Effect of Pseudomonas putida siderophore on plasma iron
ผลของไซเดอโรฟอร์ซึ่งผลิตจากแบคทีเรียสายพันธุ์ Pseudomonas putida ที่มีต่อเหล็กในพลาสมา

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ABSTRACT

The effect of siderophore produced from Pseudomonas putida on plasma iron was studied by malondialdehyde (MDA) using high performance liquid chromatography with fluorescence detection, λ_{ex} at 532 nm and λ_{em} at 552 nm. The separation and detection of MDA were carried out after derivatization by 2-thiobarbituric acid (TBA). Optimum chromatographic conditions are a mixture of methanol and 10 mM phosphate buffer (pH 7.4) at a ratio of 40:60 (v/v) as mobile phase with flow rate of 0.8 mL min^{-1} at 25 °C. A linearity correlation between peak area and MDA at the concentration range of 0.25 - 4.00 x 10^{-6} mol L^{-1} was 0.9977. The method was proved to be specific detection of MDA with or without the presence of siderophores. This method was used to investigate in 6 plasma samples. The effect of *P. putida* siderophore was compared with that of desferrioxamine B. MDA concentration concerned with plasma iron content is lowest when treated by *P. putida* siderophore. It is concluded that the *P. putida* siderophore can probably reduce the plasma iron at an average of 29.4% (n=6).

บทคัดย่อ

ศึกษาผลของไซเดอโรฟอร์จากแบคทีเรียสายพันธุ์ Pseudomonas putida ที่มีต่อปริมาณเหล็กในพลาสมา โดยการตรวจวัดปริมาณของมาลอนไดอัลดิไฮด์ด้วยเทคนิคโครมาโทกราฟีของเหลวสมรรถนะสูง และตรวจวัดด้วยฟลูออเรสเซนซ์ที่ใช้ความยาวคลื่นกระตุ้นที่ 532 นาโนเมตร และความยาวคลื่นคายแสงที่ 552 นาโนเมตร การแยกและการตรวจวัดปริมาณสารมาลอนไดอัลดิไฮด์เกิดขึ้นภายหลังจากการทำอนุพันธ์กับสาร 2-thiobarbituric acid (TBA) ได้มีการปรับปรุงสภาวะของการทดลองทางโครมาโทกราฟีสำหรับการวิเคราะห์หามาลอนไดอัลดิไฮด์ โดยใช้เฟสเคลื่อนที่เป็นสารผสมระหว่างเมทานอลและ 10 มิลลิโมลาร์ฟอสเฟตบัฟเฟอร์ (pH 7.4) ด้วยอัตราส่วน 40:60 อัตราการไหล 0.8 มิลลิลิตรต่อนาที ที่อุณหภูมิการวิเคราะห์

Key Words : Siderophore, Lipid peroxidation, Malondialdehyde
คำสำคัญ : ไซเดอโรฟอร์ ไลปิดเพอรอกซิเดชัน มาลอนไดอัลดิไฮด์

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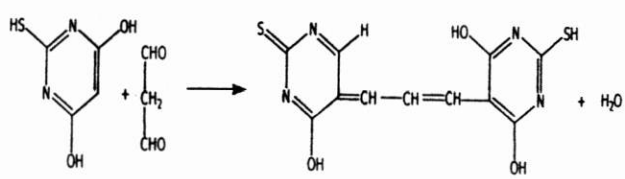
25 (C พบว่า ความสัมพันธ์เชิงเส้นตรงในช่วงความเข้มข้นของมาลอนไดอัลดิไฮด์ 0.25 - 4.00 x 10^{-6} โมลต่อลิตร มีค่าเป็น 0.9977 วิธีการนี้สามารถตรวจวัดปริมาณสารมาลอนไดอัลดิไฮล์ได้อย่างจำเพาะเจาะจงโดยไม่มีการรบกวนจากสารไซเดอโรฟอร์ วิธีการดังกล่าวใช้ทดสอบในตัวอย่างพลาสมา 6 ราย โดยเปรียบเทียบกับ desferrioxamine B พบว่าความเข้มข้นของสารมาลอนไดอัลดิไฮด์ซึ่งสัมพันธ์กับเหล็กในพลาสมาของไซเดอโรฟอร์ที่ผลิตจาก *P. putida* แสดงค่าต่ำสุด ทั้งนี้อาจสรุปได้ว่าไซเดอโรฟอร์จาก *P. putida* มีศักยภาพในการลดปริมาณเหล็กในพลาสมาได้ดีที่ค่าเฉลี่ย 29.4% เมื่อเทียบกับกลุ่มควบคุม (n=6)

Introduction

Siderophores are chelating agents which have strong specificity and affinity (K_{aff}~10^{30} M^{-1}) toward the ferric ion. They are generally synthesized and secreted by microorganisms in iron limitation conditions, which present unique chemical and physiochemical properties. A commercial siderophore, desferrioxamine B (DFO), a hydroxamate siderophore produced by Streptomyces pilosus, is an example of iron chelator which is effective and rather safe iron chelator used worldwide in patients who suffer from iron overload (Pootrakul, 1999).

Normally, iron is an essential microm olecule for life. Human body contains 0.004% of iron (Ashmead et al., 1985). While the human plasma, the liquid portion of the blood, is the complex solution containing more than 90 % of water which have the iron lower than 0.5%. Plasma iron can exist in either ferrous or ferric form. Particularly, free iron can catalyze the formation of very injurious compounds such as hydroxyl radical, which can attack with lipid and generate lipid peroxidation. Lipid peroxidation is oxidative damage that causes cell damage. Therefore, iron chelation therapy is one of the most ways to reduce the iron stored and subsequently to maintain the body iron at normal or low levels (Södergren, 2000).

Since chelators that mobilize iron may have the potential to cause oxidative stress, either by redistribution of iron, it is of interest to determine the effect of siderophores on oxidation stress of lipid peroxidation. The secondary product of lipid peroxidation, malondialdehyde, is one of the most frequently used as indicator of lipid peroxidation. Therefore, in this study, the concentration of MDA in plasma was investigated. Determination of MDA is based on the 2-thiobarbituric acid substance (TBAs) assay. Figure 1 shows the reaction of 2-thiobarbituric acid with MDA produces a chromogen (pink color) with an absorption maximum around 532-535 nm (Brown and Kelly, 1996).



TBA MDA MDA-$(TBA)_2$ complex

Figure 1 The reaction of 2-thiobarbituric acid with malondialdehyde to giving a color product.

High performance liquid chromatography (HPLC) is very appropriate for MDA determination. It is used world wide for MDA analysis with high sensitivity, selectivity, accuracy and less time of analysis. Consequently, the aim of this work was study an effect of the siderophore produced by *P. putida* on plasma iron by MDA analysis using HPLC with fluorescence detection.

Materials and Methods

Microorganism

Bacterial strain P. putida GIFU 1081 was bought from the Health Science Research Institute, Bangkok, Thailand.

Chemicals and Instruments

Liquid culture medium was modified from SA universal medium (20 g/L sucrose, 2 g/L L-asparagine, 1 g/L K_2HPO_4 and 10 mL/L $MgSO_4$). The siderophore was isolated and purified by passing supernatant through column containing Amberlite XAD-4 resin and CM Sephadex C-25, respectively (Budzikiewicz et al, 2001). Samples of human plasma from 6 donators were supported by the Blood Bank, Srinagarind Hospital, Khon Kaen University. The reagent 1,1,3,3-tetraethoxypropane (TEP), Desferrioxamine B (DFO) and butylated hydroxytoluene (BHT) were purchased from Sigma (St. Louis, MO). 2-thiobarbituric acid (TBA) was purchased from Fluka (Steinheim, Switzerland).

Chromatographic determination was performed on Perkin Elmer HPLC system with pump LC 200, integrator model 1022. The detection was carried out by fluorescence detector model FL 2000 (Thermo Separation Product, USA). The column was Microsorb-MV100 C18 (150 mm, 4.6 mm and i.d.; 5 (m) from Varian, USA.

Atomic absorption spectroscopy (AAS) analysis was carried out on AAS Perkin Elmer system with AAanalyst 100 and Fe hollow cathode lamp. AAS data was analyzed by the program of AA WinLab for AAanalyst 100/300 Version 3.2.

Preparation of MDA standards

MDA standards were prepared by acid hydrolysis (0.1 mol L-1 HCl) of TEP. All working standards were freshly prepared as mentioned by Nielsen et al., 1997.

Sample preparation and assay procedure

MDA was derivatized with TBA to form a fluorescent product (pink color). 700 (L phosphoric acid and 200 (L TBA was added to an aliquot portion of 100 (L standard or plasma sample, respectively. Then, the solution was centrifuged at 10,800 rpm for 3 min., incubated at 100 (C for 60 min. Hereafter, the sample was kept on ice and added 1:12 (v/v) of NaOH:methanol at ratio of 1:1. At that time the sample was vortex-mixed before HPLC analysis. The chromatographic analysis was performed by optimum condition of methanol : 10 mM phosphate buffer (pH 7.4) at 40:60 ratio as a mobile phase; the flow rate was 0.8 mL min-1. The temperature of the column was also controlled at 25 °C.

Results and Discussion

HPLC analysis of MDA

The HPLC analysis was developed successfully for the determination of siderophore affected by MDA. Figure 2 shows the effect of pH of mobile phase on peak area of MDA. Low level of peak area occurred at low pH and it increased and approached to constant when

the pH higher than 6.0. It was, thus, fixed that the pH of the mobile phase was adjusted to 7.4 resemble to the physiological pH. For optimization of mobile phase system, five various ratios of the mixture were carried out by gradient elution. The results were shown in Figure 3. The ratios of 50:50 and 45:55 (v/v) show asymmetric chromatograms, yet the symmetric chromatograms were occurred by the ratios of 40:60, 35:65 and 30:70. Since the chromatograms obtained from 35:65 and 30:70 ratio are broader because of diffusion phenomenon. Consequently, methanol:buffer at 40:60 ratio was used as mobile phase for MDA analysis. As studied on influence of ambient temperature between 24 and 27 °C (Fig. 4). It was indicated some significant effects on the determination of MDA, particularly peak area. Therefore, the HPLC analysis needs to be controlled by insulating the column used and kept its temperature constant at 25 °C.

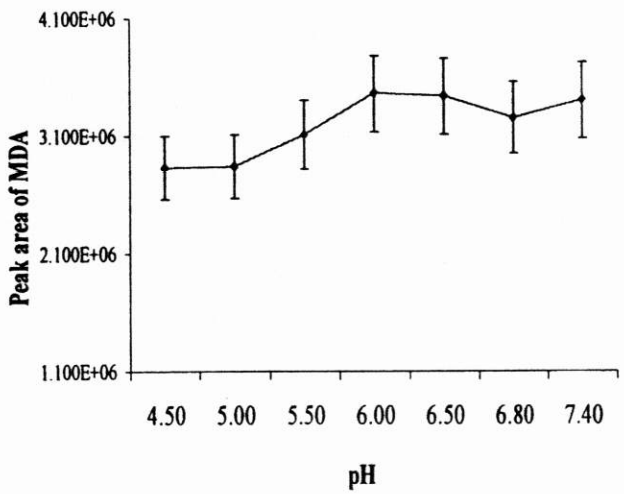


Figure 2 Effect of pH of mobile phase on peak area of MDA

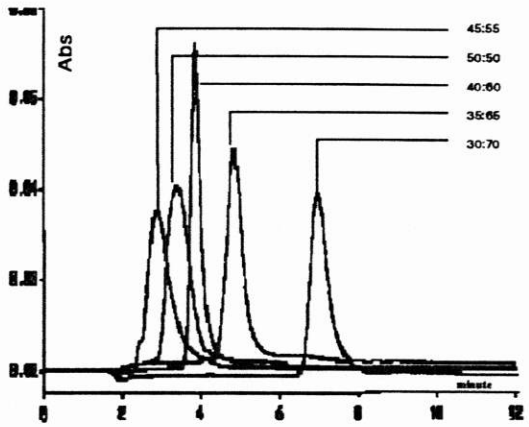


Figure 3 Chromatograms of MDA analysis with five different ratios of methanol and phosphate buffer, flow rate 0.8 mL min-1 and injection volume 20 °L

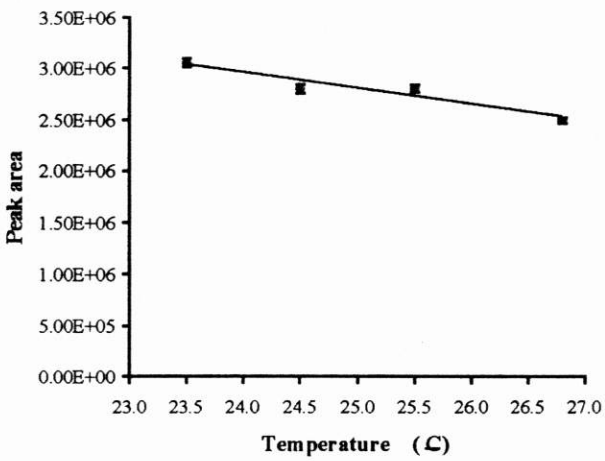


Figure 4 Effect of column temperature on peak area of MDA

With this condition, MDA was eluted at 3.8 min. as shown in Fig 5 (a) and (b). Figure 5 (a) shows the chromatogram of MDA only and (b) MDA with the presence of siderophore (1 mg/ml) in order to prove that siderophores did not interfere the determination of MDA.

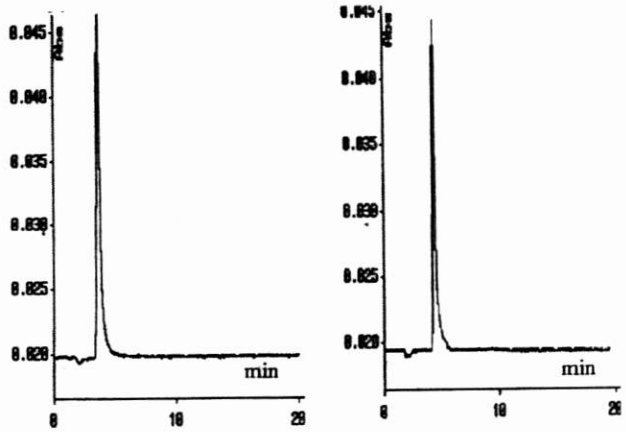


Figure 5 Chromatograms of MDA standard solution using fluorescence detection (λ^{ex} = 532 nm and λ_{em} = 552 nm); (a) MDA only, (b) MDA with 1 mg/ml of siderophore

From the graph as shown in Figure 6, there was a good correlative coefficient between concentration of MDA ranging from 0.25 - 4.00 x 10-6 mol L-1 and peak areas of MDA.

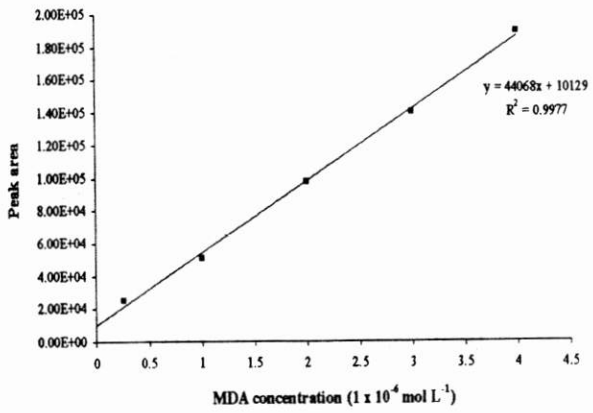


Figure 6 Linearity of MDA standard (0.25-4.00 x 10-6 mol L-1)

Effect of siderophore on plasma iron

Iron in plasma samples with 6 different donators were quantitatively determined by AAS. The plasma samples had content in the range of 0.69-1.68 mg L-1 (Table 1). These iron contents might be different due to the affecting factors such as diet, health, time of donating.

Table 1 The iron contents found in plasma samples

Donator No.	Year of donation	Iron contents (mg L^{-1})
P1	2002	1.29
P2	2002	1.68
P3	2004	1.01
P4	2004	0.81
P5	2004	0.69
P6	2004	1.16

2^7 Factorial design method was used to screen the main factors, which had large effects on the yield of MDA. The results were summarized to compare between P. putida GIFU 1081 siderophore and desferrioxamine B (DFO) as shown in Table 2. The iron quantity strongly effected on MDA analysis in both siderophore systems.

Table 2 Three main factors affecting on the yield of MDA for each siderophore

DFO	P.putida siderophore
Fe quantity	Fe quantity
DFO quantity	Plasma pH
Plasma pH	siderophore quantity

Figure 7 shows that the iron chelators (*P.putida* GIFU 1081 siderophore and DFO) had an effect on the concentration of MDA. Especially, *P.putida* GIFU 1081 siderophore can reduce MDA concentration in which directly concerned on plasma iron. This can also reduce plasma iron with an average of 29.4% (n=6).

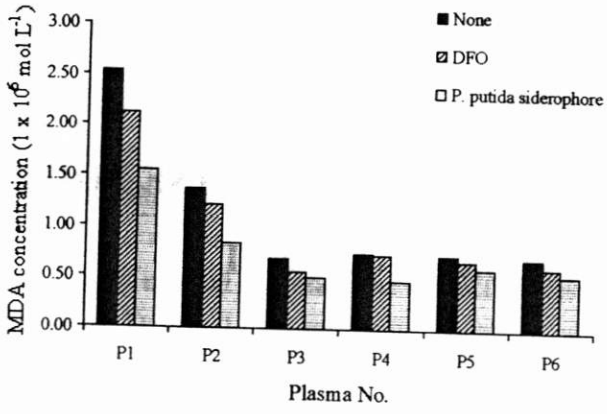


Figure 7 Effect of siderophores on MDA analysis in plasma iron

Conclusions

The result illustrated that the siderophore produced by P. putida GIFU 1081 showed the possibility in reducing the iron-catalyzed lipid peroxidation in plasma. This could be resulted from an iron chelation of the siderophore. Furthermore, HPLC with fluorescence detection promoted to be a suitable technique for lipid peroxidation product, MDA, analysis in plasma sample.

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