RRPG92041276 ( 46..P)

計畫編號：NHRI-EX92-9229SI

國家衛生研究院整合性醫藥衛生科技研究計畫

# Haptoglobin 在動脈硬化中所扮演之功能角色

計畫名稱

九十二年度成果報告

執 行 機 構：交通大學 生物科技系/生化工程研究所

計 畫 主 持 人：毛仁淡

本年度執行期間： 92 年 1 月 1 日 至 94 年 12 月 31 日

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**研究人員**：毛仁淡

**關鍵字**：Haptoglobin、動脈硬化

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# 中文摘要

這是三年計畫中的第一年計畫報告，首先我們非常感謝國家衛生研究院的支持及給予我們機會執行此計畫，目前本計畫第一年已出版 2 篇 SCI 報告與 6 篇研討會論文（其中 3 篇為國際研討會論文）。除此之外，另有 3 篇報告已經完成，並預定於 2003 年底投出。

為了探討 Haptoglobin（以下簡稱 Hp）的抗氧化特性，發展並建立簡單純化大量 Hp 的方法就顯得相當重要。共有 2 個方法在本研究中完成，第一，血紅素親和性管柱，但是此方法最終所得到的產物摻有少量的血紅素。第二，先利用抗體親和性管柱純化，再過一個 gel filtration 管柱，結果發現這是一個方便而有效的方法去除了之前純化方法傳統的缺點，如純度不夠、純化條件太嚴苛、太花時間等等。

我們也發展出一個快速辨別 Hp 類型的方法，不需要經過傳統的 starch gel，血漿（或血清）只需要先與血紅素混合，再跑個 native PAGE，再以 DAB 呈色，即可由呈色的樣式判斷 Hp 的類型，因此 3 種形式的 Hp 就可輕易的判別出來。

再者，6 株特異性辨識 Hp 的單株抗體也成功的製造出來並已對其加以分析。每一株單株抗體與 3 種形式的 Hp 之間的親和力也以藉由競爭型與非競爭型 ELISA 計算出來。其中最強的單株抗體與 Hp 1-1、2-1 及 2-2 的親和力分別為 $5.58 \times 10^9$、$2.81 \times 10^9$ 與 $2.19 \times 10^9$。由這樣的結果推測 Hp 1-1 的『功能性』表面可能較 2-1 及 2-2 為多，這樣的結構性差異可能可以解釋部分 Hp 類型與感染、動脈硬化以及一些自體免疫疾病之間的關係。

每一種 Hp 表現型 CD 的光譜圖可用於評估 Hp 的二級結構。依據這好的結果我們發現在 $\alpha$ 螺旋結構中 Hp 1-1、Hp 2-1 及 Hp 2-2 分別有 29%、22% 及 21%。Hp 1-1 比聚合體 Hp 2-1 及 Hp 2-2 的結構排序上更為整齊。

在 Cu2+及 AAPH 所誘發的氧化試驗中，Hp 比現有的抗氧化物”propucol”的抗氧化效力還強。Hp 的抗氧化效力是依據濃度的改變而改變，依據 IC 50 抗氧化效力的大小排序為 Hp 1-1 > Hp 2-1 > Hp 2-2 > probucol > vitamin E。利用化學修飾法改變 Hp 的結構來研究 Hp 的結構是否為 Hp 擁有抗氧化能力的主要因素。有趣的是，carboxymethylation 破壞分子間與分子內的雙硫鍵的鍵結，不僅改變 $\alpha$ 螺旋結構的結合，並且在 Hb 的結合分析中發現破壞 Hp-Hb 的結合能力。更特別的是還原後的 Hp 比未還原的 Hp 的抗氧化能力還強。因此 Hp 的抗氧化能力並不是與血紅素結合所產生的覆合物所導致的。

探討 Hp 在 in vivo 的抗氧化活性上，我們設計了 Hp1-1 的 cDNA 並利用 CMV 啟動子將其插入 pcDNA3.0 載體中，再將此載體轉殖到 CHO-K1 細胞中，並利用已存在選擇性標的物 (geneticin) 的培養液中培養， 接著使用競爭型 ELISA、西方點漬法及免疫化學染色法來確認已轉殖 Hp1-1 cDNA 的 CHO-K1 細胞是否有表現 Hp1-1。此細胞可以穩定的表現超過十個繼代後，轉殖的 CHO-K1 細胞再以不同濃度的過氧化氫培養 24 小時，使用 MTT 分析法分析細胞的存活率，發現轉殖的 CHO-K1 細胞抗氧化壓力的耐受度會比一般的 CHO-K1 細胞抗氧化壓力的耐受度高三倍，因此表示 Hp 可以增加細胞抗氧化壓力的耐受度。

**Abstract**

This is a first-year report for the 3-years contract grant funded by National Healthy Research Institute (NHRI). First, we deeply appreciate for the funding support from NHRI to provide us the opportunity to conduct this study. With this support, in the first year, 2 SCI papers and 6 conference reports (including 3 international conference reports) have been produced. In addition, 3 papers have been written and ready to submitted by the end of 2003.

With respect to the antioxidant characteristic of Haptoglobin (Hp), it is essential to evaluate and establish a simple method to purify large amount of Hp. Two methods have been developed. First, hemoglobin (Hb)-affinity column was used to purify Hp, however, trace amount of Hb was co-eluted with Hp. Antibody-affinity column following a gel filtration chromatography was then established for purification. This is a convenient and useful method to isolate Hp without the traditional drawbacks, such as contaminant, harsh-elution, and time-consuming.

A quick Hp-phenotyping method from human plasma without using a conventional starch gel has also been successfully developed. Human plasma (or serum) incubated with supplyment of hemoglobin was used to conduct a native PAGE, and the patterns of haptoglobin-hemoglobin complex was subsequently lit up using chromogenic substrates. Thus, 3 phenotypes of Hp 1-1, 2-1, and 2-2 could be easily determined.

Moreover, 6 lines of monoclonal antibodies (mAb) prepared against Hp have been produced and characterized. The affinities among each mAb and each phenotype Hp were also determined using competitive and non-competitive

ELISA. The highest-affinity monoclonal antibody exhibited an affinity (Ka) $5.58 \times 10^9$, $2.81 \times 10^9$, and $2.19 \times 10^9$ $M^{-1}$ against Hp 1-1, 2-1, and 2-2, respectively. The 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.

The CD spectrum of each Hp phenotype was conducted to estimate the secondary structure of Hp. According to the best of our knowledge, for the first, we show that the α-helical content of Hp 1-1, 2-1, and 2-2 was 29%, 22%, and 21%, respectively. Hp 1-1 possesses a more ordered structure than that of polymeric forms of Hp 2-1 and 2-2.

In $Cu^{2+}$- and AAPH-induced lipid peroxidation, Hp exerts more potent antioxidant activity than the known, most potent antioxidant compound, probucol. The antioxidant activity of Hp was found to be dose-dependent with an IC50 Hp 1-1> Hp 2-1> Hp 2-2> probucol> vitamin E in ranking. To determine whether or not conformation of Hp plays a role in its antioxidant property, chemical modifications on Hp were conducted to alter its conformation. Interestingly, carboxymethylation which breaks the inter- and intra- disulfide linkages, not only altered α-helical content but also abolished its formation of a Hp-Hb complex in the Hb-binding assay. Notably, the reduced form of Hp exerts even much greater antioxidant activity than native Hp. Thus, the antioxidant activity of Hp might not be resulted from forming a complex with hemoglobin.

To evaluate *in vivo* antioxidant activity of Hp, the cDNA of Hp 1-1 was

cloned and inserted into the pcDNA3.0 vector with CMV promoter. CHO-K1 cells were then transfected with the inserted vector and cultured in the presence of the selection marker, geneticin. Expression of Hp 1-1 in transfected CHO-K1 cell was confirmed by competitive ELISA, Western blot, and immunocytochemical stain. The expression was stable for more than ten passages. The transfected CHO-K1 cell was then challenged with variable amount of hydrogen peroxide ($H_2O_2$) for 24 hours. MTT assay was used to estimate the relative cell survival ratio. The transfected cell exhibited a twice-higher relative survival ratio than that untransfected CHO-K1 cell. Thus, the expressed Hp did elevate the cell tolerance against the additive oxidative stress.

## 貳、九十二年度計畫著作一覽表

註：群體計畫(PPG)者，不論是否提出各子計畫資料，都必須提出總計畫整合之資料

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| 序號 | 計 畫 產 出 名 稱 | 產出型式 | SCI/SSCI | 致謝與否 |
|---|---|---|---|---|
| | | | | |
| 1. | Liau CY, Chang TM, Pan JP, Chen WL, Mao SJ. Purification of human plasma haptoglobin by hemoglobin-affinity column chromatography. J Chromatogr B Analyt Technol Biomed Life Sci. 2003, 790(1-2):209-16 | 國外期刊 | 1.911 | **NHRI** |
| 2. | Tseng CF, Huang HY, Yang YT, Mao SJ. Purification of human haptoglobin 1-1, 2-1, and 2-2 using monoclonal antibody affinity-column chromatography. Protein Expression and Purification 2003 (in press) | 國外期刊 | 1.375 | NHRI |

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## 參、九十二年度計畫重要研究成果產出統計表

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***(係指執行九十二年度計畫之所有研究產出成果)***

| 科技論文篇數 | | | 技術移轉 | | |
|---|---|---|---|---|---|
| | 國內 | 國外 | 類型 | 經費 | 項數 |
| 期刊論文 | | 2篇 | 技術輸入 | | |
| 研討會論文 | 3篇 | 3篇 | 技術輸出 | | |
| 專著 | | | 技術擴散 | | |

| 技術報告 | 技術創新 | 著作權 | 專利權 |
|---|---|---|---|
| 篇 | 項 | (核准)項 | (核准)項 |
| | | (申請中)項 | (申請中)項 |

〔註〕:

期刊論文：指在學術性期刊上刊登之文章，其本文部份一般包含引言、方法、結果、及討論，並且一定有參考文獻部分，未在學術性期刊上刊登之文章（研究報告等）與博士或碩士論文，則不包括在內

研討會論文：指參加學術性會議所發表之論文，且尚未在學術性期刊上發表者

專　　著：為對某項學術進行專門性探討之純學術性作品

技術報告：指從事某項技術之創新、設計及製程等研究發展活動所獲致的技術性報告且未公開發表者

技術移轉：指技術由某個單位被另一個單位所擁有的過程。我國目前之技術轉移包括下列三項：一、技術輸入。二、技術輸出。三、技術擴散

技術輸入：藉僑外投資、與外國技術合作、投資國外高科技事業等方式取得先進之技術引進國內者

技術輸出：指直接供應國外買主具生產能力之應用技術、設計、顧問服務及專利等。我國技術輸出方包括整廠輸出、對外投資、對外技術合作及顧問服務等四種

技術擴散：指政府引導式的技術移轉方式，即由財團法人、國營事業或政府研究機構將其開發之技術擴散至民間企業之一種單向移轉（政府移轉民間）

技術創新：指研究執行中產生的技術，且有詳實技術資料文件者

## 肆、九十二年度計畫重要研究成果

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三、　計畫對民眾具教育宣導之研究成果

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四、　簡述全程計畫成果之討論與結論，如有技術移轉、技術推廣或業界合作，請概述情形及成效

五、　成效評估（技術面、經濟面、社會面、整合綜效）

六、　下年度工作構想及重點之妥適性

七、　檢討與展望

This is a first-year report for the 3-years contract grant funded by National Healthy Research Institute (NHRI). First, we deeply appreciate for the funding support from NHRI to provide us the opportunity to conduct this study. With this support, in the first year, 2 SCI papers and 6 conference reports (including 3 international conference reports) have been produced.

The initial goal of this proposal is to test the hypothesis whether or not the phenotypes of Hp (Hp 1-1, 2-2, and 2-1) are related to the patients with coronary artery disease (CAD) and to explore the mechanisms by which oxidized LDL stimulates the expression of Hp in SMC and macrophages. Utimately, the long-term goal is to focus on the functional role of Hp in atherogenesis. The specific aims in the next 3 years are to:

1. Develop a new and fast approach for human Hp phenotping (Hp 1-1, 2-2, and 2-1) using monoclonal antibodies, and to test the hypothesis whether or not Hp phenotypes are correlated to the patients with coronary artery disease in Taiwan (2003-2005).

2. Study the Hp mRNA expression in macrophages and SMC in the presence of oxidized LDL and lipoproteins VLDL, LDL, and HDL (2003-2004).

3. Test the hypothesis that Hp may exert an antioxidant role in macrophages and SMC transfected with sense and antisense Hp cDNA; to define the antioxidant activity of Hp phenotypes in vitro (2003-2004).

4. Investigate the functional role of Hp in the progression of atherosclerosis using cholesterol-fed rabbits and antiatherogenic effect (if any) using Hp transgenic mice (2004-2005).

With respect to the functional role of Hp, it is essential to evaluate and develop a convenient purification method of Hp. Hemoglobin (Hb)-affinity chromatography and antibody-affinity chromatography were evaluated to purified Hp, respectively. Due to the high-affinity between Hp and Hb, Hb-affinity chromatography was first used to purify Hp. However, because the interaction between the subunits of Hb was ionic interaction, Hb lost degressively. However, a better method, 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. Those two methods described above were both published.

To distinguish whether the phenotypes of Hp (Hp 1-1, 2-2, and 2-1) are related to the patients with coronary artery disease or not, a convenient method was also evaluated successfully. Plasma (or serum) was premixed with Hb and a native PAGE was then performed. Subsequently, 3,3'-diaminobenzidine (DAB) was used as chromogenic substrate. Due to the pseudo-peroxidase activity, Hb and Hp-Hb complex would be represented as brown bands in the native PAGE. According to the chromogenic pattern, the phenotypes of Hp could be identified. Since we have developed a useful typing method, about 1,000 CAD patients' serum from Taipei Veterans General Hospital were under analyzing and gathering statistics.

Moreover, 6 lines of monoclonal antibodies (mAb) prepared against Hp have also been produced and analyzed. The affinities among each mAb and each phenotype Hp were characterized using ELISA. The highest-affinity monoclonal antibody exhibited an affinity (Ka) $5.58 \times 10^9$, $2.81 \times 10^9$, and $2.19 \times 10^9$ $M^{-1}$ against Hp 1-1, 2-1, and 2-2, respectively. The 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. 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.

In addition, the CD spectrum of each Hp phenotype was determined to estimate the secondary structure of Hp. We are able to show, for the first time ,

that the α-helical content of Hp 1-1, 2-1, and 2-2 was 29%, 22%, and 21%, respectively. Hp 1-1 possesses a more ordered structure than that of polymeric forms of Hp 2-1 and 2-2. It is the first time the CD spectra of each phenotype were presented.

To investigate *in vitro* antioxidant role of Hp, thiobarbituric acid-reactive substances (TBARS) was used to estimate antioxidant activity of Hp in lipid peroxidation. We demonstrated that Hp molecule itself was an extremely potent antioxidant activity in $Cu^{2+}$- and AAPH-induced lipid peroxidation. The AAPH (a hydrophilic decomposed radical initiator) experiment could produce a similar antioxidant effect of Hp suggesting a scavenge role of Hp.

The overall three-dimensional structure of Hp proved to be not exclusively involved in maintaining its antioxidant activity, since blockage of disulfide linkages causing drastic conformational changes of Hp did not detriment the activity. Beyond our expectation, the activity was remarkably greater (4 x) than that of native Hp with a dose-dependent manner. Either phenotype of CM-Hp possessed the enhancement effect. Hypothetically, we speculate that the region(s) exerting the antioxidant activity were exposed entirely or in a large extent. However, the mechanism was unclear by which Hp 1-1 possessed differentially higher than Hp 2-1 and 2-2 when polymerization form of Hp 2-1 and 2-2 were no longer existed as shown on the gel. Because the major structural difference among the Hp phenotypes lies on the amino-acid sequence of α-chains, it would be of great interest to analyze the antioxidant property in α-chains. Identification and understanding of the biochemical basis for the differences among Hp phenotypes may lead to a rational design in intervening new pharmacological agents. A mini-Hp antioxidant can thus be proposed in light of the present finding that overall structure of Hp was not absolutely

crucial. This experiment is now in progress in our laboratory to explore such possibility.

Furthermore, the structural diversity of Hp phenotype either in amino-acid sequence or polymeric form with respect to Hp antioxidant property may explain, in part, the clinical outcome by which Hp phenotype is associated with differential susceptibility to infections, atherosclerosis, and autoimmune disorders. The correlation between the phenotype-dependent modulation of oxidative stress and prostaglandin synthesis has been reported. In general, patients with Hp 2-2 are more susceptible in developing the severity of the diseases mentioned above. For example, Hp 2-2 and 2-1 are associated with an increased risk for the development of nephropathy in patients with diabetes mellitus. Nakhoul *et al.* have postulated that the differences in the molecular shape and size between the Hp 1-1 and 2-2 are involved. Melamed-Frank *et al.* further demonstrate that the antioxidant effect of Hp 1-1 is superior to Hp 2-1 and 2-2 in hemoglobin-induced oxidation, a mechanism proposed to be mediated by forming Hp-Hb complex. Nevertheless, our study shows that Hp possessed an extraordinary antioxidant activity. The potency remarkably exceeded to that clinically used antioxidants, probucol and vitamin E.

To ascertain the cellular Hp could prevent cell damage from oxidative stress, CHO-K1 cells were transfected with the CMV promoter-driven expression vectors, which could express and secret Hp 1-1. It is of worth mentioning that CHO cells were chosen because they did not express Hp mRNA in our preliminary study. Subsequently, we show that the Hp transfected cells could express Hp in both medium (Western blot) and cytosol (immunostaining). These transfected cells ultimately elevated the ability in resisting the oxidative damage. The data further substantiate our hypothesis that Hp plays a

provocative antioxidant role.

In conclusion, the present study not only indicates that Hp is an extremely potent antioxidant molecule, but also confirms its ex vivo 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, the data suggest that in addition to its role in forming Hp-Hb complex to scavenge the free radicals generated from Hb, Hp may participate an independent antioxidant role in those cells expressing Hp. Whether or not Hp may be directly beneficial for those free-radical associated diseases, such as atherosclerosis and myocardial infarction, will be a subject of interest and challenge.

## ＜計畫對民眾具教育宣導之研究成果＞

本報告為『Haptoglobin 在動脈硬化中所扮演之功能角色』三年計畫之第一年成果報告，目前已發表二篇 SCI 國際期刊報告、二篇國際研討會論文與三篇國內研討會論文。由於 Haptoglobin 之結構複雜並有三個亞型（1-1、2-1 及 2-2），因此無法以離子交換樹脂管柱純化，在此研究報告中，我們利用血紅素及抗體親和性管柱成功純化出人類 Haptoglobin，這對未來功能性研究有極大幫助。在抗氧化特性之研究中，Haptoglobin 較已知最強之抗氧化化合物 probucol 具有更強之功能，而且經過化學修飾後，抗氧化特性更加強了 5 倍。除此之外，更利用基因重組的方式，將 Haptoglobin 表現在中國大倉鼠卵巢細胞，發現在氧化壓力存在的環境下，會表現 Haptoglobin 的細胞存活率較不會表現的細胞來的高，更直接證明了 Haptoglobin 在細胞模式中確實具有抗氧化的功能。

## 伍、九十二年度計畫所培訓之研究人員

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## 陸、參與九十二年度計畫所有人力之職級分析

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| 第二級 | 副研究員、副教授、總醫師、助教授 | 人 |
| 第三級 | 助理研究員、講師、住院醫師 | 3 人 |
| 第四級 | 研究助理、助教、實習醫師 | 9 人 |
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# Purification of human plasma haptoglobin by hemoglobin-affinity column chromatography

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**Abstract**

Haptoglobin (Hp) is an acute-phase protein; its plasma levels increase consistently in response to infection and inflammation. The concentration of human plasma Hp is ranged between 1 and 1.5 mg/ml. Similar to blood type, individual human Hp is classified as Hp 1-1, 2-1, or 2-2. The structural and functional analysis of the Hp, however, has not been studied in detail due to its difficult isolation procedure. Previously, we reported a single step for the purification of porcine Hp. In this study, we established a purification method using a high capacity hemoglobin-affinity column. Briefly, DEAE-purified human hemoglobin was first coupled to Sepharose 4B to prepare an affinity column in a 15-ml bed volume. Following a flow through of human plasma and an extensive wash, the bound material was eluted with a solution of 0.15 *M* NaCl, pH 11 (adjusted by ammonium), to remove low-affinity bound proteins. The high-affinity bound Hp was then eluted with 0.15 *M* NaCl containing 5 *M* urea, pH 11, and collected in tubes containing 100 μl of 1 *M* Tris buffer, pH 7.0. The biological activity of dialyzed Hp was retained as it formed a complex with hemoglobin on a sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Using this procedure, approximately 10 mg of Hp 1-1, with homogeneity greater than 96%, was obtained from 15 ml of human plasma. Affinity purified Hp 2-1 or 2-2, however, contained trace amounts of apoA-I with the similar approach. The Hp could be further purified by HPLC using a Superose 12 gel-permeation chromatography, if desired, to achieve 100% purity. All the phenotypes of purified Hp consisted of α and β chains on SDS–PAGE in the presence of a reducing reagent, further confirmed by a Western blot analysis. We conclude that human hemoglobin-affinity column was most suitable for the isolation of Hp 1-1 in large quantities. Whereas, one additional step using a gel-permeation was necessary for that of Hp 2-1 and 2-2.

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*Keywords:* Affinity adsorbents; Haptoglobin; Proteins; Glycoproteins

## 1. Introduction

Haptoglobin (Hp), also known as an α-2 glycoprotein, is a hemoglobin-binding protein present in plasma of all vertebrates and is believed to participate in hemoglobin transport. The concentration of Hp in human plasma is relatively high ranging from 1.0 to 1.5 mg/ml [1,2], which may increase as an acute-phase protein in response to a variety of injuries and inflammatory disease states [3,4]. For this reason, Hp is useful as a diagnostic marker and as a clinical evaluation of many inflammatory diseases. Human Hp is a tetrameric structure linked by disulfide linkages among the two α and two β chains

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doi:10.1016/S1570-0232(03)00128-4

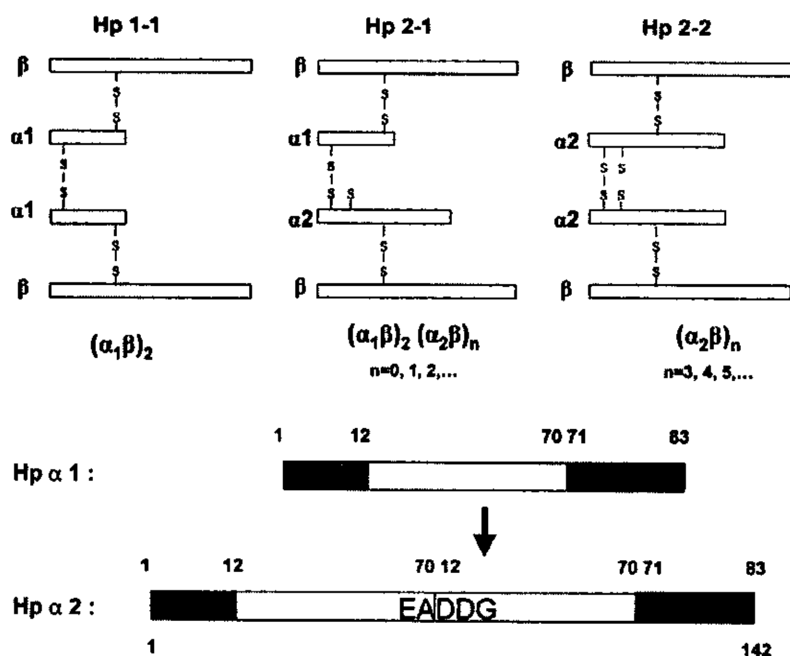


Fig. 1. Schematic drawing of the phenotype structure of human Hp 1-1, 2-1, and 2-2 (top). All three phenotypes share a common structure of β chains. The degree of polymerization within the inter-molecular arrangement is shown. The difference between α1 and α2 chains and their combinations determine the Hp phenotype (bottom). α2 is a duplicate of α1 with a repeat insert of residues 12–70. Making it simple, α2 (142 amino acids) contains two repeated domains showing a unique sequence of EADDG (residues 69–73) at the splicing site.

[5–7]. Based on the length of α chains, there are three phenotypes of Hp in the population, Hp 1-1, 2-1, and 2-2 (Fig. 1), which are similar to that of blood types. All the phenotypes share the same two β chains [each with about molecular mass ($M_r$) 40 000 including 243 amino acids and approximate 30% (w/w) carbohydrate moiety] [5–7]. A typical structure of homozygous Hp 1-1 is composed of two identical α 1 chains (each with about $M_r$ 9000 including 83 amino acids). Whereas, Hp 2-2 is composed of two identical α 2 chains (142 amino acids) as compared to that of heterozygous Hp 2-1 containing each α 2 and α 1 (Fig. 1). Likewise, the tetrameric arrangement is also found in other animal species such as rat, rabbit, and pig [3,8–10]. However, the two αβ units joined by a non-covalent interaction, rather than a disulfide bridge, are found in dog, cat, and bear [11,12].

Several functional differences between Hp phenotypes have been demonstrated, appearing to have important biological and clinical consequences [1,2,13,14]. For example, patients with phenotype Hp 1-1 are less prone to the development of inflammatory-related cardiovascular diseases and diabetics than that with Hp 2-1 and 2-2 [13–15]. Although the protein has been well characterized genetically, the exact physiological role and the biochemical mechanism by which Hp 1-1 is more resistance to those inflammatory-related diseases are not well understood. The later has been hampered by the availability of Hp, which is mainly due to the considerably difficult procedures for Hp purification. Commercially prepared Hp is not only expensive lacking the biological activity, but also heterogeneous containing the mixture of three phenotypes isolated from the plasma pools.

Currently, the most common procedures involved for the purification of human Hp are associated with electrophoresis, affinity chromatography using a monoclonal antibody, and multiple high-performance liquid chromatography (HPLC) steps [16–20]. These methods are useful but are troublesome and time-consuming, and the quantity of Hp obtained is relatively small. Affinity column purification using chicken hemoglobin has been reported [19,21]. The binding affinity of chicken hemoglobin to human Hp,

however, is less than that of human hemoglobin [1,2]. The method [21] was satisfactory for Hp purification in species other than humans, but required a few column-steps for human Hp [19]. Previously, we established a simple purification method with high yield for porcine plasma Hp. The method, however, was not practical in the isolation of human samples due to the heterogeneity of human Hp [22]. In the present report, we describe a purification procedure for human Hp 1-1, 2-1, and 2-2 using an affinity column that was immobilized with highly purified human hemoglobin. Approximately 8–10 mg of human Hp can be obtained from 15 ml of plasma. The procedure can be easily scaled up for Hp 1-1 purification. A simple hemoglobin isolation procedure using an isocratic DEAE HPLC system is also described.

## 2. Experimental

### 2.1. Materials

Goat anti-human haptoglobin was purchased from Calbiochem-Novabiochem (San Diego, CA, USA). All other chemicals were purchased from Sigma (St. Louis, MO, USA) and Merck (Darmstadt, Germany) without any further purification.

### 2.2. Preparation and purification of human hemoglobin

Fresh human blood collected in 0.1% EDTA was immediately centrifuged at 3000 *g* for 25 min, after which time plasma was removed by aspiration. The remaining red blood cells (RBCs) were washed five times with three volumes of phosphate-buffered saline (PBS) containing 0.12 *M* NaCl and 12 m*M* phosphate, pH 7.2, and then lysed with two volumes of deionized water at 4 °C. Cell debris was removed by centrifugation at 3500 *g* for 30 min. The supernatant containing mostly hemoglobin was fractionated by 50% saturated ammonium sulfate at 4 °C for 30 min followed by a centrifugation at 4500 *g* for 40 min at 4 °C. The supernatant was dialyzed against 0.02 *M* sodium phosphate, pH 8.0, at 4 °C overnight followed by a filtration through a 0.45 μm nylon fiber prior to HPLC.

The HPLC system (Waters) consisted of two pumps, an automatic sample injector, a photodiode array detector, and an interface module [23,24]. A Bio-Scale DEAE column (10×1.5 cm) packed with an anion-exchange Macro-Prep and equilibrated with 20 m*M* phosphate buffer, pH 8.0, at a flow-rate of 1 ml/min, was used for hemoglobin purification. Partially purified ammonium sulfate fraction of hemoglobin (total 50 mg in 2 ml) was applied to the column followed by an elution with the initial buffer at a flow-rate of 1 ml/min. The chromatographic profile was monitored by a photodiode array detector and read at 280 nm.

### 2.3. Preparation of human hemoglobin-affinity column

DEAE-purified human hemoglobin was first coupled to CNBr-activated Sepharose-4B (Pharmacia, Uppsala, Sweden) according to the manufacturer's procedures. Briefly, 5 g of freeze–dried Sepharose was swollen and suspended in 1 m*M* HCl and immediately washed 3× within 15 min with the same solution on a glass filter [23,25]. The gel was then washed with a coupling buffer containing 0.1 *M* $NaHCO_3$, and 0.5 *M* NaCl, pH 8.0, and subsequently degassed. A 2-ml volume of hemoglobin (25 mg/ml), pre-dialyzed in the coupling buffer, was slowly added to the gel (in 25 ml), while gently stirring for 1 h at room temperature. After coupling, the gel was washed 3× with the coupling buffer (200 ml) to remove uncoupled hemoglobin via a glass filter. Finally, the gel was treated with 0.1 *M* Tris–HCl, pH 8.0, for 2 h at room temperature to saturate the remaining reactive sites of Sepharose. The coupling efficiency of hemoglobin to gel was approximately of 98%. The degassed gel was then packed onto a 20×1.5 cm column and extensively washed with two cycles of PBS, pH 7.2, and 0.15 *M* NaCl, pH 11, which was adjusted by ammonium as previously described [25].

### 2.4. Isolation of Hp by human hemoglobin-affinity column

Initially, 15 ml of human plasma was loaded onto the hemoglobin-affinity column (15 ml in bed volume) at room temperature without incubation, fol-

lowed by an extensive wash with 200 ml of PBS. The bound materials were first eluted with three volumes of 0.15 *M* NaCl, pH 11 (adjusted by ammonium), as fraction 1 [25] and then eluted with three volumes of 5 *M* urea in 0.15 *M* NaCl, pH 11 (freshly prepared and filtered), as fraction 2. A 5-ml volume of each fraction was collected in a tube containing 0.1 ml of 1 *M* Tris–HCl, pH 7.0, to immediately neutralize the pH value. Pooled fractions containing Hp were then dialyzed at 4 °C overnight with three changes of PBS.

### 2.5. Gel electrophoresis and densitometry

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and native PAGE were performed according to the Laemmli's method [26] with some modification as previously described [22]. Samples (typically 10 μg) for SDS–PAGE were preheated at 100 °C for 10–15 min in an SDS loading buffer [50 m*M* Tris–HCl, 2% (w/v) SDS, 100 m*M* 2-mercaptoethanol, pH 6.8]. For molecular mass calibration, a subset of the following standards was included in each gel: β-galactosidase (116 000), phosphorylase B (97 000), bovine serum albumin (BSA, 66 000), ovalbumin (45 000), carbonic anhydrase (31 000), soybean trypsin inhibitor (21 500), lysozyme (14 400), and aprotinin (6500). The samples were run for 0.5 to 1 h at 120 V and stained by a Coomassie brilliant blue G-250. Densitomertic analysis of SDS–PAGE gel was performed using a Molecular Dynamics densitometer for data acquisition and Image Quant software for integration and analysis.

### 2.6. Immunoblot analysis

Following the separation of proteins by SDS–PAGE, the gel was soaked in a transfer buffer containing 50 m*M* Tris–HCl, 50 m*M* boric acid, and 1 m*M* EDTA, pH 8.2, for 30 min. The gel was then transferred to a nitrocellulose membrane (Pharmacia) at 100 mA for 1 h in a semi-dry transfer cell (Bio-Rad) containing a transfer buffer. The membrane was immersed in 1% BSA, Tween-containing Tris-buffered saline (TTBS) [20 m*M* Tris–HCl, 50 m*M* NaCl, 0.05% (w/v) Tween 20, pH 7.4] for 1 h with gentle shaking at room temperature. Following a wash with TTBS for 3 min, the membrane was incubated with a primary antibody [1:2500 dilution in TTBS containing 1% (w/v) BSA] for 1 h at room temperature and washed three times with TTBS. The membrane was then incubated with 1:5000 diluted antiserum against goat immunoglobulin G (IgG) conjugated with horseradish peroxidase for 1 h in TTBS containing 1% (w/v) BSA. Finally, the membrane was washed three times with TTBS and developed into a color immunoblot with 3,3′-diaminobenzidine (DAB)-stabilized substrate for horseradish peroxidase [22].

## 3. Results

### 3.1. Purification of human hemoglobin

A typical HPLC profile for the purification of ammonium sulfate fractionated hemoglobin is shown in Fig. 2. Both SDS–PAGE and native-PAGE analyses show that the homogeneity of purified hemoglobin was greater than 96% (Fig. 3).

### 3.2. Preparation of hemoglobin-affinity column

In theory, the purity of human hemoglobin obtained from ammonium sulfate fractionation was

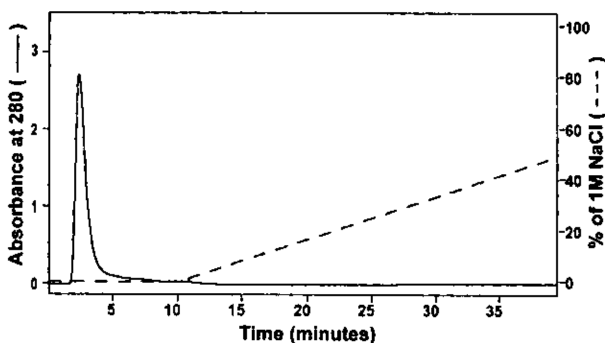


Fig. 2. Typical purification profile of human hemoglobin on DEAE HPLC. About 5 mg of 50% saturated ammonium sulfate top fraction was applied to a DEAE column (10×1.5 cm) pre-equilibrated with 20 m*M* phosphate, pH 8.0. A mobile phase containing the same buffer was run through for 10 min at a flow-rate of 1 ml/min. A linear gradient was produced from 0 to 0.3 *M* of NaCl to regenerate the DEAE resin. The same procedure was used for the purification of hemoglobin in large scale (total of 50 mg in 2 ml) as described in the Experimental section.

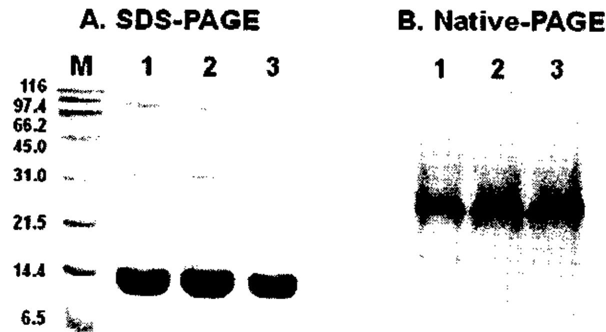


Fig. 3. Analyses of purified human hemoglobin using SDS–PAGE (A) and native-PAGE (B). (A) Lane M represents the molecular mass markers (molecular masses $\times 10^{-3}$). Lanes 1–3 represent hemoglobin obtained from the RBC lysate, top fraction of 50% saturated ammonium sulfate precipitation, and DEAE HPLC, respectively. Approximately 15 μg of each protein was loaded on an 18% SDS–PAGE in the presence of a reducing reagent. (B) Lanes 1–3 represent hemoglobin obtained from RBC lysate, top fraction of 50% saturated ammonium sulfate precipitation, and DEAE HPLC, respectively. Each protein was loaded on a 10% native-PAGE.

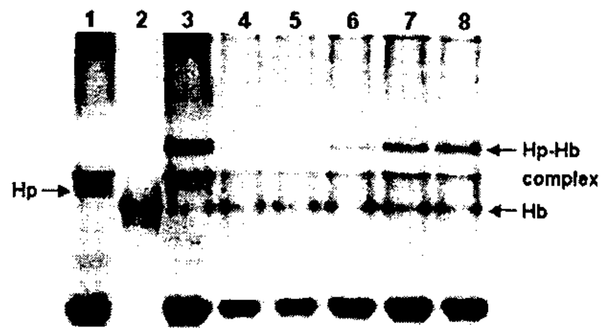


Fig. 4. Evaluation of binding capacity of hemoglobin-conjugated Sepharose to Hp in plasma. Briefly, plasma containing Hp was passed through 1 ml of conjugated Sepharose column. The pass-through fraction was collected and mixed with free hemoglobin. The unbound or remaining Hp, if any, was able to form a Hp–hemoglobin complex displaying an extra band in a 7% native-PAGE. Lanes: 1=human plasma prior to the affinity column; 2=purified hemoglobin; 3=plasma spiked with purified hemoglobin showing a Hp–hemoglobin complex; 4–8=samples of 0.25, 0.5, 0.75, 1.0, and 2.0 ml plasma passed through a hemoglobin-Sepharose containing 1 mg of hemoglobin, respectively. No Hp was detected in lanes 4 and 5 when 0.25–0.5 ml of plasma was applied onto the conjugated Sepharose. According to our calculation, 1 mg of hemoglobin coupled on Sepharose could bind about 0.75 to 1.13 mg Hp (lanes 5 and 6).

adequate in preparing affinity column for Hp purification as that described using chicken hemoglobin [19]. However in a preliminary application, we found that column immobilized with ammonium sulfate fraction of hemoglobin could produce significant plasma clots and subsequently demolished the chromatography (data not shown). This clotting effect, however, was not observed when DEAE-purified hemoglobin was employed for affinity column. Using native-PAGE to evaluate the binding capacity of Sepharose 4B immobilized with human hemoglobin, the capacity we estimated was approximately between 0.75 and 1.13 mg of Hp 1-1 per mg of hemoglobin (Fig. 4). This binding capacity was 20-times greater than that reported using chicken hemoglobin [19].

### *3.3. Isolation of human Hp by hemoglobin-affinity column chromatography*

Fig. 5 shows a typical chromatography using an affinity column conjugated with highly purified human hemoglobin. Initially, 15 ml of plasma of Hp 1-1 was applied to the column followed by an extensive wash (Fig. 5); the bound protein was first eluted with 0.15 *M* NaCl, pH 11 (fraction 1) to remove the low-affinity binding proteins such and apoA-I. The column was then eluted with 0.15 *M* NaCl containing 5 *M* urea, pH 11 (fraction 2) for high-affinity binding Hp. Each eluent was immediately neutralized in the tube containing 100 μl of 1 *M* Tris–HCl, pH 7.0 (Fig. 5). SDS–PAGE analysis on fraction 1 revealed that it contained mostly high-molecular-mass proteins and apoA-I (Figs. 6 and 7), but not in fraction 2. The purity of Hp 1-1 in fraction 2 was approximately 96%. The recovery of Hp in fraction 2 was approximately 45.5% from the plasma with a final of 77-fold purification (Table 1). Under the same condition, however, some apoA-I was found to be co-eluted in the fraction 2 of Hp 2-1 and 2-2 (Figs. 6 and 7). The contaminated apoA-I could be further removed (data not shown) using a single step on HPLC Superose 12 as previously described by us [22]. A typical Western blot analysis showing three isolated phenotypes of Hp is depicted in Fig. 7. The presence of apoA-I in Hp 2-1 and 2-2 was

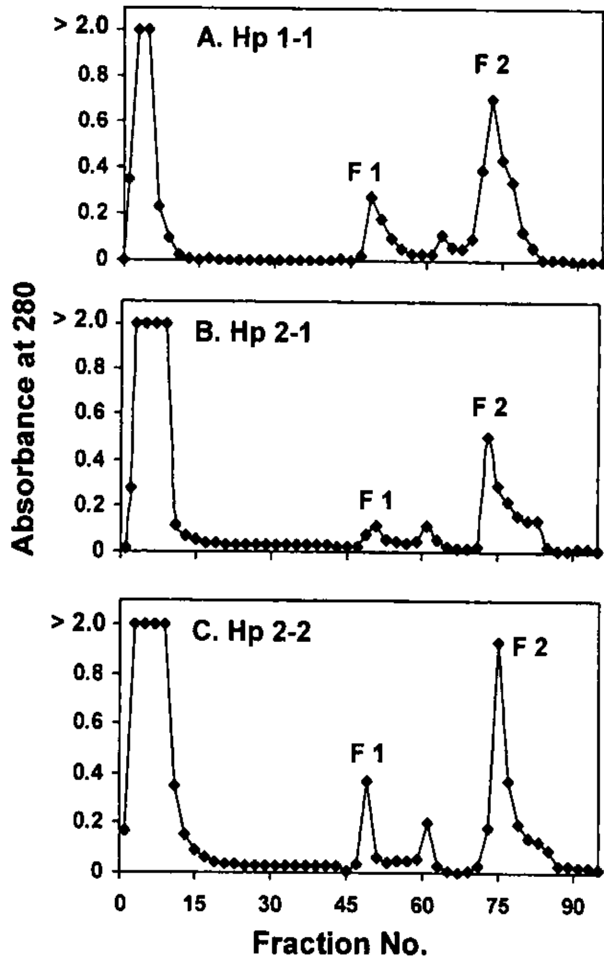


Fig. 5. Typical elution profile of hemoglobin-affinity column chromatography of plasma containing Hp 1-1 (A), Hp 2-1 (B), and Hp 2-2 (C). Initially, 15 ml of human plasma was applied to the hemoglobin-affinity column followed by an extensive wash with 200 ml of PBS. The bound materials were first eluted with three volumes of 0.15 *M* NaCl, pH 11 (adjusted by ammonium), as fraction 1 and then eluted with three volumes of freshly prepared and filtered 5 *M* urea in 0.15 *M* NaCl, pH 11, as fraction 2. A 5-ml volume of each fraction was collected in a tube containing 0.1 ml of 1 *M* Tris–HCl, pH 7.0, to immediately neutralize the pH value.

unavoidable using hemoglobin-based affinity column and was confirmed by a monoclonal antibody prepared against apoA-I (Fig. 7). Nevertheless, the major isolation procedure was simple and can be achieved within a few hours. This procedure should be widely used for the purification of Hp and particularly for 1-1 phenotype.

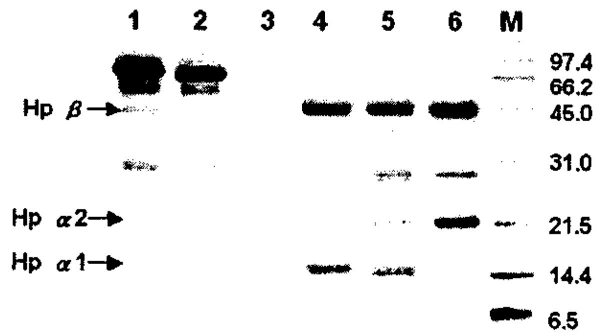


Fig. 6. Analyses of isolated Hp from affinity column on 15% SDS–PAGE. Lanes: M=molecular mass markers, 1=plasma of Hp 1-1, 2=a typical pass-through fraction (Hp 1-1 plasma) from hemoglobin-affinity chromatography, 3=a typical sample from fraction 1 (Hp 1-1) contaminated mostly apoA-I and proteins with large molecular mass. Lanes 4 and 6 represent fraction 2 of isolated Hp 1-1, 2-1, and 2-2, respectively. Notably, apoA-I is co-eluted in Hp 2-1 and 2-2.

## 4. Discussion

The acute phase serum protein, Hp, response to infection, inflammation, and trauma has been identified in a number of species. Methods designed for large isolation of human Hp have been complicated and time-consuming. We have recently described a single-step purification procedure for porcine Hp

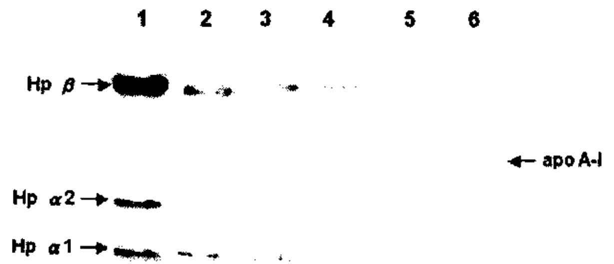


Fig. 7. Western blot analyses on affinity-purified human Hp 1-1, 2-1, and 2-2 by a goat antibody prepared against human haptoglobin. Lanes: 1=Hp standard purified from a human plasma pool, 2–4=affinity-purified Hp 1-1, 2-1, and 2-2, respectively, 5 and 6=the apoA-I co-eluted in affinity-purified Hp 2-1 and 2-2 fractions by a mouse monoclonal antibody prepared against human apoA-I. Purified Hp 1-1 did not reveal immunoreactive apoA-I (data not shown).

Table 1
Analytical recovery of haptoglobin 1-1 purified from 15 ml human plasma

| Total protein from plasma (mg) | Theoretical amount of Hp (mg) | Total Hp yield (mg) | Final yield (%) | Purity (%) | Fold purification |
|---|---|---|---|---|---|
| 1755 | 22 | 10 | 45.5 | >96 | 77 |

using HPLC gel-permeation chromatography in the presence of 5 *M* urea [22]. The procedure, however, could not be reproduced in human Hp isolation. Presumably, the human Hp structure is more complicated in its polymerization nature (Fig. 1) than that of pig. Thus, the purification for human Hp has been hampered by its structural diversity as each Hp 1-1, 2-1, and 2-2 has average molecular masses of 100 000, 220 000, and 400 000, respectively (Fig. 1). Although the procedure using a salting-out of plasma proteins followed by anion-exchange chromatography has been recommended, the reproducibility (including the yield) is rather poor due to the heterogeneity of its polymerization form of Hp 2-1 and 2-2 [20,27,28]. It is almost not feasible to isolate Hp 2-2 as a pool and to study its biochemical properties.

An immunoaffinity chromatography method to purify human Hp had been developed using a two-monoclonal antibody system [20], in which the phenotypes and the final purity of Hp were not specified. The yield, on the other hand, is limited and utilized only for the preparation of antigen and polyclonal antibodies [20].

With respect to hemoglobin-affinity column, Rademacher and Steele [19] have reported use of Sepharose immobilized with chicken hemoglobin. However, an attempt using human hemoglobin for the purification of human Hp was unsuccessful [19]. The method we employed was different from that of chicken hemoglobin-Sepharose chromatography. First, our human hemoglobin-Sepharose had a binding capacity 0.75–1.00 mg Hp/mg hemoglobin that was about 20 times greater than that of using chicken hemoglobin (Fig. 4). Second, our results demonstrated that highly purified hemoglobin via DEAE chromatography should be used for the affinity column rather than a crude extract of hemoglobin from ammonium sulfate fraction described previously [19]. Under this condition, the formation of plasma clots in the column could be eliminated. Third, the pH of each eluted fraction was immediately neutralized by a 1 *M* Tris buffer, pH 7.0, to restore the biological activity (complex formation between Hp and hemoglobin). It is worth mentioning that ammonium, rather than a high-capacity buffer solution, was used for adjusting the final pH of the saline solution (pH 11) in eluting the Hp; this was because the eluent could be easily neutralized by a Tris buffer. A similar procedure was employed previously in our laboratory [25]. Fourth, the most contaminants of proteins that bound weakly or non-specifically to the affinity column were differentially removed using pH 11 saline solution (Fig. 5, fraction 1). Fifth, unlike phenotypes Hp 2-1 and 2-2, Hp 1-1 could be isolated without apoA-I contaminant, as confirmed by a Western blot analysis (Fig. 7).

In the present study, about 8–10 mg of Hp 1-1 could be isolated from 15 ml of human plasma in one isolation. A similar yield of Hp 2-1 and 2-2 was obtained, but it required a further gel-filtration to remove apoA-I. The mechanism by which the affinity column favored the Hp 1-1 purification is not readily clear. Since Hp 2-1 and 2-2 molecules are largely polymerized by disulfide linkages with molecular weights ranging from 153 000 to 1 200 000 [6], these polymers may more accessibly "trap" the apoA-I than that of monomeric Hp 1-1. To address this assumption, we applied purified-apoA-I [25] directly to the affinity column. There was no apoA-I binding to the column suggesting that apoA-I did not interact with hemoglobin in the absence of Hp (data not shown). On the other hand, apoA-I may weakly bind to Hp and therefore was co-eluted with Hp during the purification. Regardless, the apoA-I deficient plasma, which can be easily obtained by a simple ultra-centrifugation for the removal of high-density lipoproteins [25], may be ultimately considered for the purification of all Hp phenotypes. This experimental procedure is currently in progress in our laboratory.

In conclusion, human hemoglobin could be con-

veniently isolated in large quantities by ammonium sulfate fractionation followed by a HPLC DEAE column. Immobilized human hemoglobin had a binding capacity about 20-times greater than that of chicken hemoglobin and could be more suitable for the purification of phenotype Hp 1-1. Accordingly, the procedure described in this report can be simply scaled up using a 100-ml bed affinity column for even larger Hp purification. This Hp purification procedure is currently used in our laboratory; the resulting Hp has been utilized in studying the structural and functional relationship and preparing polyclonal and monoclonal antibodies.

## Acknowledgements

This work was supported by grants NHRI-EX92-9229SI (S.J.T.M.) from the National Health Research Institute, and NSC 89-2313-B-009-001-A20 (S.J.T.M.) and NSC 90-2314-B-075-099 (J.P.P.) from the National Science Council, Taiwan. The authors thank Ms. Yu-Chi Pong for her dedicated administrative assistance.

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Protein Expression and Purification xxx (2003) xxx–xxx

Protein Expression & Purification

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# Purification of human haptoglobin 1-1, 2-1, and 2-2 using monoclonal antibody affinity chromatography

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Received 11 August 2003, and in revised form 23 September 2003

**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.

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

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 2α and 2β chains [4–6]. Based on the length of α chains, 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 2β chains (each with about $M_r$ 40,000 kDa containing 243 amino acids and approximately 30% carbohydrate). A typical structure of homozygous Hp 1-1 is composed of two identical α1 chains (each with about $M_r$ 9000 kDa containing 83 amino acids). Homozygous Hp 2-2 is composed of two identical α2 chains (each with about $M_r$ 16,500 kDa 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 two 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].

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1046-5928/$ - see front matter © 2003 Published by Elsevier Inc.
doi:10.1016/j.pep.2003.09.006

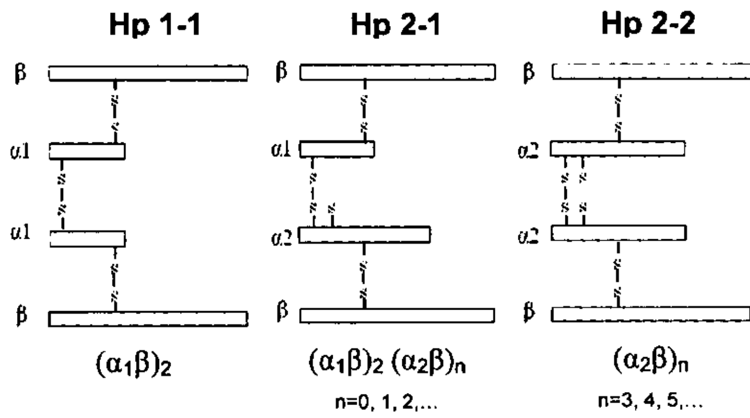


Fig. 1. Schematic drawing of proposed structure of human Hp 1-1, 2-1, and 2-2. All three phenotypes share a common structure of β chains (please also see [4]). The degree of polymerization within the inter-molecular arrangement is shown.

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. [18] 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. Meanwhile, human apolipoprotein A-I appears to be another major contaminant. Wassdal et al. [19] use rabbit hemoglobin–Sepharose; the hemoglobin is still co-eluted from the column. Travis et al. [20] employ Sephadex G-200 gel filtration, but the purified Hp is accompanied with large amounts of IgM and α-2 macroglobulin. Morimatsu et al. [21] 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). Although Katnik et al. [22] 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. 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) 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 was 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.

121 *Preparation of antibody affinity column*

122 Dialyzed monoclonal antibody was first coupled to
123 CNBr-activated Sepharose-4B (Pharmacia, Uppsala,
124 Sweden) according to manufacturer's procedures.
125 Briefly, 2.86 g of freeze-dried Sepharose (1 g of freeze-
126 dried powder gave about 3.5 ml final volume of gel)
127 was swollen and suspended in 1 mM HCl and imme-
128 diately washed with 20× volume of the same solution
129 within 15 min on a sintered glass filter [24–26]. The gel
130 was then washed with coupling buffer containing 0.1 M
131 $NaHCO_3$ and 0.5 M NaCl, pH 8.3, and degassed.
132 About 10 ml (18.7 mg/ml) of ammonium-sulfate frac-
133 tion of monoclonal antibody in coupling buffer was
134 slowly added into the gel (in 15 ml), while gently stir-
135 ring by a magnetic bar for 1 h at room temperature.
136 After coupling, the gel was washed with 10× volume of
137 PBS to remove unbound materials via a sintered glass
138 filter. The gel was then treated with a blocking solution
139 containing 0.1 M Tris-HCl and 0.5 M NaCl, pH 8.0,
140 for 2 h at room temperature to saturate the remaining
141 reactive-sites. The degassed gel was then washed with 3
142 cycles of blocking buffer and a 0.15 M NaCl solution of
143 pH 11.0 (adjusted by ammonium) according to the
144 procedures previously described by us [26]. Finally, the
145 gel was equilibrated in PBS and packed onto a
146 1.5 × 20 cm column.

147 *Purification of human Hp using antibody affinity-column*
148 *chromatography*

149 Initially, 1 ml of filtered human plasma of each Hp-
150 phenotype batch was loaded onto the antibody affinity-
151 column (10 ml in bed volume) at room temperature.
152 The column was then washed with 50 ml PBS. The
153 bound materials were washed further with 50 ml of
154 0.02 M phosphate buffer containing 0.2 M NaCl, pH
155 7.4, and then eluted with 50 ml of a freshly prepared
156 0.15 M NaCl solution with pH 11 adjusted by ammo-
157 nium [26]. Five milliliters of each fraction was collected
158 in a tube containing 0.25 ml of 1 M Tris–HCl buffer,
159 pH 6.8, to immediately neutralize the pH value. Pooled
160 fractions containing Hp were then concentrated to a
161 final volume of 1 ml using an Amicon centrifugal filter
162 (Millipore).

163 *Further purification of Hp by gel filtration column*

164 Concentrated solution with Hp was filtered with a
165 0.45 μm nylon fiber prior to HPLC. The HPLC sys-
166 tem (Waters) consisted of two pumps, an automatic
167 sample injector and a photodiode array detector.
168 Superose 12 column (1 × 30 cm) (Pharmacia) was used
169 for further Hp purification. The column was pre-
170 equilibrated with 50 mM ammonium bicarbonate.
171 Partially purified Hp (0.8 ml) was applied to the
column at a flow rate of 0.5 ml/min. Fractions con- 172
taining Hp were pooled and concentrated to a final 173
volume of 1 ml using an Amicon centrifugal filter 174
and then lyophilized. The lyophilized Hp was stored 175
at −80 °C until analyzing. 176

*Gel electrophoresis and densitometry* 177

Sodium dodecyl sulfate–polyacrylamide gel electro- 178
phoresis (SDS–PAGE) was performed according to 179
Laemmli's method [27] with some modifications in 180
using 5% polyacrylamide (w/v) on the stacking gel as 181
previously described [7]. Samples (typically 5 μg) for 182
SDS–PAGE were preheated at 100 °C for 10 min in a 183
loading buffer (12 mM Tris–HCl, pH 6.8, 0.4% SDS 184
(w/v), 5% glycerol (v/v), 2.88 mM of 2-mercap- 185
toethanol, and 0.02% bromphenol blue (w/v)). For 186
molecular weight calibration, a subset of the following 187
standards was included in each gel: myosin (200 kDa), 188
β-galactosidase (116 kDa), phosphorylase B (97 kDa), 189
serum albumin (66 kDa), ovalbumin (45 kDa), car- 190
bonic anhydrase (31 kDa), soybean trypsin inhibitor 191
(21.5 kDa), lysozyme (14.4 kDa), and aprotinin 192
(6.5 kDa). The samples were run for about 1.5 h at 193
100 V and stained using Coomassie brilliant blue R- 194
250. Densitometric analysis of SDS–PAGE was per- 195
formed using a Molecular Dynamics densitometer 196
for data acquisition and Image Quant software for 197
integration and analysis. 198

*Immunoblot analysis* 199

Following the separation of proteins by SDS– 200
PAGE, the gel and nitrocellulose- and 3MM filter- 201
papers were soaked in a transfer buffer containing 202
48 mM Tris–HCl, 39 mM glycine, 0.037% SDS (w/v), 203
and 20% methanol (v/v) at pH 8.3 for 30 min. The gel 204
was then electrotransferred to a nitrocellulose mem- 205
brane (Pharmacia) at 90 mA for 1 h in a semi-dry 206
transfer cell (Bio-Rad) containing a transfer buffer. 207
The transferred membrane was then immersed in 5% 208
skimmed milk (w/v) in PBS for 1 h at room temper- 209
ature while shaking gently. After three times washing 210
with PBS for 5 min, the membrane was incubated with 211
a primary goat polyclonal antibody against human Hp 212
(1:5000 dilution in PBS washing buffer containing 1% 213
(w/v) skimmed milk and 0.05% Tween 20 (v/v) for 1 h) 214
at room temperature and washed three times for 215
5 min. The membrane was then incubated with 216
1:10,000 diluted rabbit anti-goat IgG conjugated with 217
horseradish peroxidase in washing buffer for 1 h. In 218
addition, the membrane was washed two times with 219
washing buffer and further washed once with PBS. 220
Finally, the membrane was developed using 3,3′-di- 221
aminobenzidine (DAB) as a substrate for horseradish 222
peroxidase [7,25]. 223

### 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 spectropolarimeter. The obtained spectrum of each type of Hp was accumulated for 20 times at a scanning rate of 50 nm/min and the percentage α-helical content was estimated from the mean residue molar ellipticity ($\theta_{222}$). % α-helix $= [(\theta_{222} + 3000)/(36,000 + 3000)] \times 100$ [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 ($K_a = 5.6 \times 10^9\ M^{-1}$) 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 that 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 step of 51- to 54-fold purification (Table 1).

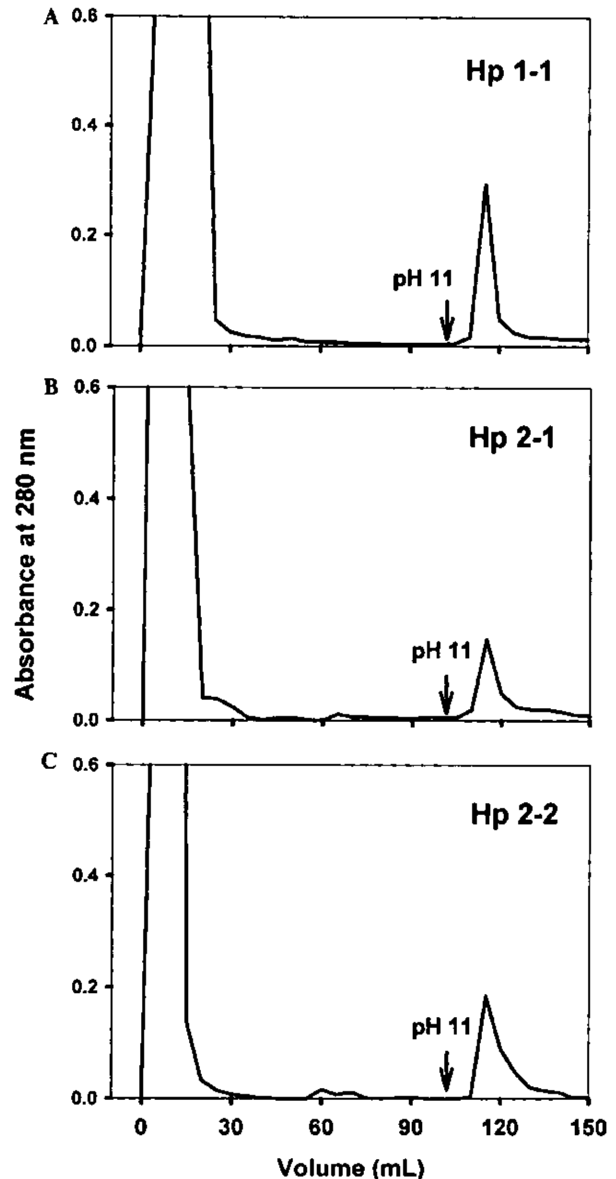


Fig. 2. Typical purification profile of human Hp on antibody affinity-column. One milliliter of human plasma was applied to an antibody affinity-column (pre-equilibrated with PBS, pH 7.4) followed by a wash in 10 mM phosphate buffer containing 0.2 M NaCl, pH 7.4. The bound Hp was then eluted in a solution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

### 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 those of Hp 2-1 and 2-2. Purity of each phenotype was then analyzed on SDS–PAGE containing the reducing reagent 2-mercaptoethanol. Homogeneity of each phenotype was greater than 95% (Fig. 6). Thus, HPLC Superose column was markedly effective in removing the apoA-I contaminant.

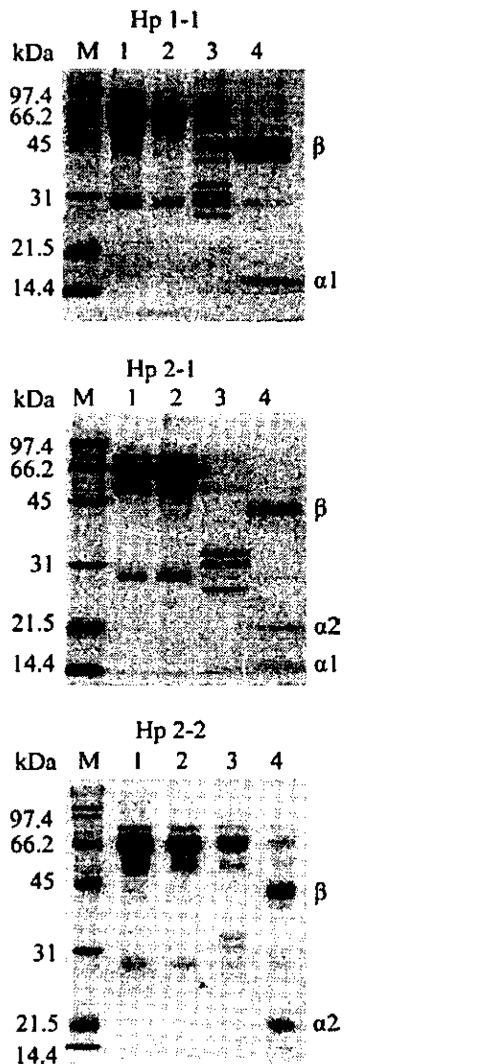


Fig. 3. Analysis of isolated Hp from affinity column using 12% SDS–PAGE in the presence of reducing reagent. Lane M: molecular markers (expressed as kDa). Lanes 1–2: plasma before and after flowing through the affinity column, respectively. Lane 3: low-affinity binding proteins washed with 10 mM phosphate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

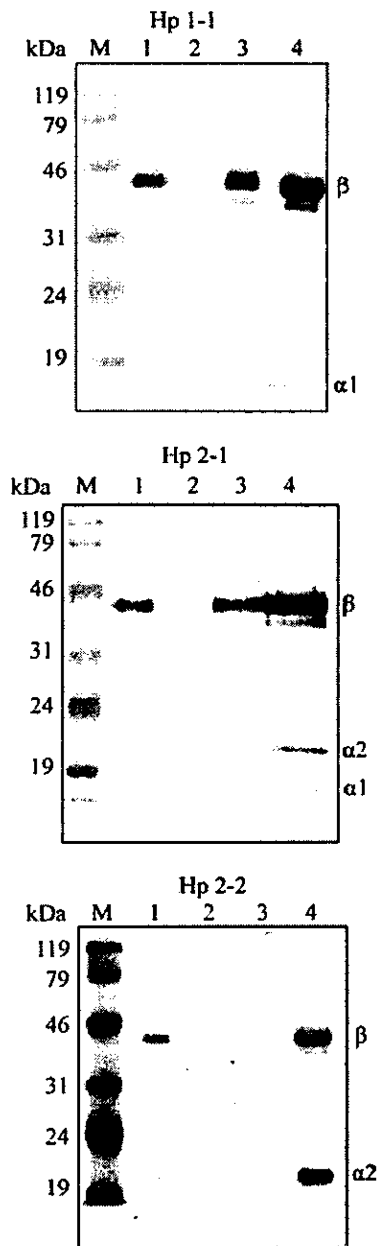


Fig. 4. Western blot analysis of Hp isolated from antibody affinity-column. Following a 12% SDS–PAGE, the separated proteins were transferred, blocked, and developed by a goat anti-human Hp. Lane M: molecular markers. Lanes 1-2: plasma before and after flowing through the affinity column, respectively. Lane 3: low-affinity binding proteins washed with 10 mM phosphate buffer containing 0.2 M NaCl, pH 7.4. Lane 4: the eluted Hp.

280 Western blot analysis in the absence of a reducing
281 reagent demonstrated that Hp 2-1 and 2-2 were all
282 polymeric (Fig. 7), in which Hp 2-2 was devoid of
283 monomer and dimer consistent with the proposed
284 structure of Hp (Fig. 1). Thus, our purification proce-
285 dures did not apparently alter the structural character-
286 istics of Hp phenotypes.

287 *The polymeric structure of isolated Hp and its binding to*
288 *hemoglobin*

289 We studied further the ionic property of isolated Hp
290 1-1, 2-1, and 2-2 on a native-PAGE; the distinct poly-
morphism of each phenotype was also observed (Fig. 8). 291
Hp 2-2 was the most basic among the Hp phenotypes. 292
Since hemoglobin (Hb) is able to bind Hp and to form a 293
Hb–Hp complex [10], Fig. 8 demonstrates that the Hb 294
could form Hb-Hp complex with each Hp phenotype we 295
isolated. 296

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

| | Plasma | Affinity column | Gel filtration |
|---|---|---|---|
| (A) Hp 1-1 | | | |
| 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 | | | |
| 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 | | | |
| 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.

297 *Circular dichroic spectra*

298 To characterize the secondary structure of each Hp
299 phenotype, we determined the conformation of Hp by
300 CD (Fig. 9). The estimated α-helical content was about
301 29, 22, and 21% for Hp 1-1, 2-1, and 2-2, respectively
302 (Table 2). Statistically, the α-helical content in Hp 1-1
303 was significantly higher than those in Hp 2-1 and 2-2
304 ($P < 0.001$).

305 **Discussion**

306 Purification of human Hp has been hampered for
307 years due to its structural diversity as described above
308 [7,18–21]. In the present study, plasma without any
309 additional manipulations (e.g., ammonium-sulfate pre-
310 cipitation) was utilized for Hp isolation. With the use
311 of a 0.2 M NaCl wash in our procedures, most of the
312 nonspecific low-affinity binding proteins were eluted
313 from the column. Nevertheless, the affinity purified Hp
314 1-1, 2-1 or 2-2 analyzed on SDS–PAGE was only 60–
315 80% pure (Fig. 3). HPLC Superose 12 column ap-
316 peared to be superior to the other methods in the
317 second-step purification since apolipoprotein A-I, a
318 major contaminant, and other unknown high molecu-
319 lar-weight proteins were almost eliminated. The solu-
320 tion property of each Hp phenotype on this Superose
321 column was consistent with its molecular forms, of
322 which Hp 1-1 was more homogeneous than the other
323 two species in size. Notably, the elution time of each
324 phenotype was also consistent with the size of Hp, in
325 which the polymeric forms of Hp 2-2 and 2-1 were
326 eluted earlier, respectively (Fig. 5). Western blot anal-
327 ysis on the polymeric structure of isolated Hp using 6%
328 SDS–PAGE without reducing reagent (Fig. 7) revealed
329 its structural identity to that originally present in
330 plasma (data not shown). It also confirmed that the
331 isolated Hp 2-1 was heterogeneous in nature containing
332 Hp monomer and dimer, while the polymeric Hp 2-2
333 contained neither.

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

343 Taking together, the Hp isolated from the antibody
344 affinity-column combined with HPLC Superose 12 ex-
345 hibited advantages over the conventional methods.
346 First, the heterogeneous particles of Hp 2-1 or 2-2 could
347 be obtained in one pool as polymeric forms. Second, the
348 co-eluted hemoglobin from hemoglobin affinity-column
349 [19] was eliminated in the present study. Using an
350 ELISA for hemoglobin assay, we could not detect he-
351 moglobin in the Hp we isolated (data not shown). Third,
352 the isolated Hp not only retained its ability to bind

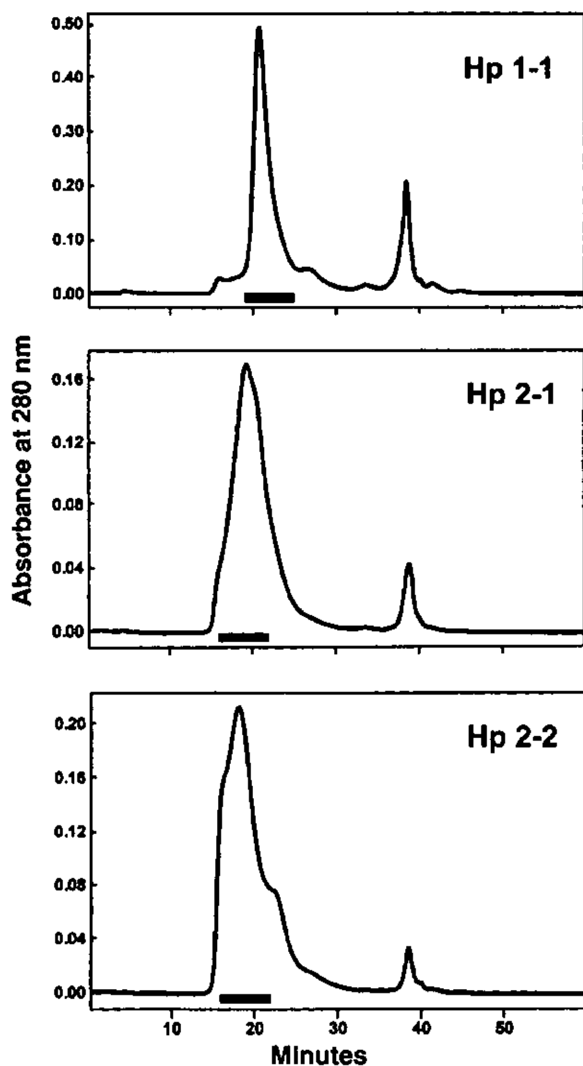


Fig. 5. Typical chromatographic profile of affinity isolated Hp on HPLC Superose 12 column. Isolated Hp 1-1, 2-1, or 2-2 from the affinity column was first concentrated and applied to a HPLC Superose 12 pre-equilibrated in 50 mM of ammonium bicarbonate, pH 8.0. The same solution was used in the mobile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

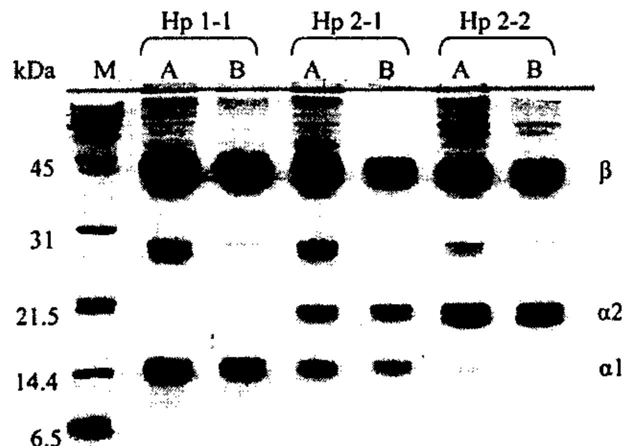


Fig. 6. Analysis of purified Hp from antibody affinity and HPLC gel filtration column on 15% SDS–PAGE in the presence of reducing reagent. M: molecular markers. (A) Isolated Hp from affinity column alone. (B) Purified Hp from an additional separation on HPLC Superose 12 column.

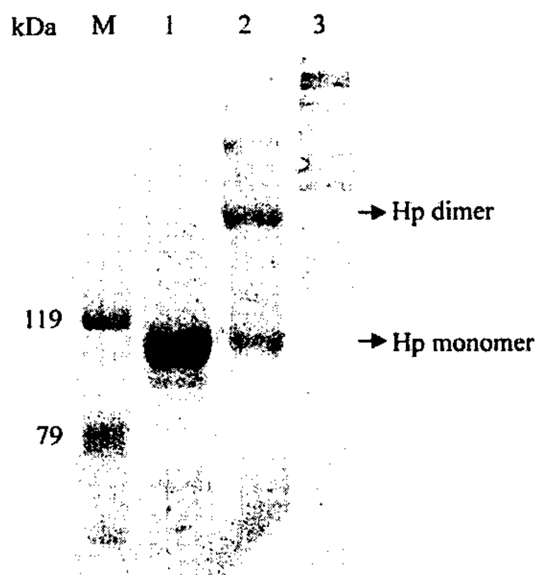


Fig. 7. Western blot analysis of polymeric structure of purified Hp on 6% SDS–PAGE in the absence of reducing reagent. Lane M: molecular markers. Lanes 1-3: purified Hp 1-1, 2-1, and 2-2, respectively. Notably, the Hp 2-1 contains monomeric and dimeric forms of Hp 1-1: whereas the Hp 2-2 is devoid of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

353 hemoglobin (Fig. 8), but also blocked the peroxidase
354 activity of hemoglobin (data not shown).
355 The CD spectrum of each Hp phenotype was deter-
356 mined to estimate the secondary structure of Hp. For
357 the first time, according to the best of our knowledge, we
358 showed that the α-helical content of Hp 1-1, 2-1, and 2-2
359 was 29, 22, and 21%, respectively. Hp 1-1 possessed a
360 more ordered structure than those of polymeric forms of
361 Hp 2-1 and 2-2. Moreover, we identified that the im-
362 munoreactivity of Hp 1-1 was also greater than those of
363 Hp 2-1 and 2-2. For example, the monoclonal antibody
364 used in our affinity column (8B1-3A) exhibited an af-
365 finity ($K_a$) of $5.58 \times 10^9$, $2.81 \times 10^9$, and $2.19 \times 10^9\ M^{-1}$
against Hp 1-1, 2-1, and 2-2, respectively. The latter 366
result suggests that the availability of "functional" sur- 367
faces of Hp 1-1 may be greater as compared to those of 368
Hp 2-1 and 2-2. Such structural differences may explain, 369
in part, the clinical outcome by which Hp phenotype is 370
associated with differential susceptibility to infections, 371
atherosclerosis, and autoimmune disorders [3,29]. These 372
effects are correlated with a phenotype-dependent 373
modulation of oxidative stress and prostaglandin 374

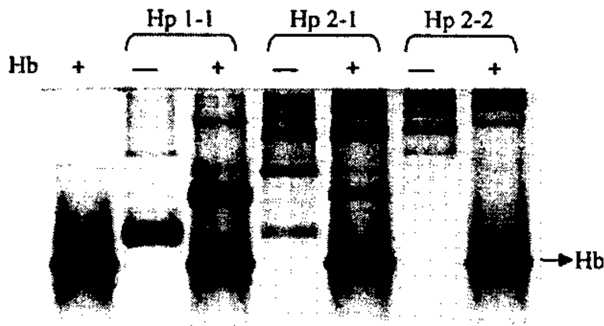


Fig. 8. Analysis of hemoglobin-binding property of purified Hp 1-1, 2-1, and 2-2 on a 7% native-PAGE. Briefly, each Hp phenotype (5 μg) was incubated with and without hemoglobin (Hb) (5 μg) at room temperature for 30 min before conducting the native-PAGE. The gel was then stained with Coomassie Blue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

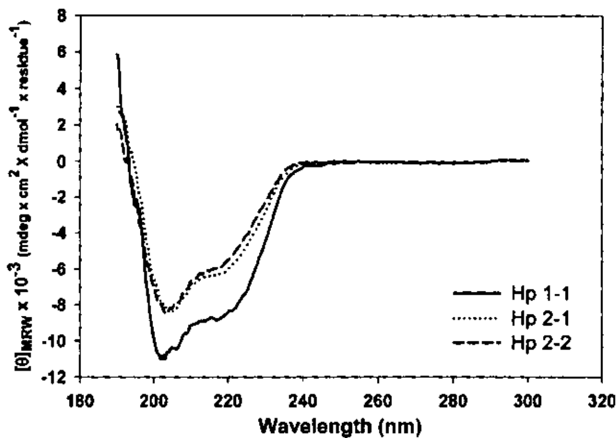


Fig. 9. Circular dichroic spectra of Hp 1-1, 2-1, and 2-2. Each Hp in 10 mM phosphate buffer, pH 7.4, at a final concentration of 0.2 mg/ml was monitored by a circular dichroism. Each spectrum represents a mean of 20× determinations.

synthesis. In general, patients with Hp 2-2 are more susceptible to develop 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].

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 ($P < 0.001$).

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.

## Acknowledgment

This work was supported by the Grant NHRI-EX92-9229SI (S.J.T.M.) from the National Health Research Institute, Taiwan, ROC.

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## 拾、九十二年度計畫執行情形

註：群體計畫(PPG)者，不論是否提出各子計畫資料，都必須提出總計畫整合之資料

若為群體計畫，請勾選本表屬於：■子計畫；　或　□總計畫(請自行整合)

1. 請簡述原計畫書中，九十二年預計達成之研究內容

Specific aims:

1. Develop a new and fast approach for human Hp phenotping (Hp 1-1, 2-2, and 2-1) using monoclonal antibodies, and to test the hypothesis whether or not Hp phenotypes are correlated to the patients with coronary artery disease in Taiwan (2003-2005).

2. Study the Hp mRNA expression in macrophages and SMC in the presence of oxidized LDL and lipoproteins VLDL, LDL, and HDL (2003-2004).

3. Test the hypothesis that Hp may exert an antioxidant role in macrophages and SMC transfected with sense and antisense Hp cDNA; to define the antioxidant activity of Hp phenotypes in vitro (2003-2004).

4. Investigate the functional role of Hp in the progression of atherosclerosis using cholesterol-fed rabbits and antiatherogenic effect (if any) using Hp transgenic mice (2004-2005).

# GENTT CHART (TIME-TABLE) FOR PROPOSED PLAN

| Task Force and Experiments | Year 1 (2003) Half | | Year 2 (2004) Half | | Year 3 (2005) Half | |
|---|---|---|---|---|---|---|
| AIM 1: | | | | | | |
| Determination of human Hp phenotypes: | | | | | | |
| Monoclonal antibody production | ■ | ■ | ■ | ■ | ■ | |
| Immunoassay using ELISA for phenotyping | ■ | ■ | | | | |
| Immunoassay using immunogold for phenotyping | ■ | ■ | | | | |
| Using Protein/Antibody chips for Hp phenotyping | | ■ | ■ | | | |
| Correlation of plasma Hp phenotypes to the domestic patients with atherosclerosis: | | | | | | |
| Patients selection | ■ | ■ | ■ | ■ | | |
| Hypothesis to be tested and statistics | | ■ | ■ | ■ | ■ | ■ |
| AIM 2: | | | | | | |
| Macrophage experiment using oxidized LDL | ■ | ■ | ■ | | | |
| Macrophage experiment using VLDL and HDL | ■ | ■ | ■ | | | |
| SMC experiment | ■ | ■ | ■ | ■ | | |
| AIM 3 | | | | | | |
| Human Hp cDNA cloning and plasmid construct | ■ | | | | | |
| Cloning of green fluorescent protein (GFPemd) as the reporter gene | ■ | | | | | |
| Cloning of Hp1-1 gene at sense and antisense orientation | ■ | | | | | |
| Antioxidant role experiments | | ■ | ■ | ■ | | |
| Transfection experiment (ex vivo) | ■ | ■ | ■ | ■ | | |
| AIM 4 | | | | | | |
| Evaluation of atherosclerosis in rabbit model | | | ■ | ■ | | |
| Transgenic Hp mouse model | | | ■ | ■ | ■ | |
| Evaluation of atherosclerosis in transgenic model | | | ■ | ■ | ■ | ■ |
| Publications | | ■ | ■ | ■ | ■ | ■ |
| Possible Patent Application | | | | | ■ | ■ |

2. 請詳述九十二年度計畫執行情形，並評估是否已達到原預期目標（請註明達成率）

Aim 1: (2003-2005) 達成率：70%

We have successfully developed a quick Hp-typing method from human plasma without a traditional starch gel. Human plasma (or serum) incubated with hemoglobin was used to perform a native PAGE, and the pattern of haptoglobin-hemoglobin complex was subsequently represented using chromogenic substrates. Phenotypes of Hp could be easily determined by the represented pattern. In addition, to investigate the correlation between Hp phenotypes and patients with coronary artery disease (CAD), about 1,000 CAD patients' serum from Taipei Veterans General Hospital were under analyzing and gathering statistics.

Aim 2: (2003-2004) 達成率：50%

Smooth muscle cell (SMC) was cultured and treated with oxidized LDL and lipoprotein VLDL, LDL, and HDL. Hp mRNA expression was under investigating.

Aim 3: (2003-2004) 達成率：80%

The *in vitro* antioxidant activity of each phenotype Hp has already defined by Cu2+- and AAPH-induced lipid peroxidation. Hp exerted an extremely

potent antioxidant activity than probucol that was a known most potent antioxidant compound. Moreover, carboxymethylated Hp possessed higher antioxidant activity than native Hp. To study the *in vivo* antioxidant activity of Hp, cDNA of Hp 1-1 was also cloned into a CMV-promoter-driven expression vector pcDNA3.0 and then the recombinant vector was transfected into CHO-K1 cell. Transfected cell exerted higher relative survival ratio than untransfected in the presence of additive oxidative stress. Thus, Hp did possess its antioxidant characteristic against oxidative stress *in vivo*.

Aim 4: (2004-2005) 達成率：20%

To investigate the functional role of Hp in the progression of atherosclerosis and antiatherogenic effect, cholesterol-fed rabbits and Hp transgenic mice were under preparing.

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# Experimental Biology 2003®
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# ABSTRACTS
## PART II

Abstracts A456.1–A886.2

Official Publication of the Federation of American Societies for Experimental Biology
Volume 17, Number 5, March 17, 2003

containing MMP-13 amino acid residues 141-228 did not compete as effectively, however, the construct in which exon 5 was deleted (amino acids 213-167) competed almost as well as the wild-type form of MMP-13. These results indicate that the binding domain for MMP-13 lies within residues 1-228, more specifically within the region 1-141.

**630.10**

**Function of the Rieske iron-sulfur protein in the cytochrome b6f complex is relatively insensitive to structure changes of the hinge region**

**Jiusheng Yan**, W. A. Cramer. Biological Sciences, Purdue University, Lilly Hall, West Lafayette, Indiana 47907

As implied by x-ray crystallography and mutational studies on the cytochrome $bc_1$ complex, a hinge region with optimal flexibility and length is required for the large amplitude rotation-translation of the Rieske iron-sulfur protein (ISP) soluble domain that mediates electron transfer from ubiquinol to cyt $c_1$. A similar but more flexible hinge region is also present in the ISP of the cytochrome $b_6f$ complex. Mutagenesis studies on the *petC* gene located in the petCA operon of *Synechococcus* sp. PCC 7002 indicated that the ISP encoded by this gene is the predominant species in the $b_6f$ complex, while the other 2 *petC* genes in the genome were non-functional. In double residue deletion mutants of the hinge region, the $b_6f$ complex was ~2-4 fold less active and relatively insensitive to $Q_p$-site inhibitors DBMIB and stigmatellin, and a four Gly insertion mutant showed increased sensitivity to the $Q_p$ site inhibitors. In contrast to the $bc_1$ complex, function of the $b_6f$ complex is little affected by changes in amino acid sequence that would increase the flexibility, rigidity, or length of the ISP hinge region. A hinge region with 4 Pro is not rigid enough to make the ISP movement the rate-limiting step. It is inferred that a hinge region with a minimum length of 7 residues is required for the proper docking of the ISP at the $Q_p$-site of the $b_6f$ complex, and its function is not sensitive to changes of its structure. (NIH GM-38323)

## METHODS: PROTEIN CHEMISTRY (631.1-631.15)

**631.1**

**Isolation and purification of eosinophil granular proteins, MBP and EPO from established eosinophilic cell lines**

**michelle patrice law**[1], ibrahim laniyan[1], ithalda uston[2], michael smith[1], theresa vaughn[2], debra parish-gause[2], kesha forrest[2], josephine awich[2], oladipo oredipe[3], paulette m furbert-harris[2]. [1]microbiology, howard university college of medicine, 520 'w' street, nw, washington, dc 20059, [2]cancer center, howard university college of medicine, washington, dc, [3]cancer center, pharmacology, washington, dc

Eosinophils are most known for their role as anti-helminthic agents and as inflammatory effectors in allergic hypersensitivity and bronchial asthma. Eosinophils have been shown to produce IFN-γ,IL-10 and IL-12,which are cytokines with very potent immunoregulatory as well as anti-cancer activity. In addition, eosinophils contain in their granules the cationic proteins, Major Basic, Eosinophil Peroxidase, Eosinophil Cationic, and Eosinophil Derived Neurotoxin. These proteins, MBP in particular, are extremely toxic to cells. Commercial vendors for these proteins have not been found. The in-house established eosinophilic cell lines provide a resource material for the isolation and purification of these proteins for further use in studies investigating their cytotoxic effects on tumor cells.

**631.2**

**Development of Methods to Discriminate Between OPN Charge Forms in Biological Fluids**

**Ian J Musselman**, Rania Al-Shami, Mary C Farach-Carson, Dan Carson. Biological Sciences, University of Delaware, 51 E Main St, Newark, DE 19711

Osteopontin (OPN) is a major non-collagenous phosphoprotein located in the bone extracellular matrix. OPN also has been found in the luminal surfaces of different glandular tissues and in many biological fluids. It is a secreted, highly acidic protein that binds to hydroxyapatite and Ca2+ in the context of mineralization and can support cell attachment/migration. Its amino acid sequence contains a conserved Gly-Arg-Gly-Asp-Ser (GRGDS) sequence, which allows it to bind effectively to the integrins. Two charge forms of OPN differing in their extent of phosphorylation have been identified in osteoblasts upon treatment with 1á, 25-dihydroxyvitamin D3. OPN-1 is the highly phosphorylated protein (pI 4.6) while OPN-2 is the less phosphorylated form (pI 5.1). It is thought that the extent of phosphorylation affects the ability of OPN to regulate crystal formation in solution. A current research focus is to establish a methodology for studying the expression of these OPN forms in human biological fluids. Examples include urine, human milk, cerebral spinal fluid, and secretions of human cell lines. Using the SELDI ProteinChip® technology, a mass spectrometry based technique, it is possible to determine a relationship between the levels of each charge form of OPN. These data then will be confirmed using Western blotting in conjunction with isoelectric focusing and 1-D and 2-D SDS-PAGE. (*Supported by NIH grant HD25235 to DDC*)

**631.3**

**Purification of Human plasma Haptoglobin using Hemoglobin-Affinity Column Chromatography**

**Simon J.T. Mao**, Chunyi Liao. Department of biological science and technology, National Chiao Tung University, 75 Po Ai St., Hsinchu, Taiwan 300 Taiwan

Haptoglobin (Hp) is an acute-phase protein and its plasma levels increase consistently in response to infection and inflammation. Similar to blood type, individual human Hp is classified as Hp 1-1, 2-1, or 2-2. The structural and functional analysis of the Hp, however, has not been studied in details due to its difficult isolation procedures. In this study, we established a purification method using a high capacity hemoglobin-affinity column. Briefly, DEAE-purified human hemoglobin was first coupled to Sepharose 4B for preparing an affinity column in a 15-ml bed volume. Following a flow through of human plasma and wash, the bound material was first eluted with a 0.15 M NaCl, pH 11.0 to remove low-affinity bound proteins. The high-affinity bound Hp was then eluted with a 0.15 M NaCl containing 5 M urea, pH 11.0. The biological activity of dialyzed Hp was retained as forming a complex with hemoglobin on a SDS-PAGE. Using this procedure approximately 10 mg of Hp 1-1, with homogeneity greater than 96 %, was obtained from a 15 ml of human plasma. Purified Hp 2-1 or 2-2, however, contained trace amount of apoA-I and could be further purified on HPLC using a Superose 12 gel-permeation chromatography. All the phenotypes of purified Hp consisted of heavy (β) and light (α) chain(s) on SDS-PAGE in the presence of reducing reagent and was further confirmed by Westernblot analysis. We conclude that human-hemoglobin affinity column was most suitable for the isolation of Hp 1-1 in large quantity.

**631.4**

**Removal and purification of trypsin-like serine proteases**

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Thrombin is commonly used for enzymatic cleavage of tags (here GST) during the production of recombinant proteins. This protease has to be removed from the protein solution in order to ensure stability of the target protein. Here we describe a simple and highly selective procedure to remove proteolytic activity or to purify specific proteases.

1. Removal of thrombin after on-column cleavage of a fusion protein.

E. coli homogenate expressing SH2-GST was loaded on a GSTrap™ FF column and non-binding proteins were washed out. To cleave off the GST-tag, thrombin was applied and incubated for two hours at RT. After on-column cleavage a HiTrap™ Benzamidine FF (high sub) 1 ml column was connected in series to GSTrap FF and the cleaved SH2 domain and thrombin were washed through both columns. HiTrap Benzamidine FF (high sub) bound the total amount of added thrombin, (shown by activity measurements. Pure SH2 was recovered in the eluate. This was completed in less than three hours.

2. Group selective capture of trypsin-like serine proteases from human plasma.

Trypsin-like serine proteases were purified from human plasma by Benzamidine Sepharose 4 FF. Plasma was loaded on a HiTrap Benzamidine FF (high sub) 1 ml column equilibrated with binding buffer. Non-binding components were washed out, and bound proteins were eluted decreased pH. Specific protease activity was much higher in

the eluted fractions than in the flow through. This was completed in less than half an hour.

631.5

**Epidemiology of haptoglobin phenotypes in chronic diseases**

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Haptoglobin (Hp) is an acute phase protein consisting of 3 phenotypes as Hp 1-1, Hp 2-1, and Hp 2-2, which are epidemiologically linked with some chronic diseases. We developed a PAGE technique that can be used for a high-though-put Hp phenotyping. In phenotyping 1194 Taiwanese, the distributions were 94 (7.9%) of 1-1, 529 (44.5%) of 2-1, and 571 (47.8%) of 2-2 type. This phenotype varies greatly among the overall world populations. We further investigated the interrelationships of Hp phenotypes with some chronic diseases. It was found that Hp 2-2 was over presented in hematological, cardiovascular and angiographically documented-coronary-artery disease, and diabetes mellitus. Hp 1-1 was over presented in essential hypertension, cancer, and allergy. Interestingly, no evidence showing that Hp 2-1 was prevailed in the resistance of any disease states we have examined. Thus, Hp phenotypes could influence the epidemiology of certain diseases among various human populations. (This study is support by NSC and NHRI grants.)

631.6

**Temperature-dependent aggregation of non-phosphorylated recombinant human β-casein and some logical mutants**

**Satish Mohan Sood**, Hongyin Bu, Charles W. Slattery. Biochemistry Division, Loma Linda University, Mortensen Hall, Loma Linda, CA 92350

Wild type (WT) recombinant human β-CN-0P was expressed in E. coli, purified, and confirmed to have the same sequence as that of native human β-CN. Single amino acid mutants at conserved positions M1 (K88E), M2 (K90E), M3 (K96E), and M4 (R98E) and three C-terminal deletion mutants, C-11, C-22 and C-31 were also prepared. As the temperature was increased from 4 to 37°C, turbidity at 400 nm monitored protein aggregation. For the recombinant WT protein, the pattern was similar to the native protein during the first heating cycle but was not similarly reproducible after cooling. The possible explanation for this is that there may be some mis-folded recombinant molecules that cannot totally disaggregate upon cooling at 4°C. For the single amino acid mutants, the turbidity at 37°C decreased in the order M1>M2>M3>WT>M4 indicating that a change in charge from positive to negative at 3 of the 4 positions either increased the hydrophobic interactions of the molecules or allowed salt-bridges to form. For the deletion mutants, turbidity at 37°C decreased in the order C-31>WT>C-22>C-11 suggesting upon analysis that the particular sequence of amino acids at the C-terminus may be important in protein aggregation.

631.7

**Fluorescence analysis of native, recombinant wild type and some logical mutants of human β-CN**

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Fluorescence intensities for the intrinsic Trp ($FI_i$) and for added ANS ($FI_e$), and Fluorescence Resonance Energy Transfer (FRET or RET) between the Trp and added ANS were measured for native, wild type recombinant (WT), four single amino acid mutants M1 (K88E), M2 (K90E), M3 (K96E) and M4 (R98E), and three C-terminal deletion mutants C-11, C-22 and C-31 of human β-CN-0P. As the temperature was increased from 4 to 37°C, there was an increase in the $FI_i$ and a significant downward shift in the emission wavelength maxima, indicating that Trp 154 (the only one present in human β-CN) is buried as temperature increases. At the same concentration, the $FI_i$ and $FI_e$ are much lower for the WT than for the native human β-CN-0P suggesting mis-folding of some WT molecules. FRET increases as T increases, indicating that hydrophobic ANS binding sites are close to the Trp. The $FI_i$ spectra of the four single aa mutants are different from those of the WT and native, suggesting that changing the charge at these locations affects protein folding and/or protein-protein interactions. Only M2 shows significant FRET at 37°C. The deletion mutants showed some changes from the WT but did not exhibit a decrease in protein-protein aggregation seen in a 20 aa C-terminal deletion in bovine β-CN.

631.8

**Prediction of secondary structure in human β-casein using the latest available algorithms**

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The latest versions of the four programs PSI, GOR IV, HHN and SOPMA have been used to predict the secondary structure of human β-CN. There were two predicted α-helical regions, one near the N-terminus (residues 11 or 12-40) and the other near the C-terminus (residues 177-188), that are consistent by all four methods. There are two β-sheet structural segments with a high probability in human β-CN involving amino acid residues 92-95 and 99-102 or 98-103. Sequence alignment of human and bovine β-CN shows a 59% identity and 62% similarity. The hydrophilicity and surface probability of the two were calculated and compared. An examination was also made using hydrophobicity cluster analysis. It was observed that despite the sequence homology, the two molecules are very different in their hydrophobicity profile. This may result in a difference in protein-protein interactions and in casein micelle structure in human and bovine milk.

631.9

**Adhesion of albumin to FDA Group I contact lenses**

**Edward O. Keith**[1], Ogine Lo[1], Denise Gonzalez[1], Jessica Smith[1], Sanober Tapia[1], Christopher Drennen[1], Andrea Janoff[2]. [1]Math, Science, and Technology, Nova Southeastern University, 3301 College Avenue, Ft. Lauderdale, FL 33314, [2]College of Optometry, Nova Southeastern University, Ft. Lauderdale, FL

Tear protein adhesion can contaminate contact lenses and reduce their effectiveness for the treatment of vision abnormalities.. Protein adhesion depends upon the type of contact lens material, and fluctuates with tear secretion rate and pathology. We examined the adhesion of albumin, a major tear protein, to FDA Group I contact lenses over a four day period. Never worn Optima FW contact lenses (Bausch and Lomb, Inc., Rochester, NY) were incubated in albumin dissolved in optical saline (0.2 mg/ml) contained in borosilicate glass vials. The concentration of albumin in the vials, and adhering to the lenses, was monitored with bicinchoninic acid (BCA). Albumin concentration in the vials decreased on day three, and then regained initial levels. After one day of incubation, albumin adhesion to lenses reached a plateau that was stable through the remainder of the incubation period. This pattern is in contrast to that of lysozyme, which adheres to lenses in an up-down-up-down pattern regardless of lens material. Both albumin and lysozyme adhered to a lesser degree to these lenses than to FDA group IV lenses. These results clearly indicate the need for further studies of this complex interaction.

631.10

**Adhesion of albumin to FDA Group IV contact lenses**

**Edward O. Keith**[1], Akashi Patidar[1], Niki Hector[1], Premal Smart[1], Sheneeza Ishmael[1], Khadija Latif[1], Andrea Janoff[2]. [1]Math, Science and Technology, Nova Southeastern University, 3301 College Avenue, Ft. Lauderdale, FL 33314, [2]College of Optometry, Nova Southeastern University, Ft. Lauderdale, FL

Contact lenses are essential for the treatment of vision abnormalities. Tear protein adhesion can contaminate lenses and reduce their effectiveness. This adhesion depends upon the type of contact lens material, and fluctuates with tear secretion rate and pathology. We examined the adhesion of albumin, a major tear protein, to FDA Group IV contact lenses over a four day period. Never worn AcuVue contact lenses (Bausch and Lomb, Inc., Rochester, NY) were incubated in albumin dissolved in optical saline (0.2 mg/ml) contained in borosilicate

# 第十八屆（九十二年度）生物醫學聯合學術年會

# The Eighteenth Joint Annual Conference of Biomedical Sciences（2003）

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## O21

### ANTIOXIDANT ACTIVITY OF ACUTE PHASE PROTEIN HAPTOGLOBIN

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Haptoglobin (Hp) is an acute phase protein in plasma, the level increases consistently in response to inflammation. Recent reports have indicated that Hp possesses an antioxidant activity by directly forming complex with Fe-containing hemoglobin. In the present study, we hypothesized that the antioxidant activity of Hp was deriving from scavenging the free radicals generated during the lipid peroxidation rather than the formation of Hp-hemoglobin complex. To test this hypothesis, copper (2+) and iron (3+) induced lipid peroxidation was conducted to determine whether or not Hp itself may remove the free radicals without the presence of hemoglobin. Our data show that Hp was an extremely potent antioxidant with an activity greater than probucol, vitamin E, and vitamin C. We conclude that Hp could scavenge Fe (3+) induced free radicals without the presence of hemoglobin during the lipid peroxidation. Finally, we attempt to delineate the structure of Hp that is associated with its antioxidant activity. The role of carbohydrate moiety and disulfide bonding in Hp will be reported (supported by a NHRI grant).

## O22

### PROTECTIVE EFFECTS OF "FU TU DAN" ON THE OXIDATIVE DAMAGE IN THE MIMETIC AGING MICE

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Chinese herbal medicine, an alternative method of therapy, was successfully applied in retarding the aging progress established on D-galactose mimetic aging animal model. There were seven herbs within the prescription and C57 BL/6J mice were employed for the experiment. The animals were divided into 5 groups: (1) phosphate buffered saline (PBS) oral and subcutaneous injection as PBS group; (2) PBS oral and 5% D-galactose injection as D-galactose group; (3) 0.5 g/kg prescription oral as low-dose group; (4) 1.0 g/kg prescription oral as medium-dose group; (5) 2.5 g/kg prescription oral as high-dose group. Each group was treated with 0.5g/kg D-galactose subcutaneous injection except the PBS group. Biochemical markers, including superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), total antioxidant status (TAS) and malondialdehyde (MDA) were determined in blood collected from animals after 60 days handling as described. The high-dose, medium-dose and PBS groups exhibited a higher level in SOD, GSH-Px and TAS while a lower level in MDA activities in blood compared with the D-galactose group. We had confirmed the effectiveness of Chinese herbal medicine to hinder decrepit progress.

## O23

### LONG-TERM MONITORING OF LEVELS OF LIPID PEROXIDATION IN CANCER PATIENTS RECEIVING CISPLATIN CHEMOTHERAPY AND RADIOTHERAPY

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Cisplatin (CPT) and ionizing radiation, which are used to treat patients with cervical cancer, can inhibit tumor growth by damaging DNA but also induce oxidative stress. Irreversible nephrotoxicity has been a major problem during CPT therapy, which could be caused by oxidative damage. In this study, we monitored levels of 15-$F_{2t}$-isoprostane ($F_{2t}$IsoP), a specific marker of lipid peroxidation *in vivo* and also a vasoconstrictor, in urine of ten patients with cervical cancer squamous cell carcinoma receiving daily radiotherapy and weekly CPT chemotherapy for four to six cycles of CPT treatment. Levels of urinary $F_{2t}$IsoP in those patients before the therapy were 53-429 pg per mg creatinine. Except two patients with only less than two-fold increase of $F_{2t}$IsoP, other eight patients had approximately two- to six-fold increase of $F_{2t}$IsoP levels at their highest peak levels, compared to pre-treatment values. However, the up-and-down patterns and time points or numbers of the peaks varied among those patients, which could be affected by the antioxidant status and other physiological conditions. Moreover, serum levels of creatinine were normal. Our study is the first one to demonstrate increased urinary $F_{2t}$IsoP levels during combined CPT chemotherapy and radiotherapy in cancer patients. The monitoring of $F_{2t}$IsoP may be useful as an indicator in improving therapeutic protocols for cervical cancer.

## O24

### EFFECTS OF *TOONA SINENSIS* ROEM EXTRACTS ON REACTIVE OXYGEN SPECIES OF HUMAN SPERM

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[1]Department of Biology, National Cheng Kung University, Tainan, [2]Department of Physiology, Kaohsiung Medical University, Kaohsiung, [3]TUBE Fertility Clinic, Tainan, Taiwan

Water extracts of Roem leaves freeze-dried into crude powder (5-4) was extracted in series with 99.5 (5-2) and 50% alcohol (5-5) to receive precipitate as 5-5R. 5-5R was extracted with water to receive the precipitate as 5-4R. Roem leaves were dried, water boiled and freeze-dried assigned as Black tea. Extracted powder of 5-4 with acetyl ethyl was assigned as 5-3. The purpose of this study was to investigate the effects of various concentrations (0.01, 0.05, 0.1, 0.5 and 1 mg/ml) of different Roem leave extracts (5-4, 5-4R, 5-2, 5-5, 5-5R, Black and 5-3) on the reactive oxygen species (ROS) levels in human sperm. Intracellular ROS was quantified, using DCFH$_2$-DA, by flow cytometry. Results indicated that ROS level was significantly decreased in sperm treated with 0.01 and 0.05 mg/ml of most extracts for 3 h ($P<0.01$). Only 0.1 mg/ml of 5-4R significantly decreased the ROS level in sperm ($P<0.01$). However, ROS level was significantly increased in sperm treated with 0.5 and 1 mg/ml of most extracts for 3 h ($P<0.01$). In conclusion, low and high concentrations of Roem leaves extracts significantly decreased and increased ROS levels in sperm, respectively.

## P173

GREEN TEA CATECHINS ENHANCE THE EXPRESSION OF OSTEOPROTEGERIN (OPG) IN PLURIPOTENT STEM CELLS

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Catechins have been applied for the treatment of cancer, cardiovascular disease, dermatological problems, dental caries and even memory loss. Prior studies found that the bone mineral density (BMD) of post-menopausal women with tea drinking habit was higher than that without tea consumption. Osteoprotegerin (OPG) can stimulate oseoblast genesis, thwart the differentiation, survival, and fusion of osteoclastic precursor cells, block the activity of mature osteoclasts and induce osteoclast apoptosis via the OPG/RANK-L/RANK system. Our results showed that among catechins, (-)-epigalloncatechin-3-gallate (EGCG) has the most powerful positive effect on mRNA expression of OPG. The OPG mRNA expressions of D1-cells were significantly increased upon 24 hr treatment of EGCG at concentrations of $10^{-6}$ and $10^{-5}$ M and the result of the semi-quantitation of OPG mRNA expression showed 2.5-fold and 4-fold increases in OPG expression of D1-cells respectively. These results suggests EGCG may enhance osteogenic and suppress osteoclastic functions through the effects of OPG.

## P174

ROLE OF 17β-ESTRADIOL IN GAP JUNCTION IN CHEMICALLY INDUCED HYPOXIC CARDIOMYOCYTES

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Gap junctions are intercellular communicating channels responsible for the synchronized electrical activation and contraction of cardiomyocytes. The cardioprotective effects of 17β-estradiol (E2) on gap junctions in cardiomyocytes are not clear. In this study, immunofluorescence microscopy showed that staining intensity of Cx43 was decreased in a time-dependent manner when cardiomyocytes were treated with 5 mM of KCN or 5% $O_2$. No significant change of Cx43 intensity was observed when cardiomyocytes were pretreated with 2 μM of E2 for 30 min followed by 10 mM of KCN for another 1 h. Functional assays by Lucifer yellow microinjection showed that dye coupling in cardiomyocytes treated with 10 mM of KCN for 1 h or 2 μM of E2 for 30 min followed by 10 mM of KCN for another 1 h fell to 55% or 83%, respectively, compared to controls. Immnuoblot analyses showed that levels of Cx43-P and Cx43-NP in cardiomyocytes treated with 10 mM of KCN for 1 h fell to 59% and 67%, respectively, compared to controls. However, levels of Cx43-P and Cx43-NP in cardiomyocytes treated with 2 μM of E2 for 30 min followed by 10 mM of KCN for another 1 h were 110% and 67%, respectively, compared to controls. These results suggest that E2 attenuates the inhibitory effect of hypoxia on gap junctional intercellular communication in cardiomyocytes by affecting the phosphorylation of Cx43.

## P175

EPIDEMIOLOGY OF HAPTOGLOBIN PHENOTYPES IN TAIWAN

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Haptoglobin (Hp) is an acute phase protein consisting of 3 phenotypes as Hp 1-1, Hp 2-1, and Hp 2-2, which are epidemiologically linked with some chronic diseases. We developed a PAGE technique that can be used for a high-though-put Hp phenotyping. From 1194 Taiwanese, the phenotype distributions were 7.9% of 1-1, 44.5% of 2-1, and 47.8% of 2-2 type. This phenotype varies greatly among the overall world populations. We further investigated the interrelationships of Hp phenotypes with some chronic diseases. Hp 2-2 was found to be over presented in hematological, cardiovascular and angiographically documented-coronary-artery disease, and diabetes mellitus. Hp 1-1 was over presented in essential hypertension, cancer, and allergy. Interestingly, no evidence showing that Hp 2-1 was prevailed in the resistance of any disease states we have examined. Thus, Hp phenotypes could influence the epidemiology of certain diseases among various human populations. (This study is support by a NHRI grants.)

## P176

FUNCTIONAL STUDIES OF HAPTOGLOBIN IN RAT SMOOTH MUSCLE CELL A-10 AND THE PRODUCTION OF MONOCLONAL ANTIBODY AGAINST HUMAN HP2-1

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Haptoglobin ( Hp ) is a hemoglobin-binding protein expressed by genetic polymorphism as three major phenotypes Hp1-1, Hp2-1, and Hp2-2. The protein is composed of two heavy β- and two light α- chains which are joined together by disulfide bonds. It also displays antioxidant, antibacterial, and angiogenic activities, we have recently found that it plays a role in the formation of atherosclerosis. In a preliminary study, we demonstrated that Hp2-1 could stimulate smooth muscle cell ( A-10 ) proliferation in a dose-dependent manner suggesting Hp itself was a growth factor in remoldeling the smooth muscle cells during the atherogenesis. In this study, monoclonal antibodies against human Hp2-1 were prepared using conventional hybridoma techniques. Seven lines of monoclonal antibodies, namely W1-11G, W9-10B, 4A2-4H, G2D-7G, P2F, 8B1-3A, and 2-9F were produced in cell culture media. Line W1-11G was proved to react with α chain of haptoglobin on Western blot analysis and the others recognized β subunit of Hp2-1. These antibodies are currently used for identifying the functional sites of Hp2-1 and for probing the epitopes on Hp molecules to explore the structural and functional relationship.