Cloning and expression of human haptoglobin subunits in *Escherichia coli*: Delineation of a major antioxidant domain

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Abstract

Human plasma haptoglobin (Hp) comprises α and β subunits. The α subunit is heterogeneous in size, therefore isolation of Hp and its subunits is particularly difficult. Using *Escherichia coli*, we show that α1, α2, β, and α2β chain was abundantly expressed and primarily present in the inclusion bodies consisting of about 30% of the cell-lysate proteins. Each cloned subunit retained its immunoreactivity as confirmed using antibodies specific to α or β chain. By circular dichroism, the structure of each expressed subunit was disordered as compared to the native Hp. The antioxidant activity was found to be associated with both α and β chains when assessed by Cu^{2+}-induced oxidation of low density lipoprotein (LDL). Of remarkable interest, the antioxidant activity of β chain was extremely potent and markedly greater than that of native Hp (3.5×), α chain (10×) and probucol (15×). The latter is a clinically proved potent compound used for antioxidant therapy. The "unrestricted" structure of β subunit may therefore render its availability for free-radical scavenge, which provides a utility for the future design of a "mini-Hp" in antioxidant therapy. It may also provide a new insight in understanding the mechanism involved in the antioxidant nature of Hp.
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Haptoglobin (Hp)[1] is an acute-phase protein present in all mammals [1–3]. One of the major functions of Hp is to bind hemoglobin [4] and thereby prevent the oxidative tissue damage mediated by free hemoglobin [5]. We have recently demonstrated that chemically modified Hp losing its binding ability to hemoglobin, but is able to directly inhibit Cu^{2+} induced LDL oxidation [6]. In humans, Hp is originally synthesized as a single polypeptide containing both α and β chains (Fig. 1A) [7]. Posttranslational cleavage between the Arg of α chain and Ile of β chain (followed by

removal of Arg) results in the formation of a single α and β chain that is subsequently linked by disulfide bridges to form mature Hp [8].

The Hp gene is characterized by two common alleles ***Hp 1*** and ***Hp 2*** corresponding to α1β and α2β polypeptide chains, resulting in three main phenotypes: Hp 1-1, 2-1 and 2-2. DNA encoding for these two chains are linearly oriented in chromosome 16q22.1 [9] with 5 and 7 introns in ***Hp 1*** and ***Hp 2***, respectively. These introns, however, are exclusively located at the region corresponding to the α chain [5]. The α2 contains a unique nonhomologous crossing-over in a part of the α1 [10]. Hp 2-1 and 2-2 form polymers due to an additional –SH group at the α2 chain, which determines its phenotype specificity [11]. All the phenotypes share the same β chains (each with 245 amino acids). The α1 chain (each with 83 amino acids) is "monovalent" forming one disulfide bridge with α and β subunit resulting in Hp dimer $(\alpha1\beta)_2$. In contrast, the α2 chain (each with 142 amino

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A   Schematic drawing of cloned human Hp
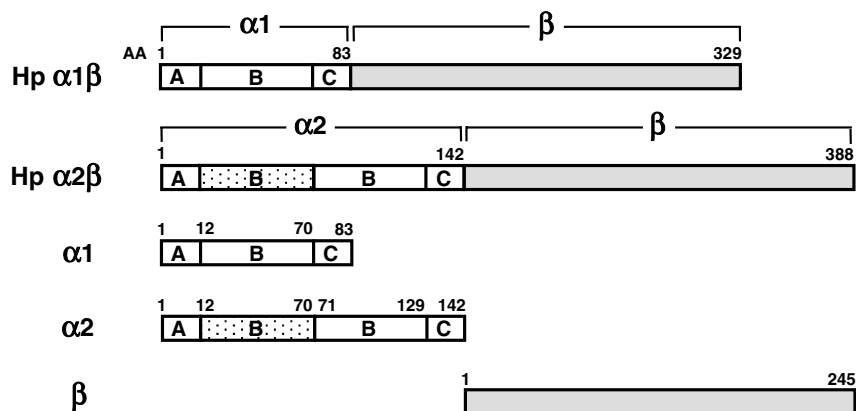
B   SDS-PAGE
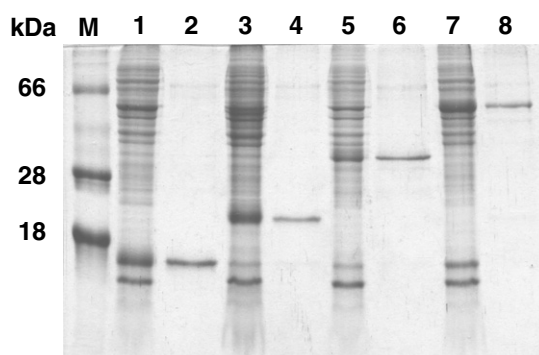
C   Western blot
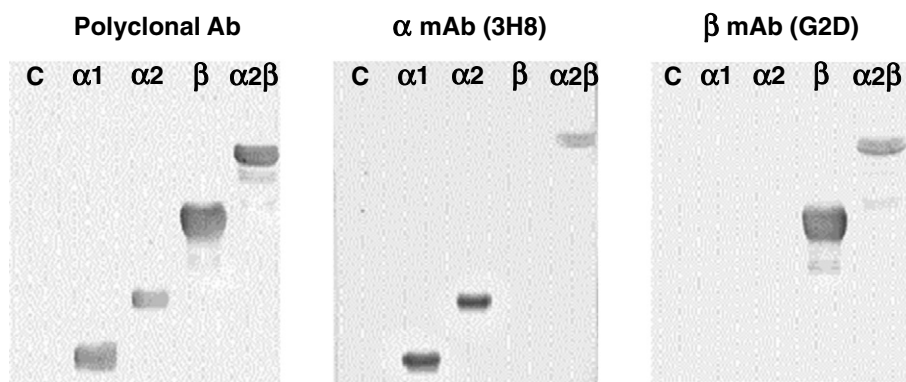

Fig. 1. Gene construction and Hp expression. (A) Schematic drawing of cloned human Hp α1, α2, β and α2β subunits in *E. coli*. Human matured mRNA corresponding to Hp 1-1 is linear oriented (α1β). The linear amino acid sequence (without signal peptide) containing residues 1–329 is shown on the top. Following a protease cleavage and a loss of the C-terminal residue Arg-84, it forms one α (residues 1–83) and one β chain (residues 1–245). Similarly, α2β forms one α2 (residues 1–142) and one β (1–245) chain. The amino acid sequence of α2 is identical to that of α1 with an insertion of repeated sequence B (dotted area or residues 12–70). It should be noted here, there are two amino acids in the inserted sequence B (dotted area) that are replaced by Asp-52 and Lys-53 in both native and our cloned sequence. (B) Expression and purification of recombinant Hp subunit in *E. coli*. Lane M, molecular markers; lanes 1, 3, 5 and 7, whole cell lysate containing expressed α1, α2, β and α2β, respectively; lanes 2, 4, 6 and 8, purified recombinant α1, α2, β and α2β, respectively. Notably, the α2β was not cleaved due to the lack of specific protease in *E. coli*. (C) Western blot of cell lysate containing α1, α2, β or α2β using Hp poly-clonal antibody and α and β chain specific mAb. Lane C, whole cell lysate control. Other lanes represent the cell lysate containing expressed α1, α2, β or α2β subunit, respectively.

acids) is "divalent" containing one extra free –SH that is able to interact with an additional α2. As such, α2 chains can bind to either α1 or α2 chains to form large polymers $[(\alpha1\beta)_2–(\alpha2\beta)_n$ in Hp2-1 and $(\alpha2\beta)_n$ in Hp2-2]. The poly-meric phenotypes have been reported to be more prevalent

in some groups of patients with certain diseases, such as diabetic and autoimmune diseases [5,12–14].

The purposes of this study were to produce recombinant Hp (rHp) α1, α2, β and α2β subunits in *Escherichia coli* expression system and to test whether *E. coli* contains a

protease that may specifically cleave the linear αβ chain as that in mammalian cells [6,15], and to determine which subunit of Hp possesses antioxidant activity. We demonstrated that *E. coli* was devoid of a specific protease responsible for the cleavage of α and β chains. Each expressed α or β subunit not only retained the immunoreactivity, but also possessed the antioxidant activity. However, a major antioxidant domain was located in the β chain with a superior potency to probucol. Remarkably interesting, the unfolded structure of Hp, based on circular dichroic spectra, dramatically increased in antioxidant activity. The present study provides a potential utility for the future design of "mini-Hp" in developing a novel potent antioxidant. It may also provide a new insight in understanding the mechanism or specific amino acids involved in the antioxidant nature of Hp.

Materials and methods

Materials

Escherichia coli JM109, M15 [pREP4], and the pQE30 expression vector were obtained from Qiagen (Hilden, Germany). Plasmid preparation and gel-extraction kits were purchased from BD Biosciences (Palo Alto, CA). The proofreading DNA-polymerase and dNTP were purchased from Invitrogen (Carlsbad, CA). All restriction enzymes were purchased from New England Biolabs (Beverly, MA). T4-DNA-ligase and HiTrap chelating column were purchased from Fermentas (Burlington, Canada) and Amersham Biosciences (Uppsala, Sweden), respectively.

Preparation of mouse mAb and human Hp

Mouse monoclonal antibodies (mAb) 3H8 and G2D specific to human Hp α or β subunit were produced in our laboratory according to the standard procedures previously established [16]. Native Hp was isolated from human plasma by an immunoaffinity column using procedures previously established by us [17].

Plasmid construction of pQE30-Hp (α1, α2, β, α2β)

Gene fragments coding for human Hp α1, α2, β and α2β subunits were amplified by PCR using proofreading DNA-polymerase and oligonucleotide primers (Fig. 1A). The primer design was based on the published cDNA sequence of human Hp [18]. The primers were prepared for Hp α1 and α2 (forward 5′-GGGGTACCATGGTGGACTCAGG CAATGATGT-3′ and reverse 5′-AACTGCAGTTACTG CACTGGGTTTGCCGGA-3′), Hp β (forward 5′-GGGG TACCATGATCCTGGGTGGACACCTGG-3′ and reverse 5′-AACTGCAGTTAGTTCTCAGCTATGGTCTTCT-3′), and Hp α2β (forward 5′-GGGGTACCATGGTGGACTC AGGCAATGATGT-3′ and reverse 5′-AACTGCAGTT AGTTCTCAGCTATGGTCTTCT-3′). Both of *Kpn*I and *Pst*I restriction sites were incorporated into the 5′ end of the forward sequence and reverse sequence primers, respectively. The cDNA of Hp α1, α2, β and α2β were ligated into the *Kpn*I/*Pst*I sites of an *E. coli* expression vector, pQE30. The plasmids were screened in JM109 and then expressed in M15 [pREP4]. Finally, the sequence of pQE30-Hp (α1, α2, β and α2β) was confirmed by DNA sequencing.

Expression of recombinant Hp subunits

Escherichia coli [M15 (pREP4)] was transformed with the recombinant plasmid and cultured in 1 L of Luria–Bertani (LB) medium containing ampicillin (100 μg/ml) at 37 °C on a rotary shaker. When the optical density reached 0.6 at 600 nm, the protein expression was induced by 1 mM IPTG at 37 °C for 2–4 h. The medium was centrifuged at 8000*g* for 5 min, and washed three times in a wash buffer containing 20 mM Tris–HCl, pH 8.0.

Disruption, wash and isolation of inclusion bodies

The induced cells were then suspended in 40 ml of wash buffer and sonicated for 5 min at 4 °C, followed by centrifugation at 20,000*g* for 20 min at 4 °C. The pellet containing the inclusion body was resuspended in 30 ml of 2 M urea containing 20 mM Tris–HCl, 0.5 M NaCl, 2% Triton X-100, pH 8.0 and sonicated as above, followed by centrifugation at 20,000*g* for 20 min at 4 °C. Finally, the pellet was washed twice in wash buffer and stored frozen for late processing.

Solubilization of rHp subunits from inclusion bodies

The inclusion bodies were dissolved in a binding buffer containing 20 mM Tris–HCl, 0.5 M NaCl, 5 mM imidazole, 6 M guanidine-HCl, and 1 mM 2-mercaptoethanol, pH 8.0. The mixture was gently stirred at 4 °C for 12 h and the insoluble material was removed by centrifugation at 20,000*g* for 20 min at 4 °C. Finally, the remaining soluble supernatant was then passed through a syringe filter (0.45 μm) and proceeded directly for purification and refolding.

Purification and refolding of rHp subunits

Initially, a 1 ml-HiTrap chelating column was washed with 5 ml distilled water using a 10 ml-syringe. After loading 1 ml of 0.1 M NiSO₄, the column was extensively equilibrated with the binding buffer. The recombinant fusion protein was subsequently loaded onto the column and washed with the binding buffer. The bound protein was then treated with 6 M urea, starting with the binding buffer mentioned above and finished at one without urea. Finally, the recombinant proteins were eluted using a 20 ml linear gradient starting with an elution buffer containing 20 mM Tris–HCl, 0.5 M NaCl, and 20 mM imidazole without 2-mercaptoethanol, pH 8.0 and ending with the same buffer containing 500 mM imidazole. Protein fractions were pooled and then desalted on a P-2 column using 0.05 M

ammonium bicarbonate, followed by lyophilization. Protein concentration was determined by the Lowry method [19], while using bovine serum albumin as a standard.

SDS–PAGE and Western blot analyses

Recombinant Hp subunits were characterized using SDS–PAGE containing 15% polyacrylamide as described previously [20]. In general, the tested sample was preheated at 100 °C for 10 min in a buffer containing 12 mM Tris–HCl, 0.4% SDS, 5% glycerol, 2.9 mM 2-mercaptoethanol and 0.02% bromphenol blue, pH 6.8, before loading to the gel. Western blot analysis was performed similar to that described previously [20]. In brief, the electrotransferred and blocked nitrocellulose was incubated with anti-Hp polyclonal or monoclonal antibodies (3H8 and G2D), followed by washes and incubation of peroxidase-conjugated anti-IgG. The membrane was developed using 3,3′-di-aminobenzidine (DAB) containing 0.01% H_2O_2 [20].

Determination of immunoreactivity of rHp subunits by enzyme-linked immunosorbent assay (ELISA)

ELISA was conducted according to the procedure previously established [20,21]. Half micrograms of each Hp subunit in 50 μl PBS (pH 7.4) was coated onto each well of an ELISA plate. After blocking and washes, 50 μl of diluted mouse mAb prepared against Hp α (3H8) or β chain (G2D) were added and incubated at room temperature for 2 h. Following washes and incubation of 50 μl of peroxidase-conjugated goat anti-IgG (1:3000 dilutions), the plate was developed using 0.04% 2,2-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) containing 0.01% H_2O_2. The assay was conducted in triplicates. In general, the standard error of the mean (SEM) was less than 2%.

Preparation of human low density lipoprotein

Human low density lipoprotein (LDL; d. 1.012–1.063 g/ml) was prepared from human plasma by sequential ultracentrifugations according to the method previously established [22,23].

Antioxidant activity using Cu^{2+}-induced LDL oxidation

Thiobarbituric acid-reactive substances (TBARS) were used as an index for the measurement of LDL oxidation [23,24]. In a typical assay, 5 μM $CuSO_4$ and 20 μg of LDL (protein) were incubated with tested samples in a final volume of 100 μl. After 2 h incubation at 37 °C, 250 μl of 20% tricholroacetic acid were added to precipitate proteins. Subsequently, 250 μl of 0.67% 2-thiobarbituric acid were added into the reaction mixtures and incubated at 80 °C for 30 min. The reaction mixtures were centrifuged at 3000*g* for 5 min. Supernatant (300 μl) in a 96-well plate was read at 540 nm [23]. The assay was conducted in triplicates. In general, the standard error of the mean was less than 2.5%.

Reduction and carboxymethylation of Hp

Tris–HCl buffer (0.01 M) containing 5.4 M urea and 1% (v/v) β-mercaptoethanol, pH 8.6, was added to 1 mg of Hp to make a final volume of 3.3 ml. The reaction mixture was flushed with nitrogen and incubated at room temperature. After 2 h, 20 mg iodoacetic acid was slowly added and maintained the pH at 8.6 by the addition of 1 M NaOH for 30 min. Finally, carboxymethylated (CM) Hp was desalted on a P2 column equilibrated with 0.1 M ammoniumbicarbonate and lyophilized [22].

Circular dichroic analysis

Lyophilized rHp subunits and CM-Hp were dissolved in 10 mM phosphate buffer (pH 7.4) with a final concentration of 0.2 mg/ml. About 300 μl aliquot was used for the analysis in a cuvette of 1 mm path length. Circular dichroic (CD) spectrum recorded between 190 and 300 nm (Jasco J-715 spectropolarimetry) was accumulated 20 times at a scanning rate of 50 nm/min [17,20].

Results and discussion

Plasmid construction and expression of rHp subunits

DNA sequencing analysis demonstrated that the cDNA of each Hp α1, α2, β or α2β (Fig. 1A) was inserted into the *E. coli* expression vector pQE30 (data not shown). The expression of $(His)_6$-tagged fusion proteins was constructed based on the T5 promoter transcription–translation system [25]. One advantage of this system is its ability to maintain target genes silently transcribed in the uninduced state, since the extremely high transcription rate initiated at the T5 promoter is efficiently regulated and repressed by the presence of high levels of the *lac* repressor. In addition, the pQE30 vector also permits the fusion of a 6 × His-tag at the N-terminus of a given recombinant protein, which is useful for rapid purification and permits the purification under denaturing conditions. Fig. 1B shows that clones containing cDNA of Hp or Hp subunit (α1, α2, β, or α2β) were abundantly and effectively induced by IPTG. Determined by densitometry on the SDS–PAGE gel, the average expressed level of each subunit was account for about 30% of the total-lysate proteins (data not shown). Thus, this expression system is considerably effective in yield. The recombinant proteins were almost exclusively expressed in the inclusion bodies. Only trace amount (<5%) of α subunits was found as a soluble form (data not shown). A full and intact length of α2β chain of Hp was expressed (Fig. 1B, lane 7). In human liver cells, however, the α2β is postranscriptionally cleaved into α and β chains between the residues Arg and Ile [26,27]. A more recent study suggests that a complement C1r-like protease is responsible for the cleavage [15]. Our data indicate that this protease is not present in the *E. coli*. The recombinant α2β expressed may, therefore, provide a unique substrate for the identification

of the specific protease involved in mammalian cells. This experiment is now in progress in our laboratory.

Purification and refolding of rHp subunits

The present study shows the rHp subunits to be primarily present in the inclusion bodies of *E. coli*. Since binding of a protein containing $6\times$ His-tag to nickel-immobilized column is not interfered by the chaotropic agent (such as urea or guanidine hydrochloride) at high concentration, $6\times$ His-tagged recombinant protein can be solubilized by the chaotropic extraction before loading to the column. Removal of contaminating materials followed by refolding the recombinant protein using nondenaturing buffer can then be performed before the elution from the column by 20 mM imidazole [28]. Using this strategy, we show that the purification for rHp subunits efficiently achieved with about 95% homogeneity as determined by a 15% SDS–PAGE (Fig. 1B). The final average yield of each recombinant purified was about 20% as calculated from the total *E. coli* proteins (Table 1). The average recovery from each step as judged by the SDS–PAGE, however, was greater than 70%.

With respect to the solubility of rHp subunits, we found that the solubility could be maintained when lyophilized protein was reconstituted into a final concentration less than 2 mg/ml by a PBS. It is of interest to point out that the eluted subunits were soluble if they were concentrated (up to 5 mg/ml) by Centricon tubes immediately following the desalting on a P-2 column, while using 0.05 M ammonium bicarbonate as a mobile phase.

Immunoreactivity of mAb for rHp subunits

To address whether the expressed proteins were immunoreactive, each subunit was examined by a Western blot analysis using Hp mAb specific to human α (3H8) and β chain (G2D) and a Hp polyclonal antibody specific to α/β chains. Fig. 1C shows that each subunit was specifically recognized by each regionally specific mAb. Using a quantitative ELISA, the immunoreactivity of native Hp was higher in some extent than that of expressed subunit (Fig. 2). Although the reason is not readily clear, it is possible that those mAb were originally prepared against the native state of the Hp structure and the antibodies preferably recognized the native conformation of Hp. Another potential explanation is that recombinant subunits might not be

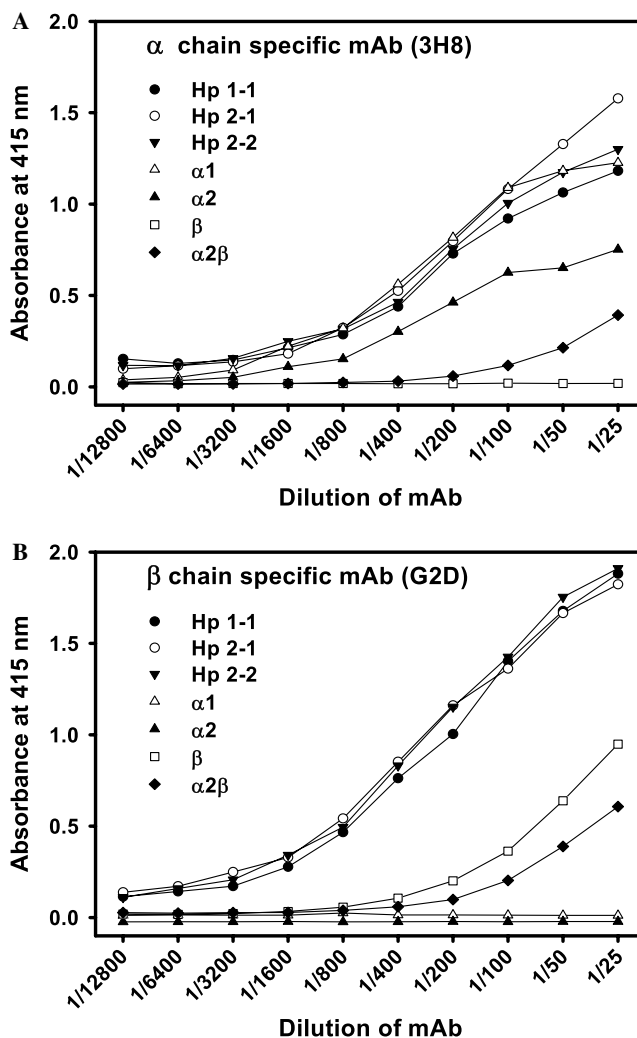
Fig. 2. Immunoreactivity of native Hp 1-1, 2-1, and 2-2 and recombinant Hp measured by ELISA using Hp α and β specific mAb. The expressed α2β possesses full length of Hp, but is not cleaved as α and β subunits. (A) The immunoreactivity of native Hp is moderately higher than that of expressed α2β subunit using α chain mAb. (B) The immunoreactivity of native Hp is greater than β and α2β subunits using β chain mAb. This could be due to the structural difference found in Fig. 4.

completely refolded as same as that in its native state (discussed below in CD spectral data). Nevertheless, the immunoreactivity of each expressed subunit was retained.

Antioxidant domain of rHp subunits

To determine the antioxidant activity of each Hp subunit, a TBARS method using Cu^{2+}-induced LDL oxidation was conducted [23]. Recombinant β subunit was extremely potent to protect LDL from oxidation and was in a dose-dependent manner. A typical example of the inhibition in TBARS is shown in Fig. 3. Ranking of the 50% of inhibitory concentration (IC_{50}) in antioxidant activity was as follow: $\alpha2\beta > \beta > \alpha1 \geqslant \alpha2 \geqslant$ probucol (Table 2). Most importantly, the antioxidant activity of α2β and β was markedly higher than that of α1 and α2 subunits. Thus, the major antioxidant domain of Hp was located in the β sub-

Table 1
Purification of recombinant Hp subunits from *E. coli*

Purification step	Yield (mg)				Purity[a](%)			
	α1	α2	β	α2β	α1	α2	β	α2β
Wet cells in 100 ml culture	410	450	420	510	37.3	32.8	38.5	23.3
Washed inclusion bodies	146	118	135	166	42.6	42.4	44.6	30.4
Ni^{2+}-column purified protein	14[b]	11[b]	5[b]	7[b]	95	95	95	95

[a] Determined by densitometry of 15% SDS–PAGE.
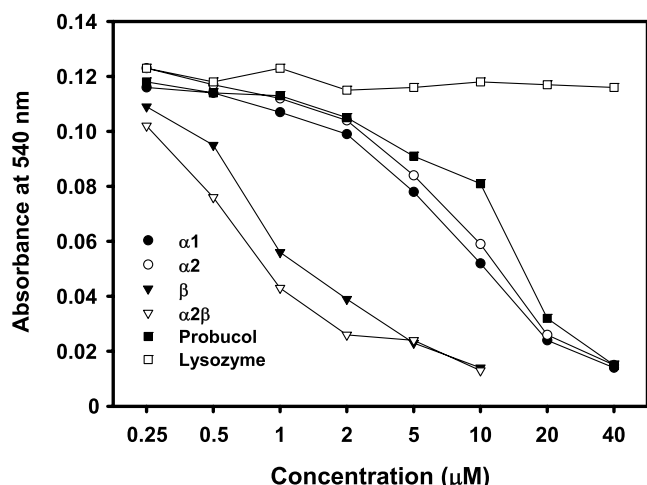[b] Determined using a Lowry method.

Fig. 3. Antioxidant activity of each recombinant subunit. The assay was evaluated using the degree of inhibition of Cu^{2+}-induced formation of TBARS from LDL. LDL (20 µg) was incubated with 5 µM Cu^{2+} in the presence of recombinant α1, α2, β, α2β or probucol at 37 °C for 2 h in a final 100 µl of PBS. Lysozyme served as a control did not reveal any antioxidant activity.

unit. It is of remarkable interest that the activity of β subunit was greater than that of native Hp molecule (Table 2). One of the possible explanations is that the random structure of the subunit may expose a neo-epitope that renders its availability for further scavenging the free radicals (discussed below). In the next experiment, we chemically modified Hp by carboxymethylation (CM) (in the presence of 5.4 M urea) to dissociate the disulfide linkages between α and β chains. The antioxidant activity of CM-Hp was substantially increased by about 3.75 folds when compared to native Hp (Table 2). The activity of CM-Hp was almost identical to β subunit. Because the carboxymethylation was conducted in the presence of 5.4 M urea, it is worth mentioning that native Hp exposed to the denaturing agent (5.4 M urea or guanidine hydrochloride) did not alter the antioxidant potency after renaturation by desalting (data not shown). The data suggest that the antioxidant domain of native Hp was exposed further, while reducing irreversibly. Another essential feature was that the antioxidant activity of β subunit was superior to probucol (15×): one of the most established potent antioxidants. Since probucol has been used for the treatment of patients with xanthoma

Table 2
Antioxidant potency in Cu^{2+}-induced LDL oxidation

	Type	Cu^{2+}-induced IC_{50}[a] (µM)
α1	Recombinant	7.50
α2	Recombinant	8.72
β	Recombinant	0.87
α2β	Recombinant	0.65
Probucol	Compound	13.70
Hp 1-1	Native	3.00
CM-Hp 1-1[b]	Native	0.80

[a] IC_{50}: the concentration that inhibited 50% of LDL oxidation.
[b] CM-Hp 1-1 was obtained by carboxymethylation to break up all the disulfide linkages.

and atherosclerosis for decades [29–33] and its analogs have been designed for antioxidant therapy [34,35], the recombinant β subunit plus the success of large expression in *E. coli* may pave the way for the future design of "mini-Hp".

Circular dichroic analysis

To address the moderately decreased immunoreactivity and high antioxidant activity in recombinant subunit were probably due to the conformational difference from the ordered structure of whole Hp molecule, the secondary structure of recombinant Hp subunits was studied by CD spectra. Fig. 4 depicts that all the α1, α2, β and α2β were typically disordered in structure (a sharp drop of ellipticity between 190 and 210 nm) as compared to native Hp. The result suggests that the conformational changes in each subunit may be responsible for the low binding to each chain specific mAb, as each mAb was originally prepared against the native Hp. Whereas, the random structure of β chain may facilitate its availability for scavenging free radicals. Furthermore, the carboxymethylation Hp also exhibited a disordered structure.

In conclusion, we demonstrate that Hp β chain is an extremely potent antioxidant directly preventing LDL against oxidation in the present study. Using a recombinant Hp cDNA without the signal sequence, the yield of each subunit was relatively high containing approximately 30% of total cell-lysate proteins. Each expressed subunit retained the immunoreactivity as confirmed by α and β chain specific mAb (3H8 and G2D). It is conceivable that expressed β subunit may provide as an initial utility for the future design of "mini-Hp" for potent antioxidant. It may also provide a new insight in understanding the mechanism or specific amino acids involved in free-radical scavenge when site-directed mutagenesis is carried out for further studies. The latter experiment is now in progress in our laboratory.
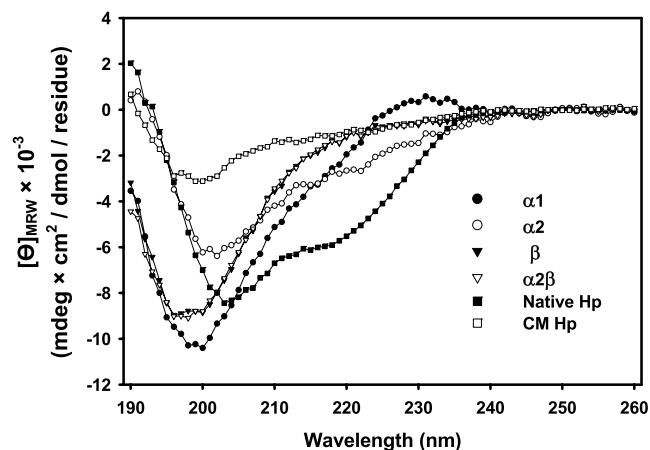

Fig. 4. Circular dichroic spectra of recombinant α1, α2, β and α2β subunits. Recombinant Hp subunit or Hp (final protein concentration 0.2 mg/ml) in 10 mM phosphate buffer, pH 7.4, was monitored by a circular dichroic spectrophotometer. Each spectrum represents a mean of 20 determinations. Essentially, recombinant α1, α2, β and α2β subunits and CM-Hp are typically random or disordered in structure (a sharp drop of ellipticity between 190 and 210 nm).

Acknowledgments

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