RESEARCH PAPER

Iron chelating activity of Pyoverdin PaII and pseudobactin 589 siderophores with plasma iron

Saengrawee Sutthiparinyanont[1] and Chalerm Ruangviriyachai[2]

[1]School of Pharmaceutical Sciences, University of Phayao, Phayao and
[2]Faculty of Science, Khon Kaen University, Khon Kaen, Thailand

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Correspondence
Saengrawee Sutthiparinyanont
School of Pharmaceutical Sciences
University of Phayao
Phayao 56000
Thailand

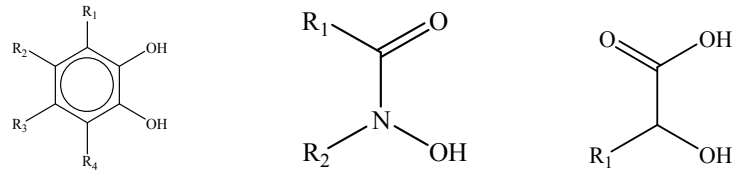
E-mail
saengrawees@yahoo.com

Abstract

Two kinds of siderophores produced from two bacterial species, *Pseudomonas aeruginosa* and *P. putida,* called pyoverdin PaII (pvd-PaII) and pseudobactin 589 (psdb-589), respectively. Both of them were investigated for their iron scavenging effects upon plasma iron and compared with commercial iron chelator named esferoxamine B (DFO-B) by malondialdehyde-2-thiobarbituric acid assay (MDA-TBA assay). These siderophores were produced by the optimized conditions using 6% sucrose, 0.4% L-asparagine and K_2HPO_4 with the incubation temperature at 25°C. They were isolated and purified by Amberlite XAD-4 and Bio-Gel P-2 columns. Each of siderphore-iron complexes was at the ratio of 1:1. The content of iron in plasma sample determined by atomic absorption spectrophotometer was in the range of 0.81- 1.68 μg/ml. The iron scavenge was represented by the reduction of lipid peroxidation in plasma measured by MDA-TBA content. At the concentration of 35.8 μM of 3 iron chelating agents, DFO-B, pvd-PaII and psdb-589, could reduce the average lipid peroxidation in plasma (n = 6) by 11.2, 27.0 and 29.4%, respectively. The reduction of plasma MDA compared between pvd-PaII and psdb-589 or pvd-PaII and DFO-B was not significantly different ($p > 0.05$). It can be concluded that the biological iron chelator of siderophore especially psdb-589 reveals capability to reduce iron which is the cause of lipid peroxidation in plasma.

Introduction

Gram negative bacteria can produce colorless fluorescent siderophores under iron deficient conditions (Cobessi *et al*, 2005). Siderophores, with a range of molecular weight of 400-2000 Da, are powerful and selective low molecular weight chelators which have strong specificity and affinity ($K_{aff} \sim 10^{30}$ M^{-1}) toward the ferric ion (Braun and Killmann, 1999). Other metals such as Cu, Cr, Cd, Ni, and Al do not interfere with the biosynthesis of the siderophores (Carrillo-Castaňeda *et al*, 2005).



Catecholates Hydroxamates Hydroxyacids

Figure 1 Three main bidentate chelating groups in siderophores

Bidentate chelating groups, e.g., cate-cholates, hydroxamates, hydro-xyl acids, shown in Figure 1, are responsible for stable hexacoordinates octahedric complex with iron (III) (Pattus and Abdallah, 2000). A fluo-rescent siderophore, pyoverdin from *Pseudomonas* spp, consists in bidentate ligand for Fe (III), a peptide chain with 2 bidentate sites and monoamide side-chain (Meyer *et al*, 2002, del Olmo *et al*, 2003, Hannauer *et al*, 2012).

Pseudomonas aeruginosa, an oppor-tunistic pathogen, synthesizes different siderophores including pyo-verdins (Budzikiewicz, 1997, Cobessi *et al*, 2005, Shinozaki-Tajiri *et al*, 2004) and pyochelin (Mislin *et al*, 2006). *P. putida*, a plant-growth promoting bacterium, was shown to produce several types of pseudobactin including psedobactins (Duijiff *et al*, 1994, Boopathi and Rao, 1999). Deferoxamine or desfero-xamine B (DFO-B) is a commercially hydroxamate siderophore produced by the actinobacteria *Streptomyces pilosus* (Morel *et al*, 1992), used clinically as an effective parenteral drug for iron overload conditions (Kontoghiorghes 1995, Pootrakul, 1999, Kontoghiorghes *et al*, 2000) and being shown to be neuro-protec-tive for hypoxic-ischemic brain injury (Hamrick *et al*, 2005).

Iron is essential in various biochemical processes including cell proliferation, electron transfer and detoxification (Neilands, 1995). How-ever, an excess iron may induce toxicity via free radicals (Kontoghior-ghes *et al*, 2000, Schafer *et al*, 2000, Roy and Enns, 2000, Dontan *et al*, 2004) causing oxidative stress (Comporti *et al*, 2002). Lipid peroxidation, a mechanism of cellular injury of living organisms, is used to measure oxidative stress in cells and tissues. Certain stress conditions, free radicals are generated resulting in reduction of iron to ferrous form. Non-protein bound iron converts hydrogen peroxide to highly reactive oxygen species (ROS), such as the hydroxyl radical, via the Fenton reaction. These ROS can initiate further injury through lipid and protein oxidation and DNA base modification (Dontan *et al*, 2004).

Tissues with high concentration of lipids and oxygen consumption rate are particularly vulnerable to this oxidative damage due to disturbance of the redox balance and increased availability of non-protein bound free iron and could affect vital organs such as the liver, heart, blood and endocrine organs as well as cancer (Minotti *et al*, 1996). Certain compounds with antioxidant properties and capacity to chelate transition metals like ferrous ion could inhibit lipid peroxidation and free-radical-mediated cell damage in skin fibroblast cultures (Campo *et al*, 2005). Also, chelators that mobilize iron were reported to exhibit a potential to cause oxidative stress. Iron chelation therapy is used to reduce oxidative damage caused by iron overload. Malondialdehyde (MDA) is one potential technique and the most frequently used to evaluate lipid peroxidation. The concentration of MDA, known as a secondary product and a biomarker of lipid peroxidation, can be measured upon reaction with 2-thiobarbituric (TBA) in the presence of lipids (Nielsen *et al*, 1997; Campo *et al*, 2005, Hermans *et al*, 2005). The condensation of MDA with two molecules of TBA forms achromogen or complex of pink color which is usually measured by spectrophotometry or by spectrofluorometry with an absorption maximum at 532-535 nm (Brown and Kelly, 1996, Hermans *et al*, 2005).

Two stains of *Pseudomonas* species, *P. aeruginosa* and *P.putida*, were used in the synthesis of siderophores. The conditions of siderophore production were optimized. The siderophores, in comparison to DFO-B, were investigated on iron complexation and lipid peroxidation using MDA-TBA assay and human plasma.

Materials and Methods

Acetic acid, ammonium chloride, ammonium sulfate, glycerol, hydrochloric acid, magnesium sulfate heptahydrate, potassium dihydrogen phosphate, potassium hydroxide and sodium acetate were obtained from Carlo Erba (Italy). Bio-Gel P-2 was purchased from Bio-Rad Laboratory, Richmond (USA). Succinic acid was bought from Ajax Chemicals (Australia). Di-sodium hydrogen phosphate, methanol, phosphoric acid, pyridine and sodium carbonate were obtained from Merck (Germany). Ferric citrate monohydrate, sodium-D gluconate, hexadecyl-triethyl ammonium bromide, 2-thiobarbituric acid (TBA) and trichloroacetic acid were from Fluka (Buchs SG, Switzerland). The desferrioxaminemesylate salt (DFO-B), 2,6-di-tert-4-methylphenol (BHT), 1,1,3,3-tetraethoxypropane (TEP), chrome azurolsufonate (CAS), L-asparagine and amberlite XAD-4 resin from Sigma (U.S.A.). Methanol and acetonitrile (HPLC grade) were from Lab Scan (Ireland). Sucrose and Glucose was obtained from Imperial General Foods Industry (Thailand) and Boot Manufacturing (Thailand).

Bacterial strains used were *P. aeruginosa* R', obtained from Srinagarind Hospital, Khon Kaen University, Khon Kaen, Thailand, and *P. putida* GIFU 1081, purchased from Health Science Research Institute, Bangkok, Thailand. The methods used to prepare and separate siderophores from these bacterial strains were previously described (Ruangviriyachai *et al*, 2000, Ruangviriyachai *et al*, 2001). Human plasma samples from 6 donators were from the Blood Bank, Srinagarind Hospital, Khon Kaen University, Khon Kaen, Thailand.

Optimization of siderophore production: Two strains of *P.*

aeruginosa R' and *P. putida* GIFU 1081 were used to produce the siderophore in this study. The siderophore from *P. aeruginosa* R' was produced in SA universal medium, isolated and purified as described by Ruangviriyachai *et al* (2000), where-. As the production of siderophore from *P. putida* GIFU 1081 was carried out by appropriate conditions as following studies. In all studies, the media were shaken at 25 ± 1°C for 48 h. The siderophore production was detected by UV-visible absorption at λ_{max} = 402 nm as well as fluorescence property under UV-light, and formation of complex with ferric ion.

Type and content of carbon (C) and nitrogen (N) were varied and formulated in SA universal medium, mixture of 20g/L sucrose, 2g/L L-asparagine anhydrous, 1g/L di-potassium hydrogen phosphate and 1g/L magnesium sulfate heptahydrate. Four different C sources including sucrose, glucose, fructose and succinic acid, as well as four different N sources of L-asparagine, urea, ammonium chloride and ammonium sulfate, were investigated. The content of each C source was varied into 0.5, 1, 2, 4, 6, 10 and 14%w/v, whereas the content of N source was varied into 0.05, 0.1, 0.2, 0.4 and 0.6 %w/v of the medium. Each single colony of bacterial stains on chrome azurolsufonate (CAS) agar plate was inoculated into each medium.

Effect of buffers, 5.74 mM Tris buffer, 5.74 mM K_2HPO_4 and K_2HPO_4:KH_2PO_4 at 1:1, 1:2 and 2:1 ratios, in modified SA medium was also investigated.

Isolation and purification: A modified method (Ruangviriyachai *et al*, 2000) was used. In brief, extra cellular proteins and any cells was precipitated by NaCl and filtrated through filter paper and the supernatant was subjected to ion exchange column of Amberlite XAD-4. The isolation was carried out step by step as followings: rinsed with the mixture of methanol and 1 M HCl (1:1 %v/v), washed with water, loaded the sample, eluted with mixture of methanol and water (9:1 %v/v), and concentrated by rotary evaporator. Crude siderophore was provided and subsequently purified by Bio-Gel P-2 column using eluting solvent of 0.01 M pyridine-acetate buffer (pH~6). A major fluorescent fraction was collected, concentrated under vacuum, and lyophilized to dry form. Dry powder siderophores from *P. aearuginosa* R' and *P. putida* GIFU 1081, called pyoverdin PaII (pvd-PaII) and pseudobactin 589 (psdb-589), respectively, were helpfully identified these structure by Dr. Budzikiewicz and his colleagues.

Complexation ratio between iron and siderophores: Stoichiometric ratios of complexation between ferric ion and the siderophores, pvd-PaII and psdb-589, were conducted by Job's method (Harvey, 2000) using fluorescence detection. Fluorescence intensities of a series of dilutions fixing siderophore at 0.1 µmol and varied ferric ion from 0 to 1 µmol, were measured and recorded over the range of λ_{em} 300-800 nm and λ_{ex} 400 nm using a spectrofluorometer (Shimadzu RF-5000, Japan).

Determination of plasma iron: Iron content in plasma sample was determined by atomic absorption spectrophotometer (AAS) after protein precipitation by trichloroacetic acid. Thereafter, mixed solution was immediately heated for 15 min, cooled and filtrated, resulting in supernatant of plasma sample. Standard addition technique used for determination was the use a series of

standard iron (0, 0.5, 1.0 and 1.5 ppm). The samples were analyzed by AAS (Perkin Elmer AAnalyst 100 using Fe hallow cathode lamp) at 248.3 nm, using C_2H_2 as the fuel gas.

Determination of lipid per-oxidation by MDA-TBA assay: The effect of pvd-PaII and psdb-589 siderophoreson plasma iron was investigated, comparing with desferrioxamine B (DFO-B) by MDA-TBA assay analyzed by HPLC with fluorescence detection. The MDA-TBA assay used was as described by Hermans *et al* (2005) that was optimized and validated prior to use.

MDA standard was prepared by acid hydrolysis, using 0.1 M HCl and TEP. All working standards were freshly prepared. The TBA reaction was carried out by mixing 100 µl of plasma samples or MDA standard solution, 1 µl of 2 mM BHT added where appropriate, 700 µl of 1% orthophosphoric acid and 200 µl of 0.67% TBA, and then centrifuged, mixed and followed by incubation at 100˚C for 1 h, cooled and centrifuged. The supernatant was diluted by a solution of NaOH:methanol (1:12) and filtered. Subsequently, the filtered sample was analyzed by HPLC (Perkin Elmer LC 200, U.S.A.) with fluorescence detector (Thermo Separation model FL 2000, U.S.A.) using Microsorb-MV 100 C18 was a column (150 mm x 4.6 mm, 5 µm, Varian, U.S.A.), mobile phase was 10 mM phosphate buffer (pH 7.4): methanol (60:40 %v/v), and the flow rate was 0.8 ml/min. The temperature of the column was controlled at 25°±1°C. The fluorescence intensities of the resultant MDA-TBA complexes were determined using λ_{ex} of 532 nm and λ_{em} of 552 nm.

Results and Discussion

Carbon and nitrogen are major sources for siderophore production, results are shown in Figure 2 (a) and (b), respectively. In comparison to others, sucrose was the optimal source of carbon ($p<0.05$). Likewise, L-asparagine presented the source of nitrogen ($p<0.05$).

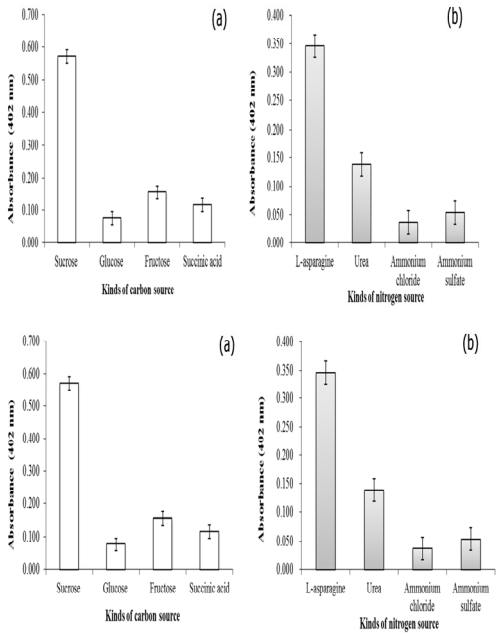


Figure 2 UV absorbance of media after incubation with *P. putida* GIFU 1081 at 402 nm for comparison of effects of (a) carbon sources and (b) nitrogen sources on siderophore production at pH 7.0 (n = 4).

The amount of sucrose and L-asparagine were then optimized. The increasing of sucrose and L-asparagine contents resulted in proportionally increase of siderophore production as shown in Figure 3(a) and 3(b). The production was almost similar in the range of 2- 6% of sucrose ($p>0.05$). However, the content of 14% or 7 folds of sucrose from original content provided the highest production which significantly

differ from the others ($p<0.05$). For N source, suitable content of nitrogen source for obtaining the highest siderophore production was 0.4% or 2 folds of L-asparagine from original content of SA medium. Consequently, 6% w/v of sucrose and 0.4% w/v of L-asparagine or the portion of 15:1 for C:N were chosen as an appropriate condition of the medium used for siderophore production. Siderophore production was found highest with K_2HPO_4, Figure 4(a) with a maximum bacterial growth in 2:1 K_2HPO_4:KH_2PO_4 (Figure 4(b)), thus, K_2HPO_4 was used. The production of siderophore from *P. putida* was finally performed using modified SA medium, containing 6% w/v sucrose, 0.4%w/v L-asparagine, 0.1% w/v K_2HPO_4, and incubated at 25°C.

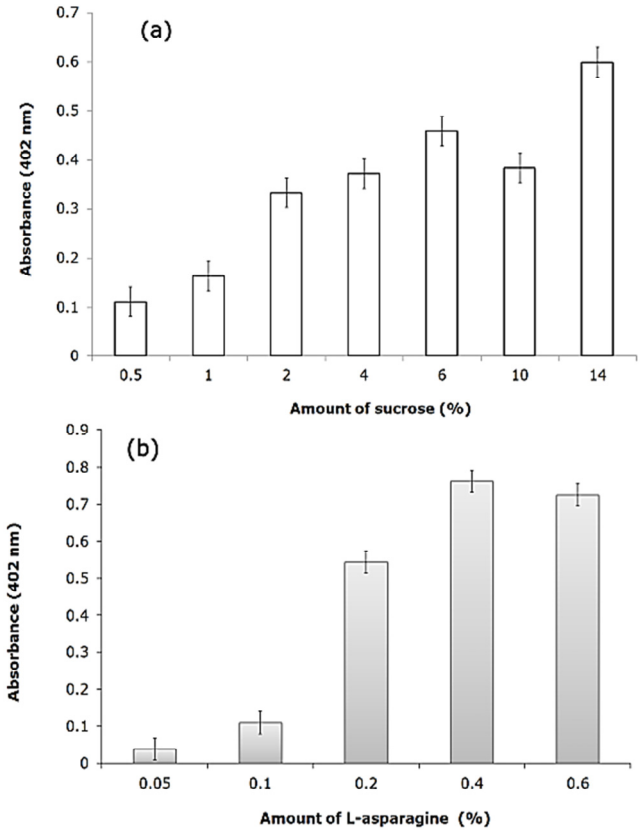


Figure 3 UV absorbance media after incubation with *P. putida* GIFU 1081 at 402 nm for comparison of effects (a) carbon and (b) nitrogen content siderophore production (A_{402}) at pH 7.0 (n = 4)

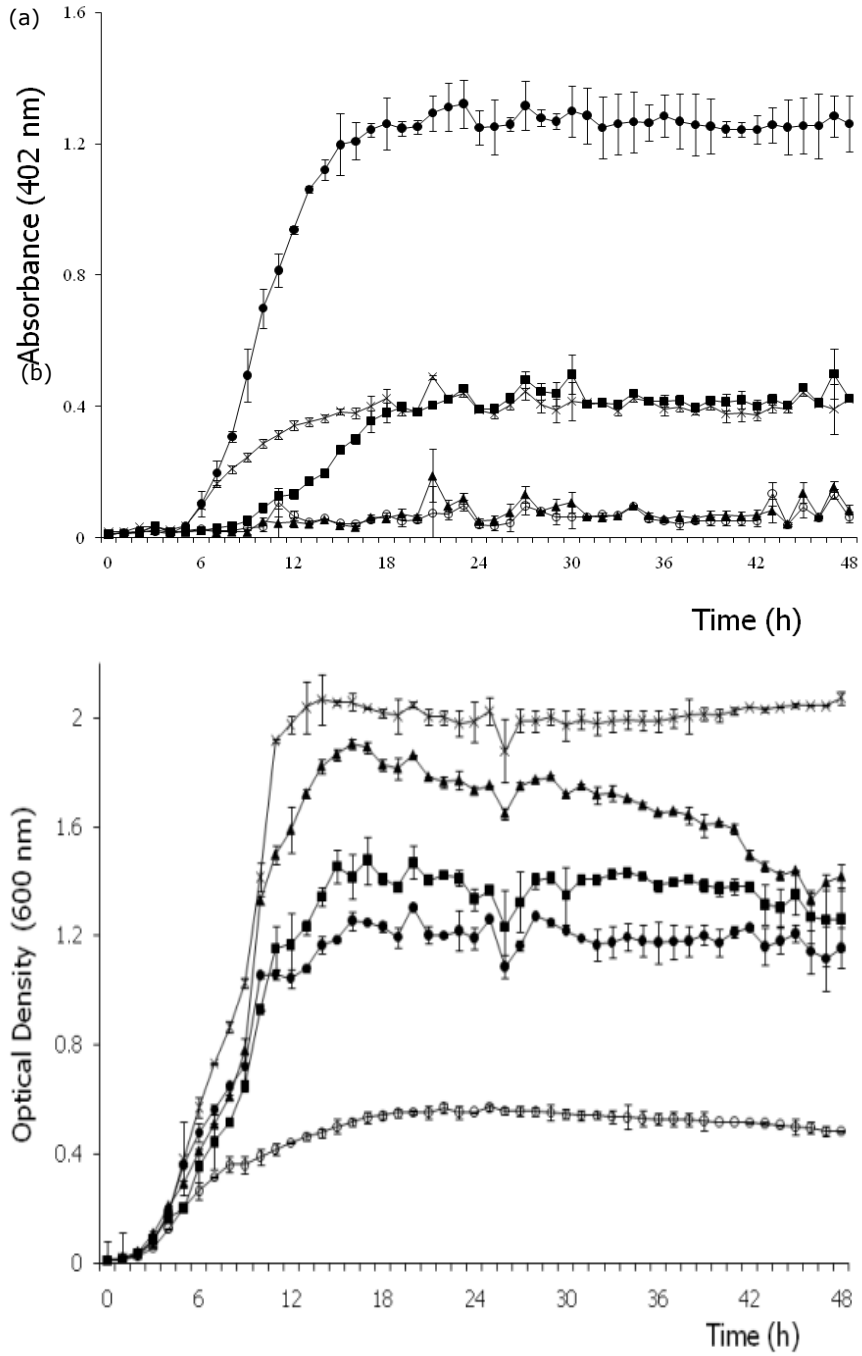


Figure 4 Effect of buffers on (a) siderophore production and (b) growth of *P. putida* GIFU 1081; detected at pH 7.0 (n = 4); × K_2HPO_4:KH_2PO_4(2:1), ● K_2HPO_4, ■ K_2HPO_4:KH_2PO_4(1:1), ▲ K_2HPO_4:KH_2PO_4 (1:2) and ○ Tris.

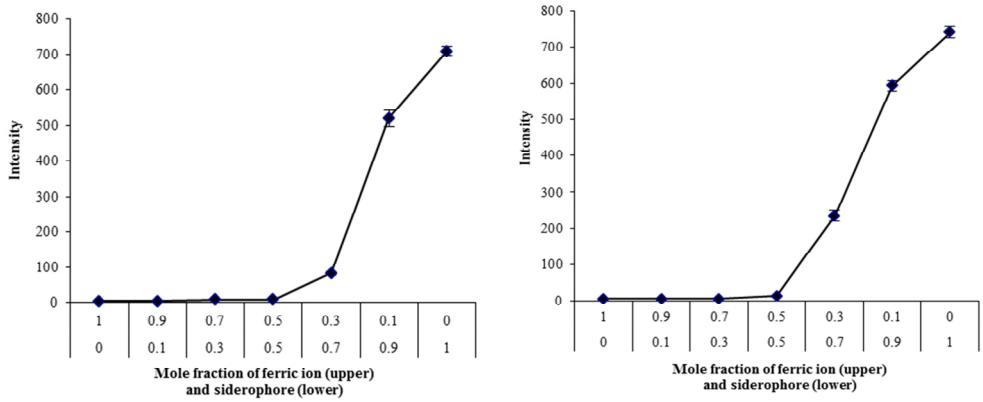


Figure 5 Stoichiometric ratio of complexation between iron and the siderophores (a) pyoverdin PaII (b) pseudobactin 589 (n=3)

Table 1 Comparison of the siderophores on plasma lipid peroxidation reduction by MDA-TBA analysis

Type of siderophores	Reduction of plasma lipid peroxidation (%)		
	Low*	Medium*	High*
Desferroxamine	11.1 ±6.4	11.2 ± 6.4	11.8 ± 8.1
PyoverdinPaII	10.0 ±6.5	27.0 ±16.0	17.1 ±14.2
Pseudobactin 589	14.0 ±9.7	29.4 ± 9.4	25.8 ±16.7

*Low=17.9 µM, Medium=35.8 µM, High = 71.6 µM concentration of the siderophores

The stoichiometric ratios of complexation between iron and siderophores produced from *P. aeruginosa* and *P. putida* were obtained from the graphs plotted between fluorescence intensities and the mole fractions of the siderophores and the ferric ions, as shown in Figure 5, suggesting that the siderophores from both bacterial strains presented the chelation with ferric ion at 1:1 molar ratio. DFO-B which has smaller structure is able to chelate the iron at the same ratio of 1:1 (Morel *et al*, 1992).

Plasma samples (n = 6) contained varied iron concentrations from 0.69-1.68 µg/ml. Table 1 shows that at 35.8 µM, pseudobactin-589 gave an average reduction of plasma lipid peroxidation of 29.4%, while pvd-PaII provided reduction of 27.0%. DFO-B reduced MDA by 11% at all concentrations used ($p>0.05$). Several factors can influence the effects of siderophore complexation to ferric ions including pH and metal ions, (Braud *et al*, 2010, Wang *et al*, 2013). Differences in hydroxamate or catecholate sites of each siderophore and mixed components of human plasma could interfere the binding efficiencies.

Conclusion

Pyoverdin PaII (pvd-PaII) and Pseudobactin 589 (psdb-589) siderophores were produced by *P. aeruginosa* R' and *P. putida* GIFU 1081, respectively, with an optimized SA and modified SA medium containing sucrose, L-

asparagine, phosphate buffer at 25°C. Effect of pvd-PaII and psdb-589 on plasma iron compared with commercial DFO-B siderophore evaluated by lipid peroxidation product of MDA revealed the highest reduction with pseudobactin 589 at 35.8 μM. Lipid peroxidation reduction of DFO-B was not dose-dependent within the range used. Further studies are required on toxicity and dose determination.

Acknowledgements

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