

Reactions of *Pseudomonas aeruginosa* pyocyanin with reduced glutathione

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Pseudomonas aeruginosa is the most common cause of chronic and recurrent lung infections in patients with cystic fibrosis (CF) whose sputa contain copious quantities of *P. aeruginosa* toxin, pyocyanin. Pyocyanin triggers tissue damage mainly by its redox cycling and induction of reactive oxygen species (ROS). The reactions between reduced glutathione (GSH) and pyocyanin were observed using absorption spectra from spectrophotometry and the reaction products analysed by nuclear magnetic resonance imaging. Pyocyanin reacted with GSH non-enzymatically at 37°C resulting in the production of red-brown products, spectrophotometrically visible as a 480 nm maximum absorption peak after 24 h of incubation. The reaction was concentration-dependent on reduced glutathione but not on pyocyanin. Minimizing the accessibility of oxygen to the reaction decreased its rate. The anti-oxidant enzyme catalase circumvented the reaction. Proton-NMR analysis demonstrated the persistence of the original aromatic ring and the methyl-group of pyocyanin in the red-brown products. Anti-oxidant agents having thiol groups produced similar spectrophotometrically visible peaks. The presence of a previously unidentified non-enzymatic GSH-dependent metabolic pathway for pyocyanin has thus been identified. The reaction between pyocyanin and GSH is concentration-, time-, and O₂-dependent. The formation of H₂O₂ as an intermediate and the thiol group in GSH seem to be important in this reaction.

Keywords: catalase, superoxide dismutase, glutathione, *Pseudomonas aeruginosa* pyocyanin, cystic fibrosis, oxidative stress, spectrophotometry, nuclear magnetic resonance imaging

INTRODUCTION

Chronic *Pseudomonas aeruginosa* infections occur frequently with the immunocompromised, the aged, and those with cystic fibrosis (CF) (Lyczak *et al.*, 2002), bronchiectasis (Khalid *et al.*, 2004), and otitis externa (Beers & Abramo, 2004). Antibiotic-resistant *P. aeruginosa* infections are a major source of

concern (Conway *et al.*, 2003). *P. aeruginosa* secretes a number of virulence factors, including elastases, rhamnolipids, alginate, exotoxin A, exoenzyme S, and phospholipase C, whose virulent properties have been elucidated well (Lyczak *et al.*, 2000; Lau *et al.*, 2004). Yet, neither the precise virulent properties, nor the *in vivo* metabolic fate of pyocyanin, a redox active blue-green phenazine pigment secreted

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Abbreviations: δ , chemical shift; CF, cystic fibrosis; DTT, dithiothreitol; ELF, extracellular lung fluid; GSH, glutathione (reduced); GSSG, glutathione (oxidized); NAC, N-acetylcysteine; NMR, nuclear magnetic resonance imaging; ROS, reactive oxygen species.

by *P. aeruginosa* in copious quantities in infected CF patients (Pitt, 1986; Wilson *et al.*, 1988), have been charted adequately.

Sputum sol phase pyocyanin concentrations from patients with CF colonized by *P. aeruginosa* can go up to 27.3 µg/ml (\approx 130 µM) (Wilson *et al.*, 1988). These concentrations are much more than adequate to inhibit ciliary beating as demonstrable *in vitro* (Wilson *et al.*, 1987). Pyocyanin exerts diverse cytotoxic effects mediated mainly through its redox activity and reactive oxygen species (ROS)-inducing capability (Mahajan-Miklos *et al.*, 1999; O'Malley *et al.*, 2004). Pyocyanin inhibits cell respiration (Sorensen & Klinger, 1987), inactivates catalase (O'Malley *et al.*, 2003), inhibits prostacyclin release (Kamath *et al.*, 1995), and disrupts lung epithelial cell growth (Wilson *et al.*, 1988), calcium homeostasis (Denning *et al.*, 1998) and ciliary function (Wilson *et al.*, 1987). Pyocyanin facilitates *P. aeruginosa* colonization of the respiratory tract by significantly reducing tracheobronchial mucus velocity (Munro *et al.*, 1989). Pyocyanin inactivates protease inhibitors and contributes to a protease-antiprotease activity imbalance, resulting in lung epithelial injury (Britigan *et al.*, 1999). Two *in vivo* murine respiratory tract infection models, acute and chronic, have demonstrated the absolute necessity of pyocyanin biosynthesis for *P. aeruginosa* virulence (Lau *et al.*, 2004).

Glutathione (GSH), a ubiquitous tripeptide intracellular and extracellular thiol, constitutes 90% of intracellular nonprotein thiols (Meister & Anderson, 1983). In a reaction catalyzed by GSH peroxidase (GPx), GSH converts hydrogen peroxide to water, and lipid peroxides to nontoxic hydroxy fatty acids (Zachara *et al.*, 2006). In addition to this most important anti-oxidant function, GSH also impedes antiprotease-degradation (Buhl *et al.*, 1990) and assists in maintaining lung surfactant (Jain *et al.*, 1992). GSH levels in the extracellular lung fluid (ELF) range from 250 to 800 µM with a mean of 429 µM for patients not under oxidative stress, and a mean of 800 µM for patients under oxidative stress (Roum *et al.*, 1993; Morrison *et al.*, 1999). This is significantly higher than the normal plasma GSH range of 2–5 µM (Samiec *et al.*, 1998). The total ELF glutathione (96% GSH + 4% glutathione disulfide GSSG) is at least 140 times more than the plasma concentration (Cantin *et al.*, 1987). Although the ELF GSH levels are slightly lower in CF patients when compared to normal individuals (Roum *et al.*, 1993), a normal level of intracellular GSH (2–10 mM) is present in CF epithelial cells (Gao *et al.*, 1999). In CF patients, both spontaneous and induced sputum samples contain higher GSH levels than samples from healthy controls (Dauletbaev *et al.*, 2004).

Since pyocyanin is produced in large amounts in CF pseudomonal infections (Pitt, 1986; Wilson *et al.*, 1988), these differences in GSH levels in the ELF and bronchoalveolar secretions could be easily and frequently perturbed by acute or chronic pyocyanin-induced epithelial and endothelial damage, resulting in GSH release from the lysed cells. Pyocyanin (12.5 µM or more, 30 min-incubation) significantly decreases human umbilical venous endothelial cell GSH level (Muller, 2002). Pyocyanin (30 µM or more, 24 hour-incubation) significantly decreases intracellular GSH in the respiratory cell-line A549 (O'Malley *et al.*, 2004). Pyocyanin concentrations of 30 µM or more significantly increase extracellular GSH export and GSSG concentrations in A549 cells (O'Malley *et al.*, 2004). Those authors demonstrated a prompt direct reaction between pyocyanin and GSH, resulting in the formation of thiyl, pyocyanin, and superoxide radicals. Both Muller (2002) and O'Malley *et al.* (2004) reported the results of experiments involving pyocyanin and GSH only that were incubated for less than an hour. Yet in CF patients, *P. aeruginosa*, secreting pyocyanin, lingers for long periods of time causing recurrent remissions and exacerbations. We simulated this scenario by demonstrating a reaction between pathophysiologically relevant concentrations of pyocyanin and GSH over a period of days, rather than minutes or hours.

In our study, we show a reaction of pyocyanin with GSH in phosphate buffer (pH 7.4), at 37°C, observed over a period of days under pathophysiologically relevant conditions using spectrophotometry. We also investigated the effect of oxygen concentration, reactant concentrations, anti-oxidant enzymes, and pH on the reaction. We also report our attempts to purify and analyze the reaction products using nuclear magnetic resonance (NMR) imaging.

METHODS

Synthesis of pyocyanin. Pyocyanin was chemically synthesized by photolysis of phenazine methosulfate (Knight *et al.*, 1979) and purified (Muller & Sorrell, 1992) as described earlier. Briefly, phenazine methosulfate (P-9625-5g, Sigma-Aldrich Pt Ltd, Sydney, Australia) was made up in 0.01 M Tris/HCl buffer (pH 7.4) in a round-bottomed Pyrex flask and exposed to fluorescent tube light (Phillips TLD 18 W/54) for 2.5 h. The resulting solution was extracted into chloroform and vacuum-dried to a powdery residue. The residue was dissolved in chloroform and acidified with an equal volume of 0.1 M HCl. The red acidic form of pyocyanin was converted into the blue form using 0.5 M NaOH. This blue form of pyocyanin, now in the aqueous phase, was extracted into chloroform and this cycle repeated 3 times. The blue form of pyocyanin which was extracted into chloroform the last time was vacuum dried, washed

as well as precipitated with hexane, trapped on a type EH 0.5 μm filter, reconstituted in chloroform and purified by thin-layer chromatography (TLC) using an equimolar chloroform/methanol mixture solvent. The pyocyanin thus purified was stored in methanol at -70°C . The purity of pyocyanin was ascertained and its concentration quantitated by utilizing its known absorption spectrum and absorption coefficient values as elucidated earlier (Watson *et al.*, 1986). Before use, the methanol solvent was removed by nitrogen insufflation and when completely dry, pyocyanin was reconstituted in saline and used.

Glutathione-pyocyanin reactions. All reactions were done in triplicate. Pyocyanin concentrations used were 0–125 μM to cover previously estimated *in vivo* concentrations (Wilson *et al.*, 1988). GSH concentrations utilized were 0–10 mM to cover concentration ranges in CF ELF (Roum *et al.*, 1993), CF epithelial cells (Gao *et al.*, 1999) and in spontaneous and induced sputa (Dauletbaev *et al.*, 2004). Most reactions had 25 μM pyocyanin and 10 mM GSH as the reactants. All incubation mixtures were incubated in well-covered 1 cm plastic cuvettes, unless otherwise specified. Incubation of all reactions was done in phosphate buffer solution (PBS, 125 mM KH_2PO_4 , pH 7.4) at 37°C in the dark. For oxygen-deprived reactions, nitrogen was bubbled through the reaction mixtures, followed by addition of a 3 cm mineral oil layer on top of each final reaction mixture before incubation. Reaction mixtures were transferred to plastic cuvettes to measure the absorbance spectra using a Shimadzu UV120 IPC spectrophotometer with the following calibrations: Measuring Mode — Abs, Recording Range — Low 0.0 to High 0.5, Wavelength Range (nm) — 800–200 nm, Scan Speed — Fast and Sampling Interval — 1 nm. The absorption spectra presented throughout this study are representatives of triplicates. The reaction rates and constants were deliberately not calculated owing to the obvious slow reaction time and the chronicity of the pathophysiological scenario.

Purification of glutathione-pyocyanin reaction products. The red-brown GSH-pyocyanin reaction products were purified from up-scaled 10 ml reaction mixtures (concentrations of reactants increased 10-fold) with the discovery that a Tris/HCl (10 mM, pH 3) wash prior to acidified (2% acetic acid) aqueous methanol elution of the GSH-pyocyanin reaction products removes glutathione, both reduced and oxidized. The presence or absence of glutathione is easily discernable by the presence or absence of the 230 nm peak or by the ninhydrin test. Each up-scaled reaction mixture consisted of 10 ml of PBS with 250 μM pyocyanin and 100 mM GSH (final pH=7.4), incubated at 37°C in a loosely capped 50 ml conical bottom Falcon polypropyl-

ene tube for 36–48 h. After several attempts, purification of the red-brown species from the reactant remnants and other products involved the following solid phase extraction (SPE) method: A polymer based SPE column (Oasis HLB 3cc cartridges from Waters company) was determined to be the best available method to purify the red-brown species by utilizing the following protocol (for each Oasis HLB 3cc cartridge): (1) CH_3OH : 1 ml, (2) Millipore water: 1 ml, (3) up-scaled pyocyanin-GSH reaction mixture: 1 ml, (4) Tris/HCl wash: 1 ml, (5) PBS (pH=7.4) wash: 5 ml, (6) Millipore water: 1 ml, (7) acidified 60% methanol elute solution: 1 ml, and (8) speed vacuum drying.

Ninhydrin test. The ninhydrin test is a test for the presence or absence of the amino ($-\text{NH}_2$) group and hence should be positive if glutathione (GSH or GSSG) is present. A crude qualitative visual test that we utilized for testing for the presence or absence of glutathione was done by heating 0.5 ml of the sample with 0.5 ml of ninhydrin (triketohydrindene hydrate; Sigma-Aldrich, St. Louis, MO, USA) in acetone with 1% acetic acid up to 45°C in a glass tube and observing color changes. In this adaptation of the ninhydrin test, a dark red-brown color change indicates the presence of abundant amino ($-\text{NH}_2$) groups in the sample. This crude test was positive only when amino groups were abundant.

Chemicals and enzymes. Glutathione (GSH and GSSG), *N*-acetylcysteine (NAC), dithiothreitol (DTT), superoxide dismutase (SOD), and catalase (CAT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). TLC plates (Silicagel 20×20 cm) were obtained from Crown Scientific (Moorebank, NSW, Australia).

Nuclear magnetic resonance imaging. All proton NMR (^1H solution) spectra were done at 25°C using a Bruker DRX 300 MHz NMR instrument. Deuterium oxide (Sigma Aldrich) was consistently used as the solvent. The internal standard was the sodium salt of 3-(trimethylsilyl) 3,3,2,2-tetradeteropropionic acid. The typical acquisition parameters utilized were spectral width 4000 Hz, repetition time 2 s and line broadening frequency 0.5 Hz.

RESULTS

Glutathione and pyocyanin reaction

GSH reacts with pyocyanin non-enzymatically after 24 h of incubation at 37°C , resulting in formation of red-brown products, visible spectrophotometrically as a 480 nm peak (Fig. 1E and G). Figure 1A and B represent 10 mM GSH-only controls under aerobic and oxygen-deprived con-

ditions, respectively. Figures 1C and D represent 25 μ M pyocyanin-only controls under aerobic and oxygen-deprived conditions, respectively.

Time-course experiments involving glutathione and pyocyanin

GSH and pyocyanin were incubated at 37°C over 96 h under normal aerobic conditions or oxygen-deprived conditions, with absorption spectra recorded 24-hourly. The production of the 480 nm peaks, coinciding with the formation of the visible red-brown products, was much faster under aero-

bic conditions (Figs. 1E and G) in contrast to oxygen-deprived conditions (Fig. 1F and H).

Dose-response experiments involving glutathione and pyocyanin

Dose-response experiments were done involving GSH and pyocyanin at 37°C over 96 h under normal aerobic conditions. Production of the 480 nm peak, coinciding with the formation of the visible red-brown products, was dependent only on the concentration of GSH (Fig. 2A) but not on the concentration of pyocyanin (Fig. 2B).

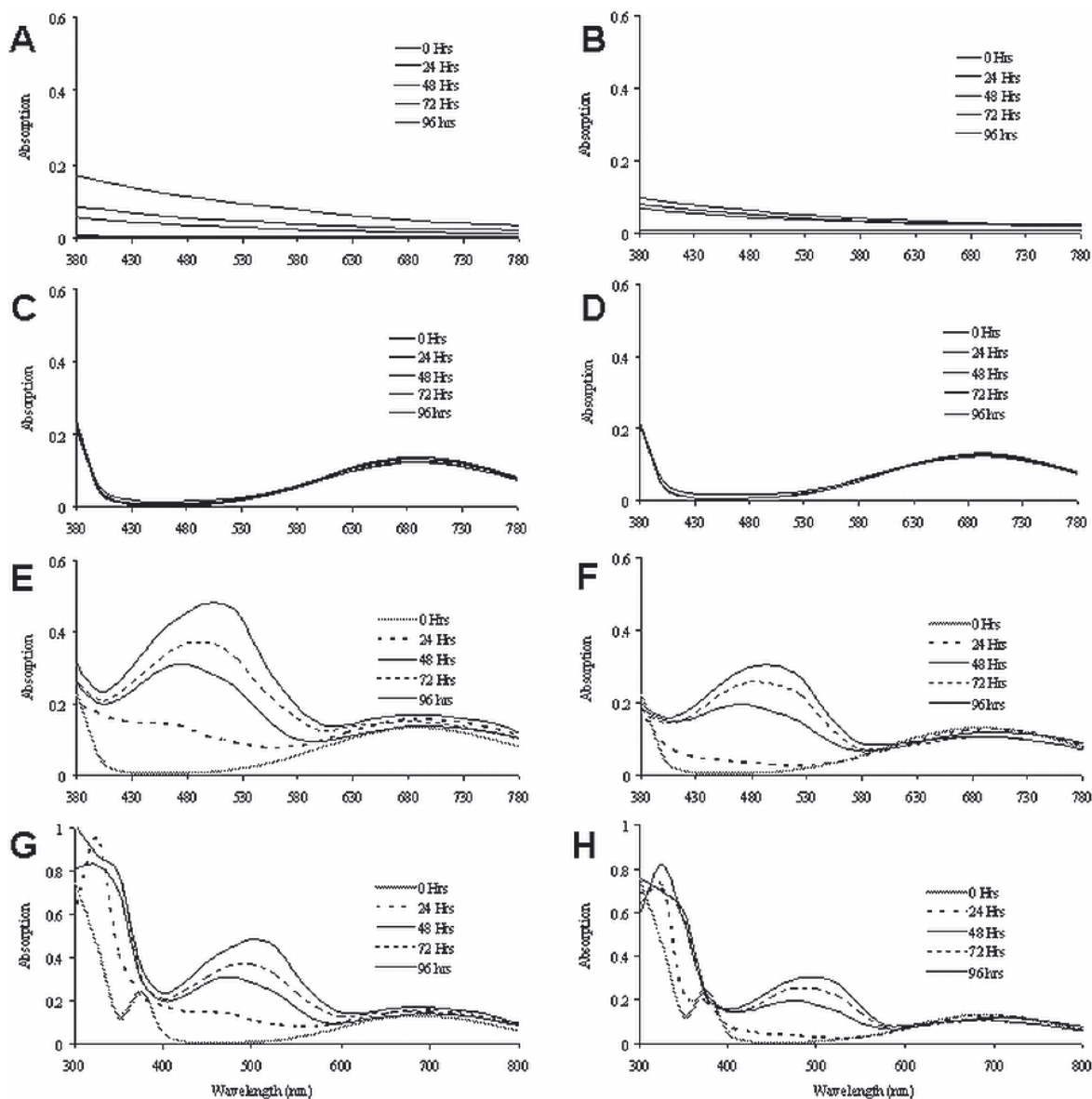


Figure 1. Time-course experiments involving GSH and pyocyanin.

GSH and pyocyanin were allowed to react at 37°C over 96 h with absorption spectra recorded 24-hourly. A and B, 10 mM GSH-only controls under aerobic and oxygen-deprived conditions, respectively. C and D, 25 μ M pyocyanin-only controls under aerobic and oxygen-deprived conditions, respectively. E and F, 25 μ M of pyocyanin reacting with 10 mM GSH under aerobic and oxygen-deprived conditions, respectively, demonstrating that the reaction producing the 480 nm absorption peak was much faster under aerobic conditions than anaerobic conditions. G and H, Complete spectra shown in part as E and F, respectively.

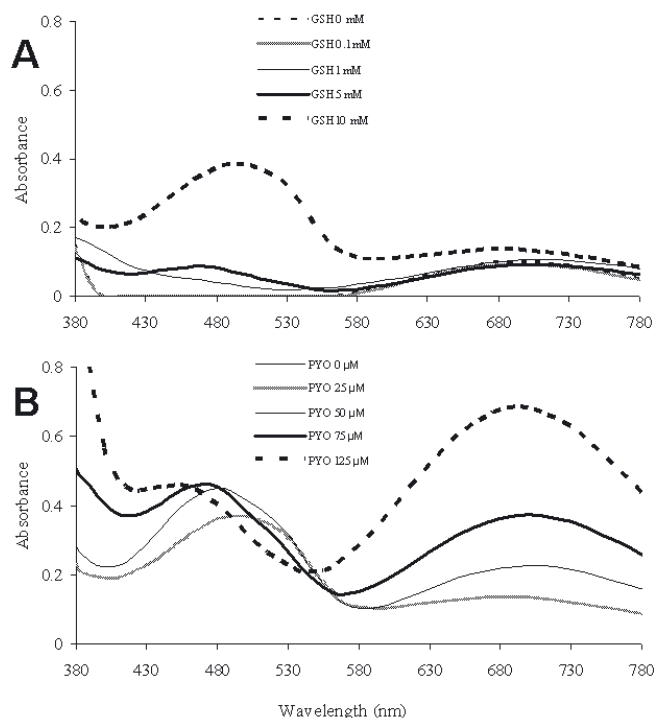


Figure 2. Dose-response experiments involving GSH and pyocyanin.

GSH and pyocyanin were allowed to react at 37°C over 96 h under aerobic conditions. A. Absorption spectra for 25 μM pyocyanin treated with increasing concentrations of GSH. Production of the 480 nm peak was dependent on the concentration of GSH. B. Absorption spectra for 10 mM GSH treated with increasing concentrations of pyocyanin. Production of the 480 nm peak did not seem to be dependent on the concentration of pyocyanin.

The effect of anti-oxidant enzymes on the glutathione-pyocyanin reaction and the effect of pH variations on the reaction products

SOD did not prevent the 72-h GSH-pyocyanin reaction from occurring but a combination of SOD and catalase did (Fig. 3A), pointing to the importance of H_2O_2 but not superoxide in the reaction. A completely and repeatedly demonstrable pH-dependant (pH 2 to 9) reversible shift in the complete absorption spectra of a diluted sample of the red-brown GSH-pyocyanin reaction products was evi-

dent (Fig. 3B), suggesting active electron resonance in the reaction products.

Purification of glutathione-pyocyanin reaction products

Solid-phase extraction procedures involving various concentrations, proportions and combinations of methanol, chloroform, ethyl acetate and acetone were done before ascertaining that acidified methanol was the best eluant for solid-phase-trapped red-brown GSH-pyocyanin reaction products. The more the aque-

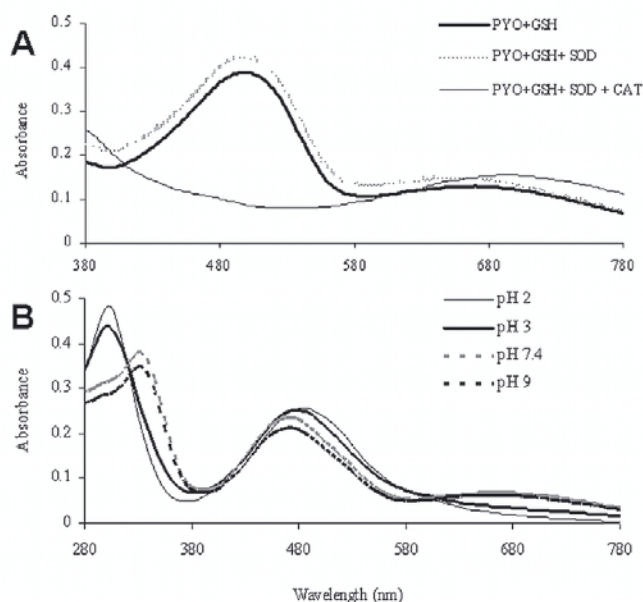


Figure 3. The effect of anti-oxidant enzymes on the GSH-pyocyanin reaction and the effect of pH variations on the reaction products.

A. SOD did not prevent the 72-h GSH-pyocyanin reaction from occurring but a combination of SOD and catalase did. B. pH-dependant reversible shift (pH 2 to 9) in absorption spectra of diluted sample of the red-brown GSH-pyocyanin reaction products.

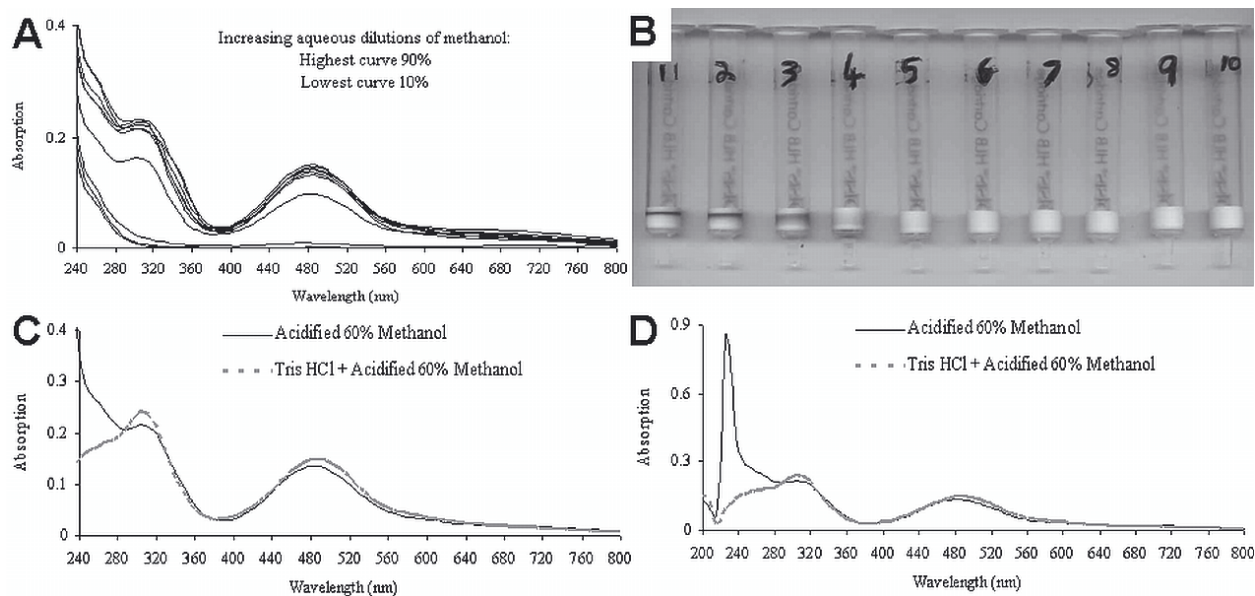


Figure 4. Purification of GSH-pyocyanin reaction products.

A and B. Demonstration of the efficacy of increasing dilutions of aqueous acidified methanol to elute the red-brown GSH-pyocyanin reaction products trapped in the Oasis HLB 3cc cartridges. B. Cartridges 1 to 10 arranged in order of increasing dilutions of acidified methanol demonstrating that more the aqueous dilution of acidified methanol, the better the elution efficacy of the trapped red-brown GSH-pyocyanin reaction products. However, owing to the ease in extracting the dry GSH-pyocyanin reaction products from 60% methanol compared to more aqueous solutions, this dilution of acidified methanol was used subsequently. C and D. Spectra of 60% acidified methanol eluted GSH-pyocyanin reaction products with or without Tris/HCl (10 mM, pH 3) removal of residual glutathione. Absence of residual glutathione was confirmed by the absence of the 230 nm absorption peak and a negative ninhydrin test. D. Complete spectra shown in part as C.

ous dilution of acidified methanol (2% acetic acid), the better the elution efficacy of the trapped red-brown GSH-pyocyanin reaction products trapped in the Oasis HLB 3cc cartridges (Figs. 4A and B). Having estab-

lished that acidified 60% methanol was the most appropriate eluant (Figs. 4A and B) owing to the ease in extracting the dry reaction products from 60% methanol compared to more aqueous solutions, the problem

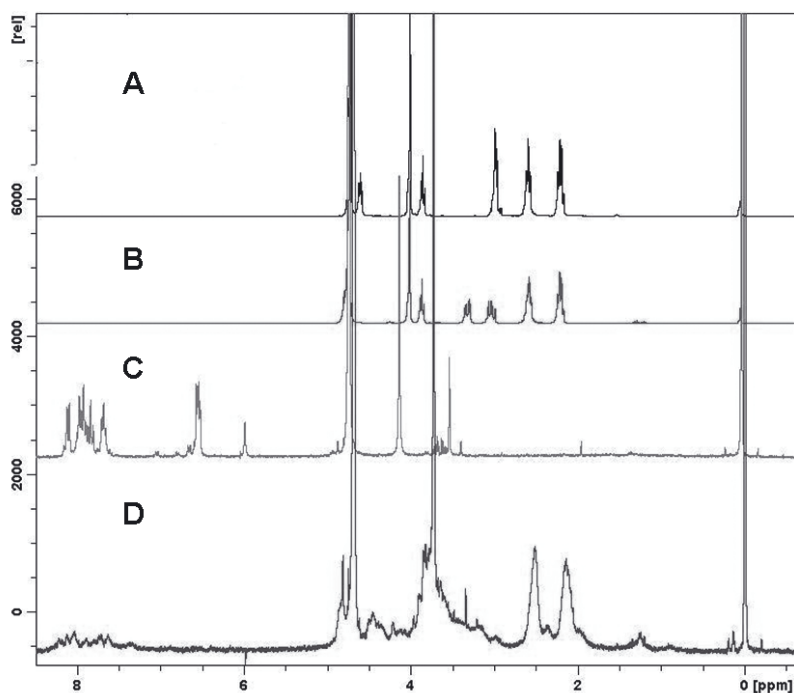


Figure 5. Proton NMR analysis of the red-brown GSH-pyocyanin reaction products.

Proton NMR spectra of GSH (A), GSSG (B), Pyocyanin (C) and red-brown GSH-pyocyanin reaction products (D). The singlet peak at δ 4.14 ppm, corresponding to the methyl group in pyocyanin (C), shifts upfield in the red-brown GSH-pyocyanin reaction products (D) with additional peaks in the latter (δ 2.14, 2.53, 3.82, 4.21 and 4.81) corresponding to GS in the red-brown GSH-pyocyanin reaction products. The multiple peaks around δ 7.64–8.21 ppm, indicative of the aromatic region of the pyocyanin (C), shifted downfield in the red-brown GSH-pyocyanin reaction products (D).

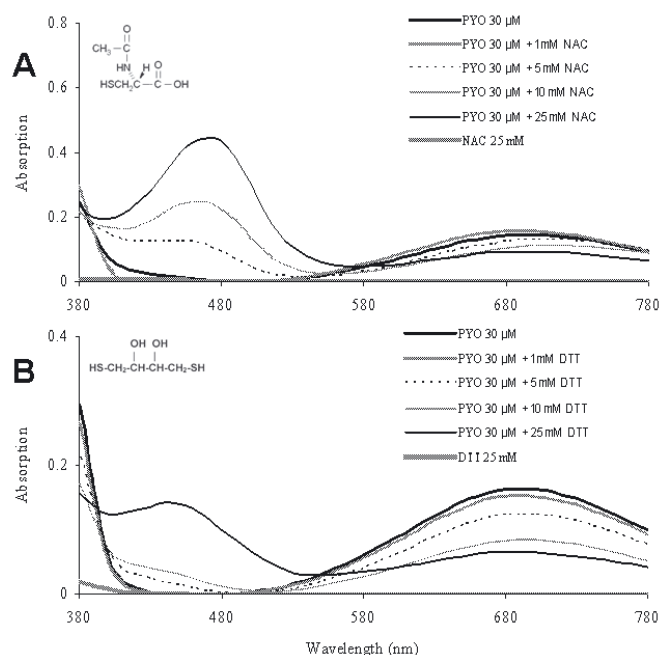


Figure 6. Reactions of pyocyanin with other thiol anti-oxidant agents

At 37°C over 96 h under aerobic conditions, thiol anti-oxidant agents *N*-acetylcysteine (A) and dithiothreitol (B) produced absorption peaks at 470 nm and 450 nm respectively, with 30 μM pyocyanin.

of co-elution of the original reactants (both reduced and oxidized glutathione) with the products was precluded only after the discovery that Tris/HCl (10 mM, pH 3) removes glutathione (GSH and GSSG). The absence of residual glutathione can be inferred from the absence of the 230 nm absorption peak (Figs. 4A and 4D; Fig. 4C is a magnified circumscribed version of Fig. 4D), after Tris/HCl treatment and subsequent acidified aqueous methanol elution of the GSH-pyocyanin reaction red-brown products. These products were also ninhydrin test-negative suggesting the absence of abundant amino group and hence, glutathione.

Properties of glutathione-pyocyanin reaction products

The purified red-brown products were insoluble in methanol, chloroform, ethyl acetate, acetone, and a number of organic solvents, but very soluble in water.

Nuclear magnetic resonance analysis of the red-brown glutathione-pyocyanin reaction products

A comparison of the proton NMR spectra of GSH (Fig. 5A), GSSG (Fig. 5B), pyocyanin (Fig. 5C)

and the red-brown GSH-pyocyanin reaction products (Fig. 5D) provides an indication of the appropriate assignments to the red-brown GSH-pyocyanin reaction products. The singlet peak at δ 4.14 ppm, which corresponds to the methyl group in pyocyanin (Fig. 5C), shifts upfield in the red-brown GSH-pyocyanin reaction products (Fig. 5D) due to the disappearance of the positive charge from the amine group. In the NMR spectra of the red-brown GSH-pyocyanin reaction products (Fig. 5D), five peaks at δ 2.14, 2.53, 3.82, 4.21 and 4.81 can be assigned to the GS bond to the pyocyanin owing to similar peaks produced by GSH and GSSG. Furthermore, the multiple peaks around δ 7.64–8.21 ppm, indicative of the aromatic region of the pyocyanin (Fig. 5C), shifted downfield in the red-brown GSH-pyocyanin reaction products (Fig. 5D). This downfield shift in the aromatic region of the NMR spectra may be the characteristic feature of the new net positive charge of the red-brown GSH-pyocyanin reaction products.

Reactions of pyocyanin with other thiol anti-oxidant agents

Thiol anti-oxidant agents *N*-acetylcysteine (Fig. 6A) and dithiothreitol (Fig. 6B) produced ab-

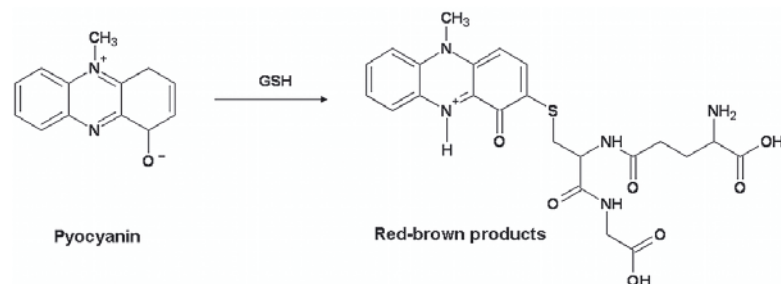


Figure 7. Proposed reaction of pyocyanin with GSH.

Hydrogen from the thiol (–SH) group of GSH reacts with the first nitrogen atom of pyocyanin. The hydroquinone pyocyanin structure is oxidized to a quinone structure, adding a whole GS molecule to the *ortho*-position of the quinone red-brown products, with a re-arranging of covalent carbon bonds.

sorption peaks with 30 μ M pyocyanin (37°C; 96 h; normal aerobic conditions) at 470 nm and 450 nm respectively, demonstrating that the thiol group may be important in these reactions.

DISCUSSION

Pyocyanin reacted non-enzymatically with GSH resulting in formation of red-brown products, visible spectrophotometrically as a 480 nm peak recorded after 24 h of incubation at 37°C. The reaction was concentration-dependent on GSH. Decreasing oxygen availability decelerated the reaction. The red-brown products also demonstrated an absorption spectral shift with pH changes. The red-brown products were insoluble in methanol, chloroform, ethyl acetate, acetone, and a number of organic solvents, but very soluble in water. Catalase circumvented the reaction demonstrating the importance of H_2O_2 in this reaction. Redox cycling of pyocyanin utilizing cellular reductants results in the production of abundant quantities of H_2O_2 which can be used in this reaction (Muller, 2002). Thiol group-containing agents such as NAC and DTT produced similar reactions showing that the thiol group may be important in this reaction. Proton-NMR analysis clearly depicted the persistence of the original aromatic ring and the methyl group of pyocyanin in the red-brown products. Hydrogen from the thiol group of GSH or a possible thiyl radical that may have formed in the reaction between pyocyanin and GSH (O'Malley *et al.*, 2004) may react with the first nitrogen atom of pyocyanin (Fig. 7). GSH probably reacts with the hydroquinone pyocyanin structure producing a quinone structure, resulting in a whole GS molecule being added to the *ortho*-position of the quinone red-brown products in the process.

Pyocyanin, which is produced in large amounts in CF pseudomonal infections (Pitt, 1986; Wilson *et al.*, 1988), exerts manifold cytotoxic effects mediated through redox cycling and ROS induction (Britigan *et al.*, 1992; Mahajan-Miklos *et al.*, 1999; Muller, 2002). Pyocyanin alters specific immune defences, and potentiates and perpetuates harmful inflammatory reactions in the infected CF lung. Yet the *in vivo* metabolic fate of pyocyanin has not been elucidated till now.

Lung epithelial, endothelial, and ELF GSH exerts significant cytoprotective antioxidant, surfactant stabilizing, anti-protease protecting and mucolytic activity (Meister & Anderson, 1983; Rahman & MacNee, 2000). Abnormal and low GSH levels can lead to crippling of the lung's antioxidant system, anti-protease system (Konstan *et al.*, 1994; Birrer, 1995), message transmission and immune recognition system (Barbero, 1994), mucolytic system (Hudson,

2001; 2004), and surfactant secretion system (Jain *et al.*, 1992). The end results of low GSH levels include pulmonary damage, exacerbation of extant pseudomonal infections and a drastically increased propensity to contract infections. Since pyocyanin has a profound ability to modulate the glutathione redox cycle and deplete intracellular GSH in endothelial cells (Muller, 2002) and intracellular and extracellular GSH in lung epithelial cells (O'Malley *et al.*, 2004), it is reasonable to expect lower GSH levels in ELF from CF patients with *P. aeruginosa* infection. Although ELF has decreased GSH levels (Gao *et al.*, 1999) compared to intracellular GSH levels in CF lung epithelia, CF sputum samples have higher GSH levels than healthy sputum samples (Dauletbaev *et al.*, 2004). The latter discrepancies could be accentuated by pyocyanin-induced epithelial (Hingley *et al.*, 1986; Wilson *et al.*, 1987) and endothelial (Britigan *et al.*, 1992; Miller *et al.*, 1996; Muller, 2002) necrosis and apoptosis, resulting in GSH release. Since normal intracellular GSH concentration ranges from 2 to 10 mM (Gao *et al.*, 1999), released cellular GSH would probably be present in high concentrations for a non-enzymatic reaction with pyocyanin in the setting of a persistent *P. aeruginosa* infection lingering in the CF lung.

While pyocyanin is very soluble in water, methanol and chloroform, the purified red-brown products were insoluble in most organic solvents. Yet the red-brown products were very soluble in water, attesting to the polarity of the same and suggesting that this reaction is probably a pyocyanin detoxification mechanism. Preliminary studies with NADPH show a significant drop in the oxidative potential of the red-brown products when compared to that of pyocyanin. Therefore the red-brown products may be the result of a previously unknown oxygen-dependent, non-enzymatic pyocyanin detoxification system involving GSH. Consequentially, the production of pyocyanin in the airways of CF patients may be much higher than previously assessed (Pitt, 1986; Wilson *et al.*, 1988), because the presence of the red-brown products has not been accounted for by the methodologies used till now. Neither the red-brown products produced during this reaction, nor the magnitude of GSH concentration fluctuations in CF patients with *P. aeruginosa* infections, have been estimated yet. This can be the next research course to pursue.

To conclude, the presence of a possible non-enzymatic GSH-dependent pathway for *P. aeruginosa* pyocyanin conversion has been identified. This pathway is time-, concentration-, and O_2 -dependent. The formation of H_2O_2 as an intermediate is necessary for this reaction. The thiol group of GSH seems to be important in this reaction. The original aromatic ring and the methyl group of pyocyanin persist in

the red-brown reaction products. Strategies to inhibit pyocyanin production and to facilitate pyocyanin metabolism to neutralize its toxicity by GSH and other antioxidant therapies may be incorporated into existing regimens for the treatment of CF (Conway, 1999; Roum *et al.*, 1999; Rahman & MacNee, 2000). If the red-brown products prove to be substantially less pathogenic than pyocyanin in future research, the utilization of aerosolized, spaced, or nebulized GSH could be viable therapeutic options in chronic pseudomonal lung infections.

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