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Purification of Eicosapentaenoic Acid (EPA) and Docosahexaenoic Acid (DHA) From Fish Oil Using HPLC Method and Investigation of Their Antibacterial Effects on Some Pathogenic Bacteria

Balık Yağındaki Eikosapentaenoik Asit (EPA) ve Dokosaheksaenoik Asit (DHA)'in Yüksek Performanslı Sıvı Kromatografi (HPLC) Yöntemi ile Saflaştırılması ve Bazı Patojenik Bakteriler Üzerine Etkisinin Incelenmesi

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ABSTRACT

The aim of this study was to purified eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) essential oils from trout oil using high performance liquid chromatography (HPLC) method, and bioconverted EPA and DHA into bioconverted EPA (bEPA) and bioconverted DHA (bDHA) extracts by *P. aeruginosa* PR3. Moreover, in vitro antibacterial activity of bEPA and bDHA was investigated using disc diffusion methods and minimum inhibitory concentration (MIC). EPA and DHA concentration in trout oil increased after HPLC optimisation. In this study, EPA and DHA enriched products were obtained which are to be used as valuable supplements for food and pharmaceutical purposes. The bioconverted EPA and DHA exhibited antibacterial activities against two Gram-positive bacteria (*Listeria monocytogenes* ATCC 7677 and *Staphylococcus aureus* ATCC 29213) and six Gram-negative bacteria (*Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC700603, *Enterococcus faecalis* ATCC 29212, *Aeromonas hydrophila* NCIMB 1135 and *Salmonella paratyphi* A NCTC 13). Inhibition zones and MIC value of bEPA and bDHA against bacterial strains ranged from 7 to 12 mm and from 350 to 2350 µg/mL, respectively. Our results suggested that the crude extracts of bioconversion of EPA and DHA by *P. aeruginosa* PR3 can be considered as promising antimicrobials in improving food safety by controlling foodborne pathogens.

Keywords: High performance liquid chromatography (HPLC), docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), minimum inhibitory concentration (MIC), *Pseudomonas aeruginosa* PR3

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ÖZET

Bu çalışmada alabalık yağlarından HPLC tekniği ile esansiyel yağ asitleri olan EPA ve DHA ω-3 yağ asitlerinin elde edilmesi ve bu yağ asitlerinin P. *aeruginosa* PR3 suşu tarafından biyodönüşümleriyle üretilen ekstraktların inhibisyon zonu ve MIC değerleri ile gıda kaynaklı patojen bakterilere karşı antimikrobiyal etkileri incelenmiştir. Ham alabalık yağında %11.1 oranında EPA ve %15.9 oranında bulunan DHA yağ asitleri, HPLC optimizasyonu sonucu %58.64 (EPA) ve %40.33 (DHA) seviyelerine yükseltilmiştir. Bu şekilde gıda ve farmasötik amaçlar için değeri yüksek destek maddesi olarak kullanılabilecek EPA ve DHA'ca zenginleşmiş ürünler elde edilmiştir. Biyodönüşümlü EPA ve DHA (bEPA, bDHA) 2 gram pozitif bakteri (*Listeria monocytogenes* (ATCC 7677) ve *Staphylococcus aureus* (ATCC 29213)) ve 6 gram negative bakteriye (*Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC700603), *Enterococcus faecalis* (ATCC 29212), *Aeromonas hydrophila* (NCIMB 1135), *Salmonella paratyphii* A (NCTC 13)) karşı antibakteriyel aktivite göstermiştir. Her iki bEPA ve bDHA büyüme inhibisyonu gram pozitif bakterilere karşı benzer sonuçlar gösterirken, bDHA ekstraktı minimum inhibitor konsantrasyonu (MIC) olarak tanımlanan gram negative bakterilere karşı bEPA'dan daha etkili olmuştur. Sonuç olarak, *P. aeruginosa* PR3 tarafından EPA ve DHA'nın biyodönüşüm ekstraktları gıda kaynaklı patojenlerin kontrolü için gıda güvenliğinin geliştirilmesinde gelecek vaad eden antimikrobiyal ajanlar olarak düşünülebilir.

Anahtar sözcükler: HPLC; DHA; EPA; MIC; *Pseudomonas aeruginosa* PR3; büyüme inhibisyonu.

1. INTRODUCTION

During the last years, interest in the nutritional and pharmacological effects of dietary polyunsaturated fatty acids and specifically of n-3 polyunsaturated fatty acids has increased (Simopoulus, 1991; Uauy et al., 1992). The ω-3 polyunsaturated fatty acids (PUFA), especially eicosapentaenoic acid (20:5, EPA), docosahexaenoic acid (22:6, DHA) are attracting increasing attention because of their importance to human health (Uauy & Valenzuela, 1992). EPA is the precursor of prostaglandins, tromboxanes and leukotrienes, which are effective anti-aggregatory substances. EPA ingestion can has effects on cardiovascular diseases through a variety of mechanisms (Glosmet, 1985) and DHA in essential for the development of the neural and vision function, mainly in neonates (Uauy et al., 1992). DHA is a main component of membrane phospholipids of brain and retina cells. Clinical studies show that DHA is essential for the growth and development of the brain in infants and for maintenance of normal brain functions in adults. Recently it has been shown that EPA and DHA supplementation has positive health effects including attenuation of coronary heart disease risk factors (hypertension, hyperlipidemia, platelet aggregation, glucose tolerance); modulation of eicosanoid synthesis (cellular immune system, dermal integrity) and tumoricidal activity. Some studies indicated that the PUFA concentrates, devoid of more saturated fatty acid, are much better than oils themselves since they

allow the daily intake of total lipid to be kept as low as possible.

Samples enriched in these fatty acids are needed to further investigate their nutritional, health and biochemical effects and to serve as secondary analytical standards. The PUFA concentrates can be produced by several methods, including fractional crystallization, urea fractionation, molecular distillation, supercritical fluid extraction, silver ion complexation and enzymatic hydrolysis and esterification reactions. Different procedures for the obtention of EPA and DHA concentrates at laboratory scale, including urea complexation and interesterification with specific lipases allowing the obtention of concentration up to 90% for both fatty acids, have been developed (Haagsma et al., 1982; Ackman et al., 1988; Haraldsson et al., 1989). However, the nutritional and pharmacological research on these fatty acids needs pure forms of either EPA and DHA.

Microbial conversion of unsaturated fatty acids has been widely exploited to produce new, value-added hydroxy products. The bioconversion reactions by *Pseudomonas aeruginosa* PR3 have been cited extensively among microbial systems that produce mono-, di- and trihydroxy fatty acid derivatives from unsaturated fatty acids (Hou and Bagby, 1991; Kuo et al., 1998, 2001; Kim et al., 2000). Strain PR3, isolated from a wastewater stream on a pig farm in Morton, Illinois, was found to convert oleic acid to a novel compound, 7,10-dihydroxy-8(E)-octadecenoic acid (DOD) (Hou and Bagby, 1991), and to convert ricinoleic acid to a novel compound, 7,10,12-trihydroxy-8(E)-octadecenoic acid (TOD) (Kuo et al., 1998).

Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are formed in animal (including fish and shellfish) tissues, but not plant tissues. DHA is a component of membrane structural lipids that are enriched in certain phospholipid components of the retina and nonmyelin membranes of the nervous system in animal. EPA is a precursor of the ω-3 eicosanoids, which have been shown to have beneficial effects in prevention of coronary heart disease, arrhythmias, and thrombosis (Kinsella et al., 1990). Although microbial bioconversion of EPA and DHA was reported by Hosokawa et al. (2003), the antimicrobial activity of bioconversion products has not been investigated so far.

In this study we describe two comparative procedure for the obtention of almost pure fractions of EPA and DHA by HPLC starting from a concentrate containing up 11.1% and 15.9% in trout oil increased in 58.64% and 40.33% after HPLC optimization (Nieto et al., 1997) and antibacterial availability of bioconversion extracts of EPA and DHA produced by *P. aeruginosa* PR3 strain as determining the inhibition zone and MIC values against a range of foodborne pathogenic bacteria. This concentrate could be used as valuable supplement for food and pharmaceutical purposes.

2. MATERIAL AND METHOD

2.1. Materials

2.1.1. Fish Oil

The rainbow trout oil used in this study was obtained from a commercial company from Adana.

2.1.2. Microorganisms

P. aeruginosa PR3, kindly provided by Dr. Hou in USDA/ ARS/NCAUR, Peoria, IL, USA, was grown at 28°C aerobically at 200 rpm on screening medium (SM) containing 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 $MnSO_4 \cdot 7H_2O$.

Eleven strains of foodborne pathogenic and spoiling bacteria including *Bacillus subtilis* (ATCC 6633), *Enterobacter aerogenes* (KCTC 2190), *Escherichia coli* (ATCC

8739), *E. coli* O157:H7 (ATCC 43888), *E. coli* O157:H7 (human), *Listeria monocytogenes* (ATCC 19166), *Pseudomonas aeruginosa* (KCTC 2004), *Salmonella enteritidis* (KCCM 12021), *S. typhimurium* (KCTC 2515), *Staphylococcus aureus* (ATCC 6538) and *S. aureus* (KCTC 1916) were obtained from the Korea Food and Drug Administration, Daegu, Korea. The stock cultures were maintained on Luria broth (LB) agar medium at 4°C. Active culture for experiments were prepared by transferring a loopful of cells from stock cultures to flasks and inoculated in the LB and incubated at 37 °C for 24 h. The cultures were diluted with fresh LB to achieve optical density of 10^5 CFU/ml for the test organisms.

2.1.3. Chemicals

EPA and DHA as substrates were purchased from Sigma-Aldrich (Germany). The purity of substrate fatty acids was over 95%. All another chemicals used in this study were purchased from Merck.

2.2. Methods

2.2.1. Purification of EPA and DHA Fractions

The mobile phase for the purification of fatty acids in rainbow trout oil was ethanol and HPLC grade ultra pure water. The separation time of total fatty acids was 20 minute. The program takes 1 minute to return to the initial condition after separation. The injection level was 5 µl and the determination was at 254 nm (Nieto, 1997).

2.2.2. Fatty Acids Analysis of Fractions

Samples were converted to their constituent fatty acid methyl esters by the method of Ichihara et al. (1996), by using 2M KOH in methanol and n-heptane with minor modifications. Twenty mg of sample was dissolved in 2 ml n-heptane followed by 4 ml of 2 M methanolic KOH. The tube was then vortexed for 2 min at room temperature. After centrifugation at 4000 rpm for 10 min, the n-heptane layer was taken for gas chromatography analyses.

2.2.3. Bioconversion reactions

Bioconversions were carried out in 50 ml of SM which was containing (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 $MnSO_4 \cdot 7H_2O$. EPA and DHA (each 0.5 g) as substrates were added to 24 h old cultures separately followed by continued incubation for an additional 72 h and then continuously shaken at 200 rpm in a Psycro Therm controlled environment shaker (New Brunswick Scientific, Edison, NJ) for specified temperature and duration and bioconversion was allowed to proceed. At the end of the bioconversion the culture broth was acidified to pH 2.0 with 6 N HCl and extracted twice with an equal volume of ethyl acetate/diethyl ether (1:1 vol/vol). The solvent was then evaporated from the combined extract with a rotary evaporator and the crude lipid extracts were obtained with the yield of 95%.

2.2.4. Antibacterial assay

Antibacterial tests were carried out by disc diffusion method (Murray et al., 1995), using 10 ml of suspension containing 10^5 CFU/ml of bacteria and poured on LB agar. The discs (6 mm in diameter) separately were impregnated with 1.5 µl (1500 µg crude lipid extracts) of bioconverted eicosapentaenoic and docosahexaenoic acids respectively and placed on the inoculated agar. The inoculated plates were incubated at 37 °C for 24 h. Antibacterial activity was evaluated by measuring the diameter of inhibition zones against the test microorganisms. Each assay for EPA and DHA in this experiment was performed in triplicate. Non-bioconverted EPA and DHA as substrates were used as negative control.

2.2.5. Minimum inhibitory concentration (MIC)

Minimum inhibitory concentrations of the crude lipid extracts of the bioconverted eicosapentaenoic and docosahexaenoic acids were tested by the two-fold dilution method (Murray et al., 1995). A loopful of the bacterial culture from the LB slant was inoculated in the Luria broth and incubated at 37 °C for 24 h, and two-fold serial dilution method was followed as below. The crude extract was first dissolved in 5% dimethyl sulfoxide (DMSO). This solution was further diluted with 5%DMSO and was added to LB to final concentration of 0, 125, 250, 350, 500, 650, 800, 1000, 2000, 3000, 4000, 5000 µg/ml. The bacterial suspensions of tested strains were inoculated in LB medium in 25 ml of cap tube and incubated for 24 h at 37 °C. The minimum concentration at which no visible growth was observed in the tube was defined as MIC, which is expressed in µg/ml. A set of tubes containing only seeded liquid medium was kept as control and 5% DMSO control was also maintained. All the tests for MIC determinations were performed in triplicate.

2.2.6. Statistical analysis

Analysis of variance was conducted with SAS program (Cary, NC, USA). Comparison of means was performed using Duncan's multiple test with significance level of α=0.05 using the same program.

3. RESULTS AND DISCUSSION

The chromatographic profile of the EPA + DHA concentrate obtained from rainbow trout oil subjected to HPLC elution has been given in Figure 1. The retention time of the desired omega-3 fatty acids was found in the peak between 3.5 and 4.7 minutes. The fraction in this range was then esterified to be injected in a gas chromatography (GC) apparatus for yield analysis after collection with HPLC fraction collector.

Table 1 shows the EPA and DHA composition of the concentrate, expressed as percentage of the total fatty acids present in the sample, compared to the original fatty acid composition of rainbow trout oil before the concentration procedure. Individual concentrations of EPA and DHA in the concentrate are 58.64% and 40.33% respectively.

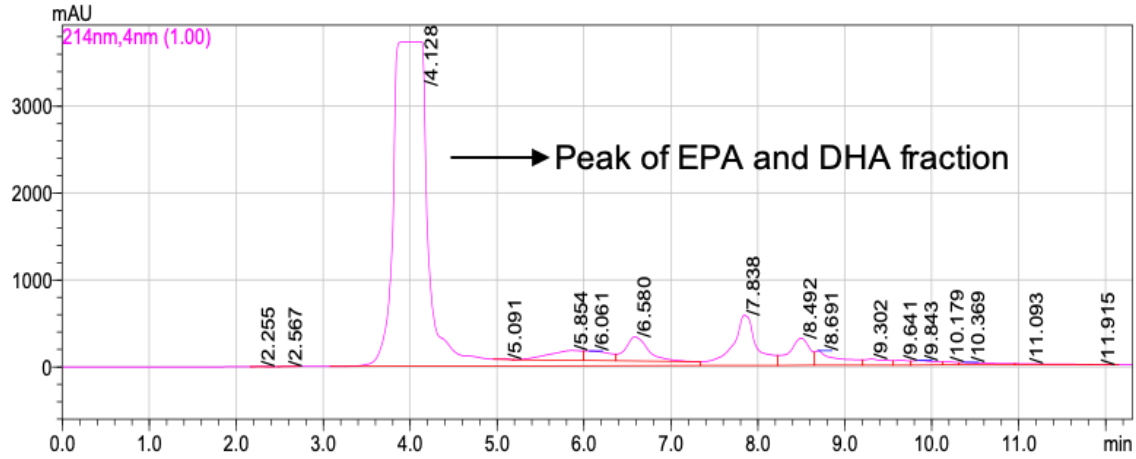

Figure 1. Peak of EPA and DHA fraction after HPLC purification

Table 1. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) composition (% methyl esters) of rainbow trout oil before and after the concentration procedure

	Before concentration procedure	**After concentration procedure**
EPA	11.1	58.64
DHA	15.9	40.33
EPA + DHA	27.0	98.97

* Results represent a typical concentration procedure.

As a result of bioconversion of EPA and DHA, total of 44 biotransformation components have been identified after GC-MS analysis. The components of this extract are carbonochloridic acid, methoxymethane, hydrazine, L-prolin, oxalic acid, luprisol, propionaldehyde, caproaldehyde, propanoic acid, valeric acid, 1,1-dimethoxyoctane, cyclooctane, sulfurous acid, butyric acid, 1,2,3,4-undecanetetrol, 2-propenoic acid, butanedioic acid, 2,2,3- triethyloxirane, acrolein, tetrahydropyrrolo, thaizole, cyclohexanecarboxylic acid, diethyl carbinol, cyclohexanone, butanoic acid, decanoic acid, 4-pentenyl butyrate, 1-heptene, cyclohexanol, silane, 1-butyne, birnenoel, trifluoroacetic acid, dodecane, pentalene-1,5-dione, 3-decen-1-ol, cyclopentaneundecanoic acid, ethyl isohexanoate, 1-pentanol, propylphosphonic acid, 4-heptenoic acid, hex-4-enoic acid, butyldimethylsilanol and 4-methylhexyl acetate. As a major components are 15,18-dihydroxy-14,17-epoxy-5 (Z), 8 (Z), 11 (Z) -eicosatrienoic acid (18.2), 17,19-dihydroxy-16,18-epoxy-4 (Z),7(Z),10(Z),13(Z)-docosatetoraenoic acid (11.96), carbonochloridic acid (2.99%), methoxymethane (40.6%), caproaldehyde (7.85%), 2-propenoic acid (8.13 %) and cyclohexanone (2.07%).

In-vitro antibacterial activities of EPA and DHA biotransformation products against foodborne pathogenic bacteria were determined qualitatively and quantitatively by inhibition zone and MIC values. According to the results given in table 2 and 3, the crude extracts of bEPA and bDHA showed high antibacterial activity against two gram positive (*L. monocytogenes* (ATCC 7677) and *S. aureus* (ATCC 29213)) and six gram negative (*E. faecalis* (ATCC 29212), *E. coli* (ATCC 25922), *P. aeruginosa* (ATCC27853), *K. pneumonia* (ATCC700603), *A. hydrophila* (NCIMB1135) and *S. paratyphii* A (NCTC13)) pathogenic bacteria. Although eicosapentaenoic acid, which is not bioconverted and used as negative control, exhibit very low antimicrobial activity, non-bioconverted doxahexaenoic acid did not showed any antibacterial effect. The inhibition zone diameters and MIC values for bacterial strains varied between 7-12 mm and 350-5000 μg/ml for crude bEPA extract and 7-12 mm and 250-4800 μg/ml for crude bDHA extract, respectively (Table 1 and 2). Control group as applied 5% DMSO used in this study did not inhibit any test bacteria. Gram-positive bacteria were more sensitive to bEPA and bDHA than gram-negatives and showed relatively higher antimicrobial activity than other strains against *L. monocytogenes* and *S. aureus* (ATCC 7677) strains (Table 2). *P. aeruginosa* (ATCC 27853) from gram negative bacteria appears to be the most sensitive bacteria to DHA.

Table 2. Growth inhibition zone produced by bacteria against bEPA and bDHA extracts

Bacteria	Inhibition Zone (mm)	
	EPA	**DHA**
Listeria monocytogenes ATCC 7677	12	13
Staphylococcus aureus ATCC 29213	10	11
Pseudomonas aeruginosa ATCC 27853	10	12
Escherichia coli ATCC 25922	8	7
Klebsiella pneumoniae ATCC700603	7	8
Enterococcus faecalis ATCC 29212	7	7
Aeromonas hydrophila NCIMB 1135	7	7
Salmonella paratyphii A NCTC 13	7	8

In the antibacterial susceptibility studies, many essential oils have been reported using against *P. aeruginosa* (Deans and Ritehie, 1987; Knobloch et al., 1989; Paster et al., 1990). These results have been substantially similar to those reported by other researchers for essential oils (Cosentino et al., 1999; Lambert et al., 2001; Karaman et al., 2003). Other gram-negative organisms had been less sensitive to the effect of antimicrobial compounds than *Pseudomonas aeruginosa*. This is probably due to the fact that their cell wall perimeter has an outer membrane (Ratledge and Wilkinson, 1988), which limits the diffusion of hydrophobic compounds along the lipopolysaccharide cover (Vaara, 1992).

The growth inhibition of bEPA and bDHA crude extracts was similar to gram positive bacteria, but DHA's bioconversion extract was more effective than EPA as the minimum inhibition concentration against gram negative bacteria (Table 3). This was closely related to experimental findings that oxidative activity of DHA's crude biotransformation extract was higher than that of EPA (Kim et al., 2006). As a result, the antibacterial properties of crude extracts converted by the biotransformation of ω-3 fatty acids such as EPA and DHA were produced by *P. aeruginosa* PR3 bacterial strain.

Table 3. MIC values of bEPA and bDHA extracts which inhibit the growth of bacteria

Bacteria	MIC (µg/mL)	
	bEPA	**bDHA**
Listeria monocytogenes ATCC 7677	350±10.00[a]	350±9.50[a]
Staphylococcus aureus ATCC 29213	500±5.00[a]	500±5.80[a]
Pseudomonas aeruginosa ATCC 27853	350±7.20[a]	250±6.30[b]
Escherichia coli ATCC 25922	2350±27.00[a]	1800±30.50[b]
Klebsiella pneumoniae ATCC700603	1800±28.60[a]	1650±29.90[b]
Enterococcus faecalis ATCC 29212	5000±32.00[a]	4800±23.50[b]
Aeromonas hydrophila NCIMB 1135	2350±18.30[a]	1650±11.80[b]
Salmonella paratyphii A NCTC 13	1800±16.40[a]	1650±12.40[b]

[a] and [b] shows statistically significant differences between bEPA and bDHA against test bacteria.

4. CONCLUSION

The results showed that *P. aeruginosa* PR3 may suggest that EPA and DHA's bioconversion extracts are promising antimicrobial agents for improving food safety for control of foodborne pathogens. In order to produce and bioprocess extruding extracts of a wide range of EPA and DHA, it is necessary to isolate the active new compounds from the crude extracts and to define their chemical structures to make them feasible and practical. As a result of this study, bEPA and bDHA extracts significantly inhibited bacterial growth.

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CONFLICT OF INTEREST

The authors declare there are no conflicts of interest.

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