Effect of Pyoverdin II on lipid peroxidation in milk by high performance liquid chromatography

ผลของไพโอเวอร์ดีน II ต่อการเกิดลิพิดเพอร์ออกซิเดชันในนม ด้วยเทคนิคโครมาโทกราฟีของเหลวสมรรถนะสูง

Maliwan Suebsadsana (มะลิวัลย์ สืบศาสนา)* Dr. Chalerm Ruangviriyachai (ดร.เฉลิม เรืองวิริยะชัย)**
Dr. Aroonsri Priprem (ดร. อรุณศรี ปรีเปรม) * Dr. Saksit Chanthai (ดร.ศักดิ์สิทธิ์ จันทร์ไทย) ******

ABSTRACT

Lipid peroxidation with iron-catalyzed deteriorates milk, resulting in malondialdehyde (MDA). The detection of MDA was done by derivatization with 2-thiobarbituric acid (TBA) prior to analysis by high-performance liquid chromatography with fluorescence detection, λ_{ex} at 532 nm and λ_{em} at 552 nm. Mobile phase was a mixture of 10 mM phosphate buffer (pH 7.0) and methanol at ratio of 60:40 (v/v) with flow rate of 0.8 mLmin-1. A linearity relationship (r^2) between peak height and MDA concentration range of 0.5-5.0 μM was 0.9950. This method was applied to detect MDA in different types of milk samples and studied to determine quality of milk. The effect of pyoverdin II obtained from *Pseudomonas aeruginosa* TISTR 1467, which is high affinity for iron (III), on lipid peroxidation in milk was studied. The results showed that pyoverdin II has effect on lipid peroxidation in milk samples

บทคัดย่อ

กระบวนการเกิดลิพิดเพอร์ออกซิเดชันเป็นสาเหตุหนึ่งของการเสื่อมคุณภาพของนม โดยมีเหล็กเป็นตัวเร่งปฏิกิริยา และได้สารมาลอนไดอัลดิไฮด์เกิดขึ้นจากปฏิกิริยา ซึ่งสามารถตรวจวัดมาลอนไดอัลดิไฮด์ได้โดยทำอนุพันธ์กับกรดไทโอบาร์บิทูริค ก่อนนำมาวิเคราะห์ด้วยเทคนิคโครมาโทกราฟีของเหลวสมรรถนะสูงที่ตรวจวัดโดยใช้เครื่องฟลูออเรสเซนต์ ด้วยความยาวคลื่นแสงกระตุ้นที่ 532 นาโนเมตรและคายแสงที่ 552 นาโนเมตร โดยใช้สารผสมระหว่าง 10 มิลลิโมลาร์ ฟอสเฟตบัฟเฟอร์ (พีเอช 7.0) กับเมทานอลในอัตราส่วน 60 ต่อ 40 โดยปริมาตร เป็นเฟสเคลื่อนที่ ด้วยอัตราการไหล 0.8 มิลลิลิตรต่อนาที จากการศึกษา พบว่าได้ความสัมพันธ์เชิงเส้นตรงระหว่างความเข้มข้นของมาลอนไดอัลดิไฮด์กับความสูงพีคอยู่ในช่วง 0.5-5.0 ไมโครโมลาร์ มีค่าเท่ากับ 0.9950 การวิเคราะห์โดยวิธีนี้สามารถหาปริมาณของมาลอนไดอัลดิไฮด์ในตัวอย่างนมชนิดต่าง ๆ เพื่อใช้ตรวจสอบคุณภาพของนม โดยศึกษาผลของไพโอเวอร์ดีน II ที่ได้จากแบคทีเรีย *Pseudomonas aeruginosa* TISTR

* *Graduate Student in Analytical Chemistry Program, Faculty of Science, Khon Kaen University*

** *Associate Professor, Department of Chemistry, Faculty of Science, Khon Kaen University*

****Associate Professor, Department of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Khon Kaen University*

*****Assistant Professor, Department of Chemistry, Faculty of Science, Khon Kaen University*

1467 ซึ่งมีสมบัติในการจับเหล็ก (III) ได้ดี ต่อการเกิดลิพิดเพอร์ออกซิเดชันในนม และพบว่าไพโอเวอร์ดีน II มีผลต่อการเกิดลิพิดเพอร์ออกซิเดชันในตัวอย่างนม

Key Words : Pyoverdin II, Lipid peroxidation, Malondialdehyde
คำสำคัญ : ไพโอเวอร์ดีน II ลิพิดเพอร์ออกซิเดชัน มาลอนไดอัลดิไฮด์

Introduction

Siderophores are natural, low molecular weight compounds with high iron (III) chelating affinity constants (10^{23}-10^{52} M^{-1}) that are responsible for solubilization and transportation of iron (III) into bacterial cells (Mucha, Rekowski and Kosakowska et al., 1999). They are generally synthesized and secreted by microorganisms in iron limitation conditions, which present unique chemical and physicochemical properties. Siderophores contain the most efficient iron-binding ligand types in nature, consisting of hydroxamate, catecholate or hydroxycarboxylate ligands that can form hexadentate Fe (III) complexes, satisfying the six coordination sites on ferric ions (Enyedy, Po'csi, and Farkas, 2004). Pyoverdin, the major endogenous siderophores, possesses a chromophore derived from 2, 3 - diamino - 6, 7-dihydroxyquinoline that confers color and fluorescence to the molecule, linked to a partly cyclic octapeptide. The peptide differs among strains by number, composition, and configuration of amino acids. Pyoverdin is a very water-soluble molecule and a powerful chelator of ferric iron, which is bound with a stoichiometry of (1:1) and a stability constant of approximately 10^{24} M^{-1} at neutral pH. Although more than 50 different pyoverdins or pyoverdin-like siderophores have been structurally characterized, the iron- chelation properties have been studied for only a few pyoverdins (Boukhalfa, Reilly and Michalczyk et al., 2006).

Iron is required for many biological processes, including oxygen distribution, electron transport and DNA synthesis. Iron in excess, however, is toxic, due to its catalysis of one-electron redox reactions, the most reactive product of which is the hydroxyl radical, which damages proteins and lipids (Buss, Neuzil and Ponka, 2002).

Lipid peroxidation is a well-established mechanism of cellular injury in both plants and animals, and is used as an indicator of oxidative stress in cells and tissues (Schafer, Qian and Buettner, 2000). The most commonly used criteria for occurrence of oxidative stress are those based either on determination of the concentration of lipid oxidation products in body fluids, or on the susceptibility of lipids to peroxidation induced, either by transition metal ions or by generators of free radicals (Emerit, Beaumont and Trivin, 2001). Lipid peroxidation is a factor in numerous pathologies, including inflammation. Therefore, udder inflammation or mastitis was shown to relate to lipid peroxidation in milk. Mastitis is caused by bacterial invasion into the udder, the so-called intramammary infection (IMI) (Suriyasathaporn, Vinitketkumnuen and Chewonarin et al., 2004). Foods with high content of polyunsaturated fatty acids (PUFA) are more susceptible to lipid peroxides have been measured in milk (Fenaille, Mottier and Turesky et al., 2001). Lipid peroxidation with iron-catalyzed is one of the main factors of milk quality deterioration during

processing and storage. Human and cow's milk are relatively low in Fe ranging from 0.2 - 0.8 mgL-1, but it is highly bioavailable mainly in human milk (Aleixo and N?brega, 2003). Milk is fortified with iron to prevent iron deficiency anaemia, through its catalytic role in Fenton reaction, (equation below) and through formation of ferryl/ perferryl radicals and lipid peroxides. Then iron is a known generator of free radicals (Almass, Rootwelt and ∅yasater et al., 1997).

$$Fe^{2+} + H_2O_2 \longrightarrow OH^- + OH^{\bullet} + Fe^{3+}$$

In previous studied, it was presented the effect of siderophore produced by *Pseudomonas putida* on iron-catalyzed lipid peroxidation in plasma, and it was found that malondialdehyde (MDA) was used as indicator of iron quantity in plasma (Sutthiparinyanont, Ruangviriyachai and Priprem et al., 2005).

MDA, the secondary product of lipid peroxidation is one of the most frequently used as indicator of the peroxidation reaction. MDA has been measured by the 2-thiobarbituric acid (TBA) method. The reaction of TBA with products of lipid peroxidation such as hydroperoxide and conjugated aldehydes interferes with TBA. This method involves two molecules of TBA and one molecule of MDA (pink color product) which can then be determined spectrophotometrically at 532-535 nm. The major problem in this method is the lack of sensitivity (Karatas, Karatepe and Baysar, 2002). The method is done by separation and measuring MDA-TBA product using high performance liquid chromatography (HPLC) with fluorescence detector.

The purpose of this study was to compare the MDA content in different types of milk samples. Moreover, the purified siderophore produced by Pseudomonas aeruginosa TISTR 1467, pyoverdin II was used to study their effect on iron in milk. Thus, pyoverdin II is a chelating agent that to be strong specificity and affinity with ferric ion. The effect was analyzed by MDA using reverse-phase HPLC with fluorescence detection.

Materials and Methods

Microorganism

Bacterial strain *Pseudomonas aeruginosa* TISTR 1467 was purchased from the Thailand Institute of Scientific and Technological Research (TISTR), Bangkok, Thailand for this study.

Chemicals and Instruments

The reagents such as hydrochloric acid, magnesium sulfate heptahydrate, potassium di-hydrogen phosphate and potassium hydroxide were obtained from Carlo Erba (Italy). Di-sodium hydrogen phosphate, methanol and pyridine were obtained from Merck (Germany). Methanol (HPLC grade) was also bought from Lab Scan (Ireland). Iron (III) nitrate AAS standard from Spectrosol (Australia), 2-thiobarbituric acid (TBA), L-asparagine anhydrous and trichloroacetic acid (TCA) were purchased from Fluka (Switzerland). 2, 6-di-tert-4-methyl phenol (BHT) and 1, 1, 3, 3- tetraethoxypropane (TEP), were purchased from Sigma (USA). Raw milk samples were collected from some cows in farm at Department of Animal Science, Faculty of Agriculture, Khon Kaen University, Thailand.

Equipments used were, as follows: the lyophilizer (Stone Ridge, USA),

spectrospectrofluorometer (Shimadzu RF-5301PC, Japan), microcentrifuge (Model Centurion Scientific 1010, Centurion Scientific Ltd., England). Atomic absorption spectroscopy (AAS) analysis was carried out on AAS Perkin Elmer system with AAanalyst 100 and Fe hollow cathode lamp, HPLC analysis was carried out on WATERS system with WATERS 600 HPLC pump equipped with WATERS 2475 Multiwavelength fluorescence detector. The column was Microsorb-MV100 C18 (150 x 4.6 mm i.d., 5 μm, Varian, USA).

Isolation and purification of Pyoverdin II

Bacterial strain *Pseudomonas aeruginosa* TISTR was used to produce pyoverdin II. For isolation and purification, Amberlite XAD-4 resin and BioGel P-2 gel columns were used. (Ruangviriyachai, Fernández and Fuchs et al., 2001).

Determination of stoichiometric ratio of iron and pyoverdin II

The concentration of pyoverdin II was fixed at 0.10 μM whereas ferric ion was varied from 0.00 to 1.00 μM. Analyzed by Job's method using fluorescence intensity was recorded by spectrofluorophotometer over the range of emission wavelength at 300-700 nm, constant exitation wavelength of 400 nm.

Determination of iron in milk using atomic absorption spectrophotometry (AAS)

Iron (III) nitrate AAS standard was used for a series of standard iron (0.5, 1.0 and 1.5 μg/mL). Precipitation of milk protein was done by adding 10% trichloroacetic acid into milk samples, homogenization and after 24 hours. The milk sample was then filtered through Whatman No.42 filter paper (Silva, Lopes and Nobrega et al., 2001). The iron content in the samples was analyzed by AAS at 248.3 nm using a hollow cathode lamp operating at 30 mA, a slit of 0.2 nm, and an acetylene-air-flame. The experiment was repeated in triplicate and compared with a standard addition method.

Determination of MDA and effect of pyoverdin II on MDA in milk samples using RP-HPLC with fluorescence detection

Preparation of MDA-TBA2 standard

MDA standard was prepared by acid hydrolysis (0.1 mol L^{-1} HCl) of 1,1,3, 3-tetraethoxy propane (TEP). All working standard solution were freshly prepared (Karatas, Karatepe and Baysar, 2002). The linearity of the response for different concentrations carried out of the MDA standard was 0.5, 1, 2, 3 and 5 μM for the MDA-TBA_2 complex.

Preparation of MDA-TBA_2 and effect of pyoverdin II on MDA-TBA_2 in milk samples

1 mL milk sample or MDA standard mixed with 1%TBA in 5% trichloroacetic acid (TCA). 0.8% BHT in methanol was homogenized and centrifuged. Heating at 80 °C for 1 hour, the supernatant was cooled, centrifuged and filtered, then analyzed by RP-HPLC. The mobile phase was 10 mM phosphate buffer (pH 7.0): methanol (60:40, v/v) at a flow rate of 0.8 $mLmin^{-1}$. The fluorescence intensities of the resultant MDA-TBA2 complexes were determined using HPLC with fluorescence detection, λ_{ex} at 532 nm and λ_{em} at 552 nm. The effect of pyoverdin II on lipid peroxidation was also carried out by addition with 2 μg/mL of pyoverdin II into the milk samples and then analyzed as method mentioned above.

Results and discussion

The structural elucidation of pyoverdin II (as shown in Figure 1) obtained from the purified of siderophore, its structure was identified by spectroscopic methods at Department of Organic Chemistry, University of Cologne, Cologne, Germany. It is found that this Pyoverdin II is comprised of a peptide chain and a fluorescent multifunctional chromophore, 2, 3-diamino-6, 7-dihydroxyquinoline. Amino acid analysis established the presence of L-Gly, L-Phe, L-Orn, L-Lys, L-Tyr plus succinic acid.

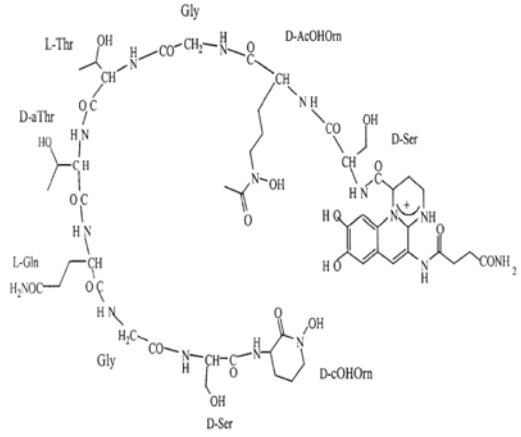

Figure 1 Structure of pyoverdin II (Fernández, Fuchs and Taraz et al., 2001)

The fluorescence intensity of pyoverdin II-ferric complexes was plotted versus the mole fraction of siderophore and ferric ion as shown in Figure 2. The results showed one molecule of the pyoverdin II chelate one molecule of ferric ion or ratio 1:1, as the same as the review reported.

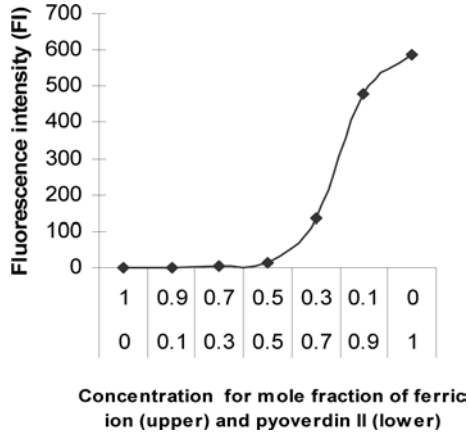

Figure 2 Stoichiometric ratio of complexation between iron (III) and pyoverdin II

According to the iron quantity in milk samples using AAS, the iron content in milk samples was found in the concentration range of 0.16-4.65 μgmL^{-1} (Table 1). The difference in iron content might be due to some factors such as milk processing, diet and health of cow (Bermejo, Dominguez and Bermejo, 1997).

Table 1 The iron contents found in milk samples

Milk samples	Average content of iron in milk ($\mu g\ mL^{-1}$)
UHT milk	1.08
Pasteurized milk	0.82
Powder infant formulas	4.65
Raw milk (mastitis 1)	0.60
Raw milk (mastitis 2)	1.10
Raw milk (normal 1)	0.48
Raw milk (normal 2)	0.62
Raw milk (normal 3)	0.16
Raw milk (normal 4)	0.76

Figure 3 shows the chromatogram with a single peak of MDA-TBA_2 complex under our HPLC optimum conditions, the retention time of the MDA-TBA_2 complex was about 3.9 min.

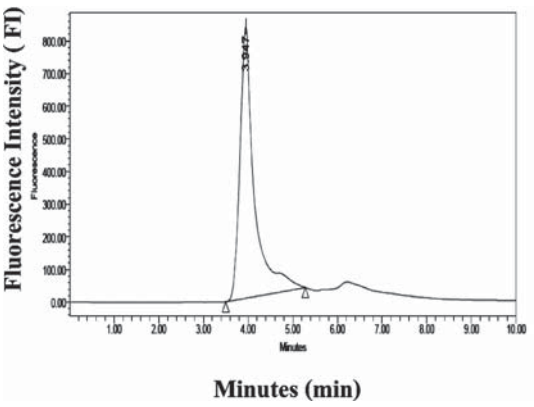

Figure 3 Chromatogram of MDA-TBA_2 standard solution using fluorescence detection (λ_{ex} at 532 nm and λ_{em} at 552 nm)

Standard calibration graph was constructed by plotting between peak height and concentration of MDA ranging from 0.5-5.0 μM. From the graph as shown in Figure 4, good correlation coefficients ($r \geq 0.99$) were obtained in the concentration range studied.

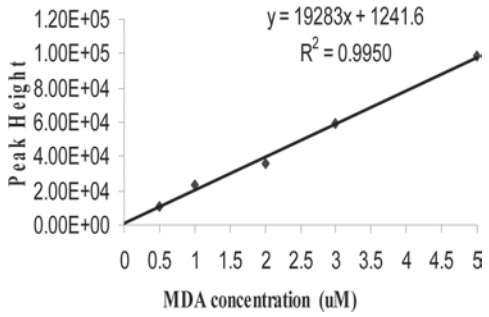

Figure 4 Linearity of MDA standard (0.5-5.0 μM)

The results showed the relation between the MDA concentration and the iron content in milk samples. In mastitis milk, MDA concentrations were higher than that in milk from normal cows. Thus, udder inflammation might be related to lipid peroxidation in milk. The purified pyoverdin II was then used to study the effect on iron-catalyzed lipid peroxidation in milk. MDA in milk samples with and without addition of pyoverdin II was determined by this method. In Figure 5, the MDA concentration decreased in the presence of pyoverdin II. It was found that pyoverdin II can reduce the lipid peroxidation in milk samples due to the complexation of pyoverdin II with iron.

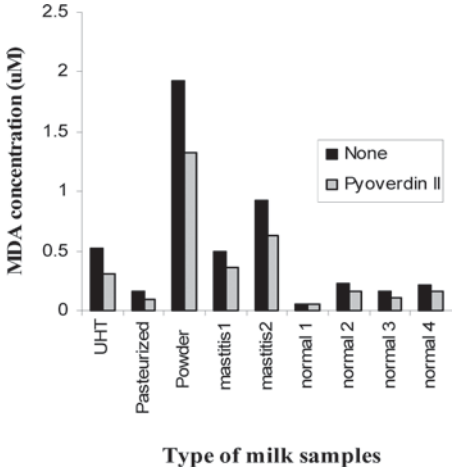

Figure 5 Effect of pyoverdin II on MDA analysis in milk samples

Conclusions

In this study, the effect of pyoverdin II on iron-catalyzed lipid peroxidation in milk was determined by HPLC with fluorescence detection. The result showed that the pyoverdin II produced by *Pseudomonas aeruginosa* TISTR 1467 can propably reduce MDA concentration in milk

samples. In addition, this method might be useful for estimating the quality of milk.

Acknowledgments

The researchers would like to thank Department of Chemistry, Faculty of Science, Khon Kaen University for chemicals and instruments; Department of Animal Science, Faculty of Agriculture, Khon Kaen University for cow's raw milk samples. We also thank the Postgraduate Education and Research Program in Chemistry (PERCH) and the National Research Council of Thailand (NRCT) for financial support.

References

Aleixo, PC., and NÓbrega, JA. 2003: Direct determination of iron and selenium in bovine milk by graphite furnace atomic absorption spectrometry. Food Chemistry 83: 457-462.

Almass, R., Rootwelt, T., Øyasæter, S. and Saugstad, O. D. 1997: Ascorbic acid enhances hydroxyl radical formation in iron-fortified infant cereals and infant formulas. European Journal of Pediatr 156: 488-492.

Bermejo, P., Dominguez, R. and Bermejo, A. 1997: Direct determination of Fe and Zn in different components of cow milk by FAAS with a high performance nebulizer. Talanta 45: 325-330.

Boukhalfa, H., Reilly, S.D., Michalczyk, R., Iyer,S. and Neu, M. 2006: Iron (III) coordination properties of a pyoverdin siderophore produced by *Pseudomonas putida* ATCC 33015. Inorganic Chemistry 45: 5607-5616.

Buss, JL., Neuzil, J. and Ponka, P. 2002: The role of oxidative stress in the toxicity of pyridoxal isonicotinoyl hydrazone (PIH) analogues. Biochemical Society Transactions 30: 755-757.

Emerit, J., Beaumont, C. and Trivin, F. 2001: Iron metabolism, free radicals, and oxidative injury. Biomedicals Pharmacother. 55: 333-339.

Enyedy, EA., Po'csi, I. and Farkas, E. 2004: Complexation of desferricoprogen with trivalent Fe, Al, Ga, In and divalent Fe, Ni, Cu, Zn metal ions: effects of the linking chain structure on the metal binding ability of hydroxamate based siderophores. Journal of Inorganic Biochemistry 98: 1957-1966.

Fenaille, F., Mottier, P., Turesky, RJ., Ali, S. and Guy, PA. 2001: Comparison of analytical techniques to quantify malondialdehyde in milk powders. Journal of Chromatography A 921: 237-245.

Fernández, DU., Fuchs, R., Taraz, K., Budzikiewicz, H., Munsch, P. and Meyer, J.M. 2001: The structure of a pyoverdine produced by a *Pseudomonas tolaasii*-like isolate. BioMetals 14: 81-84.

Karatas, F., Karatepe, M. and Baysar, A. 2002: Determination of free malondialdehyde in human serum by high-performance liquid chromatography. Analytical Biochemistry 311: 76-79.

Mucha, P., Rekowski, P., Kosakowska, A. and Kupryszewski, G. 1999: Separation of siderophores by capillary electrophoresis. Journal of Chromatography A 830: 183-189.

Ruangviriyachai, C., Fernández, DU., Fuchs, R., Meyer, J.M. and Budzikiewicz, H. 2001: A new Pyoverdin from *Pseudomonas aeruginosa* R'. Zeitschrift für Naturforschung 56c: 933-938.

Schafer, FQ., Qian, SY., and Buettner, GR. 2000: Iron and free radical oxidations in cell membranes. Cellular and Molecular Biology 46(3) : 657-662.

Silva, FV., Lopes, GS., Nobrega, JA., Souza, GB. and Nogueira, A.R.A. 2001: Study of the protein-bound fraction of calcium, iron, magnesium and zinc in bovine milk. Spectrochimica Acta, Part B 56: 1900-1916.

Suriyasathaporn, W., Vinitketkumnuen, U., Chewonarin, T., Boonyayatra, S., Kreausukon, K. and Schukken, YH. 2004: Higher somatic cell counts resulted in higher malondialdehyde concentrations in raw cows' milk. International Dairy Journal 16: 1088-1091.

Sutthiparinyanont, S., Ruangviriyachai, C., Priprem, A. and Chanthai, S. 2005: Effect of *Pseudomonas putida* siderophore on plasma iron. Khon Kaen University Research Journal (Graduate studied) 5(2): 74-79.