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Antibacterial activity of eicosapentaenoic acid (EPA) against foodborne and food spoilage microorganisms

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Abstract

Eicosapentaenoic acid (EPA), a long-chain polyunsaturated fatty acid of ω-3 type was evaluated for its antimicrobial action against the range of foodborne and food spoilage pathogens, using agar disc diffusion assay in Luria broth (LB) media. The EPA exhibited antimicrobial activity against *Bacillus subtilis* ATCC 6633, *Listeria monocytogenes* ATCC 19166, *Staphylococcus aureus* ATCC 6538, *S. aureus* KCTC 1916 and *Pseudomonas aeruginosa* KCTC 2004. The minimum inhibitory concentrations (MICs) of the EPA against the tested bacterial strains were found in the range of 500–1350 μg/ml using broth dilution method. EPA reduced the viability of *S. aureus* at 250, 125 and 62.5 μg/ml after 15 min exposure and a steep decline in colony forming units (CFUs) was observed at 125 μg/ml after 30 min exposure, while similar reduction in CFU rate was exhibited by EPA when treated with 62.5 μg/ml after 180 min. EPA also reduced the CFU numbers of *P. aeruginosa* at all the concentrations used in this study after 15 min exposure. On the other hand, scanning electron microscopy (SEM) study of bacterial cells clearly exhibited the antibacterial effect of EPA as evidenced by the damages found in the outer membrane of the cells when treated with EPA. The results demonstrated that EPA exerted significant bactericidal and bacteriostatic effects against both *P. aeruginosa* and *S. aureus*.

Keywords: Antibacterial activity; Eicosapentaenoic acid; EPA; Omega-3 fatty acid

1. Introduction

ω-3 Fatty acids are essential dietary *cis*-polyunsaturated fatty acids (PUFAs). These PUFAs include α-linolenic acid (C18: 3n-3, LNA), abundantly available in some edible plants whereas eicosapentaenoic acid (C20: 5n-3, EPA), and docosahexaenoic acid (C22: 6n-3, DHA) are associated with fish and marine vertebrates (Leaf, Kang, Xiao, & Billman, 2003). Supplementing food with ω-3 (n-3) fatty acids in the normal diet has been proved beneficial in reduction of cardiovascular disease in those who consume diet containing deep-water fish and fish oil (Siscovick, Raghunathan, & King, 2000). Further, evaluation of Eskimos diets derived a large portion of dietary calories from ω-3 fatty acids demonstrated a significantly lower rate of cardiovascular disease (von Schacky, 1987).

Fatty acids function as the key ingredients of antimicrobial food additives due to their inhibitory action on undesirable microorganisms (Freese, Shew, & Galliers, 1973). Additionally, the long-chain unsaturated fatty acids such as linoleic and oleic acids are bactericidal to important pathogenic microorganisms, including methicillin-resistant *Staphylococcus aureus* (Farrington, Brenwald, Haines, & Walpole, 1992; Kabara, Swieczkowski, Conley, & Truant, 1972; Knapp & Melly, 1986), *Helicobacter pylori* (Hazell & Graham, 1990; Sun, O'Connor, & Roberton, 2003), and *Mycobacteria* (Seidel & Taylor, 2004). The antibacterial actions of long-chain unsaturated fatty acids including oleic acid, linoleic acid, and LNA are usually attributed to inhibit against pathogenic microorganisms (Kabara et al., 1972; Knapp & Melly, 1986; Seidel & Taylor, 2004; Sun et al., 2003). However, there are no available reports on antibacterial activity of ω-3 PUFA, EPA against foodborne and food spoilage microorganisms. Hence, present report is highlighted on the antibacterial activity of EPA

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against commonly occurring foodborne and food spoilage microorganisms.

2. Materials and methods

2.1. Chemical

EPA was purchased from Nu-Chek-Prep Inc. (Elysian, MN, USA). The purity of fatty acid was over 0.99 g/ml.

2.2. Microorganisms

Eleven strains of commonly occurring genera of foodborne and food spoilage pathogens such as *Bacillus subtilis* ATCC 6633, *Enterobacter aerogenes* KCTC 2190, *Escherichia coli* ATCC 8739, *Escherichia coli* O157:H7 ATCC 43888, *Escherichia coli* O157:H7 (human), *Listeria monocytogenes* ATCC 19166, *Pseudomonas aeruginosa* KCTC 2004, *Salmonella enterica* serovar Enteritidis KCCM 12021, *S. typhimurium* KCTC 2515, *S. aureus* ATCC 6538 and *S. aureus* KCTC 1916 were obtained from Korea Food and Drug Administration, Daegu, Korea. The stock cultures were maintained on Luria broth (LB) agar medium at 4 °C.

2.3. EPA sensitivity test

The sensitivity of EPA was determined against different bacterial strains by disc diffusion method (Murray, Baron, Pfaller, Tenover, & Yolke, 1995) using 24-h-old bacterial culture suspension (10^5 colony forming unit (CFU)/ml) in LB agar media. The sterile Whatman/filter paper no. 11 discs (6 mm diameter) impregnated with pre-determined 2.0 μl (2000 μg) EPA were placed on the surface of inoculated agar plates. The plates were incubated at 37 °C for 24 h and the zones of inhibition were measured. Each assay was performed in triplicate.

2.4. Determination of minimum inhibitory concentrations (MICs)

The MICs of EPA were determined by the two-fold dilution method (Murray et al., 1995). A loopful of the each bacterial culture was inoculated in the LB broth and incubated at 37 °C for 24 h. For sample preparation, EPA was dissolved in 5% dimethyl sulfoxide (DMSO) and further diluted with 5% DMSO, added to LB broth to achieve final concentrations of 0, 125, 250, 500, 750, 1000, 1350, 3000, 4000, and 5000 μg/ml. The different concentrations of EPA were added in the pre-grown bacterial cultures and incubated for 24 h at 37 °C. The minimum concentrations of EPA at which no visible growth was observed were defined as MICs which were expressed in μg/ml. All the tests for MIC determinations were performed in triplicate.

2.5. Effect of EPA on viable counts of bacteria

For viable counts, LB broth (10 ml) was inoculated with three different concentrations of EPA containing 10 μl of active inoculum of each *S. aureus* ATCC 6538 (approximately 10^7 CFU/ml) and *P. aeruginosa* (10^5 CFU/ml) and kept at 37 °C. Samplings for viable cell counts were taken out at 0, 10, 15, 30, 1, 2, 3, 4 and 24 h. The viable plate counts were monitored as followed: 0.1 ml sample of each treatment was spread on the surface of LB agar and the colonies were counted after 24 h of incubation at 37 °C. The controls were treated without EPA for each experimental assay.

2.6. Scanning electron microscopy (SEM) analysis

SEM study of EPA with treated and non-treated bacterial cells was performed to observe the morphology of bacterial cells. The method of SEM was modified from Kockro et al. (2000). The bacterial samples were washed gently with 50 mmol/l phosphate buffer solution (pH 7.2), fixed with 2.5 g/100 ml glutaraldehyde and 1 g/100 ml osmic acid solution. The specimen was dehydrated using sequential ethanol concentrations ranging from 30 to 100 ml/100 ml with 10 min of exposure per concentration, and the ethanol was replaced by tertiary butyl alcohol. After dehydration, the specimen was dried with CO_2. Finally, the specimen was sputter coated with gold in an ion coater for 2 min followed by microscopic examinations (S-4300; Hitachi).

3. Results and discussion

The antibacterial activity of EPA against the foodborne and food spoilage microorganisms was determined by agar diffusion and broth dilution methods. As shown in Table 1, among all the strains tested, EPA significantly reduced the growth of four Gram-positive bacteria, *B. subtilis*, *L. monocytogenes*, *S. aureus* ATCC 6538, *S. aureus* KCTC 1916 and one Gram-negative bacterium, *P. aeruginosa*. The EPA exhibited the antibacterial activity as zones of inhibition and MIC values, which were noted to be in the range of 11–12 mm and 500–1350 μg/ml, respectively (Table 1). The positive control with 5% DMSO used in this study did not affect the growth of any of the tested bacterial strain. EPA did not exhibit antibacterial activity against any of the Gram-negative bacteria except *P. aeruginosa* as reported by others that long-chain unsaturated fatty acids, including linoleic acid, are well known for, not to inhibit Gram-negative bacteria such as *Escherichia coli* (Dilika, Bremner, & Meyer, 2000; Freese et al., 1973; Kabara et al., 1972; Sun et al., 2003). The antibacterial activity of unsaturated fatty acids using linoleic acid and LNA against Gram-positive bacteria against *S. aureus* and *S. pyogenes* has been well documented (Zheng, Yoo, Lee, Cho, Kim, & Kim, 2005). This large difference in the fatty acid sensitivities between

Table 1
Antibacterial activity of eicosapentaenoic acid

Bacteria tested	Inhibition zone (mm)	MIC (μg/ml)
Bacillus subtilis ATCC 6633	11±0.0[a]	500
Listeria monocytogenes ATCC 19166	12±0.0[a]	750
Staphylococcus aureus ATCC 6538	11±0.0[a]	750
Staphylococcus aureus KCTC 1916	11±0.0[a]	1350
Pseudomonas aeruginosa KCTC 2004	12±0.0[a]	500
Escherichia coli ATCC 8739	ND[b]	ND[b]
Escherichia coli O157:H7 ATCC 43888	ND[b]	ND[b]
Escherichia coli O157:H7 (human)	ND[b]	ND[b]
Enterobacter aerogenes KCTC 2190	ND[b]	ND[b]
Salmonella Enteritidis KCCM 12021	ND[b]	ND[b]
Salmonella Typhimurium KCTC 2515	ND[b]	ND[b]

[a]Values for zone of growth inhibition are presented as mean±SD.
[b]ND denoted antibacterial activity not detected up to the concentration of 5000 μg/ml.

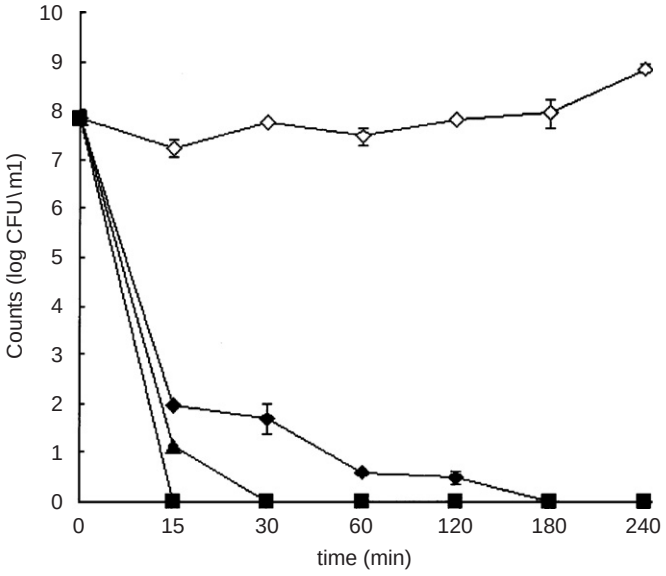

Fig. 1. Effects of EPA on viability of *Staphylococcus aureus* ATCC 6538 as affected by the addition of EPA at the lag phase. The data are expressed as means±standard deviation. (□) no EPA, (□) 62.5 μg/ml, (▲) 125 μg/ml, and (■) 250 μg/ml.

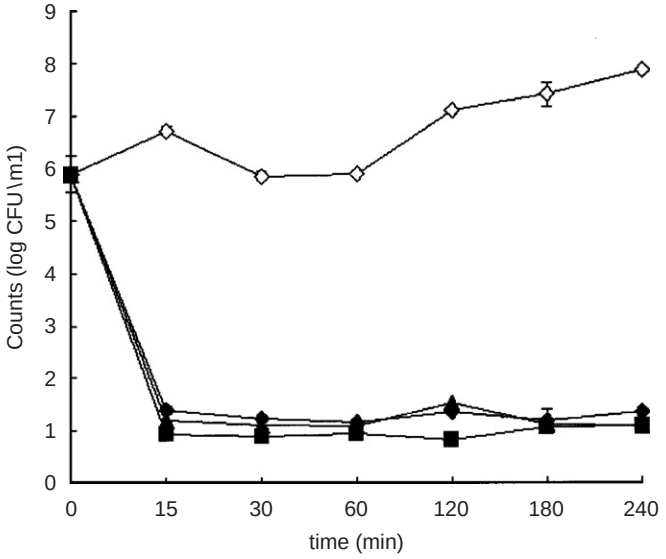

Fig. 2. Effects of EPA on viability of *Pseudomonas aeruginosa* KCTC 2004 as affected by the addition of EPA at the lag phase. The data are expressed as means±standard deviation. (□) no EPA, (□) 31.25 μg/ml, (▲) 62.5 μg/ml, and (■) 125 μg/ml.

Gram-positive and Gram-negative bacteria may result due to the impermeability of the membrane of Gram-negative bacteria, since the outer membrane acts as effective barrier against many hydrophobic substances including long-chain fatty acids as also observed by earlier workers (Galbraith & Miller, 1973; Sheu & Freese, 1973; Sheu, Salomon, Simmons, Sreevalson, & Freese, 1975).

Further, elaborative study was carried out on *S. aureus* ATCC 6538 and *P. aeruginosa,* which displayed different sensitivities of EPA. The effects of EPA on *S. aureus* growth demonstrated the reduced viability of *S. aureus* at 250, 125 and 62.5 μg/ml at 15 min exposure, further a steep decline in CFU was observed after 30 min exposure when treated with 125 μg/ml EPA, while similar reduction in CFU numbers was exhibited at 62.5 μg/ml EPA after 180 min (Fig. 1). EPA also reduced the number of CFU of *P. aeruginosa* at all the concentrations after 15 min of time exposure. Further, reduction did not correspond to exposure time but exhibited bacteriostatic effect of EPA (Fig. 2). The results revealed that *P. aeruginosa* was found more sensitive to EPA as compared to *S. aureus* at all the concentrations. After 15 min, EPA exerted its maximum bactericidal activity as evidenced by the significant reduction in microbial counts and its bacteriostatic action was pronounced till 24 h of incubation at higher EPA concentrations. Other studies revealed that the decrease in viable cells was found greater than 3 log CFU, achieved after the addition of n-6 PUFAs, which has been considered as the criterion for this bactericidal effect (Giamarellos-Bourboulis, Grecka, Dionyssiou-Asteriou, & Giamarellou, 2000). Similar to our findings, one of n-6 PUFAs also exerted an inhibitory effect against *P. aeruginosa* (Giamarellos-Bourboulis, Grecka, Dionyssiou-Asteriou, & Giamarellou, 1995; 1998). Direct effect of n-6 PUFAs on bacterial cells is prone to peroxidation ending in free radicals capable of attacking bacterial outer membrane and facilitating the action of antimicrobials (Giamarellos-Bourboulis et al., 1998). Similar mechanism cannot be ruled out in case of EPA action on *S. aureus* ATCC 6538 and *P. aeruginosa*.

To visualize the effects of EPA, SEM study of EPA-treated cells demonstrated that both *S. aureus* ATCC 6538 and *P. aeruginosa* showed their altered cell morphology in comparison to their control (Fig. 3). Control cells of both strains in the absence of the EPA showed a regular, smooth surface (Fig. 3a and c). The cells incubated with EPA at various concentrations in different groups of bacteria, corresponding to the MIC values revealed severe

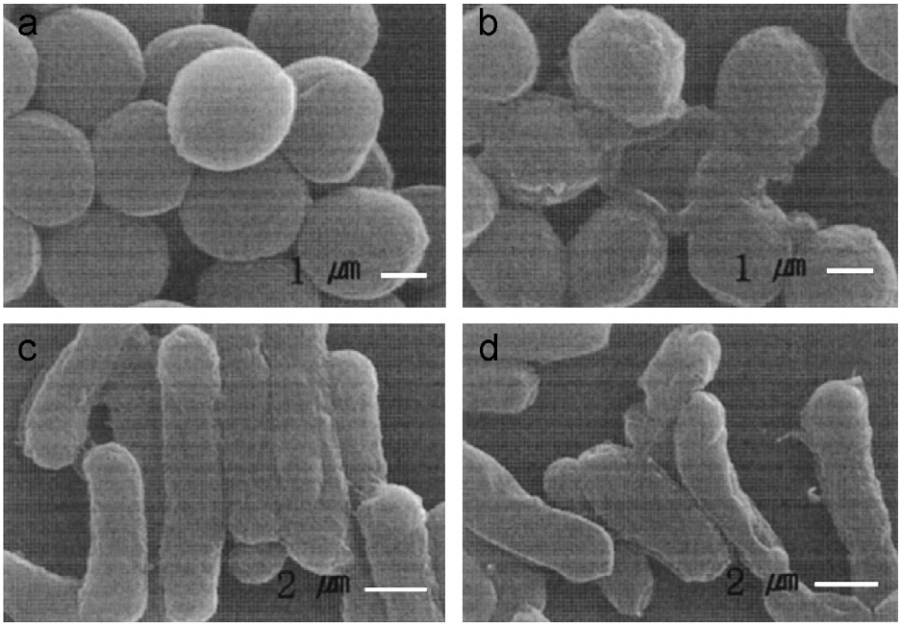

Fig. 3. Scanning electron micrographs of *S. aureus* ATCC 6538 (top) and *P. aeruginosa* KCTC 2004 (bottom) cells, in the absence of EPA (a and c), after 6 h incubation at 37°C with concentrations of the EPA corresponding to the MIC value (b and d).

membrane damages consistent with disruption of the membrane integrity (Fig. 3b and d). In fact, initial exposure of EPA to *S. aureus* revealed large surface collapse on the cells (Fig. 3b) and wrinkled abnormalities in the cells of *P. aeruginosa* with numerous small clefts, regularly distributed on the bacterial cells surface (Fig. 3d). Such morphological features in bacterial cells may be due to the lysis of outer membrane followed by loss of cellular electron dense material on the surface of treated cells (Fig. 3b). The loss of electron dense material from the cells treated with EPA indicates the loss of cell constituents and breakdown of the cell wall resulting in the release of cell materials (Fig. 3d). Such morphological abnormalities mainly occurred due to the disruption of membrane structure as evidenced by the findings of others (Koyama, Yamaguchi, Tanaka, & Motoyashiya, 1997).

4. Conclusions

In conclusion, we reported here the novel function of EPA as an antibacterial agent showing antibacterial activity against foodborne and food spoilage microorganisms. Our results suggested that use of EPA can be considered as antibacterial availability for trials in controlling food safety standards. Therefore, EPA may be useful in controlling number of foodborne and food spoilage pathogens as a traditional concern in food systems.

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