

Preparation of Stable and Highly Active Hemoglobin by Using Antiglycosylspecific Hemoglobin Polyclonal Antibodies

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Abstract- The highly purified oligosaccharide chains obtained from glycoprotein invertase were coupled with bovine hemoglobin with the help of cross linking agent. The neoglycoprotein hemoglobin was highly purified to homogeneity by column chromatography and characterized on SDS- PAGE. Rabbits were immunized to produce glycosylspecific antihemoglobin polyclonal antibodies. Antibodies were purified by $(\text{NH}_4)\text{SO}_2$ precipitation followed by DEAE cellulose chromatography. The IgG-Sepharose was prepared by covalently coupling the purified polyclonal antibodies to CNBr-activated Sepharose 4B. The large amount of hemoglobin was immobilized on IgG-Sepharose by alternate incubation of hemoglobin and glycosylspecific antihemoglobin polyclonal IgG. The immunoaffinity based layered assembled preparation were highly active. The amount of hemoglobin immobilized could be raised 55 fold after four binding cycles. A layer by layer immobilization of hemoglobin significantly increases its life span and improved in stability against high temperature and other several denaturants (pH and salicylate). The results show the excellent platform for protein immobilization with high affinity. The polyclonal antibodies as supporting material has been found challenging for immobilizing the proteins. Further antiglycosylated hemoglobin can be used as a nanoparticle for immobilizing high amount of hemoglobin with no cytotoxicity to cell and this technique enhances the incorporation of hemoglobin properties (recognition, transport and catalytic properties). The applications of the immobilized glycosylated hemoglobin are found in the field of medicine, biotechnology, nanotechnology and biosensors.

Index Terms- Immobilization, glycosyl specific antibodies, Hemoglobin, stability, Application
Abbreviation : ConA, concanavalinA

I. INTRODUCTION

Hemoglobin is an important respiratory protein in red blood cells has a molecular weight of approximately 64,500 and consists of four polypeptide chains each with electroactive iron heme group [1]. It is an iron containing oxygen transport metalloprotein used extensively as an ideal electron transfer molecule in biosensor [2], [3]. The measurement of hemoglobin is of great importance due to its significance as a mediator in clinical, pharmaceutical, and as electrochemical biosensors

[4],[5]. The polyclonal antibodies as supporting material has been found challenging for immobilizing the enzymes and proteins. In the authors laboratory, the high yield and very stable immobilized glucose oxidase preparations were obtained by immunoaffinity- layering of enzyme with polyclonal antibodies and $(\text{Fab})_2$ fragments [6],[7].

The carbohydrate specific antibodies have also been successfully employed for the high yield immobilization and stabilization of glycoenzymes [8]. In the present study an efforts have been made to obtain high yield immobilized neoglycohemoglobin preparation by assembling the proteins in layers by using glycosyl specific antihemoglobin polyclonal antibodies. These various immunoaffinity layered preparations of hemoglobin have been investigated for its stability against pH, temperatures and few more denaturants.

The procedure of bioaffinity based layering using an antibody support find its application in nanotechnology and biotechnology which give rise to newly emerging field: nanobiotechnology [9].

II. MATERIALS AND METHODS

Hemoglobin (bovine blood was purchased from sigma), Invertase (250 units /mg), Sepharose 4B were obtained from Sigma (USA), PD-10 column, Sephadex G-10 and DEAE-52 (Amergham Biosciences), Pronase (Calbiochem Novabiochem Corporation ,USA).

Preparation and purification of neoglycoprotein

Commercially available invertase (150 mg) was digested with 8.0mg of pronase at 37 °C in 5ml of 0.1M Sodium phosphate buffer, pH 7.2 for 72 hours. The pronase digest was boiled at 100°C for 10 min and then centrifuged at 1600g for 30min and was fractionated to isolate glycopeptide by loading on PD10 gel filtration column(7.5cm x 1.7cm) [6]. The preparation obtained was further purified by affinity column chromatography on the ConA-Sepharose support. The bound glycopeptides were eluted with 0.2 glycine/HCL containing 0.05 M NaCl buffer, pH 2.0, after 30min of continuous agitation. Excess glycine /HCL were removed by passing the sample through a Sephadex G-10 gel filtration column (1.8cmx20cm) [8]. Purified glycopeptides were treated with 0.5% glutaraldehyde for 5 hours at 4°C. These treated glycopeptides were then treated with 50 mg of hemoglobin dissolved in total volume of 5ml of sodium

phosphate buffer, pH 7.0. The obtained neoglycoprotein hemoglobin was separated from uncross reacted hemoglobin by loading the sample on Con A- Sepharose .The neoglycoprotein was then eluted with 0.2 M glycine / HCL buffer, and the preparation was immediately neutralized with 0.1 M NaOH [6]. Glycine /HCL were removed by passing purified neoglycoprotein through a Sephadex G-10 gel.

Production of polyclonal antibodies against neoglycoprotein.

Albino rabbits were immunized with 300µg of neoglycoprotein dissolved in 0.5ml of mM sodium phosphate buffer, pH7.2 mixed with equal volume of Freund's complete adjuvant as a first injection [10]. Booster doses of 150µg neoglycoprotein with Freund's complete adjuvant were administered weekly after resting the animal for 15 days. After each booster blood was collected from the ear vein of the animal and allowed to clot at room temperature for 3 hrs. Serum was collected by centrifugation after each booster dose and purified on DEAE cellulose column (1.2cmx10cm) [11].

Immunodiffusion

Ouchterlony double diffusion was used to detect the presence of antibodies against the glycosyl moiety of the hemoglobin. The cross reactivity of antineoglycoprotein with other proteins were performed in 1% agarose gel [12].The purified carbohydrate specific antibodies were employed for immunoaffinity layering of hemoglobin on sepharose 4B.

Preparation of layered assembly of hemoglobin by using glycosyl-specific anti hemoglobin polyclonal antibodies .

Purified polyclonal antibodies were coupled to CNBr-activated Sepharose 4B[13].The IgG bound matrix was then incubated with excess of hemoglobin in 20 ml of sodium phosphate buffer ,pH7.2 in a total volume of 3.0 ml overnight at 4°C .The matrix bound enzyme was then washed thoroughly with buffer to remove the unbound protein . The preparation thus obtained was considered as first affinity layer. This preparation was then incubated with excess and appropriate amounts of glycosyl specific antihemoglobin polyclonal IgG and hemoglobin, alternately up to four layers.

Protein Assay

Protein concentration was determined by using method of Lowery et al. [14]. Hemoglobin concentration by Drabkin et al Method [15].

III. RESULTS AND DISCUSSION

Pronase – digested preparation of commercially available invertase was used to isolate the oligosaccharide chains of the enzyme. The glycosyl moieties were purified by ConA-Sepharose chromatography and coupled to hemoglobin with the help of glutaraldehyde to synthesize the neoglycoprotein [8].The newly synthesized glycoconjugate was further purified by conA-Sepharose chromatography and characterized on SDS/PAGE by periodic acid –Schiff's staining [16].The neoglycoprotein moved as a single band on the gel .The neoglycoprotein thus obtained was highly immunogenic and precipitating antibody produced against this antigen. The glycosyl-specific antihemoglobin

antiserum showed cross reactivity with neoglycoprotein and hemoglobin, but not with the digested peptides of invertase (fig1). The results confirm that the specific antibodies were raised against peptide epitopes of neoglycoprotein and native hemoglobin. The results suggest that presence of specific antibodies for neoglycoproteins and hemoglobin. In earlier studies it has already been demonstrated that neoglycoproteins synthesized by coupling the glycosyl specific moieties of yeast glucose oxidase to BSA were highly specific. The immunoabsorbant for immobilization of hemoglobin was prepared by coupling IgG purified from the sera of rabbits immunized with neoglycoproteins on activated Sepharose-4B [8]. The activated support bound 7.6 mg of protein /g of the gel. The IgG-Sepharose preparation was incubated overnight with excess of hemoglobin at 4°C in 20 mM buffer, pH 7.2.The preparation thus obtained was considered as first layer. The IgG immobilized hemoglobin preparation was then incubated alternately with glycosyl specific polyclonal antibodies and hemoglobin .The results obtained after four affinity layers are summarized in table 1.

Enzyme immobilized on Sepharose-4B support increased gradually with successive incubation of protein antibodies and after four desired cycles, the amount associated with matrix was 55 fold as compared with protein directly bound on IgG-Sepharose4B. The effectiveness factor η also increased linearly with the formation of successive affinity layers of IgG and protein. Protein assembled in the form of layers on Sepharose - 4B exhibited enhancement in the incorporation of hemoglobin properties (recognition, transport and catalytic properties).

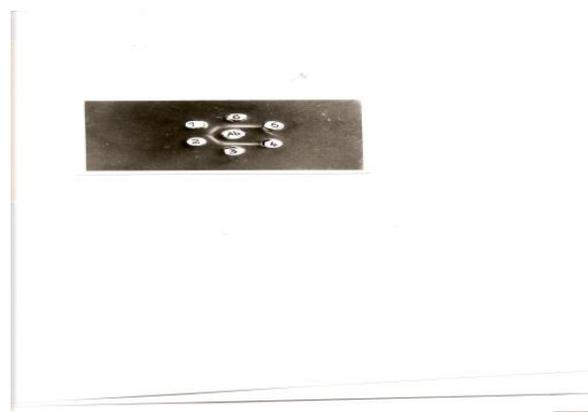


Figure 1 shows antiglycosylspecific polyclonal antibodies (Abs) at the center and peripheral well 1, 2 Hemoglobin 3,5and 6well neoglycohemoglobin and well 4 with digested invertase peptides. .

Study the properties of immunoaffinity layered glycosylated hemoglobin.

Figure 2 demonstrates the effect of temperature on stability of various immunoaffinity layered hemoglobin. The preparation bearing 4 layers was significantly superior in stability and retained nearly 85% activity while the first affinity layer preparation exhibited only 55% of the original protein activity after 2 h of pre incubation at 60°C. The native protein lost 99% of the initial activity. The increase in stability was also layer –

dependent and was of relatively high magnitude in all the immobilized preparation as compared to the soluble protein. The immunoaffinity support holds the protein at multiple points of attachment in three dimensional antigen-antibody complexes,

thus increased the thermal stability of protein. The enhancement in thermal stability was further proved to a greater extent with bioaffinity layering of proteins on the matrix [8].

Table 1

Immunoaffinity layering of hemoglobin on Sepharose -4B using purified glycosylspecificIgG. Each value represents the mean from three independent experiments performed in duplicate not exceeding 100% of the mean value.

Amount of Hemoglobin bound mg /g of the gel					
Layer	Theoretical Value (A)	Actual Value (B)	Effectiveness factor (B/A) η	Increase in binding over 1(fold)	Protein bound in (mg/g of gel)
I	10	7.6	0.76	1	7.6
II	100	60	6.0	8	60
III	250	235	23.5	31	235
IV	500	418	41.8	55	418

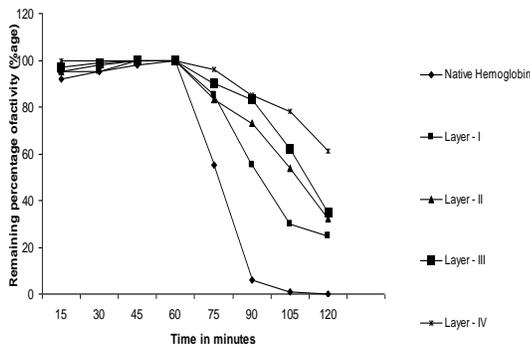


Figure2. Thermal stability plot for native and immunoaffinity bound hemoglobin. Native and immunoaffinity layered hemoglobin was incubated at 60 °C for 2 h for various times and its activity was measured as described in text by using Drabkins method [15]. The activity of unincubated hemoglobin was considered as control (100%).

Effect of pH on the immunoaffinity layered preparation of protein and native protein was investigated in pH range from pH 4 to pH 13 [Fig.3]. There was a remarkable increase in stability of immunoaffinity layered preparations of hemoglobin as compared to the native protein at highly acidic and alkaline side of the pH. Several earlier investigators have also documented for the stability of normal immobilized hemoglobin [17]. Khan et al. [18] have also shown that bioaffinity bound enzymes retained high activity at wide range of acidic and alkaline pH. It exhibited that the stability of hemoglobin enhanced due to binding of protein with antibodies at multiple sites [19],[20]. The structural basis for stabilization of hemoglobin is associated with decrease in the ionic character and

increase in the covalent nature of the bonds in the bioaffinity layered preparation of hemoglobin.

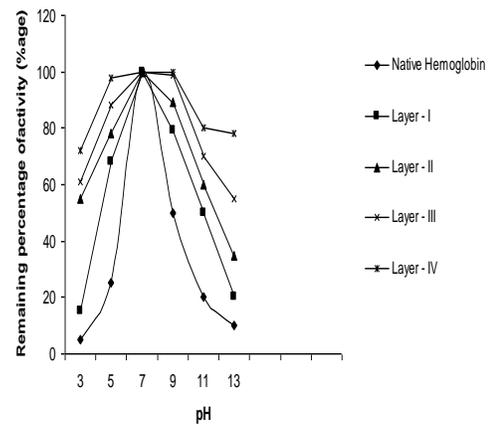


Figure 3.pH-activity profiles for native and immunoaffinity bound hemoglobin. Native and immunoaffinity bound hemoglobin were incubated in the buffers of various pH ranges for 2 h at 37°C. The activity for native and immunoaffinity layered neoglycoprotein at pH 7.0 was considered as control (100%).

Four immunoaffinity layered preparation of hemoglobin showed no sign of its precipitation when it was incubated with 0.5 M salicylate in a neutral phosphate buffer for 2 h at 37°C while the native and single layered protein preparations were precipitated when these preparations were incubated under similar experimental conditions. The 10% of precipitation was observed for one layered preparation whereas no precipitation was seen in the fourth layered preparation. The results show the dense and visible precipitation in the native protein hemoglobin

under the identical conditions. Earlier studies were documented with differences between native and denatured hemoglobin [21].

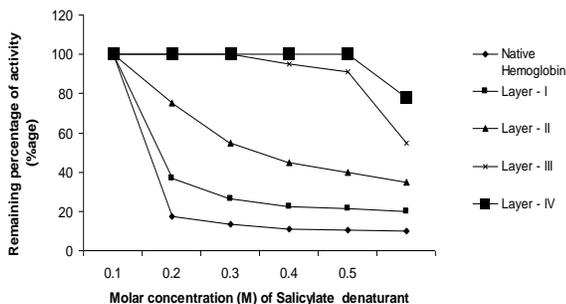


Figure 4. Stability of native and immunoaffinity layered hemoglobin against denaturant salicylate. Native and immunoaffinity layered hemoglobin preparations were incubated with various concentration of salicylate for 2 h 37°C. The activity of unincubated native and immunoaffinity bound hemoglobin was considered as control (100%).

Storage stability

Storage stability the native and the bioaffinity layered preparation of neoglycohemoglobin were stored at 37°C and 4°C for 30, 60, 90 and 120 days. In the native protein hemolytic and rapid growth of bacteria were observed after 30 days in both 4°C and 37°C. However, The bioaffinity layered preparation of hemoglobin did not show any hemolysis and growth of bacteria after a long term storage for 120 days .

Applications

The large amount of immobilized hemoglobin molecule on a small surface of matrix is one of the important factors for development of immunosensors and biosensors which is clinically relevant for diabetic patients. The denaturation of hemoglobin with denaturing adducts present in the blood can be reduced by using the immunoaffinity layered preparation of protein .The study is also applicable for the patients of family with glycated hemoglobin formed by non enzymatic reaction of glucose with reactive amine groups on hemoglobin The work done can be carried out to study the immunoaffinity matrix for development of HbA1c immunosensors.

IV. CONCLUSION

Immunoaffinity layered hemoglobin showed very high stability against extreme conditions of pH and temperature and this preparation did not exhibit any sign of precipitation when exposed to high concentration of salicylate for very long time. In view of the stability of the immobilized hemoglobin it can be suggested that these types of preparations can be used in biosensors. The proposed work showed excellent reproducibility, stability and long term storage and it indicated that such preparations can successfully be used for continuous analysis.

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