國立交通大學

生物科技學系

博 士 論 文

人類血液 Haptoglobin 之抗氧化角色

Antioxidant Role of Human Plasma Haptoglobin

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中 華 民 國 九 十 五 年 四 月

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生物科技學系

博 士 論 文

A Thesis
Submitted to Department of Biological Science and Technology
College of Biological Science and Technology
National Chiao Tung University
in Partial Fulfillment of the Requirements
for the Degree of PhD
in

Biological Science and Technology

April 2006

Hsinchu, Taiwan, Republic of China

中華民國九十五年四月

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論文名稱：＿人類血液Haptoglobin之抗氧化角色＿＿＿＿＿＿＿＿＿＿
指導教授：＿毛仁淡＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿＿

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論文名稱：＿＿人類血液Haptoglobin之抗氧化角色＿＿＿＿＿＿＿＿＿＿

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人類血液 Haptoglobin 之抗氧化角色

學生：曾繼鋒　　　　　　　　　　　　　指導教授：毛仁淡 博士

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摘　　要

人類血液中之 Haptoglobin (Hp)，與血型分類相似，可以分為三種表現型：1-1、2-1、與 2-2。然而在這些 Hp 表現型之結構與功能的關係，由於其複雜的結構與困難繁複的純化步驟，目前的瞭解仍十分有限。在此我們發展一個可以純化每一種 Hp 表現型的簡便方法。首先將血漿通過已結合可專一辨識 Hp 之單株抗體，將所得到之 Hp 再通過 gel filtration 管柱，藉由 SDS-PAGE 分析，所得到之 Hp 純度可大於 95%，並可保有其本身所具有之醣基成分與血紅素結合能力。經由 Circular dichroism 分析，Hp 1-1 (29%) 之 α-helix 組成比例高於 2-1 (22%) 與 2-2 (21%)。這個方法較現有 Hp 的純化方法有了明顯的改善與進步。為了進一步瞭解 Hp 在 in vitro 之抗氧化活性，thiobarbituric acid-reactive substances (TBARS) assay 用來估計在脂質過氧化反應中 Hp 的抗氧化活性。Hp 在銅離子所誘發之脂質過氧化反應中表現了極強之抗氧化能力。此外，在另一種親水性自由基產生者 2,2’-azobis(2-amidinopropane)-dihydrochloride (AAPH) 所誘發的脂質過氧化反應中， Hp 亦具有相似之抗氧化特性，因此推測 Hp 可能也扮演著自由基清除者的角色。為了更進一步研究結構對於其抗氧化特性的影響，

carboxymethylation 被用來阻絕在 Hp 中 cysteine 間雙硫鍵的形成，有趣地，經過修飾後的 Hp 反而較 native Hp 表現出更強之抗氧化能力，因此推論在 native 的構形中，抗氧化 domain 可能並未完全暴露在外。為了更深入研究 Hp 在細胞內的抗氧化角色，我們將 Hp 的 cDNA 放入含有 CMV 啟動子之 pcDNA 3.0 載體中，並轉殖至本身不會表現 Hp 之 Chinese Hamster Ovary (CHO) 細胞中，發現確實可以增加該細胞對於氧化壓力的耐受度，在添加雙氧水的培養條件下 24 小時，其耐受度較未轉殖之細胞高出 1 倍。因此 Hp 在 *in vitro* 與 *ex vivo* 的研究中皆表現出極佳之抗氧化能力。最後我們分析了目前普遍使用之抗氧化活性檢測方法，並且闡釋如何研發與設計防止動脈硬化之強效抗氧化藥物。首先抗氧化藥物必須可以專一地 LDL 結合。第二，必須具有高度之 bioavailability。文中並針對這些抗氧化作用機制與分析程序之原理與策略進行討論。

Antioxidant Role of Human Plasma Haptoglobin

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Abstract

Similar to blood type, human plasma haptoglobin (Hp) is classified as 3 phenotypes: Hp 1-1, 2-1, or 2-2. The structural and functional relationship between the Hp phenotypes has not been studied in detail due to their complicated structures and difficult isolation procedures. In the present study, we developed a simple protocol that can be used to purify each Hp phenotype. Plasma was first passed through an affinity column coupled with a high affinity Hp monoclonal antibody. The bound Hp was eluted and further chromatographied on a HPLC. The homogeneity of purified Hp 1-1, 2-1, or 2-2 was greater than 95% as judged by SDS polyacrylamide gel electrophoresis. It retained the carbohydrate moiety and hemoglobin-binding ability. Circular dichroic spectra showed that the α-helical content of Hp 1-1 (29%) was higher than that of Hp 2-1 (22%) and 2-2 (21%). The procedures described here represent a significant improvement in current purification methods for each Hp phenotypes. To investigate *in vitro* antioxidant role of Hp, thiobarbituric acid-reactive substances (TBARS) was used to estimate antioxidant activity of Hp in low-density lipoprotein (LDL) lipid peroxidation. We demonstrated that Hp molecule was an extremely potent antioxidant activity in Cu^{2+}-induced LDL peroxidation. Using 2,2’-azobis(2-amidinopropane)-dihydrochloride (AAPH), a

hydrophilic decomposed radical initiator, it produced a similar antioxidant effect of Hp against LDL oxidation suggesting a free radical-scavenging role of Hp. To study the structural effect in its antioxidant activity, carboxymethylation that alters the overall structure of Hp by blocking the formation of disulfide linkages between cysteine residues was used for the evaluation. Interestingly, carboxymethylated Hp exerting higher antioxidant potency than that of native Hp indicated that the antioxidant domain of Hp might not be fully exposed. To investigate antioxidant role of Hp on the cellular level, the cDNA of Hp 1-1 was cloned, constructed (containing the pcDNA3.0 vector with CMV promoter) and transfected to Chinese Hamster Ovary (CHO) cells expressing no Hp. These transfected CHO cells were able to express Hp 1-1 and significantly ($P<0.001$) elevated the tolerance against the oxidative stress. The elevation was about twice-higher than that normal CHO cells when challenged with hydrogen peroxide for 24 h. Thus, Hp plays a provocative antioxidant role as demonstrated in our *in vitro* and *ex vivo* studies. Finally, we analyzed commonly used analytical methods for measuring the antioxidant potency and outlined the critical steps as how to evaluate and design a potent antioxidant agent that can be used for the intervention of atherosclerosis. We conclude that an antioxidant should be first targeted and incorporated into human LDL. Second, the candidate compound should possess high bioavailability. The rationale and strategy for the analytical procedures are discussed.

誌　謝

在經過五年辛苦帶著甘甜、微笑伴著眼淚的博士求學過程中，特別要感謝指導教授毛仁淡老師，無論在生活上、實驗上都給予繼鋒悉心地指導與幫助，讓繼鋒得以成長，博士論文得以完成，在此獻上最誠摯地感謝。同時也謝謝張正老師，趙雲鵬老師，曾慶平老師，林志生老師，戴鏗碩老師耐心地批閱斧正，使論文得以更臻完整詳實。

這些日子以來，實驗室是我第二個家，學長以及學弟妹就像是家人一般，陪我度過這酸甜苦辣、多樣地五年光陰。阿木學長、文亮、學弟妹以祥、明誌、佳靜、心怡、怡荏、繡文、媛婷、忠義、彰威、盛龍、惠君、宏輝、俊瑩、韶嬋、珮真、文昭、立品、佩茹、中曦、冠佑、靜怡，謝謝大家給了我這麼多充實愉快地回憶，未來的日子裡，一起再從笑傲回味這一段充滿著喜怒哀樂的記憶吧。也謝謝靖婷、盈蓉、國領、千婷、政男，很難得地，我們共同從碩士班，博士班一路奮鬥至此，未來也要記住這份情誼，大家再一起打拼。

最後我要感謝我的家人及女朋友英潔，謝謝你們在背後默默地支持著我，讓我可以不斷地鼓起勇氣，去克服生活中接踵而來的挑戰，謹以此論文獻給你們，這是我們共同的榮耀。

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Overview

Coronary artery and other vascular diseases (including myocardial infarction, MI) are the leading cause of death in the Western industrialized countries [1-3]. The mortality of the diseases are the top third-fourth in Taiwan, and has since become the leading killer, despite the overall improvement in mortality of the patients treated with hypocholesterolemic drugs. One concept that has received much recent attention for the pathogenesis of atherosclerosis is the LDL-modification hypothesis [4-6], which postulates that atherosclerotic plaque resulted from the uptake of oxidized LDL by macrophages in the arterial wall followed by smooth muscle cell (SMC) migration and proliferation [7]. In this respect, an understanding of the biochemical events, which impact on the oxidation stress and SMC proliferation into the intima deserves to receive much attention. Inflammatory phenomena at sites of atherosclerotic plaques are increasingly thought to be major determinants of the progression and clinical outcome of atherosclerosis disease [7-10]. Therefore, attention is being paid to systemic markers that may reflect the inflammatory activity in the plaques. Recently we found that haptoglobin (Hp), an acute phase protein elevated sharply during the infection and inflammation, was substantially accumulated in atherosclerotic lesions []. Nevertheless, while the pathogenesis of atherosclerosis has been continuously explored, the Hp molecule that may protect against the progression of atherosclerosis has never been reported [11-15].

Hp is classified as three phenotypes, Hp 1-1, 2-1, and 2-2. The primary translation product of Hp mRNA is a polypeptide that dimerizes cotranslationally and is proteolytically cleaved while still in the endoplasmic reticulum [16,17]. All the phenotypes share the same 2 β chains (each with about Mr 40,000 dalton containing 245 amino acids and approximately 30% carbohydrate) [18-20]. A typical structure of homozygous Hp 1-1 is composed of two identical α 1 chains (each with about Mr 9,000 dalton containing 83 amino acids). Homozygous Hp 2-2 is composed of two identical α 2 chains (each with about Mr 16,500

dalton containing 142 amino acids) as compared to that of heterozygous Hp 2-1 containing one each of α 2 and α 1 [18]. Polymeric form of Hp 2-1 or 2-2 is thought to be associated with the complications of myocardial infarction [21-22], kidney failure [23-26], and diabetics [21,27,28]. Hp is also a hemoglobin-binding protein ubiquitously present in the plasma of all vertebrates and believed to participate in hemoglobin transport [29]. The antioxidant activity of Hp was thought to be related from its binding ability with hemoglobin [15].

Hp may reduce loss of hemoglobin (Hb) and iron through the formation of an Hb-Hp complex which is not filtered through the glomeruli but transported to the liver [30]. The Hb-Hp complex is cleared from circulation in the liver by recognition of a specific hepatic macrophage receptor CD163 [19,31]. Thus, the *in vivo* function of haptoglobin was established as the plasma protein responsible for capture and clearance of excellular Hb from circulation, thereby preventing its toxicity to vasculature components [32]. However, studies on Hp knockout mice demonstrated that lack of Hp does not impair clearance of Hb from plasma [33]. Indication that uptake of free hemoglobin from circulation is faster than that of its complex with haptoglobin pointed to other, more urgent functions for this protein [33]. It appears that, by binding hemoglobin, Hp can serve as a vascular antioxidant. Haptoglobin was shown to completely inhibit the oxidative activity of Hb toward lipids as well as LDL protein. Because oxidative modification of LDL plays a critical role in the pathogenesis of atherosclerosis, Hp can be considered as a central antiatherosclerotic agent.

The present dissertation is divided as three sections:

1. A novel approach for Hp purification
2. Study of antioxidant role of Hp in LDL oxidation and in transfected cells
3. Analysis of antioxidant as a therapeutic agent for atherosclerosis

Section 1: A novel approach for Hp purification

To evaluate the antioxidant role of Hp, in the present thesis, it is essential to evaluate and develop a convenient purification method of Hp. The methods currently used for the purification of Hp frequently suffer some drawbacks. For example, Rademacher et al. utilize the chicken hemoglobin-Sepharose affinity column to isolate human Hp [34]; the harsh-elution condition (8 M urea) causes the dissociation of a hemoglobin subunit from the sepharose. Meanwhile, human apolipoprotein A-I appears to be another major contaminant. Wassdal et al. use rabbit hemoglobin-Sepharose; the hemoglobin is still co-eluted from the columns [35]. Travis et al. employ Sephadex G-200 gel filtration, but the purified Hp is accompanied with large amounts of IgM and α-2 macroglobulin [36]. Morimatsu et al. provide a modified method using HPLC with anion-exchange, Sephacryl S-300, TSK Phenyl-5PW, and TSK DEAE-5PW columns together; the procedures however are time-consuming, and the yield is relatively low (2.5 mg per 130 ml acute phase serum) [37]. Although Katnik et al. have shown a single-step isolation for Hp using an antibody-affinity column, the phenotypes, final purity, and the biological properties of Hp are not fully reported [38]. Presumably, the purpose of their report was to use isolated Hp for raising monoclonal antibodies. The similar antibody affinity-column procedure was employed in our laboratory, but the isolated Hp was not pure.

Therefore, we established simple two-step procedures for each Hp 1-1, 2-1, and 2-2

purification using a monoclonal antibody affinity-column followed by a HPLC Superose 12 gel filtration. Finally, some of the biochemical and physical properties with respect to each Hp phenotype were characterized and discussed.Antibody-affinity chromatography following a gel filtration column was then developed and used for purification. The purity and recovery of purified Hp was at least 92 and 55%, respectively. Each phenotype of isolated Hp possessed the ability to bind hemoglobin and retained its carbohydrate moiety. The CD spectrum of each Hp phenotype was also determined to estimate the secondary structure of Hp. For the first time, according to the best of our knowledge, we showed that the α-helical content of Hp 1-1, 2-1, and 2-2 was 29, 22, and 21%, respectively. Therefore, Hp 1-1 possessed a more ordered structure than that of polymeric forms of Hp 2-1 and 2-2. In conclusion, due to the structural heterogeneity, it is difficult to purify Hp from human plasma, particularly with the Hp 2-1 and 2-2 polymers. The present report provides a simple method for the purification of Hp phenotypes with relatively high yield. Hp 1-1, 2-1, and 2-2 can therefore be prepared and used for the study of structural and functional relationship with the pathogenesis in the diseases of interest.

Section 2: Study of antioxidant role of Hp in LDL oxidation and in transfected cells

We found that Hp was an extremely potent antioxidant and the activity of Hp 1-1 was differentially and moderately greater than that of Hp 2-1 and 2-2. We also demonstrated that Hp 1-1 cDNA transfected Chinese hamster ovary (CHO) K1 cells (normally not expressing the Hp molecules) significantly resist against oxidative stress. The role of Hp as an antioxidant molecule is therefore identified. The clinical significance with respect to the structure and function of Hp phenotype is discussed. To determine the antioxidant potency of Hp, TBARS assay using Cu^{2+}-induced LDL oxidation was employed. Hp showed an

extremely potency against LDL oxidation with a dose-dependent manner: The potency was 5 x greater than that of probucol and almost 20 x than vitamin E, in which probucol is known as a highly potent antioxidant compound used in clinics. Notably, the antioxidant activity of monomeric Hp 1-1 was moderately higher than that of polymeric Hp 2-1 and 2-2. To study the structural effect in its antioxidant activity, while the cystein was carboxymethylated, it exerted essentially a disordered structure in circular dichroism (CD). Such conformational change, however, did not attenuate its antioxidant activity. Unexpectedly, it resulted in a markedly increase in antioxidant activity by about 4 x. Taking together, the antioxidant potency expressed as IC50 in ranking was: CM Hp1-1 > CM Hp 2-1 > CM Hp 2-2 > Hp 1-1 > Hp 2-1 > Hp 2-2 > probucol > vitamin E. Subsequently, we tested its ability for scavenging free radicals generated from a water-soluble azo-compound AAPH. The tested antioxidant activity of Hp 2-1 was shown as a dose-dependent fashion with an IC50 about 5 x greater than that of probucol.

Presumably, the binding domain of Hp to Hb is dependent on the overall three-dimensional structure of Hp. We further examined the effect of carboxymethylation of Hp on the formation of Hp-Hb complex using a HPLC technique. However, the binding was totally abolished when Hp was carboxymethylated. Since the native Hp possessed its free radical-scavenging ability and the antioxidant activity of carboxymethylated Hp was superior to native Hp, it might imply that the antioxidant nature of Hp was independent on its binding ability to Hb.

To evaluate the *ex vivo* antioxidant activity of Hp, a CHO-K1 cell line was transfected with a pcDNA3.0 vector containing CMV promoter-driven Hp 1-1 cDNA. The plasmid construct also contained a selection marker, geneticin, for conditionally expressing the Hp 1-1. RT-PCR showed that the un-transfected cells did not express endogenous Hp mRNA.

Expression of Hp 1-1 protein in the culture medium and CHO cells was confirmed by Western blot and immunocytochemical staining. Hydrogen peroxide (H_2O_2) was then added to the CHO-K1 cell culture. Cells with and without Hp 1-1transfection were treated with variable dosages of H_2O_2 for 24 h. Upon the challenge of H_2O_2 the relative cell survival ratios of transfected CHO-K1 cells were statistically and significantly higher ($P<0.001$) than that of untransfected CHO-K1 cells, especially in the presence of 1 mM H_2O_2. Thus, the expression of Hp elevated the cell tolerance against the H2O2-induced oxidative stress.

In conclusion, we demonstrated that Hp was an extremely potent antioxidant molecule and have identified its antioxidant role in cell model. Blockage of disulfide linkages of Hp resulted in a loss of its ability to form a complex with Hb and yet exerted almost 4 x greater antioxidant activity than that native Hp. Thus, in addition to its role in forming Hp-Hb complex to block the heme group, Hp may participate in an independent antioxidant role for those cells expressing Hp. Whether or not Hp may be directly beneficial for free-radical associated atherosclerosis and myocardial infarction will be a subject of interest and challenge.

Section 3: Analysis of antioxidant as a therapeutic agent for atherosclerosis

Atherosclerosis and its complications are the major causes of mortality in industrialized countries [1-3]. Research into the oxidation of lipoprotein has yielded many insights into the process underlying the development of atherosclerosis. Oxidative modification of low density lipoprotein (LDL) has been suggested as an initial step in the pathogenesis of atherosclerosis [4,6]. However, up until now, investigations of antioxidants have focused on three main dietary antioxidant vitamins (β-carotene, vitamin C, and vitamin E) [39-41] and some synthetic compounds [42-44]. Among those antioxidants described above, probucol, a

synthetic compound, has been shown to be an extremely potent and effective antioxidant in preventing against the formation of atherosclerosis in both *in vitro* and *ex vivo* studies [42,43]. The present review focuses on commonly used analytical methods for measuring the antioxidant potency and outlines the critical steps as how to evaluate and design a potent antioxidant agent that can be used for the intervention of atherosclerosis. We conclude that an antioxidant should be first targeted and incorporated into human LDL. Second, the candidate compound should possess high bioavailability.

From the atherogenesis process and we evaluated those currently-used and potential antioxidant candidates for preventing the formation of atherosclerosis. The critical consideration in designing a compound that can be effectively used for antioxidant therapy in atherosclerosis are reviewed as 5 sections: **1)** The oxidation hypothesis and atherogenesis induced by oxidized LDL; **2)** Recent antioxidant therapies for atherosclerosis; **3)** potential antioxidants as antiatherosclerotic agents; **4)** commonly used analytical methods of antioxidant potency; **5)** rational design of a synthetic antioxidant as an antiatherosclerotic agent.

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Section 1: A novel approach for Hp purification

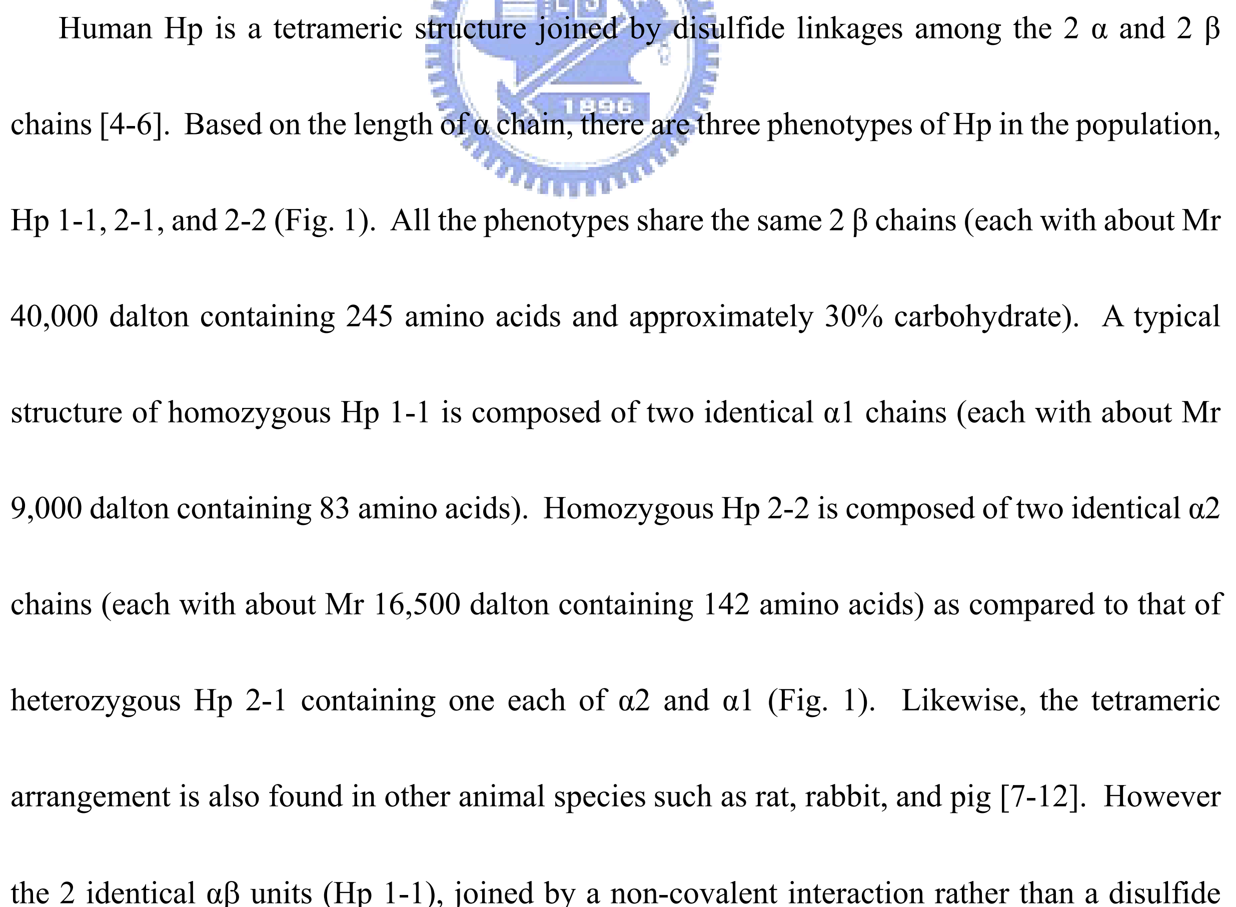
Abstract

Similar to blood type, human plasma haptoglobin (Hp) is classified as 3 phenotypes: Hp 1-1, 2-1, or 2-2. The structural and functional relationship between the phenotypes, however, has not been studied in detail due to the complicated and difficult isolation procedures. This report provides a simple protocol that can be used to purify each Hp phenotype. Plasma was first passed through an affinity column coupled with a high affinity Hp monoclonal antibody. The bound material was washed with a buffer containing 0.2 M NaCl and 0.02 M phosphate, pH 7.4, eluted at pH 11, and collected in tubes containing 1 M Tris-HCl, pH 6.8. The crude Hp fraction was then chromatographed on a HPLC Superose 12 column in 0.05 M ammonium bicarbonate at a flow rate of 0.5 ml/min. The homogeneity of purified Hp 1-1, 2-1, or 2-2 was greater than 95% as judged by SDS polyacrylamide gel electrophoresis. Essentially, each Hp isolated was not contaminated with hemoglobin and apolipoprotein A-I as that reported from the other methods, and was able to bind hemoglobin. Neuraminidase treatment demonstrated that the purified Hp possessed a carbohydrate moiety, while Western blot analysis confirmed α and β chains corresponding to each Hp 1-1, 2-1, and 2-2 phenotype. The procedures described here represent a significant improvement in current purification methods for the isolation of Hp phenotypes. Circular dichroic spectra showed that the α-helical content of Hp 1-1 (29%) was higher than that of Hp 2-1 (22%) and 2-2 (21%). The structural difference with respect to its clinical relevance is discussed.

Keywords: Human haptoglobin 1-1, 2-1, 2-2; Affinity purification; α-helix; Monoclonal antibodies

Introduction

Hp is known as an acute phase protein, and its plasma level elevates in response to inflection or inflammation. For this reason, Hp is a useful indicator for some infectious diseases [1-3]. It is also a hemoglobin-binding protein present in the plasma of all vertebrates and believed to participate in hemoglobin transport [4].



Human Hp is a tetrameric structure joined by disulfide linkages among the 2 α and 2 β chains [4-6]. Based on the length of α chain, there are three phenotypes of Hp in the population, Hp 1-1, 2-1, and 2-2 (Fig. 1). All the phenotypes share the same 2 β chains (each with about Mr 40,000 dalton containing 245 amino acids and approximately 30% carbohydrate). A typical structure of homozygous Hp 1-1 is composed of two identical α1 chains (each with about Mr 9,000 dalton containing 83 amino acids). Homozygous Hp 2-2 is composed of two identical α2 chains (each with about Mr 16,500 dalton containing 142 amino acids) as compared to that of heterozygous Hp 2-1 containing one each of α2 and α1 (Fig. 1). Likewise, the tetrameric arrangement is also found in other animal species such as rat, rabbit, and pig [7-12]. However the 2 identical αβ units (Hp 1-1), joined by a non-covalent interaction rather than a disulfide

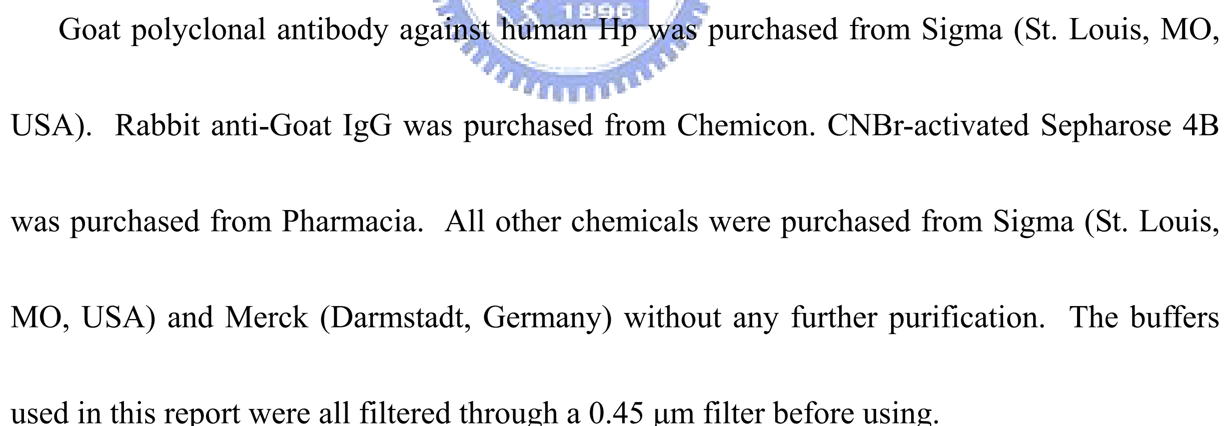
bridge, are found in dog, cat, and bear [13-14].

Clinically, polymeric form of Hp 2-1 or 2-2 is associated with the complications of myocardial infarction [15], kidney failure [16], and diabetics [17]. Presumably, this was due to the complicated structure of Hp 2-1 and 2-2 as it forms heterogeneous polymers, in which some of the biologically functional groups are not fully expressed on the surface (Fig. 1). The assumption, however, has not been tested because the structural and functional studies are hampered by lack of a straightforward isolation procedure in preparing sufficient Hp phenotypes. The methods currently used for the purification of Hp frequently suffer some drawbacks. For example, Rademacher *et al.* utilize the chicken hemoglobin-Sepharose affinity column to isolate human Hp; the harsh-elution condition (8 M urea) causes the dissociation of a hemoglobin subunit from the Sepharose [18]. Meanwhile, human apolipoprotein A-I appears to be another major contaminant. Wassdal *et al.* use rabbit hemoglobin-Sepharose; the hemoglobin is still co-eluted from the column [19]. Travis *et al.* employ Sephadex G-200 gel filtration, but the purified Hp is accompanied with large amounts of IgM and α-2 macroglobulin [20]. Morimatsu *et al.* provide a modified method using HPLC with anion-exchange, Sephacryl S-300, TSK Phenyl-5PW, and TSK DEAE-5PW columns together; the procedures however are time-consuming, and the yield is relatively low (2.5 mg per 130 ml acute phase serum) [21]. Although Katnik *et al.* have shown a single-step isolation for Hp using an antibody-affinity column, the phenotypes, final purity, and the biological properties of Hp are

not fully reported [22]. Presumably, the purpose of their report was to use isolated Hp for raising monoclonal antibodies [22]. The similar antibody affinity-column procedure [22] was employed in our laboratory, but the isolated Hp was not pure. In the present report, we established simple two-step procedures for each Hp 1-1, 2-1, and 2-2 purification using a monoclonal antibody affinity-column followed by a HPLC Superose 12 gel filtration. Finally, some of the biochemical and physical properties with respect to each Hp phenotype were characterized and discussed.

Materials and methods

Materials



Goat polyclonal antibody against human Hp was purchased from Sigma (St. Louis, MO, USA). Rabbit anti-Goat IgG was purchased from Chemicon. CNBr-activated Sepharose 4B was purchased from Pharmacia. All other chemicals were purchased from Sigma (St. Louis, MO, USA) and Merck (Darmstadt, Germany) without any further purification. The buffers used in this report were all filtered through a 0.45 μm filter before using.

Preparation of monoclonal antibody against Hp

Six monoclonal antibodies: 8B1-3A, W1-11G, 2-3H, G2D-7G, 12B-1 and 4A2-4H, against human Hp were produced and characterized according to the standard procedures established in

our laboratory [23]. Monoclonal antibody 8B1-3A, which possessed the highest binding affinity to Hp, was selected for preparation of the affinity column. Briefly, 120 ml of cultured medium from the 8B1-3A hybridoma were first precipitated in 50% saturated ammonium sulfate. The precipitate was dissolved in 12 ml of phosphate buffered saline containing 0.02 M phosphate and 0.15 M NaCl, pH 7.4 (PBS). The solution was then dialyzed exhaustively in PBS to remove the remaining ammonium sulfate, followed by a dialysis in coupling buffer containing 0.1 M $NaHCO_3$ and 0.5 M NaCl, pH 8.3.

Preparation of antibody affinity column

Dialyzed monoclonal antibody was first coupled to CNBr-activated Sepharose-4B (Pharmacia, Uppsala, Sweden) according to the manufacturer's procedures. Briefly, 2.86 g of freeze-dried Sepharose (1 g of freeze-dried powder gave about 3.5 ml final volume of gel) were swollen and suspended in 1 mM HCl and immediately washed with 20x volume of the same solution within 15 min on a sintered glass filter [24-26]. The gel was then washed with coupling buffer containing 0.1 M $NaHCO_3$ and 0.5 M NaCl, pH 8.3, and degassed. About 10 ml (18.7 mg/ml) of ammonium-sulfate fraction of monoclonal antibody in coupling buffer were slowly added into the gel (in 15 ml), while gently stirring by a magnetic bar for 1 h at room temperature. After coupling, the gel was washed 10x volume of PBS to remove unbound materials via a sintered glass filter. The gel was then treated with a blocking solution containing

0.1 M Tris-HCl and 0.5 M NaCl, pH 8.0, for 2 h at room temperature to saturate the remaining reactive-sites. The degassed gel was then washed with 3 cycles of blocking buffer and a 0.15 M NaCl solution of pH 11.0 (adjusted by ammonium) according to the procedures previously described by us [26]. Finally, the gel was equilibrated in PBS and packed onto a 1.5 x 20 cm column.

Purification of human Hp using antibody affinity-column chromatography

Initially, 1 ml of filtered human plasma of each Hp-phenotype batch was loaded onto the antibody affinity-column (10 ml in bed volume) at room temperature. The column was then washed with 50 ml of PBS. The bound materials were further washed with 50 ml of 0.02 M phosphate buffer containing 0.2 M NaCl, pH 7.4, and then eluted with 50 ml of a freshly prepared 0.15 M NaCl solution with pH 11 adjusted by ammonium [26]. Five ml of each fraction was collected in a tube containing 0.25 ml of 1 M Tris-HCl buffer, pH 6.8, to immediately neutralize the pH value. Pooled fractions containing Hp were then concentrated to a final volume of 1 ml using an Amicon centrifugal filter (Millipore).

Further purification of Hp by gel filtration column

Concentrated solution with Hp was filtered with a 0.45 μm nylon fiber prior to HPLC. The HPLC system (Waters) consisted of two pumps, an automatic sample injector, and a

photodiodearray detector. Superose 12 column (1 x 30 cm) (Pharmacia) was used for further Hp purification. The column was pre-equilibrated with 50 mM ammonium bicarbonate. Partially purified Hp (0.8 ml) was applied to the column at a flow rate of 0.5 ml/min. Fractions containing Hp were pooled and concentrated to a final volume of 1 ml using an Amicon centrifugal filter and then lyophilized. The lyophilized Hp was stored at –80℃ until analyzing.

Gel electrophoresis and densitometry

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the Laemmli's method [27] with some modification in using 5% polyacrylamide (w/v) on the stacking gel as previously described [7]. Samples (typically 5 μg) for SDS-PAGE were preheated at 100 ℃ for 10 minutes in a loading buffer [12 mM Tris-HCl, pH 6.8, 0.4% SDS (w/v), 5% glycerol (v/v), 2.88 mM 2-mercaptoethanol, 0.02% bromphenol blue (w/v)]. For molecular weight calibration, a subset of the following standards was included in each gel: myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase B (97 kDa), serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa), and aprotinin (6.5 kDa). The samples were run for about 1.5 h at 100 V and stained using Coomassie brilliant blue R-250. Densitometric analysis of SDS-PAGE was performed using a Molecular Dynamics densitometer for data acquisition and Image Quant software for integration and analysis.

Immunoblot analysis

Following the separation of proteins by SDS-PAGE, the gel and nitrocellulose- and 3MM filter- papers were soaked in a transfer buffer containing 48 mM Tris-HCl, 39 mM glycine, 0.037% SDS (w/v), and 20% methanol (v/v) at pH 8.3 for 30 min. The gel was then electrotransferred to a nitrocellulose membrane (Pharmacia) at 90 mA for 1 h in a semi-dry transfer cell (Bio-Rad) containing a transfer buffer. The transferred membrane was then immersed in 5% skim milk (w/v) in PBS for 1 hour at room temperature while shaking gently. After three times washing with PBS for 5 min, the membrane was incubated with a primary goat polyclonal antibody against human Hp [1:5000 dilution in PBS washing buffer containing 1% (w/v) skim milk and 0.05% Tween-20 (v/v) for 1 hour] at room temperature and washed three times for 5 min. The membrane was then incubated with 1:10,000 diluted rabbit anti-goat IgG conjugated with horseradish peroxidase in washing buffer for 1 h. In addition, the membrane was washed two times with washing buffer and further washed one time with PBS. Finally, the membrane was developed using 3,3'-diaminobenzidine (DAB) as a substrate for horseradish peroxidase [7, 25].

Circular dichroic spectra

The lyophilized Hp was dissolved in 10 mM phosphate buffer at pH 7.4 with a final

concentration of 0.2 mg/ml. About 300 μl of Hp solution was used to analyze within a cuvette of 1-mm path length. Circular dichroic spectrum was conducted between 190 and 300 nm in a Jasco J-715 spectropolarimetry. The obtained spectrum of each type of Hp was accumulated for 20 times at a scanning rate of 50 nm/min and the % α-helical content was estimated from the mean residue molar ellipticity (θ_{222}). % α-helix = $[(\theta_{222} + 3000)/(36000+3000)] \times 100$ [28].

Results

Preparation of monoclonal antibody against Hp

Six monoclonal antibodies prepared against Hp were characterized, in which 8B1-3A possessed the highest binding affinity (Ka=5.6×10^{9} M^{-1}) and was chosen to prepare an affinity column. The binding capacity estimated was greater than 100 μg of Hp per ml of coupled Sepharose (data not shown).

Purification of human Hp using antibody affinity column chromatography

Fig. 2 shows a typical chromatographic profile for Hp 1-1, 2-1, and 2-2 purification on the affinity column. Human plasma was applied to the column followed by an extensive wash with a phosphate buffer containing 0.2 M NaCl. It is worth mentioning, this pre-wash step differed from the conventional method in which 0.12 M NaCl was used. Using 0.2 M NaCl, most of the low-affinity binding proteins were eliminated (Fig. 3). Hp was then eluted at pH 11 and

collected in tubes containing 0.25 ml of 1 M Tris-HCl, pH 6.8, to immediately neutralize the pH. The purity of each Hp phenotype was approximately 60-80% in homogeneity as analyzed on SDS-PAGE. Apolipoprotein A-I appeared to be a major contaminant. All the phenotypes of Hp converted to α (α1 or α2 or both) and β subunits in the presence of the reducing reagent (Fig. 3). A typical Western blot analysis showing 3 isolated phenotypes is depicted in Fig. 4. The recovery of Hp at this step accounted for 75-94% of the Hp from the plasma with a final of 51-54 fold purification (Table 1).

Further purification of Hp on HPLC gel-filtration column

The obtained Hp 1-1, 2-1, or 2-2 fraction was concentrated and applied onto a gel-filtration Superose 12 column pre-equilibrated with 0.05 M of ammonium bicarbonate, pH 8.0. Chromatographic profiles (Fig. 5) revealed that the solution property of each Hp phenotype was consistent with its molecular form, in which Hp 1-1 was more homogeneous in size with longer elution time than that of Hp 2-1 and 2-2. Purity of each phenotype was then analyzed on SDS-PAGE containing reducing reagent 2-mercaptoethanol. Homogeneity of each phenotype was greater than 95% (Fig. 6). Thus, HPLC Superose column was markedly effective to remove apoA-I contaminant.

Western blot analysis in the absence of a reducing reagent demonstrated that Hp 2-1 and 2-2 were all polymeric (Fig. 7), in which Hp 2-2 was devoid of monomer and dimer consistent with

the proposed structure of Hp (Fig. 1). Thus, our purification procedures did not apparently alter the structural characteristics of Hp phenotypes.

The polymeric structure of isolated Hp and its binding to hemoglobin

We further studied the ionic property of isolated Hp 1-1, 2-1, and 2-2 on a native-PAGE; the distinct polymorphism of each phenotype was also observed (Fig. 8). Hp 2-2 was the most basic among the Hp phenotypes. Since hemoglobin (Hb) is able to bind Hp and to form a Hb-Hp complex [10], Fig. 8 demonstrates that the Hb could form Hb-Hp complex with each Hp phenotype we isolated.

Circular dichroic spectra

To characterize the secondary structure of each Hp phenotype, we determined the conformation of Hp by CD (Fig. 9). The estimated α-helical content was about 29, 22, and 21% for Hp 1-1, 2-1, and 2-2, respectively (Table 2). Statistically, the α-helical content in Hp 1-1 was significantly higher than that in Hp 2-1 and 2-2 ($P < 0.001$).

Discussion

Purification of human Hp has been hampered for years due to its structural diversity as described above [7, 18-21]. In the present study, plasma without any additional manipulations

(e.g., ammonium-sulfate precipitation) was utilized for Hp isolation. With the use of a 0.2 M NaCl wash in our procedures, most of the nonspecific low-affinity binding proteins were eluted from the column. Nevertheless, the affinity purified Hp 1-1, 2-1 or 2-2 analyzed on SDS-PAGE was only 60-80% pure (Fig. 3). HPLC Superose 12 column appeared to be superior to the other methods in the second-step purification since apolipoprotein A-I, a major contaminant, and other unknown high molecular-weight proteins were almost eliminated. The solution property of each Hp phenotype on this Superose column was consistent with its molecular forms, of which Hp 1-1 was more homogeneous than the other two species in size. Notably, the elution time of each phenotype was also consistent with the size of Hp, in which the polymeric form of Hp 2-2 and 2-1 was eluted earlier, respectively (Fig. 5). Western blot analysis on the polymeric structure of isolated Hp using 6% SDS-PAGE without reducing reagent (Fig. 7) revealed its structural identity to that originally present in plasma (data not shown). It also confirmed that the isolated Hp 2-1 was heterogeneous in nature containing Hp monomer and dimer, while the polymeric Hp 2-2 contained neither.

Subsequently, we show each phenotype of isolated Hp possessed the ability to bind hemoglobin (Fig. 8), although we cannot presently address whether or not the binding potency might be attenuated during the isolation. Meanwhile, we demonstrated the presence of carbohydrate moiety in isolated Hp as neuraminidase treatment could remove the terminal sialic acid residues from the Hp with a time-dependent manner similar to our previous study [7]

(data not shown).

Taking together, the Hp isolated from the antibody affinity-column combined with HPLC Superose 12 exhibited advantages over the conventional methods. First, the heterogeneous particles of Hp 2-1 or 2-2 could be obtained in one pool as polymeric forms. Second, the co-eluted hemoglobin from hemoglobin affinity-column [19] was eliminated in the present study. Using an ELISA for hemoglobin assay, we could not detect hemoglobin in the Hp we isolated (data not shown). Third, the isolated Hp not only retained its ability to bind hemoglobin (Fig. 8), but also blocked the peroxidase activity of hemoglobin (data not shown).

The CD spectrum of each Hp phenotype was determined to estimate the secondary structure of Hp. For the first time, according to the best of our knowledge, we showed that the α-helical content of Hp 1-1, 2-1, and 2-2 was 29, 22, and 21%, respectively. Hp 1-1 possessed a more ordered structure than that of polymeric forms of Hp 2-1 and 2-2. Moreover, we identified that the immunoreactivity of Hp 1-1 was also greater than that of Hp 2-1 and 2-2. For example, the monoclonal antibody used in our affinity column (8B1-3A) exhibited an affinity (Ka) 5.58×10^9, 2.81×10^9, and 2.19×10^9 M^{-1} against Hp 1-1, 2-1, and 2-2, respectively. The later result suggests that the availability of "functional" surfaces of Hp 1-1 may be greater as compared to Hp 2-1 and 2-2. Such structural differences may explain, in part, the clinical outcome by which Hp phenotype is associated with differential susceptibility to infections, atherosclerosis, and autoimmune disorders [3, 29]. These effects are correlated with a phenotype-dependent

modulation of oxidative stress and prostaglandin synthesis. In general, patients with Hp 2-2 are more susceptible in developing the severity of the diseases mentioned above. Identification of the biochemical basis for the differences among Hp phenotypes may lead to a rational design in intervening new pharmacological agents, such as mini-Hp, which have been recently proposed [30].

In conclusion, due to the structural heterogeneity, it is difficult to purify Hp from human plasma, particularly with the Hp 2-1 and 2-2 polymers. The present report provides a simple method for the purification of Hp phenotypes with relatively high yield. Hp 1-1, 2-1, and 2-2 can therefore be prepared and used for the study of structural and functional relationship with the pathogenesis in the diseases of interest.

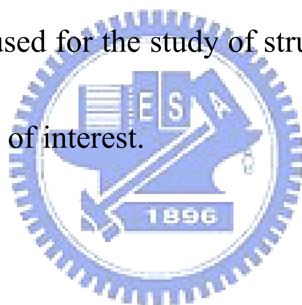


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Figure Legends

Fig. 1. Schematic drawing of proposed structure of human Hp 1-1, 2-1, and 2-2. All three phenotypes share a common structure of β chains (please also see reference 4). The degree of polymerization within the inter-molecular arrangement is shown.

Fig. 2. Typical purification profile of human Hp on antibody affinity-column. One ml of human plasma was applied to an antibody affinity-column (pre-equilibrated with PBS, pH 7.4) followed by a wash in 10 mM phosphate buffer containing 0.2 M NaCl, pH 7.4. The bound Hp was then eluted in a solution at pH 11 and collected in tubes containing 0.25 ml of 1 M Tris, pH 6.8.

Fig. 3. Analysis of isolated Hp from affinity column using 12% SDS-PAGE in the presence of reducing reagent. Lane M: molecular markers (expressed as kDa). Lanes 1-2: plasma before and after flowing through the affinity column, respectively. Lane 3: low-affinity binding proteins washed with 10 mM phosphate buffer containing 0.2 M NaCl, pH 7.4. Lane 4: the eluted Hp as described in Fig. 2.

Fig. 4. Western blot analysis of Hp isolated from antibody affinity-column. Following a 12%

SDS-PAGE, the separated proteins were transferred, blocked, and developed by a goat anti-human Hp. Lane M: molecular markers. Lanes 1-2: plasma before and after flowing through the affinity column, respectively. Lane 3: low-affinity binding proteins washed with 10 mM phosphate buffer containing 0.2 M NaCl, pH 7.4. Lane4: the eluted Hp.

Fig. 5. Typical chromatographic profile of affinity isolated Hp on HPLC Superose 12 column. Isolated Hp 1-1, 2-1, or 2-2 from the affinity column was first concentrated and applied to a HPLC Superose 12 pre-equilibrated in 50 mM of ammonium bicarbonate, pH 8.0. The same solution was used in the mobile phase at a flow rate of 0.5 ml/ml. The filled bar represents the pooled fractions corresponding to isolated Hp.

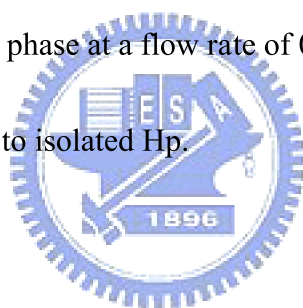
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Fig. 6. Analysis of purified Hp from antibody affinity and HPLC gel filtration column on 15% SDS-PAGE in the presence of reducing reagent. M: molecular markers. A: isolated Hp from affinity column alone. B: purified Hp from an additional separation on HPLC Superose 12 column.

Fig. 7. Western blot analysis of polymeric structure of purified Hp on 6% SDS-PAGE in the absence of reducing reagent. Lane M: molecular markers. Lanes 1-3: purified Hp 1-1, 2-1, and 2-2, respectively. Notably, the Hp 2-1 contains monomeric and dimeric forms of Hp 1-1:

whereas the Hp 2-2 is devoid of both.

Fig. 8. Analysis of hemoglobin-binding property of purified Hp 1-1, 2-1, and 2-2 on a 7% native-PAGE. Briefly, each Hp phenotype (5 μg) was incubated with and without hemoglobin (Hb) (5 μg) at room temperature for 30 minutes before conducting the native-PAGE. The gel was then stained with Coomassie Blue R-250. Hp of each phenotype was shifted to basic upon the binding of Hb.

Fig. 9. Circular dichroic spectra of Hp 1-1, 2-1, and 2-2. Each Hp in 10 mM phosphate buffer, pH 7.4, at a final concentration of 0.2 mg/ml was monitored by a circular dichroism. Each spectrum represents a mean of 20x determinations.

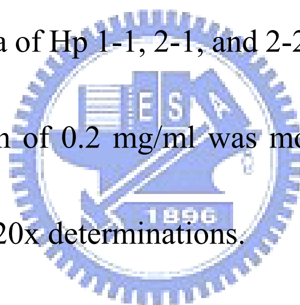
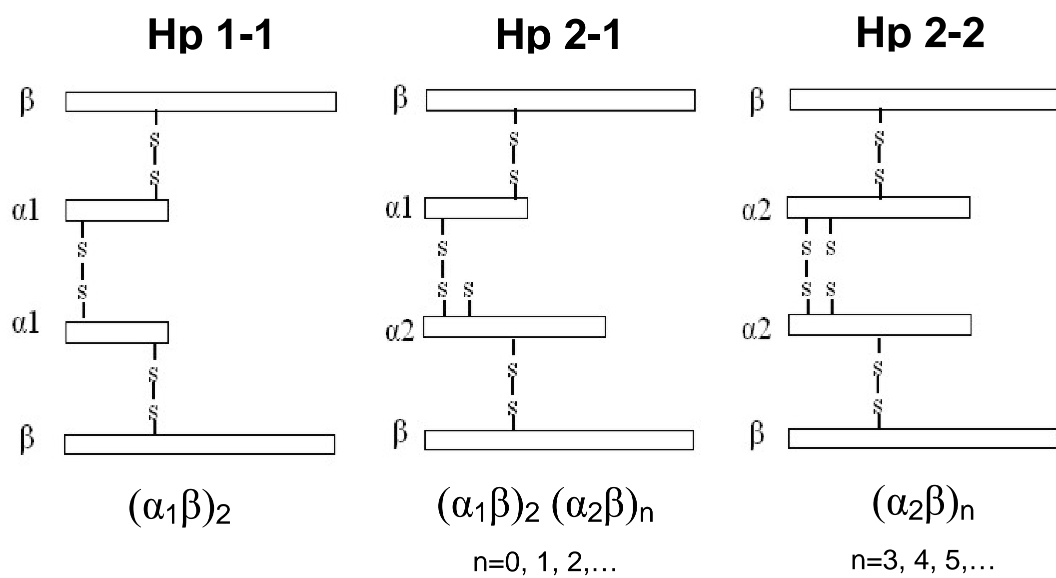
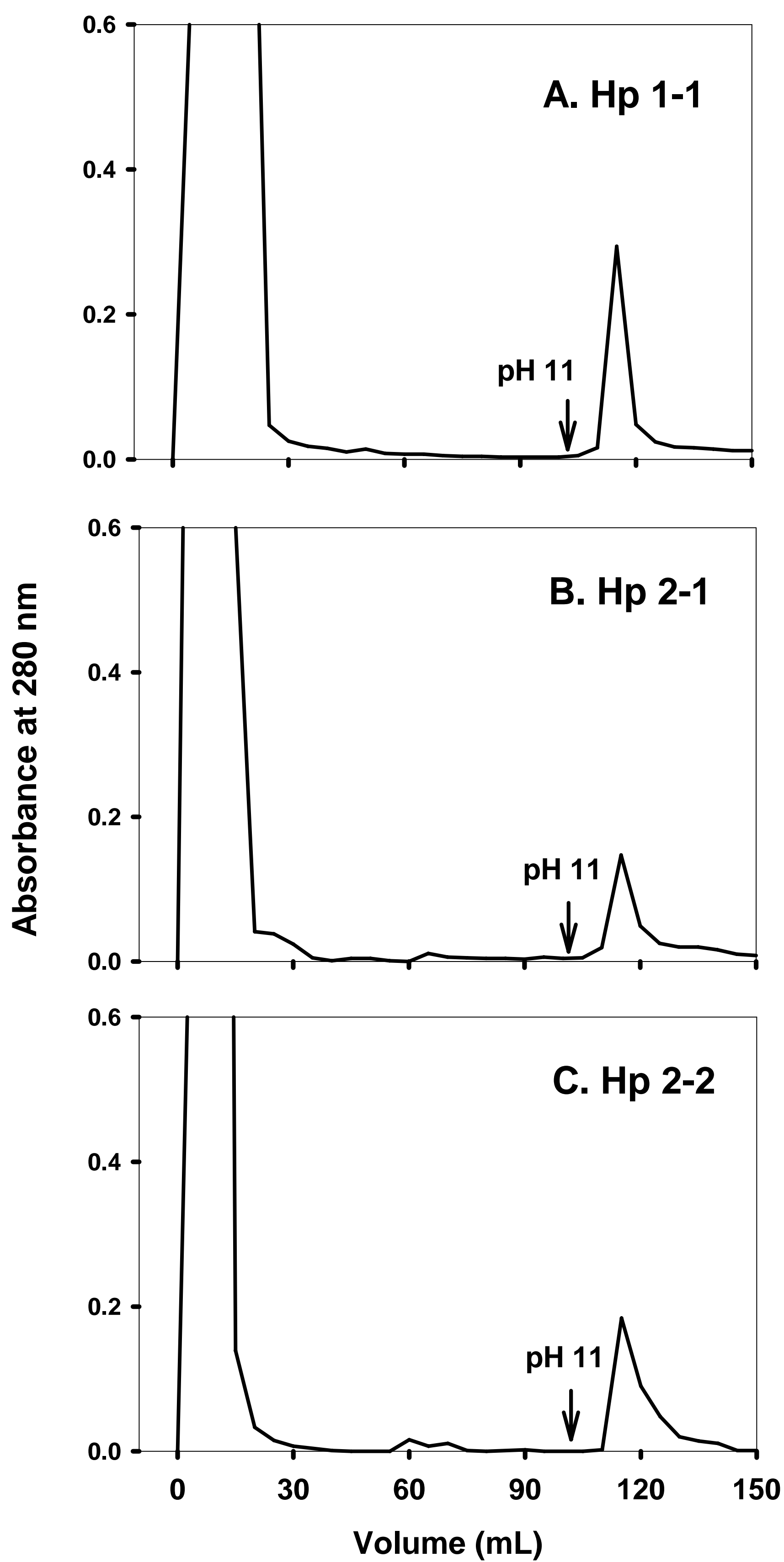


Fig 1.




A. Hp 1-1
B. Hp 2-1
C. Hp 2-2
pH 11
pH 11
pH 11
Absorbance at 280 nm
Volume (mL)
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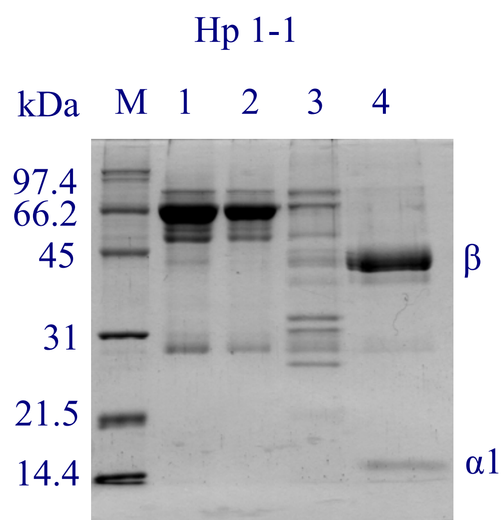
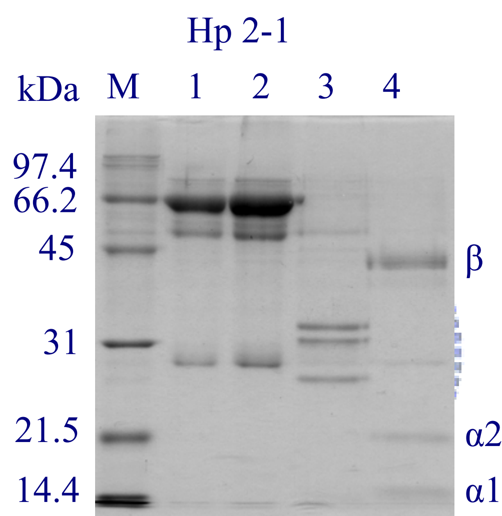
0.2
0.4
0.6
0
30
60
90
120
150

Fig 3.





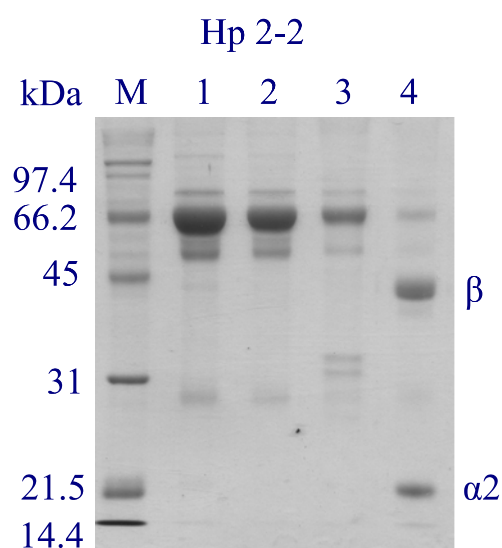
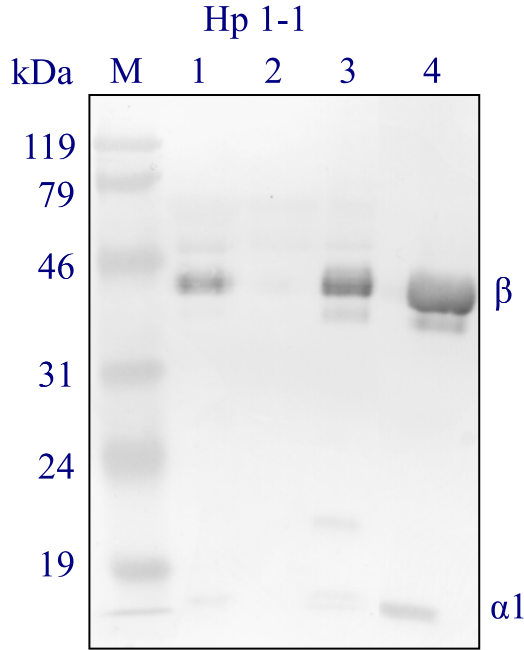
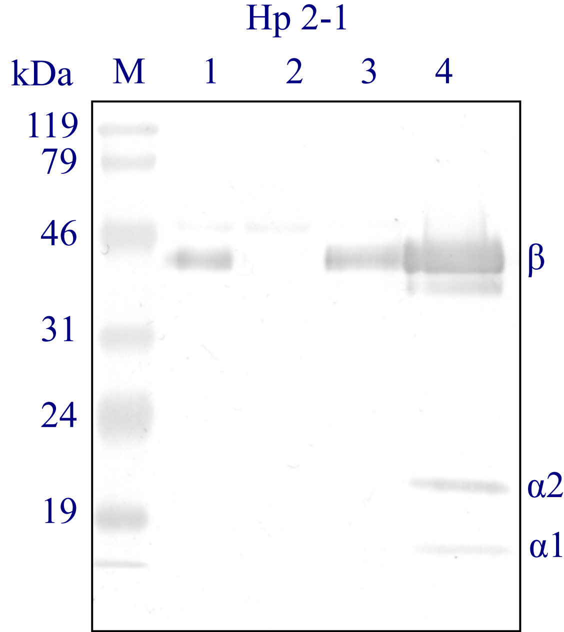


Fig 4.





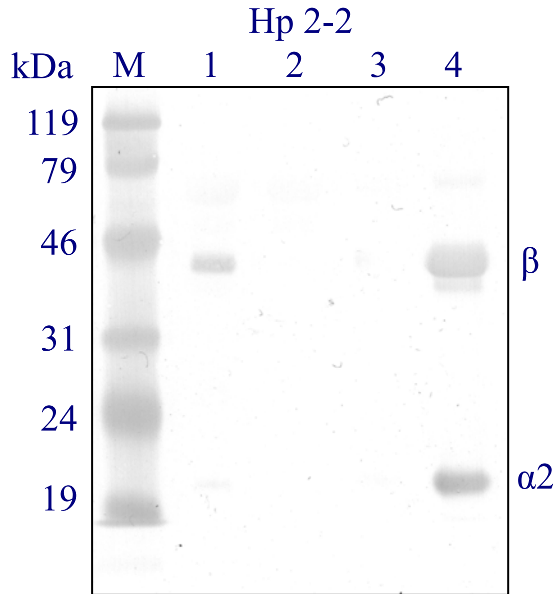


Fig 5.

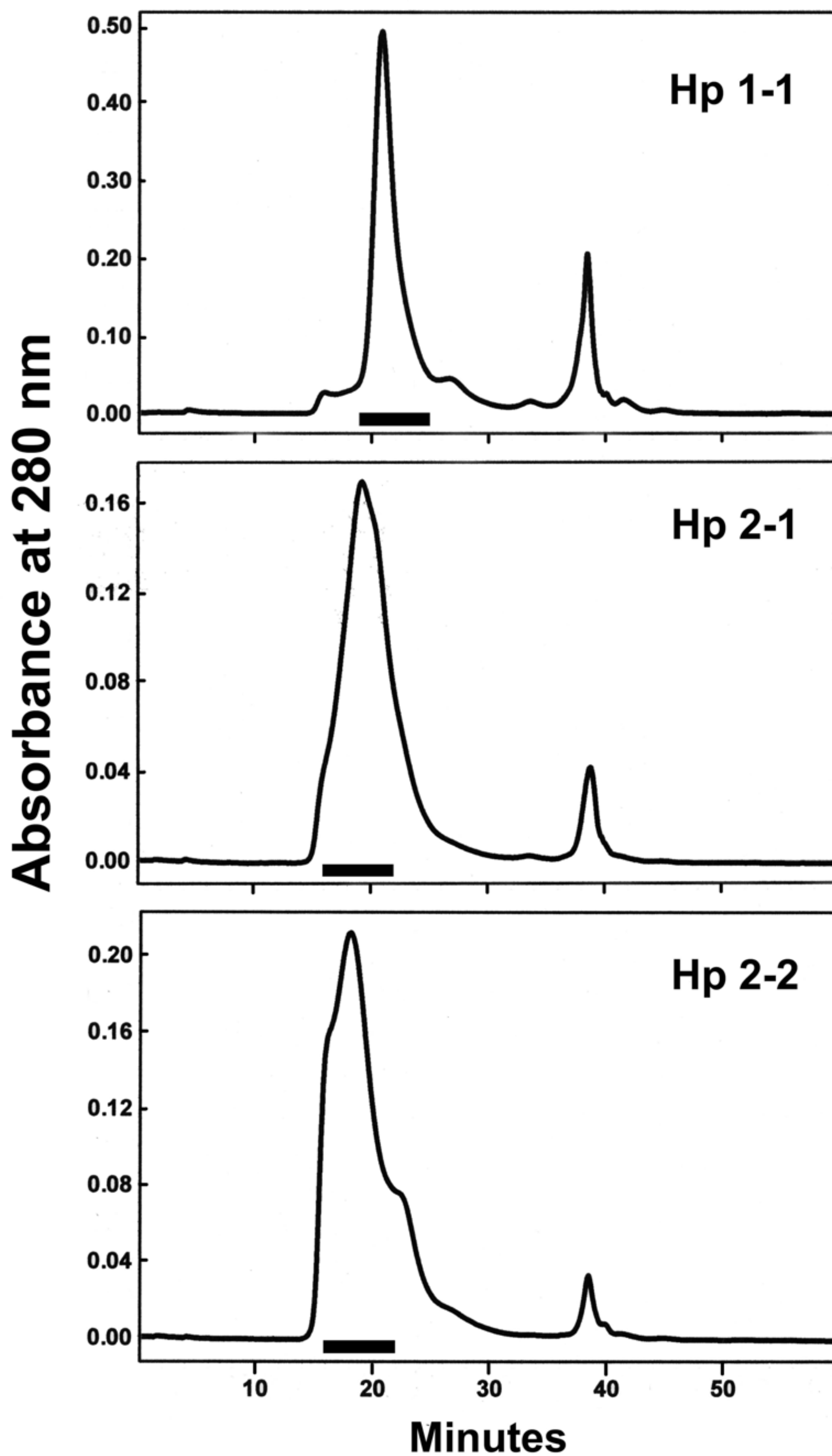


Fig 6.

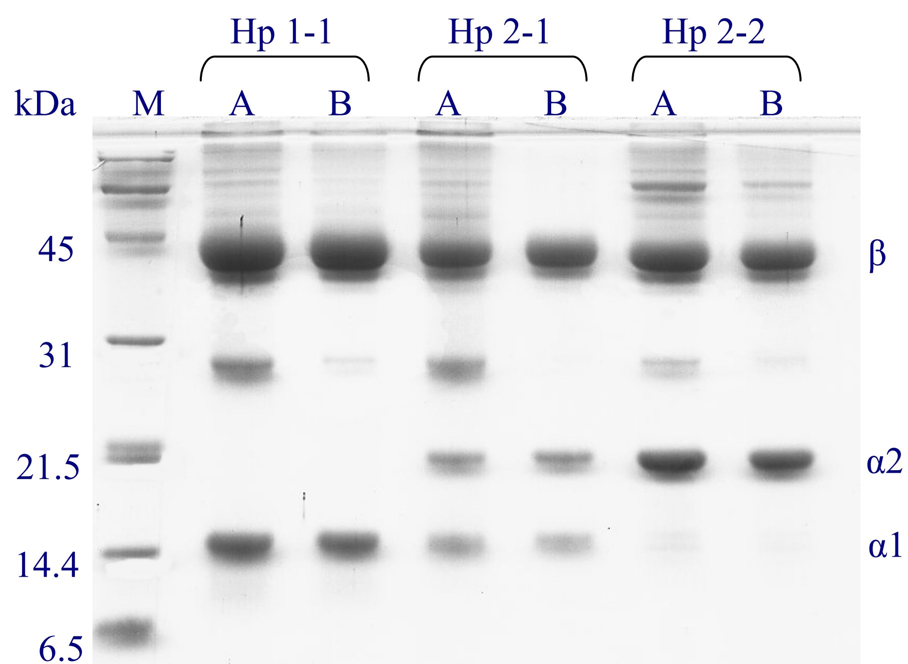


Fig 7.

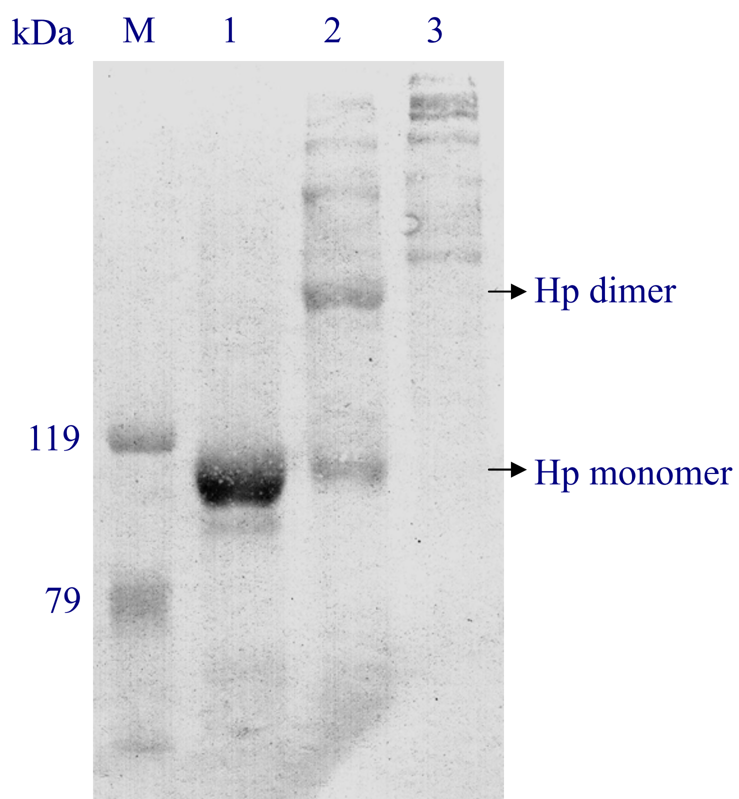


Fig 8.

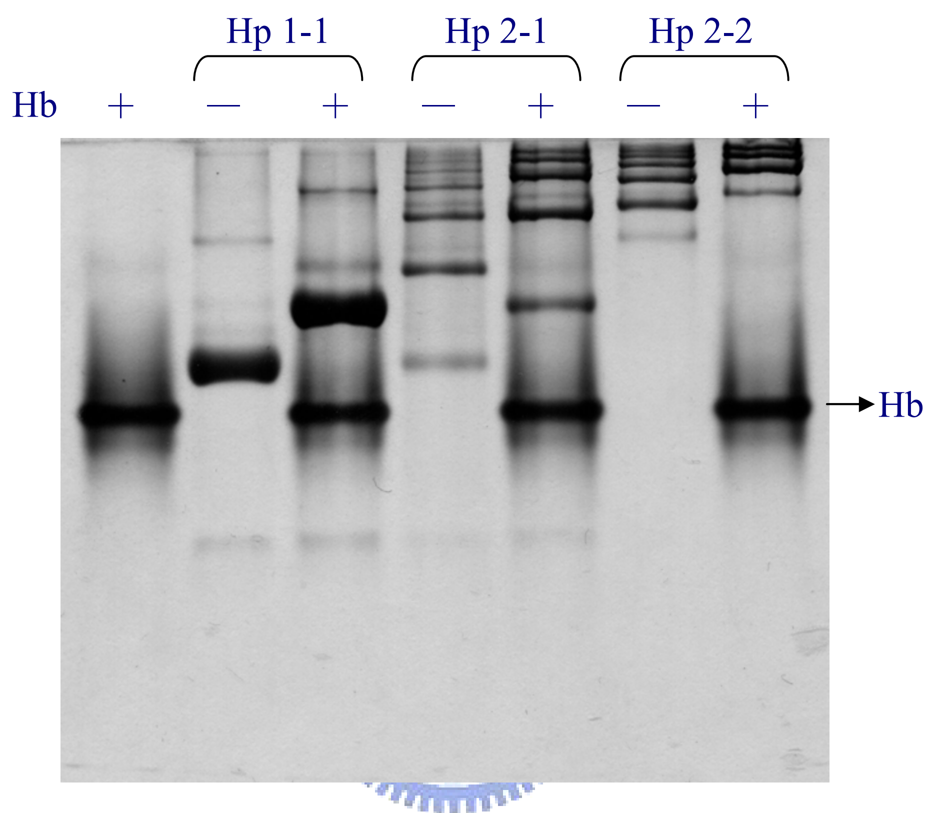


Fig 9.

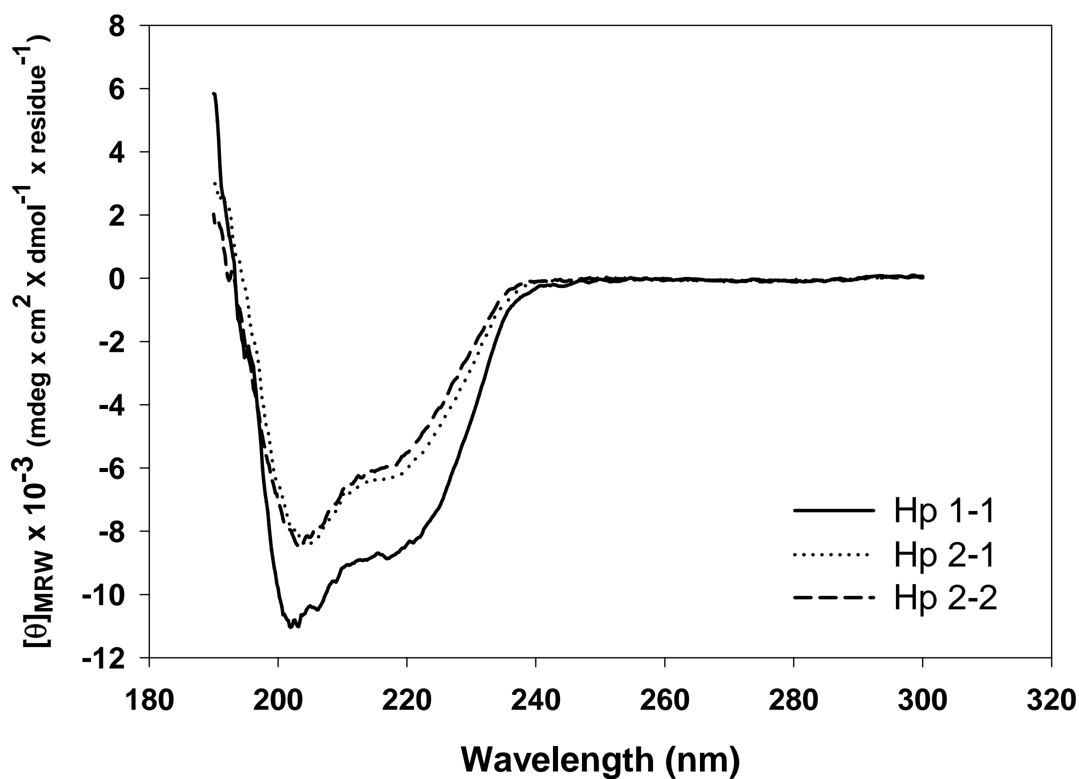


Table 1. Analytical recovery and yield of haptoglobin purified from 1 ml of human plasma

(A) Hp 1-1

	Plasma	Affinity column	Gel filtration
Total protein (mg)	102	1.41	0.88
Moiety of Hp (mg)	1.46[a]	1.09[b]	0.81[b]
Purity (%)[b]	1.43	77	92
Fold purification	1	54	64
Recovery (%)	100	75	55
Yield (mg)	—	—	0.81

(B) Hp 2-1

	Plasma	Affinity column	Gel filtration
Total protein (mg)	101	1.80	0.72
Moiety of Hp (mg)	1.17[a]	1.10[b]	0.71[b]
Purity (%)[b]	1.16	61	98
Fold purification	1	53	84
Recovery (%)	100	94	61
Yield (mg)	—	—	0.71

(C) Hp 2-2

	Plasma	Affinity column	Gel filtration
Total protein (mg)	100	1.53	0.81
Moiety of Hp (mg)	1.26[a]	0.98[b]	0.76[b]
Purity (%)[b]	1.26	64	94
Fold purification	1	51	75
Recovery (%)	100	78	60
Yield (mg)	—	—	0.76

[a] The concentration of each human Hp phenotype was determined using an ELISA currently used in our laboratory.

[b] Determined by densitometer using digital Image Quant software.

Table 2. α-helical content of each Hp phenotype as determined by CD

	Hp 1-1	Hp 2-1	Hp 2-2
α-helix	29%*	22%	21%

*Significant difference as compared to Hp 2-1 or Hp 2-2 ($P < 0.001$).

Section 2: Study of antioxidant role of Hp in LDL oxidation and in transfected cells

Abbreviations: **AAPH**, 2,2'-azobis(2-amidinopropane)-dihydrochloride; **ABTS**, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); **Hp**, haptoglobin; **Hb**, hemoglobin; **CAD**, cardiovascular disease; **CD**, circular dichroism; **CHO**, Chinese hamster ovary; **CMV**, cytomegalovirus; **CM**, carboxymethylated, **DAB**, 3,3'-diaminobenzidine; **LDL**, low density lipoprotein; **HDL**, high density lipoprotein; **HRP**, horseradish peroxidase; **MDA**, malonaldehyde; **MTT**, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **TBA**, 2-thiobarbituric acid; **TBARS**, thiobarbituric acid-reactive substance

Keywords: Haptoglobin phenotypes / Antioxidant / Hemoglobin / Haptoglobin cDNA / Physiologic role

Abstract

Similar to blood type, human plasma haptoglobin (Hp) is classified as 3 phenotypes: Hp 1-1, 2-1, and 2-2 attributed by their two common alleles 1 and 2. Clinically, the phenotype 2-2 is associated with the risk in patients with cardiovascular diseases and diabetes mellitus. In this study, we demonstrated that Hp was an extremely potent antioxidant, which directly prevented LDL from Cu^{2+}-induced oxidation. Its potency was markedly superior to probucol: one of the most potent antioxidants. The IC_{50} of antioxidant activity in ranking was: Hp 1-1> Hp 2-1> Hp 2-2> probucol> vitamin E. Blockage of disulfide linkages between Hp subunits, not only abolished the α-helical content but also diminished its ability to form a complex with hemoglobin (Hb). It exerted almost 4 x greater antioxidant activity than that of native Hp. To investigate antioxidant role of Hp on the cellular level, the cDNA of Hp 1-1 was cloned, constructed (containing the pcDNA3.0 vector with CMV promoter) and transfected to CHO-K1 cells. Following the transfection, these CHO cells were able to express Hp 1-1 protein and significantly ($P<0.001$) elevated the tolerance against the oxidative stress. The elevation was about twice-higher than that normal CHO cells when challenged with hydrogen peroxide for 24 h. Thus, Hp plays a provocative antioxidant role as demonstrated in our *in vitro* and *ex vivo* studies.

Introduction

Hemoglobin (Hb) is the most abundant and functionally important protein in erythrocytes. However, once Hb released from red blood cells, it becomes highly toxic because of the oxidative nature of iron-containing heme, which participates in the Fenton reaction to produce reactive oxygen species causing cell injury [1-2]. On the other hand, human plasma haptoglobin (Hp), known as an acute phase protein [3-5], may capture the Hb by forming a high affinity Hp-Hb complex [1, 3-4]. The complex is then metabolized through a receptor-mediated process including a recent report showing the CD 163 receptor of macrophages [6]. Therefore, Hb binding by Hp is essential in rapid clearance of Hb from the plasma [7]. For this reason, Hp plays a crucial role against Hb-induced oxidative stress by a mechanism thought to be from its high-affinity binding with Hb and prevent the iron "leaking" from the Hb. However, thus far, there is no report directly pointing out that Hp itself is an antioxidant molecule.

The different Hp phenotype 1-1, 2-1, or 2-2 in each respective individual is attributed by two common alleles 1 and 2 located at chromosome 16q22.1. Structurally, the minimal unit of Hp (β-α-α-β) is joined by disulfide linkages among the 2 α and 2 β chains [6, 8-9]. Fig. 1 shows that all the phenotypes share the same 2 β chains (each with about MW 40,000 dalton containing 245 amino acids and about 30% carbohydrate). A typical structure of homozygous Hp 1-1 (β-α1-α1-β) is composed of only two identical α1 chains (each with

about MW 9,000 dalton containing 83 amino acids). Homozygous Hp 2-2 is composed of two identical α2 chains (each with about MW 16,500 dalton containing 142 amino acids) as compared to that of heterozygous Hp 2-1 containing each α2 and α1 (Fig. 1). Due to an extra-thio group in α-2 chain, only Hp 2-1 and 2-2 form large polymers in monomeric, trimeric, tetrameric, pentameric, hexameric, and even larger arrangement through the disulfide-linkages (Fig. 1).

Clinically, diabetic patients with the Hp 1-1 type are markedly resistant to the development of diabetic retinopathy, diabetic nephropathy, and cardiovascular disease (CAD) [10-12]. In a prospective study, participants homozygous with Hp 2-2 are 5-fold increased in risk for the development of CAD as compared to Hp 1-1, whereas the risk in heterozygous Hp 2-1 are intermediate [13].

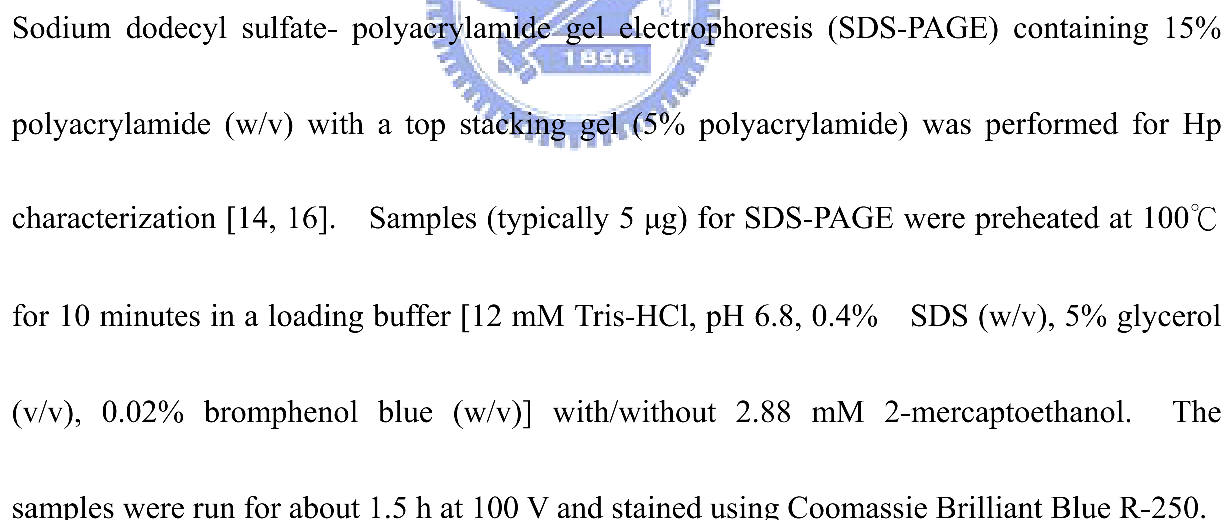
In the present study, we show that Hp was an extremely potent antioxidant and the activity of Hp 1-1 was differentially and moderately greater than that of Hp 2-1 and 2-2. We also demonstrated that Hp 1-1 cDNA transfected Chinese hamster ovary (CHO) K1 cells (normally not expressing the Hp molecules) significantly resist against oxidative stress. The role of Hp as an antioxidant molecule is therefore identified. The clinical significance with respect to the structure and function of Hp phenotype is discussed.

Materials and methods

Purification of human Hp

Hp phenotype was first identified using plasma supplemented with hemoglobin (Hb) on a native polyacrylamide gel electrophoresis (PAGE), followed by a peroxidase substrate staining [14]. The plasma of each specific Hp phenotype was then chromatographed on an antibody affinity-column followed by a gel filtration chromatography as previously described by our laboratory [14-15]. The homogeneity of each Hp species employed was greater than 95%.

Gel electrophoresis



Sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) containing 15% polyacrylamide (w/v) with a top stacking gel (5% polyacrylamide) was performed for Hp characterization [14, 16]. Samples (typically 5 μg) for SDS-PAGE were preheated at 100℃ for 10 minutes in a loading buffer [12 mM Tris-HCl, pH 6.8, 0.4% SDS (w/v), 5% glycerol (v/v), 0.02% bromphenol blue (w/v)] with/without 2.88 mM 2-mercaptoethanol. The samples were run for about 1.5 h at 100 V and stained using Coomassie Brilliant Blue R-250.

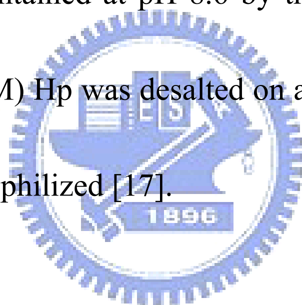
Western blot

Following the separation of proteins by SDS-PAGE, the gel soaked in a transfer buffer containing 48 mM Tris-HCl, 39 mM glycine, 0.037% SDS (w/v), and 20% methanol (v/v) at

pH 8.3 was electrotransferred to a nitrocellulose paper (Pharmacia) at 90 mA for 1 h in a semi-dry transfer cell (Bio-Rad). Immunoblotting and developing were conducted according the standard procedures previously described [14-16].

Reduction and carboxymethylation of Hp

To 1 mg of Hp was added 3.3 mL of 0.01 M Tris-HCl buffer (pH 8.6) containing 5.4 M urea, and 1% (v/v) β-mercaptoethanol. The reaction mixture was flushed with nitrogen and incubated at room temperature. After 2 h, 20 mg of iodoacetic acid were added and the reaction mixture was then maintained at pH 8.6 by the addition of 1 M NaOH for 30 min. Finally, carboxymethylated (CM) Hp was desalted on a Bio-Gel P2 column eluted with 0.1 M ammonium bicarbonate and lyophilized [17].



Preparation of LDL

Human low density lipoprotein (LDL) (d. 1.012-1.063 g/mL) was prepared from human plasma by a sequential ultracentrifugation according to the method previously established [17]. Sodium azide (0.01%) was added into plasma prior to ultracentrifugation and into LDL after isolation. Subsequently, freshly prepared LDL was dialyzed against PBS to remove EDTA and used for oxidation assays [1].

LDL oxidation

Thiobarbituric acid-reactive substances (TBARS) were used as an index to measure the LDL

oxidation [1, 18]. In a typical assay, 4 μM $CuSO_4$ and 40 μg of LDL were incubated with native Hp, CM-Hp, probucol, or vitamin E in a final volume of 100 μL. For the oxidation induced by water-soluble initiator, 5 mM of 2,2'-azobis(2-amidinopropane)-dihydrochloride (AAPH), only Hp 2-1 was employed for antioxidant activity. Incubation was carried out at 37℃ for 2 h, after which time 250 μL of 20% trichloroacetic acid (w/v) was added to precipitate proteins. Subsequently, 250 μL of 0.67% 2-thiobarbituric acid (w/v) (TBA) was added and incubated at 80℃ for 30 min. The reaction mixtures were centrifuged at 3,000*g* for 5 min, and 300 μL of supernatant in a 96-well plate were read at 540 nm [1]

Circular dichroic analysis

The lyophilized Hp was dissolved in 10 mM phosphate buffer at pH 7.4 with a final concentration of 0.2 mg/ml. About 300 μL of the aliquot in a cuvette (1-mm path length) was used for the analysis. Circular dichroic spectrum recorded between 190 and 300 nm (in Jasco J-715 spectropolarimetry) was accumulated for 20 x at a scanning rate of 50 nm/min. The α-helical content was estimated from the mean residue molar ellipticity (θ_{222}). % α-helix = [(θ_{222} + 3000)/(36000+3000)] X 100 [17].

Analysis of Hp-Hb binding complex using HPLC

A HPLC experiment was performed to examine whether or not CM-Hp retains its ability to bind Hb. Hb was purified as previously described [16]. A gel-filtration column (TSK-GEL

G2000SWXL, 7.8 x 300 mm) pre-equilibrated in PBS was used to analyze the formation of Hp-Hb complex at a flow rate of 0.5 mL/min. A final reaction mixture (100 μL) containing Hp1-1 (24 μg) or CM Hp 1-1 (24 μg) with and without Hb (16 μg) was subjected for HPLC. Typical chromatographic profiles were monitored at 280 nm, while Hb or Hb-Hp complex was monitored at 415 nm.

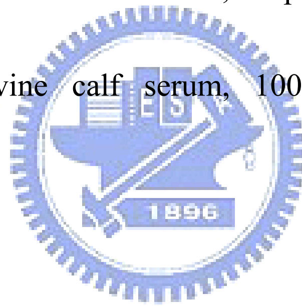
Preparation of Hp 1-1 cDNA

Total RNA was extracted from HepG2 cells using a TRIzol reagent according to the instruction provided by the manufacturer (Gibco BRL, Grand Island, NY, USA). The first-strand cDNA was synthesized using 1 μg of total RNA, 30 ng/μL of oligo dT, 0.25 mM of dNTP, and 3.5 μL of DEPC water in a 40 μL volume. The mixture was incubated at 65℃ for 5 min and chilled on ice. Subsequently, 8 μL of 5 x reaction buffer, 4 μL of 0.1 M DTT, and 2 μL of RNase inhibitor, and 1 μL of M-MLV RTase were added and incubated at 37℃ for 50 min. The reaction was terminated by heating at 70℃ for 15 min. PCR was performed in 50 μL containing 0.1 μg of cDNA product, 5μL of 10x Taq polymerase buffer, 4 μL of 2.5 mM dNTP, 2.5 units of Taq polymerase, and 50 ng of each specific primer for Hp 1-1 cDNA (forward: 5'-TC<u>GGTACC</u>ATGAGTGCCCTGGAAGCTGTCATTG-3'; reverse: 5'-TC<u>GGTACC</u>TTAGTTCTCAGCTATGGTCTTCTG-3'). *Kpn I* restriction site is shown underlined. The thermal cycling program was 96℃ for 30 s, 50℃ for 30 s, and 72℃ for 1 min with 40 cycles. The resulting PCR amplification products were visualized by ethidium

bromide in a 1% agarose gel and then recovered using NucleoSpin Extraction Kit (Clontech, CA, USA). The extracted DNA fragment and plasmid pcDNA3.0 (Invitrogen, Carlsbad, CA, USA) were subsequently digested with *Kpn I* at 37℃ for 2 h and recovered. Finally, the insert fragment and vector were ligated with a molar ratio of 3:1 at 16℃ for 16 h.

Cell cultures

Chinese hamster ovary (CHO-K1) cell line CCL-61 and human hepatoblastoma (HepG2/C3A) CRL-10741 obtained from American Type Culture Collection (Manassas, VA, USA) was cultured in DMEM/F12 and α-MEM medium, respectively, at 37℃. Both media were supplemented with 10% bovine calf serum, 100 U/mL penicillin, and 100 μg/mL streptomycin.



Immunocytochemistry

Immunostaining was performed using VECTOR® M.O.M.™ Immunodetection Kit (Burlingame, CA, USA) with the recommended experimental protocol. Briefly, 10^5 cells were cultured in a 6-well plate placed a 76 x 26 mm glass-slide overnight. After washes in PBS, 4% paraformaldehyde was added to fix cells for 30 min, the slide was then immersed in PBS containing 0.1% Tween-20 for 15 min and washed 4 x with PBS. The slide was then immersed in 3% H_2O_2 followed by 2 x washes. and blocked with M.O.M.™ Mouse Ig Blocking Reagent for 1 h with 2 x washes. Monoclonal anti-human Hp (1:5,000 dilution)

was added and incubated for 30 min. Following washes, it was incubated with biotinylated anti-mouse IgG for 10 min and washed 2 x. VECTASTAIN® ABC reagent was then added, incubated for 5 min, washed, and developed using 3,3'-diaminobenzidine (DAB) as a chromogenic substrate.

Transfection and H_2O_2 tolerance assay

The 1044-bp cDNA of Hp 1-1 obtained from human hepatoblastoma HepG2/C3A cells by RT-PCR was cloned into the pcDNA3.0 vector to generate a CMV promoter-driven Hp 1-1 construct. CHO-K1 cells were transfected by the inserted vector using SAINT-MIX™ Gene/Protein-Delivery System Kit (Groningen, Netherlands), and selected in the presence of 400 μg/mL geneticin (G418). Expression of Hp 1-1 was confirmed by Western blot, immunostaining, and competitive ELISA. About 5 x 10^3 cells were cultured in a 96-well plate for 24 h and treated with variable amount of H_2O_2 (0-5 mM) for another 24 h. MTT assay was then used to estimate the relative survival ratio [19].

Results

Antioxidant activity of Hp molecule

To determine Hp possessing an antioxidant activity, a method using Cu^{2+}-induced LDL oxidation was employed. Fig. 2 shows that Hp was an extremely potent antioxidant against LDL oxidation with a dose-dependent manner: The potency was 5 x greater than that of

probucol [1] and almost 20 x than vitamin E, in which probucol is known as a highly potent antioxidant compound used in clinics. Notably, the antioxidant activity of monomeric Hp 1-1 was moderately higher than that of polymeric Hp 2-1 and 2-2 (Fig. 2).

Effect of disulfide-linkages on Hp antioxidant property

Since the disulfide-linkages provide a provocative role in maintaining the "tetrameric" arrangement of each Hp monomer, we attempted to address whether or not the overall conformation of Hp plays a key role in its antioxidant function. First, we show that carboxymethylated reduction disassembled the α and β chains of Hp on SDS-PAGE without β-mercaptoethanol (Fig. 3). Each chain was identical to Hp subunit in the presence of β-mercaptoethanol reduction [14]. Second, using circular dichroic spectrum analysis, we observed a drastic conformational change of Hp upon the blockage of disulfide linkage. A representative spectrum is shown in Fig. 4. The estimated α-helical content before the carboxymethylation was about 29, 22, and 21% for Hp 1-1, 2-1, and 2-2, respectively. Statistically, the α-helical content in Hp 1-1 was significantly higher than that in Hp 2-1 and 2-2 ($P < 0.001$). The carboxymethylated Hp 1-1 exerted essentially a disordered structure. Such conformational change, however, did not attenuate its antioxidant activity. Unexpectedly, it resulted in a markedly increase in antioxidant activity by about 4 x (Fig. 2). Taking together, the antioxidant potency expressed as IC_{50} in ranking was: CM Hp1-1 > CM Hp 2-1 > CM Hp 2-2 > Hp 1-1 > Hp 2-1 > Hp 2-2 > probucol > vitamin E (Table 1). Bovine

serum albumin, however, did not exhibit any antioxidant activity (Data not shown).

AAPH-induced LDL oxidation

In the next experiment, we tested its ability for scavenging free radicals generated from a water-soluble azo-compound AAPH. The tested antioxidant activity of Hp 2-1 was shown as a dose-dependent fashion with an IC_{50} about 5 x greater than that of probucol (Table 1).

Hemoglobin-binding ability of carboxymethylated Hp

Hp binds Hb forming a high affinity Hp-Hb complex [6]. Presumably, the binding domain of Hp to Hb is dependent on the overall three-dimensional structure of Hp. We examined the effect of carboxymethylation of Hp on the formation of Hp-Hb complex using a HPLC technique. Fig. 5 shows that the binding was totally abolished when Hp was carboxymethylated. Since the antioxidant activity of carboxymethylated Hp was superior to native Hp (Fig. 2), the data indicate that the antioxidant nature of Hp was independent on its binding ability to Hb.

Expression of Hp 1-1 in CHO-K1 cells

To create an "antioxidant" cell that may prevent oxidative stress, a CHO-K1 cell line was transfected with a pcDNA3.0 vector containing CMV promoter-driven Hp 1-1 cDNA. The plasmid construct also contained a selection marker, geneticin, for conditionally expressing the Hp 1-1. RT-PCR shows that the un-transfected cells did not express endogenous Hp

mRNA (Fig. 6). Expression of Hp 1-1 protein in the culture medium and CHO cells was confirmed by a Western blot analysis (Fig. 7). Only the cell lysate from transfected cells revealed Hp immunoreactivity in a competitive ELISA (data not shown). The specificity of Hp expression in transfected cells was also confirmed by an immunocytochemical staining (Fig. 8). The non-immuned serum did not give an immunostaining on transfected cells (Data not shown).

Activity of Hp transfected CHO cells against oxidative stress

To explore the "antioxidant" cells created above may resist the oxidative stress, hydrogen peroxide (H_2O_2) was added to the CHO-K1 cell culture. Cells with and without Hp 1-1transfection were treated with variable dosages of H_2O_2 for 24 h. As shown in Fig. 9, upon the challenge of H_2O_2 the relative cell survival ratios of transfected CHO-K1 cells were statistically and significantly higher ($P<0.001$) than that of untransfected CHO-K1 cells, especially in the presence of 1 mM H_2O_2. Thus, the expression of Hp elevated the cell tolerance against the H_2O_2-induced oxidative stress.

Discussion

Recently, it has been demonstrated that Hp can prevent Hb-induced oxidative tissue damage by virtue of its ability to form a high-affinity complex with Hb [7]. It further indicates that the Hp 1-1 is superior to Hp 2-1 and 2-2 in binding to Hb [7]. As such, Hp is able to

attenuate the release of heme from the Hb. In addition to this mode of action of Hp mentioned, we demonstrate that Hp itself was an extremely potent antioxidant molecule in Cu^{2+}- and AAPH-induced LDL oxidation (Fig. 2 and Table 1). The rationale to choose Cu^{2+}, rather than Fe^{3+}, as a free radical initiator was both of them forming identical hydroxyl radicals in Fenton reaction [1, 20]. The same experimental condition previously reported by us [1, 21-24] could be mimicked to compare with the antioxidant compound probucol. In fact, a similar result was seen by using Fe^{3+} [25]. Furthermore, using a hydrophilic decomposed radical initiator, AAPH, produced a similar antioxidant effect using Hp 2-1 (Table 1). Essentially, the potency of Hp significantly exceeded to probucol [1], which has been used for the treatment in patients with xanthoma and atherosclerosis in the last decades [26-30]. Hypothetically, the concentrations of Hp in the cellular level may also play a key role as a natural antioxidant in protection of atherosclerosis.

It is not clear, however, why Hp 1-1 possessed antioxidant activity differentially and moderately greater than Hp 2-1 and 2-2. Evidently, it was not totally due to the polymeric forms of Hp 2-1 and 2-1, since the dissociation of polymeric forms by chemical modification (carboxymethylation) (Fig. 3) did not "equalize" the antioxidant activity among the phenotypes (Fig. 2). On the contrary, the drastic conformational changes in CM Hp (Fig. 4) resulted in even enhanced potency by 4 x (Fig. 2). We speculate that the region(s) exerting

the antioxidant activity were further exposed following the structural unfolding of Hp. Because the difference among the Hp phenotypes lies on the amino-acid sequence of α-chains, it would be essential to analyze the antioxidant domain in this region that may lead to a rational design in intervening new pharmacological agents [1, 21-23]. We are currently in progress to explore such possibility.

The diversity of antioxidant activity may explain, in part, the clinical outcome by which Hp phenotype is associated with differential susceptibility to free-radical related atherosclerosis and autoimmune disorders [5, 31]. The correlation between the phenotype-dependent modulation of oxidative stress and prostaglandin synthesis has been reported [31-34]. In general, patients with Hp 2-2 are more susceptible in developing the severity of nephropathy in diabetes mellitus. Nakhoul *et al.* [12] postulate that the differences in the molecular shape and size between the Hp 1-1 and 2-2 are involved.

Abundant evidence showing that Hp can be synthesized in the liver, lung, and some fibroblast cells [35], we have recently reported that it can also be endogenously synthesized in macrophages [36]. However, the functions of Hp on the cellular level, other than stimulating angiogenin and remodeling arterial wall [37-39], have not been fully explored. To ascertain the cellular Hp could prevent cell damage from oxidative stress, we show Hp cDNA transfected CHO-K1 cells exerting the ability in resisting the oxidative damage (Fig. 9). The

data further substantiate our hypothesis that Hp plays a provocative antioxidant role.

In conclusion, we demonstrate that Hp was an extremely potent antioxidant molecule and have identified its antioxidant role in cell model. Blockage of disulfide linkages of Hp resulted in a loss of its ability to form a complex with Hb and yet exerted almost 4 x greater antioxidant activity than that native Hp. Thus, in addition to its role in forming Hp-Hb complex to block the heme group, Hp may participate in an independent antioxidant role for those cells expressing Hp. Whether or not Hp may be directly beneficial for free-radical associated atherosclerosis and myocardial infarction [29, 31 and 40] will be a subject of interest and challenge.

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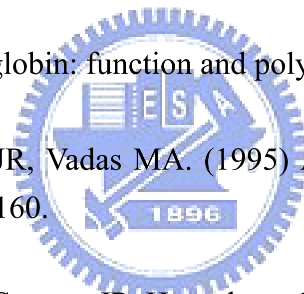


Figure Legends

Figure 1. Schematic drawing of proposed structure of human Hp 1-1, 2-1, and 2-2. All three phenotypes share a common structure of β chains, while Hp 2-1 and 2-2 are heterogeneous polymeric forms. Essentially, α2 chain represents one entire copy of α1 (1-83) plus an insertion of partial sequence of α1 (residues 12-70). The degree of polymerization within the inter-molecular arrangement is shown. Due to an extra thio group in β-chain, only Hp 2-1 and 2-2 form large polymers (please refer to Fig. 3).

Figure 2. Antioxidant activity of Hp phenotypes. The assay was evaluated using the degree of inhibition of Cu^{2+}-induced formation of thiobarbituric acid-reactive substances (TBARS) from LDL. LDL (40 μg protein) was incubated with 4 μM Cu^{2+} in the presence of Hp, carboxymethylated (CM) Hp, probucol, or vitamin E at 37℃ for 2 h with a final volume of 100 μL in PBS. Bovine serum albumin (10-100 μM) did not reveal any antioxidant activity (Data not shown).

Figure 3. Analysis of carboxymethylated Hp on 15% SDS-PAGE without reducing reagent β-mercaptoethanol. Lane M: protein marker. It demonstrates that α and β subunits are covalently linked in native Hp, but not in carboxymethylated (CM) Hp 1-1, 2-1, and 2-2 following the chemical modification.

Figure 4. Circular dichroic spectra of native Hp 1-1, 2-1, 2-2, and carboxymethylated (CM) Hp 1-1. Hp in 10 mM phosphate buffer, pH 7.4, at a final concentration of 0.2 mg/ml was monitored by a circular dichroism. Each spectrum represents a mean of 20 x determinations. The estimated α-helical content is about 29, 22, and 21% for Hp 1-1, 2-1, and 2-2, respectively. While, the structure of CM Hp 1-1 is disordered. Statistically, the α-helical content in Hp 1-1 is significantly higher than that in Hp 2-1, 2-2, and CM Hp 1-1 ($P < 0.001$).

Figure 5. HPLC profile of hemoglobin (Hb) complexed with native and carboxymethylated (CM) Hp 1-1. A HPLC gel-filtration column (TSK-GEL G2000SWXL, 7.8 x 300 mm) was used to analyze the formation of Hp-Hb complex. A final reaction mixture in 100 μL of PBS containing Hp1-1 (24 μg), CM Hp 1-1 (24 μg), or with and without Hb (16 μg) was subjected for HPLC. Hb was also monitored at 415 nm in addition to 280 nm.

Figure 6. Expression of Hp α1-β mRNA in transfected CHO-K1 cells. PCR was performed to amplify the Hp α1-β cDNA. Lane 1: 100 bp ladder; Lane 2: HepG2 control (Hp α1-β cDNA, 1044 bp); Lane 3: CHO-K1 cells; Lane 4: transfected CHO-K1 cells (Hp α1-β cDNA, 1044 bp); Lane 5: HepG2 internal standard (β-actin, 838 bp); Lanes 6 and 7: CHO-K1 internal standard (GAPDH fragment, 120 bp). The PCR products were analyzed on a 1% agarose gel.

Figure 7. Western blot of Hp expression in culture medium of transfected CHO-K1 cells. CHO-K1 cells were transfected with the pcDNA3.0 vector containing CMV promoter-driven Hp α1-β cDNA and cultured in 400 μg/ml of geneticin (G418), a selection marker. Transfected CHO-K1 cells exhibited a stable expression over at least 10 passages during more than one month of culture. The collected culture media were concentrated by 40-fold, followed by a 15% SDS-PAGE and Western blot analysis. Lane 1: protein marker. Lane 2: Hp 2-1 standard containing α1, α2, and β subunits. Lanes 3-4: culture media of untransfected (Lane 3) and transfected CHO-K1 (Lane 4) cells.

Figure 8. Immunochemical staining of Hp expressed in untransfected (Top) and transfected (Bottom) CHO-K1 cells. DAB was used for the chromogenic substrate and hematoxylin was employed for the nucleus staining. Distribution of Hp (Brown) and nucleus (Blue) were observed. The staining was negative when non-immuned antiserum was used (Data not shown).

Figure 9. Oxidative stress on CHO-K1 cells in the presence of H_2O_2. About 5 x 10^3 cells were cultured in a 96-well plate for 24 h, followed by an additional 24-h treatment with variable amount of H_2O_2 (0-5 mM). MTT assay was then used to estimate the relative survival ratio. Each bar represents a mean ± SD in seven determinations. * $P < 0.001$

Fig. 1

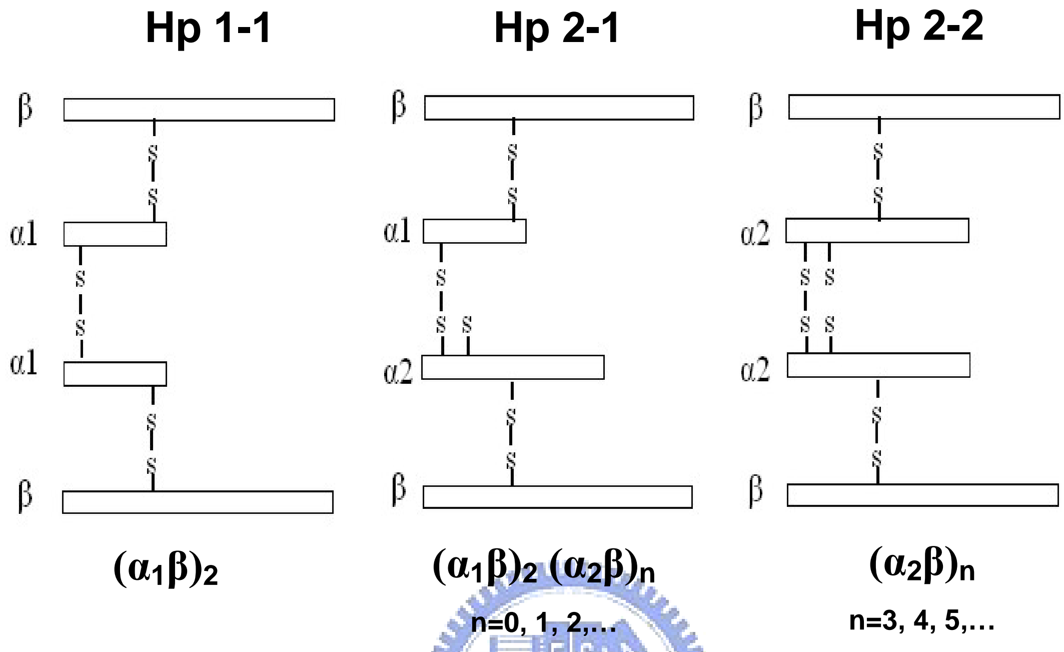
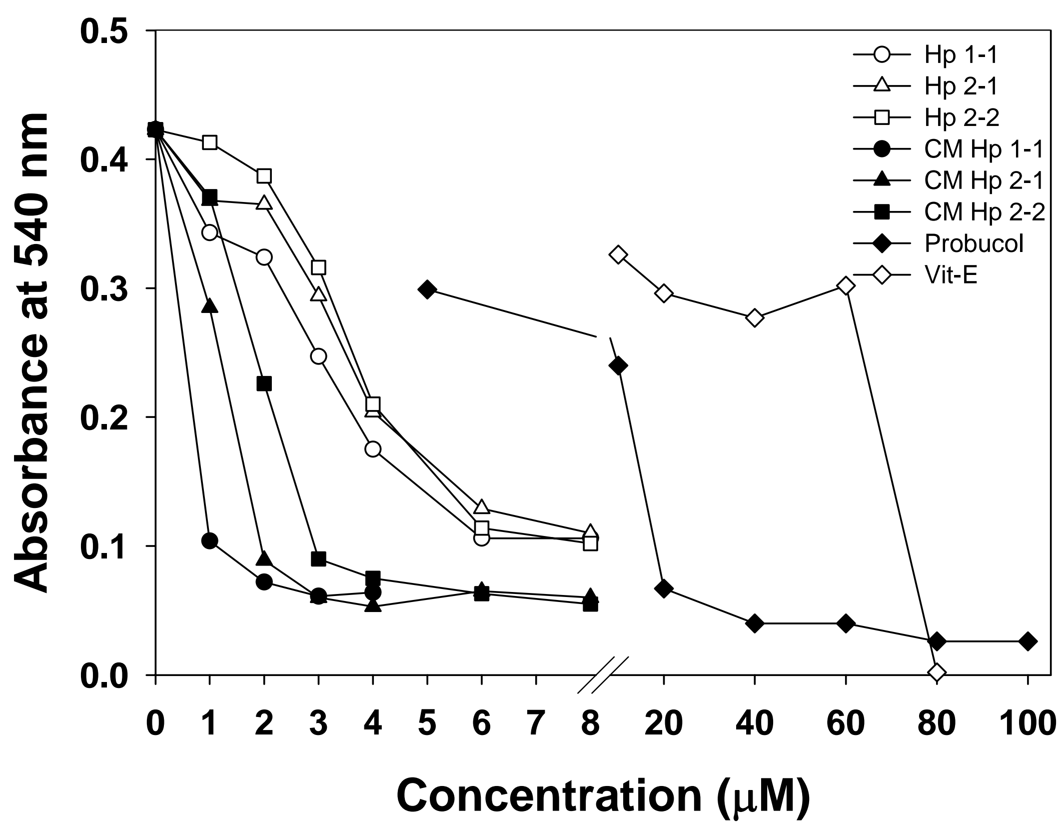


Fig. 2



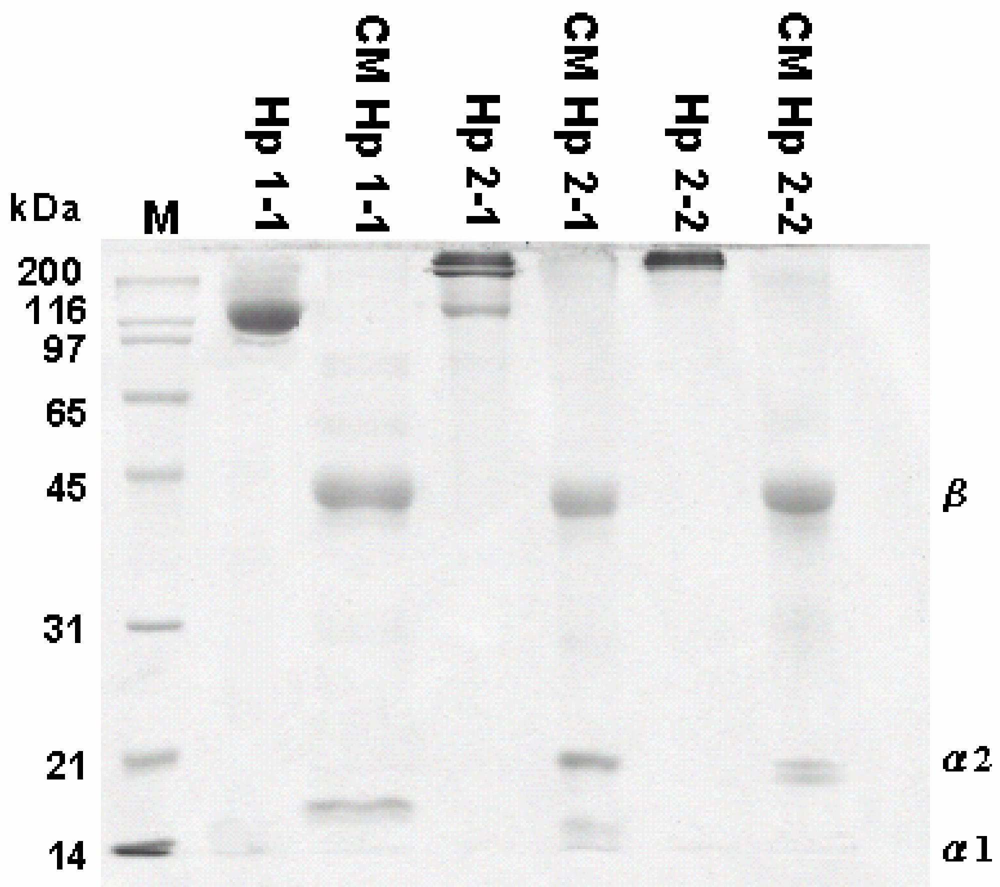


Fig. 3
kDa
M
Hp 1-1
CM Hp 1-1
Hp 2-1
CM Hp 2-1
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CM Hp 2-2
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97
65
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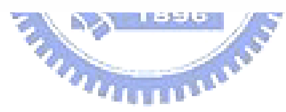
31
21
14
β
α2
α1



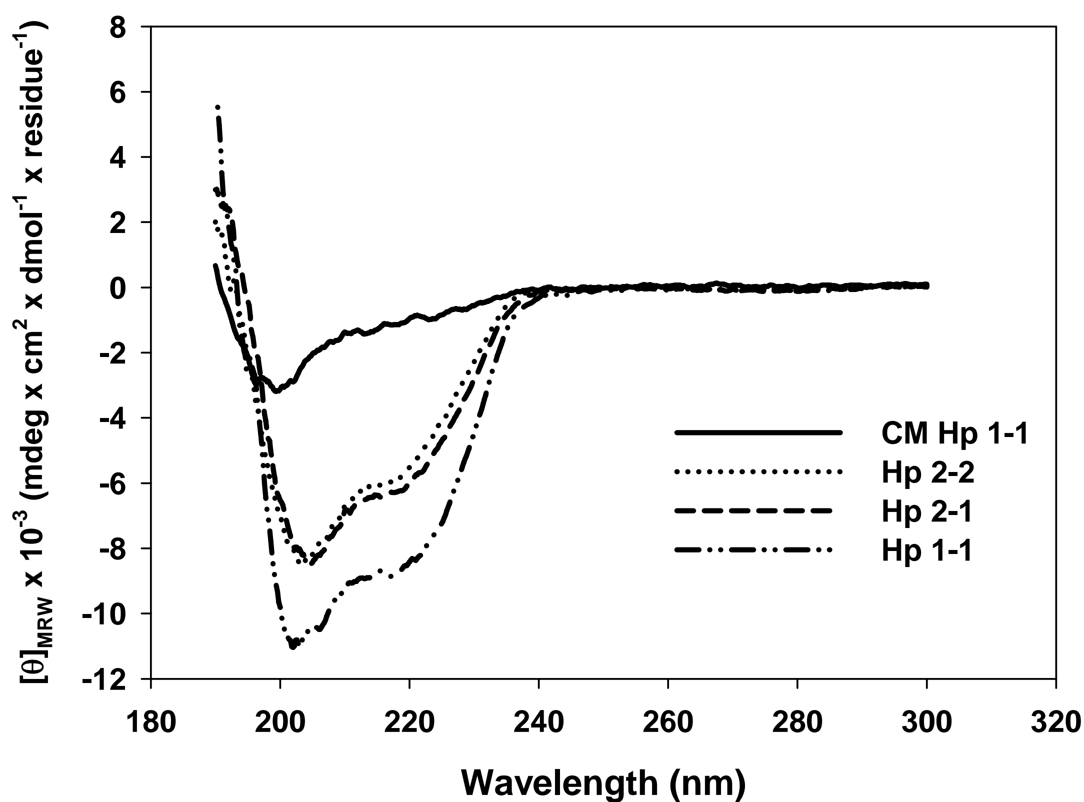


Fig. 4
$[\theta]_{MRW}$ x 10^{-3} (mdeg x cm^2 x $dmol^{-1}$ x $residue^{-1}$)
8
6
4
2
0
-2
-4
-6
-8
-10
-12
180
200
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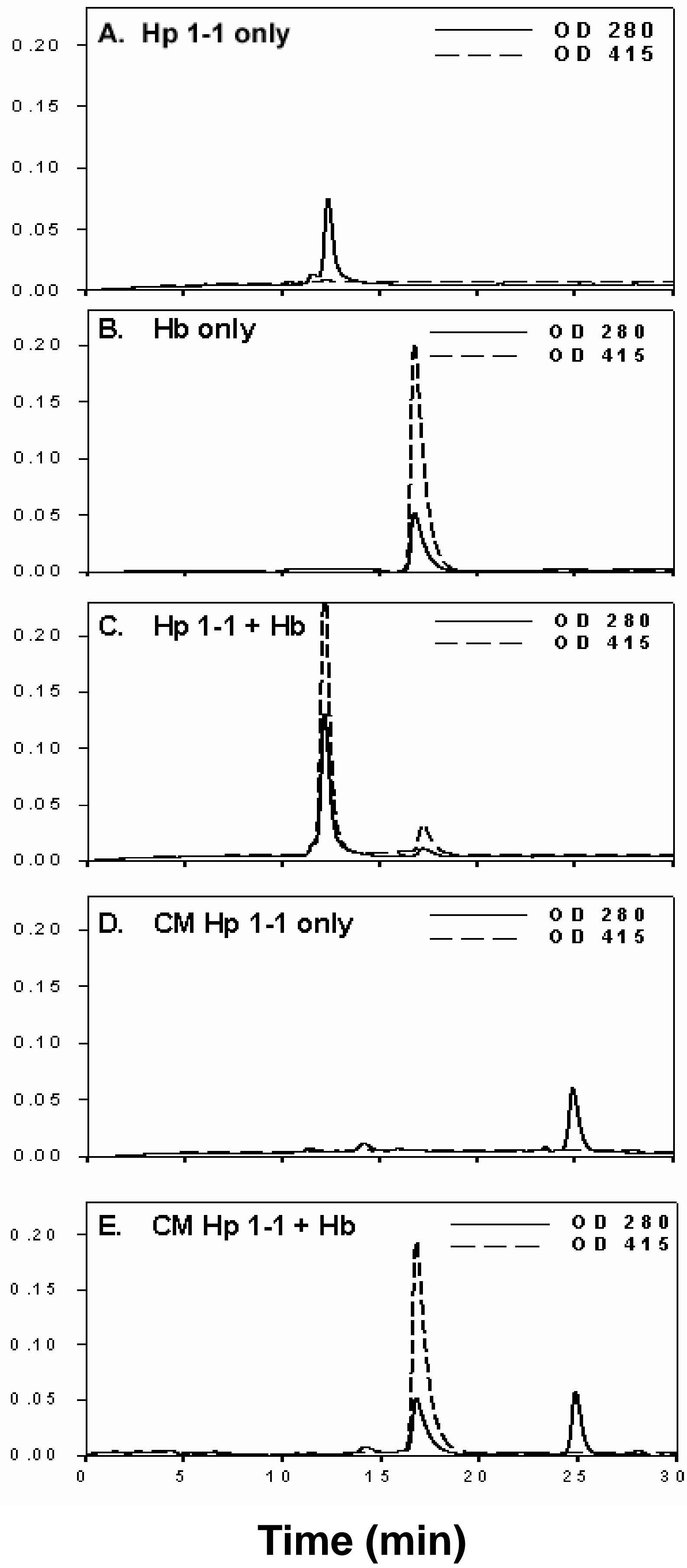
240
260
280
300
320
Wavelength (nm)
CM Hp 1-1
Hp 2-2
Hp 2-1
Hp 1-1


A. Hp 1-1 only
B. Hb only
C. Hp 1-1 + Hb
D. CM Hp 1-1 only
E. CM Hp 1-1 + Hb
OD 280
OD 415
Absorbance
Time (min)
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0.05
0.10
0.15
0.20
0
5
10
15
20
25
30

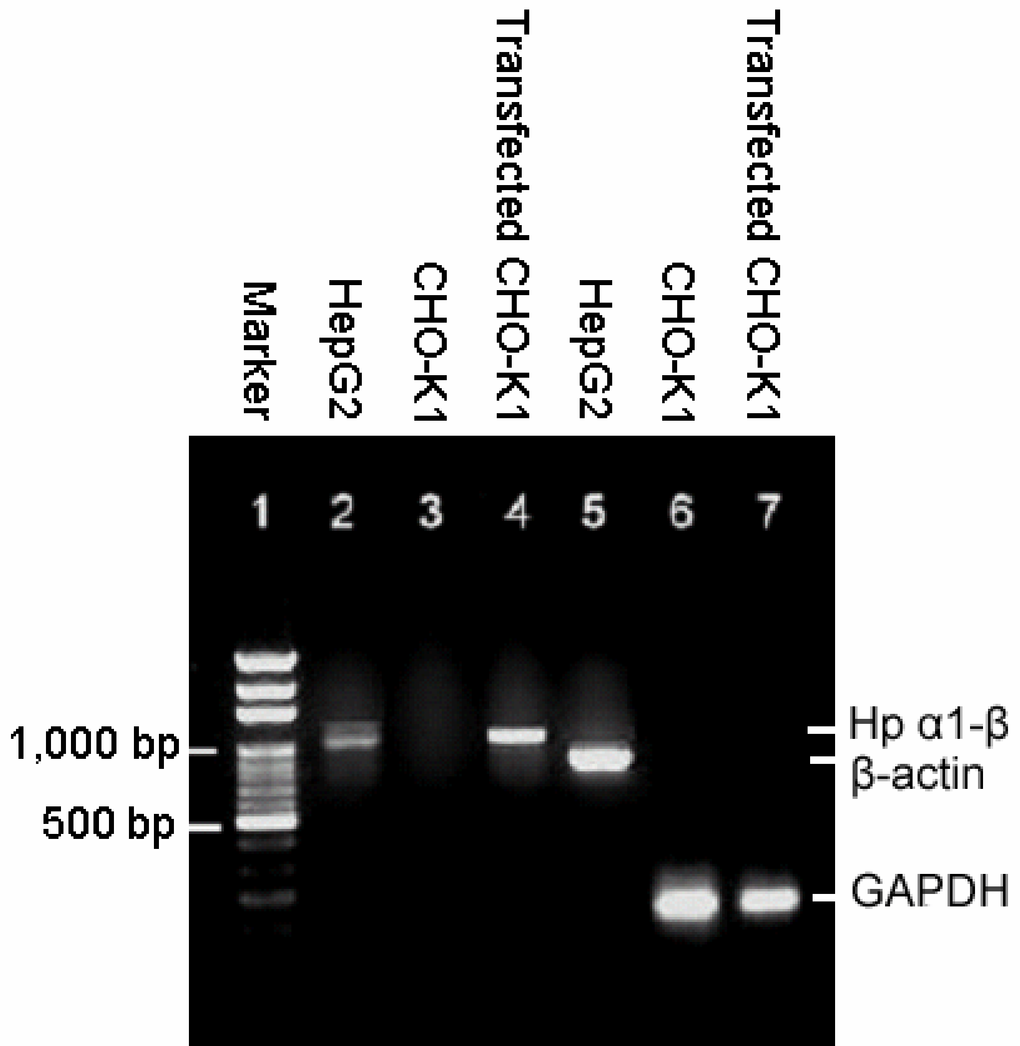


Fig. 6

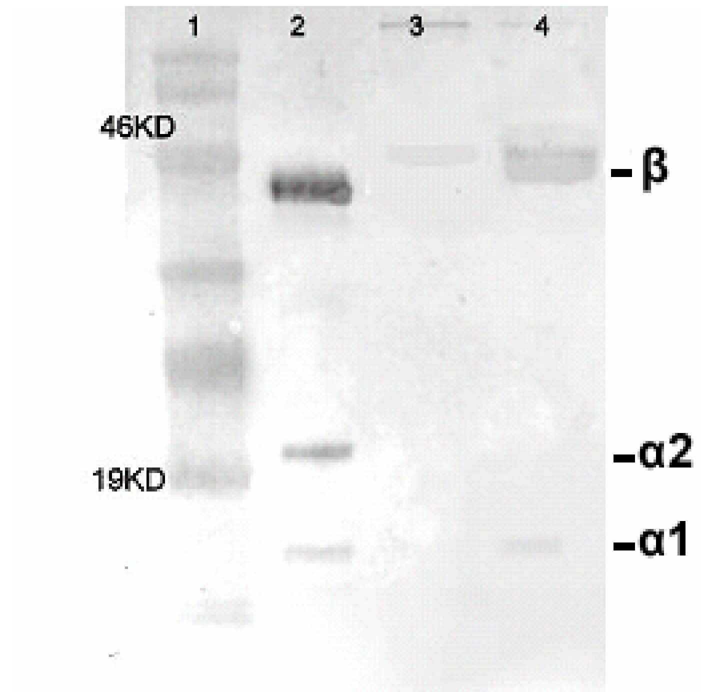
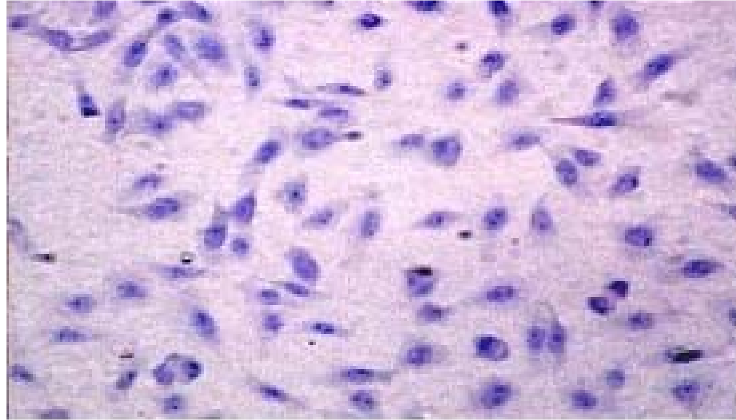


Fig. 7

Fig. 8



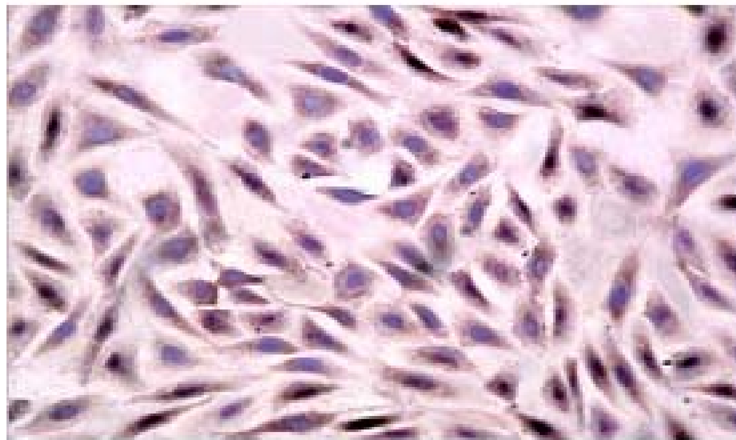


Fig. 9

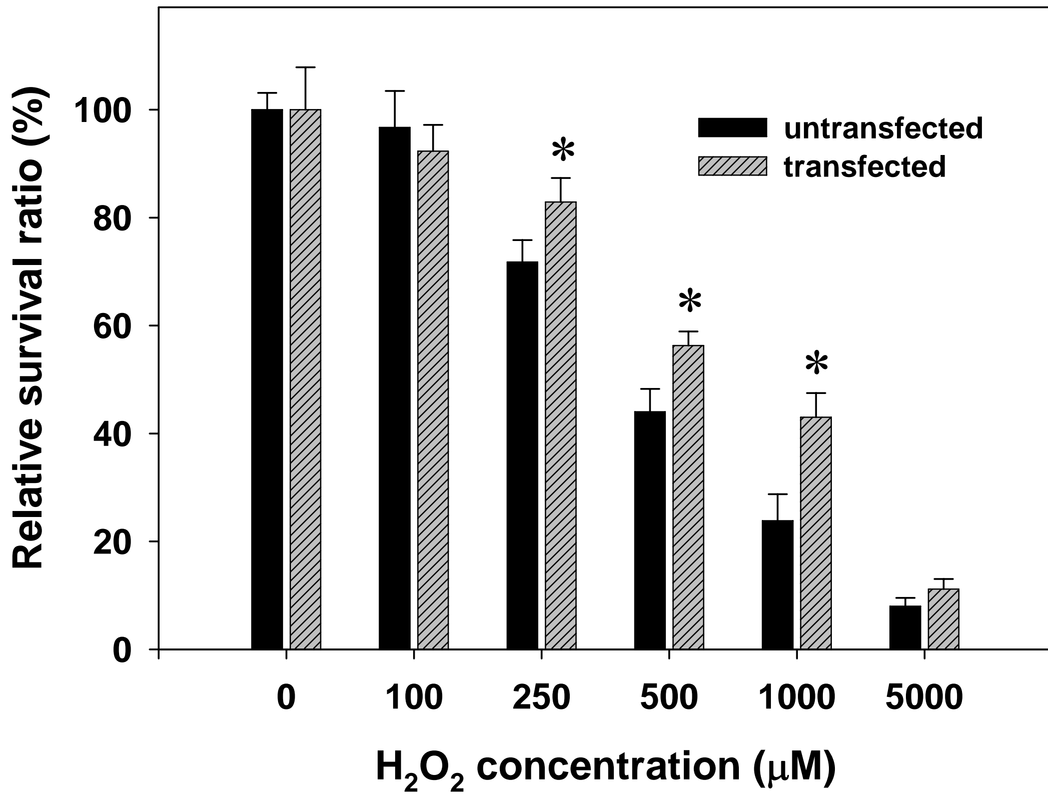


Table 1. Antioxidant activity in Cu^{2+}- and AAPH- induced LDL lipid peroxidation.

	Cu^{2+}-induced *IC_{50} (μM)	AAPH-induced *IC_{50} (μM)
CM Hp 1-1	0.8	nd
CM Hp 2-1	1.5	nd
CM Hp 2-2	2.2	nd
Hp 1-1	3.0	nd
Hp 2-1	3.5	6
Hp 2-2	3.7	nd
Probucol	15	30
Vitamin E	70	nd

nd: not determined; *IC_{50}: the concentration that inhibited 50% of LDL oxidation. See the legend of Fig. 2 for more details.

Section 3: Analysis of antioxidant as a therapeutic agent for atherosclerosis

Abstract:

Research into the oxidation of lipoprotein has yielded many insights into the underlying process of the development of atherosclerosis. Oxidative modification of low density lipoprotein (LDL) has been suggested as an initial step in the pathogenesis of atherosclerosis. However, up until now, investigations of antioxidants have mostly focused on three main dietary antioxidant vitamins (β-carotene, vitamin C, and vitamin E) and some synthetic compounds. Among those antioxidants, probucol, a synthetic compound, has been shown to be an extremely potent and effective antioxidant in preventing the formation of atherosclerosis in both *in vitro* and *in vivo* studies. The present review focuses on commonly used analytical methods for measuring the antioxidant potency and outlines the critical steps on how to evaluate and design a potent antioxidant agent that can be used for the intervention of atherosclerosis. We concluded that an antioxidant should first be targeted and incorporated into human LDL. Second, the candidate compound should possess high bioavailability. The rationale and strategy for the analytical procedures are discussed in this report.

Key Words: Antioxidant therapy, Antioxidant analysis, Atherosclerosis, Low density lipoprotein, Free radical, Pharmaceutical agent.

1. INTRODUCTION

Atherosclerosis and its complications are the major causes of mortality in industrialized countries [1-3]. Many theories of the cause of atherosclerosis have been proposed in the last decades [4-6]. Several environmental and physiological factors, such as diabetes, smoking, lack of exercise, increased low density lipoprotein (LDL), and reduced apolipoprotein A-I of high density lipoprotein (HDL) [7-12], elevate the risk of atherogenesis. It is well accepted that elevated LDL-cholesterol is a major risk factor for the development of coronary heart disease (CHD), and lipid-lowering agents (such as simvastatin or pravastatin) can reversely attenuate the atherosclerosis [13,14]. Although these agents are effective, it should be noted that approximately one-half of CHD patients have relatively normal cholesterol values [15]. For this reason an alternative therapeutic approach should be considered in addition to the lipid lowering.

1.1 Oxidation hypothesis of atherosclerosis

Since Steinberg proposed his oxidatively modified LDL hypothesis as one of the major causes of atherosclerosis in 1989 [6], there have been continuing evidence of lipid oxidation playing a central role in atherogenesis [16,17]. As shown in Fig. **1**, the infiltrated LDL is oxidized in the arterial wall and taken up by the scavenge receptors of macrophage which then trigger the biochemical and pathological changes in the artery wall [18]. The uptake of

oxidized LDL results in accumulation of large quantities of cholesterol esters or foam cells in the subendothelial space of the arterial walls leading to the formation of fatty streaks, the earliest event of atherogenesis [6,19,20].

1.2 Atherogenesis induced by oxidized LDL

LDL are detergent-like particles which transport cholesterol, cholesterol esters, and other neutral lipids in the blood. Their outer hydrophilic shell is composed mainly of phospholipids, free cholesterol, and proteins (primarily apoB-100), while their lipophilic core contains cholesterol esters and triglycerides [21,22]. During the oxidation of LDL (initiated by free radicals), polyunsaturated fatty esters of lipids are transformed into reactive lipid peroxides, many of which attach to the apolipoprotein B (apoB) [23].

A free radical is defined as any species that contains one or more unpaired electrons, the unpaired electron being one that is alone in an orbital. It donates the unpaired electron to another molecule or takes an electron from other molecules, leading to a chain reaction with damage to adjacent biological molecules (free radical propagation) [24]. Little is known about pathways of LDL oxidation *in vivo*. To date, studies on several candidates for the initiation of LDL oxidation have been carried out *in vitro* or *in vivo*. For example, transition metal ions such as copper and iron can initiate LDL oxidation using mostly cholesterol esters and phospholipids as a lipid source. In addition, these transition metals can also indirectly

modify the lysine groups of apoB in LDL or break up apoB into fragments leading to the recognition of the scavenge receptors of macrophage. Another example is that the bound metal ions of heme protein and ceruloplasmin are capable of initiating LDL oxidation by decomposition of hydroperoxides [25]. The endothelial cell, macrophage, neutrophil, and smooth muscle cell (the majors cell types found in arterial walls) can also provide endogenous superoxide ions and nitric oxide (NO·); the latter free radical promotes the production of peroxynitrite anion ($ONOO^-$) *in vivo*. The conjugated acid of peroxynitrite, peroxynitrous (ONOOH), and its decomposition products (HO· and nitrogen dioxide) further initiate lipid peroxidation without the requirement of transition metals [26,27]. However, the production of NO· from cytokine-stimulated macrophage can inhibit the oxidative modification of LDL [28]. Some oxidized phospholipids of LDL are generated by potent oxidants via the lipoxygenase and myeloperoxidase pathways [17]. Lipoxygenase, which is highly expressed in macrophage, plays an essential role in the oxidation of circulating LDL. The enzyme catalyzes the oxidation of unsaturated fatty acids to hydroperoxides and other bioactive metabolites utilizing a non-heme iron active site [29]. Myeloperoxidase (MPO), an abundant heme enzyme released by activated phagocyte, also catalyzes the formation of a number of reactive species that subsequently modify LDL [30].

These oxidized LDL can then stimulate the endothelial cell and macrophage to induce the production of several kinds of cytokines such as interleukin-1 (IL-1), interleukin-10

(IL-10), monocyte chemotactic protein-1 (MCP-1), and macrophage colony-stimulated factor (MCSF) [25,31,32]. Some of these factors are even able to induce the expression of adhesion molecules on the surface of the endothelial cell and monocyte leading to up-regulated scavenger receptors, which further results in an uncontrolled uptake of oxidized LDL. The presence of these cytokines and oxidized LDL triggers the proliferation of smooth muscle cell of intima resulting in increased thickening of the artery walls [25]. The early event of intimal thickening (Fig. **1**) and the late formation of atherosclerotic plaques eventually lead to the potential fatal clinical episodes of atherosclerosis such as thrombosis and plaque rupture [15,18,25,33].

2. RECENT ANTIOXIDANT THERAPIES FOR ATHEROSCLEROSIS

Since LDL oxidation plays a causative role in the formation of atherosclerosis, a number of treatments and preventive approaches are possible. Rather than attempting to alter lipid composition, an alternative approach is to introduce agents which protect LDL against oxidation. There are so many candidates for the inhibition of LDL oxidation that it is difficult to determine which compounds should be focused on. On the other hand, the total concentrations of endogenous antioxidants in plasma, including vitamin C (ascorbate), vitamin E (α-tocopherol), β-carotene, glutathione, ubiquinol-10 (the reduced form of coenzyme Q10), bilirubin, haptoglobin, and others, are already greater than 100 μM. We

speculate that the sum activity of these natural antioxidants is sufficient to counteract the increase in free radicals present in plasma [34]. For example, there is almost no oxidized LDL that can be found in the human plasma according to our experience [35] or very trace amount of it if so desired to find it [36]. It is doubtful such trace amount of oxidized LDL in circulation may lead to a significant uptake by macrophages in the early event of atherosclerosis. Therefore, direct incorporation of an antioxidant into LDL particles might be a better local treatment approach for atherosclerosis. A lot of experimental data obtained from *in vitro* indicates that vitamins E and C are important antioxidants that exert the ability to inhibit LDL oxidation [37,38]. They have been overwhelmingly proposed to prevent the initiation of atherosclerosis. So far however, *in vivo* studies using vitamins do not reveal an obvious reduction in atherosclerosis. In overall results of randomized human trials using vitamin E alone or vitamin cocktails, they do not effectively retard the formation of atherosclerosis [39-44]. The reasons for such failure in antioxidant treatment have several possibilities [45,46] and are discussed in the subsequent section. Nevertheless, the failure of vitamin supplements for the suppression of atherosclerosis is not sufficient to completely controvert the approach using antioxidant as a therapeutic agent, since such strategies using strong antioxidant probucol have been supported by numerous experimental data obtained from *in vitro* and animal models [35,47-52].

3. POTENTIAL ANTIOXIDANTS AS ANTIATHEROSCLEROTIC AGENTS

Antioxidants may exhibit effect by different functions, such as suppressing the formation of active species by reducing hydrogen peroxides and by sequestering metal-ion induced free radicals. It is worth mentioning that an antioxidant must sacrifice itself first during scavenging of the free radicals. In some cases, it forms a prooxidant and is able to be regenerated in the presence of other antioxidant or re-activated by defense enzymes [53]. A typical example for vitamin E is shown in Fig. **2**. The structures and chemical reaction with free radicals of some typical antioxidants are presented in Figs. **3** and **4**. Their antioxidant properties derived from the *in vitro* or *in vivo* studies are discussed as follows:

3.1 Vitamin E

Vitamin E (α-tocopherol) is typically a lipid-soluble antioxidant in cell membranes serving as a chain-breaking antioxidant [54]. In North America, the concentration of α-tocopherol in human plasma is about 20.5 ± 6.6 μmol/L [55], which is equally associated with LDL and HDL particles [56]. Each LDL particle contains about five to nine vitamin E molecules [57]. Vitamin E protects against chain propagation of lipid peroxidation by acting directly on a variety of oxygen radicals, including singlet oxygen, alkoxy radicals (LO·), lipid peroxyl radicals (LOO·), alkyl radicals (L·), and the superoxide radicals [47]. In LDL, vitamin E reacts poorly with reactive nitrogen species (RNS) and does not appear to protect

lipoprotein against hypochlorous acid or tyrosyl radicals, most likely because these radicals are targeting to apoB [58]. Long-term oral ingestion of vitamins C and E does not improve the key mechanisms involved in the pathogenesis of atherosclerosis such as endothelial dysfunction and LDL oxidation [42]. The most recent clinical trials reveal that vitamin E fails to protect heart disease [59-62]. In patients with vascular disease or diabetes mellitus, long-term vitamin E supplementation does not prevent cancer or major cardiovascular events and even increased the risk for heart failure [59].

The failure of α-tocopherol in these trials is not yet clear. Interestingly, overdose of vitamin E produces negative effect on the intervention of coronary artery disease [63]. In our view, there are three key points need to be considered. First, it is probably due to the short half-life of vitamin E with only about 4.4 hours [64]. Second, the more polar phenolic rings of vitamin E are arranged toward the surface of the LDL, while the isoprenoid side chain toward the core [47]. Because of the bulky nature of the chromanol group, it could sterically retard the insertion of its side chain into LDL. As such it does not penetrate deeply enough to effectively block the lipid peroxidation, which takes place inside the hydrophobic lipid core of LDL. Third, vitamin E donates a hydrogen ion to a radical to form a consequent tocopheroxyl radical (or prooxidant) (Fig. **2**) [65]. The tocopheroxyl radical may further participate in lipid peroxidation. Although such radicals in theory can be regenerated by vitamin C, glutathione or coenzyme Q10 (Fig. **2**) [66,67], the regeneration system is rate

dependent. The rate is limited by the concentration of other antioxidants or enzymes. Lacking sufficient vitamin C, for example, may immediately cease the regeneration or it may not be taken place in the LDL present in the arterial wall.

3.2 Vitamin C

Vitamin C (ascorbate) is water-soluble and can directly react with superoxide, hydroxyl radicals and singlet oxygen. It acts as the first defending line against oxidative stress in human bodies [68,69]. Most animals and humans lack the ability to synthesize ascorbate due to the lack of L-gulono-1,4-lactone oxidoreductase, the last enzyme required for ascorbate biosynthesis [66]. The concentration of vitamin C in human plasma is about 70 μmol/L [70,71], which is solely dependent on dietary intake. Once consumed, oxidation of ascorbate produces the short-lived radical monodehydroascorbate (MDHA), which is converted to ascorbate by MDHA reductase (MDHAR) or non-enzymically disproportionates to ascorbate and dehydroascorbate (DHA). DHA is recycled to ascorbate by dehydroascorbate reductase (DHAR), which uses glutathione (GSH) as a reductant [66,72]. Vitamin C is also important for participating in the regeneration of vitamin E with the mechanism depicted in Fig. **2**. Therefore, supplementation of vitamin C combined with vitamin E could be beneficial in atherosclerosis, at least in theory. However, clinical human trials do not reveal strong evidence for supporting such hypotheses. In addition, long-term supplementation of higher

dosage of vitamin C (greater than 1 gram per day) is accompanied with a number of side effects such as kidney stones and uricosuria [43,73].

3.3 β-Carotene

β-Carotene, a major carotenoid precursor of vitamin A, is a scavenger of singlet oxygen and peroxyl radicals [74]. The chemical structure of β-carotene is depicted in Fig. **3**. In plasma, the concentration is about 0.294 ± 0.241 μmol/L [75]. The quenching involves a physical reaction in which the energy of the excited oxygen is transferred to the carotene, forming an excited state molecule. The exact mechanism of β-carotene's antioxidant activity is not yet clearly understood. Some, but not all, studies showed a difference for *in vitro* activities of the β-carotene isomers. One study indicated that 9-*cis* β-carotene, a naturally occurring form of β-carotene, protected methyl linoleate from oxidation more efficiently than all-*trans* β-carotene [76]. However, another study demonstrated that 9-*cis* and all-*trans* β-carotene had equal antioxidant activities when assessed by enhanced human neutrophil chemiluminescence [77]. Such discrepancy might be due to the method of analysis. β-carotene can enter peripheral lymphocyte and attenuates DNA damage [78]. It improves the cell viability of hepatocyte and increases catalase activities and glutathione levels in hepatocyte from chronically ethanol-fed rats implicating that β-carotene is able to reduce the oxidative stress induced by chronic ethanol intake [79].

In a 6-year post α-tocopherol and β-carotene cancer prevention (ATBC) trial [61], 29,133 male smokers aged 50-69 years were randomized to receive α-tocopherol 50 mg, β-carotene 20 mg, both, or a placebo daily for 5-8 years for evaluating the benefit of CHD. β-Carotene seemed to increase the post-trial risk of first-ever non-fatal myocardial infarction. Although there is no plausible mechanism to explain it, the finding does not advocate the use of α-tocopherol or β-carotene supplements in prevention of CHD among male smokers. As mentioned above, the β-carotene itself forms an excited state molecule upon the quenching singlet oxygen and peroxyl radicals; it may serve as a prooxidant as that found in the vitamin E clinical trial [61]

3.4 Glutathione

Glutathione (GSH) plays a central role in the cellular defense against oxidative damage. A tripeptide- nature glutathione (L-gamma-glutamyl-L-cysteinyl-glycine) can spontaneously, or with the help of peroxidase, deliver the H necessary for the reduction of free radicals. It also serves as a co-substrate in numerous enzymic reactions catalyzed by glutathione peroxidase, an enzyme that functions to remove hydrogen peroxide [80]. In the presence of oxidant species (such as singlet oxygen, hydroxyl radical, superoxide radical, lipid hydroperoxide, peroxynitrite, and cytotoxic hydrogen peroxide), GSH can be oxidized by glutathione peroxidase to produce oxidized glutathione dimer (GSSG) [81]. Both GSH and

GSSG maintain a redox balance in the cellular environment, and the ratio serves as an index of oxidative stress. Therefore, oxidation of a trace amount of GSH to GSSG can dramatically change this ratio and affect the redox status within the cell. Under moderate oxidative stress, thiol groups of intracellular proteins are modified by the reversible formation of mixed disulfides between protein thiols and low molecular mass thiols such as GSH in a process known as S-glutathionylation [82]. The active element of GSH, thiol group of cysteine, is oxidized to cystine (or cysteine disulfide) when performing its antioxidant activity. As shown in Fig. **2**, another central role of GSH in antioxidant defense is its ability to regenerate vitamin C via the ascorbate-glutathione cycle [83,84]. GSH is present abundantly in red blood cells capable of inhibiting hemin-induced hemolysis or heme-induced lipid peroxidation based on its ability to bind and degrade hemin or heme [85,86]. In lipid-fed rabbits, plasma levels of GSH are found to be low suggesting that there is a relationship between atherosclerosis and GSH [87]. Due to the bioavailability, GSH has never been given *in vivo* to test its antiatherogenic activity thus far.

3.5 Coenzyme Q10

Coenzyme Q10 is an amphipathic (biphasic) molecule due to the hydrophilic benzoquinone ring and the hydrophobic polyisoprenoid side chain [88]. The chemical structure of this coenzyme is depicted in Fig. **3**. It belongs to a family of compounds known

as ubiquinones, refering to the ubiquitous presence in all living organisms with a chemical structure containing a functional group known as a benzoquinone. Ubiquinones are lipid-soluble molecules with anywhere from 1 to 12 isoprene (5-carbon) units. Mammals generally have 10 isoprenoid units in the tail portion (Fig. **3**), but other non-mammalian species may have fewer units. The ubiquinone of humans, ubidecaquinone or coenzyme Q10, is composed of a long tail of 10 isoprene units (a total of 50 carbons) attached to its benzoquinone "head".

Coenzyme Q10 is an unusual lipid. Because the redox-active benzoquinone ring is connected to a long isoprenoid side chain, it requires specific placements in a biological membrane [89]. It is both an essential electron carrier and an important antioxidant in the mitochondrial inner membrane. The reduced form of coenzyme Q10 (ubiquinol, $CoQH_2$) suppresses lipid peroxidation directly by breaking the chain reaction and indirectly by recycling vitamin E [90]. The antioxidant activity of ubiquinol together with its high hydrophobicity (due to its long isoprenoid tail) and superior solubility in LDL make it a unique endogenous molecule for cellular defense against oxidative stress [91]. Not only is $CoQH_2$ an attractive scavenger for lipid radicals [92,93] by continuously regenerating from CoQ and $CoQ\cdot^{-}$ [92], but it is also an effective plasma antioxidant by regenerating plasma vitamin E [94]. Whereas, dietary supplementation with coenzyme Q10 in mice showed that the total elevated coenzyme Q (in tissue homogenates and mitochondria) does not produce a

discernable effect reflecting enhanced antioxidative capacity or altered life span [95]. In apoE knock-out mice, coenzyme Q10 attenuates the atherosclerotic lesions [96], but fails to decrease intimal thickening in balloon-injured rabbits [97]. However, it appears to be effective in patients with coronary atherosclerosis by the use of combination therapy with statins [98].

3.6 Haptoglobin

Haptoglobin (Hp) is known as an acute phase protein; the plasma level of Hp elevates in response to inflection or inflammation [99,100]. In circulation, Hp captures free hemoglobin forming an Hp-hemoglobin complex for the rapid clearance of hemoglobin from the plasma [101-105]. It plays a crucial role against hemoglobin-induced oxidative stress by a mechanism thought to be from its high-affinity binding with hemoglobin in preventing the iron "leaking" from the hemoglobin [102,106, 107]. However, we have recently reported that Hp is the most potent antioxidant that can directly inhibit cooper- or free-radical induced LDL oxidation *in vitro* [106]. Its antioxidant potency is about ten times greater than probucol. Transfection of Hp cDNA into a Chinese Hamster Ovary (CHO) cell significantly elevates the tolerance of a CHO cell against oxidative stress [106]. Interestingly, remarkable levels of Hp were found to be accumulated in the lesions of cholesterol-fed rabbits (unpublished data). Because of the high levels of Hp in human plasma (about 150 mg/dL)

and macrophage, we hypothesize that Hp may directly protect the arterial wall against LDL oxidation in atherogenesis. It deserves future studies for further substantiating this hypothesis.

3.7 Probucol

Probucol, bis (3,5-di-*tert*-butyl-4-hydroxyphenylthio) propane (Fig. **3**), is a well-known, marketed lipid-lowering drug used in the treatment of hypercholesterolemia. In hyperlipidemic patients, probucol reduces total plasma cholesterol and LDL-cholesterol by 10 to 20% [49,108,109]. The symmetrical nature of probucol is lipid soluble, in which the phenolic-OH groups have easily donable hydrogens. A carbon centered radical, peroxyl radical (ROO·), alkoxy radical (RO·), or hydroxyl radical can abstract a hydrogen from the probucol. Since a hydrogen atom has only one electron, this process converts probucol itself into a free radical, probucol-O·. Notably, the bulky tertiary butyl groups shield the radicals; it is poorly reactive with water or other radicals. The metabolic pathway under free-radical induced oxidation is shown in Fig. **5** [51]. In which, the unpaired electron delocalizes into the benzene ring forming a quinone structure. The net effect of probucol's action is its free radical chain-terminating activity, which results in the inhibition of lipid peroxidation [110]. As such, probucol is a superior antioxidant against LDL oxidation. In Watanabe hypercholesterolemic rabbits, we and others have shown that probucol is relatively effective

in attenuating the atherosclerotic lesions [51,52]. In humans, a low daily dose of probucol decreases the incidence and severity of restenosis after transluminal coronary balloon angioplasty [111]. In a long-term 9-year clinical trial covering approximately 4,000 patient-years, the cholesterol lowering effect of probucol on plasma cholesterol was confirmed in 1,133 patients. On average, plasma cholesterol values decreased from 300 mg/dL to about 240 mg/dL in hypercholesterolemic subjects [112]. One of the concerns is that probucol also reduces HDL cholesterol, but there is no direct clinical evidence that such reduction increases the mortality. The morbidity and mortality rates in these 1,133 patients were compared with those of two major epidemiological surveys: the Coronary Drug Project and the WHO studies [113]. Statistical analysis was impossible, because the long-term trial did not include patients under placebo. However, no unexpected overall excess of mortality was observed with long-term probucol treatment and there was no increase in morbidity or in sudden deaths or deaths from coronary disease. These findings applied equally to patients with or without a history of coronary disease prior to their entry into the trial [112].

In a recent 10-year prospective study, 18 familial hypercholesterolemic (FH) patients with severe coronary stenosis (received LDL apheresis every 2 or 4 weeks and lipid-lowering statin therapy) were conducted and followed [114]. Probucol was given to 17 of these 18 patients. Univariate Cox regression analysis revealed that the calculated mean LDL cholesterol level could be a predictive value of treatment efficacy (mean LDL cholesterol <

140 mg/dL, hazard ratio 0.23, P = 0.028) [114]. The combination therapy with the use of probucol delayed the progression of coronary atherosclerosis and prevented a major cardiac event, although complete inhibition was limited to a small group [114]. Since probucol was used in the combination study, it is hard to directly single out its clinical benefit. However, in the Fukuoka Atherosclerosis Trial (FAST), probucol has been demonstrated to induce regression of carotid atherosclerosis [49].

3.8 Probucol analogs AGI-1067

Several probucol analogs appear to have an antiatherogenic effect in animal models as previously reported [51,52,115]. One of the analogs, MDL 29311, possesses higher antioxidant activity than probucol and appears to not reduce the HDL levels in rabbits [51]. Another compound AGI-1067, a metabolically stable analog of probucol with an antioxidant property equivalent to that of probucol, is effective in preventing atherosclerosis in all tested animal models including the LDL receptor-deficient and apolipoprotein E-knockout mice and the hypercholesterolemic primate [115]. AGI-1067 improves luminal dimensions of the percutaneous coronary intervention (PCI) site and reduces restenosis in the Canadian Antioxidant Restenosis Trial (CART-1), suggesting a direct antiatherosclerotic effect [116-119]. Because the study was carried out within 4 weeks, the ongoing multicenter CART-2 trial designed for long-term follow-up in patients with coronary artery disease may

provide valuable information with respect to the effect of the antioxidant therapy in atherosclerosis [116].

3.9 BO-653

2,3-Dihydro-5-hydroxy-2,2-dipentyl-4,6-di-*tert*-butylbenzofuran (BO-653) is designed and synthesized as a novel antiatherogenic antioxidant with a structure similar to probucol (Fig. **3**). BO-653 possesses high reactivity toward free radicals and shows great antioxidant activity in suppressing oxidation of LDL better than α-tocopherol *in vitro*. BO-653 also exerts an antiatherogenic effect in animal models [120]. It effectively suppresses the formation of hydroxyoctadecadienoic acid (HODE) that acts as a biomarker for oxidative stress *in vivo* [121]. When BO-653 was given to healthy human subjects at 400 mg twice daily for 28 days, lipids in the resulting plasma were protected from oxidation as compared with that present in subjects receiving a placebo [122]. In Watanabe hypercholesterolemic rabbits, BO-653 exhibits the highest concentration in LDL fraction of plasma due to its high affinity to LDL. The isolated LDL samples possess potent resistibility to oxidation [123]. BO-653 is readily incorporated into plasma by oral administration, and it inhibits plasma lipid peroxidation more efficiently than vitamin E regardless of the presence or absence of vitamin C [124]. However, its analogue BO-653M (2,3-dihydro-5-hydroxy-4,6-di-methyl-2,2-dipentylbenzofuran) having two methyl

substitutions in place of *tert*-butyl groups of BO-653 does not inhibit the lipid peroxidation in plasma. It clearly demonstrates that the *tert*-butyl groups at the *ortho*-position play a key role in determining the antioxidant efficacy [124].

4. COMMONLY USED ANALYTICAL METHODS OF ANTIOXIDANT POTENCY

To estimate the antioxidant potency, many analytical methods have been used. In principle, these methods are classified as two types. 1) Direct quenching or removal of free radicals. For this measure, a given antioxidant is first placed with a free radical initiator. The magnitude of radical quenching is then determined via reading the absorbance, chemiluminescence, or color change. The method however is not designed for LDL oxidation. 2) Inhibition of lipid peroxidation of LDL. For this assay, a given antioxidant is incubated with LDL in the presence of a free radical initiator, during which time lipid peroxidation is measured over time. The byproducts of lipid peroxidation, such as malondialdehyde (MDA), conjugated diene, or 4-hydroxynonenal (4-HNE), can be estimated by the addition of a chromogenic reagent or directly read by a spectrophotometer or analyzed by an HPLC [125-132]. It is worth mentioning that the second method is specifically considered for evaluating the compounds designated for the treatment of atherosclerosis. The most commonly used analytical methods are discussed below and some of the key

chemical reactions are shown in Fig. **6**.

4.1 TBARS

The most widely used assay for lipid oxidation is the measurement of the peroxidation byproduct, malondialdehyde (MDA), by reacting with thiobarbituric acid (the so-called thiobarbituric acid–reactive substances or TBARS) [125,133,134]. The principle of this method is that MDA first reacts with thiobarbituric acid (TBA) forming a chromogenic TBA-MDA-TBA complex [125,135]. The trimeric complex in pink can be measured for absorbance at 532 nm or for fluorescence at 553 nm (Fig. **6**) [129,130]. Thus, the reacting complex becomes an estimating index for the degree of oxidation. Due to the simple procedures without using a special instrument, the TBARS assay is regarded as a mostly useful measure for estimating antioxidant potency. However, some substances in the body fluid or other aldehyde byproducts generated from the oxidation process may interfere in the result of the TBARS assay [133,136-138]. Several other compounds, including cyclic peroxides, sugars, amino acids, and bilirubin, may also react with TBA to give nonspecific false values [137,139,140]. Such reactions may lead to an overestimate of the oxidative stress while measuring the human body fluid or tissue samples. Using isolated LDL as a substrate for *in vitro* studies, the influencing factors which reflect the MDA levels can be estimated by employing the negative controls performed without a free radical initiator. The

interference can be reduced to a minimum, and that is why the TBARS assay is so widely used: technically simple and works well for *in vitro* studies [50].

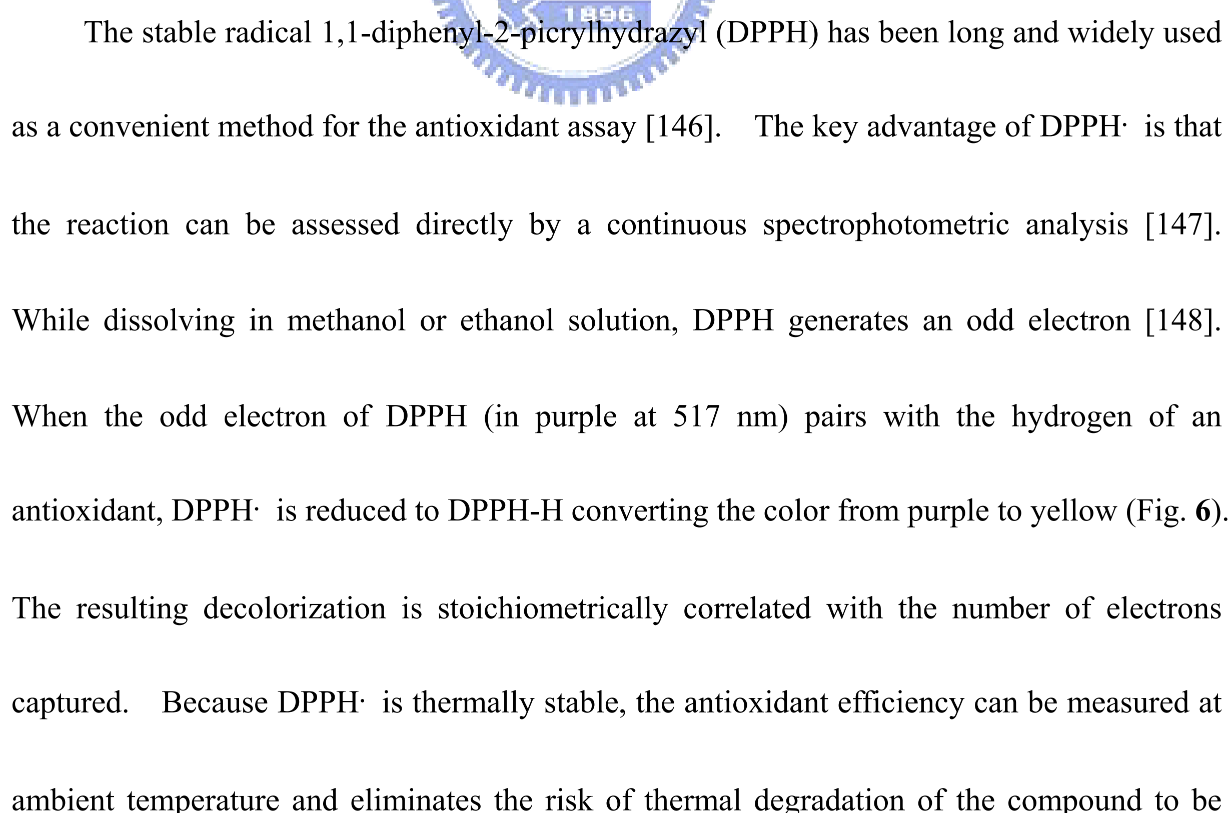
With respect to the formation of the lipid peroxides from LDL, transition metal ions are commonly employed as an initiator. Most transition metals (such as iron, copper, and cobalt) have more than one oxidation state besides the ground state. Their valence electrons may be unpaired allowing one-electron redox reactions. As such, transition metals can react with H_2O_2 to produce OH· and related oxidants [141]. In 1894, Fenton first described the oxidation of tartaric acid by Fe^{+2} and H_2O_2 [142]. The combination of H_2O_2 and a ferrous salt is named as Fenton's reagent. Forty years later, Haber and Weiss proposed a formation of a hydroxyl radical (OH·) from using Fenton reagent [143]. This highly reactive oxidizing species is now believed to be involved in cellular toxicity and leads to the lipid peroxidation [144] and DNA damage [141]. Since then, the iron (or copper) catalyzed hydrogen peroxide has been called Fenton's reaction. The reaction is further applied to be an analytical method in characterization of antioxidant activity by transition metal ion-induced lipid peroxidation. The overall chain reaction is illustrated in Fig. **7**. Thus, the extent of oxidation can be determined by the resulting lipid peroxide.

4.2 Conjugated dienes

Conjugated diene is one of the intermediate products formed during the peroxidation of

polyunsaturated fatty acids of cholesterol esters, phospholipids, and triglycerides. It is widely used for monitoring the LDL oxidation [126,145]. The principle is based on the conversion of diene double bonds into conjugated dienes during the lipid peroxidation. The conjugated diene gives a local maximal absorbance at 234 nm. The kinetics of the oxidation of human LDL can be measured continuously via the change of the absorbance [126]. The time-course curve is divided as three consecutive phases: lag, propagation, and decomposition. In lag phase, the diene absorption increases slowly. In propagation phase, a rapid arising of diene appears and finally achieves a decomposition phase [126].

4.3 Reduction of the DPPH radical



The stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) has been long and widely used as a convenient method for the antioxidant assay [146]. The key advantage of DPPH· is that the reaction can be assessed directly by a continuous spectrophotometric analysis [147]. While dissolving in methanol or ethanol solution, DPPH generates an odd electron [148]. When the odd electron of DPPH (in purple at 517 nm) pairs with the hydrogen of an antioxidant, DPPH· is reduced to DPPH-H converting the color from purple to yellow (Fig. **6**). The resulting decolorization is stoichiometrically correlated with the number of electrons captured. Because DPPH· is thermally stable, the antioxidant efficiency can be measured at ambient temperature and eliminates the risk of thermal degradation of the compound to be

tested [149]. More recently, a novel method for the determination of DPPH· has been developed. The reactive DPPH radical is measured amperometrically by the existing current, which is proportional to the concentration of DPPH· [150]. Under this condition, the higher activity of antioxidant, the lower current is observed.

4.4 FTC method

Ferric thiocyanate (FTC) method is used to determine the amount of peroxides at the initial stage of lipid peroxidation [151-153]. First, an antioxidant is mixed at 37℃ with linoleic acid which acts as a lipid source. The mixture then reacts with $FeCl_2$ and thiocyanate (SCN^-). While oxidation is taking place, lipid peroxides leads the oxidation of Fe^{+2} to Fe^{+3}. The latter ions form a complex with thiocyanate as $Fe(SCN)^{+2}$ exhibiting an absorbance at 500 nm. This reaction is allowed to proceed over time until reaching the maximal absorbance. Therefore, high absorbance indicates high oxidation of linoleic acid emulsion when the tested compound possesses a weak antioxidant activity [154,155]. Although FTC method is widely used in estimating the antioxidant activity, it is time-consuming and requires large amounts of sample.

4.5 Total Phenols Assay using Folin-Ciocalteu Reagent

Phenolic antioxidants, a specific group of secondary metabolites, play important roles in

protecting tissues against harmful oxygen radicals and other highly reactive oxygen species [156]. The sum of phenolic compounds usually acts as a reference while determining the total antioxidant capacity of natural products, such as plant extract. Initially, Folin and Ciocalteu [157] showed that a complex reagent containing molybdate (MoO_4^{-2}), tungstate (WO_4^{-2}), and phosphoric acid reacted with proteins to yield a product in blue with maximal absorbance at 745-750 nm that was approximately proportional to the tyrosine and tryptophan content. The key reaction is via the oxidation of phenols by the molybdotungstate reagent. The overall reaction is: Na_2WO_4 / Na_2MoO_4 → (phospho-$MoW_{11}O_{40}$)$^{-4}$ (**step 1**) and (phospho-$MoW_{11}O_{40}$)$^{-4}$ (yellow) + e^- → (phospho-$MoW_{11}O_{40}$)$^{-5}$ (blue) (**step 2**). This method is simple and sensitive. However, the reaction is slow at acidic pH and lacks specificity. Singleton and Rossi [158] improved the method with a molybdotungstophosphoric heteropolyanion reagent $3H_2O$-P_2O_5-$13WO_3$-$5MoO_3$-$10H_2O$ and $3H_2O$-P_2O_5-$14WO_3$-$4MoO_3$-$10H_2O$ that reacts phenols more specifically (reduction). The maximal absorbance for the product is at 765 nm [158,159]. Singleton *et al.* [160] further extended this assay to the analysis of total phenols in wine; since then the assay has gained popularity in determining the total phenolic moiety [156,161].

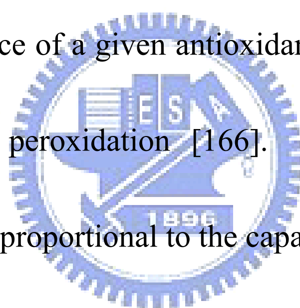
Although the Folin-Ciocalteu method is simple and useful in characterizing total phenolic content, it suffers from a number of interfering substances, such as ascorbate, sugars (glucose, fructose, and saccharose) and others that cause an overestimate of the values of

phenolics [161]. The correction can be made in some extent via endogenous subtraction [159,162], but the corresponding corrections are experimentally complicated due to the instability of the interfering compounds with fast subsequent reactions [156]. Additional common interfering substances reacting with the Folin-Ciocalteu reagent include uric acid, hydroxyammonium chloride, iron sulfate, manganese sulfate, potassium nitrite, sodium phosphate, sodium sulfite, and others [159,161-163].

4.6 TRAP Assay

The use of total radical-trapping antioxidant parameter (TRAP) assay has been originally proposed to evaluate the total plasma "antioxidant capacity", but not "antioxidant activity" [164,165]. The "antioxidant activity" corresponds to the rate constant of a single antioxidant reacting against a given free radical. The "antioxidant capacity" is the measure of the moles of a given free radical scavenged by a tested solution and is different from the antioxidant activity [166]. The test solution may be a single-compound solution or a complicated mixture, such as body fluid and plant extract. The method is based on the ability of a tested sample in trapping peroxyl radicals generated through the thermal decomposition of azo-compound (Fig. **6**), such as 2,2'-diazobis-(2-amidinopropane) hydrochloride (ABAP) or 2,2'-diazobis-(2-amidinopropane) dihydrochloride (AAPH) [166-168]. The widely used azo-compound, ABAP or AAPH, is a water-soluble peroxyl

radical initiator. While dissolving in aqueous solution, the azo-compound decomposes and releases the peroxyl radicals at a constant rate [164,166]. Wayner *et al*. proposed that the primary TRAP assay was based on the measure of oxygen consumption during a ABAP-induced lipid peroxidation reaction [164]. Subsequently, DeLange *et al*. developed a new approach based on the utilization of an external probe R-phycoerythrin (R-PE) [169]. R-PE is excited at 495 nm and generates an emission at 575 nm [170]. The decay of R-PE fluorescence is accelerated under the azo-compound induced free radicals. This improved assay can directly measure the attack of a peroxyl radical upon an external probe (such as R-PE) in the presence or absence of a given antioxidant rather than oxygen consumed during the chain reactions of lipid peroxidation [166]. Therefore, fluorescence emission is suppressed for a period of time proportional to the capacity of a given antioxidant [171].



4.7 Radical Quenching by ESR (Electronic Spin Resonance)

Electron spin (paramagnetic) resonance (ESR or EPR) spectroscopy is another method for detecting and identifying the radicals due to the presence of an unpaired electron in the molecular framework [172]. The spin of a single, unpaired electron produces a magnetic moment. Thus, electromagnetic wave radiation of an appropriate frequency under a given external magnetic field causes the excitation of unpaired electrons from the lower to the higher energy level by the interaction of the magnetic moment of the electron spin with the

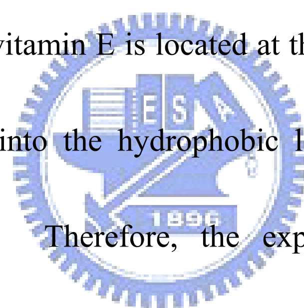
magnetic component of the electromagnetic wave (magnetic resonance) [173]. Therefore, ESR may detect an unpaired electron presenting in a free radical through the absorbance of microwave radiation spectra. Up-to-date ESR spectroscopy remains to be the most reliable technique for measuring biological free radicals and redox states because it specifically measures the paramagnetic species having unpaired electrons [173].

Continuous wave (CW) accompanying a spin-trapping technique is a method commonly used in ESR for evaluating the redox status between free radicals and antioxidants. An ESR-detectable spin trap molecule is often designed and used for rapidly reacting with highly reactive free radicals to form another ESR-silent molecule [174-176]. Such spin trap adducts have much higher stability than the primarily generated free radicals and are stable enough to persist for the complete reaction process [173,174]. The decay rate of an ESR signal can be regarded as a parameter for accessing the free radicals generated. Thus far it is the only approach reported to provide a direct evidence for the presence of a given free radical [177]. Due to the free radicals having to be relatively stable in ESR spectroscopy, "spin trapping" of the radical becomes necessary if its decay is very rapid [175,176].

5. RATIONAL DESIGN OF A SYNTHETIC ANTIOXIDANT AS AN ANTIATHEROSCLEROTIC AGENT

It is generally agreed that oxidized LDL can trigger the atherosclerosis in experimental

animals, while lipid peroxidation is considered to play a central role in LDL oxidation. Thus far, only few natural and synthetic compounds have exerted their superior antioxidant and antiatherosclerotic activities for both *in vitro* and *in vivo* models, it is necessary to explore other novel antiatherosclerotic agents. Although certain antioxidants are effective in preventing atherogenesis, some doubts still remain. For example, is it potent enough to suppress the oxidative stress in an artery wall in randomized human trials? Recent clinical trials have shown that vitamin E fails to reduce the severity of CHD, but we should keep in mind that vitamin E is only a mild antioxidant as compared to the other synthetic compounds [39-44,62,178]. In addition, vitamin E is located at the surface of LDL particles; it does not penetrate profoundly enough into the hydrophobic lipid core of LDL in preventing lipid peroxidation [68,69,179-181]. Therefore, the exploration and development of novel compounds have to be continued.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How to rationally design an ideal antioxidant with a superior antiatherogenic activity remains to be a subject of challenge. On the basis of LDL oxidation theory in atherosclerosis, some criteria for designing a novel agent, in our opinions, are as follows:

(1) The designed compound has to possess high activity to scavenge free radicals.

(2) The compound has to specifically target, incorporate, and anchor in the lipid hydrophobic core of LDL.

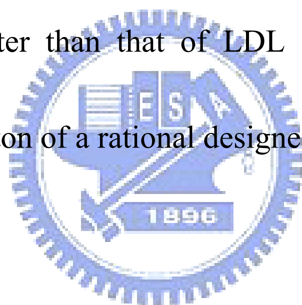
(3) The intermediate metabolites of the compound *in vivo* have to exert low prooxidant

activity.

(4) The oral antioxidant has to possess high bioavailability with long half-life.

5.1 Potent antioxidant activity

Among the considerations of a rationally designed agent for inhibiting lipid peroxidation, one important property is its antioxidant potency. Many phenolic compounds are well known by its high antioxidant activity [50,123,182]. Because of the high hydrogen-donating ability results from its low dissociation energy of the phenolic O-H bond, these compounds react toward free radicals faster than that of LDL [183]. For this reason, the phenolic structure could be a good skeleton of a rational designed agent [53,123,184,185].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5.2 Anchoring in the hydrophobic core of LDL

A compound which possesses potent antioxidant activity may exert its electron-donating ability with free radicals everywhere. Should the antioxidant not be able to specifically target and anchor to a LDL particle, the effectiveness of preventing LDL oxidation *in vivo* may be reduced substantially. For instance, the function of the long phytyl side chain of vitamin E has been thought to facilitate the incorporation of itself into LDL particles due to its hydrophobic nature. A vitamin E analog (2,2,5,7,8-pentamethyl-6-chromanol) without having a hydrophobic phytyl side chain, has the same reactivity toward free radicals [186],

but its biological activity is quite limited [183,186,187]. Even so, vitamin E is still not an ideal compound because the phytyl side chain of vitamin E may not be oriented parallelly with the phospholipid fatty acyl chains of LDL. Furthermore, the numbers of HDL particles in human plasma are more abundant than LDL (about 2:1). *In vitro*, vitamin E on LDL can be immediately redistributed onto vitamin E-free HDL with an equal molar partition suggesting that vitamin E might not be tightly associated with LDL [181,188,189]. Whereas, plasma β-carotene (the endogenous hydrophobic molecules) is abundantly present in LDL (80%), in which the β-carotene molecules do not transfer between the LDL and HDL particles [190]. Similarly, probucol is predominantly present in LDL particles following oral dosages [108,191,192]. Therefore, it is feasible to produce a novel compound that can specifically target to LDL. Presumably, a phenolic compound containing a suitable hydrocarbon chain length may facilitate its anchoring in LDL particles and have the maximal effectiveness in inhibiting LDL oxidation [68].

5.3 Low prooxidant activity

A given antioxidant must sacrifice itself in order to remove free radicals and then converts itself to another prooxidant. Vitamin E is a typical example which reacts with a peroxyl radical in a single reaction by forming a vitamin E radical via a redox reaction (Fig. **2**). If the vitamin E radical reacts with another peroxyl or vitamin E radical, the reaction

comes to termination. However, if the resulting vitamin E radical reacts with another polyunsaturated fatty acid, it will be regarded as a prooxidant role that promotes the initiation of lipid peroxidation. The prooxidant property may result from the rate constant of hydrogen-abstraction in the chromanoxyl radical from lipids or lipid hydroperoxides; a typical example is seen in trolox (a vitamin E analogue) [193-196]. To avoid such prooxidant effects, BO-653, a partial vitamin E and probucol analogue utilizing two *tert*-butyl groups on the *ortho* position of the hydroxyl group instead of the methyl groups of vitamin E, was prepared [123]. The hydrogen atom of BO-653 (-OH) is more in steric hindrance for reacting with peroxyl radicals than the methyl groups of vitamin E. The resulting phenoxyl intermediate of BO-653 is kinetically persistent until the reaction with other peroxyl radicals at the only open 7-position (Fig. **3**) [123]. Therefore, the stability of the phenoxyl intermediate of BO-653 leads to the decrease in its prooxidant characteristic [123,183]. Conclusively, the prooxidant characteristic has to be one of the key considerations while designing a novel antioxidant compound. Probucol and its analogs, however, have another unique feature. As shown in Fig. **5**, upon the oxidation *in vitro* and *in vivo*, probucol is metabolized into diphenoquinone and bisphenol [50,51], in which bisphenol is another potent antioxidant [197-200]. The subsequent metabolite diphenoquinone is not a prooxidant according to our experience. Interestingly, all the probucol analogs tested so far can all be metabolized into bisphenol [197-200].

5.4 Bioavailability and half-life

There are many natural phenolic compounds, mostly flavonoids, found in plants. Over 4,000 different flavonoids have been described; some flavonoids are highly potent against LDL oxidation *in vitro* [201,202]. It suggests that flavonoids are beneficial for protecting cardiovascular diseases. The extent of absorption of flavonoids is an important unsolved issue. Flavonoids present in diet are considered non-absorbable; many well-controlled human trials showed that only little or no flavonoids existed in human plasma [203,204] when ingesting flavonoids [205]. On the other hand, a single oral dosage of probucol may last as long as 6 months in plasma. While testing synthetic probucol analogs, we experienced that those compounds able to incorporate into LDL *in vitro* usually gave a superior bioavailability [197-200]. The high hydrophobicity of the molecules seems to facilitate them to pass through the gut wall. We speculate that lack of enough hydrophobicity of the flavonoids might account for their poor absorption. To test the incorporation ability into LDL, a given compound was first incubated with LDL at 37℃ *in vitro* followed by reisolation of LDL using a conventional KBr density flotation procedure [52]. Next, we determined the recovered compound in LDL using an HPLC analysis. The above method assisted us in predicting the bioavailability of an unknown compound.

As to determine the bioavailability of a leading compound *in vivo*, the best approach is

to verify the recovery of the compound from the plasma using small animals such as mice and rats [50,51]. The plasma concentration of the compounds and their metabolites can be analyzed by an HPLC. We also analyzed the antioxidant activity in both plasma and isolated LDL following oral ingestions of tested compounds using a TBARS assay [50]. With respect to the oral ingestion, dried compound can be evenly mixed with powdered feed before making a solid chow (produced by Purina, St. Louis, MO, via customer order). The main advantage of the procedures mentioned above is to ensure the compound is directed to the LDL with good bioavailability. Interestingly, compounds that associated with LDL usually maintain a long half-life. For example, 80% of plasma β-carotene are associated with LDL, and it possesses a half-life about 10 days [205,206].

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6. CONCLUSION

Although clinical trials using vitamin E as an antioxidant therapy failed to show a beneficial result for the treatment of coronary artery disease, we should not be discouraged by their outcome. First, vitamin E is a mild antioxidant that does not specifically target on LDL [188]. Its antioxidant activity against LDL oxidation is at least 5 times less than that of probucol [50,106]. Second, there is no evidence showing that vitamin E can preferentially penetrate into the LDL hydrophobic core to effectively inhibit LDL lipid peroxidation [47]. A rationally designed novel compound capable of anchoring the hydrophobic core of LDL

may eventually prove the clinical efficacy of an antioxidant therapy. Third, vitamin E forms a prooxidant following challenge of free radicals [65], as such vitamin E may offset its antiatherogenic role *in vivo*. Unlike the results of vitamin E in clinical trials, probucol is able to prevent coronary restenosis after balloon angioplasty in humans [111]. It also induces regression of carotid atherosclerosis in Fukuoka Atherosclerosis Trial (FAST) [116]. Many of the probucol analogs [51,52,197-200] that were designed for the specific incorporation into LDL with a superior bioavailability may be worthwhile for future studies as novel clinical leads. A recent CART-1 study in humans suggests that AGI-1067, a probucol analog, is effective in preventing atherosclerosis. A final promised outcome should wait for the ongoing CART-2 clinical trials [116-119].

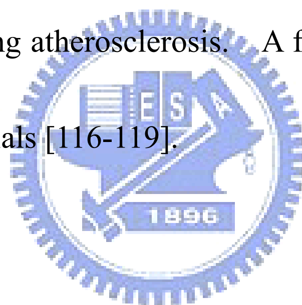

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Figure Legends:

Fig. (1). Schematic drawing of the oxidation hypothesis in atherogenesis.

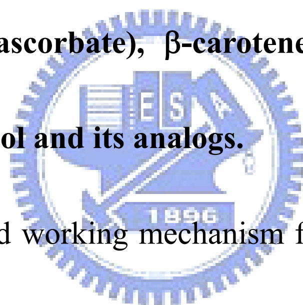
In initial atherogenesis, the infiltrated LDL are oxidized by free radicals catalyzed via the action of myeloperoxidase, lipoxygenase, or phospholipase in the arterial wall to become so-called minimal modified LDL (MM-LDL) **(step I)**. The MM-LDL injure the wall and stimulate the endothelial cell (EC) to express chemotactic factors. Monocyte chemotactic protein 1 (MCP-1) attracts monocyte and facilitates its infiltration **(step II)**. The growth factors, such as macrophage colony stimulating factor (MCSF) and granulocyte colony stimulating factor (GCSF), stimulate monocyte by transforming into macrophage. MM-LDL can be further oxidized by free radicals to become Ox-LDL **(step III)**. Macrophage rapidly takes up Ox-LDL through specific scavenger receptors leading to the formation of a foam cell **(step IV)**. The release interleukin-1 (IL-1) from macrophage then stimulates the proliferation of smooth muscle cell (SMC) with a coordinated action of platelet-derived growth factor (PDGF) **(step V)**. The accumulation of foam cells and proliferation of SMC cause the formation of the fatty streak and the initiation of atherosclerosis.

Fig. (2). Free-radical scavenging pathway of vitamin E and proposed regeneration cascade.

The odd electron in lipid peroxyl radical (LOO·) is first scavenged by α-tocopherol leading to

the formation of an α-tocopheroxyl radical in the lipid phase. The α-tocopheroxyl returns to α-tocopherol via the oxidation of ascorbate in the aqueous phase. Ascorbate is oxidized to monodehydroascorbate (MDHA), which can either renovate to ascorbate or form dehydroascorbate (DHA). DHA can be then reduced to ascorbate resulting from the sacrificing of glutathione (GSH). Finally, GSSG is reconverted to GSH at the expense of NADPH.

Fig. (3). Chemical structures of naturally occurred antioxidants vitamin E (α-tocopherol), vitamin C (ascorbate), β-carotene, glutathione, coenzyme Q10, and synthetic antioxidants probucol and its analogs.



The characteristic and proposed working mechanism for each antioxidant is described in the text. In brief, the phenolic compounds convert themselves to quinones, while glutathione (GSH) forms a GSSG dimer upon scavenging the free radicals. For β-carotene, the quenching involves a physical reaction in which the energy of the excited oxygen is transferred to the carotene forming an excited state molecule. The ability of β-carotene to quench excited oxygen is limited. At high oxygen concentrations it functions as a prooxidant; at low oxygen tension, it reacts directly with a peroxyl radical.

Fig. (4). Free-radical scavenging reaction of vitamin E, vitamin C, coenzyme Q10,

probucol, and glutathione.

The antioxidant activity of phenolic compounds, such as vitamin E, vitamin C, coenzyme Q10, and probucol, is based on its hydrogen-donating ability. The derived products become another free-radical source leading to a prooxidant effect. The reduced glutathione (GSH) exerts its free-radical scavenging activity by donating hydrogen of the SH group and forms GS·.

Fig. (5). Metabolic pathway of probucol.

Three major metabolites from probucol, namely diphenoquinone, bisphenol, and spiroquinone, are found in human plasma after oral dosages of probucol and can be easily identified by an HPLC technique [50]. Interestingly, bisphenol is another potent antioxidant.

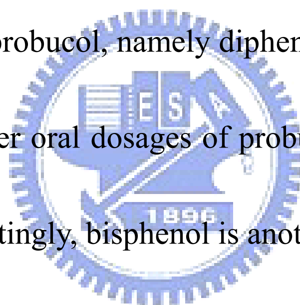


Fig. (6). Mechanisms involved in the evaluation of some antioxidant assay.

(a) TBARS assay: The principle of the assay relies on the formation of malondialdehyde (MDA), one of the major products during lipid peroxidation. One MDA reacts with two thiobarbituric acid (TBA) molecules to form a TBA-MDA-TBA adduct, giving a maximal absorbance at 532 nm. **(b)** DPPH method: DPPH· in methanol forms chromogen with an absorbance at 517 nm. It acts as a stable, free-radical donor at ambient temperature. The tested antioxidant scavenges the odd electron of DPPH· to form DPPH-H converting the

purple to yellow color with a decreased absorbance at 517 nm. **(c)** TRAP assay: The assay is based on the protection provided by antioxidants on the lag-phase fluorescence decay of R-phycoerythrin (R-PE) under a controlled peroxidation reaction. R-PE is excited at 495 nm and gives an emission at 575 nm. TRAP values are obtained from the length of the lag-phase. Trolox, an antioxidant, is commonly used as a standard.

Fig. (7). The overall of Fenton-related chain reactions.

Fenton reaction is widely applied to be an analytical method in characterization of antioxidant activity by inhibiting the transition metal-ion induced lipid peroxidation. (LH=lipid molecule; L·=alkyl radical; LO·=alkoxy radical; LOO·=lipid peroxyl radical; LOOH=lipid peroxide)

Fig. (**1**).

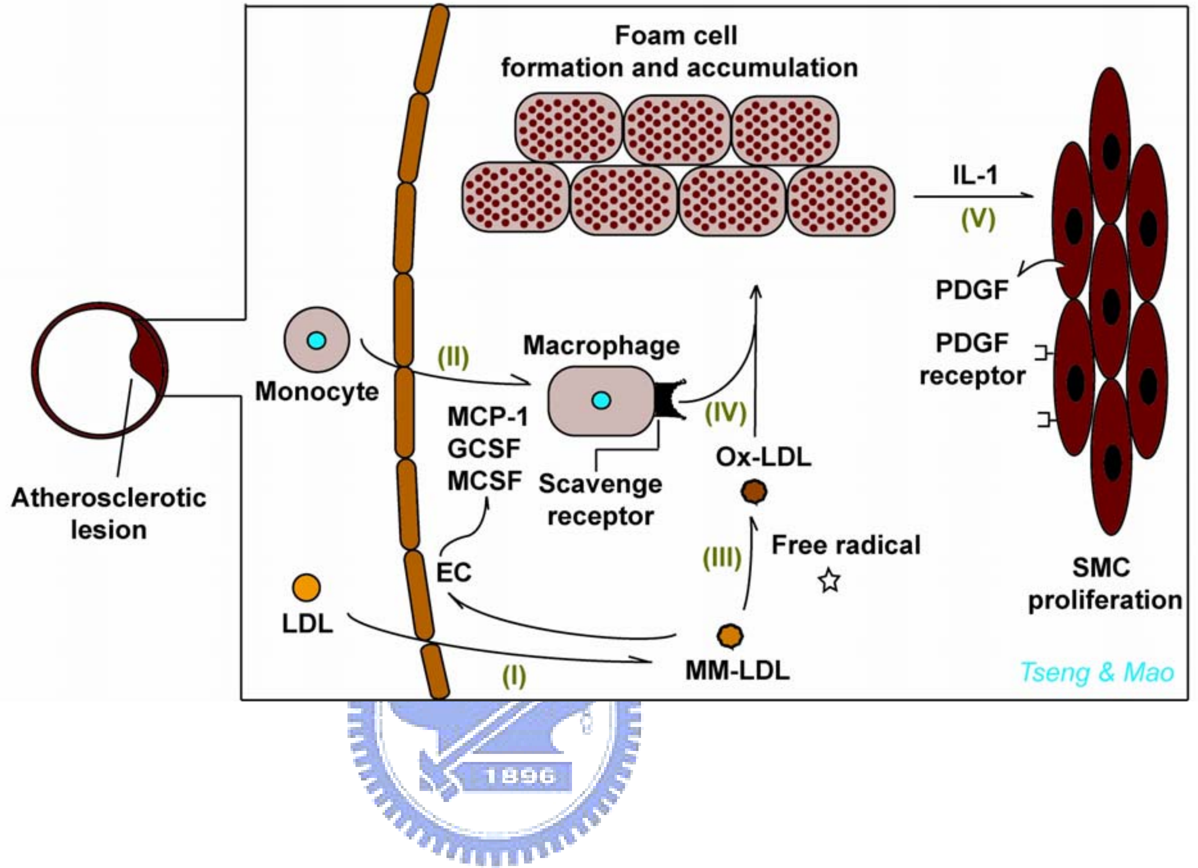


Fig. (**2**).

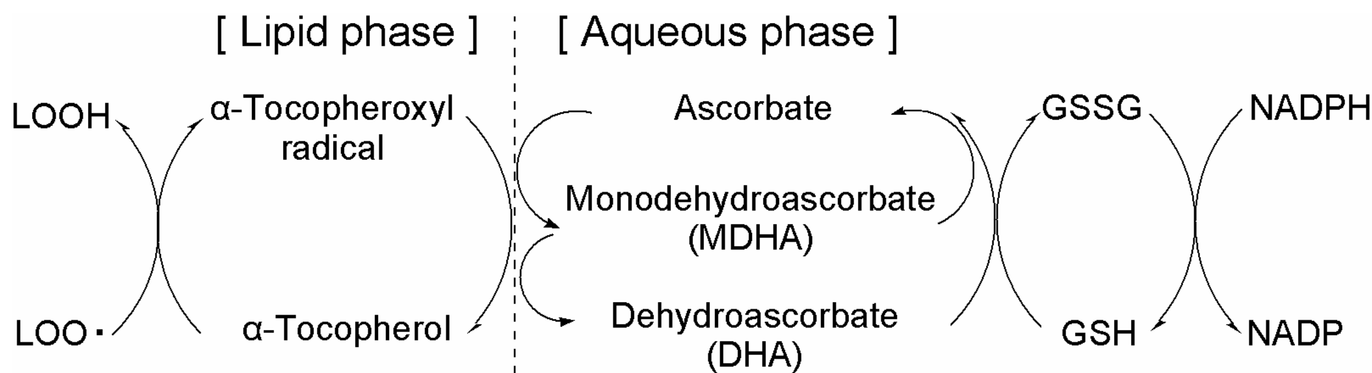
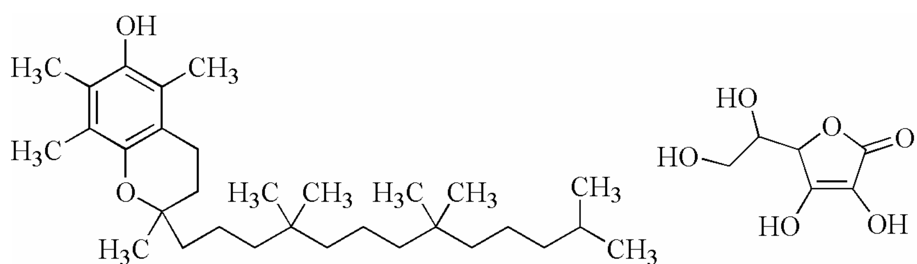
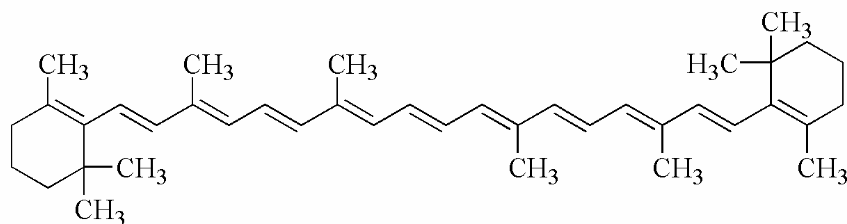


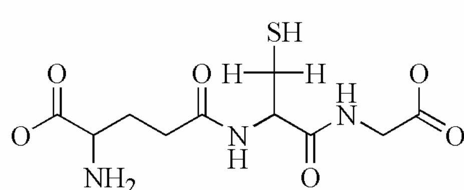
Fig. (**3**).



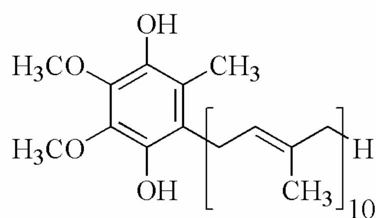
Vitamin E **Vitamin C**



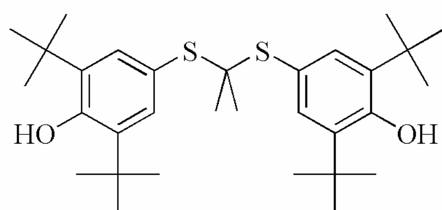
β-carotene



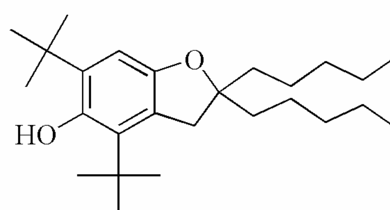
Glutathione



Coenzyme Q10 (CoQH$_2$)



Probucol



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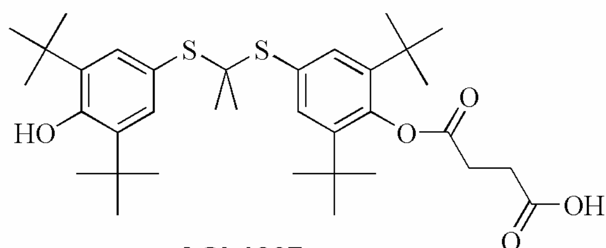

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Fig. (4).

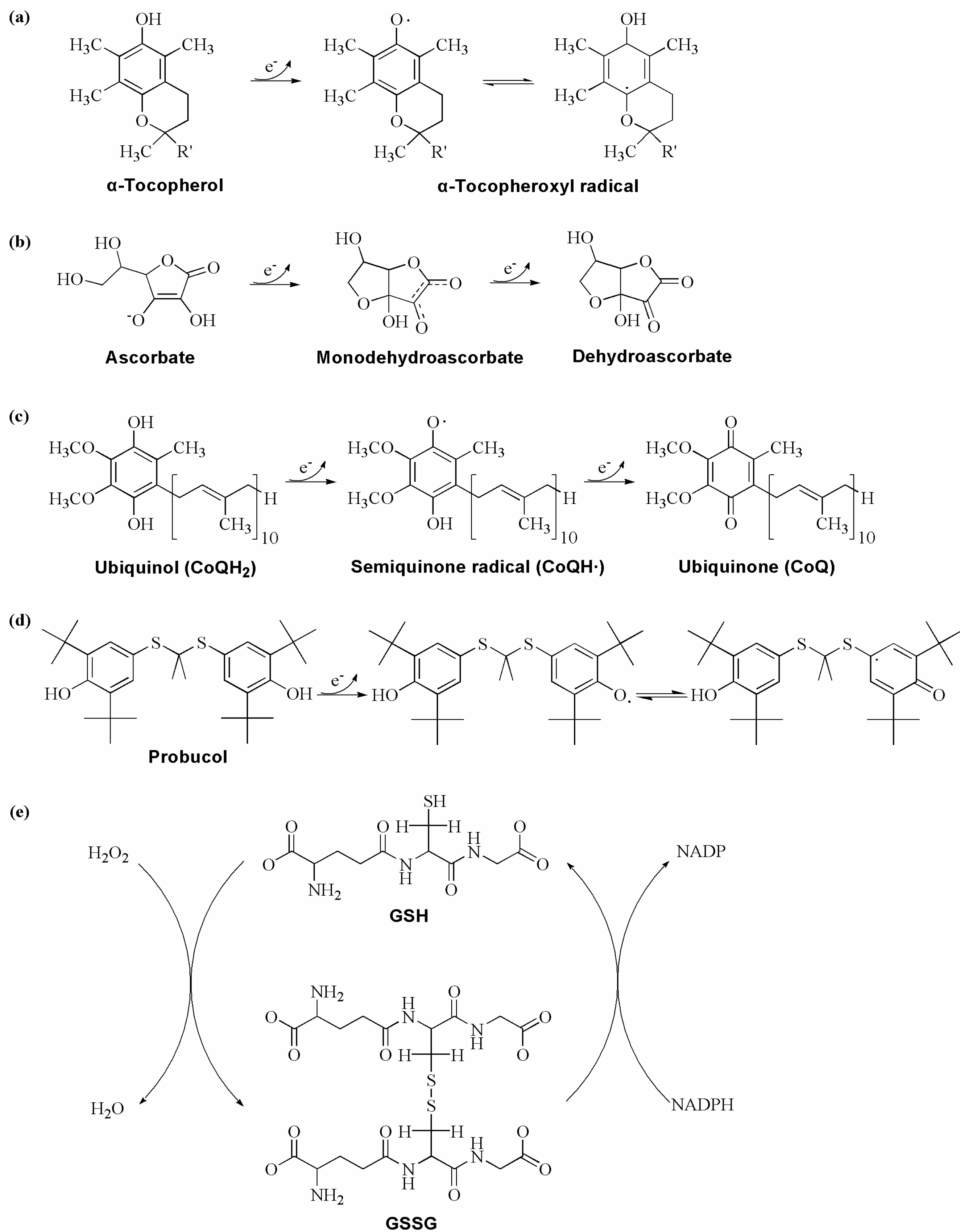


Fig. (**5**).

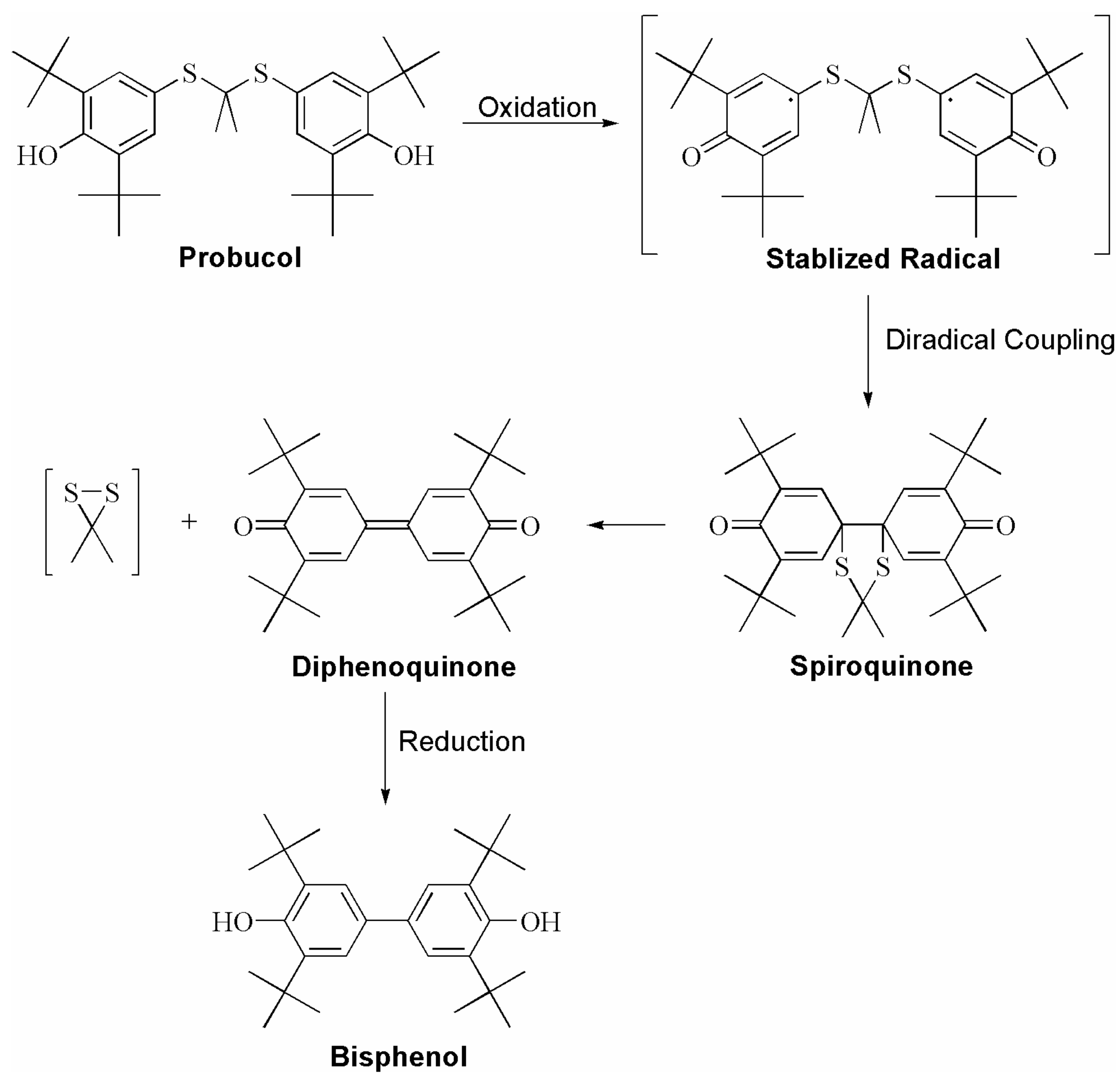
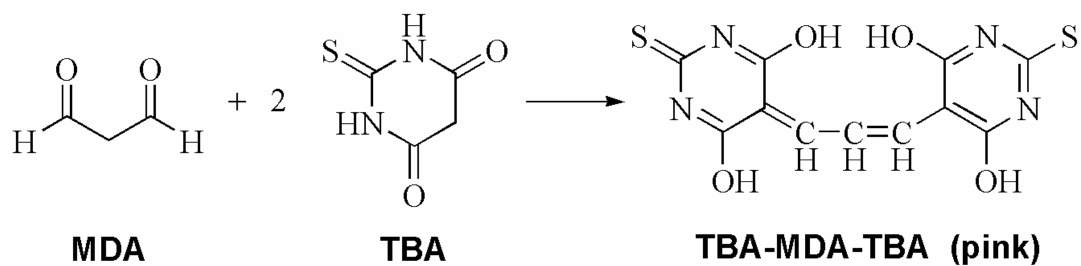
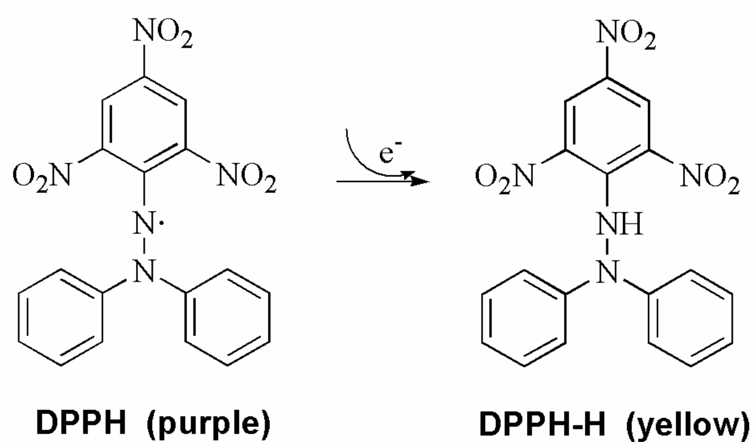


Fig. (**6**).

(a) TBARS assay



(b) DPPH method



(c) TRAP assay

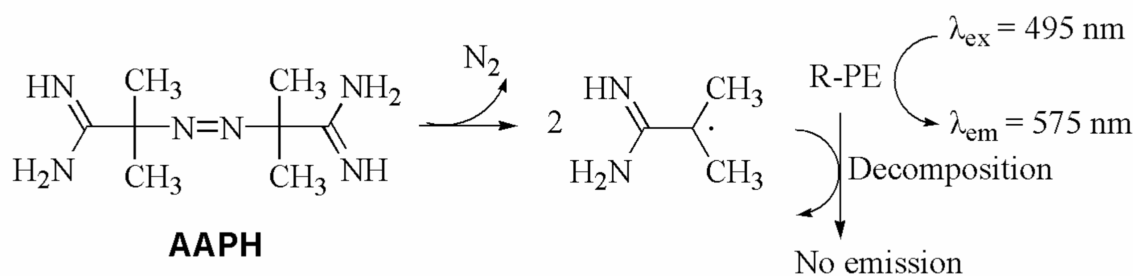
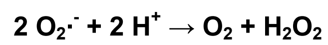
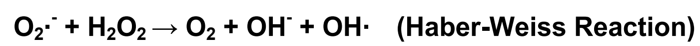


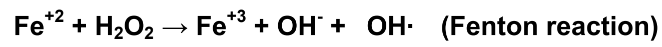
Fig. (7).



$2\ O_2\cdot^- + 2\ H^+ \rightarrow O_2 + H_2O_2$

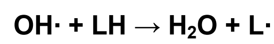


$O_2\cdot^- + H_2O_2 \rightarrow O_2 + OH^- + OH\cdot$ (Haber-Weiss Reaction)

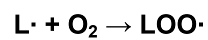


$Fe^{+2} + H_2O_2 \rightarrow Fe^{+3} + OH^- + OH\cdot$ (Fenton reaction)

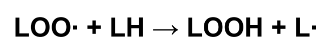
$Fe^{+3} + H_2O_2 \rightarrow Fe^{+2} + H^+ + OOH\cdot$



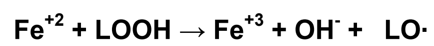
$OH\cdot + LH \rightarrow H_2O + L\cdot$



$L\cdot + O_2 \rightarrow LOO\cdot$



$LOO\cdot + LH \rightarrow LOOH + L\cdot$



$Fe^{+2} + LOOH \rightarrow Fe^{+3} + OH^- + LO\cdot$