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Biochemical and Structural Characterization of Recombinant Human Serum Transferrin from Rice (*Oryza sativa* L.)

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Abstract

The Fe³⁺ binding protein human serum transferrin (hTF) is well known for its role in cellular iron delivery via the transferrin receptor (TFR). A new application is the use of hTF as a therapy and targeted drug delivery system for a number of diseases. Recently, production of hTF in plants has been reported; such systems provide a relatively inexpensive, animal-free (eliminating potential contamination by animal pathogens) method to produce large amounts of recombinant proteins for such biopharmaceutical applications. Specifically, the production of OptiferrinTM (hTF produced in rice, *Oryza sativa*, from InVitria) has been shown to yield large amounts of functional protein for use in culture medium for cellular iron delivery to promote growth. In the present work we describe further purification (by gel filtration) and characterization of hTF produced in rice (purified OptiferrinTM) to determine its suitability in biopharmaceutical applications. The spectral, mass spectrometric, urea gel and kinetic analysis shows that purified OptiferrinTM is similar to recombinant nonglycosylated N-His tagged hTF expressed by baby hamster kidney cells and/or serum derived glycosylated hTF. Additionally, in a competitive immunoassay, iron-loaded OptiferrinTM is equivalent to iron-loaded N-His hTF in its ability to bind to the soluble portion of the TFR immobilized in an assay plate. As an essential requirement for any functional hTF, both lobes of purified OptiferrinTM bind Fe³⁺ tightly yet reversibly. Although previously shown to be capable of delivering Fe³⁺ to cells, the kinetics of iron release from iron-loaded OptiferrinTM/sTFR and iron-loaded N-His hTF/sTFR complexes differ somewhat. We conclude that the purified OptiferrinTM might be suitable for consideration in biopharmaceutical applications.

Keywords

human serum transferrin; OptiferrinTM; recombinant transferrin; mass spectrometry; iron delivery; kinetics; transferrin receptor

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1. Introduction

Cellular iron delivery is critically dependent upon the transferrin/transferrin receptor endocytic pathway. Part of a larger family comprised mainly of iron-binding proteins, human serum transferrin (hTF) is a bilobal (N-lobe and C-lobe) glycoprotein. Synthesized in the liver and secreted into the blood plasma, the primary role of hTF is to bind Fe^{3+} with high affinity ($\sim 10^{22} \text{ M}^{-1}$) [1] and carefully chaperone this reactive metal to sites of utilization (reticulocytes and other actively dividing cells). At the neutral pH of the blood (~ 7.4), two iron-bearing hTF molecules bind with nanomolar affinity to the homodimeric transferrin receptor (TFR), forming a 2:2 complex (hTF:TFR monomer). Following clathrin-dependent endocytosis of the hTF/TFR complex [2], the endocytic pH is lowered through the action of an ATP-dependent H^+ pump [3]. The significantly lower pH (~ 5.6) within the endosome initiates receptor stimulated iron release from hTF to an, as of yet, unidentified chelator. Critically, apohTF (iron-free hTF) remains bound to the TFR with high affinity at endosomal pH allowing the apohTF/TFR complex to return to the cell surface. Upon exposure to the more basic pH of the blood, apohTF is released, either through dissociation from the TFR or by displacement by an iron-containing hTF [4], and is free to bind more Fe^{3+} .

While the process of internalization and recycling of the hTF/TFR complex has been extensively studied, the fate of the released iron remains unclear. However, within the endosome the iron must be reduced to Fe^{2+} by a ferrireductase (such as Steap3 in erythroid cells) [5] before being exported out of the endosome by the divalent metal transporter DMT1 [6–8]. Due to its inherent redox properties, iron within the cell is a critical cofactor for many proteins and enzymes involved in various biological processes, including oxygen and electron transport (in the form of heme and iron-sulfur clusters, respectively). Therefore, as would be expected, iron deficiency is extremely detrimental to cellular function [9].

Given the absolute requirement of iron for cellular growth and proliferation, it is not surprising that transferrin (along with insulin and selenium) is often added to supplement serum-free mammalian cell culture medium [10]. Moreover, the use of transferrin is currently being developed for a number of biopharmaceutical applications. Due to the high iron requirements of proliferative cells (which in turn express elevated levels of the TFR), hTF is being actively exploited as an anti-cancer drug delivery molecule [11]. Specifically, the use of hTF as a ‘Trojan horse’ for targeted drug delivery within cells provides the advantage of being non-immunogenic. Furthermore, recombinant transferrin has been used to treat diseases such as thalassemia, atransferrinemia, and age-related macular degeneration [10]. Clearly, an economically feasible and readily available source of hTF is needed to support these important applications.

The natural abundance of glycosylated hTF in human serum makes isolation relatively facile as well as economical. However, the use of hTF from such biological sources has drawbacks including the potential for exposure to blood-borne pathogens and the inability to introduce mutations. Over the past two decades a number of expression systems have been utilized to produce recombinant hTF. Naturally, the common expression platform, *Escherichia coli*, was the first system tested for its ability to produce hTF. While the numerous advantages of using a bacterial expression system are well established, the production of hTF in *E. coli* met with extremely limited success due to an inability to correctly form the 19 disulfide bonds in hTF in the non-reducing prokaryotic environment [12–16]. Another commonly used expression system for recombinant proteins is the eukaryote yeast, *Pichia pastoris*. While offering several advantages (high yields and low cost), for unknown reasons very little full-length hTF was successfully produced in this system until quite recently [17]. In contrast, the use of common baker’s yeast, *Saccharomyces cerevisiae*, has produced homogenous

non-glycosylated hTF in higher yields (~1.5 g/L) than previously reported in any yeast system [18].

By far the most well-defined and well-characterized expression system used to produce recombinant N-His-tagged nonglycosylated hTF (referred to as N-His hTF) is the mammalian (baby hamster kidney, BHK) cell system developed in our laboratory [19–22]. The use of this recombinant system has provided the means to produce hTFs that are either incapable of binding (Fe_N hTF or Fe_C hTF constructs) [22] or releasing iron from one of the two lobes (Lock_N hTF or Lock_C hTF constructs)[23–25]. We have utilized these recombinant hTF constructs to develop a comprehensive scheme of all kinetic steps involved in iron release from hTF both in the absence and presence of the soluble portion of the TFR [25,26]. However, although successful in producing fully-functional hTF (~30–50 mg/L of tissue culture medium), the relatively high cost and current scale of the BHK cell system cannot provide the amount of hTF required for many biopharmaceutical applications.

Recently, given the potential of animal-derived recombinant proteins for contamination by viruses, mycoplasma and prions, the recombinant production of human proteins in plants has become an attractive alternative expression system and the focus of much research effort. Plant sources provide a relatively inexpensive method for the large-scale production of recombinant proteins while reducing the risk of potential contamination by animal pathogens. Thus far, attempts to produce recombinant hTF in tobacco (*Nicotiana tabacum*) have met with rather limited success due to low yields (estimated 0.25% total soluble protein) [27]. However, the production of recombinant hTF in rice (*Oryza sativa*) produced significantly more protein (estimated 40% total soluble protein) [28]; this hTF is now available for use as a cell culture medium supplement from InVitria under the trade name Optiferrin™.

Although recombinant hTF is commercially available from many different companies (Table S1), detailed biochemical characterization of the recombinant product is often lacking. In the current study, we have thoroughly evaluated the properties of Optiferrin™ (through direct comparison to the recombinant N-His hTF constructs produced in our BHK cell system and/or to the serum derived glycosylated hTF) using a combination of well-documented biophysical techniques many of which we have developed to characterize our recombinant transferrins and mutants thereof.

2. Materials and Methods

2.1. Materials

Dulbecco's modified Eagle's medium-Ham F-12 nutrient mixture (DMEM-F12) and fetal bovine serum (FBS) were obtained from the GIBCO-BRL Life Technologies Division of Invitrogen. Antibiotic-antimycotic solution (100X) solution and trypsin were from Mediatech, Inc. Both Pro293A-CDM serum-free medium, and L-glutamine were purchased from Lonza. All tissue culture dishes, flasks, and Corning expanded surface roller bottles were obtained from local distributors. Ultracel 30 kDa molecular weight cutoff (MWCO) membrane microconcentrator devices were manufactured by Amicon. Ni-nitrilotriacetic acid (NTA) resin came from Qiagen. Hi-prep 26/60 Sephacryl S-200HR and S-300HR columns were acquired from GE Healthcare. SDS-PAGE gels (4–15%) were purchased from BioRad. EDTA was from Fisher. Serum derived glycosylated hTF, NTA and ferrous ammonium sulfate were from Sigma (St. Louis, MO). Novex 6% Tris(hydroxymethyl)aminomethane–borate–EDTA (TBE) urea mini-gels, TBE running buffer (5X) and TBE-urea sample buffer (2X) were from Invitrogen. The 3,3',5,5'-tetramethylbenzidine (TMB) microwell peroxidase (1-component) substrate system came from Kirkegaard and Perry Laboratories (Gaithersburg, MD). The A4A6 monoclonal

antibody to the TFR was a generous gift from the laboratory of Dr. James Cook at the University of Kansas Medical Center. Removawells (Immulon 1B) were from Thermo Scientific. Lyophilized OptiferrinTM (Lot: P0207) was supplied by InVitria (Fort Collins, CO). OptiferrinTM was manufactured as described previously [28].

2.2. Peptide mapping by mass spectrometry

Both OptiferrinTM and native (serum derived glycosylated) hTF proteins were electrophoresed on a 4–20% Tris-Glycine SDS-PAGE gel. The OptiferrinTM and hTF protein bands were excised from the gel, reduced, alkylated, and subjected to in-gel tryptic digestion overnight. The resulting peptides were separated and analyzed using the Paradigm MG4 HPLC system (Michrom Bioresources, Auburn, CA), which is coupled to a Thermo Finnigan LTQ-FT Ultra ion trap mass spectrometer (Thermo Fisher) through a Michrom advance captive spray ionization source. Each sample was loaded onto a trap column (Zorbax300SB C₁₈, 5 μ m, 5 $\times$ 0.3 mm; Agilent Technologies, Santa Clara, CA) and desalted online. Peptides were then eluted from the trap and separated by a reverse-phase Michrom Magic C₁₈ AQ (200- μ m by 150-mm) capillary column at a flow rate of 2 μ l/min. The gradient used to elute the peptides had a duration of 60 minutes using two solvents, A and B (A: 0.1% formic acid and B:100% acetonitrile). Samples were resuspended in 2% acetonitrile (ACN) and 0.1% trifluoroacetic acid (TFA) as the running buffer and directly loaded onto the mass spectrometer. The mass spectrometer was operated with a spray voltage of 1.8 kV, a heated capillary temperature of 200°C, and a full scan range with a mass/charge ratio of 350 to 1,400. The parameters for data dependent tandem mass spectrometry (MS/MS) are as follows: 10MS/MS spectra for the most intense ions from the full scan with 35% collision energy for collision induced dissociation. Raw spectra data was analyzed using X!Tandem and visualized using Scaffold 3 software (Proteome Software, Portland, Oregon, USA).

2.3. Circular Dichroism Spectroscopy

Both OptiferrinTM and serum derived glycosylated hTF at a concentration of 0.5 mg/ml were dialyzed against 50 mM sodium acetate, pH 4.9 for 24 h at 4 ° C to remove bound iron followed by exchange into 25 mM sodium phosphate, pH 7.0. The concentration of each was adjusted to 0.2 mg/ml (based on an hTF ELISA assay [28]) with 25 mM sodium phosphate, pH 7.0, for circular dichroism (CD) measurements. The CD spectra were collected on a Jasco J-710 circular dichroism Spectropolarimeter at 20 °C at the Protein Facility at Iowa State University. The molar ellipticity was monitored with 1.0 nm of bandwidth at 0.2-nm wavelength decrements from 250 to 190 nm in a 0.1 cm cell. The resulting spectra were base line corrected using 25 mM sodium phosphate, pH 7.0.

2.4. Expression and Purification of hTFs and the soluble portion of the TFR (sTFR)

The expression and purification of baby hamster kidney (BHK) cell derived recombinant N-His hTFs (diferric hTF, Fe₂hTF; Fe_NhTF, monoferric N-lobe hTF in which mutation of iron binding ligands, Y426F/Y517F, prevents iron binding in the C-lobe; Fe_ChTF monoferric C-lobe hTF in which mutation of iron binding ligands, Y95F/Y188F, prevents iron binding in the N-lobe) has been previously described [29]. The production and purification of the His-tagged sTFR consisting of residues 121–760 is also as previously described [30].

2.5. Spectral analysis

To determine the visible absorption maximum, 20 mg of lyophilized OptiferrinTM was taken up in 2 mL of 100 mM NH₄HCO₃, pH 8.1 (to a nominal concentration of ~10 mg/mL). The spectrum of OptiferrinTM was collected between 500–350 nm on a Varian Cary 100 spectrophotometer. To assure saturation of the OptiferrinTM with iron, an Fe-NTA solution

was prepared by mixing an appropriate amount of 25 mM ferrous ammonium sulfate (in 0.01 N HCl) with 100 mM NTA to obtain a Fe:NTA ratio of 1:2. Following the addition of a slight molar excess of Fe^{3+} (in the form of Fe-NTA) and exchange of the sample into 100 mM NH_4HCO_3 to remove any excess Fe-NTA, the spectral properties of Optiferrin™ were reevaluated.

2.6. Additional Purification of Fe₂Optiferrin™

Further purification of the now iron saturated Optiferrin™ involved passage over a Sephacryl S-200HR (26/60) gel filtration column in 100 mM NH_4HCO_3 at 1.5 mL/min. The further purified post S-200 product is designated as purified Fe₂Optiferrin™.

2.7. Analysis of Optiferrin™ by LC/MS

Pre- and Post-S-200 samples were analyzed with LC/MS by injecting 25 µg of protein (in 50 mM NH_4HCO_3 , 0.2% formic acid) onto a reverse phase BioSuite™ pC18 2.0×150 mm (Waters, Milford, MA) HPLC column. Mobile phases of A: 0.1% formic acid and B: 0.1% formic acid in acetonitrile were used at a flow rate of 200 µL/min. After a 5 min wash with 2% B, proteins were eluted with a gradient from 10% to 60% B over 35 minutes. MS analysis was performed using a Qstar-XL (ABSciex, Framingham, MA), recording data from 1000 to 3500 m/z. MS1 data was constructed from a 5 minute window that contained signal for all proteins detected and were deconvoluted using BioAnalyst v1.1.5 (ABSciex, Framingham, MA).

2.8. Determination of molar absorption coefficient

Determination of millimolar absorption coefficients (ϵ_{280}) for both the apo and iron-containing purified Optiferrin™ followed our previously described protocol [31,32].

2.9. Steady-state tryptophan fluorescence

Also, as described previously [33], a Quantamaster-6 spectrofluorometer (Photon Technology International, South Brunswick, NJ) equipped with a 75-W xenon arc lamp excitation source, excitation/emission monochromator and a 320 nm cut-on emission filter was used to monitor the steady-state tryptophan fluorescence emission spectra of the purified Fe₂Optiferrin™. Emission scans were collected (300–400 nm) using slit widths of 1 nm (excitation) and 6 nm (emission) as the purified Optiferrin™ sample was excited at 280 nm. Purified Fe₂Optiferrin™ was added to a cuvette (1.8 mL final volume) containing 100 mM HEPES buffer, pH 7.4 at 25 °C. Incubation of an identical amount of purified Fe₂Optiferrin™ in 100 mM MES buffer, pH 5.6 containing 300 mM KCl and 4 mM EDTA for 15 min was used to produce apo-protein. A minimum of three steady-state emission scans (from which the buffer background was subtracted) were collected and averaged.

2.10. Solution-Based Competition Assay to Determine the Relative Binding Affinity of pre purified and purified Fe₂Optiferrin™

The competitive immunoassay to determine the relative binding affinity of hTF for the sTFR has been described in detail previously [34]. Briefly, Removawells coated with rabbit anti-mouse IgG (1 mg/100 mL) were used to capture the sTFR specific mAb (A4A6). Following incubation (40–60 min at 37 °C), sTFR was added to saturate the mAb binding sites. Incubation (again 40–60 min at 37 °C) was followed by addition of biotinylated N-His Fe₂hTF (20 ng/well) in the presence of or absence of unlabeled N-His Fe₂hTF. The maximum amount of biotinylated N-His Fe₂hTF that can be bound to the sTFR (B_{100}) is established by wells with no added unlabeled N-His Fe₂hTF, and the amount of biotinylated N-His Fe₂hTF that is nonspecifically bound (B_0) is determined using wells with no specific mAb added. Competition of biotinylated N-His Fe₂hTF with six different amounts of

unlabeled N-His Fe₂hTF (16–400 ng/well) was used to generate a standard curve. Following incubation, an avidin-HRP conjugate was added to all wells to bind to the biotin covalently attached to the N-His Fe₂hTF. Use of a TMB substrate system allows determination of the amount of biotinylated N-His Fe₂hTF sample bound to the sTFR. All steps are carried out in pH 7.4 buffer (50 mM Tris-HCl, containing 100 mM NaCl and 0.1% bovine serum albumin (BSA)). Between each step, incubations of 40–60 minutes at 37 °C are followed by at least three washes of 200 µl/well. Aliquots of the N-His Fe₂hTF control and pre and post purified Fe₂OptiferrinTM (each at a nominal concentration of 20 µg/mL) were assayed to determine the concentration of each using the standard curve as described [34,35,36].

2.11. OptiferrinTM /sTFR complex formation and purification

The N-His Fe₂ hTF/sTFR and purified Fe₂OptiferrinTM /sTFR complexes were prepared by adding a small molar excess (~20%) of N-His Fe₂hTF (or purified Fe₂OptiferrinTM) to 1.5 mg of sTFR. Following equilibration at room temperature for ~5 min, the N-His Fe₂hTF or purified Fe₂OptiferrinTM /sTFR complex passed over a Sephacryl S300HR gel filtration column (1.5 mL/min) in 100 mM NH₄HCO₃ to remove excess N-His Fe₂hTF or purified Fe₂OptiferrinTM. Fractions (3 mL) containing the complex were pooled and concentrated to 15 mg/mL.

2.12. Urea gel analysis

The iron status of the purified Fe₂OptiferrinTM /sTFR complex was examined by urea gel electrophoresis using Novex 6% TBE-urea mini-gels in 90 mM Tris–borate, pH 8.4, containing 16 mM EDTA as previously described [25,37]. Iron-containing samples were mixed 1:1 with 2X TBE-urea gel sample buffer (final concentration 0.5 µg/µL). To determine the extent of iron removal from purified Fe₂OptiferrinTM, samples were added to iron removal buffer (100 mM MES buffer, pH 5.6, containing 300 mM KCl and 4 mM EDTA, referred to as IRB) and incubated at room temperature for either 1, 2, 3, 5, or 10 min. The iron removal process was halted by addition of 2X TBE-urea gel sample buffer. Samples (3.0 µg) were loaded and the gel electrophoresed for 2.25 h at 125 V. Protein bands were visualized by staining with Coomassie blue.

2.13. Kinetics of iron release from purified Fe₂OptiferrinTM and the purified Fe₂OptiferrinTM/sTFR complex at pH 5.6

Iron release from the purified Fe₂OptiferrinTM and the purified Fe₂OptiferrinTM/sTFR complex was monitored at 25 °C using an Applied Photophysics SX.20MV stopped-flow spectrofluorimeter as previously described [25,37]. One syringe contained the purified Fe₂OptiferrinTM (375 nM, alone or in complex with the sTFR) in 300 mM KCl and the other syringe contained MES buffer (200 mM, pH 5.6), KCl (300 mM) and EDTA (8 mM). Rate constants were determined by fitting the change in fluorescence intensity versus time using Origin software (version 7.5) to standard models as described in detail previously [25,37]. All data were corrected to zero fluorescence intensity before fitting.

3. Results

3.1. Peptide mapping

To help verify its identity and authenticity, both OptiferrinTM and serum derived glycosylated hTF were subjected to tryptic digestion and the resulting peptides were analyzed by capillary LC tandem MS. With respect to OptiferrinTM, 130 unique peptides out of 636 mass spectra were revealed; the acquired mass of fragment ions of these 130 peptides match with the calculated peptide masses derived from the hTF sequence in the database (Uniprot_20201221_p-X2jD). The matching peptides correspond to 92% amino acid sequence coverage for OptiferrinTM (Fig. 1). Comparatively 104 unique peptides out of 504

mass spectra were revealed for serum derived glycosylated hTF, corresponding to 85% amino acid coverage. The glycosylated hTF and Optiferrin™ were shown to have 86 unique peptides in common. The observed masses of these matching peptides were plotted against their positions in the hTF amino acid sequences from N- to C-terminus, revealing that they are spread throughout and share the same observed mass (Fig. S1). These results demonstrate that the Optiferrin™ has the correct sequence.

The mapping analysis identified peptides containing the two N-glycosylation sites (Asn413 and Asn611) but only in Optiferrin™ and not in the serum derived glycosylated hTF from Sigma (Fig. 1). The peptides containing Asn413 and Asn611 have a mass that matches the calculated mass of peptides lacking glycosylation indicating that these sites are not N-glycosylated in Optiferrin™ [28].

3.2. Circular Dichroism Analysis of Optiferrin™

To investigate the secondary structure of Optiferrin™, apo Optiferrin™ was analyzed by circular dichroism spectrometry and compared with native serum derived glycosylated apohTF (Fig. S2). The CD spectra of Optiferrin™ and glycosylated hTF showed similar profiles, indicating that both TF molecules have comparable secondary structures.

3.3. UV-vis spectra of Optiferrin™

The intrinsic spectral properties of hTF originate from the interaction between the protein and the bound Fe^{3+} . Specifically, the two liganding Tyr residues in each lobe produce a ligand-to-metal charge transfer (LMCT) band centered at ~470 nm that gives hTF its characteristic salmon pink color. The iron binding properties of hTF can be assessed by monitoring this LMCT band. Moreover, a ratio of the protein absorbance at 280 nm and the absorbance of the LMCT complex (A_{280}/A_{max}) assesses the iron binding status of an hTF. As shown in Table 1, the initial spectral properties of Optiferrin™ taken up in 100 mM NH_4HCO_3 (Optiferrin™*, $\lambda_{\text{max}} = 459$ nm and A_{280}/A_{max} of 42.3) are similar to those of a N-His monoferric hTF. The addition of ferric iron in the form of Fe-NTA was required to fully iron saturate the Optiferrin™ sample (Table 1, Pre purified $\text{Fe}_2\text{Optiferrin}^{\text{TM}}$). The spectral properties of prepurified $\text{Fe}_2\text{Optiferrin}^{\text{TM}}$ ($\lambda_{\text{max}} = 465$ nm and A_{280}/A_{max} of 24.6) are similar to N-His Fe_2hTF , indicative of complete iron saturation.

3.4. Further purification of Optiferrin™

As indicated by SDS-PAGE analysis, some impurities were observed in the Optiferrin™ sample (Fig. 2). Therefore, further purification was carried out by passage of $\text{Fe}_2\text{Optiferrin}^{\text{TM}}$ over an S-200HR gel filtration column. The elution profile reveals the presence of a number of both higher and lower M_r contaminants present in the initial lyophilized Optiferrin™ sample that was taken up in 100 mM NH_4CO_3 (Fig S3). Fractions containing the purified $\text{Fe}_2\text{Optiferrin}^{\text{TM}}$ (post-S-200HR sample) were pooled, concentrated to 15 mg/mL, and the spectral properties were again analyzed (Table 1, Purified $\text{Fe}_2\text{Optiferrin}^{\text{TM}}$). The spectral properties of purified $\text{Fe}_2\text{Optiferrin}^{\text{TM}}$ ($\lambda_{\text{max}} = 464$ nm and A_{280}/A_{max} of 20.4) are closer to that of N-His Fe_2hTF indicating that purified Optiferrin™ is iron-saturated in a manner that is very similar to N-His Fe_2hTF derived from BHK cells.

3.5. MS analysis of Optiferrin™

In our experience, MS analysis of BHK derived N-His Fe_2hTF can readily be performed offline under denaturing conditions by diluting the sample into a final concentration of 30% acetonitrile, 0.1% formic acid. Surprisingly, a similar treatment of either the prepurified $\text{Fe}_2\text{Optiferrin}^{\text{TM}}$ or purified $\text{Fe}_2\text{Optiferrin}^{\text{TM}}$ resulted in a large amount of precipitation with very little protein remaining in the supernatant (see discussion).

The results of LC/MS analysis of both the pre- S-200HR and post S-200HR samples are summarized in Fig. 3. The predominant mass detected in both samples (75,147- M_r) is in good agreement with the calculated average mass for OptiferrinTM (75,143- M_r). A series of higher molecular weight masses were also detected in both the pre- and post S-200HR samples and several proteins with lower molecular weight masses of around 37 kDa were present but only in the pre purified S-200HR Fe₂OptiferrinTM sample. All further experiments were conducted with the purified Fe₂OptiferrinTM sample.

3.6. Molar absorption coefficient for purified Fe₂OptiferrinTM

Although a remarkably accurate estimate of the ϵ_{280} of a protein can be calculated using the number of Trp and Tyr residues, as well as the number of disulfide bonds [32,38], this calculation does not provide an accurate estimate of the ϵ_{280} for iron-containing hTF. Due to the disruption of the π to π^* transition energy of the two tyrosine residues in each lobe involved in the coordination of the Fe³⁺, a nonlinear increase in the A_{280} is observed when hTF binds iron [33,39]. The experimentally determined absorption coefficient value for the apo purified OptiferrinTM is within the standard error of the experimentally determined value of N-His apo hTF. Additionally, the experimentally determined ϵ_{280} for purified Fe₂OptiferrinTM is nearly identical to the experimentally determined value for N-His Fe₂hTF from BHK cells, both resulting in a ~25% increase in ϵ_{280} (Table 2).

3.7. Steady-state tryptophan fluorescence of purified OptiferrinTM

A large increase (~270%) in the intrinsic Trp fluorescence is observed following release of Fe³⁺ from hTF. The fluorescent spectra of N-His hTF and purified OptiferrinTM in the iron-bound or apo-form excited at 280 nm were determined. The increase in the fluorescent signal of purified OptiferrinTM upon transitioning from the Fe³⁺ bound to the apo-form, does not differ significantly from that observed for the N-His Fe₂hTF (data not shown).

3.8. Relative Binding Affinity of purified OptiferrinTM for the sTFR

To determine whether N-His Fe₂hTF and purified Fe₂OptiferrinTM bind to the TFR in a similar manner, the relative binding affinities of the Fe₂OptiferrinTM and purified Fe₂OptiferrinTM (post S-200) samples for the sTFR were measured using a competitive immunoassay in which a constant amount of sTFR is bound to a specific antibody (Fig. 4). Importantly, no significant differences are observed between the binding of N-His Fe₂hTF and purified Fe₂OptiferrinTM to the sTFR, *i.e.*, purified Fe₂OptiferrinTM was equal to the Fe₂hTF control in its ability to compete for binding to the sTFR. Based on these results, it is not surprising that purified Fe₂OptiferrinTM, readily formed a 2:2 (hTF:TFR) complex with the sTFR (Fig. S4).

3.9. Iron Release from purified OptiferrinTM

Kinetic rate constants for iron release at pH 5.6 obtained from the analysis of N-His Fe₂hTF and purified Fe₂OptiferrinTM in the absence of the sTFR are presented in Table 3A. Under our standard conditions (100 mM MES, pH 5.6 containing 300 mM KCl and 4 mM EDTA), iron release from N-His Fe₂hTF produces two kinetic rate constants: rapid iron release from the N-lobe (k_{1N}), followed by slow release of iron from the C-lobe (k_{2C}) [25]. In the absence of the sTFR, minimal differences are observed between N-His Fe₂hTF and purified Fe₂OptiferrinTM.

Rate constants for iron release from N-His Fe₂hTF and purified Fe₂OptiferrinTM in the presence of the sTFR at pH 5.6 are reported in Table 3B. Under our conditions, the pathway of iron release utilized the majority of the time is switched in the presence of the sTFR such that iron release occurs first from the C-lobe (k_{1C}) followed by iron release from the N-lobe

(k_{2N}). While the rate of iron release from the C-lobe was only slightly faster (~25% faster) from the purified Fe₂OptiferrinTM/sTFR complex compared to the N-His Fe₂hTF/sTFR complex, the rate of iron release from the N-lobe was significantly increased (by ~80%) (Table 3B, Fig. 5).

4. Discussion

Recombinant hTF is available from many commercial sources (Table S1). While these commercial recombinant hTFs are often tested for purity (as assessed by SDS-PAGE), functional validation by more thorough and quantitative methods is far less frequent. Prior to use the comprehensive array of spectroscopic and analytical techniques described in the current work should be used to evaluate the integrity and functionality of any recombinant hTF. A more sensitive and precise technique than SDS-PAGE is required. Specifically for nonglycosylated hTF, ESI-mass spectrometry provides an accurate mass and good indication whether contaminants are present or not. Ideally, in order to determine the overall functionality of a recombinant hTF one should analyze the intrinsic spectral properties using UV-Vis (Table 1) and fluorescence (steady-state Trp) spectroscopy, confirm high affinity binding of the hTF to the TFR (Figs. 4 and S4), and evaluate the ability of the recombinant hTF to relinquish iron under endosomal conditions (Fig. 5 and Table 3B).

In the present study, the commercially available recombinant hTF produced in the rice species *O. sativa* was examined using these methods and compared to our non-glycosylated recombinant N-His hTF produced in BHK cells. Admittedly, by design, the two constructs (purified OptiferrinTM and N-His hTF) are not identical. While both hTFs appear to be non-glycosylated, the reason for the lack of glycosylation in purified OptiferrinTM is not known since the construct retains the two asparagine linkage residues [28]. In contrast to recombinant human lactoferrin produced in rice which clearly contains at least three N-linked glycans [40], the *majority* of the OptiferrinTM does not appear to be glycosylated based on a gel mobility study [28], treatment with PNGase [28], isoelectric focusing [28], and peptide mapping analysis (Fig 1). Nevertheless, the resolution of these analyses cannot totally rule out the possibility that a small fraction of OptiferrinTM is glycosylated but falls below the level of detection. In fact, the more sensitive LC/MS analysis reveals the presence of higher molecular weight species (Fig 3). We speculate that these are derived from trace amounts of OptiferrinTM with N-linked glycosylation, O-linked glycosylation or other post-translational modification. If N-linked glycosylation is present the most likely additions are hexose (Hex) and N-acetylhexosamine (HexNAc) residues. The mass of 76,159- M_r is consistent with addition of Hex5HexNAc to 75,147- M_r and the mass of 77,169 is consistent with two such additions (Fig 3). Further study is needed to resolve this completely.

In contrast to OptiferrinTM N-His Fe₂hTF is non-glycosylated due to the intentional elimination of glycosylation by mutagenesis (N413D and N611D). Moreover, as indicated by the name, the N-His Fe₂hTF construct produced in BHK cells contains an N-terminal hexa-histidine, while purified OptiferrinTM does not. The presence of the N-terminal His-tag aids in the purification of a more homogeneous hTF protein preparation and does not interfere with either binding to the TFR (as measured by HeLa S₃ cell binding studies) or with iron release at pH 5.6 [29]. Another difference, of unknown significance, is that OptiferrinTM is lyophilized to facilitate its distribution, whereas the N-His Fe₂hTF construct produced in BHK cells is not. The difference in behavior under denaturing conditions (30% acetonitrile, 0.1% formic acid) is puzzling. In contrast to our recombinant BHK derived N-His hTF, the OptiferrinTM even after further purification almost completely precipitates out of solution indicating a significant difference in solubility. Although we conjectured that lyophilization might contribute to this disparity, we found no difference in the kinetic rate constants for iron release from either lobe after subjecting our BHK derived N-His Fe₂hTF

to lyophilization (data not shown). Alternatively, it is possible that the impurities in the Optiferrin™ sample which are not completely eliminated by gel filtration might cause precipitation.

As originally reported, previous characterization substantiated the N-terminal amino acid sequence, documented the absence of glycosylation, provided an estimated molecular weight by MALDI analysis and assessed the iron binding capabilities of Optiferrin™ [28]. Additionally, Optiferrin™ was shown to promote proliferation and antibody production of hybridoma cells [28]. In the present study, the more in-depth biochemical and structural characterization analysis including peptide mapping, and circular dichroism shows that Optiferrin™ is both biochemically and structurally similar to hTF. Although estimated to be of greater than 95% purity [28], our initial SDS-PAGE analysis indicated a number of both high and low M_r contaminants in the initial Optiferrin™ sample (Fig 2). The presence of protein contaminants was confirmed by LC/MS (Fig. 3).

Initial spectral characterization suggested that the lyophilized Optiferrin™ taken up in 100 mM NH_4CO_3 is ~50% iron-saturated. The spectral data (Table 1, Optiferrin™) and urea gel analysis of Optiferrin™ (data not shown) indicate iron is bound mainly in the C-lobe, with very little iron in the N-lobe. These results are in agreement with previously reported results [28]. However, following the addition of Fe-NTA to fully iron-saturate the Optiferrin™ sample, it is clear that Optiferrin™ is capable of binding Fe^{3+} tightly in both lobes as evidenced by the λ_{max} (Table 1) of the LMCT and the increase in the millimolar absorption coefficient (ϵ_{280}) when iron is bound (Table 2).

Essential to the delivery of iron to cells is the interaction between TF and the TFR. As shown both by our competitive binding assay and the formation of the purified $\text{Fe}_2\text{Optiferrin}^{\text{TM}}/\text{sTFR}$ complex, purified $\text{Fe}_2\text{Optiferrin}^{\text{TM}}$ binds to the sTFR with an affinity that is comparable to the binding of N-His Fe_2hTF to the sTFR (Figs. 4 and S4).

The ability of $\text{Fe}_2\text{Optiferrin}^{\text{TM}}$ to deliver iron to cells is critically dependent upon the kinetics of iron release at the putative endosomal pH (~5.6). As expected, the kinetic rate constants for iron release from $\text{Fe}_2\text{Optiferrin}^{\text{TM}}$ are not significantly different than the kinetic rate constants for iron release observed for N-His Fe_2hTF (Table 3A). Interestingly, the rate of iron release from both lobes is increased in the $\text{Fe}_2\text{Optiferrin}^{\text{TM}}/\text{sTFR}$ complex in comparison to the $\text{Fe}_2\text{hTF}/\text{sTFR}$ complex (Table 3B), however the source of these kinetic differences between the $\text{Fe}_2\text{Optiferrin}^{\text{TM}}/\text{sTFR}$ and N-His $\text{Fe}_2\text{hTF}/\text{sTFR}$ complexes remains unclear. The competitive binding experiment indicates that the source of the difference in the kinetic parameters cannot be attributed a weaker interaction. The almost 2-fold increase in the rate of iron release from the N-lobe from the $\text{Fe}_2\text{Optiferrin}^{\text{TM}}/\text{sTFR}$ appears to be consistent with the relative instability of this lobe which lacked iron in the original lyophilized sample. It may also be reflected in the insolubility issue mentioned above.

One caveat in regard to Optiferrin™ is the significant length of time required to produce transgenic rice plants stably transfected with the gene of choice. A standard protocol for microparticle bombardment of embryogenic rice tissues normally requires between 2–6 months before transgenic rice plantlets are produced [41]. Typically, successive generations of the transgenic rice plants are then cultivated for an additional 5 months before the recombinant protein can be isolated and analyzed [28,41]. Thus, to produce a mutant hTF in the *O. sativa* system requires between 12–24 months, making the production of various protein mutants using the *O. sativa* system rather impractical. In contrast, this process only requires 4–5 weeks using the BHK cell system [22,42]. The advantage of the rice expression system is its capacity for large scale production of recombinant proteins. Once genetically stable transgenic rice lines are established, Optiferrin™ can be produced at very low-cost

and unprecedented scale. Thus, the *O. sativa* expression system is an enabling technology for proteins that require large scale and low cost production.

In conclusion, overall Optiferrin™ is shown to be biochemically and structurally similar to hTF. Both lobes of Optiferrin™ appear to bind Fe³⁺ tightly yet reversibly, an essential requirement of any functional hTF. Moreover, while the kinetics of iron release from the purified Fe₂Optiferrin™/sTFR and N-His Fe₂hTF/sTFR complexes differ somewhat, no significant differences in function were observed between Fe₂Optiferrin™ and BHK-cell derived N-His Fe₂hTF. The functional similarities between Fe₂Optiferrin™ with BHK-cell derived N-His Fe₂hTF make Optiferrin™ a low-cost, alternative to other plasma-derived and recombinant forms of hTF for promoting cell growth. Effort is being made at Ventria Bioscience to process recombinant hTF from rice with higher purity in order to produce a biopharmaceutical grade non-immunogenic product for more analytical purposes or for use in pharmaceutical applications aimed at treating human diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Optiferrin™ is recombinant human transferrin capable of reversibly binding iron
- It is a low cost, pathogen free alternative suitable for tissue culture applications
- Additional purification of Optiferrin™ is required for more rigorous applications
- A template to guide evaluation of any recombinant transferrin is provided

Opti	VPDKTVRWCAVSEHEATKQCSFRDHMKSVIPSDGPSVACVKKASYLDCIR	50
hTF	VPDKTVRWCAVSEHEATKQCSFRDHMKSVIPSDGPSVACVKKASYLDCIR	
Opti	AIAANEADAVTLDAGLVYDAYLAPNNLKFVVAEFYGSKEPDQTFYYAVAV	100
hTF	AIAANEADAVTLDAGLVYDAYLAPNNLKFVVAEFYGSKEPDQTFYYAVAV	
Opti	VKKDSGFQMNQLRGKKSCHTGLGRSAGWNIPIGLLYCDLPEPRKPLEKAV	150
hTF	VKKDSGFQMNQLRGKKSCHTGLGRSAGWNIPIGLLYCDLPEPRKPLEKAV	
Opti	ANFFSGSCAPCADGTDFFPQLCQLCPGCGCSTLNQYFGYSGAFKCLKDGAG	200
hTF	ANFFSGSCAPCADGTDFFPQLCQLCPGCGCSTLNQYFGYSGAFKCLKDGAG	
Opti	DVAFVKHSTIFENLANKADRDQYELLCLDNTKPFVDEYKDLCHLAQVPSHT	250
hTF	DVAFVKHSTIFENLANKADRDQYELLCLDNTKPFVDEYKDLCHLAQVPSHT	
Opti	VVARSMGGKEDLIWELLNQAQEHFGKDKSKEPQLFSSPHGKDLLFKDSAH	300
hTF	VVARSMGGKEDLIWELLNQAQEHFGKDKSKEPQLFSSPHGKDLLFKDSAH	
Opti	GFLKVPFRMDAKMYLGYEYVTAIRNLREGTCPEAPTDECKPVKWCALSHH	350
hTF	GFLKVPFRMDAKMYLGYEYVTAIRNLREGTCPEAPTDECKPVKWCALSHH	
Opti	ERLKCDWSVNSVGKIECVSAETTEDCIAKIMNGEADAMSLDGGFVYIAG	400
hTF	ERLKCDWSVNSVGKIECVSAETTEDCIAKIMNGEADAMSLDGGFVYIAG	
Opti	KCGLVPVLAENYNKSDNCEOTPEAGYFAVAVVKKSASDLTDNLKGKKSC	450
hTF	KCGLVPVLAENYNKSDNCEOTPEAGYFAVAVVKKSASDLTDNLKGKKSC	
Opti	HTAVGRTAGWNIPMGLLYNKINHCRFDEFFSEGCAPGSKKDSLLCKLCMG	500
hTF	HTAVGRTAGWNIPMGLLYNKINHCRFDEFFSEGCAPGSKKDSLLCKLCMG	
Opti	SGLNLCEPNNKEGYGYTGAFRCLEKGDVAFVKHQTVFPQNTGGKNPDW	550
hTF	SGLNLCEPNNKEGYGYTGAFRCLEKGDVAFVKHQTVFPQNTGGKNPDW	
Opti	AKNLNEKDYELLCLDGTTRKPVVEEYANCHLARAPNHAVVTRKDKKACVHKI	600
hTF	AKNLNEKDYELLCLDGTTRKPVVEEYANCHLARAPNHAVVTRKDKKACVHKI	
Opti	LRQQHLFGSNVIDCSGNFCLFRSETKDLLFRDDTVCLAKLHDRNTYERY	650
hTF	LRQQHLFGSNVIDCSGNFCLFRSETKDLLFRDDTVCLAKLHDRNTYERY	
Opti	LGEEYVKAAGNLRKCSSTSSLEACTFRP	679
hTF	LGEEYVKAAGNLRKCSSTSSLEACTFRP	

Fig. 1. Mass spectrometric peptide mapping of OptiferrinTM and serum derived glycosylated hTF. Peptide sequences highlighted in bold indicate amino acid sequences of hTF under Swiss-Prot Accession No. P02787. The regions marked in yellow represent amino acids identified by peptide mapping of OptiferrinTM; the regions marked in blue represent amino acids identified by peptide mapping of serum derived glycosylated hTF Sigma hTF.

Fig. 2. SDS-PAGE analysis of N-His Fe₂hTF and OptiferrinTM. Samples were either reduced or non-reduced as follows: (1) 2 μ g N-His Fe₂hTF, (2) 5 μ g N-His Fe₂hTF, (3) 2 μ g OptiferrinTM, (4) 5 μ g OptiferrinTM.

Fig. 3. LC/MS analysis of pre-(red lines) and post-(black lines) S-200 OptiferrinTM samples. MS spectra were generated from the same 5 min window of each chromatogram and encompassed all proteins detected. Plots of the deconvoluted masses are shown in the insets.

Fig. 4.

Evaluation of the abilities of pre and post S-200 Fe₂Optiferrin[™] samples to bind to the sTFR. All samples were prepared at a concentration of 20 µg/mL and competed with biotinylated N-His Fe₂hTF for binding to immobilized sTFR. All values are expressed as % of control (unlabeled N-His Fe₂hTF) binding and are averages of at least three different experiments ± standard deviation.

Fig. 5.

Urea gel timecourse of the N-His Fe₂hTF/sTFR and Fe₂OptiferrinTM/sTFR complexes. Samples were electrophoresed before and after (+) incubation with iron removal buffer, designated as IRB (100 mM MES, pH 5.6, containing 300 mM KCl and 4 mM EDTA) for increasing amounts of time (1, 2, 3, 5 and 10 min, respectively). Note the migration pattern of OptiferrinTM differs slightly from Fe₂hTF due to the presence of glycosylation eliminating mutations (N413D and N611D) and possibly the hexa histidine tag in the N-His Fe₂hTF construct.

Table 1Spectral Characteristics of N-his tagged hTF and OptiferrinTM

Construct	λ_{max} (nm)	A_{280}/A_{max}
N-His Fe ₂ hTF ^a	466	21.3
N-His Fe _N hTF ^a	470	41.4
N-His Fe _C hTF ^a	461	42.2
Optiferrin ^{TMb}	459	42.3
Pre purified Fe ₂ Optiferrin ^{TMc}	465	24.6
Purified Fe ₂ Optiferrin ^{TMd}	464	20.4

^aFrom reference [22].^bInitial scan following addition of 100 mM NH₄HCO₃ to lyophilized OptiferrinTM.^cScan following the addition of Fe³⁺, pre S-200HR purification.^dScan Post S-200HR purification.

Table 2Millimolar Absorption Coefficients of Various hTFs and Optiferrin.TM

Construct	calc ^a ϵ_{280} apo	exptl ϵ_{280} apo	exptl ϵ_{280} Fe ³⁺	% increase in ϵ_{280} due to Fe ³⁺ ^b
N-His Fe ₂ hTF ^c	85.1	84.0 ± 0.2	103.9 ± 0.2	23.7
N-HisFe _N hTF ^c	82.1	81.4 ± 0.3	92.5 ± 0.3	13.6
N-His Fe _C hTF ^c	82.1	81.5 ± 0.2	92.1 ± 0.2	13.0
PurifiedFe ₂ Optiferrin TM	85.1	83.2 ± 0.8	103.8 ± 0.2	24.8

^a Apo values calculated as described [32].^b Percent increase calculated as 100 X [$\epsilon_{\text{iron}} - \epsilon_{\text{apo}}$]/ ϵ_{apo} .^c From reference [31].

Table 3A

Rate Constant for Iron Release in the Absence of the sTFR.

Construct	k_{1N} (min ⁻¹)	k_{2C} (min ⁻¹)
N-His Fe ₂ hTF ^a	17.7 ± 2.2	0.65 ± 0.06
Purified Fe ₂ Optiferrin TM	15.3 ± 0.2	0.69 ± 0.01

^aFrom reference [25]. Averages and 95% confidence intervals for kinetic runs performed on N=2–5 different days. Each day 3 kinetic traces were averaged before fitting.

Table 3B

Rate Constant for Iron Release in the Presence of the sTFR.

Construct	k_{1C} (min ⁻¹)	k_{2N} (min ⁻¹)
N-His Fe ₂ hTF/sTFR ^a	5.5 ± 0.9	1.4 ± 0.2
Purified Fe ₂ Optiferrin TM /sTFR	6.9 ± 0.2	2.5 ± 0.1

^aFrom reference [25]. Averages and 95% confidence intervals for kinetic runs performed on N=2–5 different days. Each day 3 kinetic traces were averaged before fitting.