h were obtained live from local fish farms. The fresh fish was cut open at the laboratory and the crude enzyme mixture extracted as follows: Each of the blind-ending tubes (*pyloric caecae*) originating from the *pyloric* area of the stomach were cut off, and the intestinal juice was squeezed out by hand into a cooled container. The viscous solution (approximately 50 mL) was diluted to 100 mL with 0.25 M Tris buffer, pH 8.0, stirred for 30 min in an ice bath and centrifuged at 12,000 × *g* (rotor GSA) for 20 min at 4°C. The aqueous layer was removed to another centrifuge tube and the centrifugation repeated at 25,000 × *g* (rotor SS-34) for 30 min at 4°C. The aqueous layer was transferred to an Erlenmeyer flask and quickly frozen by liquid nitrogen. In some instances, before use the mixture was thawed and purified by ultracentrifugation at 100,000 × *g* (rotor SW27) for 45 min at 4°C. The resulting enzyme preparation was a clear yellow solution.

Hydrolysis of astaxanthin diesters. Into a 10-mL round-bottomed flask was added 2 mL of the astaxanthin diester stock solution and 3 mL of lipolytic enzyme mixture from fish intestine. The flask was flushed with nitrogen before closing and then wrapped in aluminum foil, and the reaction mixture

was stirred on a magnetic stirrer at r.t. for 48 h. When finished, all the water was removed under reduced pressure (0.01 Torr) at r.t. and the residue redissolved in dichloromethane. The solution was filtered through a cotton wool plug in a Pasteur pipette and stored under nitrogen in a closed container protected from light in a freezer. Analytical TLC was used to monitor the progress of the reaction with 95:5 (vol/vol) dichloromethane/acetone as an eluent. To determine the degree of hydrolysis, samples were subjected to analytical HPLC using 70:30 (vol/vol) *n*-hexane/acetone as an eluent (flow rate: 0.15 mL/min; detector: 470 nm. Approximate retention times: astaxanthin diesters: 9 min; astaxanthin monoesters: 12 min; astaxanthin diols: 22 min.)

Astaxanthin diester hydrolysis studies by pig liver esterase and microbial lipase. Lipase or pig liver esterase (20 mg) was placed in a screw-capped reaction vessel together with astaxanthin dielaidate stock solution (2 mL) under a nitrogen atmosphere. A glass rod was used to rub lumps of enzyme into solution. The reaction vessel was wrapped in aluminum foil to protect the mixture from light and then placed on a mechanical stirrer (IKA-Vibrax-VXR); the reaction was allowed to proceed for 48 h at r.t. The workup was identical to that in the hydrolysis reaction with the lipolytic salmonoid enzyme mixture described above.

RESULTS AND DISCUSSION

The bioavailability of astaxanthin for salmon and trout in aquaculture is a complicated issue that relates to both stability and various poorly understood digestion and absorption processes (11). In nature, astaxanthin is commonly present as diesters, and esterification is known to increase stability (12). However, astaxanthin dipalmitates are largely inferior to free astaxanthins in terms of pigment retention in salmon and trout muscle (5,9,13). This is believed to relate to a lower degree of hydrolysis of such diesters in the fish intestines. It was anticipated that the FA composition of astaxanthin diesters might influence the degree of hydrolysis in the fish intestines as well as their stability and bioavailability.

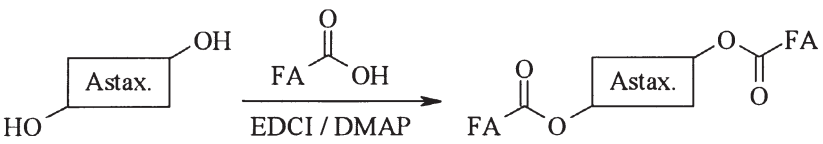
The main objective of the work described in this paper was to study a possible FA selectivity of digestive lipolytic enzymes isolated from salmon and rainbow trout intestines toward astaxanthin diesters of various FA compositions. For this purpose, a lipolytic enzyme mixture from salmonid intestines was required, and its activity had to be established *in vitro* on astaxanthin diesters. Astaxanthin diesters of various FA compositions, including n-3 PUFA, also had to be prepared.

The most important commercial source of astaxanthin is Carophyll Pink from Hoffmann-La Roche (Basel, Switzerland). This synthetic material contains approximately 75% of the natural all-*E* isomer, roughly as a 50:50 mixture of racemic optically inactive enantiomers and meso-astaxanthins (Fig. 1). This means approximately 1:1:2 of the (*R*,*R*′)- and (*S*,*S*′)-enantiomers and the meso (*R*,*S*)-diastereomer, respectively. The remaining 25% consists of *Z*-geometrical isomers, of which the 9*Z*, 13*Z*, and 15*Z* isomers are the most prominent (7). In the current studies, a synthetic astaxanthin from Alexis Biochemicals (San Diego, CA) was used. It is virtually free of any *Z*-geometrical isomers and contains a similar ratio of the all-*E* isomers as the Carophyll Pink astaxanthin material.

Lipases are known to tolerate a wide range of primary and secondary alcohols as substrates (16), and astaxanthins are among substrates reported to be tolerated by lipase (17). The screening of numerous commercially available microbial lipases and pig liver esterase was necessary for numerous reasons: first, to find and use a well-defined esterase or microbial lipase to serve as a model to set the methodology for the fish intestinal lipolytic enzyme mixture, and second, to provide a comparison between such an esterase or lipase, once found active, and the fish-based lipolytic enzymes in their astaxanthin ester hydrolysis in terms of FA selectivity. Finally, the results with such a lipase or esterase could possibly provide good evidence together with enzyme-free reactions, that the results with the fish enzymes were not an artifact related to the instability of astaxanthin diesters highly enriched with the labile EPA and DHA.

Preparation of astaxanthin diesters. In the studies described in this paper, astaxanthin from Alexis Biochemicals was used. From that material astaxanthin diesters were prepared by chemical esterification with capric, palmitic, and elaidic acids, concentrates of varying n-3 PUFA content, and virtually pure EPA and DHA. The concentrates include 30% EPA + DHA (18% EPA, 12% DHA), 55% EPA + DHA (33% EPA, 22% DHA), and 85% EPA + DHA (50% EPA, 35% DHA), the last one containing greater than 90% total n-3 PUFA content. The reaction is schematically outlined in Scheme 1.

The reactions were conducted in dichloromethane at r.t. using EDCI as a coupling agent in the presence of DMAP. EDCI is a coupling agent similar to dicyclohexylcarbodiimide but far superior when n-3 PUFA are involved (18). Its polarity and water solubility render it quite an advantage in terms of workup of the reaction and purification of the products. DMAP is presumed to serve both as a base and a catalyst for the acylation process. The same procedure was previously



SCHEME 1

used successfully to introduce pure EPA and DHA into the mid-position of 1,3-DAG (18,19).

The diesters were obtained in virtually quantitative yields and isolated in well over 90% yields after purification by silica gel chromatography. High-field ^{1}H NMR spectroscopy was used to evaluate the high degree of purity of the products. Although the astaxanthin comprised a mixture of stereoisomers, the protons located α to the carbonyl group of the cyclohexenone moiety, on the carbon possessing the hydroxyl group, resonated as a regular distinct doublet of doublets at δ 4.43 ppm. Upon acylation, a dramatic downfield shift of these protons took place to δ 5.53 ppm. This, together with a complete disappearance of the hydroxyl group proton signal, was good evidence for the formation and high purity of the astaxanthin diacyl adducts. The remaining regions of the spectra were also in excellent agreement with the postulated introduction and identity of two acyl group equivalents into the astaxanthin molecule.

Hydrolysis reactions. The astaxanthin diester hydrolysis reactions (Scheme 2) were conducted at r.t. in a Tris buffer at pH 8 using a PEG emulsifier supplied as Cremophor EL by BASF. HPLC possessing a variable wavelength detector fixed at 470 nm was used to analyze the product mixture in terms of unreacted diesters, monoesters, and free astaxanthins (diols), from which the extent of conversion was determined. The percentage conversion (%conv.) was defined and calculated according to the following equation:

$$\%\text{conv.} = \%\text{diols} + \frac{\%\text{monoesters}}{2} \qquad [1]$$

The percentage was based on area percentage on HPLC and represents molar percentage. Since the astaxanthin deposited into fish muscle is free astaxanthin, we were particularly interested in the diol content of these reactions, especially when it came to the fish-derived enzymes.

Screening of enzymes. The hydrolytic activities of numerous commercially available lipases (listed in the Experimental Procedures section) and pig liver esterase toward astaxanthin diesters of elaidic acid were determined. Analytical TLC on silica gel was used to follow the progress of the hydrolysis reactions.

Less than half of the enzymes examined displayed some activity toward the diesters, mostly generating monoesters. Active enzymes included both *Pseudomonas* lipases tested, and the *G. candidum*, *C. rugosa*, *Rhizopus delemar*, *R. oryzae*, and *P. roqueforti* lipases. The highest conversion rate was observed for the *C. rugosa* lipase, which also was the only lipase to produce diols in substantial amounts. Therefore, that lipase became an obvious choice for further hydrolysis studies.

Astaxanthin diester hydrolysis by C. rugosa *lipase.* Astaxanthin diester hydrolysis by *C. rugosa* lipase was conducted on diesters of capric, palmitic, and elaidic acids, and the 85% EPA + DHA concentrate. The reactions were followed by analytical TLC as before. They were discontinued after 42 h and analyzed by analytical HPLC. The results are presented in Table 1.

The *Candida* lipase clearly displayed preference for the saturated and less unsaturated fatty adducts as compared to the n-3 PUFA concentrate, as had been anticipated. That lipase has previously been shown to discriminate against n-3 PUFA in fish oil, DHA in particular (20,21). By far the highest degree of hydrolysis (75%) was observed with the elaidate esters. It is interesting to notice the low content of monoesters in that case, much lower than for the other esters.

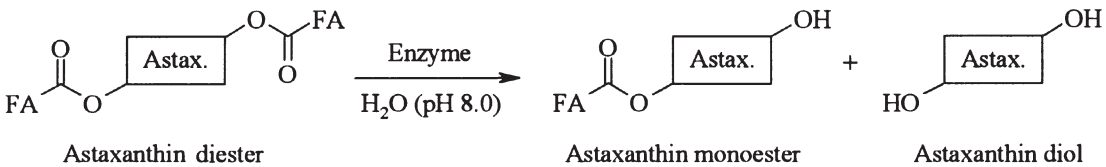
Preliminary astaxanthin diester hydrolysis by salmon intestine lipolytic enzymes. When hydrolysis studies similar to those described above for the *Candida* lipase were conducted on an enzyme mixture isolated from salmon intestines, a reversed behavior was clearly observed in terms of FA selectivity (Table 2). This time the highest activity was observed for the n-3 PUFA concentrate: 41% conversion after 45 h. This compares to 14% for the caprate esters and only 4 and 6% for the palmitate and elaidate esters, respectively. It is noteworthy that a substantial amount of free astaxanthin was produced from the n-3 PUFA astaxanthin diesters.

Astaxanthin diester hydrolysis by salmon and rainbow trout intestine lipolytic enzymes. For better insight into the FA selectivity of the crude enzyme mixture from salmon intestines, astaxanthin diesters prepared from FA mixtures containing various levels of EPA and DHA ranging from 30% EPA + DHA to pure EPA and DHA were investigated. A fresh enzyme mixture from freshly killed salmon was used. The palmitate diester was included for comparison. The results are presented in Table 3. It is noteworthy that the conversion levels were higher than those obtained in the previous experiment (Table 2). This time, however, the diol levels were significantly lower than the monoester

TABLE 1
Astaxanthin Diester Hydrolysis by *Candida rugosa* Lipase (42 h)[a]

Substrate	Conv. (%)	Diesters (%)	Monoesters (%)	Diols (%)
10:0	48	34	36	30
16:0	50	41	19	40
18:1	75	24	3	73
K85	40	39	43	18

[a]Molar percentages of the astaxanthin diesters, monoesters, and diols were determined by HPLC using detection at 470 nm. Conv., conversion calculated as the sum of astaxanthin diols and half of the astaxanthin monoesters; 10:0, capric acid; 16:0, palmitic acid; 18:1 elaidic acid (*trans*-18:1); K85, 85% EPA + DHA in approximately 3:2 ratio, respectively.



Astaxanthin diester — Astaxanthin monoester — Astaxanthin diol

SCHEME 2

TABLE 2
Astaxanthin Diester Hydrolysis by Salmon Intestinal Lipolytic Enzymes (45 h)[a]

Substrate	Conv. (%)	Diesters (%)	Monoesters (%)	Diols (%)
10:0	14	78	16	6
16:0	4	94	5	1
18:1	6	90	9	1
K85	41	43	31	25

[a]See Table 1 for abbreviations.

levels, using K85 as substrate. The difference in performance between the two extracts presumably relates to an extra purification step by ultracentrifugation as described above in "Isolation of the lipolytic enzyme preparation," resulting in higher purity as compared to the previous experiment. This may indicate that more than a single hydrolytic enzyme was responsible for the hydrolytic activity, possibly lipases, esterases, or an astaxanthin esterase. This requires further studies, but the trends clearly show a correlation between enzymatic activity and n-3 PUFA content of the diester substrate, with the 85% EPA + DHA diester and pure EPA diester offering the highest conversion.

Similar results were obtained for the rainbow trout enzyme mixture (Table 4). Significantly higher degrees of hydrolysis were obtained for all astaxanthin diesters as compared to the salmon enzymes. Again, the 85% EPA + DHA concentrate diesters displayed the highest activity and conversion (57%) as well as the highest amount of free astaxanthins (27%). From these results it is evident that astaxanthin diesters containing high levels of EPA and DHA are preferred to diesters of saturated and less unsaturated FA as substrates for the trout enzyme mixture.

No attempts were made to look into the possible selectivity of the enzymes toward individual stereoisomers of astaxanthin diesters and their possible enantiopreference. This is under investigation, but the results observed in this study uniformly indicate that the enzymes prefer the n-3 PUFA-enriched astaxanthin esters as substrates irrespective of their enantiopreference and stereoisomeric composition of the astaxanthins. The stability of the free astaxanthins and their highly enriched EPA and DHA esters during the hydrolysis reactions was of concern, although no attempts were made to evaluate their oxidative stability. The hydrolysis experiments were performed with great care, with the reaction vessels protected from light by aluminum foil, and all experiments were conducted under a nitrogen atmosphere. There was no apparent deterioration in color intensity of the astaxanthin solutions during the experiments and not much reason to believe that oxidation was taking place during the reactions to any harmful extent.

TABLE 3
Astaxanthin Diester Hydrolysis by Salmon Intestinal Lipolytic Enzymes (48 h)[a]

Substrate	Conv. (%)	Diesters (%)	Monoesters (%)	Diols (%)
16:0	10	81	19	0
K30	21	61	36	3
K55	32	42	52	6
K85	36	37	54	9
EPA	40	27	67	6
DHA	30	44	53	3

[a]K30, K55, and K85 are n-PUFA concentrates comprising 30, 55, and 85% EPA + DHA, respectively, in approximately 3:2 ratio. See Table 1 for other abbreviations.

TABLE 4
Astaxanthin Diester Hydrolysis by Rainbow Trout Intestine Lipolytic Enzymes (48 h)[a]

Substrate	Conv. (%)	Diesters (%)	Monoesters (%)	Diols (%)
16:0	20	71	18	11
K30	40	37	46	17
K55	49	25	52	23
K85	57	14	59	27
EPA	54	17	58	25
DHA	52	21	55	24

[a]See Tables 1 and 3 for abbreviations.

Fish oil hydrolysis by salmon and rainbow trout intestine lipolytic enzymes. We were also interested in investigating whether similar trends in FA selectivity of the salmon and trout lipolytic enzymes were displayed in the hydrolysis of fish oil TAG. When herring oil was incubated with the crude enzyme mixture from salmon intestines under similar conditions as those described for the astaxanthin diesters, approximately 10% conversion was obtained after 72 h. The hydrolytic enzymes clearly preferred the long-chain n-3 PUFA as substrates over saturated and less unsaturated FA, unlike commercially available lipases (22).

Similar results were obtained for the hydrolysis of cod liver oil by the crude enzyme mixture from rainbow trout intestines. Much higher hydrolysis (25%) was observed under identical conditions. As before, the enzyme mixture displayed a strong preference for EPA and DHA and other n-3 PUFA as compared to the saturated and less unsaturated FA (23). A question remains as to whether the hydrolytic activity involving both astaxanthin diesters and the fish oil TAG is solely related to lipase or to both lipase and esterase. Whatever the answer to this question, similar trends in FA selectivity were displayed in both cases. A similar FA selectivity favoring n-3 PUFA was reported by Lie and Lambertsen (24) in their studies on fish oil TAG hydrolysis using a crude hydrolytic enzyme mixture from cod intestines. Finally, a bile-salt-dependent lipase from cod intestines was observed by Gellesvik (25) to display a similar preference for long-chain n-3 PUFA up to 22 carbons in length.

Feeding experiments on salmon. In vivo studies based on the results described in this report were conducted by Norsk Hydro in Norway on farmed salmon and revealed that astaxanthin deposition in fish muscle was considerably higher when using astaxanthin diesters highly enriched with n-3 PUFA as compared to free astaxanthin (14). For the astaxanthin diesters prepared from 85% EPA + DHA and greater than 90% total n-3 PUFA, 19.7% of injected astaxanthin was deposited into fish muscle. This compares to 13.0% for fish receiving free astaxanthin, corresponding to a 50% increase in astaxanthin bioavailability. This appears to be related to the preferential activity of the hydrolytic enzymes (esterase or li-

pase) in the fish intestines for such diesters. Such an *in vivo* hydrolysis appears to increase astaxanthin bioavailability in the fish as compared to the feeding of free astaxanthin.

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