International Biodeterioration & Biodegradation xxx (2013) 1–9

Contents lists available at SciVerse ScienceDirect

International Biodeterioration & Biodegradation

journal homepage: www.elsevier.com/locate/ibiod

The action of selected isothiocyanates on bacterial biofilm prevention and control

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ARTICLE INFO

Article history:
Received 28 December 2012
Accepted 26 January 2013
Available online xxx

Keywords:
Adhesion
Biofilm control
Biofilm prevention
Allylisothiocyanate
2-Phenylethylisothiocyanate
Motility

ABSTRACT

The activity of two selected isothiocyanates (ITCs), allylisothiocyanate (AITC) and 2-phenylethylisothiocyanate (PEITC) was evaluated on the prevention and control of biofilms formed by *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Listeria monocytogenes*. In addition, the effect of ITCs was also tested on planktonic cell susceptibility, bacterial motility and adhesion. Biofilm prevention and control were tested using a microtiter plate assay and the effect of ITCs was assessed on biofilm mass and metabolic activity. The minimum bactericidal concentration for *E. coli* and *P. aeruginosa* was 1000 μg mL^{-1} (AITC) and >1000 μg mL^{-1} (PEITC), for *S. aureus* and *L. monocytogenes* was >1000 μg mL^{-1} (for both ITCs). AITC caused total inhibition of swimming (*P. aeruginosa*) and swarming (*E. coli*) motilities. PEITC caused total inhibition of swimming (*E. coli*, *P. aeruginosa* and *L. monocytogenes*) and swarming (*E. coli* and *P. aeruginosa*) motilities. Colony spreading of *S. aureus* was completely inhibited with PEITC. Adhesion assessed in terms of free energy was less favorable when bacteria were exposed to AITC for *E. coli* and *P. aeruginosa* and PEITC for *P. aeruginosa*. Both ITCs had preventive action on biofilm formation and showed a higher potential to reduce the mass of biofilms formed by the Gram-negative bacteria. AITC and PEITC promoted reductions in biofilm activity higher than 60% for all the biofilms tested. The overall study emphasizes the potential of ITCs as emergent products to inhibit bacterial motility and prevent/control biofilms of important human pathogenic bacteria.

1. Introduction

Antimicrobial resistance is one of the major challenges for the industrial, food and biomedical sectors. The increased resistance of pathogenic microorganisms to the antibacterial agents can be directly attributed to the extreme and inappropriate use of antibiotics and disinfectants (Andersson and Levin, 1999; Guillemot, 1999; Monroe and Polk, 2000; Andersson, 2003). Some infectious diseases are almost untreatable by conventional antibiotic therapy (Dalton et al., 2012). Antimicrobial resistance is worsened when the microorganisms form biofilms (Mah and O'Toole, 2001).

Biofilms are complex multicellular microbial communities irreversibly attached to a surface, enclosed in a matrix of extracellular polymeric substances (EPSs) such as proteins, nucleic acids and polysaccharides, and represent the prevalent mode of microbial life in nature, industrial processes and some infections (Hall-Stoodley et al., 2004; Cos et al., 2010; Toté et al., 2010; Jiang et al., 2011). Biofilms are an example of physiological adaptation and are one of the most important sources of bacterial resistance to antimicrobials (Simões, 2011). Bacteria embedded in biofilms are more resistant to antimicrobial products than their planktonic counterparts (Mah and O'Toole, 2001; Stewart and Costerton, 2001; Donlan and Costerton, 2002; Jagani et al., 2009; Cos et al., 2010; Simões et al., 2011b). In addition to the conventional mechanisms of antibiotic resistance found in planktonic cells (efflux pumps, modifying enzymes, and target mutations) (Walsh, 2000; Stewart and Costerton, 2001), there are several mechanisms that protect bacteria in biofilm, particularly: (i) poor penetration or inactivation of antimicrobials in the extracellular polymeric matrix; (ii) an altered (dormant) bacterial metabolic state; (iii) the presence of persister cells; (iv) resistance induced by the antimicrobial itself following the use of sublethal concentrations and the upregulation of efflux pumps (Gilbert et al., 2003; Anderson and O'Toole, 2008). Biofilm resistance is usually multifactorial and may vary from one organism to another (Gilbert et al., 2003; Aslam, 2008). Furthermore, the emergence of antimicrobial resistant bacteria and phenotypes clearly shows that new biofilm control strategies are required (Simões et al., 2006). A better understanding of bacterial tolerance and resistance to antimicrobial products has led to new interests in natural antibacterial products which restrict the ability of bacteria to adhere, communicate, and

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http://dx.doi.org/10.1016/j.ibiod.2013.01.015

form complex biofilms (Al-Sohaibani and Murugan, 2012). An important strategy to combat the resistance problem involves the discovery and development of new antimicrobials capable to suppressing bacterial resistance mechanisms (Abreu et al., 2012).

Many of the antimicrobial drugs used to effectively treat human disease have been derived from nature (Newman and Cragg, 2007; Brown and Hampton, 2011). Dietary phytochemicals (plant secondary metabolites) are potent bioactive compounds from plant sources with a wide range of effects (Holst and Williamson, 2004). Glucosinolates (GLS) are an important group of phytochemicals present exclusively in the order Capparales and very abundant in *Brassicaceae* (Syn. *Cruciferae*) family (Halkier and Du, 1997; Grubb and Abel, 2006; Barbieri et al., 2008; Al-Gendy et al., 2010). This family includes various vegetables such as cabbage, broccoli, cauliflower, horseradish, Brussels sprouts and kohlrabi (Fahey et al., 2001; Holst and Williamson, 2004). More than 120 different GLS are known to occur naturally in plants (Fahey et al., 2003; Clarke, 2010; Berhow et al., 2012). They are grouped into aliphatic, aromatic and indole glucosinolates, based on their chemical structure (Halkier and Gershenzon, 2006). These phytochemicals are usually broken down through hydrolysis catalyzed by myrosinase (β-thioglucosidase enzyme), released from damaged plant cells, in to numerous biologically active products such as isothiocyanates (ITCs), nitriles, epithionitriles and thiocyanates (Fahey et al., 2001; Hong and Kim, 2008; Aires et al., 2009b). GLS and their hydrolysis products (GHP), in particular ITCs, have long been known to have biological activities including various pharmaceutical benefits to human health (anticarcinogenic, antimicrobial and antioxidant properties) and plant defence (against insects, fungi and microbial infections) (Hong and Kim, 2008; D'Antuono et al., 2009; Saavedra et al., 2010). The effects of GLS on the quality of both human and animal foods, and the emerging evidence that brassica vegetables may have important anticarcinogenic effects associated with the biological activity of GHP provides a good reason for the increased interest in natural biosynthetic pathways of these compounds. In this work, the ability of two ITC's (allylisothiocyanate and 2-phenylethylisothiocyanate) to control biofilms formed by four bacterial species of potential biomedical concern (*Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Listeria monocytogenes*) was evaluated. In addition, these ITCs were also tested in planktonic growth control and in their ability to act on biofilm prevention, motility inhibition and on bacterial-surface free energy of adhesion.

2. Material and methods

2.1. Microorganisms and culture conditions

Two Gram-negative bacteria, *E. coli* CECT 434, *P. aeruginosa* ATCC 10145, and two Gram-positive bacteria, *S. aureus* CECT 976 and *L. monocytogenes* ATCC 15313, were used. These bacteria were previously used as model microorganisms for antimicrobial tests with phytochemical products (Simões et al., 2008a; Saavedra et al., 2010; Borges et al., 2012).

2.2. Isothiocyanates

Allylisothiocyanate (AITC) and 2-phenylethylisothiocyanate (PEITC) (Fig. 1) were obtained from Sigma–Aldrich (Portugal). Phytochemicals are routinely classified as antimicrobials on the basis of susceptibility tests that produce inhibitory concentrations in the range of 100–1000 μg mL^{-1} (Simões et al., 2009). In this work, each product was tested at a concentration of 1000 μg mL^{-1} in dimethyl sulfoxide (DMSO, Sigma), for motility, adhesion and biofilm tests. Negative controls were performed with DMSO. AITC and PEITC were selected based on their strong antimicrobial activity when compared with a panel of structurally distinct phytochemicals (Aires et al., 2009a,b; Saavedra et al., 2010).

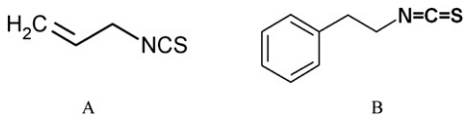

Fig. 1. Chemical structures of AITC (A) and PEITC (B).

2.3. Dose response curves

Dose response curves were performed with different concentrations (0, 100, 500 and 1000 μg mL^{-1}) of AITC and PEITC in Mueller-Hinton broth (MHB) (Merck, Germany), at 30 °C, in 96-wells flat-bottomed polystyrene (PS) tissue culture plates with a lid (Orange Scientific, USA) using a total volume of 200 μL. An inoculum of 1×10^8 CFU/mL of bacteria, in the log phase of growth, was used. After 1 h exposure to the ITCs, an aliquot of 50 μL of planktonic suspension was collected, according to the procedure described by Simões et al. (2008a). The number of bacteria in the samples was determined by making serial dilutions in saline solution (0.85% NaCl). Thirty μL of each dilution were plated on Mueller-Hinton agar (MHA) plates and incubated overnight, at 30 °C. Colonies were counted after 24 h incubation period. Three independent experiments were performed for each condition tested. The minimum bactericidal concentration (MBC) was taken as the lowest concentration of ITCs at which no CFU were detected on solid medium (Ferreira et al., 2011; Borges et al., 2012).

2.4. Motility assays

Overnight cultures grown on Luria–Bertani broth (LBB) (Merck, Germany), at 30 °C and under agitation (150 rpm) were used to characterize bacterial motility. Fifteen μL of these cultures were applied in the center of plates containing 1% tryptone, 0.25% NaCl, and 0.3%, 0.7% or 1.5% (w/v) agar for swimming/colony spreading, swarming and twitching motilities, respectively (Butler et al., 2010; Stickland et al., 2010). Colony spreading was assessed for *S. aureus* and twitching motility was only assessed for *P. aeruginosa*. The use of different concentrations of agar (the medium porosity directly related to the concentration of agar, so various levels of bacterial diffusion can be selected) enables the characterization of different types of bacterial motility. AITC and PEITC at 1000 μg mL^{-1} were incorporated in the growth medium (tempered at 45 °C). Negative controls were performed with medium without ITCs. Plates were incubated at 30 °C and the diameter (mm) of the motility halos were measured at 24, 48 and 72 h. Three plates were used to evaluate the motility of each bacterium.

2.5. Free energy of adhesion

The free energy of adhesion (ΔG_{iwl}^{Tot}) between the bacterial cells and PS surfaces was assessed according to the procedure described by Simões et al. (2008b). After overnight growth in MHB, the cells were centrifuged and resuspended in saline solution to obtain an OD_{640} of 0.2 ± 0.02 (1×10^8 CFU/mL). One hundred mL of this suspension was collected and exposed to 1000 μg mL^{-1} during 1 h. Cell suspensions without ITCs were used as controls. To ascertain the bacterial surface properties, lawns of bacteria were prepared as described by Busscher et al. (1984). PS surfaces were prepared for characterization by immersion in a solution of commercial detergent (Sonasol Pril, Henkel Ibérica S. A.) in ultrapure water for

Please cite this article in press as: Borges, A., et al., The action of selected isothiocyanates on bacterial biofilm prevention and control, International Biodeterioration & Biodegradation (2013), http://dx.doi.org/10.1016/j.ibiod.2013.01.015