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Ozone detoxification of patulin in aqueous solution and cytotoxic evaluation using human hepatic carcinoma cells

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ABSTRACT

Patulin often contaminates fruits and fruit-base products, which seriously threats the health of consumers. In this study, ozone was used to degrade patulin in aqueous solution, and investigated the cytotoxicity of patulin after ozone detoxification on human hepatic carcinoma cells (HepG2) using MTT assay and apoptosis assay. Patulin was rapidly degraded from 24.59 mg/L to 9.85 mg/L within 180 s by 10.60 mg/L of ozone at a flow rate of 90 mL/min, and reduced by 59.94%. The half maximal inhibitory concentration (IC_{50}) of patulin on HepG2 cells was 9.32 μmol/L after 24 h of exposure, and it showed a dose dependent effect. After 90 s of ozone detoxification, the cell viability of HepG2 cells obviously increased from 42.31% to 93.96%, and the total apoptotic cells significantly reduced from 22.24% to 11.18% after 180 s of ozone treatment. The results clearly show the great potential of ozone in degrading patulin in liquid foods.

1. Introduction

Patulin is a toxic secondary metabolite mainly produced by *Penicillium expansum*, which is a postharvest chemical contaminant in fruits and fruit-base products (Iqbal et al., 2018). It has been verified the carcinogenicity, teratogenicity, mutagenicity, neurotoxicity, and immunotoxicity by inducing DNA stand breaks, chromosome aberrations, and micronuclei formation in experimental animals and cells (Alves et al., 2000; Donmez-Altuntas et al., 2013; Pal et al., 2017; Puel et al., 2010; Saxena et al., 2011; Wichmann et al., 2003; Zouaoui et al., 2016). To reduce the risks of patulin to human health, physical, chemical, and biological methods have been used to remove or degrade it from contaminated foods (Hao et al., 2016; Ibarz et al., 2017; Janotová et al., 2011; Li et al., 2017; Moake et al., 2005; Peng et al., 2016; Zhang et al., 2016; Zhu et al., 2015). Among these methods, ozone as a novel method, has been studied to degrade patulin in foods by several researchers due to its advantages in high detoxification efficiency, low cost, environmentally-friendly without toxic residues, good safety, and easy operation in recent years (Diao et al., 2018b; Miller et al., 2013).

It is well known that patulin in foods is a risk to human health, while the ozonolysis products of patulin and their safety are also very concerned by people. It is therefore essential to assess the toxicity of the degradation products of patulin. Many researchers have evaluated the safety of patulin using cytotoxicity assays after being treated by UV irradiation, adding sulfhydryl compounds (cysteine and glutathione), bacterial or yeast fermentation/adsorption, enzymolysis (Appell et al., 2011; Chandra et al., 2017; Diao et al., 2018a; Lieu and Bullerman, 1978; Tannous et al., 2016; Zhu et al., 2015). However, the safety of patulin after ozone detoxification has not been evaluated. Therefore, the objective of this study was to investigate the safety of patulin in aqueous solution after ozone detoxification by cytotoxicity assay using human hepatic carcinoma cells (HepG2), to infer the IC_{50} of patulin on HepG2 cells, and to further verify the ozone degradation efficacy of patulin.

2. Materials and methods

2.1. Materials and cells

The standard Patulin [4-hydroxy-4H-fuo (3,2-c)-pyran-2-(6H)-one] (purity > 98%) and ethyl acetate were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). LC-grade acetonitrile and formic acid were obtained from Oceanpark Company (Sweden) and Kermel Chemical Reagent Company (China), respectively. Dimethyl sulfoxide (DMSO) was purchased from Sigma (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) and penicillin-streptomycin were obtained from Gibco (Thermo Fisher Scientific Grand Island, Shanghai, China). Fetal bovine serum (FBS) was provided by Corning (Medford, MA,

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USA). Trypsin-EDTA (0.25%) was obtained from Gibco (Burlington, Ontario, Canada). Phosphate-buffered saline (PBS, pH 7.4) tablets were purchased from Amresco (Solon, OH, USA). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) was obtained from Biofroxx (Einhausen, Hessen, Germany). Annexin V-FITC apoptosis detection kit was purchased from BD Biosciences (Sparks, MD, USA). Deionized water was used in all experiments. HepG2 cells were provided by the American Type Culture Collection (ATCC, Manassas, VA, USA).

2.2. Determination of patulin and ozone treatment

Determination of patulin was performed using the HPLC-UV method reported by Li et al. (2005) with little modification. Patulin aqueous solutions with and without ozone detoxification passed through 0.22 μm filter membrane and injected into the HPLC system.

The analysis was done under the followed conditions: the analytical column was Waters XBridge TM (100 × 4.6 mm i.d., 3.5 μm C18 stationary phase); mobile phase was acetonitrile–0.1% formic acid aqueous solution (5:95, v/v) with a flow rate of 0.75 mL/min; the wavelength of UV detector was set at 276 nm; the injection volume was 20 μL.

A self-developed ozone detoxification reactor was used to degrade patulin in aqueous solution (Diao et al., 2018b). The initial patulin concentration was 24.59 ± 0.22 mg/L determined by HPLC-UV. Throughout these experiments, ozone concentration and its flow rate were 10.60 mg/L and 90 mL/min, respectively, which were used to treat 2 mL of patulin solution each time for 0–180 s with an interval of 30 s at the room temperature. The sample without adding patulin was used as the control. All samples were capped and stored immediately in refrigerator at 4 °C.

2.3. Cell culture and cytotoxicity assay

The HepG2 cells were grown in DMEM medium supplemented with 10% heat-inactivated FBS and 1% antibiotic solution (100 U/mL penicillin and 100 μg/mL streptomycin) at 37 °C in the 5% CO_2 incubator (Thermo Fisher Scientific, Waltham, MA, USA). The MTT colorimetric assay was used to determine the IC_{50} value of patulin and its cytotoxicity on HepG2 cells.

Patulin solutions with different concentration (1, 2, 4, 6, 8, 10, 15, 20, and 30 μmol/L) were prepared by dissolving the DMSO solution of patulin (100 μmol/L) in DMEM medium. HepG2 cells at a concentration of 2×10^5 cells/well were seeded in 96-well flat-bottom plates containing 100 μL of DMEM medium. After adhering for 16–20 h, HepG2 cells were treated with different concentrations of patulin containing fresh DMEM medium, and incubated for an additional 24 h. Afterwards, DMEM medium was discarded and the cells received 100 μL of MTT solution (0.5 mg/mL in DMEM medium without FBS). All the plates were incubated at 37 °C for 4 h, and then the formazan crystal was dissolved in 100 μL of MTT stopping buffer for overnight. Finally, the absorbance was measured at a wavelength of 550 nm using a spectrophotometric microplate reader (Infinite M200 Pro, Tecan, Switzerland). The viability of hepG2 cells was calculated using the following formula:

$$\text{Cell viability}\ (\%) = (A_{\text{Sample}} - A_{\text{Blank}})/(A_{\text{Control}} - A_{\text{Blank}}) \times 100 \quad (1)$$

And the growth inhibition was obtained as the formula:

$$\text{Growth inhibition}\ (\%) = 100\ \% - \text{Cell viability}\ (\%) \quad (2)$$

The IC_{50}, a concentration of patulin at which 50% of growth inhibition rate of HepG2 cells, was calculated by a dose-response curve using Origin 8.0 (OriginLab corporation, USA).

2.4. Apoptosis assay

HepG2 cells (6×10^5 cells/well) were seeded in six-well plates, and cultured in the 5% CO_2 incubator at 37 °C for 24 h. Next, cells were pretreated with patulin for 24 h after ozone detoxification at different times (0–180 s with an interval of 30 s). The morphological observation of HepG2 cells was done under an inverted microscope (Olympus, Tokyo, Japan).

The above-treated HepG2 cells were digested by trypsinization with 0.25% EDTA and centrifuged at 1200 rpm for 3 min, and the cells were collected. They were washed twice with pre-cooled PBS and re-suspended in 100 μL of 1 × binding buffer. After simultaneous incubation with 5 μL of Annexin V-FITC and 5 μL of propidium iodide (PI) for 15 min in the dark, the apoptosis was detected using a C6 Plus flow cytometer (BD Biosciences), and the data were analyzed with FlowJo (ver. 10.0.7, Tree Star, Ashland, OR, USA).

2.5. Statistical analysis

All experiments were conducted in triplicate, and the results were expressed as the means ± standard deviation (SD). Analysis of variance (ANOVA) was carried out to determine any significant difference ($P < 0.05$) among the applied treatments using the SPSS 18.0 software (IBM, Chicago, USA).

3. Results

3.1. Ozone detoxification of patulin in aqueous solution

The degradation of patulin by ozone at different treatment times is shown in Fig. 1. As indicated in Fig. 1, the patulin was significantly reduced with the increase of ozone treatment times ranging from 0 to 180 s ($P < 0.05$). It was decreased from 24.59 ± 0.22 mg/L for the control to 12.28 ± 0.11 mg/L within 150 s at 10.60 mg/L of ozone concentration and 90 mL/min of flow rate, and resulted in a reduction of 50.06%. After 180 s of ozone detoxification, the residual patulin was 9.85 ± 0.21 mg/L with a reduction of 59.94%. The degradation of patulin by ozone followed a zero-order kinetic modeling with a correlation coefficient (R^2) of 0.9925. Based on the regression equation ($y = -0.0795x + 24.449$), the initial patulin in aqueous solution (24.59 mg/L) can be completely degraded by ozone within 308 s under the detoxification conditions above-mentioned.

3.2. Cytotoxicity of patulin after ozone detoxification

HepG2 cells were exposed to the increasing patulin concentrations ranging from 0 to 30 μmol/L for 24 h, and cell viability was measured by MTT assay. The results from MTT assay indicated the cytotoxic

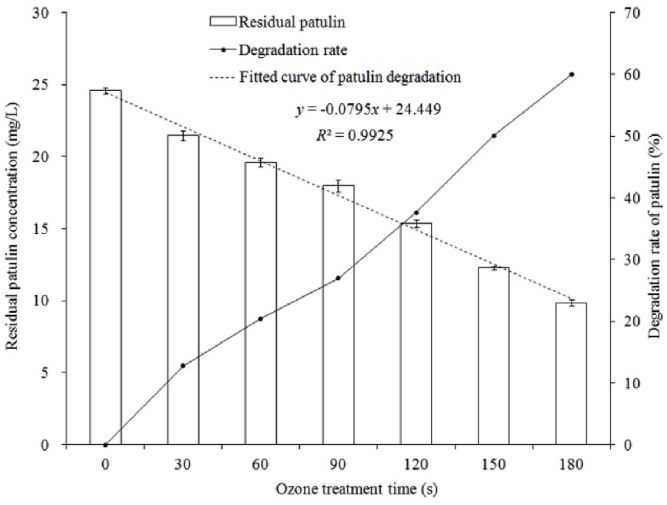

Fig. 1. Degradation of patulin by ozone at different treatment times.