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ORIGINAL PAPER

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The effect of bio-converted polyunsaturated fatty acids on the oxidation of TAG containing highly unsaturated fatty acids

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Abstract Microbial modification of vegetable fatty acids can often lead to special changes in their structure and in biological function. A bacterial strain, *Pseudomonas aeruginosa* PR3, is known to carry out multiple hydroxylations on polyunsaturated fatty acids containing 1,4-*cis, cis* diene structural units, resulting in antibacterial activity. In this paper, in an effort to understand the overall mechanism involved in the varied biological functions of the complicated metabolites of bio-converted polyunsaturated fatty acids, we performed bioconversion of several polyunsaturated fatty acids using PR3, and determined their oxidative activities against fish oil. Bio-converted linoleic acid, eicosapentanoic acid, and docosahexanoic acid promoted effectively oxidation of fish oil. It is assumed that this oxidative effect could plausibly play an important role in the antimicrobial function of these bio-converted fatty acids.

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Introduction

Microbial modification of vegetable fatty acids can often leads to specific changes in their structure and biological function. Hydroxylation of fatty acids by bacterial strains is one such modification. Hydroxy fatty acids are considered to be important industrial materials because the hydroxyl group on a fatty acid gives it special properties such as higher viscosity and reactivity, compared with other non-hydroxylated fatty acids [1–3]. Of the hydroxy fatty acids reported, 9,10,11-trihydroxy-12-octadecenoic acid, 9,10,13-trihydroxy-11(*E*)-octadecenoic acid, and 9,12,13-trihydroxy-10(*E*)-octadecenoic acid have gained special attention because they were isolated from plants, and have strong anti-fungal activity [4–6]. There are other reports about the production of biologically active trihydroxy fatty acids from various sources [7, 8].

According to the reports describing metabolic pathways involved in the conversion of linoleic acid (LA) to trihydroxy fatty acids, several intermediate reaction products are involved, such as trihydroxy-, hydroperoxy-, dihydroxy-, and hydroxyepoxy-octadecenoate [4, 5, 9]. Those metabolites of LA showed distinct biological functions according to their intermediate structures which include mono-, di-, trihydroxy-octadecenoic acid, and hydroperoxy-, epoxy-forms [4, 10–12]. Until now, however, mechanisms by which those oxidized or hydroxy lipids exert antimicrobial and/or anticancer activities have not been elucidated.

Previously, we reported that a bacterial strain, *Pseudomonas aeruginosa* PR3, was able to convert oleic acid to mono- and di-hydroxy fatty acids [13–15] and LA to trihydroxy fatty acids [16]. In this paper, in an effort to understand the overall mechanism involved in the varied biological functions of the complicated reaction metabolites of bio-converted polyunsaturated fatty

acids, we studied the oxidative activities, against fish oil, of crude extracts produced by PR3 from several polyunsaturated fatty acids.

Materials and methods

Chemicals

Linoleic acid (18:2*n*−6), eicosapentanoic acid (EPA, 20:5*n*−3), and docosahexanoic acid (DHA, 22:6*n*−3) with 99^{+}% purity by gas chromatography (GC) were purchased from NU-Check-Prep Inc. (Elysian, MN, USA). Conjugated linoleic acid (CLA, 98% purity by GC) was kindly provided by Dr. In-Hwan Kim of Korea University, Korea. A mixture of trimethylsilylimidazole (TMSI) and pyridine (1:4 v/v) was purchased from Supelco Inc. (Bellefonte, PA, USA). All other chemicals were of reagent grade and were used without further purification. Other chemicals were purchased from Sigma unless indicated otherwise.

Microorganism and bioconversion

Pseudomonas aeruginosa NRRL strain B-18602 (PR3) was kindly provided by Dr. Hou of USDA/NCAUR. The strain was grown at 28 °C aerobically in a 125 ml Erlenmeyer flask containing 50 ml of standard medium with shaking at 200 rpm. The standard medium used contained (per liter) 4 g dextrose, 2 g K_2HPO_4, 2 g $(NH_4)_2HPO_4$, 1 g NH_4NO_3, 0.5 g yeast extract, 0.014 g $ZnSO_4$, 0.01 g $FeSO_4{\cdot}7H_2O$, and 0.01 g $MnSO_4{\cdot}7H_2O$. The medium was adjusted to pH 7.0 with diluted phosphoric acid. For the production of bio-converted fatty acids, fatty acid substrates (0.5 g) were added to a 24 h-old culture followed by continued incubation for an additional 72 h. At the end of cultivation, the culture broth was acidified to pH 2 with 6 N HCl followed by immediate extraction twice with an equal volume of ethyl acetate and diethyl ether. The solvent was evaporated from the combined extracts using a rotary evaporator.

Analysis of products

The isolated reaction products were analyzed by thin-layer chromatography (TLC) and GC. The TLC plates were developed using a solvent system consisting of toluene:dioxane:acetic acid (79:14:7 v/v/v). The spots were visualized first by iodine vapor and then by spraying the plates with 50% sulfuric acid and heating in a 100 °C oven for 10 min. For GC analysis, the samples were first methylated with diazomethane for 5 min at room temperature followed by derivatization with a mixture of TMSI and pyridine (1:4 v/v) for at least 20 min at room temperature. The TMSI-derivatized sample was analyzed using a Shimadzu GC-17A (Shimadzu Seisakusho, Kyoto, Japan) equipped with a flame-ionization detector and a capillary column [SPB-1^{TM}, 15 m×0.32 mm i.d., 0.25 μm thickness (Supelco Inc., Bellefonte, PA, USA)]. GC was run with a temperature gradient of 20 °C/min from 70 to 200 °C, holding 1 min at 200 °C, and then 0.7 °C/min to 240 °C, followed by holding for 15 min at 240 °C (nitrogen flow rate = 0.67 ml/min). Injector and detector temperatures were held at 250 and 270 °C, respectively. The values presented in each experiment are the averages of duplicates. The error range was within 10% of average value.

Preparation of triacylglycerol

Sardine oil was obtained from Nippon Chemical Feed Ltd., Hakodate, Japan. The oils were passed through a column packed with a 1:1 *n*-hexane slurry mixture (w/w) of activated carbon and Celite 545 to remove tocopherols and pigments by eluting with *n*-hexane. The recovered oil was refined on a silicic acid column by eluting with *n*-hexane and a mixture of diethyl ether/*n*-hexane solution (5:95, 10:90, and 20:80 (200 ml), v/v). TAG fraction eluted with diethyl ether/*n*-hexane (10:90) showing a single spot on TLC analysis was used for the present study as fish oil-TAG. Fatty acid profiles of substrate oils were determined by GC after methylation using the sodium methoxide method. GC analysis was performed on a Shimadzu GC-14B equipped with a flame-ionization detector and a capillary column [Omegawax 320 (30 m×0.32 mm i.d.); Supelco, Bellefonte, PA, USA] at column temperature of 200 °C. The injector and detector were held at 250 and 260 °C, respectively. Helium was used as carrier gas and its flow rate was 50 kpa. Component peaks were identified by comparison with standard fatty acid methyl esters.

Oxidation and analysis

Triacylglycerol (100 mg) with or without 1% of the bio-converted fatty acid was put in a 10 ml aluminum sealed vial with a butyl-gum septum and incubated at 40 °C in the dark. Before incubation, the level of oxygen in the headspace gas of the vial was estimated by a GC (Shimadzu GC-14B). The GC was equipped with a thermal conductivity detector and a stainless steel column (3 m×3.0 mm i.d.) packed with molecular Sieve 5A (GL Science, Tokyo, Japan). The temperatures at the injection port, detector port and column oven were 100, 100 and 50 °C, respectively. The helium flow was 50 kpa. A small portion (20 μl) of the headspace gas was taken using a microsyringe at selected times of oxidation and the concentration of residual oxygen in the headspace gas of the vial was estimated by the GC method. The decrease (%) in oxygen was calculated from changes in the oxygen ratio to nitrogen as compared with that

before incubation. Each sample in the three separate vials was subjected to oxidation. For each determination there was little difference in the oxidation rate and the order of the oxidative stability of different samples was unchanged.

Results and discussion

Bioconversion of polyunsaturated fatty acids by PR3

Formation of oxygenated fatty acids, collectively named oxylipins, is observed in the plant's response to pathogen attack and as compounds acting against microbial pathogens [17]. Oxylipin formation occurs either by autoxidation or by the action of enzymes such as lipoxygenase and dioxygenase. The key unit of the substrate for the non-heme iron containing lipoxygenase is known to be a 1,4-*cis, cis*-diene unit which is present in the structure of LA [18]. This unique structural unit is also found in EPA and DHA. Based on this information, we tried to bio-convert EPA, DHA, LA, and CLA using a bacterial strain, *P. aeruginosa* PR3, which is known to generate hydroxyl groups on unsaturated fatty acids [16]. All fatty acid substrates tested, except CLA, showed complicated profiles of the bio-converted products by TLC analysis (Fig. 1). Bio-converted LA produced a smeared band in the middle-upper area of the TLC plate while EPA and DHA produced smeared bands in the lower part of the plate, suggesting that bio-converted products from EPA and DHA were relatively more polar than those from LA. However, CLA carrying one *cis* and one *trans* double bond (mixture of *c*9, *t*11; 43% and *t*10, *c*12; 55%) in the fatty acid chain did not produce any particular new bands in the TLC plate. GC analysis of all bio-converted fatty acids (except for CLA) presented complicated peak profiles containing more than 20 peaks (data not shown). These results indicated that not only LA but also EPA and DHA were successfully utilized by PR3 to produce complicated new bio-converted products and that the 1,4-*cis, cis*-diene structural unit was required for this event.

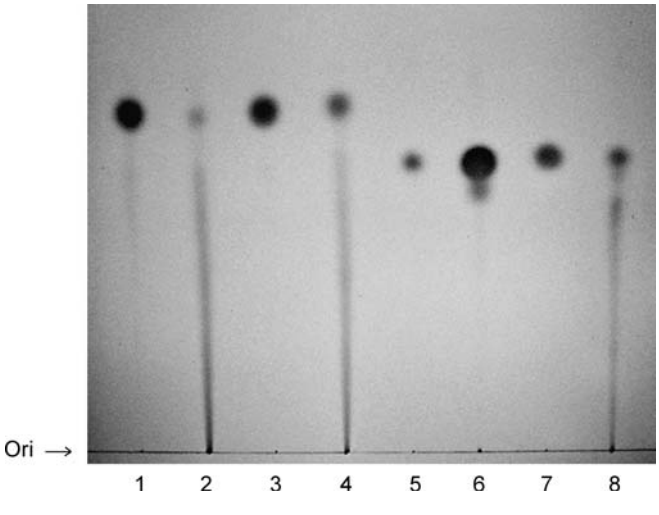

Fig. 1 Thin layer chromatography analysis of products from the bioconversion of fatty acids by PR3. *Lane numbers* represent standard DHA, bio-converted DHA, standard EPA, bio-converted EPA, standard CLA, bio-converted CLA, standard LA, and bio-converted LA. The solvent system consisted of toluene:dioxane:acetic acid = 79:14:7(v/v/v). Spots were visualized with iodine vapor

Effect of bio-converted polyunsaturated fatty acids on the oxidation of fish oil-TAG

Changes in the physical state of membranes can occur by variation in lipid types and classes of phospholipids resulting in significant impacts on not only membrane fluidity but also function of membrane proteins. Possible results of fatty acid oxidation can include changes in reactivity and three-dimensional structures since, in many cases, the oxidation of polyunsaturated fatty acids caused generation of hydroxyl or hydroperoxyl groups on the chain, loss of double bonds, and conformational change of *cis*-configuration into *trans*-form [13–16]. Among the possible changes in reactivity resulted from the oxidation of fatty acids, their oxidative capability against other lipids can be considered as important factor in the biological systems since biological cells are bounded by lipid membranes. Thus, we investigated the effect of bio-converted polyunsaturated fatty acids on the oxidation of fish oil containing a relatively high amount of polyunsaturated fatty acids (Table 1), which are often found in the phospholipids of biological membranes. The oxidation effect of bio-converted fatty acids was determined by measuring the residual oxygen concentration level in the reaction vials after the incubation of fish oil, with and without crude extracts of bio-converted fatty acids (see Materials and methods).

Fish oil, when incubated alone, exhibited autoxidation as seen by an increase in oxygen consumption. The

Table 1 Fatty acid profiles of soybean and fish oil

Fatty acid	Soybean oil (mol%)	Fish oil (mol%)
14:0	–	7.0
16:0	10.3	13.2
16:1*n*−7	–	13.3
16:2*n*−4	–	2.3
16:3*n*−4	–	2.3
18:0	3.2	1.9
18:1*n*−9	24.7	11.5
18:1*n*−7	–	3.5
18:2*n*−6	56.1	4.3
18:3*n*−3	5.1	1.1
18:4*n*−3	–	2.9
20:1*n*−9	–	1.3
20:4*n*−6	–	1.5
20:5*n*−3	–	14.5
22:5*n*−3	–	1.2
22:6*n*−3	–	6.3
Others[a]	0.6	11.9

[a]Relative proportions of unidentified peaks

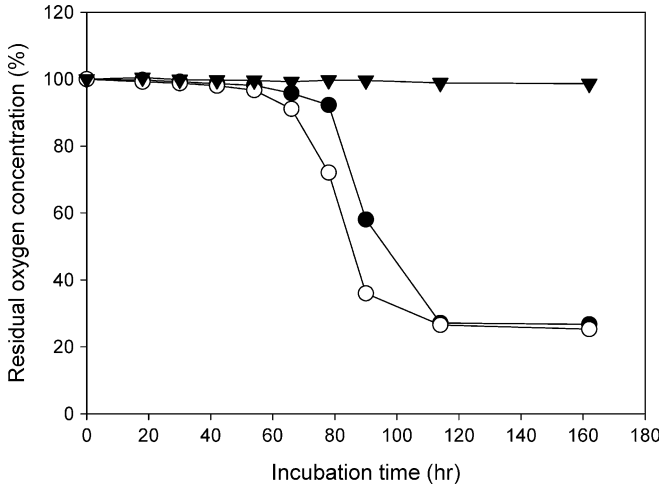

Fig. 2 Effect of bio-converted linoleic acid (*LA*) on the oxidation of fish oil-TAG. Appropriate amount of oil samples were placed into 5 ml sealed vials and all vials were then incubated at 40 °C in the dark. At selected time intervals, 20 µl of gas was withdrawn using a gas-tight syringe from each vial and the reduction of oxygen was determined by GC. *Symbols of closed circle, open circle, and inverted closed triangle* represent fish oil (100 mg) only, 1% bio-converted LA in 100 mg fish oil, and bio-converted LA (1 mg) only, respectively

oxygen consumption rate increased gradually up to 78 h with 92.3% of residual oxygen concentration, followed by an exponential increase up to 114 h representing 27% of the final residual oxygen concentration (Fig. 2). When 1% bio-converted LA, however, was added to the fish oil, the oxygen consumption rate was increased greatly, representing 72% of the residual oxygen concentration after 78 h and 36% after 90 h, compared with 92 and 58% of fish oil only, respectively. When soybean oil

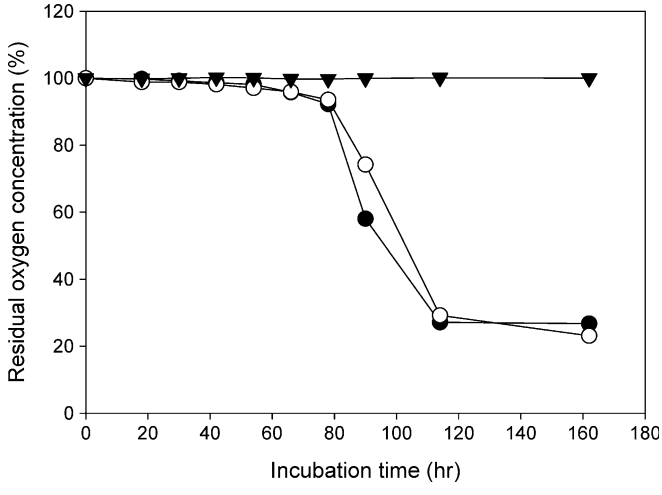

Fig. 3 Effect of bio-converted conjugated linoleic acid (*CLA*) on the oxidation of fish oil-TAG. *Symbols of closed circle, open circle, and inverted closed triangle* represent fish oil (100 mg) only, 1% bio-converted CLA in 100 mg fish oil, and bio-converted CLA (1 mg) only, respectively. Other experimental procedures were the same as those described in Fig. 2

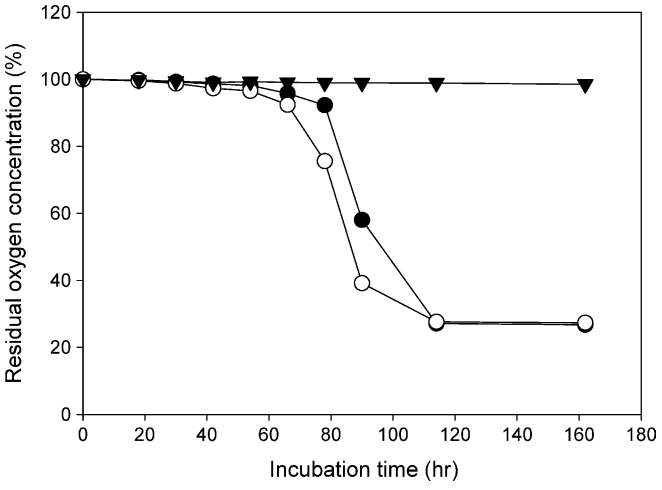

Fig. 4 Effect of bio-converted eicosapentanoic acid (*EPA*) on the oxidation of fish oil-TAG. *Symbols of closed circle, open circle, and inverted closed triangle* represent fish oil (100 mg) only, 1% bio-converted EPA in 100 mg fish oil, and bio-converted EPA (1 mg) only, respectively. Other experimental procedures were the same as those described in Fig. 2

instead of fish oil was used, the oxygen consumption rate did not change regardless of the usage of bio-converted LA (data not shown). These results suggested that mixed metabolites of bio-converted LA effectively promoted oxidation of fish oil and this oxidation effect was dependent upon the degree of unsaturation of the substrate oils. The oxidative effect of bio-converted LA was confirmed by the fact that bio-converted CLA which did not show any remarkable change in TLC band pattern did not change the oxygen consumption rate of fish oil (Fig. 3), and that intact LA did not cause those changes (data not shown). Bio-converted EPA showed a similar

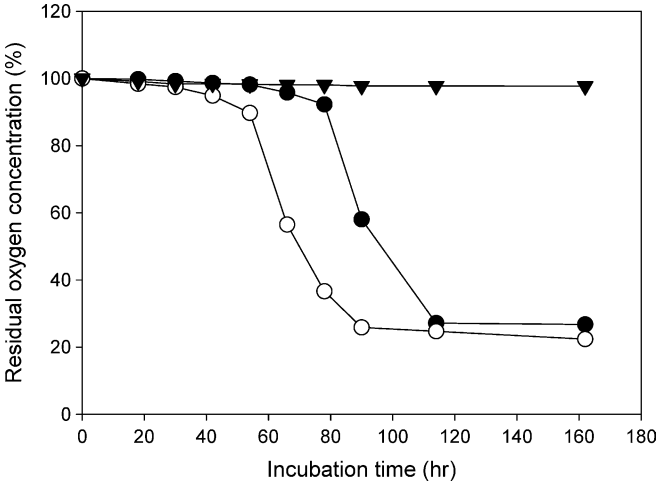

Fig. 5 Effect of bio-converted docosahexanoic acid (*DHA*) on the oxidation of fish oil-TAG. *Symbols of closed circle, open circle, and inverted closed triangle* represent fish oil (100 mg) only, 1% bio-converted DHA in 100 mg fish oil, and bio-converted DHA (1 mg) only, respectively. Other experimental procedures were the same as those described in Fig. 2

Table 2 Minimal inhibitory concentration (MIC, μg/ml) of bio-converted fatty acids for bacterial strains

Bacteria tested	LA-H	DHA-H	EPA-H	CLA-H
Bacillus subtilis ATCC 6633	750	350	350	0
Listeria monocytogenes ATCC 19166	1,500	350	350	0
Staphylococcus aureus KCTC 1916	1,500	650	500	0
Staphylococcus aureus ATCC 6538	1,500	250	500	0

LA-H, EPA-H, DHA-H, CLA-H represent bio-converted linoleic acid, eicosapentanoic acid, docosahexanoic acid, and conjugated linoleic acid, respectively. Conditions for bioconversion are the same as presented in Materials and methods

effect on fish oil oxidation as bio-converted LA did, representing 76 and 39% of residual oxygen concentration after incubation for 78 and 90 h, respectively (Fig. 4). Bio-converted DHA, however, was more effective than the other bio-converted fatty acids tested for the oxidation of fish oil. Residual oxygen concentration started to decrease from the beginning of incubation and declined to 36.6% after 78 h and 25.9% after 90 h of incubation (Fig. 5). These results, collectively, provided a hint to understand some aspect of how the bio-converted fatty acids could exert anti-microbial activities. This suggestion was confirmed partly by the fact that the bio-converted LA, EPA, and DHA, but not CLA, inhibited the growth of Gram-negative bacteria such as *Listeria monocytogenes, Staphylococcus aureus*, and *Bacillus subtilis*. Minimal inhibitory concentration (MIC) values of the bio-converted fatty acids for those bacteria are given in Table 2. In another report, a crude extract of bioconverted LA showed anti-fungal activity against *Rhizoctonia solani* and *Botrytis cinerea* [19].

In conclusion, the bacterial strain PR3 successfully converted polyunsaturated fatty acids, LA, EPA, and DHA into complicated, mixed new products and these bio-converted polyunsaturated fatty acids were very effective in promoting oxidation of fish oil-TAG. It is assumed that this oxidative effect could plausibly play an important role in the antibacterial activity exerted by these bio-converted fatty acids. Further studies should focus on isolation and structural determination of individual bio-converted products from the crude extracts and on the elucidation of their oxidative activities.

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