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Oxygen radical irradiation transforms an organic fertilizer L-tryptophan into an environment and human-friendly bactericide

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ABSTRACT

Organic fertilizers are useful in hydroponic agriculture to bring about a sustainable society with minimal pollution. Irradiation of L-tryptophan (L-Trp) solutions with electrically neutral atomic oxygen produces immediate bactericidal activity. After oxygen radical irradiation of an L-Trp-containing *Escherichia coli* suspension at pH 6.3 for 5 min, a colony-forming unit assay showed a 6-log reduction in the number of surviving *E. coli*. Kynurenine and *N*'-formylkynurenine were identified as irradiation products in the solution using liquid chromatography–mass spectroscopy and nuclear magnetic resonance spectroscopy. Tryptophan radicals (Trp•) are precursors of kynurenine and *N*'-formylkynurenine and are detected using spin-trap electron spin resonance. The bactericidal effect is mediated by central carbon metabolism via enzyme deactivation in *E. coli*. Enzymatic deactivation via the oxygen radical irradiation of a solution containing the amino acid L-Trp eventually resulted in bacterial death.

1. Introduction

Exposure of the Earth's planetary atmosphere to solar wind plasma results in a one-way energy transfer from the sun to the Earth, which involves multiple phenomena with complex, dynamic, and chemical connections. Similarly, nonthermal atmospheric-pressure plasma technology can mimic such plasma interactions using biological and aqueous fluids. The hydroponic cultivation of plants is a remarkable technology that is widely used in sustainable agriculture. However, hydroponic chemicals, such as long-lived bactericides, can have harmful effects on humans and the environment (Damalas and Eleftherohorinos, 2011; Stamati et al., 2016). Cold atmospheric plasma (CAP) and plasma-activated liquids have gained widespread attention owing to their potential for use in new types of hydroponic cultivation (Graves, 2012).

Nonequilibrium atmospheric pressure plasma (NEAPP) can generate instantaneous and short-lived bactericidal effects mediated by ultraviolet (UV) irradiation (Chawla et al., 2021), hydroxyl radicals (OH•) (Moreno-Andres et al., 2018), and ozone (O_3) (Giuliani et al., 2018). NEAPP exhibits synergistic effects involving multiple reactive ion species, electrically neutral radicals, and UV rays in

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aqua (Lukes et al., 2014; Ikawa et al., 2010, 2016). Peroxynitrite chemical reactions (Lukes et al., 2014) and hydroperoxyl radical (HOO•) formations (Ikawa et al., 2010, 2016) are catalyzed by H^+ and require an acidic environment (pH < 4.8), whereas the optimal pH range for hydroponic plant cultivation is 5.5–7.5 (Oshunsanya, 2019).

A radical source of ground-state oxygen atoms (O[^{3}P]) was developed by utilizing high-density electrons of the order of 10^{16} cm^{-3} in Ar/O_2 plasma (Iwasaki et al., 2008), which achieved a very high density of 10^{15} cm^{-3} of O(^{3}P) and a low density of $< 2.5 \times 10^{13}$ cm^{-3} of O_3, without ions or UV irradiation (Iseki et al., 2011; Hashizume et al., 2013). In a previous study, we assessed the effects of oxygen radical-activated L-phenylalanine (L-Phe) on plant growth. The length of the radish sprouts was almost double that in the oxygen radical-irradiated L-Phe solution (Iwata et al., 2019). Furthermore, oxygen radical-irradiated solutions containing L-Phe have antibacterial effects (Iwata et al., 2019). Amino acids (AAs), such as L-Phe, are organic molecules that can directly or indirectly affect physiological activities associated with the growth and development of plants (Aghaei et al., 2019). Among AAs, L-tryptophan (L-Trp) has gained attention because it is a biologically active precursor of auxin, such as indole-3-acetic acid (IAA) which is an essential plant growth regulator. The importance of using L-Trp in agriculture has been reported on previously (Mustafa et al., 2018); therefore, we focused on the effects of oxygen radical-irradiated solutions containing L-Trp.

Here, we report that radical treatments can kill *E. coli* with a 6-log reduction in survival following irradiation with L-Trp solution for 5 min at pH 6.3. The AA L-Trp skeleton comprises benzene and pyrrole or indole. To know an origin of the bactericidal effect, the species generated from L-Trp by oxygen radical irradiation in aqua have been investigated using liquid chromatography (LC)–mass spectrometry (MS), nuclear magnetic resonance (NMR) spectroscopy, and electron spin resonance (ESR) spectroscopy. As well as detection of short-lived Trp radicals, metabolic disorder and enzymatic activity disruption in *E. coli* were eventually observed. Furthermore, we show that oxygen radical irradiation can instantly transform the organic fertilizer L-Trp into a short-lived bactericide that is environmentally and human-friendly.

2. Materials and methods

2.1. Preparation of bacterial suspension containing amino acids or aromatic compounds

Escherichia coli (O1:K1:H7) was pre-cultured in 3 mL of nutrient broth (NB, Difco™, BD) using a bioshaker (BR-23FP, TATEC) at 30 °C and 250 rpm for 17 h, and the cells were then resuspended in phosphate buffer (PB; pH 6.3) after centrifugation at 5,000 g for 3 min (Iwata et al., 2019). A 0.3-mL aliquot of the bacterial suspension was mixed with 2.7 mL of PB containing various AAs (L-Ala, L-Phe, L-tyrosine (L-Tyr), or L-Trp) or aromatic compounds. To measure a viable cell count, the initial concentration of *E. coli* was fixed at approximately 1×10^7 or 1×10^9 mL^{-1}. AA reagents were purchased from Peptide Institute, Inc. (Osaka, Japan). Benzene (99.8%), pyrrole (98%), and indole (99%) were purchased from Sigma-Aldrich Co., LLC (St. Louis, MO, USA).

2.2. Electrically neutral oxygen radical irradiation

A non-equilibrium atmospheric-pressure oxygen radical generator (Tough Plasma FPA10, FUJI Corp., Tokyo, Japan) was used. The device was operated using a gas mixture of Ar (4.97 slm) and O_2 (30 sccm) at the high voltage (Vpp) of 15 kV (60 Hz). Electron and ground-state atomic oxygen [O(^3P_j)] densities in the generated Ar/O_2 plasma were previously reported to be approximately 10^{16} and 10^{15} cm^{-3}, respectively (Iwasaki et al., 2008; Iseki et al., 2011; Hashizume et al., 2013; Iwata et al., 2019). The radical generator was designed to irradiate only electrically neutral oxygen radicals, such as O(^3P_j). Irradiation targets were placed on a movable stage, scanned at a speed of 4 $mm \cdot s^{-1}$ within 15 mm. Next, 3 mL of *E. coli* suspension was dispensed into a φ38-mm dish. The distance between the irradiation slit (0.5×16 mm^2) of the radical generator and the surface of the liquid sample was set at 10 mm. A plastic chamber covering the space between the radical generator and samples was purged with Ar gas to protect the liquid samples from entering the ambient air.

2.3. Colony-forming unit assay

Immediately after radical irradiation, the treated samples were collected and subjected to 10-fold serial dilutions, and 100 μL of each diluted sample was spread onto a nutrient agar medium (Difco™, BD Biosciences, San Jose, CA, USA). After 24 h incubation at 37 °C, colony-forming unit (CFU) assays were performed.

2.4. Fractionation of components in radical-treated L-Trp solutions

UV-HPLC (Prominence, Shimadzu Corp., Kyoto, Japan) was performed using a reverse-phase column (InertSustain AQ-C18, 5 μm, 20 mm × 150 mm, GL Sciences Inc., Cheyenne, WY, USA). A total of 2 mL of a 10-min oxygen radical-irradiated L-Trp (50 mM) solution was injected into the system. Mobile phase (A) comprised deionized (DI) water, and mobile phase (B) comprised methanol (MeOH, HPLC grade, Kanto Chemical Co., Inc., Tokyo, Japan). The gradient conditions were as follows: start and hold at 0% (B) for 10.0 min, increase linearly from 0% (B) to 10% (B) over 2.5 min and hold at 10% for 5.5 min, then increase linearly to 35% (B) over 2.0 min and hold at 35% (B) for 3.0 min, finally decrease to 0% (B) over 2.0 min and hold at 0% (B). The flow rate was set to 8 $mL \cdot min^{-1}$. The eluate from the column was separated into several fractions and concentrated using a rotary evaporator (N-1210; TOKYO RIKAKIKAI Co., Ltd., Tokyo, Japan).

2.5. Bactericidal effect of extracted components

The fractions from the 10-min oxygen radical-irradiated L-Trp (50 mM) solution were re-dissolved in 2 mL of PB (pH 6.3). Next, 1.8 mL of each sample and 0.2 mL of *E. coli* suspension in PB (1×10^8 mL^{-1}) was mixed and incubated for 10 min at 30 °C and 250 rpm using a bio-shaker. The number of surviving *E. coli* cells was evaluated using the CFU assay.

2.6. LC-MS analysis

An Orbitrap mass spectrometer (Exactive Plus, Thermo Fisher Scientific, Waltham, MA, USA) connected to an Ultimate 3000 HPLC system (Thermo Fisher Scientific) with a reverse-phase column (InertSustain AQ-C18, 5 μm, 3 mm × 30 mm, GL Science Inc.) was used. Mobile phase (A) comprised DI water (purified using Milli-Q Integral 10, Merck Millipore, Rahway, NJ, USA), and mobile phase (B) comprised MeOH (LC/MS grade, WAKO CHEMICAL Co., Ltd., Richmond, VA, USA). The gradient conditions were as follows: start at 0% (B) for 2.0 min, increase linearly from 0% (B) to 10% (B) over 0.5 min, hold at 10% for 1.1 min, increase linearly to 25% (B) over 0.4 min, hold at 25% (B) for 0.6 min, and finally decrease to 0% (B) over 0.4 min and hold at 0% (B). The flow rate was set to 0.3 $mL{\cdot}min^{-1}$.

Three milliliters of L-Trp (50 mM) solution was irradiated for several minutes using a radical generator. Fractions from the 10-min oxygen radical-irradiated L-Trp solution were concentrated using a rotary evaporator and then dissolved in 1 mL of DI water. After a further 100-fold dilution of the fractions using DI water, 5 μL was injected into the LC-MS system. An electrospray ionization (ESI) source was used in the positive ion mode. ESI-MS was operated at a voltage of 3.5 kV and a capillary temperature of 350 °C. The possible elemental compositions for the gained mass numbers were estimated using Xcalibur LC-MS analysis software, ver. 4.2.47 (Thermo Fisher Scientific). ChemDraw ver. 19.0 was used to calculate the exact mass.

2.7. NMR spectroscopy

An Avance III HD instrument (600 MHz for ^1H and 151 MHz for ^{13}C; Bruker, Ettlingen, Germany) was used. Dried fractions P1 and P2 were re-dissolved in 0.75 mL of deuterium oxide (D_2O, 99.9% for NMR spectroscopy; Merck Millipore) containing 0.05% acetonitrile (CH_3CN). A total of 2.5 mg each of standard reagents of Kynurenine (KYN) and *N'*-formyl Kynurenine (FKYN) (FUJIFILM Corp.) was dissolved in 0.75 mL of D_2O containing 0.05% CH_3CN. ^1H- and ^{13}C NMR analyses were performed using 32 and 4,000 scans, respectively.

2.8. ESR spectroscopy

For ESR spectroscopy, 3 mL of PB (pH 6.3) with or without L-Trp (45 mM) and a spin-trap agent, 3,5-Dibromo-4-nitrosobenzene sulfonate (DBNBS) (10 mM), was dispensed onto a φ38-mm dish and irradiated for several minutes using an oxygen radical generator. The Fenton reaction was induced to produce OH• radicals, and the reaction between L-Trp and OH• radicals was analyzed using ESR spectroscopy. In a φ38-mm dish, 2.1 mL of PB (pH 6.3) containing L-Trp (50 mM), 0.3 mL of PB (pH 6.3) containing DBNBS (100 mM), 0.3 mL of PB (pH 6.3) containing H_2O_2 (40 mM), and 0.3 mL of PB (pH 6.3) containing $Fe(II)SO_4$ (40 mM) were mixed and incubated at room temperature for 10 min. After incubation, 150 μL of the sample was dispensed into a φ1.1-mm ESR quartz tube (Q-Band) and analyzed using an EMXplus ESR spectrometer (9.4 GHz, X-Band; Bruker).

2.9. TEM analysis

Transmission electron microscopy (TEM) analysis was performed at the Hanaichi UltraStructure Research Institute (HUSRI; Aichi, Japan). A total of 1×10^9 *E. coli* cells mL^{-1} cells were suspended in PB (pH 6.3) containing 0 or 50 mM L-Trp. Next, 3-mL samples of the *E. coli* suspension were irradiated for 0, 2, and 15 min using an oxygen radical generator. The samples were then centrifuged at 14,000 g and 4 °C for 5 min, and the pelleted *E. coli* cells were washed twice with 5 mL of DI water. PB (pH 7.4) containing 2% glutaraldehyde was added to the *E. coli* pellets, which were then kept at 4 °C until delivery to HUSRI. Post-fixation of the samples by treatment with 1% osmium tetroxide at 4 °C for 120 min was performed at HUSRI. Subsequently, *E. coli* was dehydrated using an ethanol series (30–100%) for 15 min, and the pellets were confined in epoxy resin at 60 °C for 48 h and sliced using an ultramicrotome. The samples were stained with uranyl acetate and observed under an H-7600 transmission electron microscope (Hitachi High-Tech Corp., Tokyo, Japan) at an accelerating voltage of 100 kV.

2.10. Metabolome analysis

Metabolome analysis was performed using a facility service at Human Metabolome Technologies, Inc. (Yamagata, Japan). A total of 1×10^9 *E. coli* cells mL^{-1} were suspended in PB (pH 6.3) containing 0- or 50-mM L-Trp. Next, 3-mL samples of the *E. coli* suspension were irradiated for 0, 2, and 15 min using an oxygen radical generator. After the *E. coli* pellets were washed with DI water, 3 mL of NB culture medium was added to each sample and incubated in a shaker at 250 rpm with a regulated temperature of 30 °C for 10 min *E. coli* samples were collected by centrifugation at 14,000g and 4 °C for 5 min. To extract metabolites from the *E. coli* cells, 1.6 mL of methanol was added to dissolve the cells, and then 1.1 mL of an internal standard solution for capillary electrophoresis-mass spectroscopy (CE-MS) was mixed with each sample. After centrifugation at 2300 g and 4 °C for 5 min, the supernatants were ultrafiltered at

9100 g and 4 °C for 3 h. The resulting supernatants were frozen at − 80 °C and transported to Human Metabolome Technologies, Inc. The samples were then dried and redissolved in 25 μL of DI water. Metabolites extracted from *E. coli* were quantified by CE-MS using an Agilent CE-TOF-MS system (Agilent Technologies, Santa Clara, CA, USA).

2.11. Preparation of recombinant E. coli suspension and purified GAPDH

The full-length *E. coli* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (UniProtkb entry: P0A9B2) was PCR-amplified using a T100TM Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Primer sets were designed using gene sequences (Table S1). The PCR conditions were as follows: initial denaturation at 95 °C for 4 min, followed by 34 cycles of denaturation at 95 °C for 2 min, annealing at 60 °C for 5 s, and extension at 68 °C for 10 s. The PCR products were separated on 1% agarose gel, stained with ethidium bromide, and visualized using Molecular Imager FX (Bio-Rad). The amplified *gapA* gene was inserted into a pET28a vector (Invitrogen, Carlsbad, CA, USA) and treated with EcoRI (Invitrogen). The recombinant plasmid was used to transform *E. coli* BL21 (Invitrogen) using the heat-shock method, and the transformants were selected based on kanamycin resistance in Luria-Bertani (LB) broth. The recombinant plasmid (pET28a-*gapA*) was identified by sequencing. *E. coli* cells harboring *gapA* expression plasmids were grown at 37 °C with constant shaking in LB broth supplemented with 100 μg/mL kanamycin until the optical density of the cultures at 600 nm reached 0.6. GAPDH expression was induced by adding 0.1-mM isopropyl β-D-thiogalactopyranoside (IPTG) to the medium, followed by incubation for up to 24 h at 26 °C.

The culture supernatant was discarded after centrifugation at 5,000 g for 10 min, and recombinant *E. coli* exhibiting a survival number of 1×10^9 mL^{-1} were suspended in PB containing 0- or 50-mM L-Trp. The suspension of recombinant *E. coli* was irradiated with an oxygen radical generator.

Purified GAPDH was prepared as follows: Recombinant *E. coli* cells were centrifuged at 3000 g and 20 °C for 5 min, and the pellets were resuspended in a buffer A (50 mM Tris-HCl [pH 8.0], 200 mM NaCl) containing 10% (w/v) glycerol. The cells were lysed by sonication (30 s × 6) using a Q700 sonicator (Qsonica, Melville, NY, USA). After centrifugation at 15,000 g and 4 °C for 5 min, the supernatant was collected and loaded onto a nickel affinity column (HisTrap™ FF, Cytiva, Marlborough, MA, USA) equilibrated with buffer A at 4 °C. Proteins were eluted using a 0–0.3 M imidazole gradient in buffer A, and recombinant GAPDH was purified. Purified GAPDH buffer was exchanged with PB (pH 6.3) containing 50 mM L-Trp using a desalting column (PD-10, Cytiva).

2.12. Measurements of GAPDH activity

A total of 1×10^9 wild-type (O1:K1:H8) or recombinant *E. coli* (BL21) cells mL^{-1} were suspended in PB (pH 6.3) containing 0- or 50-mM L-Trp, and 3 mL of each sample was irradiated using an oxygen radical generator. The samples were then centrifuged at 5000 g and 4 °C for 5 min, and the cells were washed with DI water. GAPDH activity was assayed using the MAK277–1KT kit (Sigma-Aldrich). For the assay, 100 μL of assay buffer contained in the kit and 1 small medicine spatula of zirconia beads were added to each *E. coli* pellet. The *E. coli* samples were then homogenized using a bead crusher (μT-01, TAITEC Corp.) at 4 °C and speed of 4600 rpm for 15 s, with intervals of 15 s repeated 12 times. The samples were centrifuged at 5000 g and 4 °C for 5 min, and the supernatants were recovered into new tubes. For the wild-type *E. coli* samples, 15 μL of the recovered supernatant was diluted with 35 μL of assay buffer in a 96-well plate. For recombinant *E. coli*, 5 μL of the recovered supernatant was diluted with 45 μL of assay buffer, and 50 μL of the reaction mix solution from the assay kit was added to each well. The samples were then incubated at 37 °C for 10 min, and absorption at 450 nm was measured using a microplate reader (SpectraMax ABS plus, Molecular Devices, LLC., San Jose, CA, USA).

2.13. Measurements of Aconitase activity

A total of 1×10^9 wild-type (O1:K1:H8) *E. coli* cells mL^{-1} were suspended in PB (pH 6.3) containing 0- or 50-mM L-Trp, and 3 mL of each sample was irradiated using the oxygen radical generator. The samples were centrifuged at 5000 g and 4 °C for 5 min, and the pelleted cells were then washed with DI water. After washing, 50 μL of assay buffer from an aconitase activity assay kit (K716–100, BioVision Inc., Waltham, MA, USA) was added to each *E. coli* pellet. The samples were centrifuged at 5000 g and 4 °C for 15 min, and the supernatants were discarded. Next, 100 μL of assay buffer and one small medicine spoon of zirconia beads were added to each *E. coli* pellet, and the cells were homogenized using a bead crusher. The samples were centrifuged at 5000 g and 4 °C for 5 min, and the supernatants were recovered into new tubes. Next, 15 μL of each recovered supernatant was diluted with 35 μL of assay buffer in a 96-well plate, and 50 μL of the reaction mix solution was added to each well. The samples were incubated at 25 °C for 10 min, after which 10 μL of the developer contained in the kit was added to each well, and the absorption at 450 nm was measured using a microplate reader.

2.14. SDS-PAGE

The remaining supernatants of wild-type or recombinant *E. coli* or GAPDH solutions irradiated with oxygen radicals with or without L-Trp (50 mM) were analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). For preparation of SDS-PAGE, 30% acrylamide (0.65 mL), DI distilled water (3.05 mL), Tris buffer (0.5 mM, 1.25 mL), 10% SDS (50 μL), 10% ammonium persulfate (APS) (25 μL), and *N*,*N*,*N*',*N*'-tetramethyl ethylenediamine (TEMED) (5 μL) were mixed and then incubated at 70 °C for 7 min. Next, 10 μL of protein extract from wild-type *E. coli* and 5 μL of extracts from recombinant *E. coli* or oxygen radical-irradiated GAPDH solutions were separated by SDS-PAGE at 200 V and 30 mA. A protein molecular weight marker kit (LMW Marker Kit,

Cytiva) was used. The SDS-PAGE gel was stained with Coomassie Brilliant Blue.

3. Results and discussion

3.1. Bactericidal effects of oxygen radical irradiation in the presence of L-Trp

An oxygen radical stream was produced by the generator. The bactericidal effect of oxygen radical irradiation of PB containing 1-mM indole or pyrrole as the skeleton structure of L-Trp (Fig. 1**a**) was observed on the survival of *E. coli* suspended in PB (Fig. 1**b**). Although oxygen radical irradiation in the presence of benzene was maintained at the initial number of survivors, suspensions with the nitrogen heterocyclic compounds, pyrrole and indole, killed *E. coli* completely because no colony formation was observed.

Next, *E. coli* cells were suspended in PB (pH 6.3) containing 1 mM of L-alanine (L-Ala), L-Phe, L-tyrosine (L-Tyr), or L-Trp. Fig. 1**c** shows the bactericidal effects of oxygen radical irradiation on the *E. coli* suspensions containing AAs (Fig. 1**d**). The number of surviving *E. coli* cells in the solution containing L-Trp declined rapidly following oxygen radical irradiation, and a 6-log reduction was achieved after 5-min of irradiation. Although we previously reported the antibacterial effect of oxygen radical-irradiated solutions containing high concentrations of L-Phe (80 mM) (Iwata et al., 2019), the bactericidal effect of L-Phe was negligible under the conditions used in this study (Fig. 1**d**).

The pH of the L-Trp (50 mM) solution changed negligibly to 6.24, after 5 min of irradiation (Fig. S1). The bactericidal effect of oxygen radical irradiation on a bacterial suspension containing L-Trp was achieved within the optimal pH range for plant growth.

3.2. Kynurenine, Trp-derived species

An L-Trp (50 mM) solution subjected to 10-min of oxygen radical irradiation was analyzed using UV high-performance liquid chromatography (HPLC). As shown in Fig. 2**a**, two peaks could be distinguished, hereafter referred to as P1 and P2, with retention times of 26.7 and 29.6 min, respectively. Fractions labeled as "R1, R2, and R3" were evaluated, and none of these fractions reduced the *E. coli* survival number (Fig. 2**b**). Fractions P1 and P2 were assigned 209.0919 and 237.0876 *m/z* (Fig. 2**d**), respectively, using LC-MS analysis (Fig. 2**c**). These ions were assigned as $C_{10}H_{12}O_3N_2 + H^+$ and $C_{11}H_{12}O_4N_2 + H^+$, respectively, and KYN ($C_{10}H_{12}O_3N_2$) and FKYN ($C_{11}H_{12}O_4N_2$) were identified as the L-Trp oxides (Savits, 2020; Stanley et al., 2019). Commercially available KYN and FKYN were analyzed using ^{1}H- and ^{13}C NMR spectroscopy, and the data were compared with the P1 and P2 results. The NMR spectra of P1 and KYN matched perfectly (Fig. S2 **[a, b]**). The spectra of P2 and FKYN were also similar. Multiple resonance peaks were observed because of the rotational isomers (Fig. S2 **[c, d]**). Combined with HPLC peaks P1 and P2 (Fig. 1**c**), KYN and FKYN were identified as

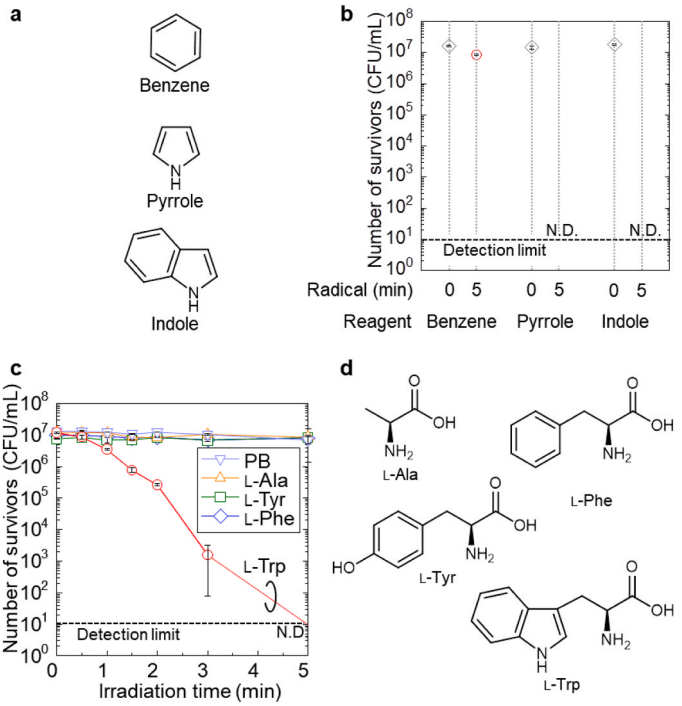

Fig. 1. Oxygen radical irradiation in conjunction with nitrogen heterocyclic compounds and amino acid L-Trp kills bacteria in neutral-pH solutions. **a**, Chemical structures of benzene, pyrrole, and indole. **b**, Number of surviving *E. coli* suspended with 1 mM benzene, pyrrole, or indole after 5-min oxygen radical irradiation (n = 3). **c**, Reduction in the number of surviving *E. coli* suspended in AA-containing solutions (n = 3) including alanine (L-Ala), phenylalanine (L-Phe), tyrosine (L-Tyr), and tryptophan (L-Trp). N.D: not detected. **d**, Chemical structures of AAs.

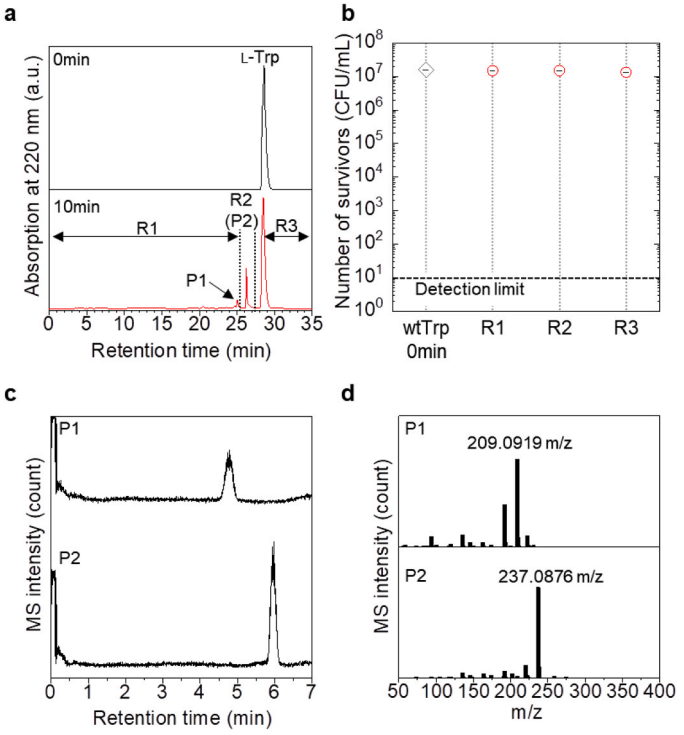

Fig. 2. HPLC analysis of an L-Trp (50 mM) solution subjected to a 10 min of oxygen radical irradiation **a**, UV chromatogram of 0- or 10-min irradiated L-Trp solutions. **b**, No bactericidal effect was observed in the collected products labelled P1 and P2. Reduction in the number of surviving *E. coli* suspended in R1, R2, or R3 fractions (n = 3). **c**, LC–MS chromatograms of fractionated P1 and P2. **d**, MS spectra of P1 and P2.

Trp-derived products resulting from oxygen radical irradiation of the L-Trp solution.

3.3. Trp radicals play a key role in the bactericidal effect

KYN and FKYN are produced by Trp oxidation via superoxide anions ($O_2^{\bullet}$) as a reactive oxygen species (ROS), according to Carrolla et al. (2018). Initially, the oxidation of Trp produces a tryptophan free radical (Trp•) with a dioxetane counterpart (Trp• + OO). The dissociation of the O–O bond in dioxetane induced the formation of FKYN and KYN.

Using a spin-trapping reagent (DBNBS), ESR analysis of a 3-min oxygen radical-irradiated L-Trp (45 mM) solution showed a distinct triplet spin adduct signal with a hyperfine coupling constant, a^N, of approximately 13 G (Gunther et al., 1995; Timmins et al., 1997) (Fig. 3**a**, Fig. S3c). The signal intensity increased with the increasing duration of oxygen radical irradiation of the L-Trp solution (Fig. S3a). Notably, signals could not be observed in the absence of L-Trp and DBNBS in the solution (Fig. S3b). Thus, the generation of Trp• during oxygen radical irradiation of the L-Trp solution was confirmed.

Overall, these data indicate that oxygen radical irradiation of L-Trp solution produces KYN and FKYN via the formation of short-

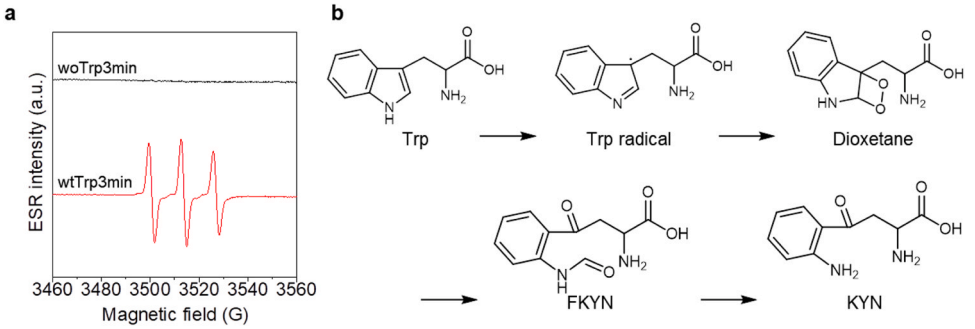

Fig. 3. Oxygen radical irradiation of L-Trp solution produces KYN and FKYN via the formation of short-lived Trp• as a bactericidal species. KYN and FKYN are produced by Trp oxidation. **A**, ESR signals of oxygen radical-irradiated PB containing DBNBS with and without 45 mM L-Trp. **B**, Pathway of KYN and FKYN production via oxygen radical irradiation.

lived Trp• as a potential candidate of bactericidal species. The path of Trp• generation by the oxygen radical irradiation of the L-Trp solution is illustrated in Fig. 3**b**.

3.4. Metabolic disorders in E. coli

Bactericidal effects were assessed by oxygen radical irradiation of 50 mM L-Trp solution containing 1×10^9 mL^{-1} *E. coli* (Fig. 4**a**). Approximately 1.1- and 6.5-log reductions in the number of *E. coli* cells occurred with oxygen radical irradiation of the L-Trp solution for 2 and 15 min, respectively. A 15-min oxygen radical irradiation of the solution without L-Trp had a negligible effect on the survival number.

Transmission electron microscopy (TEM) analysis revealed that the morphology of *E. coli* suspended in L-Trp was generally intact after 15-min of oxygen radical irradiation (Fig. 4**b**). However, the inner components of the bacteria were smaller, presumably leading to cell death.

Metabolites of *E. coli* present in solution were compared between oxygen radical-irradiated and non-irradiated *E. coli* in solution without L-Trp (Fig. 5**a**). A heatmap shows changes in the levels of the 149 detected metabolites (Fig. 5**b**) by category: (i) metabolites of the tricarboxylic acid (TCA) cycle changed significantly after 2-min of irradiation, and (ii) metabolites of glycolysis declined dramatically after 2- and 15-min of irradiation (Fig. 5**c**). Among the TCA metabolites (Fig. 5**d**), higher levels of acetyl CoA, citric acid, and malic acid were detected following 2-min of irradiation, but the levels of these metabolites declined significantly following 15-min of oxygen radical irradiation. Among the glycolysis metabolites, the levels of 3-phosphoglyceric acid, 2-phosphoglyceric acid (2-PG), and phosphoenolpyruvic acid were significantly reduced by 2- and 15-min irradiation with solutions containing L-Trp (50 mM). These data indicate that oxygen radical irradiation affects central carbon metabolism in *E. coli* suspended in solutions containing L-Trp.

3.5. Enzyme deactivation in E. coli

Enzymes involved in glycolysis and the TCA cycle, such as GAPDH and aconitase, are widely distributed in microorganisms (Rosenberg and Arnon, 1955; Beinert et al., 1996). The activities of GAPDH and aconitase in cell extracts prepared from *E. coli* cultured in oxygen radical-irradiated solution with L-Trp were significantly reduced by 2- and 15-min oxygen radical irradiation compared to those in the unirradiated solution and solution without L-Trp irradiated with oxygen radicals for 15 min (Fig. 6 **[a, b]**). Protein expression patterns in *E. coli* did not change significantly (Fig. 6**c**); thus, oxygen radical irradiation of the L-Trp solution diminished the activity of these enzymes in *E. coli*.

The number of surviving recombinant *E. coli* cells expressing GAPDH with a 6 × histidine tag was reduced by oxygen radical irradiation, as was the activity of GAPDH in cell extracts prepared from recombinant *E. coli* (Fig. 7 **[a, b]**). To evaluate the reduction in purified GAPDH activity, recombinant *E. coli* cells or cell extracts prepared from recombinant *E. coli* were suspended in PB (pH 6.3) containing 0- or 50-mM L-Trp and irradiated using an oxygen radical generator. After homogenization of recombinant *E. coli*, the cell extracts were analyzed by SDS-PAGE (Fig. 7**c**). Purified GAPDH was also analyzed (Fig. 7**d**). GAPDH expression patterns in recombinant *E. coli* did not change significantly (Fig. 7 **[c, d]**). A reduction in GAPDH activity was observed in cell extracts (Fig. 7**c**) prepared from recombinant *E. coli* in PB (pH 6.3) containing 50 mM L-Trp irradiated with oxygen radicals.(data not shown) Similar results were obtained when purified GAPDH was used as the irradiated sample (Fig. 7**d**). These results indicate that GAPDH was deactivated by oxygen radical irradiation in L-Trp solutions.

Furthermore, a reduction in aconitase activity was observed in *E. coli* cells suspended in PB (pH 6.3) containing 50 mM L-Trp after irradiation with oxygen radicals for 2- and 15-min. Aconitase catalyzes the conversion of citric acid to cis-aconitic acid during TCA

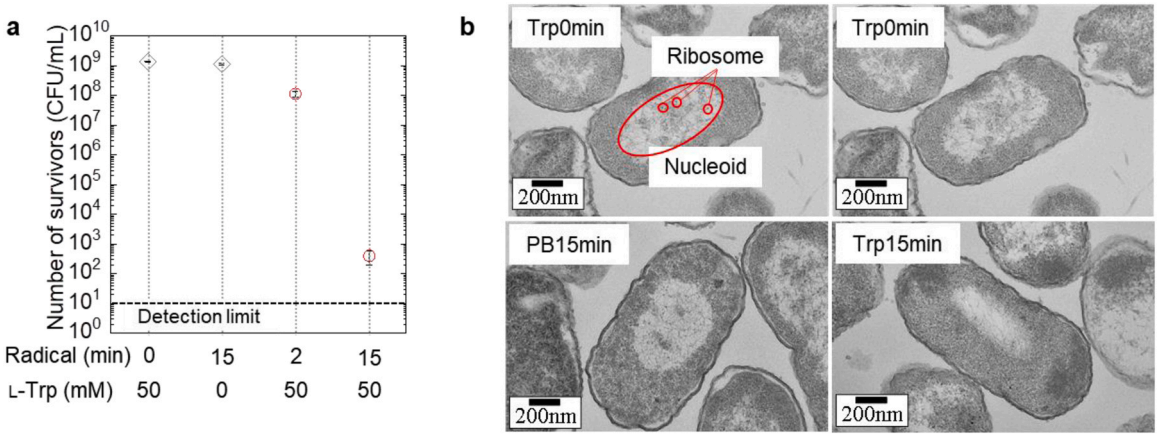

Fig. 4. The morphology of *E. coli* was intact however the inner components were altered by oxygen radical irradiation of L-Trp solution. **a**, Reduction in number of surviving *E. coli* suspended in 0- or 50-mM L-Trp solution (n = 3). Initial *E. coli* number and radical treatment time were fixed at 1×10^9 mL^{-1}. **b**, TEM images of *E. coli* after oxygen radical irradiation.

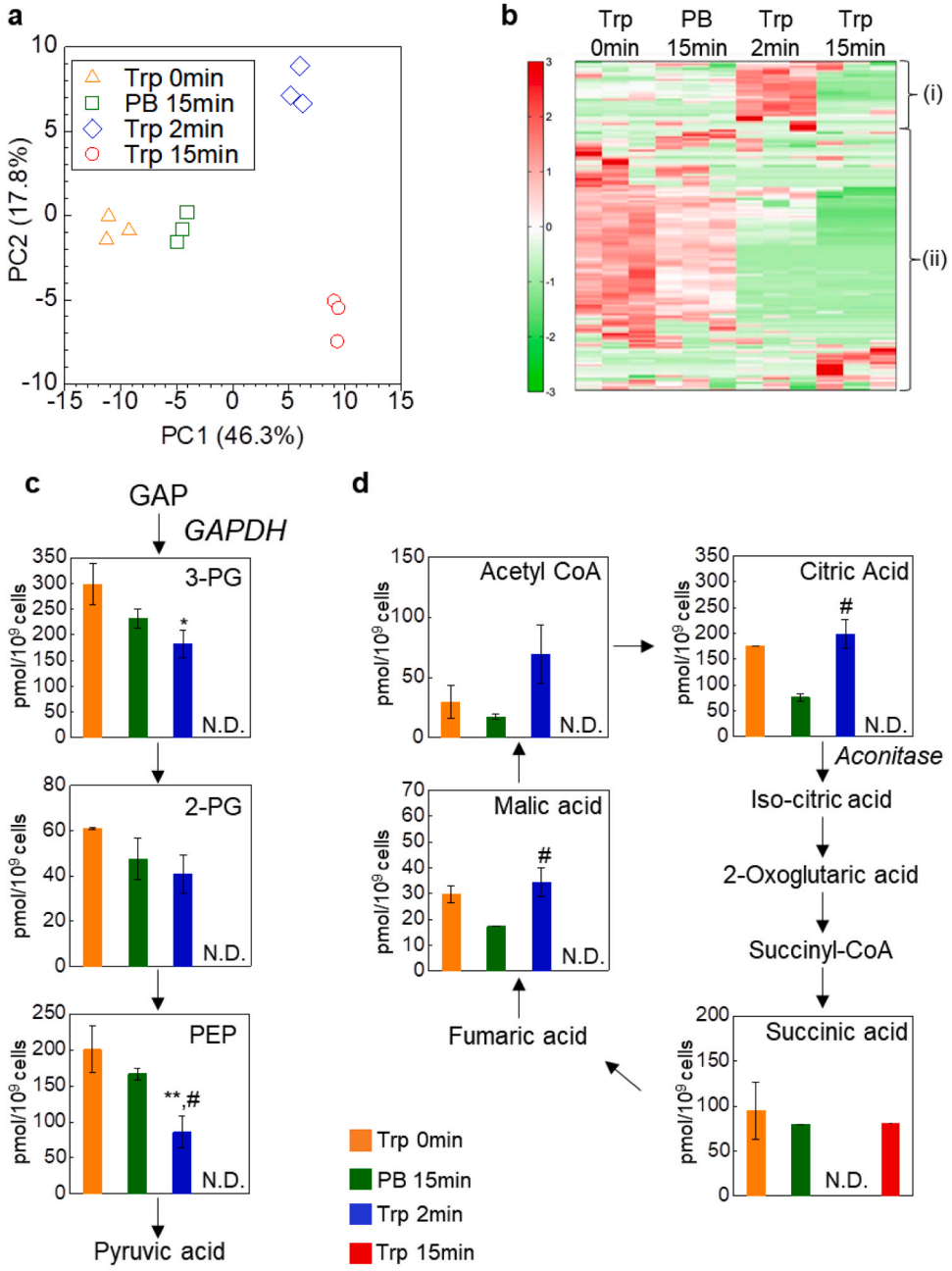

Fig. 5. Metabolic disorders in *E. coli* induced by oxygen radical irradiation in conjunction with L-Trp. **a**, PCA results of *E. coli* samples used for metabolomic analysis (n = 3). **b**, Heatmap of detected metabolites (n = 3). Green and red colors indicate a decrease and increase, respectively, in the mean value of each metabolite. **c** and **d**, Concentration (pmol/10^9 cells) of detected metabolites involved in glycolysis and the TCA cycle (n = 3). In **c** and **d**, datasets were compared using an unpaired Welch's *t*-test (* $P < 0.05$, ** $P < 0.01$ vs. 0 min with L-Trp [50 mM] and #$P < 0.05$ vs 15 min with 0 mM L-Trp).

cycle. Hydratase deactivation coincides with the stagnation of TCA cycle metabolites in *E. coli* suspended in oxygen radical-irradiated Trp-containing PB solution. In consideration of the results described so far, we concluded that the main bactericidal mechanism for oxygen radical irradiation in the presence of L-Trp was a metabolic disorder in the activities of GAPDH for glycolysis and aconitase in the TCA cycle.

Previously, we assessed the effects of oxygen radical-activated L-Phe on plant growth (Iwata et al., 2019). The simultaneous achievement of antimicrobial properties and plant growth promotion by the oxygen radical activation of amino acids can be of valuable use in agricultural applications without the use of any chemical fertilizer or agrochemicals, replacing them with electro-discharges to obtain instantaneous bactericidal effects. In other words, the use of oxygen radicals and L-Trp is promising for

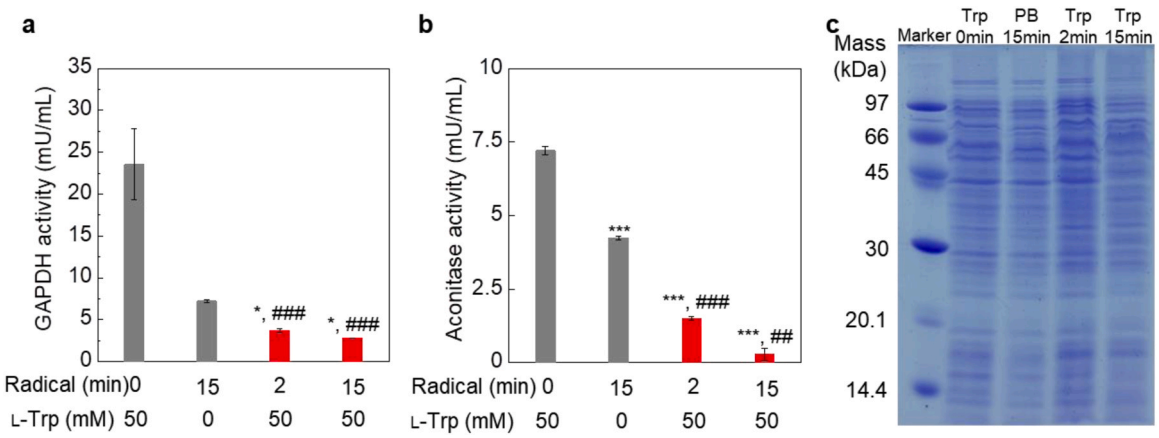

Fig. 6. Only enzymatic activity is diminished by oxygen radical irradiation of the L-Trp solution. Analysis of enzymes in *E. coli*. **a**, GAPDH activity of wild-type *E. coli* suspended with 0- or 50-mM L-Trp (n = 3). **b**, Aconitase activity of wild-type *E. coli* suspended with 0- or 50-mM L-Trp (n = 3). **c** No significant change in SDS-PAGE of cell extracts. In **a** and **b**, datasets were compared using an unpaired Welch's *t*-test (* $P < 0.05$, ** $P < 0.01$ vs 0 min with L-Trp [50 mM] and #$P < 0.05$ vs 15 min with 0 mM L-Trp). **c**, SDS-PAGE of wild-type *E. coli* samples used for metabolome analysis.

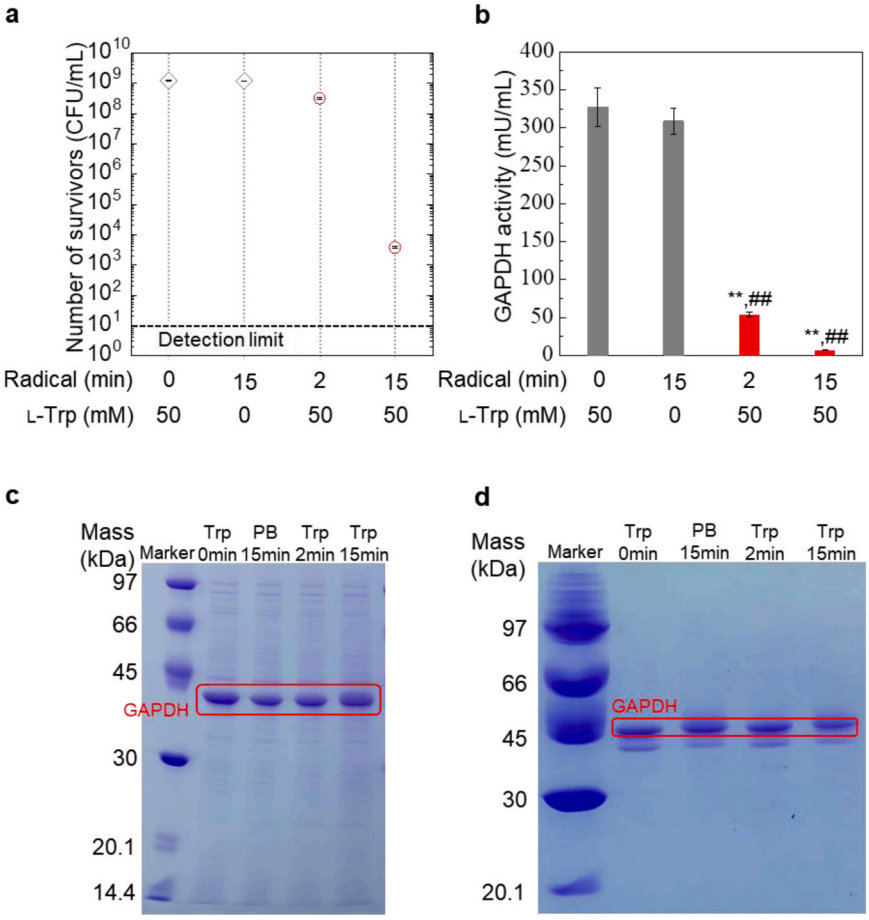

Fig. 7. Evaluation of GAPDH activity using recombinant *E. coli*. **a**, Number of surviving GAPDH-recombinant *E. coli* suspended with 0- or 50-mM L-Trp (n = 3). **b**, GAPDH activity of recombinant *E. coli* suspended with 0- or 50-mM L-Trp (n = 3). **c**, SDS-PAGE of cell extracts of GAPDH-recombinant *E. coli*. **d**, SDS-PAGE of purified GAPDH solutions containing 0- or 50-mM L-Trp.

simultaneous solutions in agriculture.

4. Conclusions

This study demonstrated the bactericidal effect of oxygen radical irradiation on bacterial suspensions in L-Trp-containing solutions. LC-MS and NMR analyses of the oxygen radical-irradiated L-Trp solution revealed that KYN and FKYN were the main products therein. A short-lived precursor (Trp•) of KYN and FKYN was detected in the oxygen radical-irradiated L-Trp solution by ESR spectroscopy and was speculated as a bactericidal species. Lethal metabolic disorders of glycolysis and TCA cycle are induced in *E. coli* by oxygen radical irradiation in the presence of L-Trp. The activities of GAPDH in glycolysis and aconitase in the TCA cycle were significantly reduced by oxygen radical irradiation in the presence of L-Trp. These metabolic disorders, which occurred via enzyme deactivation, are considered the main bactericidal mechanisms of oxygen radical irradiation in an L-Trp-containing solution.

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Author Contributions

N.I. carried out and designed the majority of the experiments in this study, including the CFU assays, HPLC, LC-MS NMR and ESR spectroscopy, evaluation of enzyme activities, SDS-PAGE, sample preparation for TEM analyses, metabolome and LC-MS analyses of enzymes, and statistical analyses. K.I. provided technical assistance for the ESR analysis. Y.N. provided advice and assistance with the HPLC, LC-MS, and NMR analyses. H.K. prepared GAPDH-recombinant *E. coli*. M.S. and M.K. provided technical advice and assistance for SDS-PAGE and LC-MS analysis of enzymes. M.I. provided technical advice regarding the selective irradiation of electrically neutral oxygen radicals. M.H. supervised N.I. and provided critical review. N.I., K.I., Y.N., and M.S. wrote the manuscript. All authors contributed to interpretation of the data.

CRediT authorship contribution statement

Kato Hiroyuki: Investigation. **Nishikawa Yasuhiro:** Resources, Investigation. **Kato Masashi:** Supervision, Investigation. **Shimizu Motoyuki:** Supervision, Investigation. **Ishikawa Kenji:** Writing – review & editing, Visualization. **Iwata Naoyuki:** Writing – original draft, Investigation. **Tanaka Hiromasa:** Resources. **Hori Masaru:** Resources, Funding acquisition. **Ito Masafumi:** Project administration, Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Kenji Ishikawa reports was provided by Nagoya University. Kenji Ishikawa reports a relationship with Nagoya University that includes: employment. Kenji Ishikawa has patent pending to Nagoya University.

Data Availability

Data will be made available on request.

Supporting Information

Bactericidal effect of oxygen-radical treatment of bacterial suspension containing pyrrolic compounds.; NMR spectra of fractionated P1 and P2.; ESR spectroscopy analysis of oxygen-radical–irradiated L-Trp solutions.; and Evaluation of GAPDH activity using recombinant *E. coli*. (PDF).

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.eti.2023.103496.

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Further reading

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