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Immobilized hemin affinity chromatography as a probe for proteins having potentiality to bind with heme

Renqiang Li [a,*], Fengyi Jiang [a], Xiaofen Zhang [a], Yao Chen [b], Ling Fang [a]

[a] *Department of Biotechnology, Jinan University, Shipai, Guangzhou 510632, China*
[b] *Department of Chemistry, Jinan University, Shipai, Guangzhou 510632, China*

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Abstract

After Sepharose 4B polymer beads were activated by using epichlorohydrin, hemin was binded with them to prepare an immobilized hemin affinity chromatography column. The coupling rate of this column was very high, more than 0.25 mg hemin could be fixed by 1 g of wet Sepharose 4B beads. The column equilibrated with deionized water and eluated with pH 3.0 NaAc–HAc buffer was applied to capture the proteins in human serum, earthworm body and Bacillus subtilis cells. Three polypeptides in human serum were captured, one of which was verified as serum albumin after comparison to the control. At least one polypeptide in earthworm body, two in Bacillus subtilis cells displayed the powerful binding specificity to hemin. Our experiments demonstrated that the immobilized hemin affinity chromatography was available as a probe for some proteins having potentiality to bind with heme.

Keywords: Hemin; Affinity chromatography; Sepharose 4B; Human serum; Earthworm; Bacillus subtilis

1. Introduction

There are many kinds of heme-containing proteins in organisms such as hemoglobins (Hb), myoglobin (Mb), cytochrome *c* (Cyt *c*), peroxidase (POD) including multi-heme cytochromes [1] and a series of heme-containing oxygenases [2]. The heme-containing proteins in organisms are very important for their various functions, and their functional mechanisms are also complex. The structure of these heme-containing proteins, especially on the point of combination between heme and polypeptide, which is the key to determine the function of the molecule, is of course the important topic for understanding the relationship between protein structure and its functions. Like the natural heme-containing proteins, the proteins that have potentiality to bind with heme could also offer us the chance for the study of heme-containing proteins. For example, the proteins combined with free hemes such as hemopexin, serum albumin and serum lipoproteins have led to many studies [3–8]. Furthermore, studies on these proteins that have potentiality to combine with heme may be helpful for probing a new functional protein molecule with heme or an abzyme, so how to find and prepare these proteins that have potentiality to combine with heme needs to be studied. Here obviously an effective and quick method to verify the existence or appearance of these proteins would be very useful. Therefore, the aim of this study is to probe or establish an available method for quickly and effectively verifying the proteins that have potentiality to bind with heme.

Based on the mechanism of affinity chromatography, immobilized hemin as a ligand attached to polymer beads could selectively adsorb those target proteins that may react to heme. To immobilize the hemin to make an affinity chromatography column, Sepharose 4B is a good candidate as polymer beads to bind with hemin because Sepharose 4B is active with its hydroxyl group after it was activated by epichlorohydrin, which would be helpful for the immobilization of hemin on Sepharose 4B beads. However, the hypothesis needs demonstration by practice.

To prove the availability and ability of the immobilized hemin column, hemopexin and serum lipoproteins should be used as the samples to pass the column, but no commercial standard is available for hemopexin and lipoprotein proteins which are well known to bind with heme, so the purified human serum albumin (HSA) and human serum are used to examine the column

* Corresponding author. Tel.: +86 20 85220219; fax: +86 20 85226262.
E-mail address: trqli@jnu.edu.cn (R. Li).

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because HSA and some proteins in human serum could combine with heme [3–7], the roles of which are to verify the function of immobilized hemin affinity chromatography column. This column is also applied to investigate the proteins in the earthworm body and in Bacillus subtilis cells. This paper described the study and reported some discussion.

2. Materials and methods

2.1. Materials

Stored Bacillus subtilis BO_4 was from the stock in our laboratory. Healthy adult human serum was kindly supplied by the hospital attached to the Medical College of our University. Earthworm (called Japanese red earthworm) with 6–8 cm length was purchased from the market. Hemin, HSA was from Sigma, and Sepharose 4B from Amersham. Histidine, leucine, epichlorohydrin, 1,4-dioxane and all other chemicals used in SDS-PAGE or for buffers were of analytical or chemical grade and were purchased from Guangzhou Chemical Co. Ltd. (Guangzhou, China). Markers for SDS-PAGE were supplied by Shanghai Sheng Zheng Biotechnology Co. Ltd. (Shanghai, China).

2.2. Preparation of immobilized hemin column

2.2.1. Activation of Sepharose 4B

Twenty grams of wet Sepharose 4B was washed using 1 mol/L NaCl and deionized water at Buchner's funnel. These polymer beads were mixed with 15 ml 2 mol/L NaOH, 4 ml epichlorohydrin and 20 ml 56% 1,4-dioxane, shaken for 2 h at 40 °C. After being washed at funnel by using deionized water, Sepharose 4B beads were saturated in 0.1 mol/L, pH 9.5 Na_2CO_3–$NaHCO_3$ buffer.

2.2.2. Combination of hemin with Sepharose 4B

Hemin was first dissolved in small quantities of 15% ammonia water and then in deionized water to make a 2 mmol/L solution, of which 25 ml was mixed with activated Sepharose 4B beads (preliminary test demonstrated hemin was excessive in this condition). The mixture was shaken at 40 °C for about 24 h and washed at funnel using deionized water to remove the hemins that were not attached to polymer beads. $O.D._{557}$ were measured for the hemin solutions in reduced state (by adding small quantities of sodium dithionite) before mixed with beads, and for the part that were not attached to polymer beads. The data was used to calculate the hemin fixed by the beads. Coupling rate between hemin and the beads was expressed using mg hemin fixed per gram of wet Sepharose 4B beads. A column with 1.5 cm × 11 cm was made using these Sepharose 4B beads to capture the hemin as a ligand. For comparison, a control column of Sepharose 4B beads without hemin was also made.

2.2.3. Determination of condition for sample loaded and eluted

The experiments were performed in two conditions. At alkaline pH, 0.2 mol/L pH 9.5 Na_2CO_3–$NaHCO_3$ buffer was used to equilibrate the column and at near neutral, deionized water (pH 6.4) was used as an equilibration solution, the loading effect of samples of HSA and human serum diluted eight-folds were measured. In these two performances, NaAc–HAc buffer with 0.2 mol/L, pH 3.0 was used as an eluant. At alkaline pH, very few proteins were found in the eluate but when the equilibration solution was deionized water, more proteins in samples were captured and was found in the eluate. So using deionized water as an equilibration solution and 0.2 mol/L pH 3.0 NaAc–HAc buffer as an eluant was the operational conditions of this immobilized hemin column.

2.3. Preparation of the samples for experiment

HSA were directly dissolved in deionized water to make a 0.05 mmol/L solution. Original human serum sample was diluted eight-folds by deionized water before performing loading to the column. Washed earthworm bodies were bled in deionized water using blender. The pH of earthworm homagenate was adjusted to 5.1, in which Lumbricus terrestris hemoglobin (LtHb) tended to precipitate due to its great molecular mass at near isoelectric point. The supernatant obtained after centrifugation of the earthworm homagenate was diluted to a certain degree before being loaded to the column.

Sterilized nutritional medium composed of 0.5% beef extract, 1% peptone and 0.5% NaCl with pH 7.4 was used to grow Bacillus subtilis cells at 37 °C for 15 h. Bacillus subtilis cells were harvested by centrifugation and weighed for calculating the productivity, and stored at −20 °C. The frozen cells paste of Bacillus subtilis were suspended in 40 mmol/L Tris–HCl buffer with pH 8.0, and sonicated for obtaining cell lysate that would be the sample for loading to the column after the dilution with deionized water to a certain degree.

2.4. Sample loaded and eluted

New black immobilized hemin column and the control column were washed fully by using pH 9.5 Na_2CO_3–$NaHCO_3$ buffer, deionized water and pH 3.0 NaAc–HAc buffer, respectively, and then equilibrated with deionized water. Three milliliters of HSA solution and 10 ml of diluted human serum were respectively loaded to immobilized hemin affinity chromatography column and the control one at 0.5 ml/min for functional verification of immobilized hemin.

Twenty-four milliliters of diluted human serum, 20 ml of earthworm protein solution and 35 ml of Bacillus subtilis cell lysate were respectively loaded to immobilized hemin affinity chromatography column at 0.5 ml/min. After being washed fully with deionized water, the eluant 0.2 ml/L pH 3.0 NaAc–HAc buffer were used to elute the column and the peak fraction at $O.D._{280}$ were collected.

2.5. SDS-PAGE

The eluate proteins obtained by chromatography were concentrated by vacuum cold drying apparatus (Heto, High Technol-

ogy of Scandinavia, Denmark) and dialyzed against deionized water before mixing with 1% SDS-mercaptoethanol solution as the SDS-PAGE sample. Gel concentration used in SDS-PAGE was 12%. Analysis of SDS-PAGE pattern was performed using Electrophoresis Image Analysis System (FR-980, Furi Company, Shanghai, China).

2.6. Experiment on reaction between amino acids and hemin

For understanding the mechanism of immobilized hemin column to capture target proteins, activity of hemin to bind with amino acids in vitro was tested. Histidine and leucine were used to bind with hemin. The mixture containing 0.30 mmol/L hemin and 6.50 mmol/L histidine (excessive for reaction) was kept at 40 °C for 10 min, to replace histidine by leucine at the same condition as the control. The absorption spectra of samples in reduced state were examined and compared to that of hemin in 450–650 nm regions.

3. Results

3.1. Function by immobilized hemin

As expected, the immobilized hemin column was obtained and the coupling rate was very high with more than 0.25 mg hemin fixed by gram of wet Sepharose 4B beads. After enough washing time by using solutions with different pH values, a stable immobilized hemin affinity chromatography column was established. When 3 ml of HSA solution was loaded to immobilized hemin column, protein was fully captured by the column, which contrasted sharply with that in control column that let almost all of HSA pass through (data not shown). Some proteins in human serum were captured by immobilized hemin but little protein was retained in control column when human serum was passed through the columns as shown in Fig. 1. Comparing to control, immobilized hemin could selectively capture the proteins in human serum, especially obvious HSA was retained largely in immobilized hemin column. These results demonstrated it was immobilized hemin as a ligand that functioned affinity chromatography.

3.2. Proteins in serum captured by immobilized hemin column

Fig. 2 showed the SDS-PAGE pattern of eluate proteins for human serum experiment. Three polypeptides that could bind with hemin in this experimental condition were found in human serum. Molecular masses of these proteins obtained after the comparison to the markers were about 69,100, 62,000 and 27,800. From these data, one polypeptide among them should be HSA. For trying to verify it, purified HSA caught by immobilized hemin was measured by SDS-PAGE and is shown in Fig. 3. From the comparison, the polypeptide with molecular mass of about 62,000 was HSA. Calculation for molecular masses in SDS-PAGE seemed to be not rigorous due to the molecular mass of HSA was actually 66,400.

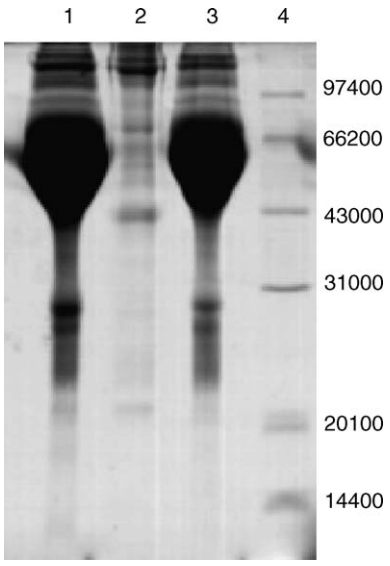

Fig. 1. SDS-PAGE pattern of human serum proteins passed through columns. Lane 3: sample loaded, lane 1: passed through control column, lane 2: passed through immobilized hemin column, lane 4: markers.

It seemed to be difficult to verify the other two polypeptides in serum captured by immobilized hemin only from calculated molecular mass because that there were so many kinds of proteins in human serum [9] and many among them, especially of lipoproteins family, could bind with heme [10] although some proteins in serum were near to the captured polypeptides in molecular mass, for example, molecular mass of apolipoprotein A I was 28,300 that was near to polypeptide 27,800. It needed actual comparison between sample and purified protein in same SDS-PAGE condition as performed for the verification of HSA. However, results of our experiment indicated that this immobilized hemin affinity chromatography could selectively capture some proteins that may bind with hemin.

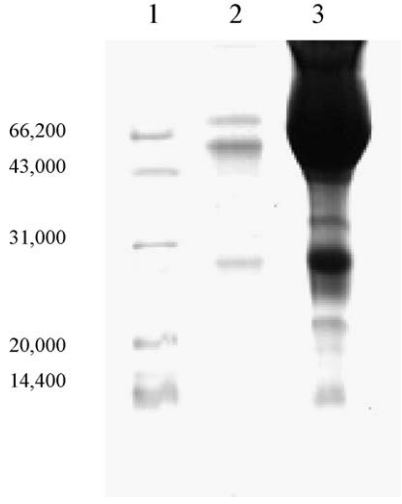

Fig. 2. SDS-PAGE pattern of human serum proteins captured by immobilized hemin column. Lane 3: sample loaded, lane 2: eluate from immobilized hemin column, lane 1: markers. Eluate from control column contained no protein.

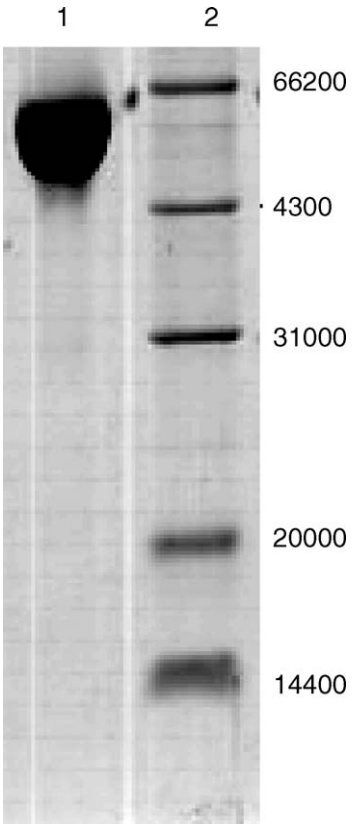

Fig. 3. SDS-PAGE pattern of purified HSA captured by immobilized hemin column. Lane 1: eluate from immobilized hemin column, lane 2: markers. Purified HSA was not captured by blank column.

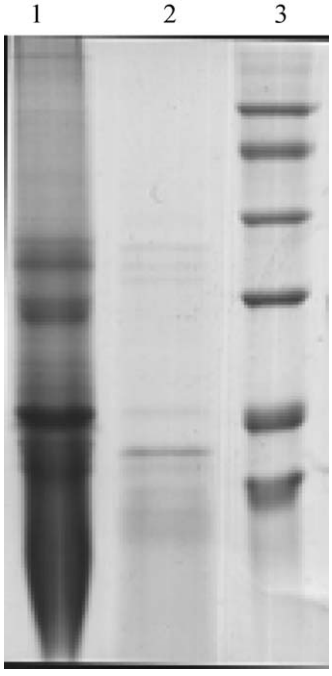

Fig. 4. SDS-PAGE pattern of earthworm body proteins captured by the column. Lane 1: sample loaded, lane 2: eluate from immobilized hemin column, lane 3: markers. Eluate from control column contained no protein.

3.3. Chromatography results for the polypeptides in earthworm body and in Bacillus subtilis cells

The polypeptides in earthworm body captured by hemin ligand at the column were shown in Fig. 4, and Fig. 5 showed the polypeptides in Bacillus subtilis cells that could be captured by hemin ligand in this chromatography column. These figures demonstrated that in earthworm body as well as in microbe body, there existed the proteins that may bind with hemin. One polypeptide from the earthworm body (Fig. 4) displayed the powerful binding ability to hemin and at least two of these polypeptides in Bacillus subtilis cells (Fig. 5) were detected. Thousands of polypeptides were there in live body, several kinds of them with the binding tendency to hemin were natural due to the great activity of heme. However, the appearance of those proteins with powerful binding ability to hemin would offer the chance to study the combination between heme and polypeptide. Experimental results explained that immobilized hemin affinity chromatography could selectively capture some proteins having potentiality to bind with heme. The column was available in practice. In detail, some weak bands in eluate of the earthworm as well as of Bacillus subtilis were showed in Figs. 4 and 5, that would be explained in the following discussion.

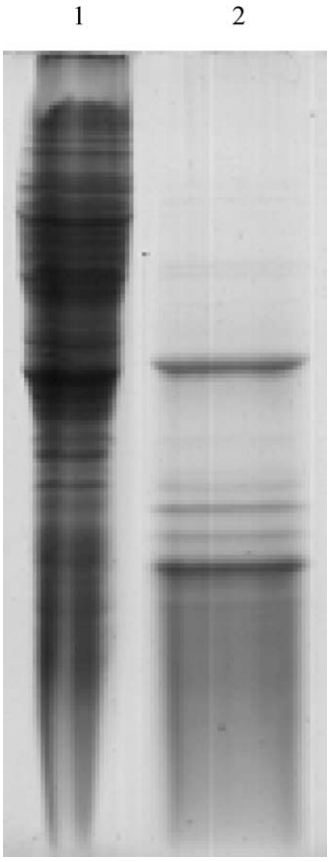

Fig. 5. SDS-PAGE pattern of Bacillus subtilis cell proteins captured by the column. Lane 1: sample loaded, lane 2: eluate from immobilized hemin column. Eluate from control column contained no protein.

4. Discussion

Based on the mechanism of affinity chromatography, this study aim to establish an available immobilized hemin affinity chromatography column, utilize hemin to capture those proteins having potentiality to bind with heme that existed in different organisms. Because of the activity of Sepharose 4B when it was activated by epichlorohydrin, it was selected as the polymer beads to fix ligand hemin. High coupling rate between hemin and the beads with more than 0.25 mg hemin were fixed per gram of wet Sepharose 4B beads in our experiment explained that binding hemin as a ligand with Sepharose 4B bead was available. However, to obtain a stable chromatography column in practice needed enough washing time for fully removing those hemins that were not attached tightly on the column by using solutions with different pH values when the column was established. Sepharose 4B beads were often used to fix protein ligand due to the fact that it was the nitrogen atom in protein molecule that formed the bond between protein and the beads, but Sepharose 4B beads had to be activated before used. Here it was deduced that it was mainly the nitrogen atom at protoporphyrin ring of hemin that liganded to the beads although thioether bond may be the candidate. So the binding between hemin and the beads was stable and tight, washing the column repeatedly could guarantee to get a good column.

During the chromatography of human serum proteins using this column, only three polypeptides could be captured by immobilized hemin (Fig. 2) although many kinds of proteins in serum have been demonstrated to combine with heme. Three main reasons may cause this result in performance condition of the present study. First, whether the protein could be captured by the column and presented in SDS-PAGE pattern was obviously dependent on the content it existed in serum. Too small quantities of the protein existed in the loaded sample, the protein was difficult to be captured in large numbers and verified in SDS-PAGE. HSA contained in serum was the largest in amount, which was helpful for its competitive binding with immobilized hemin, so it was easy to be verified. Secondly, loading condition may affect affinity and selectivity of the column. Changes of chromatography effect of this immobilized hemin column with different loading and eluting conditions need further study. Finally, it was due to the different mechanisms of hemin interaction with protein between on column and on free hemin, it was not surprising due to the fixed conformational structure of the heme complex on column.

In general, hemin was contained in protein molecule when the combination happened between hemin and polypeptide although the combination state between heme and polypeptide was complex. For example, hemin was fully inserted into the albumin or hemopexin molecule [11–16]. However, the combination state between hemin and polypeptide would certainly change if the hemins were fixed on the beads, which would hinder hemin in its entrance to the polypeptide. Hemin combined in the albumin molecule was very difficult to separate from its trap in vitro, but in this affinity chromatography column the captured proteins were very easy to be eluted down, requiring only the change of elution solution to acidic pH. Obviously the combination state between hemin and polypeptide in this column was greatly different from that between free hemin and polypeptide. For the mechanism of this immobilized hemin for selectively capture those proteins, it was deduced to be the functions of axial ligands or the fifth and sixth coordination bonds of iron at the center of the protoporphyrin ring and the thioether bonds in hemin. Several kinds of amino acid residues such as histidine or methionine were easy to link to the axial ligand of iron as happened in heme-containing protein molecules, which explained the higher activity of the fifth or sixth coordination bond of iron in heme. For understanding the activity of hemin to bind with amino acid in vitro, histidine and leucine were used to bind with hemin, test results of which were shown in Fig. 6. The blue shift found at His-hemin mixture shown in Fig. 6 may explain the formation of the fifth ligand of heme iron found in most heme-containing protein molecules. Due to the higher activity of hemin, many proteins in organisms may be captured in this immobilized hemin column, which explained why so many weak protein bands appeared in the eluate patterns of Figs. 4 and 5. However, those kinds of proteins captured in large numbers by the hemin ligand should possess their specificity to heme.

At alkaline pH, free hemin could well bind with serum albumin in vitro (data not shown), but in this column only when deionized water was used as the equilibration solution, could serum albumin be largely captured. This phenomenon may explain in practice the difference of mechanism for the combination between polypeptide and fixed hemin or between the polypeptide and free hemin, and also explain that different pH values or ion concentration may affect the affinity between hemin ligand and the target protein in this affinity chromatography.

In conclusion, this immobilized hemin affinity chromatography column was easy to operate, and was available to probe some proteins having potentiality to bind with hemin. So it is significant for the determination and purification of specific protein, and for the study of heme-containing proteins. This tool would be helpful for our further study.

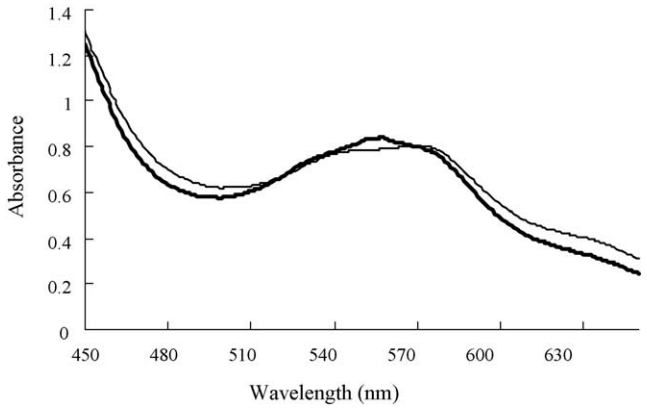

Fig. 6. Absorption spectra of hemin (thin solid line) and histidine plus hemin (bold solid line) in ferrous reduced state in 450–650 nm region. Small quantities of sodium dithionite was added to the sample to make ferrous reduced state of hemin. The concentration of samples was the same 0.15 mmol/L hemin. The spectrum of leucine plus hemin was the same as that of hemin (not shown).

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