Effective disruption of *Pseudomonas aeruginosa* biofilms using Adaptive Focused Acoustics™ (AFA)

ABSTRACT

Adaptive Focused Acoustics (AFA) was used to effectively disperse *Pseudomonas aeruginosa* biofilms and disrupt bacterial cells in a low detergent concentration lysis buffer, significantly increasing the efficiency of protein extraction. Biofilm specific staining with Calcoflour White M2R revealed that overnight cultures of *Pseudomonas aeruginosa* PA14 produce extensive biofilms, and counterstaining with the cell permeable SYTO 62 red fluorescent nucleic acid stain indicate large numbers of intact cells encapsulated in the protective biofilm. Following a brief AFA treatment, Calcoflour White staining showed nearly complete disruption of biofilms along with a decrease in nucleic acid staining indicating lysis of over 94% of the biofilm encapsulated cells.

INTRODUCTION

Pseudomonas aeruginosa is a monoflagellated gammaproteobacterium found in soil, water, and the normal human microflora. *Pseudomonas* are broadly resistant to antibiotics and are opportunistic pathogens of both plants and animals. *P. aeruginosa* is a primary cause of nosocomial infection and the leading cause of mortality in cystic fibrosis [1]. The primary exopolysaccharide (EPS) secreted by *P. aeruginosa* is a repeating polymer of mannuronic and glucuronic acid referred to as alginate (**Figure 1**). EPS biofilms anchor the cells to their substrate, protect the bacteria from the host defenses such as macrophages and antibodies, and impart antibiotic resistance [2].

The disruption of *P. aeruginosa* cells corresponds to an increased release of EPS and the formation of extensive biofilms that interfere with protein recovery and analysis. Thick biofilms dramatically increase sample viscosity making accurate liquid handling impossible. The removal of undisrupted biofilms by ultrafiltration or centrifugation results in unacceptably low protein recovery, and particularly, the loss of specific proteins associated with the biofilm [3,4]. In many cases, the need to preserve post-translational protein modifications such as glycosylation [5] and fucosylation [6,7] prohibits the use of endoglycosidases to degrade the interfering biofilms. In a clinical setting, highly efficient and highly controlled sample preparation methods are required to provide more reliable and comprehensive proteomics analyses of this clinically important microorganism.

FIGURE 1.

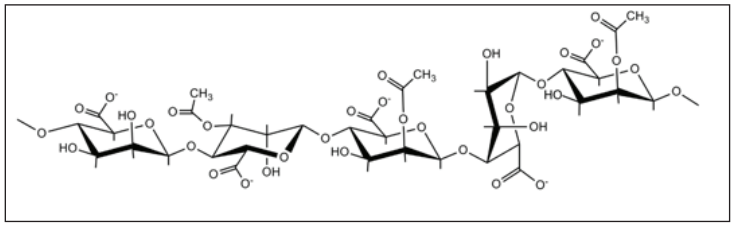

Figure 1. Possible alginate subunits arrangement proposed by Franklin et al. [8].

FIGURE 2.

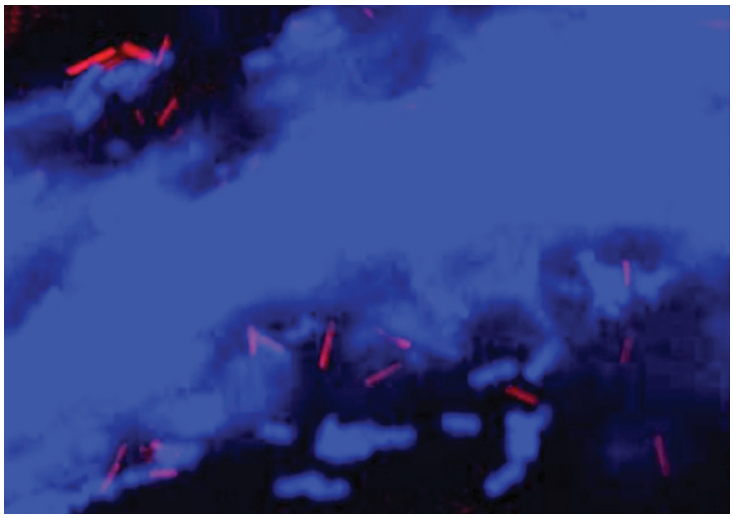

Figure 2. Merged micrographs showing PA-14 cells (red) and their associated biofilm (blue). Rod shaped structures are clusters of cells

METHODS AND MATERIALS

Bacterial cultures

P. aeruginosa PA14 cells were cultivated in 5 mL trypsin soy broth for 6 hours at 37oC. Multiple cultures were pooled into 10 mL volumes in tared 15 mL centrifuge tubes and incubated at 20oC for an additional 18 hours. Cells were recovered by centrifugation at 4,000 RCF for 30 minutes. On average, 10 mL milliliter cultures yielded 195 ± 7 mg of biomass including packed cells and their biofilms.

AFA and reagents

Where specified, cell pellets were resuspended in 2 mL Covaris Total Protein Extraction Reagent TP (Covaris, Inc., Woburn, MA, USA) supplemented with Halt™-EDTA protease inhibitors (Thermo-Pierce, Rockland, IL, USA). The relative extraction efficiencies of the Total Protein Extraction Reagent TP and 1% SDS in 100 mM Tris, 150 mM NaCl pH 7.65 were compared.

The effects of lowered chaotrope and detergent concentration were also investigated. Total protein yields were quantified from samples prepared in 3.5-7 M urea and 1- 4% (cholamidopropyl) dimethylammonio propanesulfonate (CHAPS) detergent concentration.

For each replicate sample, 1350 uL of cell suspension was transferred to a Covaris 15 x 19 mm glass tube with screw cap and the remaining 650 uL was reserved as negative control. AFA was performed at 18^0 C in the Covaris E-220 using a setting of 20% duty factor, 275W peak incidence power (PIP), 200 cycles per burst for 480 seconds.

Protein assay

Protein concentrations were estimated using the Quickstart™ Bradford Reagent (BioRad, Hercules, CA, USA)

Fluorescent staining and microscopy

Scanning laser fluorescence microscopy was performed using a Zeiss 510 META Laser Scanning Microscope (Zeiss, Oberkochen, Germany). Cells were stained for nine minutes with 20 uM SYTO-62 red fluorescent dye (Invitrogen, Carlsbad, CA, USA). The cells were then counterstained for one minute with 135 uM Calcofluor White M2R as described for biofilms [2]. Fluorescence was quantified from micrographs using the Image J open access Java-based software (NIH, Bethesda, MD).

RESULTS AND DISCUSSION

Following AFA treatment in the Covaris Reagent TP, Calcoflour White fluorescence showed that 99.6% of the biofilm was dispersed (**Figures 2 and 3**). This corresponded with a decrease in SYTO-62 fluorescence indicating 94.5% cell lysis efficiency with only a few scattered fluorescent bodies remaining visible.

FIGURE 3.

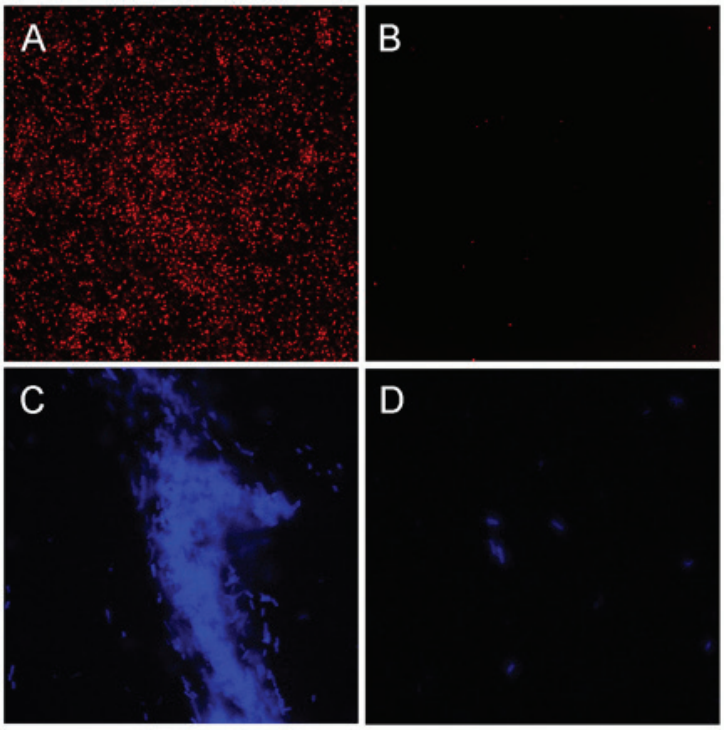

Figure 3. SYTO-62 red fluorescent staining of *P. aeruginosa* (A) before or (B) after AFA treatment. Mean fluorescence of identical areas was 11.02 and 0.04, respectively. Calcofluor White staining of extensive biofilm (C) before or (D) after AFA treatment. Mean fluorescence of identical areas was 3.82 and 0.21, respectively.

FIGURE 4.

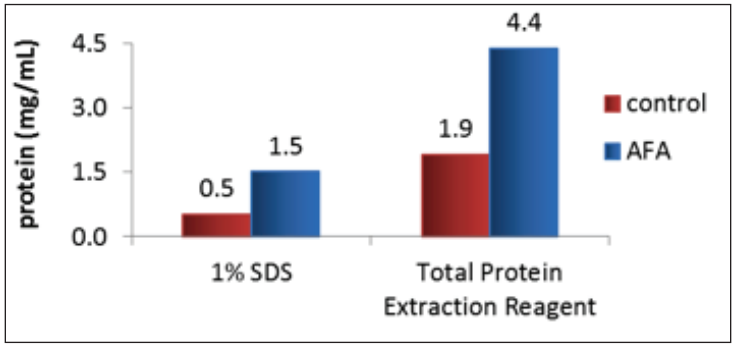

Figure 4. Comparison of total protein yields from *P. aeruginosa* PA-14 cells using either 1% SDS in 100 mM Tris, 150 mM NaCl pH 7.65 or the Covaris Total Protein Extraction Reagent TP.

Total protein recoveries from *P. aeruginosa*

When used in combination with AFA, The Covaris Total Protein Extraction Reagent (TP) yielded approximately three times more total protein from *P. aeruginosa* cells than 1% SDS (**Figure 4**).

At lowered urea concentrations, less total protein was recovered in bacterial lysates, but AFA still extracted nearly six times more protein than reagent controls extracted without AFA (**Figure 5A**). AFA increased the effectiveness of urea at lower concentrations.

AFA extracted three times more total protein than reagent controls regardless of CHAPS concentration (**Figure 5B**). Lowering the detergent requirement during sample preparation, or even eliminating it completely, decreases potential interference with downstream analyses such as LC-MS. Substituting smaller micelle size detergents such as CHAPS (micellar MW approximately 6 kDa) for SDS is also beneficial since the smaller micelles are more easily and rapidly removed from the sample than much larger SDS micelles (micellar MW 14-17 kDa).

FIGURE 5A., 5B.

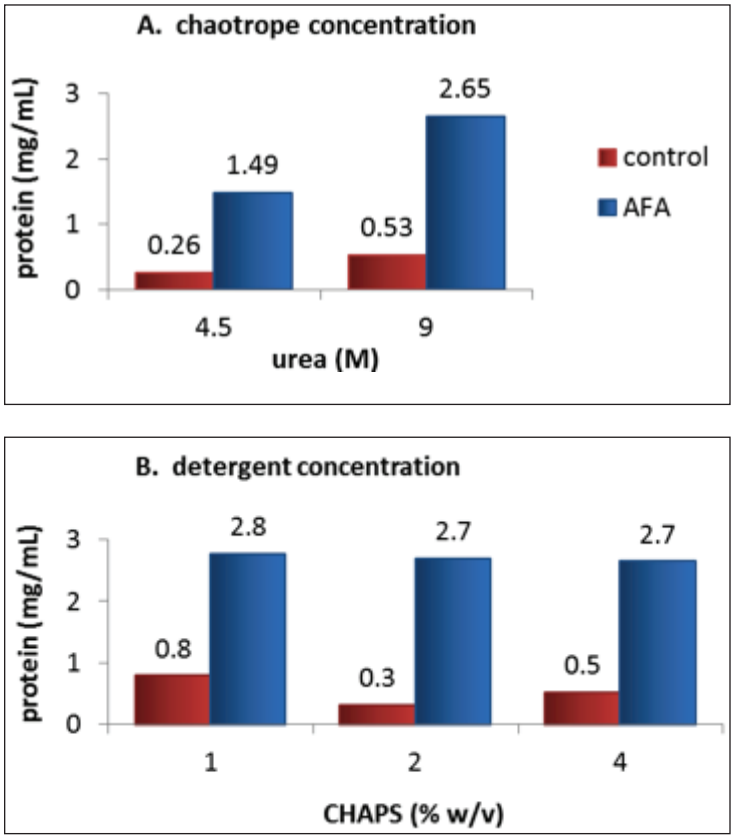

Figure 5. (A) Effects of urea concentration on total protein extracted from *P. aeruginosa* cells and biofilms. (B) Protein extraction efficiency was independent of CHAPS concentration (1%, 2%, or 4%) suggesting a lowered detergent requirement for samples when processed by AFA.

CONCLUSIONS

The AFA process, used in combination with optimized reagents, effectively disrupted bacterial cells and their associated biofilms which resulted in higher protein yields while lowering the requirement for a high detergent concentration in a protein extraction buffer.

The precise mechanism by which AFA disrupts EPS biofilms is not known. It is likely that individual polymers are not sheared, but rather that non-covalently associated polymer networks are dispersed by high velocity hydrodynamic shear forces generated during a high intensity AFA process. Since biofilms contain large number of proteins that change during the stages of biofilm development [3,4], it is critical to effectively disperse biofilms without loss of cell and biofilm proteins for accurate proteomic analysis of this clinically important microorganism.

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