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**The cell membrane-shielding function of eicosapentaenoic acid for *Escherichia coli*
against exogenously added hydrogen peroxide**

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Abstract: The colony-forming ability of catalase-deficient *Escherichia coli* genetically modified to produce eicosapentaenoic acid (EPA) showed less decrease than in a control strain producing no EPA, when treated with 0.3 mM hydrogen peroxide (H₂O₂) under nongrowth conditions. H₂O₂-induced protein carbonylation was enhanced in cells lacking EPA. The amount of fatty acids was decreased more significantly for cells lacking EPA than for those producing EPA. Much lower intracellular concentrations of H₂O₂ were detected for cells with EPA than those lacking EPA. These results suggest that cellular EPA can directly protect cells against oxidative damage by shielding the entry of exogenously added H₂O₂.

Abbreviation: CFU, colony-forming unit; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FAME, fatty acid methyl ester; H₂O₂⁺, in the presence of H₂O₂; H₂O₂⁻, in the absence of H₂O₂; LB, Luria-Bertani; OD, optical density; PUFA, polyunsaturated fatty acid; UM2EPA⁺, *E. coli* UM2 transformant producing EPA; UM2EPA⁻, *E. coli* UM2 transformant producing no EPA;

1. Introduction

Reports are accumulating on the antioxidative effects of polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids in artificial lipid suspension systems [1–3] and biological systems [4–7]. In animal systems PUFAs are metabolically converted to derivatives that could induce or promote in situ scavenging processes to eliminate and decrease reactive oxygen species (ROS) [8]. On the other hand, a more direct and structural involvement of PUFAs to protect against ROS has been proposed for artificial membrane systems. In liposomes made of phospholipids, the degree of unsaturation is inversely related to the stability of the constituent PUFAs [1,2]. Additional direct evidence has been presented that phospholipids with PUFAs such as DHA have a more packed structure in the biomembranes[9,10]. Those observations implied that the oxidative stability of PUFAs in aqueous and biological systems would depend on their inherent structures under such specific environments.

We previously presented evidence for protective effects of EPA against oxidative stress caused by exogenously added H_2O_2 using *Escherichia coli* strain DH5 α , cells that had been transformed with the genes for EPA biosynthesis derived from an EPA-producing bacterium [11,12]. Briefly, the colony-forming ability of *E. coli* DH5 α possessing EPA was similar to that of H_2O_2 -untreated cells. However, this ability was rapidly lost in the cells possessing no EPA. This was reflected in the difference in the degree of protein carbonylation between the two strains. The treatment of both cell types with H_2O_2 changed the amount of fatty acid recovered from cultures but not the fatty acid composition [11]. From those data it has been suggested that cellular EPA is stable in the

presence of H₂O₂ *in vivo* and may directly protect cells against oxidative damage.

Although the role of EPA in the membrane partially to block the penetration of H₂O₂ was proposed in that study, the direct data showing that EPA could operate as a shield for the membrane have not been available.

Here, in order to investigate the possible shielding effects of EPA against exogenously added H₂O₂ passing through the cell membrane, the intracellular concentration of H₂O₂ under nongrowth conditions was measured using a catalase-deficient *E. coli* mutant engineered to synthesize EPA as the test organism.

2. Materials and Methods

2.1. Bacterial cells and cultivation

Throughout the work the *E. coli* strain UM2 [13], a catalase-deficient mutant obtained from the *E. coli* Genetic Stock Center of the University of Yale, was used as the test organism throughout the work. This strain is a derivative of *E. coli* K12 and has a genotype of *katE2 katG2 leuB6 proC83 purE42 trpE28 his-208 argG77 ilvA681 met-160 thi-1 ara-14 lacY1 galK2 xyl-5 mtl-1 azi-6 rpsL109 tonA23 tsx-67 supE44 malA38 xthA* [14]. The recombinant strains of this bacterium were precultured by agitation at 180 rpm in Luria–Bertani (LB) medium containing ampicillin, chloramphenicol (both at 50 µg/ml), and streptomycin (30 µg/ml) at 20 °C. Nongrowing cells of *E. coli* UM2 recombinants were prepared as follows. The cells were grown until the culture had an optical density of 1.0 at 600 nm (OD₆₀₀) and were harvested by centrifugation at 3500 × *g* for 5 min at 4 °C. Collected cells were washed three times with phosphate-buffered saline (pH 7.4) and suspended in the same buffer at an OD₆₀₀ of 1.0. The cell suspension was mixed with a

solution of hydrogen peroxide (H₂O₂) normally at a final concentration of 0.3 mM and then incubated without agitation for 30 min at 20 °C. Cells were harvested at appropriate time intervals by centrifugation and used for further assay. The number of colony-forming units (CFUs) was measured as described previously [11].

2.2. *E. coli* recombinant strains

The recombinant production of EPA in *E. coli* strain UM2 was conducted as was the case in *E. coli* strain DH5 α [11]. Briefly, the host cell was transformed with pEPA Δ 1,2,3, which is a cosmid clone including four genes (*pfaA*, *pfaB*, *pfaC*, and *pfaD*) out of five genes essential for EPA biosynthesis, from *Shewanella pneumatophori* SCRC-2738, and a plasmid vector carrying *pfaE* encoding phosphopantetheinyl transferase (pSTV::*pfaE*); *pfaE* is the fifth gene needed for EPA biosynthesis [15]. When these two vectors were combined, the host strain UM2 cells produced EPA at a level of approximately 7%–8% of total fatty acids (data not shown). The UM2 reference strain, which has no ability to produce EPA, was transformed with pEPA Δ 1,2,3 and empty pSTV28.

2.3. Analytical procedures

The intracellular and extracellular concentrations of H₂O₂ were measured with the method by González-Flecha and Demple [16,17]. The *E. coli* UM2 cells suspended at an OD₆₀₀ of 1.0 with phosphate-buffered saline (pH 7.4) were treated with and without H₂O₂ and were then separated as supernatants and cell pellets by centrifugation as described above. The extracellular concentration of H₂O₂ in the supernatants was measured using a titanium-based method [18]. The cell pellets were resuspended in the same buffer at the same concentration of cells and then allowed to stand for 30 min at 20 °C. This time is

sufficient for the medium to reach the intracellular steady-state concentration of H_2O_2 on the basis of its free diffusion through the cell membrane [16]. After centrifugation the intracellular concentration of H_2O_2 was measured by assaying the concentration in the supernatants.

The carbonyl content in oxidatively modified cellular proteins was determined as described [11]. The degree of carbonylation was expressed as nmol of protein carbonyls per mg of protein. Protein was assayed by the Bradford method [19].

For analysis of cellular fatty acids, packed wet bacterial cells from 10 ml of cultures were subjected to methanolysis as described previously [11]. The resulting fatty acid methyl esters (FAMES) were purified by one-dimensional thin-layer chromatography using silica gel plates (type Silica gel 60, Merck, Germany) and a mixture of hexane–diethyl-ether–acetic acid (90:10:1, by volume). The FAME fraction extracted from gels was analyzed by gas–liquid chromatography and gas chromatography–mass spectrometry, as described previously [15]. The amount of fatty acid was expressed as micrograms of FAMES per milliliter of culture.

3. Results

3.1. Colony-forming ability of E. coli UM2 strains with and without EPA

E. coli UM2 strains that did (UM2EPA+) or did not (UM2EPA–) produce EPA were incubated under nongrowth conditions for 30 min at 20 °C in the presence (H_2O_2+) or absence (H_2O_2-) of various concentrations of H_2O_2 . The number of CFUs of each strain under H_2O_2+ and H_2O_2- conditions was represented as a relative value (%) against that at the respective baseline under H_2O_2- conditions. It was much higher for UM2EPA+ than

for UM2EPA⁻ at any concentration of H₂O₂ except for 3 mM, where no CFUs were detected.

3.2. Carbonyl contents in E. coli UM2 strains with and without EPA

Only slight increases in levels of protein carbonyls were observed for UM2EPA⁻ and UM2EPA⁺ cells treated without H₂O₂ (Fig. 1B). However, levels of protein carbonyls increased from 0.25 nmol/mg protein to 0.71 nmol/mg protein and to 0.48 nmol/mg protein for UM2EPA⁻ and UM2EPA⁺, respectively, within the first 10 min of addition of H₂O₂. Considering the baseline, the increasing rate of protein carbonyls for UM2EPA⁻ was twice that for UM2EPA⁺ under H₂O₂⁺ conditions. The maximum levels of protein carbonyls after the incubation for 30 min were 0.84 ± 0.08 nmol/mg protein for UM2EPA⁻ cells and 0.53 ± 0.11 nmol/mg protein for UM2EPA⁺ cells. These data indicate that the addition of H₂O₂ induced protein carbonylation more significantly for strain UM2EPA⁻ than for strain UM2EPA⁺, and that EPA partially protects the carbonylation of proteins caused by exogenously added H₂O₂. These results are the same as those obtained for *E. coli* DH5 α cells possessing EPA and for those not possessing EPA [11], although the latter have catalase activity and were treated with a much higher concentration of H₂O₂ (3.0 mM) under growth conditions.

3.3. Effects of H₂O₂ on fatty acid amount and composition of E. coli UM2 strains with and without EPA

The composition of fatty acids from *E. coli* UM2 transformants after incubation under nongrowth conditions for up to for 30 min in the presence and absence of H₂O₂ was examined. Treating both cell types with H₂O₂ had little effect on their fatty acid

composition (data not shown). Formation of malonaldehyde, which is a peroxidative derivative of EPA [20], was negligible in strain UM2EPA+ treated with H₂O₂ (data not shown). These data suggest that EPA, compared with other fatty acids, is not more susceptible to attack by exogenous H₂O₂ *in vivo*. However, the susceptibility to H₂O₂ treatment is much lower in UM2EPA+ cells than in UM2EPA- cells because the fatty acids recovered from 1 ml cultures scarcely changed in the UM2EPA+ cells, but decreased approximately 50% in the UM2EPA- cells after 30 min (Table 1). These results suggest that only a partial breakage of UM2EPA- cells would occur under H₂O₂+ conditions. This result was consistent with the findings in *E. coli* DH5 α transformant cells with EPA and without EPA [11].

3.4. Extracellular and intracellular concentrations of H₂O₂ in E. coli UM2 strains with and without EPA

Previously, we presented the possibility that the *E. coli* DH5 α cell membrane phospholipids composed of EPA might have a shielding effect against exogenously added H₂O₂ entering the cell [11]. Here this possibility was tested by measuring the intracellular concentration of H₂O₂ in catalase-deficient *E. coli* UM2 cells that had been treated with H₂O₂. To exclude the involvement of endogenous production of ROS during the incubation, both strains of cells were freed from nutrients and were not agitated (under nongrowth conditions). The extracellular levels of H₂O₂ when added at 0.3 mM, slightly and gradually decreased with time (Fig. 2A). The rate of decrease was a little higher for UM2EPA- cells than for UM2EPA+ cells. Intracellular concentrations of H₂O₂ less than approximately 4 μ M were consistently detected for both UM2EPA- and UM2EPA+ strains that had not been treated with H₂O₂ (Fig. 2B). The intracellular concentration of

H₂O₂ increased with time for both strains under H₂O₂+ conditions. The H₂O₂ concentrations were 35.6 ± 3.1 μM for UM2EPA– and 22.6 ± 2.3 μM for UM2EPA+ cells, 30 min after the addition of H₂O₂. Considering the baseline levels, the intracellular concentration of H₂O₂ of UM2EPA+ cells was approximately two-thirds that of UM2EPA– cells.

4. Discussion

The antioxidative effects *in vivo* of EPA against H₂O₂ were previously shown using the EPA-producing *E. coli* DH5α strains. *E. coli* DH5α cells with an EPA concentration of approximately 10% of total fatty acids were completely resistant to damage caused by exogenously added H₂O₂ at 3 mM [11]. In this study, very similar protective effects of EPA were observed for the catalase-deficient *E. coli* UM2 strains, with approximately the same levels of EPA (7%–8% of total fatty acids), that had been treated with 0.3 mM H₂O₂ under nongrowth conditions (Fig. 1).

E. coli strain UM2 cells were exposed to 0.3 mM H₂O₂, the concentration of which was only one-tenth that for *E. coli* DH5α having a normal level of catalase of 2–3 U per mg of protein [11]. The fact that the UM2EPA+ cells, even if they had EPA, only tolerated H₂O₂ at concentrations approximately less than 3 mM indicates that catalase is the most relevant factor for *E. coli* cells to overcome the challenges caused by exogenous H₂O₂. Even so, the antioxidative function of EPA is also unequivocal for *E. coli* strain UM2. The exclusion of catalase from the *in vivo* system by using transformed *E. coli* UM2 cells with EPA provided us with more direct understanding of the function of EPA. The cell-free extracts from UM2EPA+ and UM2EPA– cells had no detectable levels of H₂O₂-decomposing activity (data not shown). These results support a lack of involvement

of EPA in enhancing or inducing scavenging systems including enzymes responsible for the resistance of *E. coli* UM2 cells against oxidative damage caused by H₂O₂.

The strain *E. coli* UM2EPA+ had a higher resistance to H₂O₂ treatment not only under nongrowth (Fig. 1A) but also under growth conditions (data not shown). This is reflected in lower levels of protein carbonyls in UM2EPA+ cells compared with UM2EPA– cells under both conditions. The greater fragility of UM2EPA– cell structure, which can be assessed by the significant reduction of the amounts of fatty acid recovered from the supernatant of the cell suspension (see Table 1), also showed that the UM2EPA+ strain is more resistant against H₂O₂.

In this study the effects of H₂O₂ on the protein carbonylation of UM2EPA+ and UM2EPA– cells were examined under nongrowth conditions. A very similar trend was obtained using growing cells (data not shown); however, the levels of protein carbonyls were approximately 10 times higher in growing cells than in nongrowing cells. This could be explained from various aspects. The penetration of H₂O₂ through the membrane would be much enhanced by agitation under growth conditions. Moreover, agitation of the cultures would accelerate aerobic respiration, stimulating the cells to generate intracellular H₂O₂. Cells under growth conditions are more susceptible to various external stimuli including H₂O₂, than starved cells that were placed under nongrowth conditions [21].

The shielding effects of EPA to exogenous H₂O₂ penetrating the cell membrane were evaluated by measuring the intracellular concentration of H₂O₂. Consistently lower concentrations of H₂O₂ were found in UM2EPA+ cells than in UM2EPA– cells (Fig. 2B) implying that the entry of exogenously added H₂O₂ is hindered at least partially in

UM2EPA+ cells. Since H₂O₂ freely diffuses through the cell membrane, it is considered that 10 min is generally enough for the equilibration of H₂O₂ inside and outside of the cell membrane [16]. However, the intracellular and extracellular concentrations of H₂O₂ were greatly different when H₂O₂ was added exogenously (Fig. 2), indicating that equilibration of H₂O₂ across the cell membrane would not be attained regardless of the presence or absence of EPA in the cell membrane. Consistently lower levels of intracellular H₂O₂ in UM2EPA+ cells would be achieved by a shielding function of EPA to H₂O₂ attempting to penetrate the cell membrane. This hypothesis is supported by the finding that the profile of the increase in the intracellular levels of H₂O₂ in two strains coincided approximately with that of the decrease in the amounts of fatty acid recovered from the cultures (see Fig. 2 and Table 1).

We have no direct data to prove the structural hindrance of membrane phospholipids acylated with EPA against exogenous H₂O₂ entering through the membrane. To understand the protective mechanism of EPA against exogenous H₂O₂, it may be useful to know how EPA-lacking cells are damaged by the H₂O₂. The number of CFUs was more significantly reduced for the UM2EPA– strain than for UM2EPA+ strain in the presence of H₂O₂ under nongrowth (Fig. 1A) and also under growth conditions (data not shown). However, the OD₆₀₀ values of nongrowing cell suspensions and cultures of strains UM2EPA+ and UM2EPA– were never decreased during incubation, when they were treated with H₂O₂ (data not shown). While the amount of recovered fatty acids was significantly decreased in H₂O₂-treated UM2EPA– cells (Table 1), this should be the result of less recovery of those cells by centrifugation at 3500 × *g* for 5 min. Thus it is suggested that the treatment of UM2EPA– cells with H₂O₂ might make them so notably

leaky that the cells lose cytoplasm and become ghostlike cells. If this is correct, EPA would afford the cell a structural strength so as to prohibit H₂O₂ from partially breaking down the cell membrane.

Phospholipids with DHA or arachidonic acid have an arrangement more compact than do those with less unsaturated fatty acids, such as linoleic and linolenic acids and even saturated fatty acids, in artificial phospholipid membrane systems [9,10,22,23]. A much more rigid micellar structure of DHA was observed in its aqueous phase than in chloroform, when compared with other fatty acids [24]. According to Rajamoorthi et al. [9], a membrane consisting of phospholipids acylated with palmitic acid and DHA has a more hydrophobic interface with the phospholipid bilayers compared to that of phospholipids with two palmitic acids. From those findings it is speculated that a bilayered membrane consisting of longer PUFAs may have a more hydrophobic environment in the region between the two layers of the membrane and that such structure may hinder the entry of hydrophilic compounds such as H₂O₂. No biophysical studies characterizing physical properties of phospholipids with EPA have been available. The *E. coli* recombinant systems expressing *pfa* genes for the biosynthesis of EPA would become a useful tool for such studies.

5. Acknowledgement

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6. References

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7. Figure legends

Fig. 1. Effects of H₂O₂ on the colony-forming ability (A) and levels of protein carbonyls (B) of *E. coli* UM2 strains with and without EPA under nongrowth conditions. (A) UM2EPA⁻ (▲) and UM2EPA⁺ (●) cells suspended in phosphate-buffered saline were incubated for 30 min in the presence of 0.003, 0.03, 0.3, and 3 mM H₂O₂ under nongrowth conditions and then the number of CFUs was determined. The baseline value of each strain was that of cells treated without H₂O₂. (B) Time-dependent formation of protein carbonyls in *E. coli* UM2 strains with and without EPA that had been treated with and without H₂O₂ under nongrowth conditions. UM2EPA⁻ and UM2EPA⁺ cells suspended in phosphate-buffered saline were treated with and without 0.3 mM H₂O₂. Portions of cell suspensions were withdrawn at various time intervals and protein carbonyls analyzed. Δ, strain UM2EPA⁻ under H₂O₂⁻ conditions; ▲, UM2EPA⁻ under H₂O₂⁺ conditions; ○, UM2EPA⁺ under H₂O₂⁻ conditions; ●, UM2EPA⁺ under H₂O₂⁺ conditions. Cells were incubated at 20 °C. The data indicated are means ± standard errors for three independent experiments.

Fig. 2. Changes of extracellular (A) and intracellular (B) concentrations of H₂O₂ in *E. coli* UM2 strains with and without EPA that had been treated with and without H₂O₂ under nongrowth conditions. Portions of cell suspensions were withdrawn at various time intervals and assayed to determine extracellular and intracellular H₂O₂ concentrations. Δ, strain UM2EPA⁻ under H₂O₂⁻ conditions; ▲, UM2EPA⁻ under H₂O₂⁺ conditions; ○, UM2EPA⁺ under H₂O₂⁻ conditions; ●, UM2EPA⁺ under H₂O₂⁺ conditions. Cells were

incubated at 20 °C. The data indicated are means \pm standard errors for three independent experiments.

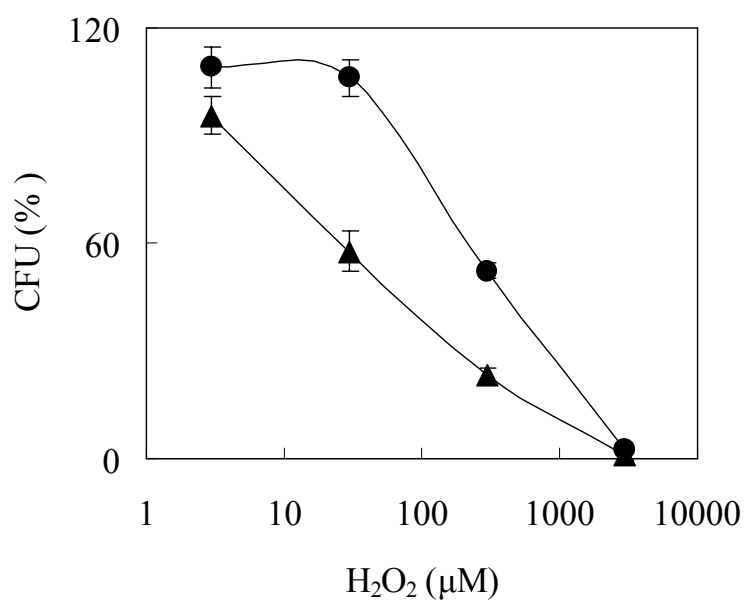
Table 1. Effects of H₂O₂ on the amount of fatty acids recovered from *E. coli* UM2

strains with and without EPA that had been treated with and without H₂O₂ under nongrowth conditions.

Time (min)	Amount of recovered fatty acids (µg/ml cell culture)			
	UM2EPA+		UM2EPA-	
	H ₂ O ₂ +	H ₂ O ₂ -	H ₂ O ₂ +	H ₂ O ₂ -
0	14.9 ± 0.2 (100.0)	14.9 ± 0.2 (100.0)	14.5 ± 1.2 (100.0)	14.5 ± 1.2 (100.0)
10	15.1 ± 0.6 (101.3)	14.6 ± 0.6 (98.2)	12.3 ± 4.0 (84.8)	15.2 ± 0.6 (104.2)
20	13.7 ± 0.4 (91.9)	14.5 ± 0.7 (97.7)	8.4 ± 1.2 (57.6)	14.2 ± 0.7 (97.8)
30	12.4 ± 1.2 (83.5)	14.9 ± 0.8 (100.4)	8.0 ± 1.3 (55.2)	15.0 ± 0.8 (103.1)

Fig. 1.

A



B

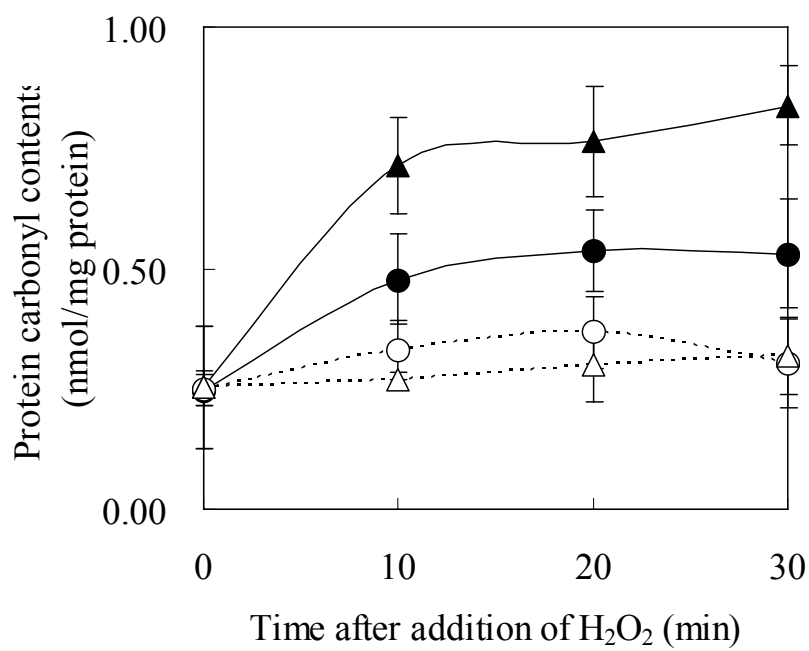
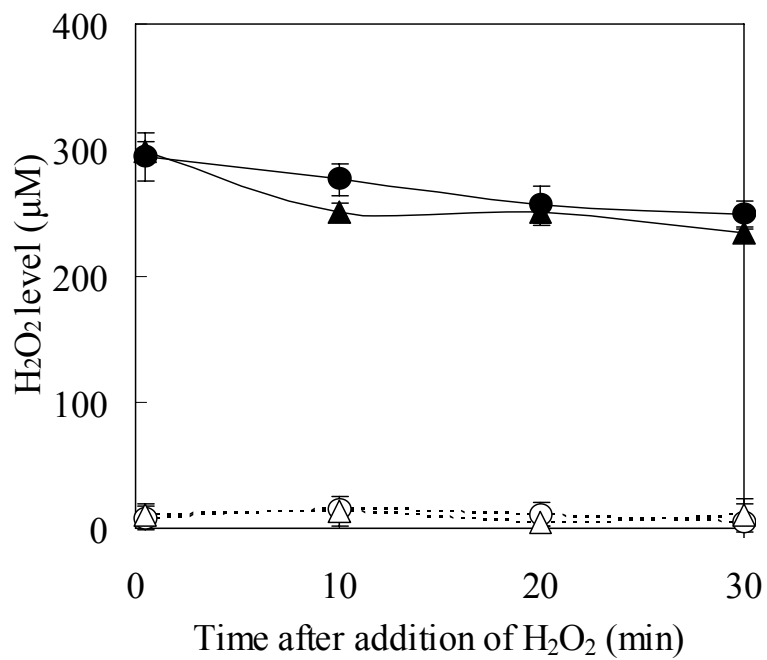


Fig. 2.

A



B

