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Review

Plant seed-derived human transferrin: expression, characterisation and applications

D Zhang*

Abstract

Introduction

Human serum transferrin plays a critical physiological role in cellular iron delivery via the transferrin receptor-mediated endocytosis pathway in nearly all eukaryotic organisms. It is widely used in mammalian cell cultures for the production of biotherapeutic proteins and vaccines and is also being explored for use as a therapy and targeted drug delivery system to treat a number of diseases. With the increasing concerns over the risk of transmission of infectious pathogenic agents of human plasma-derived transferrin, recombinant production of human serum transferrin has been pursued in various heterologous expression systems. However, high costs and limited yields of recombinant human serum transferrin remain the major challenges to many expression systems. Recently, rice seed-based expression system has been shown to produce large amounts of inexpensive and animal-free recombinant human serum transferrin. Here, we review the rice-derived recombinant human serum transferrin: its cost-effective production, molecular and functional characterisation, as well as its many potential therapeutic and clinical applications.

Conclusion

Rice seed-based expression system is shown to be able to produce large scale of recombinant human serum transferrin with high yield and at low cost. The rice-derived rhTF is shown to be biochemically, structurally and functionally similar to native hTF, and it is a low-cost alternative to other plasma-derived and recombinant forms of hTF suitable for bioprocessing and biopharmaceutical applications.

Introduction

Transferrin (TF) plays an important role in tightly controlling the cellular iron uptake, storage and transport to maintain cellular iron homeostasis in all eukaryotic organisms. TF is a single-chain glycoprotein of 679 amino acid residues and can be divided into two homologous halves, each comprising about 340 amino acid residues. The two halves fold into two distinct globular lobes, designated the N-lobe and C-lobe[1]. Each lobe comprises two dissimilar domains, which interact to form a deep hydrophilic iron-binding site. When TF is free of iron (apo-TF), both its N- and C-lobes maintain an open conformation for easy access of the ferric iron. At pH 7.4 under physiological conditions, the apo-TF binds one (monoferric TF) or two Fe^{3+} ions (diferric TF or holo-TF). The resultant iron bearing TF binds to TF receptor (TFR) on cell surface, and holo-TF has 30-fold and 500-fold higher affinity for TFR than the monoferric TF and apo-TF, respectively[2]. Then, the TF–TFR complex is endocytosed into the early endosome, where the acidic environment (pH 5.5) result in the release of iron from TF by protonation but apo-TF still remains bound to the TFR with high affinity. Finally, the apo-TF–TFR complex is recycled to the cell surface, and at pH 7.4 of the blood, the apo-TF is released from the TF-TFR complex for re-use[3,4].

The TF/TFR-mediated cellular iron uptake and transport is critical to avoid severe cell damages associated with both the iron deficiency and overload in the body. Iron deficiency can arrest cell proliferation and even cause cell death because iron is an essential element used by all eukaryotic organisms and most micro-organisms as a cofactor of numerous proteins or enzymes for respiration, DNA synthesis and many other critical metabolic processes[1]. On the other hand, excessive iron can be toxic to cells by reacting with oxygen via the Fenton reaction to produce highly reactive hydroxyl radicals that cause oxidative damage to cells[5]. The dual challenges of iron deficiency and overload can be addressed by transferrin-binding iron ions in the ferric form (Fe^{3+}) tightly yet reversibly.

Apart from the importance of maintaining iron homeostasis in cells, TF is also shown to have a wide range of therapeutic applications, which will be described later. Because the native TF derived from plasma is associated with high risk of transmission of infectious pathogenic agents, recombinant expression of human serum transferrin (hTF) has long been pursued in a variety of heterologous expression systems[6,7]. In this review, we focus on rice-derived recombinant hTF's cost-effective production, its biochemical, structural and functional properties as well as its potential clinical applications.

Discussion

The author has referenced some of his own studies in this review. The protocols of these studies have been approved by the relevant ethics committees related to the institution in which they were performed.

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Review

Cost-effective production of human transferrin in rice seeds

TF is one of the most abundant proteins in human plasma with a concentration of approximate 2 to 3 g/L. This natural abundance makes the isolation of TF relatively simple and economical. However, the therapeutic use of plasma-derived TF is limited by the risk of exposure to blood-borne infectious disease pathogens and the inability to introduce desired mutations[7]. With the rapid development of molecular biology, recombinant hTF(rhTF) has become the preferred choice for many applications.

A number of heterologous expression systems have been utilised to express rhTF. However, large quantity and cost-effective production of rhTF still remains a challenge. The commonly used *Escherichia coli* expression system proved to be impractical for producing rhTF, because the expressed rhTF remained in insoluble inclusion bodies and the renaturation process met with limited success to yield bioactive rhTF[7]. In contrast, the bioactive hTF has been expressed in the single-celled, eukaryotic yeast cells, including the baker's yeast *Saccharomyces cerevisiae*[8] and methylotrophic yeast *Pichia pastoris*[9]. As would be expected, the mammalian cell system has been successfully used to produce both fully functional rhTF and various mutant rhTF isoforms[10]. However, the mammalian cell system cannot generate sufficient amount of rhTF at a low price for many biopharmaceutical applications[7].

Plant expression systems have the potential to produce low cost and large amounts of recombinant proteins while reducing the risk of potential contamination by animal pathogens. Bioactive rhTF has been produced in rice (*Oryza sativa*) seeds at a very high expression level (estimated 40% total soluble protein or 1% seed dry weight)[6]. The tobacco (*Nicotiana tabacum*) plants also have been demonstrated to express bioactive rhTF, but the expression level is significantly lower (estimated 0.25% total soluble protein)[11]. The high expression level of rhTF, low water content (about 10%) and low amount of host cell proteins in rice grain result in a high enrichment of rhTF protein in the starting material for purification, making the purification of rhTF from rice grains simple and cost-effective. Indeed, the rhTF was purified simply by a single-step anion exchange diethylaminoethyl chromatographic process with greater than 95% purity[6] (Figure 1). Other major factors that contribute to the low cost production of rhTF in rice include: (1) Rice grains expressing rhTF can be produced by the well-established agricultural practice in large quantities, and the cost of producing the starting material is clearly the lowest among any recombinant production system. (2) Recombinant hTF expressed in rice grains can be stored under ambient temperature and dry conditions for more than 2 years without losing its stability. (3) This storage stability of rhTF in grains enables the production of starting material to be completely separated from the protein purification process, allowing for greater flexibility to scale up or down the manufacturing capacity in line with the needs. The rice-derived rhTF proves to be the most competitive commercial source of rhTF available from InVitria under the trade name Optiferrin™[7].

Biochemical, structural and functional characterisation of rice-derived rhTF

To evaluate the integrity and functionality of recombinant hTF and its potency destined for pharmaceutical use, a detailed comprehensive biochemical, structural and functional characterisation is necessary. The rice-derived rhTF has been thoroughly characterised with a range of well-documented analytical techniques for characterising recombinant proteins[6,7,12] (Table 1).

Biochemical and structural analysis demonstrates that the rice-derived rhTF is similar to native hTF. However, the majority of the Optiferrin™ does not appear to be glycosylated based on a gel mobility study, PNGase treatment, MALDI and peptide-mapping analysis. The functional properties of rhTF have been characterised by assessing its reversible iron-binding properties, its ability to bind to TFR and subsequently enter cell via TFR-mediated endocytosis, and its ability to stimulate the *in vitro* growth of mammalian cells (Table 1). The iron-binding capacity of rhTF was simply demonstrated by the characteristic salmon-pink colour of iron-bearing rhTF and the subsequent colour loss after the bound iron was removed from rhTF in an iron-sequestering buffer at pH <5. Moreover, different rhTF iron forms (holo-, monoferric- and apo-rhTF) could be distinguished by the urea-polyacrylamide gel electrophoresis. The distinctive colour of iron-loaded

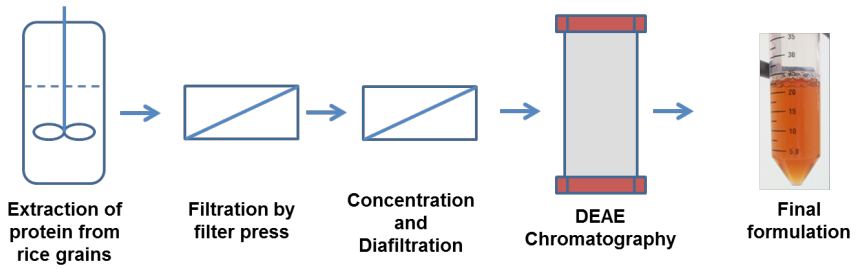

Figure 1: Diagram of the purification process of recombinant human transferrin from rice grains.

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Table 1 Comparison of rice-derived rhTF and native hTF or mammalian cell-derived rhTF.

Biochemical and functional Property	Rice-derived recombinant hTF (Optiferrin)	Native hTF or mammalian cell expressed rhTF
N-terminal sequence	VPDKTVRWCAV	VPDKTVRWCAV (native hTF)
Molecular mass	75.15 kDa	80 kDa (native hTF)
N-glycosylation	None	Yes (native hTF)
Isoelectric focusing point	p*I* 5.3	p*I* 6.3 (native hTF)
Conformation structure by RP-HPLC	Same as native hTF	Same as Optiferrin
Mass spectrometric peptide mapping	Same as native hTF	As expected (native hTF)
Circular dichroism	Similar to native hTF	Similar to Optiferrin
UV–vis spectra	Similar to mammalian cell expressed rhTF	Similar to Optiferrin
Molar absorption coefficient	Similar to mammalian cell expressed rhTF	Similar to Optiferrin
Steady-state tryptophan fluorescence	Similar to mammalian cell expressed rhTF	Similar to Optiferrin
Relative binding affinity for the sTFR	Similar to native hTF	Similar to Optiferrin
TFR-mediated endocytosis	Similar to native hTF	Similar to Optiferrin
Mammalian cell growth and antibody production	Similar to native hTF	Similar to Optiferrin

* hTF, human transferrin; rhTF, recombinant hTF; sTFR, soluble transferrin receptor; UV–vis, ultraviolet–visible

TF is attributed to the interaction of TF and the bound ferric iron, which results in a ligand-to-metal charge transfer band centred at ~470 nm. The iron-binding characteristics of rhTF as assessed by monitoring the intrinsic spectral properties using UV–vis and steady-state tryptophan fluorescence spectroscopy were shown to be similar to that of native hTF[7]. All these assays clearly show that rice-derived rhTF is capable of binding Fe^{3+} tightly yet reversibly in both lobes, which is comparable to recombinant non-glycosylated rhTF expressed by baby hamster kidney cells and/or serum derived glycosylated hTF[6,7]. Cell-based assays with HeLa human cervic carcinoma cells (CCL-2) and Caco-2 human colon carcinoma cells (HTB-37) showed that rice-derived rhTF was able to bind to TFR and subsequently enter cells via TFR-mediated endocytosis in a similar way to native hTF. The mammalian cell culture assays also showed that rhTF was similar to hTF in the delivery of iron to cells for supporting their proliferation, differentiation and physiological function of antibody production. Rice-derived rhTF was also shown to have a sufficiently long serum half-life compared with native hTF in an *in vivo* study[12].

In conclusion, overall rice-derived rhTF is shown to be biochemically, structurally and functionally similar to native hTF. Therefore, it is a low-cost alternative to other plasma-derived and recombinant forms of hTF suitable for bioprocessing and biopharmaceutical applications.

Applications of recombinant human transferrin

Recombinant transferrin has many potential applications. Recombinant hTF has been widely used as a mammalian cell culture reagent. It has been tested to treat a number of diseases such as atransferrinemia, thalassaemia, ischaemia–reperfusion (I/R) injury, bacterial infection and diabetes. In particular, it is also being actively exploited as a drug delivery vehicle.

Recombinant hTF support mammalian cell culture for production of therapeutic proteins and vaccines

With the fast advancement and development of pharmaceutical biotechnology, a vast number of protein therapeutics have been produced through mammalian cell culture[13]. Traditionally, the media used for production of protein therapeutics were supplemented with plasma serum to maximise the cell growth. However, the serum-supplemented media has many disadvantages, including difficulty in downstream purification, batch-to-batch variations and vulnerability to contamination with blood-borne pathogens. These disadvantages, especially the heightened safety concerns, have led biopharmaceutical industry to shift from serum-supplemented media to serum-free and animal product-free media.

In the development of serum-free media, TF as well as insulin and selenium were identified as three most important growth factors for many cell types[14]. TF is also a requisite component for nearly all serum-free cell culture media to ensure iron delivery to propagating cells for sustained growth in mammalian culture for the production of therapeutic proteins and vaccines[13,14]. We demonstrate that rice-derived rhTF possesses the same functionality as native hTF and

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other commercial rhTF to support mammalian cell growth and production of antibodies[6,12].

Atransferrinemia and β-thalassaemia

Atransferrinemia is an extremely rare genetic disease caused by TF gene mutations, resulting in the absence of TF in the body. It is characterised by anaemia, iron overload and increased incidence of infection. Infusion of apo-TF is shown to be able to reduce the damaging effects associated with this disease[15].

β-Thalassaemia is another genetic disease caused by β-globin gene mutations, resulting in decreased or no β-globin synthesis. It is associated with anaemia, ineffective erythropoiesis and iron overload. Transfusion has been the standard therapy for treating β-thalassaemia. However, chronic or frequent transfusions can lead to iron overload and require chelation to prevent iron overload. Recently, transferrin has been shown to be a novel and promising therapy to treat thalassaemia by using chronic treatment with apo-TF injections in a mouse model of β-thalassaemia[16].

Ischaemia–reperfusion (I/R) injury

Ischaemia is defined as inadequate blood supply to one or multiple major organs of the body. Reperfusion helps restore blood supply to an ischaemic tissue; however, it can elicit a cascade of devastating effects that paradoxically injure tissue, sometimes turning fatal. Oxidative stress is considered as one of the major causes of I/R injury. And thus, the excess free redox-active iron in the body plays a pathogenic role in I/R injury. Apo-TF administration was shown to be protective in I/R injury[17].

Bacterial infections in allogeneic stem cell transplantation

Haematopoietic stem cell transplantation (HSCT) is an effective curative therapy for a variety of disorders of the haematopoietic and immune systems. However, bacterial infection is a major complication of allogenic HSCT and causes significant morbidity and mortality. The increased susceptibility to infection in haematological patients receiving HSCT is because the HSCT-associated iron overload (increased iron availability) in the circulation promotes bacterial growth. Clinical studies showed that apo-TF administered to patients receiving HSCT reduced the free-iron level and inhibited the growth of the major causative pathogen *Staphylococcus epidermidis*[18].

Diabetes

Excess iron has been implicated in the pathogenesis of diabetes and its complications. It has been reported that elevated transferrin saturation contributes to a two- to three-fold increased risk of developing any form of diabetes[19]. Recently, recombinant apo-TF has been shown to result in protection against type 1 diabetes in animal models[20].

Targeted drug delivery

Most drugs are systemically administered and are usually distributed throughout the body evenly rather than towards the specific pathological sites. This non-specificity of drug exposure inevitably requires high dosage and causes toxic damage to healthy cells. Another drawback associated with systemic administration is the short half-life of the drugs due to the fast renal clearance and the proteolytic degradation during systemic circulation.

An ideal drug delivery strategy is to deliver a therapeutic agent to the defined target cells. TF has been employed as a targeting molecule in drug and toxin conjugates and fusion systems to deliver the drug molecules to the targeted pathogenic cells. The application of TF as a drug-delivery vehicle is based on its unique TFR-mediated endocytosis pathway and the elevated expression levels of TFR in a variety of pathogenic cells, including malignant cells[2]. Furthermore, TF can deliver drugs to the brain by crossing the blood–brain barrier, which is a major barrier for administrating sufficient drugs to reach the central nervous system[21]. The added advantage of using TF to deliver drugs is that TF is biodegradable, non-toxic and non-immunogenic.

Various TF-conjugated therapeutic agents have been shown to significantly increase the drug selectivity and efficacy while reducing side effects. For example, when adriamycin, a commonly used chemotherapeutic agent for treating human leukaemia, was conjugated to TF, it yielded a significantly more effective inhibition of tumour cell proliferation than the free adriamycin[22,23]. Another TF-conjugated anti-cancer drug, CRM107, showed 10^3 to 10^5-fold increase of drug toxicity to cancer cell lines *in vitro* and 10–100 times more effectiveness in inhibiting tumour growth with reduced side effects *in vivo* than the unconjugated CRM107. In a further clinical study, more than 50% decrease in tumour volume was found in 60% of evaluable patients treated with TF-CRM107[24].

The TF–TFR complex-mediated cellular uptake pathway has also been exploited for oral delivery of protein-based therapeutics[25]. The oral delivery of TF-conjugated therapeutic proteins can retain the advantages of oral delivery in terms of ease, lack of pain and convenience compared with invasive delivery approaches, while overcoming the disadvantages of unconjugated drugs such as limited bioavailability, instability and short half-life. Both insulin used for treating diabetes and granulocyte colony-stimulating factor (G-CSF) used for promoting neutrophil recovery following chemotherapy for malignant diseases have been chemically conjugated with TF. Each conjugate has been shown to be transcytosed across the enterocyte-like Caco-2 cell,

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which is an *in vitro* cell culture model for gastrointestinal permeability and absorption studies[26], at a rate several fold higher than that of their respective unconjugated counterparts. These results suggest that TF-conjugated drugs could be transported through the TFR-mediated transcytosis process[27]. In *in vivo* tests in diabetic rats, both the subcutaneously (s.c.) injected and orally administered insulin-TF conjugate showed a slow, prolonged and significant hypoglycaemic effect, whereas the unconjugated insulin showed a short hypoglycaemic effect when s.c. injected but no hypoglycaemic effect when administered orally[27]. In the case of G-CSF-TF conjugate, the conjugate outperformed the free G-CSF in increasing the absolute neutrophil counts (ANCs) when they were s.c. injected to BDF1 mice. When orally administered, however, only the G-CSF-TF conjugate showed a significant and dose-dependent increase in ANCs whereas the G-CSF alone did not show an effect[28]. In a separate study using recombinant G-CSF-TF fusion protein for testing, similar results were observed as that of G-CSF-TF conjugate[29]. These results indicate that TF can be used as an effective oral delivery vehicle for protein or peptide therapeutics.

Recently, the therapeutic potential of proinsulin-transferrin (ProINS-TF) fusion protein for treating diabetes was investigated by exploiting the endocytosis and recycling mechanisms of the TF–TFR pathway[30]. It has been shown that when endocytosed TF is recycled in a slow recycling pathway, it merges with the protein secretory pathway in vesicles located at the trans-Golgi network, allowing the access of endocytosed TF to secretory proteases that are responsible for the conversion and activation of prohormones. Based on this observation, Wang et al. expressed recombinant ProINS-TF fusion protein in mammalian cells, and showed that the fusion protein could be steadily converted into insulin-transferrin (INS-TF) fusion protein through a TFR-mediated entocytosis process[30]. Moreover, ProINS-TF fusion protein demonstrated enhanced and sustained *in vivo* hypoglycaemic efficacy compared with insulin and ProINS when administered to streptozotocin-induced diabetic mice[30]. These results show that ProINS-TF fusion protein is a promising long-lasting novel hypoglycaemic agent for treating diabetes. To address the challenges of the high cost and low level expression of ProINS-TF in mammalian cells, Ventria Bioscience and Shen's group at University of Southern California are collaborating to express the fusion protein in rice grains to further develop this novel hypoglycaemic agent under the support of National Institutes of Health.

Conclusion

Recombinant hTF has been widely used in mammalian cell culture for production of therapeutic drugs and vaccines and has many potential clinical applications. Rice seed-based expression system proved to be able to cost-effectively produce large amounts of rhTF. A comprehensive biochemical, structural and functional characterisation has shown that this plant-derived rhTF is similar to its native counterpart. Clearly, rice-derived rhTF is a safe, low cost and readily available source of rhTF that are able to support these important applications.

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Abbreviations list

ANC, absolute neutrophil count; apo-TF, transferrin free of iron; G-CSF, granulocyte colony-stimulating factor; HSCT, hematopoietic stem cell transplantation; hTF, human serum transferrin; rhTF, recombinant human serum transferrin; s.c., subcutaneous; TF, transferrin; TFR, transferrin receptor.

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