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Triclosan–lysozyme complex as novel antimicrobial macromolecule: A new potential of lysozyme as phenolic drug-targeting molecule

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ABSTRACT

A novel anti-infection strategy to alleviate antibiotic-resistance problem and non-specific toxicity associated with chemotherapy is explored in this study. It is based on utilizing a bacteriolytic enzyme (lysozyme) as a carrier to allow specific targeting of a potential phenolic antimicrobial drug (triclosan) to microbial cells. Lysozyme (LZ) was complexed, via electrostatic and hydrophobic condensation at alkaline pH, to various degrees with triclosan (TCS), a negatively charged phenolic antimicrobial that inhibits bacterial fatty acid synthesis. Fluorescence and absorbance spectra analysis revealed non-covalent association of TCS with the aromatic residues at the interior of LZ molecule. The conjugation greatly promoted the lytic activity of LZ as the degree of TCS derivatization increased. The complexation with LZ turned TCS into completely soluble in aqueous solution. TCS–LZ complexes showed significantly enhanced bactericidal activity against several strains of Gram-positive and Gram-negative bacteria compared to the activity of TCS or LZ alone when tested at the same molar basis. Strikingly, TCS–LZ complex, but not LZ or TCS alone, exhibited unique specificity to scavenge superoxide radicals, generated by the natural xanthine/xanthine oxidase coupling system, without affecting the catalytic function of oxidase. This finding is the first to describe that the membrane disrupting function of lysozyme can be utilized to specifically target antimicrobial drug(s) to pathogen cells and heralding a fascinating opportunity for the potential candidacy of TCS–LZ as novel antimicrobial strategy for human therapy.

1. Introduction

Infectious diseases, in particular those caused by bacterial infections, are still among the top causes of mortality in the world and represent a serious public health threat. This concern is great because of the rapidly increasing emergence of antibiotic resistance whereas certain strains of pathogens now have only one antibiotic remaining to kill them. Although substantial discoveries of antimicrobial agents have been made in the last decades, they are still bearing considerable drawbacks. The exploitation of these antimicrobial agents in human therapy has not been widespread because they have in many cases a high toxigenic potential, leading to skin irritation, allergic reactions and resorptive systemic toxicity. In addition, only few antimicrobials possess broad-spectrum activity against both Gram-positive and Gram-negative bacteria, moulds and yeast. Hence, the development of novel antimicrobial drugs, with a unique mechanism of action that bacteria have never seen before and it is difficult to develop resistance, has become an urgent matter and is becoming a great challenge for biotechnologists.

Phenolic compounds are examples of natural preservatives receiving much attraction due to their biological activities such as antimicrobial, antiviral and antioxidant effects. Although numerous studies have shown these natural polyphenols to be effective against microorganisms, they have limitations including the potential emergence of resistant strains, limited spectrum of activity by virtue of membrane exclusion, basically insoluble in water and their inherent chemical instability. Combinations of antimicrobial factors to expand the antimicrobial spectrum and minimize toxicity of phenolic antimicrobials and to prevent the emergence of resistant mutants can thus be a potential anti-infection strategy. Our strategy is to allow specific targeting of the phenolic drug agent to the microbial cells with a carrier protein that has receptors permanently present at the surface of pathogens. Lysozyme could be a potential candidate for drug targeting as it has receptors, peptidoglycan and LPS, at the surface of Gram-positive and Gram-negative

Abbreviations: nLZ, native lysozyme; TCS, triclosan; T–LZ(n), triclosan–lysozyme complex (mole ratio); NBT, nitroblue tetrazolium; X/XOD, xanthine/xanthine oxidase couple system; Trp, tryptophan; CBB, coomassie brilliant blue; Acid-PAGE, acid native polyacrylamide gel electrophoresis; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; ROS, reactive oxygen species.

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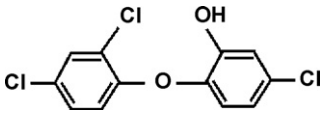
bacteria, respectively. Central to this approach is to utilize the ubiquitous antimicrobial lysozyme that specifically recognizes and able to perturb bacterial cell membrane of wide range of pathogens.

Lysozyme (1,4-β-*N*-acetylmurmidase) is a basic protein belongs to the class of enzymes that lyse the cell walls of bacteria by hydrolyzing the bond between *N*-acetylmuramic acid and *N*-acetylglucosamine of the peptidoglycan. Lysozyme (LZ) is antimicrobial protein widely distributed in various biological fluids and tissues including avian egg and animal secretions; human milk, tears, saliva, airway secretions, and secreted by polymorphonuclear leukocytes [1]. Besides its antimicrobial activity, it has many other biological functions including anti-inflammatory [2], anti-viral [3,4], immune modulatory [5], anti-histaminic [6] and anti-tumor [7] activities. Lysozyme also has the capacity to neutralize and strongly interact with LPS of Gram-negative bacteria [8]. Antimicrobial activity of LZ is limited to certain Gram-positive bacteria [9]. The molecular mechanism of antimicrobial function of LZ remained unclear until our recent findings that antimicrobial action of LZ is independent of its catalytic function [10] and both human and chicken lysozymes possess a helix–loop–helix antimicrobial peptide within their sequences [11]. Our more recent work provided the first demonstration that pepsin can fine-tune the antimicrobial potency of LZ by generating multiple antimicrobial peptide motifs, and delineated a new molecular switch of LZ in the mucosal defense systems [12]. The utilization of multi-functional LZ in therapy is, therefore, becoming more feasible particularly with the recent developments made for understanding its structure related antimicrobial function [9,10,12,13].

Lysozyme is attractive because it is endogenous microbicidal protein, specific to bacterial cell walls, and known to play important roles in the immune defense systems. Hence, LZ may offer exciting new myraid of functions beyond its antimicrobial role. Intriguingly, the unique nature of LZ molecule can be a potential drug carrier to a specific targeting of the phenolic antimicrobial drugs to pathogen cells, since receptors of LZ, peptidoglycans (PG) and lipopolysaccharides (LPS), are permanently present at the surface of Gram-positive and Gram-negative bacteria, respectively. Phenolic drug–LZ conjugates, through non-covalent affinity, can thus be a novel anti-infection strategy and drug-delivery system, to alleviate antibiotic-resistance problem and non-specific toxicity associated with chemotherapy. It has been shown that the type of interaction between protein and phenolic compounds is an important factor in producing effective delivery system. Specifically, it was demonstrated that weak binding affinity of phenolic drug to the carrier molecule is correlated with the effectiveness of drug delivery [14]. Therefore, the effectiveness of phenolic drug–LZ conjugates may be explored by identifying a phenolic antibiotic for LZ that exhibits a less degree of association. Triclosan (2,4,4′-trichloro-2′-hydroxydiphenyl ether), a new phenolic antimicrobial agent, is a hydrophobic in nature (Scheme 1). Triclosan (TCS) exerts its action by inhibiting an essential enzyme enoyl-ACP reductase (or FabI) [15] that uses NADH to reduce a double bond during each cycle of bacterial fatty acid elongation. At higher concentration, it is likely to damage the bacterial membrane [16]. TCS has also been shown to be an effective antifungal agent [17], and to have anti-inflammatory [18] and anti-carcinogenic [19] actions. TCS has a low toxicity and a very low incidence of contact sensitization [20]. However, several unfavorable biopharmaceutical properties such as poor solubility in aqueous solvents, rapid photodegradation [21] and emergence of bacterial resistance [22] limit TCS therapeutic applications. TCS was selected for our strategy of utilizing LZ as a specific drug-delivery molecule because it has a powerful antimicrobial action and structurally can be non-covalently accommodated in the hydrophobic pocket of the active site cleft of LZ. Structure examination of LZ and TCS justify that the 2,4-dichlorophenoxy ring of TCS (Scheme 1) is predicted to interact with the faces of several phenol rings (mostly Trp residues) underlying the active site cleft of LZ, a preferred conformation for compounds of this class [23].



Scheme 1. The structure of triclosan.

It is the purpose of this study to explore that the lytic enzyme LZ can be a potential phenolic antimicrobial drug (TCS) carrier to allow specific targeting to microbial cells, with special focus on developing a new microbicidal complex. The study attempts to explore the nature of molecular interaction and functional synergism of LZ with TCS that represents a potential strategy for a multi-functional macromolecule and delineate a new drug-delivery power of LZ to the treatment of emerging infectious diseases.

2. Materials and methods

2.1. Materials

Lysozyme from hen egg albumen was purchased from Innovatech Labs, Inc. (Troy, NY). Xanthine, xanthine oxidase (XOD), nitroblue tetrazolium (NBT) and *Micrococcus lysodeikticus*, the substrate of lysozyme, were from Sigma Chemicals Co. (St. Louis, MO, USA). Triclosan was provided by Ciba Specialty Chemicals (Basel, Swiss). Brain heart infusion (BHI), trypticase soya broth (TSB) and nutrient broth were from Nissui (Tokyo, Japan). Unless otherwise stated, all other chemicals were of analytical grade.

2.2. Microorganisms

Bacterial strains *Klebsiella pneumoniae* (ATCC 13883), *Salmonella typhimurium* (ATCC 14028), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus epidermidis* (ATCC 12228) and *Micrococcus luteus* (ATCC 4698) were from the American Type Culture Collection (Manassas, VA). *E. coli K12* (IFO 3301) and *Staphylococcus aureus* (IFO 14462) were obtained from the Institute of Fermentation Osaka (Japan). The wild strain of *Streptococcus zooepidemicus* was from the Institute of Bacteriology of the Veterinary Hospital, Zurich, Switzerland.

2.3. Preparation of triclosan–lysozyme complex (T–LZ)

Triclosan–lysozyme complex was prepared by adding freshly prepared TCS stock solution, in ethanol, to a 1% LZ solution, to achieve 10, 15 and 30-fold molar excess of TCS over LZ, in distilled water at pH 9.0. After stirring for 24 h at 29 °C, the mixture was directly lyophilized (these complexes were referred to as T–LZ_{10}, T–LZ_{15} and T–LZ_{30}) and a portion was dialyzed before lyophilization against distilled water at 4 °C for 72 h (referred to as T–LZ_{10}d, T–LZ_{15}d and T–LZ_{30}d). Portion of the complexes were further dialyzed against water and re-lyophilized (referred to as T–LZ_{10}cd, T–LZ_{15}cd and T–LZ_{30}cd). Control LZ (Ctrl-LZ) was treated similarly except without TCS. The protein content of lysozyme derivatives was quantitated using Bradford protein assay (Bio-Rad Protein Assay kit).

2.4. Interaction between triclosan and lysozyme

Since TCS absorbs light at UV wavelength, the degree of TCS interaction with LZ was determined by measuring absorbance at