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Quantitative analysis of the thermal stability of the gamma phage endolysin PlyG: A biophysical and kinetic approach to assaying therapeutic potential

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ABSTRACT

Endolysins are lytic enzymes encoded by bacteriophage that represent an emerging class of protein therapeutics. Considering macromolecular thermoresistance correlates with shelf life, PlyG, a *Bacillus anthracis* endolysin, was thermally characterized to further evaluate its therapeutic potential. Results from a biophysical thermal analysis revealed full-length PlyG and its isolated domains comprised thermal denaturation temperatures exceeding 63 °C. In the absence of reducing agent, PlyG was determined to be kinetically unstable, a finding hypothesized to be attributable to the chemical oxidation of cysteine and/or methionine residues. The presence of reducing agent kinetically stabilized the endolysin, with PlyG retaining at least ~50% residual lytic activity after being heated at temperatures up to 80 °C and remaining enzymatically functional after being boiled. Furthermore, the endolysin had a kinetic half-life at 50 °C and 55 °C of 35 and 5.5 h, respectively. PlyG represents a thermostable proteinaceous antibacterial with subsequent prolonged therapeutic shelf life expectancy.

Introduction

Bacillus anthracis is a Gram-positive spore-forming bacterium responsible for the zoonotic disease anthrax. In addition to causing naturally occurring anthrax, *B. anthracis* has been identified as a prominent biological warfare agent. This particular pathogen remains a public health concern due to the elevated mortality associated with the airborne transmission of sporulated *B. anthracis*, as well as diagnostic complications and the ability of *B. anthracis* to resist environmental extremes, irradiation, and antiseptics (Clery-Barraud et al., 2004). Consequently, development of diagnostic and therapeutic measures specific for *B. anthracis* remains an active area of investigation.

Bacteriophage-encoded endolysins are emerging antimicrobial agents that have been effectively exploited against Gram-positive bacteria (Nelson et al., 2012; Schmelcher et al., 2012a). During the lytic bacteriophage (*i.e.* phage) infection cycle, most endolysins accumulate in the cytosol of host cells. In response to a genetically specified signal, holin proteins produced by the phage oligomerize on the plasma membrane of the bacterium to form pores, allowing endolysins access to their peptidoglycan substrate (Fischetti, 2011; Wang et al., 2000; Young, 1992). These enzymes then cleave critical covalent bonds within the cell wall structure upon direct contact to rapidly induce osmotic lysis and simultaneous progeny virion release. Due to the extrinsic accessibility of peptidoglycan comprised by Gram-positive bacteria, the exogenous therapeutic application of endolysins is an antibacterial strategy that has been repeatedly validated *in vitro* and *in vivo* against drug-susceptible and – resistant Gram-positive bacterial strains (Nelson et al., 2001; Stark et al., 2010). The high binding affinity displayed by endolysins towards unique cognate cell wall-associated epitopes (K_A = ~10^8 to 10^9 M^{-1}) demonstrates their target specificity and thereby reduces the risk of resistance development commonly associated with broad-spectrum classical antibiotics (Schmelcher et al., 2010; Schuch et al., 2002).

The *B. anthracis* gamma phage endolysin PlyG is an N-acetylmuramoyl-L-alanine amidase, consisting of an N-terminal enzymatically active domain (EAD) linked to a C-terminal cell wall binding domain (CBD) (Kikkawa et al., 2008; Schuch et al., 2002). The CBD binds with high affinity to secondary cell wall polysaccharides explicit to both sporulated and vegetative forms of *B. anthracis*, as well as related *Bacillus cereus* strains (Ganguly et al., 2013; Yang et al., 2012). The therapeutic potential of PlyG was initially

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evaluated in 2002, which collectively involved resistance studies and determining whether vegetative bacilli and germinating spores were susceptible to the enzyme (Schuch et al., 2002). With or without the presence of the mutagenic compound methanesulphonic acid ethyl ester (EMS), repeated exposure of bacilli to low or high concentrations of PlyG did not generate any endolysin-resistant strains. In a kinetic analysis, PlyG significantly reduced *Bacillus* viability when extrinsically applied to either vegetative cells or germinating spores. The therapeutic potential of the endolysin was additionally validated *in vivo*, demonstrating an ability to rescue *Bacillus*-infected mice when intraperitoneally administered 15 min after infection (Schuch et al., 2002).

To further evaluate its potential for development as an antimicrobial agent against *B. anthracis*, we investigated the thermal properties of PlyG by circular dichroism spectroscopy, differential scanning calorimetry and thermal kinetic inactivation analysis as a platform for projecting shelf life and product stability. The intrinsic thermal stability of any protein therapeutic is a defining molecular characteristic that dictates its downstream applicability. As prescribed by the Arrhenius equation, the level of thermoresistance displayed by a macromolecule correlates with its shelf life (Anderson and Scott, 1991). In addition to the full-length endolysin, we assessed the thermal stability of the EAD and CBD modules of PlyG. Explicating the thermal properties of a protein therapeutic, such as PlyG, will provide information relevant to its future pharmaceutical or biotechnological use.

Results

Protein purification of PlyG constructs

Full-length PlyG (residues 1–233), as well as the isolated N-terminal PlyG_EAD (residues 1–160) and C-terminal PlyG_CBD (residues 156–233), were expressed and purified to homogeneity based on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis (Fig. 1). As previously described, adding the 12 amino acid leader sequence modification MRGSHHHHHHGS to the amino-terminal of each construct allowed for a single immobilized metal affinity chromatography purification step to achieve high purity (Loessner et al., 1996). The residue coordinates pertaining to each PlyG construct were selected based on findings from preceding studies, which collectively suggest each protein sample exists as a soluble monomer in solution and retains

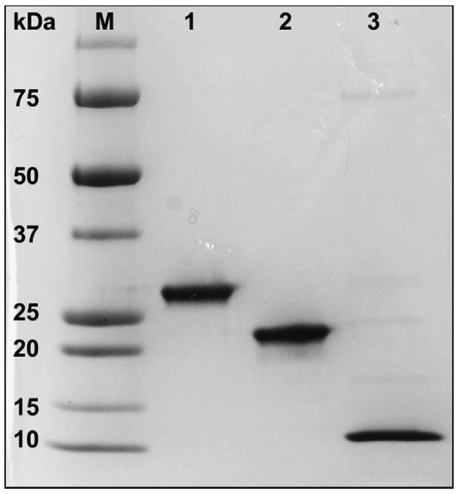

Fig. 1. SDS-PAGE analysis of the purified PlyG constructs. The homogeneity of the (1) 28 kDa full-length PlyG, (2) 19 kDa PlyG_EAD and (3) 10 kDa PlyG_CBD IMAC purified samples was assessed on a 4–15% gradient SDS-PAGE gel.

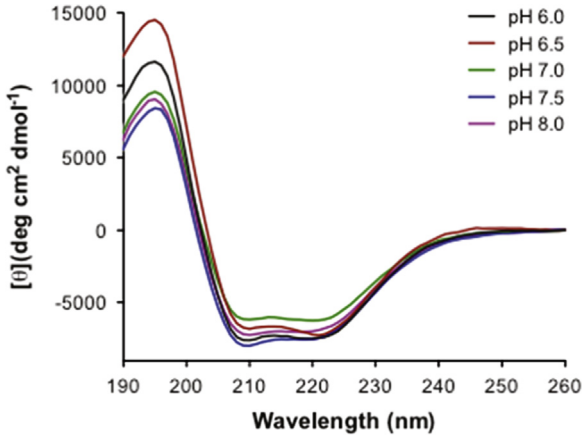

Fig. 2. Secondary structure analysis of PlyG in pH 6.0–8.0 buffers. CD spectra were obtained in the far-UV range (190–260 nm) for PlyG samples in 20 mM sodium phosphate buffer, pH 6.0–8.0. The mean residue ellipticity [θ] (deg cm^2 dmol^{-1}) was plotted against the wavelength (nm) for each sample.

biologic functionality in the presence of its cell wall receptor and/or substrate (Low et al., 2011; Mo et al., 2012; Schuch et al., 2002). Each purified sample was stored in phosphate buffered saline (PBS), pH 7.2, supplemented with 20% glycerol at −80 °C until further needed.

PlyG secondary structure composition and stability as a function of pH

Using circular dichroism (CD) spectroscopy, the influence of pH on PlyG structure and stability was investigated. First, the secondary structure composition of the endolysin was determined in 20 mM sodium phosphate buffer, pH 6.0–8.0. Significant changes in secondary structure would seemingly have implications on the thermal stability of the enzyme. In the pH environments tested, qualitative analysis of the spectra obtained by means of far-ultraviolet (UV) CD spectroscopy revealed PlyG is primarily α/β structured, with an ellipticity maximum observed at 195 nm and minima observed at 208 and 222 nm (Fig. 2). Next, the aforementioned far-UV CD spectra were deconvoluted using the CONTIN method to calculate secondary structure composition. Results suggest there were no substantial modifications in the regular α-helical (±3.5%), distorted α-helical (±1.5%), regular β-strand (±2.5%), distorted β-strand (±0.8%), turn (±2.5%) and unordered (±0.8%) structural composition of PlyG in the pH range investigated (Table 1).

The structural stability of PlyG in 20 mM sodium phosphate buffer, pH 6.0–8.0, was measured using CD thermal denaturation experiments. All of the samples displayed a single thermally-induced unfolding transition (Fig. 3). In the pH range tested, PlyG was most structurally stabilized at pH 6.0, depicting a $T_{1/2}$, which corresponds to the temperature at which the concentration of the folded and unfolded protein fractions are equal, of 64 °C. At pH 6.5, 7.0, 7.5 and 8.0, PlyG comprised respective $T_{1/2}$ values of 63 °C, 62 °C, 59 °C and 57 °C. Although the possibility exists where PlyG could display intensified or weakened structural stability at pH values exceeding pH 6.0–8.0, the $\Delta T_{1/2}$ when comparing the most stabilizing (*i.e.* pH 6.0) and destabilizing (*i.e.* pH 8.0) pH environments analyzed in this study was 7 °C. With this in mind, subsequent thermal analyses of PlyG and its domains were elucidated in pH 6.0 buffers.

PlyG_EAD and PlyG_CBD CD thermal denaturation analysis

CD experiments were next utilized to determine the secondary structure and structural stability of the EAD and CBD modules of PlyG