Structural and Functional Characterization of Recombinant Human Serum Transferrin Secreted from Pichia pastoris

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Received August 28, 2009; Accepted November 16, 2009; Online Publication, February 7, 2010
[doi:10.1271/bbb.90635]

Serum transferrin is an iron-binding glycoprotein with a bilobal structure. It binds iron ions in the blood serum and delivers them into target cells via transferrin receptor. We identified structural and functional characteristics of recombinant human transferrin which is produced in the yeast Pichia pastoris. Using the signal sequence of the α factor of the yeast Saccharomyces cerevisiae, high-level secretion was obtained, up to 30 mg/l of culture medium. Correct processing at designed sites was confirmed by N-terminal sequence analysis. Carbohydrate modification was determined by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) analysis after digestion with endo-β-N-acetylglucosaminidase H. Reflecting the secondary structure, the circular dichroism spectrum of the recombinant protein was indistinguishable from that of serum transferrin. Consequently, the recombinant product had an iron binding function just as the serum specimen has: two Fe³⁺-binding sites existed in a recombinant transferrin molecule, as estimated by titration analysis using visible absorption, fluorescence spectra, and electrophoretic behavior in urea denaturizing polyacrylamide gel electrophoresis (PAGE).

Key words: transferrin; iron transport; Pichia pastoris; carbohydrate chain

Transferrins are a homologous group of Fe³⁺-binding and transporting proteins that include serum transferrin, ovotransferrin, and lactoferrin. They are about 80 kDa single-chain bilobal proteins with one Fe³⁺-binding site in each lobe. X-Ray crystallographic data for the diferric forms of lactoferrin, serum transferrin, and ovotransferrin have indicated that the N and C-lobes have similar tertiary structures. Each lobe is made up of a pair of domains (domain 1 and domain 2), and a single high affinity Fe³⁺-binding site is located in the interdomain cleft. Four of the six Fe³⁺-coordination sites are occupied by side-chains of amino acid residues, and the remaining two by a synergistic anion that is physiologically carbonate. The crystallographic data also indicated that four Fe³⁺-coordinating amino acid residues (Asp63, Tyr95, Tyr188, and His249 in the N-lobe and Asp392, Tyr426, Tyr517, and His585 in the C-lobe of human serum transferrin) are all conserved in both lobes of serum transferrin, ovotransferrin, and lactoferrin.

Serum transferrin acts as an Fe³⁺ transporter to target cells. Transferrin-dependent Fe³⁺ delivery to the target cells occurs through the binding of two Fe³⁺ ions in the serum. Both lobes of iron-free transferrin have a domain-opened structure, and coordination of the Fe³⁺ ions to two Tyr ligands, followed by coordination to a His ligand and an Asp ligand, alter the open structure to the domain-closed structure of iron-bound transferrin. The movement of the domains can be categorized as a hinge-like motion. The hinge of each lobe substantially consists of two polypeptide chains (β-strands), and additionally van der Waals interactions in the N-lobe and a disulfide bond in the C-lobe secure the correct domain motions as "alternative hinges." A specific receptor that resides on a plasma membrane recognizes the iron-bound transferrin with a closed structure, and forms a complex with the iron-bound transferrin. The transferrin-receptor complex is then internalized in the cells, and the complex releases Fe³⁺ at acidic pH in the endosome. The release of Fe³⁺ requires simple anions such as sulfate, pyrophosphate, and chloride in vitro. The requirements of anions for Fe³⁺ release has also been demonstrated for the iron-loaded transferrin-receptor complex, indicating the involvement of anions in cellular Fe³⁺ release. To understand the mechanisms of iron release from the transferrin-receptor complex in detail, protein engineering studies should be extended on the basis of structural biology. Since targeted drug-delivery systems like Fe³⁺ transport has focused on anticancer and gene therapy, the structure and function of the transferrin molecule should be analyzed in order to utilize it as a tag for transportation.

For protein engineering study of transferrins, an efficient expression system is required. Especially, the use of a microorganism cell system should be very practical for economic and less time-consuming production of recombinant proteins, while whole molecules of human serum transferrin (hSTf) have been produced using cultured animal cells such as baby hamster kidney cells. Although previous reports have indicated that functional proteins of whole molecules of ovotransferrin, porcine lactoferrin, and equine lactoferrin can be...
produced in microorganisms (Pichia pastoris), the microorganism production system of whole molecules of Fe³⁺-transporter protein, serum transferrins has not been available. To study iron-transport mechanism, such as the binding mechanism between iron-saturated transferrin and transferrin receptor, an effective expression system for serum transferrin is prerequisite. In the study reported here, a secretory production system using a methylotrophic yeast, Pichia pastoris, was constructed for the production of the whole molecules of human serum transferrin. The chemical, spectroscopic, and functional (Fe³⁺-binding) properties of purified recombinant serum transferrin were investigated.

Materials and Methods

Reagents. Authentic hsTf was purchased from Sigma-Aldrich (St. Louis, MO). All DNA restriction enzymes were purchased from TAKARA BIO (Otsu, Japan). All other materials were from Wako Pure Chemicals (Osaka, Japan), Nacalai Tesque (Kyoto, Japan), or Sigma-Aldrich, unless stated otherwise.

Construction of a shuttle vector with original human secretion signal. A cDNA fragment encoding the precursor of hsTf was amplified from a human liver cDNA library (Marathon Ready cDNA, Clontech, Mountain View, CA) by polymerase chain reaction (PCR) with Phusion DNA polymerase (Finnzymes, Espoo, Finland) and oligonucleotides 5′ ATT AAG GCGGGCGCG AAC ATG AGC GTC GCC GTG GGA GCC (forward primer) and 5′ CGG AGG GCGGGCGCG TTA AGG TCT ACG GAA AGT GCA GCC (reverse primer). These oligonucleotides were designed to generate the restriction sites for NotI at the 5′- and 3′-ends of the PCR product (restriction sites underlined). The PCR product was incubated with NotI and isolated by digestion with the same restriction enzyme, dephosphorylated with calf intestinal alkaline phosphatase (TAKARA BIO), and extracted from 1% SeaKem GTG melting point agarose gel. The PCR fragments modified with the restriction enzymes were ligated into vector pPIC3.5K (Invitrogen, Calsbad, CA), was linearized by digestion with the same restriction enzyme, dephosphorylated with calf intestinal alkaline phosphatase (TAKARA BIO), and extracted from 1% SeaKem GTG agarose gel. The amplified fragment was ligated into linearized vector pPIC3.5K using a DNA ligation kit (TAKARA BIO), and the ligation mixture was used to transform E. coli DH5α strain. The sequence of the insert in a constructed plasmid (pPIC3.5-K/hsTf) purified from E. coli cells was confirmed as a DNA sequence.

Construction of a shuttle vector with yeast secretion signal. cDNA fragments corresponding to mature hsTf without the secretion signal were amplified from constructed plasmid pPIC3.5K/hsTf by PCR with Phusion DNA polymerase and the following oligonucleotides: 5′ GCGGCG CTCCAG AAA AGA GTC CCT GAT AAA ACT GTG AGA (forward primer for pPIC9), CCGGGCGCGGG TACGTTA GTC CCT GAT AAA ACT GTG AGA (forward primer for pPIC9), and the following restriction sites for NotI and XhoI (restriction sites in primers underlined) and isolated on a 1% SeaKem GTG agarose gel. The purified recombinant hsTf was found to be almost pure by PAGE analysis.

Transformation of P. pastoris. The colonies were selected using Tryptosome (Seikagaku Corporation, Tokyo, Japan) to lyse the cell wall. The medium and lysed cells were mixed with 0.33 volumes of an SDS-buffer (0.25 M Tris–HCl, pH 7.0, containing 4% SDS, 5% mercaptoethanol, and 40% glycerol) and then incubated in a boiling water bath. The samples, corresponding to the original cultures of 7.5 µl (medium) and 15 µl (cells), were loaded and analyzed by SDS–PAGE using 12.5% polyacrylamide gel, and then made visible by staining with CBB and by western blotting using rabbit antiserum for hsTf (Rockland, Gilbertsville, PA).

For purification of the recombinant protein. The supernatant of the cultured medium was dialyzed against 20 mM Tris–HCl buffer, pH 8.0, and loaded onto a 60-ml Q Sepharose FF (GE Healthcare) anion-exchange column (ϕ2.6 × 12 cm) that had been equilibrated with the same buffer. The column was washed with 80 ml of the same buffer and then eluted with a 100-ml gradient of NaClO₃–0.1 M in the same buffer. The fractions that contained the recombinant hsTf as analyzed by SDS–PAGE were collected and dialyzed against 20 mM Tris–HCl buffer, pH 8.0. The sample was put onto an 8-ml Mono Q (GE Healthcare) anion-exchange column (ϕ1.0 × 10 cm) equilibrated with 50 mM Tris–HCl buffer, pH 8.0, and eluted with a 60-ml gradient of NaClO₃–0.6 M in the same buffer. For purification of the recombinant hSTF was found to be almost pure by PAGE analysis.

N-Terminal sequence analysis. The purified recombinant protein was blotted on a PVDF (polyvinyliden fluoride) membrane and the N-terminal sequences were determined using a protein sequencer (Procise 492, Applied Biosystems).

Far-UV CD spectra measurement. The far-UV circular dichroism (CD) spectrum of the Fe⁺⁺-loaded form of the recombinant protein was recorded at 0.03–0.05 mg/ml at 25 °C in 50 mM Tris–HCl buffer (pH 8.0) using a spectropolarimeter (model J-720, JASCO, Tokyo, Japan) with 0.2-cm cuvettes.

Treatment of recombinant protein with endolysocidases. Ten µg of Fe⁺⁺-free recombinant hsTf was treated with 0.75 milliunits of Endo-H (Calbiochem, Gibbstown, NJ) in 10 µl of reaction buffer at 37 °C for 30 min or 24 h. The reaction buffer used for the reaction was 50 mM sodium phosphate (pH 5.5). The samples were analyzed by SDS–PAGE using 8% acrylamide gels.

Mass spectrometry. The recombinant hsTf was mixed with 3.5-dimethoxy-4-hydroxynicamic acid (sinapinic acid) and put through MALDI-TOF-MS (Voyager, Applied Biosystems, Carlsbad, CA). Bovine serum albumin (Sigma-Aldrich) was used for molecular weight calibration.

Preparation of the Fe⁺⁺-free forms and Fe⁺⁺-loaded forms. To prepare Fe⁺⁺-free forms, the proteins were put onto centrifugal filter devices (Ultra-Free, Millipore, Billerica, MA) and washed with citrate buffer (pH 4.7) or put onto a Sephadex G-25 column (NAP column, GE Healthcare) equilibrated with citrate buffer, pH 4.7, and eluted with the same buffer. After removal of the Fe⁺⁺, the citrate containing ethylene glycol and DMSO) and Solution II (a polyethylene glycol solution), and then transformed with 10 µg of Sal I-digested pPIC3.5K/hsTf, pPIC9/hsTf, or pPIC9K/hsTf. His+ cells were selected on minimal plates.

Production and purification of the recombinant protein. The culture medium was changed to 15 ml (or 60–120 ml) of Buffered Minimal Methanol (BMM) medium in which 0.1 M potassium phosphate buffer, pH 6.0, is replaced with 0.1 M Tris–HCl buffer, pH 8.0. The medium was placed in two 200-ml baffled flasks or a 2-liter baffled flask and incubated at 30 °C using the shaking incubator (250 rpm) for 3–5 d. Methanol was added to a final concentration of 0.5% every 24 h. The cultured medium was fractionated into supernatant (medium) and precipitation (cells) by centrifugation.

For assay of produced and secreted recombinant hsTf, the cell pellet was treated with Zymolase (Seikagaku Corporation, Tokyo, Japan) to lyse the cell wall. The medium and lysed cells were mixed with 0.33 volumes of an SDS-buffer (0.25 M Tris–HCl, pH 7.0, containing 4% SDS, 5% mercaptoethanol, and 40% glycerol) and then incubated in a boiling water bath. The samples, corresponding to the original cultures of 7.5 µl (medium) and 15 µl (cells), were loaded and analyzed by SDS–PAGE using 12.5% polyacrylamide gel, and then made visible by staining with CBB and by western blotting using rabbit antiserum for hsTf (Rockland, Gilbertsville, PA).
buffer was replaced with 50 mM Tris–HCl buffer (pH 8.0). To prepare Fe$^{3+}$-loaded forms, the proteins were incubated with two-10-fold molar amounts of Fe$^{3+}$-NTA and 25 mM bicarbonate in 50 mM Tris–HCl buffer (pH 8.0). Excess Fe$^{3+}$ were removed with a NAP column (GE Healthcare) equilibrated with 50 mM Tris–HCl (pH 8.0). The Fe$^{3+}$-free forms and the Fe$^{3+}$-loaded forms were concentrated using centrifugal filter devices (UltraFree-4, Millipore) when necessary.

Fluorescence emission spectra. The fluorescence spectra of the Fe$^{3+}$-free and Fe$^{3+}$-loaded forms of recombinant ovotransferrin were recorded at 0.05 mg/ml in 50 mM Tris–HCl buffer (pH 8.0) containing 25 mM NaHCO$_3$ and 25 °C using a fluorescence spectrophotometer (FP-6500, JASCO) and 0.5 cm × 0.5 cm cuvettes. The excitation wavelength used for the measurement was 295 nm and the emission spectra were recorded.

Fe$^{3+}$-Binding analysis by urea-PAGE. The proteins were mixed with Fe$^{3+}$-NTA (protein/Fe$^{3+}$ = 0–10.0) in 50 mM Tris–HCl buffer (pH 8.0) containing 5 mM bicarbonate and incubated for 30 min on ice. The samples containing 3 μg of proteins were put onto a 6.4% acrylamide slab gel (89 mm × 68 mm × 1 mm) containing 5.0 mM urea and electrophoresed at 200 V for 180 min in a refrigerator (6 °C).26 The gel after electrophoresis was stained with CBB.

Visible absorption spectra. The visible absorption spectra of the Fe$^{3+}$-free and Fe$^{3+}$-loaded forms of the recombinant protein and egg white ovotransferrin were measured at 3 mg/ml in 50 mM Tris–HCl buffer at pH 8.0) containing 25 mM NaHCO$_3$ using a spectrometer (Ultrospec 2000, GE Healthcare) and a cuvette with a 1-cm pathlength at 25 °C.

Results

Protein expression and purification

Cloning of the hsTf gene with the secretion signal was done by PCR using a human liver cDNA library as a template. The whole hsTf gene corresponding to its precursor was inserted into a P. pastoris expression vector (pPIC3.5/hsTf) which had the yeast secretion signal (S. cerevisiae, α-factor). P. pastoris (KM71) was individually transformed with each constructed vector.

P. pastoris transformed with a plasmid (pPIC3.5/hsTf) carrying the authentic secretion signal was cultured in induction medium (BMM) that contained methanol. The culture suspension was fractionated into medium and cell fractions and analyzed by SDS–PAGE. No protein was observed in the CBB-stained gel, and hsTf was not detected even by western blotting against anti hsTf antiserum either in the medium fraction or the cell fraction (data not shown).

Expression plasmids carrying the yeast secretion signal (pPIC9/hsTf and pPIC9K/hsTf) were constructed and introduced into P. pastoris in the same way. As shown in Fig. 1, the supernatant fractions were analyzed by SDS–PAGE, the CBB stained gel is in panel a and the immunoblot with anti hsTf antiserum is in panel b.

Two protein bands of molecular masses of about 79 kDa were seen in a medium fraction on the CBB stained gel (Fig. 1a, lanes 3 and 4), and western blotting analysis revealed the two bands corresponded to hsTf (Fig. 1b, lanes 3 and 4) for both systems with the two plasmids, pPIC9/hsTf and pPIC9K/hsTf. Recombinant hsTf was purified from the cultured medium by two-step anion exchange chromatography after dialysis to the respective equilibration buffers. The secretion level was 30 mg/l of culture for both systems.

![Fig. 1. Production of Recombinant hsTf by Pichia pastoris.](image)

**Yeast cells transformed by pPIC9/hsTf (lane 3) or pPIC9K/hsTf (lane 4) were cultured in BMM medium, as described in the text. The supernatant proteins in the medium fraction were prepared and analyzed by SDS–PAGE, followed by CBB staining (panel a) and western blotting using antiserum for hsTf (panel b).**

Chemical analysis

The N-terminal sequence of the purified recombinant protein derived from pPIC9/hsTf was found to be Val-Pro-Asp-Lys-Thr-Val-. The product of the other construct, pPIC9K/hsTf, showed a N-terminal of Glu-Ala-Tyr-Val-Pro- and several minor contents, Val, Leu, Pro, and Phe in the 1st–4th cycles respectively. These data indicate that a secretion signal inserted into the expression vector with the ovotransferrin gene was cleaved in a secretion process in P. pastoris. An inconsistency in the pPIC9K/hsTf construct is described below.

Spectroscopic analysis

The CD spectra of the recombinant proteins were measured (Fig. 2). They were almost the same as that of authentic hsTf. As a prerequisite, experiments on iron titration, and fluorescence spectra were recorded for the recombinant products (data not shown). They too were indistinguishable from the authentic one. Consequently, we consider the recombinant products to have the native conformation, as authentic hsTf does.

Analysis of carbohydrate chains

Purified Fe$^{3+}$-free forms of recombinant hsTf were treated with an endoglycosidase (Endo-H) and analyzed...
The present result indicates that Fe\textsuperscript{3+} must be denatured in the endoglycosidase reaction, the hsTf and pPIC9K/hsTf). Although some glycoprotein endoglycosidase treatment for both samples (pPIC9/hsTf became a single smaller band after shown in Fig. 3. It revealed that two bands of purified recombinant hsTf, the Fe\textsuperscript{3+}-free form of the recombinant protein (derived from pPIC9/hsTf) was titrated by Fe\textsuperscript{3+} and the absorbance at 460 nm (around the absorption peak of the Fe\textsuperscript{3+}-bound transferrins) was measured as shown in Fig. 5a. The calculated linear regression curves revealed that recombinant hsTf bound two Fe\textsuperscript{3+} as authentic hsTf (Fig. 5a). The slope of the regression curve above the 2 Fe\textsuperscript{3+}/protein was caused by absorption of Fe\textsuperscript{3+}-NTA, as shown in Fig. 5a. Similar titrations were done, and fluorescence intensities at 330 nm (excitation wavelength 295 nm) were measured and, are shown in Fig. 5b. Fluorescence quenching\textsuperscript{14}) by bound Fe\textsuperscript{3+} was observed, and calculated linear regression curves for the fluorescence intensities also indicated that recombinant hsTf bound two Fe\textsuperscript{3+} (Fig. 5b).

Urea-PAGE analysis was also done for recombinant hsTf mixed with Fe\textsuperscript{3+}-NTA at various Fe\textsuperscript{3+}/protein ratios. The Fe\textsuperscript{3+}-free form is denatured and the dfferic form retains the native compact structure in the gel containing 5 M urea, and these should result in lower mobility in the Fe\textsuperscript{3+}-free form than in the dfferic form. It is also known that the 1Fe\textsuperscript{3+}-bound form has medium mobility. The gel after Urea-PAGE was stained with CBB as shown in Fig. 6. The pattern of the urea-gel well indicates that the protein bound two Fe\textsuperscript{3+} via 1Fe\textsuperscript{3+}-bound intermediate. The slightly larger mobility of authentic transferrin might indicate higher stability of the authentic protein than the recombinant protein in urea solution.

### Discussion

The methylotrophic yeast *Pichia pastoris* expression system is very suitable for the production of large quantities of heterologous proteins, especially proteins of eukaryotes. In this study, we obtained correctly-folded whole (containing both N- and C-lobes) molecules of human serum transferrin (hsTf) using the *P. pastoris* expression system. Mason et al. reported production of hsTf whole molecules using baby hamster kidney cells; in that study, recombinant hsTf was secreted into a medium with an expression level up to 53 mg/l culture medium.\textsuperscript{27} The advantage of protein expression in *P. pastoris* over protein expression in the

#### Table 1. Molecular Weights of Recombinant hsTf as Determined by MALDI-TOF-MS before and after Endo-H Treatment

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Molecular Weight (daltons)</th>
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<tbody>
<tr>
<td>Before Endo-H</td>
<td>79,015.2 and 77,729.7 (difference 3,832.8)</td>
</tr>
<tr>
<td>After Endo-H</td>
<td>75,272.4 (difference 3,631.4)</td>
</tr>
<tr>
<td>Before Endo-H</td>
<td>79,405.7 and 77,830.8 (difference 3,631.4)</td>
</tr>
<tr>
<td>After Endo-H</td>
<td>75,774.3 (difference 2,056.5)</td>
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polypeptide with two different carbohydrate chains, and that removal of the carbohydrate moieties should result in a specific band with a distinct smaller molecular weight.

Fe\textsuperscript{3+} binding ability

To analyze the Fe\textsuperscript{3+} binding ability of the recombinant hsTf, the Fe\textsuperscript{3+}-free form of the recombinant protein (derived from pPIC9/hsTf) was titrated by Fe\textsuperscript{3+} and the absorbance at 460 nm (around the absorption peak of the Fe\textsuperscript{3+}-bound transferrins) was measured as shown in Fig. 5a. The calculated linear regression curves revealed that recombinant hsTf bound two Fe\textsuperscript{3+} as authentic hsTf (Fig. 5a). The slope of the regression curve above the 2 Fe\textsuperscript{3+}/protein was caused by absorption of Fe\textsuperscript{3+}-NTA, as shown in Fig. 5a. Similar titrations were done, and fluorescence intensities at 330 nm (excitation wavelength 295 nm) were measured and, are shown in Fig. 5b. Fluorescence quenching\textsuperscript{14}) by bound Fe\textsuperscript{3+} was observed, and calculated linear regression curves for the fluorescence intensities also indicated that recombinant hsTf bound two Fe\textsuperscript{3+} (Fig. 5b).

Urea-PAGE analysis was also done for recombinant hsTf mixed with Fe\textsuperscript{3+}-NTA at various Fe\textsuperscript{3+}/protein ratios. The Fe\textsuperscript{3+}-free form is denatured and the dfferic form retains the native compact structure in the gel containing 5 M urea, and these should result in lower mobility in the Fe\textsuperscript{3+}-free form than in the dfferic form. It is also known that the 1Fe\textsuperscript{3+}-bound form has medium mobility. The gel after Urea-PAGE was stained with CBB as shown in Fig. 6. The pattern of the urea-gel well indicates that the protein bound two Fe\textsuperscript{3+} via 1Fe\textsuperscript{3+}-bound intermediate. The slightly larger mobility of authentic transferrin might indicate higher stability of the authentic protein than the recombinant protein in urea solution.
cell culture system is that a relatively simple and inexpensive growth medium can be used and that the costs of production of large quantities of recombinant protein are very low. We found that the secretion level in the *P. pastoris* expression system was 30 mg/l of culture medium, comparable to that in the cell culture system.

Since the native secretion signal of hsTf did not work, as is evident in the results, we introduced a signal of the yeast *S. cerevisiae* α-factor with an hsTf gene, and recombinant hsTf was secreted into the medium. The recombinant hsTf was very easily purified from the medium using two anion exchange columns. We have reported the expression system of the whole molecule of ovotransferrin (=chicken serum transferrin) using *P. pastoris*. In that study, the signal of chicken did work in *P. pastoris*, and the expression level of the secreted protein was good (97 mg/l).21) Although we do not have enough information on the mechanisms that determine the yield of secretory production, notice that the combination of target protein and signal may critically affect it.

The 5′ region of hsTf in the plasmids was designed as shown in Fig. 7. For pPIC9/hTf, the N-terminal sequence of the recombinant protein determined using the protein sequencer was the same as that of authentic hsTf. The signal peptide was cut at the signal cleavage site for Kex2 (inserted after *XhoI* site). For pPIC9K/hTf, the hsTf gene was inserted after the *SnaBI* site, since the other *XhoI* site exist in pPIC9K/hTf. The *SnaBI* site corresponds to Tyr-Val, and the latter signal cleavage site for Ste13 was not cut in this system. Recombinant hsTf derived from pPIC9K/hsTf has redundant residues (Glu-Ala-Tyr-Val). This suggests that the yeast signal peptide and inserted amino acids for signal cleavage functioned and were processed correctly in the *P. pastoris* cells. Since the signal cleavage site for Kex2 works well, if the same sequence of hsTf is required, the pPIC9 plasmid is better for the production of recombinant protein.

The CD spectra of the recombinant hsTf (of both constructions) were almost the same as those of hsTf, which suggests that each recombinant hsTf retains the correct secondary structure. The most important function of transferrin, Fe³⁺ binding ability, was analyzed for the recombinant hsTf (derived from pPIC9/hTf) by “Urea-PAGE” and combinations of titration using Fe³⁺ and the absorption spectrum of visible light or the
fluorescence (quenching by the 2nd Fe\textsuperscript{3+} ions, identically to authentic hsTf. The titration curve at 460 nm (which come from bound Fe\textsuperscript{3+}) and visible absorption at 460 nm was recorded as described in the text. Molar extinction coefficients \(\varepsilon (\text{M}^{-1} \text{cm}^{-1})\) were calculated as absorbance/concentration (m)-path length of the cell (cm), as shown in (a). Fe\textsuperscript{3+}-NTA was also added to the buffer without proteins and is shown as crosses. For comparison, the absorbance of Fe\textsuperscript{3+}-NTA was divided by the protein concentration of the above titration as shown in the graph. Linear regression curves of recombinant (dotted lines) and native hsTf (solid lines) were calculated for Fe\textsuperscript{3+}/protein ratios of 0–2 and 2–10 as shown in (a). Fe\textsuperscript{3+}-Free recombinant (closed triangles) and native hsTf (closed circles) were titrated using Fe\textsuperscript{3+}-NTA (Fe\textsuperscript{3+}/protein ratio of 0–4). The fluorescence intensity at 329.5 nm (excitation wavelength, 295 nm) is shown in (b). Linear regression curves of recombinant (dotted lines) and native hsTf (solid lines) were calculated for Fe\textsuperscript{3+}/protein of 0–1, 1–2, and 2–4, as shown in (b).

A common disadvantage of protein expression in \textit{P. pastoris} is that recombinant proteins can be hyperglycosylated, and can have large O-linked carbohydrate chains.\textsuperscript{30} We found that the recombinant hsTf is not hyperglycosylated but does have N-linked carbohydrate chains. The carbohydrate chains of the recombinant hsTf were analyzed by SDS–PAGE after treatment with an endoglycosidase, Endo-H. The enzyme cleaved the carbohydrate chain of the recombinant hsTf in native form. The molecular weights of the carbohydrate chain of the recombinant hsTf molecules were measured using a combination of Endo-H digestion and MALDI-TOF-MS. The differences in molecular weights of the recombinant hsTf before and after digestion were 3,832.8 and 2,457.3 for pPIC9/hsTf and 3,631.4 and 2,056.5 for pPIC9K/hsTf (the molecular weights are shown in Table 1). Endo-H cut a high mannose carbohydrate chain at a bond between two GlcNAc that exist between the peptide chain (an asparagine residue) and the mannose chain, and the differences corresponded to one GlcNAc + 12 mannoses and one GlcNAc + 6 mannoses. Since typical N-linked carbohydrate chains of the protein expressed in \textit{P. pastoris} have 8–12 mannoses\textsuperscript{31} and the hsTf sequence has two putative glycosylation sites (Asn413 and Asn611), the recombinant hsTf molecules were estimated to have two N-linked carbohydrate chains mainly with two GlcNAc + 12 mannoses and 2 GlcNAc + 6 mannoses. The property that the carbohydrate chain of the recombinant hsTf is cleaved easily may help in studying the roles of the carbohydrate chain for the functions of transferrins, such as Fe\textsuperscript{3+} binding. Cleavage of the carbohydrate chain reduces the heterogeneity of the sample; the deglycosilated recombinant hsTf should be very suitable to prepare crystals for X-ray crystallography. Since deglycosilation is done without denaturing conditions, we were able to prepare the specimen for crystallization in the most intact form.

![Image](image_url)
Since the crystal structure of Fe\(^{3+}\)-bound whole molecules of the hsTf has not been identified, this expression system may help to solve the crystal structure of the whole molecule.

For this bilobal iron-binding protein, transferrin, cooperative characteristic due to the two distinct lobes are known. For example, Fe\(^{3+}\) binding in a certain lobe stabilizes iron binding in the other lobe.\(^3\) The underlying molecular mechanism is not known yet. The present paper is the first demonstration of a micro-organism production system for whole molecules of hsTf, and should help in the study of the inter-lobe communication mechanism by a site-directed mutagenesis approach.

**Acknowledgment**

This work was supported by a Grant-in-Aid for Young Scientists from The Japan Society for the Promotion of Science.

**References**