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Improved bactericidal capacity of UV-B radiation against *E. coli* strains by photosensitizing bacteria with fructosazine – An advanced Maillard reaction product

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ABSTRACT

This study investigated the effect of UV-B irradiation and the combinational effect with glucosamine caramel, fructosazine and riboflavin on the antimicrobial activities against *Bacillus subtilis* (ATCC 6633) and two strains of *Escherichia coli* (AW 1.7 and ATCC 25922). The quantum yield of fructosazine was two times less than that of tryptophan, indicating its ability to emit fluorescent light but less efficiently than tryptophan. UV-B treatment alone was efficient to achieve a bactericidal effect for both *E. coli* stains tested, however no effect was found for *Bacillus subtilis* for up to 80 mJ/cm^2 UV-B. The combination of UV-B with photosensitizers fructosazine, glucosamine caramel and riboflavin enhanced the UV-B efficacy against *E. coli* strains at lower UV-B doses, while *Bacillus subtilis* ATCC 6633 was more resistant to the treatment combinations. High-performance liquid chromatography showed the production of different fructosazine reaction products occurred during irradiation, including the possible formation of endoperoxides.

1. Introduction

Glucosamine (GlcN) is an alpha-aminocarbonyl compound (also known as a Heyns compound) which can be synthesized by reacting fructose and ammonia through the Maillard reaction or by the hydrolysis and deacetylation of chitin. A Heyns compound like GlcN is very unstable and can undergo non-enzymatic browning at moderate temperatures (37–50 °C), producing a plethora of chemical compounds. These compounds can possess interesting bioactivities (Hrynets, Bhattacherjee, Ndagijimana, Hincapie Martinez, & Betti, 2016). For instance, the formation of fructosazine (2,5-bis-(D-*arabino*-1,2,3,4-tetrahydroxybutyl)pyrazine)) and deoxyfructosazine (2-(D-*arabino*-tetrahydroxybutyl)-5-(D-*erythro*-2,3,4-trihydroxybutyl))pyrazine) *via* the condensation reaction of GlcN at 50 °C, followed by a role as antimicrobial agents were shown (Bhattacherjee, Hrynets, & Betti, 2016). Specifically, the action of fructosazine against heat resistant *E. coli* AW 1.7 at pH 5 was shown to damage the outer bacterial membrane due to acetic acid. Damage was also caused by a fructosazine-induced production of reactive oxygen species (ROS) like singlet oxygen ($^1O_2$) within bacterial cells due to a type II photochemistry process.

Chemically, fructosazines are hydroxyalkyl pyrazines and therefore can be classified as heterocyclic Maillard reaction products; these are collectively known as advanced Maillard reaction products (AMPs). Some of these compounds are recognized for their photosensitizing property under UV light (Aragon, Silva, & Morliere, 2003; Fuentealba, Galvez, Alarcon, Lissi, & Silva, 2007; Masaki, Okano, & Sakurai, 1999; Okano, Masaki, & Sakurai, 2001).

Photosensitization is a light-activated reaction where a photoexcited chromophore – a photosensitizer – reacts with molecular oxygen to generate ROS (Delcanale et al., 2016). Melanoidins are another well-studied product of the Maillard reaction which also possess strong photosensitizing properties (Argirova, 2005). Specific chromophores in glycated proteins can also function as photosensitizers under UV radiation (de La Rochette, Birlouez-Aragon, Silva, & Morlière, 2003). This photosensitizing property of pyrazines has also been reported previously (Markham & Sammes, 1976), where the formation of endoperoxide compounds through the 1,4-addition of $^1O_2$ across two double bonds was demonstrated. Hence, fluorophore AMPs may potentially serve as valuable photosenstizers that can photo-inactivate bacteria when exposed to specific wavelengths. Fructosazine and

Abbreviations: ESI, electrospray ionization; GRAS, generally recognized as safe; GlcN, glucosamine; HPLC, high-performance liquid chromatography; AMPs, advanced Maillard reaction products; MS, mass spectrometry; PDT, photodynamic therapy; ϕ, quantum yield; ROS, reactive oxygen species; SOSG, Singlet Oxygen Sensor Green; $^1O_2$, singlet oxygen; SD, standard deviation

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deoxyfructosazine are also used as flavorants in tobacco smoke and they have also been found in roasted peanuts, caramel and soy sauce (Magaletta & Ho, 1996; Moldoveanu, Byrd, & Gerardi, 2011; Tsuchida, Komoto, & Mizuno, 1990; Tsuchida, Morinaka, Fujii, Komoto, & Mizuno, 1986). Heineken, the multinational beer corporation, also suggested the use of the "colourless caramel" containing these heterocyclic compounds to stabilize beer against light induced off-flavour (Van Der Ark et al., 2013). The authors hypothesized that the ability of the fructosazine in stabilizing the flavor and color of beer is due to its ability to absorb UV light at 275–280 nm.

In general, the photochemical processes can be divided broadly into two pathways: type I and type II. Type I sensitization deals with the production of free radicals *via* electron or hydrogen transfer from the excited triplet state of the photosensitizers to the surrounding substrates. In type II, a triplet photosensitizer transfers energy to only a limited number of molecules – only those with triple state multiplicity. Triplet O_2 is often the major substrate in the type II pathway as its ground state is already in its triplet state, but with a lower energy than the photosensitizer triplet state. Hence in pathway II the photosensitizer passes the energy to the triplet O_2, forming $^1O_2$, which can then react with the surrounding biomolecules, such as proteins, nucleic acids, lipids, etc. (Argirova, 2005; de La Rochette, et al., 2003; Masaki et al., 1999). The photosensitizer-mediated process is generally termed as photodynamic therapy (PDT) and it can utilize visible or UV-A and B light energy in combination with a photosensitizing agent to induce phototoxic reactions and further assist in killing bacterial cells (Hamblin & Hasan, 2004). PDT is a promising technique and can be used against viruses, fungi and against localized infections (Buchovec et al., 2017). Acridine hydrochloride was one of the first compounds used for the photoinactivation of microorganisms. Several compounds including phenothiazine, methylene blue, toluidine blue and phthalocyanines have been successfully used for the photoinactivation of both Gram-negative and positive bacterial strains (Jori, 2006; Wainwright, Phoenix, Laycock, Wareing, & Wright, 1998). The photoinactivation of microorganisms does not only depend on phototoxic capacity of the photosensitizer, but also on the morphological characteristics of the microorganism. It has been reported that Gram-positive *Staphylococcus aureus* was more vulnerable to PDT compared to Gram-negative *Pseudomonas aeruginosa*, even though *S. aureus* possesses a thicker peptidoglycan layer (Jori, 2006; Merchat, Bertolini, Giacomini, Villanueva, & Jori, 1996). *Candida albicans*, a well-characterized yeast, also showed resistance to certain photosensitizers, due to its membrane morphology (Lambrechts, Aalders, & van Marle, 2005). To develop this strategy of bacterial control, it will be important to identify new photosensitizers that can be used against a broad spectrum of microorganisms. Also, it will be important to optimize treatment conditions to obtain maximum photoinactivation, such as dose, exposure time and light intensity. Photosensitization could offer an alternative for non-thermal sanitization methods, with effectiveness comparable to those used in the medical field; first the research must establish which particular photosensitizer food additives are both effective and "generally recognized as safe (GRAS)". It is very important that the photosensitizer is stable or, if it degrades when subjected to the light treatment, it does not produce compounds hazardous to human health.

The direct bactericidal effect of UV (especially UV-C) alone is a well-established technology in the food industry. For instance, UV-C light is used for a large variety of applications to disinfect water, air and surfaces. UV-C light is also used to treat fruit juices for pathogens. Usually, fruit juices or apple cider have pH values between 3.3 and 4.1, and until more recently, have not been considered potential vectors for foodborne pathogens, due to the bacteriostatic effects of acidity. However, there are newly emerging pathogens with acid resistance, such as some strains of *E. coli* O157:H, leading to foodborne disease outbreaks even in pasteurized fruit juices (Cody et al., 1999). Dlusskaya, McMullen, and Gänzle (2011) isolated an extremely heat-resistant (but non-pathogenic) *E. coli* from a beef processing facility named *E. coli* AW 1.7. This heat-resistant *E. coli* strain has a specific genomic island called LHR for "locus of heat resistance", which confers heat resistance and can be involved in horizontal gene transfer between bacteria (Mercer, et al., 2015). A possible scenario has been considered in which a horizontal LHR transfer occurs from *E. coli* AW 1.7 to the acid resistant *E. coli* O157:H7 strain to generate a new strain better equipped to survive common heat treatments like the pasteurization of fruit juices. As a consequence, longer times and/or greater temperature and/or time of pasteurization would become necessary, negatively impacting the nutritional and sensorial properties of the fruit juices or apple cider. For this reason, a photodynamic approach similar to the one used in photodynamic therapy, involving the interaction of a photosensitizer and UV-B or visible light, could be both effective to control microbes but also to have minimal negative nutritional and organoleptic effects. The use of UV-B, UV-A or visible light would be a better choice than using UV-C light, since these wavelengths penetrate the food product better and therefore would improve the photoinactivation of bacteria with less energy and harmful radiation.

The objectives of this study were to demonstrate the photodynamic mechanism of fructosazine and GlcN caramel under UV-B light. The dose-specific effect of UV-B radiation alone or together with photosensitizers (fructosazine, GlcN caramel and riboflavin) was determined on the photoinactivation process against *E. coli* ATCC 25922, *E. coli* AW 1.7 and *Bacillus subtilis* ATCC 6633 in broth cultures. UV-B light was chosen because fructosazine's maximum absorbance is between 275 and 280 nm, which should respond well to the safer UB-V light emission range of 280–315 nm.

2. Materials and methods

2.1. Reagents and standards

Fructosazine (> 0.98%) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). D-Glucosamine hydrochloride, riboflavin, tryptophan, Difco Luria–Bertani (LB) broth and LB agar (Miller) were from Sigma-Aldrich (St. Louis, MO). Singlet Oxygen Sensor Green (SOSG) reagent was from Thermo Fisher Scientific (Waltham, MA). MTT cell proliferation assay kit and bacterial strains of *E. coli* (ATCC 25922) and *Bacillus subtilis* (ATCC 6633) were purchased from ATCC Bioproducts (Manassas, VA). *E. coli* AW 1.7 was isolated from a beef carcass and obtained from Dr. Michael Gänzle (University of Alberta, Edmonton, Canada). All chemicals were of analytical grade and buffers were prepared with Milli-Q purified water (18.2 MΩ·cm, Millipore, Bedford, MA).

2.2. Preparation of glucosamine caramel

GlcN caramel was prepared according to Hrynets et al. (2016). Exactly, 15 g of glucosamine hydrochloride were dissolved in 100 mL of water and the pH of the resulting solution was adjusted to 7.40 with 1 M KOH. Samples were filtered (0.2 μm sterile filters) and incubated for 48 h at 50 °C. Caramel samples were then used for irradiation experiments and microbial assays.

2.3. Bacterial strains and growth conditions

E. coli ATCC 25922, *E. coli* AW 1.7, and *Bacillus subtilis* ATCC 6633 were used in the study. All bacterial strains were grown on Difco Luria–Bertani (LB) medium supplemented with 15 g/L of agar and the final pH was adjusted to 7.0 ± 0.2 for both broth and agar media before autoclaving at 121 °C for 20 min. Cells of these strains were incubated at 37 °C under aerobic conditions for 24 h. Prior to bacterial colony count assay, bacterial cell suspension was further diluted at different ratios and 100 μL bacterial suspension were grown on LB-agar (Miller) plates for 16 h at 37 °C to determine corresponding cell numbers. Cell suspension containing 1×10^6 CFU/mL was further used for