

A New Protocol for Isolation and Purification of Transferrin from Human Serum

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ABSTRACT

Transferrin acts as a vehicle for iron transportation between the absorption, storage and utilization sites in the body and, besides iron, its two high-affinity iron-binding sites are capable of binding to 29 other elements. In physiological and pathophysiological conditions, transferrin's properties are different and close attention to these properties is required. For further investigation for the above properties, it is necessary to have this protein in highly pure state. The purpose of this research was to isolate and purify the human transferrin from serum with native properties and high purity. The results of this research lead to a protocol for the isolation and purification of human transferrin which is cost-effective and time saving to produce anti-transferrin antibody for immunological kits.

INTRODUCTION

For all plants and animals as well as most microorganisms, iron, being an essential nutritional requirement, has been selected in molecular evolution to carry out a wide range of biological functions. At the neutral pH and high oxygen tension of most physiological fluids, the predominant oxidation state of the transition metal iron is Fe³⁺ (ferric). In this form iron readily undergoes hydrolysis and polymerization, resulting in a product of extreme insolubility and toxicity. Organisms have therefore been compelled to evolve specific molecule to store iron (ferritin) and to transport iron (transferrin) in a form that is both soluble and nontoxic hence remaining readily available to biological systems (1, 42).

Human transferrin is a single unit glycoprotein containing 679 amino acid residues and two N-linked glycan chains with a molecular mass of 79570 Da. Upon binding of two atoms of iron (Fe³⁺), transferrin becomes more compact or spherical in shape, and this conformational change results in an iron-laden protein being more resistant to denaturation in comparison with apotransferrin. Based on its amino acid sequences, transferrin can be divided into two homologous regions, the N-terminal domain (residues 1 to 336) and the C-terminal domain (residues 337 to 679). Each of the two domains within the transferrin molecule contains a metal-binding site. The concomitant binding of an anion, being a bicarbonate ion (HCO₃⁻) in the physiological situation, at each site is essential for iron binding (28, 32, 36). The C-terminal domain of human transferrin carries two glycan chains, attached to the asparagines residues at positions 413 and 611, respectively. Each of the oligosaccharide chains is of a complex type exhibiting considerable variation in both the degree of branching and the nature of the sugar residues at the outermost ends of the branches and each of the branches has

the possibility of ending with a negatively charged sialic acid residue (18, 23, 28, 43).

The body of an adult contains about 17 gr of transferrin, of which approximately half is found in the blood plasma. The remainder is distributed amongst wide variety of body fluids, including lymph, tears, cerebrospinal fluid, bile, amniotic fluid, milk, saliva, aqueous humor and seminal fluid. Plasma transferrin (2.5 g/L) accounts for about 4% of the protein content of this fluid and is the fourth most abundant plasma protein. Like most plasma protein, transferrin is synthesized primarily in the liver. However, important extra sites for transferrin synthesis other than liver include nervous tissue, testis, ovary, placenta, mammary gland, thymus and lymphocytes. The synthesis of transferrin by some of these not hepatic tissues may be important in the situations that cells are separated by blood barrier from plasma transferrin (18, 24, 29).

Human transferrin migrates electrophoretically with the β 1-globulin fraction of human plasma. Under normal physiological conditions, serum transferrin is about 30% saturated with iron (18, 23).

An extensive range of techniques are available in order to measure plasma transferrin concentration. Most of the original procedures were based on the capacity of this plasma protein to bind iron (TIBC) according to the following mathematical relations; but, these methods are now being progressively replaced by more direct immunological procedures using antibodies specific for human transferrin. Some observations demonstrate that plasma transferrin concentration, calculated from the TIBC value; consistently give results that are higher than those obtained by using the more direct immunological methods (34). The purpose of this research was to isolate and

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purify the human transferrin from serum with native properties and high purity. The results of this research led to a protocol for the isolation and purification of human transferrin which is cost-effective and time saving to produce anti-transferrin antibody for immunological kits.

$$\text{Tf (mg/dL)} = 0.7 \times \text{TIBC } (\mu\text{gr/dL})$$

$$\text{TIBC } (\mu\text{gr/dL}) = 1.43 \times \text{Tf (mgr/dL)}$$

$$\text{Tf saturation\%} = 100 \times \text{TIBC/amount of Iron in serum}$$

MATERIALS AND METHODS

The following methods were used for estimation of total protein: 1) Spectrophotometric Method (Warburg and Christian Method) by knowing the ratio of A280 nm /A260 nm (7); 2) Colorimetric method (Bradford Method) by knowing the amount of A595 nm (8,12).

For estimation of the amount of albumin, Fe, TIBC, immunoglobulins (IgA, IgM, IgG), and transferrin, the following methods were used, respectively:

Colorimetric method with Bromo Cresol Green (BCG) and knowing the amount of A630 nm.(13); Colorimetric method by knowing the amount of A593 nm by using Fe kit produced by Ziest Shimi Co.(14); Colorimetric method by knowing the amount of A593 nm by using TIBC kit produced by Ziest Shimi Co.(14); SRID (Single Radial Immunodiffusion) Method by using Immunoglobulins' kit produced by Biogene Co.(41); and, SRID method by using Tf kit produced by Biogene Co.(41).

As a result of this research, a new protocol was obtained. The procedures of the protocol are as follows:

- All the steps were carried out with refrigerated conditions;
- The pH and conductivity of the column effluent and its optical density at three wavelengths were monitored; the wavelengths selected in this study were 280 nm for total protein, 410 nm for heme-containing proteins, and 470 nm for transferrin(7);
- The serum was obtained from healthy donor. In previous investigations, the sample was selected from plasma, but to exclude the various coagulation factors of plasma, we selected serum as the origin sample. Furthermore, the other privilege of using serum was its lack of any anti-coagulating factors such as chelators which may interact with ferric ion and limit iron saturation (2, 3, 31).
- Saturation of transferrin by iron: It was tried to stabilize transferrin by iron saturation with ferric ion at the presence of citrate ion as a chelator and bicarbonate which is necessary for iron binding to transferrin. Since , the amount of transferrin estimated by SRID kit in the first sample was 7.5 g ,we used 10.5 mg Fe and 11.44 mg anion bicarbonate (7 μL of 0.1 M or 54 μgr NaHCO_3 and 7 μL of 0.1 M or 153.16 ferric citrate in pH=8, 4°C , 1 h)(2,3,26,33,36,44).
- Precipitation of Albumin by Rivanol (Produced by Sigma): In previous investigations, Rivanol (4%) was used as a factor to remove albumin by adding it to the sample by 3.5 V/V ratio; but, at this protocol, we added, drop by drop, a V/V ratio of Rivanol (0.6%) at 4 °C, 2 h, and pH=9.4 . Then, the solution was centrifuged in 3000 rpm for 20 min and finally filtered by filter paper Wattman No: 1(6,10,31);
- Removing of Rivanol by gel-filtration with Sephadex G-25(Produced by Sigma Co.) with elution buffer ammonium sulfate 0.025 M (4 °C)(20);

- Precipitation of Immunoglobulins by saturated ammonium sulfate 50% (Produced by Sigma Co.) in 4 °C and pH=6.5, and then after centrifugation in 3000 rpm for 10 min after which the precipitant was discarded (immunoglobulins)(9,15);
- Precipitation of other globulins such as transferrin by 80% saturated ammonium sulfate after which the supernatant was discarded (9,15);
- Dissolving of precipitation in the buffer of 0.06 M HCl-0.1 M Tris, 1 M NaCl, pH=8;
- Removing of ammonium sulfate by dialysis in 1 M NaCl, 0.06 M HCl-0.1 M Tris, pH=8(11);
- Resaturation of transferrin by Fe^{3+} (according No: 4);
- Gel-filtration with Sephadex G-100 using elution buffer of 1M NaCl with flow rate of 15ml/h (21,22);
- Exchange buffer from 1M NaCl to 0.06 M HCl-0.1 M Tris (pH=8) by dialysis (11);
- Ion-exchange chromatography by anion exchanger DEAE-Sephadex A-50 (Produced by Sigma Co.). For this process, we performed the tube test method to estimate the resin capacity and to select the dimensions of column. Then, the sample was loaded to the system with the flow rate of 15mL/h and thereafter the system was eluted by a linear concentration gradient with starting buffer (0.06 M HCL-0.1 M Tris, pH=8) and limiting buffer (0.3 M HCL- 0.5 M Tris, pH=8) by flow rate equal to 75 mL/h(25);
- Desalting with Sephadex G-25 with deionized water (20).

RESULTS

The results of each stages of the protocol are given in Table 1 and in the densitometer scanning from cellulose acetate strip converts bands to characteristic peaks of Albumin, α 1-Globulin, α 2-Globulin, β Globulin and γ Globulin. As it was shown in Table1, there was a difference between amount of transferrin calculated by TIBC and SRID methods in the serum sample.

In the stage of precipitation with Rivanol, the total amount of the Albumin existed in sample was removed .The value detected by TIBC showed a reduction regarding to the actual amount of transferrin detected by SRID showing no difference in transferrin quantity. After precipitation of immunoglobulins with 50% saturated ammonium sulfate, all of the IgG was totally removed while a trace quantity of IgM and IgA were seen. All of the globulins (transferrin and other proteins) were precipitated by 80% saturated ammonium sulfate and transferrin was not found in any amount of supernatant fluid.

By using Blue Dextran (MW=2000 kD, monitored at wavelength of 620nm),Cyanocobalamine (MW=1350 Da , monitored at wavelength of 550 nm), Hemoglobin (MW=64 kD ,monitored at wavelength of 540 nm), and β HCG subunit marked with radioactive iodine (MW=25 kD , monitored by gama counter) in the stage of Gel-Filtration by Sephadex G-100 , the molecular weight of transferrin was calculated to be 80 kD. The fraction, with molecular weight of 80 kD, was the same fraction with $\lambda_{\text{max}}=470\text{nm}$. Taking into account the result of SRID, this finding indicated that we had been encountering to the transferrin molecule with its native properties.

In the stage of ion exchange chromatography, when the linear gradient concentration was at the point with concentration equal to 0.14 M HCL-0.24 M Tris, the collected fraction contained transferrin which was verified by SRID.

The amount of TIBC (μmol) was divided by the amount of total protein (mg) and produced an index called specific activity. Also, dividing the specific activity of any stage by the first specific activity (belonging to the serum sample) produced another index called coefficient of purity. Moreover, the yield of transferrin concentration (obtained by SRID method) in each stage over the amount of serum's transferrin concentration was calculated. Furthermore, the percentage of purity of transferrin was calculated by dividing the concentration of transferrin to amount of total protein at the same stage.

As it was shown in Table 1, the overall purity of 95% and yield of 93% for transferrin were obtained.

DISCUSSION

As mentioned earlier, difference between the concentrations of transferrin calculated by TIBC and by SRID, is because of the attachment of the other serum's proteins to iron(14,34).

The reduction observed in TIBC value regarding to the actual amount of transferrin detected by SRID was a direct consequence of albumin removal in TIBC method; because, albumin with its special affinity to iron would be able to bind to a slight amount of iron.

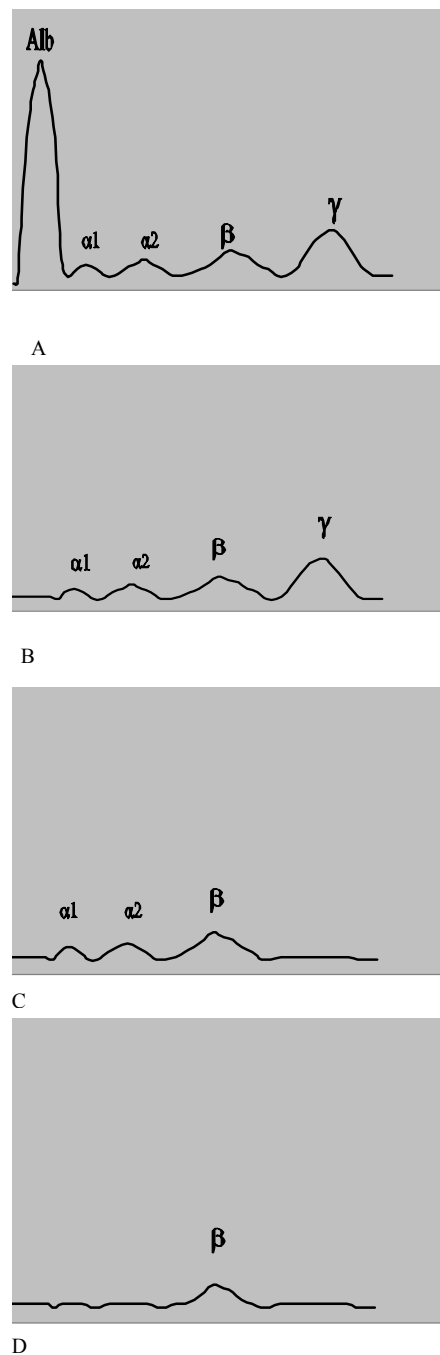
The previous investigations used charcoal to remove excess Rivanol from the supernatant solution after precipitation of the bulk of the proteins; but, in this research, we used Gel-filtration by Sephadex G-25 and this method resulted in a better yield of transferrin. After applying gel filtration with Sephadex G-25, the Rivanol was indeed removed from the solution. However, the mechanism of removal was not gel filtration but adsorption. The Rivanol could not be eluted from the column even by prolonged washing with distilled water. A diluted ammonium sulfate solution (0.025 M) gradually eluted the Rivanol upon prolonged washing(20,27,37).

The Ion-exchange chromatography and gel filtration with Sephadex G-100 were used to complete the purification. It was considered to be important to use two relatively independent methods for the final step in the purification. In this way impurities which failed to be separated by one method could probably be eliminated by the other. Gel filtration on Sephadex G-100 was applied earlier than the Ion-exchange chromatography. For this, a high salt concentration (1 M NaCl) was used as an elution buffer to minimize protein-protein interactions.

The final method used in the fractionation scheme was ion exchange chromatography. The ion exchanger, being DEAE-Sephadex A-50, has the advantages of greater capacity, greater flow rates, and fewer tendencies towards irreversible adsorption than DEAE-Cellulose. The linear concentration gradient of alkaline Tris buffer (pH=8, 0.1 to 0.5 M), was used to avoid loss of iron from the transferrin.

In this protocol, having utilized some simple and available equipments, we were able to obtain a concentration of 262 mg/dL transferrin (in its pure form) from 280 mg/dL transferrin in the serum sample in a short time while other previous protocols lacked this ability(31, 17, 19, 27, 37, 39, 40, 4, 5, 16, 30, 35, 38).

Fig. 1. Densitometer scanning from cellulose acetate strip converts bands to characteristic peaks of Albumin, α_1 -Globulin, α_2 -Globulin, β Globulin and γ Globulin in (A):serum sample, (B):After precipitation of Albumin by Rivanol, (C):After precipitation of Immunoglobulins by saturated ammonium sulfate 50%,(D):Final product after desalting with Sephadex G-25.



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