The 6th International Conference on Biochemistry and Molecular Biology

Characterization of modified crocodile (*Crocodylus siamensis*) hemoglobin using bis(3,5-dibromosalicyl) fumarate

Napaporn Roamcharern[1,2], Wisarut Payoungkiattikun[2], Nisachon Jangpromma[2,3], Sompong Klaynongsruang[1,2,*]

[1] Department of Biochemistry, Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand
[2] Protein and Proteomics Research Center for Commercial and Industrial Purposes (ProCCI), Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand
[3] Office of the Dean, Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand
*E-mail: somkly@kku.ac.th

Abstract

A variety of cross-linked cell-free hemoglobin (CL-Hb) compounds have been developed with the aim of creating novel hemoglobin-based oxygen carriers (HBOCs). In general, derived CL-Hb using different cross-linking agents exhibits a lower oxygen affinity than native hemoglobin. Therefore, crocodile (*C. siamensis*) hemoglobin (cHb) constitutes an interesting new approach in HBOC development, as it was reported to possess a higher oxygen affinity than human hemoglobin (hHb). In this study, we performed a modified cHb through cross-linking with bis(3,5-dibromosalicyl) fumarate (DBBF) and aimed to investigate its basically physiological and biological functions as comparing with hHb. The DBBF-cHb plays a different characteristic of cross-linking, since there was the absence of ββ-dimer band on SDS-PAGE. It is important to note that DBBF-cHb exhibited a higher oxygen affinity than DBBF-hHb with P50 value of 20.1 mm Hg (n = 2.5). In conclusion, cHb exhibited oxygen binding characteristics that may benefit the development of a cHb-based oxygen carrier suitable for human use in the future.

Introduction

Hemoglobin-based oxygen carriers (HBOCs) have been developed as an alternative to human blood transfusions which required ideally characteristics to accomplish many drawbacks of human blood usage such as, universal blood compatibility, no toxicity and no transmission of diseases, sufficient intravascular half-life, prolong shelf life, etc. However, isolation of hemoglobin from its natural environment, inside red blood cell, called stroma-free hemoglobin or cell-free hemoglobin exhibits many adverse effects. Outside the red blood cells, hemoglobin can be dissociated into di- and monomer forms which rapidly excreted by kidney and leaded to renal failure. Moreover, the lack of 2,3-BPG, the major oxygen affinity regulator of hemoglobin, results in increasing of oxygen binding affinity which differs from its natural characteristic.[1,2]

Recently, there are many modifications of hemoglobins which specifically aimed to overcome HBOCs development's restrictions including, pyridoxilation (decreases oxygen affinity), polymerization (reduces dissociation, oncotic pressure, and prolong intravascular half-life), intramolecular cross-linking (reduces dissociation and increases intravascular retention time), conjugation (prevents dissociation), and microencapsulation by liposome (reduces immunogenicity and prevents dissociation).[2,3] The intramolecular cross-linking using bis(3,5-dibromosalicyl) fumarate (DBBF) is well known to reduce dissociation of tetrameric

forms and prolong intravascular half-life of human hemoglobin. DBBF molecule specifically reacts with the symmetric two lysine-99(α) for deoxyhemoglobin or two lysine-82(β) for oxyhemoglobin resulting in stabilization of αα-dimer and ββ-dimer, respectively.[4]

The oxygen affinity of stroma-free hemoglobin is not completely regulated by 2,3-BPG that retained inside red blood cells. Consequently, the stroma-free human hemoglobin possesses high oxygen affinity and changes its oxygen unloading capacity. This limitation was found to be solved by the use of animal hemoglobin such as, bovine hemoglobin which its oxygen affinity is not mainly regulated by 2,3-BPG but high sensitive to chloride ion.[5] Likewise the crocodilian hemoglobin (cHb), its oxygen affinity is markedly reduced by bicarbonate ion instead of 2,3-BPG resulted a controllable oxygen releasing ability in outside red blood cells' environment.[6] Moreover, the native crocodilian hemoglobins possess high oxygen affinity therefore, passing through a strong modification process, which may lead to decrease in its biological function via an alteration on protein structure, it may prone to potentially retain oxygen affinity higher than human hemoglobin does. In recent study, we aim to investigate preliminary characteristics of DBBF cross-linked Siamese crocodile (*Crocodylus siamensis*) hemoglobin. Firstly, the cross-linking process of crocodile hemoglobin (cHb) was set along with human hemoglobin (hHb) as a comparative. Then biological characteristic was examined to answer DBBF cross-linking effects.

Methodology:

Hemoglobin extraction

The crocodile blood was supplied by a slaughterhouse of Sriracha MODA Co., LTD (Chon Buri, Thailand) and was managed by follow the method of Peta.[7] The human blood was obtained from central blood bank (faculty of medicine, Khon Kaen University). The hemoglobin was extracted from the red blood cell by the method of Jandaruang.[8] Briefly, whole blood samples were centrifuged at 8,000 × *g* for 2 min (4 °C) (High speed refrigerated centrifuge, Himac CR 22GII, Hitachi, Japan). Collected red blood cell pellets were then re-suspended in 1× phosphate buffer saline (PBS, pH 7.4) and centrifuged at 3000 × *g* for 5 min (4 °C). After red blood cell pellet was washed twice then, it was allowed to lyse in cold double distilled water with vigorously shaken and stored at 4 °C for 10 min. The hemoglobin solution was obtained after cell debris was removed by centrifugation at 10,000 × *g* for 20 min (4 °C).

Cross-linking process

Bis(3,5-dibromosalicyl) fumarate (DBBF) was purchased from Abcam (USA) and cross-linking process was performed by follow the method of Walder[9] with slightly modification. Briefly, 10 ml of 5 mg/ml hemoglobin solution was prepared in 1× PBS (pH 7.0) then mixed with 5.7 ml of 287 µM DBBF solution. The hemoglobin mixture was allowed to be shaken on ice for 2 h and then 100 µl of 1 M glycine (pH 8.8) was added to quench the reaction.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The SDS-PAGE was performed by follow the method of Dimino[10] using 14% separating gel and 4% stacking gel. The protein samples were mixed with 2× SDS loading buffer (0.5 M Tris-HCl (pH 6.8), 0.5% (w/v) bromophenol blue, 2% (w/v) SDS, 10% (v/v) glycerol, 10% (v/v) β-mercaptoethanol) at a ratio of 1:1. Then the protein samples were boiled for 5 min. The proteins were separated under electric field of 120 volts for 2 h and proteins were stained by Coomassie brilliant blue R-250. The low molecular weight calibration kit (Amersham™, GE healthcare, UK) was used to be a protein marker.

Oxygen affinity measurement

Oxygen affinity was investigated using a conventional method of Asakura[11] with minor modification. Hemoglobin samples (35 µM heme content) were prepared in 1× PBS containing 71.4 mM of Cl^- (pH 7.4). Four milliliters of hemoglobin sample was transferred to a Thunberg then degased for 30 min using vacuum pump and passed through the argon gas for 10 min, then degased again for 30 min. Before oxygen consumption has been performed, completely deoxyhemoglobin formed has been confirmed using UV-Vis spectroscopy technique (Spectronic 200, Thermo Fisher Scientific, India) at 25 °C. The known volume of the air was added into the Thunberg tube for oxygenation and incubated for 5 min then measured the absorption spectra of 400-700 nm. The changes of deoxyhemoglobin spectrum to oxyhemoglobin spectrum was observed. Percent oxygen saturation (%Y) and partial pressure of the oxygen (pO_2) were then calculated as following equations,

$$\%\mathrm{Y} = \left[\frac{D}{DHbO_2}\right] \times 100$$

$$pO_2 = \frac{1}{V_c}\left\{P_a V_0 - 760\left[\frac{1.36XY}{100}\right]\right\}$$

Where D is the absorbance of hemoglobin solution under each round of examination, $DHbO_2$ is the absorbance of hemoglobin solution at the complete oxygenation (same as atmospheric pressure), pO_2 is the partial pressure of oxygen at 25 °C, P_a is the oxygen pressure in the air at atmospheric condition (150 mmHg), V_0 is the volume of introduced air (ml), V_c is the volume of the gas phase inside the Thunberg tube (ml), and X is the weight of hemoglobin in solution (g). The oxygen affinity and cooperative activity were reported in a term of P50 and Hill's cooperativity coefficient (n value) which obtained from the oxygen equilibrium curve (OEC) and Hill's plot, respectively.

Statistical analysis

All samples were triplicated analysis. One way ANOVA and Duncan's new multiple range test were performed using SPSS ver. 19.0 (The p value of < 0.05 was considered significant).

Results and discussion

Hemoglobin cross-linking

Crocodile hemoglobin and human hemoglobin samples were extracted and yielded the average protein concentration of 30 mg/ml (950 µM heme) and 23 mg/ml (750 µM heme), respectively. To provide an intact dimeric form, hemoglobin was modified via an intramolecular cross-link with DBBF, a specific cross-linking agent for human hemoglobin. SDS-PAGE analysis (Figure 1) revealed that hHb can be easily cross-linked with DBBF resulting in an increasing in intensity of ββ-dimer band (~30.0 kDa). This result was correlated with the report of Walder[9] that DBBF potentially reacts with a specific position at symmetric two lysine-82(β) residues of oxy-hHb. However, the ββ-dimer band of cHb was not observed (Figure 1). It is speculated that the cross-linking condition may not suitable because of a replacement of arginine residue at position of 82(β) and a shift of lysine residue to position of 76(β) and 90(β) of amino acid sequence of cHb (Figure 2). Hence, the result revealed that inappropriate position and amino acid residue are important factor to accomplish the DBBF cross-linking of cHb.

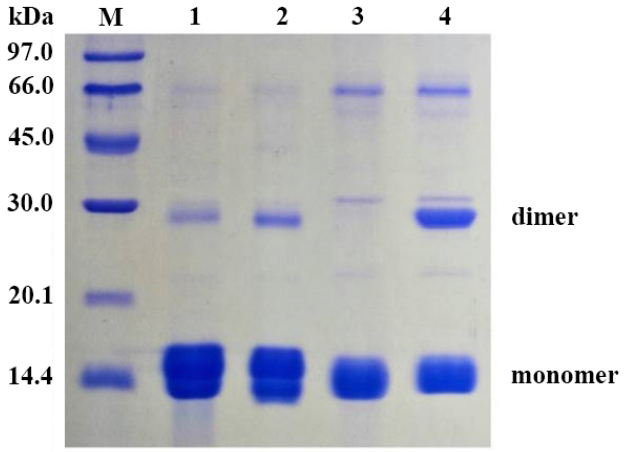

Figure 1. Protein pattern of native and cross-linked hemoglobins. Lane M: molecular weight marker, lane 1: native-cHb, lane 2: DBBF-cHb, lane 3: native-hHb, and lane 4: DBBF-hHb.

```
A

H. sapiens      VLSPADKTNVKAAWGKVGAHAGEYGAEALERMFLSFPTTKTYFPHFDLSHGSAQVKGHGK 60
C. siamensis    VLSSDDKCNVKAVWCKVAGHLEEYGAEALERMFCAYPQTKIYFPHFDLSHGSAQIRAHGK 60
                ***. ** ****.* **..*  ********** ::* ** **************::.***

H. sapiens      KVADALTNAVAHVDDMPNALSALSDLHAHKLRVDPWNFKLLSHCLLVTLAAHLPAEFTPA 120
C. siamensis    KVFAALHEAVNHIDDLPGALCRLSELHAHSLRVDPVNFKFLAQCVLVVVAIHHPGSLTPE 120
                **  ** :** *:**:*.**. **:****.***** ***:*::*:**.:* * *..:**

H. sapiens      VHASLDKFLASVSTVLTSKYR 141
C. siamensis    VHASLDKFLCAVSSVLTSKYR 141
                *********.:**:*******

B

H. sapiens      VHLTPEEKSAVTALWGKVNVDEVGGEALGRLLVVYPWTQRFFESFGDLSTPDAVMGNPKV 60
C. siamensis    SAFNPHEKQLIGDLWHKVDVAHCGGEALSRMLIVYPWKRRYFENFGDISNAQAIIHNEKV 60
                  :.*.**. :  ** **:* . *****.*:*:****.:*:**.***:*..:*:: * **
                                       ↓
H. sapiens      KAHGKKVLGAFSDGLAHLDNLKGTFATLSELHCDKLHVDPENFRLLGNVLVCVLAHHFGK 120
C. siamensis    QAHGKKVLASFGEAVKHLDSIRAHFANLSKLHCEKFHVDPENFKLLGDIIIIVLAAHYPK 120
                :*******.:*.:.: ***.::. **.**:***:*:*******:***:::: *** *: *
                              ↑             ↑
H. sapiens      EFTPPVQAAYQKVVAGVANALAHKYH 146
C. siamensis    DFGLECHAAYQKLVRQVAAALAAEYH 146
                :*    :******:*  ** *** :**
```

Figure 2. Amino acid sequence alignment of hemoglobins insisted of α-globin chain (A) and β-globin chain (B). Down arrow is position of amino acid residue which plays an important role in DBBF cross-linking reaction. Up arrows are lysine residues on β-globin chain of cHb which nearly locate to position of 82(β). Star symbol (*) indicates identical amino acid. Colon symbol (:) indicates strong conservation of amino acid based on similarity of amino acid properties. Dot symbol (.) indicates weak conservation of amino acid based on similarity of amino acid properties.

Oxygen affinity of native and cross-linked Hb

To determine an oxygen binding affinity of hemoglobin, the result demonstrated that hemoglobin spectrum exhibited a transformation of deoxyhemoglobin (single peak) to oxyhemoglobin (double peak) (Figure 3). This result was correlated to the increasing of oxygen pressure which yielding in the enhance effect of oxygen saturation. The result found that DBBF modification caused a right shift of a sigmoid curve which indicated to a decrease in oxygen binding affinity (Data not shown). In general, native-cHb possessed a higher oxygen binding

affinity (lower P50 value) than native-hHb with P50 value at 6.9 and 12.4 mm Hg, respectively. Meanwhile, DBBF modification resulted in decreasing of oxygen binding affinity for 2.9-fold (20.1 mm Hg) and 2.1-fold (25.9 mm Hg), respectively (Table 1). This lower oxygen binding affinity confirmed that DBBF caused strong alteration on heme environment. Hence, on the best of our knowledge, DBBF demonstrated the conjugation characteristic toward cHb molecule. Moreover, both of DBBF-cHb and DBBF-hHb still remained cooperative activity with Hill's cooperativity coefficient (n) higher than 1 (Table 1).

Previous report demonstrated that oxygen binding affinity was decreased via the increasing of many factors including temperature, 2,3-BPG, of H^+ and Cl^- ions concentration.[12] Here, in terms of temperature, oxygen binding affinities were investigated at 25 °C , while many reports performed at 37 °C (Table 1). Therefore, our report on oxygen binding affinities may be slightly changed which might affect to an increasing of P50 value. However, our results demonstrated a similar trend of P50 in both cHb and hHb which yielded from hemoglobin modification processes. In addition, P50 of our modified HBOCs were fall within the range of reported HBOCs from various species. As shown in Table 1, the αα-Hb (33 mm Hg) and Tm-Hb (38.7 mm Hg) are intramolecular cross-linked hHb through DBBF and trimesoyl tris(methyl phosphate), respectively.

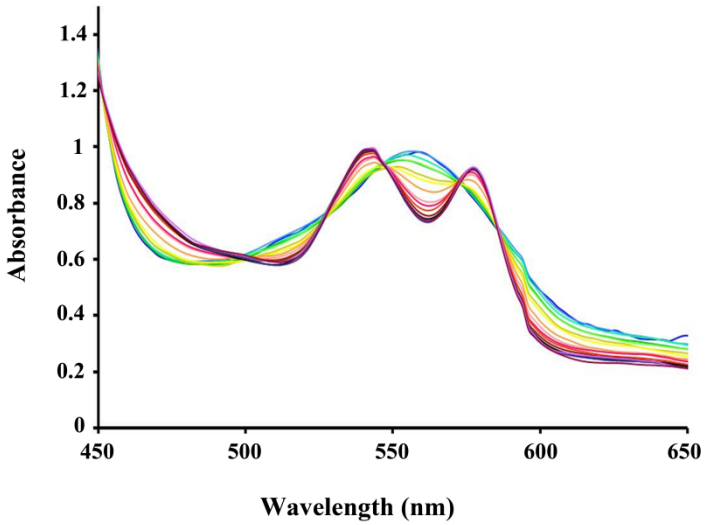

Figure 3. The transformation of crocodile hemoglobin spectra from deoxyhemoglobin (single peak) to oxyhemoglobin (double peak) by the increasing of oxygen pressure.

Table 1. P50 values and Hill's cooperativity coefficients (n) of native and modified hemoglobins.

Samples	Sources	P50 (mm Hg)[a]	n[b]	References
Native hemoglobins				
Native-cHb	Crocodile	6.9 ± 0.3 [A]	2.76	This study
Native-hHb	Human	12.4 ± 4.2 [B]	2.83	This study
Native-hHb	Human	13.8 ± 0.4	2.80	Wang Q[13]
Native-bHb	Bovine	26.9 ± 1.1	2.20	Jia Y and Alyash AI[14]
Native-pHb	Porcine	19.5 ± NA	NA	Kim HD and DUHM J[15]
Modified cell-free hemoglobins				
DBBF-cHb	Crocodile	20.1 ± 1.5 [C]	2.46	This study
DBBF-hHb	Human	25.9 ± 1.1 [D]	2.89	This study
αα-Hb	Human	33.0 ± 0.2	2.45	Rohlfs RJ[16]
Tm-Hb	Human	38.7 ± 2.0	2.80	Rohlfs RJ
PHP-Hb	Human	20.4 ± 1.3	1.64	Rohlfs RJ
PEG-Hb	Human	10.2 ± 0.6	1.39	Rohlfs RJ
o-R-poly-Hb	Bovine	52.6 ± 1.5	1.01	Rohlfs RJ
PEG5K-Hb	Human	9.4 ± 0.5	2.00	Wang Q
PolyHb-Tempol	Porcine	30.0 ± NA	NA	Wu M[17]
PEG βX-Hb	Bovine	9.67 ± 0.4	1.43	Webster KD[18]
ZL-HbBv	Bovine	6.4 ± 0.2	1.20	Jia Y and Alyash AI

[a]The P50 value is derived from oxygen equilibrium curve and refers to a partial pressure of oxygen at a half saturation of hemoglobin.

[b]The Hill's cooperativity coefficient (n) is a slope that derived from Hill's plot and refers to cooperativity of allosteric proteins (n > 1).

The different capital letters indicate to be significantly different (p value < 0.05).

NA means the data is not available.

Conclusion

This study aimed to develop the Siamese crocodile Hb to HBOC using chemically cross-linking reaction of DBBF. In addition, this study inspired and gave us the interesting evidence on cHb which exhibited a good oxygen binding characteristics after DBBF modification. Therefore, cHb may be suitable to use as alternative hemoglobin in HBOCs development.

References

1. Frietsch T, Lenz C, Waschke F. Eur J Anaesthesiol Suppl. 1998;15:571-584.
2. Buun HF, Esham WT, Bull RW. J. Exp. Med. 1969;129:909-923.
3. Inayat MS, et al. Transfus *Apher Sci. 2006;34:25-32.*
4. Snyder SR, Welty EV, Walder RY, Williams LA, Walder JA. Proc Natl Acad Sci USA. 1987;84:7280-7284.
5. Fronticelli C, Sato T, Orth C, Bucci E. Biochim. Biophys. Acta. 1986;874:76-81.
6. Komiyama NH, Miyazaki G, Tame J, Nagai K. Nature. 1995;373:244-246.
7. Peta S, et al. Dev Comp Immunol. 2011;35:545-553.
8. Jandaruang J, et al. Protein J. 2012;31:43-50.
9. Walder RY, Andracki ME, Walder JA. Methods Enzymol. 1994;231:274-280.

10. Dimino ML, Palmer AF. J Chromatogr Anal Technol Biomed Life Sci. 2007;856:535-357.
11. Asakura T, Kawai Y, Yoneyama Y, Yoshikawa H. Anal Biochem. 1964;7:393-400.
12. Bain BJ. JCS. 2017;45(4):187-193.
13. Wang Q, et al. Biochim. Biophys. Acta. 2004;1844:1201-1207.
14. Jia Y and Alyash AI. Biochim. Biophys. Acta. 2009;1794:1234-1242.
15. Kim HD and DUHM J. Am J Physiol. 1974;226(4):1001-1006.
16. Rohlfs RJ, et al. J. Biol Chem. 1998;273:12128-12134.
17. Wu M, et al. Artif Cells Nanomed Biotechnol. 2018;46:47-55.
18. Webster KD, et al. J Artif Organs. 2017;41(4):351-358.

Acknowledgements

This study was supported by Office of the Higher Education Commission and The Army Research and Development office (Thailand) and Protein and Proteomics Research Center for Commercial and Industrial Purposes (ProCCI), Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand.