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Preventing microbial colonisation of catheters: Antimicrobial and antibiofilm activities of cellobiose dehydrogenase

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ABSTRACT

The ability of cellobiose dehydrogenase (CDH) to produce hydrogen peroxide (H_2O_2) for antimicrobial and antibiofilm functionalisation of urinary catheters was investigated. A recombinantly produced CDH from *Myriococcum thermophilum* was shown to completely inhibit the growth of *Escherichia coli* and *Staphylococcus aureus* both in liquid and solid media when supplemented with either 0.8 mM or 2 mM cellobiose as substrate. Biofilm formation on silicone films was prevented by CDH when supplemented with 1 mM cellobiose. The CDH/cellobiose system also successfully inhibited many common urinary catheter-colonising micro-organisms, including multidrug-resistant *S. aureus*, *Staphylococcus epidermidis*, *Proteus mirabilis*, *Stenotrophomonas maltophilia*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa.* Interestingly, CDH was also able to produce H_2O_2 during oxidation of extracellular polysaccharides (exPS) formed by micro-organisms in the absence of cellobiose. The H_2O_2 production and consequently antimicrobial and antibiofilm activities on these exPS were enhanced by incorporation of glycoside hydrolases such as amylases. Hydrolysis of polysaccharides by these enzymes increases the number of terminal reducing sugars as substrates for CDH as well as destabilises the biofilm. Furthermore, CDH suspended in catheter lubricants killed bacteria in biofilms colonising catheters. Incorporation of the CDH/cellobiose system in the lubricant therefore makes it an easy strategy for preventing microbial colonisation of catheters.

1. Introduction

Indwelling urinary catheters are standard medical devices used the world over. Severe health problems can arise when patients are catheterised, ranging from urinary tract infections (UTIs) to life-threatening complications. Catheter-associated UTIs account for 40% of all nosocomial infections occurring in hospitals and for 80% of all nosocomial UTIs [1,2]. To prevent infection, catheters are replaced at regular intervals, causing inconvenience for the patient and increased costs for the healthcare system [3]. Catheter-associated UTIs are predominantly caused by *Escherichia*, *Klebsiella*, *Proteus*, *Pseudomonas*, *Acinetobacter* and *Enterobacter* spp. [4,5], which adhere to the catheter surface in a complex biofilm [6]. Biofilms contain extracellular polymeric substances produced by the micro-organisms, which generally comprise DNA, proteins and extracellular polysaccharides (exPS). The composition of the biofilm is highly dependent on the type of bacteria and the growth conditions and its main function is to protect the micro-organisms from negative environmental influences [7]. Several reasons, including acquisition of antibiotic resistance genetic elements from other bacteria and the formation of a diffusion barrier, make micro-organisms in biofilms more resistant to antibiotic treatment and difficult to remove once they are formed [8]. Therefore, prevention of biofilm formation and microbial colonisation is key to a successful strategy against medical device-related infections. Over the past decades, the development of antimicrobial coatings containing substances such as antibiotics, silver, gendine, nitric oxide [9,10] and, more recently, antimicrobial peptides is being intensively explored [11]. However, most of these coatings have raised issues regarding cytotoxicity and an increase of antibiotic resistance [8].

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Enzymes are gaining special interest as the new generation of antimicrobials, as summarised by Thallinger et al. [12]. Among them is glucose oxidase, which uses glucose to produce hydrogen peroxide (H_2O_2), a well-known antimicrobial agent used extensively in the food industry [12]. H_2O_2 is a strong oxidising agent that has been widely used as a disinfectant and antiseptic in low concentrations (0.25–3%). The action of H_2O_2 on microbes is due to the formation of radicals in solution that attack membrane lipids, DNA and other cell constituents important for the viability of the micro-organism [13]. Although similar studies using glucose oxidase showed promising results, the glucose needed by the enzyme as substrate is also a very good growth substrate for most micro-organisms of concern. Recently, our group has demonstrated the ability of cellobiose dehydrogenase (CDH) from *Myriococcum thermophilum* to inhibit bacterial growth [14]. CDH is an oxidoreductase that, unlike glucose oxidase, can oxidise cello-oligosaccharides (including cellobiose) and other oligosaccharides to produce H_2O_2, which are not growth substrates for many micro-organisms causing infections [15,16]. In addition, CDH oxidation of cello-oligosaccharides produces cellobionic acid, which lowers the pH thereby creating a hostile environment for bacteria [15,17]. This study therefore investigated the possibility of using CDH as an antimicrobial and antibiofilm agent to prevent urinary catheter-based infections. The CDH is incorporated into the lubricant traditionally used to minimise discomfort during catheterisation [18]. Furthermore, the potential of CDH to produce H_2O_2 for antibiofilm and antimicrobial activities directly from exPS without addition of cellobiose was assessed.

2. Materials and methods

2.1. Materials and micro-organisms

All chemicals used were of analytical grade. Media components, cellobiose and 2,6-dichlorophenolindophenol (DCIP) were purchased from Carl Roth GmbH (Karlsruhe, Germany). All other chemicals and chemicals for buffer solutions were purchased from Sigma-Aldrich (Steinheim, Germany). Commercial enzymes (α-amylase, mannanase and pulpzyme) were purchased from Novozymes (Bagsvaerd, Denmark) and endoglucanase was from Fluka (Steinheim, Germany). Recombinant *M. thermophilum* cellobiose dehydrogenase (rMtCDH) was produced as previously described [15]. Catheters and silicone sheets were provided by Degania Silicone (Israel).

Staphylococcus aureus ATCC 25923 and *Escherichia coli* strain XL1 used throughout the experiments were acquired from the culture collection of the Institute of Environmental Biotechnology of Graz University of Technology (Austria). The other clinical bacterial isolates [meticillin-resistant *S. aureus* (MRSA) U-1768/1, *Staphylococcus epidermidis* U-333/3, *Enterococcus faecalis* X-3196, *E. coli* extended-spectrum β-lactamase-positive (ESBL+) B-2718, *Klebsiella pneumoniae* ESBL+ Y-995/22, *Proteus mirabilis* U-1958/1, *Stenotrophomonas maltophilia* U-57/28, *Acinetobacter baumannii* U-1724/1 and *Pseudomonas aeruginosa* U-1662/63] were obtained from different departments (Burn, Surgical, Internal medicine and Urology) at Pirogov Hospital (Sofia, Bulgaria).

2.2. Cellobiose dehydrogenase activity assay

The activity of CDH was assayed according to Baminger et al. [19] and modified by Flitsch et al. [15]. Briefly, rMtCDH activity was measured by monitoring the decrease in absorbance of DCIP at 520 nm (ε 520 = 6.8 × 10^3/M cm), pH 4.5 and 30 °C using a Hitachi U-2900 spectrophotometer (Hitachi Corp., Tokyo, Japan). The reaction mixture contained 3 mM DCIP and 300 mM lactose in 100 mM sodium acetate buffer (pH 4.5). The reaction was started by addition of CDH and the decrease in absorbance was monitored for 2 min. Activity was calculated from the slope and was defined in units (U) as the amount of enzyme reducing 1 μmol of DCIP per minute under the above reaction conditions.

2.3. Antibiofilm activities of cellobiose dehydrogenase

The antibiofilm activities of CDH were investigated by incubation with *S. aureus* in the presence of cellobiose in a 96-well plate (Sarstedt AG & Co., Nümbrecht, Germany). Briefly, medium containing tryptic soy broth supplemented with 0.5% (w/w) glucose (TSB+) and TSB+ supplemented with 0.6% (w/w) yeast extract and 0.2% (w/w) sodium citrate was inoculated with actively growing bacteria [final optical density at 600 nm (OD_{600}) = 0.01], cellobiose (0.5–2.0 mM) and 0.33 U/mL CDH. An autoclaved silicone piece (diameter 7 mm) was also added to each well. The plate was incubated for 18 h at 37 °C without shaking. In a parallel experiment, medium, bacteria and silicone films were supplemented with varying amounts of pure H_2O_2 (125–2000 μM) and incubation was carried out under the above conditions with appropriate controls. After incubation, non-attached cells were removed by washing with distilled water, while the biofilms attached to the silicone sheets were fixed by drying at 60 °C for 1 h followed by a staining step with 100 μL of 0.1% (w/w) crystal violet for 10 min. The silicone sheets were then immersed in 100 μL of 30% acetic acid for 10 min to solubilise the crystal violet. The supernatant (50 μL) was transferred to a new 96-well plate to measure the absorbance at 595 nm in a plate reader (Infinite® M200 Pro Plate Reader; Tecan, Männedorf, Switzerland). Percent biofilm formation was calculated by dividing the absorbance values of the cellobiose-containing wells by the absorbance of the negative control.

2.4. Determination of the apparent 50% inhibitory concentration (IC_{50}) in liquid medium

IC_{50} values of cellobiose (leading to equimolar H_2O_2 production upon complete oxidation by CDH) were determined by incubating different cellobiose concentrations with *E. coli* and *S. aureus* in 96-well plates. Mueller–Hinton (MH) broth medium (100 μL) was pipetted into each well, followed by 100 μL of 50 mM cellobiose. Serial dilutions of cellobiose ranging from 0.1 mM to 25.0 mM were achieved by transferring 100 μL in each consecutive lane. A bacterial suspension containing 5 μL of 10^5 CFU/mL was added, followed by 10 μL of 1.64 U/mL CDH (final activity in each well 0.14). The respective controls, e.g. CDH only, negative and sterile control, were also prepared in the other lanes. Plates were incubated at 37 °C for 16 h and the OD_{600} was measured every 30 min in a plate reader. The apparent IC_{50} value was defined as the concentration of cellobiose inhibiting growth by 50% compared with the negative control. All determinations were performed in triplicate.

2.5. Determination of the apparent 50% inhibitory concentration on agar plates

The apparent IC_{50} on agar plates was assessed against *S. aureus* and *E. coli*. A 1 mL solution of CDH was added to every 100 mL of liquid MH agar (ca. 40 °C) in order to yield an enzyme activity of 0.33 U/plate. Then, 1 mL of 10^8 CFU/mL bacterial suspension was added together with different cellobiose concentrations ranging from 0.1 mM to 2.0 mM. Controls included plates with cellobiose only, CDH only and bacteria only. Plates were left to stand for 30 min in order for the liquid to soak before turning them upside down and incubating at 37 °C for 24 h in an incubation chamber. CFU/mL were calculated and the lowest cellobiose concentration that led to