Clinica Chimica Acta 471 (2017) 12–16

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/cca

A microscale protocol for the isolation of transferrin directly from serum

Ana Penezić*, Goran Miljuš, Bojana Milutinović, Olgica Nedić

Institute for the Application of Nuclear Energy, INEP, Banatska 31b, 11080 Belgrade, Serbia

A B S T R A C T

A microscale procedure for the isolation of transferrin directly from human serum (hTf) is described in this study. The protocol is based on three precipitation steps without application of chromatography. It lasts 90 min with the initial sample volume of 250 μL. The yield of the isolated hTf is 58%, which is considerable in biochemical terms. The purity of the isolated hTf is 97%, as assessed by three methods: electrophoresis followed by protein staining, immunoblotting and HPLC. Immunoblotting with antibodies against other major serum proteins indicated that isolated hTf does not contain albumin, immunoglobulin G or alpha-2-macroglobulin. Lectin dot-blot demonstrated that isolated hTf preserved its glycan moieties. Fluorescent emission spectroscopy of the isolated hTf has shown no changes in tertiary structure. Isolated hTf was approximately 26% saturated with iron ion, which is comparable to physiological value (although a degree of saturation decreases to some extent during isolation procedure). Finally, co-immunoprecipitation experiment confirmed that isolated hTf retained its ligand characteristics crucial for the ligand-receptor type of interaction with the hTf receptor. To conclude, the procedure described in this work, is time and cost-effective, allows multiple sample handling and provides high-purity hTf isolate with preserved structural and functional properties.

1. Introduction

Transferrin (serotransferrin, siderophilin, β_1 metal-binding globulin) belongs to a group of non-haem, metal-binding glycoproteins distributed in physiological fluids and cells of vertebrates [1,2]. Human transferrin (hTf) is a monomeric glycoprotein consisting of 679 amino acids. Different literature sources refer to its molecular mass (Mm) of approximately 79 kDa. Nineteen intra-chain disulfide bonds stabilize the protein along with four carbohydrate side chains (three N- and one O-linked) [1]. Transferrin is a powerful iron chelator, capable of binding two Fe^{3+} ions reversibly, with $K_d = 10^{-23}$ M [3].

Tf isolated from particular patients may have diagnostic potential, for example for determination of congenital disorders of glycosylation (CDG) [4–6] and alcohol abuse (prominent carbohydrate-deficient transferrin, CDT) [7,8]. Since Tf can bind other metal ions beside iron [9–11], investigation of metal-binding potential of Tf isolated from an individual can contribute to understanding of disorders associated with impaired metabolism of metal ions.

Methods for isolation and purification of Tf from human serum, employed so far, include combination of precipitation techniques (using ethanol, ammonium sulphate, rivanol) and chromatographic procedures (ion-exchange, affinity and gel filtration) [12–14], most often starting with Cohn's fraction I or IV, i.e. samples which were already processed before Tf isolation.

In the study presented here, an optimized protocol for the isolation and purification of Tf directly from human serum is described. An efficient, rapid, easy-to-perform, time-saving and cost-effective procedure was developed enabling, in contrast to most other employed procedures, a microscale isolation protocol. A microscale process becomes especially useful (or even inevitable) when it comes to clinical laboratory testing, with limited amounts of patients' samples.

2. Materials and methods

All chemicals were purchased from Sigma-Aldrich, (St. Louis, Missouri, USA) unless otherwise stated. Human serum samples were collected from healthy volunteers (10).

2.1. Biochemical analysis

Concentrations of hTf and iron ion in the initial serum samples (single or pooled) and final supernates (S3) were determined by commercial assays produced by Elitech (Puteaux, France), whereas the concentration of total proteins was measured using Bicinchoninic acid (BCA) protein quantification kit (Abcam, Cambridge, UK).

* Corresponding author at: Banatska 31b, 11080 Belgrade, Serbia.
E-mail addresses: anap@inep.co.rs (A. Penezić), goranm@inep.co.rs (G. Miljuš), bojana@inep.co.rs (B. Milutinović), olgica@inep.co.rs (O. Nedić).

http://dx.doi.org/10.1016/j.cca.2017.05.013
Received 8 May 2017; Received in revised form 10 May 2017; Accepted 10 May 2017
Available online 11 May 2017
0009-8981/

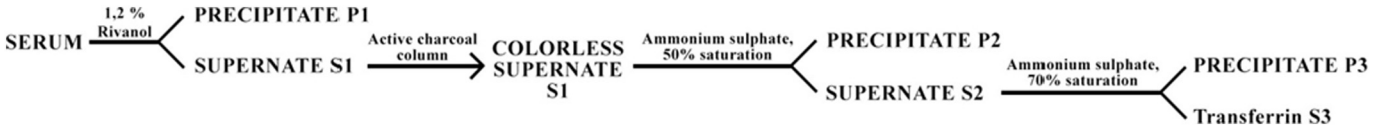

Fig. 1. Purification protocol for hTf.

2.2. Isolation and purification of hTf from human serum

The protocol for isolation and purification of Tf from human serum is shown in Fig. 1.

Either individual or a pool of serum samples (n = 10) from healthy subjects was treated with 1.2% rivanol (ethacridine lactate) aqueous solution (1:1 = vol:vol). The mixture was vortexed and centrifuged at 10000*g* for 5 min. Precipitate P1 was discarded and the supernate S1 treated again in the same manner.

Rivanol was removed from S1 by treatment with active charcoal. Active charcoal (2 g) was packed in a 5 mL plastic column and rinsed with deionized water. S1 was loaded on a column and pulled down by a syringe (in order to minimize the contact period between sample and active charcoal). Resulting flow-through fraction S1 was colorless. The volume of S1 was reduced to the initial volume of serum (250 μL) by using centrifugal filter device (Millipore, Billerica, USA) with 10 kDa cut-off (at 10000 g for 5 min).

S1 was treated with saturated ammonium sulphate solution at 4 °C (SASS, pH adjusted to 7.4) in order to achieve 50% saturation. Addition of SASS was performed very slowly, drop by drop with continuous gentle vortexing. The mixture was centrifuged at 10000*g* for 10 min. The precipitate P2 was discarded and the supernate S2 further treated with SASS in order to achieve 70% saturation. Again, the mixture was centrifuged at 10000*g* for 10 min, resulting in minute quantities of precipitate P3, which was discarded. The supernate S3 was filtered using centrifugal filter device with 100 kDa cut-off in order to remove possible impurities with higher molecular masses, and finally desalted and washed with five volumes of 0.05 M PBS pH 7.4 by using centrifugal filter device with 10 kDa cut-off. Isolation of Tf from each sample was performed in triplicate.

2.3. SDS-PAGE analysis of the isolated hTf

SDS-PAGE was performed according to the manufacturer's recommendations using a BioRad mini-PROTEAN® tetra system electrophoretic unit (Hercules, USA), on 10% gels, under reducing conditions. Following electrophoretic separation, the gel was stained by application of silver nitrate solution (Bio Rad, Hercules, USA). Pre-stained molecular mass markers ranging from 8 to 210 kDa were used.

2.4. Immunoblot analysis of the isolated hTf

Following SDS-PAGE, proteins were electrotransferred on to nitrocellulose membrane (NC, 0.45 μm, Amersham Protran, GE Healthcare UK Limited Amersham Place, Little Chalfont, UK), stained with Ponceau S and analyzed by immunoblotting, using polyclonal sheep anti-Tf antibody (INEP, Belgrade, Serbia) and biotinylated secondary horse anti-sheep IgG antibody (Vector Laboratories, Burlingame, USA). The presence of potential impurities (i.e. major serum proteins) was analyzed by immunoblotting with polyclonal rabbit anti-alpha-2-macroglobulin (α2M, AbD Serotec, Kidlington, UK), anti-serum albumin (SA, Calbiochem, San Diego, USA) and anti-IgG (INEP, Belgrade, Serbia) antibodies, coupled to HRP-conjugated secondary sheep anti-rabbit IgG antibody (AbD Serotec, Kidlington, UK). Detection of immunoreactive proteins was performed using an enhanced chemiluminescence (ECL) reagent kit (Pierce, Minneapolis, USA) and autoradiography. Commercial preparations of Tf, α2M, SA and IgG were used as biomarkers for protein positions in immunoblotting.

2.5. SE-HPLC analysis of the isolated hTf

Size-exclusion (SE) chromatography was performed on Waters HPLC system (Waters 1525 binary pump, coupled with Waters 2487 dual λ absorbance detector, Milford, USA), using BioSuite 250, 5 μm HR SEC column equilibrated with 0.05 M PBS, pH 7.2. A 200 μL samples of the commercial Tf (cTf) or isolated Tf from serum (hTf) were injected manually (at concentrations of 0.5 and 0.7 mg/mL respectively). The flow rate was 0.8 mL/min and elution was monitored by measuring absorbance at 214 nm.

2.6. Lectin dot-blot of the isolated Tf

In order to confirm the presence of glyco-residues in Tf molecules, hTf isolated from pooled serum sample and cTf (at concentration of 0.5 mg/mL, 5 μL each) were spotted on to NC membrane. The membrane was blocked in 3% BSA at room temperature for 2 h, cut in two pieces, one incubated with biotinylated Concanavalin A (ConA) and the other with *Sambucus nigra* Lectin (SNA, Vector Laboratories, Burlingame, USA) for 1 h. Membranes were then washed four times in TBST (Tris buffer saline with 0.1% Tween) and incubated with HRP-conjugated avidin (Vector Laboratories, Burlingame, USA) for 1 h. Unbound avidin was washed four times with TBST and lectin-reactive proteins were detected by using ECL reagent kit and autoradiography.

2.7. Fluorescence emission spectroscopic analysis of the isolated hTf

Fluorescence emission spectra were recorded for the isolated hTf and cTf at protein concentration of 0.2 mg/mL in the wavelength range of 300 to 450 nm following excitation at 295 nm, using quartz cell (1 cm path length) and slit widths of 4 nm. Each spectrum was recorded twice and corrected for the spectrum obtained for PBS solution.

2.8. Co-immunoprecipitation (Co-IP) of the isolated hTf

Immunoprecipitation of hTf/hTf receptor (hTfR) complexes was performed by using Pierce® Co-Immunoprecipitation Kit (Pierce Biotechnology, Rockford, USA). Following the manufacturer's instructions, AminoLink®Plus Coupling Resin (30 μL of 50% suspension) was loaded into spin columns and 10 μg of anti-CD-71 antibody (anti-hTfR, 0.2 mg/mL, Santa Cruz Biotechnology, Santa Cruz, USA), was immobilized. Solubilized cell membranes from human placental tissue were used as a source of hTfR [15,16]. hTfR containing solution (150 μL) was diluted with 150 μL of Lysis Wash buffer (LWb, provided with in the Co-IP Kit) and incubated at 4 °C overnight. Unbound proteins were separated by centrifugation at 2000*g* for 30 s, followed by washing of the column three times with LWb. The affinity matrix now contained hTfR coupled to immobilized anti-TfR antibody. In the last step, 200 μL of the isolated hTf (0.8 mg/mL) was incubated with the resin at 4 °C overnight. Unbound proteins were removed as already described, bound fraction was eluted with 110 μL of the elution buffer pH 2.8 (provided within the Co-IP Kit), separated by centrifugation and immediately neutralised with 2 μL of 2 M Tris–HCl buffer, pH 8.9. The eluted fraction (containing hTf/hTR complexes) was analyzed by dot-immunoblotting using anti-Tf and anti-hTfR antibodies (Santa Cruz Biotechnology, Santa Cruz, USA).