Oligomerization of Bovine Hemoglobin and Characterization of the Potential Blood Substitutes

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Summary: Bovine hemoglobin (HbBv) was isolated from whole blood and additionally purified by anion exchange chromatography. To obtain derivatives potentially suitable as oxygen carriers, oxidized trehalose was used as cross-linking reagent. Intra- and intermolecular bonds were formed by reaction between reactive aldehyde groups of oxidized trehalose and primary amine groups on the protein surface. After reduction of the Schiff bases formed, the HbBv derivatives were characterized by high performance liquid chromatography (HPLC), electrophoresis and by ultraviolet spectroscopy to evaluate methemoglobin contents.

Keywords: blood substitutes; bovine hemoglobin; electrophoresis; high performance liquid chromatography; oligomers

Introduction

A significant number of research groups have been working on the development of oxygen carriers, the so-called blood substitutes. These products are designed to replace whole blood in cases of severe blood loss, such as trauma and surgery. They would offer advantages over transfusion of red blood cells, including a longer shelf life, no need of previous typing and cross-matching, and minimal risk of transmittance of infectious diseases. Hemoglobin is an advantageous choice because of its ability to successfully transport oxygen to tissues in the body. Hemoglobin is a tetrameric protein consisting of two tightly bound α-β dimers held together by hydrogen bonds. In solution, tetrameric hemoglobin is in equilibrium with its α-β dimers. Modifying hemoglobin by intra- and intermolecular cross-linking prevents its dissociation, limiting renal toxicity,[1,2] and extending its half-life in circulation.[3–5] Some human and bovine hemoglobin based oxygen carriers are currently under clinical trials.[6,7] The low supply of human hemoglobin from outdated red blood cells represents a critical factor to its use in commercial products. By contrast, bovine blood is abundant and may be collected in slaughterhouses from controlled healthy animals. Bovine hemoglobin (HbBv) has been shown to be an excellent substitute for human hemoglobin (HbA) as an oxygen carrier because it can withstand heme degradation much better than HbA.[8] Studies have also shown that HbBv is capable of effectively transporting oxygen to tissues. In the presence of physiological concentrations of chloride ions, HbBv hemoglobin has an oxygen affinity similar to that of human hemoglobin completely saturated by its allosteric effector 2,3-diphosphoglycerate.[6]

Hemoglobin has been subjected to chemical modification reactions that include reductive alkylation and acylation of primary amino groups, amidation of carboxylic acid groups, and S-alkylation of sulphydryl groups. In the first case, the free aldehyde goups on both ends of glutaraldehyde were used to form intramolecular cross-links and also to produce a chain of tetrameric hemoglobins (polymerized hemoglobin, polyHb).[9] Recently, a similar

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technique has been applied to HbBv to obtain polyHb in reactions with a series of oxidized mono-, di-, tri-, and polysaccharides as cross-linking reagents.[10]

In the present work, HbBv was isolated, purified by anion exchange chromatography, and submitted to chemical modification reactions in the presence of previously oxidized trehalose (*o*-trehalose). Different conditions of *o*-trehalose/ HbBv ratio, reaction time, and reducing agent/ *o*-trehalose ratio were used. After reduction, the resulting products were characterized by high performance liquid chromatography (HPLC), electrophoresis, and UV spectroscopy.

Materials and Methods

Materials

Bovine blood was collect in an anticoagulant citrate/dextrose solution and maintained at 5 °C until purification procedures. Drabkin's reagent, the dye-binding reagent used for albumin quantification (2.5 mM bromcresol in 0.82 M lactic acid at pH 4 containing 30 mL/L of Tween 80), and hemiglobincyanide (HiCN) were purchased from Doles Reagents (Goiânia, Brazil). MW-SDS-70L Kit for Electrophoresis were purchased from Sigma Chemical Co. (Saint Louis, USA). An anion exchanger, Q Sepharose Fast Flow (Q-SFF) from Pharmacia Biotech (Wikströms, Sweden) was used after purification and conditioning. All other reagents and solvents (PA grade) were supplied by Vetec Química Fina Ltda, (Rio de Janeiro, Brazil) and used as received.

Methods

Bovine blood (200 g) was submitted to carbonylation reaction with CO gas to convert oxyhemoglobin (HbO_2) to carbonylhemoglobin (HbCO),[11] and centrifuged to eliminate leukocytes, platelets and some plasma proteins different from hemoglobin. The resulting suspension was washed with an equal weight of isotonic saline solution, and centrifuged. The washing/centrifugation procedure was repeated other four times. The presence of bovine serum albumin was investigated, by analyzing each supernatant according to the bromcresol green method at pH 4.[12] Hemolysis was carried out by sonication at 8 °C for 5 min with a 750 W Cole Parmer Processor (Vernon Hills, USA). The suspension of lysed cells was heated to 60 °C for 1 h in the dark, and centrifuged. The hemoglobin solution was recovered as the supernatant, and filtered through 0.22 μm membranes. A Q-SFF anion exchanger column was used to purify HbBv solution, which was eluted with 0.001 mol L^{-1} Tris/HCl pH 7.4. The hemiglobincyanide (HiCN) method was used to determine HbBv concentration in each collected fraction.[13] The purified HbBv solution was treated with organic solvent to extract possible residual phospholipids. Normal phase HPLC was used to verify the presence of residual phospholipids, according to the procedure described in detail elsewhere.[14]

To obtain HbBv oligomers, trehalose was oxidized by $NaIO_4$ at room temperature under N_2. Different trehalose/$NaIO_4$ molar ratios and reaction times were used. The oligomerization reactions were followed by reduction of the Schiff bases with $NaBH_4$ at different trehalose/$NaBH_4$ ratios, for 30 min. Then, the reaction mixtures were dialysed at 4 °C for 24 h. The three steps were carried out in the dark. Normal phase HPLC analyses were performed with a Pharmacia LKB-HPLC pump model 2248 (Uppsalla, Sweden) equipped with a UV-vis Shimadzu detector model SPD-10AV from Shimadzu Scientific Instruments (Columbia, USA), set at 280 nm. A Biosep Sec S3000 column from Phenomenex (Montgomeriville, USA) was used. The samples were eluted with a 0.1 mol L^{-1} Tris/HCl pH 7.4 with 0.2 mol L^{-1} $MgCl_2$. SDS-PAGE was carried out in a single-sided vertical Owl Scientific Inc. system, model P81 (Woburn, USA), equipped with a Electrophoresis Power Supply E 835 from Consort nv (Turhout, Belgium), at 2 W. Samples at 1 g L^{-1}

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Table 1.
Phopholipids concentration in isolated and purified HbBv samples.

HbBv Sample	Phospholipids concentration[a)]/(μg mL^{-1})			
	PS	PE	PC	SM
Isolated	23.5 ± 2.7	1.7 ± 0.5	3.7 ± 1.1	36.9 ± 0.9
Q-SFF purified	Nd	Nd	Nd	Nd

[a)] PS, Phosphatidylserine; PE, Phosphatidylethanolamine; PC, Phosphatidylcholine; SM, sphingomyelin.

concentration were prepared by heat denaturation at 100 °C for 5 min in an appropriate buffer.[14] The concentration of methemoglobin (met-HbBv) in the modified hemoglobin solutions was determined using an adaptation of the cyanomethemoglobin method, following the methodology described in the literature.[10,15]

Results and Discussion

After both isolation and additional purification, organic extracts from the HbBv samples were analyzed by HPLC. Table 1 shows that, although isolated HbBv solutions contained substantial amounts of phospholipids, they were eliminated by additional purification by anion exchange chromatography.

Chemical modification reactions of HbBv were carried out by intra- and intermolecular covalent bonds formation with *o*-trehalose, followed by reduction with $NaBH_4$ in the presence of $MgCl_2$, used to dissociate any noncross-linked HbBv into α,β-dimers. Table 2 shows reaction conditions and the degree of chemical modification, determined by HPLC.

There are two main sites for the formation of cross-linkings to the protein. The first corresponds to lysine 82 on the β-globin chain, which leads to a β-β bond. The second site occurs between the lysine 99 residue on the α-globin chain, and leads to a α-α bond. Figure 1 shows some HPLC chromatograms for HbBv derivatives. Peaks at 13.5 min correspond to the control of dissociated unmodified HbBv. Peaks at 13 min can be attributed to pairs of intramolecularly cross-linked α,β-dimers. Peaks at 12 and 8 min correspond to intermolecularly cross-linked tetramers and to HbBv oligomers, respectively. Sample 8 (Figure 1e) reached almost 100% conversion, and is composed of the highest content of oligomers.

Figure 2 shows SDS-PAGE electrophoresis analyses for the purified HbBv sample

Table 2.
Reaction conditions and degree of chemical modification determined by HPLC.

Sample	Reaction conditions			
	[*o*-Trehalose]/[HbBv] ratio	Reaction time/(h)	[$NaBH_4$]/[*o*-Trehalose]	% Chemical modification
1	5:1	2	2:1	2.3
2	6.36:1	6	10:1	72.0
3	20:1	2	2:1	2.8
4	20:1	4	5:1	77.4
5	20:1	4	15:1	74.3
6	40:1	3.2	10:1	47.4
7	40:1	6	1.59:1	76.0
8	40:1	6	18.4:1	~100.0
9a	40:1	6	10:1	47.5
9b	40:1	6	10:1	46.8
10	60:1	4	2:1	81.1
11	60:1	4	5:1	78.0

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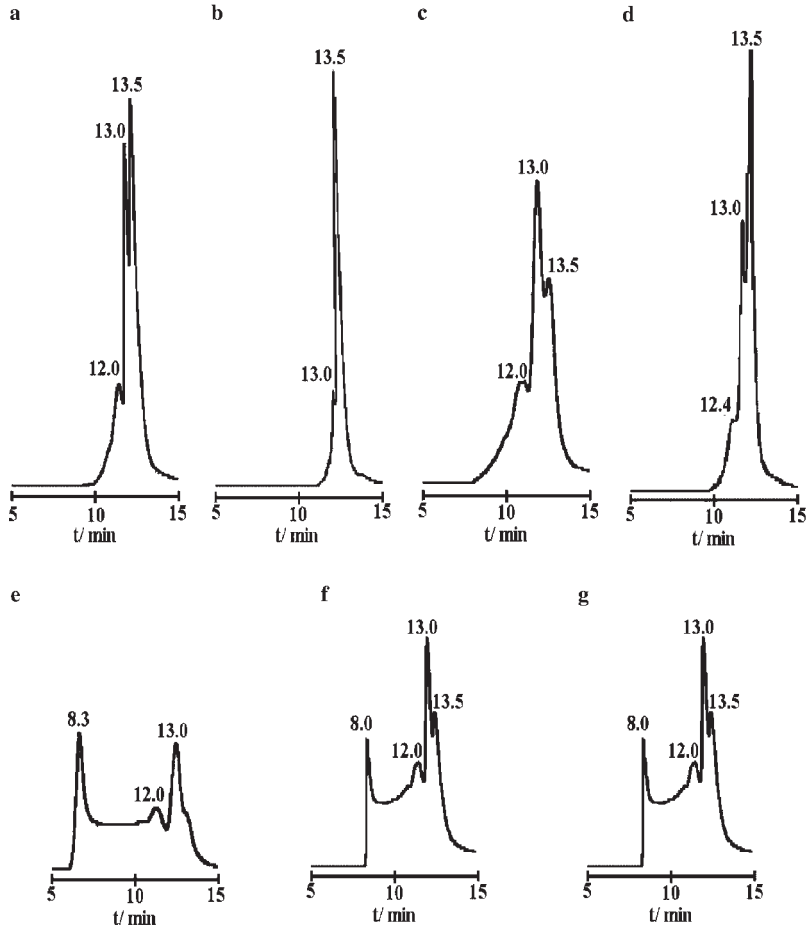

Figure 1.
HPLC chromatograms for HbBv derivatives samples 2 (a), 6 (b), 7 (c), 9a (d), 8 (e), 10 (f) and 11 (g).

(lane 2) and modified HbBv samples 2 (lane 3), 6 (lane 4), 7 (lane 5), 8 (lane 6), 9a (lane 7), 9b (lane 8), 10 (lane 9), and 11 (lane 10), in comparison with the standard solution, prepared with MW-SDS-70 markers (lane 1). The presence of only one protein species was observed in lane 2, and this band corresponds to purified HbBv. Bands around 24.0 kDa, found for modified HbBv samples, indicate formation of intramolecular covalent bonds. Bands at higher molar masses, observed in lanes 3–10, are attributed to species with intermolecular cross-linkings.

Oxidation of hemoglobin to the non-functional ferric form (met-Hb) is an important issue to be taken into consideration. Met-Hb levels greater than 10% can be toxic.[16] Table 3 shows met-HbBv levels, obtained for each sample. Met-HbBv contents were lower than 10% for the samples analyzed.

Conclusion

According to the results obtained in the present work, a high trehalose/HbBv ratio (40:1), a reaction time of 6 h, and a high $NaBH_4$/*o*-trehalose ratio (18.4:1) led to the highest content of HbBv oligomers, with a low level of methemoglobin.

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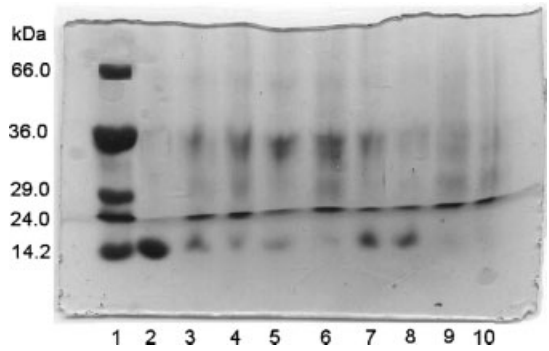

Figure 2.
SDS-PAGE electrophoresis analyses for the purified HbBv sample (lane 2) and modified HbBv samples 2 (lane 3), 6 (lane 4) 7 (lane 5) 8 (lane 6) 9a (lane 7), 9b (lane 8), 10 (lane 9), and 11 (lane 10).

Table 3.
Quantification of methemoglobin in modified HbBv samples.

Sample	1	2	3	4	5	6	7	8	9a	9b	10	11
MetHbBv (%)	7.4	7.6	–	9.9	5.6	7.2	3.7	4.7	6.8	7.0	3.1	3.1

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