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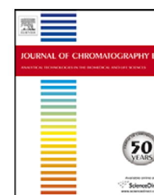

A simple method for obtaining transferrins from human plasma and porcine serum: Preparations and properties

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ABSTRACT

A simple method was described for the purification of serum transferrin (Tf) from human plasma and porcine serum with relative high yield and purity. The properties including purity, integrity, immunoreactivity and the receptor-binding ability of the proteins were studied by several assays, comprising spectrometry, SDS-PAGE, HPLC, Western blotting, urea electrophoresis, mass spectrometry and cytometry. Analysis from all the different aspects manifested that the proteins were of high purity. The two kinds of Tfs appeared to be iron-saturated as confirmed by their absorbance spectra and urea-PAGE mobility. The specific spectra of absorption of the two Tfs were both at around 465 nm. The relative molecular weights of human Tf (hTf) and porcine Tf (pTf) were determined by SDS-PAGE and further identified by MAIDI-TOF mass spectrometry with a result of 79,707 and 79,258, respectively. Immunoblotting assay showed that pTf could react with the anti-human Tf monoclonal antibody with a less level compared to hTf. FACS assays of their binding activities to Tf receptor-positive cell (K562 cell line) indicated that pTf could be recognized by the hTf receptor and internalized into cells, with a slightly less efficacy than hTf. All special property studies demonstrated that pTf was similar to hTf in physical and chemical characteristics, which gave a hint that pTf could substitute for hTf in some kinds of researches, such as using hTf as a carrier in drug targeting system.

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1. Introduction

Transferrin (Tf) comprises a family of iron-binding proteins that include serum Tf, ovotransferrin, and lactoferrin [1]. Serum Tf is a monomeric glycoprotein of 679 amino acids consisting of two subunits known as the N-lobe and the C-lobe. The fundamental role of serum Tf is to control the levels of free iron in body fluids. Delivery and cellular uptake of iron occurs through the interaction and internalization of iron-loaded Tf mediated by the Tf receptor (TfR). Besides, Tf can also bind many other metal ions of therapeutic and diagnostic interests and the Tf complexes can be recognized by many cancer cells which overexpressed TfR on cell surface. The Tf and TfR mediated drug delivery system has received much attention in the past few years [1–3]. Examples included using Tf as a carrier to deliver metallodrugs, chemotherapeutic drugs, toxic proteins, high molecular weight compounds and even genes [2,3].

Since many researchers majoring in pharmaceutics are interested in the Tf mediated drug targeting system, a large quantity of Tf is prerequisite to develop this work. At present, the commercially available Tf is very expensive, which extremely restricts the research progression in the Tf mediated drug targeting studies. Therefore, we want to produce Tf using a simple method. To our knowledge, Tf can be obtained either by recombination technology or by direct extraction from animal blood. Many attempts had been made to produce recombinant hTf in different expression systems including bacterials [4–8], mammalian cells [9–11], yeasts [12,13] and insect cells [14–16]. However, the results displaying protein inactivation or low yield were not satisfactory. Moreover, the downstream purification was also inevitable.

In human serum, the concentration of Tf is about 2.5 g/L with 30% occupied with iron [17,18]. The relative abundance of Tf in blood makes it possible and facile to purify this protein. Several methods for separating Tf from human or animal serum have been published [19–22], which usually involved several steps, whose yields were often low. Some other papers have been published concerning the purification of Tf, using by-products of human plasma fractionation as starting materials. Cost of these materials is relatively low, but to obtain them is difficult. The human serum is also not a convenient source for preparation of Tf. As Tf belongs to a conservative protein family, chemical and physical properties of Tfs from various animals possess high-level conservation [23]. In mammalian Tfs concerned, porcine Tf was reported to have more than 70% homology in amino acid sequence compared to human Tf [24]. More importantly, porcine serum is cheap and easy to get.

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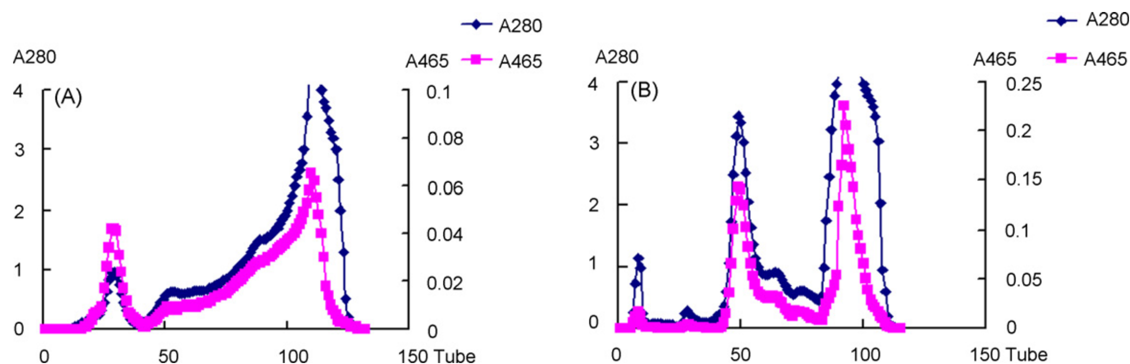

Fig. 1. To isolate transferrin, pretreated samples were loaded onto a column packed with DEAE-Fast Flow Sepharose pre-equilibrated with 20 mM Tris buffer (pH 7.8). (A) For human plasma sample (pretreated from 50 mL human plasma), transferrin was eluted from the column developed with a 60–120 mM NaCl gradient buffer. The first peak corresponded to hTf. (B) For porcine serum (pretreated from 120 mL porcine serum), transferrin was eluted by a buffer containing 50 mM NaCl. The third peak corresponded to pTf. After each elution, the column was regenerated by washing with 1 M NaCl and 1 M NaOH.

In this article, we reported a simple method to isolate Tf from human plasma as well as from porcine serum. The purification procedure for two kinds of Tfs and their detailed characterization were described. Since porcine Tf is very similar with human Tf in molecular weight, spectral property, antibody specificity and especially receptor-binding ability, it possesses potential to be used instead of human Tf. On this meaning, the present work will provide useful information for various researches concerning Tf, such as studies using Tf as a cancer-targeting carrier.

2. Experimental

2.1. Chemicals

Human apotransferrin (T-4382), 30% iron saturated transferrin (T-3309) and holotransferrin (T-4132) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Fluorescein isothiocyantae isomer I (FITC) was obtained from Sigma–Aldrich too. DEAE Sepharose™ Fast Flow and Sephadex G-25 were purchased from Amersham (Amersham Biosciences, Sweden). Mouse monoclonal [HTF-14] to transferrin was purchased from abcam (Cambridge Science Park, UK). Sheep anti-mouse horseradish peroxidase-linked IgG antibody was obtained from Amercham (GE Healthcare, UK). All other chemicals and reagents were of analytical grade, and the water was of HPLC grade.

2.2. Purification of hTf from human plasma

The starting material was human plasma collected from healthy volunteers. With the presence of $NaHCO_3$, 100 mL human plasma was saturated with excess iron by adding 2.7 mL 0.1 mol/L NTA-Fe^{3+} (0.2 mol/L NTA and 0.2 mol/L $FeCl_3$ mixed at the ratio of 1:1) under continuous gentle stirring for 4 h at 4 °C. Then the iron-saturated plasma was subjected to ammonium sulfate precipitation. Ammonium sulfate was added to supernatant with gentle stirring until the saturation degree reached 40%. After that the pH was regulated to 4.6 with drops of 6 mol/L HCl. The suspension was stored at 4 °C overnight and centrifuged at 10,000 × g for 10 min at 4 °C. Then the clear supernatant was adjusted to pH 3.7. After the second centrifugation, the light yellow precipitate was dissolved in distilled water. After a preliminary desalting by dialysis, the solution was exchanged into 20 mM Tris buffer (pH 7.8) and concentrated to less than 50 mL using a 30,000 molecular weight cutoff (MWCO) membrane (Amicon® stirred Ultrafiltration cell, model 8200; Millipore Corp., USA).

The salmon pink solution obtained above was subjected to an anion-exchange column for further treatment. The DEAE-FF Sepharose column (18 cm × 3.5 cm) was pre-equilibrated with 20 mM Tris buffer (pH 7.8). Sample was loaded onto the column, followed by four column volume of equilibration buffer. A gradient elution buffer (20 mM Tris buffer containing 60–120 mM NaCl) was imposed on the column. The fraction collector was set to shift every 2 min giving a fraction size of about 8 mL in every tube. Column was regenerated with 1 M NaCl and 1 M NaOH, and equilibrated as described above. The effluent fractions were examined on a Shimadzu UV-2450 spectrophotometer at 280 nm and 465 nm. A typical chromatography profile was shown in Fig. 1A. After being examined by electrophoresis, fractions containing pure Tf were pooled, ultrafiltrated against distilled water, concentrated and finally freeze-dried.

Total protein amount was measured at 562 nm by a spectrophotometer using a BCA protein assay kit (Kengentec, China). A serious concentration of BSA was used as a standard. The mount of specific hTf or pTf at various stages of the purification was determined by Western blot using Sigma hTf or purified pTf as a calibratin standard [16]. The details about Western blot are described in Section 2.5.

2.3. Purification of pTf from porcine serum

Porcine serum was obtained from Minhai Bio-engineering (Lanzhou National HyClone Bio-engineering Co., Ltd., China). Except for salting out process and the salt concentration in ion-exchange elution, the other procedure was the same as that for human plasma sample. Briefly, the iron saturated solution was brought to a two-step precipitation of 40% and 85% saturation with $(NH_4)_2SO_4$. The second precipitate was a pink pellet. In ion-exchange chromatography, a stationary elution buffer (20 mM Tris buffer containing 50 mM NaCl) was used to elute Tf. A typical chromatography profile was shown in Fig. 1B.

2.4. Absorption spectra

Protein samples were dissolved in distilled water to a concentration of about 1 mg/mL. Ultraviolet and visible absorption spectra (800–200 nm) were recorded on a Shimadzu UV-2450 spectrophotometer using distilled water as the reference. The extinction coefficients at 280 nm were therefore calculated for each Tf. The binding of iron to Tf is accompanied by a visible spectral signal. Iron-binding capacity of transferrin samples was then expressed as a ratio of the 280 and the maximum visible absorbances. All data given referred to the average value of three tests assayed on independent days.

2.5. SDS-PAGE and Western blotting

The purified proteins were subjected to SDS-PAGE, which was carried out using 12% polyacrylamide gel. Each electrophoretic run included one strip which contained a standard human serum Tf (Sigma) as a positive control. After electrophoresis, the gel was stained with Coomassie Blue R-250 and destained in 45:10:45 (methanol:glacial acetic acid:water). Prestained protein molecular weight marker was from Fermentas (Fermentas, EU). For Western blotting, proteins were electroblotted onto the polyvinylidene difluoride (PVDF) membrane (Millipore). The membrane was incubated with 1:5000 diluted monoclonal antibody against Tf from mouse (abcam) as primary antibody and 1:1000 diluted horseradish peroxidase-conjugated antimouse IgG from sheep (Amersham) as secondary antibody. After washing, the amount of bound HRP was visualized with a DAB kit (Boster, China).

A digital camera was used for acquisition of the digital image from the SDS-PAGE gels and the Western blotting membrane. Image data were analyzed using the gel electrophoresis image analysis system (Model JD801, Jiangsu Jieda Tech, Co. Ltd.). The optical densities (OD) were determined for each protein band and the amount of protein in each gel was estimated by contrasting the OD value with that of the control protein band. The purity of target protein was also determined by OD percentage method.

2.6. HPLC analysis

The HPLC analysis for the determination of purity of the isolated Tfs was performed on a reversed-phase protein analytical Symmetry 300^{TM} C4 column from Waters (5 μm, 300 Å, 250 mm × 4.6 mm) using a LC-10AT pump from Shimadzu and a SPD-10A UV–vis detector. At first, a C18 column was used. But results were not satisfactory, because sample peak was quite asymmetrical. So a stationary phase with a shorter chain, for instance, C8 or C4 would be more suitable for large protein. The solvent used for the analysis were solvent A (water, 0.05% trifluoroacetic acid (TFA)) and solvent B (acetonitrile, 0.05% TFA). The mobile phase consisted of a linear gradient of 30% of solvent B to 50% of solvent B in 25 min. The flow rate was set at 1.0 mL/min. The effluent was monitored at 280 nm. Human serum Tf obtained from Sigma was used as a standard control.

2.7. Urea polyacrylamide gel electrophoresis (PAGE)

The iron-binding states of the purified porcine Tf and human Tf were examined by urea Polyacrylamide gel electrophoresis (PAGE). As described by Williams et al. [25], PAGE was carried out using gels in a buffer of 90 mM Tris–borate (pH 8.4) containing 6% (w/v) acrylamide and 6 M urea. Approximately, 5 μg of each sample was loaded per lane and bands were visualized by staining with Coomassie blue. The gels were electrophoresed for 2 h at 100 V. The sample buffer was made up of 0.5 mL of 0.9 M Tris–borate (pH 8.4), 1.8 g of urea, 2.5 g of sucrose and several drops of bromophenol blue in a total volume of 5 mL and was mixed 1:1 with the sample [11]. The fully saturated, partially saturated and iron deficient Tf from Sigma were used as controls.

2.8. Mass spectrometry

Matrix-assisted laser desorption ionization (MALDI) mass spectrometry with time-of-fight (TOF) was performed using an ultraflexIITOF/TOF instrument (Bruker-Daltonics, Germany) equipped with a smartbeam laser operating at 200 Hz. A saturated solution of 2,5-dihydroxybenzoic acid (DHB) in 30% acetonitrile and 0.1% trifluoroacetic acid was prepared as a matrix solution. An aliquot (1 μL) of the sample solution was mixed with an equal aliquot of the matrix solution, and 1 μL was spotted onto the target plate and evaporated under a gentle stream of warm air. Mass spectra were acquired in positive ion reflector mode using a reflectron voltage of 25,000 V, accelerating voltage at 92.8%, lens voltage at 26% and pulsed ion extraction of 150 ns. External calibration was performed for molecular assignments using a standard of bovine serum albumin with $[M+]^+$ at 66,500.

2.9. Cell binding study

2.9.1. Cell culture

K562 cells were maintained suspended in RPMI 1640 culture medium with 10% (v/v) FCS, 2 mM glutamine, and 0.1 mg/mL of both penicillin and streptomycin. Cells were cultured in a humidified atmosphere with 5% CO_2 at 37 °C.

2.9.2. Flow cytometry

The purified hTf and pTf, as well as Sigma human holotransferrin and BSA (Amresco) were labeled with fluorescein isothiocyanate (FITC) from Sigma according to Micheel et al. [26]. The molar ratio (moles of FITC/mole of protein) of the conjugates were calculated by measuring the protein concentration using a BCA protein assay kit and FITC by its typical visible absorption at 495 nm. The fluorescein:protein ratio was 5.4 on the average. The labeled proteins were desalted and freeze-dried for further use.

Binding activities of the various coupled proteins to the specific receptor were measured as follows: K562 cells (each sample containing 1×10^6 cells) in the logarithmic growth phase were collected, washed twice in cold (4 °C) PBS, and resuspended in 100 μL cold PBS; 1 μL labeled proteins with various concentrations (1 μM, 10 μM, 50 μM and 100 μM) were added to cell suspensions and cells were incubated at 4 °C for 30 min. Labeled holotransferrin from Sigma and BSA were added as positive and negative controls, respectively. Then cells were washed twice with ice-cold PBS and resuspended in 500 μL PBS, and the amount of bound FITC-labeled protein was determined by FACS (Becton Dickinson, USA). Data were reported as mean channel fluorescence of bell-shaped histograms.

3. Results

3.1. Protein production

In the described process, samples were first saturated with iron. Then the majority of contaminant proteins were removed by ammonium sulfate fractional precipitation, followed by an ion-exchange chromatography. As depicted in Fig. 1, applying pretreated samples to the column and developing with certain NaCl resulted in separation of Tfs from other proteins, which did not elute from the column until application of 1 M NaCl. A heavily colored red band of transferrin could be observed moving down the column. Analysis of the fraction at 465 nm confirmed the presence of Tfs. All fractions with a A_{280}/A_{465} ratio below 25.0 were subjected to SDS-PAGE (data not shown). As a result, fractions with more than 90% purity judged by SDS-PAGE were collected and ultrafiltrated against distilled water and finally lyophilized. The average yields of Tf from human plasma and porcine serum were 124 mg/100 mL and 160 mg/100 mL, respectively. The typical recovery of hTf and pTf during purification was presented in Table 1.

In an effort to increase the recovery of Tfs, we evaluated whether it was possible to apply the post ion-exchange sample directly onto the Con A Sepharose 4B column. Results showed that it took a long time to load the column due to the increased volume. The gel filtration chromatography was also attempted applying a S200HR column. But for this purification step, sample volume was strictly limited. So the efficiency of the process was very low although there was a little increase in the recovery (about 55% for hTf).

Table 1
Summary of purification yield and purity for hTf and pTf after each purification step

Step	Volume (mL)	Total protein (mg)	hTf (mg)	Yield (%)	Purity (%)	Purity (fold)
Human plasma	100	6978.2	281.3	100	4.0	1
Ammonium sulfate precipitation	68	2421.2	182.1	64.7	7.5	1.9
Dialysis	98	2279.3	177.0	62.9	7.8	2.0
Anion exchange	95	168.9	153.2	54.5	90.7	22.7
Ultrafiltration	20	153.8	147.8	52.5	96.1	24.0
Freeze drying	–	149.3	145.1	51.5	97.2	24.3
Porcine serum	100	5863.3	266.8	100	4.5	1
Ammonium sulfate precipitation	69	2209.4	228.3	85.6	10.3	2.3
Dialysis	96	2091.2	225.4	84.5	10.8	2.5
Anion exchange	102	217.3	191.6	71.8	88.2	19.6
Ultrafiltration	20	190.3	183.6	68.8	96.4	21.4
Freeze drying	–	185.1	181.2	67.9	97.9	21.7

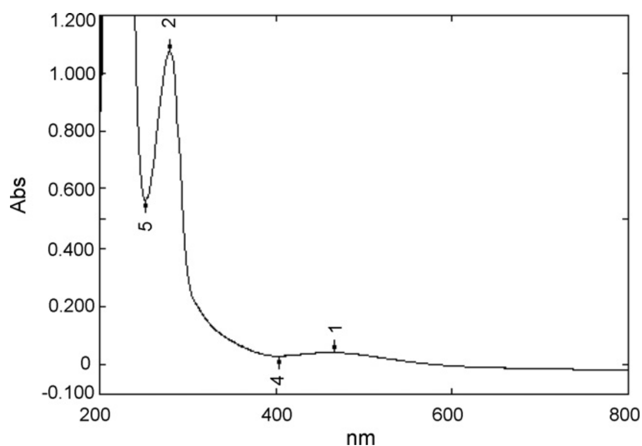

Fig. 2. Ultraviolet and visible spectra of the purified hTf. The peak at 465 nm attributed to the binding of iron to Tfs. Spectra of standard holo-hTf from Sigma and the purified pTf resembled that of the above very much and was not given here.

Based on reasons mentioned above, after ammonium sulfate precipitation, anion exchange chromatography is enough for obtaining considerable purity and efficiency when a relatively large scale of purification is required.

3.2. Purity determination

Our research on Tf conjugation requires Tf with a high degree of purity. In this study, several techniques were used to assess the purity of the isolated hTf and pTf.

The final products were examined by spectrophotometry. Absorption spectra scanning (Fig. 2) were recorded and results showed that characteristic spectra from two isolated proteins were indistinguishable. Iron uptake by Tfs resulted in a salmon pink color and gave a maximum absorption at about 465 nm in the visible spectrum. The spectral ratios of A_{280}/A_{465} were presented in Table 2. Results were consistent with previously reported $A_{280}/A_{\max\ vis}$ ratios of 20–23 [9,11,12], and suggested that the proteins were correctly folded and able to bind two iron atoms with high purity. The millimolar absorption coefficients at 280 nm were also listed. The spectral ratio and coefficient of pTf was almost identical with that of hTf.

Table 2
Spectral characterization of different transferrins[a]

Protein sources	$\lambda_{\max}$(nm)	$A_{280}/A_{\max}$	ε_{280}[b]
Human, batch1#	465	22.7	91,470
Human, batch2#	465	23.4	92,980
Porcine, batch1#	465	22.9	92,853
Porcine, batch2#	465	23.2	92,075
Human, Sigma	465	22.8	91,235

[a] Data given in the table were average values assayed in three independent experiments.
[b] The ε_{280} values were determined by spectral experiments and were expressed as the molar absorption coefficients.

It can be seen from SDS-PAGE (Fig. 3A) that hTf and pTf both appeared as almost a single band. The OD values of purified Tfs were determined using the image analysis system and the percentage of each protein indirectly represented its purity, which both were more than 95%.

Purity of Tfs was also determined with the aid of HPLC on a protein analytical column as described in Section 2. A representative HPLC chromatogram of the purified Tfs was shown in Fig. 4. The main peak at about 11.4 min corresponded to hTf. As for pTf, the main peak emerged at around 9.9 min. Samples were all of more than 97% purity represented by peak area percentage. HTf and pTf samples all displayed very similar chromatograms, demonstrating the similar retention behavior on the column. It can be inferred from these results that these proteins had similar structures or chemical properties.

3.3. Immunoreactivity

Western blotting was performed using the same electrophoresis conditions. After electrophoresis, the gel was transferred to a PVDF membrane, and the membrane was treated with mouse anti-human Tf antibody and horseradish peroxidase-linked sheep anti mouse antibody and developed with the DAB substrate system. The Western blot given in Fig. 3B showed that both hTf and pTf did react with the anti-human Tf monoclonal antibody and suggested that the two proteins had the similar domain that could be recognized by the anti-transferrin antibody, although the band corresponding to pTf was lighter than the one corresponding to hTf.

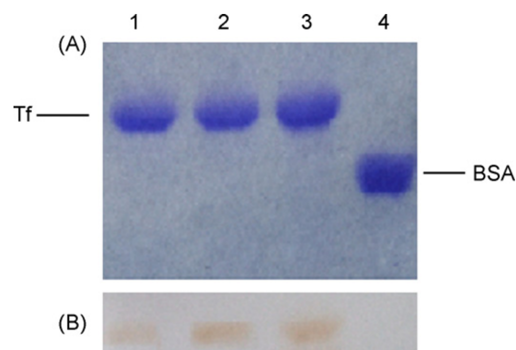

Fig. 3. SDS-PAGE of purified Tfs stained with Coomassie blue (A) and immunoblotting with anti-human serum transferrin monoclonal antibody (B) Lanes 1–3 in turns were purified pTf, purified hTf and standard holo-hTf from Sigma; lane 4: bovine serum albumin, which used as a negative control. 2 μg of protein was loaded in each lane.

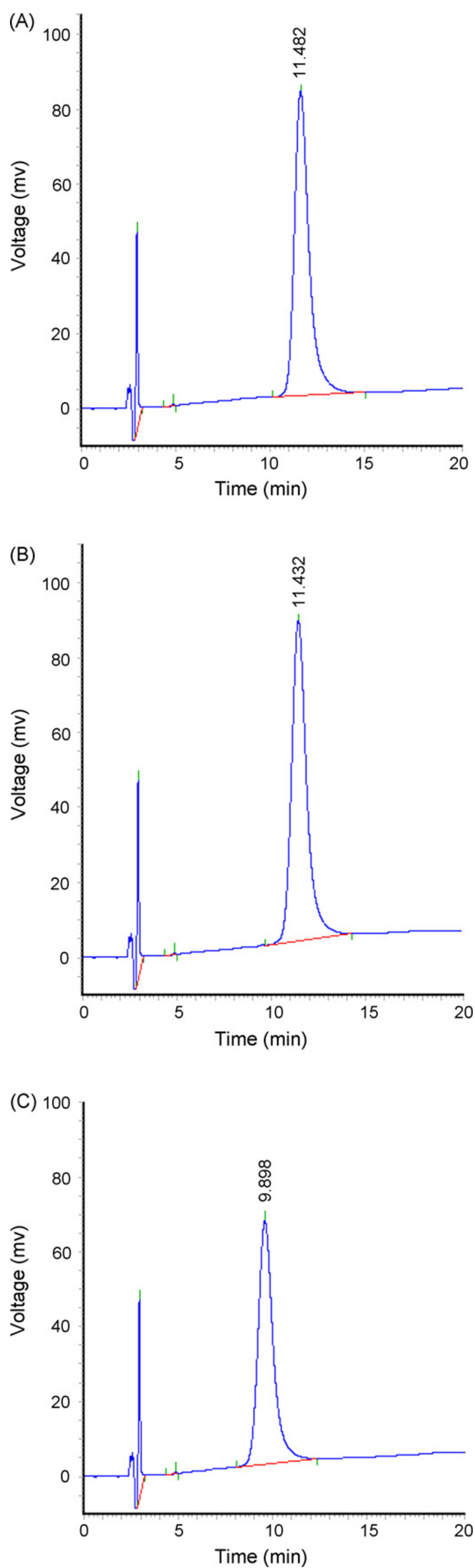

Fig. 4. HPLC chromatograms of pure Tfs. Samples: (A) hTf obtained from Sigma, (B) purified hTf and (C) purified pTf. The mobile phase consisted of a linear gradient of 30% of solvent B to 50% of solvent B in 25 min. A Symmetry 300™ C4 column (5 μm, 300 Å, 250 mm × 4.6 mm, Waters) was used for separation, column temperature was set at 25 °C.

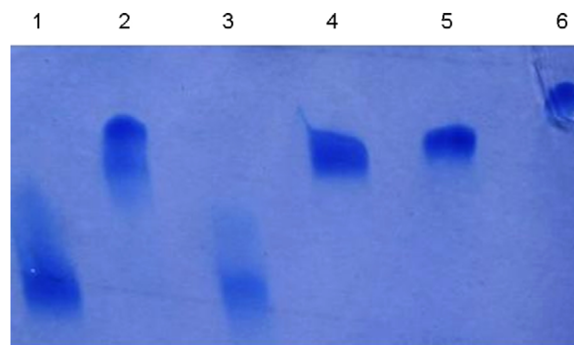

Fig. 5. Electrophoresis of Tfs in a 6 M urea/polyacrylamide gel under nonreducing conditions and visualized with Coomassie blue. Samples: (1) holo-hTf from Sigma; (2) partially saturated hTf from Sigma; (3) purified hTf; (4) purified pTf; (5) apo-hTf from Sigma; (6) apo-pTf prepared by us. Lane 2 showed there were two iron forms of transferrin corresponding to iron free transferrin and monoferric transferrin with one iron in the C or N lobe in the commercial partially saturated hTf.

3.4. Iron-binding analysis

UV–vis spectra of the purified Tfs preliminarily indicated their iron binding ability. To further demonstrate the iron-binding states, the proteins were analyzed on a 6 M urea gel to distinguish the possible forms of human transferrin [9,11,12,27]. Binding of iron resulted in both charge differences and an increase in resistance to denaturation in the presence of 6 M urea. On urea gels (Fig. 5), the diferric hTf (lane 1) ran much faster than apo-hTf (lane 5). Commercial partially saturated hTf (lane 2) showed two main bands corresponding to apo-hTf and monoferric hTf (C-lobe or N-lobe, not definitely). As for isolated pTf (lane 4), the migration was obviously slower than holo-hTf and the iron-removed pTf (lane 6) shifted more slowly than the iron-binding pTf. The urea gel results indicated that the purified hTf (lane 3) was identical with Sigma hTf, i.e. holotransferrin. We can also find that under such assay conditions, pTf took few negative charges compared to the corresponding hTf and thus migrated more slowly.

3.5. Molecular weight assay

As a simple and quick method, SDS-PAGE roughly determined the sizes of different Tf samples, which were all of approximately the same relative molecular weight of about 80,000.

MALDI-TOF mass spectrometry analysis further determined the precise molecular weights of these two proteins. Fig. 6 showed the observed mass spectra of different Tf samples. The average mass observed for the standard hTf from Sigma was 79,492. In contrast, the average molecular weight of purified hTf and pTf were 79,707 and 79,258, respectively. The MALDI-TOF analysis provided excellent mass accuracy, but it was noteworthy that the mass spectra showed a rather broad peak profile, which could be probably due to carbohydrate heterogeneity of genetic variation, as transferrin was isolated from pooled serum or plasma. The mass spectra also indicated that the two proteins we purified had very high purity, thus displaying a major peak with high intensity.

3.6. Cell-binding activity

Using flow cytometry, binding assays were performed to verify the binding activity of purified Tfs to the Tf receptor on human cell surface. Each hTf sample was labeled with FITC, and direct binding to K562 cells was measured. As a negative control, labeled BSA shown very low binding compared to Tfs, which meant few nonspecific bindings occurred. In Fig. 7, all three Tfs presented their binding activities to the Tf receptor expressed on K562 cell membrane and

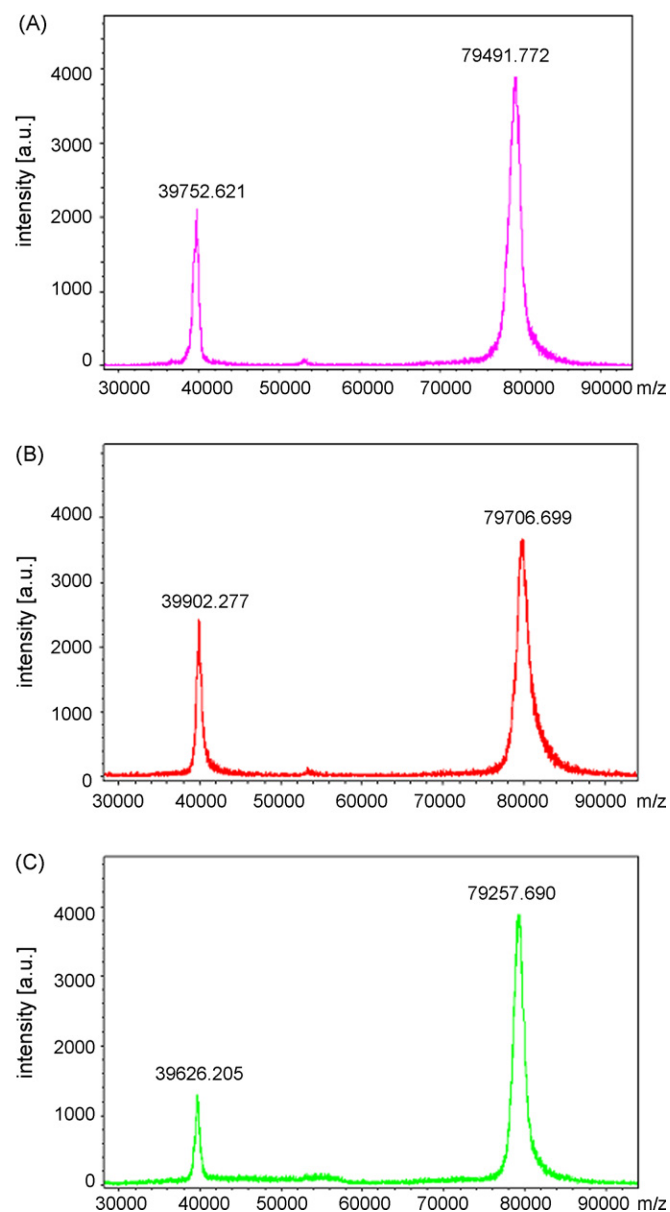

Fig. 6. MALDI-TOF mass spectrometry analysis for (A) hTf from Sigma–Aldrich; (B) hTf purified from human plasma and (C) pTf purified from porcine serum.

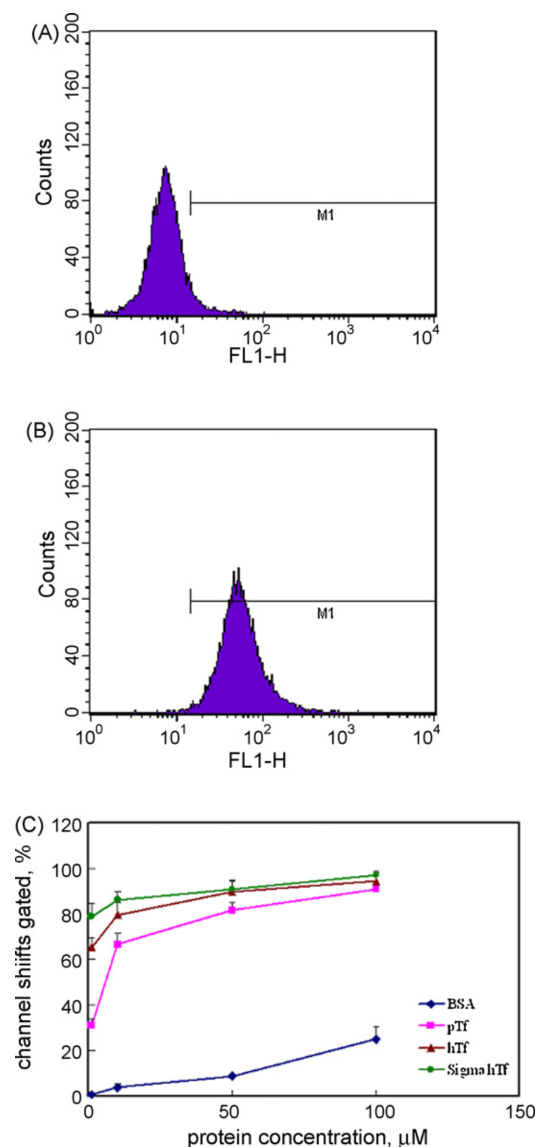

Fig. 7. Binding characteristics of hTf and pTf. K562 cells were bound with different Tf samples labeled with FITC (samples with similar conjugation ratio of about 5.4 were used in the binding assay). Aliquots of 1×10^6 cells were incubated with different concentrations of samples at 4 °C for 60 min. FITC-labeled BSA was used as negative control. Background was assessed by replacing the FITC-conjugated sample with PBS. The cell associated fluorescence of FITC was determined by flow cytometry. (A) Background staining, (B) A typical FACScan of the positive sample and (C) Comparison of binding capacity of different samples to the TfR on K562 cell surface. Data were expressed as mean channel shifts of the cell peak from the zero minus background cell fluorescence. ($n = 4$).

the difference in their affinity was observed. We could find that the binding of purified hTf sample was almost indistinguishable from binding of the Sigma hTf sample. However, the binding of purified pTf was relatively lower.

4. Discussion

Tfs are abundant in nature, making it relatively facile to purify the naturally occurring proteins. The manufacturing process described in the present paper produced pure and active holotransferrin with high yield and very few steps.

In normal human plasma, only 30% of transferrin is iron-binded [18]. Iron-saturated Tf is much more stable than the iron-free transferrin. More importantly, diferric Tf has the highest affinity for the TfR at physiological pH. On this basis, excess NTA-Fe^{3+} was added to make transferrin iron-saturated at the beginning of experiment.

The chromatographic method with ion-exchange adsorbents, which has been so successful in the separation of many protein components of serum, was employed in our work. Definition of salt concentration is very critical in the whole ion-exchange chromatography. A 0–400 mM NaCl gradient was first employed to roughly estimate under what condition of ionic strength could the target protein be eluted. Then the gradient range was further diminished until the optimal result was obtained. To purify hTf, a gradient elution buffer (20 mM Tris buffer containing 60–120 mM NaCl) was imposed on the column. But because of unequal isoelectric point, pTf could be eluted in a very pure state when a fixed salt concentration (50 mM NaCl) was employed.

In this study, the iron-saturated Tf was successfully obtained, as proved by both spectral and urea gels. The spectral ratios and migration on urea gels were completely consistent with iron binding profile of holo-transferrin. The iron-binding state of Tf is a critical property, which determines the ability to enter target cells. As

reported, diferric Tf has the highest affinity for the TfR at physiological pH. The association constant for diferric Tf and the Tf receptor (TfR) is 30-fold higher than that of monoferric Tf, and 500-fold higher than apo-Tf [28].

Despite the iron-binding ability, the capability of binding to special TfR on cell surface is the most important character. FACS assays were used to measure the receptor-binding properties of the purified transferrins. No appreciable differences were seen in the binding to K562 cell of the purified hTf and the commercial Sigma hTf. But the binding capacity of pTf observed was somewhat weaker than that of hTf. For example, at protein concentration of 50 μM, the channel shifts of purified hTf and pTf compared with Sigma hTf were 98% and 89%, respectively. These results were reasonable and well in accord with the Western blotting consequence, because porcine transferrin is not the nature ligand for the human transferrin receptor and the interaction between pTf and hTfR as well as the crossreactivity between pTf and the anti-human Tf antibody were caused by the high homology of the transferrin family [23,24]. Previous studies [29] demonstrated that reactivity among widely divergent species was observed in several instances, although specificity existed in transferrin–receptor interactions throughout the range of vertebrate animals. Structural similarities have been maintained throughout evolution. Our studies again verified this point.

5. Conclusion

The simple procedure presented in this report permits the preparations of highly purified hTf and pTf with good recovery (52% for hTf and 65% for pTf) as judged by mobility on SDS-PAGE, spectral properties, HPLC, and MALDI-TOF mass spectrometry. Initial studies on immunoreactivity and the ability to bind to the special receptors on a human K562 cell line showed that the purified pTf was a potential substitute for hTf, which had been widely used as a carrier in drug targeting system.

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References

[1] Z.M. Qian, H. Li, H. Sun, K. Ho, Pharmacol. Rev. 54 (2002) 561.
[2] H. Li, Z.M. Qian, Med. Res. Rev. 22 (2002) 225.
[3] T.R. Daniels, T. Delgado, G. Helguera, M.L. Penichet, Clin. Immunol. 121 (2006) 159.
[4] C.L. Hershberger, J.L. Larson, B. Arnold, P.R. Rosteck Jr., P. Williams, B. DeHoff, P. Dunn, K.L. O'Neal, M.W. Riemen, P.A. Tice, et al., Ann. N.Y. Acad. Sci. 646 (1991) 140.
[5] R.A. Ikeda, B.H. Bowman, F. Yang, L.K. Lokey, Gene 117 (1992) 265.
[6] L.M. Steinlein, R.A. Ikeda, Enzyme Microb. Technol. 15 (1993) 193.
[7] M.H. de Smit, P. Hoefkens, G. de Jong, J. van Duin, P.H. van Knippenberg, H.G. van Eijk, Int. J. Biochem. Cell. Biol. 27 (1995) 839.
[8] P. Hoefkens, M.H. de Smit, N.M. de Jeu-Jaspars, M.I. Huijskes-Heins, G. de Jong, H.G. van Eijk, Int. J. Biochem. Cell. Biol. 28 (1996) 975.
[9] A.B. Mason, M.K. Miller, W.D. Funk, D.K. Banfield, K.J. Savage, R.W. Oliver, B.N. Green, R.T. MacGillivray, R.C. Woodworth, Biochemistry 32 (1993) 5472.
[10] A.B. Mason, Q.Y. He, T.E. Adams, D.R. Gumerov, I.A. Kaltashov, V. Nguyen, R.T. MacGillivray, Protein Exp. Purif. 23 (2001) 142.
[11] A.B. Mason, P.J. Halbrooks, J.R. Larouche, S.K. Briggs, M.L. Moffett, J.E. Ramsey, S.A. Connolly, V.C. Smith, R.T. MacGillivray, Protein Exp. Purif. 36 (2004) 318.
[12] L.M. Steinlein, T.N. Graf, R.A. Ikeda, Protein Exp. Purif. 6 (1995) 619.
[13] A.B. Mason, R.C. Woodworth, R.W. Oliver, B.N. Green, L.N. Lin, J.F. Brandts, B.M. Tam, A. Maxwell, R.T. MacGillivray, Protein Exp. Purif. 8 (1996) 119.
[14] M.D. Retzer, A. Kabani, L.L. Button, R.H. Yu, A.B. Schryvers, J. Biol. Chem. 271 (1996) 1166.
[15] S.A. Ali, H.C. Joao, R. Csonga, F. Hammerschmid, A. Steinkasserer, Biochem. J. 319 (Pt 1) (1996) 191.
[16] H.J. Lim, Y.K. Kim, D.S. Hwang, H.J. Cha, Biotechnol. Prog. 20 (2004) 1192.
[17] A. Leibman, P. Aisen, Blood 53 (1979) 1058.
[18] J. Parkkinen, L. von Bonsdorff, F. Ebeling, L. Sahlstedt, Vox Sang 83 (Suppl. 1) (2002) 321.
[19] W. van Gelder, M.I. Huijskes-Heins, C.J. Hukshorn, C.M. de Jeu-Jaspars, W.L. van Noort, H.G. van Eijk, Comp. Biochem. Physiol. B Biochem. Mol. Biol. 111 (1995) 171.
[20] S. Blirup-Jensen, Clin. Chem. Lab. Med. 39 (2001) 1076.
[21] G.S. Baldwin, T. Bacic, R. Chandler, B. Grego, J. Pedersen, R.J. Simpson, B.H. Toh, J. Weinstock, Comp. Biochem. Physiol. B 95 (1990) 261.
[22] P.A. Werner, R.M. Galbraith, P. Arnaud, Arch. Biochem. Biophys. 226 (1983) 393.
[23] S. Welch, Comp. Biochem. Physiol. B 97 (1990) 417.
[24] G.S. Baldwin, Comp. Biochem. Physiol. B 106 (1993) 203.
[25] J. Williams, R.W. Evans, K. Moreton, Biochem. J. 173 (1978) 533.
[26] B. Micheel, P. Jantscheff, V. Bottger, G. Scharte, G. Kaiser, P. Stolley, L. Karawajew, J. Immunol. Methods 111 (1988) 89.
[27] K. Harad, A. Kuniyasu, H. Nakayama, M. Nakayama, T. Matsunaga, Y. Uji, H. Suguichi, H. Okabe, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 767 (2002) 45.
[28] S.P. Young, A. Bomford, R. Williams, Biochem. J. 219 (1984) 505.
[29] B.C. Lim, H.J. McArdle, E.H. Morgan, J. Comp. Physiol. [B] 157 (1987) 363.